



**Full Length Article**

## Differential Expression of Nitrate Transport Protein, PutNAR2.1, of *Puccinellia tenuiflora* in Response to Three Salinity Types

Guoqing Zhu, Shiya Chen, Yang Xu, Hao He, Chang Xu and Shumei Jin\*

Key Laboratory of Saline-alkali Vegetation Ecology Restoration, Ministry of Education, College of Life Sciences, Northeast Forestry University, Harbin 150040, China

\*For correspondence: jinshumei1972@163.com

Received 08 February 2020; Accepted 26 February 2020; Published 31 May 2020

### Abstract

*Puccinellia tenuiflora* can survive on saline land and is an important halophyte. *PutNAR2.1* was cloned from *P. tenuiflora* and mainly expressed in root. *PutNAR2.1* expression was significantly increased under the treatment of 300 mM NaCl, 100 mM Na<sub>2</sub>CO<sub>3</sub> or 150 mM NaHCO<sub>3</sub> stress and reached the highest at 24 h in *P. tenuiflora*. Resistance to saline and alkali has been significantly improved when the *PutNAR2.1* protein expression in *E. coli*. *PutNAR2.1* transgenic yeast cells showed the more growth trend than control under NaCl, Na<sub>2</sub>CO<sub>3</sub> or NaHCO<sub>3</sub> stress. Seeds germination and seedlings growth of wild-type was affected heavily than the *PutNAR2.1* transgenic *Arabidopsis thaliana* under NaCl, Na<sub>2</sub>CO<sub>3</sub> and NaHCO<sub>3</sub> stress. These results revealed that the expression of *PutNAR2.1* was up-regulated by salt stress treatment. © 2020 Friends Science Publishers

**Keywords:** Nitrate transport protein; Salt stress; *Puccinellia tenuiflora*; Yeast; *Arabidopsis thaliana*

### Introduction

There are the large areas of saline-alkali land around the world and this problem is continuing to deteriorate. Salt stress is a limiting factor that severely affects plant growth and development, the crop productivity is also significantly inhibited (Munns and Tester 2008). Almost all plants or crops, which are important to humans, are adversely affected by high concentration of salt (Al-Maskri *et al.* 2010; Yang and Guo 2018). Therefore, it is especially important for the research of salt-tolerant plants.

*Puccinellia tenuiflora* is a monocotyledonous halophytic species that extensively distributed in the saline land of the Songnen plain in Northeastern China (Yu *et al.* 2011). The leaves of *P. tenuiflora* were very tender and more rich in nutrition, which is a good quality forage for livestock (Wei 2016). Therefore, it is used as a typical material for the study of salt tolerance mechanism, because it is the few species that can survive under multiple salt stresses. The molecular mechanisms of *P. tenuiflora* which can adapt to salt stress deserve further research.

The high affinity nitrate transporter accessory protein (NAR2) plays a crucial role in nitrate absorption and transport. Plant absorption of nitrogen can be classified as high-affinity transport systems (HATS) and low-affinity transport systems (LATS) depending on their absorptive capacity (Kotur and Glass 2015). NRT2 and NAR2 primarily regulate HATS and are responsible for the transport of nitrates under lower nitrogen concentration in

plant growing media (Laugier *et al.* 2012). *NRT2.1* is the major contributor to total HATS activity in NRT2 family (Li *et al.* 2007). *CrNRT2.1* did not transport nitrate alone in *Chlamydomonas reinhardtii*, and required *CrNAR2* to co-regulate in transport (Quesada *et al.* 1994; Zhou *et al.* 2000). A similar result was found in *Arabidopsis thaliana*, which has *NAR2*-like genes named as *AtNRT3.1*. *AtNRT3.1*; abundantly expressed and proved to be highly sensitive to nitrate induction (Okamoto *et al.* 2006; Orsel *et al.* 2006). In *Hordeum vulgare*, three *CrNAR2*-like genes were cloned: *HvNAR2.1*, *HvNAR2.2* and *HvNAR2.3*; while NO<sup>3-</sup> is transported only if *HvNRT2.1* and *HvNAR2.3* are simultaneously present; neither *HvNRT2.1* nor *HvNAR2.3* show transport activity when present alone (Tong *et al.* 2005). These findings indicated that *NAR2* is widely found in a variety of higher plants and it will specifically interact with *NRT2* in the same species to participate the HATS. Recent studies have shown that *OsNAR2.1* overexpression manifested better drought tolerance in rice (Chen *et al.* 2019).

Salt stress can seriously affect the plant uptake of nitrogen. Transcriptional analyses of *P. tenuiflora* treated with saline-alkali water showed that *PutNAR2* was strongly up-regulated. This indicated the significance of *PutNAR2* in *P. tenuiflora*; due to which the adaptation of this species in the saline-alkali lands can never be ignored (Ye *et al.* 2019).

The expression of *PutNAR2.1* may affect the salt tolerance of *P. tenuiflora*. Through the analysis of changes in salt tolerance of yeast, protein, and plants due to

*PutNAR2.1* overexpression, we have a preliminary understanding that *PutNAR2.1* improving the salt tolerance of organisms. This research gives further insight into the study of salt-tolerant molecular mechanism *P. tenuiflora*.

## Materials and Methods

### Bioinformatics analysis of *PutNAR2.1*

Seeds of *P. tenuiflora* were obtained from saline-alkali land in Northeast China. The cDNA was obtained from total RNA of *P. tenuiflora* using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and the reverse transcription PCR kit (Takara, Tokyo, Japan). The specific primers (*PutNAR2.1F*: 5'-ATGGCTCGGCAAGGAATGGT-3' and *PutNAR2.1R*: 5'-TTAGTTGTTCTTCTTGCGCTTC-3') were designed by analyzing the *P. tenuiflora* transcriptome sequence. The PCR product was amplified with cDNA by connecting the template to plasmid pMD18-T Vector (Takara, Tokyo, Japan), and then sequenced.

### Conserved domain of *PutNAR2.1* in the NCBI database

The sequence of conserved domain of *PutNAR2.1* of cloned gene was analyzed by blast search in NCBI, and the amino acid sequence of *PutNAR2.1* protein was found highly similar with other species. The homologous amino acid sequence of protein was compared by DNAMAN software, and the phylogenetic tree was constructed by MEGA7 to observe the relationship between *PutNAR2.1* and *NAR2.1* of other species.

### Real-time quantitative PCR analysis for *PutNAR2.1* expression in *P. tenuiflora*

The expression pattern of *PutNAR2.1* under salt stress in *P. tenuiflora* was examined. Seeds were sown onto half-strength MS medium. The seedlings of one-month age were subjected to various stress treatments (300 mM NaCl, 100 mM Na<sub>2</sub>CO<sub>3</sub> or 150 mM NaHCO<sub>3</sub>) for 0, 6, 12, 24 or 36 h. The *PutNAR2.1* expression in *P. tenuiflora* seedlings under different stress treatments was detected by RT-qPCR analysis, total RNA was isolated from *P. tenuiflora* and cDNA was synthesized. Subsequently RT-qPCR analyses were carried out by SYBR green (Takara, Tokyo, Japan) and IQ5 real-time PCR equipment (Bio-Rad, Hercules, CA, USA) with the steps: 95°C for 30 s, 30 cycles of 95°C for 5 s, and 55°C for 30 s. The next steps were added to melt-curve analysis: 95°C for 15 s, followed by continuously increased from 60 to 95°C. The *PutAct2* gene expression was used as control. The forward primer sequence was ActinF: 5'-GGTAACATTGTGCTCAGTGGTGG-3' and reverse primer sequence was ActinR: 5'-AACGACCTTAATCTTCATGCTGC-3'). *PutNAR2.1* RT-qPCR primers were designed by Quant Prime Tool.

To examine the expression pattern of the *PutNAR2.1* in *P. tenuiflora* among the different organs, total RNA was

extracted from roots, shoots, leaves, flowers and seeds respectively and cDNA was synthesized. Subsequent RT-qPCR procedure was same as above.

### Construction of expression vectors and transformation

The open reading frame (ORF) of the *PutNAR2.1* gene was amplified from pMD18T-*PutNAR2.1* plasmid DNA with *Bam*H I forward primer 5'-GGATCCATGGCTCGGCAAGGAA-3' (restriction site underlined for all restriction enzymes) and *Xho*I reverse primer 5'-CTCGAGTTAGTTGTTCTTCTTGCG-3'. The PCR amplified fragment with *Bam*H I and *Xho*I was recovered and then ligated to the corresponding restriction enzyme site of the pYES2, pGEX-6p-3 and pBI121 vector, respectively. It was finally verified by double enzyme digestion and binary expression vector generating the plasmid pYES2-*PutNAR2.1*, pGEX-*PutNAR2.1* and pBI121-*PutNAR2.1*. The pGEX-*PutNAR2.1* and pGEX-6p-3 plasmid transformed *E. Coli* BL21 cells were used for the expression of *PutNAR2.1* fusion protein in *E. coli* BL21, and plasmid pGEX-6p-3 was used as the control.

The plasmid DNAs of pYES2-*PutNAR2.1* and pYES2 were transformed into the yeast strain INVSC1 (*Saccharomyces cerevisiae*) using the electric impulse method. The plasmid DNAs of pBI121-*PutNAR2.1* was transformed into the *Agrobacterium tumefaciens* strain EHA105 (Takara, Tokyo, Japan) by electro-transformation, and the *A. thaliana* (ecotype Columbia) was infected with floral dip method (Clough and Bent 1998).

### Salt stress-resistance experiments

Control strain pGEX-6p-3 vector and the transformant strain expressing *PutNAR2.1* were grown in Luria-Bertani (LB) liquid medium at 37°C until the absorbance at 600 nm was 0.5 (OD<sub>600</sub>=0.5). Expression of the *PutNAR2.1* protein was induced by 1 mM IPTG for 1 h, and separately added with 0.8 M NaCl, 0.1 M Na<sub>2</sub>CO<sub>3</sub> or 0.2 M NaHCO<sub>3</sub>. The concentrations were chosen based on the information available from a large number of previous experiments. All the strains grow well in the selected concentration). The cultures were grown with rotary shaking (160 rpm) at 37°C for 1, 2, 3, 4 and 5 h. The growth rate of strains was monitored by absorbance change at 600 nm using Spectrometer. Data are preliminary for three replicate experiments.

The pYES2-*PutNAR2.1* transgenic yeast and pYES2 were cultivated in Yeast extract peptone dextrose medium (YPD) overnight at 30°C. When the bacterial culture concentration reached OD<sub>600</sub> = 0.6, culture solutions with serial dilutions (10, 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup> and 10<sup>-4</sup>) were dripped onto YPD agar plates with no treatment (CK), 0.6 M NaCl, 0.8 M NaCl, 10 mM Na<sub>2</sub>CO<sub>3</sub>, 20 mM Na<sub>2</sub>CO<sub>3</sub>, 30 mM Na<sub>2</sub>CO<sub>3</sub>, 20 mM NaHCO<sub>3</sub>, 40 mM NaHCO<sub>3</sub> or 60 mM NaHCO<sub>3</sub>, respectively.

To observed the effects of seeds germination of T3 generation homozygous transgenic lines #1, #3 and #5 *A. thaliana* and wild-type (Fig. 7), disinfected seeds were directly placed on half-strength MS agar medium supplemented with nothing else (CK), 100 mM NaCl, 125 mM NaCl, 150 mM NaCl, 3 mM Na<sub>2</sub>CO<sub>3</sub>, 5 mM Na<sub>2</sub>CO<sub>3</sub>, 7 mM Na<sub>2</sub>CO<sub>3</sub>, 3 mM NaHCO<sub>3</sub>, 5 mM NaHCO<sub>3</sub> or 7 mM NaHCO<sub>3</sub>. The experiment was performed in triplicate independently and photographed after 14 days.

Two-week-old seedlings with similar size were arranged onto half-strength MS medium supplemented with nothing and numerous stress (125 mM NaCl, 150 mM NaCl, 175 mM NaCl, 3 mM Na<sub>2</sub>CO<sub>3</sub>, 5 mM Na<sub>2</sub>CO<sub>3</sub>, 7 mM Na<sub>2</sub>CO<sub>3</sub>, 3 mM NaHCO<sub>3</sub>, 5 mM NaHCO<sub>3</sub> or 7 mM NaHCO<sub>3</sub>). Each stress treatment (included control) had three replications mentioned above. The Petri plates were vertically positioned in order to visualize the root growth. Plants were photographed after stress treatment for 7 days.

### Statistical analysis

All treatments were performed in triplicates and data were treated for analysis of variance using SPSS for Windows version 11.5.

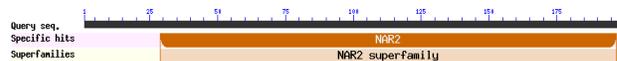
## Results

### Bioinformatics analysis of *PutNAR2.1*

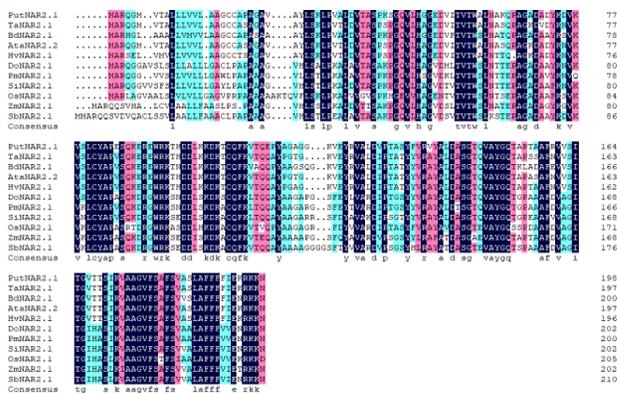
The ORF of *PutNAR2.1* was obtained from the cDNA in the *P. tenuiflora*. The full-length sequence of *PutNAR2.1* was 597 bp and encoded 199 amino acids; it contained the conserved domains of the *NAR2.1* gene family (Fig. 1). The alignment of *PutNAR2.1* amino acid sequence illustrated that it had the highest similarity (88.94%) with *TaNAR2.1* protein from *T. aestivum* (Fig. 2). Phylogenetic tree analysis was used to compare *PutNAR2.1* protein with others known homologous *NAR2.1* protein from a variety of plants, which revealed that *PutNAR2.1* was most closely related to *TaNAR2.1* from *Triticum aestivum* (Fig. 3).

### RT-qPCR analysis for *PutNAR2.1* expression in *P. tenuiflora*

The expression of *PutNAR2.1* under different salt stresses was analyzed by RT-qPCR. The results verified that the expression of *PutNAR2.1* was increased gradually and reached its the highest at 24 h under 300 mM NaCl, 100 mM Na<sub>2</sub>CO<sub>3</sub> or 150 mM NaHCO<sub>3</sub>, nearly 2.3 times higher than untreated (0 h) under 300 mM NaCl in (Fig. 4A), almost 2.7 times higher than untreated (0 h) under 100 mM Na<sub>2</sub>CO<sub>3</sub> in (Fig. 4B), over 3.1 times higher than untreated (0 h) under 150 mM NaHCO<sub>3</sub> in (Fig. 4C). *PutNAR2.1* had the highest expression in roots, followed by leaves and seeds, but was quite less in shoots and flowers (Fig. 4D).



**Fig. 1:** Analyzing the conservative domain of *PutNAR2.1* in the NCBI database

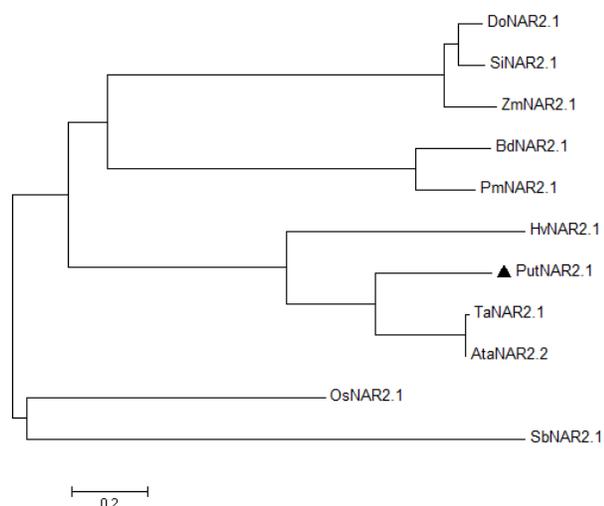


**Fig. 2:** Alignment of *PutNAR2.1* deduced amino acid sequence with other plant species *NAR2.1* protein. The amino acid sequence of this transcript had the similarity with that of the *TaNAR2.1* protein (GenBank: AAV35210.1, 88.94%) from *Triticum aestivum*, *BdNAR2.1* protein (XP\_003575282.1, 84.08%) from *Brachypodium distachyon*, *AtNAR2.2* protein (XP\_020163322.1, 85.43%) from *Aegilops tauschii* subsp. *Tauschii*, *HvNAR2.1* protein (AAP31850.1, 86.43%) from *Hordeum vulgare* subsp. *Vulgare*, *DoNAR2.1* protein (OEL38054.1, 71.51%) from *Dichanthelium oligosanthes*, *PmNAR2.1* (GenBank: RLM78325.1, 70.39%) protein from *Panicum miliaceum*, *SiNAR2.1* protein (XP\_004952978.1, 65.84%) from *Setaria italica*, *OsNAR2.1* protein (XP\_015623791.1, 65.12%) from *Oryza sativa Japonica Group*, *ZmNAR2.1* protein (GenBank: AAY40796.1, 67.05%) from *Zea mays*, *SbNAR2.1* protein (XP\_002454118.1, 68.00%) from *Sorghum bicolor*

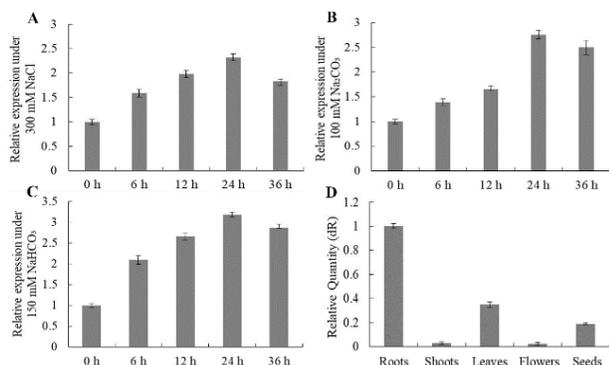
### *PutNAR2.1* expression in *E. coli* cells under salt stress

To investigate the salt response of *PutNAR2.1* in *E. coli*, the *E. coli* growth with only pGEX vector and transformants expressing pGEX-*PutNAR2.1* were compared. *PutNAR2.1* expressing strain and the pGEX vector were inoculated into LB liquid medium, adding different salt stresses respectively when both cell density was measured as OD<sub>600</sub>=0.5.

When both of the stains inoculated in LB liquid medium (CK), the OD<sub>600</sub> values of the control strain and the transgenic strain after culture for 1 h were 0.68 and 0.67, respectively, both of them had the maximum OD<sub>600</sub> of 2.0 after 5 h of incubation (Fig. 5A). Under 0.8 M NaCl treatment, the OD<sub>600</sub> values of the control strain was decreased to 0.57. However, the transgenic strain did not decrease after 1 h incubation, while the OD<sub>600</sub> values of the control strain and the transgenic strain after 5 h of culture were noted as 1.38 and 1.62, respectively (Fig. 5B). Under the treatment of 0.1 M Na<sub>2</sub>CO<sub>3</sub>, the OD<sub>600</sub> values of the



**Fig. 3:** Phylogenetic tree of 10 selected plant NAR2.1 protein. The MEGA7 program was used for the construction of phylogenetic trees. Bar represents 0.2 amino acid substitutions per site

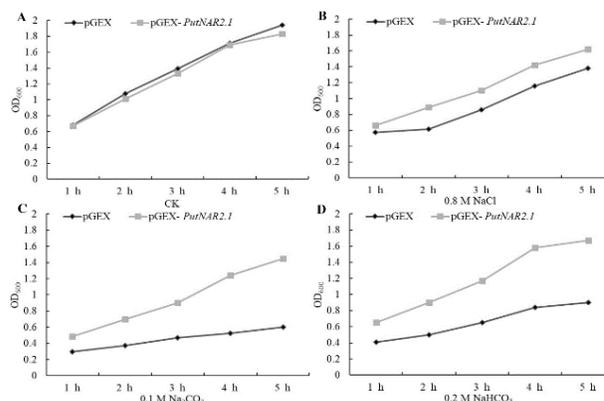


**Fig. 4:** Real-time Quantitative PCR analysis for *PutNAR2.1* expression in *P. tenuiflora*. (A) *PutNAR2.1* expression at different times under 300 mM NaCl. (B) *PutNAR2.1* expression at different times under 100 mM Na<sub>2</sub>CO<sub>3</sub>. (C) *PutNAR2.1* expression at different times under 150 mM NaHCO<sub>3</sub>. (D) *PutNAR2.1* expression in different organs of *P. tenuiflora*

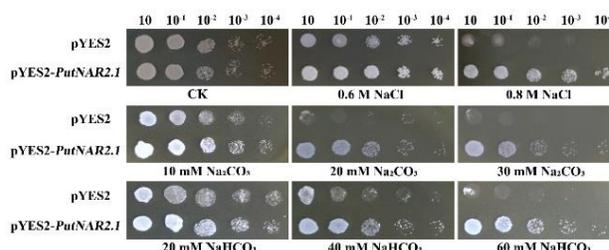
control strain and the transgenic strain were 0.29 and 0.48 after 1 h, the OD<sub>600</sub> values were 0.69 and 1.45 after 5 h, respectively (Fig. 5C). Under 0.2 M NaHCO<sub>3</sub> treatment, control and transgenic strain were cultured, with OD<sub>600</sub> values of 0.41 and 0.65 after 1 h, these values were 0.98 and 1.67 after 5 h, respectively (Fig. 5D).

#### *PutNAR2.1* transgenic yeast response to salinity

Salt stress types induced expression of *PutNAR2.1* in transgenic yeast was investigated. The growth of *PutNAR2.1* transgenic yeast cell and pYES2 was compared at five serial dilutions for different salt treatments (corresponding to five columns in each panel in Fig. 6). The control was no treatment (CK). The growth of both pYES2 (upper line) and pYES2-*PutNAR2.1* vector (lower line)



**Fig. 5:** The bacterial concentration of pGEX and pGEX-*PutNAR2.1* at different times in OD 600 nm under salt stresses. CK: no salt treatment



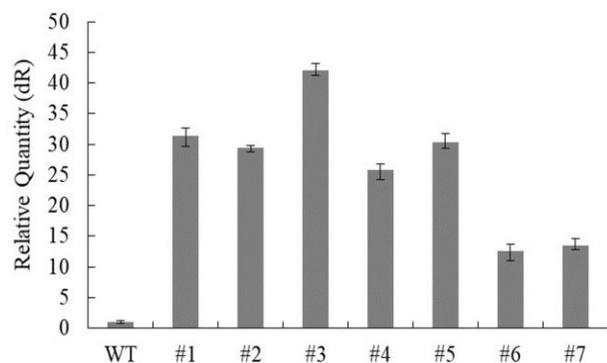
**Fig. 6:** Growth of *PutNAR2.1* transgenic yeast cells under salt stress. Ten-fold dilutions of yeast cells containing pYES2 (upper line) and pYES2-*PutNAR2.1* vector (lower line) were spotted onto solid YPG media supplemented with the indicated stresses. No treatment is a control (CK)

transgenic yeasts showed no significant difference. However, the growth of yeasts had the most drastic change when salt treatment was applied. The transgenic yeasts grew significantly better than control in 0.8 M NaCl, 20 mM Na<sub>2</sub>CO<sub>3</sub> and 40 mM NaHCO<sub>3</sub> treatments. When the concentration was increased to 30 mM Na<sub>2</sub>CO<sub>3</sub> or 60 mM NaHCO<sub>3</sub> the transgenic yeasts grew as before, but non-transgenic yeasts could hardly grow.

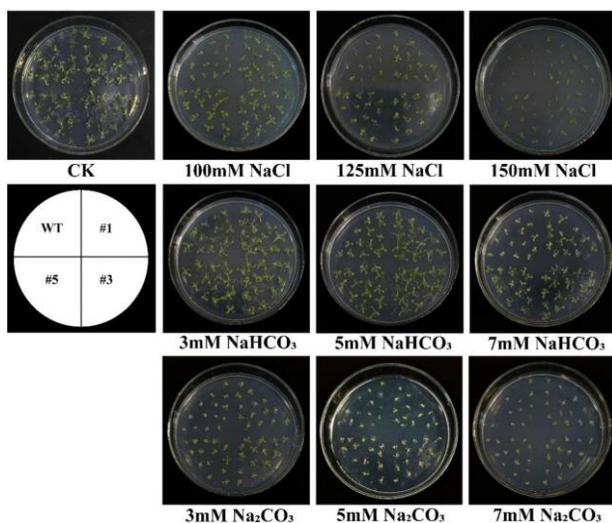
#### Identification of *PutNAR2.1* transgenic *A. thaliana* plant

The expression of *PutNAR2.1* transgenic *A. thaliana* plant was identified via RT-qPCR analysis (Fig. 7). The expression level of *PutNAR2.1* in seven randomly selected T3 transgenic *A. thaliana* was higher than its wild-type counterparts. *PutNAR2.1* expression in the transgenic lines #1- #7 had 31, 29, 42, 25, 30, 12 and 13 times higher than that in wild-type plants, respectively. Among transgenic lines, #1, #3 and #5 indicated higher expression level of *PutNAR2.1*, and were selected for further research.

The T3 *PutNAR2.1* transgenic *A. thaliana* which exhibited higher levels of *PutNAR2.1* (#1, #3, #5) and wild-type seeds were placed on half-strength MS supplemented with no stress (CK) and salt stresses (Fig. 8).

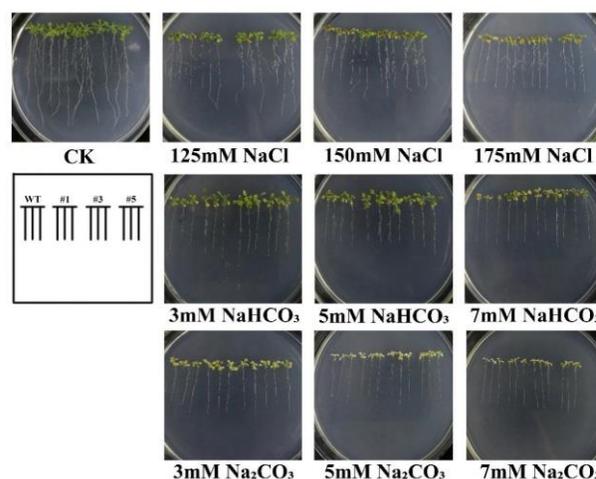


**Fig. 7:** Identification of transgenic *A. thaliana* lines by contrasting the expression of *PutNAR2.1* in the wild-type and transgenic lines using RT-qPCR. WT: wild-type. #1-#7: *PutNAR2.1* transgenic *A. thaliana* lines



**Fig. 8:** Seed germination in *A. thaliana* wild-type, *PutNAR2.1* transgenic plants under different stresses. Seed germination on medium supplemented with 0 mM (CK), 100 mM NaCl, 125 mM NaCl, 150 mM NaCl, 3 mM NaHCO<sub>3</sub>, 5 mM NaHCO<sub>3</sub> and 7 mM NaHCO<sub>3</sub>, 3 mM Na<sub>2</sub>CO<sub>3</sub>, 5 mM Na<sub>2</sub>CO<sub>3</sub> and 7 mM Na<sub>2</sub>CO<sub>3</sub>. WT: Wild-type *A. thaliana*. #1, #3 and #5: *PutNAR2.1* transgenic lines

Seeds of transgenic and wild-type plants exhibited no difference when they were germinated on half-strength MS directly (CK). With 100 mM NaCl, 3 mM NaHCO<sub>3</sub> or 5 mM NaHCO<sub>3</sub> treatment, seeds of transgenic plants had bigger leaves than the wild-type, but germination of both was similar. Transgenic plant seeds were germinated 1–2 days earlier than wild-type on the medium containing 125 mM NaCl, 3 mM Na<sub>2</sub>CO<sub>3</sub>, 5 mM Na<sub>2</sub>CO<sub>3</sub> or 7 mM NaHCO<sub>3</sub>, and the transgenic *A. thaliana* growth was obviously better. The germination of the wild-type plants was suppressed significantly under 150 mM NaCl and 7 mM Na<sub>2</sub>CO<sub>3</sub>; a few seeds did not germinate and the leaves of wild-type seedlings were severely curled with light color. However, all the seeds of the transgenic *A. thaliana* lines



**Fig. 9:** Seedlings growth between *A. thaliana* wild-type and *PutNAR2.1* transgenic plants under different stresses. Seedlings growth on half-strength MS medium supplemented with 0 mM (CK), 125 mM NaCl, 150 mM NaCl, 175 mM NaCl, 3 mM NaHCO<sub>3</sub>, 5 mM NaHCO<sub>3</sub> and 7 mM NaHCO<sub>3</sub>, 3 mM Na<sub>2</sub>CO<sub>3</sub>, 5 mM Na<sub>2</sub>CO<sub>3</sub> and 7 mM Na<sub>2</sub>CO<sub>3</sub>. WT: Wild-type *A. thaliana*. #1, #3 and #5: *PutNAR2.1* transgenic lines

germinated and remained green. These results demonstrated that transgenic lines had significantly higher salt tolerance compared to wild-type plants.

Furthermore, *PutNAR2.1* transgenic line and wild-type grown on half-strength MS medium with no treatment (CK) or salt stresses for 2 weeks were tested at the seedling stage (Fig. 9). Under normal growth condition, the *PutNAR2.1* transgenic lines and wild-type seedlings showed no significant morphological or developmental abnormalities. Under increased NaCl treatment, in the wide-type plants, the leaf margins turned brown and the color became darker. When seedlings were grown on half-strength MS medium containing Na<sub>2</sub>CO<sub>3</sub> stress, the cotyledons of wild-type seedlings were smaller compared with *PutNAR2.1* transgenic lines and most wild-type leaves turned white. Data further showed that *PutNAR2.1* was induced by salt stress and exhibited a positive response to salt stress. Therefore, the *PutNAR2.1* gene is involved in the response to salt stress, expression of *PutNAR2.1* gene can increase plant tolerance to salt stress.

## Discussion

Reportedly *P. tenuiflora* is one of the few plants that can survive on saline-alkali land (Zhang *et al.* 2013). The gene related to salt and alkali stress was cloned from the *P. tenuiflora* and the study of its gene function is helpful to explore the molecular mechanism of salt and alkali resistance of the species (Ye *et al.* 2019).

The NAR2 protein is mostly studied as nitrogen transport (Yan *et al.* 2011; Chen *et al.* 2019). In recent years, *NAR2.1* gene has been found to be related to stress

tolerance and *NAR2.1* was described as novel potato drought-responsive genes (Pieczynski et al. 2018). NaCl salinity reduces the absorption of nitrogen by roots (Rubinigg et al. 2003; Yousif et al. 2010). *PutNAR2.1* transcript level was upregulated significantly in transcriptome analyses of *P. tenuiflora* treated with water extracts from the saline-alkali soils. In this study, the possibility of *PutNAR2.1* participating in regulation of the response to salt stress was examined by using *PutNAR2.1 E. coli* transformants, *PutNAR2.1* transgenic yeasts and *PutNAR2.1* transgenic *A. thaliana* lines.

The *PutNAR2.1* has been cloned from *P. tenuiflora*. The amino acid sequence of *PutNAR2.1* had the similarity with other plant species. Drought stress can induce *OsNAR2.1* high expression (Chen et al. 2019). In this research, the expression of *PutNAR2.1* started to increase gradually at 6 h and reached the highest value at 24 h in *P. tenuiflora* under 300 mM NaCl, 100 mM Na<sub>2</sub>CO<sub>3</sub> or 150 mM NaHCO<sub>3</sub> stress, indicated its gradual induction by salt stress. *NAR2* was mainly expressed in root (Orsel et al. 2002; Lupini et al. 2016; Luo et al. 2018), while the highest expression of the *PutNAR2.1* was also found in roots of *P. tenuiflora*, under salt stresses. Thus *PutNAR2.1* may play a defense role when roots are exposed to salt stress.

It takes a long time to identify the related functions after the gene is transferred into the plant. The effect of salt stress on the growth of control strain and *PutNAR2.1* expressing strain was examined in LB medium. The OD<sub>600</sub> value of the *PutNAR2.1* expressing strain after 5 h culture was higher than that of the control strain. It is indicated that *PutNAR2.1* can protect *E. coli* to against the salt stress from the environment. However, the prokaryotes may not have the function of protein completely consistent with that in eukaryotes. The growth of control yeasts was inhibited by NaCl, Na<sub>2</sub>CO<sub>3</sub> or NaHCO<sub>3</sub> stress, while *PutNAR2.1* transgenic yeast grew well. These results revealed that the function of *PutNAR2.1* in eukaryotes was similar to that in the prokaryotes.

Chen et al. (2019) reported that *OsNAR2.1* overexpressing plant line increased the grain yield by about 26.6% compared to wild-type in limited irrigation conditions. To observe *PutNAR2.1* response to salt stress, comparison was made for seeds germination and seedlings growth between transgenic lines and wild-type counterparts. The germination and seedlings growth of wild-type were quite more reduced than that of transgenic lines under salt stress. *PutNAR2.1* transgenic line showed better growth compared with wild-type under stress of various salts at different plant development stages. The data proved that expression of *PutNAR2.1* helped the plants to resist the salt stress better.

Nitrate uptake was closely related to plant growth and development, and the ability of nitrate uptake by roots was decreased under salt stress. *PutNAR2.1* is the key gene for nitrate uptake and transport in *P. tenuiflora*. Under salt stress, *P. tenuiflora* can regulate the expression of *NAR* to

improve the ability of nitrate uptake and utilization, thus enhanced the resistance to salt stress.

## Conclusion

Tolerance of *E. coli*, yeast and plant to salt stresses could be greatly enhanced with the high expression of *PutNAR2.1*. Based on the previous research progress of *NAR2.1* gene and the results of this experiment, it is conjectured that *PutNAR2.1* may be used as an auxiliary protein to participate in the high-affinity nitrogen absorption system of plants under different salinity treatment. Consequently, *PutNAR2.1* overexpression can help plants to resist abiotic stress in the environment, but determination of specific working mechanism needs further studies.

## Acknowledgments

This work was supported by the Heilongjiang Province Nature Science Foundation (LH2019C011), Key Laboratory Open Fund of Saline-alkali Vegetation Ecology Restoration (SAVER1701) and the National Natural Science Foundation of China (No. 31070616, No. 31500317).

## Author Contributions

JSM planned the experiments and contributed reagents/materials/analysis tools; ZGQ, CSY, XY and HH performed the experiments; ZGQ statistically analyzed the data and made illustrations; JSM and ZGQ wrote the manuscript.

## References

- Al-Maskri A, L Al-Kharusi, H Al-Miqbali (2010). Effects of salinity stress on growth of lettuce (*Lactuca sativa*) under closed-recycle nutrient film technique. *Intl J Agric Biol* 12:377–380
- Chen J, T Qi, Z Hu, X Fan, L Zhu, MF Iqbal, X Yin, G Xu, X Fan (2019). *OsNAR2.1* positively regulates drought tolerance and grain yield under drought stress conditions in rice. *Front Plant Sci* 10; Article 197
- Clough SJ, AF Bent (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735–743
- Rubinigg M, F Posthumus, M Fersckhe, JTM Elzenga, I Stulen (2003). Effects of NaCl salinity on <sup>15</sup>N-nitrate fluxes and specific root length in the halophyte *Plantago maritima*. *Plant Soil* 250:201–213
- Kotur Z, AD Glass (2015). A 150 kDa plasma membrane complex of AtNRT2.5 and AtNAR2.1 is the major contributor to constitutive high-affinity nitrate influx in *Arabidopsis thaliana*. *Plant Cell Environ* 38:1490–1502
- Laugier E, E Bouguyon, A Mauries, P Tillard, A Gojon, L Lejay (2012). Regulation of high-affinity nitrate uptake in roots of *Arabidopsis* depends predominantly on posttranscriptional control of the NRT2.1/NAR2.1 transport system. *Plant Physiol* 158:1067–1068
- Li W, Y Wang, M Okamoto, NM Crawford, MY Siddiqi, AD Glass (2007). Dissection of the AtNRT2.1: AtNRT2.2 inducible high-affinity nitrate transporter gene cluster. *Plant Physiol* 143:425–433
- Luo B, J Chen, L Zhu, S Liu, B Li, H Lu, G Ye, G Xu, X Fan (2018). Overexpression of a high-affinity nitrate transporter *OsNRT2.1* increases yield and manganese accumulation in rice under alternating wet and dry condition. *Front Plant Sci* 9; Article 1192

- Lupini A, F Mercati, F Araniti, AJ Miller, F Sunseri, MR Abenavoli (2016). Nar2.1/nrt2.1 functional interaction with NO<sub>3</sub><sup>-</sup> and H<sup>+</sup> fluxes in high-affinity nitrate transport in maize root regions. *Plant Physiol Biochemol* 102:107–114
- Munns R, M Tester (2008). Mechanisms of salinity tolerance. *Annu Rev Plant Biol* 59:651–681
- Okamoto M, A Kumar, W Li, Y Wang, MY Siddiqi, NM Crawford, AD Glass (2006). High-affinity nitrate transport in roots of Arabidopsis depends on expression of the NAR2-like gene AtNRT3.1. *Plant Physiol* 140:1036–1046
- Orsel M, A Krapp, F Daniel-Vedele (2002). Analysis of the NRT2 nitrate transporter family in Arabidopsis. Structure and gene expression. *Plant Physiol* 129:886–896
- Orsel M, F Chopin, Q Leleu, SJ Smith, A Krapp, F Daniel-Vedele, AJ Miller (2006). Characterization of a two-component high affinity nitrate uptake system in Arabidopsis. Physiology and protein-protein interaction. *Plant Physiol* 142:1304–1317
- Pieczynski M, A Wyrzykowska, K Milanowska, D Boguszewska-Mankowska, B Zagdanska, W Karlowski, A Jarmolowski, Z Szweykowska-Kulinska (2018). Genomewide identification of genes involved in the potato response to drought indicates functional evolutionary conservation with Arabidopsis plants. *Plant Biotechnol J* 16:603–614
- Quesada A, A Galvan, E Fernandez (1994). Identification of nitrate transporter genes in *Chlamydomonas reinhardtii*. *Plant J* 5:407–419
- Tong Y, JJ Zhou, Z Li, AJ Miller (2005). A two-component high-affinity nitrate uptake system in barley. *Plant J* 41:442–450
- Wei XX (2016). The Identification on Drought-Resistance of 15 *Puccinellia* germplasm materials in seedling stage. *Pratacult Anim Husban* 5:18–24
- Yan M, X Fan, H Feng, AJ Miller, Q Sheng, G Xu (2011). Rice OsNAR2.1 interacts with OsNRT2.1, OsNRT2.2 and OsNRT2.3 nitrate transporters to provide uptake over high and low concentration ranges. *Plant Cell Environ* 34:1360–1372
- Yang Y, Y Guo (2018). Elucidating the molecular mechanisms mediating plant salt-stress responses. *New Phytol* 217:523–539
- Ye X, H Wang, X Cao, X Jin, F Cui, Y Bu, H Liu, W Wu, T Takano, S Liu (2019). Transcriptome profiling of *Puccinellia tenuiflora* during seed germination under a long-term saline-alkali stress. *BMC Genomics* 20; Article 589
- Yousif BS, NT Nguyen, Y Fukuda, H Hakata, Y Okamoto, Y Masaoka, H Saneoka (2010). Effect of salinity on growth, mineral composition, photosynthesis and water relations of two vegetable crops; New Zealand spinach (*Tetragonia tetragonioides*) and water spinach (*Ipomoea aquatica*). *Intl J Agric Biol* 12:211–216
- Yu J, S Chen, Q Zhao, T Wang, C Yang, C Diaz, G Sun, S Dai (2011). Physiological and proteomic analysis of salinity tolerance in *Puccinellia tenuiflora*. *J Proteome Res* 10:3852–3870
- Zhang X, L Wei, Z Wang, T Wang (2013). Physiological and molecular features of *Puccinellia tenuiflora* tolerating salt and alkaline-salt stress. *J Integr Plant Biol* 55:262–276
- Zhou JJ, E Fernandez, A Galvan, AJ Miller (2000). A high affinity nitrate transport system from *Chlamydomonas* requires two gene products. *FEBS Lett* 466: 225–227