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ROLE OF NITRATE REDUCTASE AND NITRITE REDUCTASE IN NaCl TOLERANCE IN EELGRASS (*Zostera marina* L.)

Abstract: Nitrate reductase (NR) and nitrite reductase (NiR) play important roles in nitrate assimilation in plants. Previous studies have indicated that NR and NiR in eelgrass may contribute to its NaCl tolerance. This study investigated the expression characteristics and the biological functions of NR and NiR in eelgrass (*Zostera marina*), named as ZmNR and ZmNiR, were cloned, characterised and overexpressed in both bacteria and tobacco. The open reading frames of ZmNR and ZmNiR contain 2628 and 1773 nucleotides that encode 875 and 590 amino acids respectively. Amino acid sequence alignment indicated that the purported ZmNR and ZmNiR proteins presented low homology with other plant NR and NiR sequences. Real-time quantitative PCR revealed that the expression of ZmNR and ZmNiR was suppressed when exposed to low salinity and induced by high salinity. Further physiological analyses demonstrated that blocking nitrate assimilation by adding Na_2WO_4 in eelgrass reduced its tolerance to NaCl stress. The heterologous expression of the ZmNR and ZmNiR genes in *Escherichia coli* and *Nicotiana benthamiana* could confer tolerance to NaCl stress. Physiological and growth analyses suggested that ZmNR and ZmNiR in plants could resist NaCl stress by regulating various physiological pathways and biochemical processes triggered by nitric oxide (NO). Taken together, these results suggested that NR-dependent NO synthesis may play an important role in NaCl tolerance in eelgrass.

Keywords: heterologous expression, nitrate assimilation, NaCl tolerance, *Zostera marina*

Introduction

Nitrogen is one of the most important nutrients present in cells and used to build proteins, amino acids, nucleoside phosphates, and other organic nitrogen-containing macromolecules in plants [1]. Nitrate (NO_3^-) is absorbed by the roots and then assimilated into ammonium (NH_4^+) by the sequential reductive action of the enzymes NR and NiR [2]. Ammonium ion derived from nitrate reduction is converted to glutamine by glutamine synthetase (GS) and then to glutamate by glutamate synthase (GOGAT) [3].

Our previous study showed that NR and NiR were significantly upregulated in *Z. marina* following exposure to NaCl [4]. These results suggest that the function of these key enzymes in the process of nitrate assimilation in seagrass may be underestimated. Eelgrass is a typical monocotyledonous angiosperms [5] that thrives in seawater and plays an important role in the sea ecology [6]. Eelgrass could absorb nitrogen via its roots, shoots, and leaves, and the main nitrogen source is NH_4^+ , whereas the main nitrogen source of terrestrial plants is NO_3^- [1, 7]. The NH_4^+ absorbed by eelgrass could directly enter the GS/GOGAT cycle to synthesise amino acids and proteins, and this process is more efficient

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compared with that of terrestrial plants. The nitrate assimilation process in eelgrass has been retained through a long evolutionary process, which means that its key enzymes may play a role in eelgrass adaption to the marine environment. Nitrate assimilation is affected by salt stress [8] with the effects of this stress on the expression of NR and NiR in many terrestrial plants being well documented [9, 10]. During salt stress, the enzyme activities of NR and NiR in salt-sensitive plants decreased significantly, while the activities increased in salt-tolerant plants. Therefore, we speculate that NR and NiR in eelgrass may be critical to the high salinity adaptation.

NR is not only the key enzyme in the process of nitrate assimilation, but also in the synthesis of nitric oxide (NO). In plants, NO is known as a small but important redox signaling molecule that plays an important role as a regulator of plant physiology, disease resistance, and stress tolerance [11]. It is widely accepted that NR reduces NO_3^- to NO_2^- in an NAD(P)H-dependent manner [12]. However, in vitro and in vivo experiments have also shown that NR is also able to reduce NO_2^- to NO and its derivative peroxynitrite (ONOO-) [13]. It is possible that the NO produced by the NR pathway regulates NaCl tolerance in eelgrass.

In this study, we focused on determining whether NR and NiR affect NaCl tolerance in eelgrass. To understand the expression pattern of these enzymes in nitrate assimilation, eelgrass was reared in artificial seawater with different levels of NaCl and then the transcript levels were evaluated using qRT-PCR. In addition, we examined the growth and physiological adaptation of eelgrass to different salinities after blocking the nitrate assimilation process. Chlorophyll, proline and malondialdehyde (MDA), which reflect plant growth, osmotic adjustment, and antioxidant activity, were measured. Moreover, we also investigated the biological functions of ZmNR and ZmNiR in response to NaCl stress, via the heterologous expression of both genes in *E. coli* and *N. benthamiana*. The NO content in tobacco plants overexpressing ZmNR increased significantly under NaCl treatment. Taken together our data puts forward a potential mechanism for marine angiosperms to improve salt resistance, which is to regulate NO synthesis through key enzymes of nitrate assimilation.

Materials and methods

Eelgrass growth conditions

The plants were harvested from December 2017 to May 2020 at a 2-m depth at ShuangDao Bay (latitude 37°20'-37°21.6' N longitude 122°33.2'-122°35.0' E) located in the South of Bohai. The plants were transported to the laboratory in seawater, cleaned and cultivated in artificial seawater [14]. In this study, the concentration of NaCl in artificial seawater is 24 ‰, and the total salinity of all salts (such as MgCl_2 , CaCl_2 , etc.) is 40 ‰. For the gene expression analyses, the plants were kept in aerated polyethylene tanks containing 10 L of artificial seawater with 24 ‰ NaCl (40 ‰ salinity) at a density of three specimens for 48 h, and then transferred to solutions with different levels of NaCl, with 3 replicates per treatment. The eelgrass leaves were treated for 0, 0.5, 1, and 3 h and then immediately frozen in liquid nitrogen and stored at -80 °C until the RNA extraction.

For the physiological analyses, the plants were subjected to 72 h nitrogen starvation and then transferred to artificial seawater with different forms of nitrogen. Five culture groups were set up with three replicates in each group, and cultured in artificial seawater containing NO_3^- , NO_3^- (Na_2WO_4), NH_4^+ , $\text{NO}_3^-/\text{NH}_4^+$ and $\text{NO}_3^-/\text{NH}_4^+$ (Na_2WO_4).

The concentration of NO_3^- and NH_4^+ is 10 μM . The artificial seawater was changed every three days, and the eelgrass was reared for 48 h under the following conditions: natural photoperiod on a 14-h light/10-h dark cycle with exposure to 200 $\mu\text{mol photons}/(\text{m}^2 \text{ s})$ and temperature of 19 °C.

Sequence analysis and gene expression analysis

The nucleotide sequence, the deduced amino acid sequence and the open reading frames (ORFs) of ZmNR and ZmNiR were analysed using the bioinformatic tools at the websites: <http://www.expasy.org/> and <http://www.ncbi.nlm.nih.gov/>. The deduced amino acid sequence alignments were performed in MEGA 7.0.

Table 1

PCR primers

Primer name	Primer sequences
ZmActin RT L	TGCTCCTCTGAGAGAAAGTA
ZmActin RT R	TCATACTCGGCCTTGGAATC
ZmNR RT L	ATGCTTGGATTTCGACGTAACT
ZmNR RT R	GGAGATGCGATGGAGTGATAAA
ZmNiR RT L	GAGATGCGCTGAGGCTATT
ZmNiR RT R	TTCTGGTCTTCTGGCGATTAC
ZmNR L	ATGGCGATCTTAATAAGCGA
ZmNR R	TTAGAAATATTAGTAAGGAGTGTTCAA
ZmNiR L	ATGTCTCGTTCACCTCTCTCTTC
ZmNiR R	CTAATCTTCTCCGTCTCCCT

Total RNA was extracted from the third mature leaves with different stress treatments and first-strand cDNA was synthesised with the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, Beijing, China). Quantitative RT-PCR was performed with TransStart Tip Green qPCR SuperMix (TransGen, Beijing, China) and an ABI StepOne™ Plus Real-Time PCR System, with β -actin (49G00260) used as the internal reference gene. The qRT-PCR gene expression was quantified from three technical replicates using the $\Delta\Delta\text{CT}$ comparative method. The amplification program included one cycle at 94 °C for 30 s, then at 94 °C for 5 s, 56 °C for 15 s, and 72 °C for 60 s for 40 cycles. The fluorescent products were detected in the last step of each cycle. In order to ensure proper amplification of the target fragments, we performed a melting curve analysis at the end of the 40 cycles. The qRT-PCR analysis of each gene was performed in two biological replicates, and each experiment was replicated in three technical replicates. The data are represented by the means $\pm$ SD ($n = 3$). The primers for the qRT-PCR assay were designed using Primer 5.0 software and were listed in Table 1.

Assessment of various growth, biochemical and physiological parameters

The length and width of the leaves were measured by a Vernier calliper and centimetre gauge. The original leaf length and leaf width were recorded as T_0 , the termination value was recorded as T_s , and the relative growth rate was determined as follows:

$$G_a = (T_s - T_0)/T_0 \cdot 100 \%$$

The fresh weight (f.w.) of the eelgrass was measured by an electronic balance, and the relative increase in fresh weight was calculated. The original fresh weight of the plants was

recorded as W_0 , the termination value was recorded as W_s , and the fresh weight relative growth rate was calculated as follows:

$$G_b = (W_s - W_0) / W_0 \cdot 100 \%$$

The chlorophyll content in the eelgrass leaves was determined by ethanol colorimetry following the protocol of Hiscox and Israelstam [15] and Swislowski et al. [16]. Briefly, 100 mg of the sample was extracted in 20 mL of 95 % ethanol and the absorbance was recorded at 649 and 665 nm against 95 % ethanol as a control. The total chlorophyll content was calculated and expressed in $\mu\text{g g}^{-1}$ f.w.

The proline content was estimated using the standard procedure described by Azamal [17] and Abraham et al. [18]. The leaf samples (200 mg) were extracted in 5 mL of 3 % of sulphosalicylic acid. The homogenate was centrifuged, and 2 mL of supernatant was treated with a mixture of 2 mL glacial acetic acid and 3 mL acid ninhydrin by boiling at 100 °C for 40 minutes. The samples were placed on ice to stop the reaction, and then 5 mL of toluene was added. Transfer the chromophore containing toluene to a new test tube and read the absorbance at 520 nm using toluene as a blank. The proline concentration was determined from a standard curve and expressed in $\mu\text{g g}^{-1}$ f.w.

According to the method described by Hodges et al. [19] and Zhang et al. [20], the MDA content in the samples was estimated by measuring the thiobarbituric acid reactive substances (TBARS). The control and treated samples (200 mg) were homogenised in 5 mL of 0.1 % trichloro acetic acid (TCA). Mix the homogenate with 5 mL of 0.5 % (w/v) TBA in 10 % TCA (w/v) and incubated at 100 °C for 15 min. The tubes were placed on ice to stop the reaction, which was followed by centrifugation for 15 min at 10,000 rpm (4 °C). The absorbance of the resultant supernatant was measured at 450, 532 and 600 nm. The OD600 values were subtracted from the MDA-TBA complex values at 532 nm, and the MDA concentration was calculated using the Lambert-Beer law with an extinction coefficient of $\epsilon M = 155 \text{ mM}^{-1} \text{ cm}^{-1}$. The results were presented in nmol MDA g^{-1} f.w.

NO content was quantified by using visible spectrophotometry (Griess reagent assay) as described by Zhao et al. [21] with slight modifications. Tissue powders (0.3 g) were mixed with 2 mL 4 % zinc acetate buffer (pH = 3.7) and centrifugated to collect the supernatant. Wash the pellet with 1 mL extraction buffer and centrifuge as before. Combine the two supernatants and add 0.1 g of charcoal. After vortexing and filtering, the filtrate was filtered and collected. Incubate a mixture of 1 mL filtrate and 1 mL Griess reagent at room temperature for 30 minutes. The absorbance was measured at 540 nm. The NO content was calculated by comparing with the standard curve of NaNO_2 and expressed as $\mu\text{mol/g}$.

Nitrate reductase activity and nitrite reductase activity

Nitrate reductase activity was assayed by the induction of NO_2^- , while nitrite reductase activity was assayed by the reduction of NO_2^- . Nitrate reductase activity was assayed by the method of Wang et al. [22]. The assay mixture contained 50 mM MOPS-NaOH (pH = 7.5), 2 mM EDTA, 5 mM MgCl_2 , 10 mM KNO_3 , and 150 μL of extract in a final volume of 1.15 mL. The reaction was started by the addition of 50 μL of 1 mM NADH, and then incubated at 30 °C for 15 min. The nitrite reductase activity assay mixture contained 100 mM potassium-phosphate buffer (pH = 7.5), 10 mM KNO_2 , 1.5 % methyl viologen (MV), 5 % $\text{Na}_2\text{S}_2\text{O}_4$ and 150 μL of extract in a final volume of 1.35 mL and then incubated at 25 °C for 30 min [23]. The residual NO_2^- in the supernatant was determined by

combining 200 μ L of the supernatant with 0.5 mL of 1 % sulphanilamide prepared in 14 % (v/v) HCl and 0.5 mL of 1 % N-(1-naphtyl) ethylene-diamine dihydrochloride, and the absorbance of the mixture was read at 540 nm in a spectrometer.

Heterologous expression and functional validation in *E. coli*

The overexpression vector was constructed based on the pEASY[®]-Blunt E1 Expression vector. The ORFs of the ZmNR and ZmNiR genes were amplified with specific primers. The amplification products were inserted into a pEASY[®]-Blunt E1 Expression vector downstream from a T7 lac promoter. The recombinant protein was expressed by adding 1 mM Isopropyl beta-D-thiogalactopyranoside (IPTG) for 4 h at 37 °C. The recombinant protein was induced after 4 h of treatment, and the expression of the ZmNR and ZmNiR genes in *E. coli* was analysed by SDS-PAGE. A liquid culture assay was performed to determine the function of ZmNR and ZmNiR genes under salt stress conditions. *E. coli* strain Rosetta (DE3) cells and the cells transformed with the recombinant plasmid (pEASY-Blunt E1) or the vector (pEASY) were cultured overnight in LB medium. Fresh inoculation was performed via 4 h culture and allowed to grow to an absorbance of 0.6 (OD_{600 nm}). By adding 1 mM IPTG, the transformed bacterial cells were induced to express ZmNR and ZmNiR genes. The following day, the cultures were diluted to 0.6 OD₆₀₀ and 1 mL of cells was inoculated in 50 ml LB basal medium or supplemented with 600 mM NaCl [24] and then incubated at 37 °C. The bacterial suspension was harvested from each treatment every 1 h for 12 h, and the OD_{600 nm} was measured.

Cloning and overexpression of ZmNR and ZmNiR in tobacco

The ZmNR and ZmNiR binary vectors were transformed into *Agrobacterium tumefaciens* GV3101. The leaf disc co-cultivation method was used to transform *N. benthamiana* [25, 26]. Transgenic plants were selected on MS solid medium containing 100 mg/L kanamycin and confirmed by PCR analysis using specific primers (-L/-R, Table 1) for the ZmNR and ZmNiR genes. Wild-type tobacco plants (WT) and two selected T2 transgenic lines (NR-5-1 and NiR-1-14) were used for NaCl stress tolerance analysis after five-week growth.

Statistical analysis

Two-tailed Student's *t* tests were performed to compare the leaf length, leaf width, relative growth rate of f.w., chlorophyll content, proline content, MDA content, and NO content between the control and Na₂WO₄-treated eelgrass. Two-tailed Student's *t* tests were performed to compare the chlorophyll content, carotenoid content, proline content, MDA content, and NO content between the wild-type and overexpression plants. Differences were deemed significant at $p < 0.05$ and extremely significant at $p < 0.01$.

Results

Isolation and characterization of the ZmNR and ZmNiR genes

The full-length ORFs of ZmNR and ZmNiR contain 2628 bp and 1773 bp nucleotides that encode 875 and 590 amino acid residues, with predicted molecular masses of 98.988 and 65.958 kDa, respectively. The theoretical pI of ZmNR and ZmNiR were 6.35 and 6.46, and the base composition and GC content of ZmNR and ZmNiR were

similar (Table S1, see supplementary materials). The amino acid sequence alignment indicated that the deduced amino acid sequences of ZmNR and ZmNiR presented low homology to other plants. According to the alignment, ZmNR shares 70.04, 69.90, 70.26, 73.25, 72.52, 71.12 and 71.26 % amino acid sequence identities with *Arabidopsis thaliana* (NP_174901.1), *Oryza sativa* (XP_015650300.1), *Zea mays* (AAD38068.1), *Glycine soja* (RZB71449.1), *Nelumbo nucifera* (XP_010245911.1), *Setaria italica* (XP_004973655.1), and *Nicotiana tabacum* (AEV66151.1), respectively (Fig. S1, see supplementary materials). ZmNiR shares 78.55, 73.81, 79.43, 78.25, 79.97, 78.99 and 79.05 % amino acid sequence identities with *A. thaliana* (OAP11296.1), *O. sativa* (XP_025881174.1), *Z. mays* (ACG29734.1), *G. soja* (KHN37511.1), *N. nucifera* (XP_010263547.11), *S. italica* (XP_004954025.1) and *N. tabacum* (NP_001311864.1), respectively (Fig. S2).

Tissue-specific expression of ZmNR and ZmNiR

No significant differences in the transcript levels of ZmNR were observed in different tissues (Fig. 1). The transcript levels of ZmNiR in the roots were the highest, and the levels in the leaves and stems were approximately 0.37-fold and 0.3-fold, respectively (Fig. 1).

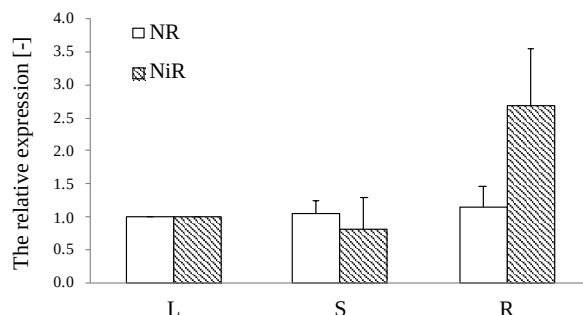


Fig. 1. Relative expression analysis of ZmNR and ZmNiR in different tissues of eelgrass: leaves (L), stems (S) and roots (R). The relative level of mRNA was normalised to that of the β -actin gene (49G00260). Values are the means $\pm$ SD ($n = 3$)

Expression of ZmNR and ZmNiR in response to NaCl

To determine whether NR and NiR might play a role in NaCl tolerance in eelgrass, qRT-PCR assay was first performed to characterise the ZmNR and ZmNiR expression patterns in response to NaCl. Eelgrass were kept in artificial seawater with 24 ‰ NaCl for 48 h, then transferred to 10 ‰ NaCl (low salinity) and 40 ‰ NaCl (high salinity). In response to the treatments, the relative expression levels of ZmNR and ZmNiR showed similar patterns (Fig. 2). When the NaCl concentration was reduced to 10 ‰, the expression levels of both ZmNR and ZmNiR decreased with the increase of treatment time (Fig. 2a and b). When the NaCl concentration was increased to 40‰, the expression of ZmNR and ZmNiR showed significant upregulation and transcript of ZmNR and ZmNiR was 5-fold and 13-fold of the control at 3 h (Fig. 2c and d), respectively.

Effect of blocking nitrate assimilation on the salt tolerance of *Z. marina*

Eelgrass growth was evaluated after blocking the activity of NR, the key enzyme in the nitrate assimilation, via the addition of Na_2WO_4 (Fig. S3). The plants were reared in

artificial seawater with NO_3^- as the sole nitrogen source, and the chlorophyll, proline and MDA content in the leaves were measured after adding 1 mM Na_2WO_4 for 3 days and 15 days. The eelgrass treated with 1 mM Na_2WO_4 presented lower chlorophyll and proline content and higher MDA content than the control (Fig. 3a, b and c). The fresh weight, leaf length, and leaf width between the control and Na_2WO_4 -treated eelgrass were not significantly different in the early days of the treatment. At the end of treatment, the growth rate of fresh weight and leaf length were significantly higher than those of the control (Fig. 3d and e).

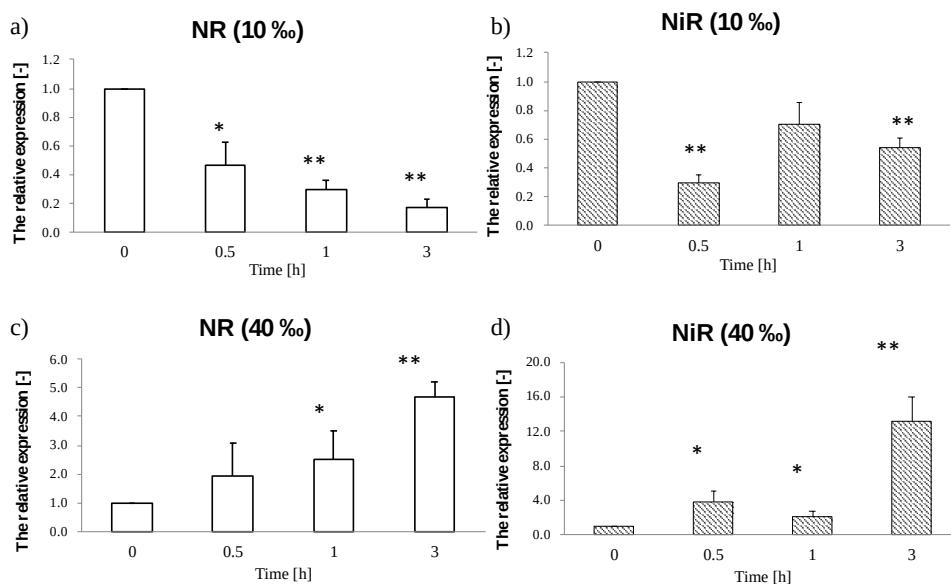


Fig. 2. Relative expression analysis of ZmNR and ZmNiR under different levels of NaCl. Eelgrass were kept in artificial seawater with 24 ‰ NaCl; a) and b) quantitative RT-PCR determination of ZmNR and ZmNiR expression in leaves exposed to 10 ‰ NaCl for the indicated times; c) and d) quantitative RT-PCR determination of ZmNR and ZmNiR expression in leaves exposed to 40 ‰ NaCl. Values are the means $\pm$ SD ($n = 3$). β -actin (49G00260) was used as a loading control; ** $p < 0.01$, * $p < 0.05$

In addition, a mixed nitrogen source (ammonium plus nitrate) was added to the eelgrass solution to observe changes in growth rates when the nitrate assimilation process was blocked (Fig. S4). Eelgrass were grown in artificial seawater with $\text{NO}_3^-/\text{NH}_4^+$, $\text{NO}_3^-/\text{NH}_4^+$ (Na_2WO_4) and NH_4^+ , and the chlorophyll, proline and MDA contents in leaves were measured after adding 1 mM Na_2WO_4 for 15 days. The eelgrass grown in NH_4^+ maintained lower chlorophyll and proline contents and higher MDA content than the mixed nitrogen source plants ($\text{NO}_3^-/\text{NH}_4^+$) (Fig. S4 a, b and c), and the eelgrass treated with Na_2WO_4 presented with reduced chlorophyll and proline contents and increased MDA content when compared to the control. The fresh weight and leaf length in the mixed nitrogen source ($\text{NO}_3^-/\text{NH}_4^+$) plants were significantly higher than those in the NH_4^+ treatment, while the fresh weight and leaf length decreased significantly after adding Na_2WO_4 (Fig. S4 d, e and f) after 15 days treatment.

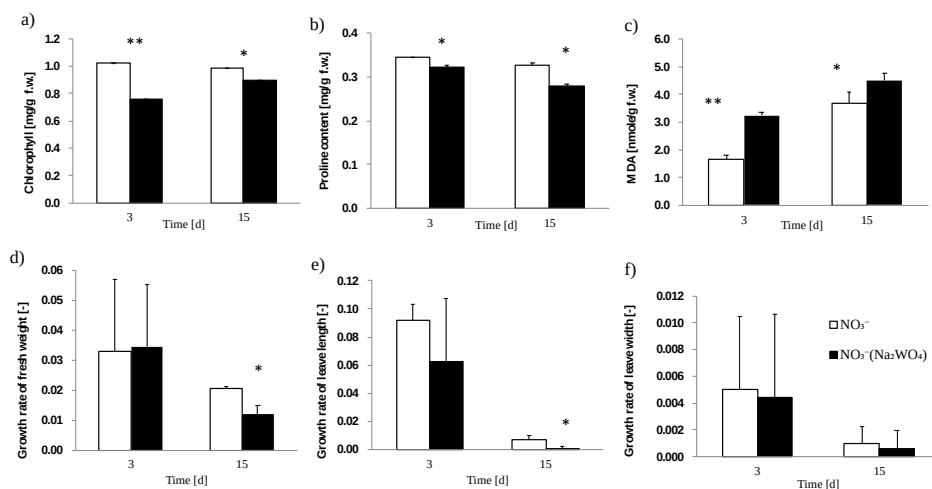


Fig. 3. Physiological and growth analysis of eelgrass after adding 1 mM Na_2WO_4 for 3 days and 15 days: a) total chlorophyll content, b) proline content, c) MDA content, d) growth rate of fresh weight, e) growth of leaf length, f) growth of leaf width; ** $p < 0.01$, * $p < 0.05$

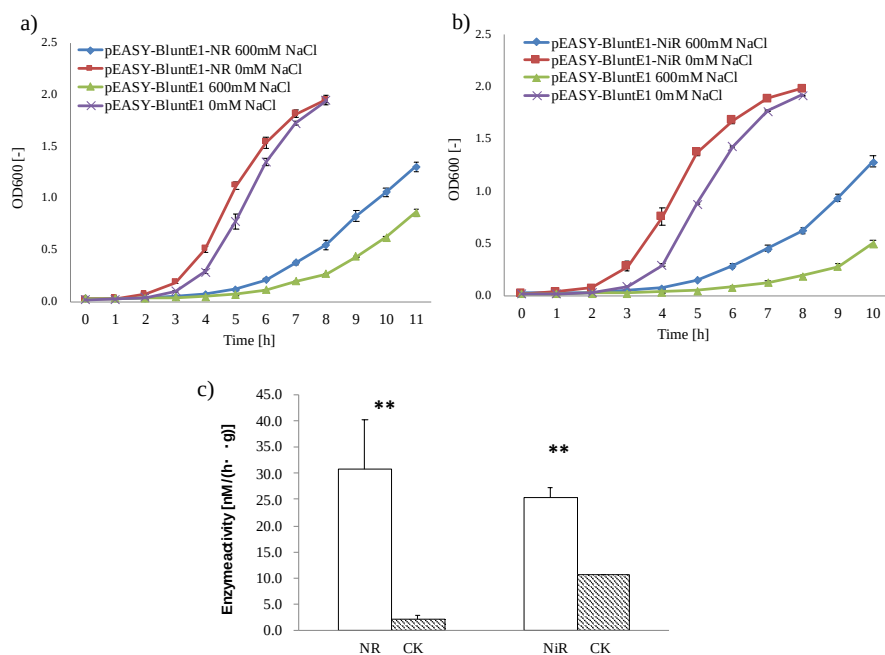


Fig. 4. Comparison of the growth and enzyme activity in recombinant *E. coli* cells (p-EASY-BluntE1-NR/NiR) and control cells (p-EASY-BluntE1); a) and b) growth analysis of recombinant *E. coli* cells and control cells performed on LB liquid medium under NaCl stress, c) NR and NiR activities of recombinant *E. coli* cells and control cells (CK). Values are the means $\pm$ SD ($n = 3$). ** $p < 0.01$

Heterologous expression and functional validation in *E. coli*

ORFs encoding functional eelgrass NR and NiR proteins were cloned into the pEASY-BluntE1 vector (Fig. S5a) and expressed in *E. coli* Rosetta (DE3) cells. Maximal expression of the recombinant protein was observed after 6 h in the presence of 1 mM IPTG and the SDS-PAGE analysis confirmed that the recombinant protein was successfully expressed in *E. coli* Rosetta cells (Fig. S5 b). The growth under NaCl stress was evaluated using a liquid culture assay, and the growth of ZmNR and ZmNiR-overexpression *E. coli* cells increased significantly compared to control (Fig. 4a and b). The activities of NR and NiR were measured in both recombinant cells and control cells, and the enzyme activities in recombinant cells were significantly higher (Fig. 4c).

Heterologous expression and functional validation in tobacco

To investigate whether heterologous expression of ZmNR and ZmNiR affected NaCl tolerance in terrestrial plants, the genes were introduced into *N. benthamiana*. The ZmNR and ZmNiR expression levels of two individual T₂ tobacco lines were determined using semi-quantitative RT-PCR (Fig. S6 a). NR-5-1 and NiR-1-14 were selected for further enzyme activity analysis, and the results showed that the enzyme activities of the transgenic lines were higher than those of the wild-type (WT) plants when exposed to NaCl stress (Fig. 5a and b).

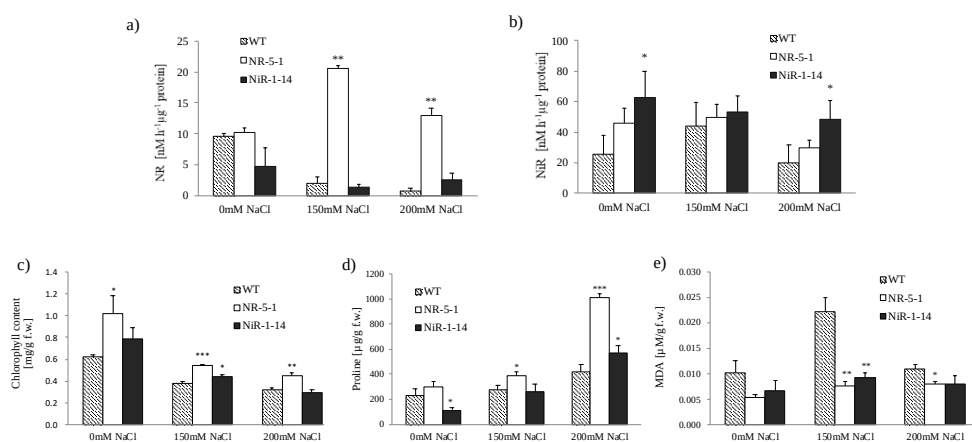


Fig. 5. a) Enzyme activities and enhanced tolerance to salt stress in transgenic plants, b) NR activities of transgenic tobacco and control plants (WT), NiR activities of transgenic tobacco and control plants (WT), c) chlorophyll content in leaves, d) Pro and e) malondialdehyde (MDA) levels. Values are the means ± SD (n = 6); ** p < 0.01, * p < 0.05

Five-week-old transgenic and WT plants were cultivated in a medium containing high levels of NaCl. WT plants were found to be more wilted than transgenic lines under NaCl stress (150 mM), while no significant difference was observed in 200 mM NaCl (Fig. S6 b). To examine the physiological changes induced by the overexpression of the ZmNR and ZmNiR genes, the chlorophyll content, proline content, and MDA content were measured in the leaves after 14 days of NaCl treatment. The results showed that the

chlorophyll and proline content of the ZmNR transgenic line (NR-5-1) was significantly higher ($p < 0.01$ or $p < 0.05$) than that of WT (Fig. 5c and d), while the chlorophyll and proline contents of the ZmNiR transgenic line (NiR-1-14) were slightly higher than those of WT. In contrast, the MDA content in the transgenic overexpressing plants were significantly lower than that of WT plants (Fig. 5e).

Nitric oxide content of eelgrass and transgenic plants

In order to explore how the ZmNR and ZmNiR regulate the physiological and growth changes in high salinity, we measured the NO content in eelgrass and transgenic tobacco under different treatment conditions. In eelgrass, the NO content increased significantly under high salinity (40 ‰ NaCl), and this response was significantly inhibited by the addition of Na_2WO_4 (Fig. 6a). In ZmNR-overexpression transgenic plants, the NO content under high salinity were higher than that of wide-type (Fig. 6b).

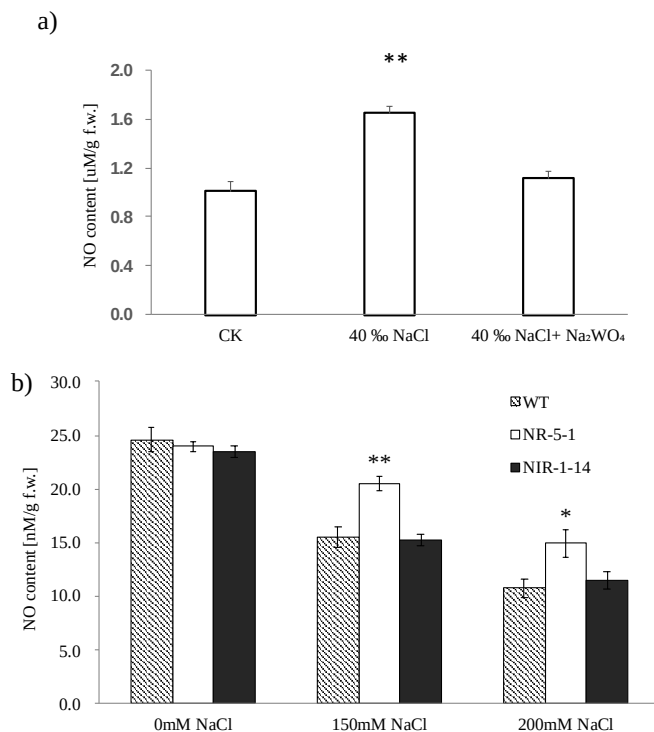


Fig. 6. a) NO contents of eelgrass in different treatments, b) NO contents of transgenic tobacco and control plants exposed to the indicated NaCl treatments for 6 h. Values are means $\pm$ SD ($n = 3$). ** $p < 0.01$, * $p < 0.05$

Discussion

Role of ZmNR and ZmNiR in adapting to NaCl tolerance

It is demonstrated that the enzyme NR and NiR play an important role in the nitrate assimilation [3]. Here, we have cloned the NR and NiR genes from eelgrass. A comprehensive analysis of the expression pattern for both genes in response to different NaCl concentration was performed. The qRT-PCR results revealed that the expression levels of the ZmNR and ZmNiR genes were downregulated at low NaCl concentrations and upregulated at high NaCl concentrations (Fig. 2), which is consistent with previous studies [4]. These results suggested that the ZmNR and ZmNiR genes were closely related to the NaCl tolerance process in eelgrass. Na_2WO_4 is an inhibitor of molybdoenzymes, commonly used in research as an NR inhibitor. After adding Na_2WO_4 to the artificial seawater, the physiological and growth indicators were evaluated in response to single nitrogen source (Fig. 3) or mixed nitrogen source (Fig. S4). We found that various physiological indicators in eelgrass changed in response to nitrate blockade whether grown using single nitrogen source or mixed nitrogen source. All physiological and growth indicators, except MDA, were significantly lower in the treatment group than in the control group (Figs. 3 and S4). These results indicated that inhibiting the enzyme activity of NR in eelgrass significantly affected its growth and physiological processes, and its NaCl tolerance decreased significantly. In addition, these assays confirmed that NR is critical to NaCl tolerance in eelgrass. Taken together, these results proved that NR plays an important role in eelgrass adaptation to the marine high-salt environment, but the role of NiR in this adaptation remains unclear.

NaCl tolerance of the ZmNR and ZmNiR transgenic bacteria and plants

The results of transcript expression analyses and NaCl tolerance assays (Figs. 4 and 5) suggested that the heterologous expression of ZmNR and ZmNiR significantly improved NaCl tolerance in both transgenic bacteria and plants. Under high salinity, the growth rate of recombinant *E. coli* cells was higher than that of control cells. The chlorophyll, proline and MDA content are considered reference parameters in plant stress resistance [27]. In the present study, the increased chlorophyll and proline contents and decreased MDA content (Fig. 5) in the transgenic plants provided strong evidence that the ZmNR and ZmNiR genes successfully increased the salt tolerance in the tobacco, and the expression of the transformed ZmNR and ZmNiR genes may explain the enhanced stress capacity of these plants. Previous studies describing plant overexpression assays have not shown that NR or NiR is closely related to plant salt tolerance in terrestrial plants. The heterologous expression and biochemical characterization of nitrate assimilation and nitrite reductase revealed that the adaptations and potential of prokaryotes are based on their cellular location and function [28]. Positional cloning of the nitrite reductase gene has been associated with improved growth and regeneration ability of calli in rice [29]. Our results showed that the ZmNR and ZmNiR genes successfully improved the salt tolerance of bacteria and tobacco, suggesting that these genes have application potential in the development of improved crop stress-tolerance. Considering the individual contribution of these two enzymes to salinity tolerance, ZmNR is more important than ZmNiR. An increasing number of genes from seagrass or halophytes will likely be cloned and subsequently expressed in crop plants (such as rice), which will help to narrow the gap between grain production and the rapidly

growing human population and provide insights into the molecular mechanism of salt tolerance in marine angiosperms.

How ZmNR and ZmNiR affect eelgrass adaptation to high salinity

The results of this study showed that NR in eelgrass is important for NaCl tolerance; however, the molecular mechanism of how NR affects NaCl tolerance requires further study. We determined various indicators of eelgrass under NaCl conditions and found that the NO content increased significantly (Fig. 6a). The NO content increased significantly under the high salinity (40 % NaCl), and decreased significantly after adding Na₂WO₄. These results indicate that the elevated NO content is closely related to NR activity and the NR pathway may be the main source of NO in eelgrass. NO is commonly found in various animals and plants and can be synthesised via the NR pathway [30]. Studies have found that NO can regulate almost all growth and development processes in plants [31, 32] and participates in the signal transmission in response to various biotic and abiotic stresses [33, 34]. In terrestrial plants, the level of NO₃⁻ in cells is 100 times that of NO₂⁻, and NR has a significant preference for NO₃⁻, which means that the level of NO produced from this pathway is very low. However, under anaerobic conditions (such as in seawater), the level of NO production is expected to be higher due to the accumulation of NO₂⁻) [13, 35].

Based on these results, we hypothesise that NO may be involved in regulating NaCl tolerance in eelgrass (Fig. 7). This may be the reason why the phenotype of ZmNR overexpression lines was more significant than that of ZmNiR overexpression lines.

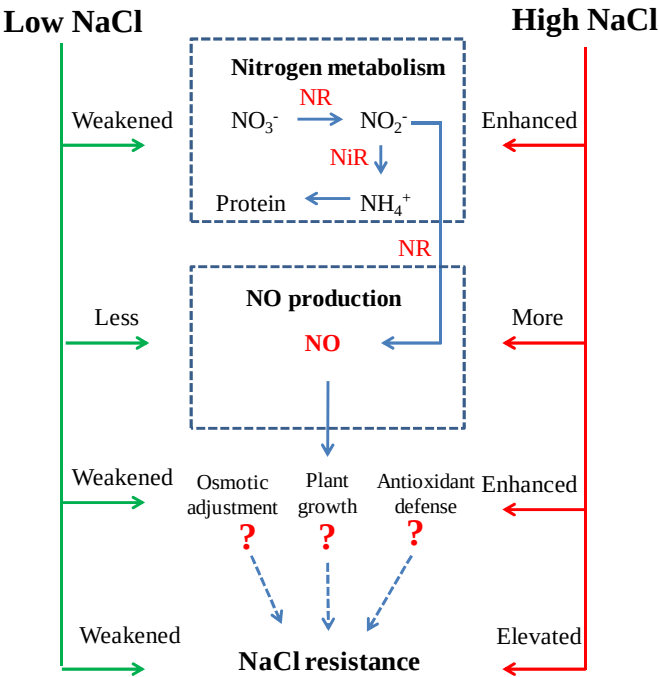


Fig. 7. The mechanism of NO effecting eelgrass adaptation to high salinity environment

In previous studies, NR and NiR has been shown to be inhibited or slightly modified in response to salt stress [36, 37], however this study found that the critical enzymes of nitrate assimilation process increased under high NaCl conditions and decreased under low NaCl conditions. Therefore, the upregulation of NR and NiR under high salinity may be unique to eelgrass, which is closely related to its adaptation under marine environment. Here, we found that both the NR and NiR genes in eelgrass have low homology to other plants (Figs. S1, and S2), which may be the reason for their unique functions under high salinity. Moreover, the analysis of these gene sequences in the terrestrial ancestors of eelgrass and other marine seagrasses may provide insights for future research.

Conclusion

In summary, ZmNR and ZmNiR were cloned and characterised in eelgrass. The expression of ZmNR and ZmNiR was induced under high NaCl conditions and suppressed under low NaCl conditions. In addition, blocking nitrate assimilation by adding Na₂WO₄ to eelgrass affected its growth and physiological processes. The heterologous expression of ZmNR and ZmNiR conferred NaCl tolerance in transgenic bacteria and tobacco. ZmNR- and ZmNiR-overexpression bacteria exhibited faster growth rates, and ZmNR- and ZmNiR-overexpression plants demonstrated increased chlorophyll and proline contents and reduced MDA content under high NaCl stress. Taken together, these results showed that ZmNR and ZmNiR play important roles in eelgrass responses to NaCl and could affect various physiological pathways to enhance the NaCl tolerance in transgenic bacteria and terrestrial plants. Nitrate reductase-dependent NO synthesis may play an important role in NaCl tolerance in eelgrass, and this study provides valuable insights for evaluating the role of nitrate assimilation in regulating stress resistance and candidate genes for enhancing salt tolerance in plants.

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