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ORIGINAL ARTICLE

Overexpression of the spinach *S*-nitrosoglutathione reductase (*SoGSNOR*) in tobacco resulted in enhanced nitrate stress tolerance

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Abstract

S-nitrosylation, the addition of nitric oxide (NO) moiety to a reactive cysteine thiol, to form an *S*-nitrosothiol (SNO), is emerging as a prototypic redox-based post-translational modification. *S*-nitrosoglutathione reductase (GSNOR) is thought to be the major regulator of total cellular SNO levels in plants. However, its role in excess nitrate stress has not been investigated in spinach. In this study, a spinach GSNOR gene (GenBank Accession No. KR381778) was amplified and designated as *SoGSNOR*. The transcript and protein level of SoGSNOR were reduced after excess nitrate treatment for 24 h. Addition of NO donor to the nitrate solution decreased the SoGSNOR expression, while supplementation inhibitor of nitrate reductase and nitric oxide synthase increased its expression. Overexpression of *SoGSNOR* in tobacco increased the germination rate of transgenic seeds, compared to the wild type (WT) under nitrate stress. Higher photosynthetic rate, transpiration rate, stomatal conductance, water use efficiency and expression level of some stress-related genes were detected in the transgenic seedlings than the WT under nitrate stress. The transgenic tobacco seedlings have lower malondialdehyde content, reactive oxygen species (ROS) fluorescence, and higher activities and transcript level of superoxide dismutase, catalase, peroxidase under nitrate stress. *SoGSNOR* transgenic tobacco plants have lower NR activity and protein level, higher GSNOR and non-symbiotic class 1 hemoglobin (nsHb) protein level than the WT plants, leading to lower NO accumulation and SNOs contents under nitrate stress. These results suggested that overexpression of *SoGSNOR* increased nitrate stress tolerance of tobacco by regulating ROS and RNS metabolism.

Key message

A spinach *S*-nitrosoglutathione reductase (*SoGSNOR*) gene was isolated from spinach root. The expression level and activities of SoGSNOR were reduced by excess nitrate treatment. Overexpression of *SoGSNOR* in tobacco increased nitrate stress tolerance by regulating ROS and RNS metabolism.

Keywords Nitrate stress · *S*-nitrosoglutathione reductase · Nitric oxide · Spinach

Introduction

Nitric oxide (NO) is an important free radical that is considered to be a general plant signal. NO regulates both normal developmental processes and biotic and abiotic stress responses (Jasid et al. 2008; Lobb et al. 2015; Qiao and Fan 2008; Zhao et al. 2007; Zheng et al. 2009). In plants, NO is produced mainly through two different enzymatic pathway, namely, nitrite reduction by nitrate reductase (NR) or by the action of NO synthase (NOS)-like activity (del Rio et al. 2004). Although NOS-like activities that are sensitive to mammalian NOS inhibitors have been detected in plant extracts, few bona fide NOS enzymes in plants have been identified. NOSes are present in a few algal species but

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appear to not be conserved in land plants (Jeandroz et al. 2016). In some cases, NO is also produced by a nonenzymatic mechanism from NO_2^- under an acidic condition in the plant apoplast (Sahay and Gupta 2017). At least three distinct NO degradation or inactivation mechanisms of plants have already been described, involving non-symbiotic hemoglobins (nsHbs), reactive oxygen species (ROS) or the formation and subsequent catabolism of *S*-nitrosothiols (SNOs) (Zuccarelli et al. 2017).

NO belongs to a family of NO-derived molecules designated reactive nitrogen species (RNS)(Chaki et al. 2011). NO regulates post-translational protein modifications, including *S*-nitrosylation of thiol groups, nitration of tyrosine and binding to metal center of proteins (Gow et al. 2004). *S*-nitrosylation with the formation of SNOs is an important feature of NO signaling regulating protein function. Glutathione (GSH), *S*-nitrosoglutathione reductase (GSNOR), and thioredoxin (Trx) have been identified as the major protein denitrosylases in mammalian cells (Sengupta and Holmgren 2013). NO is known to readily react with reduced GSH by reversibly binding to its thiol group, giving rise to S-nitrosoglutathione (GSNO). GSNOR is so far the most worked out and an established enzyme known to control nitrosylation by catalyzing the reduction of GSNO to oxidized GSH (GSSG) and ammonia in the presence of GSH (Kubienova et al. 2013).

GSNOR is involved in plant growth and development (Rodriguez-Ruiz et al. 2017). In *Arabidopsis*, allelic mutations in GSNOR1/HOT TEMPERATURES5 (HOT5)/PARAQUAT RESISTANT2 (PAR2) cause a dramatic increase in intracellular GSNO and SNOs, altered stress responses, and various developmental defects (Chen et al. 2009; Feechan et al. 2005; Kwon et al. 2012; Lee et al. 2008). GSNOR activity is down regulated during pepper fruit ripening (Rodriguez-Ruiz et al. 2017). GSNOR is also involved in plant responses to biotic and abiotic stresses (Ticha et al. 2017). Biotic stress stimuli activates *GSNOR* expression, e.g. in *Lactuca* spp. genotypes with fungal mildews (Ticha et al. 2018), in plant–herbivore interactions with jasmonate-inducible responses (Wunsche et al. 2011). Abiotic stresses such as mechanical damage (Chaki et al. 2011), heat stress (Lee et al. 2008), salt stress (Jain et al. 2018), Al treatment (Sun et al. 2017), sodic alkaline stress (Gong et al. 2015), cadmium (Barroso et al. 2006), arsenic (Leterrier et al. 2012), injury or darkness (Kubienova et al. 2014), iron toxicity (Li et al. 2019), decreased or increased *GSNOR* expression.

Nitrogen (N) is one of the important nutrients for plant growth and development. Plants mainly fulfill their N requirements by absorbing inorganic nitrate (NO_3^-) and ammonium (NH_4^+) from the soil (Kronzucker et al. 2000). It is essential to input N fertilizer to attain high crop yields, but excessive inputs lead to a waste of fertilizer, which is reflected by a decline in nutrient use efficiency and soil acidification in China (Zhu et al. 2018). It was reported that NO_3^- accounts for approximately 67–76% of the total anions in soil of the greenhouse (Ju et al. 2007; Yang et al. 2010). In *Arabidopsis thaliana*, 50 mM nitrate inhibited the primary root growth (Signora et al. 2001). Excess nitrate caused oxidative stress and increased the lipid peroxidation level in spinach (Xu et al. 2012). 80 mM $Ca(NO_3)_2$ stress significantly induced changes in the components of cell wall, anatomical structure, and expression profiles of several lignin biosynthetic genes (An et al. 2018) and inhibited the growth and photosynthetic capacity significantly in cucumber seedlings (Du et al. 2016). The tonoplast H^+-ATPase, H^+-PPase activities, malondialdehyde (MDA) and proline contents were all increased after iso-osmotic stress of $Ca(NO_3)_2$ (80 mM) and NaCl (120 mM) treatment in tomato seedlings (Shi et al. 2004), suggesting that excess nitrate stress to plants shares the similar defense pathways with NaCl stress.

Spinach (*Spinacia oleracea* L.) is an excellent source of dietary vitamins and minerals and has a tendency to absorb nitrogen fertilizer, especially nitrate. In our previous study, we found *SoGSNOR* expression decreased under nitrate stress and exogenous NO donor sodium nitroprussiate (SNP) enhances the nitrate stress tolerance of spinach (Zheng et al. 2016). To further investigate the role of SoGSNOR in response to excess nitrate and its regulation of the ROS and RNS metabolism, we cloned the full length of *SoGSNOR* sequence from spinach root, analyzed the expression and activities of SoGSNOR. Transgenic tobacco is easy to obtain to study gene function. So we investigated the function of *SoGSNOR* in the transgenic tobacco plants under nitrate stress. The main goal of the present work was to further unravel mechanism of *SoGSNOR* in response to nitrate stress.

Materials and methods

Plant materials, growth conditions and stress treatment

The seeds of spinach (*Spinacia oleracea* L., cv. 'chaoji') were obtained from Hongkong Cai Xingli agriculture Co., Ltd.. The experiment was carried out under natural conditions in greenhouse with the air temperature of 20–28 °C during the day and 13–18 °C during the night. The spinach seedlings were cultured hydroponically according to Xu et al. (2012) as control (CK). The spinach seedlings were treated with 100 mM nitrate (KNO_3 50 mM, $Ca(NO_3)_2$ 25 mM), or supplemented with 100 μM sodium nitroprusside (SNP, NO donor), NR inhibitor tungstate, or NOS inhibitor N′-Nitro-L-arginine methyl ester hydrochloride (L-NAME) for 24 h under the same conditions. Tobacco seedlings were

treated with 100 mM nitrate or without 100 mM nitrate as control (CK) for 24 h hydroponically. Then, the photosynthetic measurements, ROS and NO accumulation were assessed with fresh seedlings. Roots of spinach and tobacco seedlings were taken after different treatments, immediately frozen in liquid nitrogen and stored at − 80 °C until use.

Isolation of total RNA, synthesis of first strand cDNA, gene cloning and sequence analysis of SoGSNOR

Isolation of total RNA was done using RNAiso reagent (Takara, Dalian, China) according to the manufacturer's instructions. The first strand cDNA was synthesized with the PrimeScript TM RT-PCR (Takara, Dalian, China) according to the manufacturer's instructions.

To clone the coding sequence of GSNOR of spinach, three degenerate primers were designed according to the comparison of known plant's *GSNOR* sequences in the NCBI. The amplification of the 5′ region sequence was done by a nested PCR. The first cycle was done with F1 and R1 and the second PCR was done using F1 and R2. According to the 5′ product, F2, F3, B26 primers were designed and 3′ RACE-PCR was done to amplify the 3′ region of the *SoGSNOR* gene. The open reading frame (ORF) sequence was amplified via PCR using a pair of primers *SoGSNOR*-F-*Bam*HI and *SoGSNOR*-R-*Xho*I. Primers used in gene clone were listed in supplemental Table S1.Then the DNA sequence data was analyzed according to Xu et al. (2016).

qRT-PCR and western blot analysis

The mRNA expression level analysis was done using the EvaGreen 2 × qPCR Master Mix kit (Abm, Canada). The qRT-PCR was performed with the BioRad CFX 96 TM Real-time System. The gene- specific primers were shown in Supplemental Table S2.

Western blot analyses were done according to the method of Zheng et al. (2016). Proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). The antibody against nsHb1, GSNOR was previously made by our lab (Guo et al. 2015, 2018). For detection of NR, NR peptides were used to immunize white mice to obtain antiserum (Xu et al. 2016). After incubation with the antibody, proteins were detected using a goat peroxidase-conjugated anti-mouse antibody (1:4000; Sigma) and visualised using ECL chemiluminescence (Bio-Rad, Hercules, CA).

Plasmid construction and agrobacterium mediated transformation of tobacco plants

The coding sequence of *SoGSNOR* in the pMD18-T (Takara, Dalian, China) vectors was digested with *Bam*HI and *Xho*I, and inserted into the vector pRI101-GFP (Takara, China). The construct was introduced into *agrobacterium tumefaciens* strain LBA4404 by electroporation. The *agrobacterium*-mediated transformation into tobacco (*Nicotiana tabacum* cv. NC89) and regeneration procedures were as previously described (Kano-Murakami et al. 1993). The transgenic plants were detected using genomic DNA PCR and qRT-PCR analysis.

Seed germination analysis of SoGSNOR transgenic tobacco

Tobacco seeds were germinated on Murashige and Skoog (MS) medium with 0 (MS as control, CK) or 100 mM excess nitrate according to Xu et al. (2016). The seeds were cultivated in a growth chambers (22 °C, 16/8 h photoperiod with an irradiance of 300 μmol photons m^{-2} s^{-1}, relative humidity of 60%). Pictures were taken at the 10th day.

Photosynthetic measurements

Net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (E), in the leaves of the transgenic and WT plants were measured with an open photosynthesis system (Ciras-1, PP Systems, Hitchin, UK) in the morning after 24 h of nitrate treatment. Water-use efficiency (WUE) of the leaf was calculated as the ratio of net photosynthesis to transpiration.

Lipid peroxidation, ROS accumulation, antioxidant enzyme activities and protein concentration assay

The lipid peroxidation product of malondialdehyde (MDA) was assayed by the thiobarbituric acid-based colorimetric method (Madhava Rao and Sresty 2000). Each 0.2 g of roots was homogenised in 2 ml of 10% (w/v) trichloroacetic acid (TCA) containing 0.25% (w/v) TBA. The mixture was incubated in a water bath at 95 °C for 30 min and the reaction was terminated in an ice bath. The mixture was centrifuged at 10,000×*g* for 20 min, and the absorbance of the supernatant was measured.

The ROS accumulation was analyzed with 20 μM H_2DCFDA visualised in a fluorescence microscope and photographed (Mazel et al. 2004)

To analyze the antioxidant enzyme activities of SOD, CAT, POD, 0.2 g root sample was homogenised in 2 ml of 0.05 M sodium phosphate buffer (pH 7.8, 1.0 mM EDTA and 2% (w/v) PVP). The homogenate was centrifuged at 10,000×*g* for 20 min at 4 °C and the supernatant was used for all antioxidant enzyme activity assays. Superoxide dismutase (SOD) activity was analyzed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm (Madhava Rao and Sresty 2000). One unit of SOD activity was defined

as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction. Catalase (CAT) activity was assayed as the decline in absorbance at 240 nm due to the decline of extinction of H_2O_2 (Cakmak and Marschner 1992). Peroxidase (POD) activity was measured by the increase in absorbance at 470 nm using guaiacol as substrate (Zaharieva et al. 1999). The unit was expressed as nmol min^{-1} mg^{-1} protein. Protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) using BSA as standard.

The NO accumulation, NR and NOS-Like activity, SNOs contents, and GSNOR activity analysis

The NO accumulation was measured using the fluorescent dye diaminofluorescein-FM diacetate (DAF-FM DA, Sigma-Aldrich, USA) and then visualised in a fluorescence microscope and photographed (Zhao et al. 2009). NR activity was measured as described by Zheng et al. (2016). The nitrite formed was determined spectrophotometrically by measuring absorbance at 540 nm. The NOS-Like activity was assayed with a commercial reagent kit (Jiancheng Biotech Inc., Nanjing, China) according to the manufacture's instructions. NOS catalyzes the reaction of L-Arg and molecular oxygen to generate NO. NO and nucleophilic substances produce colored compounds. The absorbance is determined spectrophotometrically by measuring absorbance at 530 nm.

The SNOs contents were assayed using the previous method (Frungillo et al. 2013; Gong et al. 2015). The extracts of the roots were passed through Sephadex G-25 gel filtration column according to Barroso et al.(2006) and then the GSNOR activities were analyzed by detecting the oxidation of NADH at 340 nm after addition of GSNO to the reaction mixture at a final concentration of 400 μM (Zheng et al. 2016).

Statistical analysis

All data are the mean ± SD from at least three independent experiments. Data were statistically analyzed using the DPS software. Differences between the means were compared by the Duncan test ($p < 0.05$).

Results

Cloning and characterization of the spinach SoGSNOR gene

The full length of spinach *GSNOR* gene was amplified using RT-PCR and RACE technique and designated as *SoGSNOR* (GenBank Accession No. KR381778). The full-length cDNA of *SoGSNOR* was 1378 bp, with a 1140 bp open reading frame (ORF), which encoded a 379 amino acid polypeptide with a calculated molecular weight of 40.8 kDa and pI of 6.82. Comparing the predicted protein sequence with *Beta vulgaris* BvGSNOR, *Oryza sativa* OsGSNOR, *Brassica napus* BnGSNOR, *Ricinus communis* RcGSNOR, *Gossypium arboretum* GaGSNOR, *Arabidopsis thaliana* AtGSNOR, *Medicago truncatula* MtGSNOR, *Solanum lycopersicum* SlGSNOR showed that SoGSNOR has more than 90% sequence identity with these proteins (Fig. S1).

Excess nitrate inhibited GSNOR expression in spinach roots

qRT-PCR analysis showed that the *SoGSNOR* expression level decreased significantly after nitrate treatment for 24 h (Fig. 1a). Compared to the nitrate treatment, SNP addition to nitrate solution decreased the *SoGSNOR* expression. Addition of the NR and NOS inhibitor to the nitrate solution increased *SoGSNOR* expression, compared with the nitrate treatment. As shown by the western blot analysis, the SoGSNOR protein level showed the similar pattern with the *SoGSNOR* transcript level (Fig. 1b).

SoGSNOR confers enhanced nitrate stress tolerance in transgenic tobacco seeds

To reveal the biological function of *SoGSNOR,* the coding region was inserted into pRI101 under the control of 35S promoter. 20 kanamycin-resistant tobacco plants were obtained by PCR detection. The expected fragment was detected in the transgenic plants, while this gene was not detected in the WT plants (Fig. 2a). Then qRT-PCR was carried out to further confirm the transgenic tobacco lines. The kanamycin-resistant plants had significantly higher expression level of mRNA than the WT plants (Fig. 2b). Three lines (#3, #4, #12) with different *SoGSNOR* transcript level were selected for seeds germination analysis.

Sterilized seeds of #3, #4, #12 and WT plants of the T2 generation were plated on MS medium with or without excess nitrate. Without excess nitrate treatment, the germination rate of WT and the transgenic seeds were similar (Fig. 3a). When seeds were planted on medium containing excess nitrate, the germination rate was inhibited and the transgenic plants have higher germination rate than the WT. The germination rate of WT, #3, #4 and #12 were 48.1%, 93.3%, 80.5%, 93.6% on 9th day, respectively (Fig. 3d). The results suggested that overexpression of *SoGSNOR* enhanced nitrate stress tolerance.

Response of SoGSNOR transgenic seedlings to excess nitrate treatment

To further reveal the response of *SoGSNOR* overexpression transgenic seedling to nitrate stress, 4-week-old transgenic

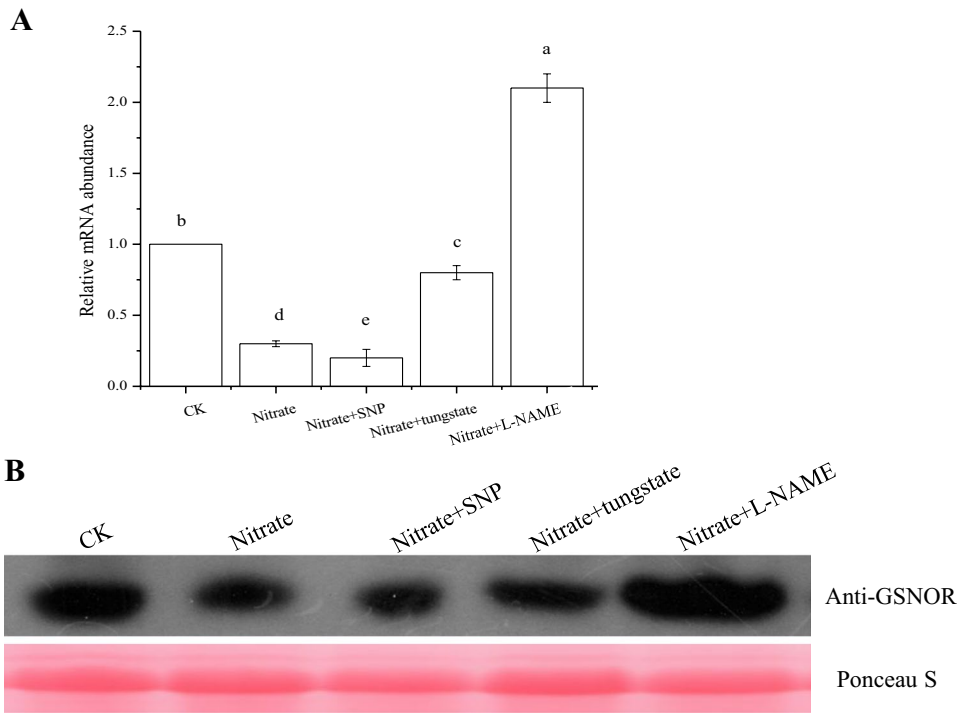

Fig. 1 Analysis of the transcript and protein level of SoGSNOR under control (CK) and nitrate stress supplemented with NO donor (SNP), NR inhibitor (tungstate) and NOS inhibitor (L-NAME) in spinach roots. **a** qRT-PCR analysis of *SoGSNOR*. **b** Western blot analysis of the SoGSNOR protein level. Data are mean values ± SD of three independent experiments. Error bars with different letters indicate significant differences ($p < 0.05$). For western blot, protein was stained with ponceau as loading control

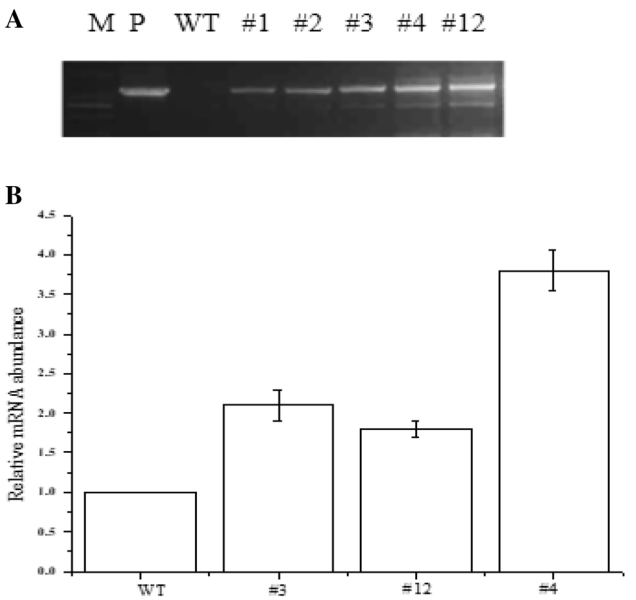

Fig. 2 Verification of SoGSNOR transgenic tobacco plants. **a** Genomic PCR of the transgenic plants. WT: wild type tobacco. P: the plasmid of pRI101-SoGSNOR. #1, #2, #3, # 4, #12: different transgenic lines. M: DNA marker. **b** Relative expression of SoGSNOR in leaves of WT and transgenic lines. Data for qRT-PCR analysis are the means of three biological replicates (± SE)

and WT plant seedlings were treated with 100 mM nitrate hydroponically for 24 h. Photosynthetic gas exchange parameters were then analyzed. There was no significant difference of the net photosynthesis rate (Pn) between the transgenic and WT plants under normal conditions (Fig. 4). The Pn decreased significantly after nitrate stress treatment in both WT and transgenic plants, while the transgenic plants have higher Pn than the WT plants. Similar results were observed in transpiration rate (E), stomatal conductance (Gs) and WUE between the transgenic and WT plants.

The transcript level of 6 genes expressed under salt and osmotic stress was evaluated with qRT-PCR, including *NtDREB2* (*Dehydration-Responsive Element-Binding Factor 2*), *NtDREB4*, *NtLEA5, NtERD10c (LEA-protein 10c), NtERD10D, NtP5CS1* (*delta1-Pyrroline-5-Carboxylate Synthetase 1*). Under normal conditions, there was no difference of the expression levels of these stress-responsive genes between WT and transgenic plants. After nitrate treatment, the expression of *NtERD10D, NtERD10c, NtDREB2, NtDREB4*, *NtP5CS1, NtLEA5* increased, and their expression in transgenic tobacco was higher than the WT (Fig. 5). The above results suggested that the *SoGSNOR* overexpression transgenic plants have higher nitrate stress tolerance than the WT plants.

Overexpression of SoGSNOR alleviated excess nitrate-induced oxidative stress in transgenic tobacco seedlings

To elucidate the mechanism of higher tolerance of *SoGSNOR*-overexpressing transgenic plants after nitrate treatment, the MDA contents and ROS accumulation were analyzed. There were no difference of MDA contents between WT and transgenic plants under normal condition. The MDA

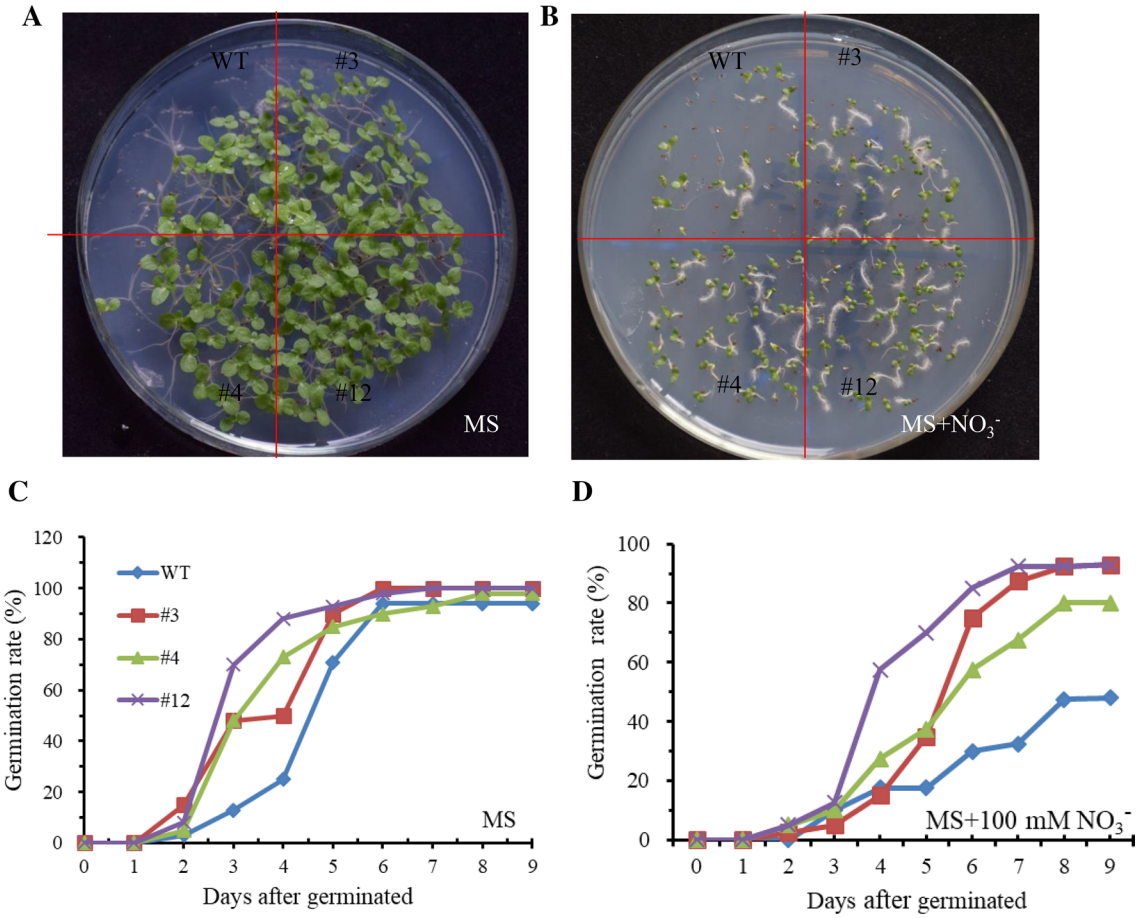

Fig. 3 Overexpression *SoGSNOR* enhances the germination rate of transgenic tobacco seeds under nitrate stress. **a**, **b** Pictures of the WT and transgenic seeds after sowing on MS or MS added 100 mM nitrate for 10 days. **c**, **d** Germination rates of WT and transgenic lines under normal and nitrate stress treatment from 1 to 9th day

contents were significantly lower in the transgenic plants than that of the WT plants after nitrate treatment (Fig. 6a) ($P < 0.05$). Overexpression of *SoGSNOR* did not affect ROS accumulation in the control. An obviously increase in ROS accumulation was observed in the roots under excess nitrate stress, especially in WT plants (Fig. 6b, c).

Then, the SOD, CAT and POD activities were analyzed in the WT and transgenic plants after nitrate treatment. The SOD, CAT and POD activities have no difference between the transgenic and WT plants under control (Fig. 7). After nitrate treatment, the activities of SOD, CAT and POD were significantly higher in the transgenic than the WT plants, especially in the transgenic line #4 ($p < 0.05$). The SOD, CAT and POD activities in #4 line were 6.63-, 2.18-, 2.41-fold (respectively) above the control after nitrate treatment. The transcript level of *NtSOD* (Mn SOD), *NtCAT* and *NtPOD* were then analyzed by qRT-PCR assay. Before the nitrate stress, there has no significant difference of the transcript levels of *NtSOD, NtCAT* and *NtPOD* between the transgenic and WT plants. After nitrate treatment, the *NtSOD, NtCAT* and *NtPOD* expression level were all significantly higher than the WT plants, especially the #4 line ($p < 0.05$) (Fig. 7).

Overexpression of SoGSNOR resulted in lower NO and SNOs contents in the tobacco seedlings

As shown in Fig. 8a, b, the NO accumulation in the transgenic plants was lower than the WT plants under the control. After nitrate treatment, the NO accumulation increased dramatically, and the NO contents in the transgenic plants were lower than the WT plants. Then, we analyzed the activities of the main NO production enzymes of NR and NOS. The NR activity in the transgenic lines was lower than the WT plants in the control. After nitrate treatment, compared to the control, the NR activity in the transgenic plants increased, but was lower than the WT. The NOS-Like activity showed no difference between the transgenic plant and WT under control and nitrate stress. Western blot analysis showed that the NR protein level in the transgenic plants was lower than

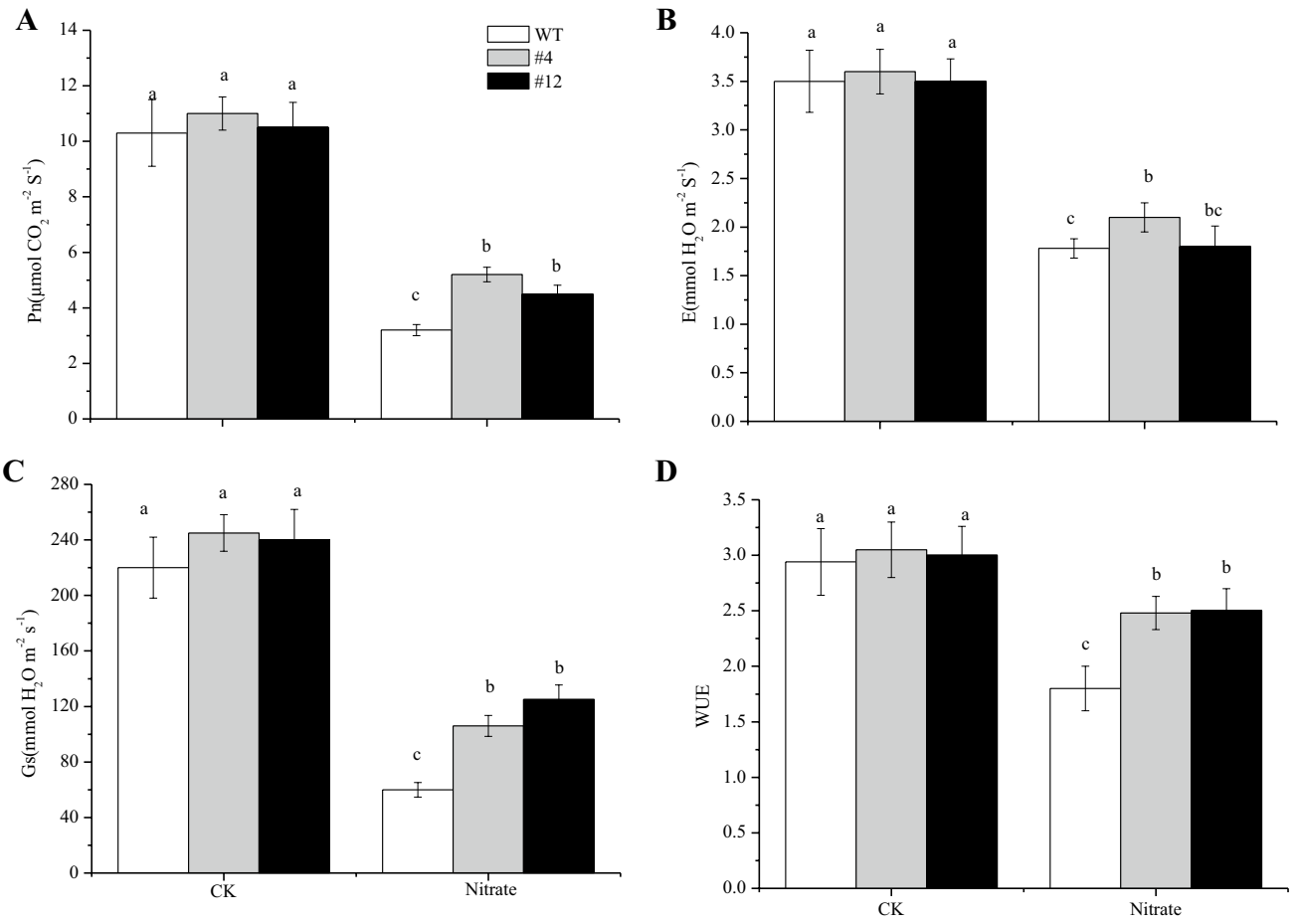

Fig. 4 Photosynthetic performance of *SoGSNOR*-transgenic and WT tobacco plants in response to nitrate stress. **a** Net photosynthetic rate. **b** Transpiration rate. **c** Stomatal conductance. **d** Water use efficiency. Data are mean values ± SD of three independent experiments. Error bars with different letters indicate significant differences ($p < 0.05$)

the WT plants in the control and after nitrate stress treatment. After nitrate stress treatment, the NR protein level increased, especially the WT and #4 line.

The GSNOR activities were significantly higher in the overexpressing transgenic plants than the WT plants ($p < 0.05$). After nitrate treatment, the SoGSNOR activities increased in the transgenic and WT plants, especially in the transgenic plants. Western blot analysis also showed that the GSNOR protein level in the transgenic plants was higher than the WT plants (Fig. 9a, c).

In the normal conditions, the SNOs contents in the transgenic plants were lower than the WT plants. After nitrate treatment, the SNOs contents increased in the transgenic and WT plants, and the *SoGSNOR* overexpressing transgenic plants have lower SNOs contents than the WT plants. The SNOs contents increased by 33.22%, 23.76%, 28.78% in the WT, #4 and #12, respectively, after nitrate treatment, compared with the control (Fig. 9b).

Plant non-symbiotic class 1 hemoglobin (nsHb1) plays role as a modulator of NO levels in plants by a dioxygenase mechanism (Hebelstrup et al. 2014). As shown in Fig. 9c, western blot analysis showed that nsHb1 protein level in the transgenic plants was higher than the WT plants. The protein level increased after nitrate stress treatment, especially in the transgenic plants, indicated the better function of scavenging NO in the *SoGSNOR* transgenic plants.

Discussion

GSNOR plays important roles in plants development, biotic and abiotic stresses. In this study, we cloned the *SoGSNOR* gene by RT-PCR and RACE-PCR. SoGSNOR showed high similarity with GSNOR of other plants (Fig. S1). A significant reduction in GSNOR activity is evident in response to salt stress in sunflower seedlings (Jain et al. 2018). In our previous study, *SlGSNOR* expression was decreased under nitrate stress in tomato (Guo et al. 2018). Similar results were reported in *Arabidopsis* exposed to paraquat-induced oxidative stress, in which GSNOR

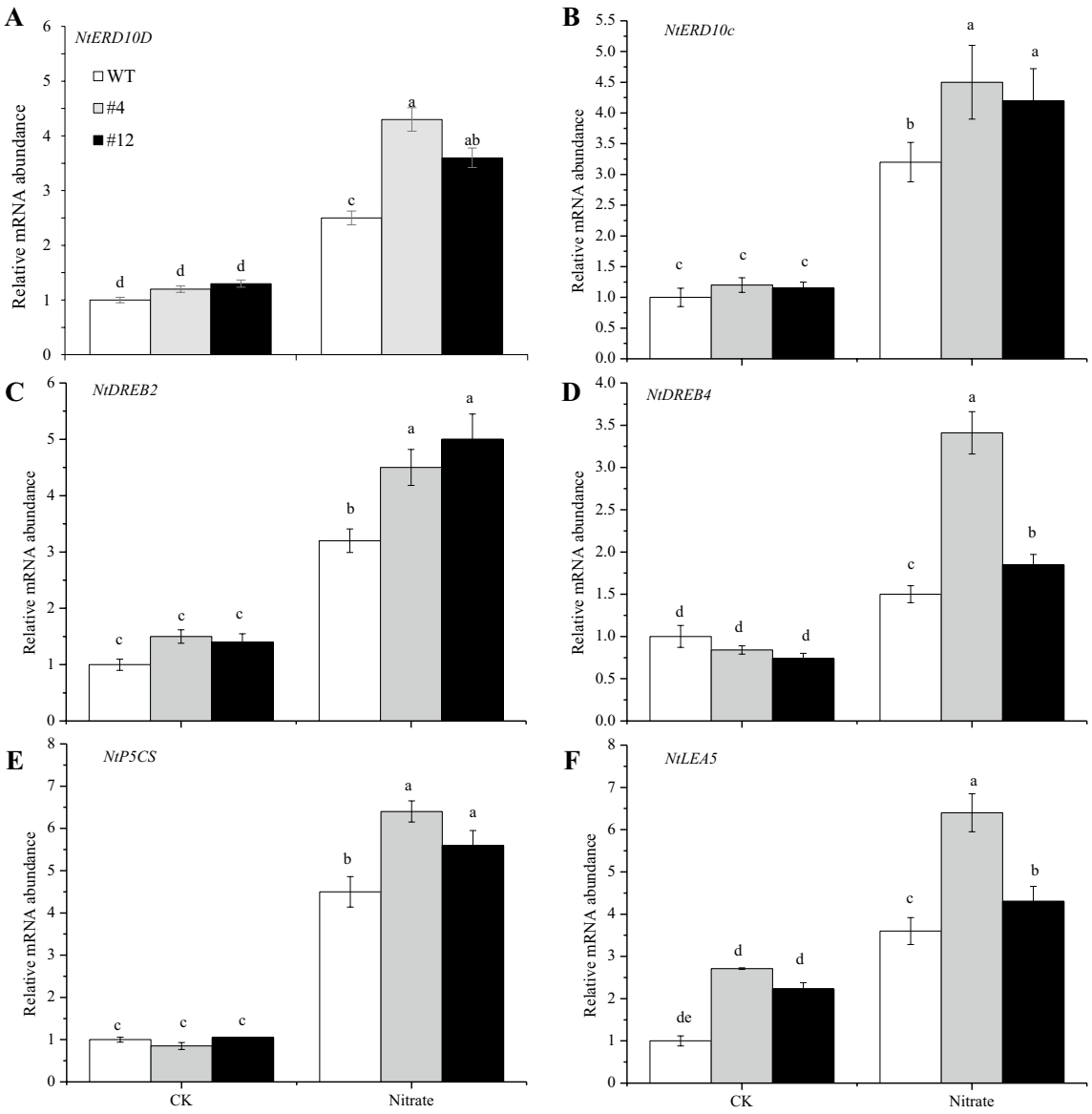

Fig. 5 Transcript levels of salt stress responsive genes in *SoGSNOR*—transgenic and WT plants. **a** *NtERD10D*. **b** *NtERD10C*. **c** *NtDREB2*. **d** *NtDREB4*. **e** *NtP5CS*. **f** *NtLEA5*. The transcript levels were normalized to *NtACTIN*. Data are mean values ± SD of three independent experiments. Error bars with different letters indicate significant differences ($p < 0.05$)

activity was inhibited with a concomitant increase in cellular SNOs (Guerra et al. 2016). In this study, we found the SoGSNOR transcript and protein level were decreased after nitrate treatment for 24 h (Fig. 1), suggesting that SoGSNOR was related to excess nitrate stress tolerance. GSNOR enzymatic activity was significantly increased by NO treatment in rice plants grown under aluminum stress (Yang et al. 2013). While, the protein level of GSNOR was decreased by NO donors (Chen et al. 2009) in *Arabidopsis*. In this experiment, exogenous NO decreased the *SoGSNOR* expression under nitrate stress, but NR and NOS inhibitor enhanced its expression. These results suggested that the activity of GSNOR in plant defense might differ with NO accumulation (Salgado et al. 2013).

To further investigate the function of GSNOR in spinach under nitrate stress, overexpression transgenic tobacco lines were obtained (Fig. 2). The *SoGSNOR*- overexpressing transgenic lines have higher germination rate than the WT plants under excess nitrate stress (Fig. 3), suggesting that *SoGSNOR*-overexpressing transgenic tobacco plants showed improvement in nitrate stress tolerance. The ability to maintain an optimal rate of photosynthesis is essential to salt acclimation. Overexpression of the tomato GSNOR can degrade the maintained redox homeostasis,

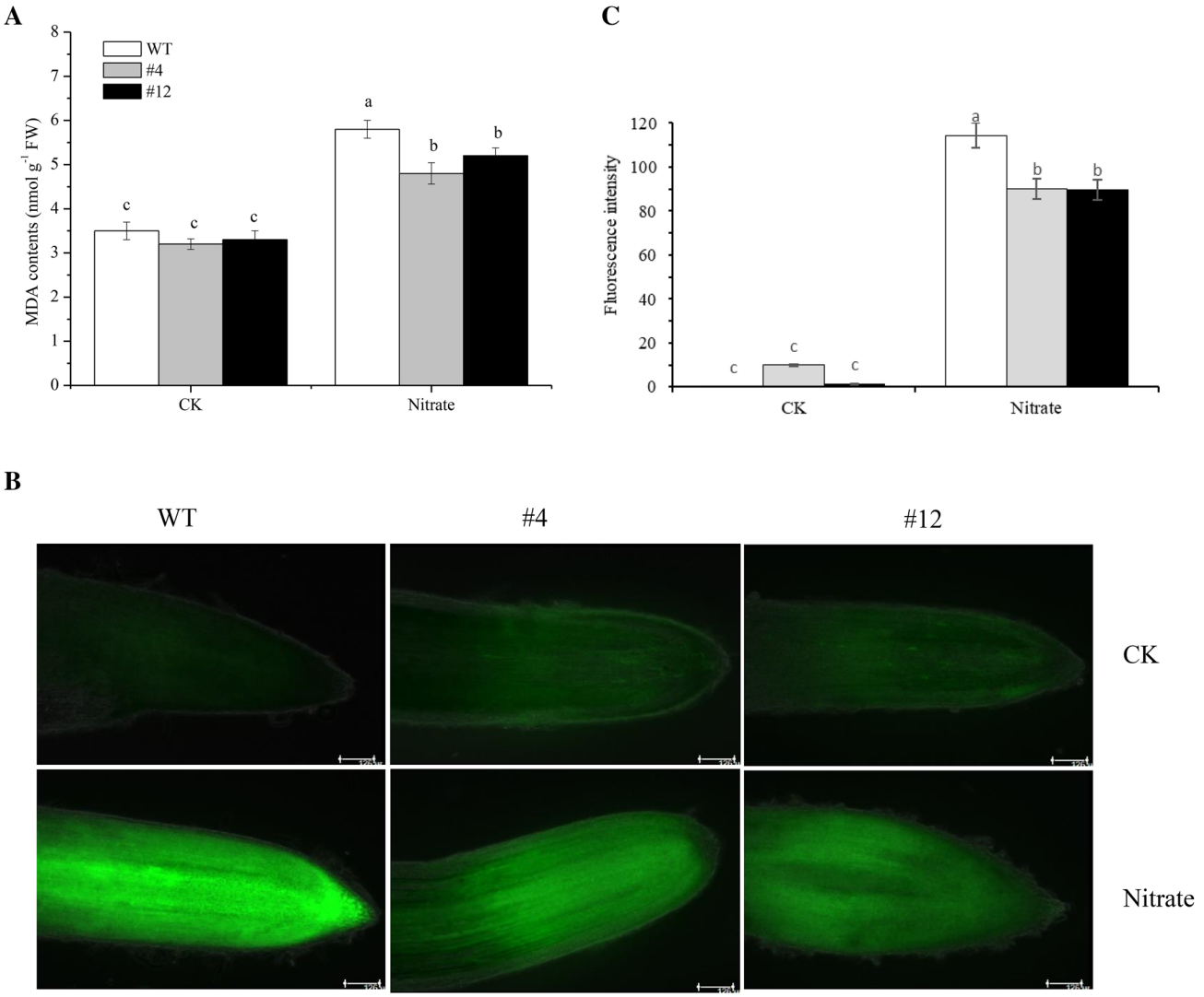

Fig. 6 Effect of excess nitrate stress on MDA contents and ROS accumulation in WT and *SoGSNOR*-transgenic tobacco seedlings. **a** MDA contents. **b** ROS accumulation. The ROS accumulation was analyzed with 20 μM H_2DCFDA and visualised in a fluorescence microscope and photographed. **c** ROS production expressed as relative fluorescence. Data are mean values ± SD of three independent experiments. Error bars with different letters indicate significant differences ($p < 0.05$). Signal intensities of green fluorescence in the images were quantified using Image J software (http://rsb.info.nih.gov/ij/)

resulting in a greater photosynthetic capacity in Fe-deficiency condition (Wen et al. 2019) and protect the photosynthetic apparatus under alkaline stress (Gong et al. 2015). In this study, the SoGSNOR transgenic tobacco plants have higher photosynthetic rate than the WT plants (Fig. 4), suggesting that overexpression of SoGSNOR can protect the photosynthetic system under nitrate stress. In our previous study, photosynthetic oxygen-evolving protein (OEC), and ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco) were found to be *S*-nitrosylated in the spinach root under nitrate stress (Zheng et al. 2016). Gong et al. (2015) provided evidence that overexpression of tomato GSNOR can degrade the intracellular RNS to reduce the photosynthetic proteins *S*-nitrosylation level, and then protects the photosynthetic apparatus under alkaline stress(Gong et al. 2015).

The expression of stress-responsive genes often increased under salinity conditions. *P5CS1* encodes a key enzyme in the biosynthesis of proline, which is important for stress tolerance (Mattioli et al. 2009; Yoshiba et al. 1999). Late embryogenesis abundant (LEA) proteins are large groups of hydrophilic proteins with major role in drought and other abiotic stresses tolerance in plants (Magwanga et al. 2018). Overexpression of a group I LEA protein increases ABA sensitivity and enhances osmotic tolerance in rice (Yu et al. 2016). ERD10 (C/D) are group 2 late embryogenesis abundant (LEA) proteins, which can partially bind water, stabilize labile enzymes, and protect

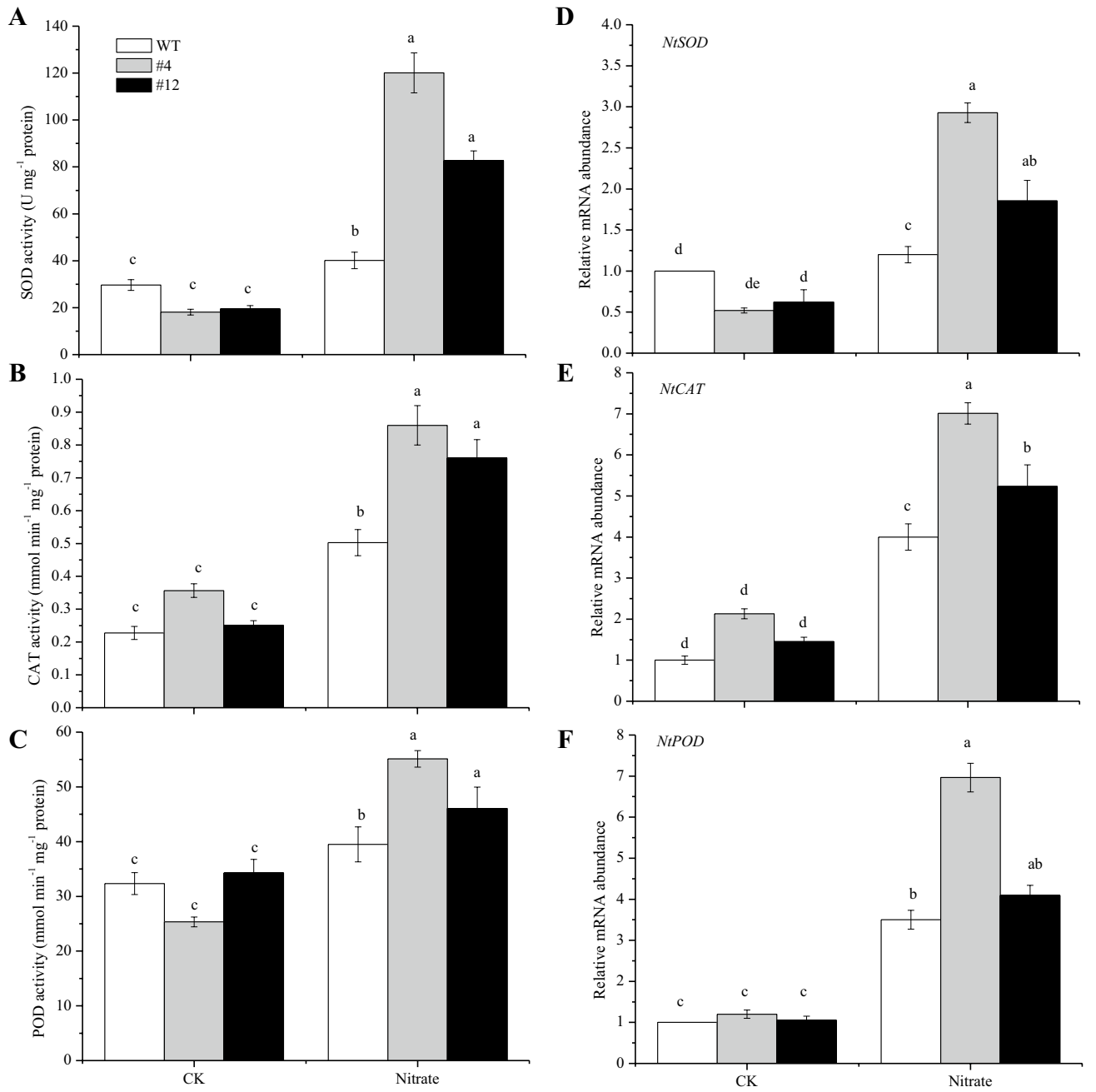

Fig. 7 Effect of excess nitrate stress on antioxidant enzyme activities and relative transcript levels of SOD, CAT and POD in *SoGSNOR*-transgenic plants and WT seedlings. Data are mean values ± SD of three independent experiments. Error bars with different letters indicate significant differences ($p < 0.05$)

cellular and macromolecular structures, reduce extensive membrane damage. In our study, the transcript level of *NtERD10D, NtERD10c, NtDREB2, NtDREB4*, *NtP5CS1, NtLEA5* increased under excess nitrate treatment in the transgenic plants, compared to the WT plants (Fig. 5), suggesting that *SoGSNOR*- overexpressing tobacco plants might enhance nitrate stress tolerance by increasing the transcript levels of these stress related genes.

GSNOR-mediated RNS levels are cross-linked with ROS homeostasis in plants (Rusterucci et al. 2007). Excessive accumulation of ROS is potentially harmful to cells and causes oxidative damage to proteins, DNA, and lipids (Apel and Hirt 2004). MDA, an important intermediate in ROS scavenging, is toxic to plant cells if it accumulates excessively, so it is often used as an indicator of oxidative attack on membrane lipids (Apel and Hirt 2004; Mittler et al.

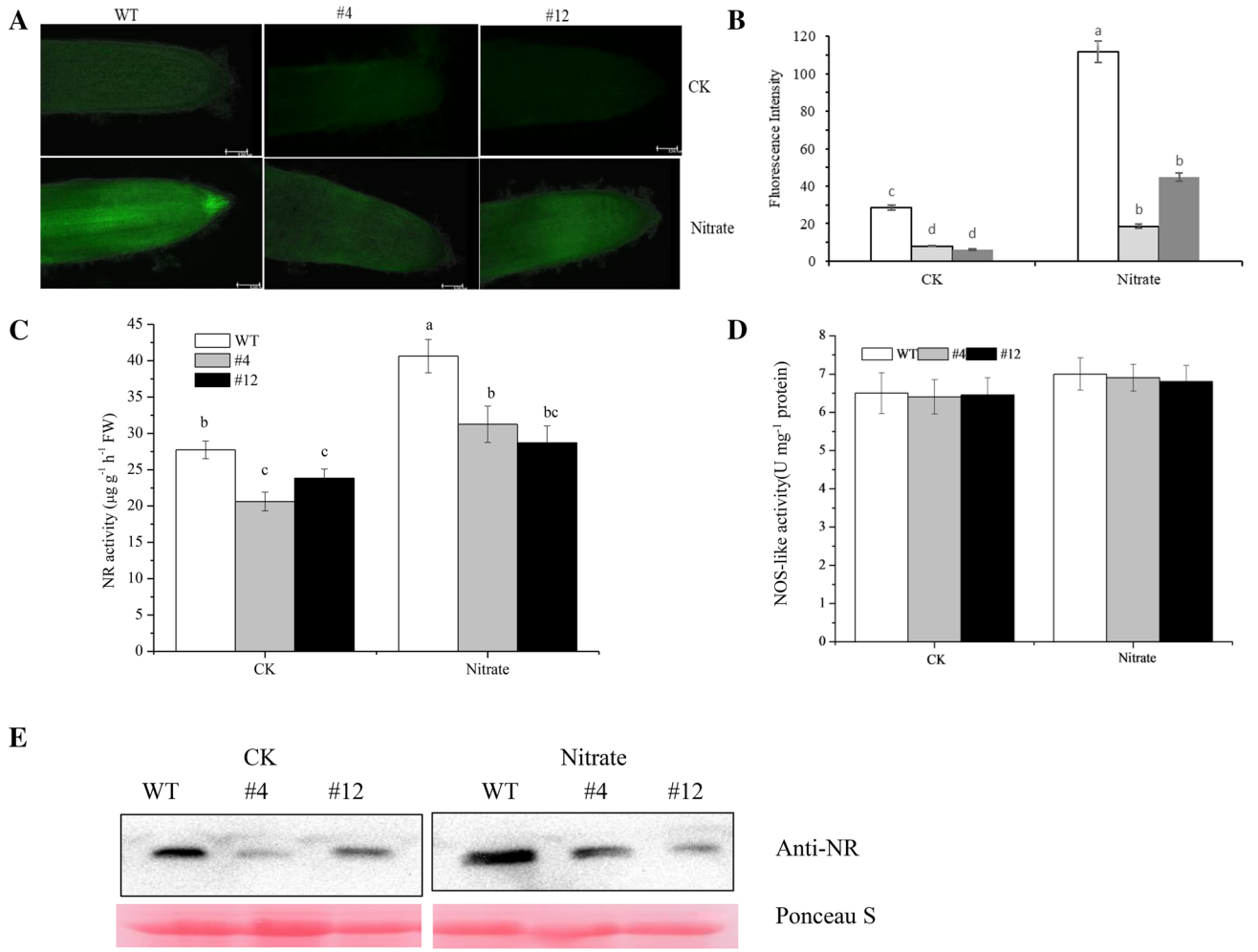

Fig. 8 Effect of excess nitrate stress on NO accumulation, NR and NOS-Like activities, NR protein level in *SoGSNOR*-transgenic and WT seedlings. **a** NO accumulation. The NO accumulation was measured using the fluorescent dye diaminofluorescein-FM diacetate and visualised in a fluorescence microscope and photographed. **b** NO production expressed as relative fluorescence. **c** NR activities. **d** NOS-Like activities. **e** NR protein level. Data are mean values ± SD of three independent experiments. The scale bar was 126 μm. Signal intensities of green fluorescence in the images were quantified using Image J software (http://rsb.info.nih.gov/ij/). For western blot, protein was stained with ponceau as loading control

2004). The MDA content and ROS fluorescence increased significantly in WT plants compared with the transgenic after nitrate stress (Fig. 6), which meant that nitrate stress produced more oxidative damage in WT tobacco plants. Increased oxidative stress were observed in wild *Solanum habrochaites,* together with increased GSNOR activity and reduced S-nitrosothiols under salinity stress (Jedelska et al. 2019). Plants have evolved efficient enzymatic and nonenzymatic detoxification mechanisms to scavenge ROS (Xu et al. 2013). Enzymatic ROS scavenging mechanisms in plants include SOD, CAT, glutathione peroxidase, ascorbate peroxidase, and peroxiredoxin (Apel and Hirt 2004; Mittler et al. 2004). The *SoGSNOR* transgenic plants have significantly higher transcript level and activities of SOD, CAT and POD than the WT plants after nitrate stress treatment (Fig. 7), indicating that *SoGSNOR* overexpression tobacco plants can enhance nitrate stress tolerance by removing the excess ROS through higher antioxidant enzyme activities. Previous studies showed that some ROS scavenging enzymes, including SOD, CAT, ascorbate peroxidase were S-nitrosylated to modulate its enzyme activities (Gong and Shi 2019). These results suggested the interactions between RNS- and ROS-mediated signaling pathways in stress tolerance.

NR and NOS are two key enzymes for NO synthesis in plants. In this study, the NR activities and protein levels were lower in the transgenic plants than the WT. The NOS-Like activities have no difference between WT and *SoGSNOR* transgenic plants under normal and nitrate stress condition (Fig. 8). Frungillo et al. (2014) reported that S-nitrosothiols regulate NO production and storage in plants through the

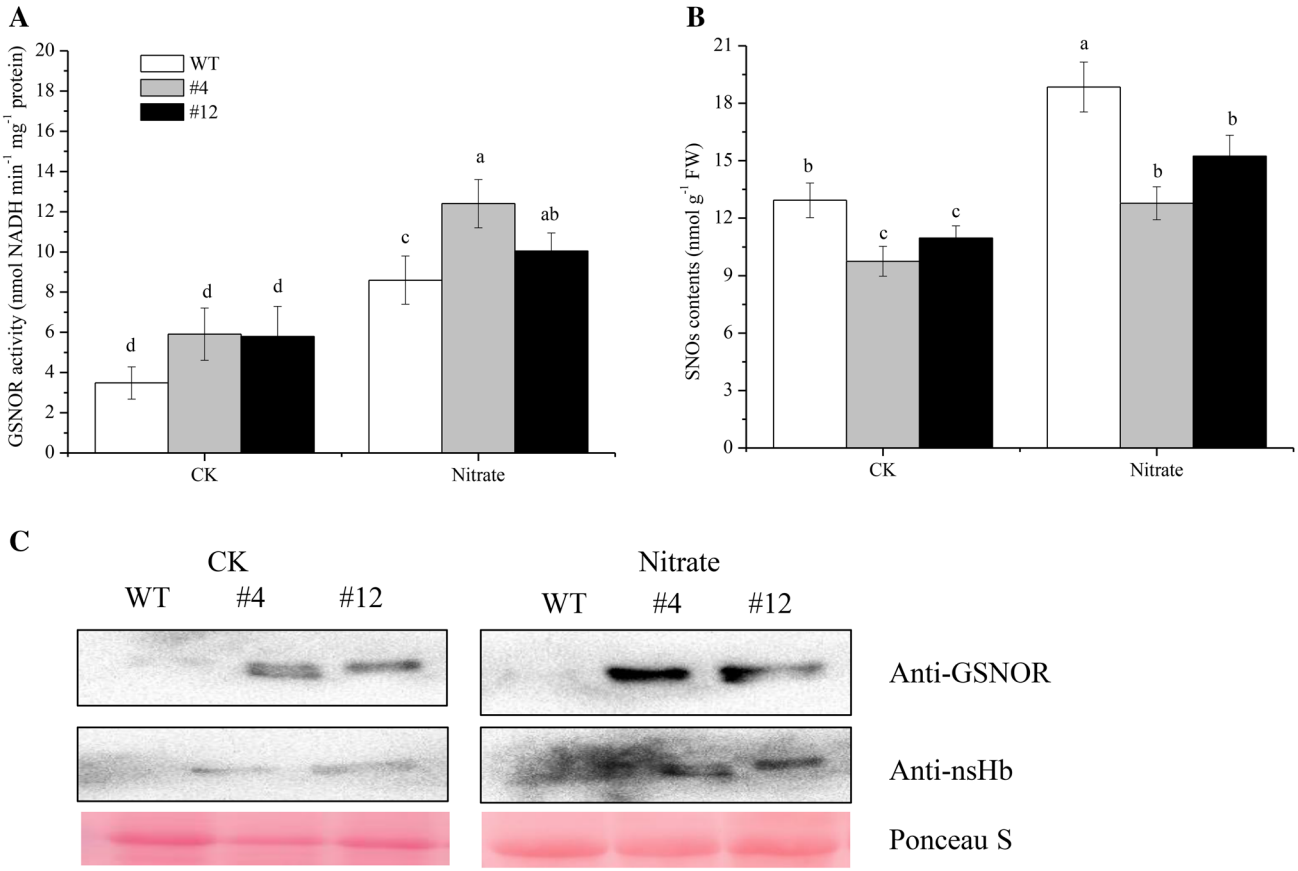

Fig. 9 Effect of excess nitrate stress on the GSNOR activity (**a**), SNOs contents (**b**), protein level of GSNOR and nsHb (**c**) in *SoGSNOR*-transgenic and WT seedlings. Data are mean values ± SD of three independent experiments. Error bars with different letters indicate significant differences ($p < 0.05$). For western blot, protein was stained with ponceau as loading control

nitrogen assimilation pathway. Our results indicated that NR might be the main enzyme of the production of NO, which was different from Zhao et al. (2007), who found that inhibition of root elongation in maize by high external nitrate is likely to result from a reduction of nitric oxide synthase-dependent endogenous NO levels in maize root apical cells.

GSNOR is a key enzyme which regulates intracellular levels of GSNO and indirectly also of protein SNOs, consequently, regulate cellular NO homeostasis (Ticha et al. 2017). SNOs play roles in signaling, transport and storage of NO. Salt stress has been demonstrated to elevate SNOs content in olive and sunflower seedling plants (Jain et al. 2018; Valderrama et al. 2007). The *Arabidopsis* mutant that is deficient in GSNOR activity is more sensitive to high temperature stress, because of high levels of SNOs (Lee et al. 2008). In this study, overexpression *SoGSNOR* enhanced the excess nitrate stress tolerance in transgenic tobacco with lower NO and SNOs contents than the WT plants (Fig. 9). Increased AtGSNOR1 activity reduced SNO formation, enhancing protection against ordinarily virulent microbial pathogens in *Arabidopsis* (Feechan et al. 2005). These results indicated that GSNOR exerts crucial roles in the homeostasis of NO and SNOs in plant cells.

Besides impacting NO homeostasis via S-nitrosylation reactions, plant Hbs can control developmental and physiological responses by modulating cellular NO levels (Hill 2012). *Arabidopsis* AtHb1 functions as a NO-dioxygenase, metabolizing NO to nitrate(Perazzolli et al. 2004). The control of NO homeostasis by hemoglobin gene expression has been reported to participate to nitrate sensing in maize roots (Trevisan et al. 2011). In our previous study, after nitrate treatment, the spinach nsHb expression induced and might control the endogenous NO contents (Zheng et al. 2016). *Mycorrhizal* fungi decrease NO content in Al-treated Medicago roots, probably via active NO scavenging system of GSNOR and Hb (Sujkowska-Rybkowska et al. 2018). In this study, the overexpression transgenic plants have higher nsHb1 expression, indicating that Hb might function in the scavenging of the excess NO accumulation.

Conclusion

In summary, we amplified the *SoGSNOR* gene from spinach root. The decreased SoGSNOR expression leads to NO accumulation under excess nitrate stress. In the *SoGSNOR*-overexpression transgenic plants, the higher expression of GSNOR and nsHb1 protein, and lower NR activity lead to lower NO and SNOs contents; the higher antioxidant enzyme activities lead to lower ROS accumulation. These results suggest that GSNOR enzyme appears to have an important role in the maintenance of ROS and RNS homeostasis.

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Author contributions H.N. X designed the project and wrote the article. M.Q.W, Y.Y. D conducted the experiments and analyzed the data. J.P.Y, Q.Q.H and K.Z. L helped in the experiments. All authors agreed on the final content of the article.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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