Carbon monoxide enhances salt tolerance by nitric oxide-mediated maintenance of ion homeostasis and up-regulation of antioxidant defence in wheat seedling roots

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ABSTRACT
Salt stress induced an increase in endogenous carbon monoxide (CO) production and the activity of the CO synthetic enzyme haem oxygenase (HO) in wheat seedling roots. In addition, a 50% CO aqueous solution, applied daily, not only resulted in the enhancement of CO release, but led to a significant reversal in dry weight (DW) and water loss caused by 150 mM NaCl treatment, which was mimicked by the application of two nitric oxide (NO) donors sodium nitroprusside (SNP) and diethylenetriamine NO adduct (DETA/NO). Further analyses showed that CO, as well as SNP, apparently up-regulated H⁺-pump and antioxidant enzyme activities or related transcripts, thus resulting in the increase of K/Na ratio and the alleviation of oxidative damage. Whereas, the CO/NO scavenger haemoglobin (Hb), NO scavenger or synthetic inhibitor methylene blue (MB) or N⁶-nitro-L-arginine methyl ester hydrochloride (L-NAME) differentially blocked these effects. Furthermore, CO was able to mimic the effect of SNP by strongly increasing NO release in the root tips, whereas the CO-induced NO signal was quenched by the addition of L-NAME or cPTIO, the specific scavenger of NO. The results suggested that CO might confer an increased tolerance to salinity stress by maintaining ion homeostasis and enhancing antioxidant system parameters in wheat seedling roots, both of which were partially mediated by NO signal.

Key-words: Triticum aestivum; antioxidant enzymes; carbon monoxide/heme oxygenase; K/Na ratio; nitric oxide signal; salt stress.

INTRODUCTION
Salinity is one of the most serious plant growth stresses in the world, and has become a major problem for the successful use of salt-affected soils for crop production. Hence, methodologies to use saline soils have received worldwide attention. In fact, plants can respond and adapt to salinity stress by altering their cellular metabolism and invoking various defence mechanisms (Zhu 2001). It was well-known that unfortunate consequences of salinity stress in plants are the growth inhibition and ionic phyto-toxicity, as well as the excessive generation of reactive oxygen species (ROS), which includes superoxide radical, hydrogen peroxide, singlet oxygen and hydroxyl radical. The overproduction of ROS during salinity stress which was usually derived from impaired electron transport processes in chloroplast and mitochondria, as well as from some metabolic pathways such as fatty acid oxidation, often resulted in an increasing oxidative burden, thus leading to the damage of lipids, proteins and even DNA. Because ROS are toxic but also act as signalling molecules to mediate various key physiological processes, plant cells possess both enzymatic and non-enzymatic mechanisms for regulating the overproduction of ROS. It has been proposed that changes observed in the levels of the transcripts of antioxidative enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR), and the activities of their isozymes in response to salinity stress may be caused, at least in part, by the altered metabolic activities of cells that lead to changes in ROS levels (Hernández et al. 1993, 2000; Sreenivasulu et al. 2000; Mittler 2002).

Several lines of evidence from biochemical and genetic studies have shown that the tolerance to salinity stress in plants is partially correlated with a more efficient antioxidant system, and most importantly is contributed to the maintenance of ion homeostasis, such as low sodium (Na⁺) and high potassium (K⁺) in the plant cells (Zhu 2003). Recent studies also showed that nitric oxide (NO), an endogenous signalling molecule in animals and plants, mediates multiple responses to abiotic and biotic stresses. In fact, although the gene(s) encoding NO synthase (NOS) protein, the NO synthetic enzyme proven in mammalian...
cells, remained to be identified in plants (Crawford et al. 2006; Zemojtel et al. 2006). NOS-like activity has been detected widely in plants, and inhibitors of mammalian NOS also inhibit NO generation in plant cells (Delledonne et al. 1998; Zhao, Tian & Zhang 2007). In addition, nitrate reductase (NR) is another enzyme responsible for NO synthesis in plants (Delledonne 2005). Interestingly, besides the activation of ROS scavenging systems (Ruan et al. 2002; Uchida et al. 2002), NO also serves as a signal in inducing plant salt tolerance by increasing the K-to-Na ratio, which was not only dependent on the increased plasma membrane H^+/H^+ -ATPase activity, but also the activation of vacuolar (V) H^+/H^+ -ATPase and H^+/PPase enzymes, thus providing the driving force for Na^+/H^+ exchange (Zhao et al. 2004; Zhang et al. 2006). More recently, Zhao et al. (2007) provided strong genetic evidence that NOS-dependent NO production in plant cells is associated with salt stress tolerance of Arabidopsis.

Carbon monoxide (CO), usually created by incomplete combustion of organic materials, has been known for a long time that produces asphyxia by reversibly combining with haemoglobin (Hb). CO is also the component of cigarette smoke and fumes produced by automobiles. Stirringly, recent investigations of animal tissues have demonstrated that CO, which was mainly generated by haem oxygenase enzymes (HOs; EC 1.14.99.3) in vivo, was known to play a major role as neurotransmitter, regulator of sinusoidal tone, inhibitor of platelet aggregation and suppressor of acute hypertensive response, and most of these effects were similar to or mediated by NO (Verma et al. 1993; Lamar, Mahesh & Brann 1996; Longo et al. 1999; Dulak & Józkowicz 2003; Watts, Ponka & Richardson 2003). Additionally, administration of a low concentration of CO gas has been proven to provide potent cytoprotective effects in the mouse, including attenuation of lung lipid peroxidation induced by oxidant (Otterbein et al. 2003).

Besides CO, HOs have been shown to catalyse the oxidative conversion of haem to biliverdin (BV) with the concomitant release of iron. In animals, there are three forms of HOs. HO-1 is inducible, while the constitutively expressed HO-2 and HO-3 display very low activity. A lot of evidence has accumulated, indicating that HO and its products such as BV might serve as key biological molecules in the adaptation and/or defence against oxidative stress (Vile & Tyrrell 1993; Abraham et al. 1995; Choi & Alam 1996; Shibahara, Kitamura & Takahashi 2002). While in plants, HO-1 has only recently been reported to play an important protective role against oxidative cell damage induced by cadmium (Cd) stress and ultraviolet-B irradiation in soy bean leaves (Noriega et al. 2004; Yamarelli et al. 2006). It is also known for its association with phytochrome and leghaemoglobin metabolisms (Davis et al. 2001; Muramoto et al. 2002; Baudouin et al. 2004; Emborg et al. 2006). Ballestrasse et al. (2006) showed that the signal transduction pathways involved in oxidative stress triggered by Cd ions were similar to those implicated in HO induction, and thus suggested a close relationship between oxidative stress generation and HO induction in higher plants.

More recently, we demonstrated that the substrate/inducer of HO hematin (a haem molecule, also termed as the CO donor in animal physiological research investigations; Lamar et al. 1996; Longo et al. 1999), and even CO aqueous solution dose-dependently alleviated oxidative damage in wheat seedling leaves and germinating rice seeds (Huang et al. 2006; Liu et al. 2007). In view of the fact that the by-product of HO BV was also involved in the antioxidant defence systems both in animals and plants (Maines 1997; Shibahara et al. 2002; Noriega et al. 2004), the possibility of the direct attenuation of oxidative stress by BV under the administration of hematin could not be easily ruled out. Additionally, another product of HO, CO can be stimulated by the different stress responses, including heat shock, oxidants, metals, lipopolysaccharide, hypoxia and hyperoxia in animals (Plantadosi 2002). However, although the presence of CO biosynthesis in plants was firstly reported by Wilks (1959), and HO was claimed as its potential source (Muramoto et al. 2002), the experimental evidence for CO as a signal or bio-effector molecule in plant tissues under multiple environmental stresses is still quite limited.

In this paper, the wheat cultivar Yangmai 158 used in our experiments, displays a moderate tolerance to 150 mM NaCl stress in comparison with other more sensitive cultivars (e.g. Chinese Spring). We firstly discovered that NaCl treatment induced an increase in CO accumulation in wheat seedling roots, which was consistent with the changes in the activities of the CO synthetic enzyme HO. Exogenous application of 50% CO-saturated aqueous solution could result in the enhancement of endogenous CO release. Furthermore, we elucidate why, when directly treated with 50% CO-saturated aqueous solution, as well as two NO donor sodium nitroprusside (SNP) and diethylenetriamine NO adduct (DETA/NO), wheat seedlings exhibited more tolerance to salt stress. Evidence is also provided to show that wheat seedling roots treated daily with exogenous CO could synthesize NO, which suggests that NO may be part of the downstream signal molecule of above CO action, and similar result has been shown to occur in animal tissues in recent years (Thom, Xu & Ischiropoulos 1997; Thom et al. 2000; Hartsfield 2002; Dulak & Józkowicz 2003).

**MATERIALS AND METHODS**

**Plant material and growth condition**

Selected seeds of wheat (Triticum aestivum L., Yangmai 158) were detoxified in 2% NaClO for 5 min, cleaned in distilled water and germinated at 25 °C in the dark for 1 d. Then, the identical buds were selected and moved into the rectangular boxes in a growth chamber (12 h light period, 25 °C, humidity 50 ± 4%; 12 h dark period, 18 °C, humidity 56 ± 5%, MGC-300B, Shanghai Yiheng Technology Co., Ltd., Shanghai, China) with modified Hoagland solution containing 3 mM KNO₃, 1 mM NH₄H₂PO₄, 0.5 mM MgSO₄, 5.5 mM Ca(NO₃)₂, 50 mg of FeEDTA per litre (10% iron), 25 μM KCl, 12.5 μM H₃BO₃, 1 μM MnSO₄, 1 μM ZnSO₄,
0.25 μM CuSO₄ and 2 μM H₂MoO₄ (Gulick & Dvořák 1987). The irradiance was approximately 300 μmol m⁻² s⁻¹ provided by fluorescent lamps. The culture solution was renewed every other day until two fully expanded leaves appeared.

Chemicals

All chemicals were obtained from Sigma (St Louis, MO, USA) unless otherwise stated. Two NO donors, SNP and DETA/NO were used at 0.1 or 0.05 mM (García-Mata & Lamattina 2001; Zhang et al. 2001; Lee et al. 2008). Hb (1.5 g L⁻¹), obtained from Shanghai Boao Ltd., Shanghai, China, was chosen as the scavenger of CO/NO (Morita et al. 1995; Lamar et al. 1996; Han et al. 2008). N⁵-nitro-L-arginine methyl ester hydrochloride (L-NAME, 0.4 mM) was used as the inhibitor of NOS. N⁵-nitro-D-arginine methyl ester hydrochloride (D-NAME, 0.4 mM) was used as an inactive stereoisomer of L-NAME (Maier et al. 2001). Methylene blue (MB, 0.1 mM), which inhibits NO production and/or action (Gouvêa et al. 1997; Zhang et al. 2006), was used as NO scavenger. NO₂⁻/NO₃⁻ (50/50 μM) and KFe₃(CN)₆/KFe₄(CN)₉ (50/50 μM) were used as the degradation product of SNP and SNP analogue (Bethke et al. 2006; Shi et al. 2007). The compound 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (pPTIO) was used at 0.2 mM as a specific NO scavenger. NO-specific fluorophore 4,5-diaminofluorescein diacetate (DAF-2 DA) and the negative probe 4-amino fluorescein diacetate (AF 4-DA) were purchased from Calbiochem (San Diego, CA, USA), both used at a final concentration of 10 μM (Pagnussat, Lanteri & Lamattina 2003; Correa-Aragunde, Graziano & Lamattina 2004).

CO-saturated aqueous solution preparation

CO gas was prepared by heating concentrated sulphuric acid (H₂SO₄) with formic acid (HCOOH) at the speed of 3–5 s per drop. In our experiment, CO-saturated aqueous solution was freshly obtained by bubbling above CO gas gently through a glass tube into 300 mL of above Hoagland solution in the presence of 150 mM NaCl for about 15 min, a duration time long enough to make the solution saturated with CO. Then, the saturated stock solution (100% of saturation) was immediately diluted with freshly prepared Hoagland solution in the presence of 150 mM NaCl to the concentration the experiment required (1, 10 and 50% of saturation).

CO production determined by gas chromatography and mass spectrometry (GC–MS)

CO production was determined by GC–MS according to the method described by Anderson & Wu (2005) and Liu et al. (2007). In our experimental conditions, the concentration of CO in the saturated stock solution was about 187 μM. Meanwhile, the half-life of CO loss from the stock solution at 30 °C was about 210 min.

HO preparation and activity determination

Preparation of crude membrane fractions and detection of HO activity were determined by the method of Liu et al. (2007) and Han et al. (2008). HO activity was calculated by measuring the formation of BV-IXα (BV). The concentration of BV was estimated using a molar absorption coefficient at 650 nm of 6.25 mm⁻¹ cm⁻¹ in 0.1 M HEPES–NaOH buffer (pH 7.2). One unit of activity (U) was calculated by taking the quantity of the enzyme to produce 1 nmol BV at 37 °C per 30 min.

Treatments

According to our previous work (Ruan et al. 2002), salinity treatment was established by adding 150 mM NaCl to the nutrient solution immediately after two fully expanded leaves appeared. Firstly, the primary experimental design consisted of a control (Con, only Hoagland solution without the addition of NaCl and other chemicals) and five treatment groups (S, Con + 150 mM NaCl; S + 100% CO, Con + 150 mM NaCl + 100% CO-saturated aqueous solution; S + 50% CO, Con + 150 mM NaCl + 50% CO-saturated aqueous solution; S + 10% CO, Con + 150 mM NaCl + 10% CO-saturated aqueous solution; S + 1% CO, Con + 150 mM NaCl + 1% CO-saturated aqueous solution). We further chose 50% CO-saturated aqueous solution containing 150 mM NaCl as the suitable treatment because of its better positive results determined by thiobarbituric acid-reactive substances (TBARS) content compared with other treatments (data not shown).

The further experimental design thus consisted of the following treatment groups: (1) Con, only Hoagland solution without the addition of NaCl and other chemicals; (2) S, Con + 150 mM NaCl; (3) S + 50% CO, Con + 150 mM NaCl + 50% CO-saturated aqueous solution; (4) S + 50% CO + Hb, Con + 150 mM NaCl + 50% CO-saturated aqueous solution + 1.5 g L⁻¹ Hb; (5) S + Hb, Con + 150 mM NaCl + 1.5 g L⁻¹ Hb; (6) S + 50% CO + L-NAME, Con + 150 mM NaCl + 50% CO-saturated aqueous solution + 0.4 mM L-NAME; (7) S + L-NAME, Con + 150 mM NaCl + 0.4 mM L-NAME; (8) S + 50% CO + D-NAME, Con + 150 mM NaCl + 50% CO-saturated aqueous solution + 0.4 mM D-NAME; (9) S + D-NAME, Con + 150 mM NaCl + 0.4 mM D-NAME; (10) S + 0.1 SNP, Con + 150 mM NaCl + 0.1 mM SNP; (11) S + DETA/NO, Con + 150 mM NaCl + 0.05 mM DETA/NO; (12) S + KFe(CN)₆/KFe(CN)₉, Con + 150 mM NaCl + 0.05 mM KFe(CN)₆ + 0.05 mM KFe(CN)₉; (13) S + NO₂⁻/NO₃⁻, Con + 150 mM NaCl + 0.05 mM NaNO₂ + 0.05 mM NaNO₃; (14) S + 0.1 SNP + MB, Con + 150 mM NaCl + 0.1 mM SNP + 0.1 mM MB; (15) S + 50% CO + 0.1 SNP, Con + 150 mM NaCl + 50% CO-saturated aqueous solution + 0.1 mM SNP; (16) S + 50% CO + 0.1 SNP + MB, Con + 150 mM NaCl + 50% CO-saturated aqueous solution + 0.1 mM SNP + 0.1 mM MB.
Relative shoot and root growth, plant growth and relative water content (RWC; %)

Relative shoot and root growths were measured following the method described by Katsuhara & Kawasaki (1996). Relative shoot or root growth was expressed as a percentage of control (Con), which regarding shoot or root growth rate of the control as 100%. Plant growth was also expressed as dry weight (DW) of the samples. Thirty individual wheat seedlings, after 14 d of treatment per treatment group and repeated in three independent experiments with five replicated measurements, were divided into shoot and root parts, and dried in an oven respectively at 75 °C to constant weight. RWC (%) was also investigated according to the method described by García-Mata & Lamattina (2001).

TBARS and PM permeability

TBARS and PM permeability were estimated as described by Zhao et al. (2004, 2007).

Antioxidant enzyme assays

Frozen wheat seedling root segments (0.5 g) were homogenized in 10 mL of 50 mm potassium phosphate buffer (pH 7.0) containing 1 mm ethylenediaminetetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP) for SOD and glutathione reductase (GR) assay, or combination with the addition of 1 mm ascorbate acid (ASC) in the case of APX, monodehydroascorbate reductase (MDHAR) and DHAR assay. The homogenate was centrifuged at 12,000 g for 20 min at 4 °C, and the supernatant was immediately used for the following antioxidant enzyme determinations.

APX (EC 1.11.1.11) activity was determined by monitoring the decrease in A320 (Nakano & Asada 1981). Total SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) following the method described by Giannopolitis & Ries (1977). GR (EC 1.6.4.2) activity was determined by following the oxidation of NADPH at 340 nm (Jiang & Zhang 2001). DHAR (EC 1.8.5.1) activity was performed by measuring the formation of ASC at 265 nm (De Tullio et al. 1998). MDHAR (EC 1.6.5.4) activity was assayed by monitoring the change in absorbance at 340 nm because of NADPH oxidation (Jiang & Zhang 2001).

Native gradient PAGE, SOD activity staining

Native gradient PAGE (5–20%) was performed for 15 h at 4 °C at a constant voltage of 150 V in Tris–Gly buffer, pH 8.3. For each lane, 30 μg of protein extract was applied. SOD activity on the gel was visualized by activity staining according to the procedure described by Beauchamp & Fridovich (1971). For the inhibition of H2O2 towards Cu/Zn–SOD and Fe–SOD (in fact, none Fe–SOD isozyme was found in wheat roots; Biemelt et al. 2000), the gels were first soaked in 5 mm H2O2 in a 100 mm potassium phosphate buffer, pH 7.0, for 30 min, followed by SOD activity staining. All staining procedures were carried out at room temperature, and the reaction mixture containing gels was shaken at 75 rpm.

H+–ATPase and PPase activity measurement

PM and tonoplast vesicles were prepared by discontinuous sucrose gradient centrifugation methods, following the method described by Yoshida, Matsuura & Etani (1989). The purity of the isolated PM and tonoplast vesicles was determined by examining H+–ATPase activity in the absence and presence of inhibitors according to the method of Kondoh et al. (1998). The PM H+–ATPase belongs to P-type characterized by vanadate inhibition; those in tonoplast and mitochondria belong to V- and F-types identified by nitrate and azide, respectively. In the present study, the enzyme activities of isolated PM and tonoplast vesicles were inhibited by vanadate or nitrate more than 75 and 70%, and both were reduced by other inhibitors less than 10%, demonstrating the vesicles collected were PM and tonoplast enriched. Finally, the H+–ATPase and H+–PPase activities were determined by quantifying the inorganic phosphate that was released from ATP and pyrophosphate, respectively (Ohnishi, Gall & Mayer 1975).

Protein content determination

Protein concentration was determined by the method of Bradford (1976) using BSA as the standard.

Total RNA isolation and semi-quantitative RT-PCR

For semi-quantitative RT-PCR, total RNA was isolated from 100 mg of fresh-weight wheat seedling root samples grinding with mortar and pestle in liquid nitrogen until a fine powder appeared and using Trizol reagent (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer’s instructions. DNA-free total RNA (5 μg) from different treatment times was used for first-strand cDNA synthesis in a 20 μL reaction volume containing 2.5 units of AMV
reverse transcriptase XL (Takara, Dalian, Liaoning, China) and 1 μM of Oligo-dT primer. PCR reactions were performed using 2 μL of a twofold dilution of the cDNA, 10 pmol of each oligonucleotide primer and 1 U of Taq polymerase (Takara) in a 20 μL reaction volume. To verify the exponential phase of PCR amplification, a different number of amplification cycles ranging from 20 to 35 was tested for each gene. Different gene-specific primers used were: APX (accession number CV066046) forward 5'-TGGGAGAAGTGCACCAAGGAGAG-3' and reverse 5'-CCAACGGCATATTACAGAGA-3' (amplifying a 380 bp fragment), DHR (accession number AY074784) forward 5'-CACGTCGGCCGACTGTCCTTT-3' and reverse 5'-CCCCCGGGGATCGGTCTTT-3' (amplifying a 578 bp fragment), Mn-SOD (accession number AF092524) forward 5'-ACCCAGACCGACCCAGGACC-3' and reverse 5'-GGCTCCCAAGACATCAATTCCCAAACAA-3' (amplifying a 417 bp fragment), Cu/Zn-SOD (accession number U69632) forward 5'-CTCCTCTTTCAAGCTTCTGGC-3' and reverse 5'-ATGAACAACAAAGCTCTCCC-3' (amplifying a 465 bp fragment), PM H⁺-ATPase (accession number AY543630) forward 5'-ATCGTCTCGTGATGCCTTGCTT-3' and reverse 5'-AGCCTCTCTACAGGCTTCTCCC-3' (amplifying a 1093 bp fragment), V H⁺-ATPase (accession number DQ344624) forward 5'-GTTCGGAGAAAAATTTGAGT-3' and reverse 5'-GAGAAATGTTGCGAGTAGAC-3' (amplifying a 337 bp fragment) and 18s rRNA (accession number AJ272181) forward 5'-CAAGGCTATCGATGTTGATAATTTC-3' and reverse 5'-CTGTTATTGCCTCAAATTC-3' (amplifying a 658 bp fragment). To standardize the results, the relative abundance of 18s rRNA was also determined and used as the internal standard. After 26 PCR cycles for 18s rRNA and 25–32 cycles for others with a primer annealing temperature at 47–54 °C, 10 μL samples of the PCR reaction products were fractionated by 1.2% (w/v) agarose gel electrophoresis and stained with ethidium bromide. Specific amplification products of the expected size were observed, and their identities were sequencing.

Determining of ion content

Roots of different treated seedling samples were harvested during 12 d after treatment. Then, root parts were oven dried for 48 h at 70 °C. The dried sample (100 mg) was heated in 10 mL of 1.4 M HNO₃ on a hot plate (100 °C) for 30 min. After cooling, the suspension was diluted 100 times with distilled water and filtered through a 0.25 μm pore size membrane filter. Na and K contents were measured by an atomic emission spectrophotometer (TAS-986; Beijing Purkinje General Instrument Co., Ltd., Beijing, China) (Rauser 1987).

X-ray microanalysis

Element ratio measurement was examined with a scanning electron microscope (SEM) (model S-3000N; Hitachi High-Technologies Corporation, Tokyo, Japan) equipped with an energy-dispersive X-ray detector (EDX; Horiba Inc., Kyoto, Japan) as described by Vázquez et al. (1999) and Zhao et al. (2004) with some modifications. After being placed vertically in the hole of an aluminium stub and immersed quickly into liquid nitrogen for freeze-drying, the tender root tips were transferred quickly into a vacuum evaporator and dried under the vacuum of 5 × 10⁻⁴ Torri for at least 12 h. The root tips were coated with a fine layer of pure evaporated carbon, and then observed. Acceleration voltage of 10 kV was used. Beam current was adjusted to a fixed (0.06 μA), and the working distance from the EDX detector was 13.5 mm. For analyses of relative elemental levels within cortical and stelar cells of root tips accurately, point analysis of each sample was conducted for at least four times. The results were calculated by expressing the atomic number for a particular element in a given point or region as a percentage of the total atomic number for all the elements measured (K, Na, calcium, magnesium, phosphorus, sulphur and chlorine) in the root tips.

Visualization of endogenous NO

Endogenous NO was monitored using either 10 μM of the positive fluorescent probe DAF-2 DA or the negative probe AF 4-DA. Roots were loaded with DAF-2 DA or AF 4-DA in 20 mM HEPES buffer (pH 7.8) for 30 min, washed in HEPES buffer three times for 15 min and then root tip parts were examined by a TCS-SP2 confocal laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany; excitation with the 488 nm, and emission using a 500–530 nm). Pictures without the addition of probe were also taken. Experiments were repeated six times, and similar results were obtained. All manipulations were performed at 25 ± 1 °C.

NO production determined by using Greiss reagent

NO production was determined using the method described by Zhou et al. (2005). Absorbance was assayed at 540 nm; NO content was calculated by comparison to a standard curve of NaNO₂.

Statistical analysis

Data are the means ± SE from at least three independent experiments. For statistical analysis, t-test (P < 0.05 or 0.01) or Tukey’s multiple range test (P < 0.05) was chosen where appropriately.

RESULTS

Salt stress causes the enhancement of CO release and NO activity in wheat seedlings roots

Since 1959, there have been several reports on the photoproduction of CO from living plants, which increases linearly with solar actinic irradiance and CO₂/O₂ ratio in the ambient.
atmosphere (Wilks 1959; Lüttge & Fischer 1980; Tarr, Miller & Zepp 1995). Interestingly, germinating seeds of rye, pea, cucumber and lettuce also produced CO at levels of 10–25 ppm, and neither light nor chlorophyll was necessary for above CO formation (Siegel, Renwick & Rosen 1962). Our findings shown in Fig. 1 extended these observations. When wheat seedlings were treated with 150 mm NaCl, CO production was measured by using GC–MS. In comparison with the control sample (Con), the time course experiments for 120 h illustrated that a fast burst of endogenous CO release at 12 h with salt stress treatment (S), then followed by a gradual decrease and reached another peak at 72 h (Fig. 1a). Interestingly, the changes in CO production are also similar to the changes in the activities of the CO synthetic enzyme HO (Fig. 1b). Thus, there might be potentially significant increases in endogenous CO and HO activity, both of which might be deduced as a part of the signalling cascade for plant salt tolerance.

CO treatment enhances the adaptive responses against salt stress
As expected, our further results showed that, in comparison with the salt-stressed sample alone, the application of exogenous 50% CO-saturated aqueous solution (S + 50% CO) could result in a sharp induction of CO production ($P < 0.01$) during an initial 3 h of treatment, then declined at 12 h and enhanced again for a prolonged period of treatment time (up to 72 h; Fig. 1a). When the CO/NO scavenger Hb was added together, the increase of CO production induced by salt stress in the presence or absence of 50% CO-saturated aqueous solution for 3 and 12 h was reversed by 86.7 and 82.1, 69.6 and 89.6%, respectively, further indicating that the majority change is CO specific, and Hb was able to scavenger CO in vitro as well as in vivo (Fig. 1c). We also noticed that the changes in the CO release except the first sharp spike were approximately similar to those for HO activity (Fig. 1a,b). These results further indicated that the first sharp spike might be only derived from the exogenous CO applied.

It was well-known that salinity stress typically inhibits plant growth and reduces the yield of many plant species. In our experimental conditions, after wheat seedlings were grown at 150 mm NaCl stress, severe symptoms of NaCl toxicity, including the growth inhibition and severe lodging (Table 1; Fig. 2), appeared in salt-stressed wheat plants (S) compared to the control sample (Con). Furthermore, a possible effect of CO on growth of wheat seedlings subjected to...
Plant dry weight (DW) or RWC (%) reflects the growth inhibition caused by salt stress and the protective role of CO or NO. Data are the means ± SE of three different experiments with five replicated measurements. Significantly different compared with salt-stressed sample (S) at \( P < 0.05 \) or 0.01 level according to t-test.

150 mM NaCl was tested. As shown in Table 1, the significant reduction \( (P < 0.05) \) in the amount of growth inhibition (DW of shoot and root parts) and water loss was observed in 50% CO-saturated aqueous solution-treated wheat seedlings \( (S + 50\% \) CO) compared to salt treatment alone. After the treatment of exogenous 50% CO-saturated aqueous solution for 14 d, the growth inhibition of wheat seedling shoot and root parts was alleviated by 65.6 and 57.1%; RWC attenuated by 77.8 and 66.7%. Combination of NaCl with NO donor SNP \( (0.1 \text{mM}, S + 0.1 \text{SNP}) \) or DETA/NO \( (0.05 \text{mM}, S + \text{DETA/NO}) \) treatment exhibited the similar positive effects. To attribute a specific role of NO in

**Table 1.** Effects of 50% carbon monoxide (CO)-saturated aqueous solution, nitric oxide (NO) donors sodium nitroprusside (SNP) and diethylenetriamine NO adduct (DETA/NO), SNP analogue K₃Fe(CN)₆/K₄Fe(CN)₉, SNP degradation product NO::/NO₃, NO synthase (NOS) inhibitor N⁰-nitro-L-arginine methyl ester hydrochloride (L-NAME) and inactive stereoisomer N⁰-nitro-D-arginine methyl ester hydrochloride (D-NAME) treatments on the growth and relative water content (RWC) (%) of 30 wheat seedling plants exposed to salt stress at 14 d

![Graphs](image-url)

**Figure 2.** Effects of 50% carbon monoxide (CO)-saturated aqueous solution and CO/NO scavenger haemoglobin (Hb) on the seedling growth (a), relative shoot (b) and root (c) growth of salt-stressed wheat plants for 5 d. Wheat seedlings were grown in Hoagland solution in the presence of 150 mM NaCl with or without 50% CO-saturated aqueous solution and/or 1.5 g L⁻¹ Hb. Plants without NaCl treatment were the control (Con). Shoot or root growth of the control (Con) is regarded as 100%. Data are the means ± SE from three independent experiments. Bars with different letters are significantly different at \( P < 0.05 \) according to Tukey’s multiple range test.
regulating wheat seedling growth, several controls such as NO₃⁻/NO₂⁻ and K₂Fe(CN)_6/K₃Fe(CN)_6 were set. Contrary to the effects of SNP, we discovered that the control chemicals exhibited the negative responses (Table 1).

Furthermore, a significant alleviation of relative shoot and root growth inhibition, as well as the resistance to lodging by CO, was observed (P < 0.05; Fig. 2). For example, salt-stressed plants treated with CO (S + 50% CO) for 48 h exhibited the significantly greater shoot or root elongation as compared with the plants without CO treatment (S, P < 0.05). In mammals, local effects of CO in animals may derive from its interaction with NO (Hartsfield 2002). Thus, to establish a link between CO and NO in salt tolerance signalling, salt-stressed wheat seedlings were treated with either 50% CO-saturated aqueous solution or SNP in the presence or absence of NOS inhibitor L-NAME (0.4 mm) or the inactive stereoisomer d-NAME (0.4 mm), MB (0.1 mm), which inhibits NO production and/or action in plants (Zhang et al. 2006).

Furthermore, we discovered that the relief of salinity toxicity by CO aqueous solution was further proven and partially related to NO, because the CO/NO scavenger Hb (1.5 g L⁻¹, S + 50% CO + Hb) or NOS inhibitor L-NAME treatments (S + 50% CO + L-NAME) differentially abolished these observed effects of CO (Fig. 2; Table 1). Meanwhile, although Hb (S + Hb) and L-NAME (S + L-NAME) approximately exhibited the same effects in comparison with the salt-stressed sample alone (S), the combination with L-NAME or Hb treatment resulted in the further aggravation of the growth inhibition of seedling shoot parts (Table 1; Fig. 2b). However, in comparison with L-NAME, the inactive stereoisomer d-NAME produced weaker effects (Table 1). Collectively, these results suggested that CO, as well as NO, be responsible for the enhancement of adaptive responses of wheat seedlings against salt stress, and endogenous NO presumably produced by putative NOS-like protein might be involved in these processes.

Lipid peroxidation and PM permeability

The supplementation with 50% CO-saturated aqueous solution alleviates salt-induced lipid peroxidation approximately in a time-dependent manner in wheat seedling roots (Fig. 3a) and leaves (data not shown), a similar response to that obtained with the NO donor SNP in plant tissues (Ruan et al. 2002; Uchida et al. 2002). Salt stress (S) for 120 h largely increased TBARS levels compared with the control group (Con), whereas this accumulation was markedly reduced by the application of 50% CO-saturated aqueous solution. Conversely, the CO-induced alleviation of TBARS level was noticeably blocked by Hb, the scavenger of CO/NO. Interestingly, Hb enhanced TBARS content caused by exposure to salt stress alone only for the first 12 h, suggesting that early treatment period of seedling roots with salt stress and Hb simultaneously has further detrimental impacts on lipid peroxidation.

To further assess the beneficial effects of CO, the PM permeability was measured, as it is another reliable marker of oxidant-induced tissue injury (Fig. 3b). Similarly, wheat seedlings exposed to 150 mM NaCl salt stress (S) for 120 h exhibited the increased PM permeability compared to the control samples (Con), which was significantly reduced (P < 0.01) in root tissues in the presence of 50% CO-saturated aqueous solution (S + 50% CO). Contrary to these observations, the combination with Hb treatment (S + 50% CO + Hb) resulted in a clear cut in the CO-inducing actions. In addition, Hb treatment (S + Hb) exhibited a slight effect on the induction of PM permeability as compared with the salinity-treated samples (S) during the first 12 h of treatment, which was consistent with the change

Figure 3. Effects of 50% carbon monoxide (CO)-saturated aqueous solution and CO/NO scavenger haemoglobin (Hb) on thiobarbituric acid-reactive substances (TBARS) content in salt-stressed wheat seedling roots (a). Time-course of changes in plasma membrane (PM) permeability of salt-stressed wheat seedlings roots exposed to 50% CO-saturated aqueous solution in the presence or absence of Hb treatments (b). Plants without NaCl treatment were the control (Con). Data are the means ± SE from at least three independent experiments. * and ** indicate significantly different between 50% CO-saturated aqueous solution plus salt-treated sample (S + 50% CO) and salt-stressed sample alone (S) at P < 0.05 or 0.01 levels according to t-test.

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of TBARS content (Fig. 3a). Both results suggest that changes in endogenous CO were likely to be related to salt-induced oxidative damage during the early treatment period, and further provide evidence that CO gas, when dissolved in aqueous solution, as well as that of the CO donor hematin (Huang et al. 2006), provides cytoprotection against salt-induced oxidative damage in plant tissues, which has been proven recently in animal systems either (Otterbein et al. 2003).

**CO induces NO production**

Thom et al. (1997) discovered that exposure of endothelial cells to low levels of CO (10–110 nm) resulted in an increase in steady-state NO releasing. However, until now, little direct evidence has been available to delineate NO actions in CO-mediated responses in plants subjected to abiotic stresses. In our further experiments, a fluorescence method with or without the specific fluorescent probe DAF-2 DA and the negative probe AF 4-DA (Fig. 4a,b), or Greiss reagent method (Fig. 4c) was used to evaluate the changes of NO signal, respectively.

A slight accumulation of DAF-2 DA-dependent green fluorescence was firstly observed in the control root tips (Fig. 4a, Con). The green fluorescence was enhanced by the addition of the NO donor SNP and reduced by the specific scavenger cPTIO, respectively, indicating that DAF-2 DA-dependent green fluorescence is associated with endogenous NO concentration. Furthermore, NaCl treatment for 24 h enhanced endogenous NO level moderately, as demonstrated by 35.7% increase of fluorescence intensity. When salt-stressed wheat seedlings were used to measure the response to either 50% CO-saturated aqueous solution or NO donor SNP for 12 and 24 h, a very strong increase of NO appeared especially at 24 h in the CO-treated root tips, which was above endogenous levels by 126% when plants were subjected to salt stress alone (Fig. 4a,c). Moreover, NO accumulation began to appear in the most apical region of the roots; above induced increase in fluorescence was not observed when AF 4-DA, the negative control probe for DAF-2 DA, was applied. Furthermore, cPTIO, a specific scavenger of NO, also inhibited CO-induced NO release (Fig. 4a). Similar inhibition was obtained when l-NAME was used as the inhibitor of NOS. Moreover, no additive effect of CO and SNP on NO production was observed (Fig. 4c). Meanwhile, both cPTIO and l-NAME could inhibit the rates of NO release under NaCl treatment, respectively (Fig. 4). We also noticed that in our experimental conditions, autofluorescence could be neglected when the positive or negative NO probe was not added (Fig. 4b).

**Antioxidant enzyme activities and their transcripts**

Previous studies have shown that NO can activate the antioxidant enzyme activities in salt-stressed plants (Ruan et al. 2002; Uchida et al. 2002). Our experimental results showed that in 150 mm NaCl-stressed seedling roots, application of the NO donor SNP (0.1 mm) was observed to increase APX, GR, SOD, MDHAR and DHAR activities significantly at 1 d (Fig. 5) and 4 d (data not shown) of treatment, in comparison with those of salt stress alone (*P* < 0.05). Meanwhile, the transcript levels of several antioxidant genes, such as APX, Mn–SOD, Cu/Zn–SOD and DHAR, were also induced (Fig. 5g). In addition, similar results were observed when 50% CO-saturated aqueous solution was applied (Fig. 5). For example, the application of 50% CO-saturated aqueous solution resulted in the differential induction of the activities and transcripts of APX, SOD and DHAR in salt-stressed seedling roots when treatment time was prolonged. However, no additive inductive effect of CO and SNP on the antioxidative enzyme activities was observed (data not shown). By using native gradient PAGE (5–20%) and activity staining, half of six SOD isozyme forms, belonging to Cu/Zn–SOD or Mn–SOD, respectively, were detected in extracts of soluble proteins from salt-stressed seedling roots for 4 d (Fig. 5d). SNP or CO treatments only induced increases in band size, and no band appeared or disappeared, which is different from the results obtained in animal cells (Frankel, Mehindate & Schipper 2000).

Interestingly, for example, either l-NAME or MB, both differentially blocked the enhancement of APX, GR, SOD, MDHAR and DHAR activities or their transcripts induced by CO, SNP or their combined treatment at 1 or 4 d, and the effect of CO on SOD isozyme activities could be partially weakened by the treatment with l-NAME (Figs 5 & 6), both of which indicating that NO produced by NOS-like enzyme might be required for the activation of the expression and the activities of antioxidant enzymes in CO signal transduction in salt-stressed plant tissues. In addition, treatment with MB produced the down-regulation of the

Figure 4. Nitric oxide (NO) accumulation in salt-stressed wheat seedling root tips induced by 50% carbon monoxide (CO)-saturated aqueous solution, as well as the NO donor sodium nitroprusside (SNP). (a) The distribution of NO in root tips was detected by fluorescence probe DAF-2 DA and negative probe AF 4-DA 24 h after different treatments under fluorescence and bright field microscopy (TCS-SP2 confocal laser scanning microscope; Leica Lasertechnik GmbH). A–H, Treatments loaded with DAF-2 DA; a–h, treatments loaded with AF 4-DA. Mean relative DAF-2 DA or AF 4-DA fluorescence densities corresponding to A–H, or a–h were given in I or i, taking control (Con, A or a) as 100%. (b) The autofluorescence of root tips without the addition of probe. Experiments were repeated six times, and similar results were obtained. Scale bar = 500 μm. (c) NO contents in 12 or 24 h of salt-stressed wheat seedling roots detected by using Greiss reagent. Mean and SE were calculated from at least four independent experiments. Different letters indicate the statistical difference at the *P* < 0.05 level among different treatments according to Tukey’s multiple range test.
CO enhances wheat salt tolerance

(a) DAF-2 DA

(b) Relative fluorescence (%)

(c) NO production (nmol g⁻¹ FW)

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The image contains graphs and figures illustrating the activities of various enzymes and proteins in different treatments. The graphs show the activity levels of APX, GR, SOD, MDHAR, and DHAR in response to various conditions, including controls and treatments involving S, CO, L-NAME, and SNP. The treatments are labeled on the x-axis, and the y-axis represents the activity levels in units of nmol min⁻¹ mg⁻¹ protein. The graphs are accompanied by images showing the expression levels of APX, Mn-SOD, Cu/Zn-SOD, and DHAR, as well as an image of 18s rRNA expression.

The data suggest that different treatments have varying effects on the activities of these enzymes, with some treatments showing significant increases or decreases compared to controls. The graphs are labeled with letters (a, b, c, d, e, f) to indicate statistical comparisons between treatments.
expression or activities of these antioxidative enzymes, which was only weakly induced by the salt stress alone at 1 d, while TBARS formation was enhanced (Table 2; Fig. 6). Meanwhile, application of t-NAME for 1 d exhibited similar results except the responses of the DHAR enzyme and transcript (Fig. 5). These results further suggest that endogenous NO produced by NOS-like enzyme might be associated with CO-induced the up-regulation of antioxidant defence in wheat seedling roots.

**K/Na ratio, related enzyme activities and their transcripts**

The ionic balance inside the plant cell is closely related to plant adaptation to salt stress, and it is possible that a high K/Na ratio is more important for many plant species than simply maintaining a low concentration of Na. Therefore, we compared K/Na ratios under salt stress in the whole roots and especially in the root tips, the most sensitive organisms in the whole plant. For this purpose, exogenous 50% CO-saturated aqueous solution was applied to examine the relationship between CO gas and K/Na ratios. Figure 7a shows the X-ray density maps of the distribution of Na and K elements in root tips of wheat seedling plants at 8 d after different treatments. Interestingly, in comparison with these of salt-stressed samples alone, more K but less Na was observed in the root tip sections of sample treated with 50% CO-saturated aqueous solution as well as SNP treatment, significantly reduced Na but increased K, and the latter was mainly observed in cortical and stelar cells of root tips, thus resulting in the enhancement of the K-to-Na ratio, especially in stelar cells of CO-treated sample (Fig. 7b). To attribute the role of NO in inducing the enrichment of K in plant root tips, t-NAME, an NOS inhibitor, was also used in our further experiment. As expected, the combination treatment with t-NAME both really reduced the enrichment of K distribution and the K/Na ratio in cortical, stelar or total cells induced by CO (Fig. 7a,b). K/Na ratio in salt-stressed root tip tissues was also decreased by the addition of t-NAME (S + t-NAME). Furthermore, the time-course of K/Na ratio in wheat roots was obviously increased with the addition of 50% CO-saturated aqueous solution treatment under salt stress (P < 0.05; Fig. 7c). Additionally, the CO-mediated effect during the early period of treatment was fully reversed when CO/NO scavenger Hb was added together.

<table>
<thead>
<tr>
<th>K/Na ratio</th>
<th>APX</th>
<th>Mn–SOD</th>
<th>Cu/Zn–SOD</th>
<th>DHAR</th>
<th>V H⁺-ATPase</th>
<th>PM H⁺-ATPase</th>
<th>18s rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

**Figure 6.** Effects of 50% carbon monoxide (CO)-saturated aqueous solution, nitric oxide (NO) donor sodium nitroprusside (SNP), NO scavenger methylene blue (MB) on the expression of *APX*, *Mn–SOD*, *Cu/Zn–SOD*, *DHAR*, *V H⁺-ATPase* and *PM H⁺-ATPase* in root tissues of wheat seedling exposed to salt stress. The transcript levels of these genes analysed by semi-quantitative RT-PCR at 1 d. Lines 1–9 represent different treatments as follows: (1) Con, only Hoagland solution without adding NaCl and other chemicals; (2) S, Con + 150 mm NaCl; (3) S + 50% CO, Con + 150 mm NaCl + 50% CO-saturated aqueous solution; (4) S + 50% CO, Con + 150 mm NaCl + 50% CO-saturated aqueous solution + 0.1 mm t-NAME; (5) S + t-NAME, Con + 150 mm NaCl + 0.4 mm t-NAME; (6) S + 0.1 SNP, Con + 150 mm NaCl + 0.1 mm SNP Lane of H₂O₂ (d) represents enzyme extract in vitro culture with 5 mm H₂O₂ to inhibit Cu/Zn–SOD isozyme. For each lane, 30 μg of protein extract was applied. (g) The transcript levels of the antioxidant genes *APX*, *Mn–SOD*, *Cu/Zn–SOD* and *DHAR* analysed by semi-quantitative RT-PCR at 24 h. Lines 1–6 were the same as these shown in (d). The relative abundance of 18s rRNA was used as the internal standard.
Table 2. Effects of 50% carbon monoxide (CO)-saturated aqueous solution, nitric oxide (NO) donor sodium nitroprusside (SNP) and NO scavenger methylene blue (MB) treatments on the thiobarbituric acid-reactive substances (TBARS) content, PM H^+-ATPase, V H^+-ATPase and V H^+-PPase activities in wheat seedling roots exposed to salt stress at 1 d

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TBARS (nmol g^(-1) FW)</th>
<th>PM H^+-ATPase (μmol Pi mg^(-1) Pro h^(-1))</th>
<th>V H^+-ATPase (μmol Pi mg^(-1) Pro h^(-1))</th>
<th>V H^+-PPase (μmol Pi mg^(-1) Pro h^(-1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>5.2 ± 0.2^b</td>
<td>93.1 ± 4.0^a</td>
<td>62.7 ± 2.1^a</td>
<td>30.9 ± 1.0^a</td>
</tr>
<tr>
<td>S</td>
<td>10.5 ± 0.2</td>
<td>104.3 ± 2.7</td>
<td>75.5 ± 1.7</td>
<td>36.3 ± 1.1</td>
</tr>
<tr>
<td>S + 50% CO</td>
<td>7.7 ± 0.3^a</td>
<td>133.7 ± 3.3^b</td>
<td>86.2 ± 1.5^a</td>
<td>42.9 ± 1.1^a</td>
</tr>
<tr>
<td>S + 50% CO + MB</td>
<td>9.3 ± 0.4</td>
<td>102.0 ± 2.5</td>
<td>65.6 ± 1.6^a</td>
<td>36.5 ± 0.8</td>
</tr>
<tr>
<td>S + 0.1 SNP</td>
<td>6.2 ± 0.3^b</td>
<td>143.8 ± 4.2^b</td>
<td>93.7 ± 2.2^b</td>
<td>47.4 ± 1.1^b</td>
</tr>
<tr>
<td>S + 0.1 SNP + MB</td>
<td>11.0 ± 0.5</td>
<td>114.4 ± 3.8</td>
<td>71.7 ± 1.7</td>
<td>38.5 ± 1.0</td>
</tr>
<tr>
<td>S + 50% CO + 0.1 SNP</td>
<td>6.7 ± 0.3^b</td>
<td>140.3 ± 3.3^b</td>
<td>90.1 ± 2.0^b</td>
<td>49.0 ± 1.2^b</td>
</tr>
<tr>
<td>S + 50% CO + 0.1 SNP + MB</td>
<td>9.7 ± 0.4</td>
<td>81.2 ± 2.9^a</td>
<td>81.6 ± 1.0^b</td>
<td>38.1 ± 0.6</td>
</tr>
<tr>
<td>S + MB</td>
<td>13.5 ± 0.6^b</td>
<td>79.2 ± 2.5^a</td>
<td>67.9 ± 1.7^a</td>
<td>29.3 ± 0.9^a</td>
</tr>
</tbody>
</table>

TBARS are recommended as an effective biomarker of lipid peroxidation and oxidative stress. Measurement of TBARS reflects the injury of wheat seedling roots under salt stress and the protective role of CO and NO. Plasma membrane (PM) H^+-ATPase, V (vacuole) H^+-ATPase, V (vacuole) H^+-PPase belong to H^+ pumps and provide electrochemical gradient of H^+ to maintain ion homeostasis in plant cells.

Data are the means ± SE of three different experiments with five replicated measurements.

^a,bSignificantly different compared with salt-stressed sample (S) at P < 0.05 or 0.01 level according to t-test.

NaCl treatment, MB reduced the expression or activities of the enzymes to the control levels.

**DISCUSSION**

In our experiment, the observation that the addition of NaCl to wheat seedlings causes the enhancement of CO release and HO activity in root tissues (Fig. 1) is new, to the best of our knowledge. Besides the presentation of the empirical evidence, one question must be asked: What is the regulatory role of CO biosynthesis induced by the addition of NaCl stress? Linked to this question is the conjecture that endogenous CO production may lead to the salt tolerance process in wheat seedlings. In the further experiment, the physiological significance of this endogenous CO production was tested by exogenous CO application.

Although it is generally agreed that CO in high concentrations normally inhibits plant growth and development (Wilks 1959), we further demonstrate that, exogenously applied 50% CO-saturated aqueous solution, which could strongly induce the appearance of the first CO spike and enhance the second spike, participated by conferring salt tolerance to wheat seedlings subjected to NaCl salt stress, as well as the application of NO donors SNP and DETA/NO, and the CO action was at least related to the induction of antioxidant system parameters and maintenance of ion homeostasis, as well as the modulation of growth regulation. Additionally, NO might be a component of the CO-induced plant responses via NOS-like enzyme, remarkably similar to those found in animals. Recently, Han et al. (2008) from our research group also confirm that CO could alleviate cadmium-induced oxidative damage by modulating glutathione metabolism in the roots of Medicago sativa.

**CO attenuates salinity toxicity**

In our experimental conditions, salinity toxicity symptoms were evaluated in wheat seedlings through the estimation of seedling growth, RWC as well as PM permeability and TBARS formation. Salinity typically led to a significant inhibition of growth measured as DW and relative shoot and root growth, all of which were partially restored by the daily application of CO for 14 or 5 d (Table 1; Fig. 2b,c). Salinity toxicity can also affect the PM permeability (Fig. 3b), and NaCl, in particular, has been reported to influence the water balance. In our experiment, it was observed that the maintenance of RWC (Table 1) and resistance to lodging (Fig. 2a) under CO treatment might represent an adaptive mechanism to avoid seedling tissue water deficits associated with salinity toxicity. Similarly,
S + 50% CO + L-NAME

K/Na ratio

(a) IM Na\(^+\) distribution K\(^+\) distribution

(b) Cortex Stele Total

(c) S S + 50% CO S + 50% CO + Hb S + Hb

K/Na ratio

Time of treatment (h)

CO enhances wheat salt tolerance
the vital protective role of NO in salt tolerance of wheat seedling roots to salinity has also been confirmed (Table 1), which has been discovered previously by several research groups (Zhao et al. 2004, 2007; Zhang et al. 2006).

**Up-regulation of antioxidant defence and maintenance of ion homeostasis: two major mechanisms responsible for CO-induced cytoprotective effects**

Salinity stress often causes oxidative stress in plant cells, and the tolerance to salinity stress is partially correlated with a more efficient antioxidant system, both of which have already been reported by our and other research groups (Tanaka et al. 1999; Hernández et al. 2000; Mittler 2002; Ruan et al. 2002; Uchida et al. 2002). For example, the transformed rice over-expressing yeast Mn–SOD showed more salinity tolerance than the wild type (Tanaka et al. 1999). Furthermore, the simplest explanation for our data illustrated that the protective effect of CO might be ascribed to CO capability to alleviate the increases in TBARS and PM permeability (Fig. 3) by enhancing the activities or transcripts of the antioxidant enzymes APX, GR, SOD, MDHAR and DHAR in wheat seedling roots (Figs 5 & 6). Thus, our results presented are consistent with data obtained in animals, in which a low concentration of CO gas can exert the protective role against oxidant-induced lung injury (Otterbein et al. 2003). However, cytotoxic and cytoprotective effects of CO in animals, at least partially, were dependent on its concentration applied as well as on the availability of iron or copper (Piantadosi 2002). In this study, the cytoprotection of CO was also dependent on its concentration. For example, the application of CO aqueous solution (1 or 100% saturation) exhibited the negative or weaker effects (data not shown).

Another important mechanism regarding salt stress to plants is attributed to ionic phyto-toxicity that is caused by excess amounts of salt ions in plants. Metabolic toxicity of Na+ is largely as a result of its ability to compete with K+ for binding sites essential for cellular function, which activated more than 50 enzymes (Bhandal & Malik 1988). High level of Na+ alters aqeous and ionic thermodynamic equilibria, resulting in hyperosmotic stress, ionic imbalance and toxicity. Thus, rebuilding ion homeostasis is essential for plants to resist salt stress. In fact, a high cytosolic K/Na ratio is important for maintaining cellular metabolism, and it was done by regulating the expression and activity of K+ and Na+ transporters, and of H+ pump that generates the driving force for transport. For instance, increased ATPase-mediated H+ translocation across the PM is a component of plant cell response to salt imposition (Ratajczak, Richter & Lüttinge 1994; Zhu 2001, 2003). Here, the application of 50% CO-saturated aqueous solution was able to mimic the effect of NO donor SNP in inducing the K-to-Na ratio in salt-stressed seedling roots or even root tips (Fig. 7), both of which were consistent with the enhanced PM H+-ATPase and V H+-ATPase transcripts or related enzyme activities, including V H+-ATPase, PM H+-ATPase and V H+-PPase (Fig. 6; Table 2). Thus, ion homeostasis is re-established so as to adapt to the plant cell to salt stress.

**A possible link between CO/HO and NO in plant salt tolerance**

In animals, it is interesting that many of the novel properties pertaining to CO have strong analogies or are mediated with the well-established biological activities elicited by NO, another gaseous molecule produced mainly by NOS both in animals and plants. In animal cells, for example, Piantadosi (2002) found that CO could cause redistribution of NO, which is consistent with the different equilibrium constants of these molecules for metal binding. Meanwhile, although there are some debates on the nature of NO in plants, NO has been previously suggested to act as messenger and regulator in plant developmental processes, stress tolerance, defence responses and symbiosis establishment (Delledonne 2005). In further investigations, it was proved that NO produced moderately during 24 h of salt treatment (Fig. 4), might serve as a signal for the weaker induction of antioxidant genes or ion homeostasis-related gene expression (Figs 5 & 6; Table 2). In contrast, when the NOS inhibitor l-NAME, NO scavenger MB or Hb was added together, the above salt-induced parameters were not only reversed, the K-to-Na ratio and DW of shoot parts were also differentially decreased, especially in total cells of root tips of the former change (Fig. 7; Table 1). The addition of an NO donor SNP (S + 0.1 SNP) strengthened some responses elicited by salinity stress (Figs 5–7; Table 2), further showing a protective action of NO to plant cell under salt stress (Zhao et al. 2004).

Furthermore, the results of Fig. 4a showed that a rapid increase of endogenous NO was measured in salt-stressed seedling root tips as a response to exogenous 50% CO-saturated aqueous solution, whereas treatment of wheat seedling roots with cPTIO resulted in an almost complete abolition of NO production induced by CO. Additionally, the combination of NOS inhibitor l-NAME or NO scavenger MB, both of which have been shown to prevent NO accumulation induced by salt stress (Zhao et al. 2004; Zhang et al. 2006), substantially blocked the increases in the expression of antioxidant genes including APX, Mn–SOD, Cu/Zn–SOD and DHAR, and the activities of antioxidant enzymes such as APX, GR, Mn–SOD, Cu/Zn–SOD, MDHAR and DHAR induced by CO, while TBARS formation was also enhanced (Figs 5 & 6). Furthermore, application of an NO donor SNP (S + 0.1 SNP) exhibited the similar induction effects elicited by CO on the parameters. Additionally, treatment with MB arrested CO- or NO-induced increases in the gene expression and activities of PM H+-ATPase and V H+-ATPase, and V H+-PPase activities (Fig. 6; Table 2). Taken together, these results indicate that NO may, at least partially, serve as a signal for...
CO-induced salt tolerance by increasing the antioxidant system parameters and maintaining ion homeostasis.

In a recent study, however, it was reported that 100 mM NaCl inhibited NOS activity in wild-type Arabidopsis in a concentration-dependent manner; also, the inhibition of NOS activity by NaCl was consistent with the reduction of the endogenous NO levels for 2 h of treatment (Zhao et al. 2007). Additionally, Zhao et al. (2004) observed that the NOS activity remained almost unchanged under 200 mM NaCl treatment after 2 d in the SR callus (salt-sensitive reed species), but increased by 22% in the DR callus (salt-resistant reed species). Similarly, in maize seedling leaves, NaCl (100 mM) enhanced endogenous NO level at 2–6 h (Zhang et al. 2006). There are several possible explanations for this disparity. One of the possibilities might be related to the different concentration of NaCl and plant species used in the experiments. Different plant varieties, which show different sensitivities to salinity (tolerance or sensitive), might exhibit an increased or reduced endogenous NO level when they were subjected to salinity stress. Because the wheat cultivar Yangmai 158 used in our experiment displays a moderate tolerance to 150 mM NaCl stress in comparison with other sensitive cultivars (e.g. Chinese Spring), it is consistent with this hypothesis that under 150 mM salt stress, Yangmai 158 seedling roots produced more NO signal in comparison with the control samples (Con). Certainly, it was also possible that kinetic change of NO signal varied with the different detection time.

It is worth noticing that in animal cells, when protection from cell death is initiated by CO, NO production and NO-induced HO-1 activity are each required for the protective effect (Zuckerbraun et al. 2003). More recently, Noriega et al. (2007) reported that 100 μM SNP could up-regulate HO-1 gene expression to protect plant leaf tissues against cadmium-induced oxidative damages. In this study, we also observed that in comparison with the salt-stressed sample alone, the application of exogenous 50% CO-saturated aqueous solution also induced the enhancement of CO release (Fig. 1a). However, the first sharp spike of CO release was earlier and stronger than the salt-stressed samples, and the second peak of CO production was consistent with the changes of HO activity. Based on our further observations that a rapid increase of endogenous NO was found in salt-stressed seedling root tips as a response to exogenous 50% CO-saturated aqueous solution (Fig. 4), it is hypothesized that CO may protect salinity-induced oxidative damages via an NO-induced HO-dependent mechanism. This hypothesis is worthy of further careful investigation in the near future, and more studies are needed to be carried out.

Collectively, our work not only raises the intriguing possibility for the potential practice use of CO gas, or its donor dissolved in the aqueous solution in the modulation of plant tolerance to salt stress, but also sheds light on many unknown aspects of CO functions in plants, including conferring tolerance/resistance to different abiotic and biotic stresses mainly mediated by the overproduction of ROS.

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