NO accumulation alleviates H$_2$O$_2$-dependent oxidative damage induced by Ca(NO$_3$)$_2$ stress in the leaves of pumpkin-grafted cucumber seedlings

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Nitric oxide (NO) and hydrogen peroxide (H$_2$O$_2$), two important signaling molecules, are stimulated in plants by abiotic stresses. In this study, we investigated the role of NO and its interplay with H$_2$O$_2$ in the response of self-grafted (S-G) and salt-tolerant pumpkin-grafted (Cucurbita maxima × C. moschata) cucumber seedlings to 80 mM Ca(NO$_3$)$_2$ stress. Endogenous NO and H$_2$O$_2$ production in S-G seedlings increased in a time-dependent manner, reaching maximum levels after 24 h of Ca(NO$_3$)$_2$ stress. In contrast, a transient increase in NO production, accompanied by H$_2$O$_2$ accumulation, was observed at 2 h in rootstock-grafted plants. N$^\omega$-Nitro-L-Arg methyl ester hydrochloride (L-NAME), an inhibitor of nitric oxide synthase (NOS), tungstate, an inhibitor of nitrate reductase (NR), and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a scavenger of NO, were found to significantly inhibit NO accumulation induced by salt stress in rootstock-grafted seedlings. H$_2$O$_2$ production was unaffected by these stress conditions. Ca(NO$_3$)$_2$ stress-induced NO accumulation was blocked by pretreatment with an H$_2$O$_2$ scavenger (dimethylthiourea, DMTU) and an inhibitor of NADPH oxidase (diphenyleneiodonium, DPI). In addition, maximum quantum yield of PSII (Fv/Fm), as well as the activities and transcript levels of antioxidant enzymes, were significantly decreased by salt stress in rootstock grafted seedlings after pretreatment with these above inhibitors; antioxidant enzyme transcript levels and activities were higher in rootstock-grafted seedlings compared with S-G seedlings. These results suggest that rootstock grafting could alleviate the oxidative damage induced by Ca(NO$_3$)$_2$ stress in cucumber seedlings, an effect that may be attributable to the involvement of NO in H$_2$O$_2$-dependent antioxidative metabolism.

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Abbreviations – APX, ascorbate peroxidase; AsA, ascorbic acid; CAT, catalase; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAB, 3,3′-diaminobenzidine; DAF-FM DA, 3-amino 4-aminomethyl-2′,7′-difluorescein diacetate; DMTU, dimethylthiourea; DPI, diphenyleneiodonium; Fm, maximal fluorescence; Fo, minimal fluorescence; Fv/Fm, maximum quantum yield of PSII; L-NAME, N$^\omega$-Nitro-L-Arg methyl ester hydrochloride; NADPH, triphosphopyridine nucleotide reduced tetrasodium salt; NBT, nitro blue tetrazolium; NOS, nitric oxide synthase; NR, nitrate reductase; POD, peroxidase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase.
Introduction

Salinity is one of the primary constraints on crop growth and productivity. In China, the main ions associated with salt stress in protected horticulture are Ca$^{2+}$ (60% of total cations) and NO$_3^-$ (67–76% of total anions) (Tong and Chen 1991, Yuan et al. 2013). Salt stress can inhibit photosynthesis, alter respiration, influence phytohormone synthesis, and disturb the homeostasis of reactive oxygen species (ROS) and antioxidant systems (Rady 2011). ROS, such as the superoxide anion radical (O$_2$•−), hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (•OH), are produced by the incomplete reduction of oxygen. When ROS levels surpass plant defence mechanisms, plant cells become exposed to secondary oxidative stress (Chaves et al. 2009). Secondary oxidative stress is highly toxic; it can reduce the stability of DNA and proteins and can induce lipid peroxidation (Halliwell and Gutteridge 1999). Plants, however, have evolved mechanisms to combat oxidative stress, including antioxidant enzymes [e.g. superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX)] and non-enzymatic pathways that scavenge different types of ROS (Apse and Blumwald 2002). Plants have also developed a signaling network that can sense and respond to unfavorable external conditions, allowing them to cope with stress (Nakashima et al. 2009). There is compelling evidence that H$_2$O$_2$, at relatively low concentrations, can function as a defence signaling molecule in plants in response to several abiotic stresses (Meneguzzo et al. 1999, Dat et al. 2000).

Cucumber (Cucumis sativus) is one of the main vegetable crops under protected cultivation in China. According to Food and Agriculture Organization of the United Nations (FAO) statistics, the cucumber cultivation area of China has increased progressively since 1997, and China is now the largest producer of cucumber in the world in terms of cultivation area value. However, cucumber is highly sensitive to salinity because of its vulnerable root system. Oxidative damage is the primary mechanism by which salt stress causes injury in plants. Rootstock grafting, which is both efficient and environmentally friendly, is a promising strategy that aims to reduce oxidative damage induced by salinity (Asins et al. 2010). The study of Wei et al. (2007) found that grafted eggplants maintained lower H$_2$O$_2$ content and higher antioxidant enzyme activity under Ca(NO$_3$)$_2$ stress compared with non-grafted plants. In addition, the favorable effect of rootstock grafting on salinity has been identified in various plant species, including cucumber, tomato and watermelon (Chen et al. 2005, Colla et al. 2006, Wei et al. 2007, Huang et al. 2009).

Salt-induced cellular changes include the increased production of nitric oxide (NO) and other reactive nitrogen species (RNS), which cause nitrosative stress in plants (Valderrama et al. 2007). In addition, NO is a ubiquitous and important endogenous molecule in plant signal transduction pathways that plays a significant role in plant responses to abiotic stress (Besson-Bard et al. 2008). In plants, NO is produced via two enzymatic pathways: one is catalyzed by nitrate reductase (NR) and the other is catalyzed by nitric oxide synthase (NOS) (Rockel et al. 2002, Guo et al. 2003). NO can also be generated by non-enzymatic pathways (Neill et al. 2003). Several studies have demonstrated that NO is involved in multiple physiological and biological processes in plants, including growth, development and defence responses to abiotic stress (García-Mata et al. 2001, Neill et al. 2003, Zhao et al. 2007). In addition, it has been suggested that NO can protect plants from salt stress-induced oxidative damage by acting as an antioxidant to scavenge ROS (Zhang et al. 2009) and enhancing the activity of antioxidant enzymes (Zhou et al. 2005). The involvement of H$_2$O$_2$ and NO in plant adaptation to salinity has also been documented (Uchida et al. 2002, Zhang et al. 2007a, 2007b). However, the role of NO and the relationship between NO and H$_2$O$_2$, in rootstock-grafted (Rs-G) cucumber seedlings in response to salt stress-induced oxidative damage is still unclear. In a previous study, it was demonstrated that application of exogenous NO reduced the harmful effects of salinity on cucumber seedlings (Fan et al. 2012). In the present study, we used NO scavenger and inhibitors [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), tungstate and N$^\omega$-Nitro-L-Arg methyl ester hydrochloride (i-NAME)] as well as H$_2$O$_2$ scavenger and inhibitor (DMUT and DPI) to investigate the relationship between NO and H$_2$O$_2$ in grafted cucumber plants under Ca(NO$_3$)$_2$ stress. We also examined NO and H$_2$O$_2$ content, antioxidant enzyme activity and gene expression in the leaves of Rs-G and self-grafted (S-G) cucumber seedlings under Ca(NO$_3$)$_2$ stress. Finally, we discuss a mechanism whereby rootstock grafting enhances the activity of antioxidant enzymes in cucumber leaves, leading to increased salt tolerance.

Materials and methods

Plant materials and grafting

Cucumber (C. sativus cv. Jinyou NO. 3) seeds were obtained from Tianjing Kerun Research Institute, China. Pumpkin (Cucurbita maxima × C. moschata) seeds were purchased from the Qingdao Agricultural Academy of Science, China. After cucumber and pumpkin germination, the seeds were sown in plastic salvers (41 × 41 × 5
cm) containing quartz sand and grown in a greenhouse at Nanjing Agricultural University, China. The average day/night temperatures were 28/18°C, and the relative humidity was 60–75%. Cucumber was grafted onto pumpkin rootstock using the ‘insertion grafting’ procedure described by Lee (1994) when the scion’s cotyledon was fully expanded and rootstock’s second true leaf of the rootstock was in the development stage. S-G cucumbers were used as a control. At the two true-leaf stage, the grafted seedlings were transferred to a 20 l container (12 seedlings per container) filled with half-strength Hoagland’s solution (pH 6.5 ± 0.1, EC 2.0–2.2 dS m⁻¹) (Hoagland and Arnon 1950). The solution was replaced every 3 days. After 5 days of cultivation, uniform grafted plants were selected for different treatments.

Treatments

**Experiment I**

This experiment was designed to study the role of NO in salt-induced antioxidant capacity in pumpkin Rs-G and S-G cucumber plants subjected to Ca(NO₃)₂ stress. To induce salt stress, Ca(NO₃)₂ (80 mM) was added to half-strength (1/2) Hoagland’s solution. S-G and Rs-G seedling leaves were pre-treated with an NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 250 μM, Beyotime, Shanghai, China), a NOS inhibitor Nw-Nitro-L-Arg methyl ester hydrochloride (L-NAME, 200 μM, Beyotime), or a NR inhibitor (tungstate, 200 μM, Beyotime) for 8 h and then exposed to 80 mM Ca(NO₃)₂ for 24 h under the same conditions as described above. The third leaves were sampled and immediately frozen under liquid nitrogen. Each experiment was repeated at least three times.

**Experiment II**

This experiment was designed to study the relationship between NO and H₂O₂ in Rs-G plants under Ca(NO₃)₂ stress. Pumpkin-grafted and S-G cucumber leaves were pre-treated for 8 h with 250 μM cPTIO, 200 μM L-NAME, 200 μM tungstate, an NADPH oxidase inhibitor diphenyleneiodonium (DPI, 100 μM, Beyotime) or an H₂O₂ scavenger dimethylthiourea (DMTU, 5 mM, Beyotime). Plants were then exposed to 80 mM Ca(NO₃)₂ for 24 h under the conditions described in Experiment I. The third leaves were sampled and immediately frozen under liquid nitrogen. Each experiment was repeated at least three times.

**NO content analysis**

For fluorometric NO quantification, the fluorophore 3-amino, 4-aminomethyl-2',7'-difuorescein diaceate (DAF-FM DA) was used according to the method of Corpas et al. (2004). Briefly, leaf segments (25 mm²) were pre-incubated with 10 μM DAF-FM DA in 20 mM PBS buffer (pH 7.4) for 3 h at 37°C in darkness and then washed three times with fresh buffer for 15 min. After washing, the samples were viewed using a confocal laser-scanning microscope (Nikon, Tokyo, Japan). DAF-FM DA fluorescence was obtained at an excitation wavelength of 495 nm and an emission wavelength of 515 nm. Fluorescence measurements were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

**Determination of H₂O₂ content**

H₂O₂ production was visualized using 3,3′-diaminobenzidine (DAB) as substrate according to Orozco-Cardenas and Ryan (1999), with minor modifications. Leaf discs were submerged into tubes containing 10 ml of DAB solution (1 mg ml⁻¹, pH 3.8) for 6 h in the dark at 25°C. The leaf discs were then decolorized in boiling 80% ethanol for 20 min. The leaves were transferred to 95% ethanol to remove chlorophyll completely and then photographed. H₂O₂ content was also measured by monitoring the formation of a titanium-peroxide complex at a wavelength of 415 nm, as described by Brennan and Frenkel (1977).

**Measurement of Fv/Fm**

An Imaging-PAM M-series fluorimeter (Heinz Walz, Germany) was used to measure minimal fluorescence (Fo) in leaves that had been dark-adapted for 20 min and maximal fluorescence (Fm) in leaves after a 0.8 s pulse of light at approximately 8000 μmol m⁻² s⁻¹. The maximum quantum yield of PSII (Fv/Fm) was calculated using the formula (Fm – Fo)/Fm.

**Assay of antioxidant enzyme activities**

Antioxidant enzyme activities were assayed using 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA. Extraction was performed at 4°C. After centrifugation at 12 000 g for 20 min, the supernatant was collected and used for the subsequent determination of enzyme activity.

SOD activity was determined by measuring the ability of the supernatant to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm according to the method of Giannopolitis and Ries (1977). One unit
of SOD activity was defined as the amount of enzyme required to inhibit NBT photo-reduction by 50%.

CAT activity was performed as described by Aebi (1974), with some modifications. CAT activity was determined by monitoring the rate of disappearance of H$_2$O$_2$ at 240 nm for 2 min. An absorbance change of 0.1 U min$^{-1}$ was defined as 1 U of CAT activity, and CAT activity was expressed as unit mg$^{-1}$ fresh weight (FW).

A modified method of Rao et al. (1996) was used to assay POD activity. After pre-incubation of the extracted supernatant in a reaction mixture containing 50 mM PBS (pH 7.0) and 20 mM guaiacol for 5 min at 25°C, 6 mM H$_2$O$_2$ was added to start the reaction. POD activity was calculated as the change in absorbance at 470 nm within 2 min. One unit of POD activity was defined as an absorbance change of 0.01 U min$^{-1}$. POD activity was expressed as unit mg$^{-1}$ FW.

APX activity was performed as described by Nakano and Asada (1981). The assay was carried out in a reaction mixture consisting of 50 mM PBS (pH 7.0), 0.5 mM AsA, 3 mM H$_2$O$_2$, and 100 ml of the enzyme extract. The change in the absorbance at 290 nm was recorded at 25°C for 1 min after the addition of H$_2$O$_2$. One unit of APX activity was defined as an absorbance change of 0.1 unit min$^{-1}$. APX activity was expressed as unit mg$^{-1}$ FW.

**RNA extraction and qRT-PCR**

Total RNA was isolated from cucumber leaves using the TRI reagent protocol (Takara Bio Inc., Tokyo, Japan) according to the manufacturer’s instructions. For real-time quantitative RT-PCR (qRT-PCR) analysis, cDNA was obtained by reverse transcription of 1 µg of total RNA using the PrimeScript$^{	ext{TM}}$ 1st strand cDNA Synthesis Kit (Takara Bio Inc.) according to the manufacturer’s instructions. Gene-specific primers were designed based on sequences available in the NCBI and cucumber databases (cucumber.genomics.org.cn) (see Table S1 in Supporting Information). Subsequently, qRT-PCR was performed using the SYBR PrimeScript$^{	ext{TM}}$ RT-PCR Kit (Takara Bio Inc.) according to the manufacturer’s instructions.

**Statistical analysis**

Statistical analysis of all data was performed using SPSS software (IBM, Statistical Product and Service Solutions, New York, NY). Values are expressed as the mean ± standard error (SE) from at least three independent experiments. Duncan’s multiple range test was used to determine statistical significance, with statistical significance set at $P < 0.05$.

**Results**

**NO and H$_2$O$_2$ accumulation in the leaves of Rs-G cucumbers under Ca(NO$_3$)$_2$ stress**

To investigate whether Ca(NO$_3$)$_2$ stress affects endogenous NO release in the leaves of Rs-G cucumber seedlings, a NO-specific fluorescent probe, DAF-FM DA, was applied to monitor NO production. As shown in Fig. 1A, significant differences in NO content were observed under 80 mM Ca(NO$_3$)$_2$ stress between S-G and Rs-G seedling leaves. The content of NO in S-G plants increased significantly in a time-dependent manner under salt stress, and a maximum increase of approximately 5.3-fold was reached after 24 h of salt treatment. However, in the leaves of Rs-G seedlings, a transient increase in NO content was observed at 2 h. At this time point, the Rs-G seedlings had a higher endogenous NO content compared with S-G seedlings. After 2 h, the NO content of Rs-G seedlings decreased but exhibited a slight increase at approximately 6 h. After 24 h of Ca(NO$_3$)$_2$ stress, NO accumulation in Rs-G seedlings was about 2.8-fold greater than in seedlings grown under normal conditions. In addition, the NO content in S-G seedlings was approximately 1.7-fold higher than in Rs-G seedlings after 24 h of salt stress.

The kinetics of H$_2$O$_2$ accumulation was similar to that observed for NO in both S-G and Rs-G cucumber seedlings exposed to salt stress (Fig. 2). Ca(NO$_3$)$_2$ stress-induced H$_2$O$_2$ accumulation in S-G seedlings increased in a time-dependent manner that reached a maximum value after 24 h. H$_2$O$_2$ content was also significantly increased in the leaves of Rs-G plants after 2 h of salt stress, although H$_2$O$_2$ content was slightly decreased at 12 h. After 24 h of Ca(NO$_3$)$_2$ stress, the content of H$_2$O$_2$ in S-G seedlings was much higher than in Rs-G seedlings.

To identify the source of Ca(NO$_3$)$_2$-induced NO production in the leaves of Rs-G seedlings, an NR inhibitor (tungstate) and a NOS inhibitor (L-NAME) were used (Fig. 3). In grafted seedlings, salt stress-induced NO production was significantly reduced in the presence of NR and NOS inhibitors. Furthermore, the NO scavenger, cPTIO, reduced the accumulation of NO in response to Ca(NO$_3$)$_2$ stress in both the Rs-G and S-G cucumber seedlings.

**H$_2$O$_2$-dependent NO accumulation in Rs-G cucumber seedlings under Ca(NO$_3$)$_2$ stress**

Next, we determined whether NO is involved in H$_2$O$_2$ accumulation induced by Ca(NO$_3$)$_2$ stress in Rs-G seedlings. We pre-treated grafted cucumber seedlings with DMTU (a chemical trap for H$_2$O$_2$) or DPI (an
Fig. 1. Endogenous nitric oxide (NO) concentrations in self-grafted (S-G) and rootstock-grafted (Rs-G) cucumber seedlings under Ca(NO₃)₂ stress. (A) NO fluorescence measured using the fluorophore 3-aminomethyl-2′,7′-difluorescein, diacetate (DAF-FM DA) and a fluorescence microscope. (B) NO accumulation expressed as relative fluorescence. Three true-leaf stage S-G and Rs-G cucumber seedlings were exposed to 80 mM Ca(NO₃)₂ and harvested at 0, 2, 6, 12 and 24 h. The results are expressed as the mean ± SE (n = 4).

Fig. 2. Hydrogen peroxide (H₂O₂) content in the leaves of self-grafted (S-G) and rootstock-grafted (Rs-G) cucumber seedlings under Ca(NO₃)₂ stress, determined by monitoring the formation of a titanium-peroxide complex at 415 nm. Three true-leaf stage S-G and Rs-G cucumber seedlings were exposed to 80 mM Ca(NO₃)₂ and harvested at 0, 2, 6, 12 and 24 h. The results are expressed as the mean ± SE (n = 3).

inhibitor of NADPH oxidase). As shown in Fig. 4A, DAB staining demonstrated that pretreatment with DMTU and DPI strongly inhibited H₂O₂ accumulation in grafted cucumber leaves under Ca(NO₃)₂ stress. In addition, Grafted seedlings were also pre-treated with tungstate, L-NAME or cPTIO. As shown in Fig. 4B, the application of tungstate, L-NAME or cPTIO had no significant effect on H₂O₂ production in grafted cucumber seedlings under salt stress. Compared with Rs-G seedlings, S-G seedlings maintained a higher H₂O₂ content under the conditions described above. The pattern of H₂O₂ production was similar when a chemical method was used to measure H₂O₂ levels (Fig. S1).

To investigate whether salt stress-induced NO accumulation requires H₂O₂, we monitored changes in NO accumulation in S-G and Rs-G cucumber plants pre-treated with DMTU or DPI. As shown in Fig. 5, after 24 h of Ca(NO₃)₂ stress, treatment with DMTU and DPI significantly decreased endogenous NO content in the leaves of S-G and Rs-G plants.

Effects of NO and H₂O₂ on Fv/Fm in the leaves of Rs-G seedlings under Ca(NO₃)₂ stress

As shown in Fig. 6, Ca(NO₃)₂ stress induced a significant decrease in the value of Fv/Fm in the leaves of S-G and Rs-G cucumber seedlings. Moreover, compared with S-G
Fig. 3. Effects of a nitric oxide (NO) scavenger (cPTIO), a nitrate reductase (NR) inhibitor (tungstate) and a nitric oxide synthase (NOS) inhibitor (L-NAME) on NO levels in self-grafted (S-G) and rootstock-grafted (Rs-G) cucumber seedlings under Ca(NO$_3$)$_2$ stress. Three true-leaf stage S-G and Rs-G cucumber seedlings were pre-treated with 250 μM cPTIO, 200 μM tungstate or 200 μM L-NAME for 8 h, followed by salt treatment for 24 h. (A) Representative images of NO fluorescence. Bar, 500 μm. (B) NO accumulation expressed as relative fluorescence. Values are means ± SE (n = 4). Values without a common letter are significantly different (P < 0.05). CK, control; N, salt stress; N + cPTIO, salt stress + cPTIO; N + tungstate, salt stress + tungstate; N + L-NAME, salt stress + L-NAME.

Plants, Rs-G seedlings exhibited a higher Fv/Fm value under salt stress. We hypothesized that the application of H$_2$O$_2$ and NO scavengers and synthesis inhibitors may further reduce the value of Fv/Fm in both S-G and Rs-G seedlings. However, there was no significant difference in Fv/Fm under these conditions.

**NO-induced changes in transcript levels and antioxidant enzyme activity under Ca(NO$_3$)$_2$ stress**

We found that NO accumulation played a vital role in protecting against salt-induced oxidative stress by increasing the transcription and activity of antioxidant enzymes. As shown in Fig. 7, after 24 h of salt stress, the activities of SOD, CAT and APX in cucumber leaves increased by 35, 189 and 54%, respectively, in Rs-G seedlings, and by 21, 53 and 21%, respectively, in S-G plants. In contrast, POD activity was much higher during salt stress in S-G seedlings compared with Rs-G seedlings. In addition, application of cPTIO, tungstate or L-NAME reduced the activities of SOD, CAT and APX in both S-G and Rs-G seedlings under salt stress but had no effect on POD activity. Changes in antioxidant enzyme transcript levels coincided with changes in their activities (Fig. 8). Rootstock grafting increased the transcript levels of SOD, CAT and APX when the seedlings were exposed to Ca(NO$_3$)$_2$ stress, the effect that was blocked by pretreatment with an NO scavenger or the inhibitors of NO synthesis. Furthermore, the effect of DMTU and DPI on antioxidant enzyme transcript levels and activities in Rs-G seedlings was similar to the effect of the NO scavenger and the NO synthesis inhibitor (Figs S1 and S2).

**Discussion**

It is well known that rootstock grafting is an important method to increase the performance of plants under abiotic stress. Physiologically, it has been demonstrated that the advantages of rootstock grafting results from alterations in growth regulation, ion homeostasis, osmolyte accumulation, and carbon and nitrogen metabolism (Colla et al. 2010, Huang et al. 2010, Li et al. 2015, Xing...
Fig. 4. Effects of a hydrogen peroxide (H$_2$O$_2$) scavenger (DMTU), an NADPH oxidase inhibitor (DPI), a nitric oxide (NO) scavenger (cPTIO), a nitrate reductase (NR) inhibitor (tungstate) and a nitric oxide synthase (NOS) inhibitor (L-NAME) on H$_2$O$_2$ production in self-grafted (S-G) and rootstock-grafted (Rs-G) cucumber seedlings under Ca(NO$_3$)$_2$ stress. (A and B) Three true-leaf stage S-G and Rs-G cucumber seedlings were pre-treated with 5 mM DMTU, 100 μM DPI, 250 μM cPTIO, 200 μM tungstate or 200 μM L-NAME for 8 h, followed by salt treatment for 24 h. H$_2$O$_2$ was detected by DAB staining in grafted cucumber seedlings exposed to the treatments described above. CK, control; N, salt stress; N+DMTU, salt stress + DMTU; N+DPI, salt stress + DPI; N+cPTIO, salt stress + cPTIO; N+tungstate, salt stress + tungstate; N+L-NAME, salt stress + L-NAME.

et al. 2015). Nevertheless, there is still a limited understanding of the NO and H$_2$O$_2$ signaling pathways that are induced in Rs-G plants under conditions of salt stress, especially under Ca(NO$_3$)$_2$ stress. The aim of this study was to evaluate the roles of and the relationship between NO and H$_2$O$_2$ in Rs-G cucumber seedlings in response to salt stress-induced oxidative damage. We found that Ca(NO$_3$)$_2$ stress induced NO and H$_2$O$_2$ accumulation in Rs-G cucumber seedlings after 2 h of salt treatment (Figs 1 and 2). In contrast, there was no significant difference in NO and H$_2$O$_2$ accumulation at 2 h; however, there was a significant increase in NO and H$_2$O$_2$ content in S-G seedlings after 24 h. It is well known that NO and H$_2$O$_2$ are extremely toxic to organisms at high concentrations. However, at relatively low concentrations, NO and H$_2$O$_2$ may act as signaling molecules, participating in a variety of cellular processes that regulate plant responses to different environmental conditions. It is essential that plants tightly control the levels of NO and H$_2$O$_2$ to limit oxidative and nitrosative stress (Sharma et al. 2012). Therefore, the time course of NO and H$_2$O$_2$ accumulation in Rs-G seedlings over 24 h of salt stress may function as an important signal that promotes salt tolerance. S-G seedlings, which display a different time course of NO and H$_2$O$_2$ accumulation, may be more susceptible to salt-induced oxidative and nitrosative stress.

The available evidence indicates that NO serves as a key messenger in plant abiotic defence (Zhao et al. 2004, Arasimowicz-Jelonek et al. 2009, Elisabeth et al. 2014). However, there are still discrepancies regarding which enzymatic pathway is primarily responsible for NO production in plants (Moreau et al. 2010). Elisabeth et al. (2014) suggested that the NR pathway is the primary source of NO in Medicago truncatula seedlings under water-deficit stress. Another study reported that NOS-like activity contributed to NO accumulation in Hibiscus moscheutos (Tian et al. 2007). In the present study, NO accumulation during salt stress was strongly inhibited by the NR inhibitor, tungstate and by the NOS-like inhibitor, L-NAME, in both S-G and Rs-G plants. These findings suggest that both NR and NOS-like enzymes are involved in NO biosynthesis in the leaves of Rs-G seedlings under salt stress (Fig. 3). This finding is consistent with the previous study of Arasimowicz-Jelonek et al. (2009). The lack of consensus about how NO is synthesized in plants may reflect species-specific, treatment-specific or time-dependent differences in responses to stress. Our data indicate that there may be an interplay between NOS and NR pathways that influence NO production in Rs-G seedlings under Ca(NO$_3$)$_2$ stress.

H$_2$O$_2$ is a vital signaling molecule that controls various physiological processes, including tolerance to environmental stress (Neill et al. 2002). However, the relationship between H$_2$O$_2$ and NO in rootstock grafting-mediated systemic defence signaling is still unclear. We found that DMTU or DPI could significantly
Fig. 5. Effects of a hydrogen peroxide (H₂O₂) scavenger (DMTU) and an NADPH oxidase inhibitor (DPI) on the nitric oxide (NO) content of self-grafted (S-G) and rootstock-grafted (Rs-G) cucumber seedlings under Ca(NO₃)₂ stress. Three true-leaf stage S-G and Rs-G cucumber seedlings were pre-treated with 5 mM DMTU or 100 μM DPI for 8 h, followed by salt treatment for 24 h. (A) Representative pictures of NO fluorescence. Bar: 500 μm. (B) NO accumulation expressed as relative fluorescence. Values are means ± SE (n = 4). Values without a common letter are significantly different (P < 0.05). CK, control; N, salt stress; N + DMTU, salt stress + DMTU; N + DPI, salt stress + DPI.

inhibit H₂O₂ production induced by Ca(NO₃)₂ stress in Rs-G plants (Fig. 4A). Several studies have demonstrated that NADPH oxidase contributes to H₂O₂ production and accumulation in plants under stressful conditions (Torres et al. 2002, Apel and Hirt 2004). Our results suggest that some salt-induced H₂O₂ accumulation can be attributed to NADPH oxidase in Rs-G plants. Importantly, we showed that the NO accumulation induced by Ca(NO₃)₂ stress was inhibited by pretreatment with DMTU or DPI (Fig. 5). This indicates that H₂O₂ may contribute to NO production in Rs-G seedling leaves exposed to salt stress. We also found that pretreatment with the NO scavenger, cPTIO, or the NO synthesis inhibitors, tungstate and L-NAME, had no effect on salt-induced H₂O₂ production in Rs-G seedlings (Fig. 4B), indicating that NO does not influence H₂O₂ accumulation in the leaves of pumpkin-grafted cucumber seedlings under Ca(NO₃)₂ stress. In S-G plants, we did not find evidence for a link between NO and H₂O₂. It is possible that the high concentrations of NO and H₂O₂ in plants after salt stress may lead to nitrosative and oxidative stress, respectively. Therefore, the NO/H₂O₂
Fig. 7. Involvement of NO in the salt stress-induced activities of antioxidant enzymes in self-grafted (S-G) and rootstock-grafted (Rs-G) cucumber seedling leaves. Three true-leaf stage S-G and Rs-G cucumber seedlings were pre-treated with a nitric oxide (NO) scavenger (250 μM cPTIO), a nitrate reductase (NR) inhibitor (200 μM tungstate) or a nitric oxide synthase (NOS) inhibitor (200 μM L-NAME) for 8 h, followed by salt treatment for 24 h. Values are means ± SE (n = 3). Values without a common letter are significantly different (P < 0.05). CK, control; N, salt stress; N + cPTIO, salt stress + cPTIO; N + tungstate, salt stress + tungstate; N + L-NAME, salt stress + L-NAME.

signaling pathway may be disordered in S-G plants. Our data suggest that NO may act downstream of H2O2 in the leaves of Rs-G seedlings under Ca(NO3)2 stress. This is consistent with the finding that H2O2 plays a key role in modulating chitosan-induced NO synthesis in the guard cells of Pisum sativum (Nupur et al. 2009). However, other studies have yielded opposite findings. For example, in tobacco leaves NO was shown to positively regulate H2O2 production, whereas increases in H2O2 had no effect on NO levels (Stefania et al. 2008). Thus, the relationship between NO and H2O2 signaling pathways in plants appears to be complex. Indeed, Kulik et al. (2015) showed that bidirectional crosstalk occurs between NO and H2O2, whereby ROS formation precedes NO accumulation, and NO subsequently regulates the levels of H2O2. Discrepancies around NO and H2O2 crosstalk may also reflect species- or treatment-specific differences in plant responses to stress. In addition, the fluorescence parameter Fv/Fm is a measure of the primary photochemistry of PSII. Because it is specifically sensitive to various environmental stresses, it is considered a reliable indicator of stress (Figueroa et al. 1997, Sheng et al. 2008). We found that treatment of H2O2 and NO scavenger and inhibitor induced a decrease in Fv/Fm in Rs-G seedlings under salt stress (Fig. 6), indicating that both NO and H2O2 are involved in Ca(NO3)2 stress-induced salt tolerance.

In brief, these results suggest that NO production occurs downstream of H2O2 signaling under conditions of Ca(NO3)2 stress, a mechanism that may mediate the salt tolerance of Rs-G seedlings.

NO scavenger and inhibitors were then used to examine the role of NO in salt tolerance by rootstock grafting, and more specifically, its effect on the transcript levels and activities of antioxidant enzymes. Our results clearly showed that pretreatment with cPTIO, tungstate or L-NAME inhibited antioxidant gene transcription and enzyme activity in Rs-G seedlings under Ca(NO3)2 stress (Figs 7 and 8). These findings suggest that NO plays an important role in increasing the expression and activities of antioxidant enzymes, resulting in the protection of the Rs-G plants from oxidative stress. This is consistent with the results of Zhou et al. (2005) and Zhang et al. (2007a, 2007b). Compared with Rs-G plants, the S-G seedlings maintained higher POD expression and activity, possibly due to the presence of elevated levels of H2O2 during salt stress; POD may catalyse the oxidative cross-linking of cell wall polymers to control and regulate cell-wall
Involvement of NO in the salt stress-induced expression of antioxidant genes in self-grafted (S-G) and rootstock-grafted (Rs-G) cucumber seedling leaves. Three true-leaf stage S-G and Rs-G cucumber seedlings were pre-treated with a nitric oxide (NO) scavenger (250 μM cPTIO), a nitrate reductase (NR) inhibitor (200 μM tungstate) or a nitric oxide synthase (NOS) inhibitor (200 μM L-NAME) for 8 h, followed by salt treatment for 24 h. Values are means ± SE (n=3). Values without a common letter are significantly different (P < 0.05). CK, control; N, salt stress; N+cPTIO, salt stress + cPTIO; N+tungstate, salt stress + tungstate; N+L-NAME, salt stress + L-NAME.

stiffening (Figs 7D and 8D) (Sun et al. 2014). In addition, our results suggest that H₂O₂ could enhance the antioxidant ability of Rs-G seedlings under Ca(NO₃)₂ stress by enhancing the expression and activities of antioxidant enzymes (see Figs S2 and S3), which indicates that NO and H₂O₂ accumulation are necessary for the expression of antioxidant enzymes. In summary, H₂O₂ is involved in the NO signaling pathway, and this mechanism could mediate the stress tolerance induced by Ca(NO₃)₂ in the leaves of pumpkin-grafted cucumber seedlings.

**Conclusion**

In summary, we demonstrate that high salinity induces a transient accumulation of NO in Rs-G cucumber plants that can be attributed to both NR and NOS enzymatic pathways and that this NO accumulation is H₂O₂-dependent. In addition, NO may play an important role in regulating the gene expression and activities of antioxidant enzymes in Rs-G plants subjected to Ca(NO₃)₂ stress. Therefore, Rs-G cucumber seedlings may be more tolerant to high salt conditions compared with S-G seedlings. Our study provides insight into the mechanisms by which Rs-G plants defend against high salinity.

**Author contributions**

L. L. conducted the experiments, analyzed the data and drafted the manuscript. Q. X. and Y. H. A. participated in plant cultivation and some experiments. S. S. modified this manuscript. J. S. and S. R. G. designed the research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Effects of a hydrogen peroxide (*H2O2*) scavenger (DMTU), an NADPH oxidase inhibitor (DPI), a nitric oxide (NO) scavenger (cPTIO), a nitrate reductase (NR) inhibitor (tungstate) and a nitric oxide synthase (NOS) inhibitor (L-NAME) on *H2O2* production in self-grafted...
(S-G) and rootstock-grafted (Rs-G) cucumber seedlings under Ca(NO₃)₂ stress.

**Fig. S2.** Involvement of H₂O₂ in the salt stress-induced activities of antioxidant enzymes in self-grafted (S-G) and rootstock-grafted (Rs-G) cucumber seedling leaves.

**Fig. S3.** Involvement of H₂O₂ in the salt stress-induced expression of antioxidant genes in self-grafted (S-G) and rootstock-grafted (Rs-G) cucumber seedling leaves.

**Table S1.** Primer sequences for real-time quantitative RT-PCR.