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Nitric Oxide and Its Interaction with Hydrogen Peroxide Enhance Plant Tolerance to Low Temperatures by Improving the Efficiency of the Calvin Cycle and the Ascorbate–Glutathione Cycle in Cucumber Seedlings

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Abstract

The present study was aimed to assess the effect of nitric oxide (NO) and the interaction of NO with hydrogen peroxide (H_2O_2) on plant tolerance to low temperatures in cucumber seedlings. Exogenous NO significantly increased the endogenous NO content, initial and total activities of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), RuBisCO carboxylation rate ($V_{c,max}$), RuBP regeneration rate (J_{max}) and the transcript levels of related genes in cucumber seedlings under low temperatures (11 °C/7 °C); however, the effect of NO was blocked by PTIO (NO scavenger). In addition, the SNP treatment significantly improved the contents of glucose, fructose, sucrose, starch, and the activities of sucrose phosphate synthase (SPS), acid invertase (AI), sucrose synthase (SS), as well as the expression levels of *SUCROSE PHOSPHATE SYNTHASE 1&2* (*SPS1*, *SPS2*), *SUCROSE TRANSPORTER 1&2* (*SUT2*, *SUT4*), β-starch hydrolase (*BAM*), and invertase gene (*INVERTASE*) in cucumber leaves under low temperatures, and the positive effect of NO was impaired by PTIO. Furthermore, we found that the H_2O_2, induced by NO, participated in NO-induced elevation of ascorbic acid (AsA), glutathione (GSH), and increased activities of related enzymes in the AsA-GSH cycle at low temperatures. However, the positive effect of NO was blocked by L-NAME (NOS inhibitor), PTIO, DPI (inhibitor of NADPH oxidase), and DMTU (reactive oxygen species scavenger). Taken together, our findings indicate that NO increased the low temperature tolerance of cucumber seedlings via H_2O_2 by improving the efficiency of the Calvin cycle, which in turn increased the carbohydrates content and accelerated the AsA-GSH cycle to enhance ROS scavenging.

Keywords Cucumber · Low temperature · Nitric oxide · Sucrose · Calvin cycle · Ascorbate–glutathione

Introduction

Most tropical and subtropical plant species lack the ability to cope with low temperatures and are typically injured by temperatures below 15 °C. Cucumber (*Cucumis sativus* L.) is a typical subtropical plant, which is susceptible to low temperature stress, particularly during off-season cultivation (September to December). Low temperature is one of the most critical abiotic factors limiting the growth and production of plants (Puyaubert and Baudouin 2014). Increasing evidence indicates that low temperatures can cause an imbalance between reactive oxygen species (ROS) production and scavenging. If not detoxified in time, the excess ROS will increase the membrane permeability and membrane lipid peroxidation, DNA damage and protein

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denaturation (Ruelland et al. 2009). In addition, excessive generation of ROS caused by low temperatures could further severely impair the photosynthetic activity, including inhibition or abnormality of ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (RuBisCO) activity, carbon fixation, and chloroplast morphologies (Ruelland et al. 2009; Zhang et al. 2014). Plants have evolved a series of strategies to cope with low-temperature stress, such as strengthening the induction of antioxidant systems and synthesis of protective molecules (e.g., sugar, reduced glutathione and polyamines) (Theocharis et al. 2012).

H_2O_2 is the most stable ROS molecule, which was thought to be a toxic byproduct of aerobic metabolism previously (Ahammed et al. 2020a; Dat et al. 2000; Wang et al. 2010). In contrast with earlier views, H_2O_2 has also been implicated in a multitude of cellular signaling networks in plants (Mittler 2017). H_2O_2 acts as signaling molecule for the activation of defense response when present at non-toxic levels (Ahammed et al. 2020b; Arfan et al. 2019; Zhou et al. 2014). In addition, studies have reported that *RBOH1*-dependent H_2O_2 production is critical for the epigallocatechin-3-gallate-induced tolerance to abiotic stress (Li et al. 2019; Zhang et al. 2020a). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the main enzymatic sources for the generation of H_2O_2 in the cell membrane of plants (Ahammed et al. 2020b). Rapid H_2O_2 production in the root tips of tolerant wheat genotype in response to aluminum (Al) stress is likely to be resulted, at least in part, from NADPH oxidase (Sun et al. 2018). Therefore, the diphenyleneodonium (DPI, an inhibitor of NADPH oxidase) is widely used to study the effects of NADPH-dependent H_2O_2 in plants.

A large body of evidence demonstrates that nitric oxide (NO) is a gaseous signaling molecule, which acts as a potential mediator of numerous biochemical and physiological processes in plants under biotic and abiotic stresses (Kotapati et al. 2017; Sharma et al. 2020a, b). Studies have reported that NO and its related RNS have the capacity to govern every single step of plant developmental process by balancing antioxidants and ROS under stress conditions (Begara-Morales et al. 2018). NO is synthesized endogenously in plants (Castello et al. 2019; Fancy et al. 2017; Zhang et al. 2020a, b). The main sites of NO production in plants are cytoplasm, chloroplasts, mitochondria, and peroxisomes, and both biotic and abiotic stresses can alter NO production (Terron-Camero et al. 2020). Nitric oxide synthase (NOS)-and nitrite reductase (NR)-based pathways are the major NO production routes, although the existence of NOS genes in plants still remains controversial (Astier et al. 2016). Notably, NO itself is a reactive nitrogen species and its roles in cells have been proven to be either as a potent oxidant or as an effective antioxidant; nonetheless, the positive effects of NO in plants largely depend on its local concentrations and spatial accumulation patterns (Fatma and Khan 2014; Sami et al. 2018). More interestingly, numerous studies have reported cross-talk between NO and other signaling molecules such as H_2O_2 during exposure of plants to environmental stress (Arfan et al. 2019; Si et al. 2017; Sun et al. 2018).

In recent years, NO along with ROS are supposed to accomplish developmental and stress responses (Nidhi et al. 2020). For instance, NO regulates the H_2O_2 generation under salt stress, and H_2O_2 potentially acts downstream of NO to regulate the activity of plasma membrane (PM) H^+-ATPase under salt stress (Mazid et al. 2011). However, in some other conditions, H_2O_2 mediates NO production, and NO as a downstream signal of H_2O_2, increases the activities of antioxidant enzymes (Sun et al. 2018). As observed by Tanou et al. (2010), the protein network interfered by H_2O_2 and NO mainly belongs to photosynthesis and especially the Calvin–Benson cycle.

In our previous study, we showed that NO could enhance the chilling tolerance of cucumber seedlings (Zhang et al. 2020b). To our knowledge, as yet, only a small number of studies have reported the effects of NO on photosynthetic carbon assimilation in response to chilling stress in cucumber seedlings. Therefore, in the present study, we investigated the effect of NO on carbohydrate metabolism and the Calvin-Benson cycle. Additionally, we analyzed the influence of NO and the interaction of NO produced by the NOS-dependent pathway with H_2O_2 on the AsA-GSH cycle in cucumber seedlings under low temperature stress.

Materials and Methods

Plant Materials

The experiments were carried out in the solar greenhouse and laboratory of the Experiment Station of the Agricultural College, (longitude 86° and latitude 44.18° N), Shihezi University, China. Cucumber (*C. sativus* L.) cultivar 'Jinyan No. 4' was used for the study. Healthy and uniform seeds of cucumber were selected and disinfected by soaking in 55 °C hot water, then the seeds were further soaked for 6 h at room temperature, followed by photophobic incubation at 28 °C. Cucumber seeds were germinated in a growth medium filled with a mixture of peat and vermiculite (2:1, v:v). The seedlings were transplanted into plastic pots (diameter × height, 120 × 110 mm) containing a mixture of peat and vermiculite (2:1 by volume, with one seedling per container), when the two cotyledons were fully expanded. Thereafter, the seedlings were irrigated daily and fertilized every three days with Hoagland's nutrient solution. When the second true leaves of seedlings were fully expended, the seedlings were transferred to RXZ intelligent artificial incubator (Ningbo,

China) and used for the experiment. The environmental conditions were as follows: light cycle 12 h, the temperature of 25 °C/20 °C (day/night), photosynthetic photon flux density (PPFD) of 120 μmol m^{-2} s^{-1}, and relative humidity was controlled at around 70%.

Experimental Procedures

To investigate the effects of NO on photosynthetic carbon assimilation and carbohydrate metabolism in cucumber seedlings under low temperature stress, the seedlings were treated with distilled water, sodium nitroprusside (SNP, 200 μmol L^{-1}), and PTIO (200 μmol L^{-1}) combined with SNP (Cui et al. 2011; Zhang et al. 2020b). The treatments were classified as presented in Table 1. After 24 h of treatment, low temperature treatment was carried out (11 °C with a 12 h-light, 7 °C with a 12 h-dark cycle, light intensity 120 μmol m^{-2} s^{-1}). The second leaves of cucumber seedlings from the bottom were harvested at various durations of low temperature stress (2 h, 5 h, 7 h, 24 h, and 48 h) and immediately frozen in liquid nitrogen for subsequent analyses.

To investigate the interrelation of NO and H_2O_2 in the low temperature-induced lipid peroxidation and redox state, the cucumber seedlings were pre-treated with 200 μmol L^{-1} L-NAME, 200 μmol L^{-1} PTIO, 100 μmol L^{-1} DPI (diphenyleneodonium, a NADPH oxidase inhibitor) or 5 mmol L^{-1} DMTU (dimethylthiourea, a H_2O_2 and OH· scavenger), and 8 h after the pre-treatment, the plants were sprayed with 200 μmol L^{-1} SNP (Cui et al. 2011). The treatments were classified as presented in Table 1. Sixteen hours after the SNP treatment, the seedlings were exposed to low temperature (11 °C with a 12 h-light, 7 °C with a 12 h-dark cycle, light intensity 120 μmol m^{-2} s^{-1}). Cucumber seedlings were harvested at various durations of low temperature stress (0 h, 2 h, 5 h, 7 h, 24 h, and 48 h) and leaf samples were immediately frozen in liquid nitrogen.

Nitric Oxide Fluorescence Detection

According to the method of Corpas et al. (2004), the DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate), a probe highly specific to NO, was used to detect the NO fluorescence. Cucumber leaf segments (approximately 20–25 mm^2) were incubated in 10 μmol L^{-1} DAF-FM DA (prepared in 10 μmol L^{-1} Tris–HCl, pH 7.4) at 25 °C for 1 h in the dark and then washed at least twice with the same buffer for 15 min each. The confocal laser scanning microscope system (ZEISS LSM 510 META, Germany) was used for detecting NO fluorescence, and the standard filters and collection modalities for DAF-FM DA green fluorescence (excitation 495 nm; emission 515 nm) were also used.

Determination of Activity and Carboxylation of RuBisCO and Regeneration Rate of RuBP

RuBisCO activity was measured according to the improved method of Lilley and Walker (1974). The assay of RuBisCO initial activity was assayed by determining the rate of change in the absorbance at 340 nm over 90 s. Added 10 μL of the enzyme solution to 0.1 mL reaction solution. The reaction mixture consisted of 5 mmol L^{-1} Heps-NaOH (pH 8.0) buffer, 1 mmol L^{-1} $NaHCO_3$, 2 mmol L^{-1} $MgCl_2$, 0.25 mmol L^{-1} DTT, 0.1 mmol L^{-1} EDTA, 1 U inositol kinase, 1 U 3-phosphoglycerate kinase, 1 U GAPDH, 0.5 mmol L^{-1} ATP, 0.015 mmol L^{-1} $NADH_2$, 0.5 mmol L^{-1} inositol phosphate and 0.06 mmol L^{-1} RuBP. The enzyme activation reaction (30 °C, 15 min) was carried out before the RuBisCO total activity measurement. The activated reaction solution contained 33 mmol L^{-1} Tris–HCl (pH 7.5), 0.67 mmol L^{-1} EDTA, 33 mmol L^{-1} $MgCl_2$, and 10 mmol L^{-1} $NaHCO_3$. After the activation, the reaction solution was added. When the temperature reached the measurement temperature, RuBP was quickly added, and the absorbance of 340 nm was recorded according to the initial viability method.

The gas exchange parameters including net photosynthetic rate (P_n), stomatal conductance (G_s), intercellular CO_2 concentration (C_i) and transpiration rate (T_r) were measured using a LI-6400 Portable Photosynthesis system (LI-COR Inc., Lincoln, NE, USA). The carboxylation rate ($V_{c,max}$) of RuBisCO and the regeneration rate (J_{max}) of RuBP were calculated by the method of Ethier and Livingston (2004) based on the gas exchange parameters.

Table 1 Pharmacological treatments used in the current study

Spraying time	Treatments					
	CK	SNP	PTIO + SNP	L-NAME + SNP	DPI + SNP	DMTU + SNP
10:00	Distilled water	Distilled water	PTIO	L-NAME	DPI	DMTU
18:00	Distilled water	SNP	SNP	SNP	SNP	SNP

SNP, 200 μmol L^{-1} sodium nitroprusside (NO donor); PTIO, 200 μmol L^{-1} 2-phenyl-4, 4, 5, 5-tetramethylimidazolinium-1-oxo-3-oxide; L-NAME, 200 μmol L^{-1} N^G-nitro-L-arginine methyl ester; DPI, 100 μmol L^{-1} diphenyleneodonium (NADPH oxidase inhibitor); DMTU, 5 mmol L^{-1} dimethylthiourea (the scavenger of H_2O_2 and OH·)

Determination of Carbohydrates and Related Enzyme Activity

The contents of carbohydrates were measured according to the method of Buysse and Merckx (1993) as described in detail previously (Zhang et al. 2020b). The carbohydrates were extracted from 0.1 g of fresh material, and incubated with 4 mL of 80% ethanol for 30 min at 85 °C, then centrifuged at room temperature for 30 min. The precipitate was repeatedly extracted three times (80% ethanol 2 mL for each time) and the supernatant was combined. After the pigment of extract was adsorbed by activated carbon (0.1 g), the extract was brought to a volume of 50 mL. The resulting solution was used to determine the content of glucose, fructose and sucrose, and the content of starch was measured using the residue after plant sugar extraction.

Sucrose phosphate synthase (SPS) and sucrose synthase (SS) were extracted at 0–4 °C, according to the method described by Lowell et al. (1989). SPS activity was measured at a wavelength of 620 nm in 37 °C. Acid invertase (AI) was extracted as described by Schaffer et al. (1989). The reaction mixture consisted of 4% sucrose, 50 mmol L^{-1} sodium acetate buffer (pH 4.5), and an aliquot of enzyme solution in a total volume of 1 mL. The activity of the AI was measured by incubating the reaction solution at 30 °C for 15 min.

Histochemical Staining of H_2O_2

In situ H_2O_2 accumulation was assayed by histochemical staining of cucumber leaves with 3,3-diaminobenzidine (DAB) according to the method of Thordal et al. (1997) as described previously (Hasan et al. 2019).

Determination of Lipid Peroxidation

Lipid peroxidation was determined by quantifying the content of leaf malondialdehyde (MDA). The content of MDA was determined based on the method of Health and Packer (1968), based on the thiobarbituric acid (TBA) reaction. The MDA contents were calculated based on absorption at 532 and 600 nm, with an extinction coefficient of 155 mM^{-1} cm^{-1}.

Determination of the Related Substances and Enzyme Activities in AsA-GSH Cycle

Frozen leaf tissues (0.3 g) were homogenized in 3 mL ice-cold 6% metaphosphoric acid containing 0.2 mmol L^{-1} EDTA. The homogenate was centrifuged at 4 °C for 15 min at 12,000×*g* and the supernatant was used for antioxidant analysis. The content of reduced ascorbate (AsA) and oxidized ascorbate (dehydroascorbate, DHA) were detected based on the method of Law et al. (1983). Absorbance was recorded at 525 nm. The contents of reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined according to the method by Griffith (1980) based on the changes in absorption at 412 nm.

Cucumber leaves (0.3 g frozen leaf tissues) were homogenized in 3 mL ice-cold phosphate (pH 7.8) buffer containing 0.2 mmol L^{-1} EDTA, 2% PVP (W/V). The homogenate was centrifuged at 4 °C for 20 min at 12,000×*g* and the extract was used for determining the activities of antioxidant enzyme. Ascorbate peroxidase (APX) activity was measured according to Nakano and Asada (1981). The reaction mixture (2 mL) contained the enzyme extract (0.1 mL), 25 mmol L^{-1} sodium phosphate buffer (pH 7.0), 0.5 mmol L^{-1} ascorbate, 0.1 mmol L^{-1} H_2O_2, and 0.1 mmol L^{-1} EDTA. The reaction was started by adding H_2O_2.

The activity of glutathione reductase (GR) was measured according to Foyer and Halliwell (1976). The activity of GR was determined by the reduction rate of NADPH at 340 nm. The reaction mixture consisted of 25 mmol L^{-1} HEPES buffer (pH 7.0), 0.5 mmol L^{-1} oxidized glutathione (GSSG), 0.12 mmol L^{-1} NADPH, 0.2 mmol L^{-1} EDTA, and 0.1 mL enzyme extract.

The activity of dehydroascorbate reductase (DHAR) was detected by measuring the increase of absorbance at 265 nm due to dehydroascorbate (DHA) formation. The reaction mixture of 2 mL contained 25 mmol L^{-1} sodium phosphate buffer (pH 7.0), 2.5 mmol L^{-1} reduced glutathione (GSH), 0.4 mmol L^{-1} DHA, and 0.1 mL enzyme extract.

The activity of monodehydroascorbate reductase (MDAR) was analyzed by determining the rate of decrease in the absorbance at 340 nm due to NADH oxidation using a UV-3900 (Japan) spectrophotometer.

Total RNA Extraction and Gene Expression Analysis

The extraction of total RNA from cucumber leaves was carried out using Trizol reagent according to the supplier's recommendation. The nucleic acid content was detected by Nanodrop 2000, and 2% agarose gel electrophoresis was performed to verify RNA integrity. Total RNA (1 μg) was reverse-transcribed using ReverTra Ace qRT-PCR first Strand cDNA Synthesis kit (TOYOBO, Japan). The gene-specific primers were designed based on the sequence of CDS, and the primers were presented in Table 2.

Quantitative real-time PCR was performed in the PCR detection system of iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The SYBR Green Realtime PCR Master Mix (TOYOBO, Japan) was used for PCRs. The PCR conditions consisted of denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min. The analysis of the

Table 2 The primer sequences for qRT-PCR

Gene	Forward primer	Reverse primer
Actin (AB010922)	5′-TACCGGCGGGATTATTAACT-3′	5′-GCTACTGCGGTACCAATCAA-3′
BAM (Csa3G751970)	5′-ACCGAATATGGCGAATTCTT-3′	5′-TGCCAATGAATTCCTGCTAC-3′
Invertase (KP727813.1)	5′-CTGGAAAGAATATGGTGGCA-3′	5′-CCCAAGAGAAGGATCGAGAG-3′
SPS1 (JX046480.1)	5′-GGTGGTCATTCCTCCTGGTA-3′	5′-TTGTAAGAAACCGCATCACATC-3′
SPS2 (JX046481.1)	5′-GGTGGTCATTCCTCCTGGTA-3′	5′-GGTGGTCATTCCTCCTGGTA-3′
SUT2 (GU356640.1)	5′-ATGGAAGCCAACCTGAACAC-3′	5′-AGGACCCAGCAAGAATGAAG-3′
SUT4 (GQ903726.1)	5′-GGTTTCTGGGGTTCTGAGCA-3′	5′-CTTAGCAATAGTTTTCCCACAGGTT-3′
RCA (FJ980456.1)	5′-GCTGACAACCCAACCAA-3′	5′-CATCCGACCATCACGAA-3′
rbcS (EF208124)	5′-CGCTGTTGCCTCTGTGAA-3′	5′-AGAGTAGAACTTGGGGCTTGTA-3′
rbcL (EF208123)	5′-TATGGTCGCCCTCTATTG-3′	5′-GCGGCTTCGGTCTTTTTC-3′
FBPase (DQ641061.1)	5′-GGGAGAGGACCAGAAAAA-3′	5′-GGCTGTAGATGCCAAAGA-3′
SBPase (NM_001280729.1)	5′-CAGGGTTATCAAATGTGG-3′	5′-TATGGTCGCCCTCTATTG-3′
FBPaldolase (GT564642.1)	5′-CTCGTGCTGCTGCTTACT-3′	5′-CTGCCCAAACTTTCTGTG-3′

dissolution profile was carried out at 65 °C to 95 °C. To minimize sample variations, mRNA expression of a target gene was normalized relative to the expression of housekeeping gene *Actin*. All experiments were repeated three times using cDNA prepared from three biological replicates of cucumber leaves. The fold changes in expression level relative to the control were expressed as $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen 2001).

Statistical Analysis

All treatments were conducted at least three replicates. The data were analyzed using SPSS version 19.0 (SPSS, Inc., Chicago, IL). Results were expressed as mean ± standard deviation (SD). The Tukey test (HSD) was used for significance analysis. Differences between treatments were considered to be significant, when the P value was less than 0.05 ($P < 0.05$).

Results

SNP Induces NO Accumulation Under Low Temperature

To investigate whether the exogenous SNP can increase endogenous NO accumulation in cucumber seedlings under low temperature stress, we pre-treated cucumber leaves with SNP and PTIO. The NO signals in cucumber leaves after 2 h, 5 h and 7 h of low temperature were shown in Fig. 1. Compared with CK, SNP treatment alone greatly increased the NO signal, but PTIO diminished the positive effect of SNP to some extent.

Effect of NO on the Carboxylation Rate of RuBisCO and Regeneration Rate of RUBP

To study the effect of NO on photosynthetic carbon assimilation after 24 h of low temperature stress, we detected the carboxylation rate ($V_{c,max}$) of RuBisCO and regeneration rate (J_{max}) of RuBP after 24 h of low temperature treatment. As presented in Table 3, compared with CK (11 °C/7 °C for 24 h), exogenous SNP significantly improved the $V_{c,max}$ and J_{max} by 25.3% and 19.9%, respectively. However, the PTIO in combination with SNP treatment, lead to a significant decrease in $V_{c,max}$ of RuBisCO and J_{max} of RuBP by 24.3% and 24.3%, respectively, when compared with the SNP treatment alone.

Effect of NO on the Activity of RuBisCO

To further determine whether NO could induce the process of carbon dioxide (CO_2) fixation after 24 h of low temperature stress, we examined the initial and total activity of RuBisCO in cucumber seedlings that were pre-treated with SNP and PTIO. As shown in Fig. 2, pre-treatment of cucumber seedlings with SNP significantly enhanced the initial (Fig. 2A) and total activities (Fig. 2B) of RuBisCO by 43.86% and 38.16% compared with CK (11 °C/7 °C for 24 h), while PTIO significantly suppressed the effect of NO on initial and total activities of RuBisCO by 65.79% and 42.54%, respectively.

Effect of NO on the Transcript Levels of Calvin–Benson Cycle-Related Genes

Next, we analyzed the changes in the transcript levels of some genes encoding for enzymes related to photosynthetic carbon assimilation in cucumber seedlings after 24 h of low

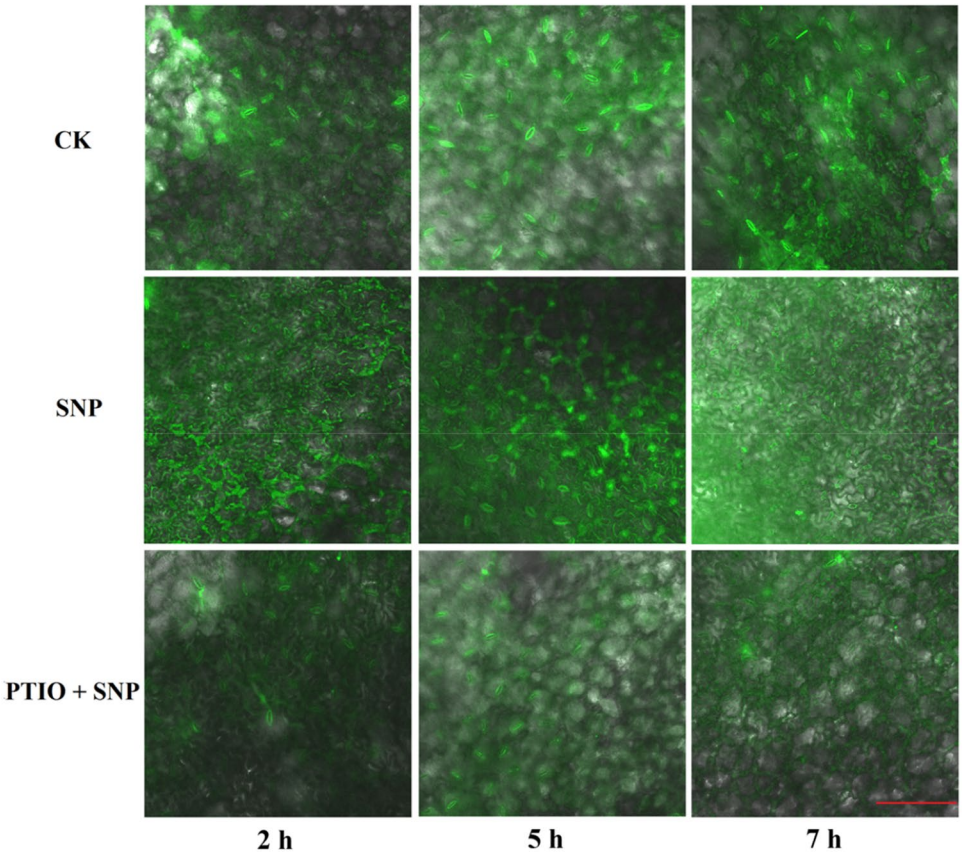

Fig. 1 Nitric oxide (NO) accumulation in cucumber leaves as influenced by SNP (NO donor) and PTIO (NO scavenger). The seedlings were treated with distilled water (CK), 200 µmol L^{-1} SNP and 200 µmol L^{-1} PTIO + 200 µmol L^{-1} SNP. After 24 h of the first spray, low temperature treatment (10 °C/12 h during the day and 6 °C/12 h at night) was imposed. Segments of the second leaves were loaded with diaminofluorescein-FM diacetate (DAF-FM DA) after 2, 5 and 7 h of low temperature treatment, and NO accumulation was detected by confocal laser scanning microscope system (scale bars = 100 µm)

Table 3 Effects of NO donor and NO scavenger on the RuBisCO carboxylation rate and RuBP regeneration rate under low temperature

Treatment	$V_{c,max}$ (µmol m^{-2} s^{-1})	J_{max} (µmol m^{-2} s^{-1})
CK	31.65 ± 2.19b	77.45 ± 3.59b
SNP	38.06 ± 1.62a	92.85 ± 3.23a
PTIO + SNP	28.81 ± 1.08b	70.32 ± 3.18c

In each column different letters mean significant differences ($P < 0.05$) between the treatments

temperature stress (Fig. 3). Compared with CK (11 °C/7 °C for 24 h), SNP pretreatment significantly increased the expression levels of *RCA*, *rbcS*, *FBPaldolase, FBPase*, and *SBPase* by 3, 3, 2.5, 2, and 1.3 fold, respectively, while the expression level of *rbcL* decreased in cucumber seedlings by 0.7 fold under low temperature stress. In addition, the PTIO in combination with SNP treatments significantly attenuated the expression levels of *RCA*, *rbcS*, *FBPase*, *SBPase*, and *FBPaldolase*, and increased the *rbcL* expression level.

Effect of NO on the Accumulation of Carbohydrates Under Low Temperature

As presented in Fig. 4, the content of glucose, fructose, sucrose, and starch in cucumber seedlings increased with the prolongation of low temperature stress. In addition, compared with the CK (11 °C/7 °C for 24 h), exogenous NO significantly increased the content of glucose, fructose, sucrose and starch in cucumber leaves by 30.1%, 22.2%, 4.8% and 27.5%, respectively at 24 h of low temperature treatment. In addition, NO induced the content of glucose, fructose, sucrose and starch by 46.5%, 50.9%, 33.6%, and 26.6% at 48 h of low temperature stress when compared to CK (11 °C/7 °C for 48 h), respectively. However, the effect of exogenous NO on the accumulation of carbohydrates was either attenuated or reversed by PTIO.

Effect of NO on the Metabolism of Sucrose

In order to clarify the role of NO in the regulation of sucrose metabolism, we determined the activity of sucrose metabolism related enzymes. Table 4 showed that NO induced the activity of sucrose phosphate synthase (SPS) by 12.7% and 8.2% at 24 h and 48 h of low temperature stress, respectively. Importantly, these results were consistent with the content of

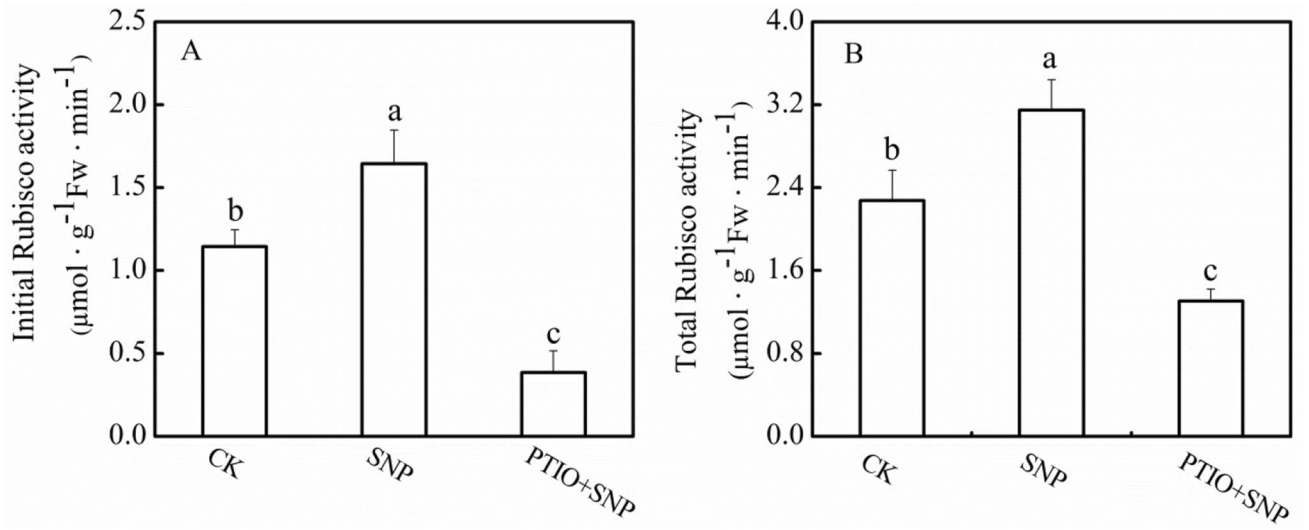

Fig. 2 The initial activity (**A**) and total activity (**B**) of RuBisCO in cucumber leaves after low temperature treatment (11 °C/7 °C for 24 h) as influenced by SNP and PTIO pre-treatments. Values are the means ± SD ($n = 3$). Means with different letters are significantly different at $P < 0.05$

sucrose in Fig. 4c. In addition, NO induced the activities of acid invertase (AI) and sucrose synthase (SS) by 15.6% and 12.4% at 24 h of low temperature stress, and by 13.6% 13.4% at 48 h of low temperature stress, respectively. However, the positive effect of NO on sucrose metabolism was attenuated by PTIO treatment.

Effect of NO on the Expression Levels of Carbohydrate Metabolism-Related Genes

To investigate the effect of NO on sucrose synthesis and transport in low temperature-stressed cucumber seedlings, we assessed the transcript levels of β-starch hydrolase gene (*BAM*) (it often plays an important role in decomposition of starch to sucrose in plants), invertase gene (*Invertase*), *SUCROSE PHOSPHATE SYNTHASE 1&2* (*SPS1*, *SPS2*), *SUCROSE TRANSPORTER 1&2* (*SUT*2, *SUT*4). As evident from Fig. 5, compared with CK, the expression levels of *SPS1*, *SPS2*, *SUT*2*,* and *SUT4* were significantly increased by NO in cucumber seedlings regardless of the duration of low temperature stress. In particular, the transcript levels of *SPS1*, *SPS2*, *SUT*2, *SUT4,* and *Invertase* increased 0.18, 1.53, 0.57, 0.54, and 0.52 fold, respectively, in SNP-pretreated seedlings at 24 h of low temperature stress. NO also induced the transcript levels of *SPS1*, *SPS2*, *SUT2* and *SUT4* by 0.54, 0.85, 0.74, and 0.89-fold at 48 h of low temperature stress, respectively. There was no significant difference in the transcript level of *Invertase* at 48 h of low temperature stress. Apart from these, NO significantly increased the expression level of *BAM* by 0.37 fold after 24 h of low temperature stress; however, the transcript levels of *BAM* significantly decreased by 0.4 fold after 48 h of low temperature stress in cucumber seedlings when compared with CK. Notably, compared with SNP treatment alone, PTIO in combination with SNP treatment significantly diminished the effect of SNP on the transcript levels of *SPS1*, *SPS2*, *SUT2* and *SUT4*.

H_2O_2 is Involved in NO-Induced Alleviation of Oxidative Stress in Cucumber Seedlings

To further determine whether H_2O_2 is involved in NO-induced low temperature tolerance of cucumber seedlings, we investigated the in situ H_2O_2 accumulation in cucumber leaves by histochemical staining with DAB. The L-NAME, PTIO, DPI, and DMTU were used to understand the relationship between NO and H_2O_2. As shown in Fig. 6, pretreatment of cucumber seedlings with SNP enhanced the H_2O_2 accumulation during the first 7 h when compared with CK, while simultaneous application of L-NAME and SNP, PTIO and SNP reduced the SNP-induced H_2O_2 production up to 7 h of low temperature treatments. However, compared to CK, the content of H_2O_2 was greatly decreased by SNP after 24 h and 48 h of low temperature treatments. But compared to SNP treatment alone, L-NAME and PTIO in combination with SNP treatments partly blocked the H_2O_2 content under low temperature for 24 h and 48 h. Notably, pretreatment of cucumber seedlings with DPI and DMTU along with SNP also attenuated the SNP-induced H_2O_2 levels under low temperature for 2 h, 5 h and 7 h when compared with SNP treatment alone.

To further investigate the effect of H_2O_2 in NO-induced tolerance to low temperature in cucumber seedlings, we analyzed the content of MDA, an important marker of lipid peroxidation and relevant oxidative stress. As shown

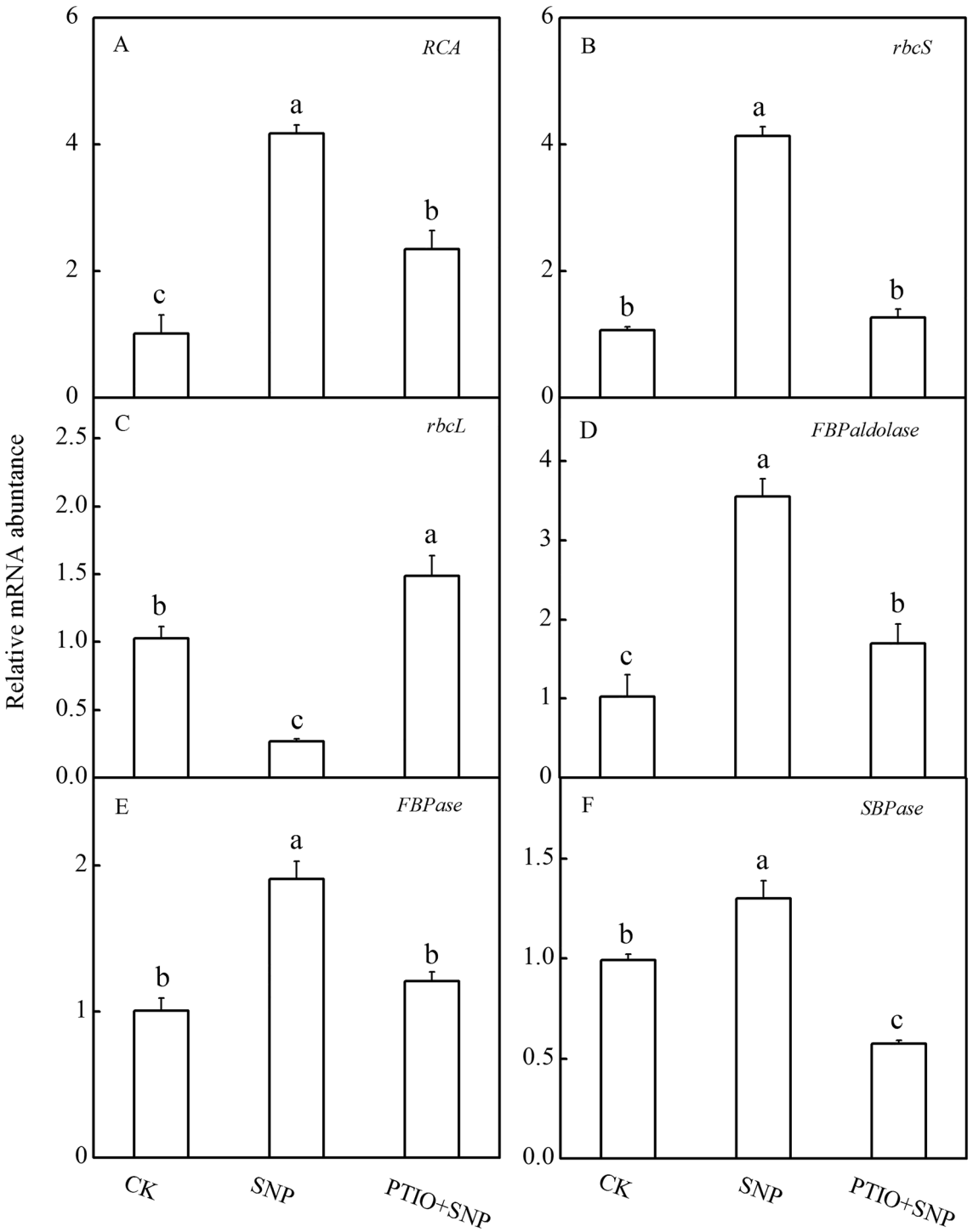

Fig. 3 Transcript levels of *RCA* (**A**), *rbcS* (**B**), *rbcL* (**C**), *FBPaldolase* (**D**), *FBPase* (**E**), and *SBPase* (**F**) in cucumber leaves as affected by SNP and PTIO treatments. Error bars represent SD ($n = 3$). Bars with a different letter indicates a significant difference at $P < 0.05$

in Fig. 7, compared with CK, pre-treatment of cucumber seedlings with SNP alone significantly reduced the MDA content by 40% and 42.1% when cucumber seedlings were exposed to low temperature for 24 h and 48 h, respectively. In addition, L-NAME in combination with SNP, and PTIO in combination with SNP, significantly increased the content of MDA, whether in normal condition or under low temperature stress, when compared with SNP alone. Intriguingly, compared with SNP, the DPI plus SNP and DMTU plus SNP also significantly increased the content

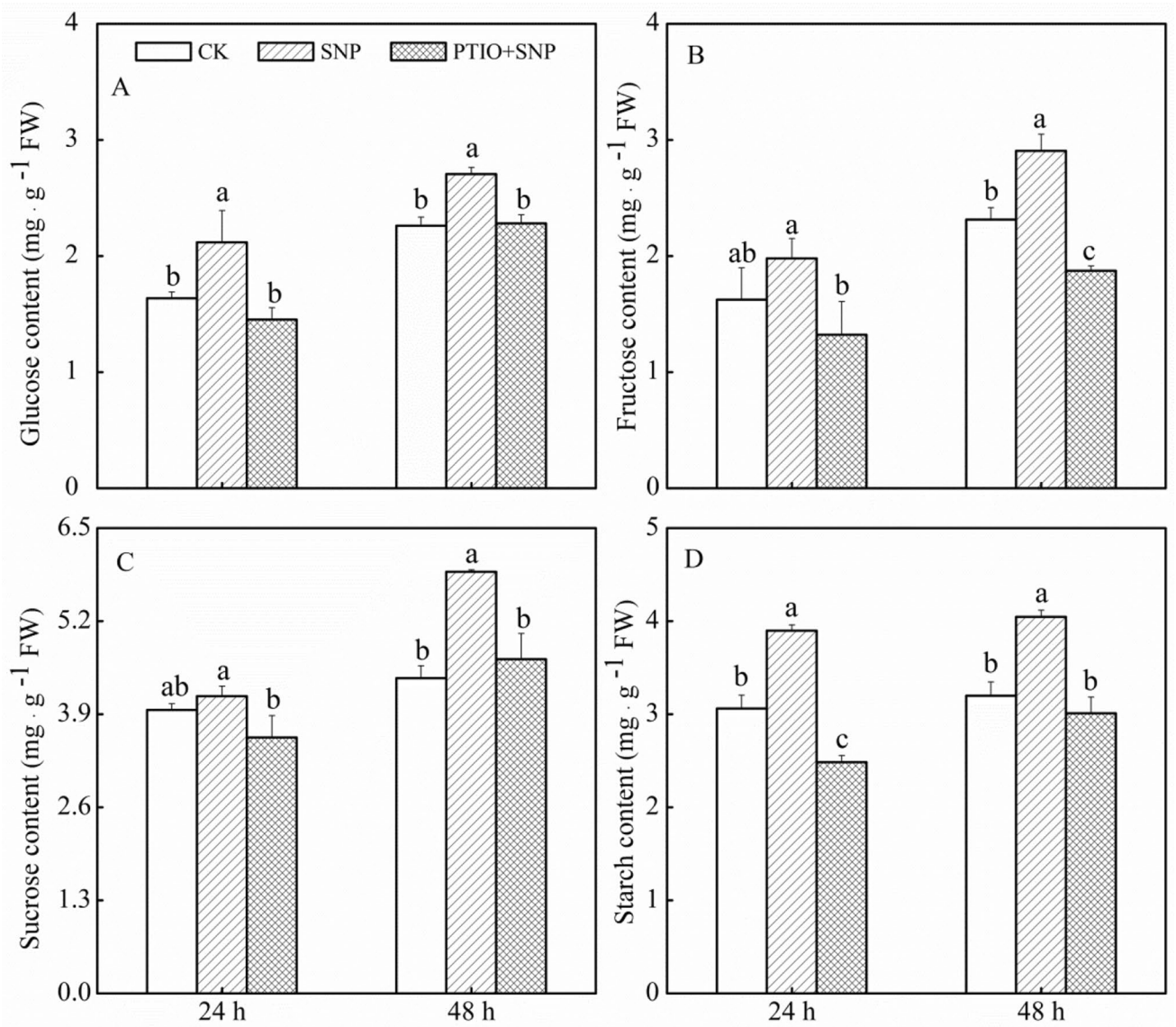

Fig. 4 The content of glucose (**A**), fructose (**B**), sucrose (**C**), and starch (**D**) in leaves of low temperature-stressed cucumber seedlings as affected by SNP and PTIO. Values are means ± SD ($n = 3$). Values with a different letter within a sampling data are significantly different ($P < 0.05$)

Table 4 Effect of different treatments on the activities of sucrose phosphate synthase (SPS), acid invertase (AI) and sucrose synthase (SS) in cucumber leaves under low temperature

Duration of low temperature stress (h)	Treatments	SPS activity (IU·L^{-1})	AI activity (IU·L^{-1})	SS activity (IU·L^{-1})
24 h	Control	153.74 ± 4.31c	18.71 ± 0.55b	21.63 ± 0.60b
	SNP	173.32 ± 3.30a	21.63 ± 0.18a	24.32 ± 0.49a
	PTIO + SNP	164.38 ± 4.81b	19.75 ± 0.74b	22.13 ± 0.27b
48 h	Control	159.14 ± 2.50b	19.27 ± 0.45c	21.57 ± 0.80b
	SNP	172.14 ± 3.04 a	21.89 ± 0.25a	24.47 ± 1.23a
	PTIO + SNP	168.60 ± 5.84a	20.21 ± 0.57b	23.42 ± 0.90ab

In each column different letters mean significant differences ($P < 0.05$) between treatments at the same time

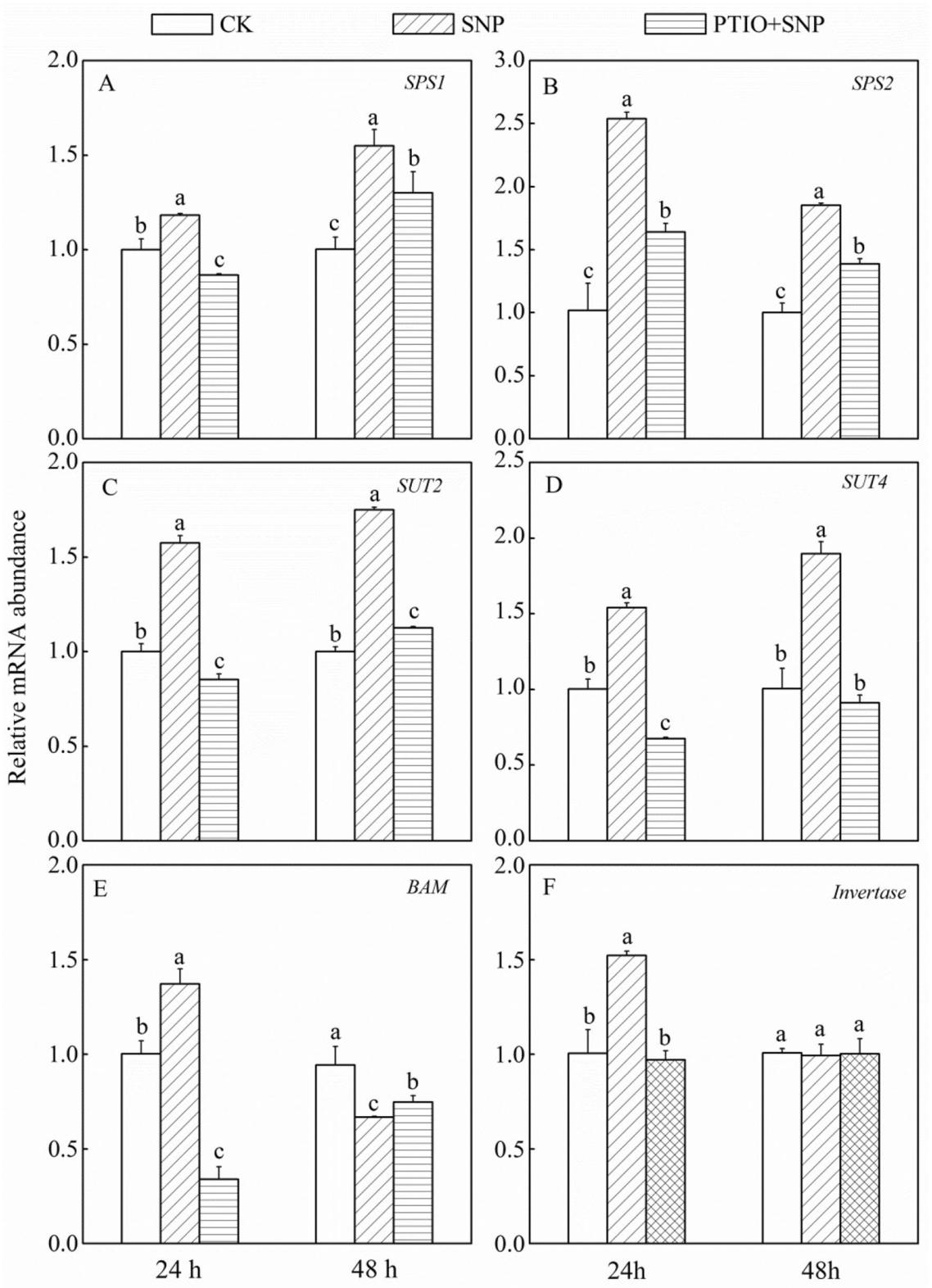

Fig. 5 Transcript levels of *SUCROSE PHOSPHATE SYNTHASE 1&2 (SPS1)* (**A**), *SPS2* (**B**), *SUCROSE TRANSPORTER 1&2 (SUT2)* (**C**), *SUT4* (**D**), *β*-starch hydrolase gene (*BAM*) (**E**), and invertase gene (*Invertase*) (**F**) in leaves of low temperature-stressed cucumber seedlings as affected by exogenous SNP and PTIO. Error bars represent SD ($n=3$). Bars with different letters within a sampling time are significantly different ($P<0.05$)

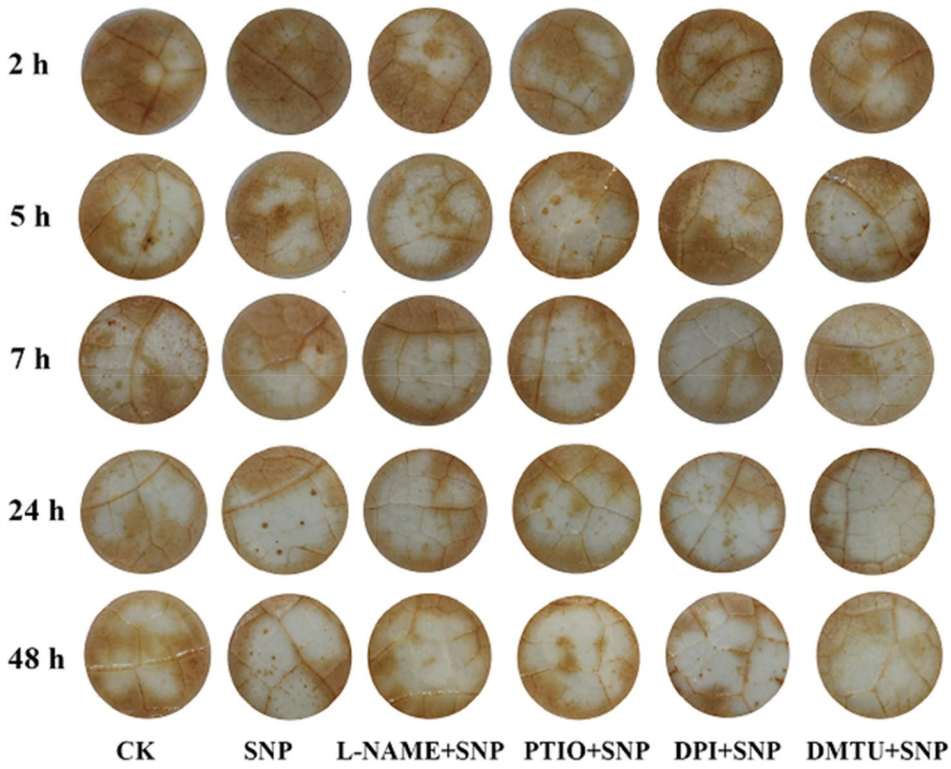

Fig. 6 Histochemical staining of cucumber leaf discs with DAB for visualization of H_2O_2 accumulation in situ in low temperature-stressed cucumber seedlings as affected by various pharmacological treatments

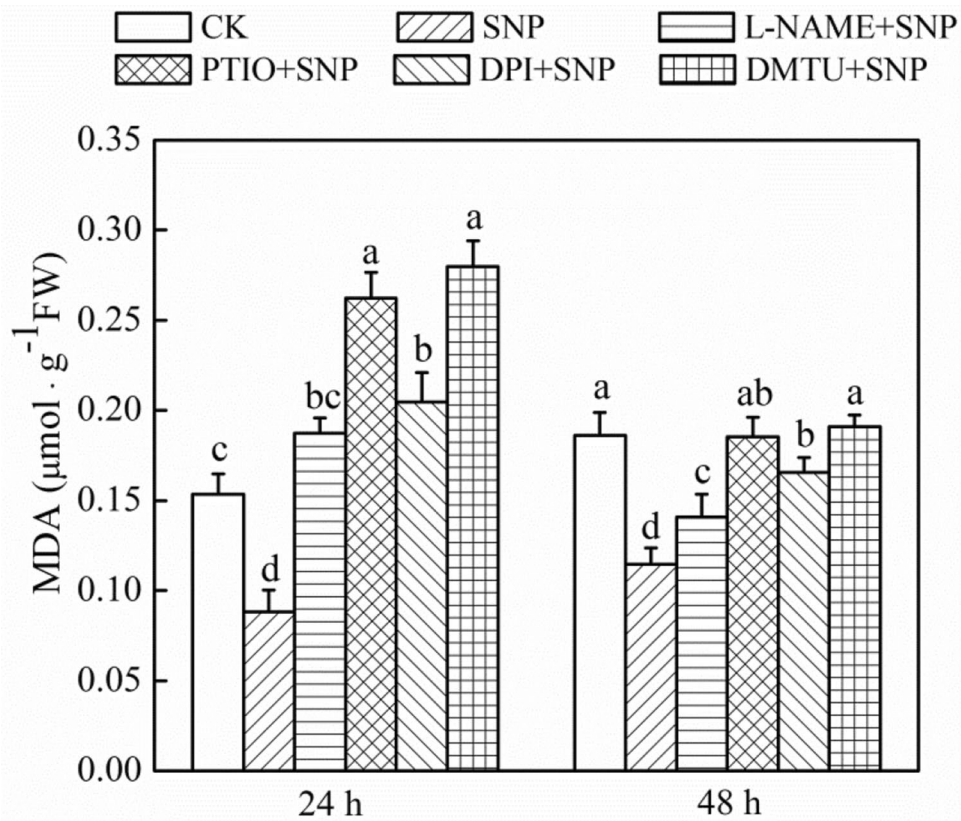

Fig. 7 Malondialdehyde (MDA) content in low temperature-stressed cucumber leaves subjected to various pharmacological treatments. Error bars represent SD ($n = 3$). Bars with a different letter within a sampling time are significantly different ($P < 0.05$)

of MDA after exposure of cucumber seedlings to low temperature for 24 h and 48 h.

H_2O_2 is Involved in NO-Induced Changes in Ascorbate–Glutathione (AsA-GSH) Cycle

To determine whether the H_2O_2 plays a role in NO-induced stress tolerance via the AsA-GSH cycle, we analyzed the effects of NO and ROS scavenger on NO-induced changes in key components of AsA-GSH cycle in cucumber seedlings. Compared with CK, SNP alleviated the low temperature-induced declines of reduced glutathione (GSH), reduced ascorbic acid (AsA), and the ratios of cellular redox status (GSH/GSSG and AsA/DHA) in cucumber leaves throughout the experiment ($P < 0.05$) (Fig. 8A, C, D, F). For instance, SNP increased the ratios of GSH/GSSG and AsA/DHA by 36.8% and 45.5% at 24 h of low temperature stress, respectively, compared with the CK. However, when the cucumber leaves were pre-treated with L-NAME and PTIO, and then treated with SNP, the effects of SNP on GSH, AsA, GSH/GSSG, and AsA/DHA were completely abolished. In addition, the content of AsA and GSH, and the ratios of GSH/GSSG and AsA/DHA in combined treatment of DPI with SNP and DMTU with SNP remarkably attenuated the SNP effect on AsA and GSH contents during the entire experiment ($P < 0.05$). In contrast, the contents of DHA and GSSG were significantly declined by SNP throughout the experiment when compared with CK. Compared with SNP treatment alone, cucumber seedlings that were pre-treated with L-NAME, PTIO, DPI and DMTU followed by SNP treatment, showed significantly increased contents of DHA and GSSG throughout the experiment.

H_2O_2 is Involved in NO-Induced Increases in Activities of Antioxidant Enzymes

Since we found that H_2O_2 is involved in NO-induced changes in GSH and AsA contents, we analyzed the temporal changes in the activities of enzymes related to the AsA-GSH cycle in cucumber seedlings under low temperature. The application of SNP alone exerted positive effects on the activity of these enzymes in cucumber seedlings during low temperature (Fig. 9). When compared with SNP, the application of L-NAME plus SNP and PTIO plus SNP significantly decreased the activity of APX, GR, MDAR, and DHAR. Moreover, treatment with DPI plus SNP and DMTU plus SNP markedly decreased the activity of APX, GR, MDAR, and DHAR in cucumber seedlings, when compared to treatment with SNP alone.

Discussion

NO-Enhanced Tolerance to Low Temperature is Attributed to Stimulation of the Carbohydrate Metabolism and Calvin–Benson Cycle

Many studies have reported that NO is an important signal for transducing information, and can alleviate the injuries of plants under low temperatures (Liu et al. 2016; Zhao et al. 2009). In the present study, we used NO-sensitive fluorescent molecular probes, DAF-FM DA, combined with laser confocal microscopy to observe the accumulation of NO in cucumber leaves after the exposure of plants to low temperature for 2 h, 5 h, and 7 h. Consistent with Cui et al. (2011) and Dong et al. (2018), our results showed that exogenous SNP could increase NO accumulation, whereas the positive effect of SNP on cucumber leaves under low-temperature stress was reversed by the addition of PTIO (Fig. 1). These results suggest that exogenous SNP could further induce the increase of endogenous NO in plants under low temperature. The rapid increases in NO levels during early hours of low temperature stress may act as a signal, which could further regulate the physiological and biochemical processes of plants, and thus enhancing the low temperature tolerance in cucumber plants.

In plants, many physiological and metabolic processes are impaired by low temperatures (Karimi and Ershadi 2015). Previous studies have shown that the accumulation of water-soluble carbohydrates and starch is an essential strategy for plant adaptations to low temperature stress (Hajihashemil et al. 2018; Shin et al. 2015; Theocharis et al. 2012; Yamdeu et al. 2016). In addition, NO could strongly stimulate the physiological processes including carbohydrates metabolism of plants under various environmental stresses (Sehar et al. 2019; Wang et al. 2017). All of these reports indicate that the increase of carbohydrate content is induced by low temperature, while NO also participates in the low temperature-induced accumulation of water-soluble carbohydrates and starch in many plants (Amooaghaie and Nikzad 2013). Consistent with previous studies, here, we showed that the content of water-soluble carbohydrates and starch increased to varying degrees at low temperature. Apart from this, exogenous NO promoted the accumulation of starch, sucrose, glucose, and fructose in cucumber leaves, but PTIO blocked the effect of NO at low temperature stress.

The initial products of photosynthesis in plants include sucrose and temporary starch that are synthesized in cytoplasmic matrix and chloroplasts, respectively. Sucrose is the main (or even only) form of carbohydrates for long-distance transport in plants. Krapp et al. (1993) reported that when the synthesis of sucrose in leaves is greater than the ability to export sucrose in plants, excessive accumulation of sucrose

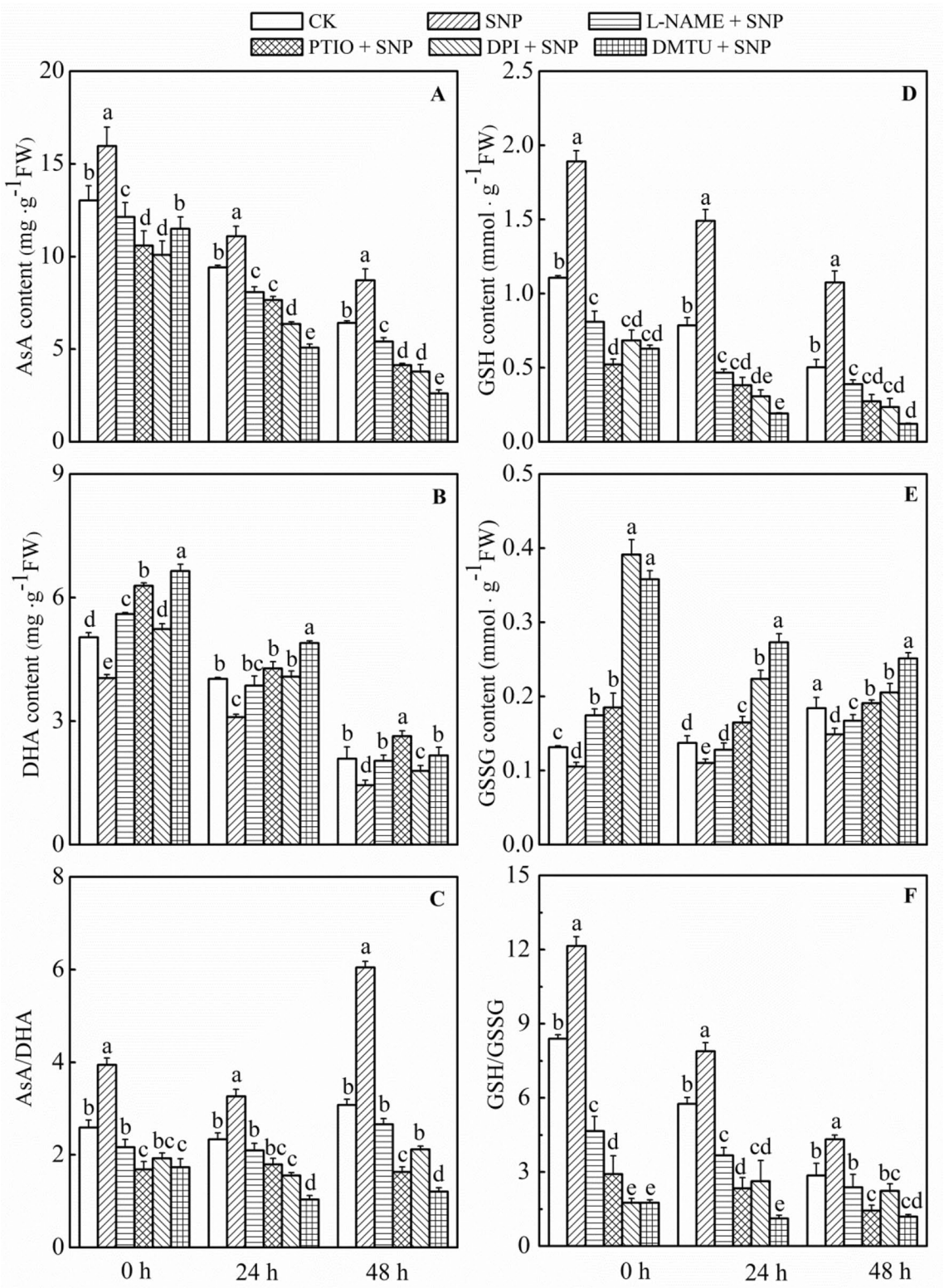

Fig. 8 Ascorbate (AsA) content (**A**), dehydroascorbate (DHA) content (**B**), Ascorbate/dehydroascorbate (AsA/DHA) rate (**C**), glutathione (GSH) content (**D**), oxidized glutathione (GSSG) content (**E**), and glutathione/oxidized glutathione GSH/GSSG (**F**) in low temperature-stressed cucumber seedlings as affected by various treatments. Error bars represent SD ($n=3$). Bars with a different letter within a sampling date are significantly different ($P<0.05$)

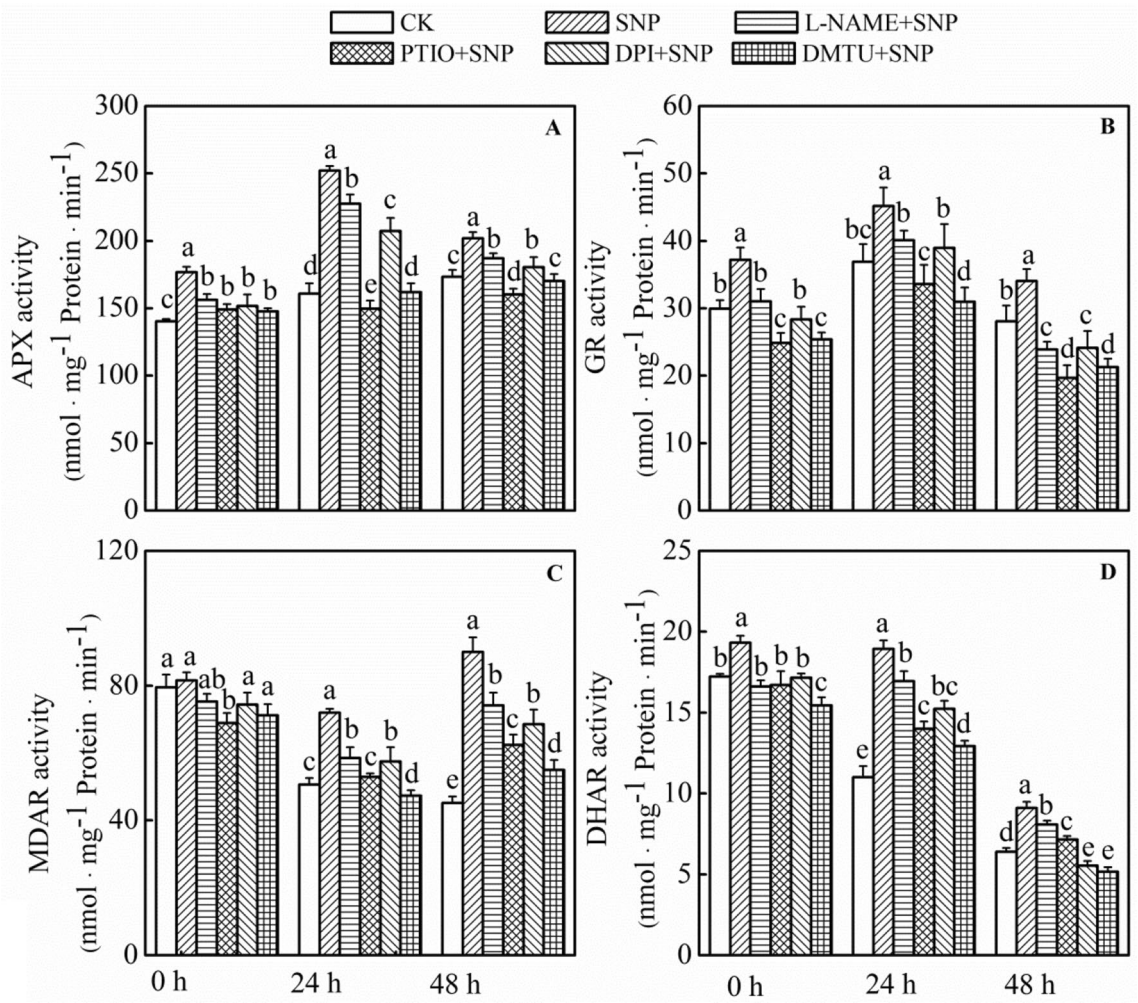

Fig. 9 Activities of Ascorbate peroxidase (APX) (**A**), glutathione reductase (GR) (**B**), monodehydroascorbate reductase (MDAR) (**C**), and dehydroascorbate reductase (DHAR) (**D**) in chilling-stressed cucumber seedlings as affected by various treatments. Error bars represent SD ($n=3$). Bars with a different letter within a sampling date are significantly different ($P<0.05$)

could inhibit the expression level of photosynthetic genes, thereby attenuating photosynthesis. Therefore, sucrose metabolism and transport are very important in regulating many physiological processes of plants. In the present study, sucrose accumulation in the cucumber leaves continued to increase under low temperature stress along with the prolongation of stress conditions. In addition, low temperature-stressed cucumber seedlings treated with exogenous NO exhibited increased content of sucrose, activities of SPS, AI and SS, and transcription levels of *SPS1*, *SPS2*, *SUT2*, and *SUT4*. These results are in agreement with the reports by Wang et al. (2013) and Yu et al. (2015), in peach fruits during cold storage. However, the positive effect of NO was offset by PTIO. So it is highly plausible that NO-induced enhancements of glucose and fructose contents under low temperature were probably caused by NO-induced increased metabolism of sucrose and starch. This also suggested that the products of photosynthesis were influenced by NO under environmental stress, and a relatively higher sucrose content was induced by NO alongside its higher transportation and decomposition activity. Besides, the inverse relationship between starch content and the expression level of *BAM* under low temperature stress may also suggest that the hydrolysis of starch is responsible for the increased levels of glucose and fructose. All these results indicated that NO participated in low temperature-induced increased carbohydrates content, sucrose metabolism, and polysaccharide hydrolysis. Moreover, an increased accumulation of water-soluble carbohydrates further contributed to enhanced tolerance to low temperature in cucumber seedlings. Our results are in conformity with an earlier study in cucumber, suggesting that NO generated by the NOS-dependent pathway may alleviate the damage caused by chilling stress through elevating the soluble sugar content (Liu et al. 2016).

The photosynthetic carbon assimilation can also affect the accumulation of carbohydrates in plants, and RuBisCO is the essential carboxylase in carbon assimilation. The improvement of carbohydrate content induced by NO can be partly explained by the changes in RuBisCO activities and the carboxylation rate in this study. The initial and total RuBisCO activity was decreased to a lesser extent than CO_2 assimilation by various environmental stresses (Chen and Cheng 2003; Li et al. 2010). However, it has been revealed that NO could play a protective role in the regulation of plant responses to abiotic stress by enhancing the RuBisCO activity (Khairy et al. 2016). The present study discovered that exogenous NO significantly improved the activity and carboxylation rate of RuBisCO, the regeneration rate of RuBP, and the expression levels of *RCA* and *rbcS*, and decreased the expression level of *rbcL* in cucumber leaves under low temperature. The differential expression of *rbcL* and *rbcS* as induced by NO may be due to the different coding positions of the two genes in plants, *rbcL* is encoded by the chloroplast gene, and *rbcS* is encoded by the nuclear gene (Silverthorne et al. 1990). However, PTIO blocked the effect of NO throughout the experiment (Table 3, Figs. 2 and 3). These results suggested that NO may play a critical role in photosynthetic carbon assimilation of cucumber seedlings by improving the RuBisCO activity and carboxylation rate, thereby enhancing the tolerance of plants to low temperature stress. In addition, Puyaubert et al. (2014) reported that the *S*-nitrosylation level of the large and small subunits of RuBisCO as well as its regulator, RuBisCO activity, is strongly increased by NO in Arabidopsis. From this point of view, it is possible that NO regulates the activity of RuBisCO and carboxylation rate through post-transcriptional modifications, thereby enhancing cold tolerance of cucumber seedlings. The activities of several enzymes such as FBPaldolase, FBPase, and SBPase play an essential role in the RuBP regeneration phase. The present study showed that exogenous NO also increased the RuBisCO regeneration rate and the expression levels of *FBPaldolase*, *FBPase*, and *SBPase* in cucumber seedlings under low temperature. However, PTIO diminished the positive effect of SNP. These results further demonstrate that NO can effectively alleviate the inhibitory effect of low temperature stress on the Calvin cycle by improving the initial and total activities of RuBisCO, RuBisCO carboxylation and the regeneration rate of RuBP in cucumber seedlings.

H_2O_2 is Involved in NO-Induced Low Temperature Tolerance of Cucumber

Previously, ROS were mainly regarded as toxic byproducts, but in contrast with earlier views, H_2O_2 has been shown to be a necessary and vital signaling molecule in normal metabolism as well as stress metabolism in plants (Ahammed et al. 2020a; Guo et al. 2019; Mittler 2017; Shi et al. 2016). Previous studies show that H_2O_2 participates in NO-induced chilling tolerance in tomato seedlings (Diao et al. 2017) and stomatal closure in *Arabidopsis* (Shi et al. 2015). In addition, Qiao et al. (2014) reported that the production of H_2O_2 and NO under abiotic stress conditions occurs in short succession to one another or parallelly. A plethora of reports evidenced that the NO is required for the H_2O_2 production. For instance, cPTIO (NO scavenger) or L-NAME could inhibit the endogenous H_2O_2 generation during the development of adventitious roots (Liao et al. 2011). Consistent with this, in the present study, the elevation of H_2O_2 in cucumber leaves was induced by NO under low temperature for 2 h, 5 h, and 7 h. However, the application of exogenous NO showed an opposite effect on H_2O_2 content after 24 h and 48 h at low temperatures as compared to the untreated ones. On the other hand, L-NAME and PTIO blocked the effect of SNP throughout the treatment period (Fig. 6). This also indicates that both exogenous NO and NO produced by the NOS-dependent pathway might mediate the increased production of H_2O_2 under short-term low-temperature stress.

The environmental stress-induced ROS accumulation could oxidize proteins and membrane lipids, resulting in abnormalities in cells (Petrov et al. 2015; Wu et al. 2017). MDA content is a well-known index that indirectly reflects the membrane fluidity, integrity and membrane peroxidation (Rui et al. 2010). In our study, the MDA content in the control seedlings increased quickly with the extension of low temperature treatment, while exogenous NO significantly inhibited the increase of MDA content (Fig. 7). Similar to our results, a study in walnut indicated that NO could decline the MDA concentration under low temperature (Dong et al. 2018). However, the application of L-NAME, PTIO, DPI and DMTU in combination with SNP aggravates the oxidative stress induced by low temperature as compared to the SNP treatment alone, implicating an enhanced oxidative degradation of lipids. These results suggest that H_2O_2 is partially involved in NO-induced potential delay of membrane lipid peroxidation, thus enhancing the low temperature tolerance of cucumber seedlings.

Plants have evolved enzymatic and non-enzymatic antioxidant defense systems to protect themselves against oxidative stress. It is well known that the AsA-GSH cycle is a key metabolic pathway to scavenge ROS, and it maintains redox equilibrium in plants. A higher efficiency of the AsA-GSH cycle under abiotic and biotic stress is indispensable for alleviating damage to plants (Asada 2006). In particular, H_2O_2 produced in plants is mainly eliminated by the ASA-GSH cycle located in the cytosol, mitochondria, chloroplast, and peroxisomes (Foyer and Noctor 2011). A recent report revealed that NO could promote the metabolism of ROS in peroxisomes (Corpas et al. 2019) and maintain the stability of the cell membrane by enhancing the reducing ability of

the ASA-GSH cycle. Notably, GSH and AsA are the vital cellular antioxidants, and high concentrations of GSH and AsA in plants are conducive to maintaining an appropriate redox environment and reducing the damage caused by abiotic and biotic stresses. Consistent with this, our study also showed that exogenous NO enhanced the tolerance to low temperature in cucumber seedlings by decreasing the content of DHA and GSSG, and increasing the content of AsA, GSH and the ratio of AsA/DHA and GSH/GSSG. Notably, DPI and DMTU diminished the effect of SNP, implying that H_2O_2 participated in NO-induced promotion of GSH and AsA content. The intracellular redox state is essential for plants to resist the damage of biotic and abiotic stress (Dietz 2008). Therefore, maintaining a high reducing power of GSH and AsA is essential for plants to remove excess ROS. To attain a high reducing power, relatively high activities of APX, GR, MDAR, and DHAR are required (Mittler 2002). In AsA-GSH cycle, APX is the most important enzyme for removing excessive H_2O_2. GR, MDAR, and DHAR are responsible for reducing the DHA and GSSG to AsA and GSH, respectively, and thus providing substrates for APX. Recently, Ma et al. (2019) reported that NO could enhance the reducing ability of the AsA-GSH cycle and maintain a high antioxidant capacity of peach during cold storage. In tomato seedlings, the activities of the enzymes related to the AsA-GSH cycle could be enhanced by the application of exogenous NO under cadmium stress (Ahmad et al. 2018). The present study also showed that the enhanced activities of APX, GR, MDAR, and DHAR were induced by NO at low temperature, while L-NAME and PTIO blocked the effect of SNP. Apart from this, as mentioned by Kolupaev et al. (2015), NO and H_2O_2 signaling pathways are closely linked in plant cells under control conditions as well as during the overall responses of plants to environmental stimuli. In the present study, we found that the application of DPI and DMTU can also diminish the activities of APX, GR, MDAR, and DHAR induced by NO at low temperature, suggesting that H_2O_2 is involved in NO-induced improvement of the reducing ability of the AsA-GSH cycle.

In conclusion, we have revealed that exogenous NO is involved in the enhancement of plant tolerance to low temperature stress in cucumber seedlings by increasing the initial and total activities of RuBisCO and RuBP regeneration rate, thereby improving the content of carbohydrates. Furthermore, NO could induce the production of H_2O_2, which plays a crucial role in stimulating the AsA-GSH cycle. H_2O_2 is involved in the process of NO-induced enhancement of GR, APX, DHAR, and MDAR activities. The NO-induced improvement in enzyme activities further contributed to the increased content of ASA, GSH, and the ratio of AsA/DHA, GSH/GSSG, thereby alleviating the oxidative damage of cucumber seedlings under low temperature. Our results suggest that H_2O_2 participates in NO-mediated signaling pathways in response to low temperature stress, which may provide an understanding of plant response mechanisms to other environmental stresses as well.

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Author Contributions This work was carried out in collaboration between all the authors. J.C., P.W., and C.X. defined the research theme and designed the experimental setup. P.W., C.X., B.H., W.Z. and Z.Y. performed the experiments. P.W. and C.X. analyzed the data, and interpreted the results. P.W. and C.X. wrote the initial draft of the manuscript. J.C., P.W., G.J.A., H.L. and H.C. revised the manuscript and edited it to present form. All authors have contributed to, read, and approved it for submission.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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