Sodium nitroprusside pretreatment alters responses of Chinese cabbage seedlings to subsequent challenging stresses

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ABSTRACT
Nitric oxide (NO) is a self-regulating gaseous molecule as well as an interacting partner with different reactive oxygen species (ROS). Endogenous NO increased, but hydrogen peroxide (H$_2$O$_2$) and superoxide radical anion (O$_2^-$) decreased in Chinese cabbage leaves treated with sodium nitroprusside (SNP) (0.5 mM). Antioxidant enzyme genes BrCat1, BrCat2, BrAPX, BrGR1, and BrGST1 expressions were transiently induced by the SNP (0.5 mM). Pretreated SNP (0.5 mM) enhanced tolerance to subsequent challenges by phytotoxic SNP (5 mM) and methyl viologen (MV) (50 μM), but accelerated cellular damage by H$_2$O$_2$ (4 M) shown by electrolyte leakage and lipid peroxidation. The SNP (0.5 mM) differentially modulated endogenous NO and ROS levels as well as antioxidant enzyme gene expressions by excess SNP, MV, and H$_2$O$_2$ stresses. These results suggest that NO as a signal molecule differentially interacts with challenging stresses in Chinese cabbage leaves, followed by differential cellular damage and antioxidant responses.

Introduction
Inducible NO accumulation was highly accelerated in plant tissues under environmental stresses such as water-deficit, mechanical wounding, heavy metal, ozone, and UV-B irradiation as well as biotic attacks by microbial pathogens and insect pests (Garcés et al. 2001; Sang et al. 2008; Zhang and Zhao 2008; Ahlfors et al. 2009; Sivakumaran et al. 2016; Solórzano et al. 2020; Xu et al. 2020). Nitrosoative stress signaling was activated by the NO burst in severely damaged plant cells (Corpas and Barroso 2013). It has been questioned whether the augmented NO promotes or suppresses plant cell death, and is finally harmful or beneficial to plants (Beligni and Lamattina 1999a; Wang et al. 2010a). Oxidative burst accompanying rapid production of reactive oxygen species (ROS) frequently occurred in plant tissues under threats by abiotic and biotic stresses, and various ROS are considered as probable cellular partners of NO to cope with the diverse external stimuli (Romero-Puertas and Sandalio 2016; Hancock and Neill 2019). However, so far knowledge has been still limited on either excess NO stress-specific signaling or crosstalk with oxidative signaling in plants, and endogenous NO or -nitrosothiols (SNOs), i.e., nitric oxide-dependent signaling or crosstalk with oxidative signaling in plants, and antioxidant enzyme gene expressions by excess SNP, MV, and H$_2$O$_2$ stresses. These results suggest that NO as a signal molecule differentially interacts with challenging stresses in Chinese cabbage leaves, followed by differential cellular damage and antioxidant responses. The SNP (0.5 mM) differentially modulated endogenous NO and ROS levels as well as antioxidant enzyme gene expressions by excess SNP, MV, and H$_2$O$_2$ stresses. These results suggest that NO as a signal molecule differentially interacts with challenging stresses in Chinese cabbage leaves, followed by differential cellular damage and antioxidant responses.

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NO-producing enzymes and/or the enzyme encoding genes have been identified in animals and microbes (Kolbert et al. 2019). Interestingly, pharmaceutical investigations using various NO donors (sodium nitroprusside [SNP], S-nitrosoglutathione [GSNO]), mammalian NO synthase inhibitor (N-nitro-L-arginine methyl ester), nitrate reductase inhibitor (sodium tungstate) and/or NO scavenger (2-phenyl-4,4,5,5, tetramethylimidazoline-3-oxide-1-oxyl) efficiently provided evidence that NO participated in plant tolerance against environmental stresses and pathogen infections especially in most crops and animals as well, in which genetic dissection using NO overproducers or low NO accumulators is technically difficult (Tian et al. 2007; Wang et al. 2010; Sun et al. 2014; Sivakumaran et al. 2016; Hasanuzzama et al. 2017; Wu et al. 2017; Dong et al. 2018; Lu et al. 2020). MV-stressed potato leaves, drought-stressed rapeseed seedlings, dehydrated wheat seedlings, and arsenic-contaminated rice roots could be rescued efficiently by exogenous NO donors (Beligni and Lamattina 1999b; Singh et al. 2009; Hasanuzzama et al. 2017). NO donors enhanced plant resistance in response to biotic stresses as well. Both SNP and GSNO pre-treatments reduced disease incidence in Rice black-streaked dwarf virus-infected rice plants and diminished activity of feeding aphids on pea seedlings (Woźniak et al. 2017; Lu et al. 2020). Aluminum-triggered cell death of peanut root tips was mitigated by treatment with SNP, whilst lead-triggered cell deaths in tobacco suspension-cultured cells were rather accelerated by SNP supplement (He et al. 2019; Wu et al. 2019). It is worthy to note that suggested roles of NO in a variety of plant species, organs, and cells were derived from different endogenous and exogenous stimuli using different doses of different NO-associated chemicals, which may lead to confusion as to the function of plant NO on the stress-induced cell deaths and interaction with ROS in all circumstances.

ROS production and regulation of antioxidant enzyme activities were associated with the NO-mediated enhanced tolerance in plants, but regulations of various antioxidant enzymes in each plant species have been quite different. The low NO Arabidopsis noa1 mutant hypersensitive to UV-B stress, accompanying high H$_2$O$_2$ production together with reduced CAT and APX activities, demonstrating suppression of cellular H$_2$O$_2$ by NO via activation of the antioxidant enzymes (Zhang et al. 2009). Activation of glutathione-dependent enzymes and regeneration of reduced antioxidants are sophisticated regulatory antioxidant mechanisms in plants to cope with oxidative stresses (Gill and Tuteja 2010). Reduced glutathione (GSH) regenerated by GR was transferred to several enzymes as a substrate through a glutathione-ascorbate cycle, or could be formed by mixed disulfides with proteinaceous cysteine through protein S-glutathionylation (Dixon et al. 2005; Gill and Tuteja 2010). Glutathione-S-transferase (GST) is one of the enzymes detoxifying xenobiots by transferring GSH into the toxic agents, and GSH-conjugated xenobiots can be delivered to the vacuole for cellular compartmentalization (Dixon et al. 2010). Both monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) enhanced the recycling of reduced ascorbate that would be used for APX working for H$_2$O$_2$ decomposition in response to oxidative stress (Li et al. 2010; Wang et al. 2010b). APX, GR, MDHAR, and DHAR activities in rapeseed seedlings during SNP-increased drought stress tolerance were higher than those in the drought-stressed seedlings, which were correlated with reduced H$_2$O$_2$ in the protected seedlings (Hasanuzzama et al. 2017). GSNO enhanced pea cytosolic APX and MDHAR enzyme activities in vitro, supporting correlation of increased NO with SNOs levels and activities of S-nitrosylated APX and MDHAR in pea plants grown under high salinity (Begara-Morales et al. 2014, 2015). But it is rarely described how they are regulated in plants during NO-mediated stress tolerance.

NO involvement in seedling development and stress tolerance was also demonstrated in Chinese cabbage plants. NO generation was dependent on leaf position or growth stages of Chinese cabbage seedlings under normal ambient environments, and the NO levels were closely associated with leaf chlorophyll contents but inversely correlated with H$_2$O$_2$ levels in the leaves of different positions (Kim et al. 2015). Exogenous SNP (0.2 and 0.5 mM) promoted leaf growth and chlorophyll contents during the early development of Chinese cabbage seedlings (Ham and Jin 2005). Application of non-phytotoxic SNP restored retarded growth of Chinese cabbage seedlings caused by high salinity, low temperature, and cadmium toxicity (López-Carrión et al. 2008; Fan et al. 2014; Su et al. 2019). By contrast, excess SNP doses (10 and 20 mM) triggered cellular damages in Chinese cabbage seedlings and it was closely associated with declined H$_2$O$_2$ as well as transcriptional activation of several antioxidant enzymes (Kim et al. 2015). Oxidative stresses by excess MV and H$_2$O$_2$ in Chinese cabbage leaf discs were attenuated by SNP (0.5–1 mM) co-treatment (Sung and Hong 2010). Pre-treated SNP (0.1 mM) conferred tolerance to detached leaves of Chinese cabbage seedlings against subsequent paraquat treatment (Na and Jin 2014). Lipid peroxidation and H$_2$O$_2$ content in the leaves were significantly reduced in the SNP-induced tolerance against paraquat, whilst enzyme activities of APX, DHAR, and GR relatively increased during the cellular protection (Na and Jin 2014).

In this study, we aimed to investigate plant cellular responses of the primary leaves of Chinese cabbage seedlings treated with non-phytotoxic SNP (0.5 mM) with or without subsequent excess SNP and ROS (MV and H$_2$O$_2$) stresses. Electrolyte leakages, lipid peroxidations, and levels of NO, H$_2$O$_2$, and O$_2^\cdot$ in the leaves were compared during the three different interactions of the SNP pre-treatment with the following challenge stresses. In addition, antioxidant enzyme gene expressions were also analysed in the non-phytotoxic SNP treated leaves as well as in the leaves under the three differential NO-ROS interactions.

**Materials and methods**

**Plant growth**

Six Chinese cabbage seedlings (Brassica rapa subsp. pekinensis cv. Tushim-eotgari) were sown and grown in a square pot (12 cm × 8 cm × 5.5 cm) containing steam-sterilised commercial soil mixtures ‘Toshil’ (Shinan Growth Co., Ltd, Jinju, Republic of Korea) under slightly modified controlled conditions of light illumination (70 μmole m$^{-2}$ s$^{-1}$) with 12-h light (8:00 am to 8:00 pm)/12-h dark photoperiod regime, temperature (26 ± 2°C) and relative humidity conditions (55 ± 5%), in a walk-in growth room as described in our previous studies (Kim et al. 2015; Jo et al. 2020). For SNP (0.5 mM) treatment alone, 12-day-old seedlings (fully expanded two
true leaves stages) were used, and the primary true leaves were harvested for molecular, cellular, and biochemical analyses. To investigate the effect of SNP (0.5 mM) pretreatment, the same growth stages of the seedlings were prepared and posttreatments with high dosages of SNP, MV, and H₂O₂ stresses were performed at 24 h after the pretreatments, and then the primary true leaves were harvested for analyses of electrolyte leakage, lipid peroxidation, NO and ROS contents, and gene expression at indicated time points in each Figure.

**Exogenous NO and ROS treatments**

The SNP (0.5 mM) was freshly prepared in distilled water as a NO donor and was evenly foliar-sprayed onto the Chinese cabbage seedlings. The SNP concentration (0.5 mM) could not lead to a phytotoxic effect on the primary true leaves of the 12-day-old Chinese cabbage seedlings as demonstrated in our previous studies (Sung and Hong 2010; Kim et al. 2015). To investigate the changes in tolerance to excess NO and ROS stresses, the seedlings were foliar-sprayed with SNP (5 mM), MV (50 µM) superoxide radical anion \( \text{O}_2^- \) generator, or H₂O₂ (4 M) at 24 h after the non-phytotoxic SNP (0.5 mM) pretreatment. The concentrations of SNP, MV, and H₂O₂ for challenging stresses were the dosages enough to cause the visible leaf damages including the collapse of the primary true leaves within 24 h after treatments. The challenge-stressed seedlings were placed under the light condition described above at 10:00 am to provoke photooxidative stresses because oxidative stresses and antioxidant responses of plants can be dependent on the timing of the chemical application. An equal volume of distilled water was foliar-sprayed onto the seedlings as a mock treatment. Six seedlings in one square pot were evenly foliar-sprayed using 10 ml of each chemical. Four independent experiments were conducted, and each experiment has four biological replicates.

**Observation of leaf autofluorescence**

Detached leaves were observed under UV-illuminated lamp (Black-Ray® B-100 AP High Intensity UV Lamp, Ultra-Violet Products, UK) to observe red autofluorescence and bright blue fluorescence emitted from healthy chlorophylls and the stress-associated phenolic compounds, respectively, as previously demonstrated (Lee and Hong 2012).

**Electrolyte leakage measurement**

Electrolyte leakage from the leaves treated with the chemicals was measured according to methods previously reported (Lee and Hong 2012; Kim et al. 2015). Four leaf discs (1 cm in diameter) were collected from the chemical-treated leaves and floated for 1 h in 10 mL of distilled water in a 50-mL conical tube. Ion conductivity of the eluates was measured after 1 h-incubation at room temperature using a conductivity meter (Model EC-40N, iSTEK Inc., Seoul, Republic of Korea). After boiling for 15 min and cooling down the leaf disc samples to room temperature, ion conductivity was re-measured to compare stress-mediated electrolyte leakage with total electrolyte contents within the leaf discs. Four independent experiments were separately conducted with four biological repeats.

**Evaluation of lipid peroxidation**

Four leaf discs were powdered in a microtube using liquid nitrogen and micro-pestle, and the fine leaf powder was mixed in 600 µl of 0.1% trichloroacetic acid (TCA). After centrifugation at 15,000 × g for 15 min at 4°C, supernatant (250 µl) was transferred into a 15-ml conical tube in an ice-bed. One ml of thiobarbituric acid (TBA) prepared in 20% TCA was added. The conical tube was incubated in a water bath pre-heated at 95°C for 30 min and immediately transferred to an ice bath to stop the reaction. The reaction mixture was transferred to a fresh microtube and centrifuged at 12,000 × g for 10 min at 4°C. For blank, a mixture of 250 µl of 0.1% TCA with 1 ml of TBA prepared in 20% TCA was simultaneously incubated at 95°C for 30 min. The supernatant was used to measure thiobarbituric acid reactive substances (TBARS) content spectrophotometrically at the absorbance of 532 and 600 nm. The OD value at 532 nm was corrected for non-specific absorption by subtracting the OD value at 600 nm. The amount of TBARS was calculated based on the extinction coefficient of 155 mM⁻¹ cm⁻¹ and expressed as TBARS nmole g FW⁻¹.

**NO and H₂O₂ quantification**

Endogenous NO contents in the leaves were analysed according to the methods described (Kim et al. 2015; Kaya et al. 2020; Yu et al. 2020). Leaf discs were macerated in zinc diacetate buffer in microtubes and centrifuged. The supernatants were mixed with Griess reagent for NO measurement spectrophotometrically and expressed as nmole g FW⁻¹. H₂O₂ contents in the leaves were also measured by experimental procedures previously described using xylenol orange as a substrate and were expressed as µmole g FW⁻¹ (Kim et al. 2015).

**Histochemical staining of H₂O₂ and O₂⁻ in the leaves**

In situ accumulation of H₂O₂ and O₂⁻ in the leaves was histochemically detected using slightly modified staining methods of 3,3-diaminobenzidine (DAB) tetrahydrochloride hydrate and nitro blue tetrazolium (NBT), respectively (Sung and Hong 2010).

DAB solution [0.1% (w/v)] was prepared in prewarm distilled water and cooled to room temperature. The detached leaves were immersed and stained in the DAB solutions with different pH values adjusted with 0.1 M NaOH for 24 h under dark conditions. The detached leaves were immersed and stained in 0.1% (w/v) NBT prepared in 10 mM sodium azide for 30 min. After DAB or NBT staining, leaf chlorophylls were removed by boiling the leaves in 95% ethanol for 15 min, and the decolourized leaves were photographed.

**Microscopic observation of H₂O₂ and O₂⁻ accumulation in the leaf tissues**

Leaf fragments (5 mm × 5 mm) were cut from the primary true leaves and stained with DAB (pH 4.3) or NBT solutions for H₂O₂ and O₂⁻ accumulation, respectively, as described above. After removing chlorophylls by boiling in 95% ethanol
for 15 min, the leaf fragments were mounted on glass slides using 50% glycerol and observed under a light microscope.

RNA isolation and semi-quantitative RT–PCR analysis

Total RNA isolation from the leaves and gene expression analyses using semi-quantitative RT–PCR were performed as demonstrated in our previous study (Kim et al. 2015). Leaf tissues were macerated in a chilled mortar and pestle using liquid nitrogen, and the frozen leaf powder (0.1 g) was transferred to microtubes. One ml of RiboEx™ (GeneAll®, GeneAll Biotechnology Co., LTD, Seoul, Republic of Korea) was added to the leaf powder and incubated for 5 min at room temperature after vigorous shaking. The samples were centrifuged at 12,000 × g for 10 min at 4°C, and the supernatant was mixed with 0.2 ml of chloroform vigorously and incubated for 2 min at room temperature. Centrifugation was performed at 12,000 × g for 15 min at 4°C, and the supernatant was harvested and gently mixed with isopropyl alcohol by inversion. After overnight incubation at −20°C, total RNAs were precipitated by centrifugation at 12,000 × g for 15 min at 4°C. RNA pellets were washed with 1 ml of 75% ethanol, harvested by centrifugation at 8,000 × g for 5 min at 4°C, and dissolved in DEPC-treated water.

Total RNA (2 μg) was reverse-transcribed into cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, MA, USA) and oligo (dT)20 following the manufacturer’s instruction. PCR amplification was conducted by initial denaturation at 95°C for 2 min followed by appropriate cycles of incubation at 95°C for 30 s, 52°C for 40 s, 72°C for 30 s, with a final extension at 72°C for 10 min. Information on Chinese cabbage antioxidant enzyme genes and their PCR primer pairs were summarized in Table 1. The PCR products stained by RedSafe™ Nucleotide Acid Staining Solution (iNtRON Biotechnology, Inc., Seongnam, Republic of Korea) in agarose gels were visualized by UV-illumination.

Statistical analyses

An analysis of variance (ANOVA) was conducted to determine the effects of chemical treatments on plant responses such as electrolyte leakage, and NO and H₂O₂ contents. Data were acquired from four independent experiments with four biological replicates in each experiment. Means were compared using the least significant difference tests. Statistical analysis was performed with the SAS software version 8.1 (SAS Institute Inc., Cary, NC, USA).

Results

SNP application led to increased endogenous NO contents in Chinese cabbage leaves

Foliar spraying SNP (0.5 mM) solution was enough to change endogenous NO contents in the leaves (Figure 1). The SNP as a NO donor increased endogenous NO within 1 h in the leaf tissues and the elevated NO level was consistently maintained until 24 h. Water spraying as a mock treatment did not change the endogenous NO contents at each time point compared to untreated leaves.

Reduced ROS in Chinese cabbage leaves by the non-phytotoxic SNP treatment

Changes in endogenous ROS (H₂O₂ and O₂·−) contents were found in the leaves by foliar spraying SNP (0.5 mM) (Figure 2). H₂O₂ contents were temporally analysed in the mock- and SNP-treated leaves (Figure 2A). The SNP resulted in decreased endogenous H₂O₂ contents within 1 h after the treatment compared to mock-treated leaves. Reduced H₂O₂ content was maintained until 24 h after the SNP treatment. The reduced H₂O₂ contents in the SNP-treated leaves were demonstrated by histochemical staining with DAB solution (Figure 2B). No H₂O₂-mediated staining was found in the mock- and SNP-treated leaves when stained in DAB solution non-adjusted (pH 2.2) or adjusted to pH 3.8. Relatively stronger staining shown by the accumulation of red-brown precipitates was observed in the mock-treated leaves by increasing pH value in DAB solution from pH 4.3 and 4.8. However, the SNP (0.5 mM) application reduced the DAB-stained H₂O₂ in the leaves at 24 h after the treatment under pH 4.3 and 4.8 conditions. Accumulation of H₂O₂ in the mock-treated leaves stained by DAB (pH 4.3) was found in whole leaves and stronger stains occurred mainly in veins and hydathodes. H₂O₂ accumulated in the veins was slightly decreased by the SNP treatment.

Histochemically detected O₂·− accumulation was also down-regulated in the leaves by the SNP treatment (Figure 3).

Table 1. Chinese cabbage genes encoding various antioxidant enzymes and nucleotide sequences of forward (F) and reverse (R) oligonucleotide primers used for semi-quantitative RT-PCR to analyse transcriptional regulation.

| Gene name | Protein product | Accession number | Sequence (′5′ to 3′) | Expected size (bp) |
|-----------|-----------------|-----------------|---------------------|------------------|
| BrSOD     | Cu/Zn superoxide dismutase | AF071112 | F: ATGGCCAAGGGAAGTGGGAGT<br>R: CCACAAGCAACCGCCCT | 437 |
| BrCat1    | Catalase 1      | JQ027695 | F: GAACCTGAGGATAGTGTGAGG<br>R: GGCTTCATACAAACCAACAGG | 413 |
| BrCat2    | Catalase 2      | JQ027696 | F: GTAGATCTTGGCTGATGGTG<br>R: CCATTACATGAACTGGGATCT | 566 |
| BrAPX     | Ascorbate peroxidase | GQ500125 | F: ATGACGAGGACCCTCACCCGCTGA<br>R: TTAAGCACTGCAAAAAACCCAGCTCA | 753 |
| BrMDHAR   | Monodehydroascorbate reductase | AY039786 | F: GGCTTCGTGTTGATTGGGAGG<br>R: GGCAACTGCAACCAACAGG | 403 |
| BrDHR     | Dehydroascorbate reductase | AF536330 | F: TTTGATGAAATGAGGTTGCTGG<br>R: CATACCTTTGGCTCAACCC | 441 |
| BrGR1     | Glutathione reductase 1 | JQ928642 | F: GCTGATCGCATCACAAACAGCT<br>R: CCAATGGGGGATCAAATTGSC | 550 |
| BrGST1    | Glutathione S-transferase | AY567976 | F: TCAATGGCGATTCAAAATG<br>R: TCACTGAAAGGATCTTCATGGG | 642 |
| BrActin7  | Actin 7         | KU851921 | F: GTGACAATTGGAACATGGGATG<br>R: AGACCGGAGGATAGCGT | 505 |
2C). Constitutive O$_2^\cdot$ production in the mock-treated leaves was transiently reduced at 12 h (under a dark condition) and restored at 24 h, whilst the O$_2^\cdot$ generation at each time point was lessened until 24 h by the SNP treatment compared to those in the mock-treated leaves. O$_2^\cdot$ production in the mock-treated leaves detected by NBT was observed in whole leaf areas except for main veins, and the SNP-reduced O$_2^\cdot$ accumulation in whole leaf areas was diminished.

Subcellular localization of ROS down-regulated by the SNP treatment

Accumulation of H$_2$O$_2$ and O$_2^\cdot$ in the mock- and SNP-treated leaves was observed under a light microscope (Figure 3). Red-brownish precipitates were mainly found as scattered spots at epidermal and mesophyll cells in the mock-treated leaves by the DAB-staining, whilst SNP treatment drastically decreased the red-brownish spots at both cell-types. DAB-stained precipitates seemed to be observed at the cellular organelles probably mitochondria within the cytosols. Violet precipitates were demonstrated found in chloroplasts at mesophyll cells not epidermal cells in the mock-treated leaves by the NBT-staining. By the SNP treatment, the violet precipitates in many chloroplasts were disappeared and only a few mesophyll cells showed the NBT precipitates.

Activation of antioxidant enzyme gene expression by the non-phytotoxic SNP treatment

The expression of various antioxidant enzyme genes was investigated in the mock- and SNP (0.5 mM)-treated leaves (Figure 4). BrSOD gene expression was not changed in the mock-treated leaves. BrCat1 gene expression in the mock-treated leaves was decreased at 3 h–6 h, more suppressed at 12 h, and returned to basal level in the untreated leaves at 24 h. BrCat2 and BrAPX expressions were hardly detected in untreated leaves but increased at 6–12 h by the mock treatment. The transiently induced BrCat2 and BrAPX expressions in the mock-treated leaves were compromised at 24 h. BrSOD, BrMDHAR, and BrDHAR genes were not responsive to the SNP application compared to those in the mock-treated leaves. But BrCat1, BrCat2, and BrAPX expressions were up-regulated at 3–6 h, and BrGR1 and BrGST1 were up-regulated at 6–12 h.

Responses of Chinese cabbage leaves to excess NO and ROS stresses differentially modulated by the non-phytotoxic SNP

Pretreatment with SNP (0.5 mM) led to differential cellular responses of Chinese cabbage leaves against excess NO and ROS stresses (Figure 5A). SNP (5 mM) and MV (50 µM) stresses triggered severe leaf damages, and these were...
mitigated by the SNP (0.5 mM) pretreatment shown as reduced tissue collapse as well as retained red chlorophyll autofluorescence observed under UV-illumination. By contrast, the pretreated SNP accelerated tissue collapses and loss of the chlorophyll autofluorescence in the leaves treated with excess H$_2$O$_2$ (4 M). In the severely damaged leaves under excess NO and MV stresses at 48 and 9 h, respectively, bright blue fluorescence was emitted from the leaf tissues. SNP pretreatment mitigated the bright blue fluorescence in the leaves treated with SNP (5 mM) and MV (50 µM). Bright blue fluorescence was also found during the accelerated tissue damage of leaves pretreated with SNP followed by excess H$_2$O$_2$ at 6 h but disappeared in highly collapsed leaves at 24 h.

Electrolyte leakage and lipid peroxidation were evaluated in the leaves during the three different interactions of SNP (0.5 mM) pretreatment with excess SNP (5 mM), MV (50 µM), and H$_2$O$_2$ (4 M) (Figure 5B). The SNP pretreatment-mitigated stress by the high NO donor in the leaves was shown by the reduced electrolyte leakage and lipid peroxidation (Figure 5B(a)). The high SNP (5 mM) resulted in increased cellular damage at 24 and 48 h showing elevated electrolyte leakage, whilst the SNP (0.5 mM) pretreatment alleviated the cellular damage indicated by reduced electrolyte leakage. The high SNP-induced lipid peroxidation at 24 h was also mitigated by the SNP (0.5 mM) pretreatment. Lipid peroxidation at 48 h after high SNP treatment was decreased compared to one at 24 h, but the SNP pretreatment more decreased lipid peroxidation against the high SNP (5 mM). The SNP-mediated tolerance to MV stress was also confirmed by decreased electrolyte leakage and lipid peroxidation (Figure 5B(b)). Electrolyte leakage was markedly increased to ca. 30.8% at 6 h after MV stress without the SNP (0.5 mM) pretreatment and elevated to 53.8% at 9 h. By contrast, the augmented electrolyte leakages at 6 and 9 h were completely compromised by SNP pretreatment. No significant change in lipid peroxidation was found in the leaves at 6 h after MV treatment with or without SNP pretreatment. The MV-induced lipid peroxidation occurred at 9 h and the SNP pretreatment returned to the basal level. Electrolyte leakage was augmented to ca. 24.7% and 37.4%, respectively, at 6 and 24 h after H$_2$O$_2$ stress (4 M) without SNP pretreatment (Figure 5B(c)). Electrolyte leakages were more accelerated at 6 and 24 h after excess H$_2$O$_2$ by the SNP pretreatment, demonstrating higher electrolyte leakage of ca. 28.6% and 49.6%, respectively, at the same time points. Lipid peroxidation was not demonstrated at 6 after the H$_2$O$_2$ stress with or without SNP pretreatment. Increased lipid peroxidation occurred at 24 h after the H$_2$O$_2$ stress, and the pretreated SNP led to more increment in the lipid peroxidation.

**Figure 3.** Microscopic observation of reactive oxygen species accumulations in the Chinese cabbage leaf tissues treated with non-phytotoxic SNP treatment. (A) H$_2$O$_2$ and (B) O$_2^−$ production in the leaf tissues were histochemically detected in the SNP (0.5 mM)-treated leaves by DAB and NBT staining, respectively, at 24 h, and observed under a light microscope. Mock, foliar-sprayed with distilled water. Ten leaf fragments were used for each staining experiment. Similar results from DAB and NBT staining were obtained in three independent experiments and the representative result was demonstrated. Bar = 25 µM.

**Figure 4.** Temporal expressions of antioxidant enzyme genes in the leaves in response to the non-phytotoxic SNP treatment. The seedlings were sprayed with distilled water (as a mock) and SNP (0.5 mM) and harvested at the indicated time points in parentheses for the isolation of total RNA and analysis of gene expression using semi-quantitative RT-PCR. Un, untreated leaves; h, hours after treatment; L, light-condition; D, dark-condition; leaf harvest times indicated below the light conditions; BrSOD, superoxide dismutase; BrCat1, catalase 1; BrCat2, catalase 2; BrAPX, ascorbate peroxidase; BrMDHAR, monodehydroascorbate reductase; BrDHAR, Dehydroascorbate reductase; BrGR1, glutathione reductase 1; BrGST1, glutathione S-transferase 1. Similar results were obtained in three independent experiments and the representative result was demonstrated.

**Figure 5.** Fluorescence emissions from the leaf tissues treated with SNP (5 mM) and MV (50 µM). Bright blue fluorescence was emitted from the leaf tissues. By contrast, the pretreated SNP accelerated tissue collapses and loss of the chlorophyll autofluorescence observed under UV-illumination. By contrast, the pretreated SNP accelerated tissue collapses and loss of the chlorophyll autofluorescence in the leaves treated with excess H$_2$O$_2$ (4 M). In the severely damaged leaves under excess NO and MV stresses at 48 and 9 h, respectively, bright blue fluorescence was emitted from the leaf tissues. SNP pretreatment mitigated the bright blue fluorescence in the leaves treated with SNP (5 mM) and MV (50 µM). Bright blue fluorescence was also found during the accelerated tissue damage of leaves pretreated with SNP followed by excess H$_2$O$_2$ at 6 h but disappeared in highly collapsed leaves at 24 h.

**Figure 6.** Enhanced tolerance to high SNP stress. The seedlings were sprayed with SNP (0.5 mM) and harvested at the indicated time points in parentheses for the isolation of total RNA and analysis of gene expression using semi-quantitative RT-PCR. Un, untreated leaves; h, hours after treatment; L, light-condition; D, dark-condition; leaf harvest times in parentheses for the isolation of total RNA and analysis of gene expression using semi-quantitative RT-PCR.
stress, a significant change in H$_2$O$_2$ production was not found in the leaves. The SNP pretreatment mitigated NO burst and reversed H$_2$O$_2$ decline during the augmented tolerance to MV stress (Figure 6A(b)). MV (50 µM) markedly induced NO generation to ca. 3.29-fold increase in the leaves at 12 h compared to the control, but the SNP pretreatment significantly reduced the MV-induced NO production. NO production was much increased at 24 h after MV alone to ca. 4.43-fold increase, compared to the control. The SNP pretreatment completely suppressed MV-triggered NO accumulation. MV (50 µM) distinctly decreased H$_2$O$_2$ production at 12 h to ca. 3.49-fold reduction compared to the. Declined H$_2$O$_2$ production was completely compensated in the leaves tolerant to the MV stress by the SNP pretreatment at 12 h, and H$_2$O$_2$ level was partially recovered in the SNP-pretreated leaves under MV stress at 24 h.

The SNP pretreatment increased endogenous NO but the H$_2$O$_2$ level remained during enhanced sensitivity to high H$_2$O$_2$ stress (Figure 6A(c)). High H$_2$O$_2$ dose (4 M) alone increased NO content at 6 h to ca. 2.01-fold induction, and the SNP pretreatment did not alter NO content. The SNP pretreatment and following the excess H$_2$O$_2$ stress noticeably increased NO contents at 24 h to ca. 4.36-fold increase, compared to the control. The SNP pretreatment alone did not affect H$_2$O$_2$ production at 6 and 24 h. However, the excess H$_2$O$_2$ rather decreased endogenous H$_2$O$_2$ production in the leaves at 6 and 24 h after treatment. H$_2$O$_2$ production in the SNP-pretreated leaves was not different from the H$_2$O$_2$ production detected in the leaves under H$_2$O$_2$ stress.

O$_2^•-$ accumulations in the leaves were different depending on the SNP pretreatment and following stresses (Figure 6B). High SNP (5 mM) reduced O$_2^•-$ production in the mock-pretreated leaves at 24 h and no O$_2^•-$ accumulation was detected at 48 h. Pretreated SNP resulted in slightly decreased O$_2^•-$ production at 24 and 48 h in the mock (water)-treated leaves. The high NO stress completely halted O$_2^•-$ production at 24 and 48 h in the SNP-pretreated leaves. Very low O$_2^•-$...
production was detected in the mock- and SNP-pretreated leaves by the mock treatment at 12 h. MV stress abolished $O_2^\cdot -$ production in the mock-pretreated leaves, but could not mediate the $O_2^\cdot -$ decrease in the SNP-pretreated leaves. These different accumulations of $O_2^\cdot -$ generations were distinct at 24 h after the mock- and MV-treatments. Excess H$_2$O$_2$ led to decreases in $O_2^\cdot -$ production in the mock- and SNP-pretreated leaves at 6 and 24 h without any difference.

Antioxidant enzyme gene expressions in Chinese cabbage leaves during the altered tolerances to high NO and ROS stresses by the non-phytotoxic SNP pretreatment

Antioxidant enzyme gene expressions were analysed in the Chinese cabbage leaves pretreated with SNP (0.5 mM) followed by the different excess SNP and ROS stresses (Figure 7).

No distinct expression changes regulated by the low SNP (0.5 mM) pretreatment and/or high SNP (5 mM) in most genes except for BrGR1 and BrGST1, and BrGR1 and BrGST1 gene expressions up-regulated by excess NO donor stress were mitigated by the SNP (0.5 mM) pretreatment (Figure 7A). At 24 h, both gene expressions were inducible by the SNP (5 mM), but the SNP pretreatment returned the increased expressions to basal levels. Only BrGST1 induction was found in the high SNP-stressed leaves at 48 h but was completely abolished by the SNP pretreatment.

Attenuated $O_2^\cdot -$ stress by the SNP pretreatment led to distinct changes in BrSOD, BrAPX, BrGR1, and BrGST1 gene expressions by MV stress (Figure 7B). These four gene expressions were inducible by MV stress, whereas the SNP pretreatment alleviated the expression levels at 12 h. Pretreated SNP-mediated reductions in BrSOD, BrGR1, and BrGST1 gene expressions were also demonstrated at 24 h.

Most antioxidant enzyme genes were regulated by excess H$_2$O$_2$ (4 M) stress, but pretreatment with SNP could not affect the gene expressions modulated by H$_2$O$_2$ in the leaves (Figure 7C). BrSOD was slightly inducible by the excess H$_2$O$_2$ at 24 h regardless of the SNP (0.5 mM) pretreatment. BrCat1, BrAPX, BrGR1, and BrGST1 gene expressions were up-regulated by the excess H$_2$O$_2$ at 6 h, whereas BrCat2
and BrDHAR genes were down-regulated at the same time point. BrMDHAR genes were not regulated by H2O2 stress. No antioxidant enzyme genes, except for BrGR1 and BrGST1, were regulated by SNP pretreatment alone at 24 h. Both gene expressions increased by the excess H2O2 were slightly reduced by the SNP pretreatment at 24 h.

Discussion

Elevated NO reduced ROS levels and activated antioxidant enzyme gene expressions before subsequent challenge stresses

Pretreated SNP (0.5 mM) rapidly increased endogenous NO (Figure 1) but reduced two ROS (H2O2 and O\(_2^-\)) accumulation (Figure 2) in the Chinese cabbage leaves, suggesting that the elevated NO could play roles as a second messenger for plant defences and/or act as an antioxidant to scavenge ROS. The slight changes in NO and ROS levels seemed to be critically important for different responses of Chinese cabbage seedlings to subsequent challenging stresses such as excess NO donor and ROS. Interestingly, the SNP pretreatment conferred enhanced tolerance to Chinese cabbage seedlings to both high NO donor and MV stresses, whilst it increased sensitivity to excess H2O2 stress (Figure 5). Recently, SNP pretreatment increased root length but not shoot length of Kandelia obovata plants under high salinity stress (Hasanuzzaman et al. 2021). It may be correlated with the fact that increased endogenous NO and reduced H2O2 in the salt-stressed plants were only found in the roots but not in shoots. Biochemical and molecular responses need to be uncovered for the three different physiological interactions of the elevated NO with excess SNP, MV, and H2O2 stresses to gain an insight into how the NO signal dealing with excess NO and ROS stresses in plants under diverse environmental threats including high salinity, drought, extreme temperature stresses.

H2O2 level in the Chinese cabbage leaves was not dependent on the day-night cycle (Figure 2A). By contrast, O\(_2^-\) accumulation in the mock-treated leaves at 4:00–10:00 pm was slightly reduced compared to 10:00 am, and the exogenous SNP (0.5 mM) led to the less O\(_2^-\) accumulation at each time points (Figure 2C). It is likely to be related to the temporal regulation of antioxidant enzyme gene expressions in the leaves during the day-night cycle. Most gene expressions in the leaves have been concurrently regulated by a circadian clock regardless of the SNP treatment under usual plant growing conditions including a light/dark regime. In particular, and BrCat1 was down-regulated, and BrCat2, BrAPX, BrGR1, and BrGST1 were up-regulated without external stimuli at these time points (Figure 4). Plants possess a biological clock for tight control of their physiology including flowering transition, plant immunity to pathogen attacks, and environmental stress tolerance (Suárez-López et al. 2001; Spoel and van Ooijen 2014). Light period-dependent antioxidant enzyme activities are indeed evident in many plant species, and diurnal cycles of light and dark were interconnected with abiotic stress tolerance in plants (Dutilleul et al. 2003; Sanchez et al. 2011). Regulatory roles of the circadian clock for the antioxidant enzymes and environmental stress tolerances in Chinese cabbage plants remain elusive. It will also need to analyse which one is a precedent event between reduced ROS and activation of antioxidant enzymes in response to the SNP application.

NO as a diffusible gaseous molecule can permeate different cellular organelles of plants to play diverse physiological roles. Plant ROS were generated in various cellular organelles such as chloroplasts, mitochondria, peroxisome, and plasma membrane (Janků et al. 2019). In the Chinese cabbage leaf tissue, H2O2 and O\(_2^-\) might be accumulated mainly at mitochondria and chloroplasts, respectively, and the NO donor decreased the two ROS levels at the cells (Figure 3). Different isoforms of the antioxidant enzyme i.e. APX can be localized...
at different subcellular compartments and may be regulated by NO and/or ROS *vice versa* individually in response to diverse external stimuli in plants (Pandy et al. 2017). Simultaneous uses of cytochemical tracking dyes for subcellular localization as well as ROS detection will give more insights on the ROS levels down-regulated by NO.

**NO may confer tolerance of Chinese cabbage seedlings against high SNP stress via priming the same defence signaling**

Induced tolerance to the environmental stresses in many plants was achieved by the application of different NO donors and they have been suggested for stress mitigators during crop production in agriculture (Seabra and Oliveira 2016; Marvasi 2017). Declined ROS accumulation was frequently found in the NO donor-protected plant tissues under adverse environmental conditions. However, it hardly demonstrated how elevated NO by exogenous NO donors mitigates the NO burst owing to the environmental stresses. The SNP (0.5 mM) pretreatment promoted more NO accumulation in the Chinese cabbage leaves under the high SNP (5 mM) stress, but distinct changes in ROS levels were not found during the enhanced tolerance. It may be due to preactivated signaling against excess NO stress that might trigger the same defence pathways separately from ROS signaling. The foliar-spraying excess SNP (more than 1 mM) causing visible leaf damages, activated BrAPX, BrGR1, and BrGST1 gene expressions in the Chinese cabbage leaves in our previous study (Kim et al. 2015). The three genes transiently up-regulated by the SNP (0.5 mM) in the present study (Figure 4) might mediate an efficient build-up of tolerance to the high NO stress (Figure 8A). The BrAPX, BrGR1, and BrGST1 may require ascorbate or glutathione for their proper enzyme activities (Hasanuzzama et al. 2019). Ascorbate and glutathione redox as well as the associated antioxidant enzymes regulated by NO remain elucidated to gain insights into the molecular roles of NO for the enzymatic- and/or non-enzymatic antioxidants in the Chinese cabbage plants.

**Elevated NO protects Chinese cabbage seedlings from O$_2^\cdot$ oxidative stress by repressing phytotoxic O$_2^\cdot$ level**

Reduced O$_2^\cdot$ level in the SNP (0.5 mM)-pretreated leaves before MV stress may directly contribute to mitigating the oxidative stress by lowering cellular O$_2^\cdot$ below the phytotoxic level (Figure 8B). Chloroplasts are the main target organelles in the MV-stressed plant cells under light illumination (Cui et al. 2019). MV caused disorder of enzymatic antioxidative enzymes bound to chloroplast stroma and thylakoid, but not cytosolic APX, were rapidly inactivated in the MV-treated spinach leaves (Mano et al. 2001). Bioinformatics analyses predicted that Chinese cabbage BrCat1 and BrAPX enzymes are located at peroxisome and peroxisome/cyttoplasm, respectively, not to the chloroplast. Chinese cabbage BrCat1 and BrAPX inducible by the SNP pretreatment may be involved in mitigating MV stress by reducing H$_2$O$_2$, at different cellular places. Precedent decreased H$_2$O$_2$ level by the SNP pretreatment before MV stress may be achieved by transient activation of BrCat1 and BrAPX. It is interesting to note that Arabidopsis mutant with suppressed cytosolic APX1 expression was hypersensitive to MV, indicating that APX1-mediated H$_2$O$_2$ detoxification in the cytosol may play a critical role in the tolerance to the MV stress in chloroplasts via signaling interaction of chloroplast with cytoplasm in Arabidopsis (Daveltova et al. 2005). Signaling cross-talks of chloroplasts with apoplast, mitochondria, or nucleus suggest that chloroplasts can cooperate with other cellular organelles for alleviating O$_2^\cdot$ stress (Shapiguzov et al. 2012; Foyer 2018; Cui et al. 2019). Both GR and GST enzymes were known to require glutathione for their decent cellular function, and glutathione-dependent stress tolerances may be closely associated with NO-conferred tolerance to MV oxidative stress (Noctor et al. 2012). Chinese cabbage BrGR1 and BrGST1 are predicted to be located at the endoplasmic reticulum and mitochondria, respectively. BrCat1, BrAPX, BrGR1, and BrGST1 may be cooperated in different cellular organelles to cope with MV stress.

**Figure 8.** Proposed model for pretreated nitric oxide (NO) donor as a modulator of high NO and oxidative stresses. By SNP (0.5 mM) pretreatment, endogenous NO was increased and ROS decreased. Transient upregulation of antioxidant enzyme genes BrCat1, BrCat2, BrAPX, BrGR1, and BrGST1 can be involved in declines of reactive oxygen species (ROS) accumulation. (A) Mitigated NO donor stress. NO defence signaling primed by the SNP pretreatment can facilitate to alleviate cell death and cellular damage rapidly in response to high NO donor stress. (B) Decreased O$_2^\cdot$ stress. Reduced O$_2^\cdot$ by the SNP pretreatment can arrest O$_2^\cdot$ below the level for oxidative burst leading to cell death and cellular damage in response to high O$_2^\cdot$ stress by MV treatment. NO decline in the SNP-pretreated leaves under MV stress can avoid the interaction with H$_2$O$_2$, which can also delay cell death and cellular damage. (C) Accelerated H$_2$O$_2$ stress. NO level is highly increased and is enough to provoke cell death and cellular damage as an interacting partner of H$_2$O$_2$ in response to H$_2$O$_2$ stress in the SNP-pretreated leaves. Red and green arrows indicate increases and decreases, respectively.
In this study, MV-inducible expressions of BrSOD, BrAPX, BrGR1, and BrGST1 were not likely to be effective at mitigating MV stress without the SNP pretreatment, because MV stress eventually led to severe tissue damages within 24 h. Compromised MV-inducible gene expressions during the SNP-induced tolerance suggests that the upregulation of these gene expressions is no longer necessary due to enhanced protection at early hours. It will be notable that pea catalase activity was inhibited by NO and S-nitrosylated by cadmium and 2,4-D stimuli, indicating post-translational regulation during oxidative stress tolerance (Ortega-Galisteo et al. 2012). Many antioxidant enzyme activities were not changed in the K. obovate roots regardless of the SNP pretreatment (Hasanuzzaman et al. 2021). By contrast, SOD, APX, and CAT activities in UV-B-irradiated bean leaves treated with SNP were relatively higher than those in leaves damaged by UV-B-irradiation alone (Shi et al. 2005). These studies suggest that we cannot exclude the regulation of antioxidant enzyme activities post-translationally modified by the SNP pretreatment in Chinese cabbage leaves.

**Elevated NO may play as a prooxidant accelerates cellular damage of Chinese cabbage leaves in cooperated with exogenous H$_2$O$_2$.**

Generally, the role of NO has been suggested as an antioxidant during plant environmental stress tolerance. UV-B-induced H$_2$O$_2$ increased much faster in a low NO mutant noa1 than wild-type, suggesting a suppressive role of NO in the UV-B-mediated H$_2$O$_2$ production in Arabidopsis (Zhang et al. 2009). Indeed, co-treatment with non-phytotoxic SNP mitigated the oxidative stress by excess H$_2$O$_2$ in the Chinese cabbage leaf discs, showing higher chlorophyll contents compared to leaf discs treated with excessive H$_2$O$_2$ alone in our previous study (Sung and Hong 2010). Unexpectedly, pretreatment with the SNP (0.5 mM) remarkably promoted cellular damage in the leaves of Chinese cabbage seedlings under the subsequent excess H$_2$O$_2$ in this study. This opposite finding may be due to different experimental methods including plant leaf status and timing and doses of SNP and H$_2$O$_2$ application in our previous and current studies. Accelerated H$_2$O$_2$-triggered cellular damage by the SNP pretreatment in the present study can be supported by the cooperation of optimal NO and H$_2$O$_2$ doses to promote cell death in microbes, animals, and plants. Co-treated SNP and H$_2$O$_2$ synergistically decreased in vitro growth of phytopathogenic bacterium Ralstonia solanacearum (Hong et al. 2013). NO synergistically mediated murine lymphoma cell death together with H$_2$O$_2$, indicating increased DNA fragmentation and cell lysis (Filep et al. 1997). Simultaneous SNP and H$_2$O$_2$ treatments caused much faster death of tobacco suspension-cultured cells compared to cell death mediated by either SNP or H$_2$O$_2$ (de Pinto et al. 2002). SNP dose leading to mild cell death in tobacco leaves under a moderate illumination, accelerated cell deaths in catalase-deficient tobacco leaves overproducing H$_2$O$_2$ under the identical plant environments (Zago et al. 2006). SNP-induced hypersensitivities to excess H$_2$O$_2$ can be mediated by the direct chemical interaction of NO with H$_2$O$_2$ via the producing cytotoxic singlet oxygen, one of the major ROS involved in photooxidative damage to plants (Noronha-Dutra et al. 1993; Triantaphylidès et al. 2008). Excess H$_2$O$_2$-inducible endogenous NO as a nitrosative stress agent may directly lead to cellular damage of Chinese cabbage leaves. In addition, drastically increased NO level, but not H$_2$O$_2$, can be a reason for the rapid tissue collapse of Chinese cabbage leaves treated with excess H$_2$O$_2$ via producing cytotoxic diverse ROS and/or RNS (Figure 8C). It is difficult to find differences in the gene expressions of antioxidant enzymes between H$_2$O$_2$ treatment alone and H$_2$O$_2$ treatment on the SNP-pretreated Chinese cabbage leaves. There is limited knowledge on the biochemical and molecular mechanisms of synergistic interactions between NO and H$_2$O$_2$ to provoke cell death in Chinese cabbage plants.

**Conclusion**

The non-phytotoxic SNP as a NO donor rendered rapid changes in endogenous NO and ROS levels within 24 h as well as differential gene expression of various antioxidant enzymes in the Chinese cabbage leaves. By the SNP pretreatment, cellular damages (electrolyte leakage and lipid peroxidation) by the excess SNP, MV, and H$_2$O$_2$ were differentially regulated. NO and ROS accumulation, and antioxidant enzyme gene expressions in the leaves were changed in the three different interactions. These findings may be derived from distinct chemical and signaling interactions of pre-elevated NO levels with RNS and/or ROS. Further investigations including small antioxidant molecules such as ascorbate, glutathione, and S-nitrosoglutathione (Silveira et al. 2017; Hasanuzzama et al. 2019; Parent et al. 2019) will provide more insights on interaction of NO with RNS or ROS during plant responses to deal with environmental stresses and pathogen infections.

**Abbreviations**

APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; GR1, glutathione reductase 1; GST1, glutathione S-transferase 1; H$_2$O$_2$, hydrogen peroxide; MDHAR, monodehydroascorbate reductase; MV, methyl viologen; NO, nitric oxide; O$_2^\cdot$-, superoxide radical anion; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase.

**Disclosure statement**

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References

Ahlfors R, Brosché M, Kollist H, Kangasjärvi J. 2009. Nitric oxide modulates ozone-induced cell death, hormone biosynthesis and gene expression in Arabidopsis thaliana. Plant J. 58:1–12.

Bai S, Li M, Yao H, Wang H, Zhang Y, Xio L, Wang J, Zhang Z, Hu Y, Liu W, He Y. 2012. Nitric oxide restrain root growth by DNA damage induced cell cycle arrest in Arabidopsis thaliana. Nitric Oxide. 26:54–60.

Begara-Morales JC, Sánchez-Calvo B, Chaki M, Mata-Pérez C, Valderrama R, Padilla MN, López-Jaramillo J, Luque F, Corpas FJ, Barroso JN. 2015. Differential molecular response of monodehydroascorbate reductase and glutathione reductase by nitration and S-nitrosylation. J Exp Bot. 66:5983–5996.

Begara-Morales JC, Sánchez-Calvo B, Chaki M, Valderrama R, Mata-Pérez C, López-Jaramillo J, Padilla MN, Carreras A, Corpas FJ, Barroso JN. 2014. Dual regulation of cytosolic ascorbate peroxidase (APX) by tyrosine nitration and S-nitrosylation. J Exp Bot. 65:527–538.

Beligni MV, Lamattina L. 1999a. Is nitric oxide toxic or protective? Trend Plant Sci. 4:299–300.

Beligni MV, Lamattina L. 1999b. Nitric oxide protects against cellular damage produced by methylviologen herbicides in potato plants. Nitric Oxide Biol Chem. 3:199–208.

Corpas FJ, Barroso JB. 2013. Nitric-oxidative stress vs oxidative or nitrosative stress in higher plants. New Phytol. 199:633–635.

Cui F, Brosché M, Shapiguzov A, He X-Q, Vainonen JP, Leppälä J, Trotta A, Kangasjärvi S, Salojarvi J, Kangasjärvi J, Overmyer K. 2019. Interaction of methyl viologen-induced chloroplast and mitochondrial signalling in Arabidopsis. Free Radiol Biol Med. 134:555–566.

Davelová S, Rízhsky L, Liang H, Shengqiang Z, Oliver DJ, Coutu J, Kolbert Z, Barroso JB, Brouquisse R, Corpas FJ, Barroso JN. 2016. Changes in the antioxidant systems as part of the signaling pathway responsible for the programmed cell death activated by nitric oxide and reactive oxygen species in tobacco bright-yellow 2 cells. Plant Physiol. 130:698–708.

Dixon DP, Skipsey M, Edwards R. 2010. Roles for glutathione transferases in plant secondary metabolism. Phytochemistry. 71:338–350.

Dixon DP, Skipsey M, Grundy NM, Edwards R. 2005. Stress-induced protein S-glutathionylation in Arabidopsis. Plant Physiol. 138:232–244.

Dong N, Li Y, Qi J, Chen Y, Hao Y. 2018. Nitric oxide synthase-dependent nitric oxide production enhances chilling tolerance of walnut shoots in vitro via involvement chlorophyll fluorescence and other physiological parameter levels. Sci Hortic. 230:68–77.

Duttilleul C, Garmier M, Noctor G, Mathieu C, Chétrit P, Foyer CH, de Pinto MC, Tommasi F, De Gara L. 2002. Changes in the antioxidant systems and nitric oxide synthase activity, and determine stress resistance through altered signaling and diurnal regulation. Plant Cell. 15:1212–1226.

Fan H, Du C, Xu Y, Wu X. 2014. Exogenous nitric oxide improved chilling tolerance of Chinese cabbage seedlings by affecting antioxidant enzymes in leaves. Hort Environ Biotechnol. 55:159–165.

Farnese FS, Menezes-Silva PE, Gusman GS, Oliveira JA. 2016. When bad guys become good ones: the key role of reactive oxygen species and nitric oxide in the plant responses to abiotic stress. Front Plant Sci. 7:471.

Filep JG, Lapierre C, Lachance S, Chan JS. 1997. Nitric oxide co-operates with hydrogen peroxide in inducing DNA fragmentation and cell lysis in murine lymphoma cells. Biochem J. 321:897–901.

Foyer CH. 2018. Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. Environ Exp Bot. 154:134–142.

Garcés H, Durzan D, Pedroso MC. 2001. Mechanical stress elicits nitric oxide formation and DNA fragmentation in Arabidopsis thaliana. Ann Bot. 87:567–574.

Gill SS, Tuteja N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem. 48:909–930.

Ham J-H, Jin C-D. 2005. The role of nitric oxide on the growth regulation of Chinese cabbage (Brassica campestris L.) primary leaves. Korean J Plant Biotechnol. 32:293–300. (In Korean).

Hancock JT, Neill SJ. 2019. Nitric oxide: its generation and interaction with other reactive signaling compounds. Plants. 8:41.

Hasanuzzaman M, Borhanuddin Bhuyan MHM, Anee TI, Parvin K, Nahar K, Mahmud JA, Fujita M. 2019. Regulation of ascorbate-glutathione pathway in mitigating oxidative damage in plants under abiotic stress. Antioxidants. 8:384.

Hasanuzzaman M, Nahar K, Hossain M, Anee TI, Parvin K, Fujita M. 2017. Nitric oxide pretreatment enhances antioxidant defense and glyoxalase systems to confer PEG-induced oxidative stress in rape. J Plant Interact. 12:323–331.

Hasanuzzaman M, Inafuku M, Nahar K, Fujita M, Oku H. 2021. Nitric oxide regulates plant growth physiology, antioxidant defense, and ion homeostasis to confer salt tolerance in the mangrove species, Kandelia obovata. Antioxidants. 10:611.

He H, Oo TI, Huang W, He L-F, Gu M. 2019. Nitric oxide acts as an antioxidant and inhibits programmed cell death induced by aluminum in the root tips of peanut (Arachis hypogaea L.). Sci Rep. 9:9516.

He H, Yang Q, Shen B, Zhang S, Peng X. 2018. OsNOA1 functions in a threshold-dependent manner to regulate chloroplast proteins in rice at lower temperatures. BMC Plant Biol. 18:44.

He Y, Tang R-H, Hao Y, Stevens RD, Cook CW, Ahn SM, Jing L, Yang Z, Chen L, Guo F, et al. 2004. Nitric oxide represses the Arabidopsis floral transition. Science. 305:1968–1971.

Hong JK, Kang SR, Kim YH, Yoon DJ, Kim DH, Kim HJ, Sungh CH, Kang HS, Choi CW, Kim SH, Kim YS. 2013. Hydrogen peroxide and nitric oxide-mediated disease control of bacterial wilt in tomato plants. Plant Pathol J. 29:386–396.

Jankó M, Luhová I, Petřívková M. 2019. On the origin and fate of reactive oxygen species in plant cell compartments. Antioxidants. 8:105.

Jo YS, Park HB, Kim JY, Choi SM, Lee DS, Kim DH, Lee YH, Park C-J, Jeun Y-C, Hong JK. 2020. Menadione sodium bisulfite-protected tomato leaves against grey mould via antifungal activity and enhanced plant immunity. Plant Pathol J. 36:335–345.

Kaya C, Ashraf M, Alyemeni MN, Ahmad P. 2020. The role of endogenous nitric oxide in salicylic acid-induced up-regulation of ascorbate-glutathione cycle in salinity tolerance of pepper (Capsicum annuum L.) plants. Plant Physiol Biochem. 147:10–20.

Kim YJ, Lee YJ, Lee H-J, Jung H, Hong JK. 2015. H2O2 production and gene expression of antioxidant enzymes in kimchi cabbage (Brassica rapa var. glabella Regel) seedlings regulated by plant development and nitrosative stress-triggered cell death. Plant Biotechnol Rep. 9:67–78.

Kolbert Z, Barroso JB, Brouquisse R, Corpas FJ, Gupta KJ, Lindermayr C, Loake GJ, Palm MA, Petřívková M, Wendehenne D, Hancock JT. 2019. A forty year journey: The generation and roles of NO in plants. Nitric Oxide. 93:57–70.

Kolbert Z, Ortega L, Erdei L. 2010. Involvement of nitrate reductase (NR) in osmotic stress-induced NO generation of Arabidopsis thaliana. J Plant Physiol. 167:77–80.

Lechón T, Sanz L, Sánchez-Vicente I, Lorenzo O. 2020. Nitric oxide overproduction by cue1 mutants differs on developmental stages and growth conditions. Plants. 9:1484.

Lee U, Wie C, Fernandez BO, Feilisch M, Vierling E. 2008. Modulation of nitrosative stress by S-nitrosothiol reductase is critical for thermotolerance and plant growth in Arabidopsis. Plant Cell. 20:786–802.

Lee YH, Hong JK. 2012. Host and non-host disease resistance of kimchi cabbage against different Xanthomonas campestris pathovars. Plant Pathol J. 28:322–329.

Li F, Wu Q-Y, Sun Y-L, Wang L-Y, Yang X-H, Meng Q-W. 2010. Overexpression of chloroplastic monodehydroascorbate reductase
enhanced tolerance to temperature and methyl viologen-mediated oxidative stresses. Physiol Plant. 139:421–434.

Liu X, Liu B, Xue S, Cai Y, Qi W, Jian C, Xu S, Wang T, Ren H. 2016. Cucumber (Cucumis sativus L.) nitric oxide synthase associated gene1 (CnNOA1) plays a role in chilling stress. Front Plant Sci. 7:1652.

López-Carrón AI, Castellano R, Rosales MA, Ruiz JM, Romero L. 2008. Role of nitric oxide under saline stress: implication on proline metabolism. Biol Plant. 52:587–591.

Lu R, Liu Z, Shao Y, Su J, Li X, Sun F, Zhang Y, Li S, Zhang Y, Cui J, et al. 2020. Nitric oxide enhances rice resistance to Rice black-streaked dwarf virus infection. Rice. 13:24.

Mano J, Ohno C, Domay A, Asada K. 2001. Chloroplastic ascorbate peroxidase is the primary target of methylviologen-induced phototoxidative stress in spinach leaves: its relevance to monodehydroascorbate radical detected with in vivo ESR. Biochem Biophys Acta. 1504:275–287.

Marvasi M. 2017. Potential use and perspectives of nitric oxide donors in agriculture. J Sci Food Agric. 97:1065–1072.

Montilla-Bascón G, Rubiales D, Hebelstrup KH, Mandon J, Harren JF, Cristescu SM, Mur LAJ, Prats E. 2017. Reduced nitric oxide levels during drought stress promotes drought tolerance in barley and is associated with elevated polyamine biosynthesis. Sci Rep. 7:13311.

Mur LAJ, Simpson C, Kumari A, Gupta AK, Gupta KJ. 2017. Moving nitrogen to the centre of plant defence against pathogens. Ann Bot. 119:703–709.

Na H-G, Jin C-D. 2014. Ectopic expression of AtCaM3 in Arabidopsis enhances rice resistance to Rice black-streaked dwarf virus infection. Rice. 13:24.

Ortega-Galisteo AP, Rodríguez-Serrano M, Pazmiño DM, Gupta DK, Noronha-Dutra A, Epperlein MM, Woolf N. 2019. CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. Nature. 410:1116–1120.

Sung CH, Hong JK. 2010. Sodium nitroprusside mediates seedling development and attenuation of oxidative stresses in Chinese cabbage. Plant Biotechnol Rep. 4:243–251.

Vitor SC, Duarte GT, Saviani EE, Vinentz MGA, Oliveira HC, Salgado I. 2013. Nitrate reductase is required for the transcriptional modulation and bactericidal activity of nitric oxide during the defense response of Arabidopsis thaliana against Pseudomonas syringae. Planta. 238:475–486.

Wang BL, Tang XY, Cheng LY, Zhang AZ, Zhang WH, Zhang FS, Liu Q, Tian Q-Y, Sun D-H, Zhang W-H. 2007. Inhibition of nitric oxide synthase (NOS) underlines aluminium-induced inhibition of root elongation in Hibiscus moscheutos. New Phytol. 174:322–331.

Wu J, Zhang Y, Hao R, Cao Y, Allan DL, Vance CP, Shen JB. 2010. Nitric oxide boosts Bemisia tabaci performance through the suppression of jasmonic acid signaling pathway in tobacco plants. Front Plant Sci. 11:847.

Xu Y, Zhou S, Wang L, Cheng Y, Zhao L. 2010. Nitric oxide functions as a signal and acts upstream of AtCaM3 in thermotolerance in Arabidopsis seedlings. Plant Physiol. 153:1895–1906.
Yu Z, Cao J, Zhu S, Zhang L, Peng Y, Shi J. 2020. Exogenous nitric oxide enhances disease resistance by nitrosylation and inhibition of S-nitrosoglutathione reductase in peach fruit. Front Plant Sci. 11:543.

Yun B-W, Skelly MJ, Yin M, Yu M, Mun B-G, Lee S-U, Hussain A, Spoel SH, Loake GJ. 2016. Nitric oxide and S-nitrosoglutathione function additively during plant immunity. New Phytol. 211:516–526.

Zago E, Morsa S, Dat JF, Alard P, Ferrarini A, Inzé D, Delledonne M, van Breusegem F. 2006. Nitric oxide- and hydrogen peroxide-responsive gene regulation during cell death induction in tobacco. Plant Physiol. 141:404–411.

Zhan N, Wang C, Chen L, Yang H, Feng J, Gong X, Ren B, Wu R, Mu J, Li Y, et al. 2018. S-Nitrosylation targets GSNO reductase for selective autophagy during hypoxia responses in plants. Mol Cell. 71:142–154.

Zhang L, Zhao L. 2008. Production of nitric oxide under ultraviolet-B irradiation is mediated by hydrogen peroxide through activation of nitric oxide synthase. J Plant Biol. 51:395–400.

Zhang L, Zhou S, Xuan Y, Sun M, Zhao L. 2009. Protective effect of nitric oxide against oxidative damage in Arabidopsis leaves under ultraviolet-B irradiation. J Plant Biol. 52:135–140.