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Original Contribution

Carbon monoxide enhances the chilling tolerance of recalcitrant Baccaurea ramiflora seeds via nitric oxide-mediated glutathione homeostasis

Xue-gui Bai [a,b,1], Jin-hui Chen [c,1], Xiang-xiang Kong [a,b,1], Christopher D. Todd [d], Yong-ping Yang [a], Xiang-yang Hu [a,b,*], De-zhu Li [a,b,**]

[a] Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650201, China
[b] Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650201, China
[c] Key Laboratory of Forest Genetics & Biotechnology, Nanjing Forestry University, Nanjing 210037, China
[d] Department of Biology, University of Saskatchewan, Saskatoon, SA, Canada S7N 5E2

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ABSTRACT

Both carbon monoxide (CO) and nitric oxide (NO) play fundamental roles in plant responses to environmental stress. Glutathione (GSH) homeostasis through the glutathione–ascorbate cycle regulates the cellular redox status and protects the plant from damage due to reactive oxygen species (ROS) or reactive nitrogen species (RNS). Most recalcitrant seeds are sensitive to chilling stress, but the roles of and cross talk among CO, NO, ROS, and GSH in recalcitrant seeds under low temperature are not well understood. Here, we report that the germination of recalcitrant *Baccaurea ramiflora* seeds shows sensitivity to chilling stress, but application of exogenous CO or NO markedly increased GSH accumulation, enhanced the activities of antioxidant enzymes involved in the glutathione–ascorbate cycle, decreased the content of H_2O_2 and RNS, and improved the tolerance of seeds to low-temperature stress. Compared to orthodox seeds such as maize, only transient accumulation of CO and NO was induced and only a moderate increase in GSH was shown in the recalcitrant *B. ramiflora* seeds. Exogenous CO or NO treatment further increased the GSH accumulation and *S*-nitrosoglutathione reductase (GSNOR) activity in *B. ramiflora* seeds under chilling stress. In contrast, suppressing CO or NO generation, removing GSH, or blocking GSNOR activity resulted in increases in ROS and RNS and impaired the germination of CO- or NO-induced seeds under chilling stress. Based on these results, we propose that CO acts as a novel regulator to improve the tolerance of recalcitrant seeds to low temperatures through NO-mediated glutathione homeostasis.

Introduction

Recalcitrant seeds, in contrast to orthodox seeds, lose viability under drying or chilling exposure once they are shed from the parent plant. Unlike orthodox seeds, recalcitrant seeds cannot be stored for a long period under low-temperature conditions. Thus, it is challenging to maintain a regular supply of these seeds for genetic conservation purposes [1,2]. Recent studies on recalcitrant seeds, including sweet chestnut [3], silver maple [4], and tea [5], demonstrate that reactive oxygen species (ROS), particularly H_2O_2, play an essential role during recalcitrant seed germination after desiccation. However, the mechanisms underlying the sensitivity of recalcitrant seeds to drying or chilling stress are poorly understood. H_2O_2 has been shown to act as a key regulator in a broad range of physiological processes, including senescence, growth, differentiation, and stress responses. An appropriate concentration of H_2O_2 is critical for seed development and germination [6], whereas higher concentrations of H_2O_2 may lead to oxidative stress and cell death. To withstand the oxidative assault imposed by ROS, plants employ an array of enzymatic and nonenzymatic oxidants for detoxification of ROS when they accumulate within cells. Glutathione (GSH) is the most crucial metabolite required to maintain the normal reduced state of the cell by counteracting the inhibitory effects of ROS-induced oxidative stress. Additionally, GSH generates another potential water-soluble antioxidant, ascorbate (AsA), via the ascorbate–glutathione cycle [7]. As this cycle operates, H_2O_2 is degraded by ascorbate peroxidase (APX), with AsA acting as an electron donor. The resulting short-lived radical, monodehydroascorbate, is reduced directly to AsA and dehydroascorbate, which, in turn, is reduced by dehydroascorbate reductase (DHAR) or monodehydroascorbate reductase (MDHAR) using GSH as the electron

* Corresponding author. Fax: +86 871 5223283.
** Corresponding author.
E-mail addresses: huxiangyang@mail.kib.ac.cn (X.-y. Hu), dzl@mail.kib.ac.cn (D.-z. Li).
[1] These authors contributed equally to this article.

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donor, producing GSSG (the oxidized form of GSH, in which two glutathione molecules are bound by a disulfide bridge). GSSG is recycled to GSH by NADPH-dependent glutathione reductase (GR). Biosynthesis of GSH can also be catalyzed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase in plants [8]. In addition to H_2O_2, reactive nitrogen species (RNS), such as nitric oxide (NO), nitrate (NO_3^-), nitrite (NO_2^-), peroxynitrite ($ONOO^-$), and 3-nitrotryosine, play direct roles in cellular signaling and plant defense responses. RNS can interact with protein or nonprotein sulfhydryl-containing compounds to form *S*-nitrosothiols (SNOs), which also carry out important biological reactions, including NO release, transnitrosation, and redox-based posttranslational modification. For example, the total SNO levels in pea were found to be upregulated by wounding and heavy metal stresses. The S-nitrosylation reaction of NO^- with GSH to form GSNO has an important physiological function in plants, as GSNO is regarded as a mobile reservoir of NO bioactivity. The enzyme GSNO reductase (GSNOR) modulates the content of GSNO in plants by catalyzing the NADH-dependent reduction of GSNO to GSSG and NH_3 [9]. The activity and expression of GSNOR can be induced by various stimuli, including wounding, low temperature, high temperature, heavy metals, and pathogen infection [10–13], but the potential roles of RNS and GSNOR in controlling germination remain unclear.

NO is a hydrophobic diffusible gaseous molecule participating in a wide spectrum of physiological processes in plants, such as germination, flowering, root initiation, stomatal closure, and programmed cell death [14]. NO has also been suggested to be involved in the regulation of plant responses to multiple stresses, including salinity, drought, mechanical injury, heavy metals, and pathogen attack [15–19]. NO synthesis in plants can potentially be carried out by NO synthase (NOS), nitrate reductase (NR), and other potential nonenzymatic sources [14]. In orthodox seeds, such as those of *Arabidopsis,* NO-mediated breakage of seed dormancy can be detected by reducing abscisic acid (ABA) accumulation [20,21]. Furthermore, exogenous NO treatment has been shown to enhance cold acclimation and freezing tolerance in *Arabidopsis* seedlings [22]. Similar to NO, carbon monoxide (CO) is an important reactive trace gas in the troposphere. CO was recently shown to be generated in animals via the action of heme oxygenase (HO; EC 1.14.99.3), and this gas plays an important role in regulating sinusoidal tones, inhibiting platelet aggregation, transmitting neuronal signals, and suppressing the acute hypertensive response [23–26]. Despite the recognition of CO biosynthesis in the plant kingdom almost 5 decades ago [27], the regulatory roles of CO in photosynthesis, stomatal closure, and adventitious root development as well as in plant responses to salt, heavy metals, and iron-deficiency stress have only recently been investigated [28–33]. CO can interact with ROS or NO to alleviate the inhibitory effect of salt on germination [20,30]; however, the available data on this topic are focused primarily on orthodox seeds, belonging to species such as rice or wheat. No detailed studies have yet been undertaken to evaluate the roles and interactions of CO, NO, and ROS in recalcitrant seeds.

To explore a new strategy for improving recalcitrant seed viability under low-temperature conditions, we first need to understand the mechanism underlying the sensitivity of recalcitrant seeds to chilling stress. In this study, we investigated the physiological and molecular response of recalcitrant *Baccaurea ramiflora* seeds exposed to low-temperature stress. We demonstrated that low-temperature treatment induced the accumulation of CO in *B. ramiflora* embryos, accompanied by increased heme oxygenase activity. Treating the seeds with a CO-saturated solution or hematin (a CO donor) significantly reversed the inhibitory effect of low temperature on seed germination. Furthermore, by adopting pharmacological and physiological approaches, we showed that CO treatment accelerates NO and GSH accumulation, which, in turn, efficiently scavenged the ROS accumulated during chilling stress. Therefore, we propose that CO enhances the tolerance of recalcitrant seeds to chilling stress through NO-mediated GSH homeostasis in *B. ramiflora.*

Materials and methods

Seed collection

Mature *B. ramiflora* (Phyllanthaceae) fruits were harvested from trees cultivated at the Xishuangbanna Tropical Botanical Garden (21°41′N, 101°25′E; 570 m altitude). The pericarps and shells were removed after harvest. The seeds were then rinsed five times with water and surface sterilized with a 5% hypochlorous acid solution. In control experiments, the seeds were placed in a growth room (22 °C, 65% relative humidity, under light). For the chilling treatment, the seeds were subjected to a temperature decrease from 22 to −2 °C at 3 °C per hour in a growth chamber. When the temperature reached −2 °C, the seeds were covered with ice crystals to induce crystallization and prevent supercooling, as described previously [34]. After 2 h at −2 °C, the temperature was lowered to −8 °C at 1 °C per hour. After the chilling treatment, the temperature was returned to 22 °C at 0.5 °C per hour to measure the germination rate. In chemical treatment experiments, fresh seeds were incubated in an aqueous solution with various chemicals for 24 h. After treatment, the seeds were washed three times in distilled water and wiped before low-temperature treatment.

CO and NO aqueous solution preparation

Pure CO gas was purchased from Standard Gas Co. (Guangzhou, China). CO gas was bubbled gently through a 3-mm (i.d.) glass tube into 500 ml of distilled water in an open tube for 15–20 min to saturate the solution with CO. The saturated stock solution (100% saturation) was immediately diluted with distilled water, with or without various chemicals, to the desired concentration (1, 10, 25, 50, or 75% saturation). Hematin, an artificial CO donor, was used to produce an aqueous solution of CO by dissolving it in 1/4 Hoagland solution at the concentrations given in a previously described method [28]. To prepare an aqueous NO solution, 2,2′-(hydroxynitrosohydrazino)-*bis*-ethanamine (NOC-18; an NO donor) was dissolved into 1/4 Hoagland solution at the indicated concentrations. For scavenger or inhibitor cotreatments, various chemicals were added to the CO or NO aqueous solutions to attain the required final working concentrations.

Germination rate measurement

Five replicates of 50 seeds were used to measure germination. After chilling or other treatments, the seeds were washed five times and placed on H_2O-saturated wet filter paper at 22 °C under a 16-h photoperiod and 50 μmol photons m^{-2} s^{-1} light intensity. The wet filter papers were replaced every 2 days. The seeds were judged to have germinated when the radicle pierced the seed coat after 6 days of germination under normal conditions (22 °C, 65% humidity) [21].

Electrolyte leakage

The electrolyte leakage test was performed according to Dong et al. [31]. Briefly, embryos from seeds either subjected to the chilling treatment or not were excised and transferred to tubes containing 10 ml of deionized water. The conductivity of the

solution was measured after shaking overnight at room temperature. After these measurements, the samples were autoclaved and shaken at room temperature for 2 h, and the conductivity of the solution was measured again. The percentage of electrolyte leakage was calculated as described previously [31].

CO and NO concentration determination

The CO content in aqueous solutions or embryos was quantified by gas chromatography and mass spectrometry (GC/MS) according to a previously reported method [28,36]. Briefly the treated embryos were quickly separated from the seeds and frozen in liquid nitrogen. Approximately 20 collected embryos (0.5 g) were crushed into a fine powder with a mortar and pestle under liquid N_2 and then placed in a sealed bottle (4 ml). Vacuum-packed frozen products were removed from the ultralow chiller and thawed in their original packages before being homogenized. To avoid foaming, 20 μl of 1-octanol was added to each aliquot, followed by 1 ml of distilled water. The bottles were immediately capped and shaken vigorously for approximately 30 s. Using a separate needle as a vent source, an aliquot of 1 ml of sulfuric acid (5 μm) was added via a syringe. Thereafter, the bottles were briefly shaken, placed in a 70 °C water bath for 3 h, and shaken again when removed from the bath. Analysis of CO was carried out by injecting a 1-ml aliquot from the headspace into the GC/MS system after the bottle and its contents had cooled to room temperature.

An Agilent 5975C GC (Agilent Technologies, Palo Alto, CA, USA) equipped with an HP-5 capillary column (30 m × 0.32-mm i.d. × 12 μm) was used for GC/MS analysis. Oxygen and nitrogen elute earlier than CO under the experimental conditions described. The samples were manually injected using a 100-μl gastight syringe into the 2-mm i.d. liner within the injection port, which was set at 150 °C. The oven temperature was held at 30 °C for 2.5 min, increased to 60 °C over the next 0.5 min, and held at 60 °C for the remaining 2 min of analysis. Helium was used as a carrier gas with a flow rate of 1.5 ml min^{-1}. The transfer line was set at 280 °C, and the Agilent 5975C inert MS was operated in electron ionization mode at 70 eV using selected ion monitoring at m/z 28.

To assay the NO content in embryo tissue, the collected embryo tissue (0.5 g) was homogenized in 5 ml of extraction buffer (10 mM Tris–Cl, pH 8.0, 10 mM $MgSO_4$, 5 mM KCl, 5 mM NaCl, 1 mM EDTA, 1% (w/v) polyvinylpyrrolidone, 10% (v/v) glycerol, and 1 mM dithiothreitol). The extracts were then centrifuged (15,000 *g*, 10min) at 4 °C, and the supernatants were used for NO determination with the Griess reagent system (G2930, Promega, Madison, WI, USA).

HO activity determination

The HO activity in the embryos was analyzed as described previously [28]. The collected embryos (0.5 g) were homogenized in 30 ml of ice-cold isolation medium (250 mM mannitol, 25 mM Hepes–Tris (pH 7.4), 1 mM EDTA, 1% (w/v) polyvinylpyrrolidone, 10% (v/v) glycerol, and 1 mM dithiothreitol) at 4 °C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 1500*g* for 30 min at 4 °C. The supernatant was removed and centrifuged at 60,000 *g* for 30 min to yield a crude membrane fraction to be used for HO activity determination. The 80-μl reaction assay mixtures contained 20 μl of enzyme solution, 10 μM hemin, 0.15 mg ml^{-1} bovine serum albumin, 50 μg ml^{-1} (4.2 μM) spinach (*Spinacia oleracea*) ferredoxin (Sigma), 0.025 U ml^{-1} spinach ferredoxin-$NADP^+$ reductase (Sigma), 5 mM ascorbate, and 2 mM desferrioxamine in 100 mM Hepes–NaOH (pH 7.2). The reaction was initiated by adding NADPH to a final concentration of 100 μM. The samples were incubated at 37 °C for 30 min, and biliverdin (BV) IXa formation was calculated by measuring the absorbance change at 650 nm. The concentration of BV was estimated using a molar absorption coefficient of 6.25 mM^{-1} cm^{-1}. One unit of activity was calculated as the quantity of enzyme needed to produce 1 nM BV per 30 min. The protein concentration was determined by the method of Bradford [43] using bovine serum albumin as the standard.

H_2O_2 content assay

The collected embryos (0.5 g) were frozen in liquid N_2 and ground to a fine powder. The frozen powdered samples were then homogenized in 5 ml of extraction buffer (10 mM Tris–Cl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 10 mM $MgSO_4$, 5 mM KCl, 5 mM NaCl, 10 μM oxyhemoglobin, and 10 U ml^{-1} of catalase). The extracts were subsequently centrifuged (15,000 *g*, 10 min) at 4 °C, and the supernatants were used for H_2O_2 determination according to Hu et al. [33].

RSNO content determination and GSNOR enzyme activity assay

The total RSNO content was determined via a chemiluminescence method with minor modifications [38]. The detection of RSNOs is based on the reductive decomposition of nitroso species by an iodine/triiodide mixture to release NO, which is subsequently measured by gas-phase chemiluminescence upon reaction with ozone. RSNOs are sensitive to mercury-induced decomposition, in contrast to other nitroso species, including nitrosamines (RNNOs) and nitrosyl hemes. Briefly, the treated samples were homogenized in the buffer containing 100 mM diethylenetetraminepentaacetic acid (1:5; w/v) and centrifuged at 3000*g* for 10 min. Then the supernatants were incubated with 10 mM *N*-ethylmaleimide for 15 min at 48 °C. For each sample, two aliquots were prepared: (i) one treated with 10 mM sulfanilamide for 15 min at 48 °C to eliminate nitrite and (ii) one treated with 10 mM sulfanilamide and 7.3 mM $HgCl_2$ for 15 min at 48 °C to eliminate nitrite and RSNOs, respectively. These samples were then analyzed in a Nitric Oxide Monitor 410 (2B Technologies, Boulder, CO, USA). The data obtained from both aliquots (i and ii) represented the total concentration of RSNOs. The entire procedure was performed under a red safety light to protect RSNOs from light-dependent decomposition.

GSNOR activity was determined spectrophotometrically at 25 °C by monitoring the oxidation of NADH at 340 nm [38]. The treated samples were quickly homogenized in liquid nitrogen and extracted with an assay mixture containing 20 mM Tris–HCl (pH 8.0), 0.2 mM NADH, and 0.5 mM EDTA at 4 °C and then centrifuged at 3000 *g* for 10 min. The supernatants were used for further assays, and the reaction was initiated by adding GSNO (Calbiochem, San Diego, CA, USA) to the supernatants at a final concentration of 400 mM. The activity was expressed as nanomoles NADH consumed per minute per milligram of protein (e340 6.22 mM^{-1} cm^{-1}).

Glutathione and GSSG content assay

The contents of glutathione in its reduced (GSH) and oxidized (GSSG) forms were determined according to a previous method [4]. Briefly, 50 mg of an embryo sample was homogenized in 5% (w/v) sulfosalicylic acid in an ice bath and then centrifuged at 15,000 *g* for 15 min. A 1-ml aliquot of the supernatant was removed and neutralized by adding 1.5 ml of 0.5 mol/L K-phosphate buffer (pH 7.5). The neutralized supernatant was used to determine the total glutathione content (GSH + GSSG). Another 1 ml of neutralized supernatant was pretreated with

0.2 ml of 2-vinylpyridine for 1.5 h at 25 °C to mask GSH and to allow determination of GSSG alone. The contents of GSH and GSSG were determined according to a previously described method [39].

Fluorescence microscopy

NO was measured in vivo in the embryo tips of treated seeds using confocal laser-scanning microscopy [37]. Epicarp-free seeds were thoroughly washed to remove traces of the treatments and then incubated with 10 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate) for 30 min. The cotyledons were removed, and NO-induced fluorescence was observed using a Zeiss confocal laser-scanning microscope (LSM 510 META, Zeiss, Oberkochen, Germany) (excitation at 488 nm, emission at 515 nm). The intensities of the green fluorescence signal in the images were quantified according to a previous method by measuring the average pixel intensity with Photoshop software [19]. The data are presented as the means of the fluorescence intensities relative to the untreated control embryos.

Antioxidant enzyme activity assays

All enzyme assays were performed in the same crude enzyme extract. All extraction procedures were carried out at 4 °C. Samples containing 100 embryos were ground in liquid nitrogen and homogenized in extraction buffer containing 50 mM sodium phosphate buffer (pH 7.0), 0.2 mM EDTA, and 2% polyvinylpolypyrrolidone, for 10 min. The homogenates were filtered through two layers of cheesecloth and centrifuged at 4 °C at 15,000*g* for 15 min. The supernatants were desalted using a Sephadex G-50 column and employed for determination of enzymatic antioxidant activities, specifically those of APX, GR, DHAR, and MDHAR, in accordance with a previous method [5].

Western blotting

The homogenates obtained for the HO activity assays were also used for Western blotting. A 50-μg sample of protein from the homogenates was separated by SDS–PAGE using 12% acrylamide resolving gels (Mini Protean III system; Bio-Rad) as described previously [39]. The separated proteins were then transferred to polyvinylidene difluoride membranes, which were then probed with the appropriate primary antibodies and a diluted (1:3000) horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Promega). The resultant signals were detected using an ECL kit (GE Healthcare, USA) [44]. The primary antibodies, which were obtained from Agrisera (Vännäs, Sweden), were diluted as follows: polyclonal antibody against plant APX, 1:2000; GR, 1:3000; and DHAR, 1:1000.

Results

Chilling treatment reduced germination, caused CO and NO generation, and increased heme oxygenase activity in B. ramiflora seeds

To characterize the sensitivity of recalcitrant *B. ramiflora* seeds to low temperatures, we first tested the effects of temperature change on germination and electrolyte leakage from the embryos, which is an important criterion for estimating the degree of damage in response to chilling stress. As shown in Fig. 1A, temperatures below 6 °C resulted in considerable inhibition of germination, with approximately 50% seed germination being observed at 2 °C. A further decrease in temperature caused complete inhibition of germination. Electrolyte leakage also increased with decreasing temperature, reaching maximum levels at −4 to −8 °C. The time duration of the low-temperature treatment also affected both germination and electrolyte leakage (Fig. 1B). A significant decrease in germination with a parallel increase in electrolyte leakage was recorded when the seeds were held at 2 °C for 2–8 h, indicating cellular membrane damage in the seeds. These data were as expected for recalcitrant seeds and validate the classification of *B. ramiflora* as having recalcitrant seeds.

Exogenous application of both CO and NO can promote germination in orthodox seeds in stressful environments [21,30]. To understand the roles of these substances in recalcitrant seeds under low-temperature stress, the generation of CO and NO was examined in *B. ramiflora* seeds exposed to low temperature. As shown in Fig. 2A and Supplementary Fig. S1, CO accumulated transiently in the embryo after 6 h at 2 °C and subsequently dropped rapidly. In contrast, the level of CO remained almost constant, below 20 nmol g^{-1} fresh wt, in the seeds under normal conditions without chilling stress. Exogenous CO, supplied by application of a 50% CO-saturated aqueous solution, further increased endogenous CO accumulation in a biphasic pattern, with the first peak occurring after 3 h of low-temperature treatment and a second peak occurring after 96 h of treatment (Fig. 2A). Supplementation with hematin (10 μM) resulted in a

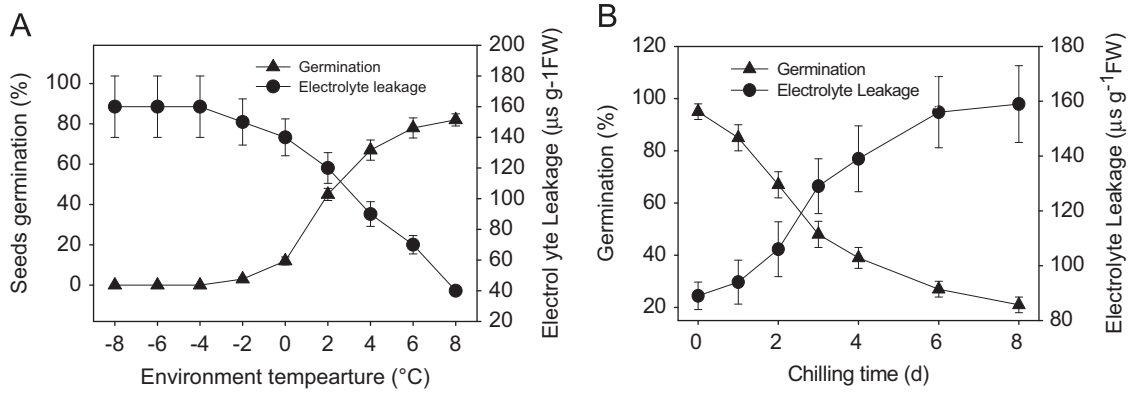

Fig. 1. Effects of various low temperatures on germination and cellular damage in *B. ramiflora* seeds. (A) Effects of environmental temperature change on the germination percentage (triangles, left axis) and electrolyte leakage (circles, right axis). Freshly collected seeds were treated at the indicated temperatures for 2 days and tested for electrolyte leakage immediately or returned to 22 °C for 6 days to test seed germination. (B) Effects of the duration of the low-temperature treatment (2 °C) on the seed germination percentage (triangles, left axis) and electrolyte leakage (circles, right axis). Freshly collected seeds were maintained at 2 °C for the indicated times and then tested for electrolyte leakage immediately or returned to 22 °C for 6 days to test germination and electrolyte leakage. Values are the means ± SE for five independent experiments.

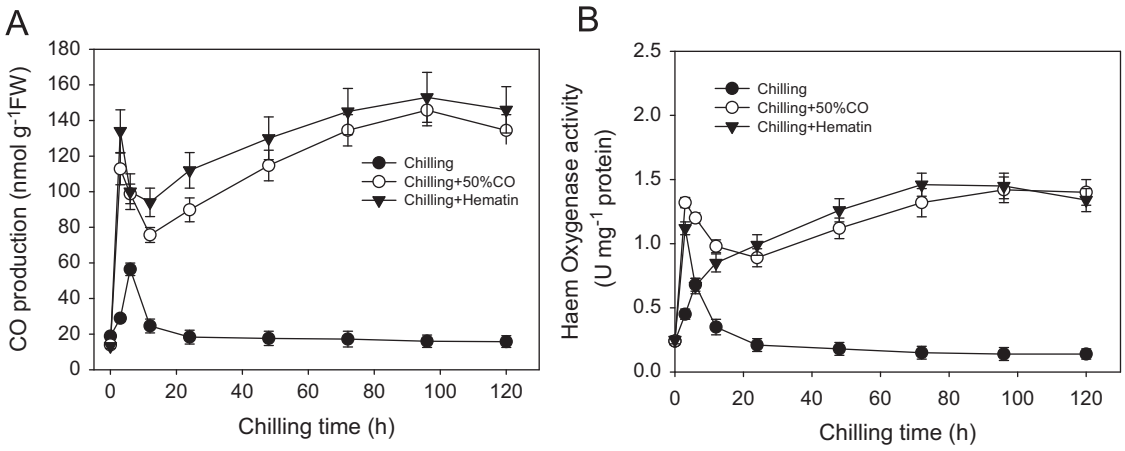

Fig. 2. Chilling induces the accumulation of CO and HO activity in *B. ramiflora* embryos. (A) Time course of CO release during chilling. Freshly collected seeds were kept at 2 °C for the indicated times, and the CO contents of the embryos were measured. For CO pretreatment, the freshly collected seeds were incubated with a CO-saturated solution (50%) or a 10 μM hematin solution for 6 h. After the seeds were washed to remove the surface solution, they were transferred to a chilling room at 2 °C for the indicated times. In control experiments, the seeds were placed in the growth room (22 °C, 65% moisture) without any treatment. (B) Time course of HO activity induced by chilling stress treatment. The treatment procedure was the same as for (A), but the HO enzyme activity in the embryos was measured. Values are the means ± SE for three independent experiments.

similar biphasic pattern of CO accumulation. The dose effects of exogenous CO or hematin on endogenous CO generation were also determined. We found that 50% CO or 10 μM hematin had obvious effects on CO accumulation (Supplementary Figs. S2 and S3). In the control seeds, exogenous CO or hematin treatment also induced CO accumulation, but not as strongly as those seeds under chilling stress (Supplementary Fig. S3). To determine if this accumulation was caused by HO, we measured HO activity under the same conditions. Similar to the pattern of CO accumulation, the HO activity responsible for CO synthesis was also enhanced after 3 h of low-temperature treatment and subsequently decreased. Addition of exogenous CO gas or hematin caused corresponding increases in HO enzyme activity (Fig. 2B).

Similar to CO, chilling treatment also induced accumulation of NO in the seeds, beginning after 6 h and then dropping to control levels (Fig. 3A). After CO treatment, a dramatic and persistent increase in NO production was observed throughout the duration of exposure (Fig. 3A). Fluorescence staining using the NO probe DAF-FM diacetate resulted in intense, bright green fluorescence in the embryo tip after the first 6 h of chilling stress, with diminished fluorescence being observed at later hours (24 h), supporting previous observations (Figs. 3B and 3C). CO treatment also caused accumulation of NO in the root tip (Fig. 3B). Pretreatment with an NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) or NR enzyme inhibitors (tungstate and glutamine (Gln)) inhibited the accumulation of NO during chilling stress. In contrast, NO generation during chilling stress was insensitive to the NOS-like enzyme inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME), and thus, NO accumulation was significantly greater (Figs. 3B and 3C). Interestingly, chilling-induced NO accumulation was abolished by the HO enzyme inhibitor zinc protoporphyrin IX (ZnPPIX; see Figs. 3B and 3C). CO treatment also induced an increase in the NO content in the control seeds not subjected to chilling stress, but to a lesser extent compared to that observed in seeds exposed to chilling stress. Compared with the recalcitrant seeds of *B. ramiflora,* orthodox seeds presented more CO and NO accumulation after chilling stress (Supplementary Fig. S5).

CO and NO enhanced the germination of B. ramiflora seeds after low-temperature treatment

Because NO accumulation was observed in response to CO supplementation during chilling stress, we then investigated whether CO or NO can enhance the efficiency of germination in *B. ramiflora* seeds after chilling treatment. As shown in Fig. 4, chilling treatment (2 days) inhibited germination compared with the control at 22 °C. Addition of the CO-saturated solution, hematin, or NOC-18 reversed the inhibitory effect of chilling stress on germination (Fig. 4, Supplementary Fig. S2). Other NO donors, such as GSNO, sodium nitroprusside, *S*-nitroso-*N*-acetyl-penicillamine (SNAP), and 10% NO-saturated solution, resulted in obvious germination enhancement under chilling stress (Supplementary Fig. S3). ZnPPIX, cPTIO, tungstate, and Gln pretreatment, but not pretreatment with L-NAME, also reversed the germination-promoting effect of CO or NO after chilling stress (Figs. 4A and 4B). Furthermore, we found that treatment with low concentrations of H_2O_2 significantly suppressed germination (Fig. 4).

CO and NO treatment activated the ascorbate–glutathione cycle and reduced H_2O_2 overaccumulation under chilling stress

Previous reports have shown that H_2O_2 might be one of the main factors blocking recalcitrant seed germination after desiccation [3,4]. Here, we measured H_2O_2 production in seeds after chilling or other treatments (Supplementary Fig. S4). Chilling stress induced the accumulation of H_2O_2 in the embryos. Exogenous application of hematin, a CO-saturated solution, or NOC-18 efficiently reduced the chilling-induced H_2O_2 accumulation, whereas the application of ZnPPIX, cPTIO, tungstate, and Gln, but not L-NAME, counteracted the inhibitory effect of CO on chilling-induced H_2O_2 accumulation (Fig. 5A). The ascorbate–glutathione pathway is a key part of a metabolic network involving enzymes and metabolites with redox properties that have the potential to detoxify ROS, thereby preventing ROS-induced oxidative damage in plants. We assayed the activities of antioxidative enzymes belonging to the ascorbate–glutathione metabolism cycle, including GR, APX, MDHAR, and DHAR (Fig. 5B). Subjecting seeds to chilling stress for 3 days significantly increased the activities of these enzymes, which were further enhanced by the application of a CO-saturated solution, hematin, or NOC-18. However, ZnPPIX was able to counteract the effect of chilling. cPTIO, tungstate, and Gln impaired the accelerated effect of CO on these enzyme activities. However, L-NAME could not abate the positive effect of CO on enzyme activities. Additionally, we determined the accumulation of these antioxidative enzymes using corresponding

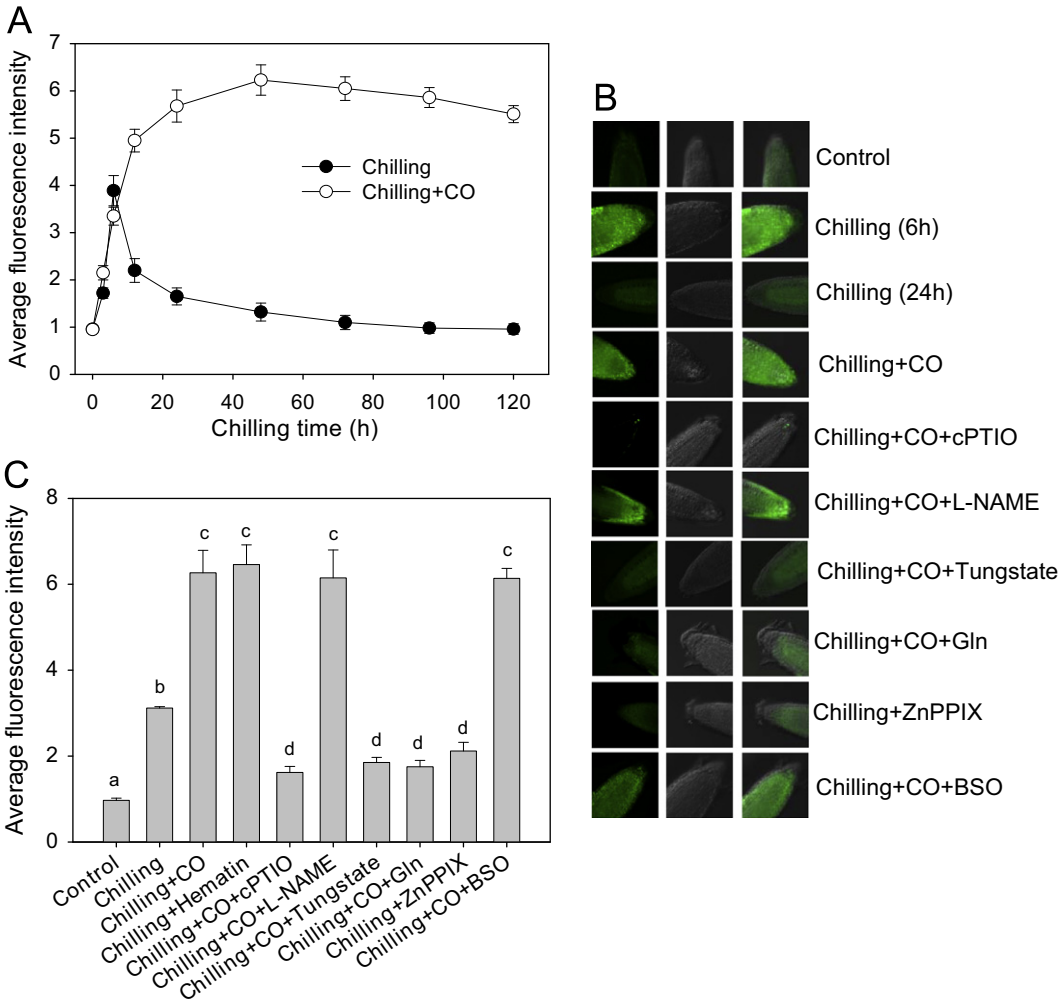

Fig. 3. Chilling stress induces NO accumulation in *B. ramiflora*. (A) Time course of effect of chilling on NO generation in embryos. Freshly collected seeds were treated at 2 °C for the indicated times, and the NO content in the embryos was measured. The CO treatment and the control experiments were the same as for Fig. 1. (B) Effect of CO and various scavengers/inhibitors on chilling-induced NO fluorescence. (C) NO accumulation in embryos after chilling stress or additional scavenger/inhibitor treatment. For both (B) and (C), freshly collected seeds were incubated in a CO-saturated solution with or without additional chemicals (50% CO-saturated solution, 50 μM cPTIO, 100 μM tungstate, 20 mM Gln, 50 μM L-NAME, 50 μM BSO, 200 μM ZnPPIX) for 6 h, followed by being subjected to chilling stress at 2 °C for 2 days, and a photograph showing NO fluorescence was taken, or the NO concentration was measured. Chilling (6 h) and chilling (24 h) mean that the photographs were taken after 6 or 24 h of chilling stress. The values shown are the means ± SE for three independent experiments. Different symbols indicate significant differences between treatments ($P < 0.05$) according to Tukey's test.

antibodies. In agreement with the enzyme activity results, the protein accumulation of these corresponding enzymes was induced by chilling stress and further reinforced by CO or SNAP treatment, whereas it was weakened by ZnPPIX, cPTIO, tungstate, and Gln, but not L-NAME (Fig. 5C).

Role of glutathione homeostasis in modulating recalcitrant seed sensitivity to chilling stress by CO and NO

Cellular reduced glutathione homeostasis is considered an important element in signaling cascades. GSH plays a role in keeping ROS to acceptable levels. Here, we observed that pretreatment with CO or NOC-18 resulted in significant increases in both total GSH content and the GSH/GSSG ratio in embryos, which were countered by the addition of cPTIO, ZnPPIX, tungstate, or Gln, but not L-NAME (Fig. 6). Furthermore, pretreatment with buthionine sulfoximine (BSO), a specific inhibitor of γ-ECS, to decrease GSH synthesis dramatically suppressed CO- or NOC-18-induced GSH accumulation. BSO treatment also inhibited CO- or NOC-18-induced germination (Fig. 4), while increasing the CO- or NO-induced inhibition of H_2O_2 produced under chilling stress (Fig. 5A). Interestingly, BSO treatment did not affect CO-induced NO generation under chilling stress (Fig. 3C). Treatment with GSH alone lessened the inhibitory effect of chilling stress on germination (Fig. 4).

Roles of SNOs and GSNOR in modulating recalcitrant seed sensitivity to chilling stress

The levels of SNOs and the activity of GSNOR affect the plant responses to various environmental stresses [10–13]. Here, we found that chilling stress increased the accumulation of SNOs and impaired the activity of GSNOR in embryos compared with the embryos not subjected to chilling stress (Fig. 7). The addition of exogenous CO or NOC-18 markedly reduced the chilling-induced increase in SNOs and enhanced the activity of GSNOR; this effect could be abated by the NO scavenger cPTIO or the NO metabolism inhibitors tungstate and Gln. BSO pretreatment also partially increased the chilling-induced accumulation of SNOs. Dodecanoic acid (DA) and 5-chloro-3-(2-[(4-ethoxyphenyl)ethylamino]-2-oxoethyl)-1*H*-indole 2-carboxylic acid (C1) are specific inhibitors of GSNOR enzyme activity [40]. Pretreatment with DA and C1

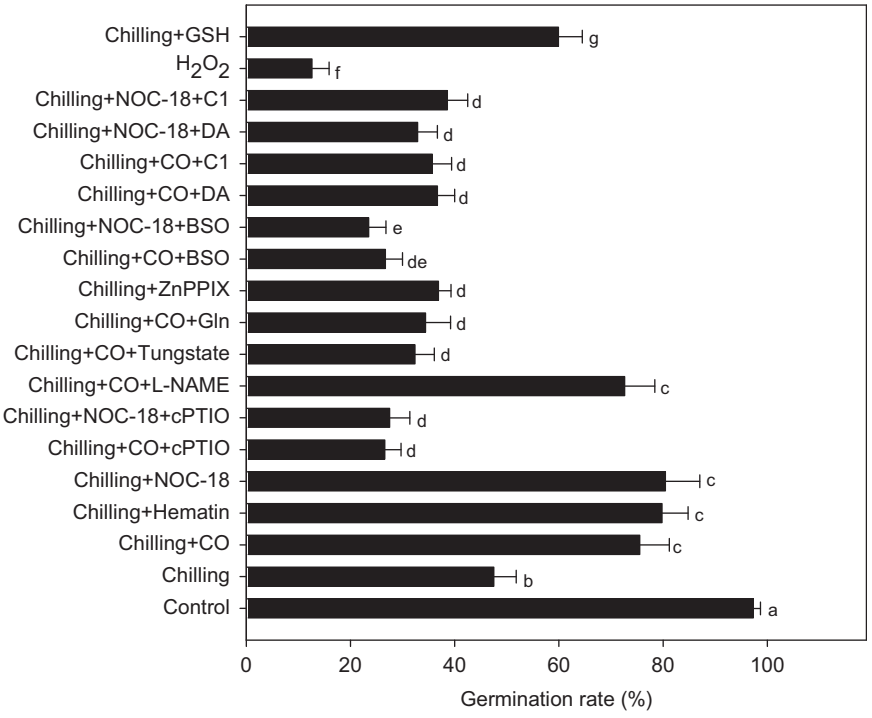

Fig. 4. Effects of CO, NO, and their corresponding inhibitors on the germination of *B. ramiflora* seeds after low-temperature treatment. Freshly collected seeds were incubated with various chemical treatments (50% CO-saturated solution, 10 μM hematin, 30 μM NOC-18, 50 μM cPTIO, 100 μM tungstate, 20 mM Gln, 50 μM L-NAME, 50 μM BSO, 200 μM ZnPPIX, 1 mM H_2O_2) for 6 h, followed by chilling at 2 °C for 2 days, and then returned to normal germination conditions. After 6 days of growth, the germination percentage was recorded. The values shown are the means ± SE for three independent experiments. Different symbols indicate significant differences between treatments ($P < 0.05$) according to Tukey's test.

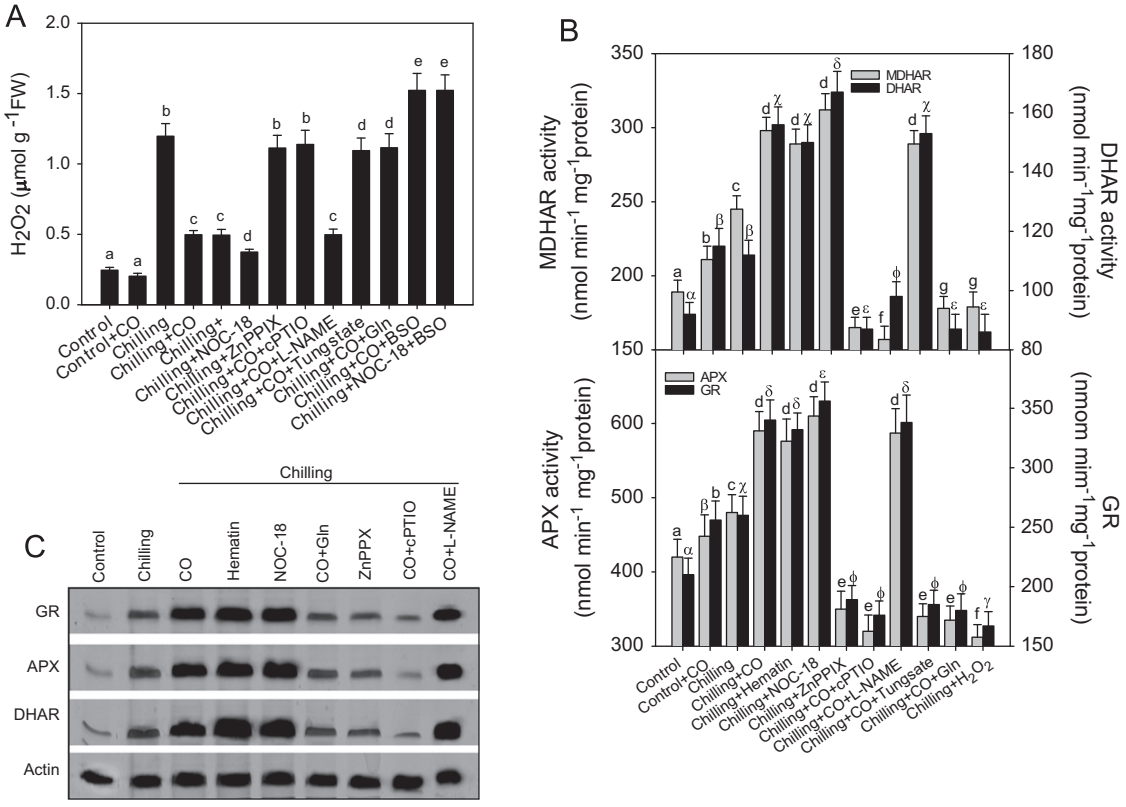

Fig. 5. Effects of CO, NO, and their corresponding inhibitors on H_2O_2 accumulation and antioxidant enzyme activity and accumulation after chilling. (A) Effects of CO and various chemicals on chilling-induced H_2O_2 accumulation in embryos. (B) Effects of CO and various chemicals on the activities of chilling-induced antioxidant enzymes, including GR, DHAR, MDHAR, and APX. (C) Effects of CO and various chemicals on antioxidant enzyme protein accumulation. Freshly collected seeds were pretreated with various chemicals (as above) for 6 h, followed by chilling stress at 2 °C. The H_2O_2 accumulation and antioxidant enzyme activities and protein accumulation in the embryos were determined after 2 days of chilling stress. The values shown are the means ± SE for three independent experiments. Different symbols indicate significant differences between treatments ($P < 0.05$) according to Tukey's test.

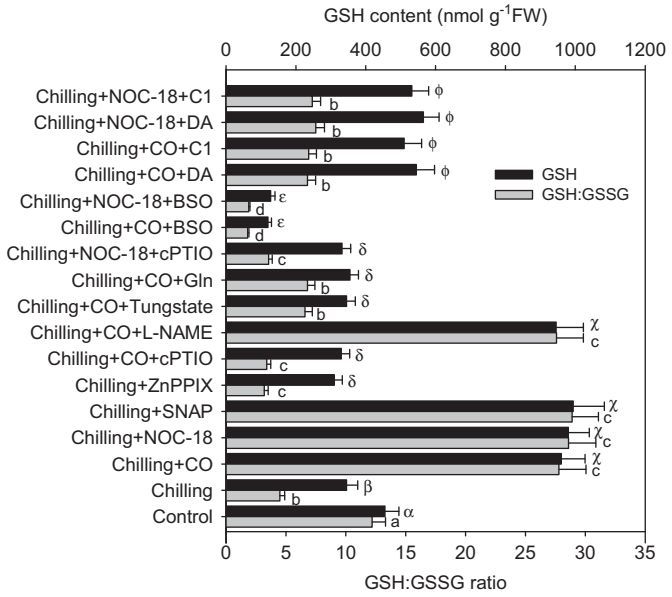

Fig. 6. Quantification of the in vivo GSH accumulation and GSH/GSSG ratio in embryos caused by various chemical pretreatments under chilling stress. Freshly collected seeds were pretreated with various chemicals (as described) for 6 h, followed by chilling stress. To measure GSH and GSH/GSSG, the seeds were collected after 2 days of chilling treatment. The values shown are the means ± SE for three independent experiments. Different symbols indicate significant differences between treatments ($P < 0.05$) according to Tukey's test.

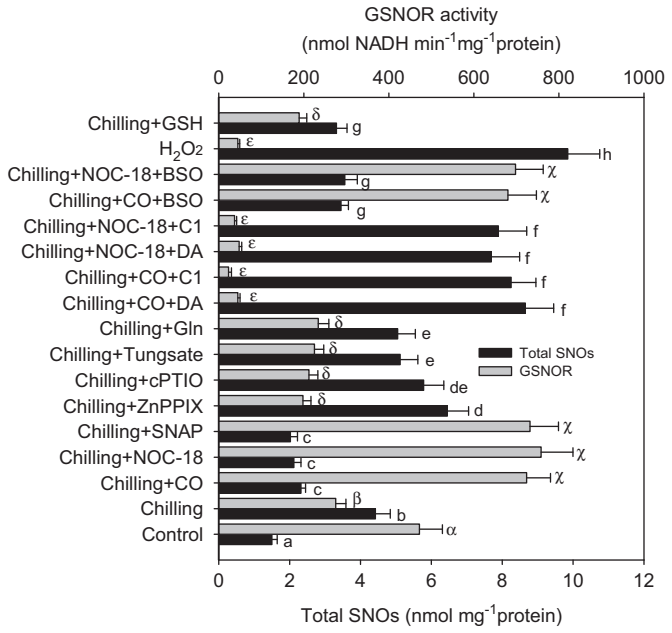

Fig. 7. The total accumulated SNO content and GSNOR activity in the embryos caused by various chemical pretreatments under chilling stress. Freshly collected seeds were pretreated with various chemicals (as described) for 6 h, followed by chilling stress. To measure the total SNO content and GSNOR activity, the seeds were collected after 2 days of chilling treatment. The values shown are the means ± SE for three independent experiments. Different symbols indicate significant differences between treatments ($P < 0.05$) according to Tukey's test.

aggravated the chilling-induced increase in SNO. In contrast, chilling suppressed the activity of GSNOR, and this suppression could be reversed by the addition of CO or NOC-18. Treatment with exogenous ZnPPIX, tungstate, Gln, and BSO also suppressed the CO-induced increase in GSNOR. Additional GSNOR pretreatment increased the accumulation of SNOs and suppressed germination of seeds subjected to either chilling stress or additional CO or NOC-18 treatment (Fig. 4).

Discussion

Recalcitrant seeds are unable to thrive after being subjected to drying or low-temperature stress in ex situ conservation procedures, e.g., seed banks [1,2], which hampers their distribution and conservation. In this study, we found that application of CO or NO can induce germination while enhancing the tolerance of recalcitrant seeds to chilling stress. We also demonstrated a previously uncharacterized signaling role of endogenous CO in enhancing the tolerance of recalcitrant seeds to chilling stress while inducing NO release and GSH homeostasis through the AsA–GSH cycle-based antioxidant enzyme system.

Endogenous heme oxidase–mediated CO production promotes germination of recalcitrant seeds under chilling conditions

CO has recently been reported to be involved in diverse biological functions, such as neural transmission in animals and defense against salinity stress in plants [25,28,30]. This study demonstrated rapid transient accumulation of CO, together with an increase in HO enzyme activity in embryos under chilling stress (Fig. 2). However, inhibition of HO resulted in decreased chilling-induced CO production, which, in turn, indicates that HO is responsible for CO synthesis under chilling stress conditions (Supplementary Fig. S2). Exogenous CO treatment further increased HO enzyme activity, which was accompanied by a biphasic increase in CO production in the treated embryos compared to the controls, in which only a single peak of CO accumulation was observed. These results suggest that exogenous CO is mainly responsible for the accumulation of the second peak and support previous reports addressing ROS accumulation under conditions of saline stress or pathogen infection [6,28].

CO treatment has been shown to accelerate wheat and rice germination under saline stress [20,30]. Here, we found that treatment with a CO-saturated solution or the CO donor hematin enhanced the germination rates of *B. ramiflora* seeds after chilling stress (Fig. 4). We propose that HO enzyme activity and endogenous CO are necessary for increasing the germination ability under chilling stress. ZnPPIX (HO enzyme inhibitor) suppressed the CO-induced HO enzyme activity and endogenous CO accumulation, resulting in an inadequate CO level that was not sufficient to trigger the defense mechanism protection against chilling stress.

CO-induced NO scavenges overaccumulated H_2O_2 to protect recalcitrant seeds from low-temperature damage

There is ample evidence supporting the regulatory role of NO in plant development and biotic stress [17,19,22]. Of particular relevance to this work, NO can break seed dormancy, stimulate germination [20,21], and enhance plant acclimation to low-temperature stress [22]. Here, we found that chilling stress induced a brief increase in the NO level in the embryo, which was completely abolished by cPTIO treatment (Fig. 3). In mammals, NO is generated from Arg through the action of NO synthase. In plants, NO synthesis from Arg using an NO synthase-type enzyme or from nitrite via nitrate reductase (NR) has been investigated previously. Another possible pathway via the nonenzymatic reduction of apoplastic nitrite has also been reported [14,21,35]. Pharmacological studies have suggested that an NOS-like enzyme and NR are responsible for NO synthesis, although the protein showing NOS-like activity in plants remains

unknown [14]. In this study, we found that pretreatment of seeds with NR inhibitors, such as tungstate and Gln, in contrast to the NOS inhibitor L-NAME, alleviated the chilling-induced NO accumulation in the embryo tip, which indicates that NR probably contributes to chilling-induced NO synthesis (Fig. 3). Similar results have been reported in *Arabidopsis* in response to low-temperature acclimation [22]. In contrast, Corpas et al. [36] detected NOS-activity-dependent NO synthesis under low-temperature stress conditions. The discrepancy regarding the source of NO synthesis between these two studies could be attributed to the difference in the experimental materials used. The NOA/RIF1 protein is proposed to be localized to the chloroplasts of leaves [37], and NOS-like enzyme activity may be much lower in seeds, particularly because they lack mature chloroplasts. We found that the NO donor NOC-18 could abate the chilling damage to seed viability by increasing the germination capability. Bethke et al. [17] reported that exogenous application of NO could break dormancy in orthodox seeds, such as those of *Arabidopsis,* in an NO-dependent manner. Liu et al. [16] reported the rapid NO-mediated reduction in ABA, which is required for breaking seed dormancy. These results agree with our finding that NO plays an important role in activating the germination of recalcitrant seeds. We found that NO-induced seed germination under chilling stress could be compromised by NR inhibitors, but not by an NOS enzyme inhibitor, suggesting the importance of NR-derived NO synthesis during germination. Previous studies have shown upregulation of NR activity in sorghum during early germination [38], and NR-mediated ABA accumulation is responsible for stomatal closure in *Arabidopsis* [17].

The involvement of CO in salt stress, auxin signaling, and germination has been demonstrated using genetic and pharmacological agents to manipulate endogenous CO levels [20,28,30]. In this study, we observed enhanced seed germination in chilling-sensitive seeds treated with exogenous application of CO. An apparent increase in CO and HO activity was inhibited by ZnPPIX pretreatment, thereby resulting in reduced seed germination during chilling stress. Based on these results, we propose that HO-dependent CO production is a mechanism protecting recalcitrant seeds from damage induced by chilling stress. These results agreed with a previous study demonstrating upregulation of HO in transgenic *Arabidopsis* associated with exogenous CO application, resulting in increased tolerance to salt stress [30].

Although the roles of CO, NO, and H_2O_2 have been investigated extensively with respect to many aspects of plant physiological processes [14,25], the cross talk between CO, NO, and H_2O_2 has remained unexplored. This is the first time that the accumulation of H_2O_2 was found to be strongly associated with other factors, such as CO and NO, contributing to the germination ability of recalcitrant seeds under chilling stress. Removal of accumulated H_2O_2 by H_2O_2 scavengers partially ameliorated the inhibitory effect of chilling stress on germination in recalcitrant tea seeds [5,14]. We found that both CO and NO treatment reduced chilling-induced H_2O_2 accumulation (Fig. 5A), which provides a mechanism by which CO and NO may enhance seed germination after chilling stress. The NO scavenger cPTIO as well as the NR inhibitors tungstate and Gln impaired the CO-induced seed germination under chilling stress. The HO inhibitor ZnPPIX suppressed NO synthesis, accompanied by reduced seed germination. These results hint at the possible action of CO as an upstream signal contributing to NO accumulation after chilling stress, consistent with the previous finding that CO acts upstream of NO to regulate root development [28]. CO-mediated H_2O_2 production has also been found to be essential for root hair development in tomato [19] and stomatal closure in *Vicia faba* guard cells [39]. Moreover, upregulation of the antioxidant system in the presence of CO has been shown to reduce ROS accumulation in wheat and rice seedling roots exposed to salt stress [20,30]. Our results showed that CO treatment can efficiently inhibit the chilling-induced accumulation of ROS, which adversely affects seed viability. These results also suggest that CO plays a central role in ROS homeostasis. Because NO can act as an antioxidant to protect plants from oxidative bursts under various stresses [14], our finding that an NO scavenger or NR inhibitor could interfere with CO also suggests that NO is active downstream of CO and can contribute to the removal of ROS in response to chilling stress.

Glutathione homeostasis plays a role in recalcitrant seed tolerance to low-temperature stress

The glutathione–ascorbate pathway is essential for scavenging ROS in cells [7]. We found that CO and NO treatment ameliorated chilling stress by enhancing the accumulation and activities of antioxidant enzymes (MDHAR, DHAR, APX, and GR) involved in the glutathione–ascorbate cycle (Fig. 5). These results support the findings of a previous study in which CO and NO treatments were shown to elevate the activities of antioxidant enzymes in the roots of wheat seedlings exposed to salt stress [30]. A similar influence of CO- and NO-mediated antioxidant enzyme activities was found in another experiment in which CO and NO treatment resulted in reduction of H_2O_2 accumulation and subsequent damage during chilling stress. However, inhibition of CO or NO production by an HO inhibitor or NO scavengers resulted in decreased antioxidant enzyme activities and greater accumulation of H_2O_2, which ultimately aggravated the damaging effects of chilling stress in the seeds.

Cellular glutathione homeostasis has long been considered to play a key role in keeping ROS below toxic levels [7]. Recently, Han et al. [25] reported CO-mediated alleviation of cadmium-induced oxidative damage via modulating glutathione metabolism in the roots of *Medicago sativa.* In this study, we observed diminished fluorescence corresponding to the GSH content in root tips under chilling stress. However, supplementation with CO or NO strongly induced the accumulation of GSH, which was inconsistent with the finding that CO- and NO-induced GR enzyme activity is responsible for GSH synthesis. Furthermore, blocking γ-ECS activity with BSO lessened the GSH content and the GSH/GSSG ratio. A significant reduction in CO- or NO-induced GSH content by BSO decreased H_2O_2 accumulation and the chilling-induced damage to the germination ability (Figs. 4 and 5). These results demonstrate the importance of GSH in maintaining H_2O_2 homeostasis and protecting the plant from oxidative damage under chilling stress. Interestingly, a reduced GSH content under BSO treatment did not affect CO-induced NO generation (Fig. 3C), but CO or NO treatment results in a remarkable increase in GSH accumulation during chilling stress, indicating that GSH functions downstream of CO or NO to modulate germination under chilling stress.

GSNOR regulates the tolerance of recalcitrant seeds to chilling stress by reducing the overaccumulation of SNOs

Reactive nitrogen species, including NO, GSNO, and $ONOO^-$, cause plant damage when they accumulation beyond certain levels; this phenomenon is known as nitrosative stress [41,42]. We found that chilling also caused a high level of total SNO accumulation in the embryo. The addition of CO and NO was able to abate this accumulation, indicating the regulatory role of CO and NO signals in chilling-induced nitrosative stress. Abolishing CO or NO accumulation using CO- and NO-metabolism inhibitors also aggravated the total increase in SNOs. GSNOR is the main enzyme responsible for cellular protection against nitrosative stress. This enzyme efficiently removes the overaccumulated

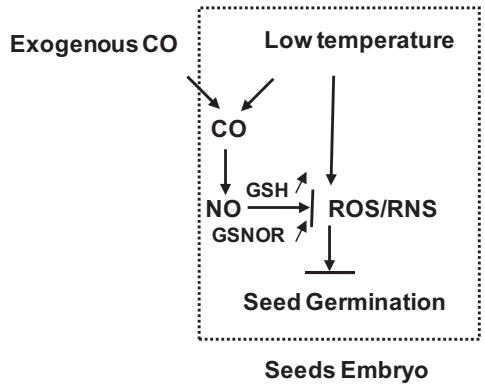

Fig. 8. Schematic representation of the signaling pathway involving CO, NO, and GSH during the germination of recalcitrant seeds under low-temperature stress.

RNS, particularly GSNO. We observed that chilling stress induced an increase in GSNOR activity, consistent with a previous report on pepper under low-temperature stress [42]. We found that both CO and NO further increase the level of GSNOR activity, accompanied by a reduction in total SNOs. In contrast, specific inhibitors of CO and NO metabolism reduced GSNOR activity under chilling stress, supporting the role of GSNOR in modulating the total RNS content under these conditions. BSO, a specific inhibitor of γ-ECS that reduces GSH synthesis, also reduced GSNOR activity and increased total SNO accumulation, whereas additional exogenous GSH reversed this effect, suggesting that GSH synthesis, or accumulation, affects the induction of the GSNOR enzyme in response to chilling, possibly by increasing the provision of GSH to accelerate GSNOR enzyme activity. These data demonstrated the dual role of GSH in regulating plant responses to environmental stress, not only by increasing antioxidant enzyme to remove overaccumulated ROS, but also by increasing GSNOR enzyme activity to remove overaccumulated RNS. Treatment with the GSNOR enzyme inhibitors DA and C1 significantly increased the total RNS accumulation and reduced germination under chilling stress, either with or without CO and NO treatment. Exogenous H_2O_2 treatment, which reduced GSNOR enzyme activity and increased the total SNO content, also suppressed germination. However, increasing GSNOR enzyme activity via CO or NO treatment increased seed germination. These data demonstrate the necessary role of GSNOR in modulating germination under chilling stress via removal of overaccumulated total SNOs.

We propose a model to illustrate the role of CO in regulating the chilling stress response in recalcitrant seeds (Fig. 8). Chilling treatment induces transient accumulation of CO, which has a stimulatory effect on NO accumulation, but this effect is weak and of a short duration, which results in poor germinability in recalcitrant seeds. Exogenous CO treatment further increased NO accumulation to activate the ascorbate–glutathione cycle and increase antioxidant enzyme activities, combating low-temperature-induced ROS accumulation. Exogenous application with CO or NO acts in the same manner to activate GSNOR, which removes overaccumulated RNS and subsequently improves the recalcitrant seeds' tolerance to chilling stress. Thus, our results reveal a novel mechanism involving CO as an important signal to enhance recalcitrant seed chilling tolerance and provide new insight regarding the use of CO in recalcitrant seed conservation.

Acknowledgments

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Appendix A. Supporting material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2012.05.042.

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