

Carbon monoxide counteracts the inhibition of seed germination and alleviates oxidative damage caused by salt stress in *Oryza sativa*

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Abstract

Although carbon monoxide (CO) has always been regarded as a toxic gas, recent reports suggested that it is one of the products of heme oxygenase (HO; EC 1.14.99.3) catalysis in animals, and could confer beneficial cytoprotection against oxidative damage. Here, we investigated the effects of exogenous putative CO donor hematin and CO aqueous solution on rice seed germination under 100 mM NaCl salt stress. Both hematin and CO aqueous solution dose-dependently attenuated the inhibition of seed germination and seedling growth caused by salinity. Moreover, 1.0 μ M hematin and 5% CO-saturated aqueous solution activated amylase activity, thus accelerating the formation of reducing sugar and total soluble sugar. Furthermore, hematin and CO aqueous solution induced catalase (CAT) and superoxide dismutase (SOD) activities, thus resulting in the alleviation of oxidative damage, as indicated by the decrease of thiobarbituric acid reactive substances (TBARS) content. Such above CO-induced effects were also confirmed by using semi-quantitative RT-PCR, including the up-regulation of expression patterns of α -amylase, CAT and Cu/Zn-SOD genes. Also, the similar function of hematin might contribute to endogenous HO-derived CO, since addition of the CO-specific synthetic inhibitor zinc protoporphyrin IX (ZnPPIX) and CO scavenger hemoglobin (Hb) reversed above effects. Meanwhile, further results also proved that treatment with hematin for 36 h could result in the potent induction of *HO-1* transcript, HO activity and CO content in the germinating seeds. Collectively, these results indicated that CO performed an advantageous effect on attenuation of inhibition of seed germination and seedling growth induced by salt stress, and alleviated oxidative damage via activating anti-oxidant enzymes.

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1. Introduction

Among various stresses, salinity is an ever-present threat to crop yields, especially in countries where irrigation is an essential aid to agriculture. Usually, salt stress leads to negative effects on seed germination, seedling growth and even plant

productivity. At the physiological level, the multitude of effects of salt stress indicates the importance of protecting the plant organism from oxidative damage caused by the overproduction of reactive oxygen species (ROS) that is directly or indirectly elicited by water deficit and increased ion uptake [1–3]. Rice (*Oryza sativa* L.), a crop of great importance in the world, is glycophyte and greatly affected by salt stress. Therefore, it is of great necessity and significance to increase rice productivity through taking all sorts of effective measures to enhance its salt tolerance.

Carbon monoxide (CO) is a diatomic gas, being odorless, tasteless and colorless. Besides its exogenous generation in the incomplete oxidation of carbon-containing materials, CO is also produced endogenously by animal cells as a product of heme, which is catalyzed by heme oxygenase (HO; EC 1.14.99.3). HO not only decomposes protoheme-IX by oxidative cleavage of its α -methene bridge and generates

Abbreviations: CAT, catalase; CO, carbon monoxide; GC, guanylyl cyclase; Hb, hemoglobin; HO, heme oxygenase; NBT, nitroblue tetrazolium; NO, nitric oxide; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; ZnPPIX, zinc protoporphyrin IX

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biliverdin-IX α (BV), which is then converted to bilirubin-IX α through the reaction of biliverdin reductase, but also expels divalent iron from the heme ring and releases a one-carbon fragment as CO. In animals, three forms of the HO isozymes have been found, including inducible HO-1, constitutively expressed HO-2 and HO-3 with very low activity [4–7]. Former studies carried out in several plant species revealed that germinating seeds produced CO gas at levels of 10–25 ppm, and neither light nor chlorophyll was required for above CO formation, which was different from the green plants [8,9]. Recently, researches of HO in *Arabidopsis* demonstrated that AtHO1 (HY1) could catalyze heme and produce CO in vitro [10–12]. In rice, *SE5*, the homologue of *HY1* has been cloned [13].

Interestingly, accumulating evidence in animal research has shown that CO involves in modulating many important physiological events, such as neurotransmission, vasodilation, the inhibition of platelet aggregation, and anti-proliferative effects on smooth muscle, all of which are also functioned by nitric oxide (NO) [4,5,14–17]. NO also acts in plants, since convincing data have been obtained by the NO-induced increase in plant tolerance or resistance against various biotic and abiotic stresses, such as pathogen [18,19], drought [20,21], salinity [22] and UV-B irradiation [23]. In animals, some activities of CO resemble those of NO [24]. For example, like NO, CO exhibits the ability to bind to the iron atom of the heme moiety associated with soluble guanylyl cyclase (GC), thereby activating the enzyme and increasing intracellular second messenger molecule cGMP production [6,14,24]. Thus, given the similarity shared by NO and CO and the analogous functions they achieved, it is thereby inevitable to consider that CO might play a regulatory role in plants against abiotic and biotic stresses.

Recently, it was convincingly reported that CO gas exerted the capability of conferring cytoprotection against oxidant-induced injury in animal cells [25]. In plants, recent report also demonstrated that the induction of HO-1 played a cytoprotective role for soybean plants against oxidative stress mediated by Cd [26]. More recently, we also discovered that application of hematin, a commonly used CO donor or HO-1 inducer in animal research, was able to alleviate the oxidative damage in salt-stressed wheat seedling leaves [27]. Despite of several former reports demonstrating the stimulatory effects of CO gas with lower concentrations on germination of dormant seed, including rice, barley and giant foxtail, the exact mechanism of above actions is still unknown yet [28,29]. In this report, we further examined the influence of CO, by using both hematin and CO aqueous solution, on the inhibition of rice seed germination and seedling growth induced by salt stress. Furthermore, the preliminary mechanism of CO-stimulated effect was discussed.

2. Materials and methods

2.1. Plant material

Seeds of rice (*O. sativa* L., Wuyujing 3), kindly provided by Jiangsu Academy of Agricultural Sciences, Jiangsu Province, PR

China, were carefully selected and sterilized with 0.1% KMnO₄ for 5 min, and then washed extensively with distilled water. These seeds were firstly soaked in distilled water for 24 h, afterwards germinated in petridishes containing filter paper wetted with 4 mL of different treatment solutions, and kept at 30 °C in growth chamber in the darkness. Germination tests were carried out on at least three independent sets of experiments with similar results. Each replicate was about at least 150 rice seeds. There were at least 50 seeds in every petridish.

2.2. Chemicals

Hematin (Ht, C₃₄H₃₃N₄O₅Fe, purchased from Sigma) was used as a putative CO donor at concentrations of 0.1, 1.0 and 10.0 μ M [30]. In 1970, Landaw et al. reported that after intravenous infusion of hematin-¹⁴C, rats produced equimolar amounts of labeled bilirubin and CO [31]. The compound zinc protoporphyrin IX (ZnPPIX, purchased from Sigma) was used at 10.0 μ M as a potent inhibitor of HO-1 [30]. Also, hemoglobin (Hb, obtained from Shanghai Boao Ltd., PR China) was chosen as the CO scavenger [14,30] at the concentration of 0.1 g L⁻¹.

2.3. CO-saturated aqueous solution preparation

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

2.4. Treatments

Treatments were performed as follows: S, 100 mM NaCl; S + Ht0.1, 100 mM NaCl + 0.1 μ M hematin; S + Ht1.0 (also termed as S + Ht), 100 mM NaCl + 1.0 μ M hematin; S + Ht10.0, 100 mM NaCl + 10.0 μ M hematin; S + Ht + Hb, 100 mM NaCl + 1.0 μ M hematin + 0.1 g L⁻¹ Hb; S + Hb, 100 mM NaCl + 0.1 g L⁻¹ Hb; S + Ht + ZnPPIX, 100 mM NaCl + 1.0 μ M hematin + 10.0 μ M ZnPPIX; S + ZnPPIX, 100 mM NaCl + 10.0 μ M ZnPPIX; S + 100% CO, 100 mM NaCl + 100% CO-saturated aqueous solution; S + 50% CO, 100 mM NaCl + 50% CO-saturated aqueous solution; S + 10% CO, 100 mM NaCl + 10% CO-saturated aqueous solution; S + 5% CO (also termed as S + CO), 100 mM NaCl + 5% CO-saturated aqueous solution; S + CO + Hb, 100 mM NaCl + 5% CO-saturated aqueous solution + 0.1 g L⁻¹ Hb. The control was set as the treatment of distilled water only, without the addition of NaCl or other chemicals. All above solutions were renewed each day to maintain unaltered concentrations. The petridishes were sealed to avoid the CO gas escaping or any possible CO gas interference. To investigate the effects of CO

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