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Heme oxygenase/carbon monoxide system participates in regulating wheat seed germination under osmotic stress involving the nitric oxide pathway

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ABSTRACT

To investigate the mechanism and signaling pathway of carbon monoxide (CO) and hematin in alleviating seed germination inhibition and lipid peroxidation, polyethylene glycol-6000 (PEG) was used to mimic osmotic stress in a series of experiments. The results showed that wheat seeds pretreated with a lower dose of PEG (12.5%) showed higher tolerance against osmotic stress as well as the up-regulation of heme oxygenase (HO, EC 1.14.99.3) and decreased lipid peroxidation during recuperation, compared to those with a higher dose of PEG (50%). Exposure of wheat seeds to 25% PEG, HO-1 inhibitor or specific scavenger of nitric oxide (NO) alone differentially led to seed germination inhibition. The PEG-induced inhibitory effects on seed germination were ameliorated by the HO-1 inducer hematin, CO or NO donor. Additionally, hematin was able to markedly boost the HO/CO system. However, the addition of the HO-1 inhibitor or the specific scavenger of NO not only reversed the protective effects conferred by hematin, but also blocked the up-regulation of HO/CO. In addition, hematin-driven NO production in wheat seeds under osmotic stress was confirmed. Based on these results, we conclude that the endogenous HO/CO signal system is required for the alleviation of osmotic stress-induced wheat seed germination inhibition and lipid peroxidation, which might have a possible interaction with NO.

Introduction

Drought is a widely-present threat to crop yields (Boyer, 1982). It has been shown to have negative effects on seed germination, seedling growth and even plant productivity. Water stress tolerance mechanisms can be analyzed from several perspectives, including at the cell level (osmotic adjustment), in tissue (water storage tissues), in organs (deep or fast growing roots), and in whole plants (water conductance). Accumulation of compatible, low-molecular-weight osmolytes, such as sugar alcohols, special amino acids, and Gly betaine, has been suggested as a major mechanism that may underlie the adaptation or tolerance of plants to osmotic stresses (Greenway and Munns, 1980; Yancey et al., 1982). LEA proteins were characterized as a set of proteins that are highly accumulated in the barley embryos in response to osmotic stress (Hong et al., 1992). The application of plant regulators such as GA_3 (Khan and Ungar, 1997, 1998), kinetin (Khan and Ungar, 1997), fusicoccin (FC) (Gul and Weber, 1998), and ethylene (Ismail, 1990) have also been shown to alleviate salinity-enforced dormancy. In addition, osmotic stress directly or indirectly causes overproduction of reactive oxygen species (ROS), resulting in oxidative damage to plants. Therefore, understanding plant responses to osmotic stress is of great importance and also a fundamental part of making crops stress-tolerant.

Heme oxygenases (HOs, EC 1.14.99.3) catalyze the oxidative conversion of heme to biliverdin (BV) with a concomitant release of carbon monoxide (CO) and free iron (Fe^{2+}). BV is then converted to bilirubin by biliverdin reductase. In animals, HO-1 is the inducible HO isozyme that responds rapidly to diverse stimuli and protects tissues against a wide range of injuries (Ryter et al., 2002).

The presence of CO biosynthesis in plants was first reported by Wilks (1959). Later, the photo-production of CO in living plants was also discovered (Lüttge and Fischer, 1980; Tarr et al., 1995). Recent results have confirmed that CO plays an important role in a number of physiological processes such as growth and developmental regulation, stomatal closure, and adaptation responses to environmental stresses (Cao et al., 2007a,b; Han et al., 2007, 2008; Song et al., 2008; Xie et al., 2008; Xuan et al., 2008). In addition, CO has been shown to be an important positive regulator of both dormancy break and seed germination (Dekker and Hargrove, 2002;

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; CO, carbon monoxide; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt; DHAR, dehydroascorbate reductase; EPR, electron paramagnetic resonance; HO, heme oxygenase; L-NAME, N_ω-nitro-L-Arg methyl ester hydrochloride; PEG-6000, polyethylene glycol-6000; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; ZnPPIX, zinc protoporphyrin.

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Xu et al., 2006; Liu et al., 2007). For example, the application of the exogenous HO-1 inducer hematin and a CO aqueous solution dose-dependently alleviated the inhibition of rice seed germination and seedling growth under salt stress, both of which were partially due to the induction of antioxidant metabolism as well as the degradation of storage reserve. However, the mechanisms and signaling of these actions are not fully understood. Further, previous studies have not explored whether the mitigation of seed germination inhibition induced by salt stress is closely associated with alterations of endogenous *HO-1* expression and CO production by the usage of HO-1 specific inhibitor zinc protoporphyrin IX (ZnPPIX), which was first found to inhibit HO activity or expression in both animals and plants (Lamar et al., 1996; Xuan et al., 2008). Additionally, the positive effects are similar to some behaviors reported for nitric oxide (NO), an important gaseous signaling molecule recently uncovered in plants (Delledonne, 2005). The relationship between NO signaling and CO response in plants has been demonstrated by some investigators. For example, CO-induced guard cell closure requires participation of NO (Cao et al., 2007a; Song et al., 2008). However, the relationship between CO and NO in seed germination has not yet been established.

In this study, we expand upon our previous data, and investigated different recovery capabilities of seed germination following recuperation after various osmotic treatments. Possible mechanisms and significance are discussed.

Materials and methods

Chemicals

All chemicals were obtained from Sigma unless stated otherwise. Polyethylene Glycol-6000 (PEG-6000) was purchased from Shanghai Medical Instrument, Ltd., The Country Medicine Group, Shanghai, China, and is generally used to imitate osmotic stress (García-Valenzuela et al., 2005). Hematin, an HO-1 inducer, applied in animal and plant research (Lamar et al., 1996; Han et al., 2007; Xuan et al., 2008), was used at concentrations of 0.1, 1.0, and 10.0 μM. The compound ZnPPIX was used as a potent inhibitor of HO-1. Sodium nitroprusside (SNP) was used as NO donor. 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO) was used as a specific NO scavenger. N_{ω}-nitro-L-Arg methyl ester hydrochloride (L-NAME) was used as a plant NOS-like enzyme inhibitor.

Plant materials, growth condition and treatments

Selected seeds of wheat (*Triticum aestivum* L., Yangmai 158) were surface sterilized with 0.1% $KMnO_4$ for 15 min, rinsed extensively in distilled water, and then dried. These seeds were transferred to Petri dishes containing 6 mL of distilled water (Con), varying concentrations of PEG, hematin, and CO aqueous solution, 50 μM ZnPPIX, 100 μM cPTIO, 0.5 mM SNP alone, or combination treatments and kept at 25 °C in a growth chamber in darkness for the indicated times. These solutions were renewed each day.

First, to evaluate the time course of the response to osmotic stress, different concentrations of wheat seeds treated with PEG for 1 d were transferred to distilled water for recovery during the indicated times. To further test whether osmotic stress-induced seed germination inhibition is related to endogenous HO/CO and NO, the effects of the CO aqueous solution (CO), HO-1 inducer (hematin, H) and its potent inhibitor (ZnPPIX), NO scavenger (cPTIO), and NO donor (SNP) on the germination percent were also investigated. All experiments were repeated at least three times. After various treatments, the seeds were sampled then immediately frozen in liquid nitrogen and stored at −80 °C until further analysis, or dried in an oven at 70 °C to constant weight for determination of dry weight (DW). Root length was measured with a ruler at the indicated times.

Germination analysis

Germination tests were carried out on at least three independent sets of experiments with similar results. Each replicate was about 180 seeds. There were 60 seeds in every Petri dish. Analysis was performed by taking the dishes from the dark and counting the number of seeds that showed emerging plantlets reaching half length of the seeds.

Determination of thiobarbituric acid reactive substances (TBARS), and reducing sugar contents

TBARS and reducing sugar content were determined according to the methods described by Liu et al. (2007).

Preparation of CO aqueous solution and CO content determination

The preparation of the CO aqueous solution and the determination of CO content in wheat seed samples by gas chromatography–mass spectrometry (GC–MS) were carried out according to the method described in previous reports (Liu et al., 2007; Xuan et al., 2008; Han et al., 2008). The CO-saturated aqueous solution was obtained by bubbling CO gas gently through a glass tube into 300 mL of 25% PEG solution for at least 45 min, a duration long enough to saturate the solution with CO. Then, the saturated stock solution (100% saturation) was diluted immediately with 25% PEG solution to the concentration required (1% and 10%, v/v). Under our experimental conditions, the concentration of CO in the saturated stock solution was about 187 μM. The half-life of CO loss from the stock solution at 30 °C was about 210 min.

Determination of enzymatic activity

Heme oxygenase (HO, EC 1.14.99.3), amylase (EC 3.2.1), catalase (CAT, EC 1.11.1.6), and superoxide dismutase (SOD, EC 1.15.1.1) activities were analyzed using the methods described in our previous reports (Zhang et al., 2003; Han et al., 2008).

Ascorbate peroxidase (APX, EC 1.11.1.11) and dehydroascorbate reductase (DHAR, EC 1.8.5.1) activities were measured as described by Jiménez et al. (1997). Protein was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Semi-quantitative RT-PCR analysis

Total RNA isolation and the RT reactions were carried out as previously described (Shen et al., 2003). cDNA was amplified by PCR using the following primers: for *HO-1* (the CA695015 and CK217201 sequences were assembled to construct the *HO-1* cluster sequence), forward (5′-TCAAGGCAGTTCAGACCTG-3′) and reverse (5′-TGAGAAGAATCCCCAAGCA-3′), amplifying a 381-bp fragment; for *18s rRNA* (accession no. AJ272181), forward (5′-CAAGCCATCGCTCTGGATACATT-3′) and reverse (5′-CCTGTTATTGCCTCAAACTTCC-3′), amplifying a 658-bp fragment. To standardize the results, the relative abundance of *18s rRNA* was determined and used as the internal standard.

The cycle numbers of the PCR reactions were adjusted for each gene to obtain visible bands in agarose gels. Aliquots of the PCR reactions were loaded on 1.5% agarose gels with the use of ethidium bromide. Specific amplification products of the expected size were observed, and their identities were confirmed by sequencing. Ethidium bromide-stained gels were scanned and analyzed