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Methane-rich water alleviates NaCl toxicity during alfalfa seed germination

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ABSTRACT

In this report, we investigated the beneficial role of methane-rich water (MRW) in the alleviation of salt toxicity in germinating alfalfa seeds. Upon NaCl stress, a progressive production of intracellular CH_4 during seed germination was observed, which was mimicked by the pretreatment with 30% MRW. Both MRW and an inducer of heme oxygenase-1 (HO-1; a ubiquitous enzyme catalyzing degradation of heme to produce carbon monoxide) could up-regulate *HO-1* gene expression, followed by the improvement of the inhibition of seed germination and seedling growth triggered by NaCl. Meanwhile, NaCl-induced lipid peroxidation and reactive oxygen species overaccumulation were reduced by MRW. This result was supported by the increases of total and isozymatic activities of representative antioxidant enzymes, and the up-regulation of corresponding transcripts. Above MRW cytoprotective responses were HO-1-dependent, since corresponding MRW-induced changes were sensitive to the potent inhibitor of HO-1, but reversed by the cotreatment with one of its catalytic by-products, carbon monoxide (CO) aqueous solution. Additionally, ion homeostasis was reestablished by MRW. Together, our evidence reveals that MRW alleviates NaCl-induced inhibition of seed germination and oxidative stress, partially by the up-regulation of *HO-1*.

1. Introduction

Salinity is one of the most important abiotic stress problems in plants that inhibit seed germination, seedling growth and reduce productivity (Kurniasih et al., 2013; Turner et al., 2013). Genetic and biochemical evidence confirms that salinity imposes both ionic imbalance and osmotic stress to plants, thus leading to nutrition disorder (Zhu, 2001, 2003; Shabala et al., 2012). Since cellular ion homeostasis is usually impaired by salinity stress (Zhu, 2003; Bazihizina et al., 2012), ion homeostasis in plant tissues should be reestablished. Another response of plants to salt stress is oxidative stress that results in lipid peroxidation of the plasma membrane in plant cells. The accumulation of reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$), causes oxidative stress and perturbance of redox status under NaCl-stressed conditions (Zhu, 2001, 2003; Zhang et al., 2012).

Since ROS act as signaling molecules to mediate some key physiological processes but also are toxic, plants exhibit various strategies to keep intracellular ROS homeostasis by modulating the production and scavenging of ROS (Liu et al., 2012). To cope with salt stress, besides non-enzymatic antioxidants (glutathione, GSH; ascorbic acid, AsA; etc), the efficient and activated enzymatic systems, including superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD) and ascorbate peroxidase (APX), etc, are beneficial for the removal of excess ROS (Evers et al., 2012; Gill et al., 2013). Thus, lipid peroxidation under salinity stress is modulated. Previous results further showed that heme oxygenase-1 (HO-1; a novel antioxidant enzyme), an inducible and major isoform of heme oxygenase (HO) which catalyzes the oxidative conversion of heme to biliverdin IXα (BV) with the concomitant

Abbreviations: APX, ascorbate peroxidase; BR, bilirubin; BV, biliverdin; CO, carbon monoxide; CH_4, methane; HO, heme oxigenase; HO-1, heme oxygenase-1; MRW, methane-rich water; *MSC27*, *Medicago sativa* cDNA 27; POD, guaiacol peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; ZnPP, zinc protoporphyrin IX.

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release of carbon monoxide (CO) and free iron (Fe^{2+}), is involved in the transduction of salinity signal to mediate salt acclimation in Arabidopsis and *Cassia obtusifolia* (Xie et al., 2008, 2011; Zhang et al., 2012). In fact, HO-1 and CO have been demonstrated to be associated with various abiotic stresses, including osmotic stress (Liu et al., 2010), ultraviolet radiation (Yannarelli et al., 2006; Xie et al., 2012), metal toxicity (Noriega et al., 2004; Han et al., 2008), and the induction of adventitious root formation (Lin et al., 2014), one of the phenotypes referred to as a stress-induced morphogenic response (SIMR; Potters et al., 2007).

Methane (CH_4) is the second most important anthropogenic greenhouse gas after carbon dioxide (CO_2) (Wang et al., 2013). From the chemical viewpoint, CH_4 is highly explosive and can cause death by asphyxiation when it is present at high concentrations. Emissions of non-microbial CH_4 have been observed in plants (Keppler et al., 2006; Wang et al., 2011), animals (Ghyczy et al., 2008), soils (Hurkuck et al., 2012), and the surface of oceans (Bange and Uher, 2005). Recently, a chemical reaction system containing iron(II/III), hydrogen peroxide (H_2O_2) and ascorbic acid (ASC) that readily forms CH_4 from organosulfur compounds under highly oxidative conditions was suggested. This result hypothesizes that this novel chemical route might mimic CH_4 formation in living aerobic organisms such as plants, fungi, algae and mammals (Althoff et al., 2014). Together, the investigation of CH_4 synthesis in plants is just the beginning.

Previously in mammals, CH_4 was suggested as a critical molecule implicated in the anti-inflammatory, and a beneficial role of CH_4 in influencing ischemia-reperfusion-induced oxidative and nitrosative stresses was firstly reported (Boros et al., 2012). In plants, by using methane-rich water (MRW) treatment (a safe, economical, and easily available method in comparison with the application of CH_4 gas directly, which is flammable and dangerous in the experiments), our recent work showed that MRW could induce cucumber adventitious rooting process in a HO-1/CO-dependent fashion (Cui et al., 2015). However, the biological functions of endogenous CH_4 in plant responses against environmental stresses are largely unknown.

Salinity is a limiting factor in irrigated alfalfa (*Medicago sativa* L.) production in many regions of the world. However, whether alfalfa plants can increase CH_4 production upon NaCl stress and its possible physiological role remain poorly understood. To address the above knowledge gaps, we firstly compared the changes of CH_4 production in alfalfa during seed germination under the normal growth condition and upon NaCl treatment. Interestingly, salinity-induced CH_4 production in vivo was firstly observed. To preliminarily mimic a physiological response of CH_4 production triggered by NaCl stress, we further investigated some physiological and biochemical events induced by MRW pretreatment followed by NaCl stress. These events included the improvement of inhibition of seed germination and seedling growth, alleviation of lipid peroxidation, and reestablishment of redox status. The involvement of HO-1, especially in the alleviation of seed germination inhibition and reestablishment of redox status, was also preliminarily confirmed by using the inducer of HO-1 and its potent inhibitor.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA). Hemin was used as an HO-1 inducer at 10 μM (Lamar et al., 1996; Xie et al., 2011). Zinc protoporphyrin IX (ZnPP), a specific inhibitor of HO-1, was used at 1 μM (Xie et al., 2011; Bai et al., 2012). 10% saturation of CO aqueous solution was prepared by the method described previously (Han et al., 2008). Since BV is easily reduced to form the potent antioxidant bilirubin (BR) by cytosolic biliverdin reductase, BR, and Fe-EDTA (Fe^{2+}), were regarded as the other two by-products of HO-1 (Wu et al., 2011; Xie et al., 2011). The concentrations used in this study were determined in pilot experiments from which the maximal modulating responses were obtained. Additionally, distilled water used in our experiments was sterilized by autoclaving.

2.2. Preparation of methane-rich water (MRW)

Purified CH_4 gas (obtained from the pure gas cylinder, 8 L, Nanjing Special Gases Factory Co., Ltd., China, gas purity was 99.9%, v/v) was bubbled into 500 ml distilled water at a rate of 160 ml min^{-1} for 30 min at 25 °C, a sufficient duration to saturate the solution with CH_4 (the concentration of CH_4 in water is no longer increased; we defined this as the saturated stock solution, 100% MRW). Afterwards, the saturated stock solution was immediately diluted to the required concentrations [1,10, 30 and 50% concentration (v/v)].

2.3. Determination of endogenous CH_4 content

For detecting endogenous CH_4 content (Cui et al., 2015), headspace sampling of gas followed by gas chromatography (GC) was used according to the method described previously (Jin et al., 2013) with minor modification, which was also used to determine endogenous CO and hydrogen gas (H_2) contents in animal and plant tissues (Renwick et al., 1964; Bernardi et al., 2008; Bruhn et al., 2009). The main changes are as follows: 0.2 g sample was homogenized and then transferred to the sealed glass vials followed by the addition of 5 ml distilled water, 5 μl octanol and 0.1 ml 5 M sulfuric acid, and finally purged with pure nitrogen gas for 2 min.

The chromatographic system (GC Agilent 7820, USA) was equipped with Poropak column (1/8 in., 8 foot) and a flame ionization detector (FID). Nitrogen gas was used as the carrier gas, and air pressure was 0.5 MPa. The GC was calibrated using a standard CH_4 mixture (2 ppm CH_4 in N_2). In our experimental conditions (25 ± 1 °C), the CH_4 content in freshly prepared MRW (100% saturation) was about 0.021 g CH_4 kg^{-1} H_2O, and maintained at a relative constant level at least 3 h.

2.4. Plant materials, growth condition and treatments

Alfalfa seeds (*M. sativa* L. cv. Biaogan) were surface-sterilized with 5% NaClO for 10 min, and then washed thoroughly with distilled water and then dried. These seeds were presoaked in distilled water, different concentrations of MRW, 1 μM ZnPP, 10% saturation of CO aqueous solution, 10 μM BR, 10 μM Fe-EDTA (Fe^{2+}) and 10 μM hemin alone, or the combinations for 12 h, and then transferred to Petri dishes containing 4 ml of distilled water (H_2O) or 100 mM NaCl (S). Treatment with distilled water (H_2O) was regarded as control (Con). All seeds were kept at 25 °C in a growth chamber in darkness for the indicated time points. Afterwards, the seedlings were washed carefully with distilled water for three times, then harvested and used directly, or frozen in liquid nitrogen immediately and stored at–80 °C for further analysis.

2.5. Germination and growth analysis

Seed germination tests were carried out using at least three replicates of 150 seeds each. There were 50 seeds in each Petri dish. Germination rate (%) in each Petri dish was counted and recorded after various treatments for the indicated time points, and alfalfa seeds were considered to have germinated when the length of