Ecotoxicology and Environmental Safety 147 (2018) 861–871

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Methane alleviates alfalfa cadmium toxicity via decreasing cadmium accumulation and reestablishing glutathione homeostasis

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ARTICLE INFO

Keywords:
Cadmium stress
Methane
Cd transporter
Glutathione homeostasis
Medicago sativa

ABSTRACT

Although methane (CH_4) generation triggered by some environmental stimuli, displays the protective response against oxidative stress in plants, whether and how CH_4 regulates plant tolerance against cadmium stress is largely unknown. Here, we discovered that cadmium (Cd) stimulated the production of CH_4 in alfalfa root tissues. The pretreatment with exogenous CH_4 could alleviate seedling growth inhibition. Less amounts of Cd accumulation was also observed. Consistently, in comparison with Cd stress alone, *miR159* transcript was down-regulated by CH_4, and expression levels of its target gene *ABC transporter* was increased. By contrast, *miR167* transcript was up-regulated, showing a relatively negative correlation with its target gene *Nramp6*. Meanwhile, Cd-triggered redox imbalance was improved by CH_4, evidenced by the reduced lipid peroxidation and hydrogen peroxide accumulation, as well as the induction of representative antioxidant genes. Further results showed that Cd-triggered decrease of the ratio of reduced/oxidized (homo)glutathione was rescued by CH_4. Additionally, CH_4-triggered alleviation of seedling growth was sensitive to a selective inhibitor of glutathione biosynthesis. Overall, above results revealed that CH_4-alleviated Cd accumulation at least partially, required the modulation of heavy metal transporters via *miR159* and *miR167*. Finally, the role of glutathione homeostasis elicited by CH_4 was preliminarily suggested.

1. Introduction

Cadmium (Cd), one of the toxic heavy metals, is not only the major pollutants in fields, but also a major limiting factor for crop productivity (Foy et al., 1978; DalCorso et al., 2008; Järup and Åkesson, 2009; Podazza et al., 2012). Cd is released into the environment as an anthropogenic pollutant since the emission from natural sources, including volcanic activities, weathering of rocks, and soil erosion, are locally limited (Sebastian and Prasad, 2014). Normally, Cd severely threats to animal and human health, since it is highly mobile and easily taken up by plant tissues, thus entering the food chain (Järup and Åkesson, 2009). Upon Cd stress, plant seedling growth is significantly inhibited (Foy et al., 1978). Strong evidence further showed that the accumulation of Cd is able to lead to alterations in plant homeostasis and even cell death (Podazza et al., 2012). Therefore, the decrease in Cd uptake is an important step for eliminating Cd toxicity.

Several genes encoding the metal transporters in Arabidopsis, including *AtPDR8*, *AtMRP3*, and *AtATM3*, have been suggested to confer heavy metal resistance in plants (Kolukisaoglu et al., 2002; Kim et al., 2006, 2007). Plant natural resistance-associated macrophage protein (Nramp) also participates in cellular Cd uptake and Cd transport within plants (Curie et al., 2000; Thomine et al., 2000; Cailliatte et al., 2009; Sasaki et al., 2012). In alfalfa, *ABC transporter* and *Nramp6* are homologous genes of *AtPDR8*, *AtNramp6* and rice *NRAMP1*, respectively. Genetic evidence showed that *AtPDR8*-over-expressing plants were more resistant to Cd than the wild-type and had lower Cd contents (Kim et al., 2007). Meanwhile, a null allele of *AtNramp6* was more tolerant to Cd toxicity, a phenotype that was reverted by expressing *AtNramp6* in the mutant background (Cailliatte et al., 2009). It was well-known that microRNAs could modulate gene expression at the transcriptional and

Abbreviations: ABC transporter, ATP-binding cassette transporter; APX, ascorbate peroxidase; BSO, L-buthionine-(*S*,*R*)-sulfoximine; Cd, cadmium; CH_4, methane; CHES, 2-(*N*-cyclohexylamino) ethane-sulphonic acid; DTNB, 5,5′dithio-bis-(2-nitrobenzoic acid); GR, glutathione reductase; hGSH, homoglutathione; ICP-OES, inductively coupled plasma-optical emission spectrometer; mBBr, monobromobimane; MCB, monochlorobimane; NEM, *N*-ethylmaleimide; Nramp, natural resistance-associated macrophage protein; POD, guaiacol peroxidase; PVP, polyvinylpyrrolidone; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid

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http://dx.doi.org/10.1016/j.ecoenv.2017.09.054
Received 6 June 2017; Received in revised form 16 September 2017; Accepted 19 September 2017
Available online 10 October 2017
0147-6513/

post-transcriptional levels via guiding target mRNA cleavage or translational inhibition (Mallory and Vaucheret, 2006; Xu et al., 2013). Among these, *miR159* and *miR167*, are involved in the Cd response process in plants, since *ABC transporter* and *Nramp* were identified as targets for *miR159* and *miR167*, respectively (Mallory and Vaucheret, 2006; Gupta et al., 2014; Xu et al., 2015).

Although the rapid overproduction of reactive oxygen species (ROS) in plant cells caused by Cd might have a role in acclimation signaling, the ROS-induced lipid peroxidation and oxidative damage in other macromolecules, and/or cell death may occur (Piqueras et al., 1999; Foyer and Noctor, 2005; DalCorso et al., 2008; Podazza et al., 2012). To keep the redox homeostasis, plant cells possess certain enzymatic and non-enzymatic antioxidant defence systems responsible for ROS scavenging. Besides enzymatic antioxidant defence systems (including superoxide dismutase, SOD; catalase, CAT; guaiacol peroxidase, POD; ascorbate peroxidase, APX; etc), reduced glutathione (GSH) and homoglutathione (hGSH; γ-glutamyl-cysteinyl-β-alanine), the most abundant of the free, low MW thiol compounds, are regarded as the part of the redox hub (Noctor and Foyer, 1998; Cobbett and Goldsbrough, 2002; Sharma and Dietz, 2009; Noctor et al., 2012). Comparatively, hGSH is more rich than GSH in leguminous plants, including soybean and alfalfa plants (Matamoros et al., 1999; Cui et al., 2012). Previous results have confirmed the functions of GSH/hGSH in plant Cd tolerance (Sharma and Dietz, 2009; Cui et al., 2014). For example, the rapid depletion of reduced (homo)glutathione (GSH and hGSH) not only caused redox imbalance, but also resulted in Cd sensitivity and/or toxicity in *Medicago sativa* (Ortega-Villasante et al., 2005).

Methane (CH_4) is the second most prevalent greenhouse gas in the earth, and its emission occurs mainly from industry, agriculture, and waste management activities (Keppler et al., 2006, 2009). Interestingly, emissions of non-microbial CH_4 have been observed in different plant species under the normal growth conditions, or stressed environment, including UV-B radiation, high temperature, and water stress (McLeod et al., 2008; Vigano et al., 2008; Messenger et al., 2009; Qaderi and Reid, 2009; Bloom et al., 2010; Wishkerman et al., 2011; Bruhn et al., 2012, 2014). Recently, we discovered that CH_4 was able to induce adventitious rooting (Cui et al., 2015; Qi et al., 2017), counteract salinity and osmotic stress (Zhu et al., 2016; Han et al., 2017), and alleviate copper and aluminum toxicity (Cui et al., 2017; Samma et al., 2017) in plants. In animals, evidence is provided that CH_4 could influence ischemia-reperfusion-induced oxidative and nitrosative stresses (Boros et al., 2012), and therefore be suggested as a therapeutic gas (Liu et al., 2012). To the best of the authors' knowledge, the impact of CH_4 on Cd toxicity in plants has never been addressed. Thus, the aim of this work was to test whether or how CH_4 acts as a regulator in preventing Cd toxicity.

First, we discovered that the beneficial role of CH_4 against Cd stress in alfalfa plants was mainly associated with its reduction of Cd accumulation. This response, at least partially, required the modulation of heavy metal transporters via *miR159* and *miR167*. Additionally, the reestablishment of glutathione homeostasis might be another explanation for the Cd tolerance triggered by CH_4.

2. Materials and methods

2.1. The preparation of CH_4 and determination of endogenous CH_4

The CH_4 gas (99.9%, v/v) from a compressed gas cylinder (Nanjing Special Gases Factory Co., Ltd, China) was bubbled into 500 ml quarter-strength Hoagland solutions with a rate of 200 ml min^{-1} for at least 40 min (Zhu et al., 2016; Han et al., 2017). The concentration of CH_4 (1.30 mM; determined by gas chromatography) in Hoagland solutions is no longer increased. We thus defined this as the saturated stock solution. Afterwards, above stock solution was immediately diluted to the required concentrations of CH_4 (0.13 and 0.65 mM), and CH_4 concentrations in these solutions were maintained at a relative constant level for at least 12 h. Similarly, in animal research, CH_4 gas was dissolved in 20 ml of physiological saline for 20 min at a speed of 200 ml min^{-1} to reach a supersaturated level (Song et al., 2010, 2015; Wu et al., 2015).

The content of CH_4 in plant samples was determined according to our previous method (Han et al., 2017; Qi et al., 2017). Firstly, seedling roots (0.4 g) were homogenized with 5 ml sterile water and transferred to a vial. 100 µl sulfuric acid (2 M) was added to digest samples, and pure nitrogen gas (99.99%, v/v) was placed into vial to displace the air. Afterwards, the chromatographic system (GC Agilent 7820, USA) equipped with Poropak column (1/8 in., 8 foot), and a flame ionization detector (FID) was applied. 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). For the measurement of CH_4 levels, 1 ml of the head space air in the vial was injected directly into carrier gas by syringe.

2.2. Plant materials, growth condition, and experimental design

Commercially available alfalfa (*M. sativa* L. cv. Biaogan) seeds were surface-sterilized with 5% (v/v) NaClO for 10 min, rinsed comprehensively in distilled water and germinated for 1 d at 25 °C in the darkness. Uniform seedlings were cultured with quarter-strength Hoagland solution in the illuminating incubator (14 h light with a light intensity of 200 µmol m^{-2} s^{-1}, 25 ± 1 °C, and 10 h dark, 23 ± 1 °C).

Four-day-old seedlings were cultured in quarter-strength Hoagland solutions containing 0.13, 0.65, and 1.30 mM CH_4, 100 µM $_L$-buthionine-(*S*,*R*)-sulfoximine (BSO, a potent and specific inhibitor of the enzyme catalyzing the first step of GSH biosynthesis; Rüegsegger et al., 1990) alone, or the combination treatment for 6 h, and then transferred to quarter-strength Hoagland solutions containing 100 µM $CdSO_4$ (diluted from the stock solution; 100 mM), for the indicated time points. The sample without chemicals was the control (Con). Afterwards, plants were photographed, and root and shoot parts were sampled and measured immediately, or rapidly frozen at −80 °C for further analysis.

2.3. Determination of Cd content

Seedlings were washed three times with 5 mM $CaSO_4$ and 10 mM EDTA-Na_2 solution, and rinsed briefly in deionized water (Han et al., 2014). Afterwards, seedlings were oven-dried at 60 °C, then digested with HNO_3 using a Microwave Digestion System (Milestone Ethos T, Italy). The Cd contents were determined using an Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Perkin Elmer Optima 2100DV).

2.4. Determination of thiobarbituric acid reactive substances (TBARS) and hydrogen peroxide (H_2O_2) contents

Lipid peroxidation was determined by measuring the amount of TBARS as previously described (Cui et al., 2014). Root tissues were crushed with 5% trichloroacetic acid (TCA; w/v) using a mortar and pestle, added with 0.5% 2-thiobarbituric acid (TBA; w/v) in 5% TCA. After incubation in 100 °C water bath for 30 min, centrifuged at 12,000*g* for 20 min, then determined by monitoring the absorbance at 450, 532, and 600 nm (extinction coefficient 155 mM^{-1} cm^{-1}). The concentration of lipid peroxides, together with oxidatively modified proteins of plants, was quantified in terms of TBARS amount and expressed as nmol g^{-1} fresh weight (FW).

The content of H_2O_2 was measured according to previous method (Ma et al., 2014). Roots were ground with a mortar and pestle, and extracted into 2 ml of 0.2 M $HClO_4$ on ice. The extract was held for 5 min followed by centrifugation (10,000*g*; 4 °C) for 10 min. Briefly, an aliquot of supernatant (500 µl) was added to 500 µl of assay reagent (0.5 mM ammonium ferrous sulfate, 50 mM H_2SO_4, 0.2 mM xylenol orange, and 200 mM sorbitol). The absorbance at 560 nm was