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Hydrogen-rich water regulates cucumber adventitious root development in a heme oxygenase-1/carbon monoxide-dependent manner

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ABSTRACT

Hydrogen gas (H_2) is an endogenous gaseous molecule in plants. Although its reputation is as a "biologically inert gas", recent results suggested that H_2 has therapeutic antioxidant properties in animals and plays fundamental roles in plant responses to environmental stresses. However, whether H_2 regulates root morphological patterns is largely unknown. In this report, hydrogen-rich water (HRW) was used to characterize H_2 physiological roles and possible signaling transduction pathways in the promotion of adventitious root (AR) formation in cucumber explants. Our results showed that a 50% concentration of HRW was able to mimic the effect of hemin, an inducer of a carbon monoxide (CO) synthetic enzyme, and heme oxygenase-1 (HO-1), in restoring AR formation in comparison with the inhibition effect conferred by auxin-depletion treatment alone. It was further shown that the inducible effect of HRW could be further blocked by the co-treatment with N-1-naphthylphtalamic acid (NPA; an auxin transport inhibitor). The HRW-induced response, at least partially, was HO-1-dependent. This conclusion was supported by the fact that the exposure of cucumber explants to HRW up-regulates cucumber HO-1 gene expression and its protein levels. HRW-mediated induction of representative target genes related to auxin signaling and AR formation, such as *CsDNAJ-1*, *CsCDPK1/5*, *CsCDC6*, *CsAUX22B-like*, and *CsAUX22D-like*, and thereafter AR formation (particularly in the AR length) was differentially sensitive to the HO-1 inhibitor zinc protoporphyrin IX (ZnPP). Above blocking actions were clearly reversed by CO, further confirming that the above response was HO-1/CO-specific. However, the addition of a well-known antioxidant, ascorbic acid (AsA), failed to influence AR formation triggered by HRW, thus ruling out the involvement of redox homeostasis in this process. Together, these results indicated that HRW-induced adventitious rooting is, at least partially, correlated with the HO-1/CO-mediated responses. We also suggested that exogenous HRW treatment on plants might be a good option to induce root organogenesis.

Introduction

It was well-known that adventitious roots (AR) are post-embryonic roots which originated from the stem, leaf petiole, and non-pericycle tissue of old roots (Blakesley, 1994). Normally, AR formation is not ordinarily expected and often caused by stress or injury. Further results implicated that AR formation is induced by many internal and external signals that converge on a set of plant hormones, signaling molecules and regulators, such as auxin (Blakesley, 1994; Eckardt, 2005; Bai et al., 2012), hydrogen peroxide (H_2O_2) (Huang et al., 2011), nitric oxide (NO) (Pagnussat et al., 2002, 2003, 2004; Xuan et al., 2012), heme compounds (hemin and hematin) and carbon monoxide (CO) (Xu et al., 2006; Xuan et al., 2008), and hydrogen sulfide (H_2S) (Lin et al., 2012a). The above factors can trigger AR initiation and developmental progress. Molecular evidence illustrated that genes of DnaJ-like protein(s), calcium-dependent protein kinases (CDPKs), and cell division-related and auxin-induced proteins are associated with the initiation and development of AR (Lanteri et al., 2006, 2008; Xuan et al., 2008; Bai et al., 2012). The complex responses regulated by these hormones, signaling molecules, and regulators are most likely to be achieved by a combinational signaling process. However, whether there are some other novel singling molecule(s) involved in AR formation remains to be investigated.

Hydrogen is the lightest and most abundance chemical element, constituting nearly 75% of the universe's elemental mass. Recently, a growing number of studies have found that hydrogen gas (H_2)

Abbreviations: AR, adventitious roots; AsA, ascorbic acid; BR, bilirubin; Fe^{2+}, $FeSO_4{\cdot}7H_2O$; H_2, hydrogen gas; H_2O_2, hydrogen peroxide; HRW, hydrogen-rich water; H_2S, hydrogen sulfide; HO-1/CO, heme oxygenase-1/carbon monoxide; NO, nitric oxide; NPA, *N*-1-naphthylphthalamic acid; ZnPP, zinc protoporphyrin IX.

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selectively reduces hydroxyl radicals and alleviates acute oxidative stress in many animal models (Ohsawa et al., 2007; Nakao et al., 2010b). As in concentrations over 5%, H_2 can form explosive mixtures with air. Therefore, hydrogen-rich water (HRW), which contains a high concentration of hydrogen, could be easily and safely applied. Subsequent experiments showed that the consumption of HRW could prevent chronic allograft nephropathy after renal transplantation (Cardinal et al., 2010), alleviate adult onset diabetes, and insulin resistance (Kajiyama et al., 2008).

The metabolism of H_2 by bacteria, green algae, and higher plants has been reported for many years (Renwick et al., 1964). Due to in vitro studies on the evolution of H_2 by isolated chloroplasts and changes of hydrogenase activity, scientists postulated the existence of hydrogenase in some higher plants, which was proven previously in green algae and bacteria (Boichenko, 1947; Torres et al., 1984, 1986; Vignais and Colbeau, 2004; Cavazza et al., 2008; Esquível et al., 2011). However, little information was known about its physiological roles and corresponding molecular mechanisms. More recently, our results showed the presence of H_2 production upon the normal growth or stressed conditions, and its physiological roles in the alfalfa and Arabidopsis subjected to abiotic stresses (Xie et al., 2012; Jin et al., 2013). For example, by using HRW pretreatment (Jin et al., 2013), we illustrated that H_2 might function as an important gaseous molecule that alleviates paraquat-induced oxidative stress in alfalfa seedlings *via* heme oxygenase-1/carbon monoxide (HO-1/CO), a well-known signaling system confirmed in animals and recently in plants (Shekhawat and Verma, 2010; Hsu et al., 2013). However, to the best of our knowledge, few studies examined the involvement of H_2 in plant growth and developmental processes.

In this report, the objective of the present study was to investigate the effect of exogenous HRW treatment on AR formation. These results may help to elucidate the regulatory effect of exogenous H_2 on plant developmental process.

Materials and methods

Chemicals

All chemicals were obtained from Sigma (St. Louis, MO, USA) unless stated otherwise. Both hemin, an inducer of heme oxygenase-1, and zinc protoporphyrin IX (ZnPP), a specific inhibitor of HO-1 (Xuan et al., 2008; Cao et al., 2011), were used at 10 μM and 1 μM, respectively. *N*-1-naphthylphthalamic acid (NPA) from Chem Service (West Chester, PA, USA), was regarded as the auxin transport inhibitor at 10 μM (Xuan et al., 2008). Both bilirubin (BR) and $FeSO_4{\cdot}7H_2O$ (Fe^{2+}) were used as the catalytic by-products of HO at a concentration of 10 μM, respectively. Ascorbic acid (AsA), a well-known antioxidant, was applied at concentrations of 1, 10, and 100 μM.

HRW preparation

According to previous reports (Xie et al., 2012; Jin et al., 2013), purified H_2 gas was generated by using a hydrogen-producing apparatus produced by Saikesaisi Hydrogen Energy Co. Ltd. (Shandong, China), then was bubbled into 500 ml distilled water at a rate of 150 ml min^{-1} for at least 30 min. Afterwards, the saturated stock solution (100% of concentration) of HRW was immediately diluted to the required concentrations (10 and 50% of concentrations [v/v]). In our experimental conditions, the H_2 concentration in freshly prepared HRW analyzed by gas chromatography (GC) was 0.22 mM, and it remained at a relative constant level in 25 °C for at least 12 h.

CO aqueous solution preparation

The preparation of CO aqueous solution was carried out according to the method described previously (Xuan et al., 2008). The saturated stock solution (100% concentration) was diluted immediately with distilled water to the required concentration with a maximal inducible response (30% concentration [v/v]).

Plant material and growth conditions

Cucumber (*Cucumis sativus* 'Lufeng') seeds were kindly supplied by Jiangsu Agricultural Institutes, Jiangsu Province, China. Selected identical seeds were germinated in petri dishes on filter papers imbibed in distilled water, then transferred to an illuminating incubator and maintained at 25 ± 1 °C for 5 d with a 14-h photoperiod at 200 μmol m^{-2} s^{-1} intensity. Cucumber seedlings were decapitated by excising the apical bud immediately above the cotyledons and incubated in the presence of 10 μM NPA (auxin-depleted) for 48 h, before removing the primary root. Cucumber explants were then maintained under the same conditions of temperature and photoperiod for another 4 d or the indicated time points in the presence of different media as indicated.

Explant treatments

After primary roots were removed, every eight cucumber explants were put into a petri dish containing 10 ml of distilled water, varying concentrations of hydrogen-rich water (HRW) or AsA, 10 μM hemin, 10 μM NPA, 1 μM ZnPP, 30% concentration of CO aqueous solution, 10 μM BR and Fe^{2+}, either alone or in the combination, and kept at 25 ± 1 °C for 4 d or the indicated time periods according to the experimental design. Previous studies (Pagnussat et al., 2002, 2003, 2004; Lanteri et al., 2006, 2008; Xuan et al., 2008; Cao et al., 2011) and our pilot experiments showed that the concentrations and the time of treatments with these chemicals are suitable for investigating the role of HO-1/CO in the adventitious root (AR) developmental signaling. Finally, excised cucumber hypocotyls (5-mm long segments of the hypocotyl base, where AR develops) (Lanteri et al., 2008) were used for the following analysis. After various treatments, the numbers and total length of AR more than 1 mm long were calculated and measured by a ruler (Drew et al., 1979; Steffens et al., 2006), and corresponding photographs were taken.

Protein immunoblot analysis for CsHO1

Rabbit polyclonal antibody was made against the mature cucumber HO-1 (mCsHO1) expression in *E. coli* (Li et al., 2011). Sixty micrograms of protein from homogenates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using a 12.5% acrylamide resolving gel (Mini Protean II System, Bio-Rad, Hertz, UK). Separated proteins were then transferred to PVDF membranes. The immunoblot was probed with rabbit polyclonal antibody as the primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG as the secondary antibody (Xuan et al., 2008). The color was developed with a solution containing 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the HRP substrate. Additionally, Coomassie Brilliant staining was used to show the equal amounts of proteins loaded.

Semi-quantitative RT-PCR analysis

Total RNA was extracted from about 100 mg (fresh-weight) excised cucumber hypocotyls, reverse-transcribed and cDNA abundance was measured by semi-quantitative RT-PCR according