Journal of Plant Physiology 195 (2016) 50–58

Contents lists available at ScienceDirect

Journal of Plant Physiology

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

Physiology

Nitric oxide is required for hydrogen gas-induced adventitious root formation in cucumber

Yongchao Zhu, Weibiao Liao*, Meng Wang, Lijuan Niu, Qingqing Xu, Xin Jin

College of Horticulture, Gansu Agricultural University, Lanzhou 730070, PR China

ARTICLE INFO

Article history:
Received 8 July 2015
Received in revised form 16 February 2016
Accepted 17 February 2016
Available online 15 March 2016

Keywords:
Hydrogen-rich water
Nitric oxide (NO)
NO synthase (NOS)
Nitrate reductase (NR)
NR genes
Enzyme

ABSTRACT

Hydrogen gas (H_2) is involved in plant development and stress responses. Cucumber explants were used to study whether nitric oxide (NO) is involved in H_2-induced adventitious root development. The results revealed that 50% and 100% hydrogen-rich water (HRW) apparently promoted the development of adventitious root in cucumber. While, the responses of HRW-induced adventitious rooting were blocked by a specific NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO), NO synthase (NOS) enzyme inhibitor N^G-nitro-L-arginine methylester hydrochloride (L-NAME) and nitrate reductase (NR) inhibitor NaN_3. HRW also increased NO content and NOS and NR activity both in a dose- and time-dependent fashion. Moreover, molecular evidence showed that HRW upregulated *NR* genes expression in explants. The results indicate the importance of NOS and NR enzymes, which might be responsible for NO production in explants during H_2-induced root organogenesis. Additionally, peroxidase (POD) and indoleacetic acid oxidase (IAAO) activity was significantly decreased in the explants treated with HRW, while HRW treatment significantly increased polyphenol oxidase (PPO) activity. In addition, cPTIO, L-NAME and NaN_3 inhibited the actions of HRW on the activity of these enzymes. Together, NO may be involved in H_2-induced adventitious rooting, and NO may be acting downstream in plant H_2 signaling cascade.

1. Introduction

The past decade has seen the rapid development of research on plant nitric oxide (NO). NO, a ubiquitous signal molecule is involved in root organogenesis and growth. The involvement of NO in inducing root development was firstly observed in *Zea mays* (Gouvěa et al., 1997). Pagnussat et al. (2002) reported significant function of NO in the auxin-induced adventitious rooting. Subsequent reports showed that cyclic guanosine monophosphate and mitogen-activated protein kinase signaling cascades were involved in NO-mediated adventitious rooting (Pagnussat et al., 2004). In addition, NO was shown to participate in lateral root development and root hair formation (Lombardo et al., 2006). Hu et al. (2005) found an asymmetric accumulation of NO in soybean (*Glycine max*) primary root in response to gravistimulation. Our previous research found that hydrogen peroxide (H_2O_2), Ca^{2+}/CaM and Ca^{2+}-dependent protein kinase were involved in adventitious rooting induced by NO (Liao et al., 2009, 2012). Xuan et al. (2012) reported that NO operated downstream of adventitious root development promoted by hemin. The mechanism of NO-induced adventitious rooting has mainly been attributed to signal transduction.

In plants, there are two major NO production pathways, one enzymatic and other non-enzymic. Nitrate reductase (NR) and NO synthase (NOS)-like enzyme are the NO-producing enzymes identified in plants (Desikan et al., 2002). It is known that a major source of NO in plants originates from nitrite regulated by NR. The expression of *NR* for NO generation in ABA-induced stomatal closure has been evidenced in *Arabidopsis* (Desikan et al., 2002). Moreover, NO accumulation in the root apex matched with the colocalization of *NR* genes in *Arabidopsis* (Stöhr and Stremlau, 2006). Trevisan et al. (2011) found that the nitrate-regulated expression of *NR* played an important role during the early perception and signaling of nitrate in maize roots. On the other hand, NOS-mediated NO generation has also been demonstrated during root organogenesis with a potential physiological role (Liao et al., 2009). Although no cloned NOS has been identified in plants until now, NOS activity has been detected in plant development and response to stress.

Molecular hydrogen (H_2) has aroused worldwide attention because of its selective reduction. It has been demonstrated that H_2 acted as a therapeutic agent in biomedical fields and clinical and experimental models of many diseases (Hong et al., 2010). At the same time, the physiological roles of H_2 and its mechanisms were

* Corresponding author.
E-mail address: liaowb@gsau.edu.cn (W. Liao).

http://dx.doi.org/10.1016/j.jplph.2016.02.018
0176-1617/

studied in higher plants. Recent results revealed that H_2 could act as an important signal with multiple functions in plant responses to salt stress (Xie et al., 2012; Xu et al., 2013), cadmium toxicity (Cui et al., 2013), mercury toxicity (Cui et al., 2014), paraquat-induced oxidative stress (Jin et al., 2013) and aluminum toxicity (Chen et al., 2013). Up to date evidence found that H_2 reestablished ROS homeostasis but exerted differential effects on anthocyanin synthesis in two varieties of radish sprouts under UV-A irradiation (Su et al., 2014). H_2 was also shown to delay fruit ripening and senescence of kiwifruit by regulating antioxidant defence (Hu et al., 2014). Recent research has indicated that H_2-induced cucumber adventitious rooting might be correlated with the heme oxygenase-1/carbon monoxide-mediated responses (Lin et al., 2014).

In animals, H_2 has been reported to induce inhibition of NO production through modulation of signal transduction and ameliorate inflammatory arthritis in mice (Itoh et al., 2011). Kashiwagi et al. (2014) found that H_2 significantly suppressed NO-induced cytotoxicity in primary cells. Although, the biological roles of H_2 and NO in plants have received worldwide interest due to their function as a signaling molecule. However, little reports have been published to support the interaction between H_2 and NO in plants. The decreasing of NO production was involved in the alleviation of aluminum-induced inhibition of root elongation by H_2 (Chen et al., 2013). Recently, there has also been some evidence that NO production may contribute to H_2-promoted stomatal closure in *Arabidopsis* (Xie et al., 2014).

Some enzymes such as peroxidase (POD), polyphenol oxidase (PPO), and indoleacetic acid oxidase (IAAO) are known to be intimately involved in indole-3-acetic acid (IAA) catabolism to modify the hormonal balance in plants, and they have different functions during root organogenesis (Smart et al., 2003). The increase of POD activity has been known to be a rooting signal during root primordium formation (Nordstrom and Eliasson, 1991). The importance of PPO in the induction of rooting is due to its effect on phenolic metabolism (Liao et al., 2010). It has been proposed that IAAO and POD had a similar effect on the occurrence of adventitious roots by changing IAA levels (Rama and Prasad, 1996).

Considering the fact that H_2 may mediate adventitious root development (Lin et al., 2014), it would be noteworthy to identify how H_2 induces downstream signaling cascades and regulates rooting. In this study, we try to determine whether NO is a second messengers involved in H_2-induced adventitious rooting. The results may provide an important foundation for future signaling pathway studies of H_2 in plants.

2. Materials and methods

2.1. Chemicals

N_G-nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma, USA) was used as NO synthase (NOS) inhibitor. NaN_3 was used as the nitrate reductase (NR) inhibitor. 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO, Sigma, USA) was used as a specific NO-scavenger. NO fluorescent probe 4-amino-5-methylamino-2′,7′-diaminofluorescein diacetate (DAF-FM DA) was used as NO specific fluorophore (San Diego, CA, USA). The solutions were prepared in complete darkness, and immediately diluted to the demanded concentrations. Unless stated otherwise, the remaining chemicals were of analytical grade which were obtained from Chinese companies.

2.2. Preparation of hydrogen-rich water (HRW)

Purified H_2 gas (99.99%, v/v) generated from a hydrogen gas generator (QL-300, Saikesaisi Hydrogen Energy Co., Ltd., China) was bubbled into 1 L distilled water at a rate of 300 mL min^{-1} for 30 min. Then, the corresponding hydrogen-rich water (HRW) was rapidly diluted to the required saturations [1%, 10% 50% and 100%, (v/v)]. H_2 concentration in freshly prepared HRW was determined with a "Dissolved hydrogen portable meter" (Trustlex Co., Led, ENH-1000, Japan), and it remained at a relative constant level in 25 °C for at least for 12 h (Fig. 1).

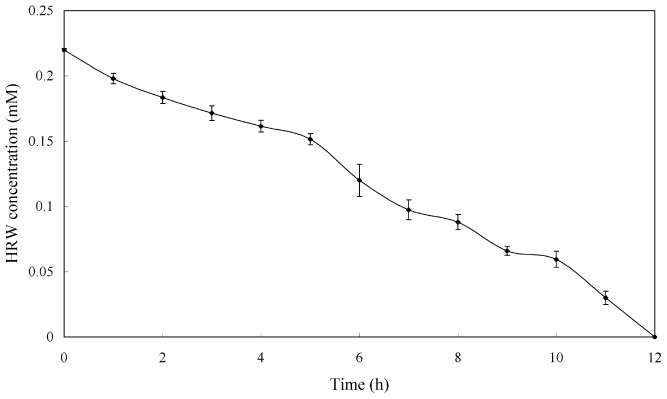

Fig. 1. The kinetics curve of H_2 releasing in HRW. Purified H_2 gas (99.99%, v/v) generated from a hydrogen gas generator (QL-300, Saikesaisi Hydrogen Energy Co., Ltd., China) was bubbled into 1 L distilled water at a rate of 300 mL min^{-1} for 30 min. Then, the corresponding hydrogen-rich water (HRW) was rapidly diluted to the required saturations [1%, 10%, 50%, and 100%, (v/v)]. H_2 concentration in freshly prepared HRW was determined with a "Dissolved hydrogen portable meter" (Trustlex Co., Led, ENH-1000, Japan), and it remained at a relative constant level in 25 °C for at least for 12 h.

2.3. Plant material and growth conditions

Selected identical seeds of cucumber (*Cucumis sativus* 'Xinchun 4'; Gansu Academy of Agricultural Sciences, Lanzhou, China) were soaked in distilled water for 5 h. The germination of seeds occurred in an illuminating incubator with a 14-h photoperiod at 200 μmol m^{-2} s^{-1} intensity and 25 ± 1 °C for 5 days. The hypocotyls of 5-d-old cucumber seedlings were excised 2 cm below the cotyledonary node to remove primary roots. The explants were placed in petri dishes containing water or different indicated chemicals under the same conditions of temperature and photoperiod described above for another 5 days.

2.4. Explants treatments

Cucumber explants were placed in Petri dish containing 6 mL of test solutions and kept at 25 ± 1 °C. The test solutions were different concentration of HRW (0, 1%, 10%, 50% and 100%), 50 μM SNP, 10 μM IBA, 200 μM cPTIO, 30 μM L-NAME and 10 μM NaN_3 alone or together with optimum concentration of HRW. The concentration of these chemicals was selected based on the results of a preliminary experiment.

2.5. Determination of the endogenous production of NO

NO content from excised cucumber hypocotyls was determined using the Greiss reagent method (Liao et al., 2011). Hypocotyls (0.2 g) were frozen in liquid nitrogen, then ground in a mortar and pestle in 4 mL of 50 mM ice-cold acetic acid buffer, pH 3.6, containing 4% (w/v) zinc diacetate. The homogenates were centrifuged at 10,000 × *g* for 15 min at 4 °C, and the supernatants were collected. For each sample, 0.1 g charcoal (Shanghai Chemical Reagent Co., Ltd.) was added. After vortex mixing and filtration, the filtrate was leached and collected. A mixture of 1 mL of filtrate and 1 mL of Greiss reagent was incubated for 30 min at room temperature