



Nitric oxide is involved in hemin-induced cucumber adventitious rooting process

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ABSTRACT

Hemin, a heme oxygenase-1 (HO-1) inducer, was shown to exert numerous beneficial physiological functions in animals. Our previous study suggests that HO-1/carbon monoxide (CO) acts as a novel downstream signal system in the auxin-induced adventitious rooting. The objective of this study was to test whether nitric oxide (NO) is involved in hemin-induced cucumber adventitious rooting. Applications of hemin or CO aqueous solution to auxin-depleted cucumber explant induced up-regulation of cucumber HO-1 transcripts (*CsHO1*), NO production, and thereafter adventitious root formation, and some above responses were blocked by the combination treatment with two nitric oxide synthase (NOS)-like enzyme inhibitors *N*^G-nitro-L-arginine methylester hydrochloride and *N*^G-nitro-L-arginine, a HO-1 specific inhibitor zinc protoporphyrin IX, and a specific NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium salt. However, these blocking responses were not observed using tungstate, an inhibitor of nitrate reductase, another NO producing enzyme in plants. Furthermore, the guanylate cyclase inhibitors 1*H*-(1,2,4)-oxadiazole[4,3-*a*]quinoxalin-1-one and 6-anilino-5,8-quinolinedione reduced root development induced by hemin, whereas the cell-permeable cyclic guanosine monophosphate (cGMP) derivative 8-Br-cGMP reversed this effect. Together, our results indicated that at least in our experimental conditions, NO might operate downstream of hemin promoting adventitious root formation probably in a cGMP-dependent manner.

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Introduction

Development of the root system, including adventitious rooting and lateral root formation, is essential for plant life. In higher plants, initiation and growth of roots are regulated by internal and external factors, including phytohormones, light, temperature, sugars or even cutting. For example, it was confirmed that adventitious root formation is affected by multiple endogenous factors, including auxin and other phytohormones, environmental factors, such as light and wounding (Sorin et al., 2005). Therefore, the discovery of signal transduction operating in root organogenesis has been recently became a major issue for scientific researchers.

Abbreviations: cGMP, cyclic guanosine monophosphate; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium salt; CO, carbon monoxide; GC, guanylyl cyclase; HO-1, heme oxygenase-1; L-NAME, *N*^G-nitro-L-arginine methylester hydrochloride; L-NNA, *N*^G-nitro-L-arginine; LSCM, laser scanning confocal microscope; LY83583, 6-anilino-5,8-quinolinedione; NO, nitric oxide; NPA, 1-naphthylphthalamic acid; NR, nitrate reductase; ODO, 1*H*-(1,2,4)-oxadiazole[4,3-*a*]quinoxalin-1-one; ZnPIX, zinc protoporphyrin IX.

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Previous results confirmed that signaling mechanisms in plants usually do not operate as the linear pathways but that extensive cross-talk occurs between various signal transduction pathways (Knight and Knight, 2001). Pagnussat et al. (2002) discovered that nitric oxide (NO) signal mediates the auxin response during adventitious root formation in cucumber explants. Subsequent reports showed that cyclic guanosine monophosphate (cGMP) and mitogen-activated protein kinase signaling cascades were involved in above process, indicating at least existing cGMP-dependent and -independent pathways (Pagnussat et al., 2003, 2004). However, yet there remain considerable gaps in our knowledge regarding a detailed description of the events and underlying signal transduction mechanisms involved in auxin-mediated adventitious rooting.

Although the presence and functions of NO have been well studied in animals and plants, the mechanism for the production of NO remains unclear in plants (Besson-Bard et al., 2008). In animals, NO is synthesized *via* the enzyme NO synthase (NOS). Although NOS-like activity (arginine (Arg)-dependent pathway) has been detected widely in plants and inhibitors of mammalian NOS inhibit NO generation in plants, the gene(s) encoding NOS protein in higher plants remains to be identified (Crawford et al., 2006; Zemojtel et al., 2006; de Montaigu et al., 2010; van Ree et al., 2011). In addition to the biosynthesis of NO by NOS-like protein, the production of NO in

plants can also be generated from nitrate reductase (NR) as well as the nonenzymatic pathway in the apoplast (Bethke et al., 2004; Zhao et al., 2009).

The commercial hemin is actually ferriprotoporphyrin IX, which is also the prosthetic moiety for a large number of proteins (including hemoglobin and plant peroxidases) that play essential roles in the transport of oxygen, mitochondrial functions, and a variety of signal transduction in both animals and plants (Conroy et al., 1982; Suzuki et al., 1995). Additionally, ferriprotoporphyrin IX and its derivatives are related to the synthesis of chlorophyll as well as phytochrome chromophore, where a ferrochelatase catalyzes heme formation by the insertion of Fe^{2+} into the protoporphyrin IX nucleus (Muramoto et al., 1999; Malkin and Niyogi, 2000; Shekhawat and Verma, 2010). It was further known that hemin up-regulates heme oxygenase-1 (HO-1), an intracellular enzyme that catalyzes the initial and rate-limiting step in the oxidative degradation of heme, and generates biliverdin (BV), free iron (Fe^{2+}), and carbon monoxide (CO) (Wakabayashi et al., 1999; Ryter et al., 2002; Devadas and Dhawan, 2006; Bilban et al., 2008). The beneficial effects of HO/CO have been reported in a variety of immune responses and inflammation. Thus, CO is regarded as a physiological messenger or bioactive molecular, and most of its effects were mediated by the activation of soluble guanylyl cyclase (sGC) or by hyperpolarizing membrane potentials through the mechanisms involving stimulation of potassium channels (Piantadosi, 2002; Dulak and Józkwicz, 2003). Similarly, some recent investigations of the functional roles of hemin and HO/CO system in plants, including the hormone-like effects on adventitious root and lateral root formation (Xuan et al., 2008; Cao et al., 2011; Lin et al., 2012) and the protection against salinity stress (Xie et al., 2008, 2011), have greatly extended our understanding of this signal system as a cellular defense mechanism against abiotic stresses-related and various other developmental processes both in animals and plants.

The depletion of endogenous auxin is regarded as a useful tool to investigate the signal transduction pathway responsible for adventitious rooting (Reed et al., 1998). Recently, using this experimental approach, we provided pharmacological, physiological, and molecular evidence that auxin rapidly activates HO activity and that the product of HO action, CO, then triggers the signal transduction events that lead to the auxin response of adventitious root formation in cucumber (Xuan et al., 2008). The cross talk between endogenous HO/CO and NO during auxin-induced adventitious rooting was also proposed. In this report, we extend our former observations, showed that hemin was able to mimic the effect of hematin (Xuan et al., 2008), leading to the induction of adventitious rooting in auxin-depleted cucumber explants. Most importantly, we further demonstrated that at least in our research conditions, NO might act as a downstream messenger in above hemin response probably by the activation of the guanylyl cyclase (GC)-catalyzed synthesis of cGMP.

Materials and methods

Plant material and growth conditions

Cucumber seeds (*Cucumis sativus* 'Lufeng') 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 $25 \pm 1^\circ\text{C}$ for 5 d with a 14-h photoperiod at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ intensity. Cucumber seedlings were decapitated by excising the apical bud immediately above the cotyledons and incubated in the presence of $10 \mu\text{M}$ 1-naphthylphthalamic acid (NPA; auxin-depleted) for 48 h, before removing primary root. Cucumber explants were then maintained under the same

conditions of temperature and photoperiod for another 5 d or the indicated time points in the presence of different media as indicated.

Chemicals

All chemicals were obtained from Sigma (St Louis, MO, USA) unless stated otherwise. Hemin (Fluka), a heme oxygenase-1 (HO-1) inducer applied in animal and plant researches (Lamar et al., 1996; Xuan et al., 2007), was used at the concentrations of 0.1, 1.0, 10.0 and $100.0 \mu\text{M}$. Zinc protoporphyrin IX (ZnPPIX), a specific inhibitor of HO-1 (Xuan et al., 2008), was used at $400 \mu\text{M}$. N^G -nitro-L-arginine methylester hydrochloride (L-NAME) and N^G -nitro-L-arginine (L-NNA, Fluka) were used as the inhibitors of NOS-like protein in plants (Wilson et al., 2008; Foresi et al., 2010; Fröhlich and Durner, 2011). Tungstate, an inhibitor of nitrate reductase or other molybdate-dependent enzymes, was used at $100 \mu\text{M}$ (Xiong et al., 2012). The compound 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium salt (cPTIO) was chosen as a specific nitric oxide (NO) scavenger. NO specific fluorophore 4,5-diaminofluorescein diacetate (DAF-2 DA) was purchased from Calbiochem (San Diego, CA, USA), used at a final concentration of $10 \mu\text{M}$. 1H-(1,2,4)-oxadiazole[4,3-a]quinoxalin-1-one (ODQ) and 6-anilino-5,8-quinolinedione (LY83583) were regarded as inhibitors of guanylyl cyclase (GC). The cell-permeable cyclic guanosine monophosphate (cGMP) derivative 8-Br-cGMP was also purchased from Fluka (Durner et al., 1998). NPA from Chem Service (West Chester, PA, USA) was regarded as the auxin transport inhibitor at $10 \mu\text{M}$ (Reed et al., 1998; Xuan et al., 2008).

Carbon monoxide (CO) aqueous solution treatment and CO content determination

The preparation of CO aqueous solution and the determination of CO content by gas chromatography and mass spectrometry (GC/MS) were carried out according to the method described in our previous report (Xuan et al., 2007). The saturated stock solution (100% saturation) was diluted immediately with distilled water to the concentrations required (10% saturation [v/v]). In our experimental conditions, the concentration of CO in the saturated stock solution was about $187 \mu\text{M}$. Meanwhile, the half-life of CO loss from the stock solution at 30°C was about 210 min. Thus, CO aqueous solution with or without other chemicals was supplied to plants by renewing the solution every 2 h over an early 6-h period, then applying daily to ensure that exogenous CO supply is continuous and sufficient, which could increase endogenous CO content approximately to the level triggered by IAA (Xuan et al., 2008).

Explant treatments

After primary roots were removed, every eight cucumber explants were each put into petri dish containing 8 ml of distilled water, varying concentrations of hemin, 10% CO aqueous solution, 1 mM of L-NAME, 500 μM (L-NNA), 100 μM tungstate, 400 μM of ZnPPIX, 200 μM cPTIO, 50 μM LY83583, 10 μM ODQ, 1 μM 8-Br-cGMP alone, or the indicated treatments and kept at $25 \pm 1^\circ\text{C}$ for 5 d or different periods according to the experiment. Previous studies showed that the concentrations and the time points of treatments with these inhibitors or scavengers were suitable for investigating the role of HO/CO or NO in the root developmental signaling (Pagnussat et al., 2002, 2003, 2004; Xuan et al., 2007, 2008; de Montaigu et al., 2010; Xu et al., 2011). Finally, excised cucumber hypocotyls (5-mm-long segments of the hypocotyl base, where adventitious root develops; Lanteri et al., 2008) were used for the following determination.

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