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Research article

Auxin-induced hydrogen sulfide generation is involved in lateral root formation in tomato

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ABSTRACT

Similar to auxin, hydrogen sulfide (H_2S), mainly produced by L-cysteine desulfhydrase (DES; EC 4.4.1.1) in plants, could induce lateral root formation. The objective of this study was to test whether H_2S is also involved in auxin-induced lateral root development in tomato (*Solanum lycopersicum* L.) seedlings. We observed that auxin depletion-induced down-regulation of transcripts of *SlDES1*, decreased DES activity and endogenous H_2S contents, and the inhibition of lateral root formation were rescued by sodium hydrosulfide (NaHS, an H_2S donor). However, No additive effects were observed when naphthalene acetic acid (NAA) was co-treated with NaHS (lower than 10 mM) in the induction of lateral root formation. Subsequent work revealed that a treatment with NAA or NaHS could simultaneously induce transcripts of *SlDES1*, DES activity and endogenous H_2S contents, and thereafter the stimulation of lateral root formation. It was further confirmed that H_2S or HS^-, not the other sulfur-containing components derived from NaHS, was attributed to the stimulative action. The inhibition of lateral root formation and decreased of H_2S metabolism caused by an H_2S scavenger hypotaurine (HT) were reversed by NaHS, but not NAA. Molecular evidence revealed that both NaHS- or NAA-induced modulation of some cell cycle regulatory genes, including the up-regulation of *SlCDKA;1*, *SlCYCA2;1*, together with simultaneous down-regulation of *SlKRP2*, were differentially reversed by HT pretreatment. To summarize, above results clearly suggested that H_2S might, at least partially, act as a downstream component of auxin signaling to trigger lateral root formation.

1. Introduction

Studies on nitric oxide (NO) and heme oxygenase-1 (HO-1)-mediated production of carbon monoxide (CO) have highlighted the possible crosstalk between gaseous signaling molecules in biology. Further evidence suggested that another novel endogenous gasotransmitter, hydrogen sulfide (H_2S), also plays important physiological functions alongside NO and CO in animal systems (Wang, 2003). For example, anti-inflammatory, metabolic, vasorelaxant, neuroprotective and psychological functions of H_2S have been described among others (Wang, 2002).

In animals, H_2S is at least partially endogenously generated from L-cysteine (L-Cys) via four pyridoxal-5′-phosphate (PLP)-dependent enzymes, especially cystathionine β-synthase (CBS; EC 4.2.1.22) hydrolyzing L-Cys to L-serine, and cystathionine γ-lyase (CSE; EC 4.4.1.1) hydrolyzing L-Cys to produce H_2S, pyruvate and ammonia (Wang, 2003; Li et al., 2011). Also, H_2S is generated in plants by L-cysteine desulfhydrase (DES), which shares homolog with CSE in animals (Papenbrock et al., 2007; Álvarez et al., 2010). Previous results discovered that elevated H_2S level of 0.25 and 0.5 μl l^{-1} exhibited promoting effects on both mRNA and protein levels of cysteine-synthesizing and degrading enzymes (Riemenschneider et al., 2005). Recently, a *Brassica napus DES* gene was isolated, and its transcript level induced by the NO-releasing compound (sodium nitroprusside, SNP) was also characterized (Xie et al., 2013).

Development of plant root system, including lateral root formation and adventitious rooting, is crucial for plant life. Lateral root formation, which exclusively orients from pericycle founder cells, is an important determinant of plant root architecture

Abbreviations: CBS, cystathionine β-synthase; CDK, Cyclin Dependent Kinase; CO, carbon monoxide; CORM-2, carbon monoxide-releasing molecule-2; CSE, cystathionine γ-lyase; Cys, cysteine; DES, L-cysteine desulfhydrase; EDTA, ethylene diamine tetraacetic acid; HO-1, heme oxygenase-1; H_2S, hydrogen sulfide; HT, hypotaurine; KRP, Kip-Related Protein; LR, lateral root; LRI, lateral root initiation; LRP, lateral root primordia; NAA, naphthalene acetic acid; NaHS, sodium hydrosulfide; NO, nitric oxide; NPA, N-1-naphthylphthalamic acid; PAG, DL-propargylglycine; PLP, pyridoxal-5′-phosphate; ThT, thiotaurine.

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(Casimiro et al., 2001). In fact, lateral root formation is largely influenced by plant hormones, especially auxin, and environmental factors, such as water and nutrient availability (Casimiro et al., 2001; Overvoorde et al., 2010). Since auxin represents a key regulator of lateral root development and first described, there has always been a close link between auxin and other phytohormones in the induction of lateral root development (De Smet et al., 2010; Casimiro et al., 2003).

Molecular evidence reveals that auxin transported from the shoot is necessary for LR formation (Casimiro et al., 2001), and the activation of the cell cycle sensitizes pericycle cells for auxin-induced lateral root initiation (LRI) (De Smet et al., 2010). In plants, auxin induces LRI by influencing the expression profiles of cell cycle regulatory genes, Cyclin Dependent Kinases (CDK) genes, such as *CDKA;1* in *Arabidopsis* (Casimiro et al., 2003). Moreover, activation of the CDKA/cyclin D complex at G1/S transition results in phosphorylation of the retinoblastoma protein (Boniotti and Gutierrez, 2001). However, one of categories of the CDK-inhibitory proteins, the Kip-Related Proteins (KRPs), is involved in inactivation of CDK/cyclin complexes (De Veylder et al., 2001). For instance, the expression level of *AtKRP2* is low in the pericycle when roots are treated with auxin, while the expression level is high upon N-1-naphthylphthalamic acid (NPA; an inhibitor of basipetal auxin efflux) administration (Casimiro et al., 2003). Previous results showed that regulation of auxin-dependent cell cycle regulatory genes encoding *CYCA2;1*, *CYCA3;1*, *CYCD3;1*, *CDKA1* and *KRP2*, was dependent on NO signaling in tomato seedlings, validating the hypothesis that the modulation of cell cycle regulatory genes by NO is involved in G1-to-S phase transition during LRI in tomato roots (Correa-Aragunde et al., 2006). Stirringly, researchers recently reported the protective effect of H_2S on counteracting oxidative stress in plants (Li et al., 2012; Wang et al., 2012; Hu et al., 2014). Besides, H_2S was found to be involved in root development (Zhang et al., 2009) and regulation of stomatal aperture (García-Mata and Lamattina, 2010). However, the molecular events involved in H_2S signaling transduction and lateral root formation are poorly understood.

The depletion of endogenous auxin by NPA is considered as a useful tool to gain a good insight into the signaling transduction pathways responsible for lateral root formation (Casimiro et al., 2001). The crosstalk between endogenous HO-1/CO and H_2S during adventitious rooting was previously proposed (Lin et al., 2012). In this context, we extended our former observations, demonstrating that NaHS is able to boost the induction of lateral root formation in auxin-depleted tomato seedlings. Most importantly, we further revealed that a linearity may exist in DES-mediated H_2S signaling downstream of auxin to trigger lateral root formation by the modulation of cell cycle regulatory genes.

2. Materials & methods

2.1. Chemicals

All chemicals were obtained from Sigma–Aldrich (St Louis, MO, USA) unless stated otherwise. Naphthalene acetic acid (NAA) was used at 100 nM. N-1-naphthylphthalamic acid (NPA), from Chem Service, was used as an inhibitor of basipetal auxin efflux at 200 nM (Casimiro et al., 2001). Sodium hydrosulfide (NaHS) was used as the H_2S donor. Hypotaurine (HT) was regarded as an H_2S scavenger at 200 μM. The concentrations of the above-mentioned chemicals were determined in pilot experiments from which the significant responses were obtained.

Other chemicals, including Na_2S, Na_2SO_4, Na_2SO_3, $NaHSO_4$ and $NaHSO_3$, were purchased from Shanghai Medical Instrument, Co., Ltd., China National Medicine (Group), Shanghai, China.

2.2. Plant materials, growth conditions and treatments

Commercially available seeds of tomato (*Solanum lycopersicum* L. cv Jiangshu 14) were surface-sterilized and rinsed extensively in distilled water. After soaking overnight, seeds were germinated on filter papers imbibed in distilled water at 25 ± 1 °C in the darkness. 2-d-old seedlings were then transferred to an illuminating incubator and maintained at 25 ± 1 °C with a 14-h photoperiod at 200 μmol m^{-2} s^{-1} intensity.

After growing for another day, the selected identical seedlings with radicals 2–3 mm were incubated with 4 ml of the various solutions as indicated for the indicated time points. Afterward, the photographs were taken. The number of lateral root (LR) and the length of all LRs (>1 mm) per seedling, as well as LR density (the number of LR per cm primary root; LRs·cm^{-1}) were quantified with Image J software. LR primordia (LRP) per seedling were observed after 1 d of treatments by root squash preparations and quantified by a light microscope (model Stemi 2000-C; Carl Zeiss, Germany). According to the previous method (Correa-Aragunde et al., 2006), the root apical meristems of tomato seedlings at the indicated time points were cut off and the shoots were removed by cutting below the root-shoot junction in order to obtain samples of only lateral root-inducible segments for the corresponding biochemical and molecular determination.

2.3. Measurement of endogenous H_2S contents

Endogenous H_2S content was determined by the formation of methylene blue from dimethyl-*p*-phenylenediamine in H_2SO_4 according to the methods described previously (Zhang et al., 2009), with some modifications. Samples (0.1 g) were ground and extracted in 5 ml 50 mM phosphate buffer solution (pH 6.8) containing 0.1 M EDTA and 0.2 M ascorbic acid (AsA). The homogenate was mixed with 0.5 ml 1 M HCl to release H_2S, and H_2S was then absorbed in a 1% (w/v) zinc acetate (0.5 ml) trap located at the bottom of the test tube. After 30 min of reaction, the trap was added with 0.3 ml 5 mM dimethyl-*p*-phenylenediamine dissolved in 3.5 mM H_2SO_4. Afterward, 0.3 ml 50 mM ferric ammonium sulfate in 100 mM H_2SO_4 was injected into the trap. The amount of H_2S in zinc acetate traps was determined colorimetrically at 667 nm after leaving the mixture for 15 min at room temperature. Blanks were prepared by the same procedures without the zinc acetate solution and known concentrations of Na_2S solution were used to make the calibration curve.

2.4. Enzyme activity measurement for DES

According to the previous method (Xie et al., 2013), the activity of DES was determined by monitoring the release of sulfide from L-Cys in a total volume of 3 ml containing 2.5 mM DL-dithiothreitol (DTT), 0.8 mM L-Cys, 100 mM Tris–HCl (pH 9.0), and enzyme extract. The reaction was initiated by the addition of L-Cys. After incubation at 37 °C for 15 min, the reaction was terminated by adding 300 μl of 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 300 μl 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 N HCl. The formation of methylene blue was determined at 670 nm. The DES enzymatic activity was calculated by comparison to a standard curve of Na_2S.

2.5. Real-time RT-PCR analysis

Total RNA was isolated from 100 mg fresh-weight samples by using Trizol reagent (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instruction. DNA-free total RNA (2 μg) from different treatments was used for cDNA synthesis in a 20-μL