In Site Bioimaging of Hydrogen Sulfide Uncovers Its Pivotal Role in Regulating Nitric Oxide-Induced Lateral Root Formation

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Abstract
Hydrogen sulfide (H2S) is an important gasotransmitter in mammals. Despite physiological changes induced by exogenous H2S donor NaHS to plants, whether and how H2S works as a true cellular signal in plants need to be examined. A self-developed specific fluorescent probe (WSP-1) was applied to track endogenous H2S in tomato (Solanum lycopersicum) roots in site. Bioimaging combined with pharmacological and biochemical approaches were used to investigate the cross-talk among H2S, nitric oxide (NO), and Ca2+ in regulating lateral root formation. Endogenous H2S accumulation was clearly associated with primordium initiation and lateral root emergence. NO donor SNP stimulated the generation of endogenous H2S and the expression of the gene coding for the enzyme responsible for endogenous H2S synthesis. Scavenging H2S or inhibiting H2S synthesis partially blocked SNP-induced lateral root formation and the expression of lateral root-related genes. The stimulatory effect of SNP on Ca2+ accumulation and CaM1 (calmodulin 1) expression could be abolished by inhibiting H2S synthesis. Ca2+ chelator or Ca2+ channel blocker attenuated NaHS-induced lateral root formation. Our study confirmed the role of H2S as a cellular signal in plants being a mediator between NO and Ca2+ in regulating lateral root formation.

Introduction
Hydrogen sulfide (H2S) is considered as the third gasotransmitter in medical biology after nitric oxide (NO) and carbon monoxide (CO) [1]. The clinical relevance of H2S as a signaling molecule has been highly appreciated in mammals [2–4]. In mammals and bacteria, two multifunctional pyridoxal 5’-phosphate (PLP)-dependent enzymes, cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS), are demonstrated to be the major sources of endogenous H2S production [5]. H2S can also be produced by 3-mercaptopyruvate sulfurtransferase (3SMT) along with cysteine aminotransferase (CAT) in brain [6]. In plants H2S is considered to be a by-product from cysteine desulfuration catalyzed by γ-cysteine desulphhydrase (LCD, EC4.4.1.1) and β-cysteine desulphhydrase (DCD, EC4.4.1.15), both of which belong to the PLP protein family [7]. Both genes (LCD and DCD) have been characterized in Arabidopsis [8]. A recent study suggests that O-acetylserine(thiol)lyase (OASTL), a cysteine synthase-like protein, also possesses the activity of cysteine desulfuration [9].

The detailed studies in the biological role of H2S in plants are very limited compared to those in mammals [10]. Exogenous application of NaHS, a H2S donor, confers the tolerance of plants to oxidative stress [11–20]. H2S is also proposed to be involved in regulating stomatal closure [21–23], photosynthesis [24], and seed germination [25,26]. However, the major challenge of identifying the nature of H2S as a plant signaling molecule is the lack of data of tracking endogenous H2S in site in plants. The traditional approaches of determining H2S from biological tissues include colorimetric in-tube assay [27], sulfide electrode assay [21], and gas chromatography/mass spectrometry [28]. These methods require tissue pre-processing (e.g. homogenization), leading to the unavoidable loss of H2S. Therefore, in the last two years, a group of chemists have developed some specific fluorescent probes for capturing and tracking H2S in vivo through instantaneous bioimaging [29], which show great potential for revealing the biological behavior of H2S. However, the application of these probes in biological study, especially for plants, is rarely reported.

NO-modulated lateral root formation is a well characterized signaling event in plants [30,31]. NO can modulate the expression of cell cycle regulatory genes (e.g. CYCD and CDK4), which are essential for lateral root initiation from primordia [32,33]. The key of lateral root formation is lateral root emergence, which is a process that new primordia break through the outer layer cells from primary roots [33]. Auxin has been confirmed as a regulatory
star in this process by positively regulating Auxin Response Factors (ARFs) (e.g. ARF4/7/19) [33–35] and endogenous NO [30]. In addition, a recent study suggests that cytosolic Ca\(^{2+}\) combined with its sensor calmodulin (CaM) acts downstream of NO during lateral root formation [36]. The auxin-NO signaling event has been considered to play a vital role in regulating lateral root growth, but the detailed regulatory network needs to be illuminated by mining novel components. The biological interplay among H\(_2\)S, NO, and Ca\(^{2+}\) has been well investigated in mammals [37,38]. Thus, it is of interest to study whether and how H\(_2\)S acts as a gasotransmitter in NO signaling cassette for the regulation of lateral root formation. WSP-1 (Washington State Probe-1) is a self-developed fluorescent probe for detecting H\(_2\)S within living cells with high-sensitivity and selectivity [39,40]. In the present study, tracking and bioimaging endogenous H\(_2\)S with WSP-1 in plant cells provide direct evidence that H\(_2\)S is a novel regulator in NO-modulated lateral root formation. This study confirmed the role of H\(_2\)S as a cellular signal molecule in plant signaling events.

**Materials and Methods**

**Plant culture and treatments**

Tomato (*Solanum lycopersicum*, Suhong2003 wild type) seeds were surface-sterilized with 1% NaClO for 10 min followed by washing with distilled water. Seeds were germinated in Petri dishes on filter papers imbibed with distilled water. Then the selected identical seedlings with radicles 1.5 cm were transferred to another Petri dish containing various treatment solutions in a chamber with a photosynthetic active radiation of 200 \(\mu\)mol/m\(^2\)/s, a photoperiod of 12 h, and the temperature at 25 ± 1°C.

SNP (sodium nitroprusside) and GSNO (S-Nitrosoglutathione) as NO donors were applied at concentrations of 0.05–0.4 mM and 2 mM, respectively. The 0.1 mM of cPTIO [2-(4-carboxy-2-phenoxy-4,5,5-tetramethylimidazoline-1-oxyl-3-oxide)] was applied as NO scavenger. The 0.2–2 mM of NaHS (sodium hydrosulphide) was applied as H\(_2\)S donor. PAG (piripropargylglycine) (0.1 mM) and HT (hypotaurine) (0.1 mM) are H\(_2\)S biosynthesis inhibitors and H\(_2\)S scavengers, respectively. Na\(_2\)SO\(_4\), Na\(_2\)SO\(_3\), and NaHSO\(_3\) at the concentration of 2 mM are applied as NaHS homologues to identify the specificity for NaHS as H\(_2\)S donor. EGTA [ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid] (0.1 mM) and LaCl\(_3\) (0.5 mM) are Ca\(^{2+}\) chelators and Ca\(^{2+}\) channel blockers, respectively. The treatment solution is composed of different chemicals as mentioned above with 488/525 nm and an excitation/emission filter set (ECLIPSE, TE2000-S, Nikon).

The cytosolic Ca\(^{2+}\) was visualized using Ca\(^{2+}\)-sensitive fluorescent probe Fluo-3 AM. Similarly, the probe was loaded to roots in 20 mM Hepes-NaOH (pH 7.5) buffer solution containing 15 \(\mu\)M of Fluo-3 AM in darkness at 25°C for 30 min. Then, the fluorescent image was captured using a fluorescence microscope with an excitation wavelength of 488/525 nm and an emission filter set (ECLIPSE, TE2000-S, Nikon).

The relative fluorescent density of the fluorescent images was analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc.).

**Analysis of transcripts**

Semi-quantitative RT-PCR was performed with the total RNA for the transcription analysis. Total RNA was extracted from root samples using Trizol (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed at 42°C in a 25 \(\mu\)l reaction mixture including 3 \(\mu\)g of RNA, 0.5 \(\mu\)g of oligo(T) primers, 12.5 nmol of dNTPs, 20 units of RNase inhibitor and 200 units of MLV. The first cDNA was used as a template for PCR to analyze the transcripts of genes. The total 25 \(\mu\)l of PCR reaction mixture in Tris-\(\mathrm{HCl}\) buffer (pH 8.3, 10 mM) were composed of 1 \(\mu\)l of normalized cDNA template, 10 pmol of sense primer, 5 nmol of dNTPs, 32.5 nmol of Mg\(^{2+}\), and 0.5 U of Tag DNA polymerase. PCR was performed as follows: 95°C for 3 min, 30 cycles at 94°C for 30 s, different annealing temperature for 30 s, 68°C for 1.5 min, and a final extension step at 68°C for 7 min. All the tested genes were retrieved from tomato genome (Sol Genomics Network, http://solgenomics.net/organism/Solanum_lycopersicum/genome) or NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). The following primers and annealing temperatures were used to amplify the genes: *CYCD3;1*, sense (Sol accession number SGN-U583476), sense 5'-TTACTTTTCTTTGTTGATATATTTAGGAGG-3' and antisense 5'-CTAGTTAATGCAGAAGAACGATACG-3' (amplifying a 526 bp fragment, 45°C); *CDKA1* (Sol accession number SGN-U572518), sense 5'-GTTATTTGTCTTCTCAGATAGTTCTT-3' and antisense 5'-CTGGTGTTGACACTGTCATGCTAAGG-3' (amplifying a 477 bp fragment, 45°C); *ARF4* (Sol accession number SGN-U569639), sense 5'-ATGTTGTGGCTTGTGGTGC-3' and antisense 5'-CTCCGTTGAGATGCTCC-3' (amplifying a 521 bp fragment, 45°C); *ACTIN (Sol accession number EF212543.1), sense 5'-TCAGAAGTTAAGGGCC-3' and antisense 5'-GACGAGGAACGAAATAAG-3' (amplifying a 373 bp fragment, 45°C); *CaM* (Sol accession number SGN-U580344), sense 5'-TGAATCTTGATGGCAACGAGG-3' and antisense 5'-TACTGCTGACCCGCTT-3' (amplifying a 338 bp fragment, 50°C); *Actin* (Sol accession number SGN-U580422), sense 5'-AGAAGCTAGT GAGCCCTCCAGATGG-3' and antisense 5'-TTAATCTTTATTGTGCTTAGGAGC-3' (amplifying a 272 bp fragment,
The relative abundance of Actin was used as an internal standard.

Statistical analysis

Each result was presented as the mean of at least three replicated measurements. The significant differences between treatments were statistically evaluated by standard deviation and one-way analysis of variance (ANOVA) using Microsoft Excel 2010 (Microsoft Corporation, USA). The data between different treatments were compared statistically by ANOVA, followed by F-test if the ANOVA result is significant at \( P < 0.05 \).

Results

NO induced lateral root formation

NO donors were used to assess the regulatory effect of NO on tomato lateral root formation. The NO donor SNP stimulated lateral root growth in both dose- and time-dependent manner (Figure 1a and b). On the contrary, treatments with NO scavenger cPTIO alone remarkably inhibited lateral root formation (Figure 1c and d). Another NO donor GSNO could stimulate lateral root formation as well (Figure 1c and d). However, the addition of cPTIO could abolish the promoting effect of both NO donors on lateral roots (Figure 1c and d). These results confirmed the promoting effect of NO on tomato lateral root formation.

WSP-1 can be used for the selective detection of H\(_2\)S in tomato root

In order to investigate the potential of WSP-1 in the detection of H\(_2\)S in plant system, tomato roots treated with NaHS at different concentrations (0.2, 0.4, and 2 mM) were loaded with WSP-1. These concentrations were within the range of those that have been used to elicit physiological responses of H\(_2\)S in plants \([19,20,24,42]\). The strong fluorescent density was observed in roots in the presence of NaHS in a dose-dependent manner (Figure 2a and b). This result was similar to the detection of H\(_2\)S with WSP-1 in mammalian system \([40]\). To further identify the selectivity of WSP-1 probe for H\(_2\)S, several kinds of reactive sulfur species (e.g. sulfane sulfur, inorganic sulfur derivatives, polysulfide, sulenic acid derivative, and S-nitrosothiol) were detected in solution. As expected, compared to the significant fluorescence signal yielded from the reaction of WSP-1 with NaHS solution, other tested reactive sulfur species did not lead to significant fluorescence increase (Figure 2c). Analysis of fluorescent density showed that several sulfur compounds (e.g. NaHSO\(_4\), Na\(_2\)SO\(_4\), Na\(_2\)S\(_2\)O\(_4\), and sulfonamide) had little fluorescence, but their values are too small as compared with NaHS (Figure 2d). These results suggested that WSP-1 could be used for the selective detection of endogenous H\(_2\)S in tomato roots.

Figure 1. NO induced lateral root formation in both dose- and time-dependent manner. (a) The roots of three-day old tomato seedlings were exposed to 0, 0.05, 0.1, 0.2, and 0.4 mM of SNP for 6 days for the measurement of the lateral root number. (b) The roots of three-day old tomato seedlings were exposed to 0.2 mM of SNP for 3–8 days for the measurement of the lateral root number. (c–d) The roots of three-day old tomato seedlings were exposed to cPTIO (0.1 mM), SNP (0.2 mM) + cPTIO (0.1 mM), GSNO (0.5 mM), and GSNO (0.5 mM) + cPTIO (0.1 mM) for 6 days for photographing root phenotype (c) and measuring the lateral root number (d). Vertical bars represent standard deviations of the mean (n = 6). Asterisk indicates that mean values are significantly different (\( P < 0.05 \)) between the treatment and the control (a, b) or between different treatments (d).

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Endogenous H$_2$S was involved in lateral root formation

Next, we investigate the link between lateral root emergence and endogenous H$_2$S. The bright green fluorescence of WSP-1 was clearly linked to the primordium initiation and lateral root emergence (Figure 3a). In a cross section of primary roots with lateral root primordium, the fluorescence of WSP-1 was clearly concentrated in the region of primordium (Figure 3b). To further ascertain the role of H$_2$S in regulating lateral root formation, we measured lateral root number by altering endogenous H$_2$S level in roots. Both H$_2$S biosynthesis inhibitor PAG and H$_2$S scavenger HT induced significant decreases in lateral root number (Figure 3c and e). However, the treatment with NaHS significantly enhanced lateral root number compared to the control (Figure 3c and e). Treatments with several homologues of Na or S (e.g. Na$_2$SO$_4$, Na$_2$SO$_3$, and NaHSO$_3$) did not affect lateral root number compared to the control (Figure 3c and e). These effects could be observed in the emergence of lateral root primordia as well (Figure 4h and i).

NO induced lateral root formation by regulating endogenous H$_2$S generation

To determine the role of H$_2$S in NO-induced lateral root formation, we first investigated the effect of NO donors on the generation of endogenous H$_2$S in roots detected by WSP-1. Treatments with NO scavenger cPTIO induced a significant decrease in endogenous H$_2$S level in roots (Figure 4a and b). Two NO donors (SNP and GSNO) stimulated the generation of endogenous H$_2$S, which could be blocked by the addition of cPTIO (Figure 4a and b). SNP stimulated the generation of endogenous NO and H$_2$S in dose-dependent manners (Figure 4c).

Since NO was able to stimulate H$_2$S generation in tomato roots, it is essential to know whether NO-governed H$_2$S generation is able to manipulate lateral root formation. The addition of NaHS reversed the inhibitory effect of cPTIO on lateral root formation (Figure 4d and f). Furthermore, the addition of PAG abolished the stimulatory effect of SNP on lateral root formation (Figure 4e and g). These effects could be observed in the emergence of lateral root primordia as well (Figure 4h and i). Then we tested the effect of the interplay between NO and H$_2$S on the expression of four genes related to lateral root emergence, including two cell cycle regulatory genes (Cyclin D3;1 and CDK4;1) and two ARF genes.
As expected, both SNP and NaHS could stimulate the expression of these genes while the addition of PAG could block the stimulatory effect of SNP (Figure 4j and k).

$\text{Ca}^{2+}/\text{CaM1}$ acted downstream of H$_2$S in NO-induced lateral root formation

By using Fluo-3 AM to detect cytosolic Ca$^{2+}$ in tomato roots, we found that both SNP and NaHS stimulated the accumulation of cytosolic Ca$^{2+}$ in roots while the addition of PAG reversed the
Figure 4. NO induced lateral root formation by regulating endogenous H$_2$S generation. (a–b) The roots of three-day old tomato seedlings were exposed to cPTIO (0.1 mM), SNP (0.2 mM), SNP (0.2 mM)+cPTIO (0.1 mM), GSNO (0.5 mM), and GSNO (0.5 mM)+cPTIO (0.1 mM) for 3 days. Then, the roots were loaded with WSP-1 for fluorescent imaging (a) and the calculation of relative fluorescent density (b). Vertical bars represent standard deviations of the mean (n = 3). (c) The roots of three-day old tomato seedlings were exposed to 0, 0.05, 0.1, 0.2, and 0.4 mM of SNP for 3 days. Then, the roots were loaded with DAF-FM DA and WSP-1 for fluorescent imaging, respectively. (d–g) The roots of three-day old tomato seedlings were exposed to cPTIO (0.1 mM), cPTIO (0.1 mM) + NaHS (2 mM), SNP (0.2 mM), and SNP (0.2 mM) + PAG (0.1 mM) for 6 days for photographing root phenotype (d–e) and measuring lateral root number (f–g). (h–i) The roots of three-day old tomato seedlings were exposed to cPTIO (0.1 mM), cPTIO (0.1 mM) + NaHS (2 mM), SNP (0.2 mM), and SNP (0.2 mM) + PAG (0.1 mM) for 2 days for the measurement of lateral root primordia. Vertical bars represent standard deviations of the mean (n = 6). (j) The roots of three-day old tomato seedlings were exposed to SNP (0.2 mM), NaHS (2 mM), and SNP (0.2 mM)+PAG (0.1 mM) for 2 days for the analysis of genes transcripts. (k) Quantitative analysis of genes transcript levels under different treatment conditions. The data were obtained by densitometric analysis of the relative abundance of the transcripts with respect to the loading control Actin. Asterisk indicates that mean values are significantly different ($P<0.05$) between the treatment and the control. Actin was used for cDNA normalization. doi:10.1371/journal.pone.0090340.g004
Figure 5. Ca\(^{2+}\) acted downstream of H\(_2\)S in the NO-induced lateral root formation. (a–c) The roots of three-day old tomato seedlings were exposed to SNP (0.2 mM), NaHS (2 mM), and SNP (0.2 mM) + PAG (0.1 mM) for 4 days. Then, the roots were loaded with Fluo-3 AM for fluorescent imaging (a) and the calculation of relative fluorescent density (b). Vertical bars represent standard deviations of the mean (n = 3). The roots were also used for the analysis of the \textit{CaM1} transcripts (c). (d) Quantitative analysis of \textit{CaM1} transcript levels under different treatment conditions. The data were obtained by densitometric analysis of the relative abundance of the transcripts with respect to the loading control \textit{Actin}. (e–f) The roots of three-day old tomato seedlings were exposed to NaHS (2 mM), NaHS (2 mM) + La\(^{3+}\) (0.5 mM), and NaHS (2 mM) + EGTA (0.1 mM) for 6 days for photographing root phenotype (e) and measuring lateral root numbers (f). Vertical bars represent standard deviations of the mean (n = 6). (g) The roots of three-day old tomato seedlings were exposed to NaHS (2 mM), NaHS (2 mM) + La\(^{3+}\) (0.5 mM), and NaHS (2 mM) + EGTA (0.1 mM) for 2 days for the analysis of genes transcripts. (h) Quantitative of genes transcript levels under different treatment conditions. The data were obtained by densitometric analysis of of the relative abundance of the transcripts with respect to the loading control \textit{Actin}. Asterisk indicates that mean values are significantly different (P < 0.05) between different treatments. \textit{Actin} was used for cDNA normalization.

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stimulatory effect of SNP and NaHS (Figure 5a and b). The changes in the CaM1 expression showed similar patterns with cytosolic Ca2+ under the above treatments (Figure 5c and d).

Next, we determined the cross-talk between H2S and Ca2+ on lateral root formation. As expected, both Ca2+ channel blocker La3+ and Ca2+ chelator EGTA could abolish the stimulatory effect of NaHS on lateral root formation (Figure 5e and f) and the expression of CTD3;1, CDK1, ARF4, and ARF7 (Figure 5g and h).

Discussion

In plants, both NO and CO have been already identified as vital signaling molecules participating in an array of intrinsic signaling events [43,44]. But the biology of H2S in plants is not well understood. Many physiological changes in plants resulting from the exposure of exogenous H2S have been summarized by Lisjak et al. [10]. In order to identify whether H2S is a true cellular signal in plants, the in situ concentration and the locality of endogenous H2S in plants need to be determined [10]. In the present study, we overcome the obstacle of tracking endogenous H2S in plants in site. The endogenous H2S in tomato roots have been successfully detected in site by specific fluorescent probe WSP-1, which provides the direct evidence supporting the interplay among NO, H2S, and Ca2+ in regulating lateral root formation. Additionally, the Ca2+ release from multiple intracellular sources rather than extracellular Ca2+ influx [38] can be proposed that Ca2+ is required for root organogenesis by functioning probably both upstream and downstream of NO. However, whether and how NO induces H2S generation by regulating LCD or DCD in plants would be an interesting topic to be investigated further.

H2S regulates various physiological processes by targeting KATP channels in mammals. H2S is an endogenous opener of KATP channels by interacting with Cys6 and Cys26 in rνSUR1 (Sulphophyrlrea Receptor 1) subunit of KATP channel complex through -sulfhydration [47]. In plants, MRP5 (Multidrug Resistance-associated Protein 5) is a homologue of mammalian SUR [48]. In Arabidopsis, AtMRP5 not only works as an auxin conjugate transporter in modulating lateral root formation but also acts as a regulator of Ca2+ channel in regulating guard cell signaling [49,50]. MRP5 can be possibly regulated by H2S due to the fact that the treatment with Gli (glibenclamide), a typical SUR inhibitor, blocks NaHS-induced stomatal closure [21]. Thus, whether H2S regulates Ca2+ signaling through -sulfhydration of MRP5 during lateral root formation needs to be further investigated. In addition, NO may induce Ca2+ influx by post-transcriptionally modified Ca2+ channel proteins directly [51,52]. Therefore, it is possible that NO may act parallelly with H2S in inducing cytosolic Ca2+.

The biology of H2S in mammals has been significantly advanced, but mining the signaling role of H2S in plants is just...
emerging. Based on our observation, a model could be proposed of the crosstalk between NO and H₂S in regulating lateral root formation (Figure 6). The current regulatory network involving NO, H₂S, and Ca²⁺ in regulating later root formation is largely unknown, but our data suggest that H₂S acting between NO and Ca²⁺ is functioning as a small intercellular signaling pathway in the complicated network for the regulation of lateral root formation. However, a possible feedback mechanism between NO and H₂S maybe operating for the induction of lateral root formation. Our present study is the first report of bioimaging endogenous H₂S in plants, which provides the direct evidence of identifying H₂S as a true cellular signaling molecule in regulating lateral root formation. These results not only propose a novel component in lateral root signaling but also shed new light on the study of the biological role of H₂S in plants.

Author Contributions
Conceived and designed the experiments: J.C.Z-Q. S.L-J.G. Performed the experiments: J.C.Y-J.L. Analyzed the data: J.C.Y-J.L. Contributed reagents/materials/analysis tools: M.X. Wrote the paper: J.C.Y-J.L. L-G.Z. F.H.

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