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Hydrogen sulfide toxicity inhibits primary root growth through the ROS-NO pathway

Ping Zhang^{1,2}, Qiong Luo¹, Ruling Wang¹ & Jin Xu¹

High concentrations of hydrogen sulfide (H₂S) are toxic to plants and inhibit their growth. Previous research indicated that high concentrations of H₂S modulate the root system architecture (RSA) by affecting auxin transport; however, the signaling pathway underlying this process remains unclear. Here, we investigated the effects of exogenous sodium hydrosulfide (NaHS), an H₂S donor, on primary root (PR) growth in *Arabidopsis* using pharmacological, physiological, and genetic approaches. H₂S toxicity repressed PR growth by triggering a signal transduction pathway involving reactive oxygen species (ROS) accumulation, *MITOGEN-ACTIVATED PROTEIN KINASE 6* (MPK6) activation, and nitric oxide (NO) production. Respiratory burst oxidase homolog mutants and an NO synthase mutant were less sensitive to NaHS, suggesting that both ROS and NO mediate the inhibitory effects of H₂S on PR growth. We found that exogenous H₂S-activated ROS production was required for NO generation and that MPK6 mediated H₂S-induced NO production. MPK6 was shown to function downstream of ROS and upstream of NO. Finally, we demonstrated that exogenous H₂S repressed the distribution of auxin and reduced the meristematic cell division potential in root tips, and NO was involved in this process.

Hydrogen sulfide (H₂S) is a colorless gas with a characteristic odor of rotten eggs. Low concentrations of H₂S improve the tolerance of plants to pathogens¹, osmotic stress, salt stress, heat shock, and heavy metal stresses^{2–6}. In plants, H₂S is predominantly produced by L-cysteine desulfhydrase (DES; EC 4.4.1.1)⁷. Endogenous H₂S plays a role in modulating plant growth and development, including seed germination, root organogenesis, stomatal closure, plant maturation and flower senescence^{8–11}. Although low concentrations of H₂S improve the tolerance of plants to abiotic and biotic stresses, high concentrations are toxic to plant growth. H₂S toxicity-induced primary root (PR) growth inhibition has been reported¹¹; however, the signaling pathway underlying H₂S toxicity-mediated root growth and development is still unclear.

Nitric oxide (NO) is a small gas molecule that mediates lateral root (LR) formation, adventitious root growth, and root hair development^{12,13}. Our previous work indicated that NO inhibits PR growth, whereas it promotes LR development¹⁴. In animals, several studies have identified possible crosstalk between H₂S and NO¹⁵. H₂S physiologically modifies the cysteines in a large number of proteins via S-sulfhydration. Thus, sulfhydration appears to be a physiological posttranslational modification of proteins¹⁶. H₂S increases NO production by inducing the S-sulfhydration of endothelial NO synthase (eNOS), promoting its phosphorylation, inhibiting its S-nitrosylation, and increasing eNOS dimerization (the activated form of eNOS)¹⁷. In plants, an interaction between H₂S and NO in modulating plant growth and development has been reported^{9,10,18}. H₂S promotes NO production and acts upstream of NO to modulate abscisic acid (ABA)-dependent stomatal closure¹⁰. H₂S acts upstream of indole-3-acetic acid (IAA) and NO to regulate root growth and development⁹; however, the signaling modulation mechanisms involved are largely unclear.

Mitogen-activated protein kinase (MAPK) cascades, which consist of MAPKKK (MEKK), MAPKK (MKK), and MAPK (MPK), are highly conserved signaling transduction pathways found in animals, plants and microbes^{19,20}. In plants, MAPK pathways are implicated in the regulation of growth and development and in responses to many environmental cues. The activation of MPKs alters their subcellular localization and their interactions with and phosphorylation of transcription factors, thereby reprogramming gene expression^{20,21}. *Arabidopsis* MPK3/6 are the most extensively studied MPKs in plants. Previous studies have revealed that

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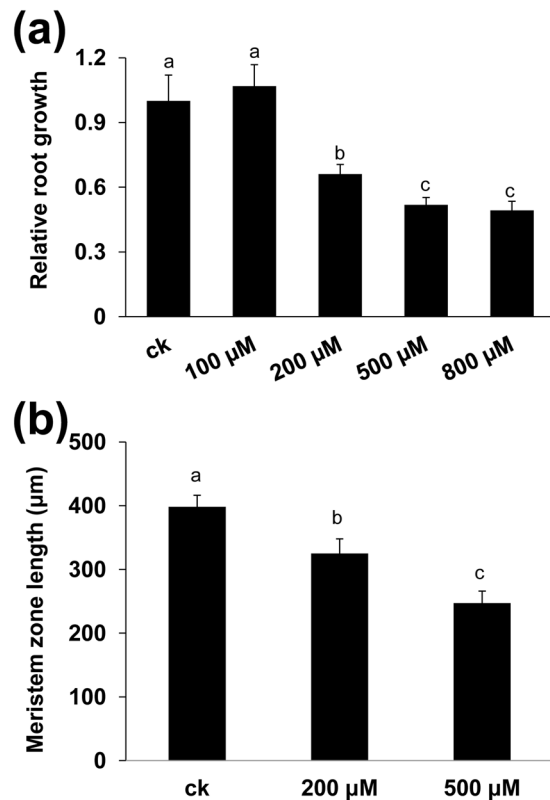


Figure 1. NaHS treatment inhibited PR growth. **(a,b)** Five-day-old wild-type seedlings grown in 1/2 MS medium were treated with 100–800 μM NaHS for 2 d, and **(a)** PR growth and **(b)** the length of the meristem zone were measured after treatment. ck, untreated control. n = 60. Error bars represent ± SD. Different letters indicate significantly different values ($P < 0.05$ by Tukey's test).

MPK3/6 modulate plant growth, development, and stress tolerance by interacting with the ABA, ethylene, jasmonate, phosphatidic acid, Ca^{2+} , and reactive oxygen species (ROS) pathways^{21–26}.

Root growth and development are largely influenced by plant hormones, especially auxin²⁷. Auxin is a central regulator of root formation. Auxin flux is essential for auxin to regulate of stem cell differentiation and root development^{28,29}. Auxin is an important phytohormone involved in controlling the balance between cell division and differentiation in the root meristem³⁰. H_2S -mediated root formation is alleviated by the IAA transport inhibitor N-1-naphthylphthalamic acid (NPA) and the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO), suggesting that both IAA and NO are involved in H_2S -mediated root system development⁹. Auxin-induced H_2S generation is involved in LR formation in tomato³¹. Recently, Jia *et al.*¹¹ showed that high levels of H_2S inhibit auxin transport and result in alterations in root system development by modulating the polar subcellular distribution of PIN proteins¹¹.

Another important pathway for regulating root system development independent of plant hormones is ROS signaling. The transcriptional regulation of ROS by the *UPBEAT1* (*UPB1*) transcription factor modulates root development by regulating cell proliferation and differentiation in root tips³⁰.

In this study, using pharmacological and genetic approaches, we analyzed the possible involvement of ROS, NO, and MPK6 in exogenous H_2S -mediated PR growth. Our results indicated that H_2S toxicity-inhibited PR growth via the ROS-MPK6-NO signaling pathway. The potential mechanisms involved in this process are discussed.

Results

H_2S toxicity inhibits PR growth by reducing the meristematic cell division potential. Five-day-old seedlings germinated on 1/2 MS medium were transferred to fresh medium with 0–800 μM NaHS, an H_2S donor, and PR growth was measured 2 d after transfer to determine how exogenous H_2S affects PR growth. With increasing NaHS concentrations of 200 μM to 800 μM, PR growth was inhibited by 34% to 51% (Fig. 1a, Fig. S1). To examine the inhibitory effects of H_2S on PR growth, we also measured the length of the meristem zone in H_2S -treated roots. As shown in Fig. 1b, the lengths of the meristematic zones decreased by 27% and 36.6% in roots treated with 200 μM and 800 μM exogenous NaHS, respectively. Because treatment with 500 μM NaHS induced an approximately 50% decrease in PR growth, we selected this concentration for use in subsequent experiments.

ROS are involved in H_2S toxicity-induced PR growth inhibition. Two independent signaling pathways regulate root growth and development: auxin signaling and the ROS signaling pathway³⁰. Jia *et al.*¹¹ found

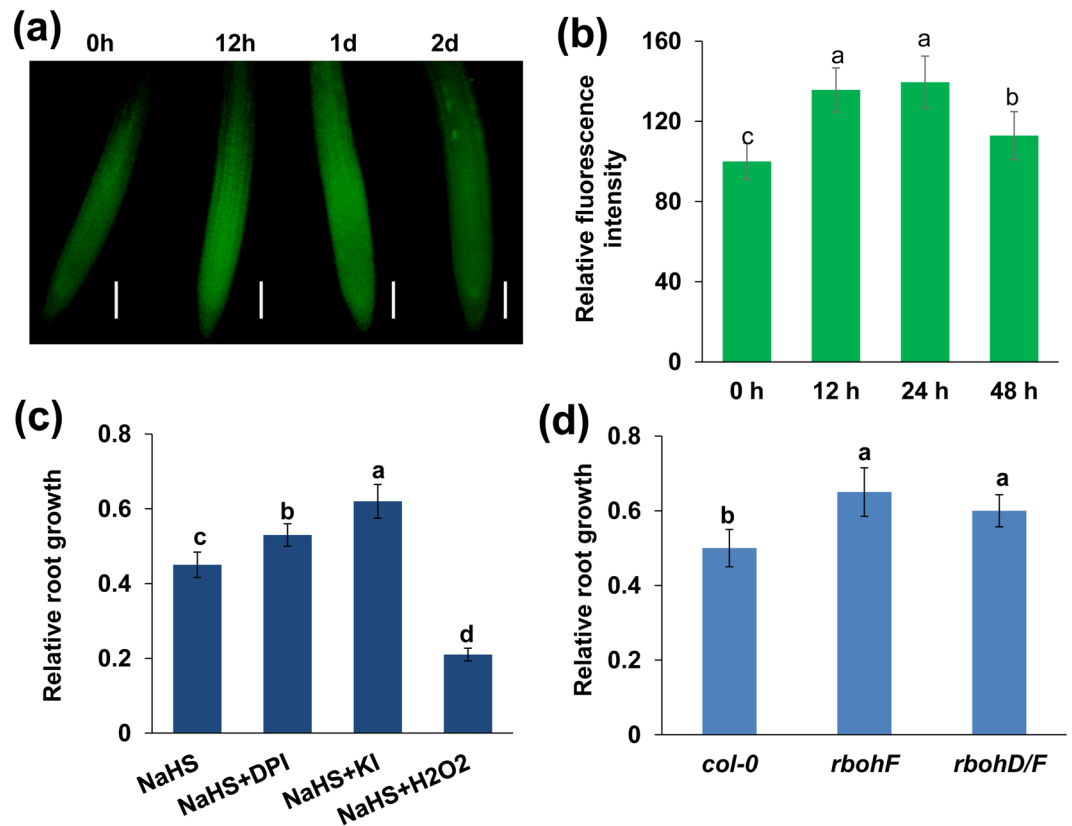


Figure 2. NaHS induces the accumulation of ROS. **(a,b)** Detection of ROS production in the roots of 5-d-old *Col-0* seedlings exposed to 500 μ M NaHS for periods of up to 2 d using the ROS-specific fluorescent probe DCFH-DA **(a)** and quantification of ROS-specific fluorescence intensities **(b)** in plants treated as described in **(a)**. The fluorescence intensity of the untreated roots was set to 100. Bars, 100 μ m. $n = 30$. **(c)** Relative root growth of *Col-0* seedlings treated with 500 μ M NaHS in the presence or absence of 1 μ M DPI, 1 mM KI, and 1 mM H₂O₂ for 2 d compared with untreated seedlings. **(d)** Relative root growth of *col-0*, *rbohF*, and *rbohD/F* seedlings treated with 500 μ M NaHS for 2 d compared with untreated seedlings. $n = 45$. Error bars represent \pm SD. Different letters indicate significantly different values ($P < 0.05$ by Tukey's test).

that H₂S toxicity inhibits auxin transport and results in alterations in root system development¹¹. To investigate whether ROS are also involved in H₂S toxicity-induced PR growth inhibition, we assessed ROS production in the roots using the ROS-specific-fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA). Exogenous H₂S increased ROS levels (Fig. 2a and b) in the roots; this increase was sustained for 24 h, after which ROS production gradually declined.

To further assess whether ROS participate in the H₂S-mediated inhibition of root growth, we next observed the effects of diphenylene iodonium (DPI), an inhibitor of NADPH oxidase, and potassium iodide (KI), an ROS scavenger, on the H₂S-mediated inhibition of root growth in the roots of wild-type plants. DPI and KI alleviated, whereas exogenous H₂O₂ increased the H₂S-induced inhibition of root growth (Fig. 2c, Fig. S2). To corroborate these pharmacological data, we further used the *Arabidopsis* ROS-biosynthesis-related respiratory burst oxidase homologue (Rboh) NADPH oxidase single and double mutants *rbohF* and *rbohD/F* and found that PR growth was 31% and 20% greater in *rbohF* and *rbohD/F* than in wild-type plants subjected to exogenous H₂S treatment (Fig. 2d and e). These data confirm the essential role of ROS in the modulation of root growth by H₂S.

NO is also required for the inhibition of PR growth by exogenous H₂S. NO is another important gaseous signaling molecule that mediates root development^{12,13}. We therefore investigated NO production in the roots using the NO-specific fluorescent probe, 4,5-diaminofluorescein diacetate (DAF-2DA). Similar to its effects on ROS production, exogenous H₂S triggered an increase in NO production in the roots; this increase was sustained for 24 h, after which NO production gradually declined (Fig. 3a and b). We further examined whether NO is involved in H₂S-mediated PR growth inhibition. As shown in Fig. 3c and Fig. S3, supplementation with the NO synthase (NOS) inhibitor N^G-nitro-L-Arg-methyl ester (L-NAME), or the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO) alleviated the NaHS-induced inhibition of root growth by 54.7% and 21.9%, respectively. Consistent with these pharmacological results, the NO biosynthesis-related *nia1-2/2-5* double mutant and the *noa1* mutant were less sensitive to exogenous H₂S treatment than were wild-type plants (Fig. 3d and e).

We found that supplementation with KI or DPI markedly inhibited NO production compared with that of the exogenous H₂S treatment alone in the roots (Fig. 4a and b), whereas supplementation with L-NAME did not alter

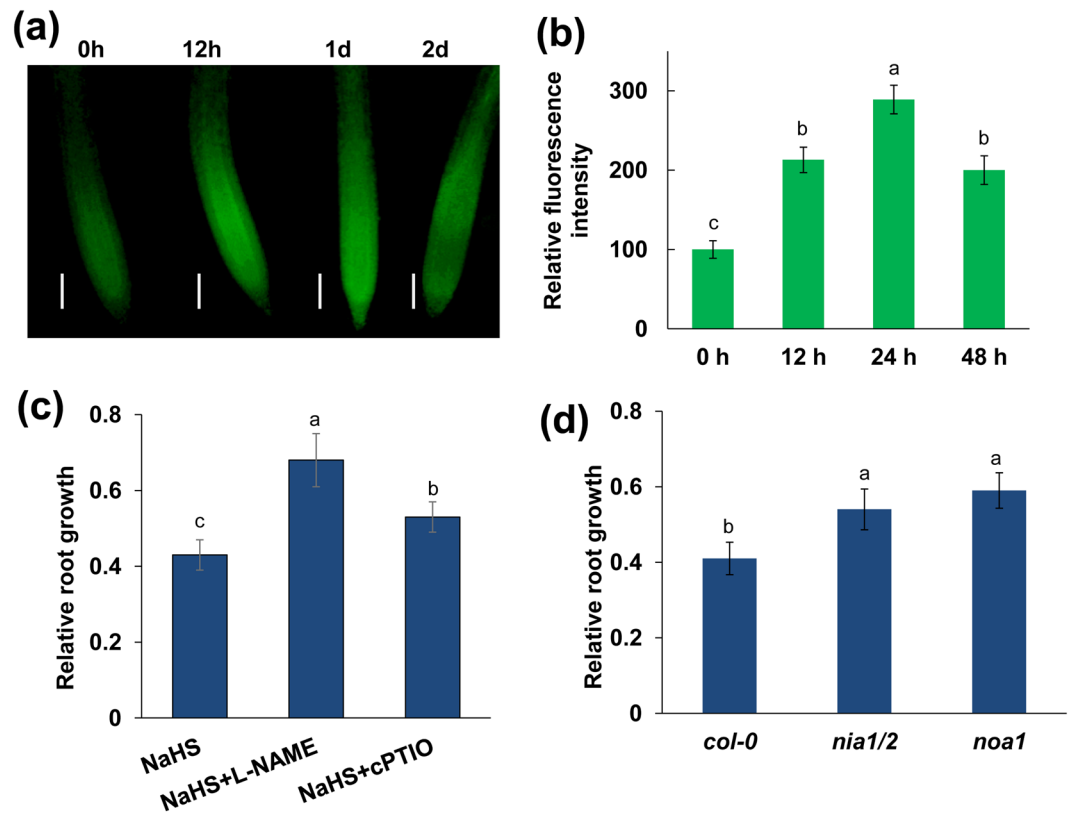


Figure 3. NO is involved in the NaHS-mediated inhibition of PR growth. **(a,b)** Detection of NO production in the roots of 5-d-old wild-type seedlings exposed to 500 μ M NaHS for periods of up to 2 d using the NO-specific fluorescence probe DAF-2 DA **(a)** and quantification of NO-specific fluorescence intensities **(b)** in plants treated as described in **(a)**. The fluorescence intensity of the untreated roots was set to 100. Bars, 100 μ m. $n = 30$. **(c)** Relative root growth of *Col-0* seedlings treated with 500 μ M NaHS in the presence or absence of 500 μ M L-NAME and 200 μ M cPTIO for 2 d compared with untreated seedlings. **(d)** Relative root growth of *Col-0*, *nia1/2*, and *noa1* seedlings treated with 500 μ M NaHS for 2 d compared with untreated seedlings. $n = 45$. Error bars represent \pm SD. Different letters indicate significantly different values ($P < 0.05$ by Tukey's test).

ROS levels in exogenous H₂S-treated roots (Fig. 4c and d). To further confirm the relationship between ROS and NO in the exogenous H₂S-mediated inhibition of root growth, we assessed the *rbohD/F*, *nia1-2/2-5*, and *noa1* mutants. We found that exogenous H₂S-induced NO production was abolished in the *rbohD/F* double mutant, while supplementation with H₂O₂ markedly induced NO production in both the *Col-0* and the *rbohD/F* double mutant (Fig. 4a and b). Meanwhile, H₂S-induced ROS production was similar in the *col-0*, *nia1-2/2-5*, and *noa1* lines (Fig. 4c and d). These data indicate that ROS is involved in exogenous H₂S-induced NO production in the roots.

We also found that supplementation with the NO donor SNAP completely reversed the insensitivity of the *rbohF*, *rbohD/F*, and *noa1* mutants to exogenous H₂S (Fig. 5a and b). Additionally, supplementation with H₂O₂ completely reversed the insensitivity of the *rbohF* and *rbohD/F* mutants to exogenous H₂S (Fig. 5a), but did not rescue the insensitivity of the *noa1* mutant (Fig. 5b). Taken together, these data suggest that exogenous H₂S-induced ROS production functions upstream of NO accumulation in the roots.

MPK6 mediates the inhibition of PR growth by exogenous H₂S. Previous studies have indicated that MAPK is involved in the ROS signaling pathway^{21,24,25}. To determine whether MAPK proteins mediate exogenous H₂S-induced PR growth inhibition, we first observed the effects of PD98059, an inhibitor of MAPK, on PR growth in wild-type *Col-0* plants. As shown in Fig. 6a and Fig. S4, supplementation with PD98059 alleviated the exogenous H₂S-induced inhibition of PR growth by 29.6%, suggesting that MAPK is involved in the modulation of PR growth by exogenous H₂S in *Arabidopsis*. To confirm the role of MAPK in the exogenous H₂S-mediated inhibition of root growth, we used the *Arabidopsis* mutant *mpk6*. As shown in Fig. 6b, the inhibition of PR growth by exogenous H₂S was alleviated by 37% in the *mpk6* mutant compared with *Col-0* seedlings. These data provide genetic evidence showing that MPK6 plays an important role in the inhibition of PR growth by exogenous H₂S. Together with the pharmacological data described above, these results clearly indicate that MPK6 is a positive regulator that mediates the exogenous H₂S-induced inhibition of PR growth in *Arabidopsis*.

MPK6 promotes NO production downstream of ROS in the exogenous H₂S-mediated inhibition of PR growth. The above results suggested that ROS, NO, and MPK6 are important intermediate signaling

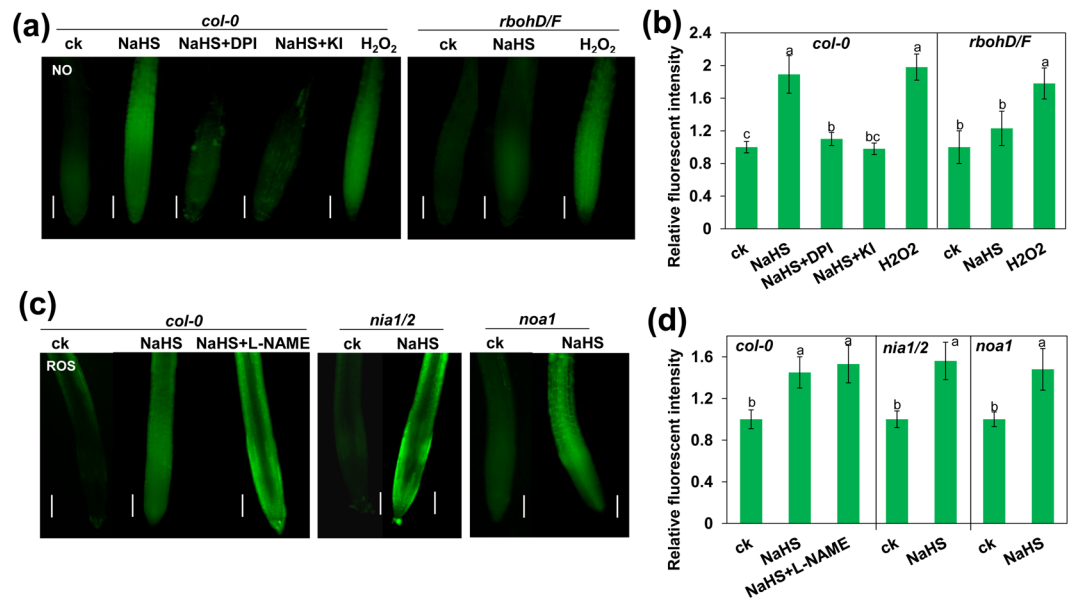


Figure 4. (a,b) Detection of NO production in the roots of 5-d-old wild-type *Col-0* and *rbohD/F* seedlings exposed to 500 μ M NaHS with or without 1 μ M DPI, 1 mM KI, and 1 mM H₂O₂ for 24 h using the NO-specific fluorescent probe DAF-2 DA (a) and quantification of NO-specific fluorescence intensities (b) in plants treated as described in (a). (c,d) Detection of H₂O₂ production in the roots of 5-d-old wild-type *Col-0*, *nia1/2*, and *noa1* seedlings exposed to 500 μ M NaHS with or without 500 μ M L-NAME for 24 h using the ROS-specific fluorescent probe DCFH-DA (c) and quantification of H₂O₂-specific fluorescence intensities (d) in plants treated as described in (c). The fluorescence intensity of the untreated roots was set to 100. Bars, 100 μ m. n = 30. ck, untreated control. Error bars represent \pm SD.

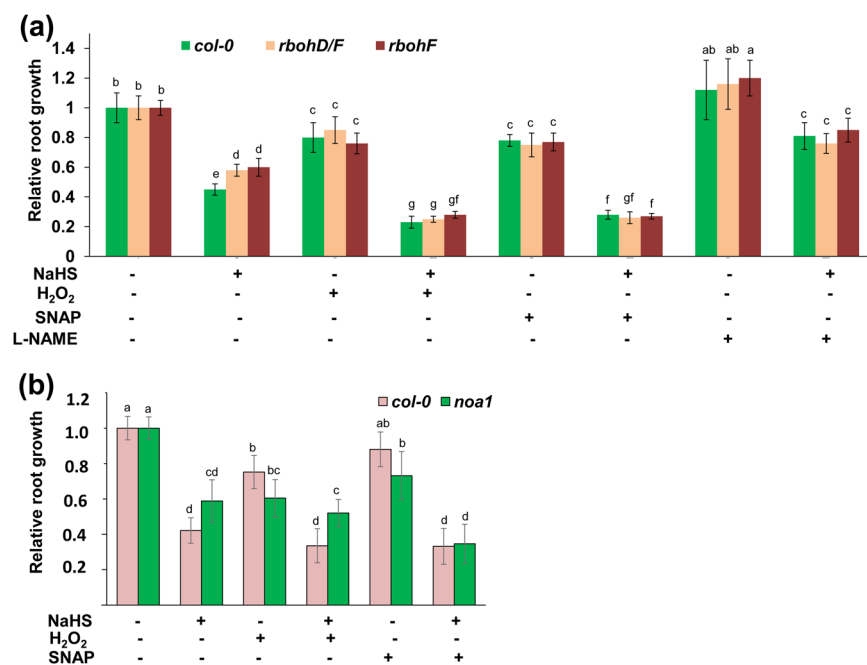


Figure 5. (a) Relative root growth of *Col-0*, *rbohF*, and *rbohD/F* seedlings exposed to 500 μ M NaHS with or without 1 mM H₂O₂, 100 μ M SNAP, and 500 μ M L-NAME for 2 d compared with untreated seedlings. (b) Relative root growth of *Col-0* and *noa1* seedlings exposed to 500 μ M NaHS with or without 1 mM H₂O₂ and 100 μ M SNAP for 2 d compared with untreated seedlings. n = 45. Error bars represent \pm SD. Different letters indicate significantly different values ($P < 0.05$ by Tukey's test).

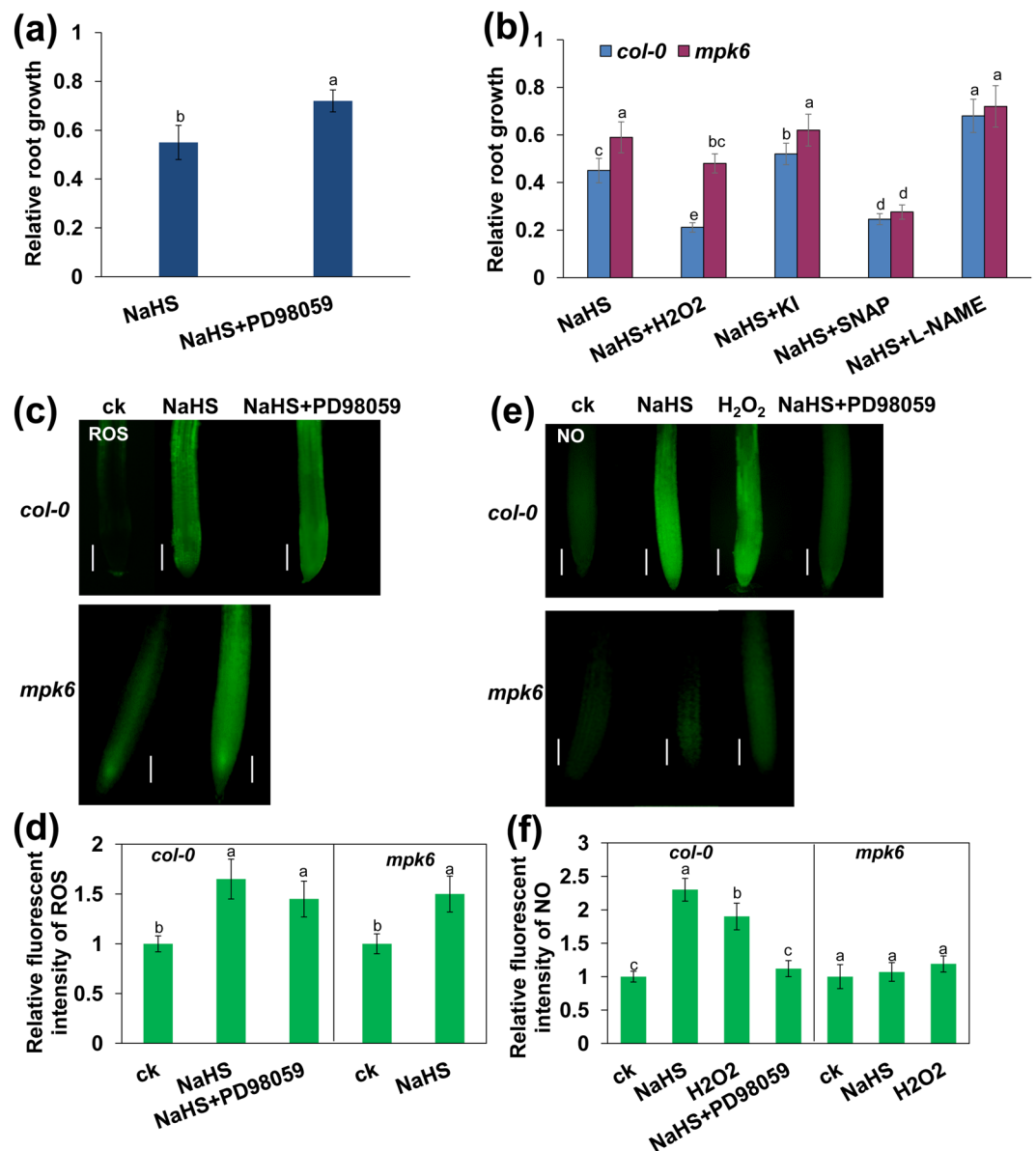


Figure 6. MPK6 mediates PR growth inhibition by NaHS. (a) Relative root growth of *Col-0* seedlings treated with 500 μ M NaHS in the presence or absence of 150 μ M PD98059 for 2 d compared with untreated seedlings. (b) Relative root growth of *Col-0* and *mpk6* seedlings exposed to 500 μ M NaHS with or without 1 mM H₂O₂, 100 μ M SNAP, 1 mM KI, and 500 μ M L-NAME for 2 d compared with untreated seedlings. n = 45. (c,d) Detection of ROS production in the roots of 5-d-old wild-type *col-0* and *mpk6* seedlings exposed to 500 μ M NaHS with or without 150 μ M PD98059 for 24 h by the ROS-specific fluorescent probe DCFH-DA (c) and the quantification of the ROS-specific fluorescence intensities (d) in plants treated as described in (c). (e,f) Detection of NO production in the roots of 5-d-old wild-type *col-0* and *mpk6* seedlings exposed to 500 μ M NaHS with or without 1 mM H₂O₂ or 150 μ M PD98059 for 24 h using the NO-specific fluorescence probe DAF-2 DA (e) and quantification of NO-specific fluorescence intensities (f) in plants treated as described in (e). n = 30. Bars, 100 μ m. ck, untreated control. Error bars represent \pm SD. Different letters indicate significantly different values ($P < 0.05$ by Tukey's test).

molecules in the modulation of PR growth by H₂S. We then examined the relationship between ROS, NO, and MPK6 in the exogenous H₂S-mediated inhibition of PR growth. For this purpose, we measured the production of ROS and NO in roots treated with exogenous H₂S. In H₂S-treated roots, the MAPK inhibitor PD98059 did not affect ROS production, but it markedly inhibited NO production (Fig. 6c–f). Additionally, in the roots of the *mpk6* mutant compared with those of *Col-0*, H₂S treatment did not affect ROS production, but NO production was abolished (Fig. 6c–f). ROS-induced NO production was also repressed in the *mpk6* mutant (Fig. 6e and f). Supplementation with KI alleviated the H₂S-mediated inhibition of PR growth by 16% in *Col-0* roots but could not further alleviate the H₂S-mediated inhibition of PR growth in *mpk6* roots. In contrast, supplementation with H₂O₂ increased the H₂S-mediated inhibition of PR growth by 51% in the *Col-0* roots; however, the inhibitory

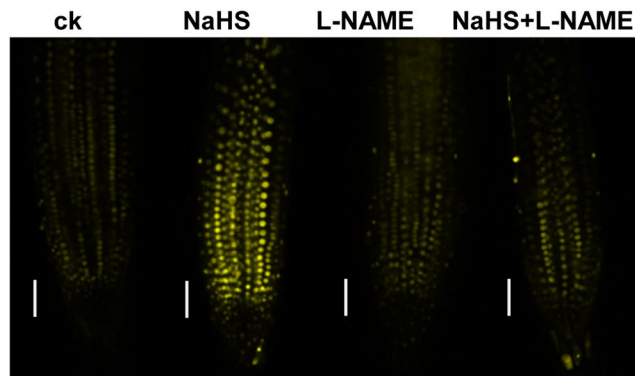


Figure 7. NO is involved in the NaHS-mediated reduction of the distribution of auxin in root tips. YFP fluorescence in the roots of 5-d-old *DII-VENUS* seedlings exposed to 500 μM NaHS with or without 500 μM L-NAME for 24 h. Bars, 50 μm . $n = 30$. ck, untreated control.

effects of H_2O_2 on PR growth were weaker in *mpk6* seedlings than in *Col-0* seedlings exposed to exogenous H_2S (Fig. 6b). These weaker effects may have occurred because the loss of MPK6 in the *mpk6* mutant resulted in the inhibition of the ROS signaling transduction pathway.

We next analyzed the effects of NO on PR growth inhibition in the *mpk6* mutant and found that supplementation with the NO donor SNAP increased the inhibitory effects of H_2S on PR growth, while supplementation with L-NAME reduced these effects in both *mpk6* and *Col-0* seedlings (Fig. 6b). Compared with *Col-0* plants, supplementation with SNAP or L-NAME rescued the insensitivity of the *mpk6* mutant to exogenous H_2S -induced PR growth inhibition (Fig. 6b). These data indicated that MPK6 mediated inhibition of PR growth downstream of ROS and upstream of NO.

NO is involved in exogenous H_2S -mediated inhibition of PR growth by regulating auxin distribution in root tips. Auxin plays a central role in modulating root growth and development³². Previous work indicated that H_2S toxicity reduced auxin accumulation in root tips¹¹. We therefore investigated whether H_2S -induced NO production is involved in the regulation of auxin distribution. For this purpose, we used an auxin-perceptive *DII-VENUS* line to monitor the possible changes in auxin distribution in H_2S -treated plants in the presence or absence of the NOS inhibitor L-NAME. Consistent with the result of Jia *et al.*¹¹, our data here showed that the *DII-VENUS* fluorescence were higher in NaHS-treated roots than in untreated plants¹¹. Supplementation with L-NAME decreased the *DII-VENUS* fluorescence in H_2S -treated seedlings (Fig. 7a), suggesting that H_2S -induced NO production affects the distribution of auxin.

Discussion

At low concentrations, H_2S is an important regulator of the stress response, which is essential for stress tolerance and survival in plants^{3,5,6}. However, high concentrations of H_2S are toxic to plants and inhibit their growth. A recent investigation indicated that high concentrations of H_2S inhibit root growth by regulating auxin accumulation in root tips¹¹; however, the molecular mechanisms underlying this process are largely unclear. In the present study, we elucidated a signaling pathway that controls H_2S toxicity-induced PR growth inhibition in *Arabidopsis*. We showed that the activation of MPK6, NADPH oxidase-dependent H_2O_2 synthesis, *Nia1/NOA1*-dependent NO production, and the regulation of auxin perception are all required for the inhibition of PR growth by exogenous H_2S . Furthermore, we found that exogenous H_2S -induced NO production is mediated by ROS and that the modulation of NO production by ROS required the activation of MPK6. We also showed that exogenous H_2S -mediated changes in auxin distribution were regulated by NO. Therefore, our study elucidates an H_2S toxicity-mediated root growth signaling pathway that involves a cascade of NADPH oxidase-dependent ROS production, which, in turn, leads to MPK6- and *Nia1/NOA1*-dependent NO production.

H_2S toxicity-stimulated ROS production inhibits PR growth. Previous reports have shown that ROS are involved in the modulation of PR growth and LR formation by phytohormones or environmental cues^{10,14,18,33}. There are different ROS sources in plants, including cell wall peroxidases, NADPH oxidases, and amine oxidase-type enzymes^{34–36}. In this study, we found that exogenous H_2S -induced ROS production was markedly inhibited by the NADPH oxidase inhibitor DPI and that the NADPH oxidase null mutants *rbohF* and *rbohD/F* were defective in exogenous H_2S -induced ROS generation and showed a smaller reduction in PR growth than did wild-type plants. In contrast, supplementation with H_2O_2 further increased H_2S -induced inhibition of root growth. These results indicated that exogenous H_2S induces ROS generation in the roots and inhibits subsequent PR growth.

ROS-activated MPK6 promotes NO production in H_2S -treated plants. In plants, MPKs are important signaling molecules that are activated in response to a variety of environmental and developmental cues^{21,26}. In this study, we showed that MPK6 plays an important role in the regulation of the H_2S toxicity-mediated inhibition of root growth. The MAPK inhibitor PD98059 alleviated the H_2S -induced inhibition of PR growth,

indicating that the activation of MAPK is required for this inhibition. The *mpk6* mutant showed reduced inhibition of PR growth following exogenous H₂S treatment, providing genetic evidence for the essential role of MPK6 in H₂S toxicity-mediated root growth.

Previous studies have indicated that MPK6 modulates plant growth and the response to stimuli by interacting with ROS and/or NO²⁶. NO promotes Cd²⁺-induced programmed cell death (PCD) by enhancing MPK6-mediated caspase-3-like activation in *Arabidopsis*³⁷. MPK6 controls H₂O₂-induced root growth by mediating the Ca²⁺ influx in *Arabidopsis*²⁶. The *mpk6* mutant produces more and LR_s than do wild-type plants after application of the NO donor sodium nitroprusside (SNP) or H₂O₂³⁸. These studies indicate that MPK6 modulates NO production and the response to ROS during root development; however, it is unknown whether and how the interactions between MPK6, ROS, and NO mediate the H₂S toxicity-induced inhibition of PR growth. In this study, we found that the H₂S toxicity-induced production of ROS and NO and inhibition of PR growth were markedly impaired in the *rbohF* and *rbohD/F* mutants. Moreover, the H₂S-induced increase in NO and inhibition of PR growth were also impaired in the *mpk6*, *nia1-2/2-5*, and *noa1* mutants. ROS rescued the defect in the H₂S-induced inhibition of PR growth in the *rbohF*, *rbohD/F*, *mpk6*, *nia1-2/2-5*, and *noa1* mutants, while only NO could rescue the defect in the H₂S-induced inhibition of PR growth in the *nia1-2/2-5* and *noa1* mutants. These data confirmed that ROS-activated MPK6 promoted NO production and thereby reduced PR growth in H₂S-treated plants. Our results show that ROS-activated MPK6 and NO production are involved in the H₂S toxicity-mediated inhibition of PR growth and suggest that the ROS-MPK6-NO pathway is a general signaling transduction pathway that regulates plant responses to abiotic stress. The phosphorylation of NIA2 by MPK6 leads to increases in nitrate reductase (NR) activity and NO production³⁸. However, how MPK6 mediates NO production via the NO synthase-associated (NOA) pathway remains unclear.

H₂S toxicity-induced NO production inhibits PR growth. Similar to its effect on ROS, we found that H₂S toxicity triggered NO accumulation in root tips within 24 h, which gradually decreased after 48 h of treatment, suggesting that both ROS and NO could act as early signaling molecules to modulate downstream gene expression, ultimately leading to PR growth inhibition.

It has been proposed that there are multiple sources of NO generation in plant cells, including NR³⁹, the NO synthase-like/NO synthase-associated (NOS-like/NOA) enzyme⁴⁰, and non-enzymatic pathways⁴¹. The roles of the NOA pathway and the NR pathway in NO generation *in vivo* have been well studied. Previous reports have shown that L-NAME, an L-arginine analog, inhibits NOS activity in plants⁴². In this study, we found that supplementation with the NOS inhibitor L-NAME markedly repressed the production of NO in H₂S-treated roots. Our genetic experiments also showed that H₂S-induced NO production and PR growth inhibition were markedly impaired in the NOA1-defective mutant *noa1* and the NR-defective *nia1-2/2-5* double mutant. These results suggested that NO production in H₂S-treated seedlings may be catalyzed through both L-Arg-dependent and NR-dependent routes. Interestingly, Lisjak *et al.*⁴³ found that the exogenous H₂S donor NaHS repressed the ABA-induced accumulation of NO in guard cells⁴³. The differences in these effects between studies may be due to the different tissues examined.

Previous studies have indicated that NO acts as a second messenger to regulate root growth via the auxin pathway^{44,45}. This prompted us to assess the possible involvement of NO in the H₂S-mediated auxin distribution. Jia *et al.*¹¹ reported that H₂S reduces auxin accumulation in root tips by disrupting the expression of auxin carriers and subsequent polar auxin transport (PAT)¹¹. Consistent with these findings, our data indicated that H₂S toxicity increased DII-VENUS fluorescence in roots, while supplementation with L-NAME decreased DII-VENUS fluorescence. These data suggest that NO accumulation is responsible for the H₂S-induced inhibition of the distribution of auxin in root tips.

Taken together, our data indicate that in addition to the auxin pathway, ROS and NO are also key players in the plant response to H₂S toxicity. ROS production in roots induced by a high concentration of H₂S inhibited PR growth, while ROS accumulation in roots activated MPK6. MPK6 then promoted NO production through both L-Arg- and NR-dependent routes. Elevated NO repressed the distribution of auxin in root tips, thereby reducing PR growth (Fig. 8). These results indicate that H₂S toxicity inhibits PR growth via both the ROS pathway and the auxin signaling pathway. These findings show that the ROS-MPK6-NO signaling pathway mediates plant responses to H₂S toxicity through morphological and physiological changes in the roots.

Methods

Plant growth and chemical treatments. The *Arabidopsis thaliana* ecotype *Columbia-0* was used in this study. The transgenic and mutant lines employed in this work included *DII-YFP*, *nia1/2*, *noa1*, *rbohF*, *rbohD/F*, and *mpk6*. Seeds were surface sterilized with 5% bleach for 5 min, then washed five times with sterile water, incubated for 2 d at 4 °C in the dark and plated onto agar medium containing half-strength MS (Sigma), pH 5.70, supplemented with 1% agar and 10% sucrose. Seedlings were grown in a growth chamber maintained at 22 °C under a 16/8 h light/dark cycle. Seeds were grown in the vertical position. Five-day-old seedlings were transferred to plates supplemented with various chemicals, including sodium hydrosulfide (NaHS), cPTIO, L-NAME, DPI, KI, SNAP, H₂O₂, and PD98059, and grown for an additional 2 d. All chemicals were obtained from Sigma-Aldrich.

Phenotypic analysis. Seeds were grown in the vertical position. After the seedlings were transferred to plates supplemented with various chemicals, root growth was measured at the same time every day. After 5 d of treatment, the PR length was measured and statistically analyzed. The length of the meristematic zone was measured from the quiescent center (QC) to the beginning of the root elongation zone as described by Liu *et al.*³². At least 15 replicate plants were measured for each treatment.

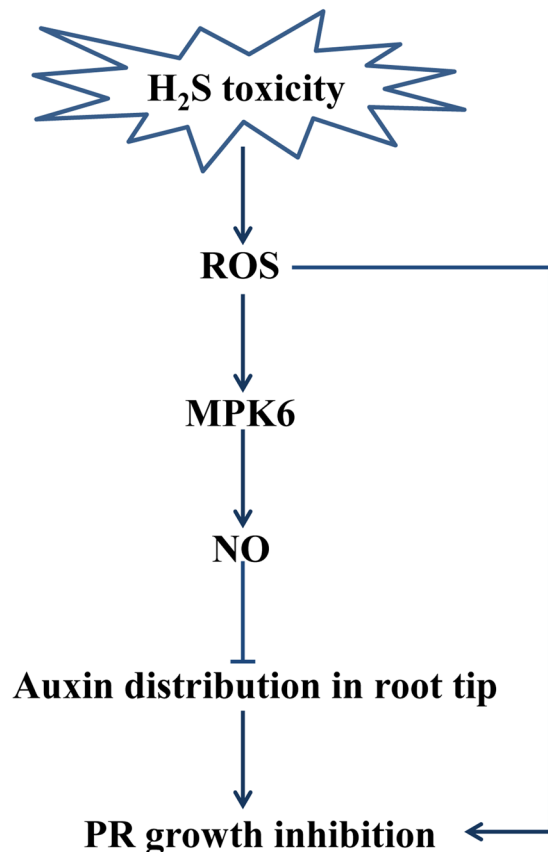


Figure 8. H₂S toxicity inhibits PR growth via the ROS-MPK6-NO signaling pathway. High-concentration H₂S induced ROS production via the NADPH oxidase pathway, which directly inhibited PR growth and activated MPK6. MPK6 then promoted NO production through both L-Arg-dependent and NR-dependent routes. Elevated NO repressed auxin distribution, ultimately inhibiting PR growth.

Measurement of NO and ROS production and fluorescence microscopy. The endogenous NO levels in root meristems were visualized using the specific NO fluorescent probe DAF-2 DA. Seedlings were incubated at 37 °C in a 5 μM staining solution for 1 h. The endogenous ROS levels in root meristems were visualized using the specific ROS fluorescent probe DCFH-DA. Seedlings were incubated at 37 °C in 10 μM staining solution for 5 min. Then, they were washed twice and viewed under a microscope. Quantitative measurement of fluorescence intensity was performed using ImageJ.

Roots were treated with DAF-2 DA and DCFH-DA and analyzed using fluorescence microscopy (Zeiss; NO: excitation 495 nm, emission 515 nm; ROS: excitation 488 nm, emission 525 nm). DII-VENUS fluorescence was observed using confocal laser scanning microscopy (Zeiss) according to the manufacturer's instructions. The excitation and emission wavelengths were 488 to 520 nm for YFP.

Statistical analysis. For the PR growth and fluorescence microscopy analysis, the experiments were repeated three times with 15–20 seedlings in each repeat. The data were analyzed using Image-Pro Plus software (version 4.5.1.29; Media Cybernetics, Carlsbad, CA) and SPSS (Statistical Package for the Social Sciences) software. The results are presented as the mean ± SD. For statistical analysis, we used Tukey's test ($P < 0.05$)^{46,47}.

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Author Contributions

J.X. designed research; Q.L., P.Z., J.P.W., and R.L.W. performed research; Q.L., P.Z., J.P.W., and J.X. analyzed data; and J.X. wrote the paper.

Additional Information

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