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Stomatal closure in sweet potato leaves induced by sulfur dioxide involves H_2S and NO signaling pathways

K.-D. HU[1], J. TANG[2], D.-L. ZHAO[2], L.-Y. HU[1], Y.-H. LI[1], Y.-S. LIU[1], R. JONES[3], and H. ZHANG[1]*

School of Biotechnology and Food Engineering, Hefei University of Technology, Hefei 230009, P.R. China[1]
Xuzhou Sweet Potato Research Center, National Sweet Potato Improvement Center, Xuzhou 221131, P.R. China[2]
Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA[3]

Abstract

Sulfur dioxide (SO_2) is a well-known and widespread air pollutant but it also acts as signaling molecule in various processes in animals. However, there is limited information on the role of SO_2 in plants except of its toxicity. Here we studied the role of SO_2 on stomatal movements in sweet potato (*Ipomoea batatas*) leaves. SO_2, generated by Na_2SO_3/$NaHSO_3$ solutions, was applied on epidermal strips. We found that the SO_2 donor induced stomatal closure in a dose-dependent manner. Rapid increases in endogenous hydrogen sulfide and nitric oxide content levels were observed in leaves after the treatment with the SO_2 donor. The SO_2-induced stomatal closure was reversed by the H_2S scavenger hypotaurine and the NO-specific scavenger cPTIO. Our results indicate that the SO_2-induced stomatal closure was likely mediated by the H_2S and NO signaling pathways.

Additional key words: abscisic acid, gaseous signals, sulfur metabolism.

Introduction

Sulfur dioxide, a waste gas and pollutant, is emitted by natural sources, such as microbial and volcanic activities, and by combustion of sulfur-containing fossil fuels. SO_2 enters plants *via* their stomata to form sulfite (SO_3^{2-}), and the plant damage is often correlated with the degree of stomatal opening (Rennenberg and Herschbach 1996, Van der Kooij *et al*. 1997). The detrimental effects of SO_2 on plants have been studied extensively in the past few decades, and it has been shown that exposure of plants to high concentrations of SO_2 leads to reduced rate of photosynthesis, chlorosis (chlorophyll destruction), necrosis, and yield reduction, whereas SO_2 at low concentration could be absorbed and used as beneficial gas (Rennenberg 1984, Van der Kooij *et al*. 1997, Noji *et al*. 2001, Legge and Krupa 2002).

Recent reports have shown that SO_2 is produced endogenously (Meng *et al*. 2007, Li *et al*. 2010, Lu *et al*. 2012) and considering the functional similarity of the gasotransimitters NO, H_2S, and SO_2 in animals (Hosoki *et al*. 1997, Li *et al*. 2010, Lu *et al*. 2012, Wang 2012), we speculate that SO_2 might also function as signaling molecule in plants rather than only as harmful gas. NO has been well established as mediator in many processes in plants, such as growth, pathogen defense, programmed cell death, and stress responses (Delledonne 2005). Further, NO has been proposed to act as signaling molecule in the abscisic acid induced stomatal closure (García-Mata and Lamattina 2002, Neill *et al*. 2002).

H_2S was found to be gaseous signal molecule both in animals and in plants (Wang 2002, 2012). In plants, H_2S is evolved during metabolism of cysteine, sulfites, and sulfates (Rennenberg 1983, 1984, Rausch and Wachter 2005). Accumulating evidence suggests that H_2S acts as signaling molecule involved in root formation, abiotic defence, and senescence (Zhang *et al*. 2008, 2009, 2011, García-Mata and Lamattina 2010, Lisjak *et al*. 2010, Jin *et al*. 2011, Hu *et al*. 2012, Shan *et al.* 2014). In guard cells, also H_2S can regulate stomatal movements (García-Mata and Lamattina 2010, Lisjak *et al*. 2010, Jin *et al*. 2011, Lisjak *et al*. 2013).

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Abbreviations: cPTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; HT - hypotaurine; SNP - sodium nitroprusside.
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* Corresponding author; fax: (+86) 551 62901043, e-mail: hzhanglab@gmail.com

SO_2 causes stomatal closure in seedlings of *Betula papyrifera*, *B. pendula*, *B. lutea*, and *B. populifolia* (Biggs and Davis 1980, Norby and Kozlowski 1982, Rao and Anderson 1983). Considering the roles of ABA, H_2S, and NO in mediating stomatal movement, we investigated whether SO_2 acts as signaling molecule in stomatal closure and its interaction with H_2S and NO in this process.

Materials and methods

Sweet potato (*Ipomoea batatas* L., cv. Xushu 18) was supplied by the Anhui Academy of Agricultural Sciences, Anhui Province, China. Seedlings were cultured at day/night temperatures of 28/18 ± 1 °C and a relative humidity of 85 %, a 12-h photoperiod, and an irradiance of 40 μmol m^{-2} s^{-1}. Na_2SO_3/$NaHSO_3$ (0 to 3.75/1.25 mM), 0.5 mM NaHS, and 0.1 mM sodium nitroprusside (SNP) were used as SO_2, H_2S, and NO donors, respectively. Further, 0.2 mM 2-(4-carboxy-phenyl)-4,4,5,5 -tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO) was used as specific NO scavenger, and 0.2 mM hypotaurine (HT) as H_2S scavenger (Ortega *et al*. 2008). All chemicals were purchased from *Sigma* (St. Louis, USA) unless speci- fically stated.

Stomatal aperture experiments were performed on epidermal strips taken from fully developed leaves of 30-d-old seedlings. Strips were cut with a scalpel and pre-incubated in an "opening" buffer (10 mM K-MES, 10 mM KCl, pH 6.1) at 25 °C for 2 h to promote stomatal opening. After pre-incubation, the strips were immersed in the same buffer (control) or treated with different concentrations of the SO_2 donor solutions for 2 h. For cPTIO experiments, epidermal strips were treated with the opening buffer with or without NaHS, SNP, or SO_2 in the presence or absence of 0.2 mM cPTIO for 2 h. For HT experiments, epidermal strips were immersed in the opening buffer with or without NaHS, SO_2, or ABA in the presence or absence of 0.2 mM HT. Stomata were photographed using a *Zeiss Observer D1* microsope, and the *Zeiss* distance measuring tool software (*Carl Zeiss MicroImaging*, Oberkochen, Germany) was used to analyze stomatal pore apertures. Stomatal pore width was monitored (90 apertures) randomly in three different epidermal strips from each treatment. The stomates were considered open with a pore width > 4.5 μm, or closed with a pore aperture < 4.5 μm.

For determinations of H_2S and NO content, epidermal strips were firstly incubated in the opening buffer for 2 h and then incubated in 2.5 mM SO_2 solutions for up to 8 h. The H_2S content was determined according to formation of methylene blue from dimethyl-*p*-phenylenediamine in H_2SO_4 using the method described by Sekiya *et al*. (1982) with some modifications. Samples (0.5 g) of epidermal strips were ground in 5 cm^3 of a 50 mM phosphate buffer (pH 6.8) containing 0.1 mM EDTA and 0.2 mM ascorbic acid. The homogenate was mixed in a test tube containing 100 mM phosphate buffer saline (PBS; pH 7.4), 10 mM L-cysteine, and 2 mM pyridoxalphosphate at room temperature, and H_2S was absorbed in a zinc acetate trap located in the bottom of the test tube. After 30 min of reaction, 0.3 cm^3 of 5 mM dimethyl-*p*-phenylene diamine dissolved in 3.5 mM H_2SO_4 was added to the trap. Then 0.3 cm^3 of 50 mM ferric ammonium sulfate in 100 mM H_2SO_4 was injected in the trap. The amount of H_2S in the zinc acetate traps was determined colorimetrically at 667 nm after incubating the mixture for 15 min at room temperature. Blanks were prepared by the same procedures without the zinc acetate solution, and a known concentration of Na_2S was used to create a calibration curve.

NO was measured according to the method of Murphy and Noack (1994). Epidermal strips were ground in 100 mM phosphate buffer saline (PBS; pH 7.4) and incubated with 100 units of catalase and 100 units of superoxide dismutase for 5 min to remove endogenous ROS before addition of oxyhaemoglobin (10 mM). After a 3-min incubation, NO was quantified by spectrophotometric measurement which reflected the conversion of oxyhaemoglobin to methaemoglobin.

Significance of differences were tested by one-way *ANOVA*, and each experiment was repeated three times. Fisher's least significant differences were calculated following the *t*-test.

Results

To test whether SO_2 plays a role in stomatal movement, epidermal strips of seedling leaves of sweet potato (*Ipomoea batatas*) were treated with different concentrations of SO_2 ranging from 0 to 5 mmol dm^{-3}. Stomata closed with increasing SO_2 concentrations; the 5 mM SO_2 treatment for 2 h induced a profound stomatal closure compared to the control (Fig. 1*A*,*B*).

To assess whether endogenous NO and H_2S are involved in SO_2/sulfite-induced stomatal closure, the endogenous content of H_2S and NO were measured in sweet potato leaves immersed in an SO_2 donor solution or in water (control). In both the control and SO_2 treatments, the content of endogenous H_2S increased in the first 1 h, but the increase was substantially larger and faster in the SO_2-donor treatment. The content of H_2S in the SO_2 treated strips increased to a maximum at 1 h, followed by a gradual decrease until 8 h. In contrast, the H_2S content in the control remained stable up to 4 h and then decreased gradually. At 1 h of the SO_2 treatment, the H_2S content of the SO_2 treated leaves was twice of that of the controls

(Fig. 1*C*).

Similar patterns were observed in the endogenous NO content which increased transiently at 0.5 h in both the treatments, but more rapidly in the SO_2 treatment. The NO content in the SO_2 treatment peaked at 2 h followed by a decline until 8 h. In the controls, the NO content increased gradually upto 1 h and then declined. The SO_2 treatment induced a higher content of endogenous NO than the control one during the whole treatment time (Fig. 1*D*). In all, our result suggests that SO_2/sulfite acted as signal to induce the release of H_2S and NO.

Since the SO_2 donor induced production of NO in sweet potato leaves, we investigated whether NO signaling is necessary for SO_2-induced stomatal closure using the NO scavenger cPTIO. Accordingly, we tested the effects of cPTIO on stomatal closure induced by the H_2S donor NaHS, the NO donor SNP, and the SO_2 donor Na_2SO_3/$NaHSO_3$. SNP, NaHS, and SO_2 induced a significant stomatal closure in sweet potato leaves (Fig. 2*A*,*B*). Stomatal closure induced by the NO donor SNP was fully blocked by cPTIO confirming the effectiveness of cPTIO as NO scavenger. Application of cPTIO dramatically counteracted the stomatal closure effects of H_2S donor and SO_2 donor indicating that NO is prerequisite in H_2S and SO_2-mediated stomatal closure and acts downstream of H_2S and SO_2 (Fig. 2*A*,*B*).

Further, we investigated whether H_2S is involved in SO_2-induced stomatal closure. The effect of NaHS on stomatal closure was blocked by the H_2S scavenger (HT) indicating the effective role of HT on H_2S scavenging. SO_2 induced stomatal closure was also counteracted by HT suggesting that H_2S is involved in SO_2-induced stomatal closure. Furthermore, ABA-dependent stomatal closure was partially blocked by HT (Fig. 2*C*,*D*). Taken together, these results suggest that SO_2/sulfite acted upstream of H_2S in stomatal closure in sweet potato leaves.

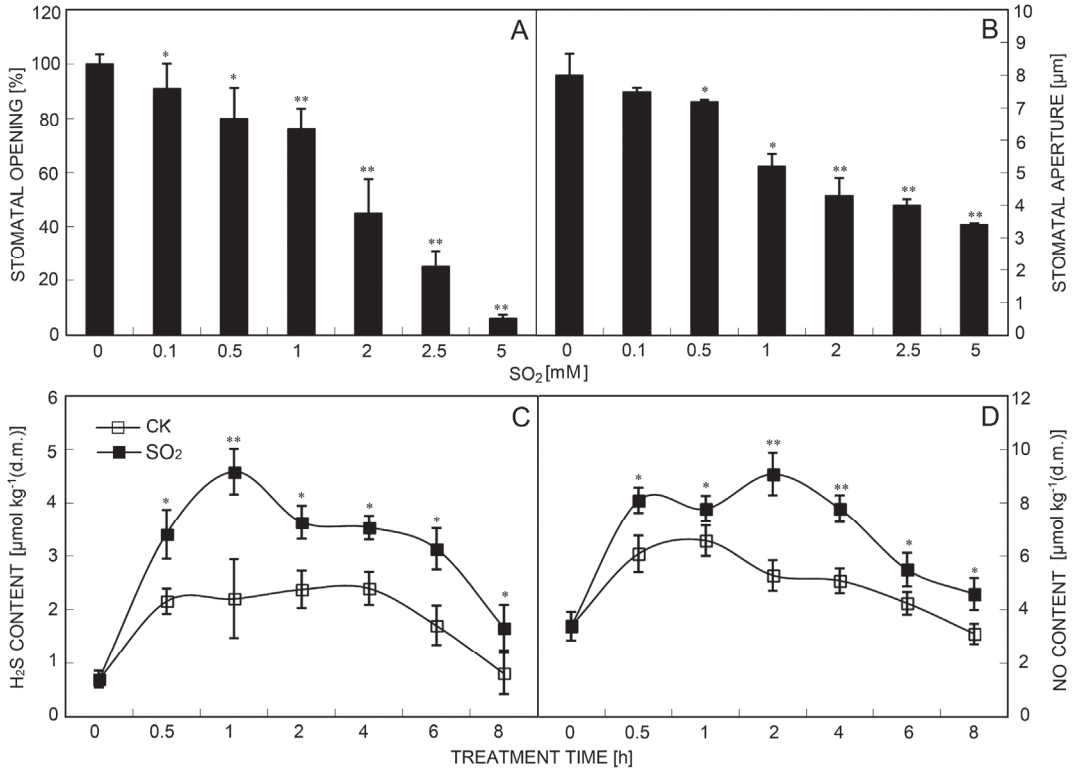

Fig. 1. Effects of SO_2 donor (Na_2SO_3/$NaHSO_3$; 3:1) on stomatal movement in epidermal strips of sweet potato leaves. Stomatal opening percentage (*A*) and stomatal aperture size (*B*) were measured. Leaves were treated with water (CK) or 2.5 mM SO_2 and sampled for H_2S (*C*) and NO (*D*) content determinations at 0, 0.5, 1, 2, 4, 6, and 8 h. Means ± SD, $n = 90$ for *A* and *B*, $n = 3$ for *C* and *D*, * and ** - significant differences between CK and SO_2 treatments at $P < 0.05$ and $P < 0.01$, respectively.

Discussion

In this report, we demonstrate that the SO_2 donor (Na_2SO_3/$NaHSO_3$) induced stomatal closure in sweet potato epidermal strips in a dose dependent manner (Fig. 1*A*,*B*). Na_2SO_3/$NaHSO_3$ has been widely used as SO_2 donor in animals (Sun *et al*. 2010) and it has also been widely used in plants to mimic the effects of SO_2 as air pollutant (Shapiro 1977). Na_2SO_3/$NaHSO_3$ dissolves in water, then dissociates to Na^+, SO_3^{2-}, and HSO_3^- in solution, SO_3^{2-} and HSO_3^- are protonated producing SO_2. Except for Na^+, all chemicals released by Na_2SO_3/$NaHSO_3$ are

derivatives of SO_2, and since Na^+ was not found to play any important role in stomatal movement in sweet potato, this justifies the use of Na_2SO_3/$NaHSO_3$ as SO_2 donor in our experiments.

SO_2 was always regarded as toxic gas in polluted atmosphere and was found to induce stomatal closure in plants (Biggs and Davis 1980, Norby and Kozlowski 1982, Sekiya *et al*. 1982, Rao and Anderson 1983). Recent studies in mammalian systems have shown that SO_2 might be a novel endogenous gaseous signaling molecule involved in the regulation of cardiovascular functions and vasorelaxation, as do NO, CO, or H_2S (Meng *et al*. 2007, Li *et al*. 2010, Lu *et al*. 2012). However, there is less evidence showing that SO_2 plays a vital role as gasotransmitter in plants. By analogy to other endogenous gaseous molecules, such as NO and H_2S, and their roles in plants and animals, it is likely that SO_2 fulfills some role in plant growth and development regulation. This is confirmed in the present study where we found that SO_2 induced stomatal closure with the increased SO_2 donor concentration and H_2S and NO were involved in this process (Figs. 1, 2).

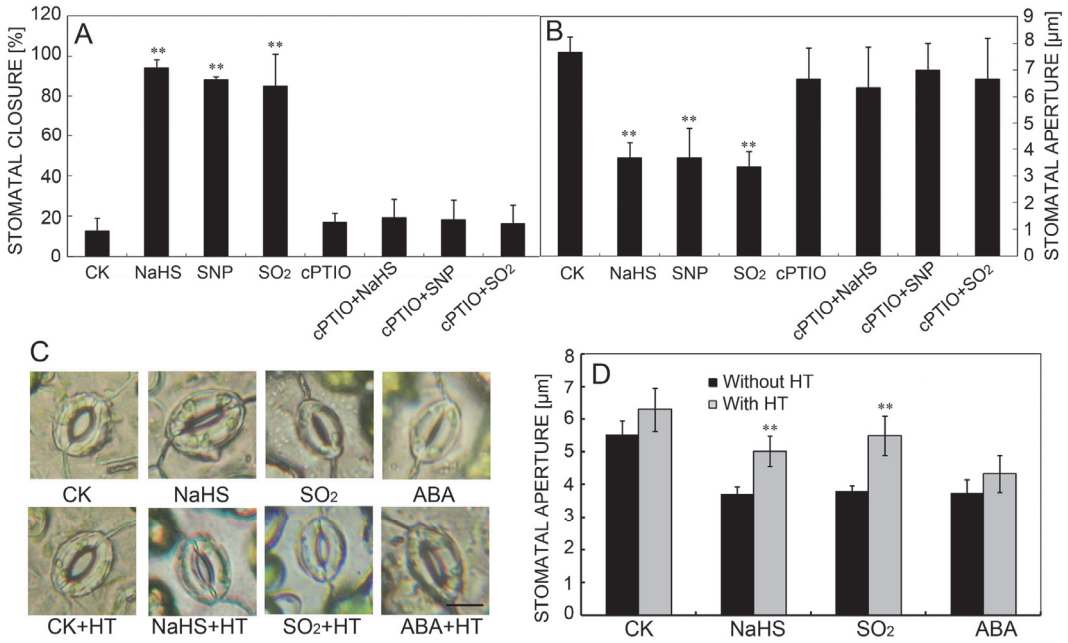

Fig. 2. Stomatal closure percentage (*A*) and stomatal aperture size (*B*) were determined in sweet potato leaves treated with an opening buffer (CK), NaHS, SNP, or 2.5 mM SO_2 in the presence or absence of an NO scavenger cPTIO. Effects of an H_2S specific scavenger hypotaurine (HT) on NaHS, SO_2 donor, or ABA induced stomatal closure in epidermal strips are shown on images of stomata (*C; bar* = 15 µm) or on a column diagram (*D*). Means ± SD, $n = 90$, * and ** - significant differences between treatments at $P < 0.05$ and $P < 0.01$, respectively.

Stomatal movements in response to changes in environmental or internal factors are very complicated processes involving a network of signaling molecules, such as ABA, CO, and NO (Araújo *et al*. 2011). NO is required for stomatal movement and acts downstream of the ABA signaling pathway (García-Mata and Lamattina 2001, 2002, Neill *et al*. 2002). Recent research has also reported the role of H_2S in stomatal movements (García-Mata and Lamattina 2002, Lisjak *et al*. 2010, Lisjak *et al*. 2013) which depends on environmental conditions and the leaf age. In our study, the SO_2 donor treatment induced stomatal closure and a rapid H_2S and NO accumulation in sweet potato leaves suggesting that SO_2 might act as signaling molecule. As cPTIO, which scavenge NO, counteracted stomatal closure induced by SO_2 and H_2S, we suggest that SO_2 and H_2S were involved in NO-induced stomatal closure.

The production of H_2S by plants is now well established. Sulfate can be activated by ATP *via* ATP sulfurylase to generate 5'-adenylysulfate (APS) which is reduced by APS reductase to form sulfite (Rausch and Wachter 2005). Sulfite is reduced to H_2S by sulfite reductase. In our study, SO_2 induced stomatal closure was blocked by the H_2S scavenger HT, also suggesting that H_2S acted downstream of SO_2 induced stomatal movement.

ABA regulation of stomatal movement has been studied extensively. In this work, we found that the H_2S scavenger HT slightly reversed stomatal closure induced by ABA. This result agrees with a previous report on the role of H_2S in guard cell signaling (García-Mata and Lamattina 2002, 2010, Lisjak *et al.* 2010, 2013). The signaling role of SO_2 was also indicated by the release of H_2S and NO after the application of SO_2 donor in sweet potato leaves demonstrating again that SO_2 acted upstream of H_2S and NO.

Taken together, these results suggest a signaling pathway from SO_2/sulfite, through H_2S and NO to induce

stomatal closure. The involvement of SO_2/sulfite in the H_2S and NO mediated signaling pathway of stomatal movement opens a more intricate field of research in the plant kingdom. However, the mechanism of SO_2 action and whether SO_2 has additional roles in plant signaling are unknown, and further investigations are needed to elucidate the network of SO_2, H_2S, and NO in plant life.

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