Title
Mechanism of SO₂-Induced Stomatal Closure Is Unshared by O₃ and CO₂ Response and Is Mediated by Non-Apoptotic Cell Death in Guard Cells

Running Head: Stomatal Response against Sulfur Dioxide

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ABSTRACT

Plants closing stomata in the presence of harmful gases is believed to be a stress avoidance mechanism. SO₂, one of the major airborne pollutants, has long been reported to induce stomatal closure, yet the mechanism remains unknown. Little is known about the stomatal response to airborne pollutants besides O₃. SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) and OPEN STOMATA 1 (OST1) were identified as genes mediating O₃-induced closure. SLAC1 and OST1 are also known to mediate stomatal closure in response to CO₂, together with RESPIRATORY BURST OXIDASE HOMOLOGs (RBOHs). The overlaying roles of these genes in response to O₃ and CO₂ suggested that plants share their molecular regulators for airborne stimuli. Here, we investigated and compared stomatal closure event induced by a wide concentration range of SO₂ in Arabidopsis through molecular genetic approaches. O₃- and CO₂-insensitive stomata mutants did not show significant differences from the wild type in stomatal sensitivity, guard cell viability and chlorophyll content revealing that SO₂-induced closure is not regulated by the same molecular mechanisms as for O₃ and CO₂. Non-apoptotic cell death is shown as the reason for SO₂-induced closure, which proposed the closure as a physicochemical process resulted from SO₂ distress, instead of a biological protection mechanism.

Keywords: sulfur dioxide, stomatal closure, airborne pollutants, non-apoptotic cell death

INTRODUCTION

Stoma, which consists of a pair of guard cells in the epidermis of vascular plants, ingeniously controls transpirational water loss and carbon dioxide (CO₂) uptake under biotic and abiotic stresses in the environment (Murata et al. 2015). Environment-polluting gases, such as ozone (O₃), nitrogen dioxide (NO₂) and sulfur dioxide (SO₂) enter leaves through stomatal pores, damage foliage, and result in crop loss and forest decline (Bobbink 1998; Cape 1998; WHO 2000). These gases are known to close stomata, and thus stomatal closure is
postulated as one of the protection mechanisms against harmful gases (McAinsh et al. 2002; Schroeder et al. 2001).

Albeit several harmful gases were reported to close stomata, molecular mechanisms of the closure have not been well investigated except for O₃. SLOW ANION CHANNEL-ASSOCIATED 1/OZONE-SENSITIVE-1 (SLAC1/OZS1) was identified as a critical factor in O₃-induced closure by genetic screening (Saji et al. 2008; Vahisalu et al. 2008). SLAC1 encodes a slow-type anion channel essential for anion efflux from the guard cells and slac1 mutant exhibits a high O₃ sensitivity owing to the insensitivity of stomata against O₃, which gives rise to augmented O₃ uptake into the leaf. OPEN STOMATA 1 (OST1/SNRK2.6/SRK2E) was initially identified by thermal screening of drought-stressed plants, of which ost1 mutants demonstrated ~1°C cooler leaf temperature as compared to wild type due to its incompetence to engender ABA-induced stomatal closure (Mustilli et al. 2002). OST1 was later identified to be participating in O₃-triggered rapid transient decrease in stomatal conductance (Vahisalu et al. 2010).

CO₂ is a gaseous stimulus that evokes stomatal closure, although it is not harmful to plants (for review, see Engineer et al. 2016). Intriguingly, the aforementioned factors SLAC1 and OST1 are also involved in CO₂-induced stomatal closure (Negi et al. 2008; Xue et al. 2011). It is tempting to assume that plants have a common molecular mechanism for the induction of stomatal closure against gaseous stimuli. In addition, loss-of-function mutation in RESPIRATORY BURST OXIDASE HOMOLOGs (RBOHs) encoding the catalytic subunit of NADPH oxidase has been shown to render stomata insensitive to CO₂ (Chater et al. 2015).

SO₂, a colorless gas with a pungent odor, is one of the major airborne pollutants, which impacts natural vegetation and crop production (WHO 2000). Global anthropogenic SO₂ emissions had been estimated to be on the rise since 1850 following economic expansion (Smith et al. 2011). Though efforts were taken in reducing the emissions, SO₂ emission remained high in the last decade (Klimont et al. 2013). The effects of SO₂ on plants have been extensively studied since 1848, reporting disrupted photosynthesis activity, suppressed plant growth, damaged chlorophyll, reduced yield and premature death in plants
(Stöckhardt 1850; Kropff 1987; Malhotra & Hocking 1976; Wilson & Murray 1990; Sprugel et al. 1980). On the other hand, reports on stomatal response against SO$_2$ are limited and inconsistent. SO$_2$ was reported to induce stomatal closure (Winner & Mooney 1980; Olszyk et al. 1981; Hu et al. 2014), meanwhile, some reported that SO$_2$ augmented the opening (Mansfield & Majernik 1970; Taylor et al. 1981; McAinsh et al. 2002). SO$_2$ dissolves in water forming three different chemical species: sulfurous acid (H$_2$SO$_3$), hydrogen bisulfite ion (HSO$_3^-$), and sulfite ion (SO$_3^{2-}$). The actual species that is responsible for SO$_2$-induced stomatal closure has yet to be determined. Furthermore, neither the molecular factors nor the signaling pathways involving in the SO$_2$-induced stomatal closure have been confirmed besides an antecedent pharmacological study (Hu et al. 2014).

"Can stomata play a part in protecting plants against air pollutants?" was a question asked in 1970, in a paper reporting CO$_2$ and SO$_2$ effects on stomata (Mansfield & Majernik 1970). It is still an open question. Today, it is recognized that stomatal closure in the presence of O$_3$ might be a protection mechanism (Merilo et al. 2013). Mutants, which are impaired in O$_3$- and CO$_2$-induced stomatal closure, can be clues in perceiving the molecular mechanisms in SO$_2$ response of stomata. Considering the partially redundant phenotypes of the mutants and the structural similarity among CO$_2$, O$_3$, and SO$_2$ (Fig. S1a), which comprised of three atoms with two oxygen atoms on both ends; we speculated that plants share parts of the regulators in response to gaseous stimuli of stomatal closure. In this study, we identified the responsible chemical species in SO$_2$ aqueous solution that induces stomatal closure, molecular biologically examined stomatal response to SO$_2$ using Arabidopsis mutants and explored the involvement of cell death in the guard cells in SO$_2$-induced stomatal closure.
MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) wild type (ecotype Columbia-0); slac1-1 (Vahisalu et al. 2008), slac1-3 (Vahisalu et al. 2008), srk2e (Yoshida et al. 2002) and rbohD/F (Kwak et al. 2003) mutant plants were grown in pots filled with 4:3 of Vermiculite GS (Nittai Co. Ltd., Osaka) and seedling soil (SK Agri, Kiryu-shi, Japan) in a growth chamber (Biotron LPH 200, NK System, Osaka) with 16-hr-light/8-hr-dark photoperiod regime at 135 µmol m⁻² s⁻¹, 23 ± 0.5 °C and 65 – 80 % relative humidity.

Chemicals

All chemicals used were guaranteed reagents or of higher grade products either from Wako Pure Chemical Industries Ltd. (Osaka, Japan) or Nakalai Tesque Inc. (Kyoto, Japan) unless otherwise stated.

Stomatal Aperture Width Measurement

Measurement of the stomatal aperture was conducted essentially according to Yin et al. (2013). In brief, excised rosette leaves of 4- to 6-week-old plants were exposed to the aqueous solution of SO₂ in the stomata opening buffer containing 5 mmol l⁻¹ KCl, 50 µmol l⁻¹ CaCl₂, and 10 mmol l⁻¹ MES-Tris (pH 5.7) for 3 hr under white light (120 µmol m⁻² s⁻¹) after a 2-hr pre-incubation in the opening buffer, unless otherwise stated. The exposed leaves were blended using a Waring blender (model BB-900, Waring Products Inc., Torrington, CT) and stomatal aperture width in the released epidermal fragments was measured under a microscope.

Chlorophyll Quantification

Chlorophyll was extracted from 3 mature rosette leaves with 1 ml of N, N-dimethylformamide for 24 – 48 hr. This procedure was repeated until all chlorophyll pigments are extracted into
the solvent at 4°C in the dark. Total chlorophyll content was determined spectrophotometrically according to the extinction coefficient reported in Porra et al. (1989).

Guard Cell Viability Test
Epidermal fragments released from leaves treated with aqueous \( \text{SO}_2 \) were double-stained with 50 ng ml\(^{-1}\) carboxyfluorescein diacetate, CFDA (Invitrogen, Carlsbad, CA, USA) for 20 min and 2 ng ml\(^{-1}\) propidium iodide, PI (Invitrogen) for 10 min in the stomata opening buffer. Stained epidermal strips were rinsed with distilled water and observed under a fluorescence microscope (either of Biozero BZ-8000 or BZ-X700, Keyence Corporation, Osaka) with two filter sets (excitation and emission wavelengths of 470/40 nm and 525/50 nm, and dichroic mirror cutoff of 495 nm for CFDA; excitation and emission wavelengths of 545/25 nm and 605/70 nm, and dichroic mirror cutoff of 565 nm for PI, respectively).

ABA Quantification
Contents of ABA in excised leaves (70 – 100 mg fresh weights) were determined by liquid chromatography-mass spectrometry (LC-MS) as described by Yin et al. (2016).

Apoptotic Cell Death Detection
Apoptotic cell death in 2-hr \( \text{H}_2\text{SO}_3 \)-treated guard cells was examined histochemically by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay according to the manufacturer’s protocol using in situ cell death detection kit, fluorescein (Roche Diagnostics GmbH, Mannheim). Epidermal tissues prepared by blending were fixed with formaldehyde and permeabilized with Triton X-100 according to Hayashi et al. (2011). The rate of guard cells positively stained with TUNEL and DAPI stainings were counted and expressed as percentage over the total number of observed guard cells. Positive control was prepared through partial digestion of DNA with DNase I recombinant (1 kU ml\(^{-1}\) in 50 mmol l\(^{-1}\) Tris-HCl, pH 7.5, 1 mg ml\(^{-1}\)), for 15 min at room temperature; after the cell wall was digested with 1% cellulase Onozuka R-10 (Yakult) and 0.1% Macerozyme R-10 (Yakult), in
phosphate-buffered saline (137 mmol l⁻¹ NaCl, 8.1 mmol l⁻¹ Na₂HPO₄, 2.68 mmol l⁻¹ KCl, 1.47 mmol l⁻¹ KH₂PO₄), 37 °C, for 30 min.

**Gaussian Fitting Analysis**

The frequency histogram of the aperture width with 0.25-µm intervals was fitted to a single-peak or a two-peak Gaussian model using the ggplot2 package of R software (version 3.2.4, R Core Team, 2016).

**RESULTS**

**Identification of Stomatal Closure-Inducing Chemical Species in Aqueous Solution of Sulfur Dioxide**

When SO₂ enters the apoplastic space in a leaf, it is readily dissolved in water and acidifies the fluid (Thomas et al., 1944); the effects of fumigation with SO₂ gas and exposure to H₂SO₃ solution on stomata are assumed to be essentially the same (Taylor et al., 1981). To investigate the effects of SO₂ on Arabidopsis stomata, we first questioned whether acidification of extracellular solution caused by H₂SO₃ exposure is the main reason for the closure. Here, we examined the effect of the external solution acidified with three acids, HCl, HNO₃ and H₂SO₃ (Fig. 1). Acidification of the stomata opening buffer with HCl and HNO₃ did not induce stomatal closure above pH 2.9 and 3.0, respectively (Fig. 1, see also Table S1), but at pH 2.0 and 2.2. Meanwhile, the aperture width reduced prominently at pH 2.9 by H₂SO₃. This result strongly suggests that H₂SO₃-induced stomatal closure is not solely attributable to the low pH of the extracellular fluid.

Three chemical species are formed when SO₂ gas is dissolved in water: H₂SO₃, HSO₃⁻, and SO₃²⁻ of which the compositions in the aqueous solution depending on the pH (Fig. 2a and Fig. S1b). We examined the chemical species in the aqueous solution of SO₂ that is responsible for the stomatal response. The dose-response of stomatal closure was assessed in a wide range of concentrations of the chemical species with different composition (Fig. 2, see Table S2 for preparation of the exact composition for each chemical...
species). Fig. 2b shows the plot of aperture width in which the X-axis indicates the concentration of SO$_3^{2-}$ in the experimental solution. Stomatal aperture was wide in the solution containing 0.2 µmol l$^{-1}$ SO$_3^{2-}$ prepared from H$_2$SO$_3$, while it was obviously narrow in the solution containing 0.2 and 0.3 µmol l$^{-1}$ SO$_3^{2-}$ prepared from the mixture of H$_2$SO$_3$ and Na$_2$SO$_3$, showing inconsistent concentration dependency. Stomata remained open with their width comparable to the solvent control in concentrations of SO$_3^{2-}$ higher than 1 µmol l$^{-1}$. Based on these observations, we considered that SO$_3^{2-}$ did not participate in the induction of stomatal closure. Fig. 2c shows the same data plotted in which the X-axis indicates the concentration of HSO$_3^{-}$. In the solution containing HSO$_3^{-}$ below 1 mmol l$^{-1}$, the stomatal aperture was comparable to the solvent control. Inconsistent stomatal response was observed at higher [HSO$_3^{-}$]. Stomatal aperture remained open wide at 2.5, 4.2 and 6.5 mmol l$^{-1}$ HSO$_3^{-}$; and obviously closed at 2.2, 3.8, 4.4, and 7.6 mmol l$^{-1}$ HSO$_3^{-}$, demonstrating discrepancies in concentration dependency. Therefore, we inferred that HSO$_3^{-}$ was not responsible for stomatal closure induction. On the other hand, stomatal closure was consistently observed in the solution containing high concentrations of H$_2$SO$_3$ (Fig. 2d). A significant decrease in aperture width was not observed below 38 µmol l$^{-1}$ H$_2$SO$_3$. Higher concentrations of H$_2$SO$_3$ in the stomata opening buffer (303 µmol l$^{-1}$, 604 µmol l$^{-1}$, 2.4 mmol l$^{-1}$ and 4.1 mmol l$^{-1}$), rendered stomatal closure in a concentration-dependent manner.

The possible involvements of Na$^+$ derived from Na$_2$SO$_3$ salt and different buffering systems (1 mmol l$^{-1}$ and 10 mmol l$^{-1}$) on stomatal aperture width were excluded by stomatal assay in the presence of NaCl (Fig. S2a) and statistical test with Mann-Whitney $u$ test between the buffering systems (Fig. S2b).

Collectively, we concluded that H$_2$SO$_3$ is the responsible chemical species for induction of stomatal closure among the three chemical species formed when leaves are exposed to an aqueous solution of SO$_2$. 
H$_2$SO$_3$-induced Stomatal Closure — An Unshared Molecular Mechanism with O$_3$ and CO$_2$

The effects of H$_2$SO$_3$ on the general appearance of excised rosette leaves were examined in several mutants with impaired stomatal response to O$_3$ and CO$_2$. *slac1* and *ost1* are O$_3$-insensitive stomata mutants that have open-stomata phenotype, which is implicated in allowing ready entry of gaseous stimuli into the leaves (Vahisalu *et al.* 2010). The stomata of rbohD/F mutant together with the aforementioned mutants are partially insensitive to CO$_2$, demonstrating closure-deficient stomatal phenotype (Negi *et al.* 2008; Chater *et al.* 2015). We thus anticipated that these mutants would also demonstrate greater sensitivity to SO$_2$ if the mechanism of stomatal closure is common among O$_3$, CO$_2$, and SO$_2$. A wide range of aqueous SO$_2$ concentrations (from 1.47 nmol l$^{-1}$ to 4.15 mmol l$^{-1}$) ranges from low concentrations (nanomolar to sub-micromolar) to high concentrations which were reported to close stomata (Taylor *et al.* 1981; Hu *et al.* 2014) was applied in this study to allow thorough understanding of stomatal response to SO$_2$.

After an exposure to high dosages of H$_2$SO$_3$ (1.2 and 4.2 mmol l$^{-1}$), the leaves were apparently wilted and paler than the control in all lines as well as the wild type (WT) (Fig. S3). Chlorophyll content in the leaves declined significantly by the exposure to 1.2 and 4.2 mmol l$^{-1}$ H$_2$SO$_3$ demonstrating no difference in the lowest effective concentration in all lines (Fig. 3a). Fig. 3b shows H$_2$SO$_3$-induced stomatal closure in the mutants. Although the width of pre-opened stomatal apertures of srk2e and rbohD/F (< 2.5 µm) were slightly narrower than WT, slac1-1, and slac1-3 (> 3.0 µm), there was not significantly difference (one-way ANOVA with Tukey’s HSD post hoc analysis, $\alpha = 0.05$). Nevertheless, no obvious insensitivity of stomata to H$_2$SO$_3$ was observed in all mutants when compared to WT. These observations suggest that SO$_2$-induced stomatal closure is regulated by a molecular mechanism distinct from O$_3$ and CO$_2$. 


H$_2$SO$_3$ Induces Cell Death in Guard Cells

Consequently, we thus hypothesized that stomatal closure at high levels of SO$_2$ is attributed to the death of guard cells, as presumed by Unsworth & Black (1981) in V. faba. CFDA and PI stainings were conducted simultaneously on SO$_2$-treated epidermal preparations to examine the viability of guard cells. CFDA stains cytosol of living cells with green fluorescence, and PI stains nuclei of dead cells with red fluorescence (Johnson et al. 2013). Representative images of CFDA/PI double-stained guard cells and the rate of viable guard cells are illustrated in Figs 4b and 4b respectively. At 1.5 nmol l$^{-1}$ H$_2$SO$_3$, 93.1 ± 2.8 % of guard cells were positively stained with CFDA. As the [H$_2$SO$_3$] increases, the number of CFDA-positive guard cells decreases, with increasing number of guard cells possessing PI-stained nuclei. Note that red autofluorescence observed in cell walls of aperture lip and PI-positive nuclei of dead epidermal pavement cells (Fig. 4a) were carefully excluded from counting. CFDA-stained guard cells were no longer observed in leaves incubated in [H$_2$SO$_3$] ≥ 0.3 mmol l$^{-1}$. Guard cell mortality rate was below 20% for treatments < 0.1 µmol l$^{-1}$. At [H$_2$SO$_3$] = 1.1 µmol l$^{-1}$, the viability rate of guard cell was 44 ± 14 %, while at [H$_2$SO$_3$] ≥ 0.3 µmol l$^{-1}$, the mortality rate was approximately 100% or equal to 100%. This indicates that H$_2$SO$_3$ kills stomatal guard cells in a concentration-dependent manner. CFDA/PI double staining assay was also conducted on guard cells incubated in HCl- and HNO$_3$-acidified stomatal opening buffer. Significant reduction in guard cell viability was not observed even at pH 2.2 suggesting that SO$_2$-induced cell death in guard cells was not mediated by acidic external pH (Fig. S4a).

H$_2$SO$_3$-induced death of guard cells was further examined by assessing the effect of fusicoccin (FC) (Fig. 4c). FC induces stomatal opening by activation of H$^+$-ATPase and increases K$^+$ conductance of the membrane in intact guard cells (Marré 1979; Blatt 1988). The stomatal width of dark-acclimated leaves was 1.1 ± 0.0 µm without FC, it increased to 3.17 ± 0.23 µm with 10 µmol l$^{-1}$ FC. Stomatal opening had reduced to 1.94 ± 0.39 µm (59% of the control) in the presence of 1.1 µmol l$^{-1}$ H$_2$SO$_3$ and no substantially opening was observed in the presence of 0.3 mmol l$^{-1}$ H$_2$SO$_3$ (0.90 ± 0.04 µm). This observation is in
accordance with that of CFDA/PI staining assay (Fig. 4b). The reduction of FC-induced stomatal opening by H$_2$SO$_3$ should not be attributed to an adverse effect of low pH on FC since FC has successfully induced stomatal opening in the solution with pH 3 in the dark (Fig. S4b).

The effect of H$_2$SO$_3$ on stomatal guard cell viability of slac1-1, slac1-3, srk2e and rbohD/F mutants was also examined (Fig. 5). The rates of CFDA-positive (viable) guard cells in the buffer solution containing equal to or less than 1.1 µmol l$^{-1}$ H$_2$SO$_3$ were above 74% in all tested lines. In parallel, the rate of PI-positive (dead) guard cells had drastically increased to 100% by H$_2$SO$_3$ with concentrations equal to or greater than 0.3 mmol l$^{-1}$. H$_2$SO$_3$ has induced similar response patterns of cell death in guard cells of the WT and mutants. This again manifests that the mode of action of H$_2$SO$_3$ on guard cells is mediated by mechanisms different from that of O$_3$ and CO$_2$.

**Kinetics of Stomatal Response to H$_2$SO$_3$**

The time courses of stomatal closure and cell death were analyzed at 1.1 µmol l$^{-1}$ and 1.2 mmol l$^{-1}$ of H$_2$SO$_3$ to gain further insight into the relationship of stomatal closure and the death of guard cells (Fig. 6a). In the absence of H$_2$SO$_3$, the stomata remained open (2.68 ± 0.42 µm); the guard cell viability rates were ranging from 87.23 ± 12.22% to 97.75 ± 3.11%. At [H$_2$SO$_3$] = 1.1 µmol l$^{-1}$, the average stomatal aperture width was steady at 2.62 ± 0.16 µm throughout the experiment. Treatment with 1.1 µmol l$^{-1}$ H$_2$SO$_3$ reduced the guard cell viability gradually from 91.72 ± 1.85% at 0 min to 56.39 ± 13.61% at 180 min. The higher concentration of H$_2$SO$_3$ (1.2 mmol l$^{-1}$) induced stomatal closure from 2.36 ± 0.48 µm to 0.70 ± 0.34 µm within the first 15 min of exposure. The stomata had remained closed throughout the rest of the experimental time, with an average aperture width of 0.50 ± 0.15 µm. A drastic decline in guard cell viability was also observed, with 100% death rate after a 15-min of H$_2$SO$_3$ incubation.

A histogram analysis was performed for stomatal aperture width in leaves incubated with H$_2$SO$_3$ for 120 min to investigate the discrepancy between stomatal aperture and guard
cell mortality (Fig. 6b). In H$_2$SO$_3$-free condition, the distribution of stomatal aperture width was apparently following a single Gaussian distribution with a peak at 2.82 ± 0.20 µm. On the contrary, two-peak Gaussian fitting reveals two apparent peaks in stomatal response to 1.1 µmol l$^{-1}$ H$_2$SO$_3$, at 0.75 and 3.60 µm (calculated means of the Gaussian curves), respectively. This suggests that at 120-min of H$_2$SO$_3$ exposure, some of the stomata had closed tightly, presumably being due to dead guard cells; while another portion of them opened wider, given the mean stomatal aperture width of 3.17 ± 0.26 µm. For 1.2 mmol l$^{-1}$ condition instead, data were densely distributed with a mean value of 0.63 ± 0.18 µm. This may be attributed to the drastic and persistent stomatal closure observed after 15-min of 1.2 mmol l$^{-1}$ H$_2$SO$_3$ treatment (Fig. 6a). We also performed a stomatal opening assay in the dark with a series of [H$_2$SO$_3$] below 1.1 µmol l$^{-1}$ (Fig. S5). Stomatal aperture width in Arabidopsis did not show significant differences among measurement from different concentrations by Dunnett’s Test ($p > 0.05$). This indicates that SO$_2$ promotes stomatal opening at low concentration in viable cells, in which the same concentration of SO$_2$ has also resulted in cell death in a certain fraction of guard cells, concurrently; this mechanism is light dependent.

**H$_2$SO$_3$-induced Stomatal Closure is Not Mediated by ABA**

Abscisic acid (ABA) is the major phytohormone known to have a vital role in plant development and tolerance to abiotic stresses (Finkelstein 2013). ABA has also been reported to be involved in stomatal closure upon SO$_2$ exposure in *Vicia faba* (Taylor *et al.* 1981). We examined the involvement of ABA in H$_2$SO$_3$-induced stomatal closure in wild type Arabidopsis by quantifying ABA contents in H$_2$SO$_3$-incubated leaves by LC-MS (Fig. 7). ABA levels did not show significant increase throughout the 180-min treatment in 1.1 µmol l$^{-1}$ and 1.2 mmol l$^{-1}$ H$_2$SO$_3$ as compared to the control, suggesting that ABA does not play a crucial role in closing stomata during H$_2$SO$_3$ exposure in Arabidopsis.
**H₂SO₃ Induces Non-Apoptotic Cell Death**

Apoptosis, that is accompanied with DNA laddering can occur as hypersensitive response (HR) to incompatible pathogens and O₃-induced HR-like lesion (Reape et al. 2008; Pasqualini et al. 2003). TUNEL assay detecting DNA laddering of the chromosome was conducted on guard cells treated with 2-hr of H₂SO₃ to explore whether the cell death was apoptotic or not (Fig. 8). The positive control, prepared from permeabilized guard cells with their nuclear DNA partially digested with DNase I, showed green fluorescence in guard cell nuclei and epidermal pavement cells, which co-localized with the DAPI-fluorescence. Similar to 0 H₂SO₃, the guard cells treated with 1.1 µmol l⁻¹ and 1.2 mmol l⁻¹ H₂SO₃ did not exhibit visible green fluorescence, indicating the absence of laddered DNA while DNA still remained in guard cell nuclei as seen by DAPI fluorescence. TUNEL-negative results observed from 1.2 mmol l⁻¹ H₂SO₃ which corresponded to 100% cell death (Fig. 4b) suggests that the death of guard cells was not caused by apoptotic mechanism.

**DISCUSSION**

**Possible Mode of Action of SO₂ in Stomatal Closure and Cell Death in Guard Cells**

The mechanism of SO₂ diffusion into the plant body and its effects on plant metabolism have long been established (Thomas et al. 1950; Malhotra & Hocking 1976; Horsman & Wellburn 1977; Kropff 1991; Muneer et al. 2014). However, the action of SO₂ in inducing stomatal closure remains concealed. Through observation of stomatal response to each chemical species formed in the aqueous solution of SO₂ (Fig. 2), we excluded the involvement of SO₃²⁻ and HSO₃⁻ in SO₂-induced stomatal closure. Our results suggest that H₂SO₃ is the only chemical species that closes stomata in the presence of SO₂. This is probably attributes to the restricted permeability of charged ions across biomembranes. Conceivably, this further indicates that the evocation of stomatal closure by H₂SO₃ is not through binding to cell surface receptors, but via intracellular recognition on the inside of the cell.

Nevertheless, SO₃²⁻ and HSO₃⁻ are immediately formed from H₂SO₃ after reaching the cytoplasm since the cytosolic pH of Arabidopsis guard cells is ~7.65 (Wang et al. 2012).
Wang et al. (2012) estimated the buffering capacity of guard cell cytosol as 84 mmol l⁻¹/pH unit. Given the volume of guard cell is 0.09 pl, a 0.53 nmol of H₂SO₃ influx into a guard cell would make 1 unit of decrease in cytosolic pH. This estimation corresponds with 5.9 mmol l⁻¹ of total aqueous SO₂ concentration in the cell and roughly matches to that in the experimental solution which induced stomatal closure. Although the critical cytosolic pH decrease for guard cell mortality is not known, a 0.5 units decrease in cytosolic pH is thought to be the reason for anoxia-induced cell death (Greenway & Gibbs 2003). The release of H⁺ could be a possible mode of action of SO₂ for the induction of cell death in guard cells, which sequentially leading to the loss of turgor and stomatal closure.

**Mechanism and Physiological Significance of SO₂-Induced Stomatal Closure**

SO₂-induced stomatal closure has been postulated to be due to cytoplasmic acidification to inhibit K⁺ influx (Olszyk & Tibbitts 1981), accumulation of ABA (Kondo & Sugahara 1978; Taylor et al. 1981) and the involvement of H₂S and NO signaling pathways (Hu et al. 2014) in V. faba, Ipomoea batatas and Pisum sativum. As opposed to these claims, our study in Arabidopsis reveals that stomatal closure in SO₂-treated leaves was a result of cell death in guard cells (Fig. 4). Quantification of ABA contents in whole leaf did not show a significant increase in ABA contents over the period of SO₂ exposure (Fig. 7). In additional, O₃- and CO₂-insensitive stomata mutants used in this study are also insensitive to ABA (Mustilli et al. 2002, Negi et al. 2008, Vahisalu et al. 2008, Kwak et al. 2003), yet they still demonstrated closure response towards SO₂ (Fig. 3b). These observations exclude the involvement of ABA in SO₂-induced closing, at least in Arabidopsis. The death of guard cells was proposed to be the reason for stomatal closure in SO₂-treated V. faba (Unsworth & Black 1981). Our study supports this hypothesis with observation of increased guard cell mortality rate. Omasa et al. (1985) reported interesting stomatal responses in attached sunflower leaves of which SO₂-induced closure was reversible in healthy leaf region, but irreversible in leaf region experiencing necrosis. The irreversible closure observed in sunflower might be the outcome of cell death in guard cells.
Taylor et al. (1978) proposed that plants obtained resistance to gaseous pollutants via “stress tolerance” and “stress avoidance” mechanisms, of which the first one involved capacity of plants to tolerate, assimilate or buffer the harmful pollutant derivatives; and the second mechanism involved the closing of stomata to avert pollutant absorption. Transcriptome analyses have disclosed the SO$_2$ detoxification process in plants, involving oxidative pathway in the peroxisomes (sulfite oxidase) and also plastid sulfur assimilation pathway localized in the chloroplasts (Brychkova et al. 2007; Hamisch et al. 2012; Randewig et al. 2012; Considine & Foyer 2015). These findings explain the metabolic changes take place in plant tolerance to non-phytotoxic levels of SO$_2$. In term of “stress avoidance” wise, we were curious if closed stomata in the presence of SO$_2$ is a protection mechanism of plant induced through apoptosis? Unlike reported by Yi et al. (2012) in V. faba, this study using TUNEL assay showed that SO$_2$-induced cell death was not apoptotic (Fig. 8). Unlike the stomatal closure induced by O$_3$, SO$_2$-induced stomatal closure is not a biological process to protect foliage against the entrance of harmful gases, but it is solely due to the killing of guard cells by the toxic effects of SO$_2$.

**Induction of Stomatal Opening by Low Dose of SO$_2$**

Apart from its effect on stomatal closure induction, SO$_2$ was reported to induce opening in V. faba at low concentrations (Mansfield & Majernik 1970; Unsworth & Black 1980; Taylor et al. 1981; Biscoe et al. 1973). This behavior was also observed in our study with Arabidopsis and it is light-dependent (Fig. 6 and Fig. S5). Taylor et al. (1981) proposed that SO$_2$-induced stomatal opening is due to an increase in osmotic pressure of guard cells resulting from the accumulation of sulfite ions, which increases cell turgor and thus promotes opening. While some other researchers attributed it to the weakening of membranes and damage in the epidermal cells surrounding the intact guard cells which lead to the wider opening of stomata (Black & Black 1979; Unsworth & Black 1981). Taking the results from the kinetic study and histogram analysis at 120-min (Fig. 6), when a portion of stomata started to close (due to death of guard cells) while another portion of them opened wider, at the guard cell mortality
rate of 38.8 ± 1.10 %. SO$_2$ induces cell death in pavement cells. We speculate that SO$_2^-$ induced stomatal aperture widening in Arabidopsis is probably due to release from the constraint by surrounding epidermal pavement cells which lost turgor. This process may not have a physiological significance.

**Do Plants Possess a Common Mechanism to Avoid Entry of Hazardous Gases?**

Several studies on air pollutants have identified similarity in the effects of O$_3$ and SO$_2$ on plants. They were thought to induce a similar signaling response in plants (Olszyk & Tingey 1986; Willekens et al. 1994; Mansfield et al. 1993). We further investigated if plants share a common mechanism in response to gaseous stimuli by exposing SO$_2$ to O$_3^-$ and CO$_2^-$ insensitive stomata mutants (Fig. 3, Fig. S3). It was demonstrated that SO$_2$-induced stomatal closure is mediated by cellular events, which are different from other gaseous stimuli.

The evolutionary development of signaling pathways in stomatal closure upon exposure to O$_3$ and elevated level of CO$_2$ is a consequence of geological history of the atmosphere of the Earth. The atmospheric ozone layer is estimated to be fully developed as early as 2 billion years ago (Walker 1978), it was 400 million years earlier than the development of stomata-like pores in land plants (Chater et al. 2017). A recent analysis of atmospheric CO$_2$ trapped in Antarctic ice cores revealed the concentration of CO$_2$ was between 170 – 300 ppm, which is not much different from the pre-industrial era back in 800,000 years ago (Bereiter et al. 2014). In contrast to that, there is no clear record of atmospheric concentration of SO$_2$ in the geological period. The prehistorical concentration of SO$_2$ in troposphere could be comparatively much lower despite the emission from active volcanic activities because the eruption plume would reach to the stratosphere from the crater in less than 10 min (Textor et al. 2004). Drastic global anthropogenic emission of SO$_2$ into the troposphere started to take place from the 1950s following industrial development (Smith et al. 2011). We postulate that these time-line differences in tropospheric concentrations of O$_3$, CO$_2$, and SO$_2$ have played decisive roles in the evolution of stomatal response mechanisms against these gases.
Hypothetically, plants have evolved a central mechanism for “stress avoidance” against hazardous gases by closing stomata. Although SO$_2$ is found to be an exception, it is supported by studies in O$_3$- and CO$_2$-induced closure. Recently, hydrogen sulfide (H$_2$S) was reported to induce stomatal closure as well although the mechanism is still elusive (Honda et al. 2015; Papanatsiou et al. 2015). Additional works on mechanism of stomatal response to other airborne gases such as H$_2$S and nitrogen oxides (NO$_x$) could possibly provide further information in revealing plant protection mechanisms against hazardous gases.

CONCLUSIONS
SO$_2$ is a major air pollutant known to induce stomatal closure. However, the responsible chemical species among the three species in aqueous SO$_2$: H$_2$SO$_3$, HSO$_3^-$, and SO$_3^{2-}$, has not been identified. In this study, we concluded that the responsible species for stomatal closure induction was H$_2$SO$_3$ by examining the stomatal response to a wide range of aqueous SO$_2$ concentrations with varied proportions of these species. SO$_2$ has been reported to induced stomatal opening at low concentrations in addition to closure induction at high concentrations. Our results suggest that SO$_2$ promotes stomatal opening in the light, while provoking cell death in the guard cells at the same time. To provide new insight into the potential common mechanisms in stress avoidance response of stomata against hazardous gases, we examined the stomatal response of O$_3$- and CO$_2$-insensitive stomata mutants to SO$_2$. It is suggested that the molecular mechanism that induced stomatal closure against SO$_2$ is different from O$_3$ and CO$_2$. We also concluded that SO$_2$-induced stomatal closure was highly correlated to non-apoptotic cell death in the guard cells.

Acknowledgments
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LITERATURE CITED


Stöckhardt J.A. (1850) Über die Einwirkung des Rauches der Silberhütten auf die benachbarte Vegetation. Polytechnisches Centralblatt 16, 257-278.


Figure 1. Effect of acidification of external solution on stomatal aperture width. pH of the stomatal opening solution was adjusted with 1 mol l⁻¹ hydrochloric acid (HCl), 1 mol l⁻¹ nitric acid (HNO₃) or 0.61 mol l⁻¹ H₂SO₃. Digits under open circles represent total concentration of H₂SO₃ in mmol l⁻¹. See Supplemental Table S1 for the relation of pH value and added H₂SO₃. Triangle (Δ) represents solvent control (water). Closed and open circles indicate data obtained separately for H₂SO₃. Data were obtained in 3 independent experiments. Twenty stomata were measured in each experiment. Error bars indicate standard errors. Some error bars are too small to be seen.
Figure 2. Induction of stomatal closure by sulfur dioxide-derived chemical species. (a)

Chemical speciation of SO₂ in aqueous solution; Stomatal aperture width in response to: (b) SO₃²⁻ (4.6 × 10⁻⁸ – 3.5 × 10⁻³ mol l⁻¹); (c) HSO₃⁻ (7.8 × 10⁻⁶ – 7.6 × 10⁻³ mol l⁻¹) and; (d) H₂SO₃ (1.5 × 10⁻⁹ – 4.2 × 10⁻³ mol l⁻¹), prepared in stomata opening buffer with two different buffering capacities (1 mmol l⁻¹ and 10 mmol l⁻¹ MES-Tris), from three different sources indicated by o: H₂SO₃ solution; △: Na₂SO₃ solution; ×: Mix solution, prepared from H₂SO₃ and Na₂SO₃ solutions in 1:1 mixture, n = 4 with 80 stomata in total.
Figure 3. Chlorophyll degradation and stomatal closure induction of H$_2$SO$_3$ in wild type (WT), carbon dioxide- and ozone-insensitive stomata mutants (*slac1-1, slac1-3, srk2e*, and *rbohD/F*). (a) Chlorophyll content in H$_2$SO$_3$-treated leaves, n = 6 individual leaf except for *rbohD/F* (n = 3). (b) Stomatal response of *Arabidopsis* lines to H$_2$SO$_3$, n = 4; 80 stomata. Asterisks (*) indicate significant differences (α = 0.05) by Dunnett's test. Error bars represent SE. Some error bars are too small to be seen.
Figure 4. H$_2$SO$_3$-induced cell death in guard cells. (a) Representative fluorescence microscopic images of CFDA- and PI-stained stomatal guard cell exposed to H$_2$SO$_3$. White arrowheads indicate representative PI-staining positive nuclei of dead pavement cells which are also seen in other PI-staining panels. (b) The rate of CFDA- and PI-stained guard cell. The viability of 100 – 140 guard cells was quantified for each concentration in every experiment. Data were from 4 independent experiments. (c) Stomatal opening induction of H$_2$SO$_3$-treated leaves by 10 μM fusicoccin (FC), 2 hr incubation, in the dark, n = 4 biological replicates (80 stomata in total). Error bars indicate SE. Some of the error bars are too small to be seen.
Figure 5. Guard cell viability of H$_2$SO$_3$-exposed wild type, carbon dioxide- and ozone-insensitive stomata mutants (slac1-1, slac1-3, srk2e, and rbohD/F). Four independent experiments with 100 – 140 guard cells observed each. Error bars represent SE. Some error bars are too small to be seen. Asterisks (*) represent significant different via one-way ANOVA followed by Dunnett’s Test ($\alpha = 0.05$).
Figure 6. Time course of H$_2$SO$_3$-induced stomatal closure/opening and cell death in guard cells. (a) Time course of stomatal aperture width and guard cell viability in a period of 180-min incubation in H$_2$SO$_3$. Bar represents stomatal aperture width; dotted line represents the rate of CFDA-stained guard cells; solid line represents the rate of PI-stained guard cells. For stomatal response, n = 6, 10 and 3 for control, 1.1 µmol l$^{-1}$ and 1.2 mmol l$^{-1}$ H$_2$SO$_3$ conditions, respectively. 20 stomata were measured in each experiment, making 120, 200 and 60 stomata measured for each condition, respectively. For viability assay, n = 4 independent experiments (400 – 560 guard cells per point). Error bars represent SE, some error bars are too small to be seen. (b) Distribution of stomatal aperture width at 120-min of H$_2$SO$_3$ treatment. Grey bars indicate the frequency of aperture width; black lines are Gaussian curves fitted to the data distribution; dotted line represents two-peak Gaussian fitting curve; black arrowhead indicates overall mean values of stomatal aperture width after a 3-hr H$_2$SO$_3$ treatment. n = 120, 200 and 60 measurements, for control, 1.1 µmol l$^{-1}$ and 1.2 mmol l$^{-1}$ H$_2$SO$_3$ conditions, respectively.
Figure 7. ABA contents in H₂SO₃ treated leaves. Mature rosette leaves of wild type plants were incubated in the buffer containing 0, 1.1 µmol l⁻¹ and 1.2 mmol l⁻¹ H₂SO₃ for 180-min under the light. Error bars represent SE.
**Figure 8.** Non-apoptotic guard cells death in the H$_2$SO$_3$-exposed epidermis. Representative fluorescence microscopy images of TUNEL-stained stomatal guard cell exposed to a 2-hr treatment of $1.1 \times 10^{-6}$ and $1.2 \times 10^{-3}$ mol l$^{-1}$ of H$_2$SO$_3$ were displayed, with 80 – 120 guard cells observed for each concentration in each experiment. [H$_2$SO$_3$] = 0 represents negative control for H$_2$SO$_3$ treatment. The positive control was prepared by partial DNA digestion with DNase I.
SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Table S1. pH of $\text{H}_2\text{SO}_3$ solutions prepared in opening buffer made up of 10 mmol l$^{-1}$ MES- Tris.

Table S2. Preparation of the exact composition of chemical species in the experimental solutions in Figure 2.

Figure S1. Molecular structure of $\text{SO}_2$, $\text{CO}_2$ and $\text{O}_3$ and chemical species ratio of the $\text{SO}_2$ aqueous solution at different pH.

Figure S2. Effects of Na$^+$ and buffering system on stomatal aperture width.

Figure S3. Leaf appearance of wild type (WT), carbon dioxide- and ozone-insensitive stomata mutants ($slac1$-$1$, $slac1$-$3$, $srk2e$, and $rbohD/F$) after $\text{H}_2\text{SO}_3$ exposure.

Figure S4. Guard cell viability in acidified solution and fusicoccin-induced stomatal opening in the dark at pH3.

Figure S5. Effect of lower concentrations of $\text{H}_2\text{SO}_3$ on the stomatal aperture in the dark.

Appendix I
Table S1. pH of H$_2$SO$_3$ solutions prepared in opening buffer made up of 10 mmol l$^{-1}$ MES-Tris.

<table>
<thead>
<tr>
<th>$C_{Total}$ (mmol l$^{-1}$)</th>
<th>pH (mean ± standard deviation)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.73 ± 0.01</td>
</tr>
<tr>
<td>0.008</td>
<td>5.72 ± 0.01</td>
</tr>
<tr>
<td>1</td>
<td>4.99 ± 0.03</td>
</tr>
<tr>
<td>2.5</td>
<td>2.86 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>2.48 ± 0.00</td>
</tr>
<tr>
<td>10</td>
<td>2.15 ± 0.07</td>
</tr>
</tbody>
</table>

$C_{Total}$ represents total concentration of H$_2$SO$_3$ added. $^a n = 3$. 
Table S2. Preparation of the exact composition of chemical species in the experimental solutions in Figure 2.

<table>
<thead>
<tr>
<th>#</th>
<th>pH of solution</th>
<th>C_{Total} (mol l^{-1})</th>
<th>Solution made of</th>
<th>Deduced concentration of species (mol l^{-1})</th>
<th>Buffering system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[SO_3^{2-}]</td>
<td>[HSO_3^{-}]</td>
</tr>
<tr>
<td>1</td>
<td>5.73</td>
<td>0</td>
<td>N/A</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>5.72</td>
<td>8.0 \times 10^{-6}</td>
<td>H_2SO_3</td>
<td>2.27 \times 10^{-7}</td>
<td>7.77 \times 10^{-5}</td>
</tr>
<tr>
<td>3</td>
<td>5.70</td>
<td>4.0 \times 10^{-6}</td>
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<tr>
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<td>4.53 \times 10^{-8}</td>
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</tr>
<tr>
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<td>9.94 \times 10^{-5}</td>
</tr>
<tr>
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<td>2.86</td>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>10</td>
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<td>6.53 \times 10^{-3}</td>
</tr>
<tr>
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<td>5.69</td>
<td>0</td>
<td>N/A</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>5.20</td>
<td>1.0 \times 10^{-3}</td>
<td>Mix</td>
<td>8.72 \times 10^{-3}</td>
<td>9.91 \times 10^{-3}</td>
</tr>
<tr>
<td>14</td>
<td>3.81</td>
<td>2.5 \times 10^{-3}</td>
<td>Mix</td>
<td>8.83 \times 10^{-3}</td>
<td>2.46 \times 10^{-3}</td>
</tr>
<tr>
<td>15</td>
<td>2.86</td>
<td>5.0 \times 10^{-3}</td>
<td>Mix</td>
<td>1.77 \times 10^{-3}</td>
<td>4.39 \times 10^{-3}</td>
</tr>
<tr>
<td>16</td>
<td>2.50</td>
<td>1.0 \times 10^{-2}</td>
<td>Mix</td>
<td>1.33 \times 10^{-3}</td>
<td>7.60 \times 10^{-3}</td>
</tr>
</tbody>
</table>

Mix indicates solution that was prepared from the mixture of H_2SO_3 and Na_2SO_3 solutions at 1:1 ratio. N/A indicates not available. The pH of each solution was measured immediately after the preparation of H_2SO_3, Na_2SO_3 or the mixture solution in the buffer using a glass electrode (F-52, Horiba, Kyoto, Japan) in triplicate at 25 °C. C_{Total} indicates total concentration of chemical(s) added.
The concentration of each chemical species were deduced using following equations:

\[
[SO_3^{2-}] = \frac{1}{1.8 \times 10^9 \cdot [H^+]^2 + 1.8 \times 10^7 \cdot [H^+] + 1} \cdot C_{\text{Total}} \quad \text{(Equation 1)}
\]

\[
[HSO_3^-] = \frac{1}{1.0 \times 10^2 \cdot [H^+] + 1 + \frac{5.6 \times 10^{-8}}{[H^+]}} \cdot C_{\text{Total}} \quad \text{(Equation 2)}
\]

\[
[H_2SO_3] = \frac{1}{1 + \frac{1.0 \times 10^{-2}}{[H^+]^2} + \frac{5.6 \times 10^{-10}}{[H^+]^2}} \cdot C_{\text{Total}} \quad \text{(Equation 3)}
\]

For derivation of Equations 1, 2 and 3, see Appendix 1.
Figure S1. Molecular structure of SO$_2$, CO$_2$ and O$_3$ and chemical species ratio of the SO$_2$ aqueous solution at different pH. (a) structure of SO$_2$, CO$_2$, and O$_3$. (b) The ratio of three chemical species (SO$_3^{2-}$, HSO$_3^-$ and H$_2$SO$_3$) in aqueous solution, calculated from pK$_{a1}$ and pK$_{a2}$ of H$_2$SO$_3$. 
Figure S2. Effects of Na⁺ and buffering system on stomatal aperture width. (a) Excised leaves were treated with indicated concentrations of Na₂SO₃ or NaCl for 3 hours in the light. Four biological replicates. One replicate is of an average of 20 stomata from the same leaf. Difference between dataset was assessed by Student T-test (α = 0.05). (b) Representation of aperture width data in Fig. 2 with different buffering systems as shown in Table S2. Four biological replicates. One replicate is of an average of 20 stomata from the same leaf. Filled circles indicate the solvent controls (Water). Statistical difference in aperture width between 1 mmol l⁻¹ and 10 mmol l⁻¹ MES-Tris buffers was assessed by Mann-Whitney u test. Error bars indicate standard errors (SE). Some error bars are too small to be seen in panel (b).
**Figure S3.** Leaf appearance of wild type (WT), carbon dioxide- and ozone-insensitive stomata mutants (*slac1-1, slac1-3, srk2e*, and *rbohD/F*) after H₂SO₃ exposure. (a) Representative images of excised mature rosette leaves exposed to aqueous solutions of SO₂ for 3 hr. (b) Representative images for leaf wilting observation.
**Figure S4.** Guard cell viability in acidified solution and fusicoccin-induced stomatal opening in the dark at pH3. (a) The viability rate of guard cells in acidified solution. Leaves were incubated for 3 hr in acidified stomata opening buffer under light (120 µmol m⁻² s⁻¹). pH was adjusted with HCl or HNO₃. n = 4, with 80 – 120 guard cells observed in each experiment, total 320 – 480 guard cells for each point. (b) Stomatal aperture width measured in acidic condition (pH3) in the dark with and without 10 µM fusicoccin (FC). Dark-adapted leaves were floated on 10 mmol l⁻¹ MES-Tris stomata opening buffer, pH 3, for 2 hr. Pre represents stomatal aperture width of pre-treatment; n = 3 independent biological replicates, total 60 stomata. Asterisks (*) indicate significant differences (α = 0.05) by Student’s t-test. Error bars indicate SE. Some error bars are too small to be seen in panel (a).
Figure S5. Effect of low concentrations of H$_2$SO$_3$ on the stomatal aperture in the dark. Dark-acclimated leaves were treated with H$_2$SO$_3$ for 3 hrs in the dark. n = 4, with 80 stomata per bar. n.s. indicates nonsignificant differences (α = 0.05) by Dunnett’s test. Error bars represent SE.
Appendix 1

Derivation of Equation 1

Definition of acid dissociation constant ($K_a$) gives Equations (1.1) and (1.2) in the relation between HSO$_3^-$ and SO$_3^{2-}$, and HSO$_3^-$ and H$_2$SO$_3$ as follow.

\[
[\text{HSO}_3^-] = \frac{[\text{SO}_3^{2-}] \cdot [\text{H}^+]}{K_{a2}} \quad (1.1)
\]

\[
[H_2\text{SO}_3] = \frac{[\text{HSO}_3^-] \cdot [\text{H}^+]}{K_{a1}} \quad (1.2)
\]

where $K_{a1}$ and $K_{a2}$ are of the dissociation constants in the following reactions and values:

\[
\text{H}_2\text{SO}_3 \Leftrightarrow \text{HSO}_3^{2-} + \text{H}^+, \quad K_{a1} = 1.0 \times 10^{-2}
\]

\[
\text{HSO}_3^- \Leftrightarrow \text{SO}_3^{2-} + \text{H}^+, \quad K_{a2} = 5.6 \times 10^{-8}
\]

From the definition of total concentration added ($C_{\text{Total}}$), [SO$_3^{2-}$] is shown as Equation (1.3).

\[
[\text{SO}_3^{2-}] = C_{\text{Total}} - [\text{HSO}_3^-] - [\text{H}_2\text{SO}_3] \quad (1.3)
\]

Assignment of Equations (1.1) and (1.2) into Equation (1.3) gives Equation (1.4).

\[
[\text{SO}_3^{2-}] = C_{\text{Total}} - \frac{[\text{SO}_3^{2-}] \cdot [\text{H}^+]}{K_{a2}} - \frac{[\text{SO}_3^{2-}] \cdot [\text{H}^+]}{K_{a1}} \quad (1.4)
\]

Rearrangement of the formula to isolate [SO$_3^{2-}$] makes Equation (1.5).

\[
[\text{SO}_3^{2-}] = \frac{1}{\frac{[\text{H}^+]^2}{K_{a1} K_{a2}} + \frac{[\text{H}^+]}{K_{a2} + 1}} \cdot C_{\text{Total}} \quad (1.5)
\]

Assignment of values of $K_{a1}$ and $K_{a2}$ into Equation (1.5) gives Equation 1.

\[
[\text{SO}_3^{2-}] = \frac{1}{1.8 \times 10^9 \cdot [\text{H}^+]^2 + 1.8 \times 10^7 \cdot [\text{H}^+] + 1} \cdot C_{\text{Total}} \quad \text{(Equation 1)}
\]
Derivation of Equation 2

Definition of $K_a$ and $C_{Total}$ gives Equations (2.1) and (2.2) as follow.

\[
[SO_3^{2-}] = \frac{K_{a2} \cdot [HSO_3^-]}{[H^+]} \quad (2.1)
\]

\[
[HSO_3^-] = C_{Total} - [SO_3^{2-}] - [H_2SO_3] \quad (2.2)
\]

Assignment of Equations (2.1) and (1.2) into Equation (2.2) gives Equation (2.3).

\[
[HSO_3^-] = C_{Total} - \frac{K_{a2} \cdot [HSO_3^-]}{[H^+]} - \frac{[HSO_3^-] \cdot [H^+]}{K_{a1}} \quad (2.3)
\]

Rearrangement of the formula to isolate $[HSO_3^-]$ makes Equation (2.4).

\[
[HSO_3^-] = \frac{1}{\frac{[H^+]}{K_{a1}} + 1 + \frac{K_{a2}}{[H^+]}} \cdot C_{Total} \quad (2.4)
\]

Assignment of values of $K_{a1}$ and $K_{a2}$ into Equation (2.4) gives Equation 2.

\[
[HSO_3^-] = \frac{1}{1.0 \times 10^{2} \cdot [H^+] + 1 + \frac{2.6 \times 10^{-8}}{[H^+]}} \cdot C_{Total} \quad \text{(Equation 2)}
\]
Derivation of Equation 3

Definition of $K_a$ and $C_{Total}$ gives Equations (3.1) as follow.

$$[\text{HSO}_3^-] = \frac{K_{a1} \cdot [\text{H}_2\text{SO}_3]}{[\text{H}^+]^1} \quad (3.1)$$

From the definition of total concentration added ($C_{Total}$), $[\text{H}_2\text{SO}_3]$ is shown as Equation (3.2).

$$[\text{H}_2\text{SO}_3] = C_{Total} - [\text{HSO}_3^-] - [\text{SO}_5^-] \quad (3.2)$$

Assignment of Equations (2.1) and (3.1) into Equation (3.2) gives Equation (3.3).

$$[\text{H}_2\text{SO}_3] = C_{Total} - \frac{K_{a1} \cdot [\text{H}_2\text{SO}_3]}{[\text{H}^+]^1} - \frac{K_{a2} \cdot \frac{K_{a1} \cdot [\text{H}_2\text{SO}_3]}{[\text{H}^+]^1}}{[\text{H}^+]^1} \quad (3.3)$$

Rearrangement of Equation (3.3) to isolate $[\text{H}_2\text{SO}_3]$ makes Equation (3.4)

$$[\text{H}_2\text{SO}_3] = \frac{1}{1 + \frac{K_{a1}}{[\text{H}^+]^1} + \frac{K_{a1} \cdot K_{a2}}{[\text{H}^+]^2}} \cdot C_{Total} \quad (3.4)$$

Assignment of values of $K_{a1}$ and $K_{a2}$ into Equation (3.4) gives Equation 3.

$$[\text{H}_2\text{SO}_3] = \frac{1}{1 + \frac{1.0 \times 10^{-2}}{[\text{H}^+]^1} + \frac{5.6 \times 10^{-18}}{[\text{H}^+]^2}} \cdot C_{Total} \quad (\text{Equation 3})$$