Title

Mechanism of SO_2-Induced Stomatal Closure Is Unshared by O_3 and CO_2 Response and Is Mediated by Non-Apoptotic Cell Death in Guard Cells

Running Head: Stomatal Response against Sulfur Dioxide

List of Authors' Names

Lia Ooi[1], Takakazu Matsuura[1], Shintaro Munemasa[2], Yoshiyuki Murata[2], Maki Katsuhara[1], Takashi Hirayama[1] and Izumi C. Mori[1]

Institute of Origin

[1]Institute of Plant Science and Resources, Okayama University, 2-20-1 Chuo, Kurashiki, 710-0046, Japan

[2]Division of Agricultural and Life Science, Graduate School of Environmental and Life Science, Okayama University, 1-1-1 Tsuhima-naka, Kitaku, Okayama, 700-8530, Japan

Correspondence to

Dr. Izumi C. Mori

Tel: +81-86-434-1215

E-mail: imori@okayama-u.ac.jp

ORCID IDs: 0000-0002-2235-0779 (I.C.M.)

ABSTRACT

Plants closing stomata in the presence of harmful gases is believed to be a stress avoidance mechanism. SO_2, 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 O_3. *SLOW ANION CHANNEL-ASSOCIATED 1* (*SLAC1*) and *OPEN STOMATA 1* (*OST1*) were identified as genes mediating O_3-induced closure. *SLAC1* and *OST1* are also known to mediate stomatal closure in response to CO_2, together with *RESPIRATORY BURST OXIDASE HOMOLOG*s (*RBOH*s). The overlaying roles of these genes in response to O_3 and CO_2 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 SO_2 in Arabidopsis through molecular genetic approaches. O_3- and CO_2-insensitive stomata mutants did not show significant differences from the wild type in stomatal sensitivity, guard cell viability and chlorophyll content revealing that SO_2-induced closure is not regulated by the same molecular mechanisms as for O_3 and CO_2. Non-apoptotic cell death is shown as the reason for SO_2-induced closure, which proposed the closure as a physicochemical process resulted from SO_2 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 (CO_2) uptake under biotic and abiotic stresses in the environment (Murata *et al.* 2015). Environment-polluting gases, such as ozone (O_3), nitrogen dioxide (NO_2) and sulfur dioxide (SO_2) 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 O_3. *SLOW ANION CHANNEL-ASSOCIATED 1/OZONE-SENSITIVE-1* (*SLAC1/OZS1*) was identified as a critical factor in O_3-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 O_3 sensitivity owing to the insensitivity of stomata against O_3, which gives rise to augmented O_3 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 O_3-triggered rapid transient decrease in stomatal conductance (Vahisalu *et al.* 2010).

CO_2 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 CO_2-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 HOMOLOG*s (*RBOH*s) encoding the catalytic subunit of NADPH oxidase has been shown to render stomata insensitive to CO_2 (Chater *et al.* 2015).

SO_2, 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 SO_2 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, SO_2 emission remained high in the last decade (Klimont *et al.* 2013). The effects of SO_2 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_2SO_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 stil 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. **S1**a), 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 SO_2 in the stomata opening buffer containing 5 mmol l^{-1} KCl, 50 μmol l^{-1} $CaCl_2$, 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 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 H_2SO_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} Na_2HPO_4, 2.68 mmol l^{-1} KCl, 1.47 mmol l^{-1} KH_2PO_4), 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 SO_2 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 SO_2 gas and exposure to H_2SO_3 solution on stomata are assumed to be essentially the same (Taylor *et al*., 1981). To investigate the effects of SO_2 on Arabidopsis stomata, we first questioned whether acidification of extracellular solution caused by H_2SO_3 exposure is the main reason for the closure. Here, we examined the effect of the external solution acidified with three acids, HCl, HNO_3 and H_2SO_3 (Fig. 1). Acidification of the stomata opening buffer with HCl and HNO_3 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 H_2SO_3. This result strongly suggests that H_2SO_3-induced stomatal closure is not solely attributable to the low pH of the extracellular fluid.

Three chemical species are formed when SO_2 gas is dissolved in water: H_2SO_3, HSO_3^-, and SO_3^{2-} of which the compositions in the aqueous solution depending on the pH (Fig. 2a and Fig. **S1**b). We examined the chemical species in the aqueous solution of SO_2 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 **S**2 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_2SO_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_2SO_3 and Na_2SO_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_2SO_3 (Fig. 2d). A significant decrease in aperture width was not observed below 38 μmol l^{-1} H_2SO_3. Higher concentrations of H_2SO_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_2SO_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. **S2**a) and statistical test with Mann-Whitney *u* test between the buffering systems (Fig. **S2**b).

Collectively, we concluded that H_2SO_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_2SO_3-induced Stomatal Closure — An Unshared Molecular Mechanism with O_3 and CO_2

The effects of H_2SO_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_2SO_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_2SO_3 demonstrating no difference in the lowest effective concentration in all lines (Fig. 3a). Fig. 3b shows H_2SO_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_2SO_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_2SO_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_2SO_3, 93.1 ± 2.8 % of guard cells were positively stained with CFDA. As the [H_2SO_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_2SO_3] ≥ 0.30 mmol l^{-1}. Guard cell mortality rate was below 20% for treatments < 0.1 µmol l^{-1}. At [H_2SO_3] = 1.1 µmol l^{-1}, the viability rate of guard cell was 44 ± 14 %, while at [H_2SO_3] ≥ 0.3 µmol l^{-1}, the mortality rate was approximately 100% or equal to 100%. This indicates that H_2SO_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. **S4**a).

H_2SO_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_2SO_3 and no substantially opening was observed in the presence of 0.3 mmol l^{-1} H_2SO_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_2SO_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. **S4**b).

The effect of H_2SO_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_2SO_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_2SO_3 with concentrations equal to or greater than 0.3 mmol l^{-1}. H_2SO_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_2SO_3 on guard cells is mediated by mechanisms different from that of O_3 and CO_2.

Kinetics of Stomatal Response to H_2SO_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_2SO_3 to gain further insight into the relationship of stomatal closure and the death of guard cells (Fig. 6a). In the absence of H_2SO_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_2SO_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_2SO_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_2SO_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_2SO_3 incubation.

A histogram analysis was performed for stomatal aperture width in leaves incubated with H_2SO_3 for 120 min to investigate the discrepancy between stomatal aperture and guard

cell mortality (Fig. 6b). In H_2SO_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_2SO_3, at 0.75 and 3.60 μm (calculated means of the Gaussian curves), respectively. This suggests that at 120-min of H_2SO_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_2SO_3 treatment (Fig. 6a). We also performed a stomatal opening assay in the dark with a series of [H_2SO_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_2SO_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_2SO_3-induced stomatal closure in wild type Arabidopsis by quantifying ABA contents in H_2SO_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_2SO_3 as compared to the control, suggesting that ABA does not play a crucial role in closing stomata during H_2SO_3 exposure in Arabidopsis.

H_2SO_3 Induces Non-Apoptotic Cell Death

Apoptosis, that is accompanied with DNA laddering can occur as hypersensitive response (HR) to incompatible pathogens and O_3-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 H_2SO_3 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 H_2SO_3, the guard cells treated with 1.1 µmol l^{-1} and 1.2 mmol l^{-1} H_2SO_3 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} H_2SO_3 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 SO_2 in Stomatal Closure and Cell Death in Guard Cells

The mechanism of SO_2 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 SO_2 in inducing stomatal closure remains concealed. Through observation of stomatal response to each chemical species formed in the aqueous solution of SO_2 (Fig. 2), we excluded the involvement of SO_3^{2-} and HSO_3^- in SO_2-induced stomatal closure. Our results suggest that H_2SO_3 is the only chemical species that closes stomata in the presence of SO_2. This is probably attributes to the restricted permeability of charged ions across biomembranes. Conceivably, this further indicates that the evocation of stomatal closure by H_2SO_3 is not through binding to cell surface receptors, but via intracellular recognition on the inside of the cell.

Nevertheless, SO_3^{2-} and HSO_3^- are immediately formed from H_2SO_3 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 H_2SO_3 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 SO_2 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 SO_2 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 SO_2-Induced Stomatal Closure

SO_2-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 H_2S 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 SO_2-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 SO_2 exposure (Fig. 7). In additional, O_3- and CO_2-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 SO_2 (Fig. 3b). These observations exclude the involvement of ABA in SO_2-induced closing, at least in Arabidopsis. The death of guard cells was proposed to be the reason for stomatal closure in SO_2-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 SO_2-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_2S) 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_2S 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_2SO_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_2SO_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

This research was supported by JSPS KAKENHI Grant-in-Aid for Scientific Research on Innovative Areas (grant No. 24114709), Japan Advanced Plant Science Research Network, and Research Grant for Encouragement of Students FY2017, Graduate School of Environmental and Life Science, Okayama University.

LITERATURE CITED

Bereiter B., Eggleston S., Schmitt J., Nehrbass-Ahles C., Stocker T.F., Fischer H., Kipfstuhl S. & Chappellaz J. (2014) Revision of the EPICA Dome C CO_2 record from 800 to 600 kyr before present. *Geophysical Research Letters* 42, 542-549.

Biscoe P.V., Unsworth M.H. & Pinckney H.R. (1973) The effects of low concentrations of sulphur dioxide on stomatal behaviour In *Vicia faba*. *New Phytologist* 72, 1299-1306.

Black C.R. & Black V.J. (1979) The effects of low concentrations of sulphur dioxide on stomatal conductance and epidermal cell survival in field bean (*Vicia faba* L.). *Journal of Experimental Botany* 30, 291-298.

Blatt M.R. (1988) Mechanisms of fusicoccin action: A dominant role for secondary transport in higher-plant cell. *Planta* 174, 187-200.

Bobbink R. (1998) Impacts of tropospheric ozone and airborne nitrogenous pollutants on natural and semi-natural ecosystems: a commentary. *New Phytologist* 139, 161-168.

Brychkova G., Xia Z., Yang G., Yesbergenova Z., Zhang Z., Davydov O., …, Sagi M. (2007) Sulfite oxide protects plants against sulfur dioxide toxicity. *The Plant Journal* 50, 696-709.

Cape J.N. (1998) Uptake and fate of gaseous pollutants in leaves. *New Phytologist* 139, 221-223.

Considine M.J. & Foyer C.H. (2015) Metabolic responses to sulfur dioxide in grapevine (*Vitis vinifera* L.): photosynthetic tissues and berries. *Frontiers in Plant Science* 6, 1-10.

Chater C.C.C., Caine R.S., Fleming A.J. & Gray J.E. (2017) Origins and evolution of stomatal development. *Plant Physiology* 174, 624-638.

Chater C., Peng K., Movahedi M., Dunn J.A., Walker H.J., Liang Y.-K., McLachlan D.H., …, Hetherington A.M. (2015) Elevated CO_2-induced responses in stomata require ABA and ABA signaling. *Current Biology* 25, 2709-2716.

Engineer C.B., Hashimoto-Sugimoto M., Negi J., Israelsson-Nordström, Azoulay-Shemer T., Rappel W.-J., …, Schroeder J.I. (2016) CO_2 sensing and CO_2 regulation of stomatal conductance: advances and open questions. *Trends in Plant Science* 21, 16-30.

Finkelstein R. (2013) Abscisic acid synthesis and response. *The Arabidopsis Book 11: e0166*

Greenway H. & Gibbs J. (2003) Mechanisms of anoxia tolerance in plants. II. Energy requirements for maintenance and energy distribution to essential processes. *Functional Plant Biology* 30, 999-1036.

Hamisch D., Randewig D., Schliesky S., Bräutigam A., Weber A.P.M., Geffers R., …, Hänsch R. (2012) Impact of SO_2 on *Arabidopsis thaliana* transcriptome in wildtype and sulfite oxidase knockout plants analyzed by RNA deep sequencing. *New Phytologist* 196, 1074-1085.

Hayashi M., Inoue S.-I., Takahashi K. & Kinoshita T. (2011) Immunohistochemical detection of blue light-induced phosphorylation of the plasma membrane H^+-ATPase in stomatal guard cells. *Plant and Cell Physiology* 52, 1238-1248.

Honda K., Yamada N., Yoshida R., Ihara H., Sawa T., Akaike T. & Iwai S. (2015) 8-Mercapto-Cyclic GMP mediates hydrogen sulfide-induced stomatal closure in Arabidopsis. *Plant and Cell Physiology* 56, 1481-1489.

Horsman D.C. & Wellburn A.R. (1977) Effect of SO_2 polluted air upon enzyme activity in plants originating from areas with different annual mean atmospheric SO_2 concentrations. *Environmental Pollution* 13, 33-39.

Hu K.-D., Tang J., Zhao D.-L., Hu L.-Y., Li Y.-H., Liu Y.-S., …, Zhang H. (2014) Stomatal closure in sweet potato leaves induced by sulfur dioxide involves H_2S and NO signaling pathway. *Biologia Plantarum* 58, 676-680.

Johnson S., Nguyen V. & Coder D. (2013) Assessment of cell viability. *Current Protocols in Cytometry* 64, 9.2.1-9.2.26.

Klimont Z., Smith S.J. & Cofala J. (2013) The last decade of global anthropogenic sulfur dioxide: 2000-2011 emissions. *Environmental Research Letters* 8, 1-6.

Kondo N. & Sugahara K. (1978) Changes in transpiration rate of SO_2-resistant and –sensitive plants with SO_2 fumigation and the participation of abscisic acid. *Plant and Cell Physiology* 19, 365-373.

Kropff M.J. (1987) The effect of SO_2 on photosynthesis and stomatal regulation of *Vicia faba* L. *Plant, Cell and Environment* 10, 753-760.

Kropff M.J. (1991) Long-term effects of SO_2 on plants, SO_2 metabolism and regulation of intracellular pH. *Plant and Soil* 131, 235-245.

Kwak J.M., Mori I.C., Pei Z.-M., Leonhardt N., Torres M.A., Dangl J.L., …, Schroeder J.I. (2003) NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. *The EMBO Journal* 22, 2623-2633.

Malhotra S.S. & Hocking D. (1976) Biochemical and cytological effects of Sulphur dioxide on plant metabolism. *New Phytologist* 67, 227-237.

Mansfield T.A. & Majernik O. (1970) Can stomata play a part in protecting plants against air pollutants? *Environmental Pollution* 1, 149-154.

Mansfield T.A., Pearson M., Atkinson C.J. & Wookey P.A. (1993) Ozone, sulphur dioxide and nitrogen oxides: some effects on the water relations of herbaceous plants and trees. *In*: Jackson M.B. & Black C.R. (Eds) Interacting Stresses on Plants in a Changing Climate *NATO ASI Series* Vol 16. Springer, Berlin, Heidelberg.

Marré E. (1979) Fusicoccin: A tool in plant physiology. *Annual Review of Plant Physiology* 30, 273-88.

McAinsh M.R., Evans N.H., Montgomery L.T. & North K.A. (2002) Calcium signaling in stomatal responses to pollutants. *New Phytologist* 153, 441-447.

Merilo E., Laanemets K., Hu H., Xue S., Jakobson L., Tulva I., …, Kollist H. (2013) PYR/RCAR receptors contribute to ozone-, reduced air humidity-, darkness-, and CO_2-induced stomatal regulation. *Plant Physiology* 162, 1652-1668.

Muneer S., Kim T.H., Choi B.C., Lee B.S. & Lee J.H. (2014) Effect of CO, NO_x and SO_2 on ROS production, photosynthesis and ascorbate-glutathione pathway to induce *Fragaria* x *annasa* as a hyperaccumulator. *Redox Biology* 2, 91-98.

Murata Y., Mori I.C. & Munemasa S. (2015) Diverse stomatal signaling and the signal integration mechanism. *Annual Review of Plant Biology* 66, 369-92.

Mustilli A.-C., Merlot S., Vavasseur A., Fenzi F. & Giraudat J. (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* 14, 3089-3099.

Negi J., Matsuda O., Nagasawa T., Oba Y., Takahashi H., Kawai-Yamada M., …, Iba K. (2008) CO_2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. *Nature* 452, 483-486.

Olszyk D.M. & Tibbitts T.W. (1981) Stomatal response and leaf injury of *Pisum sativum* L. with SO_2 and O_3 exposures. *Plant Physiology* 67, 539-544.

Olszyk D.M. & Tingey D.T. (1986) Joint action of O_3 and SO_2 in modifying plant gas exchange. *Plant Physiology* 82, 401-405.

Omasa K., Hashimoto Y., Kramer P.J., Strain B.R., Aiga I. & Kondo J. (1985) Direct observation of reversible and irreversible stomatal responses of attached sunflower leaves to SO_2. *Plant Physiology* 79, 153-158.

Papanatsiou M., Scuffi D., Blatt M.R. & Carcía-Mata C. (2015) Hydrogen sulfide regulates inward-rectifying K^+ channels in conjunction with stomatal closure. *Plant Physiology* 168, 29-35.

Pasqualini S., Piccioni C., Reale L., Ederli L., Torre G.D. & Ferranti F. (2003) Ozone-induced cell death in tobacco cultivar Bel W3 plants. The Role of Programmed Cell Death in Lesion Formation. *Plant Physiology* 133, 1122-1134.

Porra R.J., Thompson W.A. & Kriedemann P.E. (1989) Determination of accurate extinction coefficient and simultaneous equations for assaying chlorophyll *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta* 975, 384-394.

Randewig D., Hamisch D., Herschbach C., Eiblmeier M., Gehl C., Jurgeleit J., Skerra J., …, Hänsch R. (2012) Sulfite oxide controls sulfur metabolism under SO_2 exposure in *Arabidopsis thaliana*. *Plant, Cell and Environment* 35, 100-115.

Reape T.J., Molony E.M. & McCabe P.F. (2008) Programmed cell death in plants: distinguishing between different modes. *Journal of Experimental Botany* 59, 435-444.

Saji S., Bathula S., Kubo A., Tamaoki M., Kanna M., Aono M., …, Saji H. (2008) Disruption of a gene encoding C_4-dicarboxylate transporter-like protein increases ozone sensitivity through deregulation of the stomatal response in *Arabidopsis thaliana*. *Plant and Cell Physiology* 49, 2-10.

Schroeder J.I., Allen G.J., Hugouvieux V., Kwak J.M. & Waner D. (2001) Guard cell signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology* 52, 627-58.

Smith S.J., Aardenne J., Klimont Z., Andres R.J., Volke A. & Arias S.D. (2011) Anthropogenic sulfur dioxide emissions: 1850-2005. *Atmospheric Chemistry and Physics* 11, 1101-1116.

Sprugel D.G., Miller J.E., Muller R.N., Smith H.J. & Xerikos P.B. (1980) Sulfur dioxide effects on yield and seed quality in field-grown soybeans. *Phytopathology* 70, 1129-1133.

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

Taylor G.E.J. (1978) Plant and leaf resistance to gaseous air pollution stress. *New Phytologist* 80, 523-534.

Taylor J.S., Reid D.M. & Pharis R.P. (1981) Mutual antagonism of sulfur dioxide and abscisic acid in their effect on stomatal aperture in broad bean (*Vicia faba* L.). *Plant Physiology* 68, 1504-1507.

Textor C., Graf H.-F., Timmreck C. & Robock A. (2004) Emissions from volcanoes. In *Emissions of Atmospheric Trace Compounds*. (eds. Granier C., Artaxo P. & Reeves C.) pp. 269-303. Kluwer Academic Publisher, Dordrecht.

Thomas M.D., Hendricks R.H. & Hill G.R. (1944) Some chemical reactions of sulphur dioxide after absorption by alfalfa and sugar beets. *Plant Physiology* 19, 212-244.

Thomas M.D., Hendricks R.H. & Hill G.R. (1950) Sulfur metabolism of plants: effect of sulfur dioxide on vegetation. *Industrial and Engineering Chemistry* 42, 2231-2235.

Unsworth M.H. & Black V.J. (1980) Stomatal responses to sulphur dioxide and vapour pressure deficit. *Journal of Experimental Botany* 31, 667-677.

Unsworth M.H. & Black V.J. (1981) Stomatal responses to pollutants. *In*: Jarvis P.G. and Mansfield T.A. (Eds.) Stomatal physiology pp. 187-203 Cambridge University Press, London.

Vahisalu T., Kollist H., Wang Y.-F., Nishimura N., Chan W.-Y., Valerio G., …, Kangasjärvi J. (2008) SLAC1 is required for plant guard cell S-type anion channel function in stomatal signaling. *Nature* 452, 487-491.

Vahisalu T., Puzõrjova I., Brosché M., Valk E., Lepiku M., Moldau H., …, Kollist H. (2010) Ozone-triggered rapid stomatal response involves the production of reactive oxygen species, and is controlled by SLAC1 and OST1. *The Plant Journal* 62, 442-453.

Walker J.C.G. (1978) The early history of oxygen and ozone in the atmosphere. *Pure and Applied Geophysics* 117, 498-512.

Wang Y., Papanatsiou M., Eisenach C., Karnik R., Williams M., Hills A., …, Blatt M.R. (2012) Systems dynamic modeling of a guard cell Cl^- channel mutant uncovers an emergent homeostatic network regulating stomatal transpiration. *Plant Physiology* 160, 1956-1967.

WHO (2000) Effects of sulfur dioxide on vegetation: critical levels. *In* Air Quality Guidelines for Europe, 2nd Ed. Chapter 10, pp. 1-17. WHO Regional Office for Europe, Copenhagen.

Willekens H., Camp W.V., Montagu M.V., Inzé D., Langebartels C. & Sandermann H.J. (1994) Ozone, sulfur dioxide, and ultraviolet B have similar effects on mRNA accumulation of antioxidant genes in *Nicotiana plumbaginifolia* L.. *Plant Physiology* 106, 1007-1014.

Wilson S.A. & Murray F. (1990) SO_2-induced growth reductions and sulphur accumulation in wheat. *Environmental Pollution* 66, 179-191.

Winner W.E. & Mooney H.A. (1980) Responses of Hawaiian plants to volcanic sulfur dioxide: Stomatal behavior and foliar injury. *Science* 210, 789-791.

Xue S., Hu H., Ries A., Merilo E., Kollist H. & Schroeder J.I. (2011) Central functions of bicarbonate in S-type anion channel activation and OST1 protein kinase in CO_2 signal transduction in guard cell. *The EMBO Journal* 30, 1645-1658.

Yi H., Yin J., Liu X., Jing X., Fan S. & Zhang H. (2012) Sulfur dioxide induced programmed cell death in *Vicia* guard cells. *Ecotoxicology and Environmental Safety* 78, 281-286.

Yin Y., Adachi Y., Ye W., Hayashi M., Nakamura Y., Kinoshita T., …, Murata Y. (2013) Different in abscisic acid perception mechanisms between closure induction and opening inhibition of stomata. *Plant Physiology* 163, 600-610.

Yin Y., Adachi Y., Nakamura Y., Munemasa S., Mori I.C. & Murata Y. (2016) Involvement of OST1 protein kinase and PYR/PYL/RCAR receptors in Methyl Jasmonate-induced stomatal closure in Arabidopsis guard cells. *Plant and Cell Physiology* 57, 1779-1790.

Yoshida R., Hobo T., Ichimura K., Mizoguchi T., Takahashi F., Aronso J., …, Shinozaki K. (2002) ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in Arabidopsis. *Plant and Cell Physiology* 43, 1473-83.

FIGURES

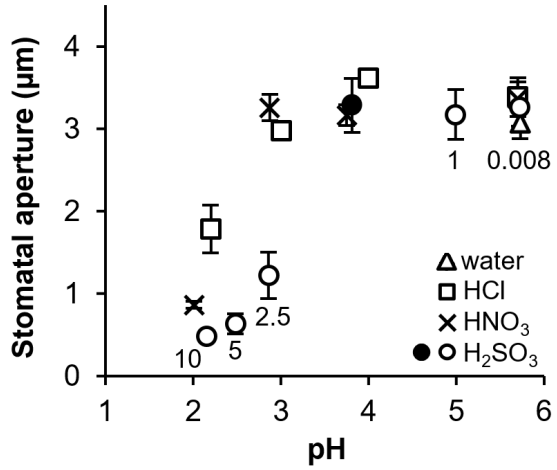

Figure 1. Effect of acidification of external solution on stomatal aperture width. pH of the stomatal opening solution was adjusted with 1 mol l^{-1} hydrochloric acid (HCl), 1 mol l^{-1} nitric acid (HNO_3) or 0.61 mol l^{-1} H_2SO_3. Digits under open circles represent total concentration of H_2SO_3 in mmol l^{-1}. See Supplemental Table S1 for the relation of pH value and added H_2SO_3. Triangle (∆) represents solvent control (water). Closed and open circles indicate data obtained separately for H_2SO_3. 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.

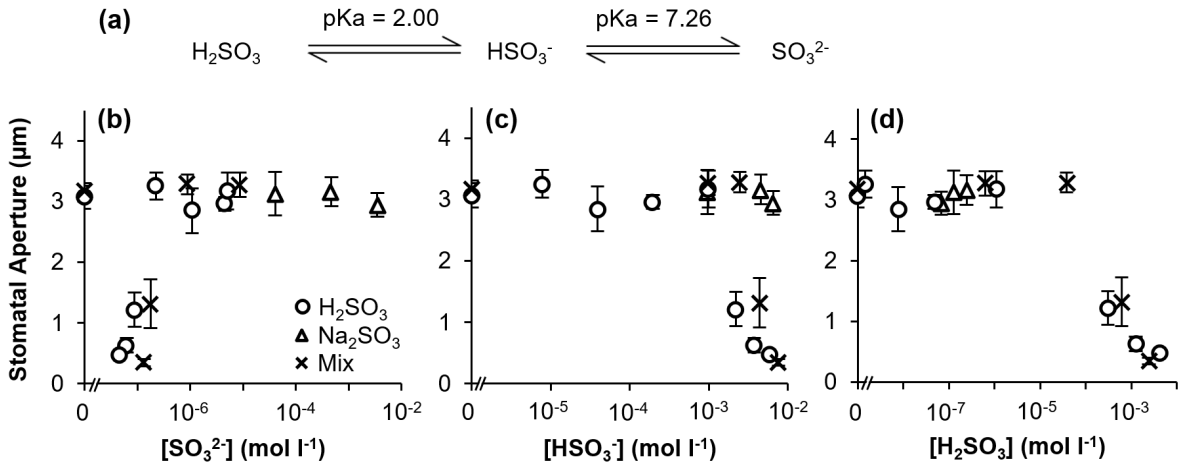

Figure 2. Induction of stomatal closure by sulfur dioxide-derived chemical species. (a) Chemical speciation of SO_2 in aqueous solution; Stomatal aperture width in response to: (b) SO_3^{2-} ($4.6 \times 10^{-8} – 3.5 \times 10^{-3}$ mol l^{-1}); (c) HSO_3^- ($7.8 \times 10^{-6} – 7.6 \times 10^{-3}$ mol l^{-1}) and; (d) H_2SO_3 ($1.5 \times 10^{-9} – 4.2 \times 10^{-3}$ 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: H_2SO_3 solution; Δ: Na_2SO_3 solution; ×: Mix solution, prepared from H_2SO_3 and Na_2SO_3 solutions in 1:1 mixture, n = 4 with 80 stomata in total.

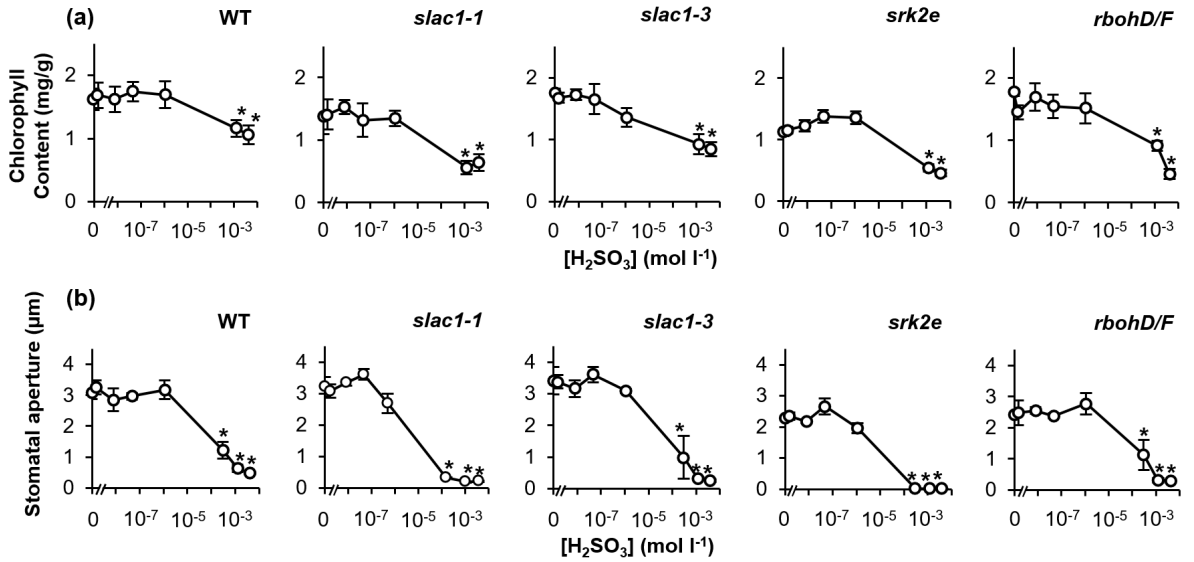

Figure 3. Chlorophyll degradation and stomatal closure induction of H_2SO_3 in wild type (WT), carbon dioxide- and ozone-insensitive stomata mutants (*slac1-1, slac1-3*, *srk2e*, and *rbohD/F*). (a) Chlorophyll content in H_2SO_3-treated leaves, n = 6 individual leaf except for *rbohD/F* (n = 3). (b) Stomatal response of *Arabidopsis* lines to H_2SO_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.

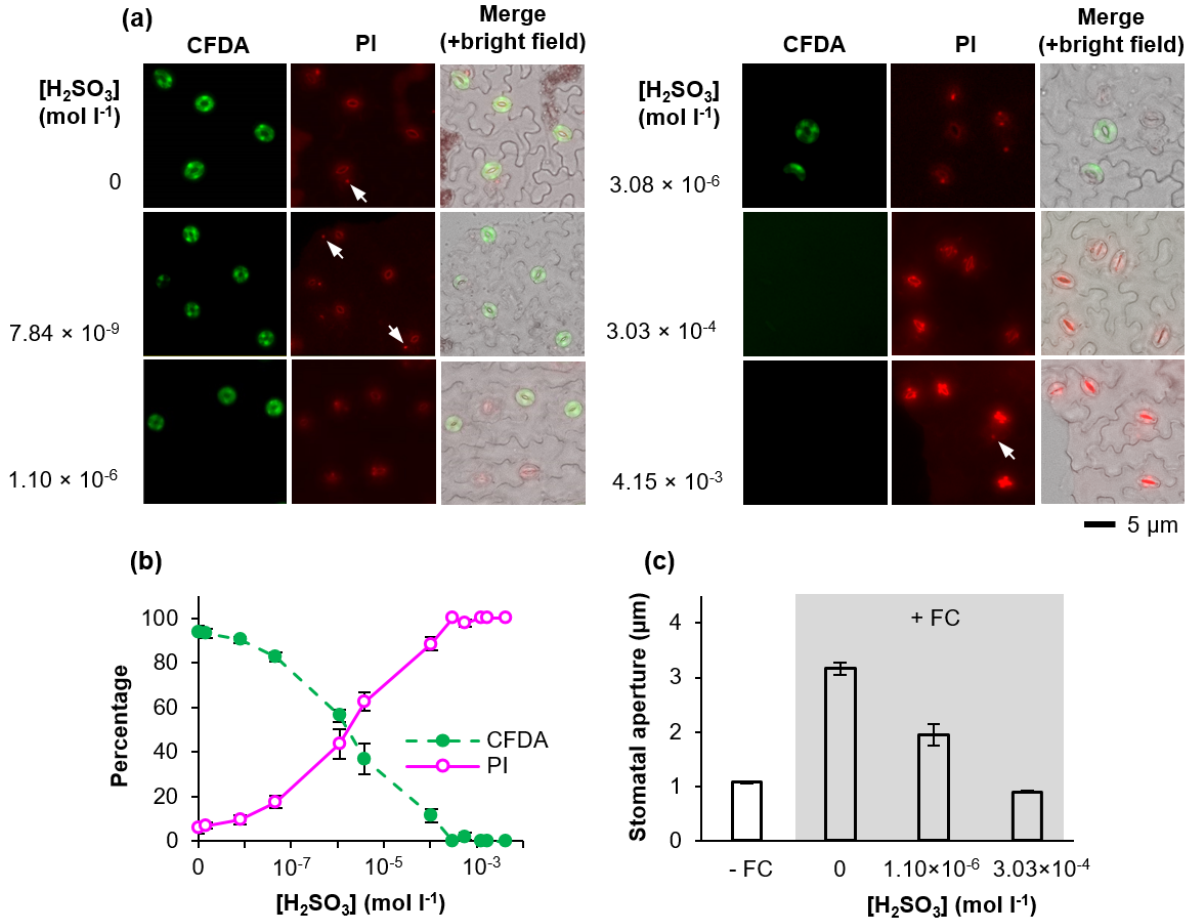

Figure 4. H_2SO_3-induced cell death in guard cells. (a) Representative fluorescence microscopic images of CFDA- and PI-stained stomatal guard cell exposed to H_2SO_3. White arrowheads indicate representative PI-staining positive nuclei of dead pavement cells which are also seen in other PI-staining 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_2SO_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.

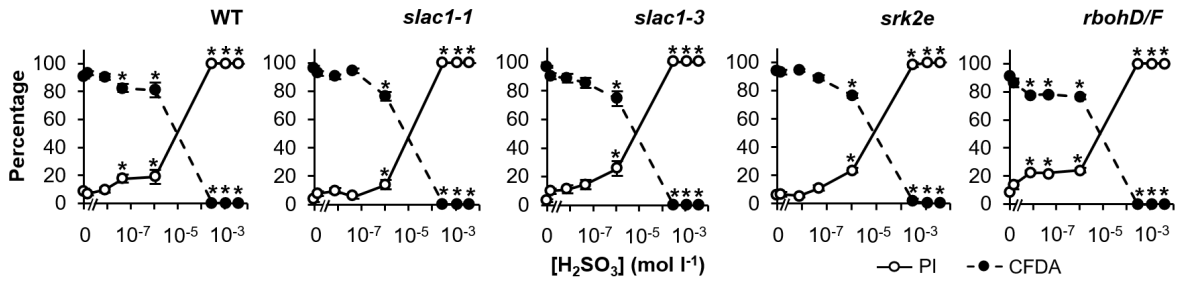

Figure 5. Guard cell viability of H_2SO_3-exposed wild type, carbon dioxide- and ozone-insensitive stomata mutants (*slac1-1, slac1-3, srk2e,* and *rbohD/F*). Four independent experiments with 100 – 140 guard cells observed each. Error bars represent SE. Some error bars are too small to be seen. Asterisks (*) represent significant different via one-way ANOVA followed by Dunnett's Test (α = 0.05).

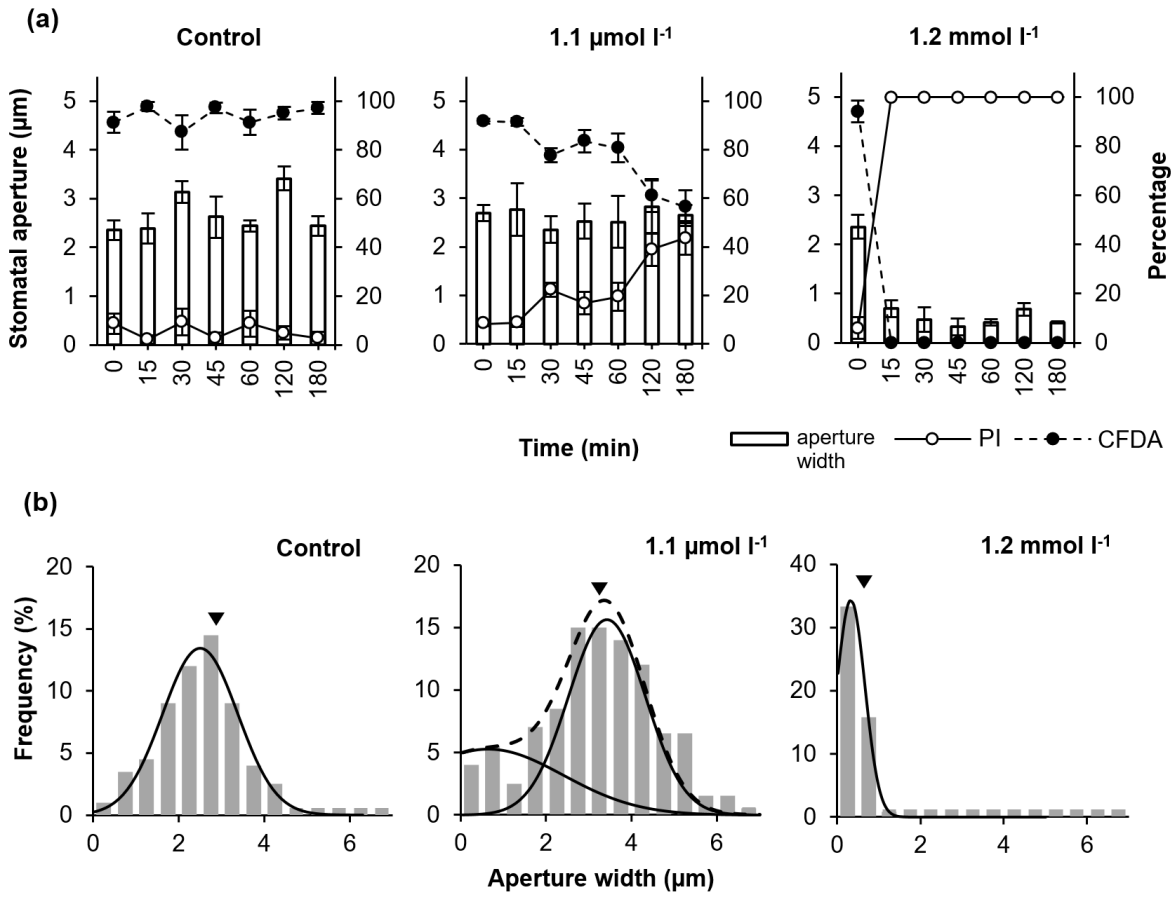

Figure 6. Time course of H_2SO_3-induced stomatal closure/opening and cell death in guard cells. (a) Time course of stomatal aperture width and guard cell viability in a period of 180-min incubation in H_2SO_3. Bar represents stomatal aperture width; dotted line represents the rate of CFDA-stained guard 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_2SO_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_2SO_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_2SO_3 treatment. n = 120, 200 and 60 measurements, for control, 1.1 µmol l^{-1} and 1.2 mmol l^{-1} H_2SO_3 conditions, respectively.

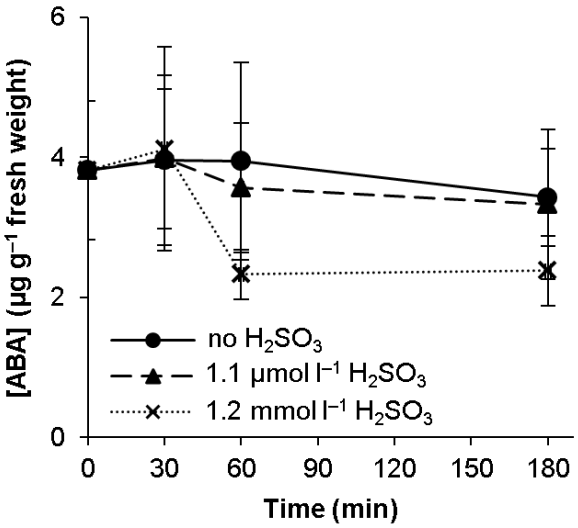

Figure 7. ABA contents in H_2SO_3 treated leaves. Mature rosette leaves of wild type plants were incubated in the buffer containing 0, 1.1 µmol l^{-1} and 1.2 mmol l^{-1} H_2SO_3 for 180-min under the light. Error bars represent SE.

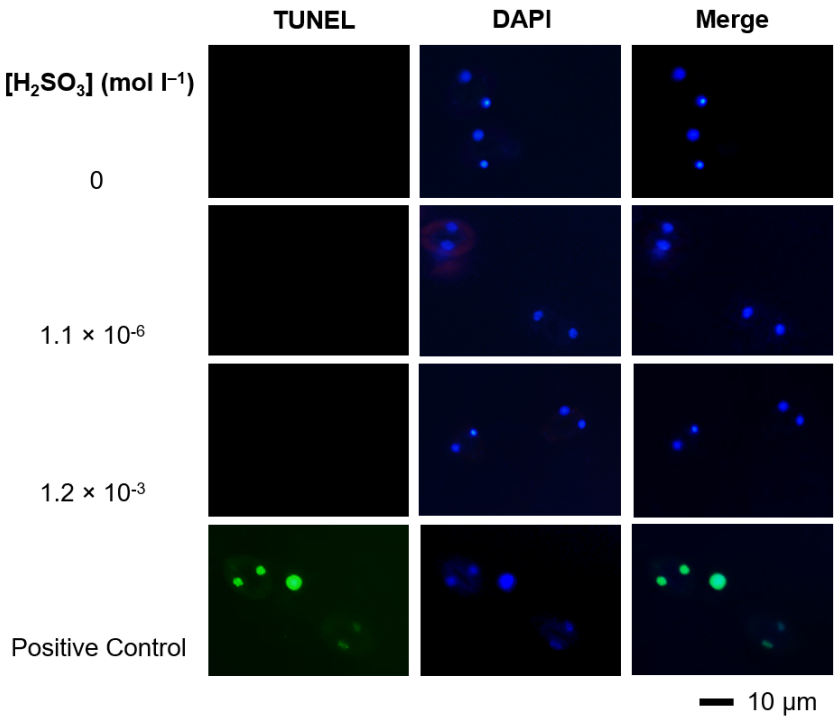

Figure 8. Non-apoptotic guard cells death in the H_2SO_3-exposed epidermis. Representative fluorescence microscopy images of TUNEL-stained stomatal guard cell exposed to a 2-hr treatment of 1.1×10^{-6} and 1.2×10^{-3} mol l^{-1} of H_2SO_3 were displayed, with 80 – 120 guard cells observed for each concentration in each experiment. $[H_2SO_3] = 0$ represents negative control for H_2SO_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 H_2SO_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 SO_2, CO_2 and O_3 and chemical species ratio of the 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 H_2SO_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 H_2SO_3 on the stomatal aperture in the dark.

Appendix I

Table S1. pH of H_2SO_3 solutions prepared in opening buffer made up of 10 mmol l^{-1} MES-Tris.

C_{Total} (mmol l^{-1})	pH (mean ± standard deviation)[a]
0	5.73 ± 0.01
0.008	5.72 ± 0.01
1	4.99 ± 0.03
2.5	2.86 ± 0.03
5	2.48 ± 0.00
10	2.15 ± 0.07

C_{Total} represents total concentration of H_2SO_3 added. [a] n = 3.

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

#	pH of solution	C_{Total} (mol l^{-1})	Solution made of	Deduced concentration of species (mol l^{-1})			Buffering system
				$[SO_3^{2-}]$	$[HSO_3^-]$	$[H_2SO_3]$	
1	5.73	0	N/A	0	0	0	10 mmol l^{-1} MES-Tris
2	5.72	8.0×10^{-6}	H_2SO_3	2.27×10^{-7}	7.77×10^{-6}	1.48×10^{-9}	10 mmol l^{-1} MES-Tris
3	5.70	4.0×10^{-5}	H_2SO_3	1.08×10^{-6}	3.89×10^{-5}	7.76×10^{-9}	10 mmol l^{-1} MES-Tris
4	5.62	2.0×10^{-4}	H_2SO_3	4.53×10^{-6}	1.95×10^{-4}	4.69×10^{-8}	10 mmol l^{-1} MES-Tris
5	4.96	1.0×10^{-3}	H_2SO_3	5.04×10^{-6}	9.94×10^{-4}	1.09×10^{-6}	10 mmol l^{-1} MES-Tris
6	2.86	2.5×10^{-3}	H_2SO_3	8.84×10^{-8}	2.20×10^{-3}	3.03×10^{-4}	10 mmol l^{-1} MES-Tris
7	2.48	5.0×10^{-3}	H_2SO_3	6.30×10^{-8}	3.76×10^{-3}	1.24×10^{-3}	10 mmol l^{-1} MES-Tris
8	2.15	1.0×10^{-2}	H_2SO_3	4.59×10^{-8}	5.85×10^{-3}	4.14×10^{-3}	10 mmol l^{-1} MES-Tris
9	5.88	1.0×10^{-3}	Na_2SO_3	4.04×10^{-5}	9.59×10^{-4}	1.26×10^{-7}	10 mmol l^{-1} MES-Tris
10	6.27	5.0×10^{-3}	Na_2SO_3	4.69×10^{-4}	4.53×10^{-3}	2.43×10^{-7}	10 mmol l^{-1} MES-Tris
11	6.98	1.0×10^{-2}	Na_2SO_3	3.47×10^{-3}	6.53×10^{-3}	6.84×10^{-8}	10 mmol l^{-1} MES-Tris
12	5.69	0	N/A	0	0	0	1 mmol l^{-1} MES-Tris
13	5.20	1.0×10^{-3}	Mix	8.72×10^{-6}	9.91×10^{-4}	6.25×10^{-7}	1 mmol l^{-1} MES-Tris
14	3.81	2.5×10^{-3}	Mix	8.83×10^{-7}	2.46×10^{-3}	3.81×10^{-5}	1 mmol l^{-1} MES-Tris
15	2.86	5.0×10^{-3}	Mix	1.77×10^{-7}	4.39×10^{-3}	6.06×10^{-4}	1 mmol l^{-1} MES-Tris
16	2.50	1.0×10^{-2}	Mix	1.33×10^{-7}	7.60×10^{-3}	2.40×10^{-3}	1 mmol l^{-1} MES-Tris

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:

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

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

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

For derivation of Equations 1, 2 and 3, see Appendix 1.

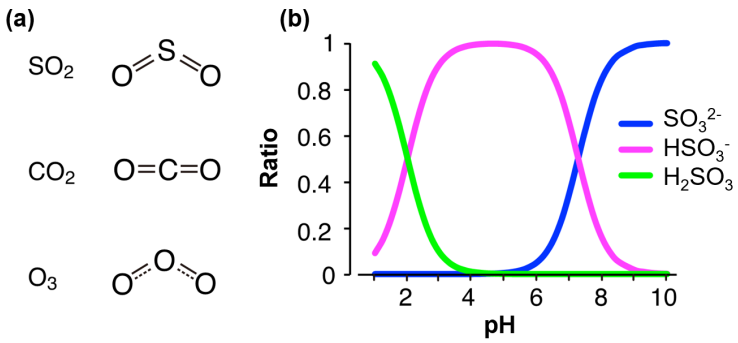

Figure S1. Molecular structure of SO_2, CO_2 and O_3 and chemical species ratio of the SO_2 aqueous solution at different pH. (a) structure of SO_2, CO_2, and O_3. (b) The ratio of three chemical species (SO_3^{2-}, HSO_3^- and H_2SO_3) in aqueous solution, calculated from pK_{a1} and pK_{a2} of H_2SO_3.

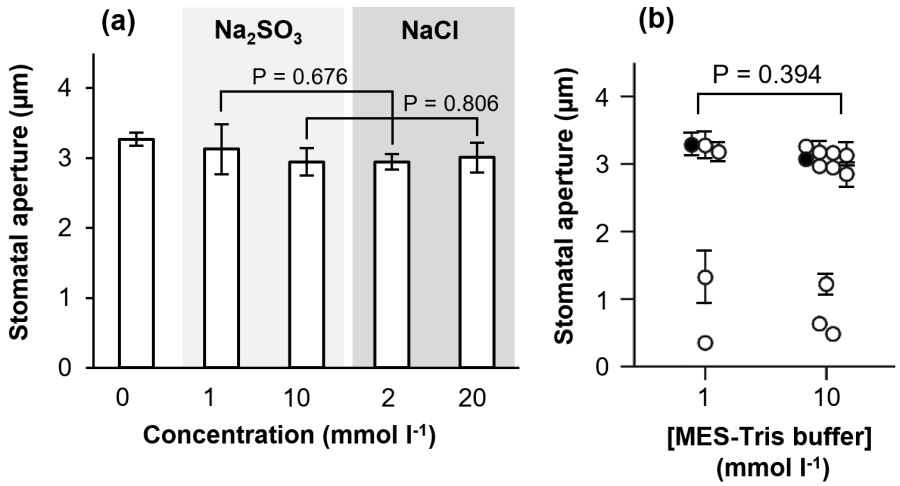

Figure S2. Effects of Na^+ and buffering system on stomatal aperture width. (a) Excised leaves were treated with indicated concentrations of Na_2SO_3 or NaCl for 3 hours in the light. Four biological replicates. One replicate is of an average of 20 stomata from the same leaf. Difference between dataset was assessed by Student T-test ($\alpha = 0.05$). (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).

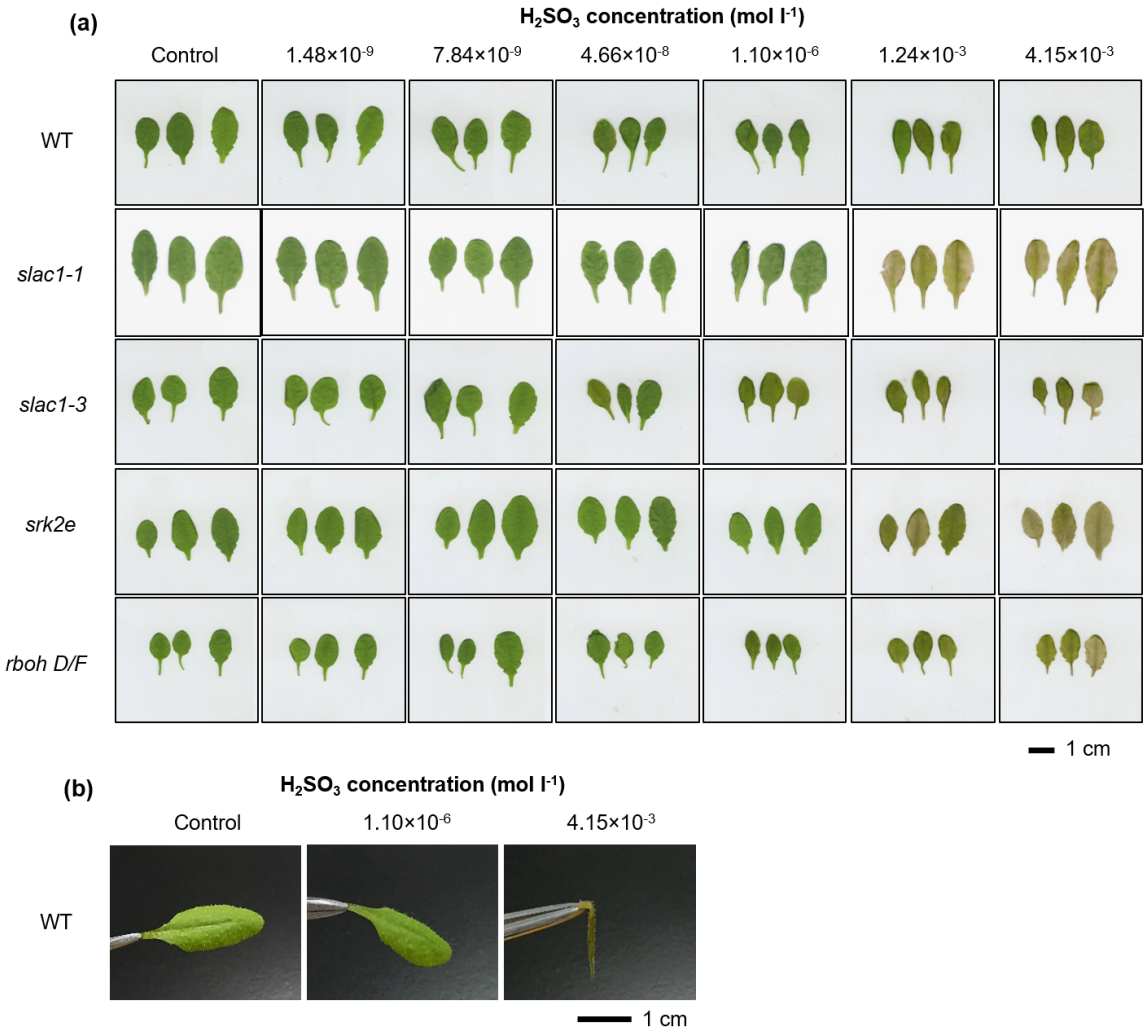

Figure S3. Leaf appearance of wild type (WT), carbon dioxide- and ozone-insensitive stomata mutants (*slac1-1, slac1-3*, *srk2e*, and *rbohD/F*) after H_2SO_3 exposure. (a) Representative images of excised mature rosette leaves exposed to aqueous solutions of SO_2 for 3 hr. (b) Representative images for leaf wilting observation.

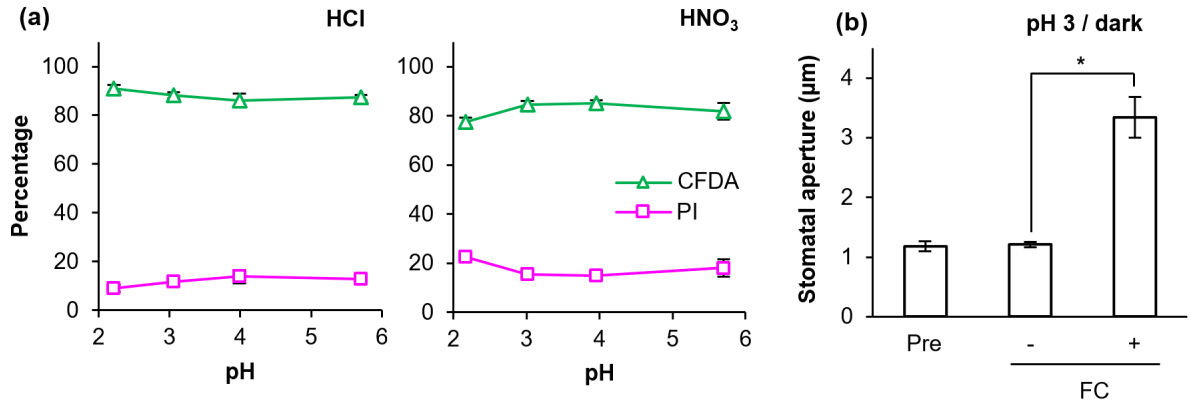

Figure S4. Guard cell viability in acidified solution and fusicoccin-induced stomatal opening in the dark at pH3. (a) The viability rate of guard cells in acidified solution. Leaves were incubated for 3 hr in acidified stomata opening buffer under light (120 μmol m^{-2} s^{-1}). pH was adjusted with HCl or HNO_3. 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).

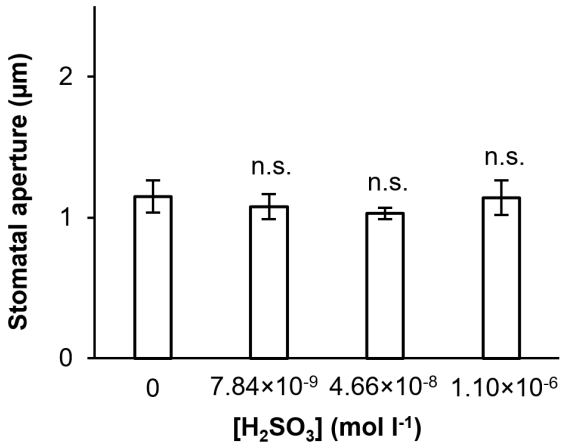

Figure S5. Effect of low concentrations of H_2SO_3 on the stomatal aperture in the dark. Dark-acclimated leaves were treated with H_2SO_3 for 3 hrs in the dark. n = 4, with 80 stomata per bar. n.s. indicates nonsignificant differences (α = 0.05) by Dunnett's test. Error bars represent SE.

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_2SO_3 as follow.

$$[\mathrm{HSO_3^-}] = \frac{[\mathrm{SO_3^{2-}}] \cdot [\mathrm{H^+}]}{K_{a2}} \quad (1.1)$$

$$[\mathrm{H_2SO_3}] = \frac{[\mathrm{HSO_3^-}] \cdot [\mathrm{H^+}]}{K_{a1}} \quad (1.2)$$

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

$$H_2SO_3 \Leftrightarrow HSO_3^{2-} + H^+, \ K_{a1} = 1.0 \times 10^{-2}$$

$$HSO_3^- \Leftrightarrow SO_3^{2-} + H^+, \ K_{a2} = 5.6 \times 10^{-8}$$

From the definition of total concentration added (C_{Total}), $[SO_3^{2-}]$ is shown as Equation (1.3).

$$[\mathrm{SO_3^{2-}}] = C_{\mathrm{Total}} - [\mathrm{HSO_3^-}] - [\mathrm{H_2SO_3}] \quad (1.3)$$

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

$$[\mathrm{SO_3^{2-}}] = C_{\mathrm{Total}} - \frac{[\mathrm{SO_3^{2-}}] \cdot [\mathrm{H^+}]}{K_{a2}} - \frac{\frac{[\mathrm{SO_3^{2-}}] \cdot [\mathrm{H^+}]}{K_{a2}} \cdot [\mathrm{H^+}]}{K_{a1}} \quad (1.4)$$

Rearrangement of the formula to isolate $[SO_3^{2-}]$ makes Equation (1.5).

$$[\mathrm{SO_3^{2-}}] = \frac{1}{\frac{[\mathrm{H^+}]^2}{K_{a1} \cdot K_{a2}} + \frac{[\mathrm{H^+}]}{K_{a2}} + 1} \cdot C_{\mathrm{Total}} \quad (1.5)$$

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

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

Derivation of Equation 2

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

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

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

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

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

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

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

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

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

Derivation of Equation 3

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

$$[\mathrm{HSO_3^-}] = \frac{\mathrm{K_{a1}} \cdot [\mathrm{H_2SO_3}]}{[\mathrm{H^+}]} \quad (3.1)$$

From the definition of total concentration added (C_{Total}), $[H_2SO_3]$ is shown as Equation (3.2).

$$[\mathrm{H_2SO_3}] = C_{\mathrm{Total}} - [\mathrm{HSO_3^-}] - [\mathrm{SO_3^{2-}}] \quad (3.2)$$

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

$$[\mathrm{H_2SO_3}] = C_{\mathrm{Total}} - \frac{K_{\mathrm{a1}} \cdot [\mathrm{H_2SO_3}]}{[\mathrm{H^+}]} - \frac{K_{\mathrm{a2}} \cdot \frac{K_{\mathrm{a1}} \cdot [\mathrm{H_2SO_3}]}{[\mathrm{H^+}]}}{[\mathrm{H^+}]} \quad (3.3)$$

Rearrangement of Equation (3.3) to isolate $[H_2SO_3]$ makes Equation (3.4)

$$[\mathrm{H_2SO_3}] = \frac{1}{1 + \frac{K_{\mathrm{a1}}}{[\mathrm{H^+}]} + \frac{K_{\mathrm{a1}} \cdot K_{\mathrm{a2}}}{[\mathrm{H^+}]^2}} \cdot C_{\mathrm{Total}} \quad (3.4)$$

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

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