STUDIES ON STOMATAL RESPONSE TO SULFUR DIOXIDE IN ARABIDOPSIS

（シロイヌナズナにおける二酸化硫黄に対する気孔応答の研究）

2018, August

OOI Lia

Graduate School of Environmental and Life Science
(Doctor's Course)
OKAYAMA UNIVERSITY

TABLE OF CONTENTS

CHAPTER 1

RESEARCH BACKGROUND

1.1 Introduction

Stoma, which consists of a pair of guard cells in the aerial parts 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). Besides, stomata play a role as the first entrance of gaseous pollutants into the plant body. Environment-polluting gases, such as ozone (O_3), nitrogen dioxide (NO_2) and sulfur dioxide (SO_2) enter leaves through stomatal pores, causing foliage destruction, and resulting in massive crop loss and forest decline (Bender & Weigel 2010; Bobbink 1998; Cape 1998; WHO 2000). O_3-induced crop loss is estimated to be several billion £ annually in Europe (Holland et al. 2002), while industrial emitted SO_2 has cost an estimated of US$ 1.43 billions of agricultural loss in China in 2008 alone (Wei et al. 2014). These gases are known to close stomata, and thus stomatal closure is postulated as a protection mechanism against harmful gases (McAinsh et al. 2002; Schroeder et al. 2001). Taylor (1978) has proposed that stomatal closure is a stress avoidance mechanism of plants to avoid damage by gaseous pollutants, yet it is unproven until today.

1.2 Molecular Mechanism for Gaseous Stimuli-Induced Stomatal Closure

Massive studies had been conducted for more than 50 years, particularly on stomatal response to ozone to understand the mechanisms of gaseous stimuli-induced stomatal closure (Hill & Littlefield 1969; Kollist et al. 2007; Mansfield 1998; McAinsh et al. 2002; Tingey & Hogsett 1985; Torsethaugen et al. 1999). Regardless of many previous studies, the molecular mechanisms for gaseous stimuli-induced stomatal closure have neither been well-investigated nor identified until the year 2008, when one of the molecular factors which

regulate stomatal closure against O_3 was reported. *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). *OPEN STOMATA 1 (OST1/SNRK2.6/SRK2E)* was later identified to be participating in the O_3-triggered rapid transient decrease in stomatal conductance (Vahisalu et al. 2010).

CO_2 is another environmental-polluting gas that is emitted in large amount into the atmosphere from anthropogenic activities. It is also a gaseous stimulus that evokes stomatal closure, although it is not harmful to plants (for review, see Engineer et al. 2016). Intriguingly, *SLAC1* and *OST1* are also involved in CO_2-induced stomatal closure (Negi et al. 2008; Xue et al. 2011). In addition, a 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). Besides these findings reported on the molecular factors mediating stomatal closure in O_3 and CO_2 response, the molecular mechanisms behind other gaseous stimuli-induced stomatal closure are unknown. Judging from the revealed molecular factors, it is tempting to assume that plants have a common molecular mechanism for the induction of stomatal closure against gaseous stimuli.

1.3 Sulfur Dioxide

SO_2, a colourless gas with a pungent odour, 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 industrial revolution and economic expansion due to fossil fuel combustion (Smith et al. 2011). SO_2 was the major contributor to global forest degradation in the 20^{th} century (Baciak et al. 2015). Though efforts were taken in reducing the emissions particularly in Europe and North America, SO_2 emission in East Asia remained high in the last decade following China's economic boom (Baciak et al. 2015; Klimont et al. 2013).

The effects of SO_2 on plants was first identified in 1848 when a German chemist reported the observation of "sickening and dying off" of forest trees in the vicinity of smelters

releasing fume containing sulfur dioxide through the smokestacks (Stöckhardt 1850). Extensive studies in the past century have reported disruption of photosynthesis activity, suppression of plant growth, damage in chlorophyll, reduction of yield and premature death in plants (Kropff 1987; Malhotra & Hocking 1976; Sprugel et al. 1980; Wilson & Murray 1990). On the other hand, there are only a handful of reports on stomatal response against SO_2, which are found to be contradicting from one another. The quantity of reports investigating stomatal response against SO_2 has decreased drastically in the past two decades following the constant decrease in SO_2 emissions, particularly in North America and Europe (Cape et al. 2003). SO_2 was reported to induce stomatal closure in multiple plant species (Hu et al. 2014; Olszyk & Tibbitts 1981; Winner & Mooney 1980). Concurrently, others reported it to augment stomatal opening (Mansfield & Majernik 1970; McAinsh et al. 2002; Taylor et al. 1981).

Even though the impacts of SO_2 on whole plants are well understood, the impacts of SO_2 on stomata remains unclear. For instance, it is known that SO_2 dissolves in water forming three different chemical species: sulfurous acid (H_2SO_3), bisulfite ion (HSO_3^-) and sulfite ion (SO_3^{2-}). Nevertheless, the actual chemical species that is responsible for SO_2-induced stomatal closure has yet to be determined. Furthermore, none of the molecular mechanisms involved in the SO_2-induced stomatal closure have been proven besides an antecedent pharmacological study (K. D. Hu et al. 2014).

1.4 Cell Signaling in Sulfur Dioxide-induced Stomatal Closure is Unknown

To date, the involvement of cell signaling pathways in SO_2-induced stomatal closure still remains debatable. A previous study using *Vicia faba* reported an increase in endogenous abscisic acid (ABA) levels in epidermal strips treated with SO_2. This suggested the involvement of hormone regulation in SO_2-indued stomatal closure (Taylor et al. 1981). In *Pisum sativum* L., SO_2-induced stomatal closure was speculated to have resulted from a combination of the immediate effects on phosphoenolpyruvate carboxylase, and NAD- and NADP-malate dehydrogenase activities in the epidermis (Rao et al. 1983). This combination

was speculated to inhibit photosynthesis activity in the mesophyll, causing CO_2 accumulation in the intracellular spaces and eventually induces the closure of stomata. Omasa et al. (1985) attributed SO_2-induced stomatal closure to a fall in guard cell turgor resulting from severe injury in the epidermis cell, in sunflower (*Helianthus annuus* L. cv Russian Mammoth). A recent study on SO_2 using *Ipomoea batatas* reported reversible stomatal closure by H_2S- and NO-scavenging chemicals, suggesting the closure event is mediated by H_2S and NO productions (K. D. Hu et al. 2014). There is no agreeable mechanism on how SO_2 induces stomatal closure so far.

1.5 Is SO_2-induced Stomatal Closure a Protection Mechanism?

"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 widely accepted that stomatal closure in the presence of O_3 is a protection mechanism (Merilo et al. 2013). "Is SO_2-induced stomatal closure a protection mechanism of plants to prevent entrance of harmful gas?" was one of my research questions.

Mutants, which are impaired in O_3- and CO_2-induced stomatal closure, can be clues in perceiving the molecular mechanisms in the SO_2 response of stomata. Considering the partial redundancy in the phenotypes of the CO_2- and O_3-insensitive stomata mutants and the structural similarity among CO_2, O_3, and SO_2 (Fig. 1.1), which are comprised by three atoms with two oxygen atoms on both ends, I postulated that plants share parts of the regulators of stomatal closure in response to gaseous stimuli. In this study, I have identified the responsible chemical species in the SO_2 aqueous solution which induces stomatal closure; molecular biologically examined stomatal response to SO_2 using Arabidopsis mutants, and explored the involvement of hormone, other signaling pathways and the death of guard cells in SO_2-induced stomatal closure.

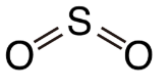
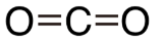
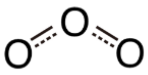
Chemical	Structure	Bond distance (Å)	Bond angle
SO_2		1.43	119°
CO_2		1.16	180°
O_3		1.28	117°

Figure 1.1 Molecular structures of SO_2, CO_2 and O_3.

CHAPTER 2

IDENTIFICATION OF CHEMICAL SPECIES THAT INDUCES STOMATAL CLOSURE IN AQUEOUS SOLUTION OF SULFUR DIOXIDE

2.1 Introduction

The mechanism of SO_2 diffusion into the plant body and its effects on plant metabolism have long been elucidated (Horsman & Wellburn 1977; Kropff 1991; Malhotra & Hocking 1976; Muneer et al. 2014; Thomas et al. 1950). However, the action of SO_2 to induce stomatal closure remained concealed. Three chemical species are formed when SO_2 gas is dissolved into water: H_2SO_3, HSO_3^- and SO_3^{2-} of which the compositions in the aqueous solution depend on the pH (Fig. 2.1). Hitherto the chemical species that is responsible for stomatal closure induction remained unknown. Reportedly, stomatal response to SO_2 was contradictive and fragmental, i.e. it was described to induce both closure and opening induction (Hu et al. 2014; McAinsh et al. 2002; Olszyk & Tibbitts 1981; Taylor et al. 1981). Here, I examined the chemical species in the aqueous solution of SO_2 that are responsible for the stomatal response in Arabidopsis and identified the potential mode of action of SO_2 in stomatal closure induction.

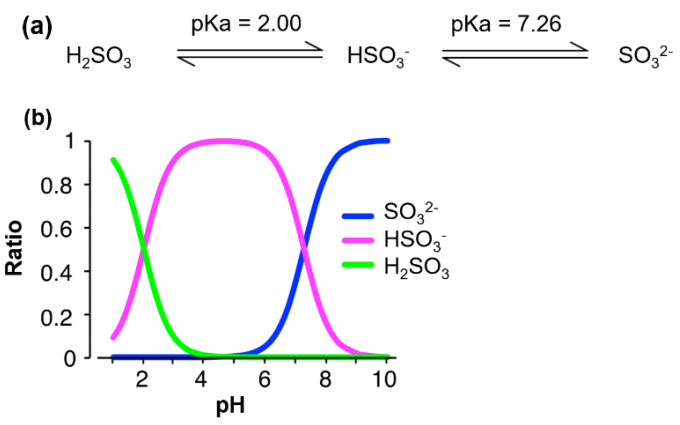


Figure 2.1 Chemical species formed in SO_2 aqueous solution at different pH. (a) Chemical speciation of SO_2 in aqueous solution; (b) Ratio of three chemical species (SO_3^{2-}, HSO_3^- and H_2SO_3) in aqueous solution of SO_2 (calculated from pKa_1 and pKa_2 of H_2SO_3).

2.2 Materials and Methods

2.2.1 Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) wild type (ecotype Columbia-0) 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.

2.2.2 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.

2.2.3 Preparation of Chemical Species in Aqueous Solution of Sulfur Dioxide

A wide range of concentrations of the three chemical species derivatized from SO_2 with different compositions was utilized to assess the dose response of stomatal closure (Table 2.1). The concentrations of each chemical species were deduced using the following equations:

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

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

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

where, C_{Total} represents the total concentration added into the solution (mol l^{-1}), $[H^+]$ of the solution was determined using glass pH electrode (F-52, Horiba, Kyoto, Japan), n = 3. For the derivation of Equations 1, 2 and 3, see Appendix 1.

Table 2.1 Preparation of the exact composition of chemical species in the experimental solutions

#	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 a 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 the total concentration of chemical(s) added.

2.2.4 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 floated on 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) supplemented with any given concentration of H_2SO_3, Na_2SO_3 or a mixture of these two chemicals for 3 hr under white light (120 μmol m^{-2} s^{-1}) following 2 hr of pre-incubation in the opening buffer, unless otherwise stated. The exposed leaves were blended by a Waring blender (model BB-900, Waring Products Inc., Torrington, CT) to release the epidermal fragments and stomatal aperture width in the epidermal fragments was measured under a microscope (DMBA-300, Shimadzu, Kyoto, Japan).

2.3 Results

2.3.1 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 SO_2 gas fumigation and H_2SO_3 solution exposure on stomatal aperture are assumed to be essentially the same (Taylor et al. 1981). During the preparation of the experimental solution, it was observed that the pH of stomata opening buffer (which acts as the background solution of the experiment) became more acidic with the addition of H_2SO_3 (Table 2.2). At total concentration, C_{Total} = 10 mmol l^{-1}, the pH of the solution has changed from the initial pH of 5.73 ± 0.01 to 2.15 ± 0.07. To investigate the effects of SO_2 on Arabidopsis stomata, I first questioned whether the acidification of extracellular solution caused by H_2SO_3 exposure is the main reason for the stomatal closure. Here, I examined the effect of the external solution acidified with three acids (HCl, HNO_3 and H_2SO_3) on stomatal aperture width (Fig. 2.2).

Table 2.2 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 solution added[a], n = 3.

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. 2.2), but did 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, other mechanisms appear to be involved in stomatal closure induction upon exposure to H_2SO_3 solution.

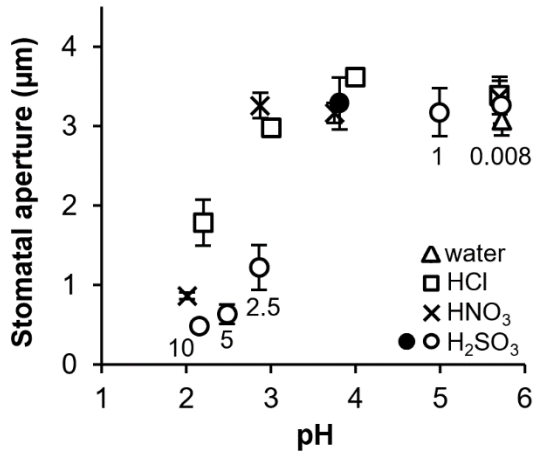


Figure 2.2 Effect of acidification of external solution on stomatal aperture width. pH of the stomata opening solution was adjusted with 1 mol l^{-1} hydrochloric acid (HCl), 1 mol l^{-1} nitric acid (HNO_3) or 0.61 mol l^{-1} H_2SO_3. Digits under open circles represent total concentration of H_2SO_3 in mmol l^{-1}. See Table 2.2 for the relation of pH value and the total concentration of H_2SO_3 added. Triangle (Δ) 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.

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 depend on the pH (Fig. 2.1). I examined the particular chemical species, if any, in the aqueous solution of SO_2 that was 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 compositions (Fig. 2.3).

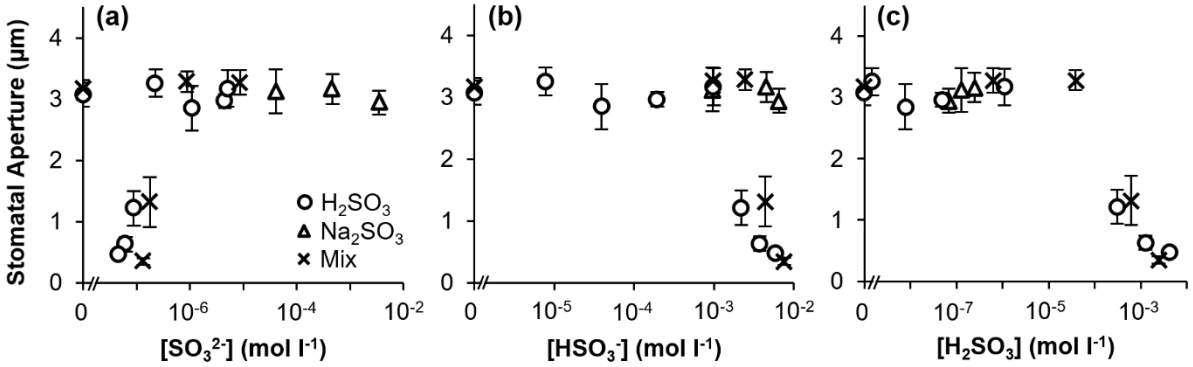


Figure 2.3 Induction of stomatal closure by sulfur dioxide-derived chemical species. (a) SO_3^{2-} (4.6×10^{-8} – 3.5×10^{-3} mol l^{-1}); (b) HSO_3^- (7.8×10^{-6} – 7.6×10^{-3} mol l^{-1}); (c) H_2SO_3 (1.5×10^{-9} – 4.2×10^{-3} mol l^{-1}), prepared in stomata opening buffer with two different buffering capacities (1 mmol l^{-1} and 10 mmol l^{-1} MES-Tris), from three different sources indicated by o: 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. Refer Table 2.1 for the preparation of the exact composition of the chemical species.

Fig. 2.3a shows the plot of aperture width in which the X-axis indicates the concentration of SO_3^{2-} in the experimental solution. The 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, I considered that SO_3^{2-} did not participate in the induction of stomatal closure. Fig. 2.3b shows the same set of 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. An inconsistent stomatal response was observed at higher [HSO_3^-]. Stomata remained opened wide at 2.5, 4.4 and 6.5 mmol l^{-1} HSO_3^-; and obviously closed at 2.2, 3.8, 4.5, and 7.6 mmol l^{-1} HSO_3^-, demonstrating discrepancies in concentration dependency. Therefore, I 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. 2.3c). 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}, 606 µmol l^{-1}, 2.4 mmol l^{-1} and 4.1 mmol l^{-1}), rendered stomatal closure in a concentration-dependent manner.

A mixture of Na_2SO_3 and H_2SO_3 was applied during the preparation for exact concentrations for each chemical species in the aqueous solution of SO_2 (*Section 2.2.3*). The possible involvements of Na^+ derived from Na_2SO_3 salt and different buffering systems (1 mmol l^{-1} and 10 mmol l^{-1} MES-Tris) on stomatal aperture width were excluded by observation of stomatal aperture width in the presence of NaCl (Fig. 2.4a) and statistical test with Mann-Whitney *u* test between the buffering systems (Fig. 2.4b).

Collectively, I 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.

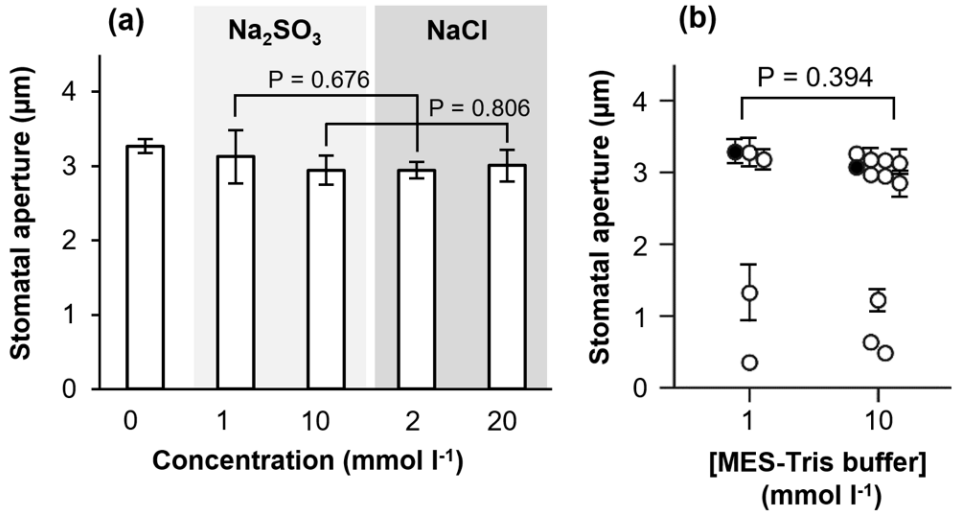


Figure 2.4 Effects of Na^+ and buffering system on stomatal aperture width. (a) Excised leaves were treated with indicated concentrations of Na_2SO_3 or NaCl for 3 hours in the light, n = 4. One replicate is of an average of 20 stomata from the same leaf. Difference between dataset was assessed by Student t-test (α = 0.05). (b) Representation of aperture width data in Fig. 2.3 with different buffering systems as shown in Table 2.1, n = 4. 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).

2.4 Discussion

2.4.1 H_2SO_3 is the Chemical Species Responsible for SO_2-induced Stomatal Closure

Through the observation of stomatal response to each chemical species formed in the aqueous solution of SO_2 (Fig. 2.3), I excluded the involvement of SO_3^{2-} and HSO_3^- in SO_2-induced stomatal closure. The results suggest that H_2SO_3 is the only SO_2-derived chemical species that closes stomata in the presence of SO_2. This is probably attributed to the restricted permeability of charged ions across biomembranes. Conceivably, this further indicates that H_2SO_3 evokes stomatal closure not by binding to cell surface receptors, but via intracellular recognition at the inside of the cell.

Hu et al. (2010) suggested that bicarbonate ion (HCO_3^-) is responsive to CO_2-induced stomatal closure, although HSO_3^- was shown not to be involved in SO_2-induced stomatal closure. This connotes that the mechanism of CO_2 response is different from that of SO_2. I further discuss the possible mode of action of SO_2 on stomata in *Chapter 5*.

CHAPTER 3

SULFUR DIOXIDE-INDUCED STOMATAL CLOSURE IS DISTINCT FROM OZONE- AND CARBON DIOXIDE-INDUCED CLOSURE

3.1 Introduction

Although studies on the impacts of gaseous pollutants on plants have been conducted since the beginning of the history of industrialization, the stomatal response mechanism to reduce the harmful effects of gaseous pollutants on plants is still concealed. In most cases, environmental-polluting gaseous stimuli were reported to induce stomatal closure or inhibit stomatal opening (McAinsh et al. 2002; Robinson et al. 1998; Torsethaugen et al. 1999). In the past decade, the first molecular factor that regulates stomatal closure against gaseous pollutant (O_3) was revealed to be *SLOW ANION CHANNEL-ASSOCIATED 1/OZONE-SENSITIVE-1* (*SLAC1/OZS1*) through genetic screening (Saji et al. 2008; Vahisalu et al. 2008). *SLAC1* encodes a slow-type anion channel essential for anion efflux in stomatal closure and *slac1* mutant exhibits a high O_3 sensitivity owing to the insensitivity of stomata against O_3, which give rise to augmented O_3 intake into the leaf. Two years later, Vahisalu et al. (2010) reported *OPEN STOMATA 1 (OST1/SNRK2.6/SRK2E)* to be involved in the O_3-triggered rapid transient decrease in stomatal conductance. *OST1* was initially identified by Mustilli et al. (2002) via 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. Both *SLAC1* and *OST1* were found to be responsible for CO_2-induced stomatal closure later (Negi et al. 2008; Xue et al. 2011). A recent study described the role of *RESPIRATORY BURST OXIDASE HOMOLOG*s (*RBOH*s) in stomatal sensitivity to CO_2 (Chater et al. 2015).

Collectively, besides the similarity in the molecular structures of O_3 and CO_2 (Fig. 1.1), it is also noticeable that plants share part of the molecular factors in regulating stomatal

closure induced by O_3 and CO_2 (Fig. 3.1). It is hypothesized that plants might have a central molecular mechanism to regulate stomatal closure in the presence of gaseous stimuli of similar molecular structures. While the chemical species that induces stomatal closure in SO_2 solution has been identified to be H_2SO_3 as discussed in *Chapter 2*, no information on the molecular factors that regulate SO_2-induced closure has yet to be identified. Here, I examined if SO_2-induced stomatal closure is regulated by the similar molecular factors which regulate O_3- and CO_2-induced closure, using Arabidopsis CO_2- and O_3-insensitive stomata mutants.

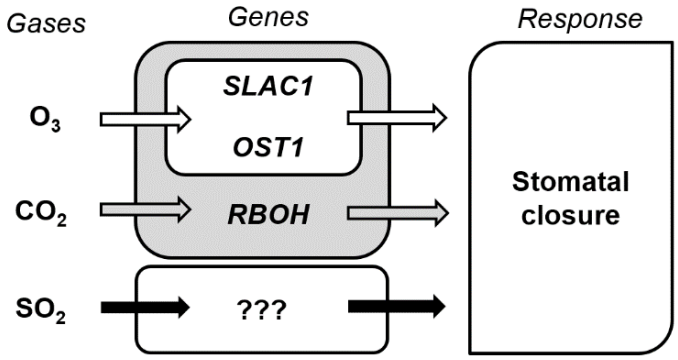


Figure 3.1 Molecular factors regulating O_3- and CO_2-induced stomatal closure. The genes that are responsible for SO_2-induced closure have remained unknown.

3.2 Materials and Methods

3.2.1 Plant Materials

Arabidopsis mutant plants of *slac1-1* (Vahisalu et al. 2008)*, slac1-3* (Vahisalu et al. 2008)*, srk2e* (Yoshida et al. 2002) and *rbohD/F* (Kwak et al. 2003) were cultivated as described in *Section 2.2.1.*

3.2.2 Chlorophyll Quantification

Chlorophyll was extracted from 3 pieces of 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).

3.2.3 *Stomatal assay*

Width of stomatal aperture was measure as described in 2.2.4.

3.3 Results

3.3.1 Sensitivity of O_3- and CO_2-insensitive Stomatal Mutants against SO_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 (Fig. 3.2a). *slac1* and *ost1* are O_3-insensitive stomata mutants that have open-stomata phenotype, which allows ready entry of gaseous stimuli into the leaves (Vahisalu et al. 2010). The stomata of *rbohD/F* mutant together with other mutants are partially insensitive to CO_2, demonstrating closure-impaired stomatal phenotype (Chater et al. 2015; Negi et al. 2008). I thus anticipated that these mutants would also demonstrate greater sensitivity to SO_2 if the mechanisms of stomatal closure were common among O_3, CO_2, and SO_2. Aqueous SO_2 concentrations applied were ranging from 1.5 nmol l^{-1} to 4.2 mmol l^{-1} (high concentrations which were reported to close stomata (Hu et al. 2014; Taylor et al. 1981). This wide range of concentrations of SO_2 was applied to allow a thorough understanding of stomatal response to SO_2.

After an exposure to 1.2 and 4.2 mmol l^{-1} H_2SO_3, the leaves were apparently wilted and paler than the control in all lines including the wild type (WT) (Fig. 3.2a and b). 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. 3.2c). This suggested that WT and mutants might not have different in sensitivity to SO_2. I further investigated the stomatal closure induction by H_2SO_3 in the mutants using the same technique as per described in *Section 2.2.4*.

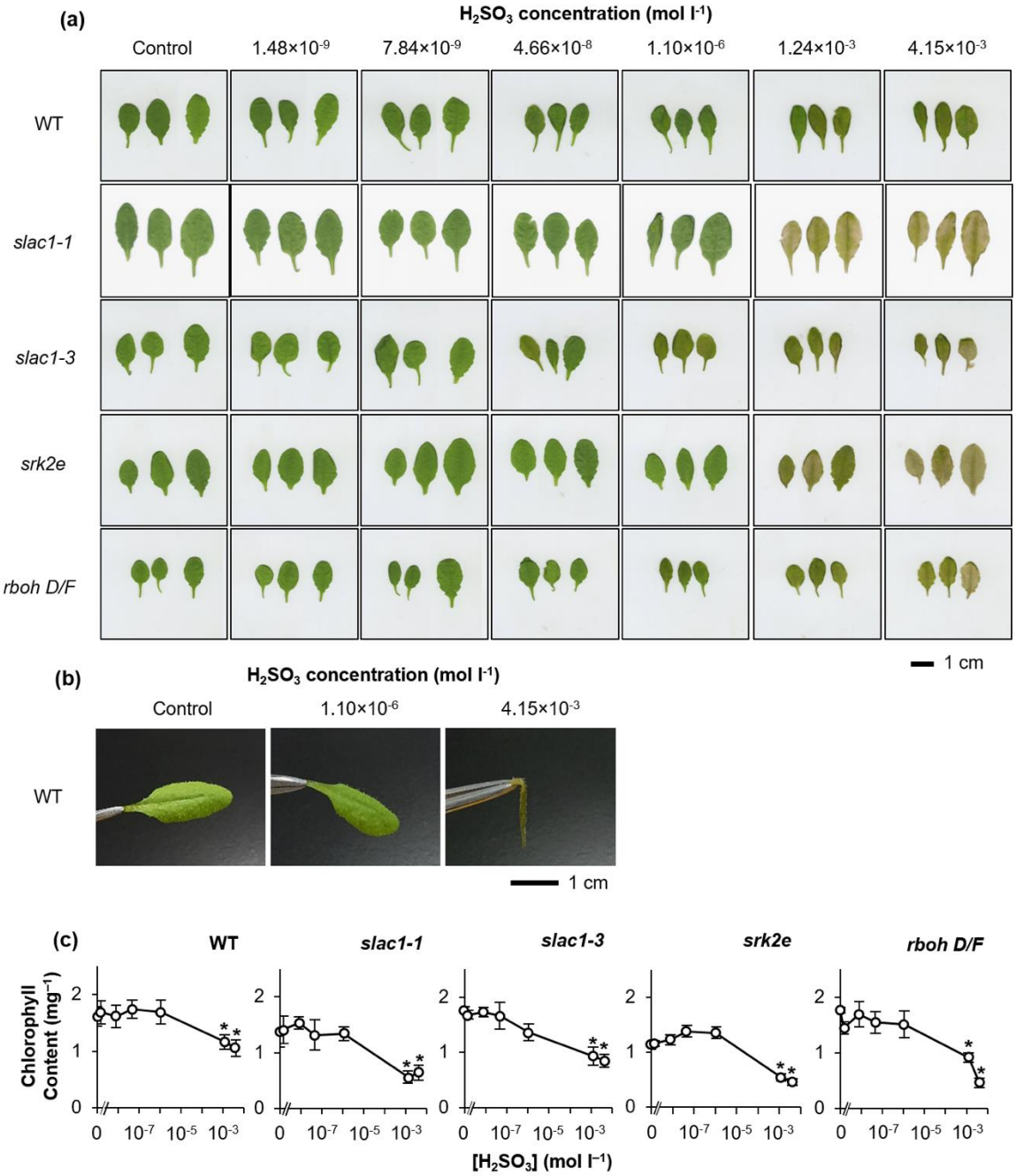


Figure 3.2 Leaf appearance of wild type (WT), carbon dioxide- and ozone-insensitive stomata mutants (*slac1-1, slac1-3*, *srk2e*, and *rbohD/F*) after H_2SO_3 exposure. (a) Representative images of excised mature rosette leaves exposed to aqueous solutions of SO_2 for 3 hr. (b) Representative images of leaves of WT after H_2SO_3 treatment. (c) Chlorophyll content in H_2SO_3-treated leaves, n = 6 individual leaf except for *rbohD/F* (n = 3). Asterisks (*) indicate significant differences (α = 0.05) by Dunnett's test. Error bars represent SE. Some error bars are too small to be seen.

3.3.2 Stomatal Closure Induction of SO_2 on O_3- and CO_2-insensitive Stomata Mutants

Fig. 3.3 shows H_2SO_3-induced stomatal closure in the WT and mutants. Although the widths 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), they were not significantly different (one-way ANOVA with Tukey's HSD post hoc analysis, α = 0.05). Nevertheless, no obvious insensitivity of stomata to H_2SO_3 was observed in all mutants when compared to WT. These observations suggested that stomatal closure induced by SO_2 is regulated by a molecular mechanism which is distinct from O_3 and CO_2.

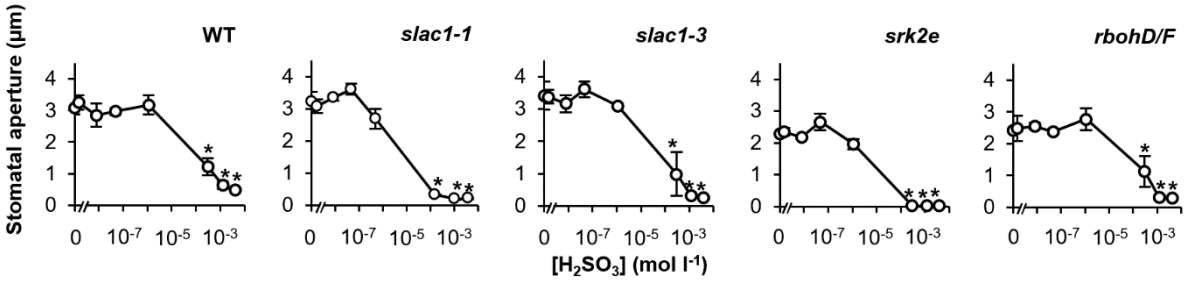


Figure 3.3 Stomatal closure induction of H_2SO_3 in wild type (WT), carbon dioxide- and ozone-insensitive stomata mutants (*slac1-1, slac1-3*, *srk2e*, and *rbohD/F*). n = 4; 80 stomata. Asterisks (*) indicate significant differences (α = 0.05) by Dunnett's test. Error bars represent SE. Some error bars are too small to be seen.

3.4 Discussion

Despite the high similarity shared in the chemical structures of SO_2 with O_3 and CO_2, chlorophyll degradation and stomatal closure induction studies did not demonstrate significant differences in the stomatal response of the mutants from WT (Fig. 3.2 and 3.3). Mutants utilized were all demonstrating open-stomata phenotype, observations of SO_2-induced stomatal closure in these mutants suggesting the involvement of other closure mechanisms that are not regulated by *SLAC1, OST1* and *RBOH*s. This study suggests that SO_2-induced stomatal closure is a distinctive event, which is different from O_3- and CO_2-induced closure. See *Chapter 5* for further discussion on the mechanism of SO_2-induced stomatal closure.

CHAPTER 4

INVOLVEMENT OF HORMONES AND GASOTRANSMITTERS IN SULFUR DIOXIDE-INDUCED STOMATAL CLOSURE

4.1 Introduction

Plants have developed numerous mechanical and chemical defense mechanisms to thrive in fluctuating environments. Phytohormones regulate defense machineries to cope with biotic and abiotic stresses, which are organized by signal networks. Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the major phytohormones that are known to play crucial roles in plant defense regulation against biotic environmental stressors, namely herbivores, pathogens and wounding. These hormones are also characterized to take part in abiotic stresses, such as water deficit, submergence and ozone (O_3) (Balbi & Devoto 2008; Devoto & Turner 2003; Gomi et al. 2005; Loake & Grant 2007). ABA is the phytohormone that plays the pivotal role in abiotic stress response and the key player in stomatal regulation. Interestingly, not only ABA, many other phytohormones: auxin (indole-3 acetic acid, IAA), cytokinins (CK), brassinosteroid (BR), ET, SA and JA are also reported to regulate stomatal apertures (Fig. 4.1) (Acharya & Assmann 2009). ABA was extensively studied due to its crucial role in regulating stomatal closure particularly to regulate water loss through transpiration, with its molecular factors identified (Nilson & Assmann 2006). Additionally, ABA was proposed to be involved in SO_2-induced stomatal closure long before the advance of genetic approaches (Black & Unsworth 1980; Kondo & Sugahara 1978; Taylor et al. 1981).

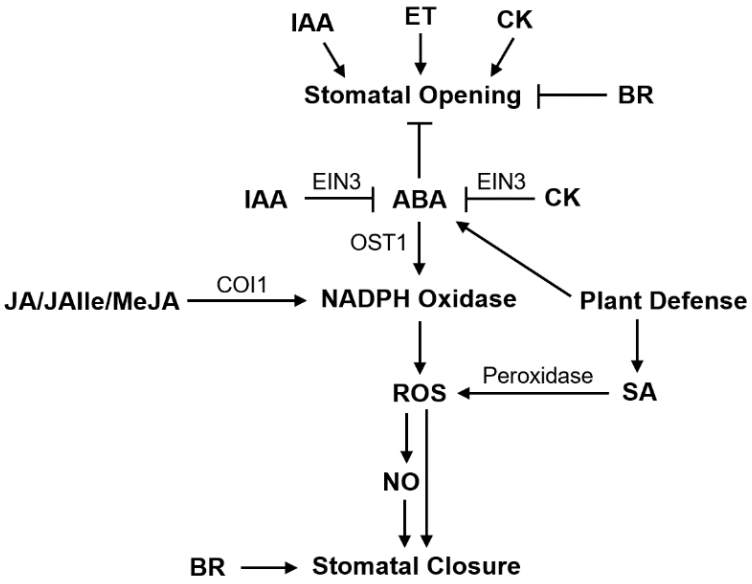


Figure 4.1 A proposed model of hormone interaction involved in stomatal regulation. ABA: abscisic acid; JA: jasmonic acid; SA: Salicylic acid; ET: ethylene; IAA: auxin; CK: cytokinin; BR: brassinosteroid. Arrows and line with bars indicate positive and negative regulatory actions respectively. Figure adopted from Acharya & Assmann, 2009.

Gasotransmitters are small endogenous gaseous molecules with significant physiological functions established in animal cells, and are recently getting recognized in plant cells as well (Wang 2003). Hydrogen sulfide (H_2S) is one of the important gasotransmitters which was found to play a critical role in various physiological processes in plants, including response against biotic and abiotic stresses (Jin et al. 2011; Lisjak et al. 2013; Wang et al. 2010). H_2S is a colourless, flammable and pungent gas which was considered to be a gaseous phytotoxin with deleterious effects on plant growth and survival in the past 300 years until the publication of the first report on its protective role on wheat germination during copper stress (Zhang et al. 2008). A recent study also identified the involvement of H_2S in inducing stomatal closure in *A. thaliana* during drought stress via interaction with ABA (Jin et al. 2013).

Nitric oxide (NO) is established as an important gasotransmitter in stomatal regulation (Desikan et al. 2002; García-Chávez et al. 2003; Guo et al. 2003). The production of NO in

guard cells is critical for ABA-, Methyl jasmonate (MeJA)- and elicitor-induced stomatal closure, following the production of reactive oxygen species (ROS) (Gayatri et al. 2017; Munemasa et al. 2011; Neill et al. 2008). In addition, NO signaling was reported to interact with other plant hormones, carbon monoxide and other molecules in the signaling pathways (Jin & Pei 2015; Lisjak et al. 2013). Lisjak et al. found evidence suggesting the interlinkage of H_2S and NO signaling in stomatal regulation (Lisjak et al. 2010; Lisjak et al. 2011), where H_2S was later recognized to be a downstream indicator of NO during ET-induced stomatal closure (Liu et al. 2011). Although these findings suggested crosstalk between H_2S and NO in plant response against stress, there is only one report available suggesting the involvement of H_2S and NO pathways in stomatal closure induction against SO_2, in *I. batatas* (Hu et al. 2014).

Here, I investigated the involvement of phytohormones in SO_2-induced stomatal closure via observation of changes in hormonal level in leaves treated with aqueous SO_2 in Arabidopsis. The involvement of gasotransmitters H_2S and NO in the regulation of stomatal closure against SO_2 was also studied.

4.2 Materials and Methods

4.2.1 Hormone Quantification

The contents of phytohormones: abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile), and auxin (IAA), in excised Arabidopsis leaf (70 – 100 mg fresh weights) after a 180-min H_2SO_3 incubation were determined by liquid chromatography-mass spectrometry (LC-MS) as described by Yin et al. (2016) and Gupta et al. (2017).

4.2.2 *Stomatal Assay*

Width of stomatal aperture was measured as described in 2.2.4.

4.3 Results

4.3.1 H_2SO_3-induced Stomatal Closure Is Not Mediated by ABA

I 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. 4.2). 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.

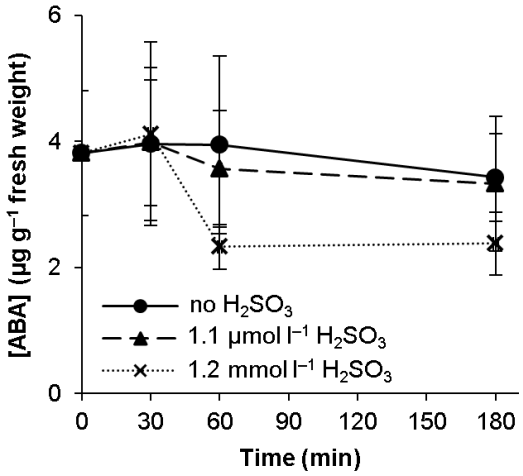


Figure 4.2 ABA contents in H_2SO_3-treated leaves. Mature rosette leaves of wild type plants were incubated in the buffer containing 0, 1.1 µmol l^{-1}, and 1.2 mmol l^{-1} H_2SO_3 for 180-min under the light, n = 3. Error bars represent SE.

4.3.2 H_2SO_3-induced Stomatal Closure Is Not Mediated by Jasmonates

Jasmonic acid (JA) signaling plays an essential role in plant defenses especially against necrotrophic pathogens and herbivores (Yan & Xie 2015). Jasmonoyl-isoleucine (JA-Ile), an amino acid conjugate of JA was later identified as the active jasmonate hormone which directly interacts with the receptor protein (Schuman et al. 2018; Wasternack & Hause 2013). To investigate the involvement of the jasmonate signaling in H_2SO_3-induced stomatal closure, here I determined the contents of JA and JA-Ile in H_2SO_3-treated leaves (Fig. 4.3). Both JA levels observed along the 180-min treatment in 1.1 µmol l^{-1} H_2SO_3 were not

substantially different from the control, except at 180-min in JA-Ile (Fig. 4.3(a)) and JA-Ile (Fig. 4.3(b)). Increments in JA and JA-Ile contents were observed in leaves treated with 1.2 mmol l^{-1} H_2SO_3 suggesting the involvement of jasmonates in stomatal closure induction against high concentration of H_2SO_3. Nevertheless, statistical analysis (Dunnett's Test, $\alpha = 0.05$) identified no significant differences in the hormone contents in H_2SO_3-treated leaves (for both 1.1 µmol l^{-1} and 1.2 mmol l^{-1}) from the control (0 mol l^{-1} H_2SO_3 at 0-min). I still question the role of jasmonates in SO_2-induced stomatal closure.

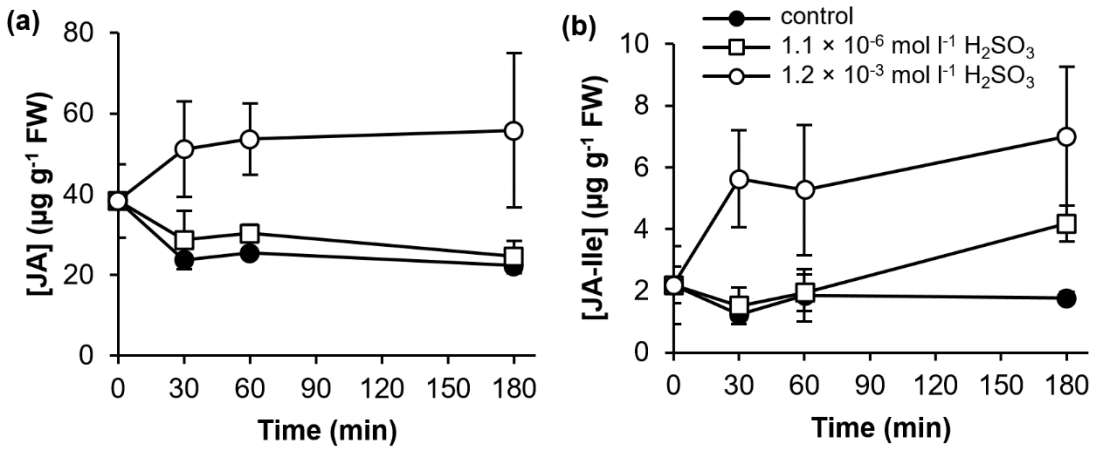


Figure 4.3 Jasmonates contents in H_2SO_3-treated leaves. JA (a) and JA-Ile (b) contents in H_2SO_3-treated leaves. Mature rosette leaves of wild type plants were incubated in the buffer containing 0, 1.1 µmol l^{-1} and 1.2 mmol l^{-1} H_2SO_3 for 180-min under the light, n = 3. Error bars represent SE. Some error bars are too small to be seen.

4.3.3 Involvement of SA in H_2SO_3-induced Stomatal Closure

SA is also a key signal regulator mediating plant tolerance and defense responses to abiotic and biotic stresses (Khan et al. 2015; Smith et al. 2009). SA is known to positively regulate stomatal closure during pathogenic bacterial invasion as an innate immune system in plants (Melotto et al. 2006; Mori et al. 2001). Here, the SA contents in H_2SO_3-treated leaves were quantified to investigate the role of SA in H_2SO_3-induced stomatal closure (Fig. 4.4). A significant increase was observed in leaves incubated in 1.2 mmol l^{-1} H_2SO_3 for 180 min.

However, the increase in SA level was not observed at other time points for treatments with 1.1 μmol l^{-1} and 1.2 mmol l^{-1} H_2SO_3 as compared to the control. The increment of SA contents in leaves treated with 180-min of 1.2 mmol l^{-1} H_2SO_3 suggested that SA could be potentially involved in the regulation of SO_2-induced stomatal closure.

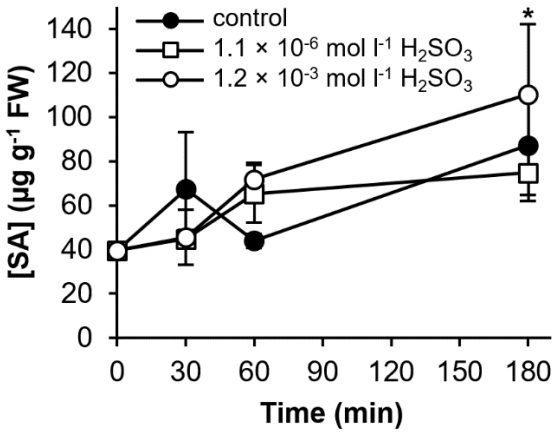


Figure 4.4 SA contents in H_2SO_3-treated leaves. Mature rosette leaves of wild type plants were incubated in the buffer containing 0, 1.1 μmol l^{-1}, and 1.2 mmol l^{-1} H_2SO_3 for 180-min under the light. Asterisk (*) indicates significant difference from the control (0 mol l^{-1} H_2SO_3 at 0-min) via Dunnett's Test (n = 3, α = 0.05). Error bars represent SE. Some error bars are too small to be seen.

4.3.4 Involvement of IAA in H_2SO_3-induced Stomatal Closure

IAA is the main natural auxin in plants known to have a positive regulatory role in stomatal opening (Levitt et al. 1987). Quantification of IAA contents in H_2SO_3-treated leaves revealed non-significant differences for the effects of 1.1 μmol l^{-1} H_2SO_3 from the control (Fig. 4.5a). Nevertheless, the treatment with 1.2 mmol l^{-1} H_2SO_3 significantly increased the IAA levels in the leaves at 30 min and decreased at 180 min. Observation of a transient increase in the IAA content in leaves at 30-min of 1.2 mmol l^{-1} H_2SO_3 treatment suggested a novel role of IAA in stomatal closure induction. To further investigate the contribution of IAA in stomata closure induction, I examined the effects of IAA on stomatal aperture width (Fig. 4.5b). No

significant differences were observed in stomatal aperture width of leaves treated with IAA (1 – 100 μmol l^{-1}). This observation rejects the apparent involvement of IAA in H_2SO_3-induced stomatal closure.

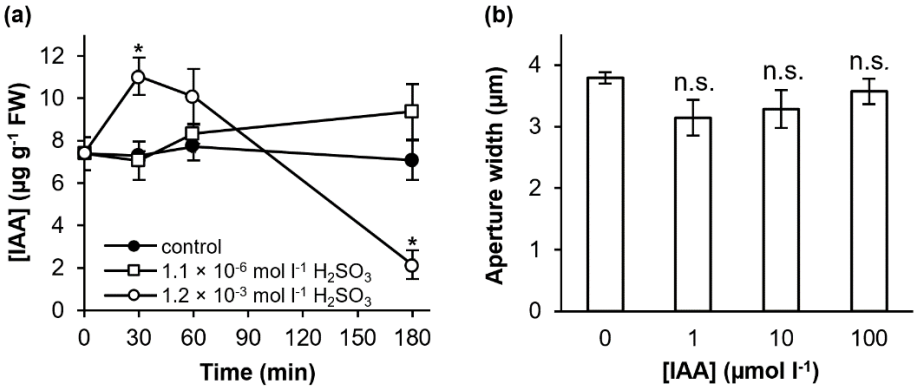


Figure 4.5 Relationship of IAA and H_2SO_3-induced stomatal closure. (a) IAA contents in H_2SO_3-treated leaves. Mature rosette leaves of wild type plants were incubated in the buffer containing 0, 1.1 μmol l^{-1} and 1.2 mmol l^{-1} H_2SO_3 for 180-min under the light. Asterisk (*) indicates significant difference from the control (0 mol l^{-1} H_2SO_3 at 0-min) via Dunnett's Test (n = 3, α = 0.05). (b) Stomatal closure induction by IAA, n = 4, with 80 stomata per bar. n.s. indicates non-significant differences (α = 0.05) from the control (0 μmol l^{-1} IAA) by Dunnett's test. Error bars represent SE. Some error bars are too small to be seen.

4.3.5 SO_2-induced Stomatal Closure Is Not Mediated by Gasotransmitters H_2S and NO

Hu et al. (2014) reported that the SO_2-induced stomatal closure is mediated by gasotransmitters H_2S and NO, through pharmacological approaches in *I. batatas*. Here, I investigated the involvement of these gasotransmitters in Arabidopsis by observing stomatal response to H_2SO_3 in the presence of H_2S or NO scavengers, hypotaurine (HT) and 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide sodium salt (cPTIO), respectively (Fig. 4.6). No significant differences from control were observed in H_2SO_3-treated leaves in the presence of HT and cPTIO unlike in *I. batatas*. This finding suggested that SO_2-induced stomatal closure does not involve H_2S- and NO-mediated signaling pathways, at least in Arabidopsis.

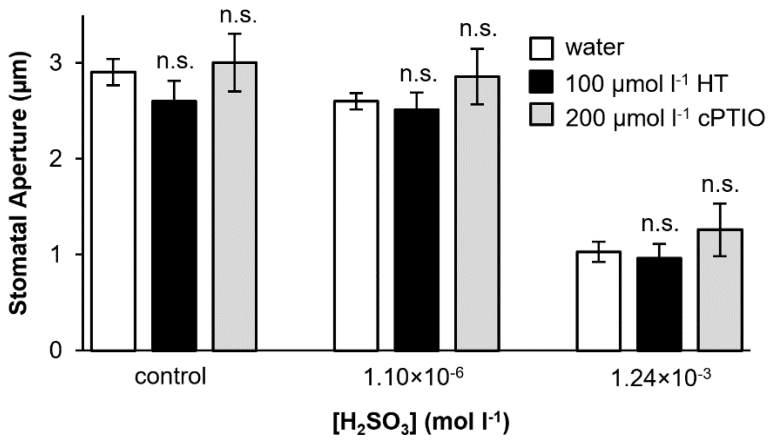


Figure 4.6 Effect of Hypotaurine (HT) and 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide sodium salt (cPTIO) on stomatal aperture width in the presence of H_2SO_3. "control" indicates condition with 0 mol l^{-1} of H_2SO_3, water treatment acts as the solvent control. Pre-opened stomata were incubated in the experimental solution containing H_2SO_3 added with 100 µmol l^{-1} HT or 200 µmol l^{-1} cPTIO, for 2 hr. n = 4, with 80 stomata per bar. Error bars indicate SE. n.s. indicates non-significant differences by Dunnett's test (α = 0.05).

4.4 Discussion

4.4.1 Hormones and Gasotransmitters Are Involved in SO_2-induced Stomatal Closure

A previous study reported that ABA is responsible for stomatal closure upon SO_2 exposure in *V. faba* (Taylor et al. 1981). In contrast, quantification of ABA contents in the whole leaf of Arabidopsis did not show significant increases in the ABA contents over the period of SO_2 exposure (Fig. 4.2). In addition, O_3- and CO_2-insensitive stomata mutants used in this study are reported to be insensitive to ABA with respect to stomatal closure induction (Kwak et al. 2003; Mustilli et al. 2002; Negi et al. 2008; Vahisalu et al. 2008), yet they still demonstrated responsiveness to SO_2 (Fig. 3.3). These observations exclude the involvement of ABA in SO_2-induced closure at least in Arabidopsis.

Hormone signal network in defense response to pathogenesis has been widely studied, where JA and SA are key factors in the signaling pathways (Robert-Seilaniantz et al.

2007). However, hormone regulatory pathways on plant defense against gaseous stimuli (hazardous gas avoidance) are not well-understood. Only a handful of studies have been reported on the involvement of SA, JA and ET in ozone response (Gomi et al. 2005; Overmyer et al. 2000). SA accumulates in Arabidopsis exposed to O_3, which in turn induces protective responses to O_3 via induction of defense genes (Sharma et al. 1996). Acute exposure to high concentration of O_3 was also reported to trigger SA production which amplifies the downstream signals and eventually leads to programmed cell death through a similar mechanism as observed in plants infected by pathogen (Tamaoki 2008). Yet, none of these studies has shown a clear relationship between these hormones with stomatal closure induction by O_3. On the other hand, a metabolomic study on the stomatal movement of rapeseed (*Brassica napus*) in elevated CO_2 conditions revealed that CO_2-induced stomatal closure is mediated by JA and JA-Ile signaling pathways (Geng et al. 2016).

As opposed to these findings, the time course study of hormonal contents of JA and JA-Ile (Fig. 4.3) in H_2SO_3-treated leaves did not demonstrate any significant difference from the control. An increase in SA content in leaves after a 180-min treatment with high concentration of H_2SO_3 (Fig. 4.4) suggests SA as a candidate in regulating SO_2-induced stomatal closure. Additional works using SA-deficient and SA-insensitive mutants (such as transgenic lines heterologously expressing the *Pseudomonas putida NahG* gene with suppressed SA accumulation (van Wees & Glazebrook 2003) or *npr* knockout mutants with impairment in SA receptor (Fu et al. 2012; Wu et al. 2012) could provide further confirmations on the role of SA in SO_2-induced stomatal closure. Nevertheless, the involvement of SA was later eliminated when the time-course profile of SA (Fig. 4.4) was found to be uncorrelated with the kinetic study of H_2SO_3 stomatal closure induction (See *Chapter 5*; Fig. 5.4). SA and JA responses against SO_2 were different from those against CO_2 and O_3 (Geng et al. 2016; Sharma et al. 1996; Tamaoki 2008), highlighting that plant response to SO_2 is distinct from CO_2 and O_3, as supported by the stomatal response studies utilizing CO_2- and O_3-insensitive stomata mutants (Fig.s 3.2; 3.3).

Although IAA has been reported to be increased in response to water stress in *Cucumis sativus* (Pustovoitova et al. 2003) and pathogen infection in Arabidopsis (Kazan & Manners 2009), there was no evidence showing that IAA was involved in stomatal closure induction in response to stresses. IAA enhances stomatal opening (Lohse & Hedrich 1992). The transient increment in IAA contents observed in the treatment with high concentration of H_2SO_3 at 30-min of exposure (Fig. 4.5a) has no significant effects on stomatal closure (Fig. 4.5b). The mechanism that triggered the increase is unknown. In this study, the involvement of hormones in H_2SO_3-induced closure was excluded based on the implication made from the kinetic profile of the plant hormones. Nonetheless, further investigation is required to confirm these findings.

Unlike the previous report in *I. batatas*, gasotransmitters H_2S and NO were not involved in stomatal closure induction against SO_2 in Arabidopsis (Fig. 4.6). The reason for the difference in these observations remains obscure. The difference in signaling pathways between Arabidopsis and *I. batatas* could be a potential reason for the differences observed in response to HT and cPTIO. SO_2 does not provoke the similar hormonal response in inducing stomatal closure as in CO_2 (Geng et al. 2016); SO_2-induced stomatal closure does not share the same molecular factors as for CO_2 and O_3 (Fig.s 3.3). Collectively, these findings indicated that the mechanism for SO_2-induced stomatal closure is not mediated by hormones and gasotransmitters H_2S and NO, and it is distinct from CO_2- and O_3-induced closure.

CHAPTER 5

SULFUR DIOXIDE-INDUCED STOMATAL CLOSURE IS MEDIATED BY NON-APOPTOTIC CELL DEATH OF GUARD CELLS

5.1 Introduction

SO_2-induced stomatal closure has been postulated to be due to cytoplasmic acidification that inhibits 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*, *I. batatas* and *P. sativum*. My previous studies had excluded the roles of hormones and gasotransmitters (H_2S and NO) in SO_2-induced stomatal closure in Arabidopsis (*Chapter 4*). I speculated that it was instead due to cell death in the guard cells, which brought to the irreversible stomatal closure. I hypothesized that H_2SO_3 kills stomatal guard cells after reaching the cytosolic liquid forming SO_3^{2-} and HSO_3^- ions. The formation of these ions leads to a decrease in cytosolic pH with the release of additional H^+ ions, which then causes the stomatal closure (*Section 2.4.1*). Here, I also investigated if low concentrations of SO_2 induces stomatal opening in Arabidopsis, as observed in *V. faba* (Black & Unsworth 1980; Taylor et al. 1981).

Taylor (1978) proposed that plants obtained resistance to gaseous pollutants via “stress tolerance” and “stress avoidance” mechanisms, of which the first one involved the capability of plants to tolerate, assimilate or buffer the harmful pollutant derivatives; the second mechanism involved the closing of stomata to avert absorption of pollutants. Transcriptome analyses have disclosed the SO_2 detoxification process in plants, which involves an oxidative pathway in the peroxisomes (sulfite oxidase) and a plastid sulfur assimilation pathway localized in the chloroplasts (Brychkova et al. 2007; Considine & Foyer 2015; Hamisch et al. 2012; Randewig et al. 2012). These findings explain the metabolic changes taking place in plant tolerance to non-phytotoxic levels of SO_2. I was curious if SO_2-

induced stomatal closure is a part of hazardous gas avoidance mechanism to protect plants from adverse effects from this gaseous pollutant.

5.2 Materials and Methods

5.2.1 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; and excitation and emission wavelengths of 545/25 nm and 605/70 nm, and dichroic mirror cutoff of 565 nm for PI, respectively).

5.2.2 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).

5.2.3 Apoptotic Cell Death Detection

Apoptotic cell death in 2-hr H_2SO_3-treated guard cells was examined histochemically through the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (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 which were positively stained with TUNEL and DAPI stains was counted and expressed as the percentage over the total number of observed guard cells.

Positive control was prepared through partial digestion of DNA with recombinant DNase I (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.

5.3 Results

5.3.1 H_2SO_3 Induces Cell Death in Guard Cells

CFDA and PI stainings were conducted simultaneously on H_2SO_3-treated epidermal preparations to examine the viability of guard cells. CFDA stains the 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 stomatal guard cells and the percentage of CFDA- and PI-positive guard cells are illustrated in Figs 5.1a and 5.1b, 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 observed. Note that red autofluorescence observed in cell walls of aperture lip and PI-positive nuclei of dead epidermal pavement cells (Fig. 5.1a) 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% (Fig. 5.1b). 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 stomata opening buffer (Fig. 5.1c, see also Fig. 2.2). 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.

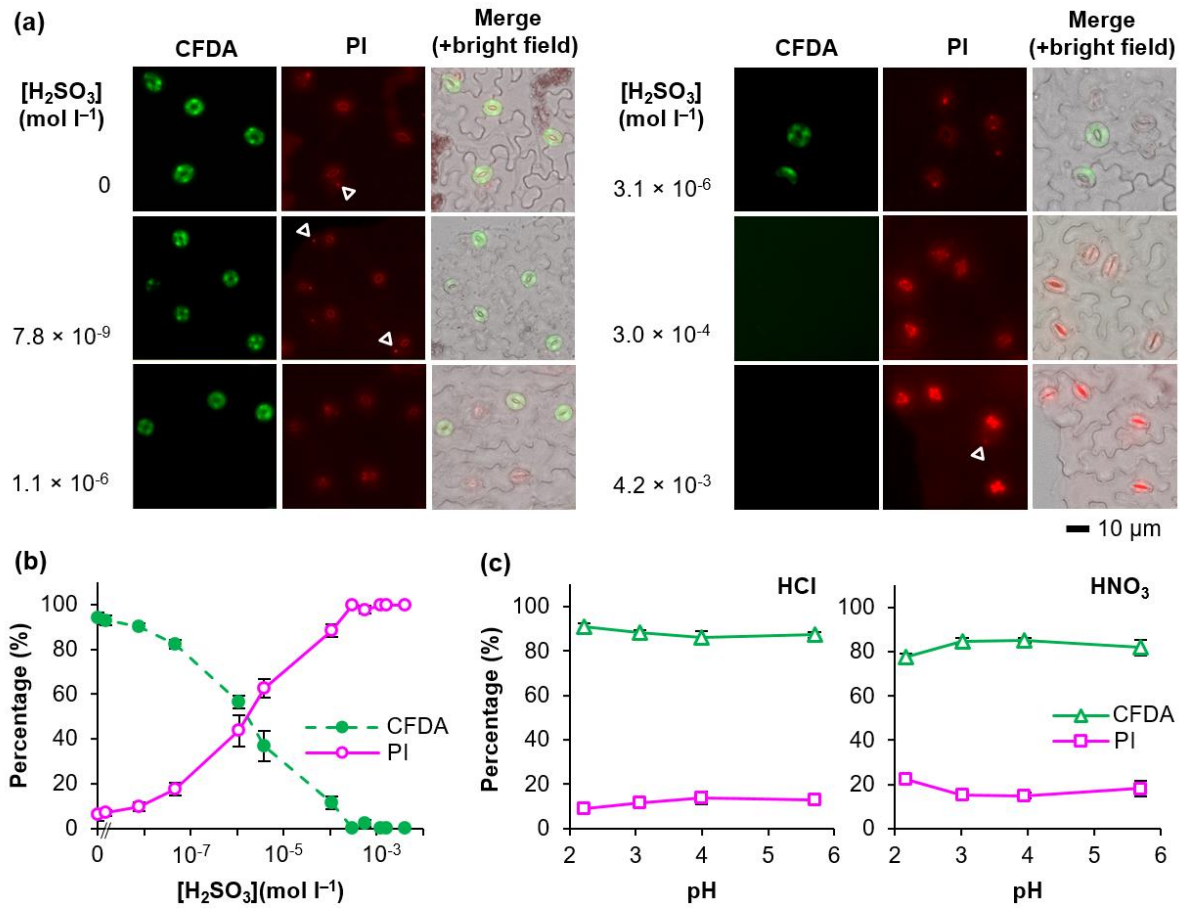


Figure 5.1 H_2SO_3-induced cell death in guard cells. (a) Representative fluorescence microscopic images of CFDA- and PI-stained stomatal guard cell exposed to H_2SO_3. White arrowheads indicate representative PI-positive nuclei of dead pavement cells which are also seen in other PI-staining panels. (b) The rate of CFDA- and PI-stained guard cells. The viability of 100 – 140 guard cells was quantified for each concentration in every experiment. Data were from 4 independent experiments. (c) 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. Error bars indicate SE. Some of the error bars are too small to be seen.

H_2SO_3-induced death of guard cells was further examined by assessing the effect of fusicoccin (FC) (Fig. 5.2a). FC induces stomatal opening by the activation of H^+-ATPase and the increase in K^+ conductance of the membrane in intact guard cells (Blatt 1988; Marrè

1979). The stomatal width of dark-acclimated leaves was 1.1 ± 0.0 μm in the absence of FC, it increased to 3.17 ± 0.23 μm with 10 μmol l^{-1} FC. The 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. No substantial 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. 5.1b). 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. 5.2b).

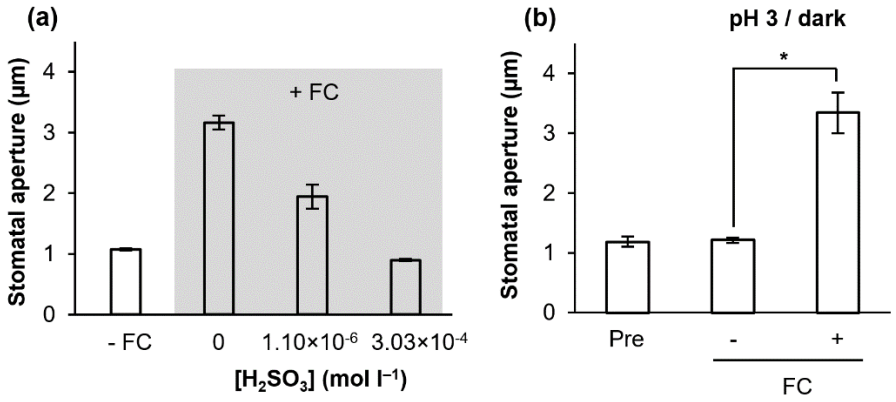


Figure 5.2 Fusicoccin-induced stomatal opening in the dark and acidified solution. (a) Stomatal opening induction of H_2SO_3-treated leaves by 10 μmol l^{-1} fusicoccin (FC), 2 hr incubation, in the dark, n = 4 biological replicates (80 stomata in total). (b) Stomatal aperture width measured in acidic condition (pH 3) in the dark with and without 10 μmol l^{-1} 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.

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. 5.3). 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 manifested that the mode of action of H_2SO_3 on guard cells is mediated by mechanism which is different from that of O_3 and CO_2.

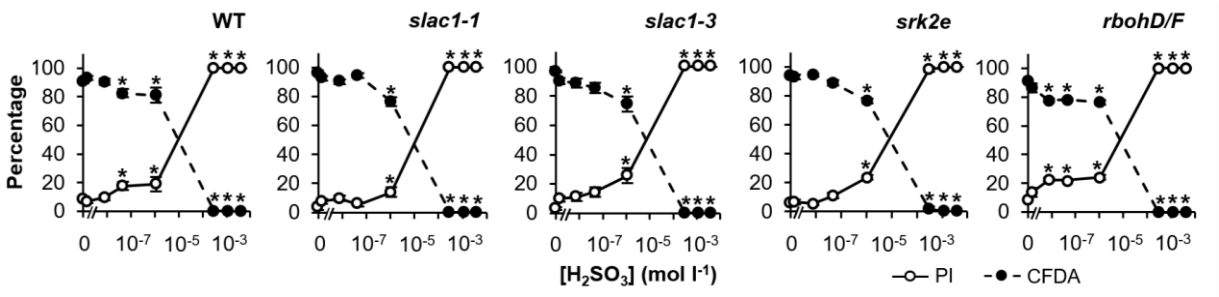


Figure 5.3 Guard cell viability of H_2SO_3-exposed wild type, carbon dioxide- and ozone-insensitive stomata mutants (*slac1-1, slac1-3, srk2e,* and *rbohD/F*). Four independent experiments with 100 – 140 guard cells were observed for each. Error bars represent SE. Some error bars are too small to be seen. Asterisks (*) represent significant different via one-way ANOVA followed by Dunnett's Test (α = 0.05).

5.3.2 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. 5.4a). 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 experimental time, with an average aperture width of 0.50 ± 0.15 µm. A drastic decline in

guard cell viability was also observed, with a 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. 5.4b). 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, a two-peak Gaussian fitting revealed 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 suggested that at 120-min of H_2SO_3 exposure, some of the stomata had closed tightly, presumably being due to the death of 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 treatment with 1.2 mmol l^{-1} H_2SO_3 (Fig. 5.4a). These results suggest that SO_2 opens stomata at lower concentrations, and induces stomatal closure at higher concentrations, in Arabidopsis.

Consequently, I also performed a stomatal opening assay in the dark with a series of [H_2SO_3] below 1.1 μmol l^{-1} (Fig. 5.5). Stomatal aperture width in Arabidopsis did not show significant differences among the measurements from different concentrations (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 also resulted in cell death in some of the guard cells, concurrently; this mechanism is light dependent (Fig. 5.5).

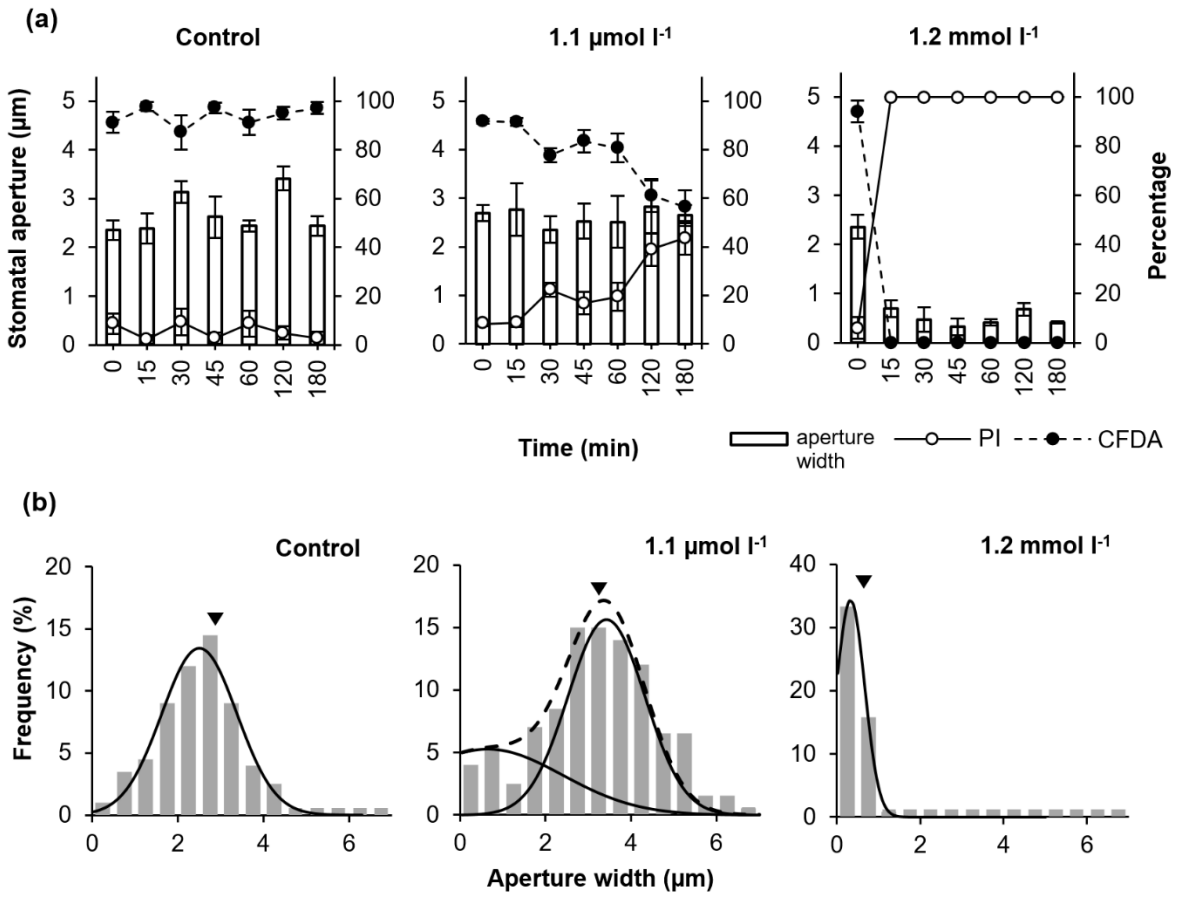


Figure 5.4 Time course of H_2SO_3-induced stomatal closure/opening and cell death in guard cells. (a) Time course of stomatal aperture width and guard cell viability in a period of 180-min incubation in H_2SO_3. Bar represents stomatal aperture width; dotted line represents the rate of CFDA-stained guard cells; solid line represents the rate of PI-stained guard cells. For the 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 arrowheads indicate 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.

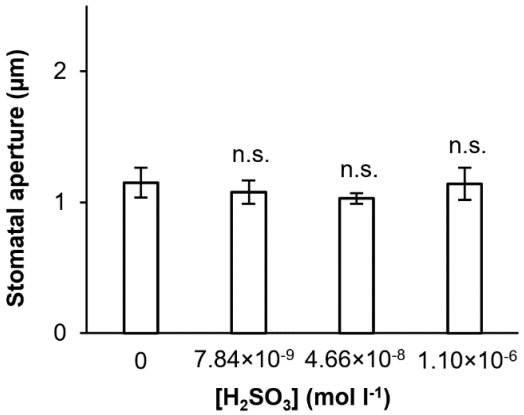


Figure 5.5 Effect of low concentrations of H_2SO_3 on the stomatal aperture in the dark. Dark-acclimated leaves were treated with H_2SO_3 for 3 hrs in the dark. n = 4, with 80 stomata per bar. n.s. indicates non-significant differences (α = 0.05) by Dunnett's test. Error bars represent SE.

5.3.3 H_2SO_3 Induces Non-Apoptotic Cell Death

Cell death plays a central role in the innate immune responses of plants in defending the invasion of pathogens (Coll et al. 2011). Apoptosis, which is accompanied by DNA laddering can occur as hypersensitive response (HR) to incompatible pathogens and O_3-induced HR-like lesion (Pasqualini et al. 2003; Reape et al. 2008). 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. 5.6). 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 mmol l^{-1} 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 the 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% of death in the guard cells (Fig. 5.1b) suggests that the death of guard cells was not caused by an apoptotic mechanism.

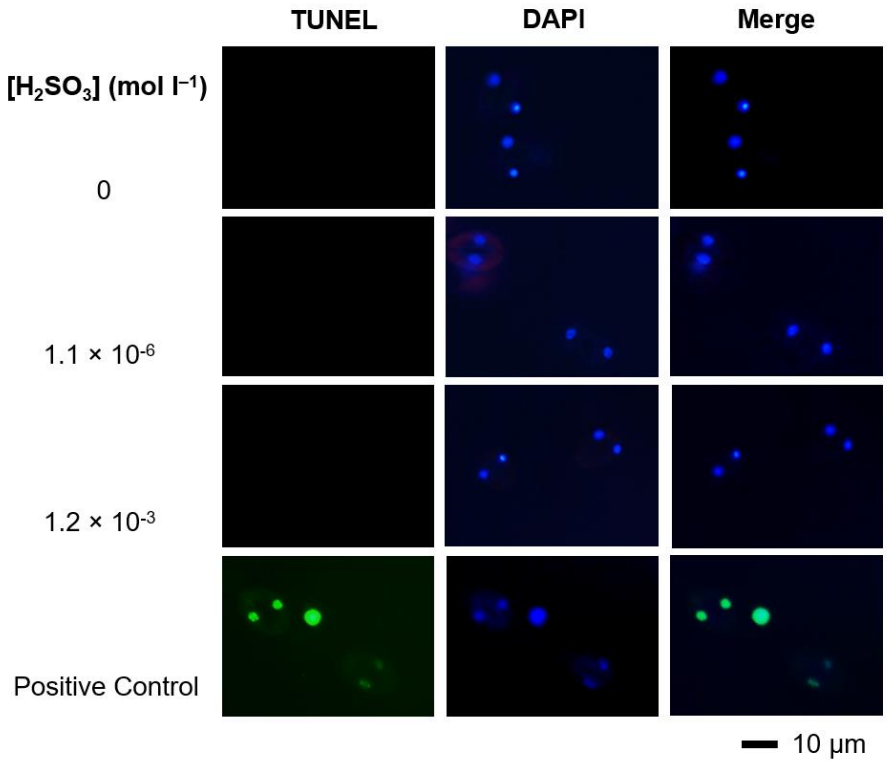


Figure 5.6 Non-apoptotic cell death of guard cells in the H_2SO_3-exposed epidermis. Representative fluorescence microscopy images of TUNEL-stained stomatal guard cells exposed to a 2-hr treatment of 1.1×10^{-6} and 1.2×10^{-3} mol l^{-1} of H_2SO_3 were displayed, with 80 – 120 guard cells observed for each concentration in each experiment. [H_2SO_3] = 0 mol l^{-1} represents negative control for H_2SO_3 treatment. The positive control was prepared by partial DNA digestion with DNase I.

5.4 Discussion

5.4.1 Possible Mode of Action of SO_2 in Stomatal Closure and Cell Death in Guard Cells

H_2SO_3 was identified as the only chemical species which induces stomatal closure, and the involvements of SO_3^{2-} and HSO_3^- were excluded (as reported in *Section 2.3*). 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 a guard cell is approximately 0.09 pl, a 0.53 nmol of H_2SO_3 influx into a guard cell would cause 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 which is roughly matches to that in the experimental solution which was observed to induce 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.

5.4.2 Mechanism and Physiological Significance of SO_2-induced Stomatal Closure

This study in Arabidopsis reveals that stomatal closure in SO_2-treated leaves was a result of the death of stomatal guard cells (Fig. 5.1). Guard cell death was once proposed to be the reason for stomatal closure in SO_2-treated *V. faba* (Unsworth & Black 1981). This study supports their hypothesis with an observation of increased guard cell mortality rate in H_2SO_3-treated leaves. 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 the guard cells.

In addition, I did not observe visible foliar damage nor stomatal closure in leaves treated with low concentrations of SO_2; SO_2-induced stomatal closure observed on leaves treated with higher concentrations of SO_2 coexists with the observation of chlorosis and wilting in the leaf, and non-apoptotic cell death in the guard cells (Fig. 2.3; 3.3; 3.4; 5.1; 5.6). These observations stipulated the failure of stomatal closure in protecting Arabidopsis plants from the harmful effects of SO_2. I speculate that SO_2-induced stomatal closure in Arabidopsis does not have any physiological meaning but it is solely an outcome from the toxic effects of SO_2. Anyway, a recent finding has identified that necrotic cell death with no DNA laddering involved could also be a form of programmed cell death which provides plant immunity to stresses (Coll et al. 2011). Additional works scrutinizing the event of SO_2-induced cell death in the guard cells could potentially confirm my speculation.

5.4.3 Induction of Stomatal Opening by Low Dose of SO_2

Apart from its effect on stomatal closure induction, SO_2 was reported to induce stomatal opening in *V. faba* at low concentrations (Biscoe et al. 1973; Black & Unsworth 1980; Mansfield & Majernik 1970; Taylor et al. 1981). This behaviour was also observed in this study with Arabidopsis and it is light dependent (Fig. 5.4 and Fig. 5.5). 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 together the results from the kinetic study and histogram analysis at 120-min (Fig. 5.4), when a portion of stomata started to close (due to the death of guard cells) while another portion of them opened wider, at the guard cell mortality rate of 38.8 ± 1.10 %, I speculate that SO_2-induced stomatal aperture widening in Arabidopsis is probably due to release from the constraint by surrounding epidermal pavement cells which have lost turgor. This process may not have physiological significance.

5.4.4 SO_2-induced Stomatal Closure is Not a Stress Avoidance Mechanism

In term of “stress avoidance” wise, I was curious if closed stomata in the presence of SO_2 was a protection mechanism of plant which is induced through programmed cell death? Unlike reported by Yi et al. (2012) in *V. faba,* this study using TUNEL assay showed that SO_2-induced cell death in the guard cells is not apoptotic (Fig. 5.6). Interestingly, 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.

CHAPTER 6

GENERAL DISCUSSION

6.1 Sulfur Dioxide, Earth, and Human Life

Sulfur dioxide (SO_2) has been known as a predominant anthropogenic airborne pollutant since the flourish of fossil fuel-driven industrial facilities resulted from The Industrial Revolution from more than 1.5 centuries ago (Stöckhardt 1850). Before the development of massive SO_2-emitting anthropogenic activities, SO_2 did exist naturally in the atmosphere emitted from volcanoes. There is no clear information on when SO_2 started to appear in the atmosphere, however, the first volcanic eruption was estimated to take place at the end of Triassic Period (~201.5 Mil BC) (Olsen 1999). The concentration of SO_2 introduced to the atmosphere by ancient volcanic eruptions and quiescent degassing during the evolution of atmosphere was estimated to be low (sulfur isotopic composition, $\Delta^{33}S \approx 0$) as it is photodissociated by deep ultraviolet radiation soon after (Farquhar & Wing 2003). Historical reconstruction of anthropogenic SO_2 emission estimated it to surge exponentially starting from year 1850, until the 21st century (Klimont et al. 2013; Smith et al. 2011). Drastic reduction in SO_2 emission was observed in Europe, Russian Federation, US and Canada in the past decade (Klimont et al. 2013), while momentous increment in SO_2 emission was observed in China and India progressively since the 20th century with continuous increment in the emission projected (Fioletov et al. 2016; Lu et al. 2011).

Global SO_2 emission has escalated for more than 55 times in the past 160 years as compared to the early industrial era (Smith et al. 2011) and its impacts on human life are not negligible. SO_2 affects human life directly and indirectly, particularly through the deterioration of human health, disruption on crop production, and destruction of the environment. Humans contact with air pollutants primarily through inhalation and ingestion, though SO_2 also forms

sulfuric acid (H_2SO_4) when in contact with the eyes. Airborne pollutants are reported to have diverse impacts on human health with effects on different organs and systems (Kampa & Castanas 2008). High concentration of atmospheric SO_2 is found to be closely related to daily mortality, and morbidity of bronchial asthma, dyspnea, bronchoconstriction, persistent phlegm and cough; irritability of the respiratory system, and the increased risk of lung cancer and DNA damage (Chen et al. 2007; Mazumdar et al. 1982; Valavanidis et al. 2008). It is ironic that the anthropogenic activities conducted aiming to improve the quality of human life through economical advances have in turn harming the well-being of human race.

6.2 SO_2-tolerant Plants for Crops and Forests?

Airborne pollutants were reported to cause massive crop loss globally. These events have not only lead to economic losses but also influence global food security (Holland et al. 2002; Wei et al. 2014). Climate change is putting more pressure on global crop production and food security, in combination with the presence of airborne pollutants (Lobell & Gourdji 2012; Sun et al. 2017). SO_2 also lead to forest declination especially in the vicinity of metropolitan cities and industrialized areas (WHO 2000).

One of the solutions to tackle the mentioned issues is by adapting tolerant plants which have greater productivity and stronger resistance when they are put under the stress from airborne pollutants. Myriads of O_3-tolerant crops have been identified, particularly in rice (Ainsworth 2017; Frei 2015), however, no SO_2-tolerant crops have yet to be introduced. The prospects and procedures for breeding plants which are tolerant to SO_2 have been described for agricultural crops as early as in 1973 (Ryder 1973). Yet, it has not been successful to the best of my knowledge. In spite of that, SO_2 tolerance was reported in several woody plants, with a reduction in photosynthesis rate observed. Tolerance to SO_2 in these plants was speculated to be either through stomatal closure or through biochemical detoxification of SO_2, depending on the species (Hwangbo et al. 2000). It is still unclear on how some woody plants could tolerate high concentrations of SO_2, while some other would experience necrosis and eventually die.

Katz (1949) reported no visible symptoms of damage on the leaves of plants exposed to long-term low concentrations of SO_2 proposing that low SO_2 could be beneficial to plants when the soil is deficient in soluble sulfates. This hypothesis has long been arguable without solid evidence. Similarly, no observable damage on leaf appearance was observed for leaves treated with low concentrations of SO_2 in this study, with pre-opened stomata remained opened (Fig. 2.3; 3.2; 3.3). This has raised an interesting question: was the stomatal opening reported in *V. faba* at low concentration of SO_2 (Biscoe et al. 1973; Black & Black 1979) a plant response in up-taking nutrient?

Studies using Arabidopsis and tomato plants identified the crucial role of sulfite oxidase in transforming SO_2-derived sulfites to non-toxic sulfate, thus protecting plants against the exposure to a non-toxic level of SO_2 (Brychkova et al. 2007; Lang et al. 2007). These results explain the tolerance of plants to low SO_2 and provide supporting information on the utilization of SO_2-derived sulfates by plants. Nevertheless, unlike O_3 tolerance in crops, the quantitative trait locus (QTLs) for SO_2 resistance remained unknown (Ainsworth 2017). More works need to be done on the identification of SO_2-tolerant plants and also on the recognition of SO_2-tolerant mechanisms to provide resolutions on the assurance of crop production, and the restoration and conservation of forests.

6.3 Can SO_2-induced Stomatal Closure Safeguard the Plants from SO_2 Attack?

SO_2 is very highly soluble in water and is readily absorbed into the cytosolic fluid through stomata, forming sulfite ions while acidifying the cytoplasm with H^+ (Refer *Section 5.4*). Three major factors determine the impacts of SO_2 on plants: (1) the SO_2 concentration; (2) the rate of SO_2 absorption; (3) the period of SO_2 exposure. As previously discussed, low concentration of SO_2 does not evoke stomatal closure or causing any observable damage on plants. The absorbed SO_2 could has been transformed into non-toxic forms (Brychkova et al. 2007) or been neutralized by the basic cytosolic fluid of the plants (Wang et al. 2012). The absorption of SO_2 is restricted by the buffering capacity of plants to neutralize the protons produced during SO_2 absorption (Ulrich 1983). On the other hand, the period of SO_2

exposure decides the tolerance of plants, of which short-term exposure to high concentration of SO_2 might have less severe impacts as compared to a long-term exposure. Other factors such as soil moisture, relative humidity, the age of plants and light intensity are also known to contribute to the uptake of SO_2 into the plants (Katz 1949).

SO_2-induced stomatal closure has been observed in a wide range of plant species (Hwangbo et al. 2000; Kropff 1987; Taylor 1978; Winner & Mooney 1980), including Arabidopsis used in this study. Taylor's proposal of "stress avoidance" mechanism by closing stomata (Taylor 1978) could be true in certain plant species and at certain concentration levels of airborne pollutants. Woody plant *Paulownia coreana* closes its stomata when it was exposed to low-level SO_2. The SO_2-treated plant shown no observation of chlorosis in the leaves suggesting the stomatal closure event is a biological mechanism in response to SO_2 exposure, which minimizes SO_2 uptake and protecting the leaf from SO_2 damage; however, complete necrosis of pavement cells was observed when it was exposed to high concentration of SO_2, suggesting that stomata closure is implausible to be strong enough for plant to survive higher SO_2 attack (Hwangbo et al. 2000).

In the same study, *Quercus serrata* and *Q. variabilis* survived the high SO_2 treatment with low stomatal inhibition rate, proposing that tolerance to high concentration of SO_2 requires other detoxification or damage repair processes. Ikeda et al. (1992) observed stomatal closure in *Quercus* plants under short- and long-term of SO_2 fumigation; long-term fumigation caused visible foliar injury on the leaves in spite of stomatal closure. Taken together, stomatal closure upon exposure to SO_2 is crucial to prevent the entrance of transient or short-term increase in ambient SO_2, but this "stress avoidance" mechanism is not practical for long-term SO_2 protection. This gives us a very useful guidance on the selection of SO_2-tolerant plants. An ideal SO_2-tolerant plant should be able to close stomata upon short-term exposure of SO_2 and be able to detoxify the harmful effects of SO_2 upon long-term exposure. If one must select one out of the two traits, one should prioritize on plants with the ability to detoxify SO_2 through biochemical processes instead of the plants with the ability to close stomata against SO_2, because long-term SO_2 tolerance is needed to ensure crop production and forest conservation.

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

Several studies on air pollutants have identified the similarity in the effects of O_3 and SO_2 on plants. They were thought to induce a similar signaling response in plants (Mansfield et al. 1993; Olszyk & Tibbitts 1981; Willekens et al. 1994). I 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.s 3.2 and 3.3). It was demonstrated that SO_2-induced stomatal closure is mediated by cellular events, which are different from the other gaseous stimuli (Fig. 6.1).

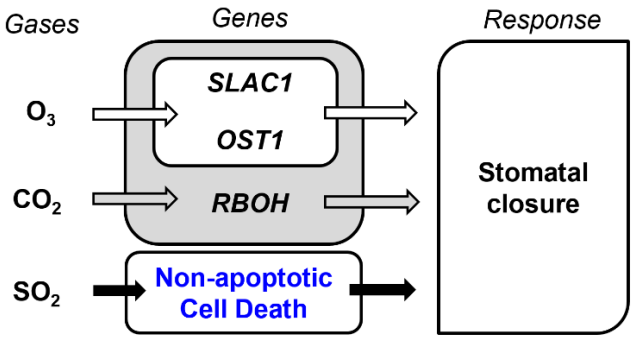


Figure 6.1 SO_2-induced stomatal closure is mediated by a different mechanism from O_3 and CO_2. Stomatal closure induction by SO_2 is a result of non-apoptotic cell death in the guard cells.

The evolutionary development of signaling pathways in stomatal closure upon the exposure to O_3 and elevated level of CO_2 is a consequence of the geological history of the Earth's atmosphere. The atmospheric ozone layer is estimated to be fully developed as early as 2 billion years ago (Walker 1978), which took place at least 400 million years earlier than the development of stomata-like pores in land plants (Chater et al. 2017). A recent analysis on the 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. 2015). 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 1850s following industrial development (Smith et al. 2011). I thus 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 through stomatal closure. Although SO_2 is found to be an exception, but 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 the 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.

SUMMARY

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, I 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 chemical species. To provide new insight into the potential common mechanisms in stress avoidance response of stomata against hazardous gases, I 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. The involvement of hormones and gasotransmitters (NO and H_2S) in SO_2-induced stomatal closure were excluded. I also concluded that SO_2-induced stomatal closure was highly correlated to non-apoptotic cell death in the guard cells. SO_2 has been reported to induce stomatal opening at low concentrations in addition to closure induction at high concentrations. My results suggest that SO_2 promotes stomatal opening in the light while provoking cell death in the guard cells at the same time.

REFERENCES

Acharya, B. R. & Assmann, S. M. 2009. Hormone interactions in stomatal function. *Plant Molecular Biology* 69(4): 451–462. doi:10.1007/s11103-008-9427-0

Ainsworth, E. A. 2017. Understanding and improving global crop response to ozone pollution. *The Plant Journal* 90(5): 886–897. doi:10.1111/tpj.13298

Baciak, M., Warmiński, K. & Bęś, A. 2015. The effect of Selected Gaseous Air Pollutants on Woody Plants. *Forest Research Papers* 76(4): 401–409. doi:10.1515/frp-2015-0039

Balbi, V. & Devoto, A. 2008. Jasmonate signalling network in *Arabidopsis thaliana*: Crucial regulatory nodes and new physiological scenarios. *New Phytologist* 177: 301–318. doi:10.1111/j.1469-8137.2007.02292.x

Bender, J. & Weigel, H. 2010. Changes in Atmospheric Chemistry and Crop Health: A Review. *Agronomy for Sustainable Development* 31: 81–89. doi:10.1051/agro/2010013

Bereiter, B., Eggleston, S., Schmitt, J., Nehrbass-Ahles, C., Stocker, T. F., Fischer, H., Kipfstuhl, S., et al. 2015. Revision of the EPICA Dome C CO_2 Record from 800 to 600 kyr Before Present. *Geophysical Research Letters* 42: 542–549. doi:10.1002/2014GL061957

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. doi:10.1111/j.1469-8137.1973.tb02106.x

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(115): 291–298. doi:10.1093/jxb/30.2.291

Black, V. S. & Unsworth, M. H. 1980. Stomatal Responses to Sulphur Dioxide and Vapour Pressure Deficit. *Journal of Experimental Botany* 31(2): 667–677.

Blatt, M. R. 1988. Mechanisms of fusicoccin action: A dominant role for secondary transport in a higher-plant cell. *Planta* 174: 187–200. doi:10.1007/BF00394771

Bobbink, R. 1998. Impacts of Tropospheric Ozone and Airborne Nitrogenous Pollutants on Natural and Semi-natural Ecosystems: A Commentary. *New Phytologist* 139(1): 161–168. doi:10.1046/j.1469-8137.1998.00175.x

Brychkova, G., Xia, Z., Yang, G., Yesbergenova, Z., Zhang, Z., Davydov, O., Fluhr, R., et al. 2007. Sulfite Oxidase Protects Plants against Sulfur Dioxide Toxicity. *The Plant Journal* 50: 696–709. doi:10.1111/j.1365-313X.2007.03080.x

Cape, J. N. 1998. Uptake and Fate of Gaseous Pollutants in Leaves. *New Phytologist* 139(1): 221–223. doi:10.1046/j.1469-8137.1998.00170.x

Cape, J. N., Fowler, D. & Davison, A. 2003. Ecological effects of sulfur dioxide, fluorides, and minor air pollutants: Recent trends and research needs. *Environment International* 29(2–3): 201–211. doi:10.1016/S0160-4120(02)00180-0

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. doi:10.1104/pp.17.00183

Chater, C., Peng, K., Movahedi, M., Dunn, J. A., Walker, H. J., Liang, Y. K., McLachlan, D. H., et al. 2015. Elevated CO_2-Induced Responses in Stomata Require ABA and ABA Signaling. *Current Biology* 25: 2709–2716. doi:10.1016/j.cub.2015.09.013

Chen, T.-M., Kuschner, W. G., Gokhale, J. & Shofer, S. 2007. Outdoor Air Pollution: Nitrogen Dioxide, Sulfur Dioxide, and Carbon Monoxide Health Effects. *The American Journal of the Medical Sciences* 333(4): 249–256. doi:10.1097/MAJ.0b013e31803b900f

Coll, N. S., Epple, P. & Dangl, J. L. 2011. Programmed cell death in the plant immune system. *Cell Death and Differentiation* 18: 1247–1256. doi:10.1038/cdd.2011.37

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(February): 1–10. doi:10.3389/fpls.2015.00060

Desikan, R., Griffiths, R., Hancock, J. & Neill, S. 2002. A new role for an old enzyme: Nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*. *PNAS* 99(25): 16314–16318.

Devoto, A. & Turner, J. G. 2003. Regulation of jasmonate-mediated plant responses in Arabidopsis. *Annals of Botany* 92: 329–337. doi:10.1093/aob/mcg151

Engineer, C. B., Hashimoto-Sugimoto, M., Negi, J., Israelsson-Nordström, M., Azoulay-Shemer, T., Rappel, W. J., Iba, K., et al. 2016. CO_2 Sensing and CO_2 Regulation of Stomatal Conductance: Advances and Open Questions. *Trends in Plant Science* 21(1): 16–30. doi:10.1016/j.tplants.2015.08.014

Farquhar, J. & Wing, B. A. 2003. Multiple sulfur isotopes and the evolution of the atmosphere. *Earth and Planetary Science Letters* 213(1–2): 1–13. doi:10.1016/S0012-821X(03)00296-6

Fioletov, V. E., McLinden, C. A., Krotkov, N., Li, C., Joiner, J., Theys, N., Carn, S., et al. 2016. A global catalogue of large SO_2 sources and emissions derived from the Ozone

Monitoring Instrument. *Atmospheric Chemistry and Physics* 16(18): 11497–11519. doi:10.5194/acp-16-11497-2016

Frei, M. 2015. Breeding of ozone resistant rice: Relevance, approaches and challenges. *Environmental Pollution* 197: 144–155. doi:10.1016/j.envpol.2014.12.011

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., Mohan, R., et al. 2012. NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486(7402): 228–232. doi:10.1038/nature11162.NPR3

García-Chávez, E., Santamaría, A., Díaz-Barriga, F., Mandeville, P., Juárez, B. I. & Jiménez-Capdeville, M. E. 2003. Arsenite-induced formation of hydroxyl radical in the striatum of awake rats. *Brain research* 976(1): 82–9.

Gayatri, G., Agurla, S., Kuchitsu, K., Anil, K., Podile, A. R. & Raghavendra, A. S. 2017. Stomatal Closure and Rise in ROS/NO of Arabidopsis Guard Cells by Tobacco Microbial Elicitors: Cryptogein and Harpin. *Frontiers in Plant Science* 8: 1–10. doi:10.3389/fpls.2017.01096

Geng, S., Misra, B. B., de Armas, E., Huhman, D. V., Alborn, H. T., Sumner, L. W. & Chen, S. 2016. Jasmonate-mediated stomatal closure under elevated CO_2 revealed by time-resolved metabolomics. *The Plant Journal* 88: 947–962. doi:10.1111/tpj.13296

Gomi, K., Ogawa, D., Katou, S., Kamada, H., Nakajima, N., Saji, H., Soyano, T., et al. 2005. A mitogen-activated protein kinase NtMPK4 activated by SIPKK is required for jasmonic acid signaling and involved in ozone tolerance via stomatal movement in tobacco. *Plant and Cell Physiology* 46(12): 1902–1914. doi:10.1093/pcp/pci211

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. doi:10.1071/PP98096

Guo, F.-Q., Okamoto, M. & Crawford, N. M. 2003. Identification of a Plant Nitric Oxide Synthase Gene Involved in Hormonal Signaling. *Science* 302(October): 100–103.

Gupta, A., Hisano, H., Hojo, Y., Matsuura, T., Ikeda, Y., Mori, I. C. & Senthil-Kumar, M. 2017. Global profiling of phytohormone dynamics during combined drought and pathogen stress in *Arabidopsis thaliana* reveals ABA and JA as major regulators. *Scientific Reports* 7: 1–13. doi:10.1038/s41598-017-03907-2

Hamisch, D., Randewig, D., Schliesky, S., Bräutigam, A., Weber, A. P. M., Geffers, R., Herschbach, C., et al. 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. doi:10.1111/j.1469-8137.2012.04331.x

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(7): 1238–1248. doi:10.1093/pcp/pcr072

Hill, A. C. & Littlefield, N. 1969. Ozone. Effect on Apparent Photosynthesis, Rate of Transpiration, and Stomatal Closure in Plants. *Environmental Science and Technology* 3(1): 52–56. doi:10.1021/es60024a002

Holland, M., Mills, G., Hayes, F., Buse, A., Emberson, L., Cambridge, H., Cinderby, S., et al. 2002. *Economic Assessment of Crop Yield Losses from Ozone Exposure*. *ICP Vegetation*. Gwynedd, Wales.

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(8): 1481–1489. doi:10.1093/pcp/pcv069

Horsman, D. C. & Wellburn, A. R. 1977. Effect of SO2 Polluted Air Upon Enzyme Activity in Plants Originating from Areas with Different Annual Mean Atmospheric SO_2 Concentrations. *Environmental Pollution* 13: 33–39.

Hu, H., Boisson-dernier, A., Israelsson-nordström, M., Böhmer, M., Xue, S., Ries, A., Godoski, J., et al. 2010. Carbonic anhydrases are upstream regulators of CO_2-controlled stomatal movements in guard cells. *Nature Cell Biology* 12(1): 87–93. doi:10.1038/ncb2009

Hu, K. D., Tang, J., Zhao, D. L., Hu, L. Y., Li, Y. H., Liu, Y. S., Jones, R., et al. 2014. Stomatal closure in sweet potato leaves induced by sulfur dioxide involves H_2S and NO signaling pathways. *Biologia Plantarum* 58(4): 676–680. doi:10.1007/s10535-014-0440-7

Hwangbo, J., Lee, C. & Kim, J. 2000. Tolerance of several woody plants to sulphur dioxide. *Korean Journal of Biological Sciences* 4: 337–340. doi:10.1080/12265071.2000.9647566

Ikeda, H., Natori, T., Totsuka, T. & Iwaki, H. 1992. High SO_2 resistance of *Clethra barbinervis* established in a smoke-polluted area of Ashio, Tochigi Prefecture, Japan. *Ecological Research* 7(3): 363–370. doi:10.1007/BF02347103

Jin, Z. & Pei, Y. 2015. Physiological implications of hydrogen sulfide in plants: Pleasant exploration behind its unpleasant odour. *Oxidative Medicine and Cellular Longevity*

2015: 1–6. doi:10.1155/2015/397502

Jin, Z., Shen, J., Qiao, Z., Yang, G., Wang, R. & Pei, Y. 2011. Hydrogen sulfide improves drought resistance in *Arabidopsis thaliana*. *Biochemical and Biophysical Research Communications* 414: 481–486. doi:10.1016/j.bbrc.2011.09.090

Jin, Z., Xue, S., Luo, Y., Tian, B., Fang, H., Li, H. & Pei, Y. 2013. Hydrogen sulfide interacting with abscisic acid in stomatal regulation responses to drought stress in Arabidopsis. *Plant Physiology and Biochemistry* 62: 41–46. doi:10.1016/j.plaphy.2012.10.017

Johnson, S., Nguyen, V. & Coder, D. 2013. Assessment of cell viability. *Current Protocols in Cytometry*. doi:10.1002/0471142956.cy0902s64

Kampa, M. & Castanas, E. 2008. Human health effects of air pollution. *Environmental Pollution* 151(2): 362–367. doi:10.1016/j.envpol.2007.06.012

Katz, M. 1949. Sulfur Dioxide in the Atmosphere and Its Relation to Plant Life. *Industrial & Engineering Chemistry* 41(11): 2450–2465.

Kazan, K. & Manners, J. M. 2009. Linking development to defense: auxin in plant-pathogen interactions. *Trends in Plant Science* 14(7): 373–382. doi:10.1016/j.tplants.2009.04.005

Khan, M. I. R., Fatma, M., Per, T. S., Anjum, N. A. & Khan, N. A. 2015. Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. *Frontiers in Plant Science* 6: 1–17. doi:10.3389/fpls.2015.00462

Klimont, Z., Smith, S. J. & Cofala, J. 2013. The Last Decade of Global Anthropogenic Sulfur Dioxide: 2000-2011 Emissions. *Environmental Research Letters* 8(1). doi:10.1088/1748-9326/8/1/014003

Kollist, T., Moldau, H., Rasulov, B., Oja, V., Rämma, H., Hüve, K., Jaspers, P., et al. 2007. A novel device detects a rapid ozone-induced transient stomatal closure in intact Arabidopsis and its absence in abi2 mutant. *Physiologia Plantarum* 129(4): 796–803. doi:10.1111/j.1399-3054.2006.00851.x

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(3): 365–373. doi:10.1093/oxfordjournals.pcp.a075604

Kropff, M. J. 1987. Physiological effects of sulfur dioxide. 1. the effect of SO_2 on photosynthesis and stomatal regulation of *Vicia faba* L. *Plant, Cell and Environment* 10: 753–760. doi:10.1111/1365-3040.ep11604762

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., Leonhard, N., Angel Torres, M., Dangl, J. L., Bloom, R. E., et al. 2003. NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. *The EMBO Journal* 22: 2623–2633. doi:10.1093/emboj/cdg277

Lang, C., Popko, J., Wirtz, M., Hell, R., Herschbach, C., Kreuzwieser, J., Rennenberg, H., et al. 2007. Sulphite oxidase as key enzyme for protecting plants against sulphur dioxide. *Plant, Cell and Environment* 30(4): 447–455. doi:10.1111/j.1365-3040.2006.01632.x

Levitt, L. K., Stein, D. B. & Rubinstein, B. 1987. Promotion of Stomatal Opening by Indoleacetic Acid and Ethrel in Epidermal Strips of *Vicia faba* L. *Plant Physiology* 85: 318–321.

Lisjak, M., Srivastava, N., Teklic, T., Civale, L., Lewandowski, K., Wilson, I., Wood, M. E., et al. 2010. A novel hydrogen sulfide donor causes stomatal opening and reduces nitric oxide accumulation. *Plant Physiology and Biochemistry* 48: 931–935. doi:10.1016/j.plaphy.2010.09.016

Lisjak, M., Teklic, T., Wilson, I. D., Whiteman, M. & Hancock, J. T. 2013. Hydrogen sulfide: Environmental factor or signalling molecule? *Plant, Cell and Environment* 36: 1607–1616. doi:10.1111/pce.12073

Lisjak, M., Teklic, T., Wilson, I. D., Wood, M. E., Whiteman, M. & Hancock, J. T. 2011. Hydrogen sulfide effects on stomatal apertures. *Plant Signaling and Behavior* 6(10): 1444–1446. doi:10.1104/pp.010707.5.

Liu, J., Hou, L., Liu, G., Liu, X. & Wang, X. 2011. Hydrogen sulfide induced by nitric oxide mediates ethylene-induced stomatal closure of *Arabidopsis thaliana*. *Chinese Science Bulletin* 56(33): 3547–3553. doi:10.1007/s11434-011-4819-y

Loake, G. & Grant, M. 2007. Salicylic acid in plant defence-the players and protagonists. *Current Opinion in Plant Biology* 10(5): 466–472. doi:10.1016/j.pbi.2007.08.008

Lobell, D. B. & Gourdji, S. M. 2012. The Influence of Climate Change on Global Crop Productivity. *Plant Physiology* 160(4): 1686–1697. doi:10.1104/pp.112.208298

Lohse, G. & Hedrich, R. 1992. Characterization of the Plasma-Membrane H^+-ATpase from *Vicia Faba* Guard Cells - Modulation by Extracellular Factors and Seasonal Changes. *Planta* 188(2): 206–214. doi:Doi 10.1007/Bf00216815

Lu, Z., Zhang, Q. & Streets, D. G. 2011. Sulfur dioxide and primary carbonaceous aerosol emissions in China and India, 1996-2010. *Atmospheric Chemistry and Physics* 11(18): 9839–9864. doi:10.5194/acp-11-9839-2011

Malhotra, S. S. & Hocking, D. 1976. Biochemical and cytological effects of sulfur dioxide on plant metabolism. *New Phytologist* 76: 227–237.

Mansfield, T. A. 1998. Stomata and plant water relations: Does air pollution create problems? *Environmental Pollution* 101(1): 1–11. doi:10.1016/S0269-7491(98)00076-1

Mansfield, T. A. & Majernik, O. 1970. Can stomata play a part in protecting plants against air pollutants? *Environmental Pollution* 1(2): 149–154. doi:10.1016/0013-9327(70)90015-7

Mansfield, T. A., Person, M., Atkinson, C. J. & Wookey, P. A. 1993. Ozone, Sulphur Dioxide and Nitrogen Oxides: Some Effect on the Water Relations of Herbaceous Plants and Trees. *Interacting Stresses on Plants in a Changing Climate* I: 77–88.

Marrè, E. 1979. Fusicoccin: A Tool in Plant Physiology. *Annual Review of Plant Physiology* 30: 273–288. doi:10.1146/annurev.pp.30.060179.001421

Mazumdar, S., Schimmel, H. & Higgins, I. T. T. 1982. Relation of daily mortality to air pollution: An analysis of 14 London winters, 1958/59–1971/72. *Archives of Environmental Health* 37(4): 213–220. doi:10.1080/00039896.1982.10667567

McAinsh, M. R., Evans, N. H., Montgomery, L. T. & North, K. A. 2002. Calcium Signalling in Stomatal Responses to Pollutants. *New Phytologist* 153(3): 441–447. doi:10.1046/j.0028-646X.2001.00336.x

Melotto, M., Underwood, W., Koczan, J., Nomura, K. & He, S. Y. 2006. Plant Stomata Function in Innate Immunity against Bacterial Invasion. *Cell* 126: 969–980. doi:10.1016/j.cell.2006.06.054

Merilo, E., Laanemets, K., Hu, H., Xue, S., Jakobson, L., Tulva, I., Gonzalez-Guzman, M., et al. 2013. PYR/RCAR Receptors Contribute to Ozone-, Reduced Air Humidity-, Darkness-, and CO_2-Induced Stomatal Regulation. *Plant Physiology* 162: 1652–1668. doi:10.1104/pp.113.220608

Mori, I. C., Pinontoan, R., Kawano, T. & Muto, S. 2001. Involvement of Superoxide Generation in Salicylic Acid-Induced Stomatal Closure in Vicia faba. *Plant and Cell Physiology* 42(12): 1383–1388. doi:10.1093/pcp/pce176

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×annasa as a hyperaccumulator. *Redox Biology* 2: 91–98. doi:10.1016/j.redox.2013.12.006

Munemasa, S., Mori, I. C. & Murata, Y. 2011. Methyl jasmonate signaling and signal crosstalk between methyl jasmonate and abscisic acid in guard cells. *Plant Signaling and Behavior* 6(7): 939–941. doi:10.4161/psb.6.7.15439

Murata, Y., Mori, I. C. & Munemasa, S. 2015. Diverse Stomatal Signaling and the Signal Integration Mechanism. *Annual Review of Plant Biology* 66(1): 369–392. doi:10.1146/annurev-arplant-043014-114707

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. *The Plant Cell* 14: 3089–3099. doi:10.1105/tpc.007906.ABA

Negi, J., Matsuda, O., Nagasawa, T., Oba, Y., Takahashi, H., Kawai-Yamada, M., Uchimiya, H., et al. 2008. CO2 Regulator SLAC1 and its Homologues are Essential for Anion Homeostasis in Plant Cells. *Nature* 452: 483–486. doi:10.1038/nature06720

Neill, S., Barros, R., Bright, J., Desikan, R., Hancock, J., Harrison, J., Morris, P., et al. 2008. Nitric oxide, stomatal closure, and abiotic stress. *Journal of Experimental Botany* 59(2): 165–176. doi:10.1093/jxb/erm293

Nilson, S. E. & Assmann, S. M. 2006. The Control of Transpiration. Insights from Arabidopsis. *Plant Physiology* 143: 19–27. doi:10.1104/pp.106.093161

Olsen, P. E. 1999. Giant Lava Flows, Mass Extinctions, and Mantle Plumes. In. William E. Winner, Mooney, & Goldstein (editors). *Sulfur Dioxide and Vegetation: Physiology, Ecology, and Policy Issues*, 1st Edi., Vol. 284, 604–605. Stanford, California: Stanford University Press.

Olszyk, D. M. & Tibbitts, T. W. 1981. Stomatal Response and Leaf Injury of *Pisum sativum* L. with SO_2 and O_3 Exposure. *Plant Physiology* 67: 539–544.

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. doi:10.1104/pp.79.1.153

Overmyer, K., Tuominen, H., Kettunen, R., Betz, C., Langebartels, C., Sandermann, H. & Kangasjärvi, J. 2000. Ozone-sensitive arabidopsis *rcd1* mutant reveals opposite roles for ethylene and jasmonate signaling pathways in regulating superoxide-dependent cell

death. *The Plant Cell* 12: 1849–1862. doi:10.1105/tpc.12.10.1849

Papanatsiou, M., Scuffi, D., Blatt, M. R. & García-Mata, C. 2015. Hydrogen Sulfide Regulates Inward-Rectifying K^+ Channels in Conjunction with Stomatal Closure. *Plant Physiology* 168: 29–35. doi:10.1104/pp.114.256057

Pasqualini, S., Piccioni, C., Reale, L., Ederli, L., Torre, G. Della & 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. doi:10.1104/pp.103.026591

Porra, R. J., Thompson, W. A. & Kriedemann, P. E. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls 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. doi:10.1016/S0005-2728(89)80347-0

Pustovoitova, T. N., Drozdova, I. S., Zhdanova, N. E. & Zholkevich, V. N. 2003. Leaf Growth, Photosynthetic Rate, and Phytohormone Contents in *Cucumis sativus* Plants under Progressive Soil Drought. *Russian Journal of Plant Physiology* 50(4): 441–443. doi:10.1023/A:1024752219336

Randewig, D., Hamisch, D., Herschbach, C., Eiblmeier, M., Gehl, C., Jurgeleit, J., Skerra, J., et al. 2012. Sulfite Oxidase Controls Sulfur Metabolism under SO_2 Exposure in *Arabidopsis thaliana*. *Plant, Cell and Environment* 35: 100–115. doi:10.1111/j.1365-3040.2011.02420.x

Rao, I. M., Amundson, R. G., Alscher-Herman, R. & Anderson, L. E. 1983. Effects of SO_2 on Stomatal Metabolism in *Pisum sativum* L. *Plant Physiology* 72(2): 573–577. doi:10.1104/pp.72.2.573

Reape, T. J., Molony, E. M. & McCabe, P. F. 2008. Programmed cell death in plants: Distinguishing between different modes. *Journal of Experimental Botany* 59(3): 435–444. doi:10.1093/jxb/erm258

Robert-Seilaniantz, A., Navarro, L., Bari, R. & Jones, J. D. 2007. Pathological hormone imbalances. *Current Opinion in Plant Biology* 10: 372–379. doi:10.1016/j.pbi.2007.06.003

Robinson, M. F., Heath, J. & Mansfield, T. a. 1998. Disturbances in Stomatal Behaviour Caused by Air Pollutants. *Journal of Experimental Botany* 49 (Special Issue): 461–469. doi:10.1093/jxb/49.Special_Issue.461

Ryder, E. J. 1973. Selecting and Breeding Plants for Increased Resistance to Air Pollutants. *Advances in Chemistry* 122: 75–84.

Saji, S., Bathula, S., Kubo, A., Tamaoki, M., Kanna, M., Aono, M., Nakajima, N., et al. 2008. Disruption of a Gene Encoding C4-dicarboxylate Transporter-like Protein Increases Ozone Sensitivity through Deregulation of The Stomatal Response in *Arabidopsis thaliana*. *Plant and Cell Physiology* 49(1): 2–10. doi:10.1093/pcp/pcm174

Schroeder, J., Allen, G., Hugouvieux, V., Kwak, J. M. & Waner, D. 2001. Guard cell signal transduction. *Annual Review of Plant Physiologist* 52: 627–658.

Schuman, M. C., Meldau, S., Gaquerel, E., Diezel, C., McGale, E., Greenfield, S. & Baldwin, I. T. 2018. The active jasmonate JA-Ile regulates a specific subset of plant jasmonate-mediated resistance to herbivores in nature. *Frontiers in Plant Science* 9: 1–19. doi:10.3389/fpls.2018.00787

Sharma, Y. K., Leon, J., Raskin, I. & Davis, K. R. 1996. Ozone-induced responses in *Arabidopsis thaliana*: The role of salicylic acid in the accumulation of defense-related transcripts and induced resistance. *Proceedings of the National Academy of Sciences of the United States of America* 93: 5099–5104. doi:10.1073/pnas.93.10.5099

Smith, J. L., De Moraes, C. M. & Mescher, M. C. 2009. Jasmonate- and salicylate-mediated plant defense responses to insect herbivores, pathogens and parasitic plants. *Pest Management Science* 65: 497–503. doi:10.1002/ps.1714

Smith, S. J., Van Aardenne, J., Klimont, Z., Andres, R. J., Volke, A. & Delgado Arias, S. 2011. Anthropogenic sulfur dioxide emissions: 1850-2005. *Atmospheric Chemistry and Physics* 11(3): 1101–1116. doi:10.5194/acp-11-1101-2011

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(12): 1129–1133. doi:10.1094/Phyto-70-1129

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

Sun, F., DAI, Y. & Yu, X. 2017. Air pollution, food production and food security: A review from the perspective of food system. *Journal of Integrative Agriculture* 16(12): 2945–2962. doi:10.1016/S2095-3119(17)61814-8

Tamaoki, M. 2008. The role of phytohormone signaling in ozone-induced cell death in plants. *Plant Signaling and Behavior* 3(3): 166–174. doi:10.4161/psb.3.3.5538

Taylor, G. E. 1978. Plant and Leaf Resistance To Gaseous Air Pollution Stress. *New Phytologist* 80(3): 523–534. doi:10.1111/j.1469-8137.1978.tb01584.x

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.) Epidermal Strips. *Plant Physiology* 68(81): 1504–1507. doi:10.1104/pp.68.6.1504

Textor, C., Graf, H.-F., Timmreck, C. & Robock, A. 2004. Emissions from Volcanoes. *Emissions of Chemical Compounds and Aerosols in the Atmosphere*, hlm. 269--303. doi:10.1007/978-1-4020-2167-1_7

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* (8): 212–226.

Thomas, M. D., Hendricks, R. H. & Hill, G. R. 1950. Sulfur Metabolism of Plants: Effect of Sulfur Dioxide on Vegetation. *Industrial & Engineering Chemistry* 42(11): 2231–2235. doi:10.1021/ie50491a023

Tingey, D. T. & Hogsett, W. E. 1985. Water Stress Reduces Ozone Injury via a Stomatal Mechanism. *Plant physiology* 77(4): 944–7. doi:10.1104/pp.77.4.944

Torsethaugen, G., Pell, E. J. & Assmann, S. M. 1999. Ozone inhibits Guard Cell K^+ Channels Implicated in Stomatal Opening. *PNAS* 96(23): 13577–13582. doi:10.1073/pnas.96.23.13577

Ulrich, B. 1983. Interaction of Forest Canopies with Atmospheric Constituents: SO_2, Alkali and Earth Alkali Cations and Chloride. In B. Ulrich & Pankrath (editors). *Effects of Accumulation of Air Pollutants in Forest Ecosystems*, pp. 33–45. D. Reidel Publishing Company.

Unsworth, M. H. & Black, V. J. 1981. Stomatal Responses to Pollutants. In Jarvis & Mansfield (editors). *Stomatal Physiology*, pp. 187–203. Cambridge, London: Cambridge University Press.

Vahisalu, T., Kollist, H., Wang, Y. F., Nishimura, N., Chan, W. Y., Valerio, G., Lamminmäki, A., et al. 2008. SLAC1 is Required for Plant Guard Cell S-type Anion Channel Function in Stomatal Signalling. *Nature* 452(7186): 487–491. doi:10.1038/nature06608

Vahisalu, T., Puzõrjova, I., Brosché, M., Valk, E., Lepiku, M., Moldau, H., Pechter, P., et al. 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. doi:10.1111/j.1365-313X.2010.04159.x

Valavanidis, A., Fiotakis, K. & Vlachogianni, T. 2008. Airborne particulate matter and human health: Toxicological assessment and importance of size and composition of particles for oxidative damage and carcinogenic mechanisms. *Journal of Environmental Science and Health - Part C Environmental Carcinogenesis and Ecotoxicology Reviews* 26(4): 339–362. doi:10.1080/10590500802494538

van Wees, S. C. M. & Glazebrook, J. 2003. Loss of non-host resistance of Arabidopsis NahG to *Pseudomonas syringae* pv. *phaseolicola* is due to degradation products of salicylic acid. *Plant Journal* 33(4): 733–742. doi:10.1046/j.1365-313X.2003.01665.x

Walker, J. C. G. 1978. The Early History of Oxygen and Ozone in the Atmosphere. *Pure and Applied Geophysics (PAGEOPH)* 117(3): 498–512. doi:10.1007/BF00876630

Wang, B.-L., Shi, L., Li, Y.-X. & Zhang, W.-H. 2010. Boron toxicity is alleviated by hydrogen sulfide in cucumber (*Cucumis sativus* L.) seedlings. *Planta* 231: 1301–1309. doi:10.1007/s00425-010-1134-9

Wang, R. 2003. The Gasotransmitter Role of Hydrogen Sulfide. *Antioxidants & Redox Signaling* 5(4): 493–501. doi:10.1089/152308603768295249

Wang, Y., Papanatsiou, M., Eisenach, C., Karnik, R., Williams, M., Hills, A., Lew, V. L., et al. 2012. Systems Dynamic Modeling of a Guard Cell Cl^- Channel Mutant Uncovers an Emergent Homeostatic Network Regulating Stomatal Transpiration. *Plant Physiology* 160: 1956–1967. doi:10.1104/pp.112.207704

Wasternack, C. & Hause, B. 2013. Jasmonates: Biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. *Annals of Botany* 111(6): 1021–1058. doi:10.1093/aob/mct067

Wei, J., Guo, X., Marinova, D. & Fan, J. 2014. Industrial SO_2 Pollution and Agricultural Losses in China: Evidence from Heavy Air Polluters. *Journal of Cleaner Production* 64: 404–413. doi:10.1016/j.jclepro.2013.10.027

WHO. 2000. Effects of Sulfur Dioxide on Vegetation : Critical Levels. *Air Quality Guidelines*, 2nd Ed., pp. 1–17. Copenhagen, Denmark: WHO Regional Office for Europe.

Willekens, H., Van Camp, W., Van Montagu, M., Inze, D., Langebartels, C. & Sandermann, H. 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. doi:10.1104/pp.106.3.1007

Wilson, S. A. & Murray, F. 1990. SO_2-Induced Growth Reductions and Sulphur Accumulation in Wheat. *Environmental Pollution* 66(2): 179–191. doi:10.1016/0269-7491(90)90120-2

Winner, W. E. & Mooney, H. A. 1980. Responses of Hawaiian plants to volcanic sulfur dioxide: stomatal behavior and foliar injury. *Science* 210(4471): 789–791. doi:10.1126/science.210.4471.789

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., De Luca, V., et al. 2012. The Arabidopsis NPR1 Protein Is a Receptor for the Plant Defense Hormone Salicylic Acid. *Cell Reports* 1(6): 639–647. doi:10.1016/j.celrep.2012.05.008

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(8): 1645–1658. doi:10.1038/emboj.2011.68

Yan, C. & Xie, D. 2015. Jasmonate in plant defence: Sentinel or double agent? *Plant Biotechnology Journal* 13: 1233–1240. doi:10.1111/pbi.12417

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. doi:10.1016/j.ecoenv.2011.11.035

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(8): 1779–1790. doi:10.1093/pcp/pcw102

Yin, Y., Adachi, Y., Ye, W., Hayashi, M., Nakamura, Y., Kinoshita, T., Mori, I. C., et al. 2013. Difference in Abscisic Acid Perception Mechanisms between Closure Induction and Opening Inhibition of Stomata. *Plant Physiology* 163: 600–610. doi:10.1104/pp.113.223826

Yoshida, R., Hobo, T., Ichimura, K., Mizoguchi, T., Takahashi, F., Aronso, J., Ecker, J. R., et al. 2002. ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in Arabidopsis. *Plant and Cell Physiology* 43(12): 1473–1483. doi:10.1093/pcp/pcf188

Zhang, H., Hu, L. Y., Hu, K. Di, He, Y. D., Wang, S. H. & Luo, J. P. 2008. Hydrogen sulfide promotes wheat seed germination and alleviates oxidative damage against copper stress. *Journal of Integrative Plant Biology* 50(12): 1518–1529. doi:10.1111/j.1744-7909.2008.00769.x

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my academic advisor, Associate Professor Mori Izumi for the continuous support of my PhD study and related research, for his patience, motivation, and immense knowledge. His guidance not only helped me in all the time of research and writing but also in dealing with unforeseen challenges in life as a research student. I could not have imagined having a better advisor and mentor for my PhD study in Japan.

Besides my main supervisor, I would also like to thank my respected co-supervisors: Professor Takashi Hirayama and Professor Wataru Sakamoto, for their insightful comments, encouragement and questions which incented me to widen my research from various perspectives.

My sincere thanks go to Professor Yoshiyuki Murata, Professor Maki Katsuhara, Associate Professor Shintaro Munemasa and Mr Takakazu Matsuura, who had provided me with constructive suggestions, research resources and technical assistance in developing my research. Without their precious supports, it would not be possible for me to complete this research as it is.

My gratitude also goes to Assistant Professor Yoko Ikeda, my fellow lab members in Group of Environmental Response Systems and Plant Light Acclimation Research Group, fellow researchers and friends in Institute of Plant Science and Resources, and my heartfelt friends particularly in the Graduate School of Environmental and Life Science for their morale supports and kindness. Thank you for lending me your ears and helping hands when I was overwhelmed and trapped in a love-hurt relationship with my PhD study (*Nature* 550, 549-552, Graduate Study: A love-hurt relationship).

I thank the Japanese Government for providing The Ministry of Education, Culture, Sports, Science and Technology (MEXT) Scholarship throughout my PhD study at Okayama University. I would also like to express my greatest gratitude to the Graduate School of Environmental and Life Science, Okayama University, for providing me with Research Grant for Encouragement of Students FY2017 and FY2018 to support my research activities.

Last but not least, I am deeply thankful to my beloved family: my soulmate Dr Tiong Teck Yaw, my superhero Mr Ooi Thean Chor and my brother Mr Ooi Tat Chung for their unconditional love and spiritual supports throughout my PhD study. Words cannot express how grateful I am for all of the sacrifices that you have made on my behalf.

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.

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

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

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.

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

Derivation of Equation 3

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

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

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

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

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

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

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

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

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

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