Title: Cytosolic acidification and oxidation are the toxic mechanisms of SO_2 in Arabidopsis guard cells.

Short title: SO_2 toxicity in plant cells

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ABSTRACT

SO_2/H_2SO_3 can damage plants. However, its toxic mechanism has still been controversial. Two models have been proposed, cytosolic acidification model and cellular oxidation model. Here, we assessed the toxic mechanism of H_2SO_3 in three cell types of *Arabidopsis thaliana*, mesophyll cells, guard cells and petal cells. The sensitivity of guard cells of *CHLORIDE CHANNEL a* (*CLCa*)-knockout mutants to H_2SO_3 was significantly lower than those of wildtype plants. Expression of other *CLC* genes in mesophyll cells and petal cells were different from guard cells. Treatment with antioxidant, disodium 4,5-dihydroxy-1,3-benzenedisulfonate (tiron), increased the median lethal concentration (LC_{50}) of H_2SO_3 in guard cells indicating the involvement of cellular oxidation, while the effect was negligible in mesophyll cells and petal cells. These results indicate that there are two toxic mechanisms of SO_2 to Arabidopsis cells: cytosolic acidification and cellular oxidation, and the toxic mechanism may vary among cell types.

Introduction

Sulfur dioxide (SO_2) emission is projected to increase by up to 1.4 times by 2060. The most worrying and striking impact of air pollution is the large number of premature deaths. Not only human health, but high levels of concentration of SO_2 devastate forests and reduce agricultural productivity. Reportedly, wheat and oil seeds are more affected than the other crops (OECD 2016). Until the mid-20th century, SO_2 was thought to be a beneficial source of sulfur for plants (e.g. Roberts and Koehler 1965). However, it is now recognized that SO_2 emission by volcanic activity and from industry, such as power plant and smelting, lead to commercial losses in agricultural productions (Smith *et al*. 2011, Wei *et al*. 2014). SO_2 fumigation caused wilting, chlorosis, browning of leaves and reduction of transpiration in tomato, radish, perilla and spinach (Kondo and Sugahara 1978). A strong decrease in photosynthesis occurred in *Vicia faba* when fumigated with SO_2 (Kropff 1987). SO_2 exposure induced reduction in stomatal conductance and formation of necrotic lesion in *Pisum sativum* leaves (Olszyk *et al.* 1981) and reduction in stomatal conductance, leaf area and dry mass in *Phaseolus vulgaris* (Temple *et al*. 1985). SO_2 exposure caused the inhibition of the vegetative and reproductive growth in a grass, *Phleum pretense* (Clapperton and Reid 1994).

Plants possess mechanisms to cope with SO_2 stress. Two classes of defense response

54 against gaseous toxicants were proposed, stress avoidance mechanism by closing stomata
55 and stress tolerance by metabolizing toxic gas (Taylor 1978). Kondo and Sugahara (1978)
56 reported that reduction in stomatal conductance, high abscisic acid (ABA) level and
57 strong SO_2 resistance were related. They proposed ABA-induced stomatal closure is an
58 avoidance mechanism for SO_2 resistance. Essentially the same conclusion was reported
59 by Taylor *et al*. (1981). Alternatively, oxidation of SO_2 to sulfate by peroxisomal sulfite
60 oxidase and sulfur assimilation in chloroplasts are thought to be the tolerance mechanism
61 against SO_2 toxicity (Brychkova *et al*. 2007, Hamisch *et al*. 2012, Randewig *et al*. 2012,
62 Considine and Foyer 2015). In contrast to the advances in plant resistance mechanism,
63 SO_2 toxic mechanism in plants has not been well elucidated.

64 Recently, it was revealed that H_2SO_3, the hydrated form of SO_2, is the responsible
65 chemical species which caused stomatal closure during SO_2 exposure (Ooi *et al*. 20019).
66 We proposed that the toxic mechanism of SO_2 was suggested to be cytosolic acidification
67 (Ooi *et al*. 2019). On the other hand, the production of reactive oxygen species (ROS) has
68 also been proposed for the toxic mechanism (Pnueli *et al*. 2003, Muneer *et al*. 2014).
69 Currently, two toxic mechanisms are argued. In this study we aimed at evaluating these
70 two toxic mechanisms, cytosolic acidification model and cellular oxidation model.

71 CLC proteins function as chloride channels or proton/anion exchanger in plants. In

Arabidopsis, there are seven isoforms of CLCs (Hechenberger *et al*. 1996). CLCa is involved in NO_3^- transport from the cytoplasm into the vacular lumen (De Angeli *et al*. 2006; Bergsdorf *et al*. 2009). It is also involved in pH homeostasis in the cytoplasm (Demes *et al*. 2020). CLCb also transports NO_3^-, while the visible phenotype of the knockout mutants seems to be apparent only during nitrate starvation (Von der Fecht-Bartenbach *et al*. 2010; Shi *et al* 2023). CLCc may transport Cl^- preferentially across the tonoplast as is involved in salt stress (Jossier *et al*. 2010). However, it was shown that it plays also a role in regulation of nitrate levels (Harada *et al*. 2004). CLCd and CLCf were reported to function in pH regulation in the trans-Golgi network (Von der Fecht-Bartenbach *et al*. 2007; Marmagne *et al*. 2007; Scholl *et al*. 2021). CLCe is localized in the thylakoids of chloroplasts and is involved in the regulation of photosynthetic electron transport (Marmagne *et al*. 2007; Herdean *et al*. 2016). CLCg was reported to involved in Cl^- tolerance (Nguyen *et al*. 2016). It is noteworthy that CLCa is the only CLC characterized as anion/H^+ transporter (De Angeli *et al*. 2006; Bergsdorf *et al*. 2009; Hodin *et al*. 2023) while CLCb, CLCc, CLCd and CLCf have been assumed to be as well exchangers (Zifarelli and Pusch 2010; Scholl *et al*. 2021). Consequently, these later CLCs could have a function in pH homeostasis, as it has been reported for CLCa (Demes *et al*. 2020).

Earlier studies, which have revealed the involvement of ROS in the SO_2 toxicity, largely rely on the findings of antioxidation enzymes (Lee *et al*. 2017). Pnueli *et al*. (2003) analyzed phenotype of ascorbate peroxidase (*APX*) mutants. Muneer *et al*. (2014) analyzed the generation of ROS and decrease in antioxidating enzyme activities. The analysis of knock-out mutants may cause in ectopic effects on SO_2 sensitivity. Observing the effects of low molecular ROS scavenging agents can compensate such earlier studies.

In this study, we investigated the involvement of two hypothetical models for SO_2/H_2SO_3 toxicity, namely cytosolic acidification and cellular oxidation in *Arabidopsis thaliana*. We examined viability of three cell types, mesophyll cell protoplasts, guard cells and petal cells to evaluate the toxic mechanisms, since it may vary among cell types. To assess the acidification model, we compared the difference in H_2SO_3 sensitivity between wild type and *clca* mutants in which cytosol pH homeostasis is perturbed. The effects of antioxidants, tiron and *N*-acetylcysteine (NAC) on viability of these cells after H_2SO_3 exposure to test another model, cellular oxidation.

Materials and methods:

Plant materials and growth condition

Arabidopsis thaliana ecotypes Wassilewskija (WS) and Columbia-0 (Col), were

used as wild types. *clca2* is a loss-of-function mutant of *CLCa* in WS background (Wege *et al*. 2014). *clca3* is a knock-out T-DNA mutant from the GABI-KAT collection (GABI-KAT_634E03; Figure S1). To check the level of *CLCa* expression, the consequences of the mutation on shoot fresh weight and nitrate content were checked on plants grown for six weeks on Jiffy® peat pellet with 16h-light/8h-dark photoperiod. For other experiments, the seeds were sown in pots filled with Vermiculite GS (Nittai Co. Ltd., Osaka) and seedling soil (Setogahara flower garden, Kiryu-shi, Japan) at 1:2 ratio after a stratification at 4 °C for 4 days. Plants were grown in a growth chamber (Biotron LPH 200, NK System, Osaka, Japan) with 16 h-light/8 h-dark photoperiod regime at 135 µmol m^{-2} s^{-1} photon flux, at 23 °C.

Mesophyll cells viability test

Viability of isolated mesophyll cell protoplasts (MCP) was examined by double staining with fluorescein diacetate (FDA) and propidium iodide (PI). Rosette leaves of 4 to 6-week-old Arabidopsis plants were chopped into approximately 1-mm-width stripes with a razor blade and treated with the cellulase solution containing 1% Onozuka cellulase R10, 0.5% Macerozyme R10, 0.1 mM KCl, 0.1 mM $CaCl_2$, and 0.5 M mannitol (pH 5.5), and agitated at 60 rpm for 3 h at 30°C with a rotary shaker. MCP were exposed to H_2SO_3

126 in the treatment solution containing 0.5 M mannitol, 10 mM MES, 0.1 mM KCl and 0.1
127 mM $CaCl_2$. pH of the treatment solution was adjusted to 5.5 with KOH. H_2SO_3 exposure
128 was conducted at any given concentration and carried out at 25 °C for 1 h under an
129 illumination with light emitting diodes (LED, model ISC-201-2 and ISL 150X150-RB,
130 CCS co., Kyoto, Japan) at 170 μmol m^{-2} s^{-1} with 470 nm and 230 μmol m^{-2} s^{-1} with 660
131 nm. Following the exposure, MCP were washed twice by centrifuging at 300 *g* for 10 min
132 at 4 °C and resuspended in treatment solution to remove H_2SO_3. Entering the apoplastic
133 space, SO_2 readily form H_2SO_3. Therefore, the exposure with SO_2 gas and H_2SO_3 solution
134 are deemed to be essentially the same (Taylor and Tingey 1981). In this study, we treated
135 cells with solution containing varying concentrations of H_2SO_3. After resuspension, the
136 cells were stained with 50 ng ml^{-1} FDA (Sigma Aldrich, Burlington, MA, USA) and 2 ng
137 ml^{-1} PI (Life Technologies, Carlsbad, CA, USA) for 10 min at room temperature. Then,
138 fluorescence of FDA and PI was observed with a fluorescent microscope (Biozero BZ-
139 X700, Keyence Corporation, Osaka, Japan) with 2 filter sets (OP-87763 for FDA and OP-
140 87764 for PI, Keyence Corporation). Cells stained with FDA and PI were counted as alive
141 and dead, respectively.
142
143 **Guard cells viability test**

144 Viability of guard cell (GC) was examined by the double staining with FDA and PI
145 according to the previous method (Ooi et al. 2019). In brief, epidermal fragments were
146 released from Arabidopsis rosettes by blending with a Waring Bar Blender (model
147 36BL60, Waring Commercial product, Stamford, CT, USA). They were collected on a
148 nylon net and transferred to Petri dishes containing GC treatment solution (10 mM MES-
149 Tris buffer, 5mM KCl and 0.1 mM $CaCl_2$). H_2SO_3 at any given concentration was added
150 to the GC treatment solution, and the epidermal fragments were incubated for 1 h under
151 a LED at 25 °C as mentioned above. After H_2SO_3 exposure, epidermal fragments were
152 collected on a nylon net (100-μm opening) and thoroughly washed with distilled water.
153 The epidermal preparations were successively stained with FDA and PI and observed
154 with a fluorescence microscope as mentioned above.
155
156 **Petal cell viability test**
157 Viability of cells in the abaxial layer of petal was assessed with the FDA/PI double
158 staining. Abaxial layer specimens of petals were prepared by the tape-peel method (Figure
159 S2). A petal was carefully detached from the flower by using forceps under a
160 stereomicroscope. It was gently stuck between two pieced of transparent adhesive tapes
161 (UF-096A, Strix Design Inc., Tokyo). Then, stuck tapes were separated slowly. The tape

stuck with the abaxial side of the petal was immediately floated on the petal treatment solution (10 mM MES, 0.1 mM KCl, and 0.1 mM $CaCl_2$) of which pH was adjusted to 5.5 with KOH. Abaxial layer of the petal with the tape was transferred to the petal treatment solution containing a given concentration of H_2SO_3 and incubated for 1 h under a LED as described above. After incubation, abaxial layer stuck on the tape was washed with the petal treatment solution followed by the double staining with FDA and PI and observed under a fluorescence microscope as described above.

Estimation of median lethal concentration (LC_{50})

LC_{50} was estimated by the binomial logistic regression using the *glm* function in R software (version 4.2.2, R Core Team, 2022). To assess the logit model fit, we determined Nagelkerke's pseudo-R^2 as described elsewhere (Nagelkerke 1991).

Semi-quantitative expression analysis of *CLC* transcripts by reverse transcription-PCR and RT-qPCR for *CLCa*

Total RNA was isolated from 5 or 6-week-old Col plants. Whole leaf, MCP, epidermal fragment in which GC were enriched, and petal were frozen in liquid nitrogen and ground in a mortar. MCP and epidermal fragments were isolated as mentioned above.

Ground tissues were suspended in TRIzol reagent (Life Technologies, Carlsbad, California, USA). RNA was purified according to the manufacture's manual of TRIzol reagent. Contaminated polysaccharides and DNA were removed by LiCl precipitation and DNA-free DNA removal kit (Invitrogen, Vilnius, Lithuania). LiCl precipitation procedure was critical for RNA qualities when isolated from epidermis and petal tissues. Single strand complementary DNA was synthesized with PrimeScript II Reverse Transcriptase (Takara Bio Inc., Kusatsu, Japan). Polymerase chain reaction was conducted with Takara Ex-Taq (Takara Bio Inc.) in a Thermal cycler (T100, Bio-Rad Laboratories, Hercules, California, USA). Nucleotide sequences of primers were listed in Table S1.

For the RT-qPCR to characterize the *clca3* mutant, mRNA extraction, cDNA synthesis and quantitative PCR were performed as described in Hodin *et al*. (2023).

Nitrate content

For shoot nitrate measurement contents, nitrate was extracted and quantified as described in Hodin *et al*. (2023).

Results

Measurement of viability of petal cells

198 We first developed a method for viability test of petal cells. Damages in petal is
199 critical for successful pollination of insect-pollinated flowers and commercial flower
200 productions. Therefore, we examined the toxicity of SO_2 to petal cells in addition to
201 mesophyll cells, the major sites of photosynthesis and guard cells forming stomata, the
202 entry gates for hazardous gas to the leaf. Whole petal cells were hardly stained either with
203 FDA or PI, when applied directly. We investigated whether peeled petal cell layers could
204 be stained with FDA and PI or not (Figure S3). Petal adaxial layer cells, after isolating by
205 the tape-peeled method (Figure S2, for detail see Materials and Methods), resulted in
206 uneven staining image both in the control and boiled tissues (Figure S3b and c). In
207 contrast, the abaxial layer specimen was evenly stained by both FDA and PI (Figure S3d
208 and e). Therefore, we used petal abaxial cell layer for successive viability test for petal
209 tissue.
210
211 **Different sensitivity among Arabidopsis tissues to H_2SO_3**
212 Viability of MCP, GC and petal cells were determined in Col after a 1h-exposure to
213 H_2SO_3 (Figure 1, Figure S4, and Figure S3f). Mesophyll cells exhibited the lowest
214 sensitivity, leading to fewer PI-stained dead cells being observed. Petal abaxial layer cells
215 demonstrated the highest sensitivity. Similar trend was also observed in WS (Table 1).

One possible explanation is that the sensitivity of cells to H_2SO_3 varies significantly among different cell types. It is also possible to explain this variability that the difference in apparent LC_{50} is attributed to the distinct specimen preparation procedures for each cell types.

Loss-of-function in *CLCa* rendered reduced H_2SO_3 sensitivity in guard cells.

Viability of MCP, GC and petal abaxial layer cells of *clca* mutants were compared with wild types (Figure 2). *clca2* mutant was previously well characterized (De Angeli *et al*. 2006; Wege *et al*. 2014; Hodin *et al*. 2023) which was not the case for *clca3.* We first checked the absence of *CLCa* expression induced a decrease in nitrate content (Figure S1). However, this KO mutation does not decrease plant growth in these growth conditions compared to wild-type as in *clca2* (Hodin *et al*. 2023). There was no apparent difference in viability after a 1h exposure to H_2SO_3 between wild types and corresponding *clca* mutants in MCP and Petal cells. In GC, *clca* mutants showed significantly lower sensitivity to H_2SO_3 (Figure 2b). CLCa is a H^+/NO_3^- antiporter localized in tonoplasts. Its loss-of-function mutants are known to show abnormal pH homeostasis in GC (Demes et al., 2020). This result may be attributed to a smaller pH change in the cytosol of mutants' GC compared to wild types when exposed to H_2SO_3.

234

235 **Differential expression of *CLC* genes among tissues**

236 Expression of *CLC* transcripts was examined by semi-quantitative RT-PCR (Figure
237 3). In whole leaf, all seven *CLC* genes' transcripts were abundantly detected. In isolated
238 mesophyll cell protoplasts, *CLCb*, *CLCe* and *CLCg* transcripts were hardly detected. Only
239 *CLCa* and *CLCc* were detected in GC-enriched epidermis, indicating only limited *CLC*
240 gene species were expressed in GC. In petal, *CLCa*, *CLCc*, *CLCd* and *CLCg* were
241 detected. The difference in *CLC* genes' expression among whole leaf, MCP and GC
242 indicates that *CLCb*, *CLCe* and *CLCg* may play a role except in MCP and GC, such as
243 vascular tissues in Arabidopsis leaf. The expression of *CLCd* and *CLCf* in mesophyll cells
244 and *CLCd* and *CLCg* in petal cells can be postulated to compensate the role of *CLCa*.
245 These differences in *CLC* genes' expression pattern among tissues may explain the result
246 that only GC showed a difference in H_2SO_3 sensitivity between wild types and *clca*
247 mutants in contrast with MCP and petal cells.

248

249 **Effects of antioxidant agents on H_2SO_3 toxicity**

250 To elucidate the involvement of cellular oxidation in H_2SO_3-induced cell death,
251 viability of MCP, GC and petal cells were determined in the absence and presence of

252 reactive oxygen scavengers, tiron and NAC using Arabidopsis plants ecotypes Col
253 (Figure 4). For unknown reason, the addition of 1 mM NAC killed petal abaxial layer
254 cells (Figure S5). We thus examined the effect of tiron, but not that of NAC, in petal
255 abaxial layer cells. LC_{50} of H_2SO_3 in MCP was not affected by the addition of tiron nor
256 NAC. GC viability rate did not change when NAC was supplemented. However, the LC_{50}
257 of H_2SO_3 in GC increased significantly by the addition of 5 mM tiron, suggesting cellular
258 oxidation was a cause of the toxic mechanism. There was no apparent effect of tiron on
259 H_2SO_3 sensitivity in petal abaxial layer cells.
260
261 **Discussion**
262 In earlier studies, the sensitivity of various plant species to SO_2 have been discussed
263 (Kondo and Sugahara 1978, Hu *et al*. 2014). On the other hand, the difference in SO_2
264 sensitivity among cell types in a species has not been examined well. Our results suggest
265 that SO_2 sensitivity was highly divergent among cell types in Arabidopsis (Figure 1). This
266 divergence in sensitivity might reflect the difference in toxic mechanisms of SO_2 from
267 one cell type to another, even within the same species. It may also be possible to infer this
268 difference by the cell capacity for the resistance to SO_2 toxicity, such as ROS scavenging
269 enzyme activities or cytosolic pH buffering capacity among cell types. We need to

consider the possibility of alternative explanations. MCP, GC-enriched epidermis and petal abaxial layer were prepared by distinctive experimental procedures, enzymatic digestion for MCP, blending leaves and collecting residual epidermis to observe guard cells, and the tape-peel method for petal cells. These differences may cause in alterations in the sensitivity due to characteristics of the specimen, such as absence and presence of cell walls and antecedent damages during the preparation.

Comparison of *clca* mutants suggested that cytosolic acidification is one of the toxic mechanisms of SO_2 in GC (Figure 2). CLCa is a proton/nitrate antiporter localized in the tonoplast and contributing pH homeostasis in guard cells (Demes 2020). In animal cell systems, proton-coupled membrane transporters, such as NHE proton/sodium exchangers, primarily play a role in the cytosolic pH homeostasis in addition to the contribution of buffering capacity of intracellular biological molecules (Doyen *et al*. 2022). In plant cell systems, plasma membrane ATPase, vacuolar-type ATPase and vacuolar pyrophosphatase play primary roles in pH homeostasis (Cosse and Seidel 2021). Furthermore, proton-coupled ion transporters in the vacuole cooperate to balance the cytosolic pH as well (Cosse and Seidel 2021). CLCa is postulated to function in pH homeostasis in Arabidopsis GC (Demes *et al*. 2020). We found that GC of *clca* mutants demonstrated lower sensitivity to H_2SO_3 (Figure 2). The defect in the CLCa function potentially disrupt the balance of

intracellular pH in *clca* mutants after a H_2SO_3 exposure. This interpretation supports the cytosolic acidification model for SO_2 toxicity. In MCP and petal cells, a difference in SO_2 sensitivity between wild types and *clca* mutants was not observed (Figure 2). This lack of the difference in SO_2 sensitivity might not necessarily be inferred as the denial of cytosolic acidification mechanism in these cell types. The RT-PCR analysis revealed that *CLCa* and *CLCc* were the dominant *CLCs* expressed in GC-enriched epidermal samples as it was previously demonstrated (Figure 3; Jossier *et al*. 2010). In MCP and petal cells, other *CLC* genes, such as *CLCd*, *CLCf* and *CLCg* were found to be expressed in addition to *CLCa* and *CLCc* (Figure 3). The presence of these CLCs could compensate the lack of CLCa function in MCP and petal cells.

We interpret the contribution of the acidification in SO_2 toxicity based on viability phenotype of *clca* mutants. Drawback of using of *clc* mutants is that CLC has multiple roles, such as nitrate accumulation, osmotic response, and salt stress sensitivity besides pH regulation (Geelen *et al*. 2000, Von der Fecht-Bartenbach *et al*. 2007 and 2010, Bergsdorf 2009, Jossier *et al*. 2010, Nguyen *et al*. 2015). The mutants would exhibit other adverse effect due to other roles of CLCs besides pH homeostasis. Further multifaceted analysis would be necessary to clarify the contribution of cytosolic acidification in SO_2 toxicity. Amongst others, a mutation converting CLCa from an anion/proton exchanger

306 to a channel could be used to test the change in cytosolic pH in response to H_2SO_3 (Hodin
307 *et al*. 2023).

308 An earlier study proposed that cellular oxidation is one of the toxic mechanisms of
309 SO_2 in plant cells (Pnueli *et al*. 2003, Muneer *et al*. 2014). Pnueli *et al*. (2003)
310 demonstrated that the loss-of-function mutation in an ascorbate peroxidase (*APX*) resulted
311 in lower photosynthesis rates, retarded growth compared to the wild type after SO_2
312 exposure. It should be noticed that APX has a role for stress-induced gene regulation
313 (Maruta *et al*. 2010). Therefore, the loss-of-function of an *APX* gene could cause in an
314 ectopic adverse phenotype. Therefore, in this study, we assessed the involvement of
315 oxidative stress in H_2SO_3 sensitivity utilizing exogenous antioxidant application (Figure
316 4). Our findings indicate that cellular oxidation is also a toxic mechanism in addition to
317 acidification. The addition of tiron significantly reduced cell mortality when H_2SO_3 was
318 challenged to GC (Figure 4). Interestingly, NAC did not affect the mortality. Tiron and
319 NAC are antioxidants, which are capable of inhibiting ROS-induced apoptosis (Han and
320 Park 2009, Halasi *et al*. 2013). Tiron has high specificities to both O_2^{-}• and OH• hydroxyl
321 radical among ROS (Greenstock *et al*. 1975). NAC is rather selective to OH• (Aruoma *et*
322 *al*. 1989, Aldini *et al*. 2018). In the earlier study, it was shown that APX is contributed to
323 the removal of ROS induced by SO_2 (Pnueli *et al*. 2003). APX has a selective reactivity

to H_2O_2 (Asada 1999, Shigeoka *et al*. 2002, Foyer and Shigeoka 2011). Collectively, these results show the involvement of O_2^{-}• and H_2O_2 in toxic mechanism of SO_2 to plants could be anticipated (Pnueli *et al*. 2003, Figure 4). The contribution of OH• was not clearly demonstrated in this study inferred from the effect of NAC (Figure 4).

Kondo and Sugahara (1978) demonstrated that SO_2 resistance and abscisic acid (ABA) levels are associated. They interpreted that stomatal closure elicited by ABA caused the avoidance of SO_2 entry to the inner tissue. ABA induces the activity of antioxidant systems in plants in addition to the induction of stomatal closure, such as upregulation of catalase, superoxide dismutase and APX (Williamson and Scandalios 1992, Zhu and Scandalios 1994, Sakamoto *et al*. 1995, Kaminaka *et al*. 1999, Ozfidan et al. 2012). If one of toxic mechanisms of SO_2 is oxidation, higher level of ABA may result in elevated antioxidant system. This can also be an explanation for the association of SO_2 resistance and ABA level in addition to stomatal closure hypothesis.

Our findings provide evidence for the involvement of cytosolic acidification and cellular oxidation in SO_2 toxicity in GC. We observed that MCP were notably less sensitive to SO_2 as compared to GC (320 to 740 fold in Col and WS respectively). This difference could be attributed to MCP's robust antioxidation mechanism that alleviates oxidative stress, unlike GC. Additionally, MCP's enhanced pH stabilization, facilitated

342 by its large central vacuole compared to GC and petal cells, and dose effects of CLC
343 transporters, may further contribute to its lower sensitivity. Petal abaxial layer cells
344 showed the least resistance to H_2SO_3 and no apparent effect of an antioxidant, tiron
345 (Figure 4). It has been shown in petals of daylily that antioxidation enzyme activity
346 sharply decreased as flowers open (Panavas *et al*. 1998). Since ROS has already been
347 highly accumulated in petal cells, the additional effect of H_2SO_3-induced cellular
348 oxidation might be negligible.

349 By understanding the toxic mechanism of SO_2, defensive measure can be established
350 to prevent damage and yield loss of crops. Rapeseed is a major source of vegetable oil.
351 The knowledge obtained using Arabidopsis would be applicable to the rapeseed (*Brassica*
352 *napus*), since these species belong to the same taxonomical family. Our findings allow
353 for the evaluation of the effect of air pollution by SO_2 in the environment establishment
354 of risk management, potentially contributing to the development of environmental
355 protection measures.

356

357 **Data availability**

358 The data underlying this article are available in the article and its online supplementary material.

359

Author contribution

I.C.M and L.O. conceived and designed the research. M.M. and C.E. performed research. M.M. and I.C.M. analyzed the data. S.F. provided biological materials and commented on the manuscript. M.M. and I.C.M. wrote the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

References

377 Aldini G, Altomare A, Baron G *et al*. N-Acetylcysteine as an antioxidant and disulphide
378 breaking agent: the reasons why. *Free Radic Res* 2018;**52**:751–62.
379 Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal
380 transduction. *Annu Rev Plant Biol* 2004:**55**:373–99.
381 Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine:
382 its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous
383 acid. *Free Radic Biol Med* 1989;**6**,593–7.
384 Asada K. The water–water cycle in chloroplasts: scavenging of active oxygen and
385 dissipation of excess photons. *Annu Rev Plant Physiol Plant Mol Biol* 1999;**50**:601–
386 39.
387 Bergsdorf EY, Zdebik AA, Jentsch TJ. Residues important for nitrate/proton coupling in
388 plant and mammalian CLC transporters. *J Biol Chem* 2009;**284**:11184–93.
389 Brychkova G, Xia Z, Yang G *et al*. Sulfite oxidase protects plants against sulfur dioxide
390 toxicity. *Plant J* 2007;**50**:696–709.
391 Clapperton MJ, Reid DM. Effects of sulphur dioxide (SO_2) on growth and flowering of
392 SO_2-tolerant and non-tolerant genotypes of *Phleum pretense*. *Environ Pollut*
393 1994;**86**:251–8.
394 Considine MJ, Foyer CH. Metabolic responses to sulfur dioxide in grapevine (*Vitis*
395 *vinifera* L.): photosynthetic tissues and berries. *Front Plant Sci* 2015;**6**:1–10.
396 Cosse M, Seidel T. Plant proton pumps and cytosolic pH-homeostasis. *Front Plant Sci*
397 2021;**12**:672873.
398 De Angeli A, Mochachello D, Ephritikhine G *et al*. The nitrate/proton antiporter AtCLCa
399 mediates nitrate accumulaltion in plant vacuoles. *Nature* 2006;**442**:939–42.

De Angeli A, Monachello D, Ephritikhine G *et al*. CLC-mediated anion transport in plant cells. *Phil Trans R Soc B* 2009;**364**:195–201.

Demes E, Besse L, Cubero-Font P *et al*. Dynamic measurement of cytosolic pH and NO_3^- uncovers the role of the vacuolar transporter AtCLCa in cytosolic pH homeostasis. 2020 *Proc Natl Acad Sci USA* 2020;**117**:15343–53.

Doyen D, Poët M, Jarretou G *et al*. Intracellular pH control by membrane transport in mammalian cells. Insights into the selective advantages of functional redundancy. *Front Mol Biosci* 2022;**9**:825028.

Foyer CH, Halliwell B. Purification and properties of dehydroascorbate reductase from spinach leaves. *Phytochem* 1977;**16**:1347–50.

Foyer CH, Shigeoka S. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiol* 2011;**155**:93–100.

Geelen D, Lurin C, Bouchez D *et al*. Disruption of putative anion channel gene *AtCLC-a* in *Arabidopsis* suggests a role in the regulation of nitrate content. *Plant J* 2000;**21**:259–67.

Greenstock CL, Miller RW. The oxidation of tiron by superoxide anion kinetics of the reaction in aqueous solution and in chloroplasts. *Biochim Biophys Acta* 1975;**396**,11–6.

Groden D, Beck E. H_2O_2 destruction by ascorbate-dependent systems from chloroplasts. *Biochim Biophys Acta Bioenerg* 1979;**546**:426–35.

Halasi M, Wang M, Chavan TS *et al*. ROS inhibitor *N*-acetyl-L-cysteine antagonizes the activity of proteasome inhibitors. *Biochem J* 2013;**454**:201–8.

Hamisch D, Randewig D, Schliesky S *et al*. Impact of SO_2 on *Arabidopsis thaliana* transcriptome in wildtype and sulfite oxidase knockout plants analyzed by RNA deep

424 sequencing. *New Phytol* 2012;**196**:1074–85.
425 Han YH, Park WH. Tiron, a ROS scavenger, protects human lung cancer Calu-6 cells
426 against antimycin A-induced cell death. *Oncol Rep* 2009;**21**:253–61.
427 Harada H., Kuromori T., Hirayama T *et al*. Quantitative trait loci analysis of nitrate
428 storage in *Arabidopsis* leading to an investigation of the contribution of the anion
429 channel gene, *AtCLC-c*, to variation in nitrate levels. *J Exp Bot* 2004;**55**:2005–14.
430 Hechenberger M, Schwappach B, Fischer WN *et al*. A family of putative chloride
431 channels from Arabidopsis and functional complementation of a yeast strain with a
432 CLC gene disruption. *J Biol Chem* 1996;**271**:33632–8.
433 Herdean A, Nziengui H, Zsiros O. The Arabidopsis thylakoid chloride channel AtCLCe
434 functions in chloride homeostasis and regulation of photosynthetic electron transport.
435 *Front Plant Sci* 2016;**7**:115.
436 Hodin J, Lind C, Marmagne A *et al*. Proton exchange by the vacuolar nitrate transporter
437 CLCa is required for plant growth and nitrogen use efficiency. *Plant Cell*
438 2023;**35**:318–35.
439 Hu KD, Tang J, Zhao DL *et al*. Stomatal closure in sweet potato leaves induced by sulfur
440 dioxide involves H_2S and NO signaling pathways. *Biol Plant* 2014;**58**:676-80.
441 Jossier M, Kroniewicz L, Dalmas F *et al*. The Arabidopsis vacuolar anion transporter,
442 AtCLCc, is involved in the regulation of stomatal movements and contributes to salt
443 tolerance. *Plant J* 2010;**64**:563–76.
444 Kaminaka H, Morita S, Tokumoto M *et al*. Differential gene expression of rice superoxide
445 dismutase isoforms to oxidative and environmental stresses. *Free Radic Res*
446 1999;**31**:S219–25.

447 Kondo N, Sugahara K. Changes in transpiration rate of SO_2-resistant and -sensitive plants
448 with SO_2 fumigation and the participation of abscisic acid. *Plant Cell Physiol*
449 1978;**19**:365–73.
450 Kropff MJ. Physiological effects of sulphur dioxide. 1. The effect of SO_2 on
451 photosynthesis and stomatal regulation of *Vicia faba* L. *Plant Cell Environ*
452 1987;**10**:753–60.
453 Lee HK, Khaine I, Kwak MJ *et al*. The relationship between SO_2 exposure and plant
454 physiology: A Mini Review. *Hortic Environ Biotechnol* 2017;**58**:523–9.
455 Li L, Yi H. Effect of sulfur dioxide on ROS production, gene expression and antioxidant
456 enzyme activity in Arabidopsis plants. *Plant Physiol Biochem* 2012;**58**:46–53.
457 Lv Q, Tang R, Liu H *et al*. Cloning and molecular analyses of the *Arabidopsis thaliana*
458 chloride channel gene family. *Plant Sci* 2009;**176**:650–61.
459 Marmagne A, Vinauger-Douard M, Monachello D *et al*. Two members of the Arabidopsis
460 CLC (chloride channel) family, AtCLCe and AtCLCf, are associated with thylakoid
461 and Golgi membranes, respectively. *J Exp Bot* 2007;**58**:3385–93.
462 Maruta T, Tanouchi A, Tamoi M *et al*. Arabidopsis chloroplastic ascorbate peroxidase
463 isoenzymes play a dual role in photoprotection and gene regulation under
464 photooxidative stress. *Plant Cell Physiol* 2010;**51**:190–200.
465 Mittler R. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci*
466 2002;**7**:405–10.
467 Nagelkerker NJD. A note on a general definition of the coefficient of determination.
468 *Biometrika* 1991;**78**:691–2
469 Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in
470 spinach chloroplasts. *Plant Cell Physiol* 1981;**22**:867–80.

Nguyen CT, Agorio A, Jossier M *et al*. Characterization of the chloride channel-like, AtCLCg, involved in chloride tolerance in *Arabidopsis thaliana*. *Plant Cell Physiol* 2015;**57**:764–75.

OECD. The economic consequences of outdoor air pollution https://www.oecd.org/environment/indicators-modelling-outlooks/Policy-Highlights-Economic-consequences-of-outdoor-air-pollution-web.pdf .

Olszyk DM, Tibbitts TW. Stomatal response and leaf injury of *Pisum sativum* L. with SO_2 and O_3 Exposures: I. Influence of pollutant level and leaf maturity. *Plant Physiol* 1981;**67**:539–44.

Ooi L, Matsuura T, Munemasa S *et al*. The mechanism of SO_2-induced stomatal closure differs from O_3 and CO_2 responses and is mediated by nonapoptotic cell death in guard cells. *Plant Cell Environ* 2019;**42**:437–47.

Örvar BL, McPherson J, Ellis BE. Pre-activating wounding response in tobacco prior to high-level ozone exposure prevents necrotic injury. *Plant J* 1997;**11**:203–12.

Ozfidan C, Turkan I, Sekmen AH *et al*. Abscisic acid-regulated responses of *aba2-1* under osmotic stress: the abscisic acid-inducible antioxidant defense system and reactive oxygen species production. *Plant Biol* 2012;**14**:337–46.

Panavas T, Walker EL, Rubinstein B. Possible involvement of abscisic acid in senescence of daylily petals. *J Exp Bot* 1998;**49**:1987–97.

Pnueli L, Liang H, Rozenberg M *et al*. Growth suppression, altered stomatal responses, and augmented induction of heat shock proteins in cytosolic ascorbate peroxidase *Apx1*-deficient Arabidopsis plants. *Plant J* 2003;**34**:187–203.

R Core Team. R: A language and environment for statistical computing. R foundati on for statistical computing, Vienna, Austria. 2022;https://www.R-project.org/.

Randewig D, Hamisch D, Herschbach C *et al*. Sulfite oxidase controls sulfur metabolism under SO_2 exposure in *Arabidopsis thaliana*. *Plant Cell Environ* 2012;**35**:100–15.

Roberts S, Koehler FE. Sulfur dioxide as a source of sulfur for wheat. *Soil Sci Soc Am Proc* 1965;**29**:696–8.

Roychowdhury R, Khan MH, Choudhury S. Physiological and molecular responses for metalloid stress in rice, a comprehensive overview. In: Hasanuzzaman M, Nahar K, Fujita M, Biwas JK (ed). Advances in rice research for abiotic stress tolerance. Sawston: Woodhead Publishing. 2019;341–69.

Rubinstein B. Regulation of cell death in flower petals. *Plant Mol Biol* 2000;**44**:303–18.

Sakamoto A, Okumura T, Kaminata H *et al*. Structure and differential response to abscisic acid of two promoters for the cytosolic copper/zinc-superoxide dismutase genes, *SodCc1* and *SodCc2*, in rice protoplasts. *FEBS Lett* 1995;**358**:62–6.

Shi Y, Liu D, He Y *et al*. CHLORIDE CHANNEL-b mediates vacuolar nitrate efflux to improve low nitrogen adaptation in Arabidopsis. *Plant Physiol* 2023;**193**:1987–2002.

Shigeoka S, Ishikawa T, Tamoi M *et al*. Regulation and function of ascorbate peroxidase isoenzymes. *J Exp Bot* 2002;**53**:1305–19.

Scholl S, Hillmer S, Krebs M *et al*. ClCd and ClCf act redundantly at the trans-Golgi network/early endosome and prevent acidification of the Golgi stack. *J Cell Sci* 2021;**134**:jcs258807.

Smith SJ, van Aardenne J, Klimont Z *et al*. Anthropogenic sulfur dioxide emissions. *Atmos Chem Phys* 2011;**11**:1850–2005.

Taylor Jr GE. Plant and leaf resistance to gaseous air pollution stress. *New Phytol* 1978;**80**:523–34.

518 Taylor Jr GE, Tingey DT. Physiology of ecotypic plant response to sulfur dioxide in
519 *Geranium carolinianum* L. *Oecologia*. 1981;**49**:76–82.
520 Temple J, Hong FC, Taylor OC. Effects of SO_2 on stomatal conductance and growth of
521 *Phaseolus vulgaris*. *Environmental Pollution.* 1985;**37**:267–79.
522 Von der Fecht-Bartenbach J, Bogner M, Krebs M *et al*. Function of the anion transporter
523 AtCLC-d in the trans-Golgi network. *Plant J* 2007;**50**:466–74.
524 Von der Fecht-Bartenbach J, Bogner M *et al*. CLC-b-mediated NO_3^-/H^+ exchange across
525 the tonoplast of Arabidopsis vacuoles. *Plant Cell Physiol* 2010;**51**:960–8.
526 Wege S, De Angeli A, Droillard MJ *et al*. Phosphorylation of the vacuolar anion
527 exchanger AtCLCa is required for the stomatal response to abscisic acid. *Sci Signal*
528 2014;**7**:ra65.
529 Wei J, Guo X, Marinova D *et al*. Industrial SO_2 pollution and agricultural losses in China:
530 evidence from heavy air polluters. *J Clean Prod* 2014;**64**:404–13.
531 Williamson JD, Scandalios JG. Differential response of maize catalase to abscisic acid:
532 Vp1 transcriptional activator is not required for abscisic acid-regulated *Cat1*
533 expression. *Proc Natl Acad Sci USA* 1992;**89**:8842–6.
534 Zhu D, Scandalios JG. Differential accumulation of manganese-superoxide dismutase
535 transcripts in maize in response to abscisic acid and high osmoticum. *Plant Physiol*
536 1994;**106**:173–8.
537 Zifarelli G, Pusch M. CLC transport proteins in plants. *FEBS Lett* 2010;**584**:2122–7.
538

539 **Figure legends.**

540 **Figure 1**. Difference in H_2SO_3 sensitivity among different cell types in Arabidopsis. (a)
541 Isolated mesophyll cell protoplasts, (b) guard cell, (c) petal abaxial layer cell. Tissues
542 were prepared from 4- to 6-week-old Col plants. Each datum was from a measurement of
543 50 to 200 cells. Biological replicates were 4 to 6.

544

545 **Figure 2.** Comparison of median lethal concentration (LC_{50}) for wild types and *clca*
546 mutants exposed to H_2SO_3 in (a) isolated mesophyll cell protoplasts (MCP), (b) guard
547 cells (GC) and (c) petal abaxial layer cells (Pe) . Col, Columbia-0. WS, Wassilewskija.
548 Each datum was from a measurement of 50 to 200 cells. Biological replicates were 4 to
549 7. Asterisks indicate significance at $P < 0.05$ by Wilcoxon signed-rank sum test between
550 a wild type and the corresponding *clca* mutant. ns indicates not significant.

551

552 **Figure 3.** Semi-quantitative RT-PCR analysis of *CLC* gene expression. Total RNA was
553 extracted from whole rosette leaf (WL), isolated mesophyll cell protoplasts (MCPs),
554 guard cell-enriched epidermis (GC), and petal (Pe) of 5-week-old Col-0 plants. The
555 numbers correspond to the length of expected DNA fragment sizes in base pairs (bp).
556 Cycle number of PCR was 35. Ten ng of total RNA was subjected to reverse-transcription

reaction for each sample. Essentially the same trend was obtained with a slightly reduced band intensity by RT-PCR with 34 cycles. AGI gene identifiers are At5G40890 (*CLCa*), At3g27170 (*CLCb*), At5g49890 (*CLCc*), At5g26240 (*CLCd*), At4g35440 (*CLCe*), At1g55620 (*CLCf*), At5g33280 (*CLCg*), and At5g09810 (*ACTIN*).

Figure 4. Effects of antioxidants on H_2SO_3 toxicity. (a) Isolated mesophyll cell protoplasts (MCP). (b) Guard cells (GC). (c) Petal abaxial layer cells (Pe). Five mM tiron and one mM *N*-acetylcysteine (NAC) were added at the same time with H_2SO_3 exposure. Each datum was from a measurement of 50 to 200 cells. Biological replicates were 5 to 6. Different letters indicate significant difference (Tukey test, $P < 0.05$).

Graphical abstract caption

There are two toxic mechanisms of SO_2 to plant cells: cytosolic acidification and cellular oxidation.