Research Article

Dual Role of Hydrogen Peroxide in *Arabidopsis* Guard Cells in Response to Sulfur Dioxide

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Sulfur dioxide (SO_2) is a major air pollutant and has significant impacts on plant physiology. Plant can adapt to SO_2 stress by controlling stomatal movement, gene expression, and metabolic changes. Here we show clear evidences that SO_2 -triggered hydrogen peroxide (H_2O_2) production mediated stomatal closure and cell death in *Arabidopsis* leaves. High levels of SO_2 caused irreversible stomatal closure and decline in guard cell viability, but low levels of SO_2 caused reversible stomatal closure. Exogenous antioxidants ascorbic acid (AsA) and catalase (CAT) or Ca^{2+} antagonists EGTA and LaCl_3 blocked SO_2 -induced stomatal closure and decline in viability. AsA and CAT also blocked SO_2 -induced H_2O_2 and $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation. However, EGTA and LaCl_3 inhibited SO_2 -induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase but did not suppress SO_2 -induced H_2O_2 elevation. These results indicate that H_2O_2 elevation triggered stomatal closure and cell death via $[\text{Ca}^{2+}]_{\text{cyt}}$ signaling in SO_2 -stimulated *Arabidopsis* guard cells. NADPH oxidase inhibitor DPI blocked SO_2 -induced cell death but not the stomatal closure triggered by low levels of SO_2 , indicating that NADPH oxidase-dependent H_2O_2 production plays critical role in SO_2 toxicity but is not necessary for SO_2 -induced stomatal closure. Our results suggest that H_2O_2 production and accumulation in SO_2 -stimulated plants trigger plant adaptation and toxicity via reactive oxygen species mediating Ca^{2+} signaling.

1. Introduction

Sulfur dioxide (SO_2) is a harmful gas that is emitted largely from burning coal, high-sulfur oil, and fuels. During the past few decades, the concentration of SO_2 in the atmosphere has increased in many areas of the world, especially in the developing countries. High levels of SO_2 can injure many plant species and varieties, resulting in photosynthesis decline, growth inhibition, and even death [1–4]. Sulfur dioxide enters plants mainly through the open stomata [5]. Once it enters the leaf, SO_2 is hydrated to form HSO_3^- and SO_3^{2-} . The toxicity of SO_2 is derived from molecular species sulfite (SO_3^{2-}) and bisulfite (HSO_3^-) generated after SO_2 is dissolved in cellular fluid [6]. Sulfite oxidation, which is the detoxification reaction of sulfite to sulfate (SO_4^{2-}), leads to the formation of reactive oxygen species (ROS) in plant cells [7, 8]. The production and accumulation of ROS are one of the key events in plant response to SO_2 [9–12].

ROS have been proposed as central components of plant response to both biotic and abiotic stresses. Under such conditions, ROS may play two very different roles: exacerbating damage or signaling the activation of defense responses [13, 14]. It has been reported that ROS act as signaling molecules mediating a variety of physiological responses, including stomatal movement and gene expression [15–17], although they can attack biomolecules such as nucleic acids, proteins, and lipids leading to cell damage and death [16, 18, 19]. Results of previous studies have shown that plants can adapt to SO_2 stress by controlling stomatal movement and gene transcription [11, 12, 20]. Stomatal closure could protect the leaves against further entry of the environmental SO_2 , while differential gene expression could regulate the metabolic routes of plant cells providing long-term adaptation to environmental stress. However, up to now, it is not clear if the ROS production is closely associated with the initial physiological mechanisms responsible for plant responses to SO_2 stress.

ROS overproduction can trigger plant adaptation or cell damage during abiotic stress. However, there has been little overlap between these two fields of research. In the present study, guard cells of *A. thaliana* leaves, which are a well-developed model cell system for studying the signal transduction in plant cells [21–23], were employed to investigate the cellular mechanism of plant response to SO₂ stress. To the best of our knowledge, this is the first report of H₂O₂ mediating both adaptation response (stomatal movement) and cytotoxicity (cell death) in plant response to SO₂ stress. Our results show that H₂O₂ elevation triggered both stomatal closure and cell viability loss via Ca²⁺ signaling in SO₂-treated plants.

2. Materials and Methods

2.1. Plant Material Preparation. Plants of *Arabidopsis thaliana* (L.) ecotype Columbia (Col-0) were grown in soil (Klasmann-Deilmann) in temperature-controlled growth rooms at 22°C with an average light intensity of 240 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a 16 h photoperiod per day, and 60% relative humidity.

Young fully expanded leaves were harvested from 4-week-old *Arabidopsis* plants. The abaxial epidermes were peeled from the underside of each leaf and cut into small pieces. The isolated epidermal strips were immediately floated in 10 mM 2-(N-morpholino)ethanesulfonic acid (MES; Bio Basic Inc.) buffer (10 mM MES-Tris, pH 7.0, and 50 mM KCl).

2.2. Determination of Stomatal Aperture. The isolated epidermal strips were incubated in 10 mM MES buffer for 2 h, for stomata-opening under continuous light of 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 22°C or for stomata-closing in the dark, and then incubated for 2 h in MES buffer containing a certain amount of SO₂ hydrates (a mixture of sodium sulfite and sodium bisulfite, 3:1 mM/mM, prepared freshly before use) under continuous illumination of 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 22°C. Control samples were treated under the same conditions with 10 mM MES buffer. The isolated epidermal strips were treated with a mixture of SO₂ hydrates and a certain amount of antagonists which include NADPH oxidase inhibitor diphenylene iodonium (DPI, Sigma), antioxidants catalase (CAT, Sigma), and ascorbic acid (AsA, Sigma) and Ca²⁺ antagonists LaCl₃ and ethylene glycol tetraacetic acid (EGTA, Sigma) to examine the protective effects. After 2 h of chemical exposure, the epidermal strips were mounted on a microscopy slide, moistened with 10 mM MES buffer, and covered with a slip. Stomatal aperture was measured by using a digital microscope camera system (DP72, Olympus) and an attached DP2-BSW software. At least three leaves and 300 stomata per leaf were measured in each treatment and all of the experiments were independently repeated at least three times. For the recovery groups, after 2 h of chemical exposure, the isolated strips were resuspended in MES buffer for 2 h under continuous light followed by stomatal aperture measurement. For time-course experiment, the stomatal apertures were examined every five minutes after isolated strips were incubated in SO₂ hydrates.

2.3. Determination of Cell Viability. Cell viability was assessed by using the method of double staining with fluorescein diacetate (FDA; Bio Basic Inc.) and propidium iodide (PI; Sigma). After 2 h of chemical exposure, the epidermal strips were simultaneously stained with 0.1 mg·L⁻¹ FDA and 10 μM PI for visualizing cell viability. At least three leaves and 300 guard cells per leaf were observed in each treatment and all of the experiments were independently repeated three times.

2.4. Measurement of Reactive Oxygen Species. Reactive oxygen species in guard cells of epidermal peels were detected using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime) according to the methods described by Yi et al. [24]. After 2 h of exposure to the chemicals, the epidermal strips were incubated in 20 μM DCFH-DA for 30 min in the dark. The mean value of fluorescence intensity, resulting from 600 guard cells in three independent experiments, represents the intracellular H₂O₂ level for each treatment.

2.5. Measurement of Intracellular Ca²⁺. The concentration of intracellular Ca²⁺ ([Ca²⁺]_{cyt}) was measured using fluo-3 acetomethoxyester (Fluo-3 AM; Beyotime) as described in our previous report [24]. The average fluorescence intensity of Fluo-3 AM obtained from three different leaves and 200 guard cells represents the [Ca²⁺]_{cyt} level for each treatment. All experiments were independently repeated three times.

2.6. Statistical Analysis. All values of mean and standard deviation (SD) were obtained from three independent experiments. Analysis of variance (ANOVA) and Dunnett's *t*-test were used to determine the significant differences among the control and a series of treatment groups.

3. Results

3.1. SO₂-Evoked H₂O₂ Production Is Involved in the Regulation of Stomatal Movement. As shown in Figure 1, SO₂ hydrates promote stomatal closure and inhibit light-promoted stomatal opening in *Arabidopsis* leaves. The width of stomatal aperture decreased in a concentration-dependent manner and showed a significant decrease after the isolated strips were exposed for 2 h to SO₂ hydrates at concentrations of 10 to 500 μM . However, stomatal closure evoked by low SO₂ concentrations (below 200 μM) could be reversed completely after SO₂ removal; otherwise the decline of stomatal aperture in high SO₂ concentration (500 μM) group could be partly reversed.

Time course analysis of stomatal movement showed that the diameter of the stomatal aperture decreased markedly within the first 30 minutes of exposure to 10 μM SO₂ hydrates and continued to decline at a slower rate in the remaining 90 minutes of SO₂ exposure (Figure 1(c)).

SO₂-induced stomatal closure was associated with an elevated H₂O₂ level in *Arabidopsis* guard cells. Exposure to SO₂ hydrates not only caused smaller stomatal apertures

FIGURE 1: Promotion of stomatal closure by SO₂ and its reversibility upon SO₂ removal. Stomata of *Arabidopsis* leaf epidermis were allowed to open in light [(a) and (c)] or to close in the dark (b) for 2 h and then were incubated for 2 h in SO₂ hydrates. b, A significant difference at $P < 0.05$ from control. c, A significant difference at $P < 0.05$ between SO₂ treatment and its recovery group.

(Figure 1) but also evoked increased H₂O₂ level (1.2- to 1.7-fold) in *Arabidopsis* guard cells (Figure 2). When H₂O₂ elevation was blocked by exogenous H₂O₂ scavengers CAT or AsA, SO₂-induced stomatal closure was efficiently reversed (Figure 2). These results indicate that H₂O₂ production in SO₂-stimulated guard cells mediates stomatal movement upon SO₂ stress.

3.2. SO₂-Evoked H₂O₂ Elevation Triggered Cell Death. To investigate the role of H₂O₂ in irreversible stomatal closure in response to SO₂, we detect the viability of *Arabidopsis* guard cells by double staining with fluorescein diacetate (FDA) and propidium iodide (PI). The results showed that SO₂ induced guard cell death in a concentration-dependent manner (Figure 3). Cell death reached 24% in 6 mM SO₂

hydrates treatment group, but no obvious cell death could be observed in 10 to 200 μM SO₂ hydrates treatment groups. The H₂O₂ level of guard cells exposed to 2 to 6 mM SO₂ hydrates showed a statistically significant increase (1.90- to 2.30-fold), which is higher than those exposed to 10 to 500 μM SO₂ hydrates. Moreover, exposure to SO₂ hydrates simultaneously with 200 U·mL⁻¹ CAT or 0.1 mM AsA, SO₂-induced cell death was efficiently blocked, associated with a significant decrease in H₂O₂ level of guard cells (Figure 3). These results clearly demonstrate that an elevated H₂O₂ level can trigger guard cell death leading to stomatal dysfunction and irreversible stomatal closure in SO₂-treated *Arabidopsis* leaves.

To further confirm the role of H₂O₂ in SO₂ toxicity, we investigate the protective effects of CAT on cell viability

FIGURE 2: Effects of antioxidants and calcium antagonists on SO₂-induced stomatal closure and H₂O₂ elevation in *Arabidopsis* guard cells. Different superscript letters indicate significant differences ($P < 0.01$). The same letters indicate no significant difference. The green fluorescence (DCFH-DA) of guard cells indicates H₂O₂ content.

in SO₂-treated samples. The results showed that application of 1000 U·mL⁻¹ CAT could completely block the cell death evoked by 2 mM and lower concentrations of SO₂ hydrates, but the cytotoxicity evoked by 6 mM SO₂ hydrates was only partly blocked (Figure 4). These results clearly demonstrate that H₂O₂ production, which may work together with other molecules, is enough to trigger SO₂ toxicity.

3.3. H₂O₂ Action Is Dependent on Its Concentrations and Spatial Generation Patterns. As shown above, SO₂ can cause stomatal closing and decline in cell viability, which is dependent on SO₂ concentrations and H₂O₂ level. However, there was no clear dividing line between a safe level and a toxic level. In order to understand what constitutes a safe level, an inhibitor of NADPH oxidase was used to detect its inhibitory effects. The results show that application of 20 μM NADPH oxidase inhibitor DPI for 2 h markedly blocked cell

death evoked by 2 mM SO₂ hydrates. But 20 μM DPI did not inhibit stomatal closing evoked by 10 μM SO₂ hydrates (Figure 4). These findings indicate that SO₂-triggered stomatal closing can be driven by NADPH oxidase-dependent and -independent H₂O₂ generation, but NADPH oxidase-dependent H₂O₂ generation is involved in SO₂-caused cytotoxicity.

3.4. H₂O₂ Acts Upstream of Ca²⁺ Signaling in Response to SO₂ Stress. SO₂ exposure enhanced the fluorescence intensity of Fluo-3 AM (Ca²⁺ indicator) in *Arabidopsis* guard cells. The relative fluorescence intensity of Fluo-3 AM, resulting from more than 600 guard cells per treatment group in three independent experiments, increased obviously in guard cells exposed to 10 μM to 6 mM SO₂ hydrates for 2 h but decreased markedly when isolated strips were treated simultaneously with SO₂ hydrates and 0.1 mM calcium channel blocker LaCl₃

FIGURE 3: Sulfur dioxide caused viability decline associated with H₂O₂ elevation in *Arabidopsis* guard cells. Different superscript letters indicate significant differences ($P < 0.01$). The same letters indicate no significant difference.

(Figure 5). These results indicate that SO₂ exposure evokes an elevation of $[Ca^{2+}]_{cyt}$ level, and Ca²⁺ influx through Ca²⁺ channels in the plasma membrane results in $[Ca^{2+}]_{cyt}$ elevation.

As shown in Figures 2 and 5, the addition of LaCl₃ and calcium chelator EGTA to SO₂ hydrates blocked $[Ca^{2+}]_{cyt}$ elevation, stomatal closure, and cell death evoked by SO₂. Stomatal closure and cell death occurred with increased $[Ca^{2+}]_{cyt}$ in SO₂-treated samples, but both of them were significantly suppressed by 0.1 mM LaCl₃ or EGTA. These results indicate that elevated $[Ca^{2+}]_{cyt}$ level is an important stimulus driving stomatal movement and cytotoxicity.

To understand the pathways to plant responses, we study the signaling pathways in *Arabidopsis* guard cells. As shown in Figures 2, 3, and 5, both antioxidants AsA and CAT and Ca²⁺ antagonists LaCl₃ and EGTA can suppress SO₂-induced

stomatal closure and cell death, but application of H₂O₂ scavenger CAT (200 U·mL⁻¹) decreased $[Ca^{2+}]_{cyt}$ elevation evoked by SO₂, whereas application of Ca²⁺ channel inhibitor LaCl₃ (0.1 mM) did not affect H₂O₂ elevation evoked by SO₂. These results confirm the involvement of Ca²⁺ downstream of H₂O₂ production, indicating that H₂O₂ triggers stomatal movement and cell death via Ca²⁺ signaling in plant response to SO₂ stress.

4. Discussion

Plant could adapt to environmental challenges through various means. Our recent findings showed that SO₂ fumigation caused an increased ROS production accompanied with differential gene expression and stomatal closure in *Arabidopsis* plants [12, 20]. The results of the present study

FIGURE 4: Hydrogen peroxide scavenger and NADPH oxidase inhibitor DPI blocked SO₂-induced cell death, but DPI cannot block SO₂-induced stomatal closure. Different superscript letters indicate significant difference at $P < 0.01$. The same letters indicate no significant difference.

show the evidences that H₂O₂ acts as an important signaling molecule triggering stomatal movement and cell death in plant response to SO₂. SO₂-caused cell viability decline could interfere with the normal function of stomata to further affect plant physiology under environmental stress. However, the occurrence of SO₂-caused guard cell death was associated with decreased stomatal aperture, suggesting the existence of a complex signaling network in plant responses to environmental stress.

Environmental challenges including biotic and abiotic stresses could induce ROS production in plant cells [25]. It has been documented that ROS play an important role in signal transduction of stomatal movement regulation and gene expression activation in plant response to environmental stresses [26–29]. ROS, which can be used as rapid long-distance autopropagating signals that are transferred throughout the plant in response to different environmental conditions, are widely considered to be an important player in guard cell signaling [26, 30]. The data presented above indicate a requirement for H₂O₂ production in plant response to SO₂ stress. First, SO₂ induces stomatal closure and cell death associated with an increased intracellular H₂O₂ level (Figures 2 and 3). Second, two types of H₂O₂ scavengers CAT and AsA could block SO₂-evoked stomatal closure and cell death; in particular, the effects of SO₂ at low concentrations could be completely reversed by CAT (Figures 2 and 4). Third, H₂O₂ scavenger blocks the SO₂-induced increase in intracellular Ca²⁺ required for stomatal closure and toxicity (Figure 5). Our study, validating the strong positive correlation between intracellular H₂O₂ level and stomatal closure/cell death, demonstrates a key role of H₂O₂ as trigger of stomatal closure and/or guard cell death in response to SO₂.

Sulfur dioxide inhibited light-promoted stomatal opening and promoted stomatal closure leading to the smaller size of *Arabidopsis* stomata. High concentrations of SO₂ also caused viability loss of *Arabidopsis* guard cells. The time course experiments show that H₂O₂ level of guard cells increased gradually during SO₂ exposure (date not shown); therefore, an increased H₂O₂ level in SO₂-stimulated *Arabidopsis* cells might trigger stomatal closure firstly and then cell viability loss through ROS-mediated cell death pathway as shown in *V. faba* cells [24] or through the gradual accumulation of free radical damage to biomolecules during SO₂ exposure.

There are two primary sources of H₂O₂ in guard cells: chloroplasts and plasma membrane-associated NADPH oxidase [22, 31, 32]. The results of the time course experiments with single cell assays using the fluorescent probe DCFH-DA showed that H₂O₂ generation was dependent on SO₂ concentration and that the increase in fluorescence intensity of chloroplasts occurred significantly earlier than within the other regions of guard cells (date not shown), demonstrating an enhanced H₂O₂ production in chloroplasts of guard cells. NADPH oxidase in plasma membrane contributes to O₂^{·-} generation and ROS elevation in plant responses to abiotic stresses [33]. Application of DPI, which is widely accepted as a relatively specific direct inhibitor of NADPH oxidase, markedly blocked SO₂-induced cell death but cannot suppress stomatal closure evoked by low concentrations of SO₂. These findings indicate that H₂O₂-mediated stomatal closure in *Arabidopsis* leaves exposed to low concentrations of SO₂ is not dependent on the activity of plasma membrane NADPH oxidase, but cell death evoked by high concentrations of SO₂ is NADPH oxidase-dependent.

FIGURE 5: Effects of antioxidants and Ca²⁺ antagonists on SO₂-induced Ca²⁺ elevation and cell death in *Arabidopsis* guard cells. Different superscript letters indicate significant differences ($P < 0.01$). The same letters indicate no significant difference.

SO₂ exposure caused stomatal closure and guard cell death associated with [Ca²⁺]_{cyt} elevation, whereas application of either Ca²⁺ chelator EGTA or Ca²⁺ channel inhibitor LaCl₃ blocking SO₂-evoked [Ca²⁺]_{cyt} elevation, stomatal closure and cell death evoked by SO₂ were effectively blocked. These results demonstrate that a channel-mediated Ca²⁺ influx across the plasma membrane contributes to the elevation of [Ca²⁺]_{cyt} and subsequent stomatal closure and cell death in SO₂-stimulated guard cells. However, it is not clear how Ca²⁺ signaling recognizes the different situations of cells to mediate appropriate processes such as stomatal closure and cell death.

It has been found that H₂O₂ could activate plasma membrane Ca²⁺ channels leading to [Ca²⁺]_{cyt} increase in plant cells [34–36]. Therefore, H₂O₂ activation of plasma

membrane Ca²⁺ channels may be a central step in SO₂-induced stomatal closure and/or cell death. The results of our present study also showed that application of antioxidant CAT and AsA significantly decreased SO₂-evoked [Ca²⁺]_{cyt} elevation, but application of Ca²⁺ channel blocker LaCl₃ did not affect SO₂-evoked H₂O₂ increase, indicating that H₂O₂ acts upstream of Ca²⁺ signaling in SO₂-induced stomatal closure and/or cell death. These observations were consistent with other previous reports that a [Ca²⁺]_{cyt} increase was linked to H₂O₂ production and was involved in ROS-mediated stomatal closure/cell death [37–39]. Therefore, H₂O₂ elevation and subsequent activation of Ca²⁺ channels are events occurring in SO₂-induced stomatal closure/cell death. Ca²⁺ influx from extracellular

region results in $[Ca^{2+}]_{\text{cyt}}$ increase, and then $[Ca^{2+}]_{\text{cyt}}$ elevation mediates subsequent stomatal closure/cell death. These results suggested that H_2O_2 mediates SO_2 -induced stomatal movement/cytotoxicity by targeting Ca^{2+} channels in the plasma membrane.

Briefly, environmental SO_2 has a remarkable effect on the size of the stomatal aperture. Exposure to SO_2 induced the overproduction of H_2O_2 in guard cells, as shown in other plant cells exposed to environmental challenges [40, 41]. Elevated H_2O_2 acts in conjunction with other factors to activate stomatal movement and cell death under SO_2 stress. Guard cells are a well-developed model system for characterizing early signal transduction mechanisms in plants. In this study, the dual role of H_2O_2 in plant cells in response to air pollutant was clearly displayed in *Arabidopsis* guard cells, which additionally broaden the role of stomatal guard cells in cytotoxicity study. Our results suggest that guard cells are a valuable model system for the study of cytotoxicity in plant cells.

5. Conclusion

Sulfur dioxide exposure caused an elevated H_2O_2 level in *Arabidopsis* guard cells. H_2O_2 elevation triggered by SO_2 mediated both stomatal closure and cell death via Ca^{2+} signaling. Intracellular Ca^{2+} increase is necessary for stomatal closure and cell death in *Arabidopsis* guard cells in response to SO_2 . H_2O_2 production by NADPH oxidase plays a critical role in SO_2 toxicity; however NADPH oxidase activation is sufficient but not necessary for SO_2 -triggered stomatal closure. Both stomatal closing and stomatal opening inhibition evoked by SO_2 lead to a decline in stomatal aperture, protecting the leaf against further entry of the pollutant but also curtailing photosynthesis. Cell death evoked by high concentrations of SO_2 indicated the cytotoxicity of SO_2 , but might provide appropriate protection by reducing ROS production in *Arabidopsis* plants.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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