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Sulfur dioxide acts as an antioxidant and delays programmed cell death in wheat aleurone layers upstream of H_2S and NO signaling pathways

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Abstract

Sulfur dioxide is a widespread air pollutant and it also acts as a signaling molecule in various processes in mammals. However, the role of SO_2 in programmed cell death (PCD) in plants is unclear. Here we studied the role of SO_2 in gibberellin (GA)-treated wheat aleurone layers. The results showed that 100 μM SO_2 donor ($NaHSO_3$/Na_2SO_3) could effectively delay PCD and inhibit the coalescence of small protein storage vacuoles (PSVs) in aleurone cells treated with GA. Also, SO_2 could reduce the accumulation of hydrogen peroxide and superoxide anion in GA-treated aleurone layers. In this process, SO_2 could sustain higher activities of catalase, guaiacol peroxidase, ascorbate peroxidase, and superoxide dismutase and lower activities of lipoxygenase and polyphenol oxidase by comparing with GA alone. In addition, an induction of endogenous H_2S and NO was observed in SO_2-treated aleurone layers. The application of NO scavenger cPTIO could accelerate PCD in SO_2 or H_2S treated aleurone cells, suggesting that NO alleviated PCD by acting downstream of SO_2 and H_2S. In conclusion, these results imply that SO_2 could delay PCD in GA-treated wheat aleurone layers by enhancing cellular antioxidative capacity, and H_2S/NO signals act downstream of SO_2.

Additional key words: ascorbate peroxidase, catalase, gibberellic acid, protein storage vacuoles, guaiacol peroxidase, reactive oxygen species, superoxide dismutase.

Introduction

Cereal grain aleurone layers play a critical role in synthesizing and secreting hydrolytic enzymes that degrade storage reserves in the endosperm to nourish the enlarging and differentiating embryo (Jones and Jacobsen 1991). After hydrolase synthesis is completed, aleurone cells undergo programmed cell death (PCD), a process which is tightly regulated by gibberellic acid (GA) and abscisic acid (ABA). ABA slows down the process of aleurone cell death, while GA triggers it (Bethke *et al.* 1999). During germination, protein storage vacuoles (PSVs) rapidly hydrolyze vacuolar storage proteins and lipids (Kuo *et al.* 1996). Meanwhile, smaller PSVs coalesce with each other and finally form a single large vacuole which occupies almost the entire volume of the cell. Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), also play key roles in PCD of aleurone cells (Del Río *et al.* 1998, Jiménez *et al.* 1998). The activities of ROS scavenging enzymes decrease and ROS accumulates in GA-treated aleurone cells, implying that reduced ability to scavenge ROS is the cause of GA-induced PCD (Fath *et al.* 2001).

Sulfur dioxide is a widespread air pollutant generated as a by-product of industrial processes and accumulating evidence has shown that SO_2 plays multifaceted biological functions in animals (Zhang *et al.* 2011). Sulfur is a nutritional element in plants and small amount of SO_2 can be absorbed *via* stomata and assimilated during synthesis of amino acids, proteins, and vitamins

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Abbreviations: ABA - abscisic acid; APX - ascorbate peroxidase; CAT - catalase; cPTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA - 3-amino, 4-aminomethyl-2',7'-difluorescein; DCHF-DA - 2',7'-dichlorodifluorescein diacetate; GA - gibberellic acid; LOX - lipoxygenase; PCD - programmed cell death; POD - guaiacol peroxidase; PSVs - protein storage vacuoles; ROS - reactive oxygen species; SOD - superoxide dismutase; WSP-1 - 3'-methoxy-3-oxo-3H-spiro [isobenzofuran-1,9'-xanthen]-6'-yl 2-(pyridin-2-yldisulfanyl) benzoate.

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(Rausch and Wachter 2005, Haworth *et al.* 2010). Sulfate can also be absorbed by roots *via* high-affinity transporters and reduced to sulfite (Rausch and Wachter 2005). In plants, exposure to high doses of SO_2 can lead to chlorophyll destruction, tissue death, and yield reduction (Van der Kooij *et al*. 1997, Noji *et al*. 2001). More recently, it has been shown that SO_2 can be involved in transcriptome reprogramming in grape (Giraud *et al.* 2012) and in stress responses to aluminum and cadmium in wheat (Hu *et al*. 2015, Zhu *et al.* 2015). These studies suggest that SO_2 might act as a signaling molecule in plants.

Nitric oxide, carbon monoxide, and hydrogen sulfide have been identified as signaling molecules involved in antioxidative defense in plants (Delledonne 2005, Sa *et al*. 2007, Hu *et al*. 2012). Several reports showed that NO, CO, and H_2S could also delay PCD in aleurone cells of barley and wheat grain (Beligni *et al*. 2002, Wu *et al*. 2011, Xie *et al*. 2014, Zhang *et al*. 2015, Zhu *et al*. 2015). Given the functional similarity between SO_2 and H_2S, or NO in animal cells, we speculate that rather than just acting only as a harmful gas, SO_2 might also act as a signaling molecule in regulating plant PCD. In this study, we investigated the alleviating effect of SO_2 treatment on GA-induced PCD in wheat aleurone cells by analyzing the antioxidative system. The interactions between the H_2S, NO, and SO_2 in aleurone PCD are also considered.

Materials and methods

Wheat (*Triticum aestivum* L.) seeds were kindly supplied by the *Anhui Aidi Agricultural Technology Company*, Hefei, Anhui province, China. $NaHSO_3/Na_2SO_3$ (1:3) was used as sulfur dioxide (SO_2) donor according to Laisk *et al.* (1988). SO_2 donor and gibberellin A3 (GA_3, abbreviated as GA) were purchased from *Sigma* (St. Louis, USA). Grains were surface-sterilized as described by Chrispeels and Varner (1967). Embryo end of the grain was removed and de-embryonated half-grains were imbibed in Petri dishes in sterile water at 25 °C for 2 d. Aleurone layers were gently isolated by scraping away the starchy endosperm with metal spatulas and were incubated in a medium containing 20 mM $CaCl_2$ and 20 μM GA with various concentrations of SO_2 donor for time indicated in figures.

For cell viability assay and visualization of PSVs, aleurone layers after incubation in sterile water at 25 °C for 2 d were treated with SO_2 donor (0, 10, 50, 100, 200, 500 μM) + 20 μM GA + 20 mM $CaCl_2$ for 24, 72, and 120 h. To determine the number of dead cells, aleurone layers were stained with 0.4 % trypan blue for 10 min and washed extensively with 20 mM $CaCl_2$. The percentage of dead cells was determined by calculating the percentage of blue or purple cells. The PSVs were observed with a *Nikon ECLIPSE 80i* microscope (*Nikon Corporation*, Kyoto, Japan).

To determine H_2O_2 and $O_2^{\bullet-}$, the isolated wheat aleurone layers were incubated in 20 μM GA or GA + 100 μM SO_2 donor at 25 °C for 120 h. H_2O_2 content and $O_2^{\bullet-}$ production were determined every 24 h according to Zhu *et al.* (2015). Total ROS content was visualized following the method of LeBel *et al.* (1992). Aleurone layers were stained with 5 μM ROS fluorescent probe 2',7'-dichlorodifluorescein diacetate (DCHF-DA) in 20 mM $CaCl_2$ at 37 °C in the dark for 20 min and rinsed three times with 20 mM $CaCl_2$. The fluo-rescence was observed by a *Nikon Eclipse 80i* micro-scope (excitation at 488 nm and emission at 525 nm).

Twenty aleurone layers were ground to fine powder in liquid nitrogen and then extracted in 2 cm^3 of 50 mM phosphate buffer (pH 7.8), 1 mM ethylene diamine tetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 12 000 *g* and 4 °C for 30 min, and the supernatant was used for the enzyme activity assay. Activities of catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (POD, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11), and lipoxygenase (LOX, EC 1.13.11.12) were determined every 24 h till 120 h by the procedures described by Hu *et al.* (2012). Activity of superoxide dismutase (SOD, EC 1.15.1.1) was assayed according to Wang and Yang (2006) and polyphenol oxidase (PPO) activity was measured according to Benjamin and Montgomery (1973). One unit of CAT, POD, APX, SOD, LOX, and PPO activities was defined as an increase or decrease of absorbance by 0.01 value per minute.

To detect endogenous H_2S and NO in wheat aleurone layers, protoplasts were prepared as described previously (Bethke *et al.* 1999). Fresh protoplasts were treated with 20 μM GA or 20 μM GA + 100 μM SO_2 donor for 24 and 48 h, respectively. The isolated protoplasts were incubated in 10 μM H_2S fluorescent probe 3'-methoxy-3-oxo-3H-spiro [isobenzofuran-1,9'-xanthen]-6'-yl 2-(pyridin-2-yldisulfanyl) benzoate (WSP-1, kindly bestowed by Prof. Jian Chen, Jiangsu Academy of Agricultural Sciences, Nanjing, China) according to the method of Li *et al.* (2014) or in 10 μM NO fluorescent probe 3-amino, 4-amino-methyl-2',7'-difluorescein (DAF-FM DA, *Beyotime*, Shanghai, China) at 37 °C in the dark for 20 min, then washed three times with distilled water. The *in situ* fluorescence of WSP-1 (excitation at 465 nm, emission at 515 nm) or DAF-FM DA (excitation at 495 nm, emission at 515 nm) was observed using a *Nikon Eclipse 80i* fluorescence microscope.

NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetra-methylimidazoline-1-oxyl-3-oxide (cPTIO; 300 μM) was used to scavenge NO in wheat aleurone layers. Aleurone

layers were randomly classified into 6 groups with 20 layers in each and subjected to different culture conditions and 50 μM NaHS was used as H_2S donor. The first group was treated with 20 μM GA + 20 mM $CaCl_2$, the second group with 20 μM GA + 20 mM $CaCl_2$ + 50 μM H_2S donor, the third group with 20 μM GA + 20 mM $CaCl_2$ + 100 μM SO_2 donor, fourth group with 300 μM cPTIO + 20 μM GA + 20 mM $CaCl_2$, fifth group with 300 μM cPTIO + 20 μM GA + 20 mM $CaCl_2$ + 50 μM H_2S donor, and sixth group with 300 μM cPTIO + 20 μM GA + 20 mM $CaCl_2$ + 100 μM SO_2 donor. Each group was incubated at above conditions at 25 °C for 24 h. Aleurone layers were stained with trypan blue and photographed for cell viability determination.

Statistical significance was tested by one-way *ANOVA* and the results are expressed as the means ± standard deviations (SDs) of three independent experiments. Fisher's least significant differences (LSDs) were calculated following a *t*-test (at $P< 0.01$ or $P< 0.05$).

Results

To investigate the effect of SO_2 on GA-induced PCD in wheat aleurone layers, different concentrations of the SO_2 donor $NaHSO_3/Na_2SO_3$ (0, 10, 50, 100, 200 and 500 μM) were applied. Wheat aleurone cells were stained with trypan blue to determine cell viability (Fig. 1*A*, Fig. 1 Suppl.). Treatment of wheat aleurone with GA for 24 h resulted in 21 % of dead cells, whereas SO_2 donor treatment ranging from 10 to 200 μM led to about 10 % cell death. However, comparable dead cell percentage to GA group was observed in 500 μM SO_2 plus GA treatment. The percentage of dead cells in GA group increased from 26 to 44 % when treatment duration increased from 72 to 120 h. However, the percentage of dead cells with GA + 100 μM SO_2 treatment was only 16 and 26 % after 72 and 120 h, respectively (Fig. 1*A*). Thus, 100 μM SO_2 could effectively delay GA-induced PCD process in wheat aleurone layers, and this concentration was used in following experiments.

GA induces coalescence and enlargement of PSV in aleurone protoplasts whereas ABA inhibits this process (Bethke *et al.* 1999). Cell death occurs only after cells become highly vacuolated. As shown in Fig. 1*B*, PSVs in wheat aleurone cells were enlarged already after GA treatment for 24 h. However, PSVs remained smaller and dispersed after GA + SO_2 treatment for 24 h. After 72 h incubation, many aleurone cells in GA group contained large vacuoles, while there were still a lot of granular PSVs in GA + SO_2 group. This indicates that SO_2 can inhibit the coalescence of PSV and maintain the stability of cell structure.

In aleurone cells, ROS, especially H_2O_2, were found to stimulate the process of PCD (Bethke and Jones 2001) and Fig. 2*A,B* shows the changes of H_2O_2 content and $O_2^{\cdot-}$ production in wheat aleurone cells under GA or GA + SO_2 treatments. H_2O_2 content under GA treatment rapidly increased and reached the maximum at 48 h, followed by a decline at 96 h. An increase in H_2O_2 content was also observed in GA + SO_2-treated cells followed by a drop at 72 h and it was significantly ($P < 0.5$) lower than that under GA alone (at 72 and 96 h). The $\cdot O_2^{\cdot-}$ production maintained at a stable level during the first 72 h in GA + SO_2 treatment, and then declined at 96 h followed by a slight increase. The production of $O_2^{\cdot-}$ in GA treatment was significantly higher than that at GA + SO_2 at 48 and 96 h. Moreover, DCHF-DA fluorescence steadily increased in GA treatment during 96 h of

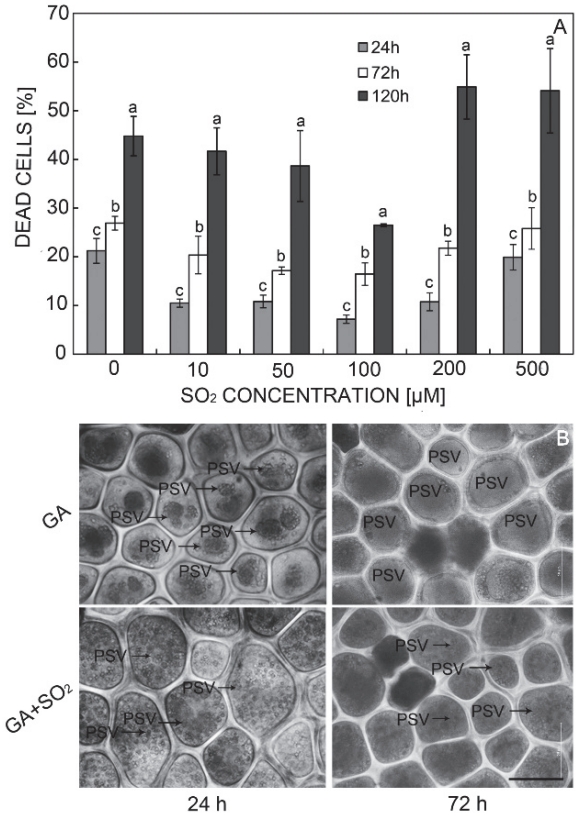

Fig. 1. Effects of SO_2 donor on cell viability and protein storage vacuoles (PSVs) of 20 μM GA-treated wheat aleurone layers. Aleurone layers were also treated with different concentrations of SO_2 donor (0, 10, 50, 100, 200, and 500 μM) for 24, 72, and 120 h. *A* - The percentage of dead cells after staining with trypan blue (at each time point 100 cells were counted, and different letters indicate statistically significant differences at $P < 0.05$). *B* - Effects of SO_2 donor on PSVs in wheat aleurone cells incubated in 20 μM GA alone (GA) or in 20 μM GA + 100 μM SO_2 (GA+SO_2) for 24 and 72 h. PSVs were observed with the *Nikon ECLIPSE 80i* microscope. *Bar* = 50 μm.

incubation, whereas the fluorescence was weakened in GA+SO_2-treated aleurone cells (Fig. 2 Suppl.). Thus, SO_2 could effectively inhibit ROS accumulation in wheat aleurone cells.

Further, to study the effects of SO_2 on antioxidative capability in cells, activities of antioxidant enzymes were measured. The CAT activity increased rapidly under both treatments during the first 48 h followed by a decrease. However, the CAT activity sustained significantly higher under GA+SO_2 treatment at 24, 72, 96, 120 h (Fig. 2*C)*. The POD activities in GA-treated aleurone layers increased gradually during the entire period of incubation, while under GA+SO_2 treatment, the POD activity was increased till 72 h followed by a decline. For instance, POD activity in GA+SO_2 treated layers was about two-fold of that in GA treated at 48 and 72 h (Fig. 2*D*). Regardless of treatments, APX activities increased quickly and peaked at 48 h, followed by a decline. However, the activity of APX under GA+SO_2 treatment was maintained higher than under GA alone since 72 h (Fig. 2*E*). The SOD activity under both treatments increased steadily till 48 h followed by a decrease. However, SOD activity under GA+SO_2 treatment was significantly higher than that under GA alone at 48, 72, and 120 h (Fig. 2*F*). However, the LOX activity under GA+SO_2 treatment decreased steadily until

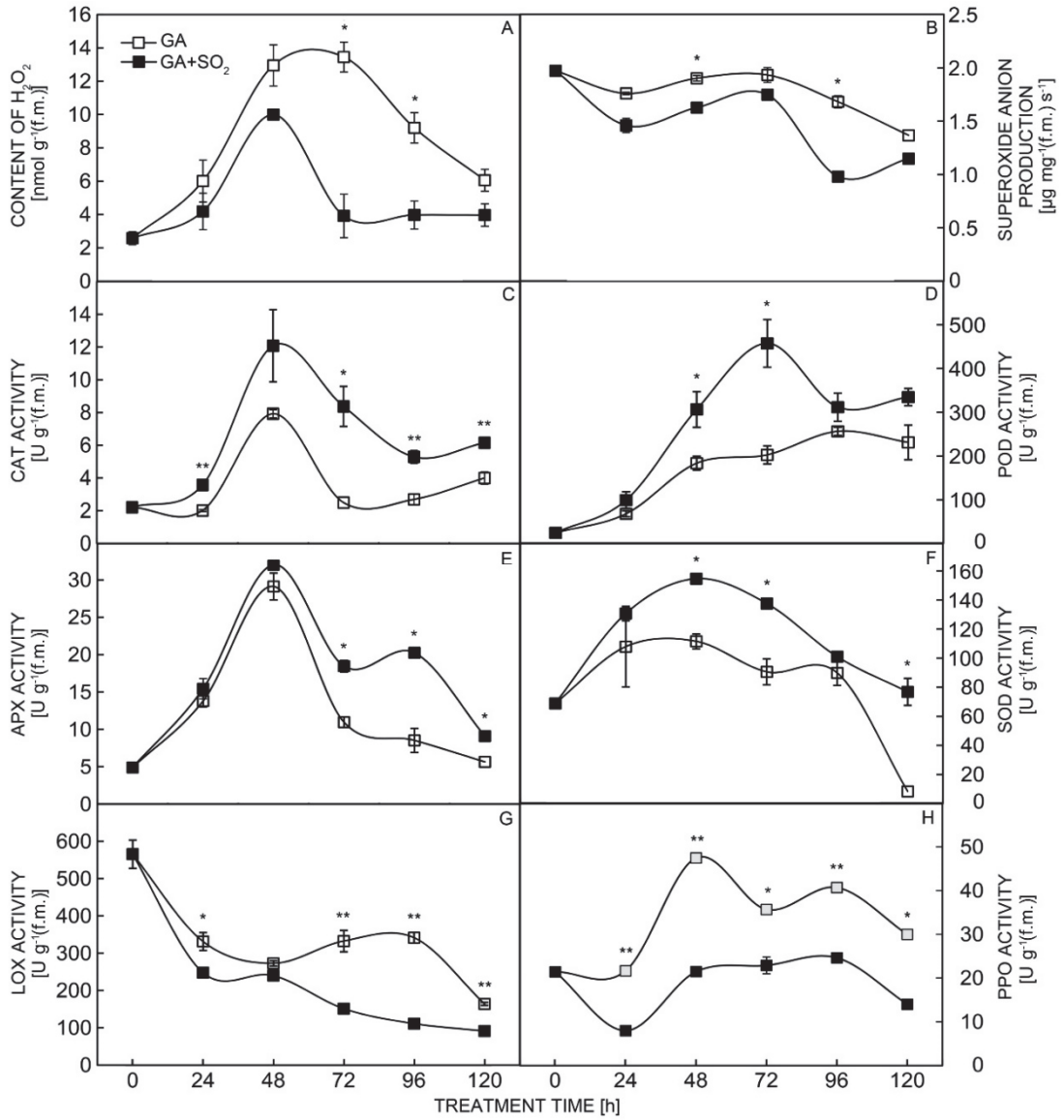

Fig. 2. Effects of SO_2 on the content of hydrogen peroxide (H_2O_2; *A*), the production of superoxide anion ($O_2^{\bullet-}$; *B*), and the activities of catalase (CAT; *C*), guaiacol peroxidase (POD; *D*), ascorbate peroxidase (APX; *E*), superoxide dismutase (SOD; *F*), lipoxygenase (LOX; *G*), and polyphenol oxidase (PPO; *H*) in wheat aleurone layers. Aleurone layers were treated with 20 µM GA (GA) or 20 µM GA + 100 µM SO_2 donor (GA+SO_2) for 0, 24, 48, 72, 96 and 120 h. Means ± SDs (n = 3). The symbols * and ** stand for significant differences between GA and GA+SO_2 at $P < 0.05$ and $P < 0.01$, respectively.

120 h and was significantly lower than that under GA treatment during the whole incubation period except 48 h (Fig. 2*G*). The PPO activity increased rapidly and peaked at 48 h under GA treatment followed by a decrease at 120 h. Under GA+SO_2 treatment, the PPO activity was stable from 48 h to 96 h followed by a decline (Fig. 2*H*). In general, GA+SO_2 treatment increased the activities of CAT, POD, APX, and SOD and decreased the activities of LOX and PPO.

Fluorescent probes WSP-1 and DAF-FM DA were used to detect endogenous content of H_2S and NO, respectively. The fluorescence showing the presence of H_2S in wheat aleurone protoplasts treated with GA+SO_2 was higher compared to those treated with GA alone at 24 and 48 h of incubation (Fig. 3*A*). Similarly, GA+SO_2 treatment induced higher NO fluorescence in aleurone protoplasts compared with GA treatment at 24 and 48 h (Fig. 3*B*). The results suggest that SO_2 treatment induced production of H_2S and NO in wheat aleurone cells.

NO was found to delay GA-induced PCD in barley aleurone layers (Beligni *et al.* 2002). To study whether NO is also involved in the role of SO_2 in alleviating PCD of wheat aleurone layers, cPTIO, a scavenger of NO, was applied. As shown in Fig. 3*C,D*, the percentage of dead cells in GA treatment reached 17 %, which was significantly higher than that under GA+H_2S (6 %) and GA+SO_2 (9 %) treatment. When cPTIO was applied, dead cell percentage increased significantly to 13 and 17 % for GA+H_2S and GA+SO_2 groups, respectively. The results suggest the involvement of NO in the PCD-alleviating effect of SO_2 and H_2S.

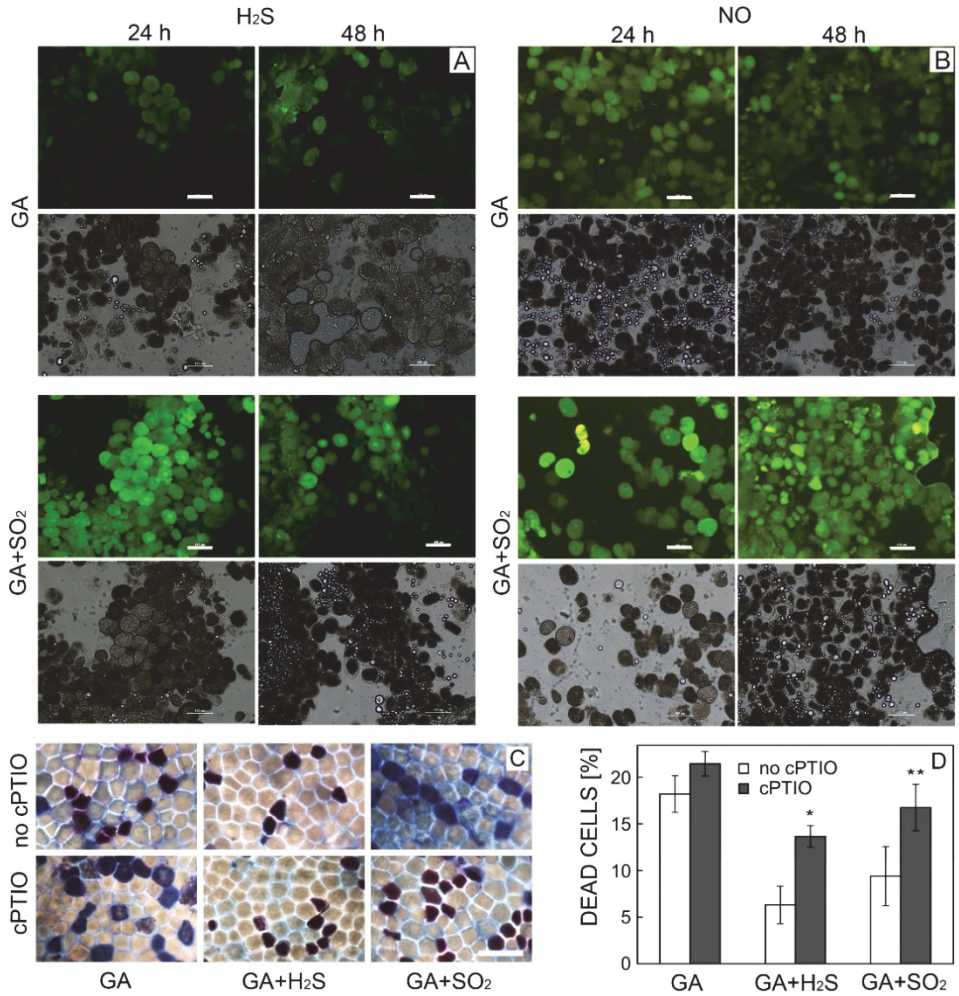

Fig. 3. Effects of SO_2 donor on accumulation of H_2S and NO in wheat aleurone layers and the application of NO scavenger cPTIO on aleurone cell viability. Fresh protoplasts treated with 20 μM GA (GA) or 20 μM GA + 100 μM SO_2 donor (GA+SO_2) for 24 or 48 h were incubated with the probes WSP-1 or DAF-FM DA to detect endogenous H_2S (*A*) and NO (*B*) by fluorescence microscopy. Wheat aleurone layers were treated with 20 μM GA (GA), 20 μM GA + 50 μM H_2S donor (GA+H_2S), 20 μM GA + 100 μM SO_2 (GA+SO_2), with or without NO scavenger cPTIO for 24 h. Aleurone layers were stained by trypan blue and photographed (*C*). Percentages of dead cells (*D*). The data at each time point are from three aleurone layers in which 100 cells were counted. The symbols * and ** stand for significant differences between treatments with and without cPTIO at $P < 0.05$ and $P < 0.01$, respectively. *Bar* = 100 μm.

Discussion

As a plant macronutrient, sulfur (S) is important for crop yield. Sulfur can be absorbed by roots in the form of sulfate or atmospheric SO_2 *via* the stomata (Rausch and Wachter 2005). Sulfite, a less oxidized form than sulfate, is an intermediate in the assimilation of S and it is cytotoxic causing decline of photosynthesis and cell damage, even apoptosis (Leustek *et al.* 2000). Low concentrations of SO_2 have been found to participate in various physiological processes in animals (Zhang *et al.* 2011). In our previous study, SO_2 could alleviate aluminum stress in wheat grains by increasing their antioxidative capability (Zhu *et al.* 2015). Cereal aleurone layers undergo GA-stimulated PCD during germination and many signals are found to regulate PCD in cells including NO, ROS, and H_2S (Fath *et al.* 2002, Ishibashi *et al.* 2012, Xie *et al.* 2014). In the present work, SO_2 donor effectively alleviated PCD in GA-treated wheat aleurone layers and the optimal concentration was 100 μM. Enlarged PSVs are one of the significant characteristics of PCD (Bethke *et al.* 1999). Consistently, we observed that many PSVs under GA treatment coalesced and formed large vacuoles after 72 h, whereas SO_2 signifi-cantly alleviated this process, similarly to ABA, which can also postpone PCD in aleurone layers (Lee *et al*. 2015).

The ROS are found to function as signal molecules in plants, especially H_2O_2 is involved in PCD in aleurone cells (Bethke and Jones 2001). Consistent with the previous observations (Fath *et al.* 2002), our results showed that GA treatment caused overproduction of ROS in wheat aleurone cells and SO_2 significantly alleviated the accumulation of H_2O_2 (Fig. 2*A*, Fig. 2 Suppl.) and the production of $O_2^{\bullet-}$ (Fig. 2*B*, Fig. 2 Suppl.). The data indicated that SO_2 could reduce oxidative stress in wheat aleurone layers and so alleviate their PCD.

Reduced ability to scavenge ROS contributes to PCD in GA-treated aleurone cells (Fath *et al.* 2002). Thus, a suite of enzymes that scavenge ROS were determined in wheat aleurone cells and higher activities of CAT, APX, SOD, and POD were observed in GA+SO_2 group (Fig. 2*C*-*F*). The elevated activities of antioxidative enzymes eliminated ROS accumulation and maintained more balanced redox state in cells. The LOX, which catalyzes oxygenation of polyunsaturated fatty acids into lipids, is an important indicator of lipid peroxidation (Andreou and Feussner 2009). In the present study, we found that aleurone cells treated with GA+SO_2 sustained lower LOX activity compared with GA group (Fig. 2*G*), suggesting less lipid peroxidation in cells. The PPO is an important enzyme in the catalytic oxidation of phenolics to brown-colored pigments (Nguyen *et al*. 2003). The PPO activity in wheat aleurone layer was lower under GA+SO_2 treatment than under GA alone, implicating that SO_2 postponed the browning process in aleurone cells (Fig. 2*H*). Taken together, these data imply that SO_2 donor delayed cell death by modulation of the antioxidant system. Similarly, endogenous SO_2 plays a protective role in the pathogenesis of animals (Jin *et al*. 2008).

Previous reports showed that NO and H_2S delay PCD in barley and wheat aleurone cells (Beligni *et al.* 2002, Xie *et al.* 2014). Besides, Wei *et al*. (2015) reported that NO is involved in the apoptosis induced by SO_2 in *Tagetes erecta* guard cells and *Pinus silvestris* needles emit H_2S when trees are subjected to relatively low concentration of SO_2 in the field (Hällgren and Fredriksson 1982). Further, sulfite can be reduced by sulfite reductase to produce the fully reduced sulfide form (Rausch and Wachter 2005). In this study, we found that SO_2 donor enhanced content of NO and H_2S, suggesting that NO and H_2S might participate in PCD alleviation caused by SO_2. The NO scavenger cPTIO was used to eliminate the endogenous NO, and the result proved that endogenous NO was involved in the PCD-alleviating role of SO_2. Thus we speculated that SO_2 delayed PCD by production of H_2S and SO_2, and H_2S could coordinately delay PCD in wheat aleurone cells. In all, the results suggest that SO_2 acts upstream of H_2S and NO in alleviating PCD in wheat aleurone cells.

In summary, our data showed that the SO_2 donor at certain concentrations alleviated PCD of GA-treated wheat aleurone cells by reducing ROS accumulation through enhanced activities of antioxidant enzymes and decreased LOX. The involvement of H_2S and NO in SO_2-treated aleurone layers suggest the interplay among SO_2, H_2S, and NO signaling pathways.

It is speculated that the delayed PCD of aleurone cells due to SO_2 could be positive for germination and embryo development, because SO_2 is able to stimulate wheat seed germination under abiotic stresses (Zhang *et al.* 2015). Similarly, H_2S was found to promote seed germination and meanwhile delayed PCD in wheat aleurone layers (Xie *et al.* 2014).

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