Involvement of NADPH oxidase in sulfur dioxide-induced oxidative stress in plant cells

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Bisulfite, a major form of SO_2 in aqueous phase of apoplast, may reduce photosynthesis rate and thereby crop yield through inducing reactive oxygen species (ROS). In this study, ROS production was directly detected in a living cell of leaf of spinach (*Spinacia oleracea* L.) using laser scanning confocal microscopes with the assistance of the fluorescence probe dichlorofluorescin diacetate (H_2DCF-DA). Results showed that, under bisulfite stress, a large quantity of ROS indicated by DCF fluorescence was produced in epidermic tissue. The role of plasma membrane (PM) NADPH oxidase in bisulfite-induced ROS production was also investigated. Treatment with bisulfite resulted in a significant increase in the content of ROS and the activity of PM NADPH oxidase in spinach leaves. The effects caused by bisulfite were inhibited pronouncedly by pretreatment with two widely used NADPH oxidase inhibitors (diphenyleneiodonium and quinacrine). Moreover, the change patterns of the bisulfite-induced increase and inhibitor-caused decrease in the two parameters were quite similar. Additionally, only a small amount of ROS could be observed on *in vitro* chloroplasts under bisulfite stress. Based on all the results, we conclude that ROS is involved in bisulfite-induced stress, and the bisulfite-induced enhancements in levels of ROS originate mainly from PM NADPH oxidase.

Introduction

The widespread use of coal and petroleum as energy sources for industries has led to the emission of large quantities of SO_2 into the atmosphere. However, it is very toxic at high concentration, so attention has been paid to the phenomenon of destroyed enzyme function and photosynthesis by SO_2, which inhibits plant growth and development and thereby markedly decreases crop yield.[1,2]

The exchange of gases between the atmosphere and a cellular system is a free diffusion process. SO_2, whose pK values are 1.78 and 6.99 (first and second deprotonation reaction), enters leaves through stomata and quickly dissolves in the aqueous apoplast to form HSO_3^-, SO_3^{2-}, H^+.[3] Bisulfite (HSO_3^-), which inhibits photosynthetic CO_2 fixation, is the major form of dissolved SO_2 in water under normal physiological pH. Therefore bisulfite could be used to replace SO_2 fumigation for studying SO_2 stress.[4,5]

Phytotoxicity from SO_2 may be related to the production of reactive oxygen species (ROS). It has been reported that the level of vitamin E and glutathione were enhanced upon exposure to SO_2 in needles of fir and spruce.[6] Additionally, superoxide dismutase activity increased in leaves of pea exposed to SO_2.[7] Moreover, sulfite-induced lipid peroxidation could be reduced in spinach by exogenous ascorbate, which reacted with superoxide radical (O_2^-) and hydroxyl radical (OH^-).[8] Although the activity of the antioxidant system can be upregulated by ROS production and also increases under bisulfite stress in many plants,[6,7,8] there is no further direct evidence indicating that ROS is involved in bisulfite stress. Also, the molecular mechanism for ROS generation under bisulfie stress is unknown, to a large extent.

It has been reported that the main site of ROS formation was chloroplasts under abiotic stresses.[9] However, increasing evidence obtained in many recent experiments is emerging to support the idea that plant plasma membrane (PM) NADPH oxidase, a homologue of the mammal gp91^{phox} in neutrophils, is an important source of ROS in plants under many stresses.[10,11] PM NADPH oxidase transfers electrons from cytoplasmic NAD(P)H to O_2 to form O_2^-, followed by dismutation of O_2^- to H_2O_2. It has been well documented that PM NADPH oxidase was involved in plant growth and development,[12,13,14] in plant defense reactions to pathogen or elicitor attack,[10,11] and in response to a few abiotic stresses such as cold, UVB, ozone and salt stress.[15] Therefore, PM NADPH oxidase may be involved in bisulfite stress though Peiser *et al.* supposed that SO_2 mainly induced chloroplasts to produce ROS.[8]

In this work, we directly detected ROS with dichlorofluorescin diacetate (H_2DCF-DA) under a laser scanning confocal microscope, and attempted to reveal the mechanism for ROS under bisulte stress. Our data showed that bisulfite-induced enhancements in levels of ROS originated mainly from PM NADPH oxidase, and thus PM NADPH oxidase was involved in bisulfite-induced ROS generation.

Materials and methods

Plant material and chloroplast isolation

The plant *Spinacia oleracea* L. was grown in soil in a controlled growth chamber (Conviron, model E7/2, Winnipeg, Canada) under a 10-h-light and 14-h-dark cycle, a photon fluency rate of 200 μmol m^{-2} s^{-1}, and day/night temperature cycle of 20 ± 0.5 °C

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and 17 ± 0.5 °C, respectively. Pots were watered every 2 to 3 days. Fully expanded leaves of 4-week to 6-week-old plants were used for bioassay.

Chloroplasts were isolated from expanded leaves of *Spinacia oleracea* L. Intact chloroplasts were prepared largely as described by Jensen and Bassham.[16] The percentage of intact chloroplasts in the chloroplast suspensions was measured by the ferricyanide method.[17] It ranged between 70 and 90%.

Measurement of photosynthesis rate (Pn)

Pn of intact leaves was determined in an atmosphere of 380 μmol mol^{-1} CO_2 or as indicated and a saturating irradiance of 1000 μmol photons m^{-2} s^{-1} using a LI-COR 6400 portable photosynthetic system (Model: LI-COR 6400 LI-COR, Lincoln, NE, USA). Entire plants were placed inside a humidified plant growth chamber (Conviron, model E7/2, Winnipeg, Canada), and leaf temperature was increased or decreased after reaching steady-state photosynthesis at the optimal temperature by changing the temperature of both leaf cuvette and the growth chamber.

Dye loading

Abaxial epidermis strips were peeled after 2.5 h of illumination and incubated for 30 min in loading buffer (Tris–KCI at 10 mM, pH 6.1) to remove any ROS that stripping might have caused.[18] Then the epidermal strips were placed into a small Petri dish containing 3 mL of the loading buffer and 30 μL of H_2DCF-DA from a 100 mM stock in DMSO for 30 min. The higher concentrations (30–50 μM) of the dye were used with some strips that had stronger autofluorescence. However, at these concentrations the dye was sometimes sequestered, as indicated by a continuous increase in fluorescence. Therefore, we limited the dye concentration below 10 μM but increased the incubation period to 30 min. Then the peels were removed, and floated on a dish of fresh buffer to wash off excess dye.

Isolation of plasma membranes

Microsomal fractions from spinach were isolated as described previously,[19] except that the homogenization buffer contained 100 mM KCI, 3 mM NaCI, 1 mM ATP, 3.5 mM $MgCI_2$, 10 μM GTP (γ) S, 5 mM sucrose, 1 mM phenylmethyl sulfonylfluoride, 2 mM DTT, and 10 mM PIPES, pH 7.3. Microsomes were resuspended in 200 pL of phase suspension medium containing 250 mM sucrose, 5 mM potassium phosphate buffer, pH 7.0, and 1 mM DTT. Plasma membranes were obtained by phase partitioning microsomal preparations for three cycles in an 8 or 4 g phase system with a final composition of 6.5% (w/w) dextran T500, 6.5% (w/w) polyethylene glycol (molecular weight of 3350), 250 mM sucrose, 3 mM KCI, 5 mM potassium phosphate, pH 7.8, and 1 mM DTT. The resultant PM fraction was used immediately for further analysis.

Determination of PM NADPH oxidase activity

Production of O_2^- by NADPH oxidase was monitored at 25 °C by following the superoxide dismutase-inhibitable and NADPH-dependent reduction of cytochrome c at 550 nm detected with a UV/VIS spectrometer (Lambda 35, Perkin-Elmer, UK), as described by Xing *et al.*[20] The reaction mixtures contained 0.1 mM cytochrome c, 6.5 mM $MgCl_2$, 87 mM KCl, 2.6 mM NaCl, pH 7.3, l0 μM GTP (γ) S, 0.16 mM NADPH, and 10–15 μg of plasma membrane proteins in a total volume of 1 mL. The control assay contained 50 μg of superoxide dismutase to account for non-O_2^--dependent reduction of cytochrome c.

Laser scanning confocal microscope

Examination of the epidermis was performed on a commercial laser scanning microscope (LSM510/ConfoCor2) combination system (Zeiss, Jena, Germany). A green argon-ion laser (488 nm) set on 3% power was used for excitation, with 525 nm emission. The viability of the cells within the epidermis under these media conditions was >95% for guard cells and 80% for epidermal cells, as tested by fluorescein diacetate staining. Images were captured over a time course, with laser scanning at set time points to avoid photo bleaching of the dye. Elicitors (no greater than a 50 μL volume) were added directly to the buffer during the time course. To quantify the results, the images of emission intensities were processing with Zeiss Rel 3.2 image processing software (Zeiss, Germany).

Luminescence spectrometer

For fluorometry of whole tissue, an abaxial epidermic strip loaded as described above was placed flat onto a plastic holder and affixed at both ends with silicon grease.[21] The holder was inserted into a 3 mL quartz cuvette containing 2 mL of aerated loading buffer. The luminescence spectrometer (LS55, Perkin-Elmer, UK) was set to an excitation of 488 nm and an emission of 525 nm, with slit width at 2.5 nm. The cuvette was then placed into the luminescence spectrometer after pharmacological agents were added. For statistical purposes, fluorometry experiments were performed in triplicate.

Results

Effect of bisulfite on photosynthesis rate

It has been reported that a high concentration of SO_2 reduced net photosynthetic CO_2 assimilation.[2] Therefore we first investigated the effect of a high concentration of bisulfite on Pn of spinach.

As shown in Fig. 1, treatment of spinach leaves with bisulfite significantly reduced Pn. The depression of bisulfite on Pn exhibited a dose-dependent manner, since bisulfite at 5 mM inhibited Pn by 15% for 30 min, and bisulfite at 10 and 100 mM obviously reduced Pn by 59 and 95% for 30 min, respectively (Fig. 1a). Similarly, the decrease in Pn was dependent on exposure time under bisulfite since 10 mM bisulfite at 30 and 60 min reduced Pn by 57 and 87%, respectively (Fig. 1b).

Effect of bisulfite on CO_2-response curves

CO_2-response curves reflect Pn for CO_2 at different concentrations. At low CO_2 concentration, the slope of the curve is restricted by the activity and quantity of Rubisco, since Pn is limited by the concentration of CO_2. At the stage of saturated CO_2, the regeneration rate of ribulose bisphosphate becomes the factor which influences Pn.

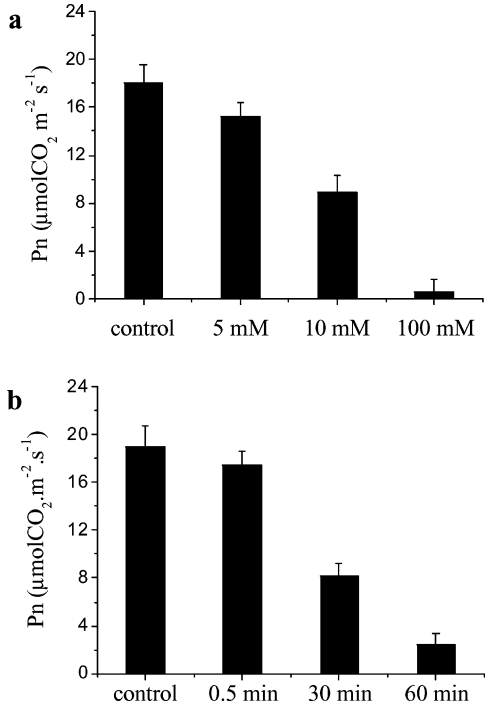


Fig. 1 Effect of bisulfite on Pn. Bisulfite was sprinkled on spinach leaves. The measurement was determined under a saturating irradiance of 1000 μmol photons m^{-2} s^{-1} and 380 μmol mol^{-1} CO_2. Each data point represents an average value ± SE from six samples with three repeated measurement. (a) Effect of different concentration of bisulfite on Pn. The measurement was determined 30 min after treatment. (b) Effect of different treating time of bisulfite on Pn. The concentration of bisulfite was 10 mM.

At low CO_2 concentration, the slope of the curves decreased under 10 mM bisulfite stress for 30 min, which indicated that the activity of Rubisco was inhibited by bisulfite stress. At the CO_2 saturated stage, the maximum of Pn obviously decreased because of the low regeneration rate of ribulose bisphosphate caused by the low activity of Rubisco (Fig. 2). The result indicated that Rubisco activity became low under bisulfite stress.

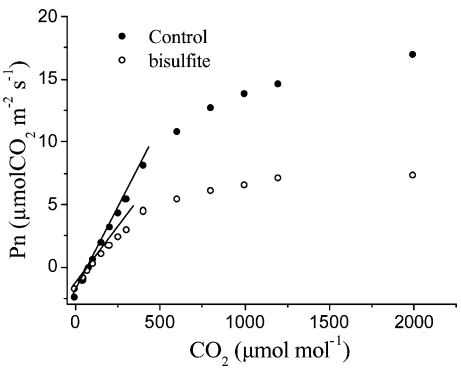


Fig. 2 Effect of sulfite on CO_2–photosynthetic curve. Bisulfite was sprinkled on spinach leaves. The measurement was determined under 10 mM bisulfite, treatment for 30 min with a saturating irradiance of 1000 μmol photons m^{-2} s^{-1} and 380 μmol mol^{-1} CO_2.

ROS directly detected and dynamics change

There would be more than one reason why Rubisco activity became low under bisulfite stress. It has been reported that the activity of free radical scavengers increased under SO_2 stress,[6,7,8] which indicated that ROS was produced in large amounts under the stress. To confirm whether ROS was involved in decreasing Rubisco acitivity under bisulfite stress, we detected ROS *in situ* with H_2DCF-DA.

In Fig. 3, treatment of epidermis with bisulfite caused a significant increase in ROS quantity after 4–7 min. Moreover, the significant increase lasted for about 23–26 min, followed by a slight increase up to 33 min which was the longest time we tested. The results clearly showed that bisulfite led to pronounced enhancement in ROS quantity in spinach. To further confirm the method in which ROS was determined by the intensity of DCF fluorescence, we detected ROS under cadmium stress and paraquat stress. In Fig. 4, ROS production increased both in epidermic tissue under 5 mM cadmium stress and in mesophyllic cells under 5 mM paraquat stress, which was consistent with previous reports.[21,22]

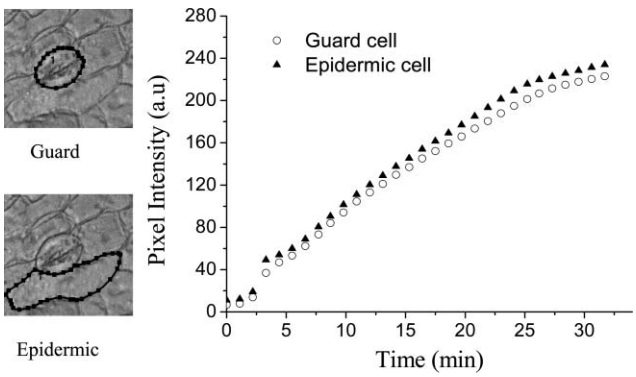


Fig. 3 Dynamics of DCF fluorescence intensity in living cells after bisulfite treatment. Epidermal tissue was loaded with H_2DCF-DA, washed, and examined by laser scanning confocal microscopy. During a time course of image acquisition, 25 μL sulfite from a 200 mM stock solution was added directly to a Petri dish containing 500 μL loading buffer.

However, the manner of ROS generation induced by bisulfite was similar in epidermal cells without chloroplasts and guard cells with chloroplasts (Fig. 3), indicating that the mechanism of ROS generation in the two kinds of cells was same or similar though they had different structures. More importantly, we guessed that chloroplasts in spinach only produced a small amount of ROS and did not play an important role in ROS production under bisulfite stress, which was different from the view that SO_2 stress mainly induced chloroplasts to produce ROS.[8]

Chloroplasts produced a small amount of ROS

To confirm whether chloroplasts produced ROS under bisulfite stress, *in vitro* chloroplasts of spinach were examined under stress.

In vitro chloroplasts produced a small amount of ROS under bisulfite stress. The ROS quantity in bisulfite-treated spinach

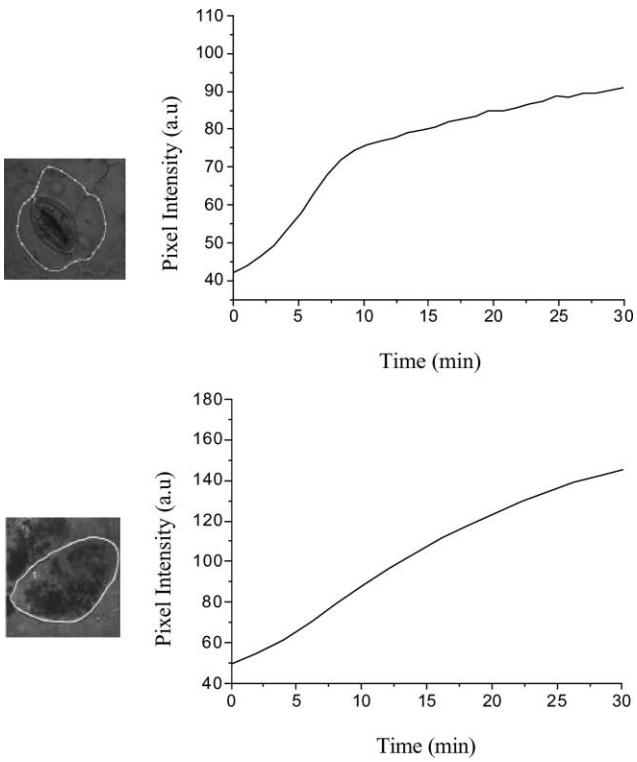


Fig. 4 Dynamics of DCF fluorescence intensity in living cells after stress treatment. Epidermal tissue was loaded with H_2DCF-DA, washed, and examined by laser scanning confocal microscopy. During a time course of image acquisition, 12.5 μL cadmium and paraquat from a 200 mM stock solution was added directly to a Petri dish containing 500 μL loading buffer.

epidermis peels increased remarkably from 5 to 20 min, then changed slightly and maintained at steady high level up to 30 min. In contrast, the ROS quantity of *in vitro* chloroplasts only altered slightly over the time course we tested (Fig. 5). These results

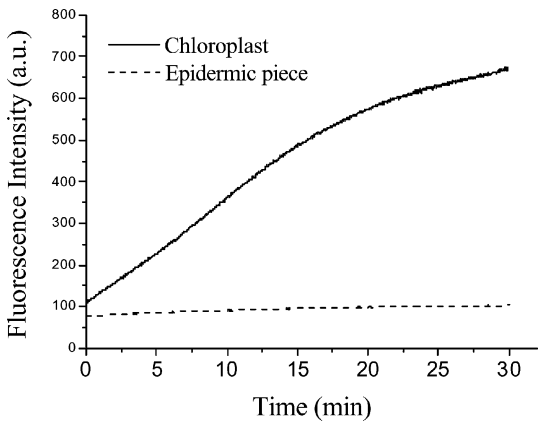


Fig. 5 Dynamics of DCF fluorescence for *in vitro* chloroplasts under bisulfite stress. Epidermal tissue and *in vitro* chloroplasts were loaded with H_2DCF-DA, washed, and examined by luminescence spectroscopy. After adding 25 μL sulfite from a 200 mM stock solution to the cuvette containing 500 μL loading buffer, the detection began with an excitation of 488 nm, an emission of 525 nm and the slit width was 2.5 nm.

clearly indicated that the chloroplasts were not the main site of ROS production induced by bisulfite. And these results were not consistent with the supposition of Peiser *et al.*[8] The mechanism of ROS generation induced by bisulfite may be different from other abiotic stress.

ROS produced by PM NADPH oxidase

Biotic stresses and a few abiotic stresses (cold, UVB, ozone and salt stress) could induce PM NADPH oxidase to produce ROS.[15] To further investigate the mechanism of ROS generation induced by bisulfite, we examined the effects of two widely used NADPH oxidase inhibitors, diphenyleneiodonium (DPI) and quinacrine (QA),[23,24,25] on ROS quantity to determine whether PM NADPH oxidase was involved in sulfite stress.

The ROS quantity in bisulfite-treated spinach epidermis peels increased remarkably from 5–20 min then changed slightly and was maintained at a steady high level up to 30 min. Pretreatment with DPI at 5 μM and QA at 100 μM resulted in ROS generation at a low rate, and the total ROS quantity was reduced. Pretreatment with DPI at 50 μM and QA at 1 mM altered DCF fluorescence slightly, which indicated that the inhibition was extremely obvious (Fig. 6a and b).

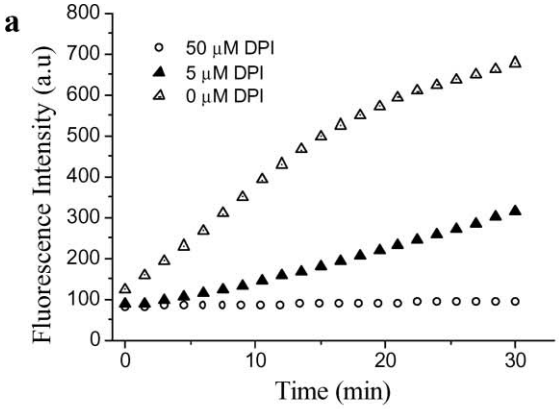
Pretreatment with inhibitors significantly reduced the quantity of ROS induced by bisulfite. The inhibition of DPI was very sensitive, since low concentration of DPI (5 μM) obviously inhibited the enhancement of ROS formation induced by bisulfite, and DPI at 50 μM reduced the ROS quantity induced by bisulfite by 84%. Furthermore, the inhibitory effects of DPI exhibited a dose-dependent manner. Meanwhile, pretreatment with QA also inhibited the enhancement of ROS formation induced by bisulfite. QA at 100 μM and 1 mM suppressed the increase of ROS quantity by 65 and 83%, respectively (Fig. 6c). These results suggested that PM NADPH oxidase was involved in the production of ROS resulting from bisulfite stress.

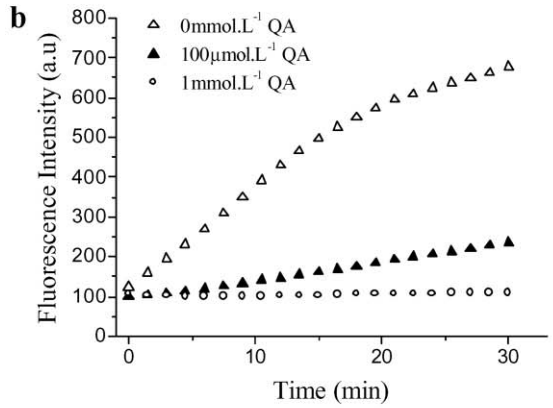
ROS production sited in the plasma membrane

To further identify that PM NADPH oxidase was involved in ROS generation induced by bisulfite, we detected the site of ROS formation *in situ* at 30 min under bisulfite stress. It was clear that DCF fluorescence intensity around plasma membrane was higher than other areas in epidermic cells under bisulfite stress (Fig. 7), which suggested that the main site of ROS formation was plasma membrane. However, guard cells were full of uncharacteristic distribution DCF. It might be due to the course of the aqueous phase diffusion which happened in the stoma opening progress (Fig. 7). The result indicated that the site of bisulfite-induced ROS formation was the PM, which confirmed that PM NADPH oxidase was involved in ROS production induced by bisulfite.

Effect of bisulfite and inhibitors on PM NADPH oxidase activity

To further identity the relationship between the bisulfite-induced increase in ROS quantity and PM NADPH oxidase activity, we detected PM NADPH oxidase activity under bisulfite stress or pretreatment with DPI and QA.





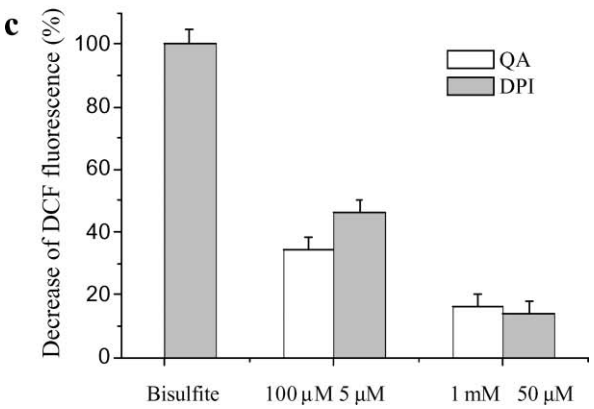


Fig. 6 Effect of DPI and QA on ROS production for spinach under bisulfite stress. The spinach was pretreated at the roots in a growth chamber with the full nutrient solution supplemented with 5 and 50 μM DPI, 0.1 and 1 mM QA for 1 h, respectively. Epidermal tissue was loaded with H_2DCF-DA, washed, and examined using a luminescence spectrometer. After adding 25 μL sulfite from a 200 mM stock solution to the cuvette containing 500 μL loading buffer, the detection began with an excitation of 488 nm, an emission of 525 nm and the slit width was 2.5 nm. (a) Effect of DPI on dynamics of DCF fluorescence under bisulfite stress. (b) Effect of QA on dynamics of DCF fluorescence under bisulfite stress. (c) Effect of DPI and QA on total fluorescence of DCF.

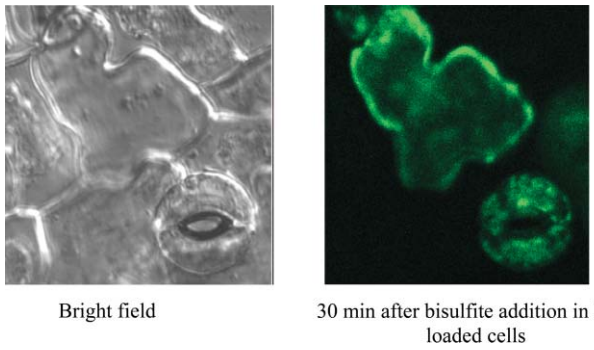


Fig. 7 The site of ROS formation. Epidermal tissue was loaded with H_2DCF-DA, washed, and examined by laser scanning confocal microscopy under 10 mM bisulfite stress at 30 min.

Treatment with bisulfite significantly increased PM NADPH oxidase activity in spinach leaves. The PM NADPH oxidase activity treated with bisulfite was about fourfold higher than that in the control sample. Pretreatment with PM NADPH oxidase inhibitors obviously inhibited the bisulfite-induced increase in PM NADPH oxidase activity. DPI at 5 μM and QA at 0.1 mM suppressed the enhancement of PM NADPH oxidase activity by 20 and 35%, respectively. However, DPI at 50 μM and QA at 1 mM blocked the increase of PM NADPH oxidase activity by 70 and 65%, respectively. Moreover, the inhibition on PM NADPH oxidase activity exhibited a dose-dependent manner (Fig. 8). The effect of bisulfite and NADPH oxidase inhibitors on PM NADPH oxidase activity was consisted with the effect on ROS quantity. Data showed above suggested that PM NADPH oxidase was involved in the production of ROS resulting from bisulfite stress.

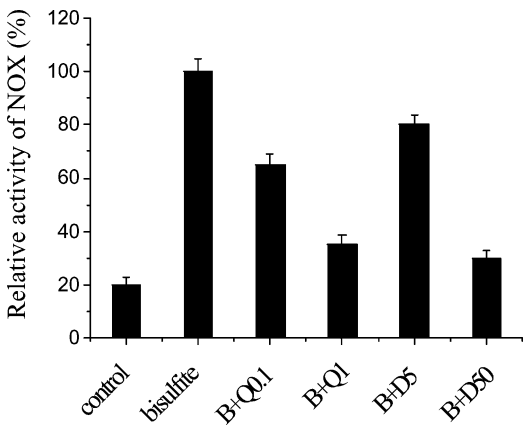


Fig. 8 Effect of NADPH oxidase inhibitors on NADPH oxidase activity under bisulfite stress in spinach leaves. The bisulfite-induced increase in values of NADPH oxidase activity was set as 100%. B + D5, B + D50, B + Q0.1 and B + Q1 represents spinach pretreated at the roots in a growth chamber with the full nutrient solution supplemented with 5 and 50 μM DPI, or 0.1 and 1 mM QA for 1 h, respectively, then exposed to 10 mM bisulfite for 30 min. All values are the average ± SE of three independent experiments.

Discussion

Pn was significantly inhibited by bisulfite, consistent with the result of Lüttge *et al.*[4] The variation of Pn depended on bisulfite

concentration and exposure time (Fig. 1). The slope of CO_2–response curves decreased at low CO_2 under 10 mM bisulfite stress for 30 min, which indicated that the activity of Rubisco was inhibited (Fig. 2). These results suggested that bisulfite significantly decreased Pn through deactivating Rubisco activity.

It has been reported that ROS was involved in SO_2 stress with indirect evidence of the level of vitamin E or superoxide dismutase activity.[6,7] In this experiment, ROS could be directly detected *in situ* using H_2DCF-DA under laser scanning confocal microscopes. Dichlorofluorescin enters cells in the diacetate form (H_2DCF-DA). Then, it is hydrolyzed and trapped as dichlorofluorescin, a nonfluorescent compound. Subsequent oxidation of H_2DCF by H_2O_2, catalyzed by peroxidases, yields the highly fluorescent DCF.[26] Results suggested that a large quantity of ROS, induced by bisulfite in epidermal tissue of spinach leaves, was an important reason to lose Rubisco activity (Fig. 3). The result indicated that a large quantity of ROS was produced and involved in bisulfite stress of spinach.

Peiser *et al.* reported that SO_2 stress mainly induced chloroplasts to produce ROS.[8] But current inspection has demonstrated that epidermic cells and guard cells had similar trends of ROS production though epidermic cells were without chloroplasts (Fig. 3). Moreover, *in vitro* experiments clearly showed that chloroplasts only produced a small amount of ROS under bisulfite stress (Fig. 5), Hence, chloroplasts were not the main site of ROS formation under bisulfite stress. Bisulfite-induced ROS generation may be different from other abiotic stress.

However, bisulfite-induced ROS production significantly decreased by pretreatment with the widely used PM NADPH oxidase inhibitors DPI and QA (Fig. 6). The result suggested that PM NADPH oxidase was involved in ROS production induced by bisulfite stress. To understand the origin of bisulfite-induced ROS, we further investigated the changes of PM NADPH oxidase activity in spinach leaves. Bisulfite induced a pronounced increase in the activity of PM NADPH oxidase in spinach leaves, and this increase could be obviously inhibited by pretreatment of DPI and QA (Fig. 8). Moreover, the patterns in the bisulfite-induced increase and inhibitors-caused decrease in ROS production and PM NADPH oxidase actiovity were quite similar. The inhibition of DPI was very sensitive since DPI at a concentration as low as 5 μM obviously inhibited the enhancement of the two parameters above. Furthermore, the inhibitory effects of DPI exhibited a dose-dependent manner. Likewise, another NADPH oxidase inhibitor QA also inhibited the increase of the two parameters induced by bisulfite (Fig. 6 and 8). In addition, the *in situ* assay also confirmed that PM NADPH oxidase was involved in bisulfite stress (Fig. 7). Although the absolute specificity of each inhibitor used in the present study could be questioned, similar results obtained with different NADPH oxidase inhibitors, together with the bisulfite-induced significant enhancement in ROS quantity and PM NADPH oxidase activity as well as the findings of an *in situ* assay, clearly revealed that PM NADPH oxidase was involved in the ROS generation induced by bisulfite for spinach.

Based on all of these results, we conclude that bisulfite-induced enhancements in levels of ROS originated mainly from PM NADPH oxidase, and PM NADPH oxidase was involved in bisulfite-induced ROS generation. Moreover, all the evidence obtained in this study suggests that PM NADPH oxidase may play an important role in response to SO_2 stress.

Abbreviations

ROS	Reactive oxygen species
O_2^-	Superoxide radical
OH^-	Hydroxyl radical
PM	Plasma membrane
H_2DCF-DA	Dichlorofluorescin diacetate
Tris	Tris(hydroxymethyl)aminoethane
DMSO	Dimethyl sulfoxide
GTP	Guanosine triphosphate
DTT	Dithiothreitol
Pn	Photosynthesis rate
DPI	Diphenyleneiodonium
QA	Quinacrine

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References

1 R. B. Bressan, L. G. Wilson and P. Filner, Mechanisms of resistance to sulfur dioxide in the Cucurbitaceae, *Plant Physiol.*, 1978, **61**, 761–767.
2 N. M. Darrall, The effect of air pollutants on physiological processes in plants, *Plant Cell Environ.*, 1989, **12**, 1–30.
3 H. Pfanz and U. Heber, Buffer capacities of leaves, leaf cells, and leaf cell organelles in relation to fluxes of potentially acidic gases, *Plant Physiol.*, 1986, **81**, 597–602.
4 U. Lüttge, C. B. Osmond, E. Ball, E. Brinckmann and G. Kinze, Bisulfite compounds as metabolic inhibitors: nonspecific effects on membranes, *Plant Cell Physiol.*, 1972, **13**, 505–514.
5 H. Pfanz, E. Martinoia, L. Otto-Ludwig and U. Heber, Flux of SO_2 into leaf cells and cellular acidification by SO_2, *Plant Physiol.*, 1987, **85**, 928–933.
6 H. Mehlhorn, G. Seufert, A. Schmidt and K. J. Kunert, Effect of SO_2 and O_3 on production of antioxidants in conifers, *Plant Physiol.*, 1986, **82**, 336–338.
7 N. R. Madamanchi and R. G. Alscher, Metabolic bases for differences in sensitivity of two pea cultivars to sulfur dioxide, *Plant Physiol.*, 1991, **97**, 88–93.
8 G. D. Peiser, C. C. Lizada and S. F. Shang, Sulfite-induced lipid peroxidation in chloroplasts as determined by ethane production, *Plant Physiol.*, 1982, **70**, 994–998.
9 K. Apel and H. Hirt, Reactive oxygen species: metabolism, oxidative stress, and signal transduction, *Annu. Rev. Plant Biol.*, 2004, **55**, 373–399.
10 D. Takemoto, A. Tanaka and B. Scott, A $p67^{Phox}$-like regulator is recruited to control hyphal branching in a fungal-grass mutualistic symbiosis, *Plant Cell*, 2006, **18**, 2807–2821.
11 A. Tanaka, M. J. Christensen, D. Takemoto, P. Park and B. Scott, Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction, *Plant Cell*, 2006, **18**, 1052–1066.
12 R. Shin, R. H. Berg and D. P. Schachtman, Reactive oxygen species and roots hairs in *Arabidopsis* root response to nitrogen, phosphorus and potassium Deficiency, *Plant Cell Physiol.*, 2005, **46**, 1350–1357.
13 R. Desikan, Man-kim Cheung, J. Bright, D. Heson, J. T. Hancock and S. J. Neill, ABA, hydrogen peroxide and nitric oxide signaling in stomatal guard cell, *J. Exp. Bot.*, 2003, **55**, 205–212.
14 J. Forman, V. Demidchik and J. H. Bothwell, Reactive oxygen species produced by NADPH oxidase regulate plant cell growth, *Nature*, 2003, **27**, 422–446.

15M. Sagi and R. Fluhr, Production of reactive oxygen species by plant NADPH oxidase, *Plant Physiol.*, 2006, **141**, 336–340.
16R. G. Jensen and J. A. Bassham, Photosynthesis by isolated chloroplasts, *Proc. Natl. Acad. Sci. U. S. A.*, 1966, **56**, 1095–1101.
17U. Heber and K. A. Santarius, Direct and indirect transport of ATP and ADP across the chloroplast envelope, *Z. Naturforsch.*, 1970, **25**, 718–728.
18Ki-Youb Park, Ji-Yul Jung, J. Park, Jae-Ung Hwang, Yong-Woo Kim, I. Hwang and Y. Lee, A role for phosphatidylinositol 3-phosphate in abscisic acid-induced reactive oxygen species generation in guard cells, *Plant Physiol.*, 2003, **132**, 92–98.
19J. E. Benna, J. M. Ruedi and B. M. Babior, Cytosolic guanine nucleotide-binding protein rac2 operates *in vivo* as a component of the neutrophil respiratory burst oxidase, *J. Biol. Chem.*, 1994, **269**, 6729–6734.
20T. Xing, V. J. Higgins and E. Blumwald, Race-specific elicitors of *Cladosporium fulvum* promote translocation of cytosolic components of NADPH oxidase to the plasma membrane of tomato cells, *Plant Cell*, 1997, **9**, 249–259.
21C. A. Andrew and F. Robert, Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells, *Plant Cell*, 1997, **9**, 1559–1572.
22E. Olmos, J. R. Martinez-Solano, A. Piqueras and E. Hellin, Early steps in the oxidative burst induced by cadmium in cultured tobacco cells (BY-2 line), *J. Exp. Bot.*, 2003, **54**, 291–301.
23P. V. Gestelen, H. Asard and R. J. Caubergs, Solubilization and separation of a plant plasma membrane NADPH, synthase from other NAD(P)H oOxidoreductases, *Plant Physiol.*, 1997, **115**, 543–550.
24G. P. Bowlwell, D. R. Davies, C. Gerrish, Chung-Kyoon Auh and T. M. Murphy, Comparative biochemistry of the oxidative burst produced by rose and french bean cells reveals two distinct mechanisms, *Plant Physiol.*, 1998, **116**, 1379–1385.
25I. Boldogh, A. Bacsi, B. K. Chouchury, N. Dharajiya, R. Alam, T. K. Hazra, S. Mitra, R. M. Goldblum and S. Sur, ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation, *J. Clin. Invest.*, 2005, **115**, 2169–2179.
26R. Cathcart, E. Schwiers and B. N. Ames, Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay, *Anal. Biochem.*, 1983, **134**, 111–116.