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Sulfur dioxide induced programmed cell death in *Vicia* guard cells

Huilan Yi [a,*], Jingjing Yin [a], Xin Liu [a], Xiuqing Jing [a], Sanhong Fan [a], Hufang Zhang [b]

[a] School of Life Science, Shanxi University, Taiyuan 030006, China
[b] Department of Biology, Taiyuan Normal University, Taiyuan 030012, China

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ABSTRACT

Sulfur dioxide (SO_2) induced nuclear condensation and nuclear fragmentation and rapid loss of guard cell viability in detached epidermis of *Vicia* leaves at concentrations of 1 mM and higher (3 h exposure). Caspase inhibitors Z–Asp–CH_2–DCB (0.1 mM) and TLCK (0.1 mM) markedly suppressed SO_2-induced cell death. The typical nuclear morphological changes and the inhibition effects of caspase inhibitors suggest the activation of a programmed cell death (PCD) pathway. SO_2-induced cell death can be blocked by either antioxidants (0.1 mM AsA or 200 U/mL CAT) or Ca^{2+} antagonists (0.1 mM EGTA or $LaCl_3$). AsA and CAT also blocked SO_2-induced ROS production and $[Ca^{2+}]_{cyt}$ increase. However, EGTA and $LaCl_3$ can inhibit SO_2-induced $[Ca^{2+}]_{cyt}$ increase, but cannot suppress SO_2-induced ROS production. Our results indicate that high concentrations of SO_2 induce guard cell death via a PCD pathway through ROS mediating $[Ca^{2+}]_{cyt}$ elevation, which causes harmful effects to plants.

1. Introduction

Sulfur dioxide (SO_2) is a common air pollutant, produced by combustion and processing of sulfur-containing fossil fuels. Exposure to high concentrations of SO_2 can cause visible foliar damage, photosynthesis decline, plant growth inhibition and even death (Hogetsu and Shishikura, 1994; Noji et al., 2001; Rakwal et al., 2003). The production of reactive oxygen species (ROS) is one of the key events in plant response to SO_2 (Hänsch and Mendel, 2005; Shimazaki et al., 1980). ROS could act as signal molecules, mediating a variety of responses including stress signaling (Neill et al., 2002; Laloi et al., 2004; Shao et al., 2008), although they can attack a variety of biomolecules leading to cellular damage and death. Previous studies have shown that SO_2 exposure caused a selective increase of antioxidant enzyme activity, different gene expression patterns and DNA damage in plant cells (Deltoro et al., 1999; Willekens et al., 1994; Yi et al., 2005, 2009; Li et al., 2008). However, up to now, it is not clear whether and how ROS regulates the responses of plants to SO_2 stress.

Guard cells respond dynamically to multiple internal and external signals to regulate gas exchange. Change of guard cell viability would affect stomatal physiology and subsequent responses of plant to environment stress. It has been found that plant could adapt to SO_2 stress by controlling stomatal movement (Haworth et al., 2010; Li et al., 2008), but exposure to high concentrations of SO_2 caused a structural disorganization or death of guard cells (Black and Black, 1979; Yin et al., 2010). However, the exact mechanism of SO_2 toxicity is not yet fully understood and the precise mechanism by which death occurs is not clear. In the present study, guard cells of *Vicia faba* leaves, which are commonly used as a model system for studying the signal transduction in stomatal movement under environmental stress (Desikan et al., 2004; Hetherington, 2001; Zhang et al., 2001; Schroeder et al., 2001), were employed to investigate the cellular mechanism of SO_2 toxicity in plant cells. Our results show that SO_2 induced programmed cell death (PCD) through ROS mediating Ca^{2+} signal in SO_2-treated *Vicia* guard cells. This is the first time SO_2-induced programmed cell death has been found in higher plant cells.

2. Materials and methods

2.1. Plant materials

Seeds of *Vicia faba* L. (Jin) were grown in a greenhouse under sunlight at 21–28 °C with a 16 h photoperiod and a relative humidity of 45–90%. Supplemental light was provided to keep a 16 h/d photoperiod at 240 μmol m^{-2} s^{-1} by means of 400 W HPS lamps.

2.2. Preparation of epidermal strips

Young fully expanded leaves were harvested from 4-week-old *V. faba* plants. Epidermis was striped off with forceps and immediately floated in 10 mM MES buffer (a mixture of 10 mM 2-(N-morpholino)ethanesulfonic acid (MES; Bio Basic Inc), 50 mM KCl and 0.1 M tris-(hydroxymethyl) aminomethane (Tris), pH 7.0).

* Corresponding author.
E-mail address: yihuilan@yahoo.co.cn (H. Yi).

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2.3. Determination of cell viability

Epidermal strips were incubated in 10 mM MES buffer containing 1–4 mM SO_2 hydrates (a mixture of sodium sulfite and sodium bisulfite, 3:1 mM/mM, prepared freshly before use) for 3 h in white light at 23 °C as the treatment groups. Control samples were treated under the same conditions with 10 mM MES buffer but without SO_2 hydrates. The isolated strips were treated with a mixture of 3 mM SO_2 hydrates and a certain amount of antagonist to examine the protective effects of some antagonists of various death mediators. In the present study, caspase inhibitors Z–Asp-2, 6-dichlorobenzoyloxymethylketone (Z–Asp–CH_2–DCB; Alexis) and Nα–ρ-tosyl-L-lysine chloromethylketone (TLCK; AppliChem), antioxidants ascorbic acid (AsA, Sigma) and catalase (CAT, Sigma), and Ca^{2+} antagonists ethyleneglycoltetraacetic acid (EGTA, Sigma) and $LaCl_3$ were selected for the examination. After 3 h of chemicals exposure, the strips were stained with 0.1 mg/mL fluorescein diacetate (FDA; Bio Basic Inc). Cell viability was quantified by counting the percentage of fluorescent guard cells relative to total guard cells observed under fluorescence microscopy. At least three leaves and 150 guard cells per leaf were observed in each treatment and all of the experiments were independently repeated at least three times. Digital images were acquired on an Olympus microscope (BX51, Olympus, Japan) using an attached digital microscope camera (DP72, Olympus, Japan).

2.4. Measurement of reactive oxygen species

ROS generation in guard cells of epidermal peels was detected using 2′,7′-dichlorofluorescein diacetate (DCF-DA; Abcam) according to the methods described by Pei (Pei et al. ,2000). After 3 h of chemicals exposure, the strips were incubated in 20 μM DCF-DA for 30 min in the dark. Using an excitation wavelength of 488 nm together with 525-nm emission, images were captured under an Olympus DP72 microscope digital camera system using the Image Pro Plus (IPP) 6.0 software. The fluorescence signal of single guard cells was measured as pixel intensity. The mean values of DCF intensity were obtained from at least six leaves and 300 guard cells to estimate relative cellular levels of H_2O_2.

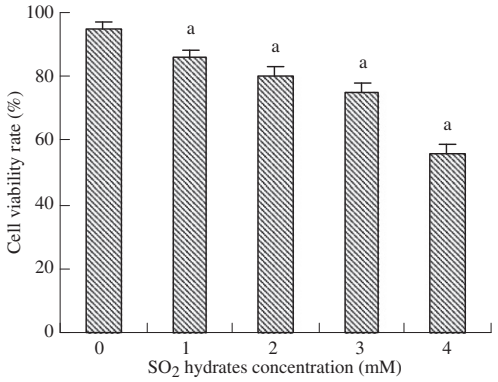

Fig. 1. SO_2-induced cell viability decrease in *V. faba* guard cells. a indicates a significant difference at the $P < 0.01$ level.

2.5. Measurement of intracellular Ca^{2+}

The concentration of intracellular Ca^{2+} ($[Ca^{2+}]_{cyt}$) was measured using fluo-3 acetomethoxyester (Fluo-3 AM; Abcam), a calcium fluorescent indicator. After 3 h of chemicals exposure, the strips were floated in 10 μM Fluo-3 AM for 50 min in the dark. Fluorescein fluorescence was excited at 488 nm and measured at 525 nm. The fluorescence signal of single guard cells was measured as pixel intensity. The mean values of Fluo-3 fluorescence intensity were obtained from at least six leaves and 300 guard cells to estimate $[Ca^{2+}]_{cyt}$.

2.6. Nuclear shape observation

Both propidium iodide (PI; Sigma) and Schiff's reagent were used to visualize the shape of guard cell nuclei. After 3 h of chemicals exposure, the strips were floated in 10 μM PI for 50 min in the dark, or stained with Schiff's reagent for 60 min. The cells are then observed under fluorescence microscopy.

2.7. 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. SO_2 induces programmed cell death features

Exposure for 3 h to 1–4 mM SO_2 hydrates not only caused a significant decrease in guard cell viability (Fig. 1and suppl. Fig. 1), but also induced the formation of abnormal cellular structures in guard cells in detached epidermis of *V. faba* leaves. The experiments were independently repeated three times with similar results. After staining with either PI or Schiff's reagent, some typical morphological features of PCD such as cytoplasmic shrinkage, nuclear condensation and nuclear fragmentation were observed in *Vicia* guard cells when epidermal strips were incubated in 1 to 4 mM SO_2 hydrates for 3 h (Fig. 2), suggesting a process of programmed cell death in SO_2-treated guard cells.

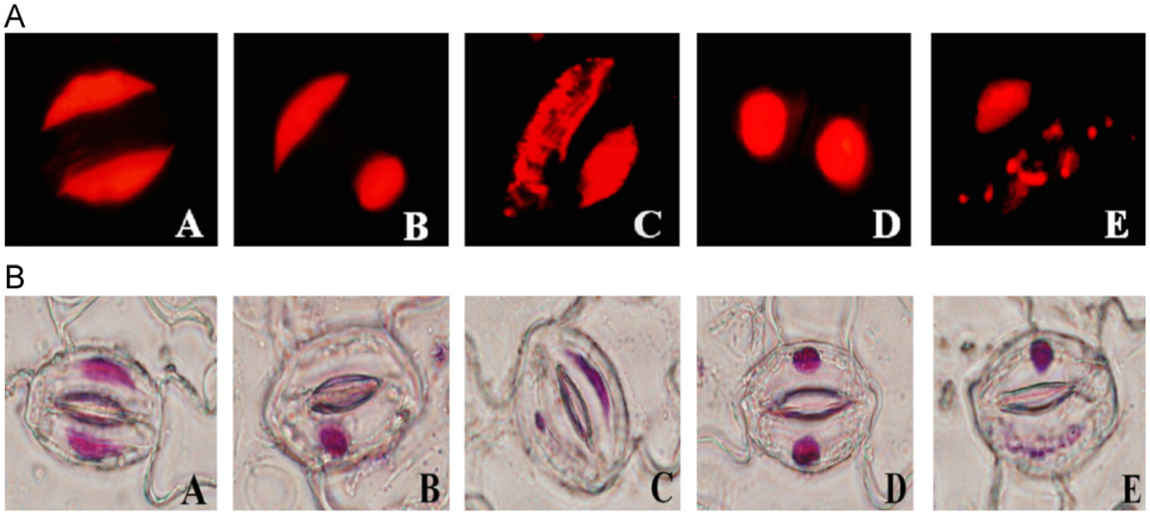

Fig. 2. Programmed cell death features in *V. faba* guard cells stained with either propidium iodide (PI, Fig. 1A) or Schiff's reagent (Fig. 1B) in control samples (A) and in the presence of 3 mM SO_2 hydrates (B,C,D and E). A—control. B and D—nuclear condensation. C and E—nuclear fragmentation. The same letters indicate the similar morphological features.