Effects of Hydrogen Sulfide on Alternative Pathway Respiration and Induction of Alternative Oxidase Gene Expression in Rice Suspension Cells

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The toxic effects of H_2S on plants are well documented. However, the molecular mechanisms reponsible for inhibition of plants by H_2S are still not completely understood. We determined the effects of NaHS in the range of 0.5–10 mM on the growth of rice suspension culture cells, as well as on the expression of the alternative oxidase (AOX) gene. AOX is the terminal oxidase of the alternative pathway (AP) and exists in plant mitochondria. The results showed that H_2S treatment enhanced the AP activity. During the process of H_2S treatment for 4 h, the AP activity increased dramatically and achieved the peak value at a concentration of 2 mM NaHS. Then it declined at higher concentrations of NaHS (5–10 mM) and maintained a steady level. The *AOX1* gene transcript level also showed a similar change as the AP activity. Interestingly, different NaHS concentrations seemed to have different effects on the expression of *AOX1a*, *AOX1b*, and *AOX1c*. The induction of *AOX* expression by low concentrations of NaHS was inferred through a reactive oxygen species (ROS)-independent pathway. At the same time, rice cells grown in culture were very sensitive to H_2S, different H_2S concentrations induced an increase in the cell viability. These results indicate that the H_2S-induced *AOX* induction might play a role in inhibiting the ROS production and have an influence on cell viability.

Key words: Alternative Pathway, Alternative Oxidase, Hydrogen Sulfide

Introduction

The mitochondria in higher plants have two respiratory pathways, a cyanide-sensitive cytochrome pathway (CP) and a cyanide-insensitive alternative pathway (AP). AP respiration is connected with the respiratory chain by an additional terminal oxidase-alternative oxidase (AOX). AOX is part of the branched respiratory electron transport chain. AOX accepts electrons from the ubiquinone pool and reduces oxygen to water (Millenaar and Lambers, 2003). The AP bypasses two of the three energy-conserving sites (complexes III and IV), and the potential energy of the system is lost as heat. So the accumulation of the AOX protein and enhancement of the AP respiration were initially considered to cause the increase in the temperature of plant tissues (Siedow and Umbach, 1995). Moreover, AP and AOX have many other functions, such as maintaining the stabilization of the respiratory electron chain and the tricarboxylic acid (TCA) cycle (Vanlerberghe and McIntosh, 1994), scavenging reactive oxygen species (ROS) in plant cells (Maxwell *et al.*, 1999; Møller, 2001), playing a role in the process of programmed cell death (PCD) (Vanlerberghe *et al.*, 2002), and maintaining plant growth rate homeostasis (Hansen *et al.*, 2002; Moore *et al.*, 2002). These facts suggest that the AP of higher plants is involved in responses to environmental stresses.

AOX is present in angiosperms; many algae and some fungi also contain the genetic capacity to express this respiratory pathway (Vanlerberghe and McIntosh, 1997). By the use of some techniques such as cDNA library and polymerase chain reaction (PCR), *AOX* is encoded by a small family of nuclear genes among a wide variety of non-thermogenic monocotyledon eudicotyledon plants such as tobacco (*Nicotiana rustinca* L.) (Vanlerberghe and McIntosh, 1994), *Arabidopsis* (Saisho *et al.*, 2001; Clifton *et al.*, 2005), soybean (*Glycine max*) (McCabe *et al.*, 1998), rice (*Oryza sativa* L.) (Saika *et al.*, 2002), and wheat (*Triticum*

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aestivum L.) (Takumi *et al.*, 2002). Differential expression of the *AOX* family genes in response to developmental cues and environmental perturbation raise a possibility that AOXs in higher plants have different roles and regulation mechanisms (Considine *et al.*, 2002; Clifton *et al.*, 2005).

Hydrogen sulfide (H_2S) is a well-known gas characterized by a peculiar rotten egg smell, representing a primary chemical hazard in natural gas production, in sewage treatment, and in certain industrial manufacturings (Patacchini *et al.*, 2004). Hydrogen sulfide is recognized to be a potent inhibitor of cytochrome c oxidase, the terminal enzyme of oxidative phosphorylation, and this is generally considered to be its primary mechanism of toxicity in animals (Reiffenstein *et al.*, 1992; Dorman *et al.*, 2002) and in plants (Kumazawa, 1984). The proposed mechanism for the inhibition of cytochrome c oxidase by H_2S is similar to that of hydrogen cyanide (HCN) and involves binding to the heme iron of the enzyme, with the greatest affinity being for the oxidized (Fe^{3+}) state (Eghbal *et al.*, 2004). However, we now know that H_2S is regarded as an important physiological messenger or bioactive molecule involved in many biological functions (Wang, 2002, 2003), such as smooth muscle relaxation (Zhao *et al.*, 2001; Eto *et al.*, 2002), facilitation of long-term potentiation (Abe and Kimura, 1996), modulation of smooth muscle tone with nitric oxide activity (Hosoki *et al.*, 1997), induction of cyclic AMP and modulation of NMDA receptors (Kimura, 2000), decreased release of corticotrophin releasing hormone (Russo *et al.*, 2000), and playing a major role in mediating the cytoprotection against oxidant-induced injury (Kimura *et al.*, 2006).

Based on previous studies, we know that H_2S plays an important physiological function in animals, and H_2S can inhibit cytochrome oxidase underlining the importance of cytochrome oxidase in the mechanism of HS^- (Khan, 1989). In this work, we investigated the activity of AOX induced or inhibited by H_2S. In addition, although there are numerous studies on differential expressions of the *AOX* family genes in response to developmental cues and environmental perturbations (Considine *et al.*, 2002; Clifton *et al.*, 2005), few researches concentrated on the relationship between the expression of the *AOX* family genes and H_2S. As we know, AOX plays a role in lowering ROS formation in plant mitochondria, and in animal cells H_2S protects neuron cells from oxidative stress (Kimura *et al.*, 2006). The purpose of the present study was to investigate the effect of H_2S on the respiratory capacity of plant mitochondria, especially to provide a more comprehensive rationalization of the differential expression of the *AOX1* family genes in rice suspension cells with H_2S treatment, and provide a new biological function of H_2S except of toxicity in plant cells.

Material and Methods

Plant material and treatment

Rice cultivars were provided by Zhonghua 11 (*Oryza sativa* L. ssp. *japonica*). Seeds were dehulled and sterilized in 70% ethanol for 1 min and then in 50% commercial bleach with a few drops of Tween 20 for 30 min. The sterilized seeds were washed 5 times with sterile distilled water and placed on LS medium (Thompson *et al.*, 1986) supplemented with 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 3 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), 3% sucrose, and 0.7% agar for induction of calli in the dark at 26 °C. Friable calli were selected and transferred to liquid AA medium (Thompson *et al.*, 1986) supplemented with 2 mg L^{-1} 2,4-D, 3 mM MES, and 3% sucrose for 7 weeks. Cell suspensions were subcultured every 7 d in fresh culture medium [1:5 (v/v) dilution ratio] and shaken on a rotary shaker at 140 rpm and (26 ± 1) °C in the dark. The cell lines were maintained on each medium for at least 2 months before growth rates were measured. These cell lines were used for the experiments.

By treatment of suspension cells wih the H_2S donor sodium hydrogen sulfide (NaHS), inhibition of cytochrome oxidase took place in the dark. NaHS was filter-sterilized immediately before its addition. To minimize loss and spontaneous oxidation of volatile, redox-labile HS^-, vented caps were quickly replaced with non-vented caps for the entire length of the experimental incubation. For treatment experiments, cells were assayed 3 d after subculture. Cells were incubated with NaHS at concentrations of 0.5, 1.0, 2.0, 5.0, and 10.0 mM for 4 h, respectively. Control samples were treated with sterile water parallel to NaHS-treated samples.

Rice suspension cells respiration measurement

After pretreated with NaHS, rice suspension cells [approx. 0.5 mg dry weight (DW) mL^{-1} cul-

ture medium] were washed 5 times with sterile distilled water and placed on a cuvette. The capacity of alternative respiratory pathway was determined at 26 °C using a Clark-type electrode (Institute of Plant Physiology & Ecology, Chinese Academy of Sciences, Shanghai) by measuring the cyanide-insensitive oxygen uptake, which was corrected by remitting residual respiration (Møller *et al.*, 1988; Bingham and Farrar, 1989). The capacity of cyanide-resistant respiration was calculated as the difference between the total respiration in the presence of 1 mM KCN and the residual respiration. The residual respiration was measured in the presence of 5 mM SHAM plus 1 mM KCN as inhibitors. 5 mM SHAM inhibited the cyanide-resistant respiration by about 80% and 1 mM KCN inhibited the O_2 consumption by the cytochrome pathway by about 78%. Results represent the average of five independent experiments.

RNA extraction and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of AOX family's expression

The samples of rice suspension cells (~100 mg) were grounds in liquid nitrogen, then homogenized in 1 mL of ice-cold TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted and treated with RNase-free Dnase (TaKaRa, Dalian, China). The total RNA concentration and purity were determined by electrophoresis on an ethidium bromide-stained 1.5% agarose gel followed by UV illumination and measurement of absorbance at 260 and 280 nm. cDNA for RT-PCR was synthesized from each 3 μg of total RNA with M-MLV reverse transcriptase (Invitrogen) in 20 μL reaction volumes. The cDNA was diluted 1:10 and 10-μL aliquots of cDNA were used as template for PCR amplification in 50-μL standard reactions. Each reaction mixture contained 10 μL cDNA as template, 5 μL of 5× RT-PCR buffer, 0.8 μL of dNTP mix (400 μM), 1.2 μL of each primer (0.6 μM), 0.5 μL of rTaq Polymerase (TaKaRa), and RNase-free water in a final volume of 50 μL. Rice *β-actin* served as an internal control gene. The RT-PCR profile was one cycle of cDNA synthesis at 37 °C for 50 min and one cycle of initial PCR activation at 95 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s, and extension at 72 °C for 30 s, followed by one cycle of final extension at 72 °C for 10 min. After RT-PCR, aliquots of the RT-PCR products were electrophoresed through 1.2% agarose gels (Bio-Rad, Hercules, CA, USA) containing 0.5 μg μL^{-1} of ethidium bromide (Bio-Rad), and gels were visualized under UV light and photographed. Semi-quantitative analysis of blots was obtained using Gelanalysis software (Syngene, Cambridge, UK). The primers P1 and P2 were used to obtain a probe specific for *OsAOX1a*, P3 and P4 for *OsAOX1b*, P5 and P6 for *OsAOX1c*, and P7 and P8 for *β-actin* (accession numbers of OsAOX1a, OsAOX1b, OsAOX1c, and β-actin are AB007452, AB004865 and AB074005, X16280, respectively):
P1, 5'-TTTGTCTACTGCCGAGGATT-3';
P2, 5'-ACTCAGCTGCCAATAGTTCA-3';
P3, 5'-ATCATCAACCGGAAGTCAAG-3';
P4, 5'-CCTTCTTGTTCTGCGGGTC-3';
P5, 5'-AGCAGCGTCTCCACGATC-3';
P6, 5'-GGGACTCGCAGTCGCACT-3';
P7, 5'-ATGACCCAGATCATGTTTGAG-3;
P8, 5'-CACTGAGAACGATGTTGCCAT-3'.

Determination of H_2O_2 content

Hydrogen peroxide levels were determined according to Sergiev *et al.* (1997). Rice suspension cells (approx. 0.5 mg DW) were homogenized in an ice bath with 5 mL 0.1% (w/v) TCA. The homogenate was centrifuged at 12,000 × *g* for 15 min, and 0.5 mL of the supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M KI. The absorbance of the supernatant was read at 390 nm. The content of H_2O_2 was given on a standard curve.

Viability assay procedures

Triphenyltetrazolium chloride (TTC) reduction assay

The TTC (2,3,5-triphenyltetrazolium chloride) reduction assay was modified from the method of Steponkus and Lanphear (1967). The aliquots of cell culture, containing 25 mg fresh weigh (FW) of cells, were incubated in 1 mL of TTC solution (23.9 mM TTC in 0.05 M potassium phosphate buffer, pH 7.5) for 4 h at 23 °C in the dark. The samples were then centrifuged for 5 min at 300 × *g*. The TTC solution was aspirated, and cells were subsequently incubated in 1 mL of 98% ethanol for 15 h at 55 °C. After centrifugation for 2 min at 8,800 × *g*, the absorbance of supernatant was read at 485 nm.

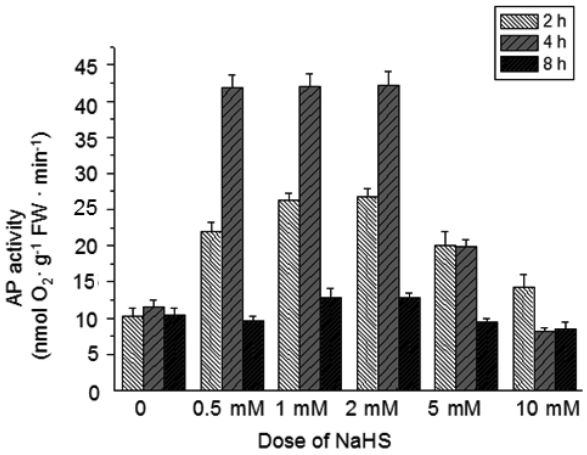

Fig. 1. Rice suspension cells (a 3-d-old culture) were supplied with culture medium containing different concentrations of NaHS to determine the AP activity periodically for an 8-h period. Bars represent the standard deviations of five independent replicates.

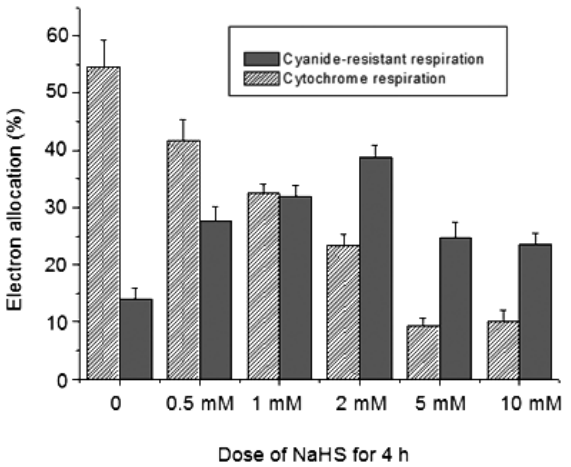

Fig. 2. Effects of NaHS on the allocation of respiration electron flux between the CP and AP. The allocation of respiration electron flux between the CP and AP was recorded by the percentages of the CP and AP taken from total respiration. Bars represent the standard deviations of five independent replicates.

Evans blue uptake

The aliquots of cell culture, containing 50 mg FW of cells, were incubated for 15 min with 0.05% Evans blue solution and then washed extensively to remove excess of unbound dye. Dye bound to dead cells was solubilized in a solution of 50% methanol and 1% SDS for 30 min at 50 °C and quantified through absorbance at 600 nm (Levine *et al.*, 1994). The viability of NaHS-treated cells, analysed by both methods, was expressed as percent fraction compared with control cells (100%).

Statistical analysis

The results were means of five independent measurements and were statistically evaluated using the standard deviation and t-test methods. The difference was considered to be statistically significant when $P < 0.05$.

Results

Effect of H_2S treatment on the AP activity

The AP activity respresents the ability of the plant to transport a respiratory electron flow along AP respiration. As shown in Fig. 1, rice suspension cells (a 3-d-old culture) were supplied with culture medium containing different concentrations of NaHS and the AP activity was determined periodically for an 8-h period. H_2S enhanced the activity of the AP in rice suspension cells for 2 and 4 h. During the process of H_2S treatment, the AP activity increased dramatically and achieved the peak value after 4 h of treatment, and then declined gradually till 8 h. Based on these data, we choose the rice suspension cells treated with H_2S for 4 h to analyse the respiration electron flux between the AP and the CP, the AOX1 multigene family transcription level.

Effects of H_2S treatment on the allocation of respiration electron flux between the CP and AP

Since the AP is an electron transport chain which branches to the site of ubiquinone and shares the common ubiquinone pool with the CP, they may impact on each other. We compared the changes in contributions of the CP and AP to total respiration during H_2S treatment for 4 h in order to observe the effects of H_2S on allocation of respiration electron flux between the CP and AP (Fig. 2). In the control, the portion of electron flux contributed by the CP was much higher than that of the AP, suggesting that the CP was the main electron transport pathway in rice suspension cells under normal growth conditions. However, as the concentration of NaHS increased, the portion of the CP declined quickly and reached the rock bottom at a concentration of 5 mM. On

the contrary, the portion of the AP was obviously enhanced by H_2S and achieved the peak value at a concentration of 2 mM.

Effects of H_2S treatment on the AOX1 family genes transcription level

As shown in Fig. 3, NaHS treatment changed the level of *AOX1* family genes transcripts. During NaHS incubation for 4 h, the *AOX1* family genes transcription levels were different. In the control, the transciption levels of *AOX1a* and *AOX1b* were detected but that of *AOX1c* was not detected. During NaHS incubation, the *AOX1a* transcription level increased quickly at the concentration of 0.5 mM and then remained at the stable level. The *AOX1b* transcription level increased and achieved the peak value at the concentration of 2 mM, with a level about 2-fold that of the beginning. But it decreased steadily until no band was detected at the concentration of 10 mM. However, the level of *AOX1c* mRNA increased quickly when the concentration of NaHS increased, and it achieved the peak value at the concentration of 10 mM.

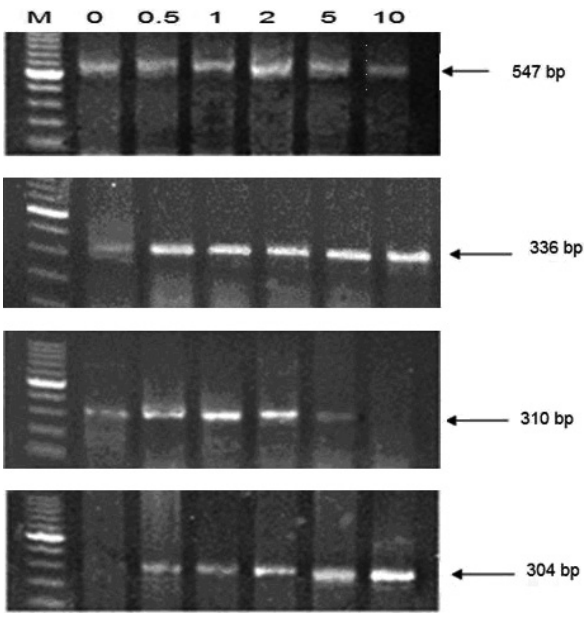

Fig. 3. Induction of *AOX1* gene expression in rice suspension cells by NaHS. Cell suspension cultures were treated with different concentrations (0, 0.5, 1, 2, 5, 10 mM) of NaHS (H_2S donor). Cells were collected after 4 h of incubation for quantiative reverse transcription-polymerase chain reaction (RT-PCR). The marker was 100-bp ladder. Representative results are presented from five independent experiments.

Determination of H_2O_2 content

As shown in Fig. 4, NaHS treatment changed the rate of H_2O_2 production dramatically. A significant decrease in intracellular H_2O_2 formation was found after 4 h of exposure to different concentrations of NaHS. At the concentration of 2 mM, the production of H_2O_2 reached the rock bottom. With the increment of exposure concentration, the H_2O_2 content of treated cells increased but reached not the level of control cells.

Effect of NaHS on the viability of suspension culture cells of rice

The viability of rice suspension cells in the presence of various concentrations of NaHS was analysed by two independent methods: triphenyltetrazolium chloride (TTC) reduction assay and Evans blue uptake (Figs. 5A, B). The data obtained by both methods showed that NaHS at the concentrations ranging from 0.5 to 2 mM did not cause a decrease in cell viability. At the concentration of 2 mM, the viability of rice suspension cells reached the peak value. By means of the TTC reduction assay, the rate of the cell viability increase was significant, the viability of

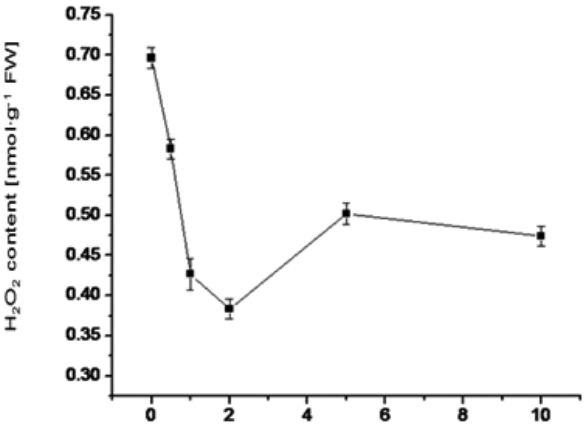

Fig. 4. Changes of hydrogen peroxide (H_2O_2) contents in rice suspension cells after 4 h exposure to different concentrations (from 0 to 10 mM) of NaHS. Bars represent the standard deviations of five independent replicates.

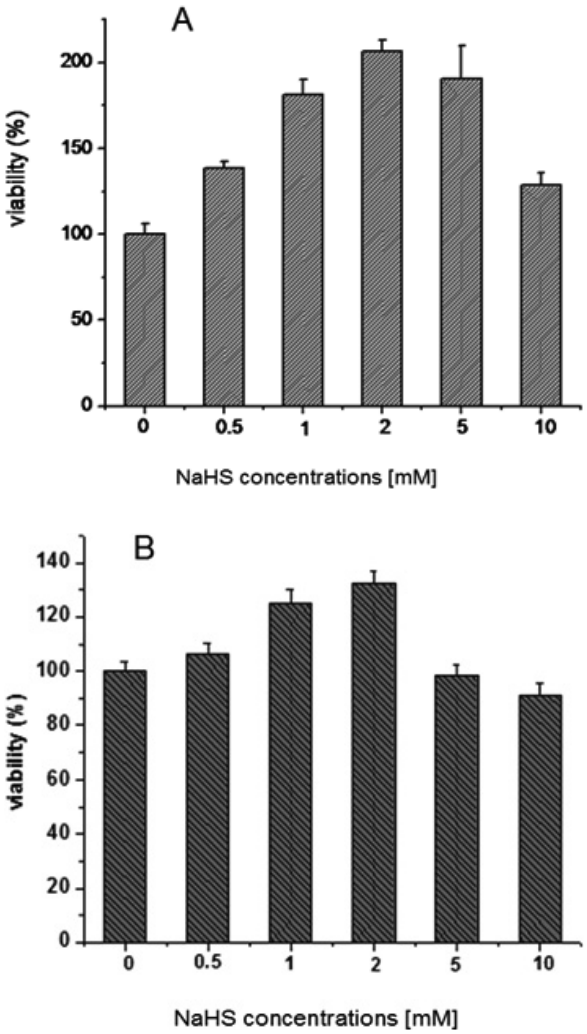

Fig. 5. Viability of rice suspension cells after 4 h of treatment with various concentrations of NaHS, analysed by (A) the TTC reduction assay and (B) Evans blue uptake. Viability of NaHS-treated cells analysed by both methods is expressed as percent of control cells' viability (100%). Bars represent the standard deviations of five independent replicates.

the cells increased above 2-fold in response to 2 mM NaHS treatment. With the increment of exposure concentration, the viability of treated cells decreased.

Discussion

H_2S has been generally considered as a toxic gas found in the contaminated environmental atmosphere. Its major toxic effects are toxication of the central nervous system in animals and inhibition of oxidative phosphorylation both in animals and plants (Beauchamp *et al.*, 1984; Guidotti, 1996; Park *et al.*, 2000; Nicholls, 1975). However, until recently, people came to know the physiological effect of H_2S. H_2S is endogenously generated via non-enzymatic and enzymatic pathways in mammals. Cystathionine b-synthase in the central nervous system and cystathionine g-lyase in the cardiovascular and gastrointestinal systems are two enzymes mostly responsible for H_2S generation from cysteine. The endogenous production of H_2S and its possible physiological functions, including membrane hyperpolarization and smooth muscle cell relaxation (Hosoki *et al.*,1997; Zhao *et al.*, 2001), position this gas well in the family of gastransmitters together with nitric oxide (NO) and carbon monoxide (CO). Therefore, H_2S can be considered a double-edged sword.

Many studies demonstrated that H_2S is an inhibitor of cytochrome oxidase. In higher plants, the mitochondria have two respiratory pathways, a cyanide-sensitive cytochrome pathway and a cyanide-insensitive alternative pathway. However, there is little information in the literature concerning the possible role of H_2S in plants. Thus, our study showed that a low dose of H_2S can inhibit the activity of cytochrome oxidase and stimulate the AOX activity as well as improve the cell viability.

In our experiment, we used incubation media containing NaHS which were used as a source of H_2S for the following reasons. First, NaHS dissociates to Na^+ and HS^- in solution, then HS^- associates with H^+ and produces H_2S. It does not matter whether the H_2S solution is prepared, by bubbling H_2S gas or by dissolving NaHS. In physiological saline, approx. one third of the H_2S exists as undissociated form (H_2S), and the remaining two thirds exist as HS^- at equilibrium with H_2S (Reiffenstein *et al.*, 1992). The use of NaHS enables us to define the concentration of H_2S in solution more accurately and reproducibly than bubbling H_2S gas. For these reasons, NaHS has been widely used for studies on H_2S (Beauchamp *et al.*, 1984; Warenycia *et al.*, 1989; Kombian *et al.*, 1993).

We all know that plant mitochondria possess AOX. From Fig. 2, we observed that AOX is resistant to H_2S when cytochrome oxidase was greatly inhibited. Results showed that during the process of H_2S treatment, the AP increased with a peak value at 2 mM NaHS. Consequently, we pre-

sume that AOX may play a role in the observed H_2S tolerance of higher plants.

Considering the hypothesis that AOX counteracts the action of H_2S, we speculated whether H_2S could actually have a stimulating effect on AOX activity and/or *AOX* transcription. In order to distinguish among the three *AOX* genes of the *AOX1* family present in the rice genome, we constructed specific probes as previously reported (Ohtsu *et al.*, 2002). From Fig. 3, in the control cells, the mRNAs of *AOX1a* and *AOX1b* could be detected while the *AOX1c* mRNA could not be detected. During the NaHS treatment, the mRNA levels of AOX1 family genes increased. However, different NaHS concentrations seems to have differential effects on transcription level. The stimulation of *AOX1a* transcription was significantly increased by the low concentration of NaHS and remained at high level when the concentration increased. Depending on the concentration, the *AOX1c* transcription level increased gradually. Although the *AOX1b* transcription was increased by the low concentrantion of NaHS, it was inhibited at high concentration. In our experiments, the expressions of *AOX1a* and *AOX1c* were obviously increased at the concentration of 10 mM, but *AOX1b* was hardly detected. Combining this observation with our results, *AOX* transcripts would be up-regulated by H_2S treatment. Consequently, the treatment of rice suspension cells with NaHS caused a significant shift of electrons from cytochrome to the AP and an increase of the activity of AP respiration (Fig. 2). But the reasons of differential effects on the *AOX1* family genes transcription level at the high concentration are still needed to be confirmed.

In general, the AP may dampen the generation of ROS during periods of rapid respiration (Maxwell *et al.*, 1999). In the present study, H_2S induced an increased AP activity and *AOX* transcription level, but production of H_2O_2 was decreased. Therefore, we considered that H_2S in our work regulated the *AOX* transcription mainly through the ROS-independent pathway, and AOX can dampen the generation of ROS.

Data presented here indicate that rice cells grown in culture are very sensitive to H_2S, different H_2S concentrations induced an increase in cell viability (Fig. 5). The data obtained by both methods showed that the lower concentration of NaHS (0.5, 1, 2 mM) caused an increase in cell viability, especially at the concentration of 2 mM. However, the changes of the cell viability caused by the TTC reduction assay were more significant than caused by Evans blue uptake. An increase in cell viability was observed in the presence of high concentration (5, 10 mM) of NaHS as compared to control cells by means of the TTC reduction assay, whereas the cell viability at high concentration (5, 10 mM) of NaHS was lower than that of control cells as evaluated by the Evans blue uptake. The different methods could play different roles in the assay of cell viability. The TTC method is based on the ability of mitochondria to reduce TTC to formazon by mitochondrial respiration in living cells (Engelmann, 1991). On the other hand, uptake of Evans blue is an indicator of the loss of the plasma membrane integrity as Evans blue is a non-permeating dye in living cells (Yamaguchi *et al.*, 1999). Hence, the decrease in Evans blue uptake by rice suspension cells in the presence of low concentration of NaHS (0.5, 1, 2 mM) may be therefore connected with a decrease in cell membrane permeability. In other words, the high concentration of NaHS (5, 10 mM) may damage the plasma membrane integrity and increase the capacity to uptake Evans blue.

In conclusion, it has been shown that suspension-cultured rice cells respond differentially to various H_2S concentrations: lower (0.5–2 mM) and higer (≥5 mM) doses of NaHS have opposite effects on cell viability and expression of *AOX1* genes family. The H_2S-induced *AOX* induction might play a role in inhibiting the ROS production and improving cell viability. In other words, the low concentration of H_2S may therefore play a significant protective role in rice suspension cells. This function might be very important, because the effects of H_2S on the plant is beneficial at lower concentration whereas toxic at higher concentration. The data presented here would constitute a starting point to more advanced study that will focus on the effects of H_2S on signal transduction and protection cells from oxidative stresses.

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