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 results showed that H$_2$S treatment enhanced the AP activity. During the process of H$_2$S 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$_2$S, different H$_2$S concentrations induced an increase in the cell viability. These results indicate that the H$_2$S-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
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$_2$S) 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., 2004). However, we now know that H$_2$S 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$_2$S plays an important physiological function in animals, and H$_2$S 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$_2$S. 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$_2$S. As we know, AOX plays a role in lowering ROS formation in plant mitochondria, and in animal cells H$_2$S protects neuron cells from oxidative stress (Kimura et al., 2006). The purpose of the present study was to investigate the effect of H$_2$S 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$_2$S treatment, and provide a new biological function of H$_2$S 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 dehusled 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-dichlorophenoxacytic 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 with the H$_2$S 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-
rive 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 grown 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 photography. The residual respiration was measured in the presence of 5 mM KCN and the residual respiration was inhibited 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.

**Determination of H$_2$O$_2$ 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$_2$O$_2$ 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 weight (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.
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$_2$S and achieved the peak value at a concentration of 2 mM.

Effects of H$_2$S 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 transcription 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.

Determination of H$_2$O$_2$ content

As shown in Fig. 4, NaHS treatment changed the rate of H$_2$O$_2$ production dramatically. A significant decrease in intracellular H$_2$O$_2$ formation was found after 4 h of exposure to different concentrations of NaHS. At the concentration of 2 mM, the production of H$_2$O$_2$ reached the rock bottom. With the increment of exposure concentration, the H$_2$O$_2$ 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

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**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$_2$S donor). Cells were collected after 4 h of incubation for quantitative reverse transcription-polymerase chain reaction (RT-PCR). The marker was 100-bp ladder. Representative results are presented from five independent experiments.

**Fig. 4.** Changes of hydrogen peroxide (H$_2$O$_2$) 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.
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$_2$S. H$_2$S 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$_2$S generation from cysteine. The endogenous production of H$_2$S 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 transmitters together with nitric oxide (NO) and carbon monoxide (CO). Therefore, H$_2$S can be considered a double-edged sword.

Many studies demonstrated that H$_2$S 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$_2$S in plants. Thus, our study showed that a low dose of H$_2$S 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$_2$S for the following reasons. First, NaHS dissociates to Na$^+$ and HS$^-$ in solution, then HS$^-$ associates with H$^+$ and produces H$_2$S. It does not matter whether the H$_2$S solution is prepared, by bubbling H$_2$S gas or by dissolving NaHS. In physiological saline, approx. one third of the H$_2$S exists as undissociated form (H$_2$S), and the remaining two thirds exist as HS$^-$ at equilibrium with H$_2$S (Reiffenstein et al., 1992). The use of NaHS enables us to define the concentration of H$_2$S in solution more accurately and reproducibly than bubbling H$_2$S gas. For these reasons, NaHS has been widely used for studies on H$_2$S (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$_2$S when cytochrome oxidase was greatly inhibited. Results showed that during the process of H$_2$S 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$_2$S tolerance of higher plants.

Considering the hypothesis that AOX counteracts the action of H$_2$S, we speculated whether H$_2$S 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 seem 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 concentration 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$_2$S 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$_2$S induced an increased AP activity and AOX transcription level, but production of H$_2$O$_2$ was decreased. Therefore, we considered that H$_2$S 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$_2$S, different H$_2$S 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 formazan 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$_2$S concentrations: lower (0.5–2 mM) and higher (≥5 mM) doses of NaHS have opposite effects on cell viability and expression of AOX1 genes family. The H$_2$S-induced AOX induction might play a role in inhibiting the ROS production and improving cell viability. In other words, the low concentration of H$_2$S may therefore play a significant protective role in rice suspension cells. This function might be very important, because the effects of H$_2$S 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$_2$S on signal transduction and protection cells from oxidative stresses.

Acknowledgement

This work was supported by grants from National Natural Science Foundation of China (No. 30471133 and No. 30670070).
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