Enhanced Expression of Alternative Oxidase Genes Is Involved in the Tolerance of Rice (*Oryza sativa* L.) Seedlings to Drought Stress

Han-Qing Feng[a,b,*], Hong-Yu Li[b,*], and Kun Sun[a,*]

[a] College of Life Science, Northwest Normal University, 730070, China.
Fax: 86-931-7971207. E-mail: fenghanq@nwnu.edu.cn
[b] MOE Key Laboratory of Arid and Grassland Ecology, School of Life Sciences, Lanzhou University, 730000, China

* Authors for correspondence and reprint requests

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Drought stress significantly enhanced the capacity of the alternative respiratory pathway and induced AOX1a and AOX1b transcripts in rice seedling leaves. The drought-stressed seedlings pretreated with the inhibitor of the alternative respiratory pathway, 1 mM salicylhydroxamic acid, had a lower level of relative water content than the seedlings either subjected to drought or salicylhydroxamic acid treatment alone. This observation suggests that the alternative respiratory pathway could play a role in the tolerance of rice seedlings to drought stress. Pretreatment with exogenous hydrogen peroxide, salicylic acid, and abscisic acid alone mitigated the water loss of rice leaves exposed to drought stress. Exogenous application of hydrogen peroxide and salicylic acid increased the capacity of the alternative respiratory pathway and induced AOX1a and AOX1b transcripts, while exogenous abscisic acid failed to induce any expression of AOX1 genes. These observations suggest that rice AOX1a and AOX1b genes may be responsive especially to drought stress but not be induced by all of the stress signals related to drought.

Key words: Rice, Alternative Respiratory Pathway, Drought Stress, Stress Signals

Introduction

Drought stress is one of the major environmental factors that affect plant growth and development (Boyer, 1982). To survive, plants can respond and adapt to drought stress by altering their cellular metabolism and invoking various defence mechanisms (Bohnert and Jensen, 1996). Mitochondrial respiration has a central role in controlling the energy and carbon metabolism of higher plants. However, the knowledge about respiratory responses to drought stress is limited and this is considered to be an important issue that needs to be addressed in the near future (Flexas *et al*., 2005).

Plant mitochondria are different from animal mitochondria in that they have an alternative oxidase (AOX). It is well known that AOX catalyzes the cyanide (CN)-resistant respiration (alternative respiratory pathway or AOX pathway), which branches from the main respiratory chain at the level of ubiquinone and thus bypasses two of three sites of energy conservation supporting oxidative phosphorylation (Millenaar and Lambers, 2003). Based on the studies of molecular distinction among AOX from different plant species, it is found that there exist two discrete AOX gene subfamilies: AOX1-type and AOX2-type genes. Generally, AOX1 is most widely known for its induction by stress stimuli in many tissues and is present in both monocot and eudicot plant species, but AOX2 is usually constitutive or developmentally expressed in eudicot species and is absent from the genomes of all monocot species (Considine *et al*., 2002).

Much works have shown that the levels of the alternative respiratory pathway and AOX protein amounts are increased during various stressful conditions including chilling (Vanlerberghe and McIntosh, 1992), phosphate limitation (González-Meler *et al*., 2001), and plant diseases (Simons *et al*., 1999). Recently, using wheat leaves, Bartoli *et al*. (2005) found that the levels of both the re-

Abbreviations: ABA, abscisic acid; AOX, alternative oxidase; ROS, reactive oxygen species; RWC, relative water content; SA, salicylic acid; SHAM, salicylhydroxamic acid.

duced and oxidized forms of the AOX protein increased in the process of dehydration, consequently resulting in an increase in the capacity of the alternative respiratory pathway. However, in soybean leaves, drought stress only caused a significant shift of electrons from cytochrome to the alternative pathway but did not affect the level of AOX protein (Ribas-Carbo *et al.*, 2005). Considering the difference in the results above, it is needed to further investigate whether drought stress regulates the alternative pathway in the transcription level or not. In addition, whether the alternative pathway contributes to plant resistance to drought is not fully understood yet.

Plants can adapt to drought stress by generating and transmitting various defense signals (Dat *et al.*, 2000). The ABA (abscisic acid) level increases as a result of drought stress and plays an important role in regulating the responses of plant to drought stress (Shinozaki and Yamaguchi-Shinozaki, 1997). Drought stress also leads to a marked increase of ROS (reactive oxygen species) production, in which hydrogen peroxide (H_2O_2) is believed as an acclamatory signal to induce plant defence against drought stress (Dat *et al.*, 2000). Recent works showed that salicylic acid (SA) also functions in inducing plant responses to drought, high salt and other osmotic stresses (Senaratna *et al.*, 2000; Borsani *et al.*, 2001). These various defense signals selectively induce defense mechanisms in plants for an optimal resistance being attained. However, the signal for regulating the AOX expression under drought stress remains unknown.

In the present work, the specific probes for rice AOX1a, AOX1b, and AOX1c were used to reveal the effects of drought stress on the expression of the rice AOX1 multigene family. The research reported here also investigated the contribution of the alternative respiratory pathway to drought tolerance. Simultaneously, an effort was made to demonstrate possible regulations of the alternative pathway under drought stress.

Material and Methods

Plant material and treatment

Rice (*Oryza sativa* L.) seeds (Luoyang Academy of Agricultural Sciences, China) were treated with 1% NaOCl for 10 min and swollen in distilled water for 10 h at room temperature. The rice seeds were germinated at 26 °C for 24 h and then planted in plastic pots each containing 10 cultivars. Germinated seeds were grown and well irrigated for 12 d at (26 ± 1) °C with 12 h light/12 h dark cycles.

Twelve-day-old seedlings were placed in distilled water. The ends of the stems were covered with filter paper. For exogenous application of stress signals, the leaves were supplied with 1 mM SA, 5 mM H_2O_2, or 1 mM ABA, respectively, for 24 h under dark conditions. For inhibition of AOX activity, the leaves of seedlings were supplied with 1 mM salicylhydroxamic acid (SHAM) for 4 h under dark conditions. Control experiments showed that supplements of the solvent of these chemical solutions alone to leaves had no significant effects on any of the experimental parameters measured (data not shown). After these treatments, the seedlings were transferred to a beaker containing PEG6000 solutions at –0.7 MPa for drought stress treatment or to a beaker containing distilled water as a control. These seedlings were kept in the dark for 24 h.

Respiration of leaves

The apical 2 cm of leaves from 3~4 plants (separately grown in individual pots with the same treatment) were detached for a single measurement of the cyanide-insensitive oxygen uptake [*i.e.*, the capacity of the alternative respiratory pathway; Vanlerberghe and McIntosh (1992)]. The detached leaves were weighed and cut with razor blades into small pieces. The pieces were transferred into an air-tight cuvette. The capacity of the alternative respiratory pathway (V_{alt}) was measured at 26 °C using a Clark-type electrode (Institute of Plant Physiology & Ecology, Chinese Academy of Sciences) by measuring the cyanide-insensitive oxygen uptake, which was corrected by remitting residual respiration (Bingham and Farrar, 1989). V_{alt} was calculated as the difference between the total respiration in the presence of 1 mM KCN and the residual respiration (V_{res}) which was measured in the presence of two inhibitors: 5 mM SHAM and 1 mM KCN. Results represent the average of four independent experiments.

Preparation of the special probes for AOX1a, AOX1b, and AOX1c

The probes for AOX1a, AOX1b, and AOX1c were made by PCR.

Primers 1 and 2 (P1: 5'-GATGTTTGTCTACTGCCGAGGATTT-3'; P2: 5'-ATGTAGTATATATAACTCAGCTGCC-3') were used to obtain a specific probe for AOX1a; primers 3 and 4 (P3: 5'-TCATCATTCATCAACGGGCGATGC-3'; P4: 5'-TGTGCACGGGTCAGCCAACGGCCA-3') were used to obtain a specific probe for AOX1b; and primers 5 and 6 (P5: 5'-CTGAAGAAATCTTACGGCGG-3'; P6: 5'-CCAAACAGATAACAGGACGC-3') were used to obtain a specific probe for AOX1c. The rice total DNA was extracted from the leaves as template. PCR with *Taq* DNA Polymerase (Sangon Inc., Shanghai, China) was carried out for 37 cycles, each consisting of 30 s at 95 °C, 30 s at 45 °C, and 1 min at 72 °C. The products were extended at 72 °C for 5 min and held at 4 °C for 1 min. The PCR products were cloned into the Pgt vector and were transformed into the DH5α strain. Nucleotide sequences of the inserts were determined by using an automatic DNA sequencer (Beckman Co., Palo Alto, USA). The DNA sequencing data of PCR production was analyzed with the chromas1.45 software.

Extraction of the total RNA and Northern hybridization

Total RNA was extracted using the Total RNA Trizol Extraction Kit (Sangon Inc., Shanghai, China). Total RNA was quantified using the UV-VIS spectrophotometer Tu-1800 (Purkinje General Inc., Beijing, China). Equal amounts of RNA (based on OD260) were loaded per lane in 0.8% agarose gel with 1×Tris-acetate-EDTA (TAE) buffer, separated by electrophoresis. Northern hybridization was performed with the ECL DNA Labeling and Detection Kit (Enzo Diagnostics Inc., Buckinghamshire, UK) following the manufacturer's instructions.

Determination of relative water contents

The relative water content (RWC) was measured to monitor the leaf water status and was calculated according to: RWC = (FW–DW)/(TW–DW), where FW is the fresh weight, DW is the dry weight obtained after over-drying the leaf samples for 24 h at 70 °C, and TW represents the turgid fresh weight. Leaf segments were hydrated to full turgidity by floating in distilled water for 3 h.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Data was analyzed using the Kruskall-Wallis one-way analysis of variance test. $P < 0.05$ was considered statistically significant.

Results

Effects of drought stress on the capacity of the alternative respiration pathway and AOX1s expression in rice seedling leaves

Drought stress caused a significant increase in the capacity of the alternative pathway of rice leaves. The present work shows that the capacity of the alternative pathway (V_{alt}) increased from 0.187 μmol O_2 g^{-1} FW min^{-1} in well-irrigated plants to 0.274 μmol O_2 g^{-1} FW min^{-1} after drought stress treatment.

In the control leaves, no steady level of AOX mRNA was detected. Under the conditions of drought stress, both AOX1a and AOX1b transcripts were obviously increased (Fig. 1). And, the level of AOX1a mRNA seemed higher than that of AOX1b under the conditions of drought stress. But AOX1c transcripts were hardly detected in the same samples (Fig. 1).

Inhibition of the alternative respiration pathway by SHAM

SHAM, a special inhibitor of AOX activity, has been used in AOX studies on intact tissues (Chivasa and Carr, 1998; Naylor *et al.*, 1998; Bartoli *et al.*, 2005). To determine the concentration of SHAM used, we mimicked a titration experiment simi-

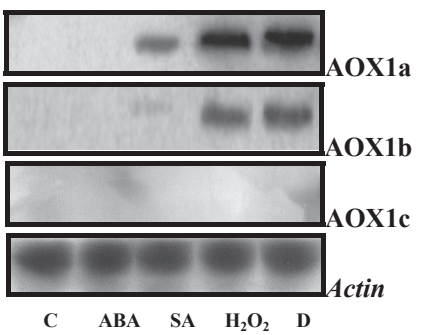

Fig. 1. The effect of exogenous ABA, SA, H_2O_2, and drought stress (D) on the AOX1 transcripts levels in leaves of rice seedlings. There was no AOX gene expression detected in all of the control leaves (C).

lar to that done by Bartoli *et al.* (2005). *In vivo* the cyanide-insensitive respiration oxygen uptake was reduced by 27–83% when 12-day-old leaves grown under a light/dark cycle were treated with 0.1 to 10 mM SHAM. Treatment with 1 mM of the SHAM inhibited the cyanide-insensitive oxygen uptake by 68.4% (Fig. 2). The inhibition of the AOX pathway did not increase linearly with the increase of the SHAM concentration, although higher concentrations of SHAM led to further inhibition of the AOX pathway. This result appeared to be similar with the observations by Bartoli *et al.* (2005). Moreover, 1 mM SHAM is sufficiently low to avoid possible side effects observed with higher levels of this AOX inhibitor or during relative long-term assays (Møller *et al.*, 1988; Bartoli *et al.*, 2005). Therefore, 1 mM of SHAM was used in the subsequent experiments.

Effects of SHAM on the RWC in rice seedling leaves

In well-irrigated seedlings, treatment with 1 mM SHAM had no significant influence on the RWC of leaves (Fig. 3). Drought stress caused a significant decrease in the leaf RWC. Drought-stressed seedlings pretreated with 1 mM SHAM had a lower RWC than seedlings either subjected to drought or SHAM treatment alone (Fig. 3), indicating that SHAM pretreatment resulted in an additional loss of RWC under drought stress.

Effects of ABA, SA, and H_2O_2 on the RWC of rice seedling leaves

The steady-states mRNA of AOX1a, AOX1b, and AOX1c were almost undetectable in leaves without chemical treatments (Fig. 1). Treatment with exogenous 5 mM H_2O_2 or 1 mM SA increased the capacity of the alternative pathway and induced the expression of AOX1a and AOX1b (Figs. 1 and 4). The transcript abundances of AOX1a and AOX1b in leaves treated with 5 mM H_2O_2 seemed higher than those in leaves treated with 1 mM SA. But neither exogenous H_2O_2 nor SA led to AOX1c induction (Fig. 1). Exogenous 1 mM ABA slightly (but not significantly) increased the capacity of the alternative pathway (Fig. 4) but failed to induce any expression of AOX1 genes (Fig. 1).

Compared with the drought-stressed seedlings without chemical treatments, pretreatment with

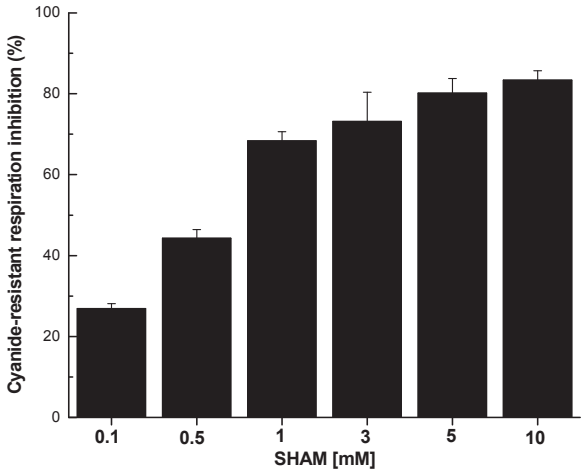

Fig. 2. The *in vivo* inhibition of the AOX pathway in rice leaves treated with 0.1 to 10 mM SHAM. Oxygen uptake by leaves treated with 1 mM KCN in the absence of SHAM was denoted as the 100% value for maximal AOX capacity. Each value represents the mean ± SD (vertical bars) of three independent experiments.

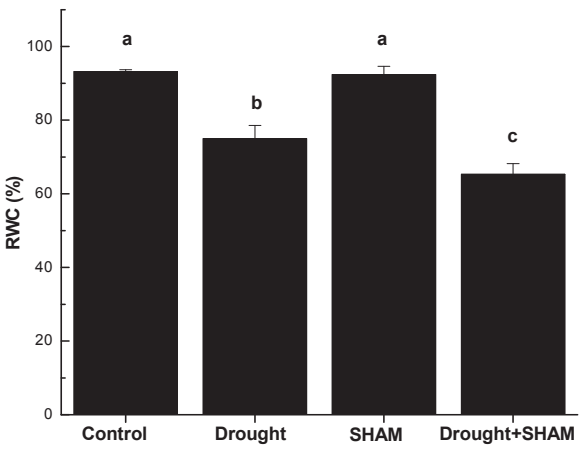

Fig. 3. The relative water content (RWC) measured in well-irrigated (Control) and drought-stressed plants (Drought), and also in well-irrigated plants following treatment with 1 mM SHAM (SHAM) and drought-stressed plants pretreated with 1 mM SHAM (Drought + SHAM). These were individual samples taken during four different experiments. Results are mean values ± SD. Means denoted by the same letter did not significantly differ at $P < 0.05$.

1 mM ABA, 1 mM SA, or 5 mM H_2O_2 effectively mitigated the reduction of the RWC found under drought stress (Fig. 5).

Discussion

In the natural environment, drought is one of the major abiotic factors that influence the development and survival of rice, especially in seedling stage (Zeng and Shannon, 2000). In our experiments, the capacity of the alternative pathway (V_{alt}) increased about 1.5-fold after rice seedlings were exposed to drought stress. Bartoli *et al.* (2005), using wheat leaves, found that the level of both the reduced and oxidized forms of the AOX protein increased in the process of dehydration, and the increase in the reduced form of the AOX protein was more dramatic than that observed in the oxidized form. To provide a more comprehensive rationalization of the expression of AOX genes under drought stress, the specific probes for rice, AOX1a, AOX1b, and AOX1c, were used to reveal the expression of the rice AOX1 multigene family in the transcription level. The expressions of the AOX1a and AOX1b genes were obviously increased under drought stress, but that of AOX1c was hardly detected (Fig. 1). Combining our results with the observations by Bartoli *et al.* (2005), AOX transcripts and protein expression would be up-regulated by drought stress.

However, Ribas-Carbo *et al.* (2005) reported that drought stress on soybean leaves caused a significant increase in the AOX activity but did not affect the amounts of mitochondrial AOX proteins. It should be noted that in greening soybean cotyledons, AOX protein amounts were stable, while stable AOX protein contents were accompanied by the increased transcription of AOX2a and the decrease of AOX2b (Finnegan *et al.*, 1997; Ribas-Carbo *et al.*, 2000). Thus, the case of drought-stressed soybean leaves still couldn't exclude the possibility that drought stress has effects on AOX transcription in eudicot plant species.

Under stressful conditions, the alternative respiration has been suggested to play an important role in preventing the formation of harmful ROS (Maxwell *et al.*, 1999) or might be involved in the

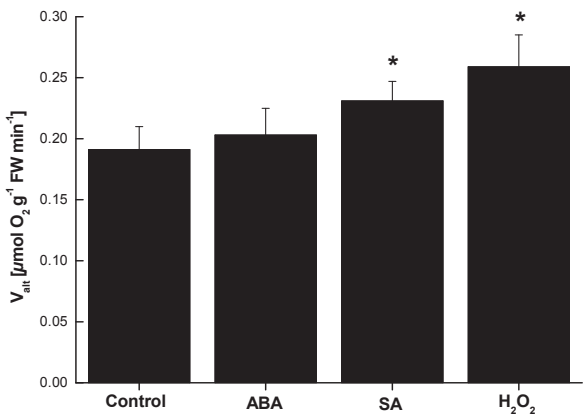

Fig. 4. The effect of exogenous ABA, SA, and H_2O_2 on the capacity of the alternative pathway (V_{alt}). Each value represents the mean ± SD (vertical bars) of four independent experiments. Asterisks indicate that the measured V_{alt} had statistically significant difference from the control leaves ($P < 0.05$).

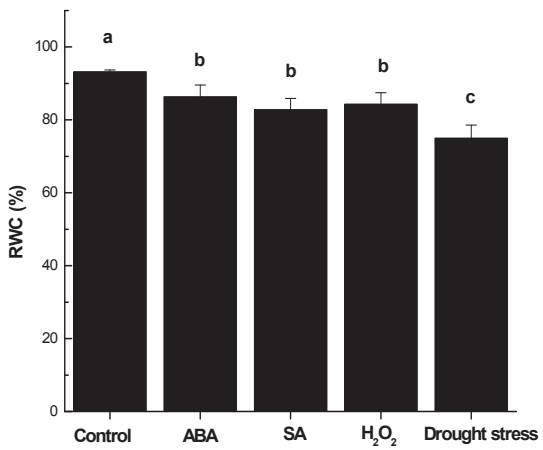

Fig. 5. The relative water content (RWC) of rice seedlings under drought stress with and without chemical pretreatment. Control, well-irrigated seedlings without chemical pretreatment; ABA, seedlings pretreated with SA were exposed to drought stress; SA, seedlings pretreated with SA were exposed to drought stress; H_2O_2, seedlings pretreated with H_2O_2 were exposed to drought stress; drought stress, drought-stressed seedlings without chemical pretreatment. Values are means ± SD calculated from four independent experiments. Bars marked with the same letter are not significantly different ($P < 0.05$).

programmed cell death during infection (Ordog *et al.*, 2002). However, whether the AOX pathway contributes to plant resistance to drought is not fully understood. 1 mM SHAM is sufficiently low to minimize possible side effects (Møller *et al.*, 1988; Bartoli *et al.*, 2005) and thus is used in the present work to inhibit the AOX activity (Fig. 2). The results showed that the drought-stressed seedlings pretreated with 1 mM SHAM had a lower RWC than the seedlings either subjected to drought or SHAM treatment alone (Fig. 3). Although the absolute specificity of SHAM used here can always be questioned and complete abolishment of AOX activity is hardly attained, this also provided evidence implying that the alternative respiratory pathway could play a role in rice tolerance to drought stress.

As introduced in this paper, ABA, H_2O_2 and SA can be used for acclamatory signals to induce plant defense responses against drought stress. In the present work, pretreatment with exogenous H_2O_2, SA, and ABA mitigated the water loss of rice leaves exposed to drought stress (Fig. 5). Our results show that exogenous application of these signal molecules can enhance the ability of rice seedling to hold the water status under the condition of drought stress. Treatment with H_2O_2 and SA not only increased the levels of the AOX1a and AOX1b transcriptions but also enhanced the capacity of the alternative pathway (Figs. 1 and 4). It should be noted that there was no AOX genes expression detected in the leaves without chemical treatments (Fig. 1), suggesting that endogenous SA or H_2O_2 in initial levels would not result in the expression of AOX1 genes. Exogenous SA or H_2O_2 supplement in these experiments might lead to levels of these compounds being higher than the initial level *in vivo* and so activating the AOX1s transcriptions.

Although there were differences in transcript abundances of AOX1a and AOX1b, the expression pattern of rice AOX1s under H_2O_2 or SA application was similar to that under drought stress (Fig. 1). It is possible that the expression of AOX1a and AOX1b under drought stress is up-regulated by H_2O_2 or SA. Xie and Chen (1999) reported that exogenous SA application increased the endogenous H_2O_2 level. Moreover, a recent work also showed that the promoter of *Arabidopsis* AOX1a is responsive to H_2O_2, rather than SA (Ho *et al.*, 2008). Thus, SA could act as the upstream of H_2O_2 signaling to induce AOX expression.

Logically, if the activations of AOX1a and AOX1b are primarily due to the accumulation of endogenous H_2O_2 (as our finding above suggests), then one might expect that ABA will induce AOX1a or AOX1b expression, because exogenous ABA treatment will result in the endogenous production of H_2O_2 (Pei *et al.*, 2000; Guan *et al.*, 2000; Jiang and Zhang, 2002). However, we found this not to be the case. ABA treatment failed to induce any increase in the expression of AOX1 genes (Fig. 1), although its application increased the tolerance of rice seedlings to drought stress (Fig. 5). This indicates that an elevated H_2O_2 level alone may be insufficient to alter the AOX genes expression directly. Hence, AOX1a and AOX1b may be responsive especially to drought stress but not induced by all of the stress signals related to drought. The signals regulating AOX genes expression might be more complex than expected.

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