Alternative Respiratory Pathway under Drought is Partially Mediated by Hydrogen Peroxide and Contributes to Antioxidant Protection in Wheat Leaves

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Abstract: Water stress significantly enhanced the capacity of alternative respiratory pathway and induced AOX1 transcript in wheat (Triticum aestivum L.) leaves. The water-stressed seedlings pretreated with 1 mM salicylhydroxamic acid (SHAM) had higher level of production of reactive oxygen species (ROS) than the seedlings either subjected to drought or SHAM treatment alone did. This observation suggests that cyanide-resistant respiration could play a role in antioxidant protection under the condition of drought. Exogenous application of hydrogen peroxide effectively increased the capacity of alternative respiratory pathway and induced AOX1 transcription. Pretreatment with ROS scavengers, such as 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron) and dimethylthiourea (DMTU), arrested the increase of ROS and partly inhibited the induction of both cyanide-resistant respiration and AOX1 transcript under water stress. These results suggest that the enhancement of cyanide-resistant respiration under drought might be partially mediated by hydrogen peroxide.

Key words: Antioxidant defence system, Cyanide-resistant respiration, Reactive oxygen species, Water stress, Wheat.

Cyanide-resistant respiration is connected with the presence in the respiratory chain of an additional terminal oxidase-alternative oxidase (AOX) (Vanlerberghe and McIntosh, 1997). AOX, located in mitochondrial inner membrane, branches from the main respiratory chain at the level of ubiquinone and catalyzes the four-electron reduction of oxygen to water (Millenaar and Lambers, 2003). Currently, the only confirmed function for respiration with AOX engagement is the thermogenesis in Araceae species where the heat produced during anthesis mobilises aromatic compounds to attract pollinators (Skubatz et al., 1993).

Drought is one of the most important environmental factors that affect plant growth, development and production (Boyer, 1982). Although much works have revealed the changes of plant respiration under various stress conditions including low temperature, wounding, and plant diseases attack (Uritani and Asahi, 1980; Vanlerberghe and McIntosh, 1992; Purvis and Shewfelt, 1993), the lack of knowledge about respiratory responses to water stress is remarkable and this is considered to be an important issue that needs to be addressed in the near future (Flexas et al., 2005). In the last years, alternative respiratory pathway has been the focus of much attention in the study of plant respiratory metabolism under drought. Recent works reported that water stress caused a significant shift of electrons from the cytochrome to the alternative pathway (Ribas-Carbo et al., 2005), and the increased AOX capacity might contribute to enhance photosynthetic electron transport under drought (Bartoli et al., 2005).

In general, water stress leads to a marked increase of ROS (reactive oxygen species) production and therefore alters oxidative balance of cell (Dat et al., 2000). Thus, it is thought that survival under drought condition is closely associated with the plant’s antioxidant ability (Lascano et al., 2001). There are many reports in the literatures showing that activation of matrix antioxidant enzymes (such as SOD, superoxide dismutase and CAT, catalase) under drought represents important response that protects plant against harmful effects of ROS (Dat et al., 2000). Unfortunately, studies examining whether respiration could be involved in the prevention of ROS formation under this stress are few, despite the facts that mitochondria is a major point of ROS production under drought (Bartoli et al., 2004) and mitochondria itself has a potential function in avoidance of damage.
of ROS by regulating the electron shift between alternative pathway and cytochrome pathway (Maxwell et al., 1999).

As well, plants can adapt to water stress by generating and transmitting various defense signals (Dat et al., 2000). It has been proposed that ROS itself can be used for acclamatory signal to induce plant antioxidant defence (Smirnoff, 1993; Prasad et al., 1994). ABA (abscisic acid), regarded as a general signal under drought (Davies and Zhang, 1991; Shinozaki and Yamaguchi-Shinozaki, 1997), can function in triggering the increased generation of ROS (Guan et al., 2000; Pei et al., 2000) and consequently regulate the expression of antioxidant genes encoding SOD and CAT (Jiang and Zhang, 2002).

In several studies, H$_2$O$_2$ (hydrogen peroxide) is considered as second messenger in inducing AOX activity by directly oxidizing transcription factors or modulating phosphorylation processes (Wagner, 1995; Wagner and Krab, 1995; Neill et al., 2002). But, it was reported that Antimycin A caused the fastest induction of AOX1 while H$_2$O$_2$, the slowest (Maxwell et al., 2002), showing that there was no clear correlation between the ROS formation and accumulation of AOX1 transcript. Maxwell et al. (2002) suggested that increased AOX pathway under oxidant stress might be as a result of non-specific disruption of cellular function. Ribas-Carbo et al. (2005) also proposed that the increase of AOX pathway under drought could be caused by the inhibition of cytochrome pathway. However, Bartoli et al. (2005) observed that the increased AOX pathway under drought was companied with an enhancement of cytochrome pathway. Thus, current knowledge to the precise regulation and function of alternative respiration under drought is only partially elucidated. To date, no single study has critically investigated the mode of action of ROS in the induction of AOX pathway under water stress.

In this study, we investigated the effect of drought on the alternative respiratory pathway and AOX1 gene expression. And, an effort was also made to demonstrate possible regulation and function of alternative respiration under drought.

Material and Methods

1. Experimental material and treatments

Wheat (Triticum aestivum L. cv. Longchun16) seeds were sterilized with 1% (v/v) NaClO for 10 min and then washed clean with water. Thereafter, seeds were imbibed in distilled water for 12 hr at room temperature and were germinated at 26±1°C for 24 hr. The germinated seeds were planted in sterilized sands at 26±1°C on a 12 hr light (100 μmol m$^{-2}$ s$^{-1}$)/12 hr dark. Seedlings were watered twice per day.

Two-wk-old seedlings were transferred out from sands carefully and were excised at the ends of the stems that were simultaneously sprayed with distilled water to eliminate possible wound damage. The detached plants above were placed in distilled water for 1 hr to adapt to the change of condition and then were placed in 10 mM 4, 5-dihydroxy-2, 3-benzene disulfonic acid (Tiron, a specific O$_2^-$ scavenger, Kawano et al., 1998), 5 mM dimethylthioureia (DMTU, a trap for H$_2$O$_2$, Casano et al., 2001), or 10 mM Tiron plus 5 mM DMTU for 12 hr, respectively, as described by Jiang and Zhang (2002). The concentrations of these ROS scavengers used here have been suggested by previous reports (Jiang and Zhang, 2002; Hu et al., 2005) and were also effective in this work by a titration experiment. After these pretreatments, the ends of the stems were exposed to -0.7 MPa polyethylene glycol (PEG 6000) solutions (for osmotic pressure, Money, 1989) until the relative water content (RWC) in the leaves was maintained at the level same to that in the seedling leaves only subjected to water stress (about 78%). The detached plants treated with distilled water for the whole period were served as control. All of these seedlings were still grown under previous growth condition.

In order to study the effect of AOX pathway inhibitor, 1 mM of Salicylhydroxamic acid (SHAM) was freshly prepared (1 M stock solution in methoxyl ethanol). The seedling leaves (attached to the excised plants placed in distilled water) were supplied with 1 mM SHAM plus 440 mM mannitol (to supplement the loss of leaf water potential due to the use of respiratory inhibitor; Bartoli et al., 2005) through the cut tips of leaves and were maintained at 26±1°C for 4 hr. Thereafter, these seedlings pretreated with SHAM were transferred to water stress or not, as the methods described above. Control experiment showed that supply of the solvent alone to leaves had no significant effects on any of the experimental parameters being measured (data not shown).

To investigate the effects of exogenous H$_2$O$_2$ leaves of well-irrigated plants were sprayed with 200 mM H$_2$O$_2$ for 20 min. After this treatment, leaves were taken at room temperature for 0, 3, 6, 9 and 12 hr respectively before leaf samples were collected and immediately washed with water to remove the remaining H$_2$O$_2$ for following use. Control plants were sprayed with water.

2. Relative water content (RWC) determination

Relative water content (RWC) was measured to monitor leaf water status and was calculated as: (FW –DW)/(TW–DW), where FW is the fresh weight, DW is the dry weight, and TW represents the turgid fresh weight. Leaf segments were hydrated to full turgidity by floating in distilled water for 3 hr.

3. O$_2$ consumption assays

Total respiration, cyanide-resistant respiration and the ratio of cyanide-resistant to total respiration were measured and calculated with a Clark-type oxygen
electrode at 25°C in a dark room as described by Bingham and Farrar (1989). Capacity of alternative pathway (Valt) was calculated as the difference between respiration in the presence of 1 mM KCN and residual respiration. Residual respiration (Vr) was measured in the presence of inhibitors of both the 3 mM SHAM plus 1 mM KCN.

4. The determination of generating rate of O₂⁻ (superoxide radical) and H₂O₂ content

O₂⁻ production was measured as suggested by Elstner and Heupel (1976) by monitoring the nitrate formation from hydroxyl amine with some modifications by Wang and Luo (1990). The plant material was homogenized in 6 ml of 65 mM phosphate buffer (pH 7.8) at 5000 g for 10 min. The reaction mixture contained 0.9 ml of 65 mM phosphate buffer, 0.1 ml of 10 mM hydroxyl amine hydrochloride and 1 ml of the supernatant plant extract. After incubation at room temperature (25°C) for 20 min, 0.5 ml of 17 mM sulphanilamide and 0.5 ml of 7 mM α-naphthyl were added into 0.5 ml of n-Butanol ether was added and centrifuged at 1500 g for 5 min and the absorbancy was read at 530 nm. A standard curve with NO₂⁻ was established to calculate the production rate of O₂⁻ from the chemical reaction of O₂⁻ and hydroxylamine.

The content of H₂O₂ was measured according to the method described by Brennan and Frenkel (1977) with some modifications. H₂O₂ was extracted by homogenizing 0.05 g leaf tissue with 3 mL of cold acetone. The homogenate was centrifuged at 6000 g for 25 min. A 1-mL sample of supernatant was mixed with 0.1 mL of 5% (w/v) Ti (SO₄)₂ and 0.2 mL NH₄OH. The mixture was then centrifuged at 6000 g for 15 min at room temperature and the precipitate solubilized in 3 mL of 2 M H₂SO₄. The optical absorption of the supernatant was measured spectrophotometrically at 415 nm to determine the H₂O₂ content. Absorbance values were calibrated to a standard graph generated with known concentrations of H₂O₂.

5. Extract the total RNA and Northern hybridization

Total RNA was extracted by Total RNA Trizol Extraction Kit (Sangon Inc., CHN). Extracted total RNA was quantitated using the UV-VIS Spectrophotometer Tu-1800 (Purkinje General Inc, CHN). 50 μg RNA (based on OD260 and actin probe hybridization) were loaded per lane in 0.8% agarose gel with 1×TAE buffer, separated by electrophoresis. Northern hybridization was performed with the ECL DNA Labeling and Detection Kit (Enzo. Diagnostics Inc., UK), according to the manufacturer instructions. The probe was provided by Prof. Whelan (Australian National University). This probe is 1,134 bp cDNA clone that was isolated from tobacco (Whelan et al., 1995) and contains conserved-region that is high identity with other AOX genes from other species including wheat (Takumi et al., 2002).

6. Electrolyte leakage

Leaf discs from leaves were collected immediately at the end of treatment. The leaf discs were washed and immersed in double-distilled water at 26°C for 12 hr. After incubation, the electrical conductivity of the solution was measured. Samples were then heated in boiling water for 20 min and then cooled to room temperature. The conductivity was measured again. The conductivity was assessed by measuring 260-nm-absorbance released to the medium (Lunde and Kubo, 2000). Electrolyte leakage was calculated as the ratio of the value of conductivity measured.

Results

1. Effect of water stress on AOX pathway in the wheat seedlings leaves

Total respiration rate (Vr), capacity of alternative pathway (Valt), and values of Valt/Vr were measured and calculated in the dehydrated process. Total respiration (Vr) increased from 48.5 μmol O₂ g⁻¹ DW h⁻¹ in well-irrigated plants to 70.8 μmol O₂ g⁻¹ DW h⁻¹ when the water availability of plants was restricted. The capacity of alternative pathway (Valt) increased from 11.6 μmol O₂ g⁻¹ DW h⁻¹ in control to 22.6 μmol O₂ g⁻¹ DW h⁻¹ after water tress treatment (Table 1). Consequently, the calculated value of Valt/Vr in well-irrigated plants

|                             | Control     | Drought   |
|-----------------------------|-------------|-----------|
| V_r (μmol O₂ g⁻¹ DW h⁻¹)    | 48.5±3.9    | 70.8±6.2  |
| V_alt (μmol O₂ g⁻¹ DW h⁻¹)  | 11.6±1.2    | 22.6±2.2  |
| V_alt / V_r (%)             | 24          | 32        |

Table 1. Total respiration rate (V_r), capacity of alternative pathway (V_alt) and V_alt / V_r (the capacity of alternative pathway to total respiration rate ratio) of wheat seedling leaves under the condition of water stress. These were individual samples taken during four different experiments. Results are mean values ± SD. V_alt / V_r was calculated from the average values of V_r and V_alt experiments carried out separately.
was approximately 24% in control and was near 32% after drought treatment (Table 1), indicating a relative contribution of the alternative pathway to total respiration under drought.

2. Alternative respiratory pathway under drought contributes to antioxidant protection of wheat leaves

In the present work, 1 mM of SHAM slightly, but not significantly, enhanced both O$_2^-$ (superoxide) (Fig. 1a) and H$_2$O$_2$ generations (Fig. 1b) in the well-irrigated plants, suggesting that this low concentration of SHAM did not obviously stimulate the production of ROS under normal (well-irrigated) condition. Water stress caused the increases of both O$_2^-$ and H$_2$O$_2$ production (Fig. 1a and b). Fig. 1a and b showed that the water-stressed seedlings pretreated with 1 mM SHAM had higher ROS production (including O$_2^-$ and H$_2$O$_2$) than the seedlings either subjected to drought or SHAM treatment alone did, indicating that SHAM pretreatment resulted in an additional production of ROS under drought.

3. H$_2$O$_2$ induce AOX pathway

In order to investigate the mode of action of H$_2$O$_2$ in the induction of AOX pathway under drought, the leaves of well-irrigated plants were treated with 200 mM H$_2$O$_2$. The capacity of AOX pathway (Fig. 2a) and $AOX1$ expression (Fig. 2b) were obviously increased after the plants were treated with exogenous H$_2$O$_2$. We observed that this exogenous H$_2$O$_2$ application did not significantly alter the endogenous H$_2$O$_2$ contents (Fig. 3a) and electrolyte leakage of leaves (Fig. 3b), compared with the plants untreated with H$_2$O$_2$.

4. The effect of ROS scavengers on the AOX pathway and $AOX1$ expression in the water stressed leaves

To further study whether H$_2$O$_2$ would be involved in the induction of AOX pathway under drought
condition, 10 mM Tiron and 5 mM dimethylthiourea (DMTU) were used in this work to inhibit increase of ROS under drought.

The well-irrigated plants were pretreated with Tiron and DMTU and then were subjected to water stress. Although the generating rate of \( \text{O}_2^- \) in water-stressed leaves was not significantly affected by the DMTU (a scavenger of \( \text{H}_2\text{O}_2 \)) treatment, the increase in the level of ROS caused by water stress was almost fully inhibited by the other ROS scavengers applications (Fig. 4a and b). A decrease in AOX pathway was observed in the water-stressed plants pretreated with these ROS scavengers, compared with the water-stressed plants without chemical treatments (Fig. 4a and b).
The probe for AOX1 gene was used to investigate possible change of AOX1 expression with these treatments. After water stress treatment, AOX1 transcript was obviously increased (Fig. 5). Combined with the results showed in Table 1, it seemed that the enhancement of AOX pathway capacity under drought could be caused, at least in part, by the increased AOX1 transcript. Similar as the change of AOX pathway, ROS scavengers’ applications decreased the level of AOX1 transcript in the water-stressed plants (Fig. 5).

Discussion

Water-stressed wheat leaves had higher level of alternative pathway than the well-irrigated ones had (Table 1), suggesting that drought might induce the operation of alternative pathway. Bartoli et al. (2005), using wheat leaves, found that the levels of both the reduced and oxidized forms of the AOX protein increased in dehydrated process (Bartoli et al., 2005). In our experiments, an increase in steady-state mRNA of AOX1 was observed after drought treatment (Fig. 5). Therefore, from these results it is supposed that drought could induce the transcription of AOX gene, consequently resulting in the increases in the amount of the AOX protein and the capacity of AOX pathway.

But in soybean leaves, water stress caused a significant shift of electrons from the cytochrome to the alternative pathway but did not affect the level of alternative oxidase protein (Ribas-Carbo et al., 2005). Based on this observation, Ribas-Carbo et al. (2005) suggested that a biochemical regulation (other than protein synthesis) led to an increased alternative pathway under drought. However, it is noted that AOX of eudicotyledon plants is encoded by the family of nuclear genes that at least include AOX1 and AOX2, while AOX2 and AOX3 are absent from the genomes of all monocot species examined to date (Considine et al., 2002). And, in greening soybean cotyledons, stable AOX protein amounts were companied with the increased transcription of AOX2 and the decrease of AOX3 (Finnegan et al., 1997; Ribas-Carbo et al., 2000). Thus, the case of the water-stressed soybean leaves still couldn’t excluded that water stress has effects on AOX transcription. It is possible that regulation in the level of AOX gene transcription is still involved in the induction of AOX pathway in water-stressed soybean but a different expression of AOX genes multi-family could cause the no observed change in AOX protein amounts, as in the case during greening (Ribas-Carbo et al., 2005). Further work is needed to confirm this idea.

Survival under drought condition is closely associated with the plant’s antioxidant ability (Lascano et al., 2001). Although using transgenic tobacco cells with varying levels of AOX expression provided molecular evidence that AOX can limit the formation of ROS (Maxwell et al., 1999), the importance of AOX pathway in the antioxidant defence under drought has not been extensively studied to date.

Salicylhydroxamic acid (SHAM), a well-known inhibitor of AOX activity, has been widely used on intact tissues to inhibit AOX activity (Chivasa et al., 1997; Chivasa and Carr, 1998; Naylor et al., 1998). In a recent work (Bartoli et al., 2005), treatment with 1 mM of SHAM inhibited cyanide-insensitive oxygen uptake by near 70% in both well-irrigated and water-stressed wheat leaves. Moreover, this concentration is sufficiently low to avoid the possible side effects observed with higher levels of this AOX inhibitor (Moller et al., 1988) or during relatively long-term (hours) of assays (Bartoli et al., 2005). In the present work, the water-stressed seedlings pretreated with 1 mM SHAM had more generation of ROS, compared with the seedlings either subjected to drought or SHAM treatment alone did (Fig. 1a and b). Amanda et al. (2000) found that 1 mM of SHAM was not an effective concentration to affect the activities of xanthine oxidase and peroxidases in tobacco suspension cells. We also observed that 1 mM of SHAM did not significantly change the activity of peroxidases in the wheat leaves experiencing dehydrated process but inhibit most of cyanide-insensitive oxygen uptake (data not shown). It seems that inhibition of AOX pathway will lead to additional ROS production under drought. These observations suggest that the enhancement of AOX pathway under water stress might play a role of antioxidant enzyme in limiting the production of ROS.

With use of wheat leaves, Bartoli et al. (2005) found that the increase in AOX pathway under drought was companied with an enhancement of cytochrome pathway. This observation indicates that a signal transduction rather than mitochondrial dysfunction might be involved in the increase of alternative protein synthesis.
respiratory pathway under drought. We firstly investigated whether H₂O₂ could be used to induce AOX pathway. In the present work, exogenous 200 mM H₂O₂ led to a obvious increase in the levels of both AOX pathway (Fig. 2a) and AOX1 expression (Fig. 2b). It was noted in the experiments here that there was no obvious increase in the level of endogenous H₂O₂ content during 0-12 hr after the exogenous H₂O₂ application (Fig. 3a). And, electrolytes leakage, which is generally correlated with cellular dysfunction (Dhindsa et al., 1981) and the degree of oxidative stress (Yu et al., 2003), was also not affected obviously under same treatment (Fig. 3b). Similar to our observation, Wagner (1995) found that an enhanced AOX activity in Petunia hybrida cell was maintained under same treatment (Fig. 3b). It was noted in the experiments here that there was no obvious increase in the level of endogenous H₂O₂ content during 0-12 hr after the exogenous H₂O₂ application (Fig. 3a). And, electrolytes leakage, which is generally correlated with cellular dysfunction (Dhindsa et al., 1981) and the degree of oxidative stress (Yu et al., 2003), was also not affected obviously under same treatment (Fig. 3b). Similar to our observation, Wagner (1995) found that an enhanced AOX activity in Petunia hybrida cell was maintained under same treatment (Fig. 3b). It was noted in the experiments here that there was no obvious increase in the level of endogenous H₂O₂ content during 0-12 hr after the exogenous H₂O₂ application (Fig. 3a). And, electrolytes leakage, which is generally correlated with cellular dysfunction (Dhindsa et al., 1981) and the degree of oxidative stress (Yu et al., 2003), was also not affected obviously under same treatment (Fig. 3b). Similar to our observation, Wagner (1995) found that an enhanced AOX activity in Petunia hybrida cell was maintained under same treatment (Fig. 3b).

We further studied whether there might exist a ROS signaling pathway to regulate AOX expression and AOX pathway even under drought condition. The experiments reported here showed that ROS scavengers not only inhibited the increase of ROS production under drought (Fig. 4a and b) but also decreased the levels of alternative pathway (Fig. 4c) and AOX expression induced by water stress (Fig. 5). Although the absolute specificity of each ROS scavenger used here can always be questioned and complete abolishment of ROS produced from all cellular compartments is hardly attained, this also provided the evidence implying that the enhancement of AOX pathway under drought is mediated, at least in part, by the ROS.

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