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ABA Controls H_2O_2 Accumulation Through the Induction of *OsCATB* in Rice Leaves Under Water Stress

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The production of both ABA and H_2O_2 is induced by drought and can act as signals under stress conditions. We investigated the relationships between ABA, H_2O_2 and catalase (CAT) in rice leaves when rice seedlings were treated with polyethylene glycol as water stress treatment. As a key gene in ABA biosynthesis, *OsNCED3* was significantly induced in rice by water stress treatment and such induction preceded the rapid increase in ABA. Water stress inhibited the expression of *CATA* and *CATC* but substantially enhanced the expression of *CATB*. Exogenously applied ABA promoted the expression of *CATB* also and inhibited the expression of *CATC* in a concentration-dependent manner. When ABA production was inhibited by using ABA biosynthesis inhibitors nordihydroguaiaretic acid and tungstate, expression of *CATB* was also subdued while *CATC* was enhanced under the water stress. Accumulation of H_2O_2 was also reduced when endogenous ABA production was inhibited and showed a correlation with the total activity of catalases. Our results suggest that water stress-induced ABA prevents the excessive accumulation of H_2O_2, through the induction of the expression of *CATB* gene during water stress.

Keywords: ABA • Catalases • Hydrogen peroxide • Reactive oxygen species • Rice (*Oryza sativa*) • Water stress.

Abbreviations: ABA, abscisic acid; ABRE, ABA responsive element; ARE, antioxidant responsive element; CAT, catalase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NDGA, nordihydroguaiaretic acid; PEG, polyethylene glycol; RIA, radioimmunoassay; ROS, reactive oxygen species; SOD, superoxide dismutase.

Introduction

Reactive oxygen species (ROS) are produced in plant cells primarily as a by-product of aerobic metabolism (Slesak et al. 2007). The production of ROS is increased by exposing plants to biotic and abiotic stress conditions, such as drought, salinity, chilling, pathogen invasion, etc., which result in lipid peroxidation, protein oxidation, enzyme inhibition and DNA and RNA damage (Miller et al. 2008). Among the ROS, hydrogen peroxide (H_2O_2), produced in both enzymatic and non-enzymatic reactions, is the most important. Many investigations have proved that H_2O_2, as a signaling molecule, is involved in various processes including pathogen defense, programmed cell death, stress defense, hormonal responses, photosynthesis regulation, and growth and development in plants (Neill et al. 2002, Apel and Hirt 2004, Laloi et al. 2004, Mittler et al. 2004, Foyer and Noctor 2005, Torres and Dangl 2005). It should be noted that excessive production of H_2O_2 has been shown to be cytotoxic and the generation of H_2O_2 should be under strict control by plants (Ushio-Fukai 2006). Indeed, the regulation of antioxidant activity is not only a strategy in plant adaptation to environmental stresses but also a mechanism in modulating cellular signal transduction (Veal et al. 2007).

Catalase (CAT) (EC. 1.11.1.6) is a major and essential antioxidant enzyme that catalyses the decomposition of H_2O_2 into oxygen and water. Plant CATs are encoded by a small gene family usually composed of three isozyme genes that exhibit fairly complex spatial and temporal patterns of expression throughout the plant life cycle (Scandalios et al. 1997, Willekens et al. 1997). The presence of a G-box or ABA responsive element (ABRE) and antioxidant responsive element (ARE) in the maize *CAT1* promoter and an ARE in the *CAT3* promoter underlines the important protective role of *CAT* in response to osmotic and oxidative stresses (Polidoros and Scandalios 1999, Guan and Scandalios, 2000). In rice, there are three isoenzyme genes *OsCATA*, *OsCATB* and *OsCATC* (Morita et al. 1994, Higo and Higo 1996, Agrawal et al. 2001). In seedlings, the *CATA*, *CATB* and *CATC* genes are highly expressed in leaf sheath, root and leaf blade, respectively. The expression of *CATA* is regulated by a circadian rhythm in leaf sheath, and diurnal oscillations of *CATC* expression are detected in leaf blade (Iwamoto et al. 2000). Interestingly, the expression of *CATC* is suppressed by ABA whereas in maize, *CAT1*, an ortholog of

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OsCATB, is significantly induced by ABA (Guan et al. 2000, Agrawal et al. 2001).

ABA plays critical roles in various physiological processes during the plant life cycle, including seed dormancy, germination and adaptive responses to environmental stress (Shinozaki and Yamaguchi-Shinozaki 2000, Schroeder et al. 2001, Zhu 2002, Himmelbach et al. 2003, Zhu et al. 2009). Water stress can trigger the accumulation of both ABA and ROS, and the action of ABA is associated with the production of ROS in plant cells (Zhu 2002). Exogenously applied ABA can also cause the generation of H_2O_2 in plant cells or tissues (Pei et al. 2000, Jiang and Zhang 2001, Zhang et al. 2001, Kwak et al. 2003, Hu et al. 2005). Using histochemical and cytochemical localization techniques, Hu et al. (2006) have proved that ABA is a key inducer of H_2O_2 production in maize leaves under water stress.

H_2O_2 as a signaling molecule is involved in the ABA signaling pathway while as a cytotoxic molecule on the other hand H_2O_2 content is under strict control by the plant itself (Ushio-Fukai 2006). Does ABA perform a dual function in inducing and controlling H_2O_2 content under drought conditions? What is the role of CAT in this process? The interrelationships between ABA production, H_2O_2 accumulation and CAT activities are still obscure. In this study, the expression profiles and patterns of *OsCAT* genes in rice leaves under water stress were examined. The effects of exogenous and endogenous ABA on the expression of *OsCAT* genes were also determined. The results indicate that *OsCATB* is significantly induced by ABA and plays a key role in controlling H_2O_2 accumulation under water stress.

Results

Water stress induced rapid accumulation of ABA in rice leaves

It is well recognized that ABA is induced by water stress. In order to further illustrate the dynamic accumulation of ABA during water stress, ABA content was determined using the radioimmunoassay (RIA) method during a cycle of water stress and rehydration. ABA content increased within 1 h and reached a peak after 4 h when the seedlings were in severe water stress (**Fig. 1A**). The ABA level decreased quickly to the basal line within 1 h during rehydration. Gene expression analysis shows that *OsNCED3*, a key gene for ABA biosynthesis and *OsABAox2*, a key gene for ABA catabolism, were responsible for the accumulation and decline of ABA during the water stress and rehydration cycle (**Fig. 1B, C**).

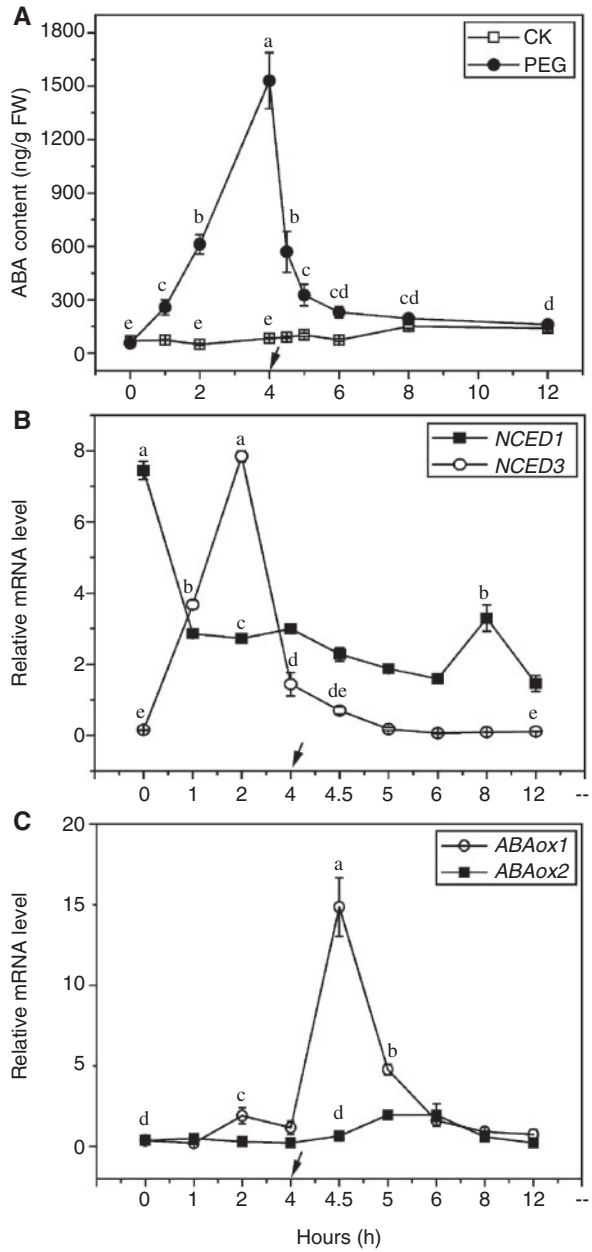

Fig. 1 ABA accumulation and expression changes of *OsNCED* and *OsABAOX* genes in rice leaf during water stress treatment and rehydration (indicated by the arrows). Seedlings were grown in nutrient solution. Two-week-old seedlings were treated with 15% PEG for 4 h, and then transferred to nutrient solution for rehydration. ABA content and gene transcript levels were quantified by RIA and qRT-PCR, respectively, as described in Materials and Methods. Values are means ± SD ($n = 3$). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

Water stress induced accumulation of H_2O_2 mediated by ABA as a key regulator in rice leaves

How is water stress-induced ABA related to H_2O_2 production under stress? Here we used two ABA biosynthesis inhibitors, nordihydroguaiaretic acid (NDGA) and tungstate, to estimate the effect of ABA on H_2O_2 accumulation. As shown in **Fig. 2A**, ABA content increased significantly in rice seedlings treated with exogenous ABA and polyethylene glycol (PEG) for 4 h, and PEG-induced accumulation was effectively suppressed by these two inhibitors (**Fig. 8A**). H_2O_2 content of rice leaves was also enhanced by ABA, H_2O_2 and PEG. However, NDGA and

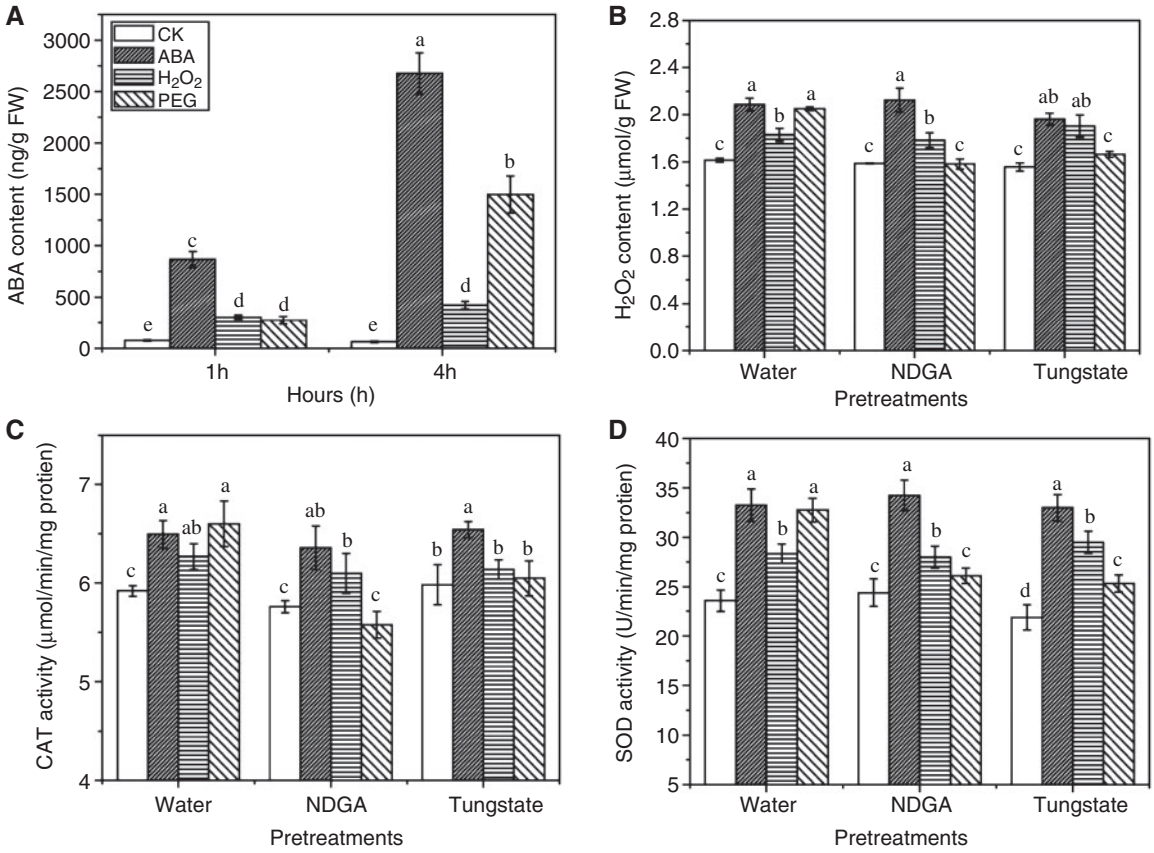

Fig. 2 ABA content (A), H_2O_2 content (B), CAT activity (C) and SOD activity (D) in rice leaves. Sample were pretreated with water, 100 μM NDGA or 2 mM tungstate for 6 h and then exposed to water, 50 μmol ABA, 20 mM H_2O_2 or 15% PEG for 4 h. H_2O_2 detection was performed immediately after sampling as described in Materials and Methods. Error bars show ± SD ($n = 3$). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

tungstate only inhibited the increase in H_2O_2 in PEG treatment, but did not affect exogenous ABA and H_2O_2 treatments (**Fig. 2B**). The effect on CAT and superoxide dismutase (SOD) activity of these two inhibitors was consistent with that on H_2O_2 accumulation (**Fig. 2C, D**). The results indicate that inhibition of ABA synthesis may subsequently reduce H_2O_2 occurrence and ABA is indispensible in mediating the accumulation of H_2O_2 under water stress.

Polygenetic analysis of the rice *CAT* gene family relative to other plant species

Fig. 2 seems to suggest that stress-induced ABA increase leads to H_2O_2 production, which in turn enhances the activity of CAT. However, the detailed interrelations between ABA, H_2O_2 and CAT could be more complicated (Veal et al. 2007). It is crucial to investigate the function of rice *CAT* genes under water stress. **Fig. 3** shows the polygenetic analysis of *OsCAT* genes in comparison with *CAT* gene families of other plant species. Except for the dicot Arabidopsis *CAT* genes, the other monocot *CAT* genes are classified into three groups. Interestingly, despite the high identity of protein sequence, the expression patterns of *CAT* genes are different (Frugoli et al. 1996, Iwamoto et al. 2000, Du et al. 2008). By analyzing their protein sequences, we found a peroxisomal targeting signal, Ser-Arg-Leu, at the C terminus of groups B and C (Gould et al. 1989), which indicates that both CATB and CATC are located in the peroxisome and the other isoenzyme, CATA, is in the cytoplasm. Such difference in location may imply different functions.

Water stress changes the expression pattern of rice *CAT* genes

Although both CATB and CATC are predicted to be located in the peroxisome, it is still not certain which protein functions in the photorespiration pathway like *AtCAT2* does in Arabidopsis (Hu et al. 2010). Here we analyzed the transcription profiles of *CAT* genes in different tissues of rice. The results show that *CATA* is abundant in leaf sheath and moderately expressed in leaf blade and root but not in seed. Interestingly, the *CATB* gene is mainly expressed in seed and root with rather a low level of expression in rice leaves. On the other hand, *CATC* gene was found with extremely high transcription level in leaf blade and

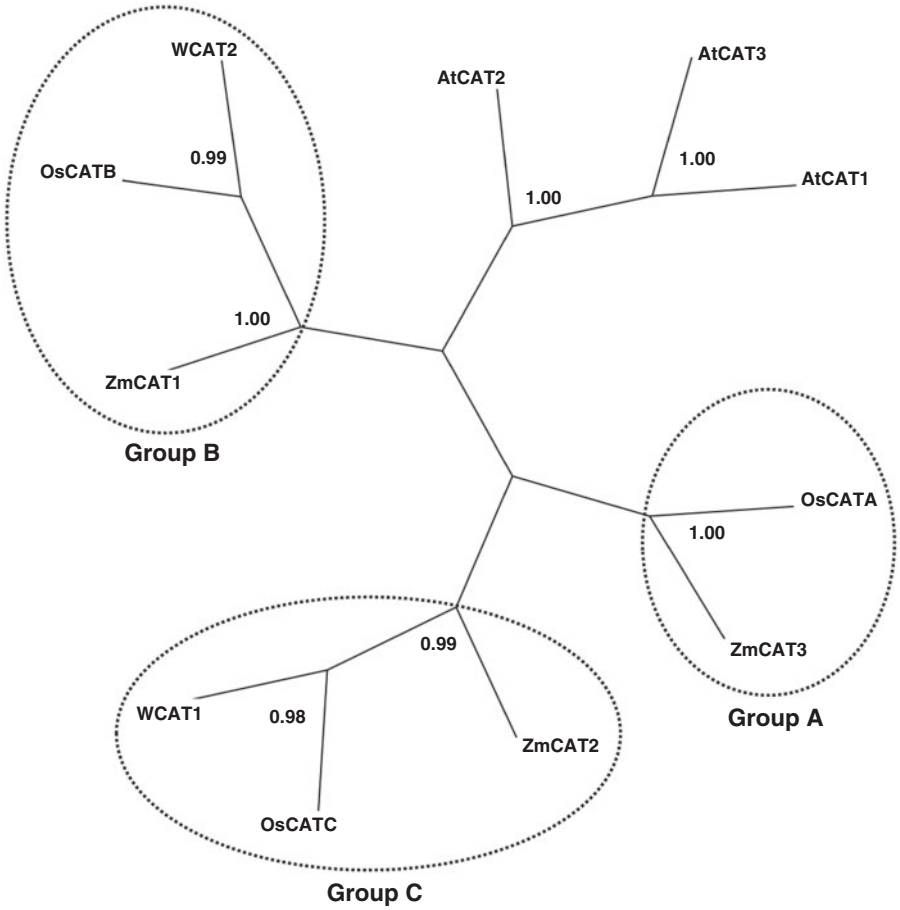

Fig. 3 Phylogenetic analysis of CAT protein sequences. The phylogenetic tree was derived from the program MrBayes version 3.0 (Ronquist and Huelsenbeck 2003). The sequences used for generating the phylogenetic tree and their GenBank accession numbers are as follows: *AtCAT1* (NP_564121.1), *AtCAT2* (NP_195235.1), *AtCAT3* (NP_564120.1), *OsCATA* (NP_001045673.1), *OsCATB* (NP_001058635.1), *OsCATC* (NP_001048861.1), *WCAT1* (Q43206.1), *WCAT2* (P55313.1), *ZmCAT1* (CAA42720.1), *ZmCAT2* (NP_001105310.1), *ZmCAT3* (NP_001105416.1).

barely expressed in other organs (**Fig. 4A**). Together with the previous results, we speculate that OsCATC is the photorespiration CAT located in the peroxisome while OsCATB, which also bears a peroxisomal targeting signal near the C terminus, tends to be expressed in root and seed; the decomposition of photorespiration-associated H_2O_2 seems unlikely to be the result of *OsCATB* gene expression.

Previous work has revealed that the expression pattern of wheat *CAT* genes is regulated by drought stress (Luna et al. 2004). Our results indicate that water stress treatment also changed the expression patterns of *CAT* genes in rice leaves (**Fig. 4B, C**). **Fig. 4B** demonstrates the diurnal cycle of rice *CAT* gene expression. The abundance of *CATA* transcript in rice leaf sheath changed during the day/night cycle and peaked late in the light period under normal environmental conditions. *CATB* showed little response to the day/night cycle due to its low abundance in rice leaves. As the most abundant *CAT* in leaf blade, *CATC* displayed an oscillation expression pattern and reached the maximum early in the light period. When the seedlings were transferred into continuous darkness, the expression pattern was only slightly changed (**Fig. 4B**). Results here indicate that the expression of both *CATA* and *CATC* is controlled by a circadian rhythm.

The effect of water stress on the expression pattern of *CAT* genes is different from that of the variation of diurnal rhythm. The expression of both *CATA* and *CATC* is significantly suppressed by water stress whereas the *CATB* transcript is induced by water stress (**Fig. 4C**). This result indicates that the *OsCATB* gene may play an important role under water stress.

ABA changed the expression of *CATB* and *CATC* in rice leaf

The above results prove that ABA is responsible for water stress-induced H_2O_2 accumulation and both ABA and *CAT* respond to water stress. To clarify their relationship further, we tested the effect of different concentrations of ABA and H_2O_2 on the expression of *CAT* genes in rice leaf blade. Both ABA and H_2O_2 failed to change the transcript level of *CATA* (**Fig. 5**). Similarly to the effect of water stress on *CAT* genes, ABA

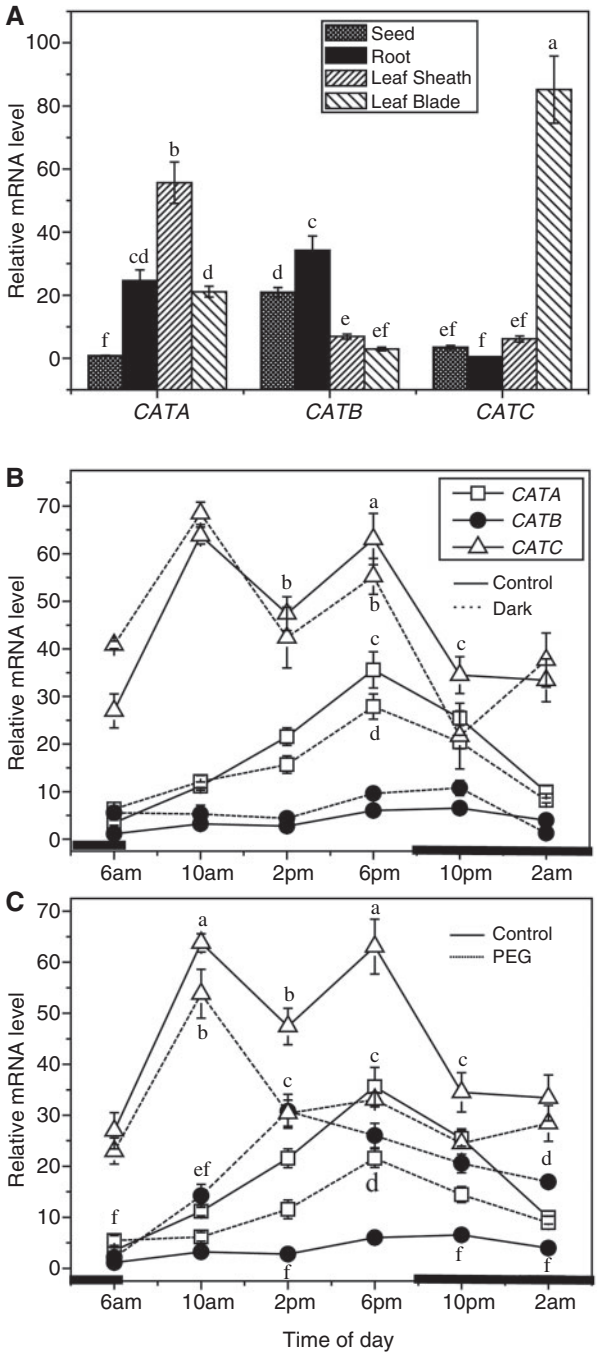

Fig. 4 Spatial (A) and temporal (B) expression of *CAT* genes and expression changes (C) of *CAT* genes in rice exposed to water stress. Two-week-old seedlings were used for RNA extraction. In (B and C), RNA from leaf sheath was used for *CATA* expression analysis, leaf blade RNA was used for expression analysis of *CATB* and *CATC* genes. Solid lines mean samples grown in normal environmental conditions while dashed lines stand for plants grown in continuous darkness (B) or in 12% PEG (C). Bold lines indicate night in a regular day (B and C). Values are means ± SD ($n = 3$). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

increased *CATB* expression profile while suppressing the expression of *CATC* and functioned in a concentration-dependent manner (**Fig. 5A**). Interestingly, H_2O_2 slightly induced the expression of *CATB* and *CATC* in either low or high concentration in rice leaf blade (**Fig. 5B**). These results indicate that it is ABA rather than H_2O_2 that regulates the transcript profiles of *CAT* genes. The activities of CAT and SOD in leaves were upregulated by a high concentration of ABA (**Fig. 6A, C**). Although H_2O_2 did not significantly change the expression of *CAT* genes, it still enhanced CAT and SOD activities at high concentration (**Fig. 6B, D**); probably because H_2O_2 treatment had perturbed the redox homeostasis in rice leaves.

ABA upregulated *OsCATB* to contain H_2O_2 content under water stress

To further demonstrate the dynamic effect of different treatments on *CAT* gene expression in rice blade, a time-course experiment was performed. The result showed that both exogenous ABA and water stress treatment had a similar dynamic effect on *CATB* and *CATC* (**Fig. 7A, B**). However, the responses of these two genes to ABA treatment were earlier than the PEG treatment, indicating that the expression of *CATB* and *CATC* genes could be induced by the accumulation of endogenous ABA, and the increased expression of *CATB* may play an important role in scavenging ROS during water stress. Such responses were not found in the H_2O_2 treatment (**Fig. 7C**).

To detect whether endogenous ABA is the inducer of *CATB* gene expression, ABA biosynthesis inhibitors, NDGA and tungstate, were applied. As shown in **Fig. 8A**, the accumulation of endogenous ABA was effectively retarded by these inhibitors. Pretreatment with these inhibitors also blocked the induction of *CATB* gene and eliminated the suppression of *CATC* gene under water stress (**Fig. 8B**). The variations in H_2O_2 accumulation and CAT activity were consistent with the ABA content in both pretreated samples and control (**Fig. 8C, D**). However, the effect of inhibitors on *CAT* gene expression, H_2O_2 accumulation and CAT activity was altogether weakened by exogenous ABA (**Fig. 8B–D**). All these results indicate that ABA not only mediates the accumulation of H_2O_2, but also indirectly controls H_2O_2 level by regulating the expression of *OsCATB*.

Discussion

Despite its cytotoxicity, H_2O_2 is involved in many developmental processes and stress responses acting as a signaling molecule (Neill et al. 2002, Ushio-Fukai 2006, Veal et al. 2007). To perform such a function, H_2O_2 production and accumulation must be well regulated. Water stress induced ABA and H_2O_2 production and H_2O_2 is mediated by endogenous ABA (Jiang and Zhang 2001, Hu et al. 2006). In this study, results prove that ABA not only mediates the accumulation of H_2O_2, but also controls the production of H_2O_2 by changing the expression pattern of the antioxidant gene, the rice *CAT* gene family, under water stress.

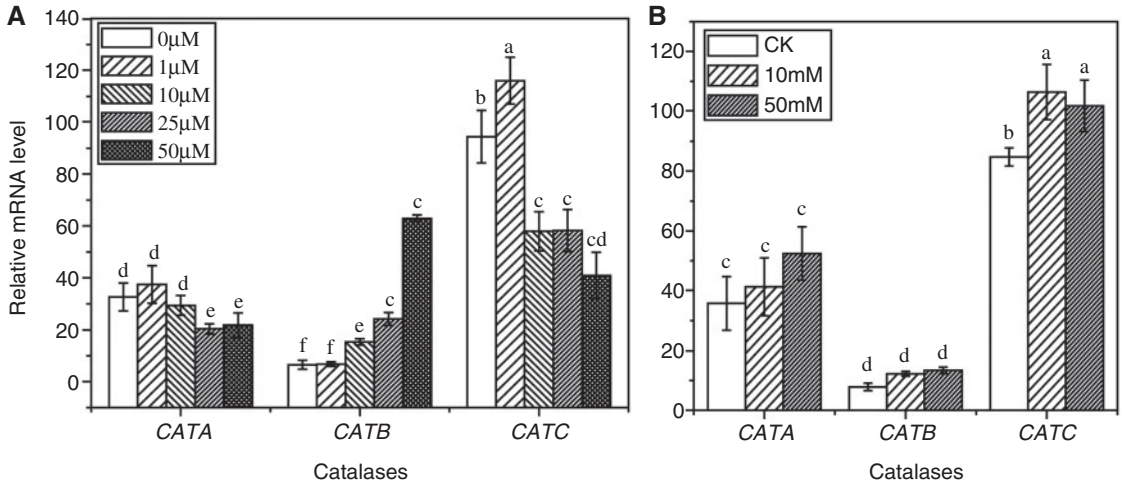

Fig. 5 Effects of ABA (A) and H_2O_2 (B) at different concentrations on expression of *CAT* genes. Two-week-old rice seedlings were treated with different concentrations of ABA and H_2O_2 for 4 h and then the leaf blade of seedlings was used for RNA purification. Transcript levels were quantified by qRT-PCR, as described in Materials and Methods. Values are means ± SD ($n = 3$). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

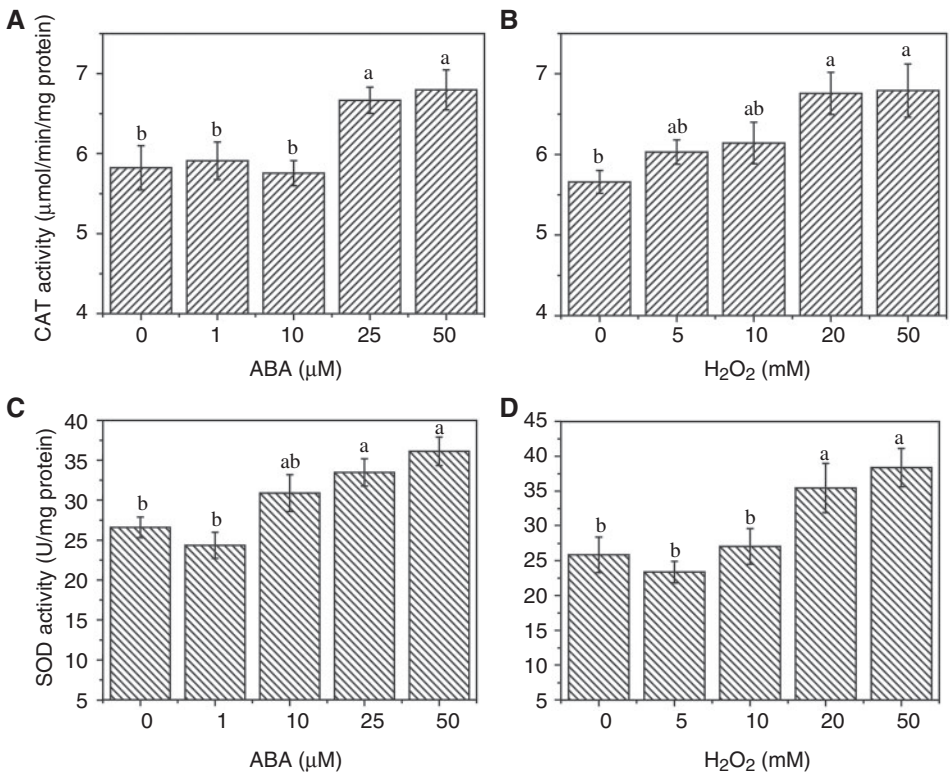

Fig. 6 Effect of ABA and H_2O_2 at different concentrations on CAT activity (A and B) and SOD activity (C and D). Two-week-old seedlings were treated by different concentrations of ABA and H_2O_2 for 4 h, and then 0.2 g of leaf blade was used for enzyme assays. Results were from triplicates, values are means ± SD ($n = 3$). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

Using the RIA method, we demonstrated the dynamic accumulation and degradation of ABA during a dehydration and rehydration cycle in rice seedlings (**Fig. 1A**), which provided a clue to analyzing the effect of ABA on the expression of *CAT* genes. Interestingly, the *OsNCED1* gene, which has the highest expression level in rice leaf and is the housekeeping gene in normal conditions, was significantly suppressed by water stress (**Fig. 1B**). This may lead to the conclusion that

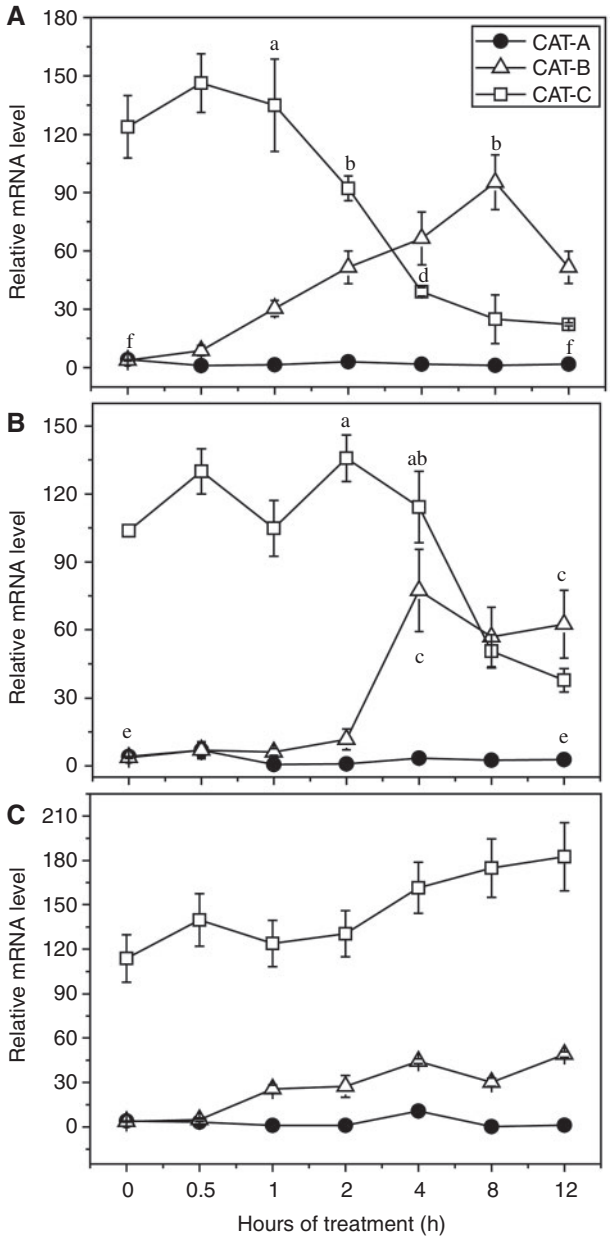

Fig. 7 Time-course of ABA (A), water stress (B) and H_2O_2 (C) treatments on expression of *CAT* genes in rice leaf blade. Leaf blade of rice seedlings from various treatment times were used from RNA purification. Values are means ± SD ($n = 3$). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

ABA accumulation has a feedback effect on the expression of *OsNCED1* gene (Tian et al. 2004).

Previous work proved the involvement of H_2O_2 in ABA-mediated stomatal closing under water stress conditions (Pei et al. 2000). Both endogenous and exogenous ABA can cause the generation of H_2O_2 in plant cells or tissues (Zhang et al. 2001, Kwak et al. 2003, Hu et al. 2005). Hu et al. (2006) further demonstrated that ABA mediates the accumulation of H_2O_2 in leaves of maize plants exposed to water stress. In this report, our findings are consistent with previous studies. The production of H_2O_2 was induced by endogenous ABA, which was induced by PEG treatment and exogenous ABA (**Fig. 2B**). The accumulation of H_2O_2 and enhancement of CAT and SOD activities required endogenous ABA because such effects were abolished by treatment with ABA biosynthesis inhibitors (**Fig. 2C, D**), suggesting that endogenous ABA accumulation is indispensable to H_2O_2 production. These results indicate the crucial role of ABA as a key regulator of redox homeostasis in rice leaves exposed to water stress.

Though CATs have been studied biochemically for over 100 years (Nicholls et al. 2000), their functions and interrelationship under stress conditions are still obscure because of their fairly complex spatial and temporal expression patterns (Scandalios et al. 1997, Willekens et al. 1997). In this study, we systematically analyzed the expression of the rice *CAT* gene family under water deficit conditions. Our results show that *CATA* is expressed mainly in leaf sheath and *CATC* is abundant in leaf blade (**Fig. 4A**); both of them are controlled by a circadian rhythm, which was not shifted but weakened under water stress. In contrast, expression of *CATB* was significantly induced in rice leaves exposed to water stress (**Fig. 4C**). Two orthologs of *OsCATB*, *CAT1* from maize and *CAT2* from wheat, were proved to be induced by water stress (Guan et al. 2000, Luna et al. 2004). These results indicate that *CATC* gene is responsible for scavenging photorespiration-associated H_2O_2 production and *CATB* gene plays a pivotal role in response to water stress. In plant leaves, there are at least six CAT enzymes and a CAT enzyme can be assembled from the same subunits or different subunits (Frugoli et al. 1996). The different responses of *CAT* genes imply that the plant has evolved with a mechanism to induce certain *CAT* genes to cope with stress conditions, which is also found in *NCED* genes under water stress (**Fig. 1B**).

It is well known that water stress induces the accumulation of H_2O_2 and CAT activity (Jiang and Zhang 2001, 2002). However, little is known about the mechanism of water stress-induced *CAT* gene expression. In this report, we provide results that the expression of the *CATB* gene is induced by ABA in a concentration-dependent manner and that *CATC* gene is suppressed by ABA (**Fig. 5A**). Expression of these two genes was not changed in plants exposed to H_2O_2 treatment (**Fig. 5B**), although applying H_2O_2 can also increase the total H_2O_2 content in rice leaf (**Fig. 2C**). These results indicate that it is ABA rather than H_2O_2 that regulates the expression of *CAT* genes under water stress. Results from the time-course experiment show that responses of *CAT* genes to water stress and ABA are similar. However, the effect of exogenous ABA treatment was faster than that of water stress treatment on the expression of *CAT* genes (**Fig. 7A, B**) due to the rapid increase in ABA. Pretreatment with ABA biosynthesis inhibitors NDGA and tungstate could arrest the expression of *CATB* and *CATC* genes under water deficit (**Fig. 8B**). The enhancement of CAT activity was also blocked by NDGA and tungstate (**Fig. 8D**). Together with previous results (Hu et al. 2006), this study

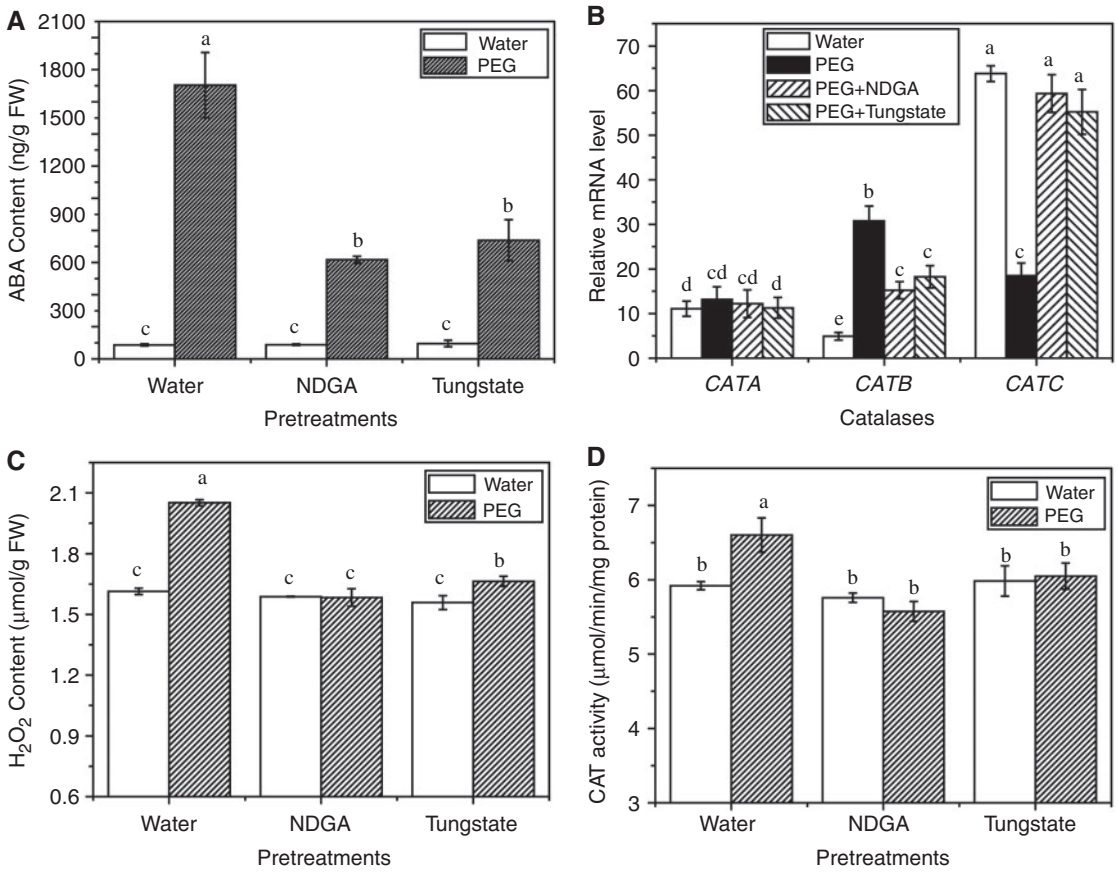

Fig. 8 Effect of ABA biosynthesis inhibitors on ABA accumulation (A), *CAT* gene expression (B), H_2O_2 accumulation (C) and CAT activity (D) under water stress in rice leaf blade. Rice seedlings were pretreated with water, 100 μM NDGA or 2 mM tungstate for 6 h and then exposed to water or 15 % PEG for 4 h. Error bars show ±SD ($n = 3$). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

demonstrates that endogenous ABA regulates both H_2O_2 production and *CAT* gene expression, which in turn keep H_2O_2 as a signaling molecule rather than a cytotoxic chemical.

In conclusion, our results suggest that water stress-induced ABA prevents the excessive accumulation of H_2O_2, through the induction of the expression of *CATB* gene during water stress. Such fine control of H_2O_2 production by ABA indicates its pivotal role when plants are under stress conditions.

Materials and Methods

Plant materials and treatments

Rice seeds (*Oryza sativa* L. cv. Yangdao 6) were surface sterilized by $NaClO_3$ for 20 min and soaked in distilled water overnight, and then germinated in a Petri dish with filter papers in darkness at 28°C for 2–3 d till the root measured 1 cm. Germinated seedlings were transferred to a black mesh and grown in Kimura B nutrient solution with a renewal every 3 d in a greenhouse at 25–30°C with 14-h/10-h (day/night) photoperiod shift. Two-week-old seedlings were prepared for further experimental use.

Water stress was imposed on seedlings in the presence of 15% PEG solution (−0.8 MPa) by immersing the root in the solution. In addition, plants were also treated with ABA (1, 10, 25 and 50 μM) and H_2O_2 (5, 10, 20 and 50 mM) solutions for various lengths of time (1, 4 and 6 h). To estimate the effect of ABA biosynthesis inhibitor, rice seedlings were pretreated with 100 μM NDGA or 2 mM tungstate by immersing the roots in these solutions for 6 h and spraying on the leaves every 2 h. Samples of rice leaves under different treatments were stored at −80°C for ABA determination, antioxidant enzyme assays and RNA isolation. The collection of samples and the extraction of H_2O_2 were conducted as soon as possible in order to prevent H_2O_2 degradation. Triplicates were set for all the experiments.

Measurement of endogenous ABA level

For the measurement of endogenous ABA levels, leaves were ground in liquid nitrogen, with the addition of 1 ml of water per 200 mg frozen ground tissue, and then shaken at 4°C overnight. The homogenates were centrifuged at 12 000 × *g* for 20 min at 4°C and the supernatant was used directly for ABA assay. ABA analysis was carried out using the RIA method as described by

Quarrie et al. (1988). The 450-µl reaction mixture contained 200 µl of phosphate buffer (pH 6.0), 100 µl of diluted antibody (Mac 252) solution, 100 µl of [^{3}H]ABA (about 8000 cpm) solution and 50 µl of crude extract. The mixture was then incubated at 4°C for 45 min and the bound radioactivity was measured in 50% saturated $(NH_4)_2SO_4$-precipitated pellets with a liquid scintillation counter (Ren et al. 2007).

Enzyme assays

Frozen leaf segments (0.2 g) were crushed into fine powder in a mortar and pestle in liquid nitrogen. Soluble proteins were extracted by homogenizing the powder in 1 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 15 000 × *g* for 20 min at 4°C and the supernatant was used for the following enzyme assays. Protein content was determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Total SOD (EC 1.15.1.1) activity was assayed by monitoring inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). The 2.5-ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 2 mM riboflavin, 0.1 mM EDTA and 20 µl of enzyme extract. The reaction mixtures were illuminated for 15 min at a light intensity of 5000 lx. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

CAT (EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm for 1 min (Aebi, 1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 50 µl of enzyme extract in a 3-ml volume.

H_2O_2 measurement

An Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Carlsbad, CA, USA) was used to measure H_2O_2 production in 2-week-old plants. Leaves were ground in liquid nitrogen. Then 500 µl of phosphate buffer (20 mM K_2HPO_4, pH 6.5) was added to 50 mg of ground frozen tissue. After centrifugation, 50 µl of the supernatant was incubated with 0.2 U ml^{-1} horseradish peroxidase and 100 µM Amplex Red reagent (10-acetyl-3,7-dihydrophenoxazine) at room temperature for 30 min under dark conditions. The fluorescence was quantified using Infinite® 200 PRO microplate reader (Tecan, Switzerland) (excitation at 560 nm and emission at 590 nm) (Xing et al. 2008).

RNA isolation and quantitative real time (qRT)-PCR

Total RNA was extracted from rice seeds with a Plant RNA Isolation Mini Kit (Agilent Technologies, USA) and then digested with RNase-free DNase I (Amersham, USA) to eliminate genomic DNA contamination. First-strand cDNA was synthesized with oligo(dT) primers using a SuperScript first-strand synthesis system according to the manufacturer's instructions (Invitrogen, USA). Transcript levels of each gene were measured by qRT-PCR using a Mx3000p QPCR System (Agilent, USA) with iQ SYBR Green Supermix (Bio-Rad, USA). The data were normalized to the amplification of a rice *ACTIN* gene. For each sample, the mean value from three qRT-PCRs was adopted to calculate the transcript abundance, and the mean values were then plotted with the standard deviation (SDs). Primer sequences of *ACTIN* and *OsCAT* genes used for qRT-PCR are listed in **Table 1**.

Table 1 Sequence of primers for *ACTIN* and *OsCAT* genes used for qRT-PCR

Gene	GenBank accession no.	Primers
ACTIN	AY212324	F: 5′-GGTATTGTTAGCAACTGGGATG-3′ R: 5′-GATGAAAGAGGGCTGGAAGA-3′
OsCATA	AK099923	F: 5′-AGGAGGCAGAAGGCGACGATACA-3′ R: 5′-TCTTCACATGCTTGGCTTCACGTT-3′
OsCATB	AK069446	F: 5′-GGCTGTCGGGAAAAGTGTGTCATTG-3′ R: 5′-TTTCAGGTTGAGACGTGAAGCCAGC-3′
OsCATC	AK066378	F: 5′-TCAAGAGATGGATCGACGCACTCTC-3′ R: 5′-GAAGCAGATTGCAACGCTGATCG-3′

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References

Aebi, H. (1984) Catalase in vitro. *Methods Enzymol.* 105: 121–126.

Agrawal, G.K., Rakwal, R. and Jwa, N.S. (2001) Stress signaling molecules involved in defense and protein phosphatase 2A inhibitors modulate *OsCATC* expression in rice (*Oryza sativa*) seedlings. *J. Plant Physiol.* 158: 1349–1355.

Apel, K. and Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55: 373–399.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Du, Y.Y., Wang, P.C., Chen, J. and Song, C.P. (2008) Comprehensive functional analysis of the catalase gene family in *Arabidopsis thaliana*. *J. Integr. Plant Biol.* 50: 1318–1326.

Foyer, C.H. and Noctor, G. (2005) Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. *Plant, Cell Environ.* 28: 1056–1071.

Frugoli, J.A., Zhong, H.H., Nuccio, M.L., McCourt, P., McPeek, M.A., Thomas, T.L. et al. (1996) Catalase is encoded by a multigene family in *Arabidopsis thaliana* (L.) Heynh. *Plant Physiol.* 112: 327–336.

Giannopolitis, C.N. and Ries, S.K. (1977) Superoxide dismutase. I. Occurrence in higher plants. *Plant Physiol.* 59: 309–314.

Gould, S.J., Keller, G.A., Hosken, N., Wilkinson, J. and Subramani, S. (1989) A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* 108: 1657–1664.

Guan, L. and Scandalios, J. (2000) Hydrogen peroxide-mediated catalase gene expression in response to wounding. *Free Radic. Biol. Med.* 28: 1182–1190.

Guan, L., Zhao, J. and Scandalios, J.G. (2000) Cis-elements and trans-factors that regulate expression of the maize *Cat1* antioxidant gene in response to ABA and osmotic stress: H_2O_2 is the likely intermediary signaling molecule for the response. *Plant J.* 22: 87–95.

Higo, K. and Higo, H. (1996) Cloning and characterization of the rice *CatA* catalase gene, a homologue of the maize *Cat3* gene. *Plant Mol. Biol.* 30: 913–924.

Himmelbach, A., Yang, Y. and Grill, E. (2003) Relay and control of abscisic acid signaling. *Curr. Opin. Plant Biol.* 6: 470–479.

Hu, X., Jiang, M., Zhang, A. and Lu, J. (2005) Abscisic acid-induced apoplastic H_2O_2 accumulation up-regulates the activities of chloroplastic and cytosolic antioxidant enzymes in maize leaves. *Planta* 223: 57–68.

Hu, X., Zhang, A., Zhang, J. and Jiang, M. (2006) Abscisic acid is a key inducer of hydrogen peroxide production in leaves of maize plants exposed to water stress. *J. Exp. Bot.* 47: 1484–1495.

Hu, Y.Q., Liu, S., Yuan, H.M., Li, J., Yan, D.W., Zhang, J.F. et al. (2010) Functional comparison of catalase genes in the elimination of photorespiratory H_2O_2 using promoter- and 3′-untranslated region exchange experiments in the *Arabidopsis cat2* photorespiratory mutant. *Plant, Cell Environ.* 33: 1656–1670.

Iwamoto, M., Higo, H. and Higo, K. (2000) Differential diurnal expression of rice catalase genes: the 5′-flanking region of CatA is not sufficient for circadian control. *Plant Sci.* 151: 39–46.

Jiang, M. and Zhang, J. (2001) Effect of abscisic acid on active oxygen species, antioxidative defence system and oxidative damage in leaves of maize seedlings. *Plant Cell Physiol.* 42: 1265–1273.

Jiang, M. and Zhang, J. (2002) Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. *J. Exp. Bot.* 53: 2401–2410.

Kwak, J.M., Mori, I.C., Pei, Z.M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D. and Schroeder, J.I. (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. *EMBO J.* 22: 2623–2633.

Laloi, C., Apel, K. and Danon, A. (2004) Reactive oxygen signaling: the latest news. *Curr. Opin. Plant Biol.* 7: 323–328.

Luna, C.M., Pastrori, G.M., Driscoll, S., Groten, K., Bernard, S. and Foyer, C.H. (2004) Drought controls on H_2O_2 accumulation, catalase (CAT) activity and *CAT* gene expression in wheat. *J. Exp. Bot.* 58: 417–423.

Miller, G., Shulaev, V. and Mittler, R. (2008) Reactive oxygen signaling and abiotic stress. *Physiol. Plant.* 133: 481–489.

Mittler, R., Vanderauwera, S., Gollery, M. and Van-Breusegem, F. (2004) Reactive oxygen gene network of plants. *Trends Plant Sci.* 9: 490–498.

Morita, S., Tasaka, M., Fujisawa, H., Ushimaru, T. and Tsuji, H. (1994) A cDNA clone encoding a rice catalase lsozyme. *Plant Physiol.* 105: 1015–1016.

Neill, S.J., Desikan, R. and Hancock, J.T. (2002) Hydrogen peroxide signaling. *Curr. Opin. Plant Biol.* 5: 388–395.

Nicholls, P., Fita, I. and Loewen, P. (2000) Enzymology and structure of catalases. *Adv. Inorg. Chem.* 51: 51–106.

Pei, Z.M., Murata, Y., Benning, G., Thomine, S., Klusener., B., Allen., G.J., Grill, E. et al. (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signaling in guard cells. *Nature* 406: 731–734.

Polidoros, A.N. and Scandalios, J.G. (1999) Role of hydrogen peroxide and different classes of antioxidants in the regulation of catalase and glutathione S-transferase gene expression in maize (*Zea mays* L.). *Physiol. Plant.* 106: 112–120.

Quarrie, S.A., Whitford, P.N., Appleford, N.E.J., Wang, T.L., Cook, S.K., Henson, I.E. et al. (1988) A monoclonal antibody to (S)-abscisic acid: its characterization and use in a radioimmunoassay for measuring abscisic acid in crude extracts of cereal and lupin leaves. *Planta* 173: 330–339.

Ren, H., Gao, Z., Chen, L., Wei, K., Liu, J., Fan, Y. et al. (2007) Dynamic analysis of ABA accumulation in relation to the rate of ABA catabolism in maize tissues under water deficit. *J. Exp. Bot.* 58: 211–219.

Ronquist, F. and Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

Scandalios, J.G., Guan, L. and Polidoros, A.N. (1997) Catalases in plants: gene structure, properties, regulation and expression. *In* Oxidative Stress and the Molecular Biology of Antioxidant Defences. Edited by Scandalios, J.G. pp. 343–406. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M. and Waner, D. (2001) Guard cell signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52: 627–658.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Molecular responses to dehydration and low temperature: differences and crosstalk between two stress signaling pathways. *Curr. Opin. Plant Biol.* 3: 217–223.

Slesak, I., Libik, M., Karpinska, B., Karpinski, S. and Miszalski, Z. (2007) The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. *Acta Biochim. Pol.* 54: 39–50.

Tian, L., DellaPenna, D. and Zeevaart, J.A.D. (2004) Effects of hydroxylated carotenoid deficiency on ABA accumulation in *Arabidopsis*. *Physiol. Plant.* 122: 314–320.

Torres, M.A. and Dangl, J.L. (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. *Curr. Opin. Plant Biol.* 8: 397–403.

Ushio-Fukai, M. (2006) Localizing NADPH oxidase-derived ROS. *Sci. STKE* 2006: re8.

Veal, E.A., Day, A.M. and Morgan, B.A. (2007) Hydrogen peroxide sensing and signaling. *Mol. Cell* 26: 1–14.

Willekens, H., Chamnongpol, S., Davey, M., Schraudner, M., Langebartels, C., Van-Montagu, M. et al. (1997) Catalase is a sink for H_2O_2 and is indispensable for stress defence in C3 plants. *EMBO J.* 16: 4806–4816.

Xing, Y., Jia, W.S. and Zhang, J.H. (2008) *AtMKK1* mediates ABA-induced *CAT1* expression and H_2O_2 produced via AtMPK6-coupled signaling in Arabidopsis. *Plant J.* 54: 440–451.

Zhang, X., Zhang, L., Dong, F., Gao, J., Galbraith, D.W. and Song, C.P. (2001) Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *Plant Physiol.* 126: 1438–1448.

Zhu, G., Ye, N. and Zhang, J. (2009) Glucose-induced delay of seed germination in rice is mediated by the suppression of ABA catabolism rather than an enhancement of ABA biosynthesis. *Plant Cell Physiol.* 50: 644–651.

Zhu, J.K. (2002) Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* 53: 247–273.