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Research article

Differential responses of antioxidative system to chilling and drought in four rice cultivars differing in sensitivity

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Abstract

Responses of antioxidative defense systems to chilling and drought stresses were comparatively studied in four cultivars of rice (*Oryza sativa* L.) differing in sensitivity, two of them (Xiangnuo no. 1 and Zimanuo) are tolerant to chilling but sensitive to drought and the other two (Xiangzhongxian no. 2 and IR50) are tolerant to drought but sensitive to chilling. The seedlings of rice were transferred into growth chamber for 5 d at 8 °C as chilling treatment, or at 28 °C as control, or at 28 °C but cultured in 23% PEG-6000 solution as drought stress treatment. Under drought stress the elevated levels of electrolyte leakage, contents of H_2O_2 and total thiobarbituric acid-reacting substances (TBARS) in Xiangzhongxian no. 2 and IR50 are lower than those in Xiangnuo no. 1 and Zimanuo. On the contrary, Xiangnuo no. 1 and Zimanuo have much lower level of electrolyte leakage, H_2O_2 and TBARS than Xiangzhongxian no. 2 and IR50 under chilling stress. Activities of antioxidant enzymes (superoxide dismutase (SOD), catalase, and ascorbate-peroxidase (APX)) and contents of antioxidants (ascorbaic acid and reduced glutathione) were measured during the stress treatments. All of them were enhanced greatly until 3 d after drought stress in the two drought-tolerant cultivars, or after chilling stress in the two chilling-tolerant cultivars. They all were decreased at 5 d after stress treatments. On the other hand, activities of antioxidant enzymes and contents of antioxidants were decreased greatly in the drought-sensitive cultivars after drought stress, or in the chilling-sensitive cultivars after chilling stress. The results indicated that tolerance to drought or chilling in rice is well associated with the enhanced capacity of antioxidative system under drought or chilling condition, and that the sensitivity of rice to drought or chilling is linear correlated to the decreased capacity of antioxidative system.

Keywords: Antioxidative system; Chilling; Drought; Rice

1. Introduction

Abiotic stresses are major limiting factors in crops production. Much of the injury to plants caused by abiotic stresses is associated with oxidative damage at cellular level [2]. Oxidative stress may be a significant factor in relation to chilling and drought induced injury [6,26,29,36]. Higher plants have active oxygen-scavenging systems consisting of several antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate-peroxidase (APX), glutathione reductase (GR) and catalase (CAT), and some low molecules of non-enzyme antioxidants, such as ascorbic acid (AsA) and reduced glutathione (GSH) [2]. These systems protect membranes from the deleterious effects of ROS, such as superoxide radicals, hydrogen peroxide (H_2O_2), hydroxyl radicals and singlet oxygen, which are produced at elevated rates when plants are exposed to abiotic stress conditions [2,25]. The superoxide radical is dismutated to H_2O_2 by SOD, and CAT and APX metabolize H_2O_2 into H_2O. AsA is the substrate of APX. Glutathione reductase and GSH are involved in the regeneration of AsA from monodehydroascorbate. In addition, AsA and GSH can directly detoxify superoxide and hydroxyl radical and thus contribute to non-enzymatic ROS scavenging [25].

Numerous studies indicated that activities of antioxidant enzymes are correlated with plant tolerance to abiotic stresses. The drought-induced damage was negatively correlated with

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capacity for the increase of SOD and CAT activities in mosses cultivars differing in drought tolerance [4]. A correlation between antioxidant enzyme activity and water stress or salinity tolerance was demonstrated by comparison of a tolerant-cultivar with a sensitive cultivar in several plant species, such as rice [5,36,37], foxtail millet (*Setaria italica*) [35], and tomato [24]. Likewise, chilling-tolerant cultivars have higher activities of antioxidant enzymes than susceptible cultivars in several crops, such as rice [11,31], cucumber [13,33], and maize [9,10]. ABA-enhanced antioxidant enzyme activity induces tolerance of many crops to chilling and water stress [23,38]. Chilling tolerance induced by heat shock treatment in rice is also correlated with elevated antioxidant enzyme activity [14].

In a previous study, quite different tolerance to chilling and drought among rice cultivars was observed. Xiangnuo no. 1 and Zimanuo are tolerant to chilling, but sensitive to drought stress [22]. This study was to investigate the differential responses of antioxidant system to chilling and drought stress, by use of two cultivars which are tolerant to chilling but sensitive to drought, or tolerant to drought but sensitive to chilling. Activities of SOD, CAT, APX and contents of AsA and GSH were determined from the seedlings subjected to chilling or drought stress. We show that the tolerance of rice to drought or chilling is well associated with the enhanced capacity of antioxidative system under stresses, and that the sensitivity of rice to drought or chilling is linear correlated to the decreased capacity of antioxidative system.

2. Results

2.1. Electrolyte leakage, H_2O_2 content and lipid peroxidation under drought and chilling stresses

Electrolyte leakage reflected the damage of stresses to the plasmalemma. It showed minor variations in tolerant cultivars, Xiangnuo no. 1 and Zimanuo, after chilling stress, whereas it increased in sensitive cultivars, Xiangzhongxian no. 2 and IR-50 (Fig. 1). The results indicated that Xiangnuo no. 1 and Zimanuo were less damaged under chilling stress than Xiangzhongxian no. 2 and IR-50. Under drought stress, Xiangzhongxian no. 2 and IR-50 had a slight increase of electrolyte leakage at 3 d and a great increase at 5 d, whereas electrolyte leakage in drought-sensitive cultivars, Xiangnuo no. 1 and Zimanuo, increased greatly (Fig. 1), indicating that Xiangzhongxian no. 2 and IR-50 were less damaged under drought stress than Xiangnuo no. 1 and Zimanuo.

Hydrogen peroxide level increased after plants were subjected to drought or chilling stress. At 5 d following chilling stress, Xiangnuo no. 1, Zimanuo and Xiangzhongxian no. 2 had a reduced H_2O_2 accumulation than IR-50 (Fig. 2). Under drought stress, H_2O_2 level increased greatly in the drought-sensitive cultivars, Xiangnuo no. 1 and Zimanuo, whereas in Xiangzhongxian no. 2 and IR-50 it was low (Fig. 2). Total amount of thiobarbituric acid-reacting substances (TBARS) reflects the level of lipid peroxidation resulting from oxidative stress. TBARS content in all tested cultivars increased during

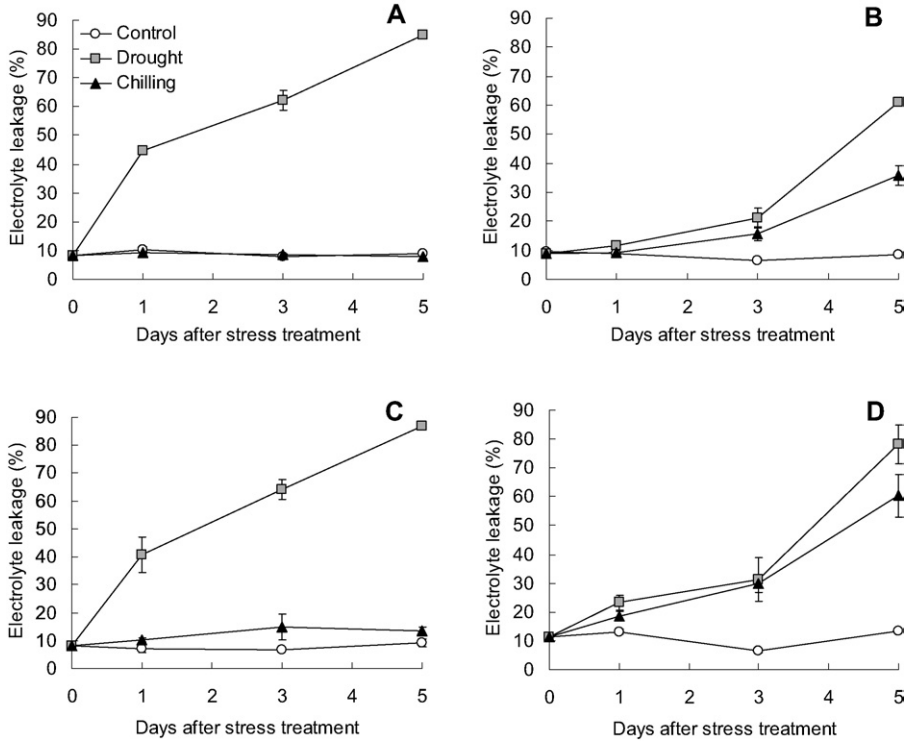

Fig. 1. Electrolyte leakage of four cultivars of rice (A. Xiangnuo no. 1; B. Xiangzhongxian no. 2; C. Zimanuo; D. IR50) under PEG-induced drought stress and chilling stress. The data were means of three measurements from independent samples. Electrolyte leakage was measured at 0, 1, 3, and 5 d after the seedlings were treated by either drought or chilling stress, or normal growth condition as a control.

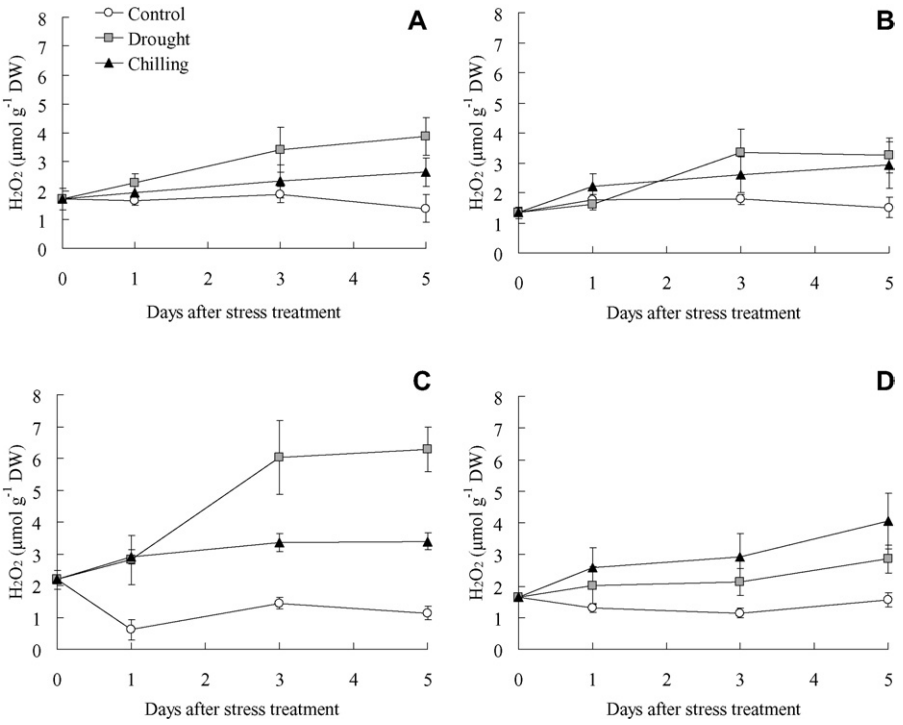

Fig. 2. Hydrogen peroxide content of four cultivars of rice (A. Xiangnuo no. 1; B. Xiangzhongxian no. 2; C. Zimanuo; D. IR50) under PEG-induced drought stress and chilling stress. The data were means of three measurements from independent samples. The fresh materials were used for measurements of hydrogen peroxide content at 0, 1, 3, and 5 d after the seedlings were treated by either drought or chilling stress, or normal growth condition as a control.

chilling stress. However, in comparison to their controls, the chilling-sensitive cultivars, Xiangzhongxian no. 2 and IR 50, increased more TBARS than the two chilling-tolerant cultivars, Xiangnuo no. 1 and Zimanuo. Xiangzhongxian no. 2 and IR 50 accumulated 2.71 and 2.8-folds more TBARS than their controls, respectively, at 5 d following chilling treatment, whereas Xiangnuo no. 1 and Zimanuo accumulated 1.97 and 1.85-folds more TBARS than their control plants, respectively (Fig. 3).

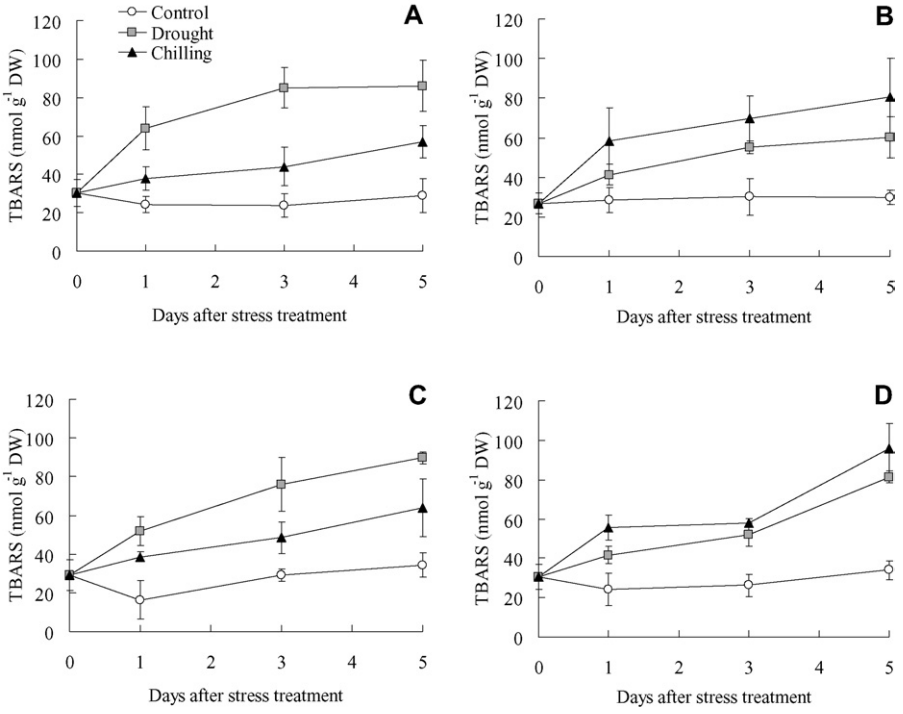

Fig. 3. TBARS content of four cultivars of rice (A. Xiangnuo no. 1; B. Xiangzhongxian no. 2; C. Zimanuo; D. IR50) under PEG-induced drought stress and chilling stress. The data were means of three measurements from independent samples. The fresh materials were used for measurements of TBARS content at 0, 1, 3, and 5 d after the seedlings were treated by either drought or chilling stress, or normal growth condition as a control.

TBARS in Xiangzhongxian no. 2 and IR-50 increased slightly and gradually after drought stress. The increased TBARS content was positively correlated with the increased electrolyte leakage at r=0.775 and 0.991, respectively. TBARS in sensitive cultivars, Xiangnuo no. 1 and Zimanuo, increased greatly and rapidly after drought stress (Fig. 3), which was positively correlated with the increased electrolyte leakage at r=0.960, 0.996, respectively.

2.2. Changes of antioxidative enzymes activity under drought and chilling stresses

SOD activity of four cultivars showed different changes. At 3 d after drought stress SOD activity in Xiangzhongxian no. 2 and IR50 was slightly higher than their control, while that in Xiangnuo no. 1 and Zimanuo decreased greatly after drought stress and had only 39.29% and 38.04% of their control plants at 5 d (Fig. 4). The decreased SOD activity in Xiangnuo no. 1 and Zimanuo showed a linear negative correlation (r=–0.938, –0.993, respectively) with the increased electrolyte leakage.

SOD activity in the chilling-tolerant cultivars, Xiangnuo no. 1 and Zimanuo, increased by 17% and 19% at 3 d after chilling stress. On the contrary, SOD activity in IR50 and Xiangzongxian no. 2 decreased after chilling stress and had only 68% and 67% of their control plants at 5 d (Fig. 5). The decline of SOD activity showed linear negative correlation (r=–0.669, –0.977) with the increased electrolyte leakage in Xiangzhongxian no. 2 and IR-50.

CAT activity increased greatly in Xiangzhongxian no. 2 and IR50 after drought stress and had 48% and 33% higher activity than their control plants at 3 d. It decreased at 5 d when the plants were damaged. CAT activity in Xiangnuo no. 1 and Zimanuo decreased greatly until it could not be determined at 5 d (Fig. 5). The decline of CAT activity showed a linear negative correlation with the increased electrolyte leakage at r=–0.93 and –0.998 in Xiangnuo no. 1 and Zimanuo, respectively.

Under chilling stress, the chilling-tolerant cultivars, Xiangnuo no. 1 and Zimanuo, maintained higher CAT activity than their control plants, while CAT activity in Xiangzhongxian no. 2 and IR-50 continuously decreased (Fig. 5). The decline of CAT activity showed a linear negative correlation with the increased electrolyte leakage at r=–0.885 and –0.933, respectively.

APX activity in the four cultivars under either drought or chilling stress showed similar patterns to CAT activity. APX activity increased at 3 d after drought by 38% and 24% in Xiangzhongxian no. 2 and IR50, respectively, and then decreased at 5 d, while that in Xiangnuo no. 1 and Zimanuo decreased continuously (Fig. 6), with a linear negative correlation with increased electrolyte leakage at r=–0.946 and –0.987. Chilling-tolerant cultivars, Xiangnuo no. 1 and Zimanuo, maintained higher APX activity under chilling stress, while APX activity in Xiangzhongxian no. 2 and IR-50 decreased greatly (Fig. 6), with a linear correlation with increased electrolyte leakage at r=–0.838 and –0.964.

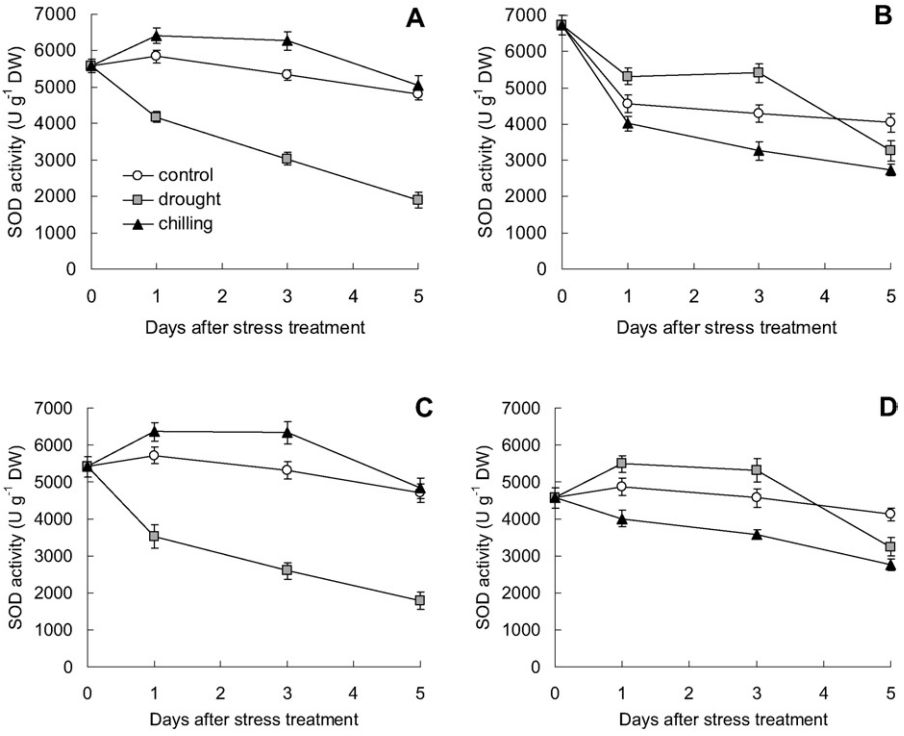

Fig. 4. SOD activity of four cultivars of rice (A. Xiangnuo no. 1; B. Xiangzhongxian no. 2; C. Zimanuo; D. IR50) under PEG-induced drought stress and chilling stress. The data were means of three measurements from independent samples. The fresh materials were used for measurements of SOD activity at 0, 1, 3, and 5 d after the seedlings were treated by either drought or chilling stress, or normal growth condition as a control.

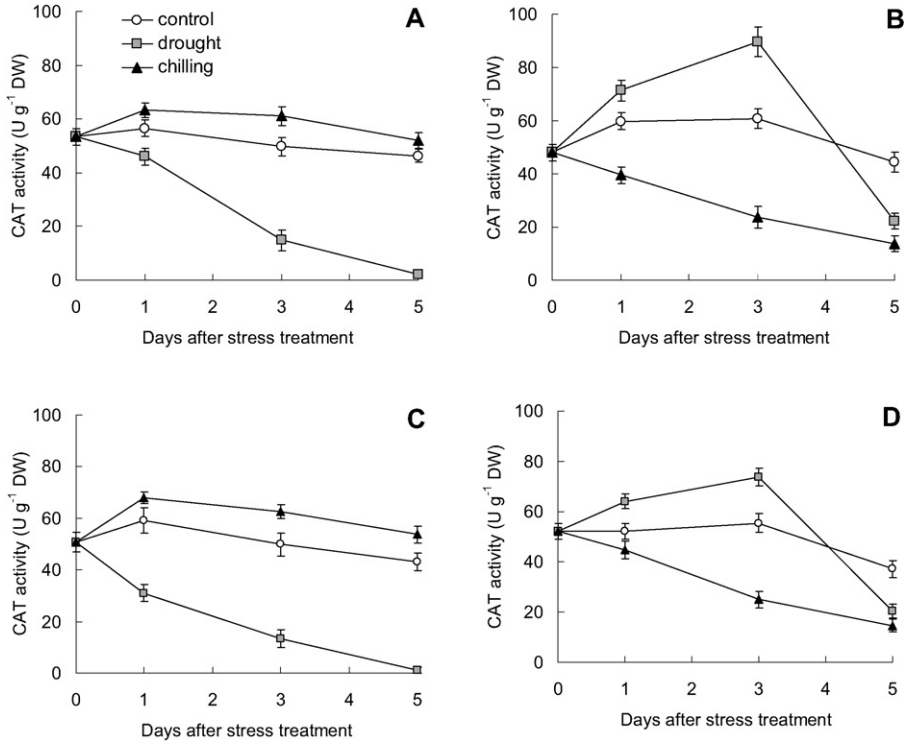

Fig. 5. Catalase (CAT) activity of four cultivars of rice (A. Xiangnuo no. 1; B. Xiangzhongxian no. 2; C. Zimanuo; D. IR50) under PEG-induced drought stress and chilling stress. The data were means of three measurements from independent samples. The fresh materials were used for measurements of CAT activity at 0, 1, 3, and 5 d after the seedlings were treated by either drought or chilling stress, or normal growth condition as a control.

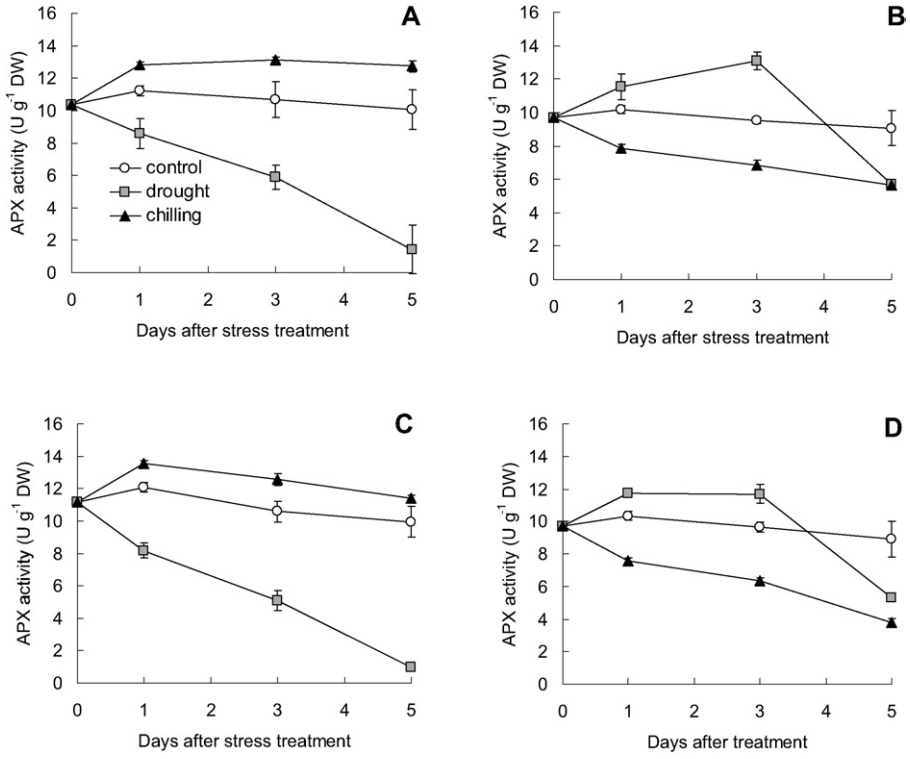

Fig. 6. APX activity of four cultivars of rice (A. Xiangnuo no. 1; B. Xiangzhongxian no. 2; C. Zimanuo; D. IR50) under PEG-induced drought stress and chilling stress. The data were means of three measurements from independent samples. The fresh materials were used for measurements of APX activity at 0, 1, 3, and 5 d after the seedlings were treated by either drought or chilling stress, or normal growth condition as a control.

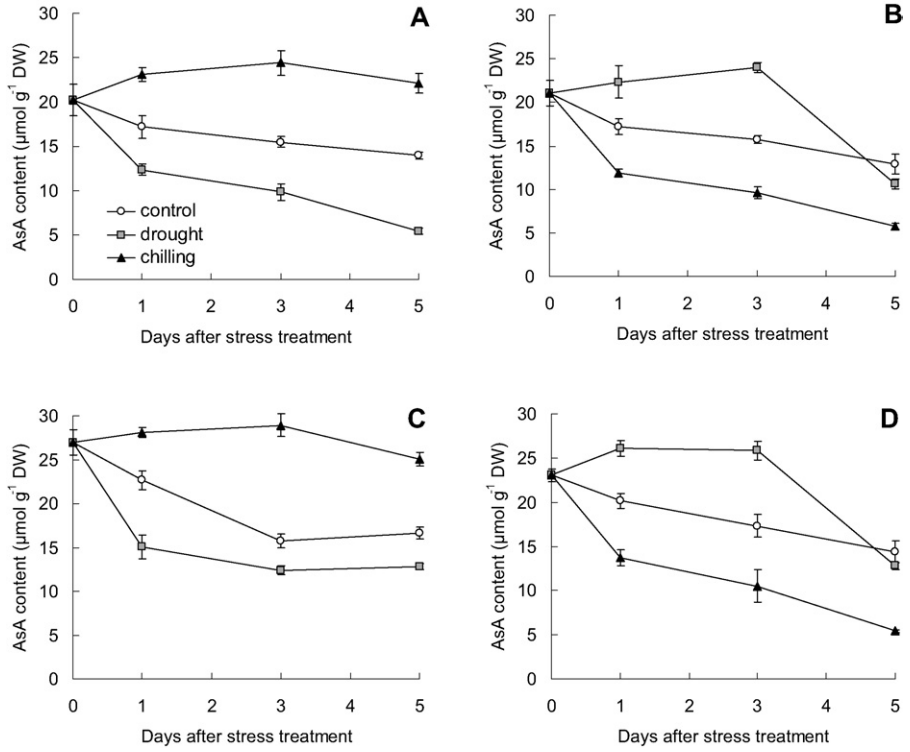

Fig. 7. AsA content of four cultivars of rice (A. Xiangnuo no. 1; B. Xiangzhongxian no. 2; C. Zimanuo; D. IR50) under PEG-induced drought stress and chilling stress. The data were means of three measurements from independent samples. The fresh materials were used for measurements of AsA content at 0, 1, 3, and 5 d after the seedlings were treated by either drought or chilling stress, or normal growth condition as a control.

2.3. Changes of antioxidants in four rice cultivars under chilling and drought stresses

AsA and GSH content increased in Xiangzhongxian no. 2 and IR50 after drought stress and were 49–62% higher than their control plants at 3 d, while those in Xiangnuo no. 1 and Zimanuo decreased gradually (Figs. 7 and 8), and showed a linear negative correlation with the increased electrolyte leakage at r=–0.998 and –0.892 for AsA and at r=–0.979 and –0.984 for GSH, respectively. Under chilling stress, AsA and GSH content increased greatly in chilling-tolerant cultivars, Xiangnuo no. 1 and Zimanuo, and were 58–92% higher than their control plants at 3 d. AsA and GSH content in Xiangzhongxian no. 2 and IR50 decreased under chilling stress (Figs. 7 and 8), which showed a linear negative correlation with the increased electrolyte leakage at r=–0.756 and –0.887 for AsA and r=–0.797 and –0.936 for GSH.

3. Discussion

In our previous study Xiangzhongxian no. 2 was screened as a drought-tolerant cultivar, and Xiangnuo no. 1 and Zimanuo were screened as chilling-tolerant but drought-sensitive cultivars [22]. IR50 is very sensitive to chilling [11]. In consistence, electrolyte leakage in Xiangnuo no. 1 and Zimanuo was little altered under chilling stress, but increased greatly under drought stress, while that in Xiangzhongxian no. 2 and IR50 showed less enhanced, but they are sensitive to chilling stress (Fig. 1). Therefore, experiments were conducted, with Xiangnuo no. 1 and Zimanuo as both chilling-tolerant and drought-sensitive cultivars, while Xiangzhongxian no. 2 and IR50 as both chilling-sensitive and drought-tolerant cultivars, to investigate the correlation of antioxidant systems and stress tolerance.

Hydrogen peroxide is one type of ROS. Its level increased after rice seedlings were subjected to drought or chilling stress, indicating the oxidative stress occurred. TBARS is an indicator of lipid peroxidation and oxidative damages on membranes. Drought-tolerant cultivar, Xiangzhongxian no. 2 and IR50, accumulated less H_2O_2 and TBARS than the sensitive cultivars, Xiangnuo no. 1 and Zimanuo, under drought stress, whereas the chilling-tolerant cultivars, Xiangnu no. 1 and Zimanuo, had less H_2O_2 and TBARS accumulation under chilling stress. The increased electrolyte leakage showed positively correlated to accumulation of TBARS, indicating that the plasmalemma injury caused by chilling or drought stress is resulted from the oxidative damage. Numerous investigations have demonstrated that the cellular injury to plants by abiotic stresses is oxidative damage [1,2]. It was similarly observed in rice [11] and maize [3,12] that chilling or drought-tolerant cultivars showed higher tolerance to oxidative damage.

SOD dismutes active oxygen radical ($O_2^{-\bullet}$) into H_2O_2 and plays a key role in cellular defense against reactive oxygen species (ROS). Its activity in the two drought- or chilling-sensitive cultivars decreased after the plants were subjected to drought or chilling stresses and showed linear negative correlation to increased electrolyte leakage under stresses, suggesting

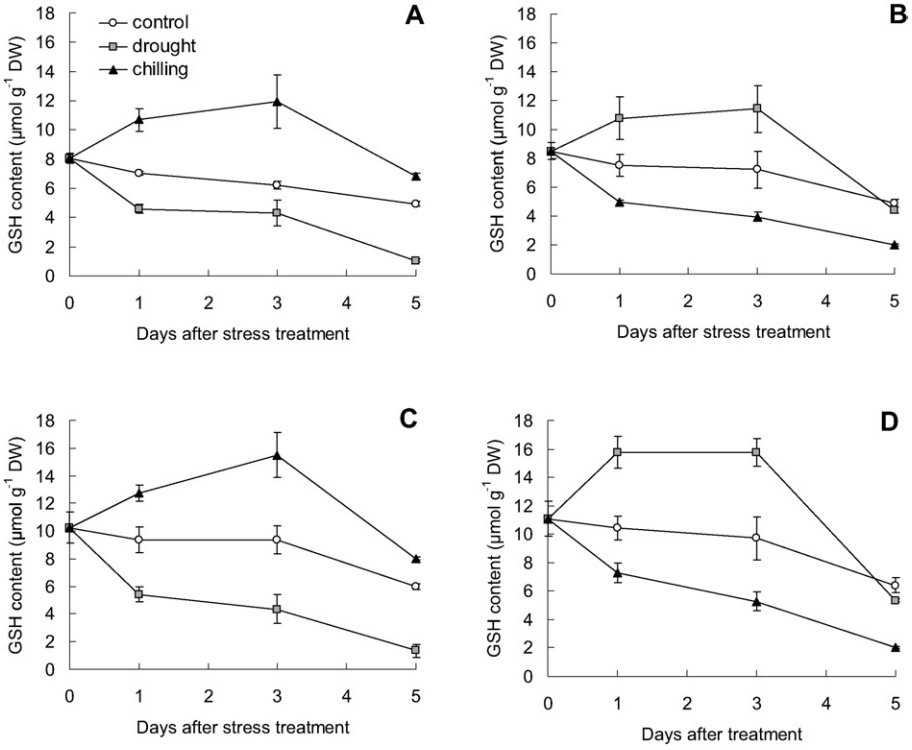

Fig. 8. Reduced glutathione (GSH) content of four cultivars of rice (A. Xiangnuo no. 1; B. Xiangzhongxian no. 2; C. Zimanuo; D. IR50) under PEG-induced drought stress and chilling stress. The data were means of three measurements from independent samples. The fresh materials were used for measurements of GSH content at 0, 1, 3, and 5 d after the seedlings were treated by either drought or chilling stress, or normal growth condition as a control.

that the association of decreased SOD activity with the stress damages. The two chilling-tolerant rice cultivars or drought-tolerant cultivars had higher SOD activity than their sensitive cultivars under chilling or drought stress. Higher SOD activity in tolerant cultivars indicated higher $O_2^{-\bullet}$ scavenging activity under stresses. Our results are in consistence with the reports that higher SOD activity have shown that in chilling-tolerant cultivars, such as that of maize, cucumber and rice, than in chilling-susceptive cultivars when plants were subjected to chilling stress [11,12,33]. Similarly, drought-tolerant cultivars of wheat had much higher SOD activity than drought-susceptible cultivars under drought stress [20,30].

Hydrogen peroxide is still a ROS and even more toxic than $O_2^{-\bullet}$ to cells. Therefore, it is important that H_2O_2 is scavenged rapidly by the antioxidative defense system. Catalase and APX are primary H_2O_2 scavenging enzymes. Glutathione reductase and GSH involved in the regeneration of AsA, the substrate of APX. These enzymes and antioxidants have important roles in protection of plants from oxidative damage. Transgenic plants elevating APX or GR improved the recovery of cotton after chilling treatment [28]. Heat shock enhanced chilling resistance was attributed to the induction of APX gene in rice [32] and elevated levels of SOD, CAT and APX in cucumber [14]. Application of exogenous AsA increased the tolerance of rice to chilling and water stress [8] and improved the tolerance of *Cassia angustifolia* to water stress [34]. Increasing GSH synthesis or exogenous GSH treatment increased chilling tolerance of maize, while inhibiting GSH synthesis reduced chilling tolerance [16,17]. In this work, the antioxidants involved in H_2O_2 scavenging, CAT, APX and GR activities and AsA and GSH contents, had similar responses to stresses. They increased in both drought- and chilling-tolerant cultivars, but greatly decreased in both sensitive cultivars under drought or chilling stress. This result was confirmed by our previous observation that CAT, APX and GR activities and AsA contents were higher in Xiangnuo no. 1 than those in IR-50 under chilling stress [11]. Higher antioxidant system activity was also observed in the drought-tolerant cultivars of wheat [20,30] and coffea [21], compared to their susceptible cultivars. The enhanced scavenging ability for H_2O_2 in tolerant cultivars inhibited the accumulation of ROS and thus protected the plants from lipid peroxidation of membrane systems and oxidative damages under chilling or drought stress.

Antioxidative system in Xiangnuo no. 1 and Zimanuo, as chilling tolerant cultivars, was induced at the beginning days under chilling stress, and that in Xiangzhongxian no. 2 and IR-50, as drought cultivars, was induced under drought stress as well. In contrast, antioxidative system in Xiangnuo no. 1 and Zimanuo, as drought-sensitive cultivars, decreased continuously under drought stress. Likewise, antioxidative system in Xiangzhongxian no. 2 and IR-50, as chilling sensitive cultivars, decreased continuously under chilling stress as well. The results indicated that the tolerance for a specific cultivar of rice to stresses, such as drought or chilling, depends upon its responses of antioxidative system. The decreased antioxidative system is linear correlated with the enhanced electrolyte leakage, suggesting that the decline of antioxidative system induced by drought and chilling is associated with the sensitiv-

ity of rice cultivars to the stresses. It is unclear what caused the induction of antioxidant system in tolerant-cultivar. Abscisic acid induces the activities of antioxidative enzymes [18,38] and is involved in the water stress induced antioxidative enzymes activity in maize [19]. In comparison to the sensitive cultivars, more ABA was accumulated in drought-tolerant cultivars under drought stress, and in chilling-tolerant cultivars under chilling stress [22]. It is probably that ABA involved in the induction of antioxidative system in the tolerant cultivars of rice under stresses.

4. Materials and methods

4.1. Plant materials and treatments

The seedlings of four rice (*Oryza sativa* L.) cultivars, Xiangnuo no. 1 Xiangzhongxian no. 2, Zimanuo and IR-50 were cultured in Kimura B nutrient solution in a glasshouse under natural light conditions for 12 d at 25–30 °C as described before [8]. The seedlings were then grown for 5 d in growth chambers at 70% humidity, and 12-h photoperiod at 165 μmol m^{-2} s^{-1} photosynthetic photon flux density. The growth chamber temperatures were 8 °C for the chilling treatment and 28 °C for drought stress treatment and the control.

4.2. Determination of electrolyte leakage

The electrolyte leakage was determined as described before [11]. Shoots of four seedlings were immersed in 15 ml of distilled water in a test tube overnight at room temperature. The initial conductivity was determined using a conductivity meter (Model DDS-IIA, Shanghai Leici Instrument Inc., Shanghai, China). The tubes were then placed in boiling water for 15 min, and cooled to room temperature. Conductivity was again determined. The electrolyte leakage was calculated as the ratio of conductivity before boiling to that after boiling.

4.3. Determinations of enzymes activity and lipid peroxidation

Fresh shoots (0.5 g) were ground in a mortar with pestle in 5 ml of 50 mM phosphate buffer (pH 7.8) at 4 °C. The homogenate was centrifuged at 13,000×*g* for 15 min. The supernatant was recovered for determinations of SOD and CAT activities as well as concentrations of lipid peroxides, expressed as total thiobarbituric acid-reacting substances (TBARS), as described before [8].

The 3-ml reaction solution of SOD contained 13 μM methionine, 63 μM ρ-nitro blue tetrazolium chloride, 1.3 μM riboflavin, 50 mM phosphate buffer (pH 7.8), and enzyme extract. The reaction solution was incubated for 10 min under fluorescent light with 80 μmol m^{-2} s^{-1}. Absorbance was determined at 560 nm with a spectrophotometer (Model UV-2010, Hitachi, Japan). One unit of SOD activity was defined as the amount of enzyme required for inhibition of photochemical reduction of ρ-nitro blue tetrazolium chloride by 50%.

The 3-ml reaction solution of CAT contained 15 mM H_2O_2, 50 mM phosphate buffer (pH 7.0), and 50 μl of enzyme extracts. The reaction was initiated by adding enzyme extracts. The decrease of absorbance of H_2O_2 (extinction coefficient 0.00394 mM^{-1} cm^{-1}) within 1 min at 240 nm was recorded. One unit of CAT activity was defined as the amount of enzyme required for oxidize 1 μmol of H_2O_2 per minute.

The extraction and assay of APX activity were performed as described before [38]. The 3-ml reaction solution of APX contained 50 mM phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, and 0.1 ml enzyme extracts. APX activity was calculated by following the decrease in absorbance of AsA (extinction coefficient 2.8 mM^{-1} cm^{-1}) within 1 min at 290 nm. Glutathione reductase (GR) activity was measured as described by Gamble and Burke [7]. Shoots (0.3 g) were extracted in 3 ml of 0.1 M Tricine–NaOH (pH 7.8). The oxidation of NADPH (extinction coefficient 6.22×10^6 cm^2 mol^{-1}) was monitored at 340 nm in 2 min. One unit of APX and GR was defined as the amount of enzyme required for catalyze the conversion of 1 μmol of substrate, AsA or GSSG per minute.

4.4. Determination of AsA and GSH content

Fresh shoots (0.5 g) were ground in a mortar and pestle in 5 ml of trichloroacetic acid at 4 °C. The homogenates were centrifuged at 13,000×*g* for 15 min. The supernatant was recovered and used for AsA determination according to the method as described before [8], and for GSH determination according to the method as described before [38].

4.5. Determination of H_2O_2

Hydrogen peroxide was determined as described before [8]. Fresh leaves (0.5 g) were frozen in liquid nitrogen and ground in a mortar and pestle in 4 ml of 5% (v/v) trichloroacetic acid. The homogenate was centrifuged at 10,000×*g* for 20 min at 4 °C. The supernatant was adjusted to pH 8.4 with 17 M ammonia solution. The volume of the supernatant after the adjustment was recorded. 0.1 g activated charcoal was added to the supernatant and mixed thoroughly. The mixture was filtered. The filtrate was divided into aliquots of 1 ml. Ten micrograms of catalase was added into one of the aliquots as a blank. The blank was kept at room temperature for 10 min, together with the other filtrate aliquots without catalase [27]. H_2O_2 was determined according to the method described by Kitaoka et al. [15]. One milliliter of colorimetric reagent was added to both series. Colorimetric reagent contained 10 mg 4-aminoantipyrine, 10 mg phenol, and 5 mg peroxidase (150 U mg^{-1}), and all dissolved in 55 ml 100 mM acetate buffer (pH 5.5). The reaction solution was incubated for 10 min at 30 °C. Absorbance was determined at 500 nm by a spectrophotometer. Hydrogen peroxide concentration was calculated by comparison to a standard curve.

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