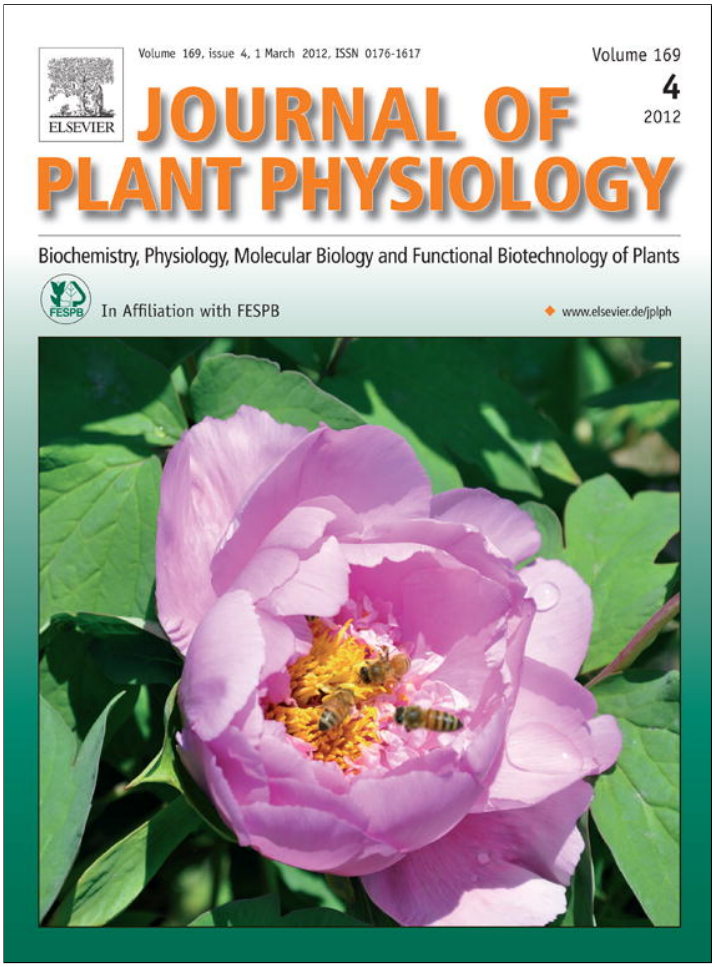

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Drought-responsive mechanisms in rice genotypes with contrasting drought tolerance during reproductive stage

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ABSTRACT

Water status is the main factor affecting rice production. In order to understand rice strategies in response to drought condition in the field, the drought-responsive mechanisms at the physiological and molecular levels were studied in two rice genotypes with contrasting susceptibility to drought stress at reproductive stage. After 20 d of drought treatment, the osmotic potential of leaves reduced 78% and 8% in drought susceptible rice cultivar *Zhenshan97B* and tolerant rice cultivar *IRAT109*, respectively. The panicle lengths had no obvious changes in drought stressed *Zhenshan97B* and *IRAT109*, suggesting that drought stress impose less effect on assimilate translocation from leaf to vegetative growth of panicles. *IRAT109* showed more extensive deeper root growth that could be considered a second line of defense against drought stress. The C_i/C_a ratio exhibited enhancement over reduction of g_s in both cultivars, reflecting the non-stomatal limitation to photosynthesis occurred during drought stress. Orthophosphate dikinase, glycine dehydrogenase, ribulose bisphosphate carboxylase (Rubisco), glycine hydroxymethyltransferase and ATP synthase were down-regulated for *Zhenshan97B* in response to drought stress, suggesting the reduction of capacity of carbon assimilation in this rice cultivar. In drought-stressed *IRAT109*, transketolase, Rubisco were down-regulated, however, Rubisco activase and peptidyl-prolyl *cis–trans* isomerase, which might alleviate the damage on Rubisco by drought stress, were up-regulated. The increased abundances of chloroplastic superoxide dismutase [Cu–Zn] and dehydroascorbate reductase might provide antioxidant protection for *IRAT109* against damage by dehydration.

Introduction

Rice-growing areas span the tropics, subtropics, semi-arid tropics and temperate regions of the world. More than 90% of the world's rice is grown and consumed in Asia, where rice is grown in 135 million ha with an annual production of 516 million tones (Roy and Misra, 2002). The predominantly rice-growing areas in Asia are often threatened by severe abiotic stresses, of which the most common is drought. Water-deficit may occur early in the growing season or any time from flowering to grain filling, and the intensity of the stress depends on the duration and frequency of water-deficit (Wade et al., 1999). Drought stress suppresses leaf expansion, tillering and midday photosynthesis (Kramer and Boyer, 1995) and reduces photosynthetic rate and leaf area due to early senescence (Nooden, 1988). All of these factors are responsible for a reduction in grain yield under drought condition. The phenology, particularly at the reproductive stage, is a major determinant of grain yield in rain fed lowland rice, and any attempt to screen for drought resistance needs to consider variation at reproductive stage (Pantuwan et al., 2001). Therefore, to identify traits that confer drought resistance from the different genotypes with contrasting drought tolerance will bring us novel insights for future breeding of rice.

Rice is most susceptible to drought stress at the reproductive stage (Pantuwan et al., 2002). Dramatic reduction of grain yield occurs when drought stress coincides with the irreversible reproductive processes (Price and Courtois, 1999; Pantuwan et al., 2002). Meanwhile, fundamental research has provided significant insights in the understanding of the physiological and molecular responses of plants to water deficits, but there is still a large gap between yields in optimal and stress conditions (Park et al., 2011). Minimizing the 'yield gap' and increasing yield stability under different stress conditions are of strategic importance in guaranteeing food for the future.

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Rice is a notoriously drought-susceptible crop due in part to its small root system, rapid stomatal closure and little circular wax during mild water stress (Hirasawa, 1999). Reduction of photosynthetic activity, accumulation of organic acids and osmolytes, and changes in carbohydrate metabolism, are typical physiological and biochemical responses to drought stress (Tabaeizadeh, 1998). Water deficit also increases the formation of reactive oxygen species (ROS) resulting in lipid peroxidation, protein denaturation and nucleic acid damage with severe consequences on overall metabolism (Hansen et al., 2006). In a previous study, lowland rice and upland rice were characterized as drought avoidance and drought tolerance, respectively (Lian et al., 2004). So the comparison of upland rice and lowland rice appears to be a paradigm for studying the molecular mechanisms in drought resistance. The understanding of the biological function of the novel genes is a more difficult proposition than obtaining just the sequences. This challenge is because the amount of information on amino acid sequences of known proteins in the database does not match the wealth of information on nucleotide sequences being generated through genome projects. Hence, an understanding of gene expression on a global scale would lend considerable insight into the molecular mechanisms of plant development. During the last several years, the field of proteomics has evolved considerably, and has been employed to analyze protein changes in response to environmental changes.

Comparative analysis of drought-responsive mechanisms between drought-tolerant and drought-sensitive rice cultivars will unravel novel regulatory mechanisms involved in stress tolerance. *Zhenshan97B* (*Oryza sativa* L. ssp. *indica*), considered to be drought susceptible, is a popular lowland rice variety in China, while *IRAT109* (*Oryza sativa* L. ssp. *japonica*), considered to be drought tolerant, is an up-land *japonica* rice variety originally developed in the Ivory Coast and is often used as a drought resistant donor in the breeding program (Nemoto et al., 1998). It was reported that upland cultivar *IRAT109* has higher values in the important traits of relative performance such as relative yield, relative spikelet fertility, relative biomass, relative grain weight, and relative harvest index than those of lowland cultivar *Zhenshan97* under drought stress (Yue et al., 2006). In this study, we explored the changes in leaf osmotic potential, growth, yielding, photosynthesis and their interrelationships using *Zhenshan97B* and *IRAT109* at the reproductive stage to analyze their responses to soil water deficit. In an effort to create a resource for protein biomarkers of rice leaf responsive to drought and to understand molecular processes that are related to drought tolerance during reproductive growth, we have analyzed the proteomic changes in flag leaves of *Zhenshan97B* and *IRAT109* at the reproductive stage. Protein expression profiles of flag leaves were studied by running two-dimensional electrophoresis (2-DE). Differential protein expressions in flag leaves between *Zhenshan97B* and *IRAT109* were compared by image analysis, which allowed the identification of 47 significantly different gel spots. These spots were further verified with matrix-assisted laser desorption/ionization time of flight spectrometry (MALDI-TOF/MS) and 20 of them were confirmed to be rice proteins.

Materials and methods

Drought stress treatments and measurements

Drought susceptible cultivar *Zhenshan97B* (*Oryza sativa* L. ssp. *indica*) and tolerant cultivar *IRAT109* (*Oryza sativa* L. ssp. *japonica*) were used in this study. *Zhenshan97B* is the maintainer line for a number of elite hybrids widely cultivated in China, and *IRAT109* was introduced from Cote d'Ivoire.

Field experiments were conducted at the experimental farm (Lingshui, Hainan province, China, 110°02′E, 18°48′N) of Shanghai Agrobiological Gene Center, from December of 2007 to March of 2008. The seeds of *Zhenshan97B* and *IRAT109* were sown in January 20th, 2008 and December 20th, 2007, respectively, in order to make the boot stage occur simultaneously for both cultivars (30 d seeding interval). The experiments were conducted in polyvinyl chloride (PVC) pipes loaded with a plastic bag filled with the local lateritic soil, one plant per pipe, under a rain-out shelter with movable roofs. The pipe was 20 cm in diameter and 120 cm in length with holes on two sides at 75 cm from the top.

Sowing time was staggered between the lines to synchronize flowering on the basis of the heading dates of the cultivars observed in 2006. Three to five germinated seeds were directly sown in each pipe and only one healthy plant was kept for 30 d after sowing. The plants were fully irrigated by watering every day until the drought treatment. Drought stress was individually applied to each plant at the booting stage. To apply drought stress, water was added to the full capacity of the pipe. Then, the plugs on the pipe were removed, and small holes were punched on the plastic bag to drain the water slowly.

The experiment was laid out in completely randomized design, where each treatment replicated 10 times. For comparison, a well-watered treatment was also included in the experiment as control treatment. The drought-stressed plants were not watered for 20 d. At the end of this drought treatment, control and drought-stressed plants were sampled. Plant height, panicle length, grain yield/plant, rate of filled grains, 1000-grain weight, maximum root depth and root weight were measured at the beginning and the end of the drought stress treatment, respectively. The plant height was measured from the stem base to the highest leaf tip. Panicle length was measured as the length from the neck node to the tip of the uppermost spikelet. Filled grain per panicle was measured by counting the average from panicles of primary, secondary and tertiary tillers. Sterility percentage was calculated from filled and unfilled grains per panicle. Total filled grains from selected tillers were weighed and converted into 1000-grain weight.

Volumetric soil water contents were measured before and after drought stressed treatment by time domain reflectometry (TDR) in the pipes with stressed plants to determine the soil water status of the root-zone. Soil water contents in the well watered (WW) pipes were considered as saturated water content.

Leaf rolling was determined based on a standard chart presented by O'Toole and Cruz (1980). A visual score was taken of the degree of leaf rolling as made on the sample leaf using a 1 to 5 scale with 1 being the first evidence of rolling and 5 being a closed cylinder. Plants under normal irrigation in the same period were used as controls.

Measurements of photosynthetic parameters and leaf osmotic potential

Photosynthetic rate (P_n), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were measured, using a LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE), between 10 AM and noon under saturating PPFD of 1500 $\mu mol\, m^{-2}\, s^{-1}$. Flow rate was maintained at 200–250 $\mu mol\, s^{-1}$ so that the relative humidity inside the chamber should be similar to ambient conditions. Measurements were made for 20 s immediately after a stable decrease in CO_2 concentration inside the chamber was achieved.

For measurement of osmotic potential, leaves of drought-stressed plants and controls were harvested and put in a syringe and frozen in a freezer. The leaf juice was squeezed and then the osmotic potential was measured with a Vapor Pressure Osmometer 5520 (Wescor, USA).

Table 1
The means (ten replicates) of leaf osmotic potential, soil water content, phenotype and gas exchange under well-watered (WW) and drought stress (DS) conditions.

Character	Zhenshan97B			IRAT109		
	WW	DS	Rate of change (%)	WW	DS	Rate of change (%)
Leaf osmotic potential (MPa)	−0.58 ± 0.02	−1.03 ± 0.08*	−78	−0.60 ± 0.02	−0.65 ± 0.01*	−8
Soil water content (%)	46	7.2	−84	46	5.2	−89
Leaf rolling score		5			2	
Plant height (cm)	82.40 ± 3.8	72.30 ± 5.3*	−12	98.50 ± 4.9	95.20 ± 3.7	−3
Grain yield/plant (g)	8.86 ± 1.93	6.43 ± 0.71	−27	10.35 ± 2.62	8.48 ± 1.78	−18
Rate of filled grain (%)	92.74 ± 4.37	85.52 ± 9.19	−8	94.18 ± 4.28	88.77 ± 10.83	−6
1000-grain weight (g)	27.38 ± 2.11	24.95 ± 2.55	−9	39.60 ± 3.66	38.22 ± 2.83	−6
Panicle length (cm)	20.64 ± 1.17	20.60 ± 1.70	0	21.38 ± 0.45	22.99 ± 0.96	+8
Maximum root depth (cm)	65.25 ± 1.5	76.20 ± 4.4*	+17	64.00 ± 5.7	87.75 ± 2.8*	+37
Root dry weight (0–30 cm) (g)	6.36 ± 1.2	2.47 ± 0.4*	−61	7.15 ± 0.4	4.07 ± 0.4*	−43
Root dry weight (30–90 cm) (g)	1.17 ± 0.5	1.00 ± 0.4	−14	1.25 ± 0.08	2.15 ± 0.6	+72
Photosynthetic rate (P_n, μmol CO_2 $m^{-2}s^{-1}$)	12.37 ± 1.72	5.62 ± 2.32*	−47	12.31 ± 1.97	3.72 ± 1.01*	−60
Stomatal conductance (g_s, mol H_2O $m^{-2}s^{-1}$)	0.24 ± 0.01	0.20 ± 0.04	−19	0.20 ± 0.04	0.13 ± 0.01*	−58
Intercellular CO_2 concentration (C_i, μmol CO_2 mol^{-1})	260 ± 8	302 ± 14*	16	281 ± 11	324 ± 10*	15
Ambient CO_2 concentration (C_a, μmol CO_2 mol^{-1})	389 ± 1.7	387 ± 3.3		389 ± 1.1	388 ± 3.7	
C_i/C_a	0.69	0.78	13	0.72	0.84	17

Asterisks indicate significant difference from control ($P < 0.05$).

Protein extraction

Protein extraction was performed according to Damerval et al. (1986) with some modifications. The flag leaves were ground in liquid nitrogen and suspended in 10% (w/v) tricarboxylic acid in acetone with 0.07% (w/v) dithiothreitol (DTT) at −20 °C for 1 h, followed by centrifugation at 35,000 × *g* at 4 °C for 20 min. The pellets were washed 3 times with ice-cold acetone containing 0.07% DTT, incubated at −20 °C for 1 h and centrifuged again at 4 °C. The pellets were vacuum-dried. The dried powder was dissolved in lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) Ampholine pH 3.5–10 and 1% (w/v) DTT.

Two-dimension electrophoresis and gel staining

Two-dimensional electrophoresis (2-DE) and gel staining were carried out according to Shen et al. (2003). The first dimensional isoelectric focusing (IEF) was performed in a 13 cm long glass tube with a diameter of 3 mm. The gel solution consisted of 8 M urea, 3.6% acrylamide, 2% NP-40 and 5% Ampholines (1 part pH 3.5–10, 1 part pH 5–8). IEF was performed at 200 V for 30 min, followed by 400 V for 15 h and finally 800 V for 1 h. After IEF, the focused strips were equilibrated in equilibration buffer (50 mM Tris–HCl pH 6.8, 2.5% sodium dodecyl sulfate (SDS), 10% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol) for 15 min twice. The second dimensional electrophoresis was performed on 15% resolving gels and 5% stacking gels (175 mm × 200 mm × 1 mm). The gels were stained with Coomassie brilliant blue (CBB) R-250.

Image analysis and protein identification

The 2-DE gels were scanned at a 300 dpi resolution with UMAX Power Look 2100XL scanner (Maxium Tech., Taipei, China). The spot detection and gel comparison were made with ImageMaster™ 2D Platinum, version 5.01 (GE Healthcare Bio-Science, Little Chalfont, UK).

The protein spots, which were differentially displayed under the drought stress, were excised from the CBB stained gels. Protein digestion and MALDI-TOF-MS analysis were performed according to Shen et al. (2003) with a little modification. Briefly, each small gel piece with protein was destained with 50 mM NH_4HCO_3 in 50% ethanol for 2 h at 40 °C. The protein in the gel piece was reduced with the solution containing 10 mM ethylene diamine tetraacetic acid (EDTA), 10 mM DTT and 100 mM NH_4HCO_3 for 1 h at 60 °C and then incubated with the solution containing 10 mM EDTA, 40 mM iodoacetamide and 100 mM NH_4HCO_3 for 30 min at room temperature in darkness. The gel pieces were minced and vacuum-dried, then rehydrated in 100 mM NH_4HCO_3 with 10 ng sequencing grade modified trypsin (Promega, USA) at 37 °C overnight. After digestion, the protein peptides were collected, and the gel pieces were washed with 0.1% TFA in 50% acetonitrile three times to collect the remaining peptides. The supernatants were pulled up together and vacuum-dried to final volumes of 3–5 μl. Tryptic peptide mass was measured with a MALDI-TOF mass spectrometer (Shimadzu Biotech, Japan).

The peptide mass fingerprints (PMFs) were searched against rice data in the NCBInr, SwissProt and MSDB databases using MASCOT software (Matrix Science, London). To confirm the identification results, we used MOWSE score (provided by the software), sequence coverage as the criteria. Generally, the score of the identified protein must be higher than 64, 61 and 47 on NCBInr, MSDB and SwissProt, respectively, and the sequence coverage of the identified protein must be higher than 12%. The identified proteins were assigned to functional groups according to their functions and involved mechanisms reported previously in the literature.

Statistical analysis

The physiological results were exported to SPSS Version 13.0 (Lead Technologies, USA) and the Student's t test was used for statistical analysis.

Results

Phenotypic variations

The phenotypic changes in drought-stressed *Zhenshan97B* and *IRAT109* were summarized in Table 1. Leaf rolling, a visible sign of drought stress, was seen both in *Zhenshan97B* and *IRAT109* after 20 d of the drought treatment. The controls remained unrolled while the degree of leaf rolling in the two cultivars became progressively more pronounced with the drought stress. Responses of leaf rolling score to soil water stress at booting stage are shown in Table 1. These results indicated that *IRAT109* had more capability to cope with the drought stress than *Zhenshan97B*. Plant height was obviously reduced by the drought stress compared to the well watered plants. The plant height decreased 12% and 3% with the drought stress in *Zhenshan97B* and *IRAT109*, respectively (Table 1), indicating that *IRAT109* had better growth in leaf and stem elongation under the drought stress than *Zhenshan97B*.

Water deficit during reproductive stage led to decreases of 27% and 18% in grain yield of *Zhenshan97B* and *IRAT109*, respectively (Table 1). The rate of filled grain and 1000-grain weight were reduced under the drought stress in both cultivars. The changes of the rate of filled grain were consistent with 1000-grain weights in *Zhenshan97B* and *IRAT109* (Table 1). Compared to the well watered control, panicle length remained unchanged in *Zhenshan97B*, but it increased 8% in *IRAT109*.

Drought-induced root growth in depth can be indicated by the difference of maximum root depths under drought and control conditions. The maximum root depths under the drought stress were increased 11.25 cm and 23.75 cm in *Zhenshan97B* and *IRAT109*, respectively (Table 1), showing almost twice more drought-induced root growth in depth for *IRAT109* in comparison with that of *Zhenshan97B*. The drought stress caused reduction of 61% and 43% in root mass (0–30 cm) for *Zhenshan97B* and *IRAT109*, respectively (Table 1). The root mass (30–90 cm) decreased 14% in *Zhenshan97B*, but increased 72% in *IRAT109* (Table 1).

Acclimation to water stress: leaf osmotic potential and photosynthetic parameters

The drought treatment to plants of *Zhenshan97B* and *IRAT109* resulted in water deficit. The responses of flag leaves of *Zhenshan97B* and *IRAT109* towards water deficit were compared by analyzing leaf osmotic potential (ψ_s). After 20 d of drought treatment, the ψ_s reduced 78% from −0.58 MPa to −1.03 MPa, and 8% from −0.60 MPa to −0.65 MPa in *Zhenshan97B* and *IRAT109*, respectively (Table 1).

The photosynthetic rate (P_n) of flag leaves decreased 47% from 12.37 to 5.62 μmol CO_2 m^{-2} s^{-1} in *Zhenshan97B*, and 60% from 12.31 to 3.72 μmol CO_2 m^{-2} s^{-1} in *IRAT109* under the drought stress (Table 1). The P_n of *Zhenshan97B* was similar to that of *IRAT109* under well-watered conditions. Stomatal conductance (g_s) decreased in both cultivars with respect to controls after drought stress treatment. However, the drought-stressed plants of *IRAT109* showed lower rates of g_s than those of *Zhenshan97B* (Table 1). Therefore, more intensive stomatal closure was observed in *IRAT109*, which exhibited 58% decrease in g_s (Table 1). However, the g_s of *Zhenshan97B* decreased 19% (Table 1). Intercellular CO_2 concentration (C_i) values were increased after drought treatment in both cultivars, resulting in the values of C_i/C_a being increased 13% and 17% for *Zhenshan97B* and *IRAT109*, respectively (Table 1).

Protein expression

Proteins were extracted from flag leaves, separated by 2D-PAGE and stained by CBB. Digital image analysis of the CBB stained gel identified about 600 proteins. Seventeen proteins responded in *Zhenshan97B* after the drought stress by up- or down-regulation. Of which, the levels of 2 proteins were elevated, but 15 proteins declined in abundance as a result of drought stress (Fig. 1). For *IRAT109*, twenty proteins responded after the drought stress by up- or down-regulation, of which, the levels of 14 proteins were elevated, while 6 proteins declined in abundance as a result in response to the drought stress (Fig. 2).

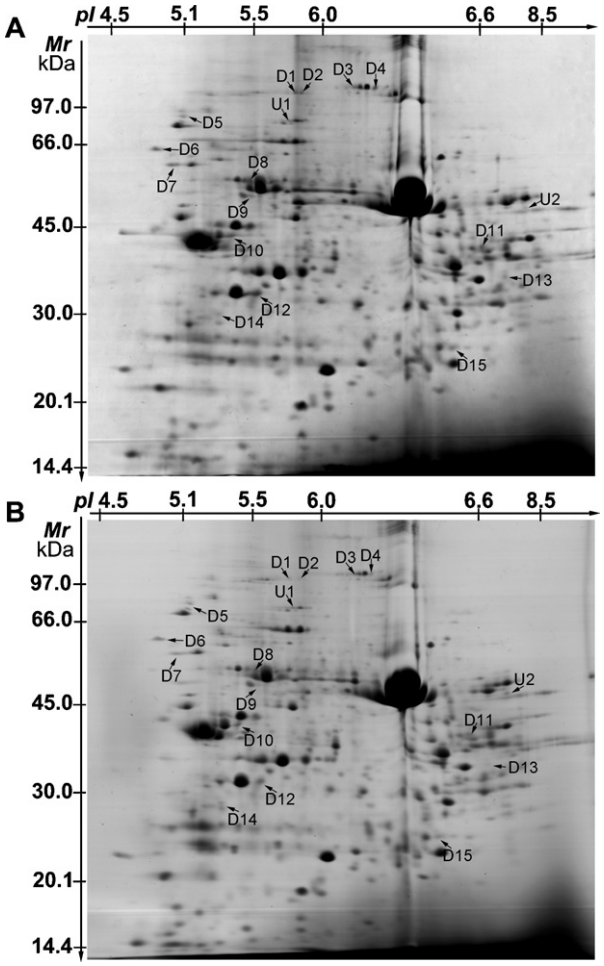

Fig. 1. Two-dimension gel analysis of proteins extracted from flag leaves of well-watered (A) and drought-stressed (B) *Zhenshan97B* harvested after 20 d of drought treatment. The arrows indicate proteins that changed reproducibly and significantly in drought-stressed plants compared with well-watered controls. D, stands for down-regulated protein; U, stands for up-regulated protein.

All the changed protein spots under the drought stress were analyzed by MALDI-TOF MS. The proteins identified from *Zhenshan97B* and *IRAT109* are listed in Tables 2 and 3, respectively. Two proteins (spots D5 and D13) from *Zhenshan97B* were annotated in the database as OsI_31743 (D5) and Os12g0420200 (D13), while those (spots D5 and U9) from *IRAT109* were annotated in the database as OsI_28519 (D5), Os02g0328300 (spot U9). From a BLAST search, OsI_31743 (spot D5 from *Zhenshan97B*) and Os12g0420200 (spot D13 from *Zhenshan97B*) were assigned as heat shock protein and NAD^+(P)-binding protein, respectively.

In this study, the abundance of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit was reduced in flag leaves under the drought stress for both *Zhenshan97B* (Fig. 1 and Table 2) and *IRAT109* (Fig. 2 and Table 3). However, the expression of ATP synthase beta subunit in flag leaves showed reverse behavior under the drought stress between *Zhenshan97B* and *IRAT109*. The

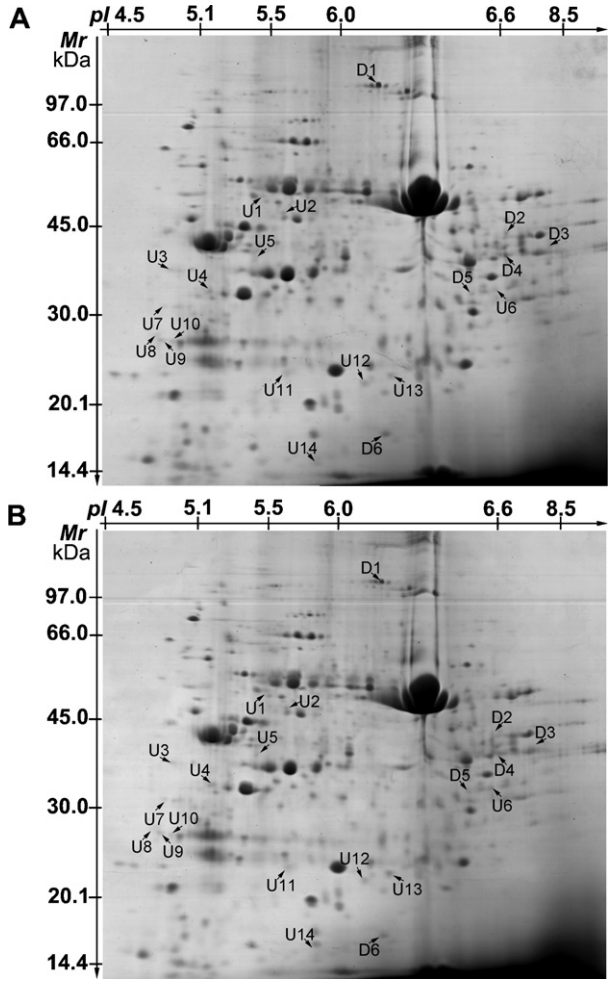

Fig. 2. Two-dimension gel analysis of proteins extracted from flag leaves of well-watered (A) and drought-stressed (B) *IRAT109* harvested after 20 d of drought treatment. The arrows indicate proteins that changed reproducibly and significantly in drought-stressed plants compared with well-watered controls. D, stands for down-regulated protein; U, stands for up-regulated protein.

abundance of ATP synthase beta subunit decreased in *Zhenshan97B*, whereas that increased in *IRAT109* (Fig. 3).

Among 17 changed protein spots from drought susceptible cultivar *Zhenshan97B*, orthophosphate dikinase, glycine dehydrogenase, ribulose bisphosphate carboxylase/oxygenase, ATP synthase CF_1 and mitochondrial F_1-ATPase were down-regulated, whereas glycine hydroxymethyltransferase was up-regulated under the drought stress (Table 2 and Fig. 3). Among 20 changed protein spots from drought tolerant cultivar *IRAT109*, six of them were reduced, but 14 of them were enhanced under the drought stress (Figs. 2 and 3). The results of protein identification showed transketolase and ribulose bisphosphate carboxylase large chain were reduced, however, ATP synthase beta subunit, peptidyl-prolyl *cis–trans* isomerase, ribulose bisphosphate carboxylase/oxygenase activase, dehydroascorbate reductase and superoxide dismutase were enhanced under the drought stress (Table 3 and Fig. 3).

Discussion

Phenotypic variations between Zhenshan97B and IRAT109 under drought stress

Leaf rolling is usually associated with soil water deficit as an effective mechanism to reduce transpiration loss. Rolling rapidly reduces effective leaf area and transpiration, and thus is a useful drought-avoidance mechanism in arid areas (Clarke, 1986). The leaf rolling scores in this study were positively associated with the reduction of plant height (Table 1), indicating that tall genotypes were impaired under drought stress that developed at booting stage. In addition, drought stress slows down carbohydrate synthesis and/or weakens the sink strength at reproductive stages and abortion of fertilized ovaries (Rahman et al., 2002). The significant reduction in 1000-grain yield in *Zhenshan97B* and *IRAT109* suggested that drought stress during the reproductive period affects assimilate translocation from leaf to grain, via altering source-sink relationships. The reduction in leaf cell expansion would decrease sink strength for vegetative growth and lessen the competition with panicle growth for assimilates. This effect might be due to a decrease in translocation of assimilates towards reproductive organs (Hsiao and Xu, 2000). In contrast, our results showed that the panicle lengths had no obvious changes in *Zhenshan97B*, and even increased about 8% in *IRAT109,* suggesting that the drought stress impose less effect on assimilate translocation from leaf to vegetative growth of panicles. The 1000-grain yield and the rate of filled grain were highly correlated with each other, indicating that the yield loss of rice under the drought stress at reproductive stage was associated with the reduction in spikelet fertility and grain weight.

IRAT109 showed more drought-induced root growth in depth than *Zhenshan97B* did. Thereafter, *IRAT109* showed less percentage reduction in root weight for roots grown to 30 cm depth than *Zhenshan97B*. The reverse performance was observed between *Zhenshan97B* and *IRAT109* for the root weight (30–90 cm). The root weight (30–90 cm) of drought-stressed *Zhenshan97B* was 14% less than that of the control (Table 1). In contrast, the root weight (30–90 cm) of drought-stressed *IRAT109* was 72% higher than that of the control (Table 1). The fact that *IRAT109* had deeper and wider roots than *Zhenshan97B* might be beneficial to absorb more, deeper underground water in order to supply adequate water for aerial parts when drought occurred. *IRAT109* showed a lesser degree of leaf rolling than *Zhenshan97B*, performing a greater degree of dehydration avoidance by the development of deep roots. These results suggested that *Zhenshan97B* resists drought stress mainly using drought tolerance strategies, and deeper root growth into wet soil could be considered a second line of defense against drought stress in *IRAT109*. The root growth may be considered as the adaptive mechanism that alleviates the water uptake under drought conditions as a result of extra root growth which enables plants to obtain more soil water (van Keuken and Seligman, 1987).

Drought-responsive mechanisms in Zhenshan97B

Water status is the main factor affecting the plants' growth and development. Decreasing external water potential produces a net accumulation of solutes in cells, which lowers the cell osmotic potential necessary for maintaining the turgor pressure (Navarro et al., 2003). In this study, *Zhenshan97B* adjusted its Ψ_s to much more negative levels than *IRAT109* (Table 1), indicating more turgor pressure to expend in *Zhenshan97B*. Osmotic adjustment (OA), the lowering of osmotic potential by a net increase of intracellular solutes, is recognized as an adaptive mechanism to water stress (Hsiao et al., 1984). With respect to OA, it is possible that this factor is not very important as a mechanism associated with resistance to water deficit. However, the drought susceptible cultivar *Zhenshan97B* would present significant OA under the drought treatment. This response was observed by a significant reduction in the leaf osmotic potential (Ψ_s) in response to the drought stress. Thus, the ability of plants to keep the cell turgor through OA could be advantageous in *Zhenshan97B* under the drought stress. It has, however, been argued that OA probably does not allow the plant to

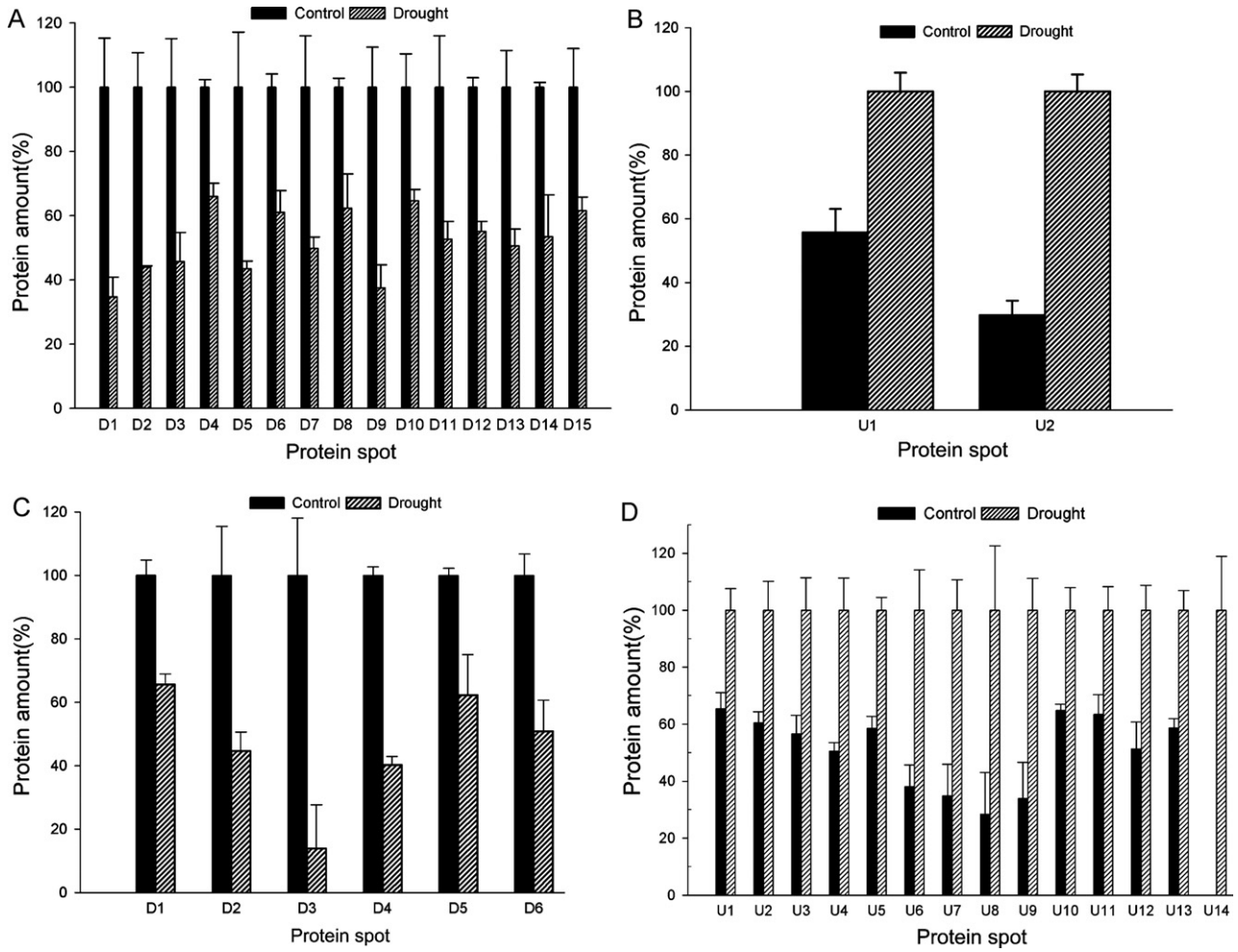

Fig. 3. Quantitative changes of the down-regulated proteins (A), up-regulated proteins (B) in drought-stressed *Zhenshan97B*, and down-regulated proteins (C) and up-regulated proteins (D) in drought-stressed *IRAT109*. Data was representative of three independent experiments and shown as mean + SE.

Table 2
Identification of proteins from *Zhenshan97B* responsive to drought stress by using MALDI-TOF MS.

Spot no.	Protein name	Theoretical *Mr* (D)/p*I*	Experimental *Mr* (kD)/p*I*	Score[a]	M/U[b]	SC(%)[c]	Accession no.
Carbon assimilation							
D[d]1	Orthophosphate dikinase	103,592/5.98	105/5.85	108	7/20	13	BAA22419
D2	Orthophosphate dikinase	103,592/5.98	105/5.9	68	8/28	12	BAA22419
D3	Putative glycine dehydrogenase	112,426/6.35	110/6.1	85	6/23	11	BAD35509
D4	Putative glycine dehydrogenase	112,427/6.35	110/6.2	147	11/36	21	BAD35509
D11	Ribulose bisphosphate carboxylase large chain	53,418/6.22	43/6.5	57*	6/26	20	P0C510
U[e]2	Putative glycine hydroxymethyltransferase	48,240/9.01	48/8.4	70	4/23	20	AAR07090
Energy							
D8	ATP synthase CF1 beta subunit	54,037/5.47	54/5.4	121	8/24	29	NP_039390
D9	Mitochondrial F1-ATPase beta subunit	59,137/6.30	53/5.5	65	4/11	13	BAA01372
Protein transport, folding and assembly							
D5	Hypothetical protein OsI_31743	89924/4.98	87/5.1	77	7/25	13	EEC84746
D13	Os12g0420200	41,621/8.59	36/8.3	80	5/20	24	NP_001066657

[a] MOWSE score.
[b] Number of matched peptides/searched peptides.
[c] Sequence coverage.
[d] D stands for down-regulated protein.
[e] U stands for up-regulated protein.
* Search results from swissprot database.

Table 3
Identification of proteins from *IRAT109* responsive to drought stress by using MALDI-TOF MS.

Spot no.	Protein name	Theoretical *M*r (D)/p*I*	Experimental *M*r (kD)/p*I*	Score[a]	M/U[b]	SC (%)[c]	Accession no.
Carbon assimilation							
D[d]1	Putative transketolase	80,549/6.12	110/6.3	76	6/22	14	AAO33154
D4	Ribulose bisphosphate carboxylase large chain precursor, putative, expressed	40,763/8.51	41/6.6	81	9/40	29	ABB47308
U5	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor, putative, expressed	47,699/5.85	40/5.45	66	4/24	17	ABG22614
Energy							
U[e]1	Putative ATP synthase beta subunit	45,265/5.26	49/5.3	132	8/17	28	BAD82522
Antioxidation and detoxification							
U13	Dehydroascorbate reductase	23,726/5.81	23/6.1	80**	5/19	33	Q84UH5
U14	Superoxide dismutase [Cu-Zn] chloroplastic	21,402/5.79	16/5.7	49*	3/40	28	P93407
Protein transport, folding and assembly							
U4	Putative peptidyl-prolyl *cis–trans* isomerase protein	50,098/5.64	35/5.2	73	4/19	20	BAC79666
Unknown							
U3	Unknown protein DS12 from 2D-PAGE of leaf, chloroplastic; flags: precursor	30,418/5.72	37/5.0	110	6/15	31	P83643
D5	Hypothetical protein OsI_28519	29,408/5.37	32/6.3	68	4/14	23	EEC83230
U9	Os02g0328300	30,791/5.44	28/4.9	111	7/21	33	NP_001046714

[a] MOWSE score.
[b] Number of matched peptides/searched peptides.
[c] Sequence coverage.
[d] D stands for down-regulated protein.
[e] U stands for up-regulated protein.
* Search results from Swiss-Prot database.
** Search result from MSDB database.

draw much extra water from the soil and that this could come at a cost in yield potential (Serraj and Sinclair, 2002). In rice, despite much previous research focus and investments, the usefulness of OA in improving grain yield under drought stress has not been documented. The failure to demonstrate any tangible benefit of OA on yield is probably related to the fact that the hypothetical benefits are expressed only when crop survival is threatened. Therefore, OA in *Zhenshan97B* would be associated with survival mechanisms under a drought stressed environment.

Drought susceptible cultivar *Zhenshan97B* seriously suffered from photoinhibition and the P_n of flag leaves decreased 47% (Table 1) after the drought treatment. The level of reduction of g_s in the flag leaves of *Zhenshan97B* subjected to the drought stress was much slighter than that of P_n. Stomata play a paramount role in the control of water loss and gas exchange in leaves. During the onset of drought, stomatal conductivity declines before photosynthesis, and the inhibition of photosynthesis during mild stress is mainly due to the reduction of CO_2 diffusion (Lawlor, 2002). However, the appearance of non-stomatal limitation to photosynthesis was evident in *Zhenshan97B* as deduced from an increase in C_i/C_a ratio. The C_i/C_a ratio (Table 1) exhibited enhancement over reduction of g_s, reflecting the onset of a non-stomatal limitation to photosynthesis under drought stress. A similar increase in C_i/C_a has also been observed in other species under severe water stressed conditions (Martin and Ruiz-Torres, 1992; Brodribb, 1996; Rouhi et al., 2007). Photosynthesis is not only restricted by stomatal limitations but also by nonstomatal limitations that impair metabolic reactions such as RuBP synthesis, ATP synthesis, and electron transfer (Cossins and Chen, 1997). In this study, the drought responsive proteins relative to carbon metabolism and energy transduction were down-regulated in the range of 35∼60% in *Zhenshan97B* under the drought stress (Fig. 1 and Fig. 3).

Orthophosphate dikinase (PPDK), which catalyzes the regeneration of the primary CO_2 acceptor phophoenolpyruvate in the chloroplast stroma of leaf mesophyll-cells, was initially discovered in C4 leaves. It is also present in C3 plants, and, likewise, this isoform is not believed to participate directly in photosynthesis. Glycine dehydrogenase (glycine decarboxylase) in conjunction with serine methylhydroxy transferase catalyzes the conversion of glycine to serine releasing one molecule of CO_2 and NH_3 (Tolbert, 1971). It is generally agreed that the majority of CO_2 is released when glycine is converted to serine, although additional CO_2 is released by the oxidation of glyoxylate and formate (Douce et al., 2001). Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the fixation of one molecule of CO_2 to the pentose-bisphosphate sugar ribulose-1,5-bisphosphate (RuBP), yielding two molecules of the three-carbon phosphoglyceric acid (PGA) (Cleland et al., 1998). Phosphoglyceric acid is then reduced to glyceraldehyde-3-phosphate by ATP and NADPH produced in the light reactions.

In this study, Orthophosphate dikinase (spots D1 and D2), glycine dehydrogenase (spots D3 and D4), ribulose bisphosphate carboxylase large chain (spot D11), ATP synthase CF_1 beta subunit (spot D8) and mitochondrial F_1-ATPase beta subunit (spot D9) were found to be down regulated in *Zhenshan97B* under the drought stress (Fig. 1 and Fig. 3). The significantly decreased abundances of the enzymes presumably reflect damages on chloroplast and mitochondria under the drought stress. The down regulations of orthophosphate dikinase and glycine dehydrogenase might lead to lower CO_2 concentration available for assimilation through the C3 cycle, and the down regulation of Rubisco and ATP synthase might lead to decrease in capacity for RuBP regeneration and reduction of ATP pools, respectively. Therefore, the decreased abundances of these enzymes in *Zhenshan97B* might result in reduction of CO_2 assimilation rate. The results of photosynthetic parameters and proteomic changes observed in water-stressed plants suggested that the components of carbon metabolism and ATP synthase be impaired as a result of nonstomatal inhibition of photosynthesis in drought susceptible cultivar *Zhenshan97B*.

Nevertheless, glycine hydroxymethyltransferase (spot U2), which is related to photorespiratory metabolism, was up-regulated in *Zhenshan97B* under drought stress. Glycine hydroxymethyltransferase catalyzes the reversible interconversion of serine and glycine with tetrahydro pteroyl polyglutamate (H4PteGlun) serving as the one-carbon (C_1) carrier (Kochetov, 2001). C_1 transfers are absolutely central to the massive photorespiratory fluxes that occur in C3 plants (Douce and Neuburger, 1999; Wingler et al., 2000). In drought-stressed leaves, the reduction of CO_2 assimilation driven by diffusive and metabolic constraints reduces the electron consumption by photosynthesis (Flexas et al., 2004). Photorespiration enhancement may, at least partially, use the excess of electrons generated by light capture through photochemical reactions. A direct involvement of the photorespiratory pathway, acting as an electron sink for the protection of the photosynthetic apparatus from electron induced photo-damage, has been reported in drought-stressed C3 plants (Wingler et al., 1999; Guan et al., 2004). The up-regulation of glycine hydroxymethyltransferase reflected that drought stress induce non-stomatal limitations to photosynthesis, which may in turn result in a positive feedback on photorespiration in drought susceptible cultivar *Zhenshan97B*.

Drought-responsive mechanisms in IRAT109

In contrast to the performance of *Zhenshan97B*, the Ψ_s of *IRAT109* was slightly changed (Table 1), indicating that *IRAT109* had less ability of OA than *Zhenshan97B*. The positive relationship between the changes of Ψ_s and the results related to the root growth indicated that the maintenance of Ψ_s involved water uptake by root systems. The negative relationship between the changes of Ψ_s and g_s indicated that the maintenance of Ψ_s involved water conservation by control of transpirational water loss through the stomata. It is well established that there is an abscisic acid (ABA) regulated drought-induced root-to-leaf signaling, promoted by soil drying and reaching the leaves through the transpiration stream, which induces closure of stomata (Davies and Zhang, 1991). Our results suggested that turgor loss avoidance due to enhancement of root growth and stomatal closure may be the main physiological mechanisms for drought resistance in *IRAT109*.

Stomatal closure is generally accepted to be the main determinant for decreased photosynthesis under mild to moderate drought stress (Medrano et al., 2002). In this study, the decrease in g_s from 0.20 to 0.13 mol H_2O $m^{-2}s^{-1}$ was paralleled by a decline in P_n (Table 1). Therefore, stomatal closure seems to be the main cause of decreased photosynthesis in drought tolerant cultivar *IRAT109*. This did not mean that non-stomatal limitations were absent, but simply that they were not dominant factors limiting photosynthesis. The C_i/C_a ratio (Table 1) exhibited enhancement similar to *Zhenshan97B*, also reflecting the onset of a non-stomatal limitation to photosynthesis under drought stress. Transketolase and Rubisco, which are enzymes of the Calvin cycle of photosynthesis in plants, were down-regulated in *IRAT109* (Figs. 2 and 3) under the drought stress, indicating that carbon metabolism was also suppressed under drought stress.

However, some protective mechanisms might be induced by drought stress, since the abundances of ATP synthase (spot U1), peptidyl-prolyl *cis–trans* isomerase (PPIases) (spot U4) and Rubisco activase (spot U5) were enhanced under the drought stress. PPIases catalyzes isomerization around peptidyl-prolyl imide bonds in peptides and proteins serving as protein folding catalysts (Handschumacher et al., 1984). The presence of PPIases in plants is comparable with the heat shock proteins (Schiene-Fischer and Yu, 2001). Rubisco activase is a chloroplast protein that activates and maintains Rubisco in an active state by facilitating removal of various sugar phosphates that either block substrate binding or prevent carbamoylation. Rubisco activase rescues Rubisco sites from dead end inhibition by promoting ATP-dependent conformational changes that open closed sites, making them more accessible to solvent and facilitating the dissociation of inhibitory sugar phosphates (Robinson and Portis, 1989; Wang and Portis, 1992). In this way, Rubisco activase is a molecular chaperone, controlling the switching of Rubisco conformation from inactive to active (Salin, 1988; Spreitzer and Salvucci, 2002). The up-regulation of these enzymes might alleviate the damage on Rubisco by drought stress.

Water stress increases the formation of ROS resulting in lipid peroxidation, denaturation of proteins and nucleic acid damage with severe consequences on overall metabolism (Hansen et al., 2006). ROS are scavenged (i) chemically by antioxidant molecules such as ascorbate, glutathione, tocopherols, carotenoids, polyphenols and flavonoids, and (ii) enzymatically by SOD and ascorbate peroxidase (APX) which scavenge O_2^- and H_2O_2, respectively. SOD is the first line of defense against oxidative stress. It catalyzes the disproportionation of O_2 to H_2O_2 and dioxygen (McCord and Fridovich, 1969). In this study, chloroplastic Cu-Zn SOD (spot U14) was up-regulated (Fig. 2 and Fig. 3) under drought stress, suggesting that an antioxidant system be induced in *IRAT109* in response to dehydration.

Removal of H_2O_2 from cells is accomplished by several enzymes, depending on cellular compartment. Various peroxidases link the detoxification of H_2O_2 to the oxidation of specific substrates, of which ascorbate is one of the most important. Ascorbate functions not only as an antioxidant but also as a substrate for ascorbate peroxidase (APX) and violaxanthin de-epoxidase in chloroplasts (Asada, 1999). APX catalyzes the decomposition of hydrogen peroxide in the active oxygen scavenging system, and protects plants from oxidative stress. In reactions catalyzed by APX, ascorbate is oxidized to monodehydroascorbate (MDA) and then dehydroascorbate (DHA) is produced via the spontaneous disproportionation of MDA. Hence, the regeneration of ascorbate is essential for the maintenance of the activity of the active oxygen-scavenging system. Dehydroascorbate reductase (spot U13), which catalyzes the re-reduction of DHA to ascorbate by glutathione, was enhanced in *IRAT109* under drought stress (Figs. 2 and 3). The increased levels of chloroplastic Cu-Zn SOD and DHAR might be expected to provide antioxidant protection for *IRAT109* against damage by dehydration.In conclusion, *IRAT109* appeared to be more resistant to drought stress than *Zhenshan97B*. This was possibly attributed to its greater capacity to obtain more soil water and induction of antioxidant systems to protect plants from impairment during drought stress, and consequently to reduce the damage to the enzymes relative to carbon assimilation. However, the two cultivars exhibited different responses of stomatal opening to drought stress. Stomatal conductance was possibly the major limitation to P_n under drought stress in *IRAT109*. However, a pronounced non-stomatal limitation, which was achieved by reducing the abundance of enzymes relative to carbon assimilation, occurred in *Zhenshan97B* under drought stress that may also lead to impairment of photosynthetic activity. The increased abundance of chloroplastic Cu-Zn SOD accompanied with increased abundance of dehydroascorbate reductase under drought seemed to provide an important protective mechanism for *IRAT109* against damage by dehydration. The up-regulation of glycine hydroxymethyltransferase in *Zhenshan97B* was helpful to increase photorespiration, which can protect plants from photoinhibition. The identified genotypes, physiological parameters and stress responsive enzymes could be used in breeding programs and/or genetic engineering for enhanced drought tolerance in rice.

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