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Different drought-stress responses in photosynthesis and reactive oxygen metabolism between autotetraploid and diploid rice

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Abstract

Photosynthetic light curve, chlorophyll (Chl) content, Chl fluorescence parameters, malondialdehyde (MDA) content, phospho*enol*pyruvate carboxylase (PEPC) activity and reactive oxygen metabolism were studied under drought stress in two autotetraploid rice lines and corresponding diploid rice lines. Net photosynthetic rate decreased dramatically, especially under severe drought stress and under high photosynthetic active radiation in diploid rice, while it declined less under the same conditions in autotetraploid lines. Compared with the corresponding diploid lines, the Chl content, maximum photochemical efficiency of photosystem (PS) II, and actual photochemical efficiency of PSII were reduced less in autotetraploid lines. PEPC activities were higher in autotetraploid rice lines. PEPC could alleviate inhibition of photosynthesis caused by drought stress. The chromosome-doubling enhanced rice photoinhibition tolerance under drought stress. The lower MDA content and superoxide anion production rate was found in the autotetraploid rice indicating low peroxidation level of cell membranes. At the same time, the superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities were higher in autotetraploid rice lines. SOD, POD, and CAT could effectively diminish the reactive oxygen species and reduced the membrane lipid peroxidation.

Additional key words: chlorophyll fluorescence; *Oryza sativa*; photosynthetic light curve; reactive oxygen species.

Introduction

Blakeslee and Avery (1937) obtained autopolyploid plants by colchicine treatment of plant seeds. Many researchers focused on the autopolyploid plants because of their particular characteristics, *e.g.*, vigorous plant, high biological yield, little seeds, and stress resistance. Genome doubling has been studied extensively to improve photosynthesis and stress resistance (Chen *et al.* 1987, Song and Zhang 1992, Cai *et al.* 2005). The autopolyploid plants have been widely used in agriculture, *e.g.*, autotriploid watermelon, autotriploid beet, and autotetraploid lily. Rice (*Oryza sativa* L.) is one of the staple food crops in Asian countries. Cultivated rice is the plant with diploid genomes (AA, 2n = 2x = 24). Diploid rice becomes autotetraploid rice (AAAA, 2n = 4x = 48) when its chromosomes are doubled. Some high seed-set, autopolyploid lines were chosen in the past few years (Cai *et al.* 2005, Tu *et al.* 2007).

Drought stress is one of the major environmental factors affecting rice growth and productivity. It induces various physiological, biochemical, and molecular responses in plants, *e.g.*, stomata closure and enzyme activity changes (Cornic 1994). Stomata closure limits CO_2 uptake of leaves, which leads to the increased susceptibility to photodamage (Cornic 1994, Powles 1984, Valentini *et al.* 1995) and finally to decline in photosynthetic rate.

In recent years, drought has become the main obstacle for rice production owing to increasing water shortage and uneven distribution of rainfall. As water availability is reduced, grain yield can be sharply lowered (Kutschera and Kohler 1993, Puliga *et al.* 1996, Pantuwan *et al.* 2002).

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Abbreviations: CAT – catalase; Chl – chlorophyll; DM – dry mass; F_v/F_m – maximum photochemical efficiency of PSII; FM – fresh mass; MDA – malondialdehyde; $O_2^{\bullet-}$ – superoxide anion; PEPC – phospho*enol*pyruvate carboxylase; P_N – net photosynthetic rate; POD – peroxidase; PS – photosystem; ROS – reactive oxygen species; SOD – superoxide dismutase; Φ_{PSII} – actual photochemical efficiency of PSII.
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We have observed that autotetraploid rice had stronger drought tolerance in field than corresponding diploid rice. Until now, no physiological and biochemical evidence of the better drought tolerance of autotetraploid rice has been reported. Therefore we explored whether autotetraploid rice responded in a different way to increasing drought stress compared with diploid rice.

Materials and methods

Plants and experimental design: Autotetraploid rice lines SP-4x and 630-4x, and corresponding diploid rice lines SP-2x and 630-2x were used. The research was done outside, close to Henan Institute of Science and Technology (35°18'N, 113°52'E), during May–October 2011. The germinated seeds were sown in a seedling bed on May 7, 2011 and seedlings were transplanted to plastic pots (40 × 60 × 20 cm, 30 pots per line and 2 plants per pot) with the soil mixture of 65% sand and 35% commercial soil on June 7, 2011. The pots were watered to soil saturation every day and a multipurpose fertilizer was applied every week together with irrigation. After 60 d of growth, each line was divided into 3 groups, 10 pots per group. Irrigation was ceased in 2 groups, while the 3rd group was maintained as the control treatment with continuing irrigation. Thus, finally, 3 groups of the plants maintained soil water potentials of 0 kPa, –20 kPa, and –40 kPa, respectively. The experiment was repeated in 2012.

Photosynthetic light curve: *LI6400* portable photosynthesis system (*LI-COR Co*., USA) equipped with a light source was used to measure the photosynthesis *vs.* light curve of the flag leaves. All measurements were done at the air temperature of 30°C, the ambient CO_2 concentration of about 350 μmol mol^{-1}, and about 70% of relative air humidity. Each point was the mean of 3 replications.

Chl content and Chl fluorescence: Leaf Chl content was measured following the method of Hipkins and Baker (1986) and it was expressed as mg g^{-1} of fresh mass (FM). Samples of approximately 0.5 g (M) were cut from mid-section of the flag leaves. Each sample was grinded in 5 mL of 80% acetone (with little $CaCO_3$ and quartz sand), and then stored in the dark for 5 min. The solution was filtered into 50 mL (V) volumetric flask. The residues were grinded and filtered again in the same manner. Finally, the volumetric flasks were made up to the mark with 50 mL by 80% acetone. The absorbance of the extract was measured at 645 and 663 nm using 80% acetone as a blank with a spectrophotometer *UV-4802* (*Yuanxi Co.*, Shanghai, China). Chl content was calculated using the following equation:

$$\text{Total Chl} = [20.29\ (A_{645}) + 8.04\ (A_{663})] \times (V/1{,}000\ M).$$

The Chl fluorescence of leaves was measured according to Genty (1989) with a portable chlorophyll fluorometer (*Mini-PAM*, *Heinz Walz GmbH*, Effeltrich, Germany). The mean values of the leaf Chl content, F_v/F_m, and Φ_{PSII}, were calculated as: $F_v/F_m = (F_m - F_o)/F_m$, $\Phi_{PSII} = (F_m' - F_s)/F_m'$ (Genty *et al*.1989).

MDA content and $O_2^{\bullet-}$ production rate: The amount of MDA derived from unsaturated fatty acid peroxidation of the membrane lipids was measured using the method of Yoshida (1994) and Gueta-Dahan (1977). Leaf tissues (0.1 g, M) were homogenized under liquid nitrogen, hydrated in 1 ml of 2.5% (w/v) trichloroacetate acid (TCA) solution. The homogenate was centrifuged at 12,000 × *g* at 4°C for 20 min. Then, the supernatants were mixed with a solution containing 0.5% (w/v) thiobarbituric acid (TBA) solution containing 20% (w/v) TCA. The mixture was incubated at 100°C for 30 min and centrifuged at 12,000 × *g* at 4°C for 10 min. The absorbance values of the supernatants were measured at 532 nm and 600 nm. MDA content was estimated using the absorbance coefficient of 1.56×10^5 and it was calculated using the following equation: MDA content [mmol g^{-1}(dry mass, DM)] = $6.452 \times [(A_{532}) - (A_{600})] \times V/M$, where V is a volume of the supernatant.

Superoxide anion production rate was measured by monitoring the nitrite formation from hydroxylamine in the presence of $O_2^{\bullet-}$, as described by Wang and Luo (1990). Segments of 0.5 g were harvested from the mid-section of the flag leaf and were homogenized at 4°C with 3 ml of 65 mM potassium phosphate buffer (pH 7.8) containing 1% (w/v) polyvinylpyrrolidone and then centrifuged at 5,000 × *g* and 4°C for 15 min. The supernatant (0.5 ml) was mixed with 0.5 ml of 65 mM potassium phosphate buffer (pH 7.8) and 0.1 ml of 10 mM hydroxylamine hydrochloride, and then incubated at 25°C for 30 min. The incubated solution (1 ml) was added to 1 ml of 58 mM 3-aminobenzene sulfonic acid and 1 ml of 7 mM 1-naphthylamine, and then further kept at 25°C for 20 min. The absorbance was recorded at 530 nm. A standard curve was used to calculate the $O_2^{\bullet-}$ production rate from the reaction equation of $O_2^{\bullet-}$ with hydroxylamine. The mean values of both MDA content and $O_2^{\bullet-}$ production rate were determined by measuring 10 leaf samples from each water condition.

Enzyme assay: Segments of about 1 cm were harvested from the mid-section of the flag leaf and they were immediately frozen in liquid nitrogen and stored until use. The samples were ground with a chilled mortar and pestle in extraction buffer containing: 100 mM Tris-HCl, pH 7.5, 5 mM Na-phosphate, 50 mM NaF, 10 mM EDTA, 14 mM 2-mercaptoethanol, 2 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μg ml^{-1} chymostatin, 1 μM microcystin-LR, 5% (v/v) ethylene glycol, 5% (w/v) glycerol, and 5% (w/v) insoluble polyvinylpyrrolidone, with a small amount of acid washed, reagent grade sand. After maceration, the homogenate was

centrifuged at 15,000 × *g* for 5 min at 4°C and the supernatant was collected as a total leaf soluble protein extract. Protein was determined by the method of Bradford (1976) with bovine serum albumin as the standard. PEPC (EC 4.1.1.31) activity was determined according to the methods of Duff and Chollet (1995) and Ding *et al*. (2012). The activity of PEPC in the leaf extracts was measured at 30°C in an assay mixture contained: 100 mM Hepes-NaOH, pH 7.5, 1 mM $NaHCO_3$, 10 mM $MgCl_2$, 4 mM PEP, 5 mM glucose-6-phosphate, 0.2 mM NADH, and 12 units ml^{-1} malate dehydrogenase. The reaction was started by adding 1/25 volume of 100 mM PEP (pH 7.0–7.5 with NaOH) and the oxidation of NADH was monitored by absorbance at 340 nm. SOD (EC 1.15.1.1) activity was determined according to the method of Giannopolitis and Ries (1977). The reaction mixture (30.25 mL) contained 100 mM potassium phosphate buffer (pH 7.8), 9.9×10^{-3} M methionine, 5.7×10^{-5} M nitroblue tetrazolium (NBT), 2.5×10^{-2} % (w/v) Triton X-100, and the required amount of the plant enzyme extract. The reaction was initiated by illumination. One unit of SOD was defined as the amount of enzyme that catalysed a 50% decrease in SOD-inhibitable NBT reduction. CAT (EC 1.11.1.6) activity was determined according to the method of Cakmak and Marschner (1992). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 30 mM H_2O_2, and the enzyme extract. Decomposition of H_2O_2 was measured as the decrease in absorbance at 240 nm. The activity was then calculated using the extinction coefficient of 39.4 mM^{-1} cm^{-1}. POD (EC 1.11.1.7) activity was determined in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.3 mM guaiacol, and the enzyme extract. The reaction was started by the addition of 0.1 mM H_2O_2, with an increase in absorbance recorded due to the formation of tetraguaiacol at 470 nm. Activity was determined using the extinction coefficient of 6.39 mM^{-1} cm^{-1}, following the method of Pütter (1974). PEPC, SOD, CAT, and POD activities were expressed as μmol mg^{-1}(protein) s^{-1}. Each result was the mean of 3 replications.

Statistical analysis: The data of this experiment were analyzed using the General Linear Models Procedure in the *SAS* package (*SAS Institute, Inc*., Cary, NC, USA). Difference was considered significant when $P<0.05$.

Results

Analysis of data variance between 2011 and 2012: One-way analysis of variance (*ANOVA*) was used to indentify differences in all traits between the two years. The differences were not significant for each trait under the same treatment.

Effects of drought stress on P_N: Under 0 kPa, the P_N of autotetraploid rice was higher than that of corresponding diploid rice when light intensity was <400 μmol mol^{-2} s^{-1} or >1,600 μmol mol^{-2} s^{-1}. The P_N of autotetraploid rice was lower than that of corresponding diploid rice when light intensity in range of 800–1,600 μmol mol^{-2} s^{-1}, but the differences of P_N between autotetraploid rice and corresponding diploid rice were not significant (Fig. 1). Drought stress reduced P_N of all rice lines, but the P_N in diploid rice decreased sharper then that of corresponding autotetraploid rice as drought stress increased. The P_N of autotetraploid rice was significantly higher than those of corresponding diploid rice under drought stress and under high light intensity (>800 μmol mol^{-2} s^{-1}) (Fig. 1).

Chl content, Chl fluorescence, and PEPC activity: Under control water conditions (0 kPa), Chl content of SP-4x was higher than that of SP-2x, while Chl content of 630-4x was lower than that of 630-2x. However, the Chl content showed no significant differences between autotetraploid rice and corresponding diploid rice (Fig. 2). Under severe drought (–40 kPa), Chl content of autotetraploid rice was higher than that of corresponding diploid rice and significant differences were found between autotetraploid and corresponding diploid rice.

The F_v/F_m and Φ_{PSII} of all rice lines decreased with the decrease of soil water potential (Fig. 3), but F_v/F_m and Φ_{PSII} of diploid rice decreased sharper then those of corresponding autotetraploid rice. The F_v/F_m ratios showed significant differences between autotetraploid rice and corresponding diploid rice under water stress of –40 kPa (Fig. 3*A*). Φ_{PSII} changed similarly as F_v/F_m. The Φ_{PSII} showed significant differences between autotetraploid and corresponding diploid rice under drought stress (Fig. 3*B*).

Drought stress increased PEPC activities of all rice lines (Fig. 4). However, PEPC of autotetraploid rice was more sensitive to drought stress treatment compared with the corresponding diploid rice. PEPC activities of autotetraploid rice were significantly higher than those of corresponding diploid rice.

MDA and $O_2^{\bullet -}$: The MDA content and $O_2^{\bullet -}$ production rate of all rice lines increased with drought stress severity (Fig. 5). MDA content and $O_2^{\bullet -}$ production rate in autotetraploid rice were lower than those in corresponding diploid rice. The MDA content and $O_2^{\bullet -}$ production rate showed significant differences between autotetraploid and corresponding diploid rice under drought stress.

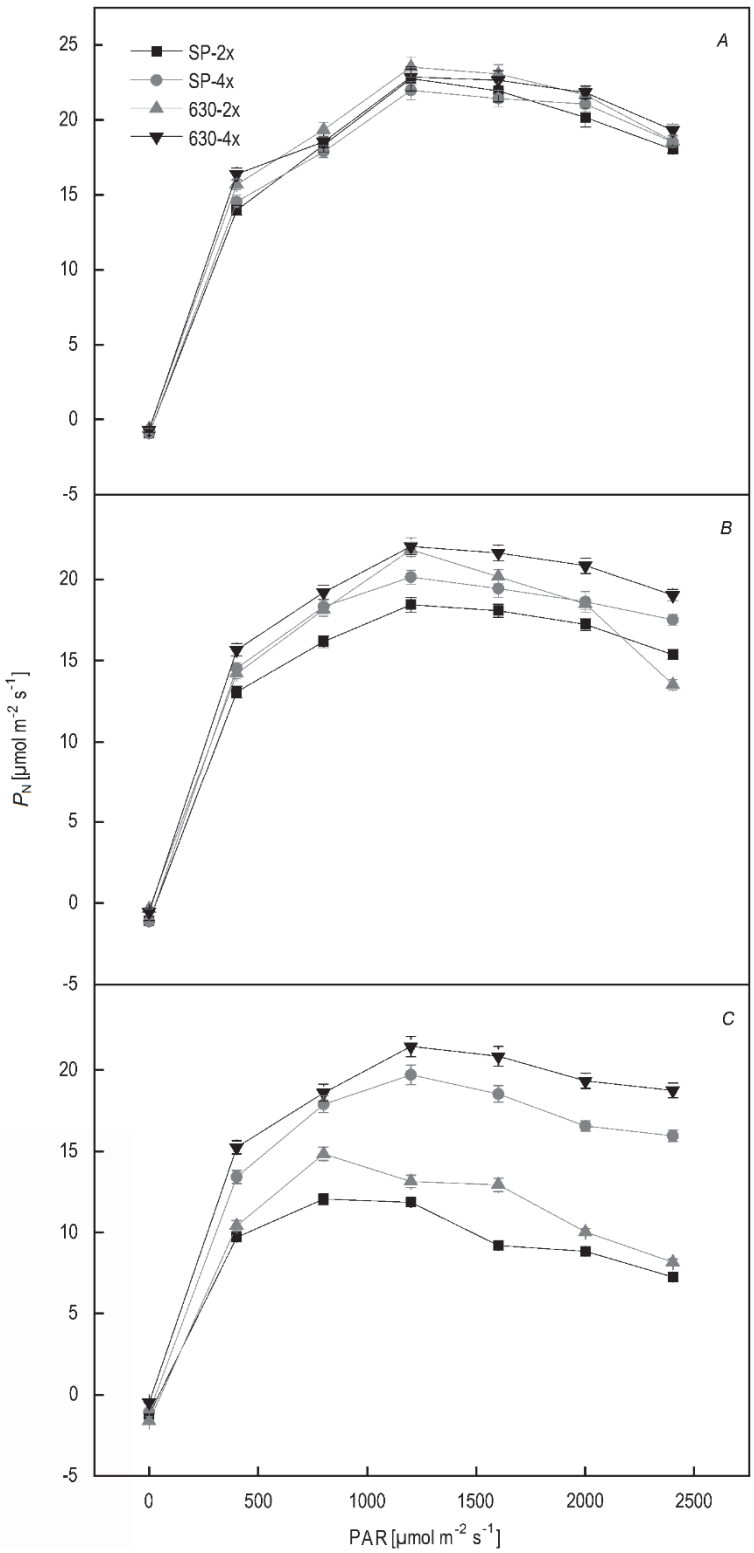

Fig. 1. Photosynthetic-light curves of flag leaves in diploid and autotetraploid rice under different drought stresses. *A*: water potential was 0 kPa; *B*: water potential was –20 kPa; *C*: water potential was –40 kPa. 2x – diploid rice line; 4x – autotetraploid rice line. Error bars show SE, $n = 3$. P_N – net photosynthetic rate; PAR – photosynthetically active radiation.

SOD, POD, and CAT activities: The activities of SOD, POD, and CAT were significantly higher in autotetraploid rice than those in corresponding diploid rice under control water condition (Fig. 6). Drought stress increased SOD,

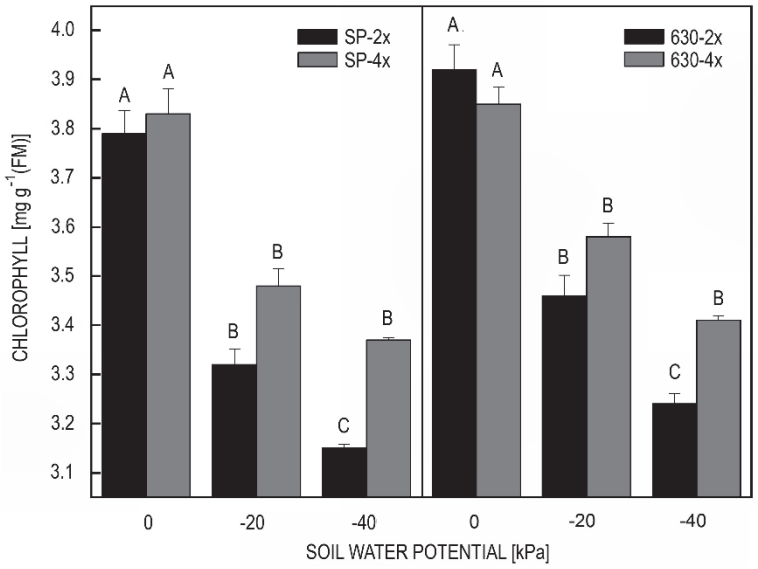

Fig. 2. Chlorophyll content in flag leaves of diploid and autotetraploid rice under different severity of drought stress. 2x – diploid rice line; 4x – autotetraploid rice line. Error bars show SE, n = 10. One-way analysis of variance (*ANOVA*) was used to indentify differences between diploid and corresponding autotetraploid in each treatment. Values with *different letters* are significantly different (P<0.05).

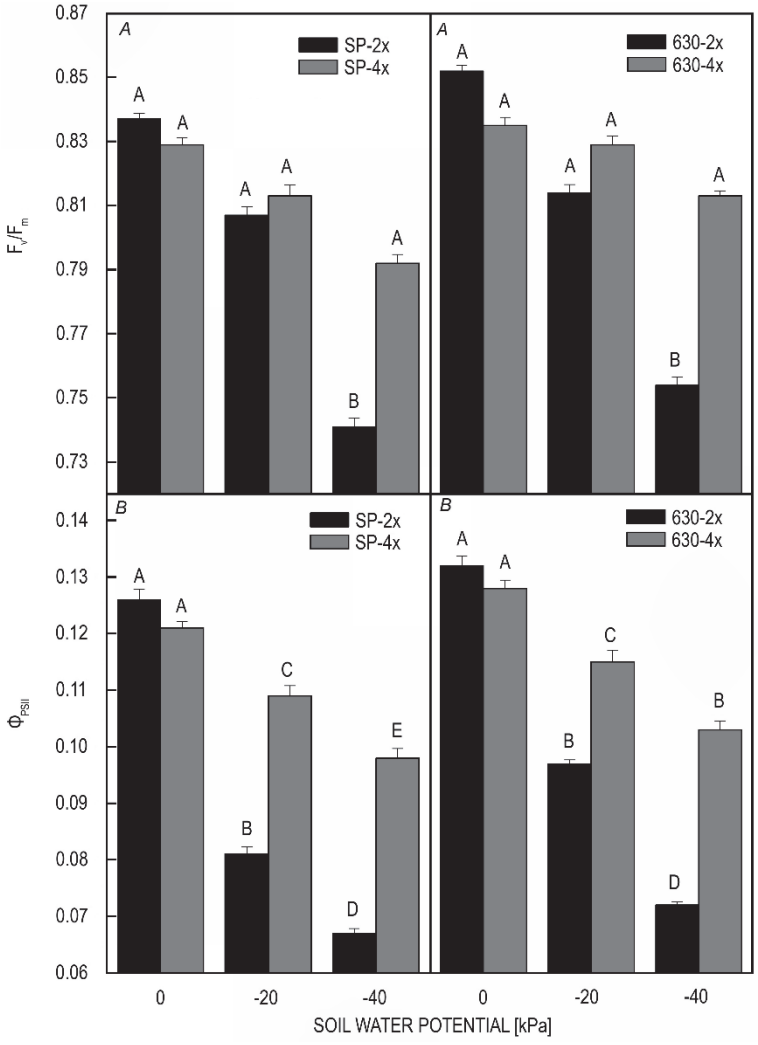

Fig. 3. Chlorophyll fluorescence parameters of flag leaves in diploid and autotetraploid rice under different severity of drought stress. 2x – diploid rice line; 4x – autotetraploid rice line; F_v/F_m – maximum photochemical efficiency of PSII; Φ_{PSII} – actual photochemical efficiency of PSII. Error bars show SE, n = 10. One-way analysis of variance (*ANOVA*) was used to indentify differences between diploid and corresponding autotetraploid in each treatment. Values with *different letters* are significantly different (P<0.05).

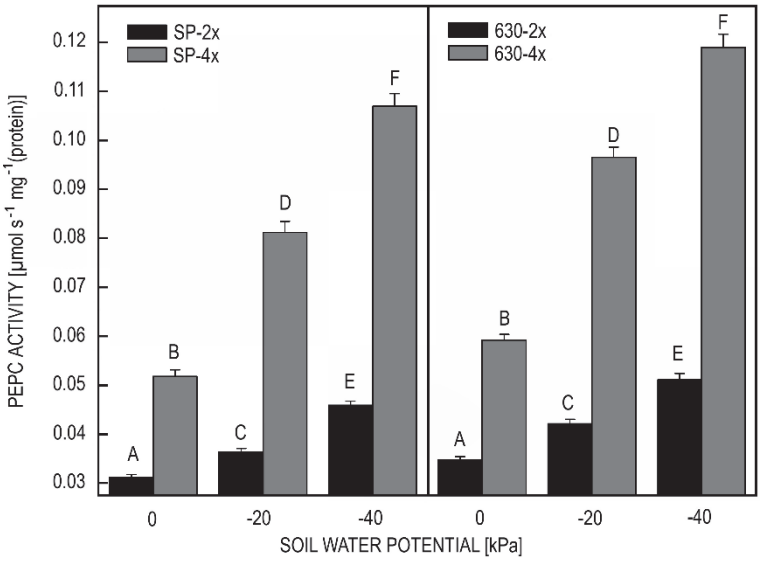

Fig. 4. Phospho*enol*pyruvate carboxylase (PEPC) activities in flag leaves of diploid and autotetraploid rice under different drought stress. 2x – diploid rice line; 4x – autotetraploid rice line. Error bars show SE, n = 3. One-way analysis of variance (*ANOVA*) was used to indentify differences between diploid and corresponding autotetraploid in each treatment. Values with *different letters* are significantly different (P<0.05).

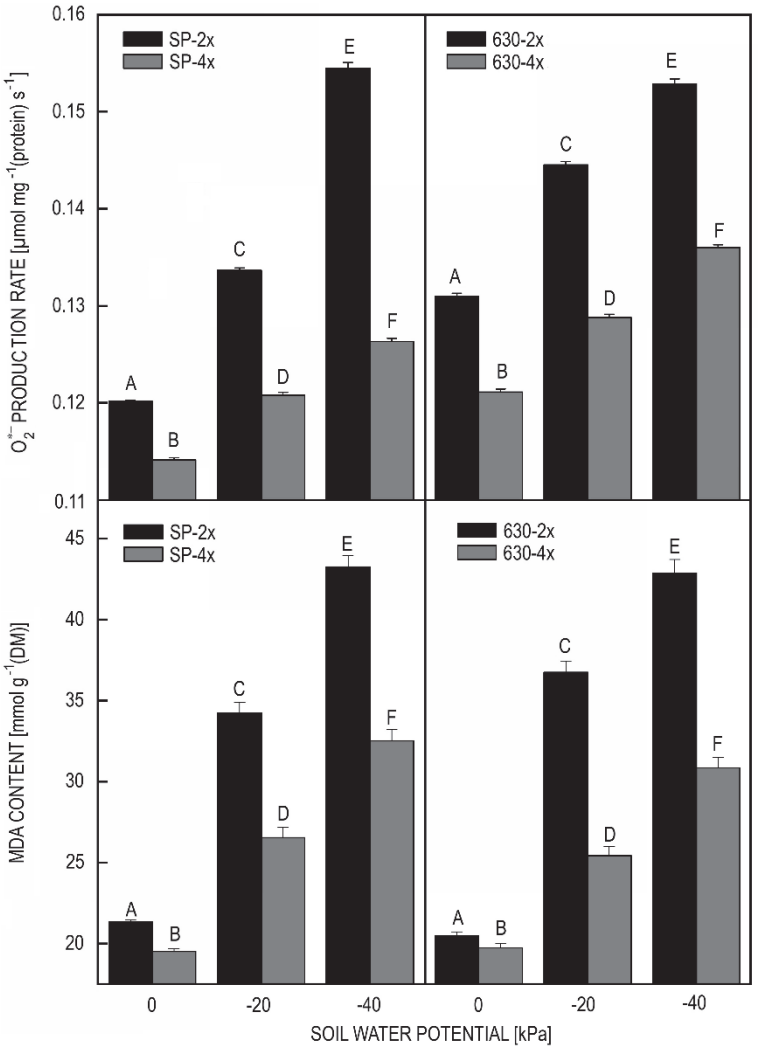

Fig. 5. Superoxide anion production rate and malondialdehyde content of rice flag leaves under different drought stresses. 2x – diploid rice line; 4x – autotetraploid rice line; $O_2^{\bullet -}$- superoxide anion; MDA – malondialdehyde. Error bars show SE, n = 10. One-way analysis of variance (*ANOVA*) was used to indentify differences between diploid and corresponding autotetraploid in each treatment. Values with *different letters* are significantly different (P<0.05).

POD, and CAT activities in all rice lines. However, SOD, POD, and CAT activities in autotetraploid rice increased more then those in corresponding diploid rice and the SOD, POD, and CAT activities in autotetraploid rice were significantly higher than those in corresponding diploid rice.

Discussion

Photosynthesis is known to be very sensitive to drought stress. As water availability is reduced, many plants show reductions in photosynthetic rate. Our results showed that especially P_N decreased dramatically under severe drought stress under high photosynthetically active radiation in diploid lines, while it decreased less in corresponding autotetraploid lines [even when photosynthetic active radiation was higher than 1,200 µmol(photon) m^{-2} s^{-1}]. The result could be explained as follows.

Nonstomatal limitation effect on photosynthetic rate: Drought stress often leads to stomatal and nonstomatal

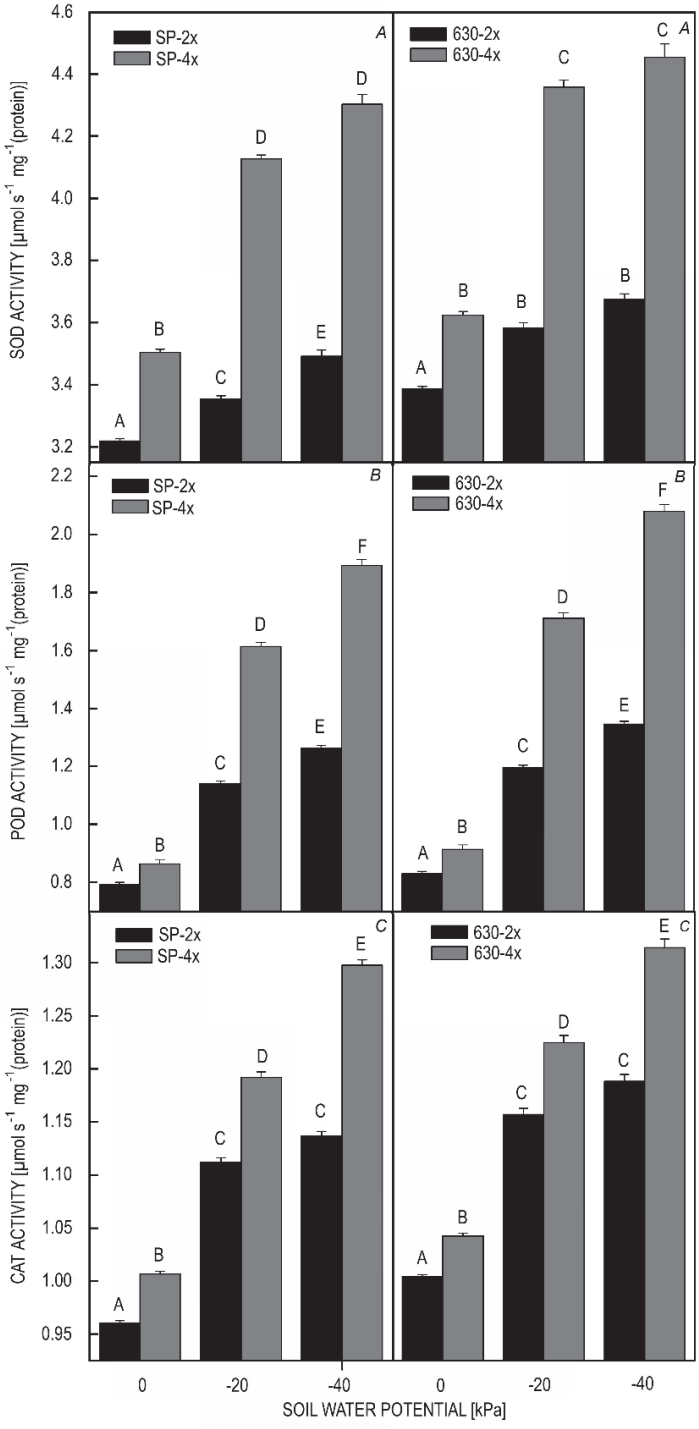

Fig. 6. Antioxidant enzyme activities of diploid and autotetraploid rice flag leaves under different drought stresses. 2x – diploid rice line; 4x – autotetraploid rice line; SOD – superoxide dismutase, POD – peroxidase, CAT – catalase. Error bars show SE, n = 3. One-way analysis of variance (*ANOVA*) was used to indentify differences between diploid and corresponding autotetraploid in each treatment. Values with *different letters* are significantly different (P<0.05).

limitation effects. Nonstomatal limitation may play a dominant role in photosynthetic rate decrease as stress develops, although drought stress often results in stomata closure, which is the primary cause of the photosynthetic rate decrease (Cornic and Briantais 1991, Chaves 1991, Yordanov *et al.* 2000). Under mild drought stress, photosynthetic capacity and quantum yield show little or no change (Yordanov *et al.* 2003). Moderate and severe drought stress caused nonstomatal limitations. Nonstomatal limitations inhibit activity of enzymes related to Calvin cycle, futher inhibit activity of PSII, eventually reduce carboxylation (Maroco *et al.* 2002, Lawlor and Cornic 2002, Dias and Brüggemann 2007). Severe drought damages photosynthetic apparatus even if plants possess efficient protective mechanisms (Valladares and Pearcy 2002). Our results were coincident with the above-mentioned findings.

The effect of PEPC, Chl content, F_v/F_m, and $\phi_{PS\,II}$ on photosynthetic rate: PEPC plays an important role in plant photosynthetic carbon fixation and carbon anaplerosis of TCA cycle, but it also alleviates photosynthesis inhibition under drought stress (Jeanneau *et al.* 2002, Jiao *et al.* 2002, 2005; Gonzalez *et al.* 2003, Sanchez and Cejudo 2003, Sanchez *et al.* 2006, Bandyopadhyay *et al.* 2007, Lebouteiller *et al.* 2007, Fang *et al.* 2008, Doubnerová and Ryšlavá 2011, Zhou *et al.* 2011). Our results showed that drought stress increased PEPC activity in all rice lines. The PEPC activity of autotetraploid lines was stimulated more by drought compared with their corresponding diploid lines.

Chl fluorescence enables to monitor and quantify the changes induced in the photosynthetic apparatus sensitively and reliably (Naumann *et al.* 2007, Oukarroum *et al.* 2007, 2009; van Heerden *et al.* 2007).

Drought declined Chl content, F_v/F_m, Φ_{PSII}, and P_N and the decrease was dependent on stress severity. Chl content, F_v/F_m, Φ_{PSII}, and P_N decreased dramatically with soil water potentials declining in diploid lines, in contrast to parameters of autotetraploid lines. The autotetraploid lines maintained the higher Chl content, F_v/F_m, Φ_{PSII}, and P_N than corresponding diploid lines and the gap between them widened further with the increasing stress severity.

The autotetraploid lines showed enhanced photoinhibition tolerance and maintained higher P_N with the higher PEPC activity, Chl content, F_v/F_m, and Φ_{PSII} than the corresponding diploid lines.

Antioxidative enzymes, reactive oxygen species (ROS), and MDA: Among other ROS, $O_2^{\bullet-}$ has greater toxicity to biomolecules and membranes (Scandalios 1993). To eliminate oxidative stress injury, plants possess antioxidant defense systems that involve antioxidant enzymes, such as SOD and CAT. The defense systems keep ROS concentration low by scavenging excessive ROS and by enhancing the activation of enzymatic and nonenzymatic antioxidants (Foyer and Noctor 2000, 2003, Yoshida 1994). In the ROS scavenging process, CAT and POD play an essential protective role together with SOD. SOD converts $O_2^{\bullet-}$ into H_2O_2 and O_2. CAT converts H_2O_2 into water and O_2, whereas POD decomposes H_2O_2 by oxidation of cosubstrates, such as phenolic compounds and/or antioxidants. Antioxidant contents and the activities of ROS scavenging enzymes have been correlated with the tolerance to environmental stresses, while MDA content could reflect the peroxidation level of plant cell membrane (Ding *et al.* 2012).

Our experiment showed that the $O_2^{\bullet-}$ production rate and MDA content increased with the increase of drought stress in all rice lines. However, the $O_2^{\bullet-}$ production rate and MDA content were relatively low in autotetraploid lines compared with their corresponding diploid lines.

The lower $O_2^{\bullet-}$ production rate and MDA content, and higher SOD, POD, and CAT activities suggested the reduced damage to membranes and more effective scavenging ROS in the autotetraploid lines. This could indicate higher drought tolerance of the autotetraploid lines.

The gene dose effect on physiological and biochemical traits of autotetraploid rice: For a plant species, physiological and biochemical traits are genetically specific. Therefore, physiological and biochemical variation of the autotetraploid reflected the effect of chromosome doubling (gene dosage). The substantial difference was observed between the autotetraploid and diploid rice in drought resistance; both autotetraploid rice lines proved the higher tolerance to drought stress than the corresponding diploid rice lines. The present study clearly showed that the autotetraploid rice lines were superior with respect to their defense systems and they should be more drought-tolerant than the corresponding diploid rice lines due to the higher PEPC activity, the lower MDA content, the lower $O_2^{\bullet-}$ production rate, and higher ROS-scavenging systems. Chromosome doubling could cause the gene dosage effects. The physiological and biochemical alteration of the autotetraploid plants might be caused by the gene dosage effects. These alterations led to drought resistance in the autotetraploid plants and it might give rise to various difference compared with the diploid plants. Such differences might be more adaptable to various environments, especially to the extreme environments, where such an adaptability could become more apparent. This explains to a certain degree why polyploid plants occur in areas of extreme climates. Naturally, the extreme climate itself causes polyploidy because it induces more abnormal gametes, finally resulting in polyploids.

Conclusion: The higher Chl content, F_v/F_m, Φ_{PSII}, and SOD, POD, CAT, and PEPC activities, lower MDA content, and the $O_2^{\bullet-}$ production rate under drought stress made the autotetraploid rice more tolerant to drought stress than the corresponding diploid rice. The gene dose effect might be one of the reasons why the autotetraploid rice varied in physiological and biochemical traits.

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