Field assessment of *CaMsrB2* transgenic lines in a drought stress environment

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Abstract: Two drought-tolerant transgenic rice lines, L-8 (single copy) and L-23 (two copy), expressing the *Capsicum annuum methionine sulfoxide reductase B2* (*CaMsrB2*) gene were selected for stress tolerance phenotyping under drought stress conditions. The two transgenic lines were selected on the basis of laboratory experiments and for performing well against drought. Field assessment of *CaMsrB2* transgenic lines L-23 and L-8 in a drought stress environment was conducted. For the assessments, small plots were prepared at the Department of Botany of the University of Karachi to test the physiological response of transgenic lines. Relative water content, quantum yield (Fv/Fm ratio), photochemical quenching (qP) photosynthetic pigments, and performance index were high in transgenic lines compared to the wild type (WT). Antioxidant enzymes as represented by catalase and superoxide dismutase activities were increased while H_2O_2 production was decreased in transgenic lines compared to the WT. The results are discussed with special reference to physiological response of the transgenic lines against drought in field conditions.

Key words: *CaMsrB2*, L-23 lines, rice, drought, field

1. Introduction

Drought and salinity have long been known to be the most prevalent abiotic stresses in arid and semiarid regions, restraining growth and productivity of many plant species across the world (Qadir, 2008; Naz, et al., 2010). With limited water resources and substantial increase in the human population, conventional crop practices are not be able to meet growing food demand. In such a challenging situation, effective measures need to be adopted to maximize crop production in order to assure access to food.

In arid environments, plant populations are mainly affected by multiple stresses simultaneously, and especially drought and salinity, which cause significant reductions in crop yield (Zia et al., 2007; Tariq et al., 2009). In this scenario, biotechnology has proven to be an excellent tool to fulfill the food demand in diverse and extreme environments. Therefore, utilization of genetically modified crops has increased drastically in the last decade (Siddiqui et al., 2014).

In abiotic stress, methionine is the main target of reactive oxygen species (ROS), which modify protein configuration through oxidation–reduction reactions (Siddiqui et al., 2014). Generally, in plants, the methionine oxidation–reduction reaction is switched on by methionine sulfoxide reductase (MSR) enzymes. The two genes *MsrA* and *MsrB* were recognized for methionine sulfoxide reductase and isolated from *Brassica napus* (Sadanandom et al., 2000). The role of the *MsrB2* gene in biotic stress as a defense regulator was first reported in pepper plant (Oh et al., 2010) and then in transgenic rice under abiotic stress (Siddiqui et al., 2014). Most living organisms exhibit MSRs that not only play a key role in the cellular response against oxidative stress, but also reduce the concentration of oxidized methionine (MetO) as it returns to its original (Met) state (Vogt, 1995; Moskovitz, 2005; Kwon et al., 2007). The physiological evaluation of transgenic rice expressing *CaMsrB2* has been well documented against drought tolerance in laboratory experiments, but transgenic rice expressing the *CaMsrB2* gene in field environments has not been tested to date. It was observed that field conditions are entirely different from the laboratory environment as they are uncontrollable. It is often seen that most transgenic plants

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do not produce substantial results in field conditions. The reason behind the nonspecific response of transgenic plants in the field might be the multiple stresses that have to be faced by plants in fields. Therefore, we conducted a field experiment on *CaMsrB2* transgenic rice plants under drought stress conditions.

2. Materials and methods

2.1. Plant materials
Seeds of the wild type (WT), Ilmi, and two *CaMsrB2* transgenic rice plants, L-8 and L-23, were provided by the National Center for GM Crops, National Academy of Agricultural Science, Rural Development Administration, Korea, and washed with distilled water several times before sowing. Seeds were allowed to germinate and were grown in plastic pots. Fifteen-day-old equally sized seedlings were transferred to a field having 12 plots (four plot repetitions for each of WT, L-8 and L-23). Plants were allowed to grow in natural conditions. The studied area was characterized by high light intensities, often reaching 35,000–40,000 lx at 1300 hours. The plants were irrigated for 4 weeks. After 4 weeks, the plants were subjected to drought by stopping watering for the next 7 days when water fraction volume reached 5%. Three plots for each transgenic line and the WT were represented as replicates.

2.2. Real-time quantitative PCR
To analyze the two *CaMsrB2* transgenic rice plants, L-8 and L-23, used in all experiments, we performed copy number assays and gene expression analyses. The procedures for TaqMan copy number assay experiments and quantitative real-time PCR were performed by previously described methods (Siddiqui et el., 2014). Total RNA was isolated from leaf samples of the WT, Ilmi, and the two *CaMsrB2* transgenic rice plants using the RNeasy Plant Mini Kit and RNase-Free DNase Set (QIAGEN) and reverse-transcribed using the amfiRivert Platinum cDNA Synthesis Master Mix (GenDEPOT). The diluted cDNA was used for analyzing the expression level of the *CaMsrB2* gene in the two CaMsrB2 transgenic rice plants. For quantitative real-time PCR, the expression level of *OsActin1* was used for the normalization of real-time PCR results (Fukao et al., 2011) and all reactions were performed in triplicate using the StepOnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The expression level of *CaMsrB2* was analyzed by the comparative cycle threshold ($\Delta\Delta Ct$) method and StepOne Software version 2.2.2 (Applied Biosystems). The gene-specific primers used for quantitative real-time PCR for *CaMsrB2* were 5'-AGTTTACACCGGCAAATTCCTA-3' and 5'-GAAAGCGCAAGGCTTAAAAGTA-3' and for *OsActin1* were 5'-ACAGGTATTGTGTTGGACTCTGG-3' and 5'-AGTAACCACGCTCCGTCAGG-3'.

2.3. Photosynthetic pigment extraction and estimation
A sample of 500 mg was harvested from each transgenic (L-8 and L-23) and the nontransgenic (WT) plant leaf and homogenized in 96% ethanol. The whole contents were centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatants were than separated and the optical density was read at 666, 653, and 470 nm. Total photosynthetic pigments were calculated and expressed as fresh weight (Lichtenthaler and Wellburn, 1985)

2.4. Relative water content
Ten randomly selected leaves were sampled and 4 cm^2 from the midveins and the edge sections of leaves were cut with scissors from each treatment or control plant. After fresh weight measurement, each sample was placed in a 90-mm airtight plastic petri plate containing distilled water for the next 12 h. Later, the leaf samples were taken out, surface moisture was removed by quickly but lightly applying filter/tissue paper, and the samples were immediately weighed to obtain the fully turgid weight. Leaf samples were then oven-dried at 60 °C for 48 h and weighed to determine the dry weight. Later, the relative water content (RWC) was measured

2.5. Quantum yield and stomatal conductance
Measurements of chlorophyll fluorescence emission from the 20 randomly selected leaves were monitored with a fluorescence monitoring system (Handy PEA) in the pulse amplitude modulation mode. A leaf, adapted to dark conditions for 30 min using leaf clips, was initially exposed to the modulated measuring beam of far-red light (LED source with typical peak at wavelength 735 nm). The original (F0) and maximum (Fm) fluorescence yields were measured under weak modulated red light (0.5 µmol m^{-2} s^{-1}) with pulses of saturating light of 1.6 s (6.8 µmol m^{-2} s^{-1} PAR). The variable fluorescence yield (Fv) was calculated by the equation Fm – F0. The ratio of the variable to maximum fluorescence (Fv/Fm) was calculated as the dark-adapted quantum yield of PSII photochemistry and performance index and nonphotochemical quenching were calculated as described by Maxwell and Johnson (2000). Likewise, the stomatal conductance of 20 randomly selected leaves of each treated and control plant were examined using a leaf porometer (Model SC-1, Decagon).

2.6. H$_2$O$_2$ production
Hydrogen peroxide content was measured according to the procedure of Velikova et al. (2000). Freshly harvested leaf samples (100 mg) were homogenized with 3 mL of 0.1% (w/v) trichloroacetic acid in an ice bath and the homogenate was centrifuged at 12,000 × g for 15 min. Later, 0.5 mL of 10 mM phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI) were added to 0.5 mL of the supernatant. The absorbance of the supernatant was read at 390 nm. The amount of H$_2$O$_2$ was calculated using the extinction coefficient and expressed as µmol g^{-1} FW.

2.7. Extraction

Randomly collected leaf samples of 500 mg were crushed in liquid nitrogen at 4 °C and homogenized in 10 mL of protein extraction buffer containing Tris-HCl (pH 6.8), 50 mg of PVP, 10 mL of DDT, and 0.1 mM EDTA. The contents were centrifuged at 10,000 rpm for 15 min. Total protein was estimated by the method of Bradford (1976).

2.8. Catalase (enzyme number by NC IUBMB: EC 1.11.1.6)

Catalase (CAT) activity was estimated by the method of Patterson et al. (1984). The decomposition of H_2O_2 was measured at 240 nm taking OD at 240 nm as 43.6 mM cm^{-1}. The reaction mixture (3.0 mL) consisted of 10.5 mM H_2O_2 in 0.05 M potassium phosphate buffer (pH 7.0) and the reaction was initiated after the addition of 0.1 mL enzyme extract at 25 °C. The decrease in absorbance at 240 nm was used to calculate the CAT activity. One unit of CAT activity is defined as the amount of enzyme that catalyzes the conversion of 1 mM of H_2O_2 min^{-1} at 25 °C.

2.9. Superoxide dismutase (enzyme number by NC IUBMB: EC 1.15.1.1)

The assay for superoxide dismutase (SOD) activity was performed by following the method of Beyer and Fridovich (1987). The assay mixture consisted of 27.0 mL of 0.05 M potassium phosphate buffer (pH 7.8), 1.5 mL of L-methionine (300 mg per 2.7 mL), 1.0 mL of nitroblue tetrazolium salt (14.4 mg per 10 mL), and 0.75 mL of Triton X-100. Aliquots (1.0 mL) of this mixture were added to small glass tubes, followed by the addition of 20 mL of enzyme extract and 10 mL of riboflavin (4.4 mg per 100 mL). The cocktail was mixed and then illuminated for 15 min in an aluminum foil-lined box, containing 25-W fluorescent tubes. In a control tube the sample was replaced by 20 mL of buffer and the absorbance was measured at 560 nm. The reaction was stopped by switching off the light and placing the tubes in the dark. Increase in absorbance due to formation of formazan was measured at 560 nm. Under the described conditions, the increase in absorbance in the control was taken as 100% and the enzyme activity in the samples was calculated by determining the percentage inhibition per minute. One unit of SOD is the amount of enzyme that causes a 50% inhibition of the rate for reduction of nitro blue tetrazolium salt under the conditions of the assay

2.10. Statistical analysis

Both treatments and the control were replicated four times and subjected to statistical analysis using SPSS 17.0. Values were compared using a paired t-test with mean standard error. Significant differences (P = 0.05) from the paired t-test were expressed on bar graphs as different letters. A correlation analysis was done between the enzymatic and nonenzymatic antioxidant versus H_2O_2 production and expressed with a line graph.

3. Results

Two transgenic lines along with the WT were evaluated in the field under drought conditions. Biomass accumulation and relative water content were significantly affected by drought (Figure 1). Transgenic lines L-23 and L-8 had more biomass accumulation compared to wild-type Ilmi. Turgid and dry weights were substantially higher in L-23 compared to L-8 and the WT. Similarly, more than 70% RWC was observed in both transgenic lines compared to the WT.

Photosynthetic pigments such as chlorophyll a and b and total chlorophyll were substantially higher in the transgenic lines compared to the WT (Figure 2). Similarly, accessory pigments like carotenoids increased in L-23 as compared to the WT under a stressful environment. The production of H_2O_2 decreased in the transgenic lines compared to the WT. However, greater decrease in H_2O_2 was observed in L-23 compared to the WT.

Photosynthetic performance, dark-adapted quantum yield, photochemical quenching, and stomatal conductance were substantially higher in transgenic lines L-8 and L-23 compared to WT (Figure 3). Substantial increases in quantum yield (Fv/Fm ratio), photosynthetic performance
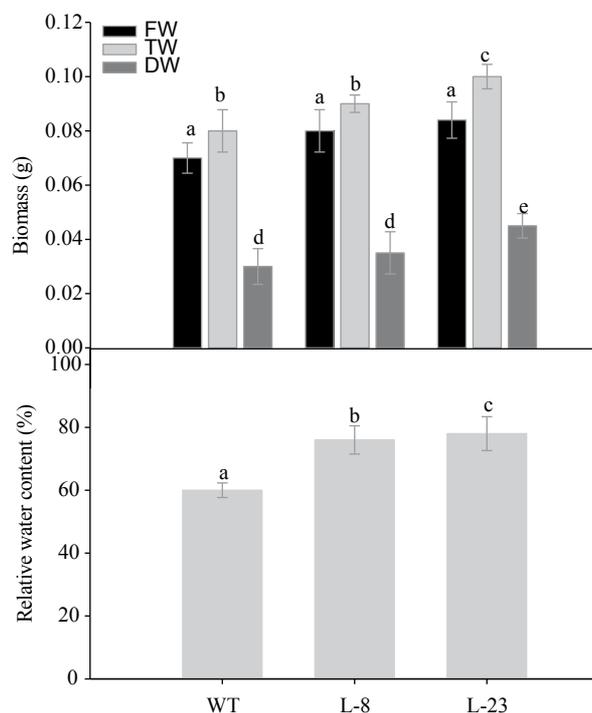

Figure 1. Mean biomass accumulation and relative water content of *CaMsrB2* transgenic rice in the field under a drought stress environment. Vertical lines on the bars represent standard error (±). Similar letters on the error bars show that the t-test was nonsignificant at P = 0.05. FW = fresh weight, DW = dry weight, TW = turgid weight.
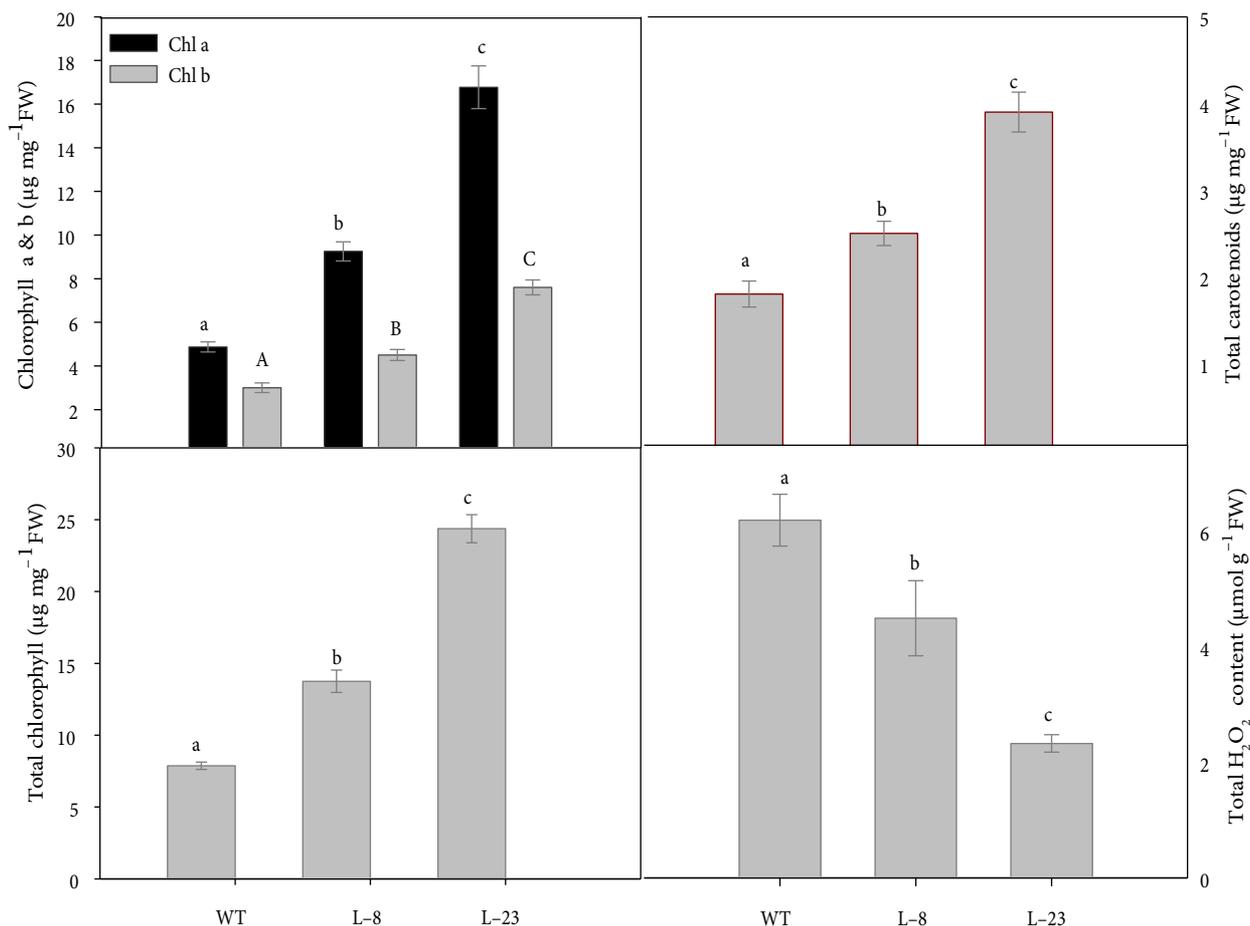
Figure 2. Chlorophyll, carotenoid, and H_2O_2 contents of *CaMsrB2* transgenic rice in the field under a drought stress environment. Vertical lines on the bars represent standard error (±). Similar letters on the error bars show that the t-test was nonsignificant at P = 0.05.

index (PI_{ABS}), photochemical quenching (qP), and stomatal conductance (gs) in L-23 were observed in the drought stress environment. It was observed that plants having two copy lines (L-23) had better performance against drought in field compared to those plants with a single copy (L-8).

Examination of enzymatic and nonenzymatic antioxidants in terms of antioxidant enzyme activity like that of SOD and CAT and total carotenoid concentration in transgenic lines in drought showed that CAT and carotenoid contents were increased in transgenic line L-23, particularly in comparison to the WT, under stress conditions (Figure 4). However, more CAT activity was observed in L-23 compared to L-8 and the WT. SOD activity was nonsignificantly higher in the transgenic lines.

Correlation analysis of enzymatic and nonenzymatic antioxidant compounds like carotenoid, CAT, and SOD versus H_2O_2 production was conducted, showing significant correlation between CAT and H_2O_2 compared to SOD and H_2O_2 (Figure 5). However, in transgenic line L-23, substantial decline in H_2O_2 production due to CAT and carotenoids was observed.

4. Discussion
It was concluded that *CaMsrB2* transgenic lines showed better performance in terms of growth and related physiological parameters in field conditions under drought stress. Transgenic line L-23 had better drought tolerance compared to L-8 and the WT, showing substantially higher photosynthetic performance, pigments, and relative water contents. Better performances of *CaMsrB2* transgenic lines in drought stress are mainly due to increased antioxidant enzyme activity, which lowers the damaging effects of H_2O_2.

The two-copy line L-23 had a better ability to retain water than the single-copy line L-8 under drought stress in a small field (1.2 × 1.2 m). It was observed that RWC and the ability to retain more water in leaves are important physiological strategies in stressful environments. It has been earlier reported that during droughts, tolerant plants maintain more than 50% of their RWC due to biomass accumulation (Pilon-Smits et al., 1995; Rezaei et al., 2006; Siddiqui, 2013). Likewise, it was also observed that greater accumulation of water-soluble compounds was found in
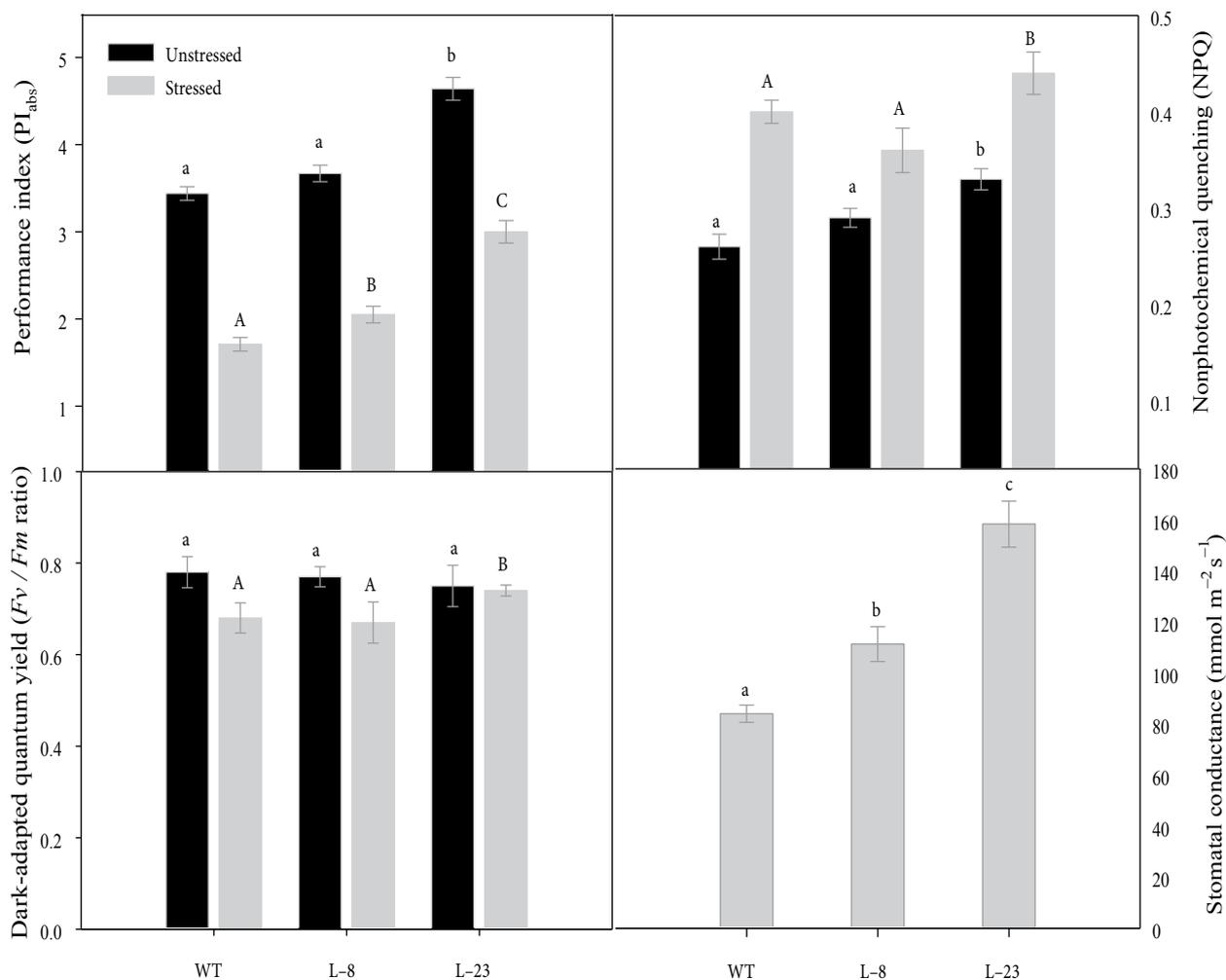
Figure 3. Dark-adapted quantum yield, photosynthetic performance index, photochemical quenching, and stomatal conductance of *CaMsrB2* transgenic rice in the field under a drought stress environment. Vertical lines on the bars represent standard error (±). Similar letters on the error bars show that the t-test was nonsignificant at P = 0.05.

stress-resistant than stress-sensitive genotypes (Martin et al., 2002). Accumulation of solute either actively or passively is an important adaptive mechanism that helps to draw more water inside the leaves of plants subjected to water deficits and high salinity levels (Rezaei et al., 2006; Cho et al., 2014). It has been examined that, in response to stress conditions, plants switch to the synthesis of compatible solutes that help to keep water within cells or protect cellular components from the injury caused by dehydration. Substantial water holding capacity of plants plays a key role in stress tolerance and has been reported to be high in many stress-tolerant plants (Munns, 2002; Yang et al., 2003; Siddiqui et al., 2008; Siddiqui and Khan, 2011; Siddiqui, 2013).

CaMsrB2 transgenic lines maintained significant quantum yield (Fv/Fm ratio), better photosynthetic performance, better photochemical quenching, and more photosynthetic pigments compared to the WT during drought stress. The two T-DNA insertion line, L-23, showed better photosynthetic performance than L-8. A significant positive correlation between quantum yield and chlorophyll in transgenic lines was observed (Figure 5). Photosynthesis and its related components like dark-adapted quantum yield, quenching, and pigments are important physiological attributes reflecting the photosynthetic ability of plants in stressful environments. It is presumed that increases in photochemical quenching in transgenic lines may be attributed to the lowering of the
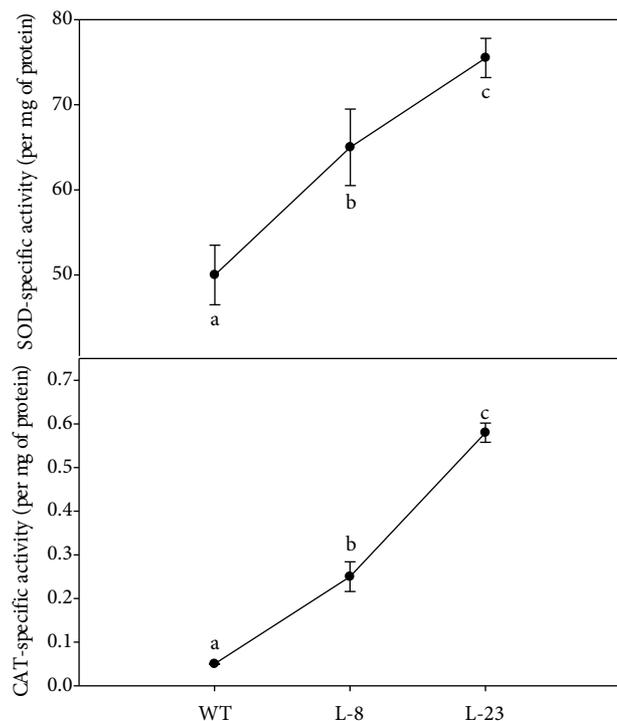
Figure 4. Activities of antioxidant enzymes CAT and SOD of *CaMsrB2* transgenic rice in the field under a drought stress environment. Vertical lines on the bars represent standard error (±). Similar letters on the error bars show that the t-test was nonsignificant at P = 0.05.

production of ROS or oxygen radicals. It was reported that nonphotochemical and photochemical quenching helps to minimize the production of oxygen radicals (Muller et al., 2001). Further, photosynthetic pigments and the quantum yield (Fv/Fm ratio) of *CaMsrB2* transgenic lines may be linked to the overexpression of the chlorophyll complex-encoded *cab* gene family, chiral macroaggregates of light-harvesting chlorophyll a or b pigment protein complexes that protect the photosynthetic apparatus (Gustavsson et al., 2002), or the reduction of oxidative damage in chloroplast lipids, pigments, and proteins (Tambussi et al., 2000).

Moreover, carotenoids and antioxidant enzyme activities were increased while H_2O_2 was decreased in transgenic lines compared to the WT. It was presumed that the L-23 transgenic line had better protective mechanisms, showing relatively higher antioxidant enzyme activity, photochemical quenching, and carotenoid concentration and lower H_2O_2 contents. Results of the present study clearly showed that drought stress-induced reduction in the quantum yield of PSII was less in the transgenic line and was maintained up to 0.8, which is similar to the results of some previous studies (Colom and Vazzana, 2002; Parida et al., 2003; Waseem et al., 2006; Siddiqui

et al. 2008; Makbul et al., 2011). Two possibilities can be predicted from the results: 1) the *CaMsrB2* gene may increase carotenoid concentrations, which may not only enhance the antioxidant ability of the plant but also lower the production of ROS via photochemical quenching; 2) increased chlorophyll provides a continuous and substantial energy supply to maintain quantum yield in drought stress and may be utilized in the synthesis of antioxidant enzymes like CAT and SOD.

CaMsrB2 transgenic rice plants showed higher stomatal conductance than the WT. The L-23 transgenic line had better stomatal conductance than L-8. Sensitivity, tolerance, and physiological responses of plants against drought fluctuate among species. For instance, species that grow slowly have been found to be more sensitive than fast-growing species (Waseem et al., 2006). This was observed in abiotic stresses like water stress; some drought-tolerant plants developed fitness by reducing leaf area and stomatal conductance to transpiration (Nativ et al., 1999; Ares et al., 2000). Therefore, plants might adapt physiologically to drought conditions by reducing stomatal conductance to water vapor, thus increasing their water-use efficiency. However, tolerant plants have been observed to acclimatize to two different strategies during droughts. For example, the annuals that live longer and perennials decrease their leaf size and/or stomatal conductance (Geber and Dawson, 1997; Querejeta et al., 2003), while shorter-lived annuals maximize fitness by increasing stomatal conductance to increase carbon gain and avoid drought stress. This strategy seems to be physiologically well adapted as it lets these species grow rapidly, flower early, and increase yield before the start of substantial soil drying (Geber and Dawson, 1997; McKay et al., 2003). Thus, it is assumed that the *CaMsrB2* gene might favor the early developmental strategy, enhancing drought tolerance in the current transgenic rice.

The present study showed that CAT and SOD activities of transgenic lines were negatively correlated with H_2O_2 concentrations compared to the WT. It has been reported that plant tolerance can be maintained by controlling the ROS production through either enzyme activity like peroxidase, SOD, or catalase activities (Ahmed et al., 2010; Terzi et al., 2010; Siddiqui, 2013) or through nonenzymatic mechanisms, including vitamins, glutathione, carotenoids, and phenolic compounds (Prochazkova et al., 2001; Jaleel et al., 2009). It is presumed that the lower H_2O_2 production in *CaMsrB2* transgenic lines may be either due to enzyme activities or related to higher carotenoid synthesis, which may lower H_2O_2 production in chloroplasts, avoiding oxidative damages.
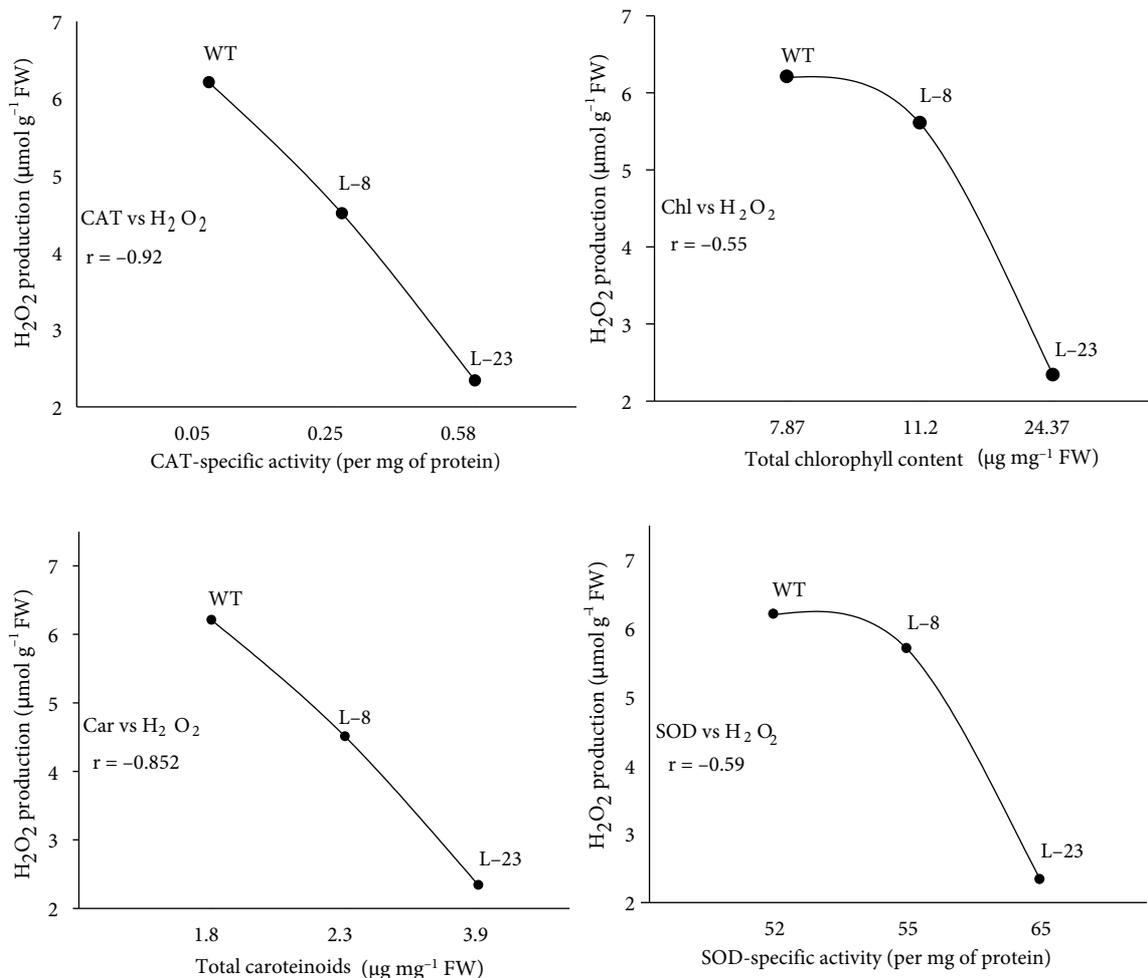
Figure 5. Relationship between enzymatic and nonenzymatic antioxidant systems versus H_2O_2 contents of *CaMsrB2* transgenic rice in the field under a drought stress environment. Lines represent r = correlation values based on mean of four replicates each for transgenic lines L-8 and L-23 and the WT.

It was concluded that the *CaMsrB2* gene provoked substantial physiological changes in rice plants, which not only maintained adequate leaf water content and effective stomatal regulation and caused optimal photosynthesis performance, but also effectively controlled H_2O_2 production during drought stress in field conditions.

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