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Short-running cycle: Anti-oxidant enzymes and photosynthetic pigments in pearl millet

Does a terminal drought tolerance QTL contribute to differences in ROS scavenging enzymes and photosynthetic pigments in pearl millet exposed to drought?

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Abstract

The control of reactive oxygen species (ROS) and the stability of photosynthetic pigments under stress conditions are hypothesized to contribute to drought tolerance. Here we studied how ascorbic peroxidase (APX), superoxide dismutase (SOD) catalase (CAT) isozyme activities and chlorophyll *a, b* (Chl *a, b*) and carotenoids (Car) contents responded to water stress and whether they related to presence of a terminal drought tolerance QTL in pearl millet. We used PRLT2/89-33 (QTL donor), H77/833-2 (sensitive), and near-isogenic lines (QTL-NILs) introgressed with the QTL in H77/833-2 background. Under water stress there was no significant change in the total APX activity; only the proportional APX5 activity increased, with higher band intensity in tolerant genotypes. There were no significant changes in total activities of CAT and SOD under water stress, with similar band intensities in all genotypes, and a new CAT isozyme was induced in all genotypes. The photosynthetic pigment content decreased under water stress, although not differently in any genotype. Under water stress, the activities of most APX, CAT and SOD isozymes were closely related to the total chlorophyll/carotenoids ratio. Overall, besides APX5, water stress did not lead to major changes in the profile of isoenzymes involved in ROS scavenging. Similarly, the pigment content under stress did not discriminate genotypes according to the presence/absence of the QTL. This absence of discrimination for the ROS scavenging enzymes and for the pigment content under stress suggests that these traits may not play a key role in terminal drought tolerance in pearl millet.

Keywords: Pearl millet, drought, anti-oxidant enzymes, photosynthetic pigments

Abbreviations: Ascorbic peroxidase (APX), Carotenoids (Car), Catalase (CAT), Chlorophyll (Chl), Dimethyl sulphoxide (DMSO), Reactive oxygen species (ROS), Superoxide dismutase (SOD), Vapor pressure deficit (VPD)

1. Introduction

Water deficit is one of the major factors limiting crops production in the world. Despite the fact that pearl millet is considered as a drought tolerant crop *per se*, it suffers substantial yield losses due to stress occurring at the end of growing season (terminal drought), and there exist genotypic variations in these losses (Bidinger and Hash, 2004). Thus, the yield enhancement under stress condition requires a better understanding of the mechanisms involved in drought tolerant genotypes.

Abiotic stresses are proven to cause oxidative stress by contributing to reactive oxygen species (ROS) formation (Haber and Weiss, 1934; Procházková et al., 2001; Apel and Hirt, 2004; Zimmermann and Zentgraf, 2005), which strongly react with organic molecules and so disrupt basic metabolic pathways and cell structures (Apel and Hirt, 2004; Møller et al., 2007). However, ROS may also serve as a molecules transducting drought signal and so enhance the plant stress defense mechanisms (Guan and Scandalios, 1998; Guan et al., 2000; Yoshimura et al., 2000; Jiang and Zhang et al., 2002; Noctor, 2005; Suzuki and Mittler, 2006). There are basically two detoxification mechanisms plants have developed to scavenge free ROS (Scandalios, 1997; Shalata and Tal, 1998; Procházková et al., 2001) (i) Non-enzymatic radical scavengers, e.g. carotenoids, glutathione, mannitol, ascorbate, tocopherol, flavonoids and some alkaloids; (ii) Enzymatic anti-oxidants of the Hallivel-Asada cycle (Asada, 1994), which involves ROS reactions with superoxide dismutase, ascorbic peroxidase (APX) and catalase (CAT).

There is evidence in some plant species (wheat, mangrove, sesame) that tolerance to abiotic stresses is correlated with enhanced capacity to scavenge ROS in tolerant genotypes (Sairam and Srivastava, 2001; Parida et al., 2004; Fazeli et al., 2007; Khanna-Chopra and Selote, 2007). From the other hand, the enhanced ROS scavenging capacity could be the response to higher ROS production of sensitive genotypes compared to tolerant once as showed in winter wheat (Simova-Stoilova et al., 2009). Other studies on jute, alfalfa, tomato and wheat have reported declining or unchanged levels of anti-oxidative enzymes under drought (Irigoyen et al., 1992; Űnyayar et al., 2005; Nikolaeva et al., 2010), and in drought tolerant transgenic groundnut, ROS scavenging enzymes

activities were not linked to tolerance-related traits (Bhatnagar-Mathur et al., 2009). In pearl millet Patil et al., (2005) found that all these enzymes increased under drought in field conditions, although SOD increased only in later drought phases, whereas APX and CAT activity rose soon after stress imposition. So, the relationship between the plant's capacity to tolerate stress and the activity of ROS scavenging mechanisms is still not clear and seems to be highly variable depending on species, developmental and metabolic state of plant, and the duration of stress (Razmjoo et al., 2008, Nikolaeva et al., 2010). For example, in Patil and colleagues, there was no indication of the soil water content where anti-oxidative enzyme started to increase. Any information about ROS scavenging mechanisms is, moreover, very limited in pearl millet.

It has been shown that the main site of ROS formation is in mitochondria and chloroplast electron transport chains. In thylakoids it happens due to over-reduction of both photosystems (PS), which are no longer able to accept excess of excitation energy from light-harvesting chlorophyll protein complexes (LHCP) (Demmig-Adams and Adams, 1992; Reddy et al., 2004; Schmid, 2008). Such electron surplus that can lead to ROS formation can be avoided: (i) by degrading chlorophyll to primarily avoid ROS formation as in poikilochlorophyllous plants (Maslova and Popova, 1993; Keiper et al., 1998; This et al., 2000); (ii) by retaining chlorophyll as in homoiochlorophyllous plants and simultaneously triggering the anti-oxidative scavenging mechanisms listed above to avoid further cell structure damage (Farrant et al., 2003). Many reports emphasize the role of carotenoids (especially xanthophylls) in direct deactivation of ROS in homoiochlorophyllous plants (e.g. Demmig-Adams and Adams, 1992; MunneÂ-Bosch and Alegre, 2000; Takano et al., 2005; Telfer, 2005; Farooq et al., 2009). Xanthophylls were shown to be spatially associated with chlorophylls (Schmid, 2008) where they can account up to 50% of total carotenoids content and contribute sustaining the photochemical functions (Demmig-Adams and Adams, 1992; Farooq et al., 2009). How ROS scavenging mechanisms and photosynthetic pigments interact under water stress is not known in pearl millet.

Drought stress generally causes decrease in the total chlorophyll (Chl) content (Terzi and Kadioglu, 2006; Kiani et al., 2008; Farooq et al., 2009) while the Chl *a/b* ratio usually increases (Ashraf et al., 2001) as shown in vascular plants like e.g. *Ctenante*

setosa, Pennisetum americanum, *Phlomis fructicosa,* and some desert plants like *Plantago albicans, Zygophyllum album etc.*. In some species like rosemary or maize Chl/Car ratio was described to decline under drought (MunneÂ-Bosch and Alegre, 2000; Mohammadkhani and Heidari, 2007) although another reports showed Chl/Car ratio increased in *Betula papyrifera* and *Arabidopsis thaliana* (Richardson et al., 2004; Zhang et al., 2008). In maize, wheat and groundnut drought tolerance was correlated with stability of pigments (Pastori and Trippi, 1992; Arunyanark et al., 2008). However, as mentioned above, in some plants like eucalyptus (*Eucalyptus microcystis*), certain wheat genotypes and barley, loss of photosynthetic pigments is likely to be understood as an adaptive feature preventing ROS formation (Maslova and Popova, 1993; Keiper et al., 1998; This et al., 2000). Therefore, the traditional recognition of chlorophyll content stability as a drought tolerance character should be considered with care.

The objectives of this work were: (i) to test whether selected biochemical traits related to the ROS scavenging machinery were increased by exposure to controlled water stress, although our purpose was not to attempt any cellular localization; (ii) assess the genotypic variability in pearl millet in photosynthetic pigment's content in well-watered and water-stressed conditions and (iii) to analyze whether this variability could be linked with the presence/absence of a drought tolerance QTL.

2. Materials and methods

2.1. Plant material

Two pearl millet [*Pennisetum glaucum* (L.) R. Br.] genotypes contrasting in tolerance to drought stress; (PRLT 2/89-33 (tolerant) and H 77/833-2 (sensitive)) and 3 QTL-introgression lines (ICMR 01029, ICMR 01031, ICMR 02041) were selected from our previous experiments (Serraj et al., 2005). Work was carried out on test-cross hybrids of these genotypes, developed by crossing the inbred parental lines and QTL-NILs to the male sterile line tester 843A (for the reasons described in Yadav et al., 2004). To develop QTL introgression lines in the background of H 77/833-2, the latter was crossed to PRLT 2/89-33, followed with 4 backcrosses with H 77/833-2. At each backcross, the assessment for the presence or absence of the terminal drought tolerance QTL was made using flanking markers on pearl millet linkage group 2 (RFLP markers *Xpsmp2059,*

Xpsmp2066 and *Xpsmp2237* once these were available). Two steps of selfing were performed to generate QTL-NILs inbreds homozygous for various parts of introgressed QTL target region. Tolerance of hybrids of the QTL-NILs had previously been assessed from yield maintenance under terminal drought stress in several years of field trials, and on the panicle harvest index (PNHI), an index that assess the success of spikelet reproduction and the degree of grain filling (Bidinger et al., 1987). The three QTL-NILs selected for the present study had the best performance among those fields tested (Serraj et al., 2005).

2.2. Plant growth and response to drought

Plants were grown in 20 cm diameter pots, one plant per pot, filled with 5 kg of soil [Alfisol (Sandy clay loam) collected from ICRISAT's farm, and mixed with sand and manure (5:3:1)] during July 2007 in the glasshouse under optimal conditions (day/night temperature 32/25°C, with relative humidity oscillating between 50 and 80%, and vapor pressure deficit (VPD) range of 2.38 to 0.63kPa.

The response of plants to progressive exposure to water deficit was assessed at vegetative stage (25 days after sowing (DAS) when panicles had not emerged yet). Fourteen plants per genotype were grown under well-watered conditions until 25 DAS. Prior to imposing the water treatments, pots with plants were saturated with water and allowed to drain overnight. The following morning, pots were bagged into plastic bag and plants wrapped around the stem and pots were subsequently weighed. Pot weight was thereafter taken every day in the morning. Half of the plants were then maintained under well-watered (WW) conditions, by re-watering the pot to 80% field capacity (approximately 100 g below the saturated weight). The other half of the plants was gradually exposed to water stress (WS) by partially compensating water loss from transpiration, i.e. pots were allowed to lose no more than 70 g on each day. Therefore, any transpiration in excess of 70 g was added back to the pots, as previously described (Vadez and Sinclair, 2001). When the transpiration of stressed plants reached 10% of control plants' transpiration, the experiment was terminated. After harvest, the fraction of transpirable soil water (FTSW) was calculated for each day of the experiment. The FTSW values represented the portion of remaining soil water available for transpiration

during the course of the experiment and were used as our indicator of stress. FTSW of day *n* was calculated as:

(Pot weight of day *n* – Final pot weight)/(Initial pot weight – Final pot weight).

The estimation of biochemical characteristics was done when symptoms of drought on plants were obvious. The experimental conditions that are used here to assess possible differences in pigment contents and ROS scavenging mechanisms among pearl millet holding or not a terminal drought tolerance QTL were those successfully used previously to identify contrast in water-conserving mechanisms in these same materials (Kholová et al., 2010a, b). There, the fraction of transpirable soil water (FTSW), our index of stress and a measure of soil volumetric water content, was below 20% of the total transpirable soil water (FTSW = initial pot weight – final pot weight) and similar in all genotypes. Then, transpiration had dropped to approx. 50% of well-watered plants (when FTSW was close to 20%). At this stage the samples of leaf tissue for the study of photosynthetic pigments content (chlorophyll *a*, *b* and carotenoids) were collected and stored in a deep-freezer (-80^{o}C) until the analysis. The following day, when the relative transpiration had dropped to approximately 35% (and FTSW was close to 15%), another set of leaf samples was collected for an immediate assessment of anti-oxidative enzymes activities. Both these FTSW level for leaf sampling (20% and 15%) represented fairly severe water stress.

2.3. Anti-oxidative enzymes activities

For isozymes study of SOD, APX and CAT equal quantity of leaf tissue were sampled from each replicated plant. Then the seven replicated samples for each genotype and treatment were pooled together and one gram used for isozyme identification. Here, our intention was to provide a qualitative assessment of the different isozyme composition in each genotype, rather than an exhaustive quantitative measurement, and to assess the response to WS.

The leaf samples were taken from the middle portion of the top first fully developed leaves. Leaves samples were ground with liquid nitrogen and extracted in buffer containing 0.1 M Tris-HCl, dithiothreitol (DTT), 1 mM EDTA, 5 mM ascorbate, 4% polyvinypyrrolidone and Tritone-X. Extract was centrifuged at 14,000 g for 10 min and clear supernatant collected. Amount of proteins in each supernatant was estimated following Bradford (1976). Separation of isozymes by native-PAGE followed the technique of Laemmli (1970). APX and SOD were separated on 7.5% polyacrylamide gels and for CAT polyacrylamide gradient gels (6-13%) were used. Equal amounts of protein in extracts were loaded into each well. SOD activity staining followed the method of Beuchamp and Fridovich (1971), which is based on color change of nitroblue tetrazolium chloride (NBT) when reacting with superoxide radicals. Particular Cu/Zn-, Fe- and Mn-SOD isoforms were distinguished based on a previous report from pearl millet (Mukhopadhyay, 2006). APX isozymes were separated and stained following Mittlers and Zilinskas (1993). APX staining is also based on NBT reaction, but uses H_2O_2 as APX substrate. For CAT visualization the method of Woodbury (1971) was used, where isozyme patterns appears after incubation with H_2O_2 followed by $FeCl_3$ and $K_3Fe(CN)_6$. The gel images were first transformed into black and white format. Furthermore, bands light intensities were evaluated using picture analyzing program package (KODAK Molecular Imaging Software, Version 4.0). Absolute values of isozyme activities and activities expressed as relative values (absolute value of isozyme activity/total enzyme activity of particular genotype) were later used for correlation analysis with pigment characteristics.

2.4. Photosynthetic pigments content

Second fully developed leaves from the top were used for determination of Chl *a*, Chl *b* and total Car (including xanthophylls) content following Wellburn (1994). Three leaf discs from each plant (total leaf area 115.45 mm^2) were extracted in DMSO. There were seven replicated samples taken for each genotype and treatment combination.

2.5. Statistical analysis

The experimental design used was a completely randomized design with two water treatments (WW and WS) as main factors and genotypes as sub-factors, with seven replications. ANOVA analyses were done with the statistical program package CoStat version 6.204 (CoHort Software, Monterey, CA, USA). Two-way ANOVA was carried out to compare treatment differences across genotypes for photosynthetic pigments. One-way ANOVA was carried out to test genotypic differences within treatment and genotype means across treatments. The means were analyzed using Tukey-Kramer test. Correlation analysis was done to evaluate the relation of particular isoenzymes means with the means of photosynthetic pigments. To compare the mean proportion of total band intensities across genotypes between treatments (WW and WS), the genotype mean proportion value for each isozyme were used as replicates and resulting means (n=5) compared between treatments.

3. Results

3.1. ROS scavenging system

We identified 9 APX isozymes in all the genotypes and in both treatments (Picture 1, Table 1). Water stress didn't cause significant change in sum of APX band intensities. Only small changes of absolute APX activity were found under water stress, except in H 77/833-2 that showed about ten-unit increase in the sum of band intensities. The proportion of each isozyme did not significantly increase under water stress, except for APX isozyme 5. This one significantly increased from 14.5% under WW to 21.5% under WS. Moreover, the APX 5 isozyme activity expressed as a proportion to the total activity (proportional activity) had higher value under WS treatment than in WW treatment in all tolerant genotypes (parent PRLT 2/89-33 and all QTL-NILs ICMR 01029, ICMR 01031 and ICMR 02041), whereas proportional APX5 activity was not notably higher under WS than under WW in sensitive H 77/833-2. In the meanwhile, the mean proportional activity of APX1 and APX8 decreased significantly under WS. However the proportional activities values were very similar in all genotypes.

Drought stress didn't significantly increase total CAT activity across genotypes. However, CAT activity showed 17-units increase under drought stress in H 77/833-2 and 9-units increase in PRLT 2/89-33 (Picture 2, Table 2). We found that under stress conditions CAT 1 isozyme was induced in all the genotypes. However, the average activity of that new isoform was only 6.5 % of the total activity.

We could visualize 8 SOD isozymes; Mn-SOD 1-4 (band 1-4), Fe-SOD (band 5), Cu/Zn-SOD 1-3 (band 6-8). None of the different SOD isoenzymes changed significantly under WS (Picture 3, Table 3), neither the sum of band intensities, nor the mean proportional activities.

3.2. Photosynthetic pigments

Across all genotypes, the total chlorophyll content decreased significantly due to water stress. Nevertheless, when values in WW / WS conditions of particular genotypes were analyzed by one-way ANOVA, this decrease under WS was significant in the QTL-NILs only. Under WW conditions, the total chlorophyll content in tolerant parent genotype PRLT 2/89-33 was significantly lower than in the QTL-NILs, whereas no significant differences occurred between sensitive H 77/833-2 and QTL-NILs. Under WS conditions, there were no significant differences in total chlorophyll content between genotypes (Fig.1a). The decrease in total chlorophyll content in WS conditions was due to a significant reduction in both chlorophyll *a* (Chl *a*) (Fig.1b) and chlorophyll *b* (Chl *b*) (Fig.1c) in all genotypes tested. As for total chlorophyll content, Chl *a* in WW conditions was lower in PRLT 2/89-33 than in QTL-NILs, but values of H 77/833-2 did not differ from the QTL-NILs. Under WS, there were no significant Chl *a* differences between genotypes. The Chl *b* content did not differ between genotypes, either under WW or WS conditions (Fig.1b). A one-way ANOVA within genotypes showed a significant decline under WS in Chl *b* in genotype ICMR 01029 only.

Across all genotypes, the total carotenoids contents declined significantly under WS conditions. The one-way ANOVA within genotypes showed that this decrease was significant only in the QTL-NILs (ICMR 01029, ICMR 01031, ICMR 02041), but not in the parental genotypes (PRLT 2/89-33, H 77/833-2) (Fig.1d). No significant genotypic differences both in well-watered and water stress conditions were found.

The ratios of photosynthetic pigments (Chl *a*/Chl *b* and total Chl/Car) are tightly regulated in the plant photosynthesis apparatus and their proportional changes may affect photosynthesis. Across all genotypes, there was no significant difference in the Chl *a*/Chl *b* ratio between WW and WS conditions (Fig. 1e). Only one QTL-NIL (ICMR 01029) showed significant increase of Chl *a*/*b* ratio under stress conditions. The analysis across all genotypes showed that the total Chl/Car ratio increased significantly under drought-stressed conditions. The one-way ANOVA showed a significant increase of total Chl/Car under WS in all genotypes except sensitive H 77/833-2 (Fig.1f). No significant differences in Chl/Car were found between particular genotypes under either WW or WS conditions.

3.3. Correlation analysis

Under WW conditions, negative correlations were found between Chl/Car ratio and absolute values of APX5, APX6 and total APX activity. Under WS conditions, the Chl/Car ratio showed positive correlations with the APX2, APX4 and APX9 activity. Furthermore, carotenoids content was negatively associated with the proportional activity of APX8 (Table 4).

Under WW conditions, no correlation was found between CAT activities and particular photosynthetic pigment parameters. Under WS conditions, a positive correlation was found between Chl/Car ratio and both CAT isozymes (Table 4).

Under WW conditions, no correlation was found between SOD activities and any of the photosynthetic pigments. Under WS conditions, the Chl/Car ratio was significantly and positively related to the relative SOD activities of Mn-SOD2 and Mn-SOD3 (Table 4).

Interestingly, while most isozyme activities within the enzyme type were positively related to one another, several APX isoenzymes showed negative correlation (in WW: APX7 and APX6, APX2 and APX 9 whereas in WS: APX2 and APX3, APX3 and APX9, APX5 and APX 9; data not shown). Activities of Mn-SOD1 and Cu/Zn-SOD1 isozymes were negatively related to each other under both treatments (Fig. 2a, b).

4. Discussion

Except for the increase in APX 5 under water stress, there were no major differences in the isozyme activity of selected anti-oxidant enzymes under well-watered and water stressed conditions, despite the fact that the stress was severe when leaf samples were collected. The photosynthetic pigments generally decreased under water stress but neither this decrease, nor their absolute values could discriminate genotypes holding a terminal drought tolerance QTL from donor parent PRLT 2/89/33 and sensitive parent H77/833-2. We conclude that neither the anti-oxidative machinery, nor the photosynthetic pigment content appear to have a causal relation to the QTL introgression event in genotypes used for this study. However, the anti-oxidative machinery appeared to be closely linked to the balance between carotenoids and chlorophyll, proxied by the Chl/Car ratio.

4.1. Change in anti-oxidative isozymes' spectrum

Usually, APX increases with drought treatment in various plant species; e.g. maize, wheat, beans, rice, alfalfa (Pastori and Trippi, 1992; Kele and Oncel, 2002; Rubio et al., 2002; Sharma and Dubey, 2005; Naya et al., 2007; Torres-Franklin et al., 2008). APX is referred as an polygene family encoded enzyme with strong affinity to its substrate H_2O_2 (e.g. Caldwell et al., 1998) and it was suggested that even slight increase in APX activity may play crucial role in allowing ROS scavenging capacity (Mittler and Zilinskas, 1994). Furthermore, especially the cytosolic APX isoforms were suggested to play the role in co-coordinating the expression of photooxidative stress responsive genes including APX itself (Yoshimura et al., 2000; Suzuki and Mittler, 2006; Reddy et al., 2009). Here we found higher total APX5 activity under WS conditions compared to WW. These observations are similar to the study made on spinach where various abiotic stresses caused stimulation of only few APX isozymes activities from full APX isozymatic spectrum (Yoshimura et al., 2000). Furthermore, there was a notable difference in APX5 activity between sensitive and tolerant genotypes. Lower proportional APX5 activity under WS was found in H 77/833-2 (sensitive genotype) than in PRLT 2/89-33 and QTL-NILs. It is unlikely that the lower APX5 activity in H 77/833-2 could be explained by a delayed stimulation by water stress since this genotype also showed an earlier decline in

transpiration upon progressive exposure to water stress treatment compared to the tolerant genotypes (Kholová et al., 2010a). We interpret that this isozyme may simply not respond to the stress treatment in this genotype. In any case, APX5 isoenzymatic bands were more intense in drought tolerant compared to drought sensitive genotypes, therefore APX5 expression might be linked to the introgressed QTL genome portions involved in terminal drought tolerance. Differential roles of various isozymes are well documented (e.g. Foyer et al., 1994; Fadzilla et al., 1997; Yoshimura et al., 2000; Suzuki and Mittler, 2006), although we are not aware of any work emphasizing the importance of particular APX isozymes for better plants` adaptation to drought stress conditions.

Contrary to APX, CAT has low affinity to H_2O_2 which suggests its restricted role in counteracting the oxidative damage to cells (Smirnoff, 1993; Cruz de Carvalho, 2008). Even reports on CAT activity under drought are very heterogeneous. CAT was shown increased under drought treatment in e.g *Prunus*, tomato, sesame, alfalfa or wheat (Rubio et al., 2002; Luna et al., 2004; Sofo et al., 2005; Űnayayar, 2005; Naya et al., 2007; Fazeli et al., 2007), but decreased or unchanged in sunflower, pea and some grasses (Iturbe-Ormaetxe et al., 1998; Fu and Huang, 2001). In our piece, a new CAT isozyme was induced under drought conditions, but the total CAT activity did not increase significantly under drought stress. This was in part because the new CAT isoform accounted in average for only 6.5% of the total CAT activity. Similar induction of CAT isozyme was documented in rice exposed to severe drought stress (Srivalli et al., 2003). Furthermore, the proportional isozyme activities between genotypes were very similar under drought conditions. Therefore, our results suggest that based on CAT activity we could not discriminate genotypes on the basis of the absence or presence of a drought tolerance QTL.

Our data obtained on SOD isoenzymatic activities vary from the previous study made on pearl millet by Patil et al., (2005). They reported increased SOD activities during the late stages of drought imposition, although well after activities of APX and CAT had increased. Unfortunately, this field study did not document the soil water content that would permit a rigorous comparison with our findings. Our findings are, however, similar to studies on alfalfa, *Arabidopsis thaliana*, wheat, pea, *Ctenante setosa,* tomato and maize where no SOD activity increment was documented in leaves tissues under severe

water stress (Irigoyen et al., 1992, Iturbe-Ormaetxe et al., 1998; Bartoli et al., 1999; Borsani et al., 2001; Ünyayar et al., 2005; Terzi and Kadioglu, 2006, Bai et al., 2006). In any case, none of the SOD measurements could discriminate QTL-NILs lines from H77/833-2, suggesting that SOD activity and isoenzymatic composition are probably not causally related to the presence/absence of QTL in pearl millet genotypes included in the present study.

4.2. Contribution of photosynthetic pigments:

The analysis of photosynthetic pigments content generally agreed with most previous studies. Stress caused a significant decline in total chlorophyll and carotenoids content in the magnitude usually described as "non-lethal" (roughly 10-30%) under harsh drought stress in pearl millet (Ashraf et al., 2001) and other species (Terzi and Kadioglu, 2006; Kiani et al., 2008; Farooq et al., 2009). In certain species like rosemary and maize retaining of carotenoids level and so decrease of Chl/Car ratio due to drought conditions was reported (MunneÂ-Bosch and Alegre, 2000; Mohammadkhani and Heidari, 2007). In contrast to these results we found an increased Chl/Car ratio suggesting proportionally higher loss of carotenoids compared to chlorophylls and therefore the involvement of other strong ROS scavenging mechanisms additional to carotenoids. Similarly, increased Chl/Car ratio due to drought was previously found in *Betula papyrifera* and *Arabidopsis thaliana* (Richardson et al., 2004; Zhang et al., 2008). Although in pearl millet a significant increase in the Chl *a/b* ratio was previously reported (Ashraf et al., 2001), we found only an insignificant increment in Chl *a/b* ratio in all genotypes under stress treatment.

The major finding was that none of these changes could clearly discriminate QTL holding genotypes from H77/833-2. Usually, no significant differences were found between parental genotypes. In several cases QTL-NILs showed even higher trait values (Chl *a*, total Chl and Car) compared to both parental genotypes. Although a relationship between photosynthetic pigments stability and drought tolerance has been proposed in other species like peanut, wheat or maize (Pastori and Trippi, 1992; Arunyanark et al., 2008) our data suggest there is no evident relationship between the maintenance of

photosynthetic pigments or their ratios, or their changes under drought, with presence/absence of terminal drought tolerance QTL in the pearl millet genotypes tested.

4.3. Correlation analysis

We found that the two CAT, two SOD, and three APX isozymes correlated positively with the Chl/Car ratio under drought conditions, whereas two APX isozymes had negative associations with the Chl/Car ratio under well-watered conditions. This agrees with the hypotheses presented by Farrant et al., (2003) who described, that chlorophyll maintenance under drought should be balanced by ROS scavenging mechanisms. Indeed, we found that both total chlorophyll and carotenoids decreased under drought stress conditions. Furthermore, the increase in the ratio of chlorophyll content (potential source of ROS)/carotenoids (ROS scavengers) – indicated that the carotenoids content decreased relatively more than the chlorophyll content. Hypothetically, disrupted photosynthetic pigment ratios could lead to higher production of harmful ROS, and in such case, the ROS may exceed the scavenging capacity of carotenoids some of which act as direct scavengers of ROS produced via chlorophyll as described previously (e.g. Demmig-Adams and Adams, 1992; Richardson et al., 2004; Farooq et al., 2009). The significant positive correlations between the Chl/Car ratio and several isozymes of CAT and SOD under WS then suggest that these isozymes may play this additional ROS scavenging role to maintain the Chl content in stress conditions.

5. Conclusion

Although the APX5 isozyme activity increased under water stress and showed large qualitative differences between the sensitive H 77/833-2 and the group of genotypes holding a drought tolerance QTL, most anti-oxidant isozyme activities showed no change under water stress and band intensities were similar in all genotypes. Similar findings were obtained for the photosynthetic pigment concentration and changes under drought. This absence of relationship between the presence/absence of the QTL and a differential response in the ROS scavenging and the photosynthetic pigment was likely not related to

the experimental conditions, which were previously successfully used to discriminate genotypes for water-conserving mechanisms in a clear relation to the presence/absence of that QTL in the very same materials (Kholová et al., 2010 a, b). These results suggest that the anti-oxidant machinery or the response of photosynthetic pigments to water stress may not play a direct causal role on the terminal drought tolerance of pearl millet that is conferred by the QTL.

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Captions

Pics. 1 to 3. Native-PAGE displaying band intensities of ascorbic peroxidase, APX (Picture 1), catalase, CAT (Picture 2), and superoxid dismutase, SOD (Picture 3). The order of samples is: H77/833-2, PRLT-2/89-33, ICMR 01029, ICMR 01031, ICMR 02041 testcross hybrids in well-watered (WW) conditions (first 5 lanes) and H77/833-2, PRLT 2/89-33, ICMR 01029, ICMR 01031, ICMR 02041 testcross hybrids in water stress (WS) conditions (last 5 lanes). Each plant extract was a blend of seven leaves, each one sampled from each replicated plants of all genotype/treatment combination. Leaves were sampled during vegetative stage of development. In each well equal concentration of protein extract was loaded (150 µg/ well).

Figs. 1 a to f. Average of total chlorophyll (a), chlorophyll *a* (b), chlorophyll *b* (c) and carotenoids (d) content and ratio of chlorophyll *a*/*b* (e) and total chlorophyll/carotenoids (f) in two parental pearl millet testcross hybrids; H77/833-2 (sensitive), PRLT 2/89-33 (tolerant) and drought tolerant QTL-NILs (ICMR 01029, ICMR 01031, ICMR 02041) in well-watered (WW) and water stressed (WS) conditions during vegetative stage of development. Bars indicate SE (n=7). For each treatment (WW, WS), bars having same letter indicate no significant genotypic difference in that particular treatment.

Figs. 2 a and b. Negative relationship between absolute activities of Mn-SOD1 and Cu/Zn-SOD1 isoforms in control conditions (a) and under drought treatment (b). Absolute activities came from the assessment of staining intensities after incubation with respective substrate (see material and methods). For the correlations, we used the mean values of two parental pearl millet testcross genotypes (H77/833-2– sensitive, PRLT 2/89-33– tolerant) and their drought tolerant QTL-NILs (testcrosses of ICMR 01029, ICMR 01031, ICMR 02041) during vegetative stage of development.

Supplementary Table 1

Analysis of several ascorbic peroxidase (APX) isozymes intensities, as percentage of total intensity (%) and sum of band intensities, assessed from gels presented in Picture 1. The means within each water treatment (WW and WS) were calculated for each isozyme taking the genotypes as replication (*n*=5) and means followed by same letter indicate there is not any significant difference between treatments (Tukey-Kramer test).

Treat-ment	**Well watered (WW)**						**Water stress (WS)**						
Isozyme intensity (%)	**843A × H 77/833-2**	**843A× PRLT 2/89-33**	**843A× ICMR 01029**	**843A× ICMR 01031**	**843A× ICMR 02041**	**Mean (%)**	**843A× H 77/833-2**	**843A× PRLT 2/89-33**	**843A× ICMR 01029**	**843A× ICMR 01031**	**843A× ICMR 02041**	**Mean (%)**
APX 1	7.82	8.74	5.23	7.29	5.30	**6.88** ±0.70 **a**	6.72	5.25	3.88	4.58	2.55	**4.60** ±0.70 **b**
APX 2	4.89	5.94	4.02	3.01	4.45	**4.46** ±0.48 **a**	4.54	3.58	4.02	3.81	3.78	**3.95** ±0.16 **a**
APX 3	5.53	4.14	5.05	6.08	3.40	**4.84** ±0.48 **a**	3.17	5.91	5.01	5.31	4.46	**4.77** ±0.46 **a**
APX 4	5.68	7.11	9.25	6.66	6.32	**7.00** ±0.61 **a**	8.39	5.42	6.34	5.31	3.93	**5.99** ±0.83 **a**
APX 5	11.14	14.41	16.30	11.75	19.10	**14.54** ±1.47 **b**	13.26	23.50	24.42	23.12	23.18	**21.50** ±2.07 **a**
APX 6	15.50	15.48	17.52	16.84	16.53	**16.38** ±0.39 **a**	15.07	16.97	17.13	14.42	16.54	**16.02** ±0.54 **a**
APX 7	17.76	17.46	12.54	14.12	14.65	**15.30** ±1.00 **a**	18.31	13.88	14.08	16.57	17.66	**16.10** ±0.91 **a**
APX 8	28.12	24.60	26.06	29.35	26.96	**27.02** ±0.81 **a**	26.34	23.56	23.00	24.45	25.36	**24.54** ±0.60 **b**
APX 9	3.55	2.13	4.04	4.90	3.27	**3.58** ±0.45 **a**	3.68	1.92	2.13	2.44	2.54	**2.54** ±0.31 **a**
SUM of band intensities	***31.5***	***46.2***	***41.3***	***46.9***	***45.2***	***42.3*** *±2.86* ***a***	***41.8***	***52.5***	***45.9***	***40.6***	***39.8***	***44.1*** *±2.35* ***a***

Supplementary Table 2

Analysis of several catalase (CAT) isozymes intensities, as percentage of total intensity (%) and sum of band intensities, assessed from gels presented in Picture 2. The means within each water treatment (WW and WS) were calculated for each isozyme taking the genotypes as replication (*n*=5) and means followed by same letter indicate there is not any significant difference between treatments (Tukey-Kramer test).

Treatment	**Control**						**Drought**					
Isozyme intensity (%)	**843A× H 77/833-2**	**843A× PRLT 2/89-33**	**843A× ICMR 01029**	**843A× ICMR 01031**	**843A× ICMR 02041**	**Mean (%)**	**843A× H 77/833-2**	**843A× PRLT 2/89-33**	**843A× ICMR 01029**	**843A× ICMR 01031**	**843A× ICMR 02041**	**Mean (%)**
CAT 1	negligible	negligible	negligible	negligible	negligible	**negligible**	8.52	7.06	6.12	5.86	4.72	**6.46** ±0.63
CAT 2	100	100	100	100	100	**100**	91.48	92.94	93.88	94.14	95.28	**93.5**±0.63
SUM of band intensities	**38.3**	**36.5**	**47.9**	**50.35**	**52.7**	**45.1** ±3.30**a**	**55.1**	**44.7**	**49.8**	**48.7**	**42.3**	**48.1** ±3.30**a**

Supplementary Table 3

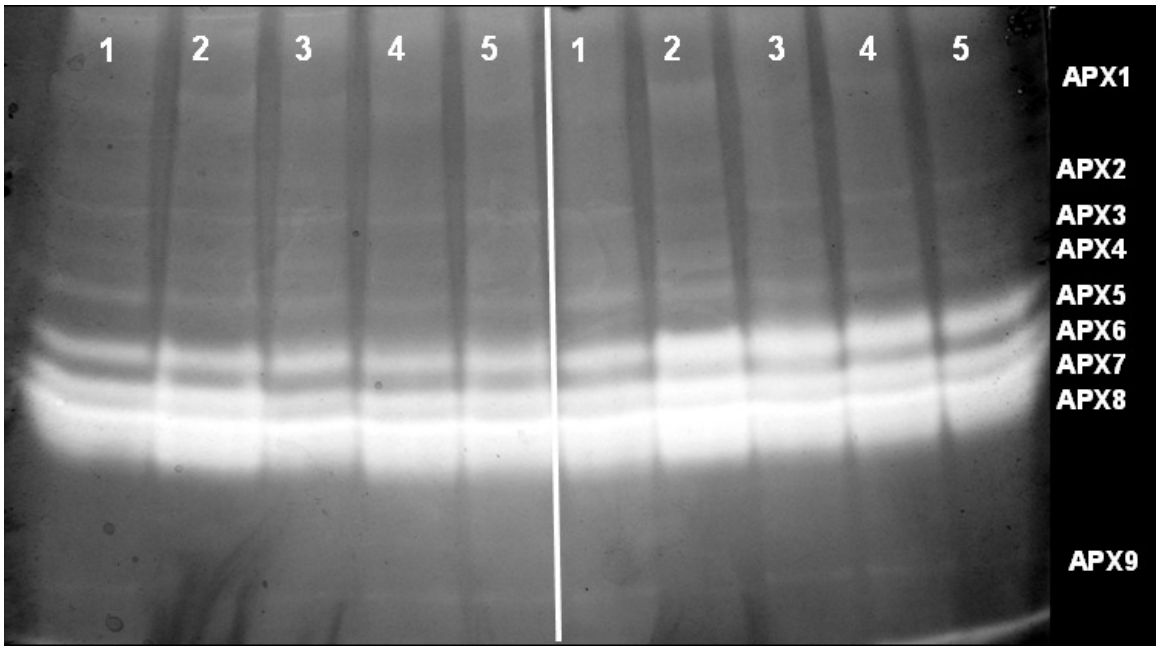
Analysis of super oxide dismutase (SOD) isozymes intensities, as percentage of total intensity (%) and sum of band intensities, assessed from gels presented in Picture 3. The means within each water treatment (WW and WS) were calculated for each isozyme taking the genotypes as replication (*n*=5) and means followed by same letter indicate there is not any significant difference between treatments (Tukey-Kramer test).

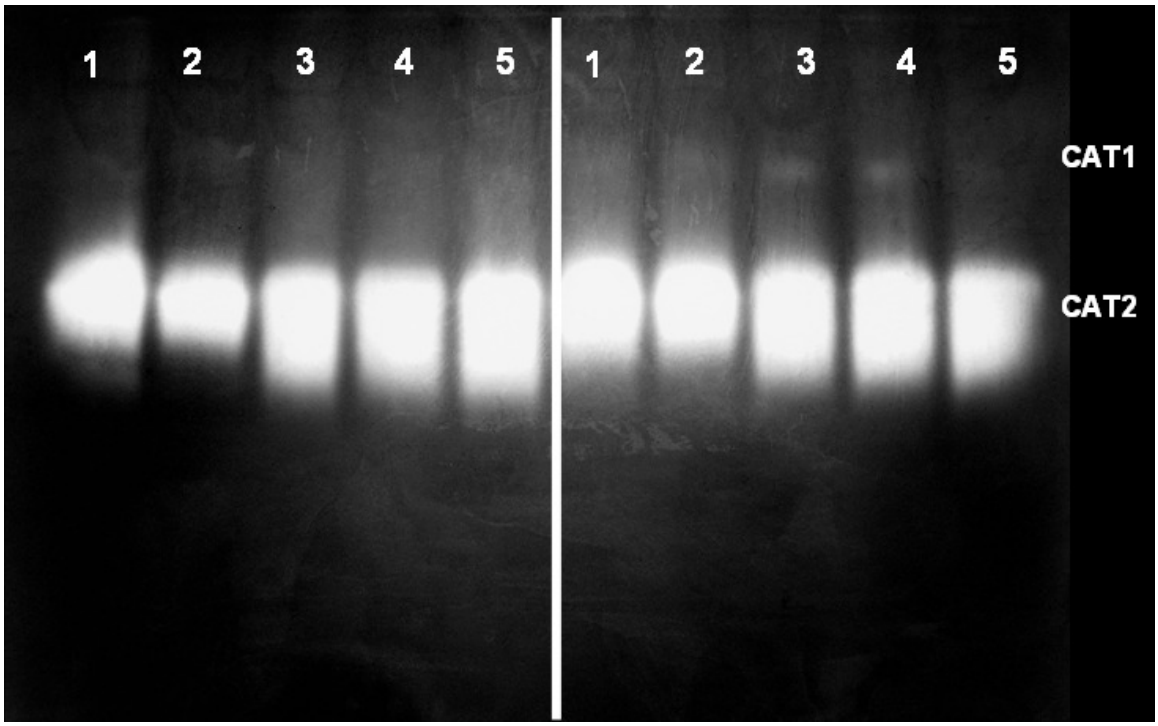
treatment	**Control**						**Drought**					
Isozyme intensity (%)	**843A× H 77/833-2**	**843A× PRLT 2/89-33**	**843A× ICMR 01029**	**843A× ICMR 01031**	**843A× ICMR 02041**	**Mean (%)**	**843A× H 77/833-2**	**843A× PRLT 2/89-33**	**843A× ICMR 01029**	**843A× ICMR 01031**	**843A× ICMR 02041**	**Mean (%)**
Mn-SOD1	20.96	23.22	17.98	21.14	17.40	**20.14** ±1.08**a**	19.83	19.94	18.79	22.02	19.18	**19.95** ±0.56**a**
Mn-SOD2	11.24	9.72	11.15	11.65	12.15	**11.18** ±0.41**a**	11.92	9.78	10.70	10.28	9.33	**10.40**± 0.44**a**
Mn-SOD3	11.57	9.91	11.11	11.83	12.54	**11.39** ±0.43**a**	11.15	10.19	10.54	9.97	9.43	**10.26** ±0.29**a**
Mn-SOD4	17.22	18.09	15.65	17.30	16.11	**16.88** ±0.44**a**	16.79	17.49	15.22	16.77	16.00	**16.46** ±0.39**a**
Fe-SOD	2.04	3.83	6.39	1.23	5.18	**3.73** ±0.96**a**	1.51	5.72	1.51	1.19	4.06	**2.80** ±0.90**a**
Cu/Zn-SOD1	4.10	3.21	7.15	4.34	7.52	**5.26** ±0.87**a**	6.18	5.71	6.45	4.15	6.73	**5.84** ±0.46**a**
Cu/Zn-SOD2	20.49	10.61	17.96	18.58	16.46	**18.82** ±0.79**a**	18.96	19.11	21.33	21.10	22.01	**20.50** ±0.62**a**
Cu/Zn-SOD3	12.38	11.40	12.58	13.93	12.65	**12.59** ±0.40**a**	13.67	12.06	15.47	14.53	13.27	**13.80** ±0.58**a**
SUM of band intensities	**723.8**	**662.5**	**972.2**	**726.1**	**1054.8**	**827.9** ±77.73**a**	**759.3**	**893.2**	**759.1**	**687.0**	**785.5**	**784.0** ±33.23**a**

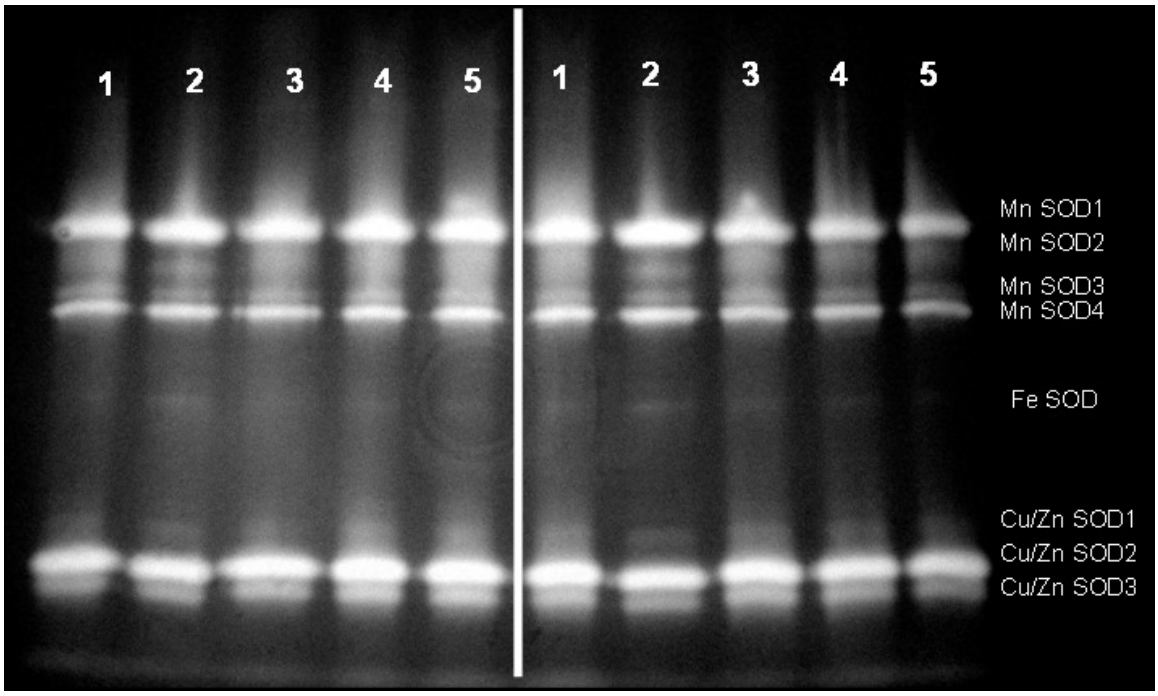
Supplementary Table 4

Correlation analysis between enzymatic activities and pigments contents and ratios, using the means of absolute values of band intensities (normal font) and of proportional values (absolute value of band intensity/total band intensity of particular genotype; bold font) of enzymatic activities and means of Chl *a*, Chl *b*, Car and their ratios of two parental testcross genotypes (H77/833-2– sensitive, PRLT 2/89-33– tolerant) and their drought tolerant QTL-NILs (testcrosses of ICMR 01029, ICMR 01031, ICMR 02041) during vegetative stage of development. The significance of relation is shown as * for $p<0.05$ and ** for $p<0.01$.

			Water stress		
	Chl a	***Chl b***	***Car***	***Chl a/Chl b***	***Chl/Car***
APX					
APX2	*ns*	*ns*	*ns*	*ns*	**0.9344***
APX4	*ns*	*ns*	*ns*	*ns*	0.9037*/**0.9881****
APX8	*ns*	*ns*	**-0.9104***	*ns*	*ns*
APX9	*ns*	*ns*	*ns*	*ns*	0.8929*
SOD					
Mn-SOD2	*ns*	*ns*	*ns*	*ns*	**0.969****
Mn-SOD3	*ns*	*ns*	*ns*	*ns*	**0.9516***
CAT					
CAT1	*ns*	*ns*	*ns*	*ns*	0.9439*
CAT2	*ns*	*ns*	*ns*	*ns*	0.8911*
			Well-watered		
	Chl a	***Chl b***	***Car***	***Chl a/Chl b***	***Chl/Car***
APX					
APX5	*ns*	*ns*	*ns*	*ns*	-0.8821*
APX6	*ns*	*ns*	*ns*	*ns*	-0.8972*
APX sum	*ns*	*ns*	*ns*	*ns*	-0.9417*


1
2
3
4
5
1
2
3
4
5
APX1
APX2
APX3
APX4
APX5
APX6
APX7
APX8
APX9


1
2
3
4
5
1
2
3
4
5
CAT1
CAT2


1
2
3
4
5
1
2
3
4
5
Mn SOD1
Mn SOD2
Mn SOD3
Mn SOD4
Fe SOD
Cu/Zn SOD1
Cu/Zn SOD2
Cu/Zn SOD3