
Expression of Boiling-stable Peroxidase (PRX) isoenzymes under combined effect of drought and heat in different tissues of *Triticum aestivum*

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In dry or semi-dry areas, plants encounter a combination of environmental stress that include drought or heat shock. Although drought and heat shock have been extensively studied, their combination affect on plants is still a matter of conjuncture. Antioxidant enzymes (like SOD, CAT and POD) are widely found in plants having intracellular and extracellular antioxidant activities. These enzymes are known to maintain oxidative stress induced-ROS at sub-lethal levels in plants under abiotic stress conditions but the role of boiling stable antioxidant enzymes in adaptation to combined stresses has not been critically evaluated. In this study, the combined effect of drought stress and heat shock on the induction of boiling stable proteins in general and boiling stable acid antioxidant enzymes (PRX,SOD,CAT) in particular were studied in 3-day old tissues (shoot, seed and root) of wheat. Imposition of combined stresses resulted in sharp decrease in tissue water content in tissues. The profile of boiling stable protein was outlined via SDS electrophoresis of tissue extracts. Many boiling stable protein bands were detected under different stress treatments in seeds samples only. Additionally, many differential boiling stable protein bands were detected in the shoot samples only under simultaneously imposed drought/heat stresses (DH). Zymography (in-gel activity) analysis revealed that among various antioxidant enzymes like SOD and CAT, only PRX was detected as a boiling stable protein. The in-gel activity analysis revealed sharp induction of BsPRX isoforms (BsPRX 1,2,3) during combined drought and heat stress (DH) conditions in seeds and shoots as compared to separately applied heat (H) and drought treatments (D). This indicates their role in water stress adaptation under simultaneous applied abiotic stress conditions. Alteration in boiling stable protein expression was more pronounced in seeds as compared to shoots of both the cultivars. Based upon these observations, the possible role of boiling stable proteins (hydrophilins) in combined stress tolerance is discussed.

Key words: Boling stable proteins, combined drought/heat stress, Peroxidase, wheat

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Introduction

Being sessile in nature, plants are exposed to adverse environmental conditions such as drought, heat, cold and flooding that dramatically affect plant survival and limit productivity (Chaves *et al.*, 2003). Most cultivated crop plants are highly sensitive and either die or display reduced productivity after they are exposed to long periods of abiotic stresses. Among all stresses, heat and drought are the major factors which affect plant development (Santos *et al.*, 2009). Extensive study has been made on each stress during last decade (Ahuja *et al.*, 2010), but little is known about their combined impact (Rizhsky *et al.*, 2002; Mittler, 2006). It has been demonstrated that under field conditions, plants are often simultaneously exposed to number of different stress simultaneously such as high irradiance, drought, salt, heat and low temperature. Among all, combined heat and drought type of conditions are very common in arid and semi-arid regions across the world which affects plant productivity dramatically (Sumesh *et al.*, 2008). These two stress factors could create water deficiency in plant tissues, which in turn may affect the synthesis of stress-induced proteins. Analyzing the effect of single stress on plants can be very different from conditions encountered by plants in the field in which a number of different stresses may occur simultaneously. These can alter plant metabolism in a novel manner that may be different from that caused by each of the different stresses applied individually (Mittler, 2006). It may require a new type of response that would not have been induced by each of the individual stresses. Some earlier studies have shown that plant response to combined drought and heat stress differed from the individual stress studies (Mittler, 2006; Rizhsky *et al.*, 2002). It was also observed that combination of drought and heat provoked cessation of conventional protein synthesis accompanied by dramatic enhancement in heat shock proteins and other stress related proteins (Lin *et al.*, 2008; Caeiro *et al.*, 2008, Mittler, 2006). Therefore, response of plants to individual stresses can be very different from conditions faced by plants in fields where different stresses can occur simultaneously.

Extensive studies on oxidative stress-induced damage have indicated that adverse abiotic stress conditions like high temp, drought, heat, cold aggravated the production of ROS (Reactive Oxygen Species) due to altered cellular metabolism accompanied by cellular redox equilibrium (Gao *et al.*, 2008). However, under optimal conditions ROS are inevitable byproducts from the essential aerobic metabolisms including chloroplastic, mitochondrial and plasma membrane linked electron transport systems (Bi *et al.*, 2009) and they need to be maintained under sub-lethal levels for normal plant growth. It was demonstrated that under water stress conditions photosynthesis is reduced either through stomatal closure or metabolic impairment (Reddy *et al.*, 2011).

All these changes in mitochondrial respiration and the photosynthetic electron transport lead to the generation of highly toxic ROS. ROS mainly includes superoxide, hydrogen peroxide, and hydroxyl radicals (Apel and Hirt, 2004). ROS are highly reactive and in the absence of any protective mechanism can cause serious oxidative damage to DNA, lipids, enzymes, chloroplast pigments and proteins leading to subsequent cell death (Mittler, 2002; Baruah *et al.*, 2009). To combat oxidative damage initiated by ROS, plants have evolved antioxidant defense mechanisms comprising of enzymes SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), POD (EC 1.11.1.7) which work in concert to detoxify ROS as well as non-enzymatic molecules like ascorbate, glutathione, carotenoids, and anthocyanins which remove, neutralize and scavenge the ROS inside plant systems in order to protect the key enzymes from ROS (Mittler, 2006, Diao *et al.*, 2011). Earlier studies have indicated that additional compounds such as osmolytes, proteins (e.g. peroxiredoxin) and amphiphilic molecules (e.g. tocopherol) can also function as ROS scavengers (Bowler *et al.*, 1992; Noctor and Foyer, 1998; Horling *et al.*, 2003). Among different antioxidant enzymes, SODs represent a group of multimeric metalloenzymes that catalyzes the disproportion of superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) and dioxygen (O_2) in different cellular compartments. Thus, SOD is the first defense against the damage that is caused by ROS. CATs and PODs are important scavengers of H_2O_2 in plant cells (Willekens *et al.*, 1997). CATs are tetrameric homoproteins that exist as multiple isozymes and are located mostly in peroxisomes and glyoxisomes (Kumari *et al.*, 2006). CATs oxidize H_2O_2 to generate H_2O and O_2 . PODs are grouped in a superfamily utilizing guaiacol as electron donor, located in cytosol, cell wall and involved in decomposition of H_2O_2 through the oxidation of phenolic compounds. Further supporting evidence on the involvement of antioxidant enzymes was documented by transgenic plants with enhanced or reduced activities of antioxidant enzymes (Yan *et al.*, 2003; McKersie *et al.*, 2001; Matsumura *et al.*, 2002). Earlier it was demonstrated that among many stress induced proteins, some ABA-inducible (responsive to ABA, or RAB proteins) and water-stress inducible- proteins (e.g. dehydrins, HSPs, LEAs) are highly hydrophilic and remain soluble even after boiling, a characteristic that has been termed as “boiling stable proteins” (BSPs) (Jacobsen and Shaw, 1989). Under drought stress, even some of the proteins detected in total protein extracts are lost in boiling treated extracts (Pelah *et al.*, 1995). Earlier research also indicated that hydrophilins represent less than 0.2% of the total protein of a given genome, however, it represents the most significant part of proteome in regulating tolerance to abiotic stresses (Battaglia *et al.*, 2008). Hence, to better elucidate the role of these boiling stable proteins (BSPs) in water-limited conditions, it is

a prerequisite to examine their expression not only under water stress, but also after boiling the extracts. Although extensive studies have been made on antioxidant enzymes under various abiotic stresses the role of boiling stable antioxidant enzymes (hydrophilins) under combined drought and heat stress is not well documented. Therefore, in the continuation of our previous studies (Sharma *et al.*, 2012 a,b), the present study, we analyzed boiling stable antioxidant isoenzymes expression analysis and (BSP) profiles in wheat tissues under combined drought and heat stress conditions. A combination of drought and heat shock can represent the conditions encountered by many plant and crops growing in arid and semiarid environments (Mittler, 2006) therefore, its understanding may be critical for the development of new strategies and tools to enhance stress tolerance via genetic manipulations. Wheat is one of the most important crops in arid and semi arid areas worldwide and is sensitive to drought and temperature stress. In view of this, wheat was chosen as an important tropical crop for the present investigation. To facilitate the detection of BSPs, the focus was on heat stable (HS) fractions that resist coagulation upon heating at 100°C. By this method, the soluble protein extract containing hydrophilic proteins could enrich the BSPs and devoid storage proteins.

Materials and methods

Seed germination and growth conditions

Seeds (*Triticum aestivum* cv. PBW. 550) were thoroughly rinsed with deionized water and allowed to imbibe water for 6 hours. After imbibition, the seeds were placed in Petri plates containing sterile filter sheets, moistened with water. The plates were incubated at 25 ±1°C in a seed germinator in darkness and allowed to grow for 3 days (Sharma *et al.*, 2012 a,b). Stress treatments were performed on 3 M Whatman filter paper. For combined drought stress and heat shock treatment, 3-day old seedlings were exposed to 3-day drought stress followed by 2-h heat shock (42°C). Individual drought stress was imposed to 3-day old seedlings for 3 days. Heat treatment was imposed to 6-day old seedlings for 2-h at 42°C. Control plants were irrigated with deionized water for 6 days. The tissues (seeds/shoots/roots) from all treatments were harvested and pooled for further analysis. Tissue water content (TWC) was measured after imposing stress treatments. Immediately the tissues were sealed in a plastic bag and quickly transferred to the laboratory. Fresh weights were determined within 2 hours after collection. Dry weights were obtained after oven drying the samples for 72 h at 70°C. TWC was calculated from the given equation: $TWC(\%) = (\text{fresh weight} - \text{dry weight} / \text{fresh weight}) \times 100$.

Extraction of Boiling Stable Proteins

The boiling stable proteins were extracted as described previously (Sharma *et al.*, 2012 a.b). Briefly, tissues were homogenized with chilled mortar and pestle in extraction buffer [50 mM Tris buffer (pH 7.0)]. Crude extracts were centrifuged at 10,000 g for 10 min. The total extract obtained was boiled for 15 min in order to get boiling stable protein fractions. The total soluble protein content in the supernatant was determined according to Bradford (1976) using BSA as a standard. Protein samples were resolved in 12% (w/v) polyacrylamide gel (SDS-PAGE) and visualized by Coomassie brilliant blue as described in Sambrook *et al.* (1989).

Zymogram analysis (in-gel activity analysis)

Boiling stable proteins were extracted as described above. For in-gel activity analysis, the proteins were separated by a non-denaturing 12% polyacrylamide gel electrophoresis as described by Sambrook *et al.* (1989).

When electrophoresis was complete, the gel was washed three times in 50 mM sodium acetate buffer (pH 5.0). Peroxidase activity was visualized by incubating the gel in 50 ml solution having 50 mM sodium acetate buffer (pH 5.0), 330 μ l of guaiacol (9M) and 1.5 ml of 6.6 % H₂O₂. The gel was incubated at room temperature in dark until reddish-brown bands appeared. The gel was washed in distilled water and used for further analysis. In order to detect SOD activity, the gel was first soaked in 25 ml of 1.23 mM NBT for 15 min, briefly washed, then soaked in the dark in 30 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 28 mM TEMED and 2.8 x 10⁻² mM riboflavin for another 15 min. The gel was briefly washed again and then illuminated on gel viewer under white light to initiate photochemical reaction. All the procedures were carried out at room temperature. For catalase activity, after electrophoresis the gel was incubated in 50 mM potassium phosphate buffer (pH 7.0) for 15 min and then in 0.03% H₂O₂ solution for 10 min. The gel was rinsed twice with water and then incubated in a mixture of freshly prepared 2% potassium ferricyanide and 2% ferric chloride (1:1). The gel became greenish-blue while zones of catalase were yellow.

Statistical analysis

Statview ANOVA program was used for statistical analysis of the data. Values for different treatments within each tissue were compared using one-way analysis of variance with repeated measures and student's *t*-test for

differences between pairs of data if the ANOVA ($LSD_{0.05}$) revealed significance. Means were tested by LSD at P 0.05 level ($LSD_{0.05}$).

Results and discussions

Effect of combined DH treatment on TWC

Individual drought stress and heat shock may affect plant metabolism in a different manner, however, it is not entirely clear how they affect plant metabolism when occurring simultaneously. Inasmuch in the field or in nature plants are often exposed to a combination of stresses such as drought and heat, studying the response of plants to a combination of different stresses may be critical to our understanding of stress tolerance in plants. Thus stress combinations such as drought and cold, heat shock and light, or drought and heat shock should be studied before a successful manipulation of plant metabolism can be achieved to artificially enhance stress tolerance. Earlier, Mittler (2006) claimed that simultaneous exposure to different stresses would result in co-activation of various stress response pathways with synergetic or antagonistic effects and that their combination should be regarded as new state of abiotic stress in plants. Hence, in the present study effect of individual drought (D), heat (H) and combined drought/heat (DH) treatments on boiling stable antioxidant enzymes and boiling stable protein profile were studied in the different tissues of wheat seedlings. In order to mimic the conditions encountered by the plants during the period of drought stress, accompanied by a brief exposure to heat shock which often occurs from midday to late afternoon, the plants were subjected to drought stressed and heat shock treatment (42°C) for 2 h. Well watered plants served as “control”, drought stressed plants that were not subjected to heat shock as “drought stress” and well watered plants that were subjected to heat shock as “heat shock”. Upon imposition of D and DH treatments, a significant decrease in TWC of seeds was observed (Fig. 1). Taken together, it is likely that TWC is controlled in a tissue specific manner. This comparative study on drought and heat stress (applied separately and in combination) confirmed their effect on TWC and protein expression in consonance with earlier studies (Jiang *et al.*, 2002; Mittler, 2006). However, imposition of DH treatment did not alter any change in the TWC of shoot and roots (Fig 1). Similar observation was also recorded for heat stressed tissues. Taken together, it can be speculated that this kind of response may be a sort of protection given by osmolytes under water stress. As indicated earlier Pinheiro *et al.* (2004) also observed no change in tissue water content upon drought stress. Same authors demonstrated that this response is associated with

protection given by assimilates mainly fructose, glucose and sucrose whose levels doubles under water deficits.

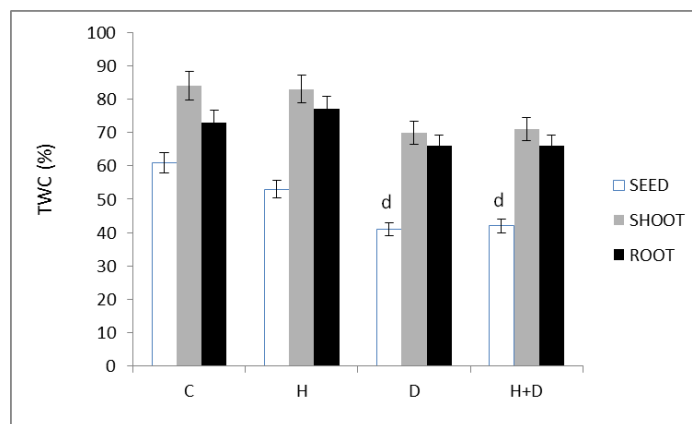


Fig. 1. Tissue Water Content (TWC, %) of wheat tissues (seeds, shoots and roots) under different stress treatments. Symbol used: C-Control; H-heat shock; D-drought stress; DH-combined drought/heat stress. Data shown are average \pm SE of three replicates. ^dindicates significant difference at $P \leq 0.05$.

Combined DH-induced changes in BSPs

The BSP expression under combined drought and heat stresses was also examined on a 12% SDS-PAGE and analyzed (Fig. 2 A and B). In all the tissues (seed, shoot and root) examined, as compared to un-boiled samples, many low and high molecular weight proteins disappeared upon boiling under H, D and DH conditions (Fig. 2 A and B), indicating boiling stable (hydrophilic) nature of existing peptides after boiling treatment. From the above observations, it was conceivable that as a stress response, protein metabolism of the cells undergoes changes in terms of acquiring specific stress proteins which are either sometimes not detected or present in low amounts. As compared to other tissues like shoot and roots, maximum protein bands in the un-boiled as well boiled protein samples were detected in the seeds, indicating tissue specific induction of proteins. Such observation is quite expected, since it is well known that an intense proteins synthesis take place in the reproductive plant structures (Duck *et al.*, 1989). In seeds, several groups have proposed that proteins are critical for protection of cellular components during seed development. Further, the results strongly suggest that the effect of this combination (DH) on plants is very different from that of drought and heat shock applied individually. As compared to controls, DH treatment elicited some sharp and barely detectable minor boiling-stable protein bands (20-66 kDa) on SDS-PAGE. Curiously enough, as compare to controls, imposition of

DH treatment elicited many differential boiling stable protein bands in shoots. The absence of these differential bands in C, D and H conditions indicates that the regulation of these proteins differ from that of number of other constitutive, heat/water stressed-induced proteins. Further studies may be required to characterize and annotate biochemical roles to these DH-induced differential boiling stable protein bands.

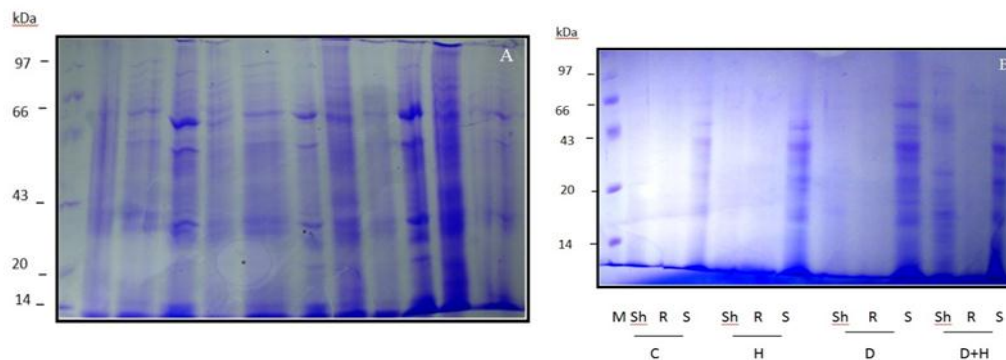


Fig. 2. An SDS-PAGE profile of un-boiled (A) and boiled (B) proteins samples of different tissues of wheat under different stress treatments. Symbol used: M-marker, S-seed; Sh-shoot; R: root. C-Control; H-heat shock; D-drought stress; DH-combined drought/heat stress. Arrow indicates differential bands.

Effect of combined DH treatment on PRX isoenzymes

Normally, abiotic stresses like heat and drought affect the physiology and biochemistry of plant cells under *in vitro* and *in vivo* conditions. These stresses have also been reported to enhance expression of antioxidant enzymes like SOD, PRX and CAT (Mittler, 2002). However, the exact role of boiling stable antioxidant enzymes under combined drought and heat stresses is still not well documented. Interestingly, in this study except PRX, no boiling stable in-gel activities of SOD and CAT were detected (data not shown) under combined DH and individual D and H treatments, implying specific regulation of hydrophilic antioxidant enzymes in a stress type dependent manner. Peroxidases (PRXs) are important enzymes of the antioxidative system for the reduction of H_2O_2 to water. As an adaptive enzyme in antioxidant system, PRXs play an important role in protecting membrane lipids from peroxidation and in reducing the cell damage being caused by oxidative stress in plants. Besides H_2O_2 detoxification, several other roles e.g in the modification of cell wall, cross-linking of structural non-enzymatic proteins like suberin polymerization and ability to cleave cell wall polysaccharides, leaf expansion, fruit growth, seed germination

and nodulation, have been attributed to plant PRXs in response to biotic and abiotic stresses (Cosio and Dunand, 2010). Even though there is apparent functional redundancy, the cellular localization and functions of most of the isoenzymes coded by different PRX genes remain incompletely understood. They may have diverse roles possibly due to large number of isoforms (isoenzymes). As indicated in Fig 3, when applied alone, H treatment did not provoke any induction in BsPRX in-gel activity (represented as BsPRX and hereafter) in any tissue as compared to respective control. When applied alone, D treatment aggravated a sharp increase in the expression of BsPRX1 isoform in a tissue dependent manner (Fig. 3). However curiously enough, a drastic many fold increase in seed BsPRX1 isoform expression was observed in seeds and shoots of plants subjected to combined DH treatment as compared to the relevant controls. In addition, as compared to individual C, D, and H treatments, two minor differential boiling stable PRX isoforms (BsPRX2 and BsPRX3) were observed only in shoots under combined DH treatment. The utilization of multiple isoforms in shoots may be one of the primary control mechanisms in plants to detoxify ROS. However, little data is available on regulation of boiling stable PRX isoform expression under combined DH stress. Hence, more studies on isoform-specific responses of antioxidant enzymes deserve to have considerable attention under combined stresses. Taken together accumulative evidences have shown that different regulatory pathways are involved in controlling expression of BsPRX isoforms under combined DH treatments. All together, no BsPRX in-gel activity was observed in roots under all C, H, D and combined DH treatments (Fig. 3) implying spatial regulation of BsPRX isoforms. Further, it can be argued that, as compared to other enzymes like SOD and CAT, PRX is the major enzyme involved in ROS detoxification in wheat tissues under combined DH stresses, thus averting cellular damage under unfavorable conditions. This notion was further supported by the observation that in *Arabidopsis thaliana*, the overexpression of its POD (AtPox) or the tobacco POD (NtPox) increases the stress resistance of the transgenic plants against some abiotic stresses (Ezaki *et al.*, 2000).

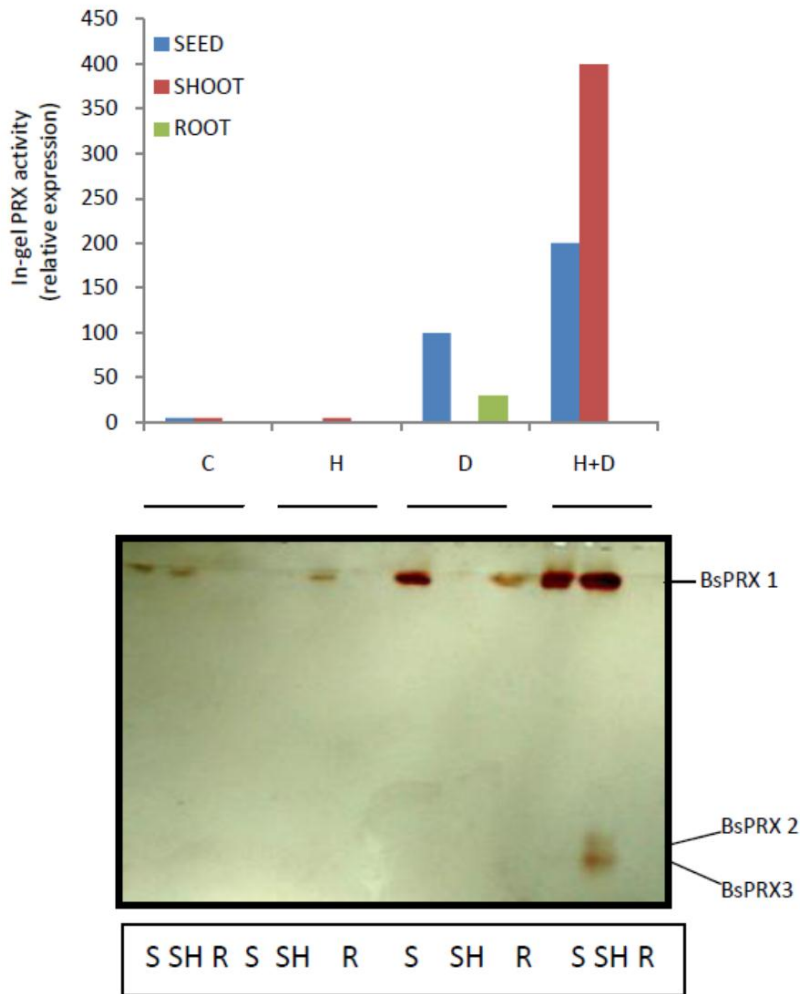


Fig. 3. In-gel activity analysis of BsPRX isoenzymes in different tissues of wheat under different stress treatments. Symbol used: M-marker, S-seed; Sh-shoot; R: root. C-Control; H-heat shock; D-drought stress; DH-combined drought/heat stress. Arrow indicates differential bands.

To conclude, the high expression of BSPs along with BsPRX in-gel activities confirmed its role for survival under combined stress conditions. They are hydrophilic and remain soluble upon boiling like LEA-type proteins, representing a new class of plant proteins involved in the plant's responses to abiotic stress. These hydrophilins may be necessary to maintain protein function during this specific type of abiotic stresses. Based on the present study findings, it can be postulated out that the interactions of PRX, CAT and SOD

with their involvement in scavenging ROS is very complex. The extent of oxidative stress causing membrane and cellular damage might possibly differ depending on the natural abiotic conditions imposed. Plants may respond in different ways, as shown in this experiment (Fig. 3). Detailed studies with more drought tolerant and susceptible cultivars will reveal the potential of this protein as a marker for drought tolerance. The results may provide entry point and a reference to future analysis of gene expression during combination of stresses. In addition, the results can suggest possible targets for the enhancement of stress tolerance in crops by genetic engineering. Therefore, a simple extrapolation of the data obtained after the imposition of one of the stress (heat or drought) separately will not produce a reliable basis to predict the effects of their combination. Such observation was also expressed by Mittler (2006) who also demonstrated that simultaneous imposition of different stress would result in co-activation of various stress response pathways. Although several environmental stresses in plants are known to induce the expression and/or increasing levels of antioxidative enzymes and their mRNAs, the mechanism by which these boiling stable activated oxygen-scavenging enzymes can work co-operatively in response to abiotic conditions is not yet known. Further analysis of the regulation of gene expression of these enzymes should elucidate the mechanism of different temperature tolerances.

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