

Transcript profiling and biochemical characterization of mitochondrial superoxide dismutase (mtSOD) in wheat (*Triticum aestivum*) under different exogenous stresses**Ranjeet R. Kumar^{1*}, Sushil K. Sharma¹, Suneha Goswami¹, Khushboo Singh¹, Kritika A. Gadpayle¹, Gyanendra P. Singh², Himanshu Pathak³, Raj D. Rai¹**¹Division of Biochemistry, Indian Agricultural Research Institute, New Delhi, India-110012²Division of Genetics, Indian Agricultural Research Institute, New Delhi, India-110012³Division of Environmental Science, Indian Agricultural Research Institute, New Delhi, India-110012

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Abstract

Amongst various antioxidant enzymes, superoxide dismutase (SOD) has been found to play most important role in the tolerance mechanism of the plants to the abiotic stresses. In this study, full length cDNA of 733 bp encoding superoxide dismutase, designated as *TaSOD*, was isolated and characterized from wheat (*Triticum aestivum*). A manganese iron-binding domain was observed in the sequence using CD search tool of NCBI. Mapping of wheat genome by southern blotting showed the presence of three copies of *TaSOD* gene. Quantitative transcript profiling of superoxide dismutase showed 1.3, 1.8, 1.9 fold increase in thermotolerant genotype (HDR77), 1.1, 1.3, 1.5 fold increase in thermosusceptible (PBW343) at pollination, milky dough and seed hardening stages respectively in leaves because of diurnal changes in environmental temperature. Abundance of *TaSOD* transcript and high SOD activity, total antioxidant capacity and cell membrane stability index were observed in HDR77 in response to the exogenous application of H₂O₂ (10 mmole L⁻¹) compared to that of salicylic acid (5 mmole L⁻¹) under differential heat shock. Isoenzymes analysis of SOD showed expression of three prominent isoenzymes in HDR77 and only two in PBW343 under the heat stress, with maximum activity at pollination and seed-hardening stages in both the cultivars. Thermotolerance capacity was analyzed using cell membrane stability and total antioxidant capacity; both the parameters were high in thermotolerant (HDR77) where abundance of SOD transcript was also observed as compared to thermosusceptible (PBW343). Superoxide dismutase regulates the expression of various heat stress associated genes through balancing the accumulation of H₂O₂ inside cells, which also act as signaling molecules. However, there is a greater need to further characterize SODs and over-express in the desirable crop-cultivars with a view to enhance the tolerance capacity against various abiotic stresses.

Keywords: Abiotic stress; Heat stress; Isoenzymes; Native PAGE; Protein profiling; Superoxide dismutase; *Triticum aestivum*; Wheat.**Abbreviations:** AOC-Antioxidant capacity; AOS-Active oxygen species; HS-Heat stress; HSP-Heat shock protein; qRT-PCR-Quantitative real time PCR; ROS-Reactive oxygen species; SOD-Superoxide dismutase.**Introduction**

Reactive oxygen species (ROS) also referred to as active oxygen species (AOS) are produced in plants under normal as well as stressed conditions. However plants have evolved different defense mechanisms to counter the excess production of ROS and their removal in order to balance the amount inside cells. Under the normal conditions, the ROS are in the optimum level. However, the related defense system gets activated once the ROS level increases inside cells. The increase in ROS production is mainly due to different abiotic and biotic factors. In most of the cases the defense mechanisms of the plants are not well developed to control the increase in the production of ROS; in such cases, there is a need for exogenous application of certain chemicals/hormones which enhance the activity of the key components involved in the defense mechanism against these stresses and hence protects the cells from the damage. Plants, being sessile, experience continuous exposure to various biotic and abiotic stresses in their natural habitats (Fujita et al., 2006) and their growth and yield are specially affected by the heat stress as it damages the functions of cells, tissues, and whole plants. For instance, high temperature causes modifications of membrane fluidity, permeability and

stability (Sangwan et al., 2002), and electrolyte loss resulting from heat-induced cell membrane is considered a measure of stress-cellular damage (Fokar et al., 1998). Several studies have provided evidence that the genetic variability observed in stress responses among wheat genotypes is mainly due to differential expression of stress-responsive genes and significant correlations have been observed between the acquisition of thermotolerance and the synthesis and accumulation of antioxidant enzymes and HSPs, although the intricate mechanisms underlying the thermal-tolerance are yet to completely deciphered (Kumar et al., 2007). However, plants have evolved a complex and coordinated series of antioxidant enzyme systems to scavenge ROS to protect the native proteins and cellular organelles from injury due to the oxidative stress. Superoxide dismutase (SOD; EC1.15.1.1), one of the most effective antioxidants in plants, catalyzes the dismutation of superoxide anion radicals into hydrogen peroxide and oxygen, specifically eliminating the ROS and balancing free oxygen radicals (Scandalios, 1993). SOD is the first and very crucial defense line in the anti-oxidative system to controls oxygen toxicity inside cells; it plays very important role in the development as well as stress resistance

Table 1. List of primers used for quantitative real time PCR (qRT-PCR) and reverse transcriptase PCR (RT-PCR) amplification of superoxide dismutase gene

Primers	Sequence (5'-3')	T _m value (°C)
SOD-F	5'-CACACACCAAACCACACTATCCA-3'	58.4° C
SOD-R	5'-TGTCTACTCGGACAAATCATGC-3'	58.4° C
qSOD-F	5'-TCCTTTGACTGGCCCTAATG-3'	63.8° C
qSOD-R	5'-CTTCCACCAGCATTTCAGT-3'	64° C
Tub-F	5'-TCGATGATCTCCAACCTCCACCAGT-3'	62.7° C
Tub-R	5'-TCGTCTGAACCTCAGCACCACACTTCT-3'	62.7° C

*q-quantitative real time PCR primer, F-forward primer, R-Reverse primer, Tub-Tubulin

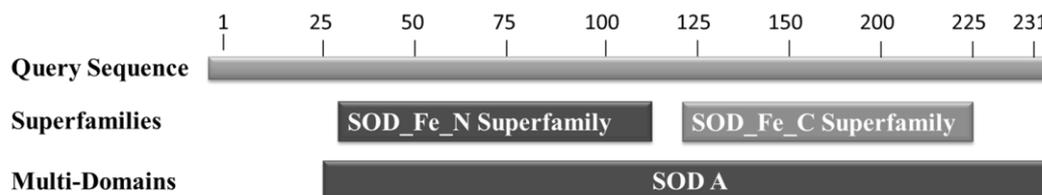


Fig 1. Conserved domain analysis of superoxide dismutase (SOD) using CD search tool, manganese iron binding domain was observed in SOD sequence.

in plants. The first plant *SOD* gene was cloned from maize in 1987 (Cannon et al., 1987). Presently, a number of *SOD* genes have been cloned in several plant species such as tomato (Perl-Treves et al., 1988), rice (Kaminaka et al., 1999), Arabidopsis (Kliebenstein et al., 1999), lotus (Dong et al., 2009) etc. Because of the different metal ions bound to the SOD it has been classified into three different groups: Cu/ZnSOD, MnSOD and FeSOD (Grace et al., 1990); while FeSOD and MnSOD are mainly found in the lower plants, Cu/ZnSOD abounds in higher plants. It is mainly localized in the cytoplasm and chloroplast as against MnSOD in the mitochondria and FeSOD in the chloroplast. Numerous isoenzymic forms of SODs have been reported from plants like maize (9 isoenzymes; Baum and Scandalios, 1979), Arabidopsis (7 isoenzymes; Kliebenstein et al., 1999) etc.

Hydrogen peroxide (H₂O₂), was initially considered as a toxic ROS causing severe damage to various cellular structures in plants, is now known as a potent signaling molecule regulating diverse physiological phenomena inside the plant system (Petrov and Breusegem, 2012); it is produced because of reduction of molecular oxygen and has relatively long lifespan compared to other ROS. It is also required for triggering the 'oxidative burst' that is a part of the defense mechanism of plants against abiotic stresses (Miller et al., 2010). However, a network of antioxidant enzymes is always there to regulate the concentration of hydrogen peroxide inside the cells to provide the effective scavenging system (Miller et al., 2010). A major impact of the environmental stress on plants is change in cellular membrane resulting into perturbations in its function. Hence, the estimation of membrane dysfunction under stress by measuring cellular electrolyte leakage from the affected leaf tissue into an aqueous medium is finding greater use as a measure of the cell membrane stability and also as a tool to screen genotype for stress resistance. Salicylic acid (SA) is a plant growth regulator which plays very important role in signaling pathway induced by various biotic and abiotic stresses (Ashraf et al., 2010). It has been characterized as an endogenous regulatory signal molecule for the activation of general defense mechanisms of plants. Exogenous application of SA has been observed to induce plant stress tolerance especially against salinity, drought and high temperatures; its application has been reported to modulate the activities of intracellular antioxidant enzymes like SOD and POD inside the plant system (Sakhabutdinova et al.,

2004). Wheat (*Triticum spp.*) is highly sensitive to the heat-stress. Some of the problems associated with heat stress in wheat are: drooping of leaves, pollen sterility, drying of stigmatic exudates, pseudo-seed setting, small starch granules, and shriveled seeds. In the present study, we report cloning of the Mn-SOD gene from *Triticum aestivum* (Gen Bank accession no JN257665) and investigate its expression profile at different stages of growth (vegetative, pollination, milky-dough and seed-hardening) and also in response to differential heat shock treatments. An attempt has also been made to study the effect of exogenous elicitors (H₂O₂ & salicylic acid) on transcript profile of SOD and other biochemical parameters under the heat stress to lend support to the underlying hypothesis of the present investigation.

Results and Discussion

RT-PCR and in silico characterization of superoxide dismutase gene

A Mn-SOD gene of ~733 bp was amplified and cloned from a thermotolerant cultivar, HDR77 of wheat. The nucleotide sequence was submitted in NCBI Gen Bank with accession no JN257665. BLASTn search showed 98% homology with that of superoxide dismutase (SOD) reported from *Triticum aestivum*. It has an open reading frame of 231aa with 5' UTR region of 21bp and 3' UTR region of 16 bp. Conserved domain (CD) search showed the presence of iron manganese N-terminal domain (accession no pfam00081) and iron manganese C-terminal domain (accession no pfam02777) which is a mixed alpha/beta fold involve in catalyzing the conversion of superoxide radicals to hydrogen peroxide and molecular oxygen (Fig. 1). Two cytosolic copper zinc superoxide dismutases (cytCuZnSOD) were identified from *Nelumbo nucifera* by Dong et al., (2011). Although, MnSOD and FeSOD shared high homology in terms of sequence and structure, no homology could be found with Cu/ZnSOD (Ma and Zhu, 2003). A signal peptide of 22aa was observed from N-terminal end targeted to mitochondria followed by conserved sequences from 30-111aa and 120-226aa characterized as Sod-Fe-N domain and Sod-Fe-C domain. Domains like VA, G(X) H (G/V) (F/N) H, PH, (V/L) H and (G/W) (G/E) HX (L/Y) were observed highly conserved (Fig. 2). Iron manganese domain present in the superoxide dismutase was represented by the conserved

sequence (G/W) (G/E) HX (L/Y) which is the main catalytic site for the conversion of peroxide radicals to hydrogen peroxide. Phylogeny study of superoxide dismutase was carried out using sequence alignment of SODs reported from *Saccharum officinarum* (GQ246460.1), *T. aestivum* (U73172.1, AF092524.1, U72212.1, AK333204.1), *O. sativa* (I19436.1, I34038.1), *B. distachyon* (XM_003568626.1), *Zea mays* (M33119.1, EU958076.1, L19463.1, L19461.1, L19462.1), *B. oldhamii* (GQ925542.1), *H. vulgare* (AK355761.1), along with *SOD* (accession no JN257665) isolated in present investigation. Based on sequence alignment and phylogenetic evolution, SODs has been classified into three broad families (I, II & III; Fig. 3). The *SOD* gene isolated in present investigation belongs to family III and share sequence homology with other SODs reported from wheat and sorghum.

Genomic organization of *TaSOD* gene

The genomic DNA isolated from HDR77 cultivar of wheat was restricted with *EcoRI*, *HinDIII* and *BamHI* in order to know the copy number of *TaSOD* gene using α - ^{32}P labeled probe. Three strongly hybridized bands were observed for *EcoRI* and *HinDIII* restricted lane, whereas only two bands were observed for *BamHI* restricted lane (Fig. 4). The band or bands that appear on a southern blot correspond to the fragment(s) of DNA overlapped by the probe. In case of *BamHI* restricted sample, the probe falls within a double *BamHI* fragment, thus two bands are visible after hybridization in background. This makes us to conclude that *TaSOD* gene has three copy numbers in the wheat genome. Similarly, two *MnSOD* genes were observed in peach (Bagnoli et al., 2002) and lotus (Li et al., 2009), whereas in maize the corresponding genes have been reported to belong to small multigene families (Zhu and Scandalios, 1994). The existence of three *TaSOD* copies could provide an explanation for the variation in the number of Mn-SOD isoforms found in the wheat.

Quantitative real time expression profiling of superoxide dismutase

Quantitative transcript profiling of superoxide dismutase showed 1.3, 1.8 and 1.9 fold (HDR77) and 1.1, 1.3 and 1.5 fold increase (PBW343) in *SOD* transcript at pollination, milky-dough and seed hardening stages (Fig. 5a). Previous research has shown that members of antioxidant gene families are differentially regulated in response to different stresses (Secenji et al., 2010). The expression of *cytCuZnSODs* was observed more in the leaf stalks and young leaves and lowest in the roots (Dong et al., 2011). An 1.5, 1.8, 1.9 and 2.1 fold (HDR77) and 1.2, 1.5, 1.8 and 1.9 fold increase (PBW343) in the *SOD* transcript was observed against differential heat shock treatment of 30°, 35°C for 2h and 40°C for 2 & 4h (Fig. 5b). Although, much difference in the fold change expression of *SOD* was not observed in tolerant and susceptible cultivars of wheat but keeping in mind the importance of superoxide dismutase, even a slight change in expression has large impact on overall thermotolerance capacity of the plant. Accumulation of *HbCCHI* (copper chaperone) transcripts in different tissues examined showed constitutive expression of this gene in all the tested tissues (Li et al., 2011). Several studies indicated that an expression increase of *MnSOD* gene in plants enhanced tolerance to many environmental stresses (Zhang et al., 2010). Gachon et al., (2004) found that

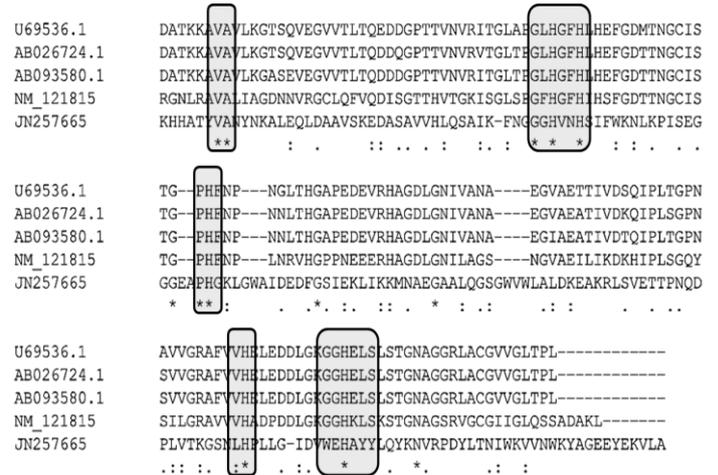


Fig 2. Multiple Clustal W alignment of different superoxide dismutase reported from *T. aestivum* (U69536.1), *O. sativa* (AB026724.1), *Zea mays* (AB093580.1), *A. thaliana* (NM121815), along with *SOD* (JN257665) isolated in present investigation, regions like VA, G(X)H(G/V)(F/N)H, PH, (V/L)H and (G/W)(G/E)HX(L/Y) were observed highly conserved as shown with asterisk marks inside shade boxes.

transgenic Brassica plants over-expressed wheat *MnSOD* gene were more tolerant to oxidative stress and aluminum toxicity. Lu et al., (2010) found that over-expression of *Cu/ZnSOD* could enhance tolerance and recovery of potato from drought stress. Semi-quantitative RT-PCR showed marked variation in the expression of *SOD* gene on the gel compared to that of β -tubulin gene which was used as endogenous control (Fig. 6).

Effect of exogenous stress on expression of superoxide dismutase gene under differential heat shock

Exogenous treatment of H_2O_2 (10 mmol L^{-1}) leads to 1.1, 1.7 & 2.4 fold (HDR77) and 0.75, 1.2 & 1.4 fold (PBW343) increase in the expression of *TaSOD* in response to 30°, 35° & 40°C for 2h (Fig. 7a). H_2O_2 acts as signaling molecule and enhances the expression of various stress associated genes under different abiotic stresses. The fold change in the transcript was observed maximum under treatment of 40°C for 2h in thermotolerant cultivar. Similarly, 0.6, 0.85, 0.7 fold (HDR77) and 0.5, 0.6, 0.43 fold (PBW343) increase in the expression of *TaSOD* was observed in response to 30°, 35° & 40°C for 2h under exogenous application of SA (5 mmol L^{-1} ; Fig 7b). The fold change in expression was maximum under treatment of 35° for 2h in susceptible cultivar. The percentage increase in the transcript of *TaSOD* was more in response to exogenous treatment of H_2O_2 compared to SA.

Electrophoretic mobility profiling of superoxide dismutase

Isoenzymic profile of *SOD* revealed the expression of three (HDR77) and two (PBW343) *SOD* isoenzymes when plant were exposed to heat shock treatment of 30°C for 2h (Fig. 8). The new isoenzymes are predicted to counter the harmful effect of active oxygen species (AOS) produced because of heat stress. *SOD* profiling was also carried out in wheat at different stages of growth and development (Fig. 9). In case of PBW343, three prominent isoenzymes were observed at vegetative stage and further a decrease in the expression of

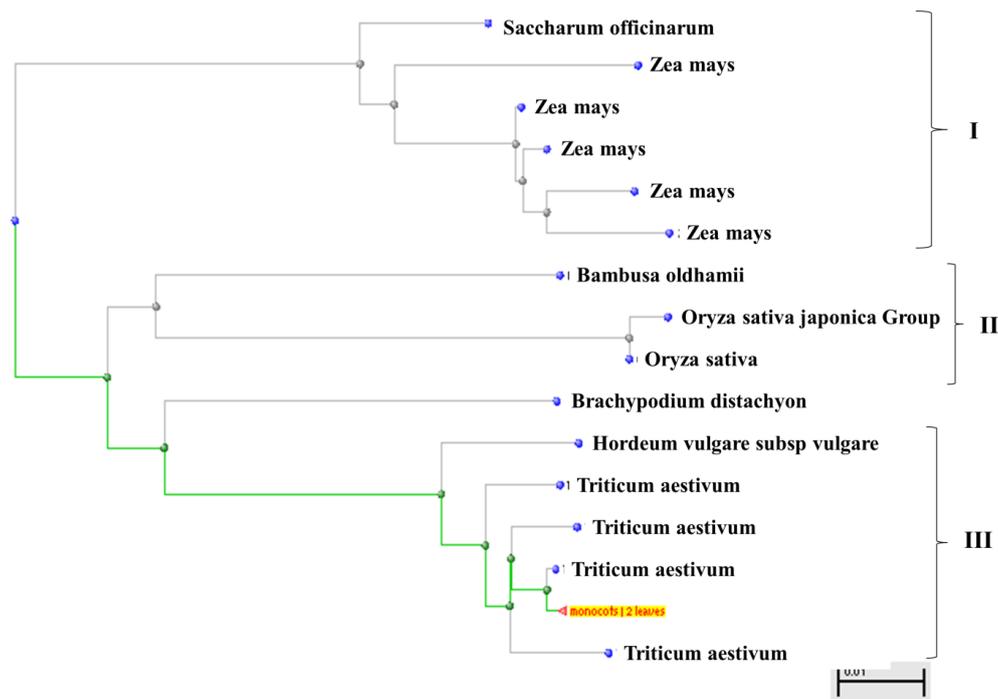


Fig 3. Phylogeny tree analysis of superoxide dismutase (SOD, accession no JN257665) isolated in present investigation generated using sequence alignment of SODs reported from *Saccharum officinarum* (GQ246460.1), *T. aestivum* (U73172.1, AF092524.1, U72212.1, AK333204.1), *O. sativa* (I19436.1, I34038.1), *B. distachyon* (XM_003568626.1), *Zea mays* (M33119.1, EU958076.1, L19463.1, L19461.1, L19462.1), *B. oldhamii* (GQ925542.1), *H. vulgare* (AK355761.1), based on sequence alignment, SODs has been classified into three broad families (I, II & III).

these isoenzymes was observed at pollination, milky dough and seed hardening stages. The expression of three prominent isoenzymes was observed in case of HDR77 at all the stages of growth with maximum activity at vegetative and milky dough stages (Fig. 9). In proteomic studies, the levels of different SOD isoforms were found up-regulated under drought (Taylor et al., 2005), ozone stress (Agarwal et al., 2002), high light (HL) (Nam et al., 2003), As stress (Requejo and Tena, 2005) and plant hormone treatment (Rakwal and Komatsu, 2004).

Alteration in superoxide dismutase (SOD) activity at different stages of growth and under differential heat shock

The SOD activity in HDR77 was 12.2 (vegetative), 12.6 (pollination), 23.5 (milky-dough) and 23.7 (seed-hardening) Umg^{-1} protein (Fig. 10a). The SOD activity was observed maximum during seed hardening stage, but no significant difference was observed in the activities observed during milky-dough and seed hardening stages. The SOD activity observed in PBW343 was 5.5 (vegetative), 6.16 (pollination), 10.7 (milky dough) and 11.5 (seed hardening) Umg^{-1} protein (Fig. 10a). The maximum activity was observed during seed hardening stage. The overall activity of SOD was low in susceptible wheat cultivar compared to tolerant at different stages of growth. Almeselmani et al., (2009) reported greater activity of SOD in heat-tolerant genotypes of wheat subjected to heat stress (35/25°C). A 0.48 fold decrease in SOD activity was observed in susceptible wheat cultivar at seed hardening stage. Similarly, in case of differential heat shock at vegetative stage, the SOD activity was 12.6 (control), 16.6 (30°C for 2h), 22.1 (35°C for 2h) and 21.1 (40°C for 2h) Umg^{-1} in HDR77 compared to 7.8 (control), 12.2 (30°C for 2h), 15.8 (35°C for 2h) and 15.9 (40°C for 2h) Umg^{-1} in PBW343 (Fig. 10b). The maximum SOD activity was observed in response to heat shock of 35°C for 2h (HDR77)

and 40°C for 2h (PBW343). At 45/40°C, the SOD activity decreased slightly over 40/35°C with no significant differences among maize and wheat genotypes (Kumar et al., 2007). Similarly, He and Huang (2007) reported that the heat-tolerant genotype of Kentucky grass (*Poa pratensis*) had significantly higher SOD activity than its sensitive genotype.

Effect of exogenous stress on SOD activity under differential heat shock

The change in SOD activity was estimated in tolerant and susceptible wheat cultivars in response to exogenous application of H_2O_2 and SA under differential HS (Fig. 12). The activity observed was 12.6, 17.8, 24.6 and 24.4 Umg^{-1} (HDR77) and 7.2, 14.0, 16.4 and 19.3 Umg^{-1} (PBW343) in response to 22°, 30°, 35° and 40°C for 2h when seedlings were exposed to exogenous application of H_2O_2 (10 mmole L^{-1} ; Fig. 12a). Similarly, SOD activity of 11.7, 16.4, 23.4 and 23.4 (HDR77) and 6.1, 12.3, 15.4 and 17.6 Umg^{-1} (PBW343) were observed in response to 22°, 30°, 35° and 40°C for 2h when plants were treated with exogenous application of SA (5 mmole L^{-1} ; Fig. 12b). The percentage increase in SOD activity under differential HS was more in response to exogenous application of H_2O_2 compared to SA and tolerant cultivar (HDR77) was observed more responsive to exogenous treatment compared to susceptible.

Change in total antioxidant capacity at different stages of growth and under differential heat shock

Total antioxidant capacity (AOC) was 155 (vegetative), 187.6 (pollination), 210.3 (milky dough) and 204.6 (seed hardening) $\mu\text{M Fe}/100\mu\text{l}$ in HDR77 compared to 112.6 (vegetative), 136 (Pollination), 139.6 (milky-dough) and 143.3 (seed hardening) $\mu\text{M Fe}/100\mu\text{l}$ in PBW343 (Fig. 11a). The maximum antioxidant capacity was observed during

milky dough (HDR77) and seed hardening stages (PBW343). Total AOC was very low in susceptible cultivar compared to tolerant at different stages of growth. An increased antioxidant capacity as a result of application of SA can reduce oxidative damage, thereby increasing MTS and reducing leaf dark respiration rates (Mohammed and Tarpley, 2009). Under differential HS at vegetative stage, the total AOC was 145 (control, 22°C), 180 (30°C for 2h), 204.6 (35°C for 2h) and 233.6 (40°C for 2h) $\mu\text{M Fe}/100\mu\text{l}$ in HDR77 compared to 118.3 (control), 123 (30°C for 2h), 137.6 (35°C for 2h) and 151 (40°C for 2h) $\mu\text{M Fe}/100\mu\text{l}$ in PBW343 (Fig. 11b). The maximum total AOC was observed under differential heat shock of 40°C for 2h in both the cultivars. A 0.64 fold decrease in total AOC was observed in PBW343 (susceptible) compared to HDR77 (tolerant) under differential HS.

Effect of exogenous stress on total antioxidant capacity under differential heat shock

A study was also conducted to analyze the effect of exogenous application of hydrogen peroxide (10 mmole L^{-1}) and SA (5 mmole L^{-1}) on total antioxidant capacity (AOC) of wheat under differential HS. The AOC observed in plant samples treated with H_2O_2 was 153, 187.3, 223.3 and 274.3 $\mu\text{mole Fe}/100\mu\text{l}$ (HDR77) and 91.6, 111.6, 148.6 and 169 $\mu\text{mole Fe}/100\mu\text{l}$ (PBW343) under differential HS of 22°, 30°, 35° and 40°C for 2h (Fig. 12c). Under SA treatment, the AOC observed was 139.6, 170.3, 211.6 and 254 $\mu\text{mole Fe}/100\mu\text{l}$ (HDR77) and 77.3, 110.3, 140.3 and 161 $\mu\text{mole Fe}/100\mu\text{l}$ (PBW343) under differential HS of 22°, 30°, 35° and 40°C for 2h (Fig. 12d). A significant increase in the AOC was observed in both cultivars in response to exogenous application of H_2O_2 and SA under differential HS. The H_2O_2 was more effective in enhancing the AOC compared to SA under HS. HDR77 was more responsive to hydrogen peroxide in term of change in AOC than the PBW343 under HS.

Screening for cell membrane stability (CMS)

HDR77 and PBW343 wheat genotypes were characterized for both basal and acquired thermotolerance at different stages of growth (vegetative, pollination, milky dough and seed hardening stages) by evaluation of cell membrane stability which is considered as a standard method to evaluate thermotolerance (Fokar et al., 1998). HDR77 showed the highest cell membrane stability index (72%) compared to PBW343 (48%). A decrease in the cell membrane stability was observed in both the cultivars at different stages of growth, but then the percentage decrease in CMS was more in case of PBW343 (Fig. 13a). Milky dough stage is more prone to terminal heat stress causing maximum damage to the wheat crops. Cell membrane stability index at milky dough stage was 66% (HDR77) and 40% (PBW343) which is the main reason behind the tolerance nature of HDR77 cultivar of wheat. The increased solute leakage, as an indication of decreased cell membrane thermo stability (CMT), has long been used as an indirect measure of heat-stress tolerance in diverse plant species, including soybean (Martineau et al., 1979), potato and tomato (Chen et al., 1998), wheat (Blum et al., 2001) etc. The effect of exogenous treatment of H_2O_2 and SA on CMS was also studied in tolerant and susceptible wheat cultivars at vegetative stage (Fig. 13b). The change in CMS under H_2O_2 and SA treatment was non-significant in both HDR77 and PBW343. A decrease in CMS

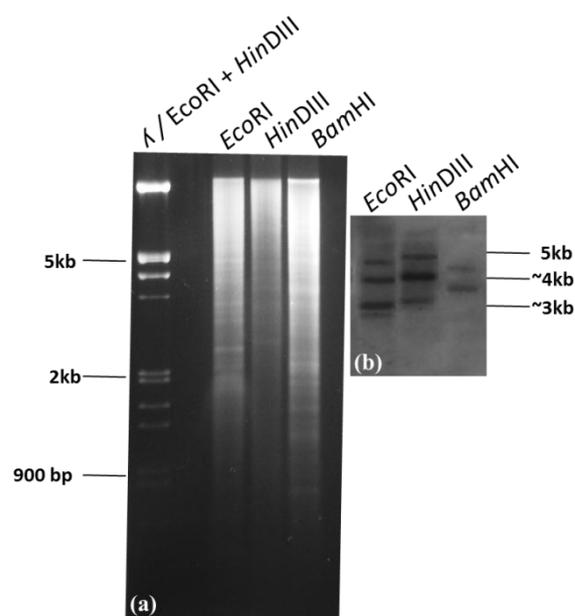


Fig 4. Southern blot analysis of genomic DNA isolated from HDR77 cultivar of wheat, (a) Wheat genomic DNA was digested with *EcoRI*, *HinDIII* and *BamHI*, separated by agarose-gel electrophoresis (0.8%), transferred to nylon membranes using iblotter (Invitrogen, UK), (b) probed with mitochondrial (*TaSOD*) gene labelled with α - ^{32}P -dCTP.

was observed against H_2O_2 and SA treatment in HDR77 whereas, slight increase was observed in case of PBW343.

Proposed model for central role of SODs in heat stress tolerance

Based on the results obtained in the present investigation, a modified mechanism of heat stress tolerance in plants, partly adopted from Sung *et al.*, (2003) has been proposed (Fig. 14). Heat stress alters the CMS by increasing the electrolyte leakage. The heat stress perceived by the different sensors and kinases induces various signaling pathways in order to activate the defense mechanism of the plants which then leads to the outburst of ROS as well as proportionate increase in the H_2O_2 accumulation. As a signaling molecule, H_2O_2 activates various stress associated TFs correlated with different defense pathways. These TFs regulate the expression of different stress associated genes like HSPs, LEA, SODs, APX, CAT etc. The abrupt increase in functional proteins and their activities help in balancing the accumulation of ROS, signaling molecules as well as osmolytes culminating into increase in the thermotolerance.

Materials and Methods

Plant materials

Seeds of HDR77 (thermotolerant) and PBW343 (thermosusceptible) cultivars of wheat (*Triticum aestivum*) were collected from Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi. Seeds of both the cultivars were sown in 20 pots each (in sets of three) inside the regulated chamber in National Phytotron Facility (temperature $22 \pm 2^\circ\text{C}$ and RH of 75% with light/dark condition of 16/8h). Autoclaved mixture of sand and alluvial soil of equal quantity was used in each pot. Samples were

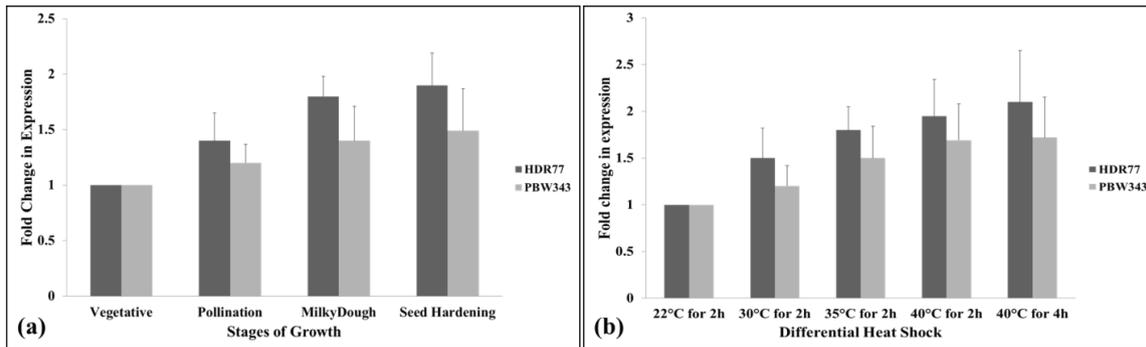


Fig 5. Quantitative real time expression profiling of superoxide dismutase (SOD) in thermotolerant (HDR77) and susceptible (PBW343) cultivars of wheat, (a) SOD expression profiling at different stages of growth, and (b) SOD transcript profiling under differential HS.

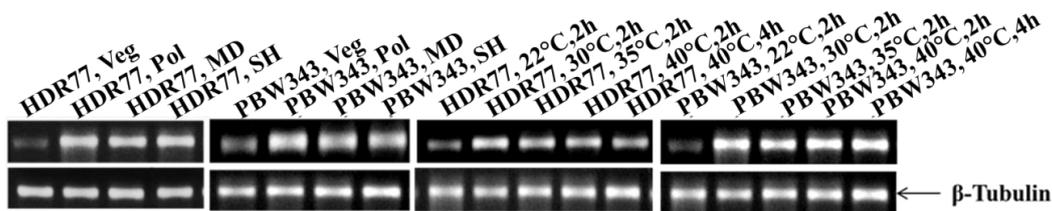


Fig 6. Semi-quantitative real time expression profiling of superoxide dismutase (SOD) in thermotolerant (HDR77) and susceptible (PBW343) cultivars of wheat at different stages of growth and against differential HS treatment, β -Tubulin gene expression was used as an endogenous control to normalized the expression, 2% agarose gel was used.

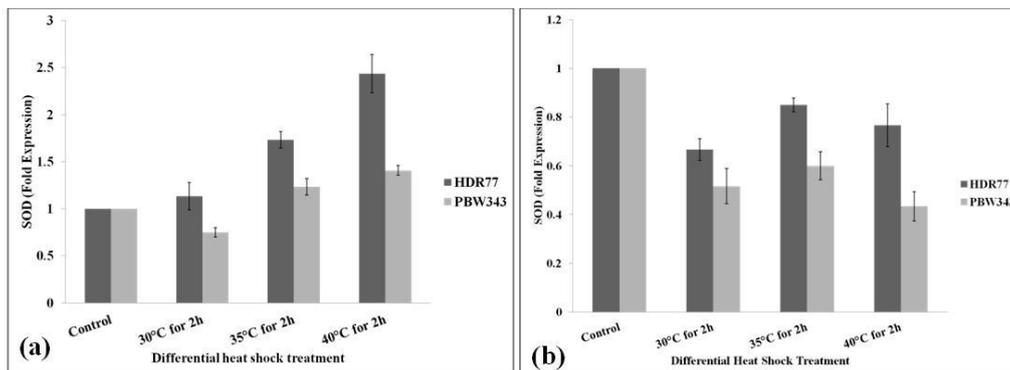


Fig 7. Effect of exogenous stress on alteration in expression profile of *TaSOD* gene isolated in present investigation under differential heat shock (22°, 30°, 35° and 40°), (a) Hydrogen peroxide (10 mmol L⁻¹) and, (b) Salicylic acid (5 mmol L⁻¹), expression of β -Tubulin gene was used as endogenous control to normalize the C_t value.

collected in triplicates from each stages of growth (vegetative, pollination, milky dough and seed hardening). One group of plants were exposed to differential heat shock (30°, 35° and 40°C for 2h) at different stages of growth. Collected samples were frozen in liq. nitrogen and stored at -72°C in deep freezer until RNA extraction.

Exogenous application of H₂O₂ and Salicylic acid

One group of plants (both tolerant and susceptible) were sprayed with hydrogen peroxide (10 mmol L⁻¹) and Salicylic acid (5 mmol L⁻¹) (Que et al., 2009) at vegetative stage and further samples were collected in three replications. The collected samples were analyzed for various biochemical parameters like abundance of SOD and their activities, total AOC & CMS in response to exogenous H₂O₂ and SA.

cDNA synthesis and RT-PCR amplification

100 mg of samples (seedling leaf for vegetative and flag leaf for other stages analysis) were crushed into powder form using liquid nitrogen and further Trizol reagent was used for total RNA isolation (Invitrogen, USA). The isolated RNA was checked for its purity by running it on 1.5% agarose gel with 800 μ l of 10% guanidine thiocyanate (GTC). The RNA isolated was used for the cDNA synthesis using the kit of Ambion and the instructions given by the manufacturers were followed. Superoxide dismutase genes specific degenerate primers were designed from conserved regions of different SOD genes reported from plant and non- plant sources using Gene Fischer primers designing software and it was custom synthesized commercially (Table 1). The 5 ng of cDNA synthesized were used as a template along with SOD gene specific primers (50 pmole each) and PCR was carried out using 10x PCR buffer(5 μ l), 30mM dNTP mix(3 μ l), Taq pol

(0.5 μ l) and final volume was made up to 50 μ l. The PCR cycle followed was 94°C for 4 min, 93°C for 45 sec, 58°C for 30 sec, 72°C for 1 min. 35 cycles were given followed by final extension at 72°C for 10 min. The amplified product was run on 0.8% agarose gel in order to check the amplification along with λ *HinDIII/EcoRI* marker. The amplicon was cloned in pGEMT_{Easy} vector (Promega) and it was transformed using DH5 α strain of *E. coli*. The positive clones were selected by blue white screening and were further sequenced using Sangers Di-deoxy method. The nucleotide sequence was *in silico* characterized using different bio-informatic tools like BLASTN, BLASTX, Exspasy tool, all in one sequence analyzer etc.

Mapping of superoxide dismutase gene using southern blotting

Genomic DNA was isolated from seedlings of HDR77 cultivar of wheat (thermotolerant) using the cetyl-trimethyl-ammonium bromide method (Proconier et al., 1990). Five micrograms of genomic DNA was digested with the restriction endonucleases *EcoRI*, *HinDIII*, and *BamHI* (HF restriction enzyme, NEB, UK) at 37°C overnight, and the resulting fragments were resolved in a 0.8% agarose gel and transferred onto a piece of nylon membrane (Hybond-N+, Amersham Biosciences, Uppsala, Sweden). The iBlot™ (Invitrogen) was used for southern transfer of DNA from gels. DNA on the blot was then denatured with an alkaline buffer (0.5 N NaOH, 1.5 M NaCl) for 2 min, soaked in a neutralizing buffer (0.5 M Tris HCl [pH 7.5], 1.5 M NaCl) for 2 min, and baked at 75°C for 30 min. The filter was pre-hybridized in a hybridization buffer (1% sodium dodecyl sulfate [SDS], 1.5 M NaCl, 10% dextran sulfate) containing 100 μ g of salmon sperm DNA (Pharmacia, Uppsala, Sweden) per ml at 65°C for 4h. The *TaSOD* DNA was 5' end labeled with α -[³²P]-dCTP (BRIT, Bhabha Atomic Research Centre, India) and was used as a probe for hybridization of processed filter at 65°C for 15 h. In the high stringency condition, the membrane was hybridized at 65°C for 14 h and washed twice with 0.1% sodium dodecyl sulfate (SDS) in 2 x standard saline citrate (SSC) (150 mmol/L NaCl, 15 mmol/L sodium citrate) solution for 15 min, and then twice with 0.1% SDS in 0.2 x SSC solution for 15 min at 50°C. Signals were quantified using automatic developer machine.

RNA extraction and Quantitative Real Time PCR (qRT-PCR)

The total RNA isolated by Trizol method was quantified using Qubit™ 2.0 fluorometer (Invitrogen). RNA integrity was verified in 1.2% agarose gels. First strand cDNA synthesis was performed using oligo dT primers and the Superscript II reverse transcriptase (Invitrogen, UK), according to the manufacturer's instructions. First-strand cDNA was diluted to a final concentration of 1.0 ng/ μ l. Primers for quantitative real time PCR reactions were designed from the deduced sequence corresponding to the wheat superoxide dismutase gene using prime 3 primers designing software (Premier Biosoft, USA) (Table 1). For each stress condition as well as for controls, expression measurements were performed using duplicate biological replications and three technical replications. Quantitative PCR was performed in 20 μ l reactions using gene specific primers, 1 μ l of cDNA as template and the SYBRGreenER qPCR SuperMix Universal (Invitrogen, UK). Reactions were performed on the CFX96 Real-Time PCR system (Bio Rad, UK). The thermal profile for qPCR was: 3 min at 95°C,

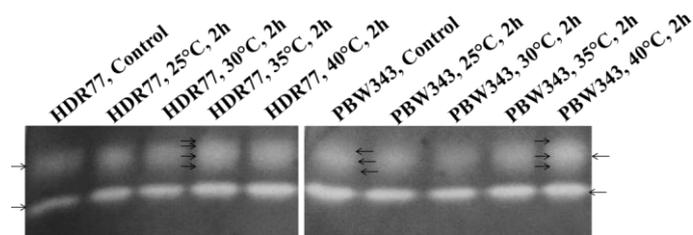


Fig 8. Isoenzymic pattern profiling of superoxide dismutase in HDR77 and PBW343 cultivars of wheat in response to differential heat shock treatment, clear region on gel depict the activity of SOD isoenzymes as shown by arrows.

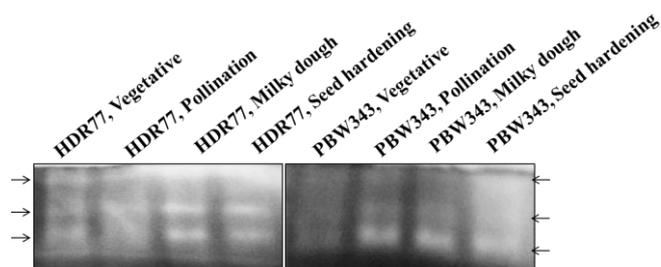


Fig 9. Electrophoretic mobility profiling of superoxide dismutase isoenzyme in HDR77 and PBW343 cultivars of wheat at different stages of growth (vegetative, pollination, milky dough and seed hardening), Clear zones depict the activity of antioxidant isoenzymes on the gel.

followed by 35 cycles each consisting of 95°C for 15 sec, 60°C for 30 sec and 72°C for 15 sec. The primer specificity and the formation of primer-dimers were monitored by dissociation curve analysis and agarose gel electrophoresis on a 3% agarose gel. The expression levels of wheat tubulin gene were used as internal standards for normalization of cDNA template quantity using tubulin-specific primers (Gen Bank accession no TAU76544) as shown in the Table 1. Data analysis was performed using software provided by Bio Rad, UK. The Comparative C_t (2^{- $\Delta\Delta$ C_t}) method (pfafl, 2001) was used to calculate the changes in gene expression as a relative fold difference between an experiment and calibrator sample.

Electrophoretic mobility profiling of superoxide dismutase isoenzyme

The crude extracts of collected samples were prepared in phosphate buffer (pH 7.5) and 20 μ g of proteins (estimated by Bradford method) were loaded on to each well of polyacrylamide gel (10%) for native PAGE. The protocol of Roychoudhuri et al., (2003) was followed for the isoenzymic study of superoxide dismutase. Illumination was discontinued when maximum contrast between achromatic zones and the general blue color was achieved. The gel was maintained in distilled water till photographed.

SOD enzyme activity assay

Leaf materials (1 g) were ground in 6 ml of ice cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium-EDTA and 1% (w/v) polyvinyl-polypyrrolidone (PVP). The protein was estimated in extracted samples (Bradford, 1976) before using it for SOD enzyme activity assay. Superoxide dismutase (SOD) activity was determined

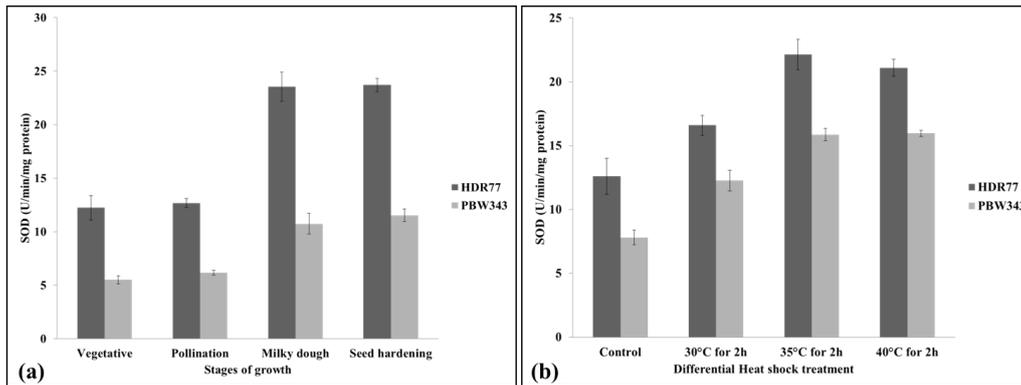


Fig 10. Superoxide dismutase activity assay in thermotolerant (HDR77) and susceptible (PBW343) wheat cultivars, (a) activity at different stages of growth and (b) activity under differential heat shock, vertical bars indicate s.e. ($n=3$).

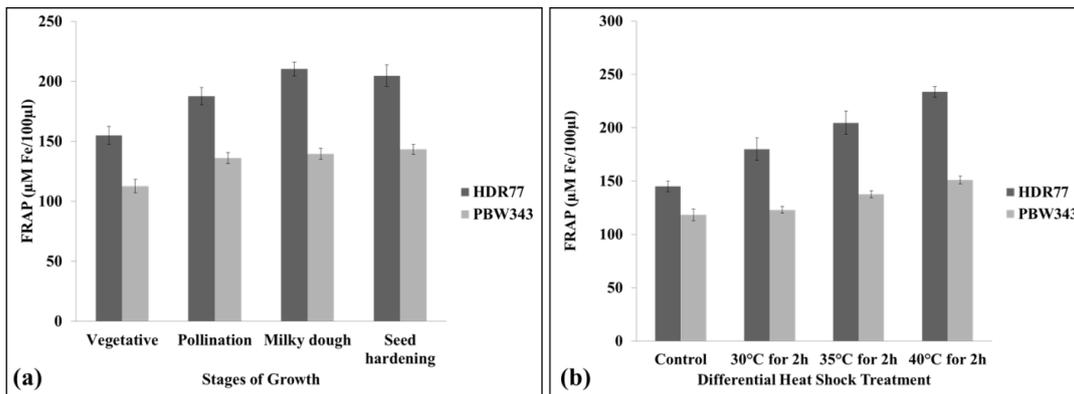


Fig 11. Total antioxidant capacity (AOC) estimated in thermotolerant (HDR77) and susceptible (PBW343) wheat cultivars using FRAP method, (a) AOC at different stages of growth and (b) AOC in response to differential heat shock treatment, vertical bars indicate s.e. ($n=3$).

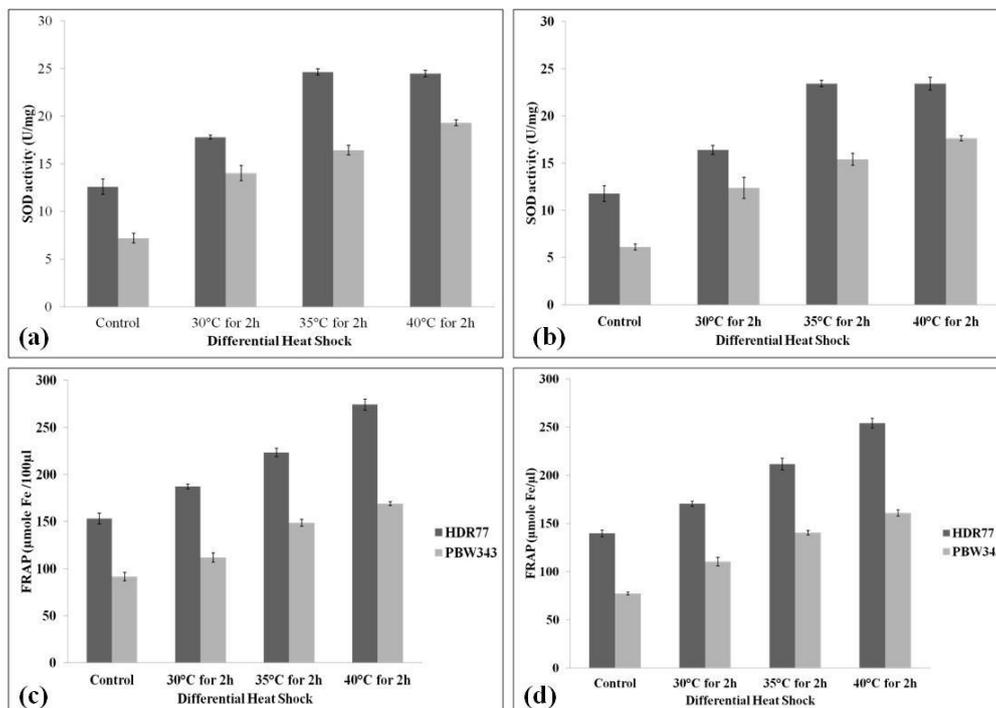


Fig 12. Effect of exogenous application of hydrogen peroxide and SA on SOD activity and total antioxidant capacity (AOC) in HDR77 (tolerant) and PBW343 (susceptible) wheat cultivars, (a) SOD activity in HDR77 and PBW343 in response to H₂O₂ (10 mmole L⁻¹), (b) SOD activity in HDR77 and PBW343 in response to SA (5 mmole L⁻¹), (c) Total antioxidant capacity in HDR77 and PBW343 in response to H₂O₂ (10 mmole L⁻¹), (d) AOC in HDR77 and PBW343 in response to SA (5 mmole L⁻¹), vertical bars indicate s.e. ($n=3$).

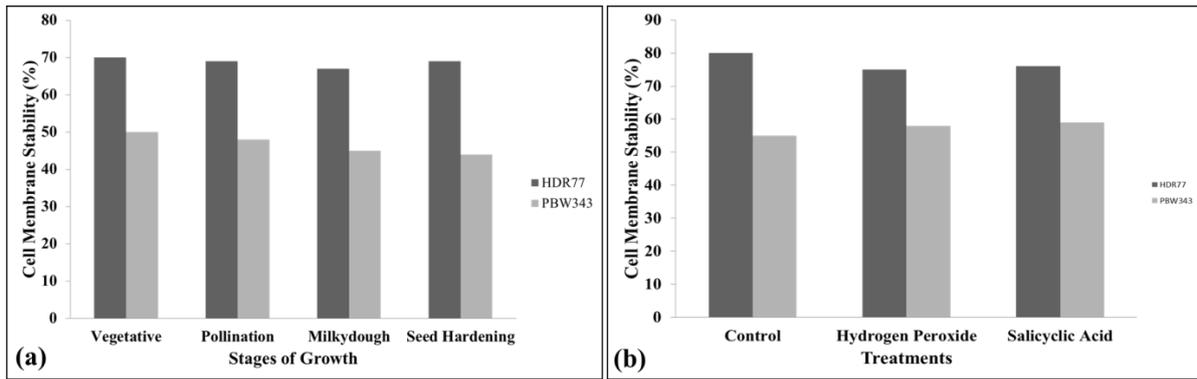


Fig 13. Cell membrane stability index of HDR77 (thermotolerant) and PBW343 (susceptible) cultivars of wheat, (a) CMS at different stages of growth, (b) CMS in response to exogenous application of H_2O_2 (10 mmole L^{-1}) and SA (5 mmole L^{-1}), Fokar's method was used for the estimation.

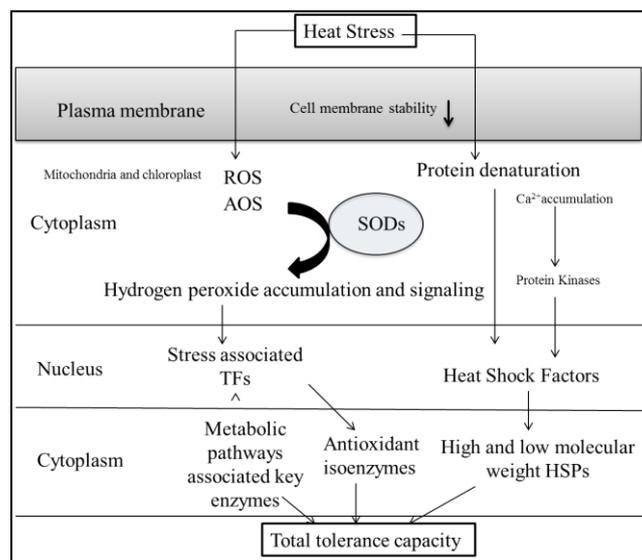


Fig 14. Proposed heat-stress tolerance mechanism in plants and the central role of superoxide dismutase in modulating the total thermo-tolerance capacity

in crude extract by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) in the presence of riboflavin in light (Giannopolitis and Ries, 1977). One unit of enzyme activity was determined as the amount of the enzyme needed for the inhibition of 50% NBT reduction rate by monitoring absorbance at 560 nm with spectrophotometer.

Estimation of total antioxidant activity

The FRAP (Ferric reducing antioxidant power assay) procedure described by Benzie and Strain (Benzie and Strain, 1999) was followed for the total antioxidant activity assay. This method is based on the reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants. The values were expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of $1 \text{ mmol L}^{-1} \text{ FeSO}_4$.

Evaluation of cell membrane stability (CMS)

Leaves of uniform size were collected and used to measure cell membrane stability (CMS) using the method of Fokar et al., (1998).

Statistical analysis

The experiment was conducted in a completely randomized design. The uppermost fully expanded leaves were collected randomly as three replicates for each treatment. Data were analyzed using one-way analysis of variance (one-way ANOVA). The standard errors were given in histograms.

Conclusions

We have cloned superoxide dismutase (*TaSOD*) gene of 733bp from wheat (*Triticum aestivum*). Mapping of wheat genome showed the presence of three copies of *TaSOD* gene. Quantitative RT-PCR showed abundance of transcript at the milky-dough and seed hardening stages in HDR77 cultivar. Exogenous application of H_2O_2 (10 mmole L^{-1}) showed increase in the transcript and activity of SOD and overall AOC in both the cultivars compared to that of SA treatment (5 mmole L^{-1}) under the differential HS. AOC and CMS were observed highest in thermotolerant HDR77. SODs play a central role in regulating the expression of various stress associated genes by manipulating the accumulation of H_2O_2 . Hydrogen peroxide in turn regulates the expression of *TaSOD* as shown by qRT-PCR. However, there is a need to

further identify and characterize different genes as well as isoenzymes of SODs with improved biochemical kinetics for their use in developing tolerance in the desirable wheat cultivars to cope up with the problem of terminal heat stress.

Acknowledgments

The authors sincerely thank the Indian Council of Agriculture Research (ICAR) and Indian Agricultural Research Institute (IARI) for providing financial assistance under the National Initiative for Climate Resilient Agriculture (NICRA) project.

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