Antioxidant response and Lea genes expression under salt stress and combined salt plus water stress in two wheat cultivars contrasting in drought tolerance

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Two wheat cultivars, C306 and PBW343 contrasting in drought tolerance were compared for their antioxidant response and Lea genes’ expression under salt stress (SS) and combined stress (CS) of salt stress plus water stress during seedlings growth. The drought susceptible cultivar (PBW343) behaved different towards SS/CS than towards WS. It accumulated more dry masses in shoots, more ascorbate, had higher ascorbate to dehydroascorbate ratio, lesser dehydroascorbate, lesser malondialdehyde (MDA), more proline and higher antioxidant enzymes under SS than under WS. CS increased dry masses, ascorbate, ascorbate to dehydroascorbate ratio, antioxidant enzymes and decreased dehydroascorbate and MDA contents from levels under WS. The drought tolerant cultivar (C306) though showed higher levels of ascorbate, ascorbate to dehydroascorbate ratio, lower levels of dehydroascorbate, showed lesser dry biomasses in shoots, higher MDA and lesser ascorbate peroxidase and catalase activities under SS than under WS and these features were improved on combining WS with SS. All lea genes were induced under all stresses in both cultivars except Wrab17 in C306 only, was not induced under any stress. Eight Lea genes out of ten were induced higher under WS than SS in C306 but induced same in PBW343. Wdhn13 gene was higher salt-responsive than other lea genes in both cultivars.

Keywords: ABA, Antioxidant, LEA, Salt stress, Water stress, Wheat

Plant responds to abiotic stresses through many stress-related pathways among which some constitute the mechanism of cross-tolerance while some are unique to each type of stress. Water stress (WS) and salt stress (SS) are two major abiotic stresses affecting different crops and their yields worldwide. Most of the time, plant under natural environment experiences many stresses together, so it becomes necessary to study plant responses to combined stress besides studying the effects of individual stresses. Moreover, response of plant towards combined stress is reported to be unique which can not be extrapolated from responses under individual stresses. In water stress and salt stress, common effect is the generation of osmotic stress but salt stress also produces ion toxicity. In the present study, two wheat cultivars, C306 (ABA-higher sensitive and drought tolerant) and PBW343 (ABA-lesser sensitive and drought susceptible) have been compared under salt stress and combined (water and salt) stress for their growth, antioxidant potential and lea genes’ expression. These two cultivars were already compared for same parameters under exogenous ABA supply and water stress earlier. Objectives of this study are (i) to compare both cultivars under salt stress using same experimental background to see if cultivars behave same towards salt stress as towards water stress, (ii) to check ABA-related effects under salt stress and (iii) to compare response of combined stress with individual stresses.

Materials and Methods

Plant materials—The experiments were conducted on two bread wheat (Triticum aestivum) cultivars PBW343 and C306. Their seedlings were developed in dark at 25 °C. Briefly, seedlings were grown on autoclaved distilled water for four days and stress was applied on 4th day using 6% mannitol for WS, 300 mM NaCl for SS and 6% mannitol in 300 mM NaCl for CS where seedlings growing on water were used as control (CT). Data were collected from 4 stages; 0, 24, 48 and 72 h after stress treatment.

Growth measurement—Growth was measured in dry masses of 25 seedlings in triplicates.

Antioxidant enzymes— Antioxidant enzymes were extracted and estimated. In brief, these were extracted in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 2% PVP, 0.05% triton-X-100.
Ascorbate peroxidase was assayed in 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.3 mM ascorbate, 1 mM H₂O₂ at 290 nm. Catalase was assayed in 50 mM potassium phosphate buffer (pH 7.0) and 25 mM H₂O₂ at 240 nm. Guaiacol peroxidase was assayed in 100 mM potassium phosphate buffer (pH 6.5), 50 mM guaiacol, 32 mM H₂O₂ at 470 nm. Glutathione reductase was assayed in 50 mM potassium phosphate buffer (pH 7.0), 0.7 mM GSSG, 0.07 mM NADPH at 320 nm. All enzymes were extracted in triplicates and values calculated as mean±SD.

Antioxidants and other metabolites—Ascorbate, H₂O₂, dehydroascorbate, proline and MDA contents were measured. Briefly, H₂O₂ was extracted in 0.1% TCA and was estimated using 2 M potassium iodide and 50 mM potassium phosphate buffer (pH 7.0) at 390 nm. Ascorbate was extracted in 5% TCA and estimated by using bipyridyl reagent and measuring at 525 nm. Dehydroascorbate was extracted in 5% metaphosphoric acid with 1% thiourea and estimated by using dinitrophenyl hydrazine reagent and then reading at 530 nm. Proline was extracted in 3% sulphasalicylic acid and estimated using ninhydrin reagent at 520 nm. MDA was extracted in 0.1% TCA and estimated by reacting with thiobarbituric acid and then reading at 532 nm and 600 nm. All contents were extracted in triplicates and values calculated as mean±SD.

Statistical analysis—Data were analyzed by Duncan’s Multiple Test (DMT) at P ≤ 0.05 to test for statistical differences among samples using DSAASTAT software version 1.101.

Semiquantitative RT-PCR—Same primers of LEA genes as used by Kaur et al. were used in the present study. Semi-quantitative RT-PCR was done in shoots of both cultivars at 24 and 48 h stages. Briefly, the protocol followed was as follows: first, total RNA was isolated and then treated with DNase I to remove contaminating DNA. Approximately 1 µg of total RNA was used as a template to make cDNA using reverse transcriptase. PCR was performed on cDNA. Cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of wheat was used as an internal control. Intensities of PCR bands were measured and ratio for each gene in each sample was calculated by dividing intensity of band of gene by intensity of band of GAPDH. Data are presented with T/C value (fold amplification of gene in stressed sample over control sample).

Results

Effect of treatments on growth—Dry biomass of shoots of PBW343 first increased significantly (at 24 h) and then decreased significantly or non-significantly (at 48 and 72 h) under all three stresses (Fig. 1A) but
biomasses were higher under SS/CS than under WS. Dry biomasses of roots of PBW343 were not altered significantly under all three stresses (Fig. 1B).

Dry biomasses of shoots of C306 were not altered significantly throughout the stress period under WS/CS but were decreased significantly under SS (at 48 and 72 h of stress) (Fig. 1C). Dry biomasses of roots of C306 (Fig. 1D) were maintained almost equivalent to control values throughout the stress period under all three stresses except these were increased significantly at 24 h under CS only.

Effects on antioxidants and other metabolites—In shoots of PBW343, ascorbate contents first increased significantly (at 24 h) then decreased significantly or non-significantly (at 48 and 72 h) from control values under all three stresses however contents were maintained higher under SS/CS than under WS (Fig. 2A). In roots of PBW343, ascorbate contents were decreased under stresses but contents were higher under SS than under WS/CS (at 24 and at 48 h) (Fig. 2B). In shoots of C306, ascorbate contents were increased significantly under SS/CS but were maintained equivalent to control values under WS throughout the stress period (Fig. 2C). Ascorbate contents were either unaltered or decreased under all stresses throughout the stress period in roots of C306 (Fig. 2D).

In shoots of PBW343, dehydroascorbate contents were not increased at any stage under SS/CS but were increased non-significantly under WS at 48 h stage (Fig. 2E). Ascorbate to dehydroascorbate ratios (inset Fig. 2E) were either higher or equivalent to CT under SS/CS but were decreased under WS (at 48 and 72 h). In roots of PBW343, dehydroascorbate contents were unaltered or decreased under SS/CS but were increased significantly (at 48 and 72 h) under WS as compared to CT (Fig. 2F. Ratios (inset Fig. 2F) were higher under SS/CS than under WS (at 24 and 48 h). In shoots of C306, dehydroascorbate contents were decreased or unaltered from CT under all stresses throughout the stress period (Fig. 2G). Ascorbate to dehydroascorbate ratios (inset Fig. 2G) were higher under SS/CS and equivalent to CT under WS during stress period. In roots of C306, dehydroascorbate contents were decreased under all three stresses (Fig. 2H) throughout the stress period. Ascorbate to dehydroascorbate ratios were almost equivalent to CT (at 24 and 48 h) under WS but higher than CT (specially at 72 h) under SS/CS (inset Fig. 2H).

H$_2$O$_2$ contents were either unaltered or decreased under all stresses in shoots of PBW343 (Fig. 3A) but contents were maintained higher under SS/CS than under WS. In roots of PBW343, H$_2$O$_2$ contents were decreased under all three stresses (Fig. 3B). In shoots of C306, H$_2$O$_2$ contents were increased significantly under all three stresses at 24 h and were maintained equivalent to control values throughout the stress period except under CS at 72 h stage where these contents were decreased significantly (Fig. 3C). In roots of C306, H$_2$O$_2$ contents were increased significantly at 24 h but were decreased later at 48 and 72 h from corresponding control values under all three stresses (Fig. 3D).

In shoots of PBW343, proline contents were increased (at 48 and 72 h) under all three stresses where increases were higher (Fig. 4A) under SS/CS than under WS. In roots of PBW343, proline contents were either unaltered or increased under all three stresses where contents were higher (at 24 and 72 h) under SS than under WS/CS (Fig. 4B). In shoots of C306, proline levels (Fig. 4C) were almost unaltered under WS and were increased significantly or non-significantly under SS/CS throughout the stress period. In roots of C306, proline contents (Fig. 4D) were either unaltered or decreased under all three stresses except these were increased significantly under SS/WS at 72 h.

MDA contents in shoots of PBW343 (Fig. 4E) were increased (significantly or non-significantly) at 24 h but were decreased later (at 48 and 72 h) under all three stresses. MDA contents were increased significantly only under WS at 48 and 72 h and CS at 48 h (Fig. 4F) otherwise these were not increased under any stress at any stage in roots of PBW343. In shoots of C306, MDA contents (Fig. 4G) were significantly higher under all three stresses at 24 h but after that these were decreased to CT values under WS but remained significantly or non-significantly higher under SS/CS. In roots of C306, MDA contents were increased significantly at 24 h under WS only while under other stresses and at other stages, these contents were maintained lower than control values (Fig. 4H).

Effects on antioxidant enzymes—In shoots of PBW343, ascorbate peroxidase (APX) activities (Fig. 5A) were either unaltered or decreased under stresses but were maintained higher under SS/CS than under WS (at 48 h). In roots of PBW343, APX activities (Fig. 5B) were decreased under all three stresses throughout the stress period. In shoots of C306, APX activities (Fig. 5C) were increased significantly under
Fig. 2—Effect of different stresses (WS, SS, CS) on ascorbate contents (mg g\(^{-1}\)DW) in PBW343 shoots (A), PBW343 roots (B), C306 shoots (C), C306 roots (D); on dehydroascorbate contents (mg g\(^{-1}\)DW) in PBW343 shoots (E), PBW343 roots (F), C306 shoots (G), C306 roots (H) at 0, 24, 48 and 72 h of stress given to 4 day-old seedlings where CT is water control. Different letters indicate significant differences among stressed and control samples at each stage (Duncan’s Multiple test at \(P < 0.05\)). Inset figures represent ratio of ascorbate to dehydroascorbate in same samples.
CS only at 24 h otherwise activities were maintained almost equivalent to CT or decreased under stresses. In roots of C306, APX activities (Fig. 5D) were decreased significantly under SS but unaltered under other stresses at 24 h while at 48 and 72 h, activities were decreased under all stresses.

Catalase (CAT) activities in shoots of PBW343 (Fig. 5E) were decreased under stresses but were significantly or non-significantly higher under CS than under WS/SS. In roots of PBW343, CAT activities were either unaltered or decreased under stresses (Fig. 5F). In shoots of C306 (Fig. 5G), CAT activities were either unaltered or decreased under stresses but decreases were higher under SS than under WS/CS (at 24 h). In roots of C306 (Fig. 5H), CAT activities were decreased under stresses but activities were higher under WS/CS than under SS (at 48 h).

In shoots of PBW343 (Fig. 6A), guaiacol peroxidase (GPOX) activities were decreased significantly at 24 h but then increased significantly or non-significantly at 48 h under all three stresses where increase was higher under SS than under WS/CS. In roots of PBW343, GPOX activities (Fig. 6B) were decreased under all stresses but were higher under SS/CS than under WS at 48 h. In shoots of C306, GPOX activities (Fig. 6C) were maintained equivalent to control values under all stresses except these were increased significantly at 24 h under CS and were decreased significantly at 72 h under WS. In roots of C306 (Fig. 6D), GPOX activities were decreased significantly under stresses except these were increased under SS at 24 h and maintained equivalent to CT under SS at 24 and 48 h.

Glutathione reductase (GR) activities in shoots of PBW343 (Fig. 6E) were decreased significantly or non-significantly throughout the stress period under all stresses except under SS and CS where these were increased significantly from CT but decreases in GR activities under SS/CS were lesser than under WS. In roots of PBW343, GR activities were decreased under all stresses throughout the stress period (Fig. 6F). In shoots of C306 (Fig. 6G), GR activities were increased significantly only under CS at 24 h and under SS at 48 h otherwise activities were maintained or decreased under stresses throughout the stress period but GR levels under SS/CS were higher than under WS. In roots of C306, GR activities were either decreased or unaltered from CT under all stresses throughout the stress period (Fig. 6H).
Fig. 4—Effect of different stresses (WS, SS, CS) on proline contents (µmole g\(^{-1}\) DW) in PBW343 shoots (A), PBW343 roots (B), C306 shoots (C), C306 roots (D); on malondialdehyde (MDA) contents (µmole g\(^{-1}\) DW) in PBW343 shoots (E), PBW343 roots (F), C306 shoots (G), C306 roots (H) at 0, 24, 48 and 72 h of stress given to 4 day-old seedlings where CT is water control. Different letters indicate significant differences among stressed and control samples at each stage (Duncan’s Multiple test at P<0.05).
Fig. 5—Effect of different stresses (WS, SS, CS) on ascorbate peroxidase (APX) activities (µmole of ascorbate changed min⁻¹ g⁻¹ DW) in PBW343 shoots (A), PBW343 roots (B), C306 shoots (C), C306 roots (D); on catalase (CAT) activities (mmole of H₂O₂ changed min⁻¹ g⁻¹ DW) in PBW343 shoots (E), PBW343 roots (F), C306 shoots (G), C306 roots (H) at 0, 24, 48 and 72 h of stress given to 4 day-old seedlings where CT is water control. Different letters indicate significant differences among stressed and control samples at each stage (Duncan’s Multiple test at \( P < 0.05 \)).
Fig. 6—Effect of different stresses (WS, SS, CS) on guaiacol peroxidase (GPOX) activities (mmole of tetraguaiacol changed min\(^{-1}\) g\(^{-1}\) DW) in PBW343 shoots (A), PBW343 roots (B), C306 shoots (C), C306 roots (D); glutathione reductase (GR) activities (µmole of NADPH2 changed min\(^{-1}\) g\(^{-1}\) DW) in PBW343 shoots (E), PBW343 roots (F), C306 shoots (G), C306 roots (H) at 0, 24, 48 and 72 h of stress given to 4 day-old seedlings where CT is water control. Different letters indicate significant differences among stressed and control samples at each stage (Duncan’s Multiple test at \(P < 0.05\)).
Effect on LEA genes’ expression—LEA genes belonging to three groups; group 2, group 3, group 4 were studied for their expression level by semi-quantitative RT-PCR in shoots of both cultivars at 24 and 48 h (Fig. 7). There were total ten LEA genes used for these measurements in which four (Wdh113, Wcor410c, Td27e, Td16) were belonging to LEA group 2, one (Td29) to LEA group 4 and five (Wrab19, Wrab18, Wrab17, Wrab15, Ta-LEAgp3-like) to LEA group 3. Gene ID (NCBI Accession no.), gene name and primer sequences were same as summarized in Kaur et al7.

Td27e, Td16, Wcor410c, Td29e genes were induced same under WS and under SS in PBW343 (Fig. 7) at both 24 and 48 h but in C306, these four were induced later at 48 h and higher under WS/CS than under SS. Wdh113 was induced at 24 h and only under SS not under other stresses in PBW343. In C306, this gene was induced under all three stresses at both 24 and 48 h but induction level was higher under SS than under WS.

In PBW343, Wrab19, Wrab18, group3-like, Wrab17 were induced under SS at 24 h and under WS/CS at 48 h; Wrab 15 was induced at 48 h under all three stresses (Fig. 7). In C306, Wrab19, Wrab18 were induced at 48 h and higher under WS/CS than under SS; Wrab17 was not induced under any stress; Wrab 15 was induced under all stresses at 24 h and under WS only at 48 h; GP3-like was induced at 48 h and only under WS.

Discussion

Biomass maintenance under stress is a tolerant feature4,8. Dry biomasses of shoots were affected more than of roots in both cultivars under all three stresses (Fig. 1). Plant adapts to stresses by partitioning more biomass towards roots than towards shoots8. Biomass of shoots was comparatively less affected under SS than under WS in PBW343 and CS improved biomass from its level under WS (Fig. 1). In
ROS scavenging enzymes are shown to be involved in rather a toxic molecule in cells under stresses and recently, $\text{H}_2\text{O}_2$ shoots of PBW343 can be related to similar increases significant increases in MDA under SS at 24 h in shoots of C306 and working under SS at 24 h in shoots of C306 to salt stress. Non-significant increases in MDA were higher under SS than under WS in shoots of both cultivars. Higher levels of GR under SS than under WS in PBW343 can be correlated with higher ascorbate to dehydroascorbate ratios along with decreased dehydroascorbate contents and improved performance of the cultivar under SS than under WS. GR activities were higher under SS than under WS in C306 also, which though not related to its salt sensitivity but accompanied with higher ascorbate to dehydroascorbate ratios and lesser dehydroascorbate contents under SS in this cultivar. Higher GR and glutathione contents are related to resistance of plants under various stresses. Guaiacol peroxidases were also observed to be higher under SS than under WS in shoots of PBW343 (Fig. 6).

Among all Lea genes, Wdhn13 was the only gene which was induced more under SS than under WS in both cultivars, hence can be salt-responsive (Fig. 7). Previously, this gene was induced under water stress and was ABA-responsive in C306 cultivar only not in PBW343. In literature, Wdhn13 is reported to be induced under salt stress, low temperature and exogenous ABA and its expression has been correlated with ABA-responsiveness and abiotic stress (drought and salt) tolerance in wheat. Four LEA genes of group 2 (Td16, Td27e Wcor410c, Td29) and three LEA genes of group 3 (Wrab19, Wrab 18 and Wrab 15) were induced higher under WS and than under SS (Fig. 7) in C306 only. Previously, these same 7 genes were induced lesser under ABA than under WS, moreover ABA plus WS treatment was decreasing their levels from levels under WS in C306. In PBW343, same seven genes were induced almost same under WS and SS in the present study.

C306, biomass of shoots was comparatively affected higher under SS than under WS and CS had improved levels under SS (Fig. 1). More biomass maintenance under SS than under WS in PBW343 indicated that PBW343 may not be sensitive to salt stress. Rice genotype resistant to chilling stress, was sensitive to salt stress as compared to chilling-sensitive rice genotype. Less biomass maintenance under SS than under SS in C306 indicated the possibility of its salt susceptibility.

Maintaining or increasing ascorbate and decreasing dehydroascorbate and improving ascorbate to dehydroascorbate ratio are involved in tolerance mechanism which seems to be working under all three stresses in C306 and working under SS but not working under WS in PBW343. As CS had improved these features from their levels under WS in both roots and shoots of PBW343 (Fig. 2), it can be beneficial over WS when given alone.

MDA was higher under WS than under SS in roots of PBW343 (Fig. 4), which can be related to drought sensitivity but not salt sensitivity of the cultivar. Combined stress had significantly decreased MDA from levels under WS in roots of PBW343 (Fig. 4). MDA was higher under SS than under WS in shoots of C306 (Fig. 4), can be correlated to corresponding more declines in dry matter of shoots of the same cultivar under SS than under WS, hence sensitivity of C306 to salt stress. Non-significant increases in MDA under all three stresses at 24 h in shoots of C306 and significant increases in MDA under SS at 24 h in shoots of PBW343 can be related to similar increases in $\text{H}_2\text{O}_2$ contents in same tissues (Figs 3 and 4) otherwise no correlation was observed between MDA and $\text{H}_2\text{O}_2$. $\text{H}_2\text{O}_2$ has been reported in many reports as a non-toxic ROS molecule and its level has not been found to be correlated with MDA contents. Recently, $\text{H}_2\text{O}_2$ is considered as a signalling molecule rather a toxic molecule in cells under stresses and ROS scavenging enzymes are shown to be involved in signalling in addition to their more traditional functions in cellular protection. Certain levels of $\text{H}_2\text{O}_2$ are required to be maintained in cytosol of the cells under stresses so as to initiate ROS ($\text{H}_2\text{O}_2$-) signalling for producing stress response. In this study and also in our previous study, $\text{H}_2\text{O}_2$ contents were not increased rather decreased under WS in shoots of PBW343 (Fig. 3) hence may be related to lack of enough $\text{H}_2\text{O}_2$ signalling to produce stress response in this cultivar. However, under SS, $\text{H}_2\text{O}_2$ contents were increased and maintained higher than under WS in the same cultivar (Fig. 3), which can be related to better performance of this cultivar under SS as well as under combined stress. Proline contents were increased more under SS than under WS in shoots of both cultivars (Fig. 4). Proline contents are reported to be increased more under salt stress than under water stress.

Among antioxidant enzymes, ascorbate peroxidase and improving ascorbate to dehydroascorbate ratio are involved in tolerance mechanism which seems to be working under all three stresses in C306 and working under SS but not working under WS in PBW343. As CS had improved these features from their levels under WS in both roots and shoots of PBW343 (Fig. 2), it can be beneficial over WS when given alone.
and were induced almost same under ABA and WS as in previous study. This indicates that these genes though are upregulated by ABA but some pathway of ABA is inhibiting their expression and that pathway is coming only in C306 which can be due to ABA-higher sensitive nature of the cultivar. Presence of this pathway under salt stress not under water stress in this cultivar may be responsible for its susceptibility to salt stress. In literature, ABA higher sensitive cultivars were reported to be salt sensitive as compared to ABA-lesser sensitive cultivars in rice, A. thaliana, Brassica species. ABA-sensitivity is associated with salt-sensitivity as well as sugar sensitivity and this may involve ABI4-mediated pathway of ABA. Comparatively higher sensitivity of C306 towards salt stress as compared to PBW343 in this study may be related to some ABA-higher sensitive pathway/s of this cultivar.

Overall results obtained in the present study indicated that PBW343, drought susceptible cultivar might not be sensitive to salt stress. Higher biomass accumulation of PBW343 under SS than under WS was accompanied with higher ascorbate, higher ascorbate to dehydroascorbate ratio, significant decreases in dehydroascorbate, significant increases in proline, significantly lesser accumulation of MDA, significantly higher GPOX, higher GR under SS than under WS. CS had improved almost all these features from levels under WS. Secondly, C306, drought resistant cultivar was not found to be salt resistant as it showed lesser dry mass accumulation, comparatively lesser antioxidant enzymes like APX and CAT, more MDA levels, lesser induction levels of LEA transcripts than levels under WS. CS had improved these features from levels under SS. This differential behaviour of these cultivars towards WS and SS can be due to differential ABA-signalling under these stresses.

References