Molecular analysis of thermomemory-related genes and high-temperature tolerance in wheat genotypes

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Abstract

High-temperature stress adversely affects plant processes and has become a recurrent event. Identifying wheat genotypes that combine basal tolerance with thermomemory mechanisms without significant yield penalties is desirable. Here, we examined six wheat genotypes for their tolerance to high-temperature (HT) stress at the jointing stage. HT stress negatively affected net photosynthesis rate, membrane stability index and yield, but it enhanced canopy temperature depression, hydrogen peroxide content, malondialdehyde level, percentage radical scavenging capacity and transcript levels of thermomemory-related genes.

Using stress indices for yield component and their average ranking in combination with other traits, we ranked genotypes for their tolerance to high-temperature stress. C306 was identified as tolerant and PBW343 as susceptible to high-temperature stress treatment. The results of this study contribute to the confirmation of the expression of thermomemory-related genes in these wheat genotypes and the identified contrasting genotypes are useful for future thermomemory experiments.

Keywords: Heat, Memory, Stress index, Wheat.

Introduction

Climate change poses serious risk to global agriculture and food security.[8] The Intergovernmental Panel on Climate Change (IPCC) projects an increase in global mean temperature (2.6 – 4.8°C) and frequent incidence of intense heat waves.[21] Over the last century, the increase in ambient temperature has disturbed plant growth and development, affected overall crop productivity, especially in cool-season crops.[31]

Wheat is the second most important staple food crop of the world feeding above 35% of the world population. With an annual production of 771 million tones, wheat plays an essential role in food security.[15] Nevertheless, wheat productivity in many regions of the world is challenged by the adverse effects of rising temperature and other abiotic stresses.[60] Under High-Temperature (HT) stress condition, photosynthesis rate decreases rapidly leading to the gradual buildup of Reactive Oxygen Species (ROS)[50] which causes oxidative stress. In turn, ROS can react with membrane lipids, proteins and nucleic acids to cause lipid peroxidation, protein denaturation, membrane leakage and possibly DNA alteration.[26] Plant antioxidant mechanism composing of enzymatic and non-enzymatic antioxidants naturally reduces the level of ROS to homeostasis,[43] but under HT stress condition the balance is greatly disturbed.[3] However, higher radical scavenging ability is induced in tolerant genotypes to minimize accumulation of ROS beyond homeostasis and oxidative damage.[1]

HT stress also initiates the inactivation of heat-labile proteins by damaging their conformation and activity.[13] In cascade, it causes the loss of stability of several proteins, membrane lipids, cytoskeletal structures and enzymatic reaction efficiencies.[45] Furthermore, denatured and aggregated proteins could hamper biochemical processes and cause cytotoxicity.[60] Plants respond to HT by rapidly activating Heat Stress Response (HSR) which includes the activation of heat shock transcription factors (HSFs) stimulating the expression of heat shock proteins (HSPs). They act as molecular chaperones that stabilize other proteins against heat stress by preventing their aggregation, misfolding and denaturation.[24,53]

In many studies, over-expression of plant HSPs has been correlated with improved thermotolerance.[17] Similarly, for decades, immense attention has been given to advance transcription factors research for understanding the regulation of gene expression and accumulation of HSP to forestall the adverse effect of HT.[24]

In exploring the mechanism of heat stress tolerance, it is quite vital to evaluate the expression of heat-stress inducible genes.[25] In *Arabidopsis*, certain genes were previously identified which show sustained expression during recovery from heat stress.[32,47,48] These are generally referred to as thermomemory-associated genes because they can enhance faster response to a subsequent reoccurrence of stress.[12]

ROF1 and *ROF2* expressions have been reported in seedlings, vascular tissues and flowers, but highly expressed under heat stress.[7] Both are homologs of peptidyl-prolyl cis/trans isomerase with 85% similarities, but they have been reported to play both positive and negative roles respectively in regulating the activity of HSFA2.[36] ROF1 has been suggested to have a role in extending thermotolerance by maintaining the level of sHSPs, which are important for HT

stress survival. Under normal conditions, ROF1 binds HSPs HSP90.1 and localizes in the cytoplasm, but with exposure to heat stress, ROF 1 – HSP 90.1 complex is nuclear-localized, where HSFA2 interacts with HSP 90.1 and synthesizes small HSP transcripts.[36]

Sedaghatmehr et al[47] noted that FtsH6 negatively regulates thermo-memory through cpHSP21 abundance which showed that HSP21 in *Arabidopsis* is essential for increasing thermomemory capacity. The memory of heat stress rapidly reduces in the absence of HSA32,[11] which affects long-term but not short-term acclimation. HSP101 protein has been identified upstream of HSA32 enhancing its translation during heat stress recovery.[32]

In wheat, many studies in relation to HT stress-tolerant mechanisms have been carried out with a focus on basal and acquired tolerance mechanism.[35,41] Recently, some studies have reported stress memory in wheat.[37,56,60] This study evaluated selected wheat genotypes for their tolerance mechanism in response to HT stress and expression of thermomemory-related genes in order to identify contrasting genotypes which can be proposed for future thermomemory-related experiments in wheat.

Material and Methods

Plant Materials and temperature treatment: Six (6) spring wheat (*Triticum aestivum* L.) genotypes i.e. C 306, PBW 343, WL 711, Chiriya 7, Raj 3765 and Dharwar Dry, previously characterized for heat and drought stress tolerance[5,23] were sown in 6 inches pots in December 2017, under ambient temperature condition in the net house, from germination stage until jointing stage (i.e. Feeke's scale 6 – 7 approx. 65 – 72 days after sowing). Using the climate controlled facility of the Division of Plant Physiology, one set of three (3) replications was subjected to the HT stress of 45°C for 4 hours (between 10:30 am – 2:30 pm) daily for 5 consecutive days. Meanwhile, the second set of pots in three (3) replications was kept in ambient temperature as a control. The same light intensity, humidity, irrigation, fertilizer application and other agronomy practices were applied to both control and heat-treated pots.

Canopy Temperature Depression: Canopy temperature was measured by using a hand-held Infrared thermometer (AmiciKart® Digital Laser IR Infrared Thermometer-GM320) and measurements were taken with the thermometer held at an appropriate angle and distance from the edge of the pots. On the 5th day during stress, an infrared thermometer was used to measure the temperature of the leaves and a mercury thermometer was used to measure the ambient and control chamber temperatures. Canopy Temperature Depression (CTD) was calculated according to Reynolds et al.[44]

Net photosynthesis and SPAD Chlorophyll: Net photosynthetic rate (Pn) of the topmost fully expanded leaf was measured on the 5th day during stress with a portable photosynthesis system (LI-6400, LI-COR, Inc., USA). Measurements were performed under light-saturated conditions (1200 mmol photon m^{-2} s^{-1}) at a constant flow rate of 500 mL min^{-1} and block temperature was adjusted according to the temperature of the controlled chamber. The greenness of the same leaf samples was measured by using Minolta chlorophyll meter (SPAD-502DL Plus Konica Minolta Sensing Inc. Japan).

Hydrogen Peroxide: The determination of hydrogen peroxide content was based on the formation of titanium-hydro peroxide complex.[39] Fresh wheat leaves (0.1g) collected from the most fully expanded leaf on the 5th day during heat stress were ground in liquid nitrogen and homogenized in 5 ml chilled acetone. The homogenate was filtered with Whatmann No. 1 filter paper; 2 ml of titanium reagent and 2.5 ml of ammonium hydroxide solution were added to the filtrate for the formation of titanium-hydro peroxide complex. The reaction mixture was centrifuged at 10,000 × g for 10 min and the precipitate formed was dissolved in 2 M Conc. sulphuric acid (5ml) and then centrifuged again. The absorbance of the supernatant was measured using a spectrophotometer at 415 nm wavelength against the blank sample. Hydrogen peroxide content was expressed as μmol g^{-1} fresh weight.

Lipid peroxidation: Oxidative damage or peroxidation of lipid membranes commonly expressed as the concentration of malondialdehyde (MDA) was determined by TBARs (thiobarbituric acid reactive substances) assay and carried out as per given protocol.[19] Leaf samples (0.1 g) collected from the most fully expanded leaf on the 5th day during heat stress were crushed with liquid nitrogen and homogenized in 2 ml trichloroacetic acid (0.1%). The homogenate was centrifuged at 12,000×g for 10 min and 1 ml of MDA extract from the supernatant was added to 4 ml of 20% trichloroacetic acid containing 0.5% thiobarbituric acid. The mixture was incubated at 95°C in a water bath for 30 min and the reaction was stopped by placing the reaction tubes on ice-water.

The solution was centrifuged at 12,000×g for 10 min and the absorbance of the supernatant was measured at 532 nm and 600 nm. The concentration of MDA was determined by subtracting value for non-specific absorption at 600 nm from the absorbance at 532 nm and MDA-TBA complex (red pigment) was calculated from the extinction coefficient value (155mM^{-1} cm^{-1}).

Membrane stability index (MSI): MSI was estimated according to Sairam[46] using electrical conductivity meter (LabMan Scientific Instruments Pvt. Ltd.). 100 mg of leaf sample collected from the most fully expanded leaf on the 5th day during heat stress was kept in 10 ml double-distilled water in two sets. One set of control and heat-stressed samples was heated at 40°C for 30 min in a water bath and the electrical conductivity was estimated as C1. The second set was boiled at 100°C for 10 min and the electrical

conductivity was estimated as C2. MSI was calculated using the formula:

$$MSI = [1 - (C1/C2)] \times 100$$

Total antioxidant activity: DPPH (2, 2-Diphenyl-1-picrylhydrazyl) was used to determine the total antioxidant activity[9] by evaluating the antiradical activity of treated and control samples. Leaf sample of 0.1 g collected from the most fully expanded leaf on the 5th day during heat stress was extracted in 1 ml methanol and centrifuged at 4,000 g for 10 min and 0.1 ml of the extract was added to 3.9 ml of 0.06 mM DPPH solution. The mixture was incubated in dark and at room temperature for 30 min, after which the absorbance was recorded at 515 nm. Radical scavenging activity of DPPH in percentage was estimated using the formula below:

$$\%\ \text{DPPH radical scavenging activity} = 1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control(DPPH)}} \times 100$$

Gene expression analysis: Based on available literature, five (5) thermomemory-related genes were selected for expression analysis,[47,49,57] these include; Small HSP *TaHSP26.*6 (AF097656.1), Heat shock associated protein TaHSA32 (BJ290222.1 and CJ674683.1), TaHSP101 (AF174433.1), Metalloprotease TaFtsH2 (KX037456.1) and rotamase FK506-binding protein ROF1 (TaFKBP62c-2B; KU350629.1). Real-time quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) analysis was performed to evaluate their expression under temperature treatment in wheat genotypes.

From the most fully expanded leaf for each biological replicate on the 5th day, heat stress samples were collected immediately after 4 hours HT stress, flash-frozen with liquid nitrogen and stored in -80°C prior to RNA isolation. Using trizol method (RNAiso Plus, TaKaRa Bio Inc.) total RNA was isolated from each replicate, 5 µg of total RNA were subjected to DNase I treatment (Promega) and used for 1st strand cDNA synthesis with Oligo (dt) primer of PrimeScript IV (TaKaRa). The cDNA was quantified via NanoDrop and diluted to 20ng μl^{-1} for qRT-PCR reactions in 48-well plates in a Real-Time PCR system (Applied Biosystems, Foster City, CA) using KAPA SYBR® FAST qPCR Master Mix (2X) Kit.

Gene of interest sequences was obtained from Genbank based on blast results. Primers were designed using IDT primer designing tools (https://eu.idtdna.com/pages), the quality of primers was checked with Oligo Analyser (IDT, USA) and synthesis was done commercially.

Each qRT-PCR reaction (10 µl) consists of 3.1 µl water, 0.3 µl (200 nM) each of forward and reverse primers, 5 µl of 2X SYBR Green Master Mix and 1 µl (20ng/ul) of cDNA. The qRT-PCR program consisted of one cycle at 95 °C for 3 min followed by 40 cycles at 95 °C for 10s and 60 °C for 30 s.

After amplification, melt curve analysis was carried out using a program with one cycle of 95 °C for 15s, 60 °C for 1 min and 95 °C held in the step acquisition mode. A negative control without cDNA template was added to each plate to evaluate the overall specificity. For normalization of the total cDNA in each reaction, *TaActin* (Accession number: AB181991.1) was co-amplified as an internal control and its expression was used to normalize all data.

Each sample was biologically replicated twice with two technical replications. Data were analyzed using control samples as the calibrator for relative levels of gene expression using the $2^{-\Delta\Delta C_T}$ method.[33] An online tool, CIMminer was used to generate the heat map (https://discover.nci.nih.gov/cimminer/home.do). The sequence of primers used in this study is presented in online resources 1.

Yield-related parameters: At physiological maturity, five randomly selected wheat plants from each treatment were harvested manually, dried to constant weight and manually threshed to determine the harvest index, 1000-seed weight and grain yield per plant.

To determine the tolerant genotype, based on different index iPASTIC toolkit[40] was used to calculate and derive nine (6) yield-based indices (*viz*. tolerance index (TOL), relative stress index (RSI), yield stability index (YSI), stress susceptibility index (SSI), stress tolerance index (STI) and yield index (YI)) and the ranking for each index was derived based on Spearman's rank-order correlation coefficients.[51]

Statistical Analysis: Data obtained from the experiment were statistically analyzed using SPSS Version 20.0 (SPSS Inc., Chicago, IL, USA). Significant differences observed among means of treatments were determined by Duncan's multiple range test (DMRT) at $P < 0.05$. At least three replicates per treatments were used.

Results and Discussion

HT stress is usually recurrent in nature, hence, arises the need for plants to adapt to fluctuating conditions as sessile organisms. The objective of this study was to test the hypothesis that wheat genotypes can acquire tolerance to HT stress and also express thermomemory associated genes.

To achieve this aim, we selected six spring wheat genotypes and subjected them to HT stress i.e. 45°C for 4 hours per day for five (5) consecutive days at jointing stage and then these plants were grown at normal temperature till maturity.

Non-destructive phenotyping: In response to heat stress, the plant increases transpiration rate to reduce leaf temperature and in turn, reduce the adverse effect of stress. CTD was significantly higher in PBW343, WL711, Chiriya

7 and Dharwar dry under HT, while in C306 and Raj 3765, the CTD was almost the same under normal and HT stress condition (Fig. 1a).

Canopy temperature is an efficient index of screening plant under heat stress condition. Evapotranspiration has been shown to play a protective role against HT stress through cooling of the leaf.[56]

In recent studies, researchers proposed that the better cooling effect noted in tolerant genotypes might be due to acclimation of stomata such as higher stomatal density, bigger stomatal and pore size under heat stress.[37,61]

This is in good agreement with our results that CTD increased under HT stress and significant increment was observed in Chiriya 7, WL 711 and Dharwar Dry (Fig. 1a). These results showed that genotypic variations in CTD under HT stress observed in this study are in accordance with previous reports.[51]

Soil Plant Analysis Development (SPAD) chlorophyll evaluation was unable to statistically differentiate between the high temperature stressed plants and their controls (Fig. 1b), except for Dharwar Dry with a relatively higher greenness index (SPAD index) in control than in high temperature stressed plants.

High-temperature stress reduced the net photosynthesis significantly in all genotypes (Fig. 1c). Under control condition (i.e. ambient temperature) Chiriya 7 and Dharwar Dry had the highest net photosynthesis rate while under HT, Raj 3765 showed highest photosynthesis rate followed by Chiriya 7 and C306 respectively. In general, the reduction of net photosynthesis rate was highest in PBW 343 and lowest in two varieties (i.e. C306 and Raj 3765).

Leaves with warmer temperature than the optimum required for growth and development usually lead to perturbed physiological activities. It is widely reported that processes of photosynthesis are highly affected by HT stress and of the components of photosynthetic apparatus, the most susceptible component is photosystem II repair mechanism.[2]

As speculated by Wang et al,[56] the decline in net photosynthesis is ascribable to the reduction of photosystem II efficiency resulting from the observable changes in chlorophyll fluorescence. Similarly, under stressful condition, Greer et al[16] noted a considerable decrease in maximal photochemical efficiency of PS II.

Our result also indicates that HT stress reduced Pn (net photosynthesis rate) significantly in all the genotypes (Fig. 1c). Nevertheless, C306 relatively maintained higher Pn under stress condition. This shows that tolerant genotype could effectively minimize the adverse impact of HT stress on photosynthesis apparatus and it was previously highlighted by Allakhverdiev et al.[2]

Biochemical and physiological analysis: ROS is known to increase above homeostasis under HT or other stresses, as a result of the decline in photosynthetic capacity.[6,29,50] Hydrogen peroxide content was significantly enhanced in PBW343, WL711, Raj 3765 and Dharwar dry (Fig. 2a), but not in C306 and Chiriya 7 under HT stress as compared with control. Significantly high H_2O_2 content was observed in Raj 3765 and Dharwar Dry under HT stress.

Over-accumulation of electrons resulting from the block in photosynthetic electron transport in reaction centres of PSI and PSII and electron acceptors caused an increase in ROS production.[6] Previous report showed that HT sensitive genotypes accumulate hydrogen peroxide content (H_2O_2) more than that of tolerant genotypes under HT stress.[4]

In the present study, we also noticed a significant increase in H_2O_2 content under heat stress in most of the genotypes (Fig. 2a) suggesting that oxidative stress was experienced by the plants subjected to HT stress. Nonetheless, C306 maintained the least H_2O_2 content under HT stress.

Accumulated ROS can react with cellular membranes or thereby cause lipid peroxidation.[26] Lipid peroxidation increased significantly under HT stress (Fig. 2b) in PBW343, WL711 and Chiriya7 while in other genotypes (C306, Raj3765 and Dharwar Dry) the increase in MDA content was not significantly higher when compared with control.

MDA, a product of peroxidation of unsaturated fatty acids in phospholipids is frequently used as an indicator of that cell membrane damage caused by ROS. It is worth noting that WL711, Chiriya 7 and PBW343 had a significant increase in MDA content under HT stress (Fig. 2b). This could imply that they probably suffer more membrane damages than other genotypes *viz.* C306, Raj3756 and Dharwar dry, which had only a slight increase in MDA under HT stress condition as compared to control.

Membrane stability reduced significantly under stress condition in all the genotypes (Fig. 2c). Under HT stress, the MSI of PBW343 and WL711 were severely reduced while those of C306 and Chiriya7 maintained higher MSI (%). Under HT stress, plasmalemma lipid bilayer becomes highly fluid especially in susceptible genotypes, but lipid saturation of the membrane in tolerant genotypes enhances better membrane stability.[28]

This was found in our study where the membrane stability of WL711 and PBW343 genotypes were severely affected by the impact of the HT stress (Fig. 2c). Hence, this finding supports our observation about higher lipid peroxidation in the same genotypes.

In defence from toxic effects of ROS, plant utilizes different antioxidant pathways (enzymatic and non-enzymatic) to check excessive ROS accumulation.[43]

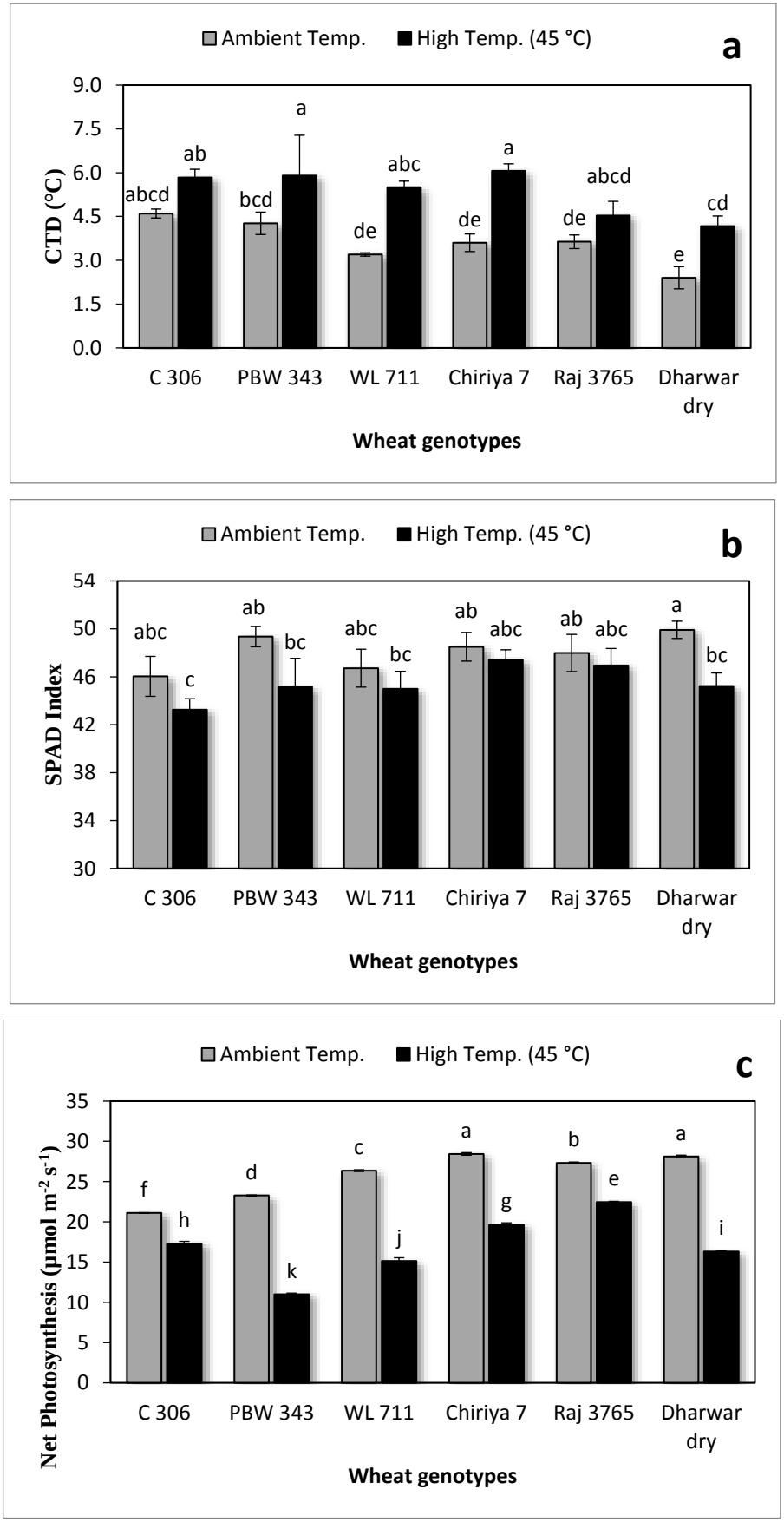


Fig. 1: (a) Canopy Temperature Depression (CTD), (b) SPAD Chlorophyll, (c) Net Photosynthesis of wheat genotypes under Ambient (Control) and High-Temperature (Treatment) conditions. Mean ± S.E (results are the average of three replications). Different lowercase letter on top of the graph indicate significantly different means (DMRT post-hoc test, $P < 0.05$)

Total antioxidant capacity (TAC) was estimated as the percentage DPPH scavenging activity of plants subjected to high and ambient temperature conditions. TAC was similar in all genotypes under non-stress conditions (Fig. 2d). HT stress increased the radical scavenging activity significantly ($p < 0.05$) in all the genotypes except WL 711 and Chiriya 7. In general, the highest total antioxidant capacity was induced by HT stress in C306 and Raj 3765. This was apparent in our findings (Fig. 2d) where the free radical scavenging activity of DPPH was greater in HT stressed plants as compared to control. Such an association was also demonstrated to occur in earlier studies.[10,22] Similarly, C306 and Raj 3756 genotypes had the highest total antioxidant activity which could probably account for the basis of lower H_2O_2 content observed in C306 genotype. A recent review by Suzuki et al also concluded that the tolerance of plants to stress combinations is resulting from the association of lower ROS accumulation or higher antioxidant capacity.

Expression of thermomemory genes: We hypothesized that subjecting wheat to HT stress i.e. 45°C for 4 hours per day for five (5) consecutive days with the recovery of approximately 16 hours each day would induce tolerance mechanism and expression of some thermomemory-related genes. Hence, expressions of thermomemory-related genes were analyzed.

ROF1 has been suggested to have a role in extending thermotolerance by maintaining the level of sHSPs which are important for HT stress survival. Under normal conditions, ROF1 binds HSP90.1 and localizes in the cytoplasm, but with exposure to heat stress, ROF 1 – HSP 90.1 complex is nuclear-localized, where HSFA2 interacts with HSP 90.1 and synthesize small HSP transcripts.[36] *TaRof1* gene was significantly up-regulated in Raj 3765 (~5.0 fold increase) and Dharwar Dry (~2.3 fold increase), while in other genotypes, HT did not alter the expression level *TaRof1* (Fig. 3a).

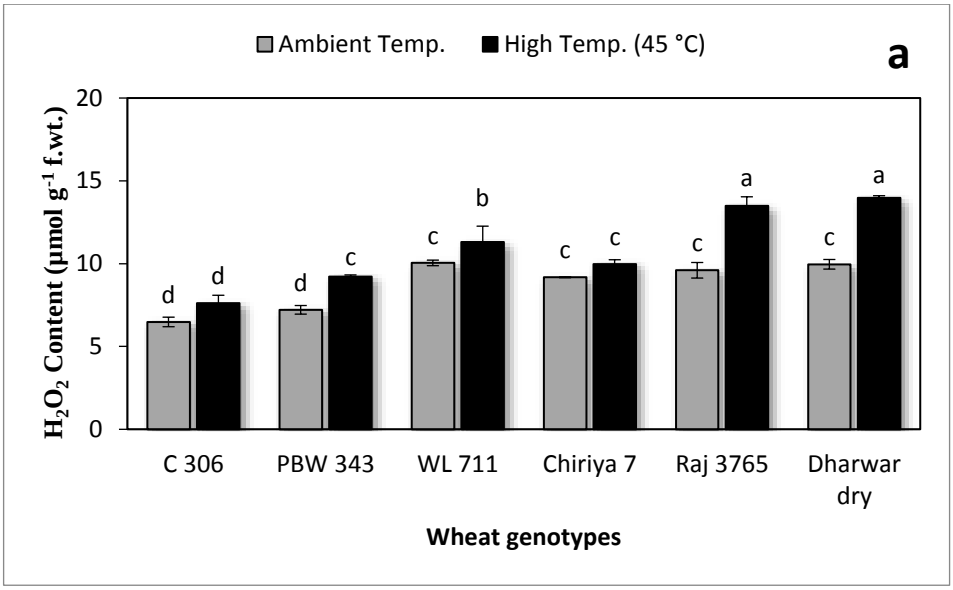
Similarly, *TaFtsH2* gene was also significantly enhanced only in two genotypes *viz.* Raj 3765 (~6.2 folds) and Dharwar Dry (~4.6 folds) under HT stress (Fig. 3b), but given the same condition, its expression in other genotypes was statistically not significant. We noted a similar expression pattern in *FtsH2* and *Rof1*, where both genes were significantly higher in the same two genotypes (Fig. 3a,b) and we speculate that this observed expression difference is a genotypic variation. Sedaghatmehr et al[47], noted that FtsH6 negatively regulate thermo-memory through cpHSP21 abundance in *Arabidopsis*. Hence, it would be essential to detect the cause upregulation in these genotypes (Raj 3765 and Dharwar Dry) per adventure, this could, in turn, lead to more protein accumulation in the absence of other post-transcriptional regulations. In plants, the first mode of defence against exposure to HT stress is proposed to be Small HSPs.[18] HSPs are associated with denaturing proteins to protect them from aggregation which can lead to proteotoxicity. They present denaturing proteins to ATP-dependent HSP100 and HSP70 chaperones, their essential role is to reduce aggregation, disaggregate and to enable effective folding during recovery.[38] In the present study, the expression of high molecular weight *TaHSP101* showed a significant increase in C306 (~2.5 folds) and PBW 343 (~2.4 folds) under HT stress (Fig. 3c) while *HSP101* was not significantly expressed in other genotypes under the same condition. Implying that, under HT stress, these two genotypes could probably prevent proteotoxic stress more than others. Although to validate this assumption, we require more experimental evidence.

The memory of heat stress rapidly reduces in the absence of HSA32[11] which affects long-term but not short-term acclimation. HSP101 protein has been identified as an upstream regulator of HSA32, as it enhances HSA32 translation during heat stress recovery.[32] Furthermore, HSA32 intensifies the stability of HSP101 and vice versa, to prevent each other from decay, suggesting a feedback loop.[57] In our experiment, the expression of *TaHSA32* was significantly up-regulated by HT treatment in C306 (8.5 fold), Dharwar Dry (~3.2 fold) and Raj 3765 (~3.0 fold) (Fig. 3d).

It is revealing that wheat genotype C306 showed a significant up-regulation of *HSA32* under HT stress. This could imply that the genotype C306 possibly will be able to acquire long-term acclimation than the other genotypes. We understand that transcript levels of a gene may not faithfully represent the abundance of proteins they encode, thus, advanced investigations focused on protein abundance. Post-transcriptional regulations are proposed.

TaHSP26.6, an orthologue of AtHSP21 is a chloroplast-localized small HSP. Lee et al[30] suggested that OsHSP21 protects chloroplast from oxidative stress. Wang et al[55] observed the maintenance of high HSP21 as a result of salicylic acid treatment during recovery from heat stress. Similarly, Sedaghatmehr et al[47] recently proved that HSP21 in *Arabidopsis* is essential for increasing thermomemory capacity. Expression of *TaHSP26.6* was significantly induced by HT in Raj3765 (~6.5 folds), C306 (~3.7 folds) and Dharwar Dry (~2.9 folds) (Fig. 3e).

Furthermore, under HT stress, denaturation of proteins is usually observed and chaperones like HSPs are induced in tolerant genotypes to reduce aggregation or misfolding of proteins.[54] Implying that lower expression of *TaHSP21* probably results in detrimental effects of cytotoxicity, this may be a reason for the highest percentage increase of both MDA content and MSI in the same two genotypes (PBW343 and WL711). However, the limitation of our treatment protocol (45°C for 4h daily and 5 consecutive days) in this experiment is focused on heat stress rather than thermomemory and prevents us from making specific assertion regarding thermomemory ability of the genes, therefore, further study to compare wheat genotypes using thermomemory treatment protocol is in progress.


Ambient Temp.
High Temp. (45 °C)
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H_2O_2 Content (µmol g^{-1} f.wt.)
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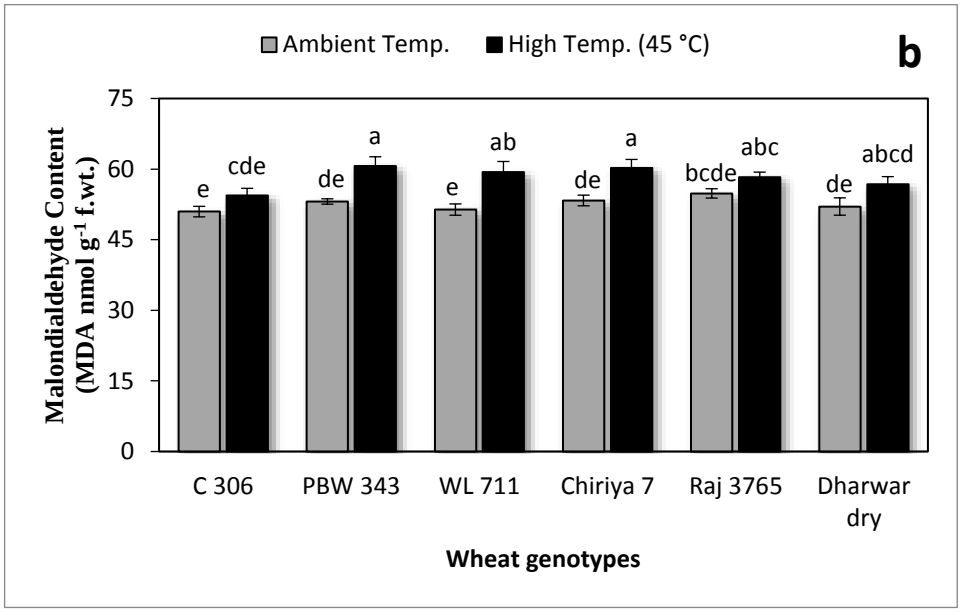
Chiriya 7
Raj 3765
Dharwar dry
Wheat genotypes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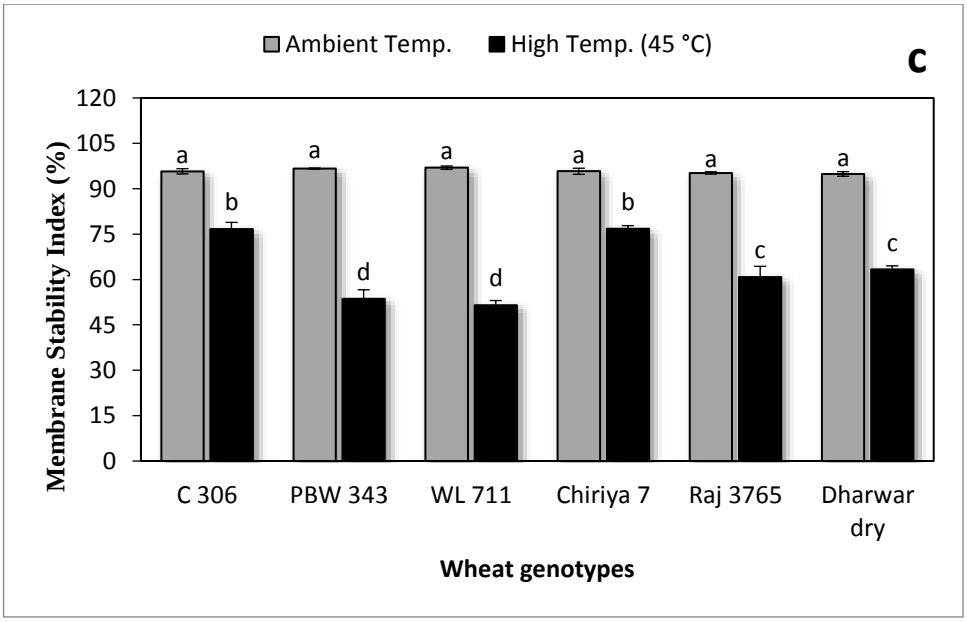
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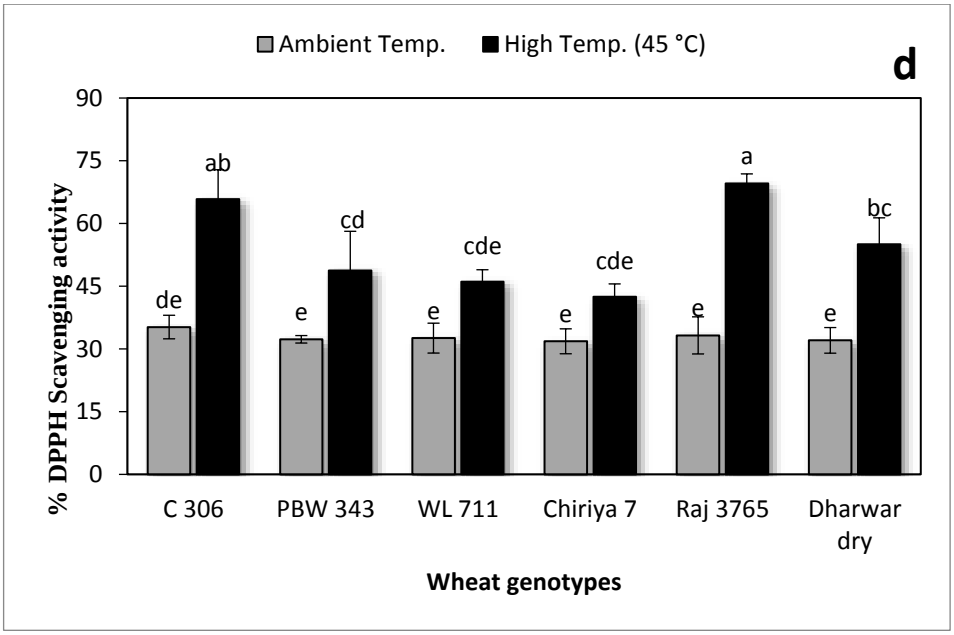
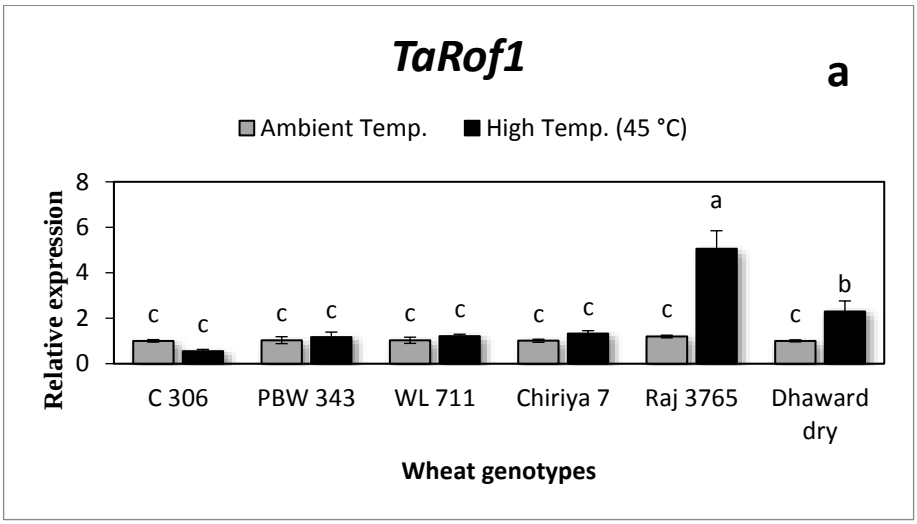
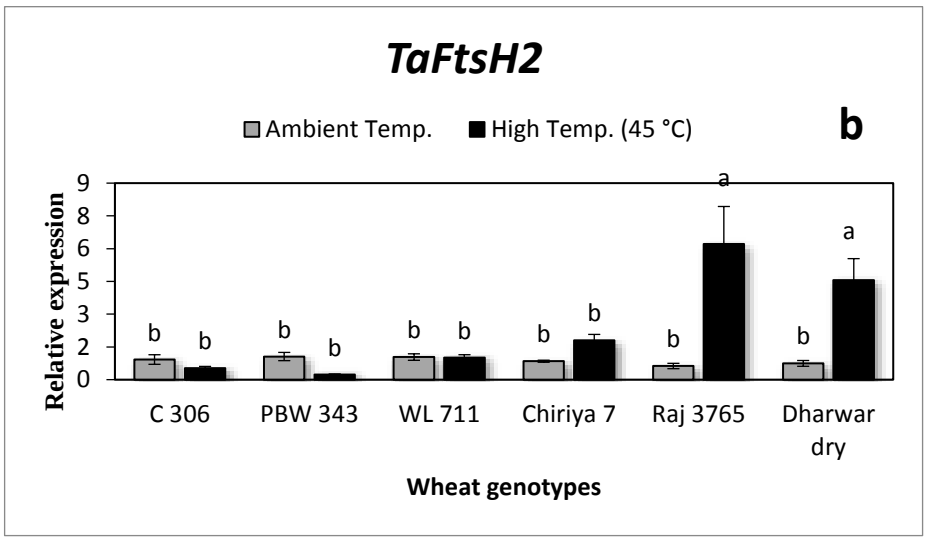


Fig. 2: (a) Hydrogen Peroxide content, (b) Lipid Peroxidation, (c) Membrane Stability Index and (d) Total antioxidant capacity of wheat genotypes under Ambient (Control) and High-Temperature (Treatment) conditions. Mean ± S.E (results are the average of three replications).
Different lowercase letter on top of the graph indicate significantly different means (DMRT post-hoc test, P < 0.05)





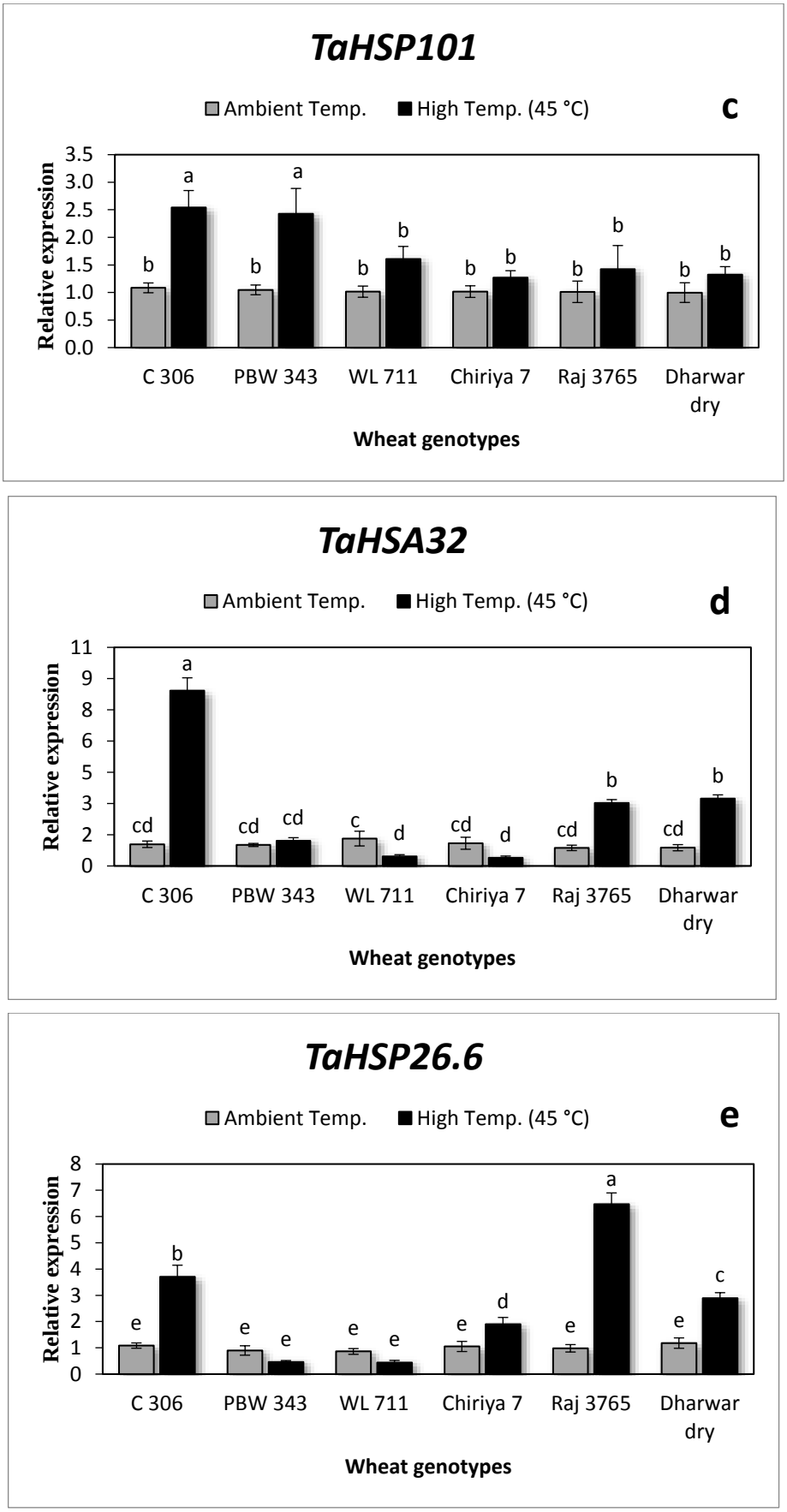


Fig. 3: Expression analysis of thermomemory-related genes; (a) *TaRof1*, (b) *TaFtsH2*, (c) *TaHSP101*, (d) *TaHSA32* and (e) *TaHSP26.6* of wheat genotypes under Ambient (Control) and High-Temperature (Treatment) conditions. Mean ± S.E (results are the average of three biological replications and two technical replications). Different lowercase letter on top of the graph indicate significantly different means (DMRT post-hoc test, P < 0.05)

Yield and its components: HT stress treatment reduced the yield of all the genotypes as compared with control (Fig. 4a). Percentage reduction in yield was quite less in C306 and Raj 3765 with approximately 14% and 18% respectively. Meanwhile, PBW 343 and Chiriya 7 had the highest yield reduction percentage of 33% and 26% respectively. The test weight of thousand grains (Fig. 4b) and harvest index (Fig. 4c) both showed a similar trend as stated above, there was no significant difference in the effect of HT stress, but the genotypic difference was apparent.

Using iPASTIC, six yield based indices were calculated (Table 1) using the yield of high temperature stressed plants and that of plants in ambient condition (i.e. controlled plants) for the six genotypes evaluated in this study. Relative change in yield resulting from HT stress for each genotype showed that C306, Raj 3765 and Dharwar Dry had the lowest percentage changes i.e. 14.17%, 18.03% and 24.73% respectively lower than their controls. Likewise, the Tolerance Index (TOL) with lower values are C306 (2.39), PBW343 (3.69), Raj 3765 (3.73) and Dharwar Dry (4.31).

Similarly, genotypes with the highest values in stress tolerance index (STI) in this study are Chiriya 7, Raj 3765 and C306. Stress susceptibility index (SSI) showed genotypes (C306, Raj 3765 and Dharwar Dry) having minimal reduction under HT stress compared to the controlled condition as tolerant genotypes. To further evaluate genotypic stability in both stressed (high temperature) and controlled condition. Yield index (YI), yield stability index (YSI) and relative stress index (RSI) were used and genotypes with the highest values are C306, Raj 3765 and Dharwar Dry.

The yield performance in ranks (Table 2) calculated for each index based on Spearman's rank-order correlation coefficients contains some additional data such as sum of ranks (SR), average sum of ranks (AR) and standard deviation (SD) which can be used to eliminate the problem that may emanate from selecting a tolerant genotype by using a single index. Therefore, average sum of ranks (ASR) provides a suitable summary of all the nine indexes. Based upon the lower values of SR, we categorize; Raj 3765, C306 and Chiriya 7 as tolerant and PBW343, WL711 and Dharwar Dry as susceptible genotypes.

Several yield-based stress tolerance and susceptibility indices have been developed to accurately estimate stress tolerance of genotypes under stressful environmental conditions.[40] In this present study, we used nine (9) indices to identify tolerant and susceptible genotypes. From the result of these indices, we observed a huge variation in the outcome of each index, making it obvious that different genotypes were identified as tolerant and susceptible. Meanwhile, our goal was to use all the indices to identify the most tolerant and susceptible genotype based on yield data.

Table 1
Yield performance of wheat genotypes under normal and high temperature condition.

Wheat Genotypes	Yp (±SE) g/plant	Ys (±SE) g/plant	RC (%)	TOL	SSI	STI	YI	YSI	RSI
C 306	16.87±0.95	14.48±0.91	14.17	2.39	0.61	0.78	1.07	0.86	1.12
PBW 343	11.07±0.76	7.38±1.01	33.33	3.69	1.43	0.26	0.54	0.67	0.87
WL 711	16.64±1.06	12.33±1.96	25.90	4.31	1.11	0.66	0.91	0.74	0.97
Chiriya 7	23.42±3.35	17.11±0.72	26.94	6.31	1.16	1.28	1.26	0.73	0.95
Raj 3765	20.69±2.11	16.96±0.73	18.03	3.73	0.77	1.12	1.25	0.82	1.07
Dharwar Dry	17.43±0.62	13.12±0.88	24.73	4.31	1.06	0.73	0.97	0.75	0.98

Control (Yp), Treatment (Ys), Relative change (RC), tolerance index (TOL), stress susceptibility index (SSI), stress tolerance index (STI), yield index (YI), yield stability index (YSI) and relative stress index (RSI)

Table 2
Ranking of wheat genotypes based on various stress index.

Wheat Genotypes	Yp	Ys	TOL	SSI	STI	YI	YSI	RSI	SR	ASR	SD
C 306	4	3	1	1	3	3	1	1	17	2.13	1.25
PBW 343	6	6	2	6	6	6	6	6	44	5.50	1.41
WL 711	5	5	4	4	5	5	4	4	36	4.50	0.53
Chiriya 7	1	1	6	5	1	1	5	5	25	3.13	2.30
Raj 3765	2	2	3	2	2	2	2	2	17	2.13	0.35
Dharwar Dry	3	4	4	3	4	4	3	3	28	3.50	0.53

Control (Yp), Treatment (Ys), Tolerance index (TOL), stress susceptibility index (SSI), stress tolerance index (STI), yield index (YI), yield stability index (YSI), relative stress index (RSI), sum of ranks (SR), average sum of ranks (ASR) and standard deviation (SD)

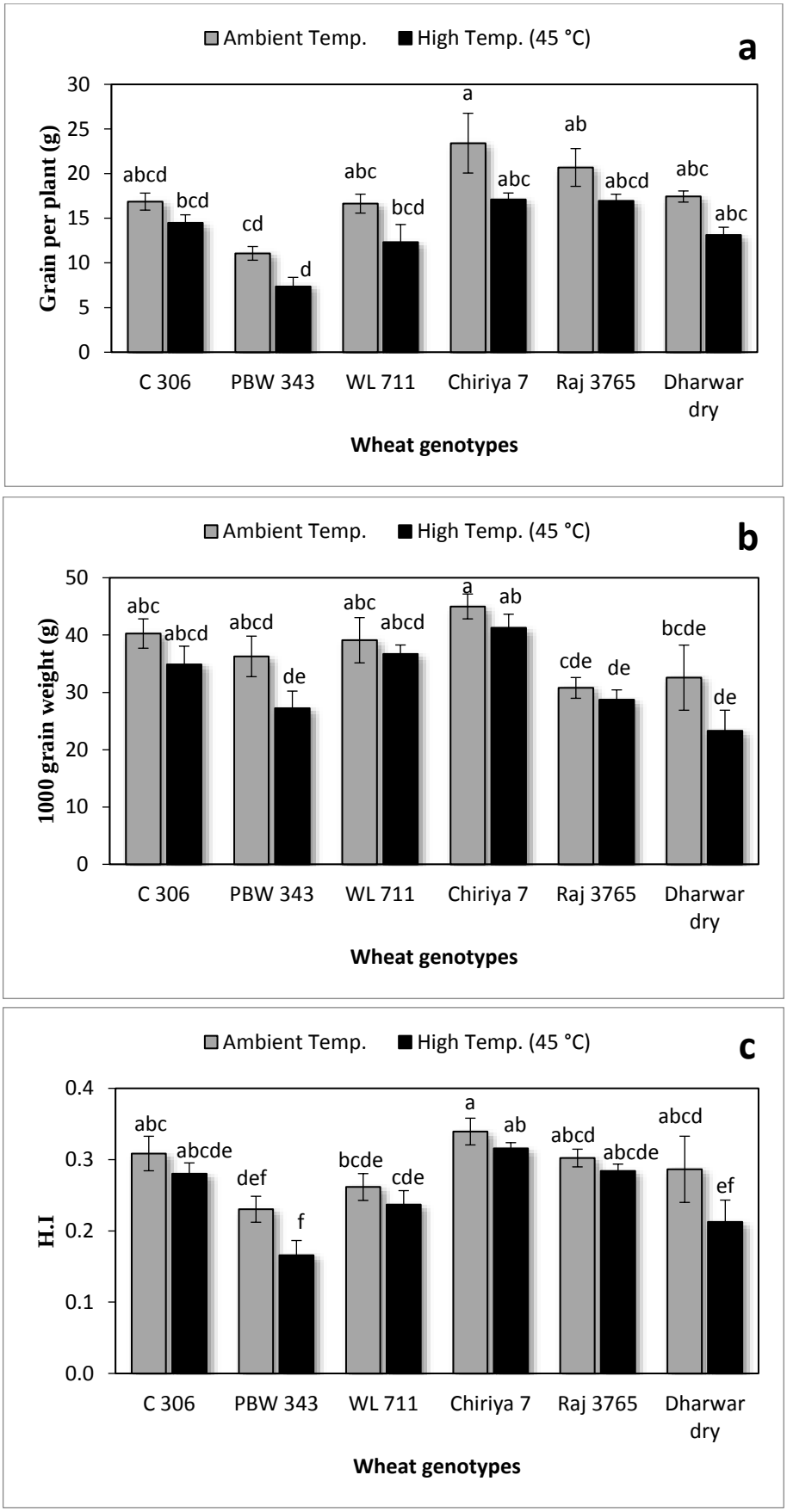


Fig. 4: Yield and its components; (a) Yield, (b) 1000 seed weight and (c) Harvest index (H.I), of wheat genotypes under Ambient (Control) and High-Temperature (Treatment) conditions. Mean ± S.E (results are the average of three replications). Different lowercase letter on top of the graph indicate significantly different means (DMRT post-hoc test, P < 0.05)

In order, to eliminate this difficulty, we used the Average Sum of Rank (ASR) of all the indices[14] provided in table 2. With this, we narrowed down to the two (2) lowest ASR value; Raj 3765 and C306, predicted as two most tolerant genotype and the highest ASR; PBW343 and WL711 as the two most susceptible genotypes.

Conclusion

Taken together all the results of our experiment, we identify C306 and PBW343 as tolerant and susceptible genotypes respectively as of interest for further thermomemory-related research. Study on the synergetic effect of salicylic acid and thermomemory treatment on these selected genotypes is currently underway to further evaluate their abilities to retain stress memory.

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