Effect of drought acclimation on oxidative stress and transcript expression in wheat (*Triticum aestivum* L.)

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**ABSTRACT**

The effects of drought acclimation on plant growth and water relations, membrane status, photosynthetic activity, proline content, membrane lipid peroxidation, enzyme antioxidant activity, and drought-responsive gene expression patterns were investigated under progressive drought conditions with drought acclimation (DA) and non-acclimation (NA) treatments in four wheat genotypes. Initial water stress applied at 10 days after seedling transfer induced acclimation to subsequent water stress (S2), following re-watering. A reversible decline in plant growth and water relations, membrane stability, and photosynthetic activity resulted in increased lipid peroxidation, reactive oxygen species, membrane injury, enzyme antioxidant activities, and 

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1. Introduction

Wheat (*Triticum aestivum* L.) is an important staple crop that is cultivated globally. The demand for wheat is estimated to increase by 60% by 2050 (Aprile et al. 2009). Wheat productivity is hampered by various abiotic stresses such as drought, salinity, and heat (Da Costa et al. 2011). Under arid and semi-arid climatic conditions, it is estimated that the detrimental effect of periodic drought stress during the growth and development seasons, adversely affect production and reduce the yield of wheat (Dhandha et al. 2004). Therefore, engineering and breeding of more efficient and improved drought-adapted wheat cultivars is important to expand the range of wheat production to arid and semi-arid regions (Lobato et al. 2009).

Drought is an abiotic stress factor that contributes globally to significant yield reduction. Water deficit induces oxidative damage and increases membrane lipid peroxidation (Jaleel et al. 2008). Plants alleviate drought-stress-mediated consequences through adaptive capacity, usually governed by the efficiency of the antioxidant defense system, including (ROS) scavenging enzymes (such as superoxide dismutase (SOD), catalase (CAT), peroxidase, (POD), and ascorbate peroxidase (APX)) and non-enzymatic components such as proline and carotenoids (Reddy et al. 2004; Vardhini and Anjum 2015). During drought stress, plant growth and water relations play a key role in the modulation of the antioxidant defense mechanism (Selote et al. 2004).

Over the years, the effect of drought stress has been well documented in many crop species (Xu et al. 2009). However, the effect of acclimation and rate of stress development based on physiological and molecular responses to progressive drought need to be thoroughly investigated. In most studies, plants were subjected to either single water deficit stress recovery cycle Bian and Jiang (2009) or two cycles of similar stress intensity without emphasis on the drought acclimation response. In nature, plants often experience cycles of water stress. Thus, plants exposed to initial water stress in the early period of seedling growth may become less sensitive to subsequent water stress of severe intensity that might occur in the later period of growth and development, a phenomenon called acclimation. Wilson and Franklin (2002), also defined acclimation as a proximal phenotypic response of a single genotype to changes in the environment, which involves reprogramming of development, physiology, and metabolism to reach a new state that improves fitness and survival.

Previously, drought acclimation treatment has been reported to limit the shoot and root accumulation of O$_2$ and membrane damage efficiently than non-acclimated seedlings during the exposure of wheat seedlings to subsequent severe water stress, following their exposure to initial stress of mild intensity (Selote et al. 2004). While this has been accepted (Khanna-Chopra and Selote 2007; Selote and Khanna-Chopra 2010), considering antioxidant defense and cellular membrane status, limited studies have addressed plant growth and water relations, membrane stability, oxidative stress, antioxidative process, and osmolyte dynamics during drought acclimation are limited.

Furthermore, one of the strategies to overcome water deficit involves the development of drought tolerant crops through advanced molecular breeding techniques and biotechnological approaches, which includes identification of responsive genes to enable manipulation (Zhu 2002). Transcription factors (TFs) are key players in the regulation of plant responses to drought stress. They are activated or suppressed by protein kinases or phosphatases and interact with specific cis-elements in the promoter region of targeted genes (Le Hir et al. 2017). The NAC TFs modulate downstream drought-inducible early response to dehydration gene
transcription by interacting with the NAC recognition sequence having a CACG core-DNA binding motif in its promoter and are involved in abiotic stress and disease resistance (Shao et al. 2015). WRKY and bZIP genes play diverse roles regulating the growth and development of plants in response to drought (Llorca et al. 2014). In addition, the basic helix–loop–helix (bHLH) and the MYB TFs are involved in a wide range of developmental processes under multiple biotic and abiotic stresses, as well as specialized metabolic pathways (Le Hir et al. 2017; Qin et al. 2012).

This study focuses on certain triggered physiological, biochemical, and molecular aspects under drought acclimation and non-acclimation in wheat. We hypothesize that firstly, the exposure of wheat seedlings to initial drought stress, will enable tolerance to subsequent water deficit. Secondly, the metabolic changes associated with the initial stress, will be greater than that with the subsequent one. Finally, the change in magnitude of investigated molecular and physiological drought response parameters during the subsequent stress period, would be greater in non-acclimated plants than in drought acclimated plants. Thus, the physiological and molecular basis of genotypic variation under drought acclimation were investigated, considering seedlings of four wheat genotypes with differential response to drought stress.

2. Materials and methods

2.1. Plant growth and treatments

Seeds of ‘Keumgang’ (National Agrobiodiversity Centre, RDA, Korea; accession no. IT 213100) and three Recombinant Inbred Lines (RILs; F5:9) of PL 337, PL 371, and PL 257 derived from the crosses between ‘Keumgang’ (KG) (female parent) and TAM201 (PI 578256), Seri82 (PI 591774), and Siete Cerros66 (PI 338921), respectively, were investigated in this study.

Seeds were surface sterilized in 70% ethyl alcohol for 2 min, treated with 0.1% HgCl for 15 min, then washed five times with sterile distilled. The seedlings were then grown in a growth chamber set to a photocycle of 13:11 h (day:times with sterile distilled. The seedlings were then grown in a growth chamber set to a photocycle of 13:11 h (day:night), 25/22°C (day/night), 80% relative humidity, and active in a growth chamber set to a photocycle of 13:11 h (day:

The seedlings were grouped into three treatments, namely, control (CK), drought acclimated (DA), and non-acclimated (NA) as shown in Figure 1. Control plants received ½ strength Hoagland solution every alternate day (i.e. from 10 to 17 days after treatment [DAT]), and sampling was performed to obtain data as CK1, CK2, CK3, concurrently with DA1, DA2, and DA3 and NA3 of the different treatment groups. First, water stress (S1) was induced by applying 10% PEG to the DA group at the fully expanded 2nd leaf stage of wheat seedlings. The water stress treatment was induced for 24 h. After 24 h, sampling was performed from the treatments at 11 DAT to obtain data as CK1 and DA1, followed by re-watering (R1) in fresh Hoagland solution for five days. Re-watering (R1) occurred between 11–15 DAT, and during this period, the solution was changed every day. At the end of R1, sampling was performed at 15 DAT to obtain data as CK2 and DA2. Following R1, there was a second stress period (S2) to determine the acclimation responses of the wheat seedlings. During this process, both droughts acclimated (DA3) and non-acclimated (NA3) plants were subjected to a 20% PEG induced-drought stress for 48 h. Sampling was performed immediately afterward to obtain data as CK3, DA3, and NA3 at the end of the treatment with CK3 as the control, DA3 as the drought acclimated plants, and NA3 as the non-acclimation plants.

2.2. Plant growth and water content

Total shoot, leaf and root dry weights, and relative water content of the control, drought acclimated, and non-acclimated plants were measured at different periods of water stress Figure 1. To assay leaf relative water content (RWC), the fresh (FW), dry (DW), and turgid (TW) weights of the leaves were estimated. All the plant samples (i.e. leaves) were heated at 105°C for 30 min and dried at 70°C for 48 h, then DW was recorded. RWC of leaves was calculated as:

\[
\text{RWC} (%) = \frac{(\text{FW} − \text{DW})}{(\text{TW} − \text{DW})} × 100
\]  

2.3. Membrane stability index (MSI) and electrolyte leakages (EL)

MSI and EL were determined indirectly by measuring electrical conductivity according to procedures described by Kocheva et al. (2005). The electrical conductivity was measured using a Waterproof Combo Meter (HM Digital Inc, Manhattan, USA). Leaf MSI and EL were estimated using the Equations (2) and (3), respectively.

\[
\text{MSI} (%) = \left[ 1 − \left( \frac{C_1}{C_2} \right) \right] × 100
\]

\[
\text{EL} (%) = \left( \frac{C_1}{C_2} \right) × 100
\]
where $C_1$ and $C_2$ represent initial and final electrical conductivity values, respectively.

### 2.4. Malondialdehyde (MDA) and H$_2$O$_2$ accumulation

MDA content was measured according to procedures previously described by Zheng et al. (2008). Leaf samples were homogenized in 1 mL 0.1% trichloroacetic acid (TCA) and centrifuged at 12 000 × g for 10 min. A 0.5 mL supernatant was mixed with 0.5 mL 0.5% thiobarbituric acid (in 20% TCA) and the mixture was heated at 100°C for 20 min.

H$_2$O$_2$ content was determined according to the procedures described by Yin et al. (2010) with some modifications. The leaves (100 mg) were extracted with 1.0 mL of TCA (0.1%, w/v) and centrifuged at 12 000 × g for 5 min. The supernatant (0.5 mL) was carefully collected, and 0.5 mL of phosphate buffer (pH 7.0) along with 1.0 mL of potassium iodide (1 M) was added. The absorbance of the mixture was read at 390 nm. H$_2$O$_2$ concentration was expressed as µmol g$^{-1}$ FW.

### 2.5. Chlorophyll, carotenoid, and proline contents

The pigments were extracted from frozen leaf samples (approximately 0.2 g) using 80% acetone on a shaker at 25°C until the tissue was completely bleached. The extract was centrifuged at 12 000 × g for 5 min, and the supernatant was gathered for absorbance measurement at 646, 470, and 663 nm using a spectrophotometer (UV-2550, Shimadzu, Japan). The concentration of each pigment was calculated according to the method described by Warren (2008). The chlorophyll and carotenoid content (µmol g$^{-1}$ FW) were then calculated.

Proline content was assessed using the ninhydrin method described by Maghsoodi et al. (2018). A 0.1 g leaf sample was homogenized in 1 mL of 3% sulfosalicylic acid. After centrifugation at 12 000 × g for 5 min, 0.5 mL of the supernatant was added to 0.5 mL of glacial acetic acid and 0.5 mL ninhydrin solution. The mixture was then heated at 100°C for 1 h, and quickly cooled on ice for 30 min, followed by the addition of 1 mL of toluene, extraction of the organic phase and reading of absorbance read at 520 nm. Proline concentration was determined using the calibration curve and expressed as µmol proline g$^{-1}$ FW.

### 2.6. Assay of antioxidant enzyme activity

To estimate the antioxidant enzymatic assays, ground leaf samples (100 mg) were homogenized in 1 mL of 0.2 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA at 4°C. After centrifugation at 15 000 × g for 20 min, the supernatant was used to determine the antioxidant enzyme (SOD, CAT, POD, and APX) activities. The methods for the enzyme activity assays are described below. Total protein content was quantified using the Bradford assay, using BSA as a standard (Bradford 1976). The activity of APX was measured using the method described by Kim et al. (2015). The reaction mixture comprised of 50 mM HEPES, 0.1 mM EDTA, 1 mM H$_2$O$_2$, and 0.6 mM ascorbic acid. The oxidation of ascorbic acid was considered as a decrease in the absorbance at 290 nm, 1 min after the start of the reaction ($e = 2.6$ M$^{-1}$ cm$^{-1}$).

CAT activity was assayed spectrophotometrically by monitoring a decrease in the absorbance of H$_2$O$_2$ at 240 nm using the method described by Behnmannia et al. (2009). The assay contained 50 mM potassium phosphate buffer (pH 7.0) and 15 mM H$_2$O$_2$. The reaction was started by the addition of 60 µL enzyme extract to the reaction mixture. The change in absorbance was determined at 1 min after the start of the reaction ($e = 43.6$ M$^{-1}$ cm$^{-1}$).

POD activity was determined using the guaiacol test, as reported by Behnmannia et al. (2009). The tetraguaiacol formed in the reaction had a maximum absorption at 470 nm, which enabled the reaction to be readily followed spectrophotometrically. The enzyme was assayed in a solution containing 50 mM phosphate buffer (pH 7.0), H$_2$O$_2$ (0.3%), and guaiacol (1%). The reaction was started by adding 40 µL enzyme extract at 25°C ($e = 25.5$ M$^{-1}$ cm$^{-1}$).

The SOD reaction solution (3 mL) comprised 50 µM p-nitro blue tetrazolium chloride, 1.3 µM riboflavin, 13 mM methionine, 75 mM EDTA, 50 mM phosphate buffer (pH 7.8), and 20–50 µL of the enzyme extract. Test tubes containing the reaction solution were irradiated under light at 78 µmol m$^{-2}$ s$^{-1}$ for 30 min. The absorbance of the irradiated solution was read at 560 nm using a spectrophotometer (APEL PD 303 UV-Vis Spectrophotometer, Tokyo, Japan). One unit of SOD activity was defined as the amount of enzyme that inhibited 50% p-nitro blue tetrazolium chloride (NBT) photoreduction (Kim et al. 2015).

### 2.7. RNA extraction, cDNA synthesis, and real-time PCR analysis

To elucidate the expression pattern of drought-responsive genes under drought acclimation, total RNA from leaf samples was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RNase free DNase (Promega, Madison, WI, USA), according to the manufacturer’s instruction.

RNA purity was checked using a nanophotometer (Implen Inc., Westlake Village, CA, USA). The purity of total RNA was assayed with ethidium- bromide stain analyses using agarose gel electrophoresis. DNA free total RNA was reverse transcribed into first strand cDNA with a Power cDNA synthesis kit (Intron Biotechnology Inc, South Korea).

Quantitative real PCR (qPCR) was performed using a CFX 96 Real-Time system (Bio-Rad, Richmond, CA, USA) with SYBR-green fluorescence and the results were analyzed using the ΔΔCT method. Gene-specific primers (Table 1) for qPCR were applied to evaluate their activity under progressive drought stress and acclimation conditions. The thermal cycle employed was 95°C for 5 min and 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s. All experiments were conducted with three biological replicates and the relative transcript levels were standardized with Actin as the internal control.

**Table 1.** List of primers used for quantitative real polymerase chain reaction (qPCR).

| TF family name | Gene identity | Primer sequence |
|---------------|---------------|-----------------|
| TabHLH1       | TraesCS3B01G553100 F: 5’ – AGTGCATCACCCAGTCAGGCAC – 3’ | \ R: 3’ – TGATCGCTGTTGCTTGGGTGT – 5’ |
| TaNAC1        | TraesCSU01G135000 F: 5’ – ACGTGGTGCTACGGGCTA – 3’ | \ R: 3’ – CTGATCTCTTCTGCTGGG – 5’ |
| TabZIP1       | TraesCSA01G409800 F: 5’ – TCTGAAACGGGGCCATTTTG – 3’ | \ R: 3’ – TGGACGCTTACAGGATCTGCT – 5’ |
| TaWRKY2       | TraesCS2A01G189700 F: 5’ – GTCGGTGTGTGGTTGTTGTTG – 3’ | \ R: 3’ – ACCATGCCACGCGCAATTTAC – 5’ |
| TaMYB2        | TraesCS2B01G118200 F: 5’ – GAATCAGCCCAACAACAGCA – 3’ | \ R: 3’ – CCCATCTTCTGTTAAACGG – 5’ |
| TaActin       | TraesCS1B02G024500 F: 5’ – ACTGTGCCCATTTACGAAGG – 3’ | \ R: 3’ – TCCTCAAGCGAGGAGTTT – 5’ |
2.8. Statistical analysis

All experiments were conducted in triplicate and the results are reported as mean ± SE. Means with different letters are significantly different (P < 0.05) according to the Duncan’s multiple range test using the R (v.3.5.1).

3. Results

Physiological response of wheat genotypes under initial drought stress (S1), re-watering (R1), and second stress (S2)

3.1. Morphological features

No visible difference was observed between the control and drought stressed plants of wheat genotypes under S1 and R1 regimes (supplementary Fig. S1A-B). However, during the exposure of plants to subsequent stress (S2), DA3 plants of PL 337 showed stunted growth and exhibited an anemic look in comparison to the control (CK3) and non-acclimated plants (NA3) (supplementary Fig. S1C).

3.2. Plant growth, water relation, and photosynthetic pigments chlorophyll and carotenoid content

S1 significantly (P < 0.05) reduced the total shoot, leaf RWC, leaf dry weight (LDW), root dry weight (RDW), and chlorophyll and carotenoid content of DA1 plants in all the wheat genotypes (Figure 2A-F). Among the wheat genotypes examined, PL 371 had the highest decline in total shoot at, 11.34%, while PL 257 had the lowest, at 5.8% (Figure 2A). PL 337 had the highest decline in leaf RWC, at 28.93%, while PL 257 had the lowest at 8.64% (Figure 2B). The LDW ranged from 35.71% in PL 371 – 5.88% in PL 257 (Figure 2C). PL 371 had the highest decline in RDW at 67.79%, while the decline in PL 257 was 20% (Figure 2D). In relation to the decline in the photosynthetic pigments, the chlorophyll content ranged from 43% in KG to 27.82% in PL 257 (Figure 2E). The carotenoid content also showed the highest decline in PL 371, at 32.78%, while KG showed the lowest, at 6.71% (Figure 2F).

Following the initial stress treatment, DA1 plants were exposed to recovery treatment for five days through the reintroduction of water (R1). The comparative evaluation of the DA2 plants of all wheat genotypes under R1 indicated that R1 caused significant (P < 0.05) reduction in plant growth, water relation, and photosynthetic pigments of wheat genotypes compared to that in the control treatment plants (Figure 3). Comparison based on total shoot MSI indicated a significant (P < 0.05) decline, ranging from 9.79% in PL 257–7.497% in PL 371 (Figure 3A). The leaf RWC of plants further declined in all wheat genotypes except in PL 371, where the leaf RWC recovered to the level in control plants (Figure 3B). In contrast, PL 371 had the highest reduction in LDW at 36.84%, while PL 257 had the lowest at 4.75% (Figure 3C). Furthermore, the RDW of PL 257 was similar to that of the control plants, relative to a 44.45% decline in RDW recorded for KG (Figure 3D). The decline in chlorophyll content ranged from 15.96% in PL 257 – 5.65% in KG (Figure 3E), while PL 371 showed the highest decline in carotenoid content, at 19.96%, and PL 337 showed the lowest, at 11.40% (Figure 3F).

In addition, S2 caused a significant (P < 0.05) reduction in the total shoot, leaf RWC, LDW, RDW, and chlorophyll and carotenoid content in both drought-acclimated (DA3) and non-acclimated (NA3) plants compared with that in the control plants (Figure 4A-F). The exception to this was that the RDW of DA3 plants of PL 371 was similar to that of the control (CK3) plants (Figure 4D).

3.3. Membrane stability, H2O2 accumulation, lipid peroxidation, and osmolyte concentration

Estimation of membrane status, ROS and osmolyte accumulation, and membrane lipid peroxidation in the shoot tissues of DA1 plants of wheat genotypes under S1 revealed that...

Figure 2. Effect of initial drought stress (S1) on total shoot (A), leaf relative water content (RWC) (B), leaf dry weight (C), root dry weight (D), chlorophyll content (E), and carotenoid content (F) of control (CK1) and drought acclimation (DA1) plants of four wheat genotypes. Values are presented as mean (± S.E.) independent triplicate (n = 3). Means with different letters are significantly different (P < 0.05) according to Duncan’s multiple range test using the ‘R’ software. Stress legends are indicated in Figure 1.
increased EL, H$_2$O$_2$, accumulation, MDA, and proline content was maximum at 64.20% (PL 337), 25.67% (KG), 54.49% (PL 337), and 14.49% (PL 257), respectively, while the minimum increase in these parameters were recorded at 43.01% (PL 257), 5.61% (PL 337), 25.81% (PL 371), and 5.6% (PL 371), respectively (Figure 5B–E). Overall, the maximum decline in MSI was recorded at 19.79% in KG, while PL 371 showed the minimum decrease in MSI at 8.7% (Figure 5A).

Furthermore, R1 resulted in the recovery of MSI and EL in PL 337 and PL 371 (Figure 6A and B), H$_2$O$_2$ in all wheat genotypes except KG (Figure 6C), and proline content in PL 371 to the level in the control plants (Figure 6E). The exception to this was that the MDA content consistently and significantly increased in DA2 plants, with the maximum increase observed at 40.75% for PL 337, while the minimum increase was recorded for PL 371 at 15.13% (Figure 6D).

Similarly, during S2, both DA3 and NA3 plants experienced a significant increase in membrane status, ROS and osmolyte accumulation, and lipid peroxidation (Figure 7B–E). For instance, the maximum increase in EL (19.81%), H$_2$O$_2$ (21.69%), MDA (44.78%), and proline content (35.47%) were observed in DA3 plants of KG and PL 337, while the maximum increase in EL (71.99%), H$_2$O$_2$ (5.54%), MDA (67.35%), and proline (45.51%) were recorded...
in NA3 plants of KG and PL 371 compared to the levels in the control (CK3) plants (Figure 7B–E).

3.4. Enzyme antioxidant activities

Exposure of seedlings to S1, led to significant (P < 0.05) enhancement in the activities of antioxidant enzymes in DA1 plants (Figure 8A–D). For instance, SOD activity increased by 16.25% (KG), 13.30% (PL 337), 22.73% (PL 371), and 24.29% (PL 257); CAT activity recorded an increase of 15.13% (KG), 24.13% (PL 337), 24.17% (PL 371), and 14.37% (PL 257); POD showed an increase of 52.61% (KG), 52.88% (PL 337), 55.73% (PL 371), and 60.27% (PL 257); while APX activity also recorded an increase of 55.37% (KG), 58.86% (PL 337), 65.01% (PL 371), and 58.90% (PL 257) compared with that in the control (CK1) plants.

Unlike CAT and APX activity, R1 resulted in the recovery of the activity of SOD in DA2 plants of PL 337 and PL 371, as well as POD activity in DA2 plant of PL 371 to the level of the control (CK2) plants (Figure 9A–D).

Similarly, NA3 plants exhibited higher SOD, CAT, POD, and APX activity than DA3 plants during S2 (Figure 10A–D). Thus, NA3 plants showed a significant increase of 38.57% (KG), 28.06% (PL 337), 51.88% (PL 371), and 49.15% (PL 257) for SOD; 22.01% (KG), 24.56% (PL 337), 25.34% (PL 371), and 21.10% (PL 257) for CAT; 26.71% (KG), 17.91% (PL 337), 8.56% (PL 371), and 14.77% (PL 257) for POD; and 59.05% (KG), 58.87% (PL 337), 65.03% (PL 371), and 60.30% (PL 257) for APX activity.
257) and 37% for (POD); and 44.57% (KG), 58.65% (PL 337), 60.78% (PL 371), and 54.89% (PL 257) for APX. Conversely, DA3 plants recorded 14.91% (KG), 20.06% (PL 371), 40.88% (PL 371), and 20.14% (PL 257) increase for SOD; 11.86% (KG), 14.13% (PL 371), 14.49% (PL 337), and 8.09% (PL 371) increase for CAT; 22.46% (KG), 5.80% (PL 337), 4.37% (PL 371), and 17.57% (PL 257) increase for POD; and 24.45% (KG), 38.96% (PL 337), 25.10% (PL 371), and 15.23% (PL 257) increase for APX activity (Figure 10A–D).

3.5. Gene expression studies

Drought stress regulates the expression of myriad genes Vishwakarma et al. (2017). In the present study, qPCR was performed to evaluate the expression pattern of drought-responsive genes, under drought stress and acclimation (Table 1). Results showed that the expression pattern of drought-responsive genes was significantly ($P < 0.05$) induced during the exposure of DA1 plants of wheat genotypes to S1 (Figure 11). For instance, the expression of TaWRKY2, increased by 2.8-, 2.1-, 1.6-, and 2.5-fold in DA1 plants of KG, PL 337, PL 371, and PL 257 wheat genotypes, respectively (Figure 11A). The TaNAC1 gene also showed a significant ($P < 0.05$) 2.9-, 1.2-, 2.6-, and 2.4-fold increase in KG, PL 337, PL 371, and PL 257 wheat genotypes, respectively (Figure 11A). The TaNAC1 gene also showed a significant ($P < 0.05$) 2.9-, 1.2-, 2.6-, and 2.4-fold increase in KG, PL 337, PL 371, and PL 257 wheat genotypes, respectively (Figure 11A). The TaMYB2 gene also showed a significant ($P < 0.05$) 2.9-, 1.2-, 2.6-, and 2.4-fold increase in KG, PL 337, PL 371, and PL 257 wheat genotypes, respectively (Figure 11B). Consistently, the expression of TabHLH1, TabZIP1, and TaMYB2 increased in a similar pattern as that of TaNAC1 and TaWRKY2 (Figure 11A). Interestingly, the highest gene expression level (3.2-fold) was recorded for the TaMYB2 gene in the DA1 plants of PL

![Figure 7](image)

Figure 7. Effect of second drought stress (S2) on membrane stability index (A), electrolyte leakage (B), H$_2$O$_2$ content (C), malondialdehyde (MDA) (D), and proline content (E) of control (CK3) and drought acclimation (DA3), and non-acclimation (NA3) plants of four wheat genotypes. Values are presented as mean ± S.E. of three independent experiments ($n = 3$).

![Figure 8](image)

Figure 8. Effect of initial drought stress (S1) on the activities of superoxide dismutase (SOD) (A), catalase (CAT) (B), peroxidase (POD) (C), and ascorbate peroxidase (APX) (D) of control (CK1) and drought acclimation (DA1) plants of four wheat genotypes. Values are presented as mean ± S.E. of three independent experiments ($n = 3$).
257, while the TabHLH1 gene showed the least increase (0.98-fold) in the DA1 plants of PL 337 (Figure 11E). Similarly, the expression level of the drought-responsive genes used in the study significantly increased in wheat genotypes after re-watering (Figure 12A–E).

Furthermore, drought-responsive genes were significantly (P < 0.05) induced in both DA3 and NA3 plants of wheat genotypes during S2 (Figure 13A, B, D, and E). The TabHLH1 gene was relatively higher (4.5-fold increase) in DA3 plants of PL 257, whereas TaNAC1 showed the lowest expression in DA3 plants of PL 257 (Figure 13B and E). It is noteworthy that although the drought responsive- genes were significantly and differentially induced in both DA3 and NA3 plants of wheat genotypes during S2, the magnitude of expression of these genes (TaWRKY2, TaNAC1, TaMYB2, TabZIP1, and TabHLH1) was greater in NA3 plants than that in the DA3 plants (Figure 13A–E). Specifically, the maximum expression pattern for TaWRKY2, TaNAC1, TaMYB2, TabZIP1, and TabHLH1 was recorded at 2.5- (PL 257), 0.21- (KG), 0.29- (KG), 0.19- (PL 337), and 0.35- (PL 337) fold, respectively, in DA3 plants compared to the control (CK3) levels, while a maximum expression at 3.5- (KG), 2.6- (KG), 3.10- (KG), 3.5- (PL 337), and 3.4- (PL 337) fold for TaWRKY2, TaNAC1, TaMYB2, TabZIP1, and TabHLH1, respectively, was observed in NA3 plants compared to the level in the control (CK3) plants (Figure 13 A–E).

4. Discussion

4.1. DA plants maintained enhanced plant growth and leaf water relation

In the present study, PEG-induced drought stress (S1) and acclimation (S2), significantly affected the growth and water content of drought acclimated (DA3) plants. Assessment of the total shoot, leaf dry weight, and leaf RWC are recognized as the most important screening indices for evaluating plant growth and water status, and for discriminating between

Figure 9. Effect of re-watering (R1) on the activities of superoxide dismutase (A), catalase (B), peroxidase (C), and ascorbate peroxidase (APX) of control (CK2) and drought acclimation (DA2) plants of four wheat genotypes. Values are presented as mean ± S.E. of three independent experiments (n = 3).

Figure 10. Effect of second stress (S2) on the activities of superoxide dismutase (A), catalase (B), peroxidase (C), and ascorbate peroxidase (APX) of control (CK3), drought acclimation (DA3), and non-acclimation (NA3) plants of four wheat genotypes. Values are presented as mean ± S.E. of three independent experiments (n = 3).
drought tolerant wheat genotypes (Bayoumi et al. 2008). In this study, initial drought treatment (S1), significantly and differentially reduced the total shoot, leaf and root dry weight, and RWC of DA1 plants of wheat genotypes (Figure 2A–D). However, during subsequent water deficit (S2) following re-watering (R1), non-significant increase in the total shoot and leaf dry weight, but not in leaf RWC, was observed in drought acclimated (DA3) plants compared to that in the NA3 plants (Figure 4A, B, C, and D). In relation to the leaf RWC, although wheat genotypes showed a differential leaf RWC during S2, the leaf RWC of DA3 plants of PL 371 was similar to that of the control (CK3) (Figure 4B). This indicates that acclimation treatment enabled DA3 plants, particularly PL 371, to retain higher water content than NA3 plants. This also formed the initial line of response to acclimation. Consistent with our data, Khanna-Chopra and Selote (2007) reported enhanced plant water relation in drought acclimated plants than non-acclimation plants. Similarly, Selote and Khanna-Chopra (2010) observed that the exposure of wheat seedlings to subsequent water deficit, prior to an initial one, enabled drought-acclimated plants to maintain a higher root RWC than the non-acclimated plants. In addition, as previously reported by Efeoğlu et al. (2009), maintenance of a good water relation under water deficiency conditions is a good indicator of physiological functioning and growth in plants. Therefore, it can be argued that the higher plant water relation in DA3 is a true indication of a better control of stomatal water loss, enhanced osmotic accumulation and adjustment towards the maintenance of tissue turgor and physiological activities than in NA3 plants.

In addition, morphological attributes of the wheat genotypes under control, drought acclimation and non-acclimation

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**Figure 11.** qRT-PCR analysis of expression patterns of *TaWRY2* (A), *TaNAC1* (B), *TaMYB2*, *TabZIP1* (C), and *TabHLH1* (D) genes under first water stress (S1) in control (CK1), drought acclimation (DA1) of four wheat genotypes. Values are presented as mean ± S.E. of three independent experiments (*n* = 3).

**Figure 12.** qRT-PCR analysis of the expression patterns of *TaWRY2* (A), *TaNAC1* (B), *TaMYB2*, *TabZIP1* (C), and *TabHLH1* (D) genes under re-watering (R1) in control (CK2), drought acclimation (DA2) of four wheat genotypes. Values are presented as mean ± S.E. of three independent experiments (*n* = 3).
condition indicated a noticeable effect of drought stress (S2) on the growth and development of DA3 plants of the PL 337 wheat genotype (supplementary Fig. S1 A–C). This suggests that genotypic variation exists between the wheat genotypes in response to drought stress and acclimation.

4.2. Drought acclimated plants suffered less membrane damage

ROS have been considered as a central component of the plant’s adaptive response to water deficit. Water stress is intimately related to ROS production, particularly H$_2$O$_2$ and O$_2^-$ under drought conditions, resulting in membrane damage and electrolyte leakage (Bian and Jiang 2009; Kumar et al. 2015). Even so, the accumulation of ROS has been reported to impede succinic acid oxidation, decrease membrane potential, and enhance protein oxidation in the leaves of wheat (Bartoli et al. 2004). Therefore, MSI, EL, H$_2$O$_2$ accumulation, and lipid peroxidation (MDA level) have been extensively used for the assessment of drought tolerant ability in wheat genotypes (Figures 5–7 A–D). In this study, the exposure of wheat seedlings to S2 led to differential membrane damage (MSI and EL) and ROS accumulation (Figure 7A–D). The maximum decrease (6.8%) and minimum increase (8.43%) in MSI and EL, respectively, were observed in DA3 plants of PL 337 and KG, respectively, compared to that in NA3 plant (Figure 7A and B). Consistent with Khanna-Chopra and Selote (2007); Kumar et al. (2017) better membrane stability and lower membrane damage have been shown in drought acclimation of plants in drought-resistance wheat genotype exposed to drought stress and acclimation treatment compared to that in the control plants (Figure 7A and B).

Furthermore, the MDA levels in DA3 plants remained similar to the levels in control plants of PL 371 when plants were exposed to S2 treatment while drought stress caused a significant increase in the MDA content of NA3 plants (Figure 7D). MDA has been reported as a marker of lipid peroxidation in plant cells and has been used to evaluate plant response to the effect of drought stress Shi et al. (2011). From their study, Khanna-Chopra and Selote (2007) reported that drought acclimation treatment by the exposure of wheat plants to initial water deficit enabled wheat plants to limit the accumulation of H$_2$O$_2$ during exposure to subsequent water stress treatment. In addition, S1 treatment increased the H$_2$O$_2$ levels, eased the lethal effect of salinity on seedling growth by a minimal change in growth parameters, water potential, and membrane permeability (Wahid et al. 2007).

Based on our findings, the reduced membrane damage in drought acclimated (DA3) plants may be due to a lower production of ROS, particularly H$_2$O$_2$ (Figure 7C and D), protecting the DA3 plants from S2 treatment by improving ROS scavenging or prevention of cellular damage associated with drought stress.

4.3. Chlorophyll, carotenoid, and proline content under drought acclimation

Chlorophyll, a photosynthetic pigment, is involved in light absorption and plays an important role in plant photosynthesis. As drought stress can accelerate chlorophyll decomposition, chlorophyll content has been reported as the most frequently used metrics for ascertaining the severity of drought stress (Efeoğlu et al. 2009; Ying et al. 2015). As expected, we observed that the chlorophyll content in DA1 plants significantly decreased under S1 (Figure 2E). Furthermore, we found a significant positive correlation between chlorophyll content, initial drought stress, and drought recovery. In addition, during S2, DA3 plants comparatively maintained significantly higher chlorophyll content than that in NA3 plants (Figure 4E). Although the effect of drought stress has been well documented in wheat, little is known about the role of this photosynthetic pigment under drought acclimation condition. Aranjuelo et al. (2010) reported that the maintenance of higher chlorophyll content under severe drought stress may help plants reduce photo-oxidative damage, which occurs when photosynthesis is inhibited and light excitation energy is in excess. Our study, therefore,
provides a new insight into the physiological role of chlorophyll during plant shoot growth under drought acclimation and non-acclimation conditions (Figure 4F).

Carotenoid performs an important role in heat dissipation of excess excitation energy in the photosynthetic apparatus which helps prevent the initial formation of superoxide in plants receiving excess light energy as photosynthesis declines under drought conditions (Reddy et al. 2004). In this study, the higher carotenoid content exhibited by DA3 plants than NA3 plants under S2 (Figure 4F), indicates the possible role of carotenoids in preventing ROS accumulation in the chloroplast through photoprotection of the photosystem. Consistent with the results of the study, Abid et al. (2018), reported an increased in the carotenoid content of wheat seedlings exposed to severe water stress than those exposed to moderate stress.

Proline can accumulate in high concentrations without any detrimental effect on cellular macromolecules. Proline acts as a compatible osmolyte and provides protection against membrane damage and protein denaturation during severe drought stress (Ain-Lhout et al. 2001). In this study, a noticeable increase in proline was observed in DA1 plants and NA3 plants under S1 and S2, respectively (Figures 5–7E). Abid et al. (2018) reported elevation in the proline content of drought tolerant wheat genotypes under moderate stress than under severe stress condition, which does not support our data. Based on our observations, initial drought stress enables tolerance to subsequent water stress in drought acclimated plants, evidenced by a lower proline content in DA3 than that in NA3 plants.

4.4. Antioxidant enzyme activity under drought acclimation

Antioxidant enzymes, such SOD, CAT, POD, and APX can rapidly scavenge ROS to minimize oxidative stress damage (Ashraf 2009). SOD constitutes the first line of defense against ROS in plants by catalyzing the detoxification of superoxide O2 \(^{-}\) to H\(_2\)O\(_2\) and O\(_{2}\) \(^{-}\) (Alschner et al. 2002; Ashraf 2009). In this study, an increase in SOD activity was observed in DA1 plants and NA3 plants under S1 and S2, respectively (Figures 8A, 10A). A significant upregulation of SOD activity was observed in the roots of non-acclimated plants resulting in higher H\(_2\)O\(_2\) levels in non-acclimated than drought-acclimation wheat seedlings under episodic drought stress and recovery treatment Selote and Khanna-Chopra (2010) which is consistent with our results. Enhanced SOD enzyme activity in drought acclimated plants indicated better protection capacity against oxidative damage.

Furthermore, initial water stress (S1) led to an elevation in the activity of CAT in drought acclimated (DA1) plants and re-watering (R1) resulted in a further increase in CAT activity in DA2 plants (Figure 9B). However, the imposition of S2 caused further elevation of CAT in non-acclimated plants (NA3) than in DA3 plants (Figure 10B). Consistent with the results of this study, Khanna-Chopra and Selote (2007) reported a greater increase in CAT activity in non-acclimation plants than drought acclimated plants of drought tolerant wheat genotypes, when seedlings were subjected to subsequent water stress of higher intensity after a mild one. Enhancement in CAT activity could explain the oxidation of harmful substances, resulting in the restoration of growth after re-watering. Moumeni et al. (2011) suggested that exposure of tomato plants to initial drought enhanced their tolerance to subsequent water deficit oxidative stress by increasing the activity of CAT in the fruits of non-acclimated than that of drought acclimated tomato plants. The results of this study indicate that the exposure of DA1 plants to initial drought protected them from subsequent damage associated with S2, by enhancement of CAT activity.

Members of the oxidoreductase family responsible for catalyzing hydrogen peroxide-dependent oxidation reaction are the peroxidases. Peroxidases are known for their numerous physiological and biochemical functions such as protection of tissues against physical damage and defense against pathogens and stress (Aijla and Rao 2009). In this study, POD and APX activity significantly increased in DA1 plants under S1 and in NA3 plants during S2 compared to that in their respective controls (Figures 8–10C and D). Previously, increase in POD and APX activity has been reported to act as mechanism of plant tolerance to drought (Zoz et al. 2013). Selote and Khanna-Chopra (2010) showed an elevation in POD and APX enzymes activity in the roots of drought acclimated and non-acclimated plants during the initial and second stress period, respectively, which is consistent with the findings from our study. From their study on the response of drought tolerant and susceptible wheat genotypes to the effect of drought acclimation, relatively, a greater magnitude of increase in POD and APX activities was observed in non-acclimated plants of drought tolerant wheat genotypes than in drought acclimated plants compared to the control which shows minimum oxidative damage to cellular components through a well-coordinated antioxidant system (Khanna-Chopra and Selote 2007).

4.5. Drought responsive gene expression pattern under drought acclimation

WRKY, one of the largest and important TF families in plants, is involved in various stress response and tolerance against drought (Karkute et al. 2015); (Yamasaki et al. 2013). Previous reports have shown the involvement of wheat WRKY gene (TaWRKY2) in multiple aspects of plant growth, development and stress responses (Niu et al. 2012). From their study on drought tolerance in Arabidopsis, Jiang et al. (2012) reported a higher expression of WRKY21 under drought stress, which contributed to improved drought tolerance by elevating the ABA level. In this study, the expression of WRKY2 increased in DA1 plants of wheat genotypes under S1, while NA3 plants experienced a higher expression level of WRKY2 than that in DA3 plants under S2 (Figures 11–13A) and was even induced at a greater magnitude in all wheat genotypes except in PL337, enabling NA3 plants to withstand the damaging effects associated with S2, different from the enhanced tolerant response to S2 in DA3 plants, evidenced from their acclimation to S1 (Figures 11–13A). Consistent with the findings of our study, Todaka et al. (2012) reported that the overexpression of TaNAC2 and TaNAC29 in Arabidopsis resulted in enhanced drought, salinity, and cold stress tolerance along with higher transcript levels of stress responsive genes and improved physiological parameters.

The NAC gene family is the largest plant specific TF (Shao et al. 2015). Previously, NAC genes have been reported to be induced during early stages in rice and have also been shown
to have differential expression patterns such as tissue-specific, developmental stage-specific or stress-specific expression, thereby suggesting their active involvement in the complex signaling networks during plant stress responses (Hong et al. 2016). In this study, the expression of the NAC gene (TaNAC1) increased under initial and subsequent drought stress in DA1 and NA3, respectively. Similarly, in comparison with the expression of NAC1 gene in both DA3 and NA3 plants, the magnitude of expression of this drought inducible protein was relatively greater in NA3 plants of the different wheat genotypes than in DA3 plants (Figures 11–13B). Thus, the onset of S2 enhanced NAC1 transcript accumulation making the gene, in part, responsible for drought tolerance in drought acclimation treated plants.

MYBs comprise a large family of TFs in plants and regulate plant specific processes and biological functions such as phenylpropanoid metabolism, biotic and abiotic stresses, and plant defense (Hichri et al. 2011; Segarra et al. 2009). Previously, a member of the MYB TF has been reported to be upregulated during drought, ABA, and salt treatments (Qin et al. 2012). Furthermore, the ectopic over-expression of TaMYB33 in Arabidopsis enhanced tolerance to salt and drought stresses with no growth inhibition in wheat seedlings (Yang et al. 2012). In this study, because the drought acclimated plants were exposed to successive stresses, the expression of TaMYB2 was reduced in DA3 plants in comparison to that in NA3 plants during S2 treatment (Figures 11–13C). This indicates that TaMYB2 might be a potential candidate drought tolerant gene in wheat. Consistent with our data, Yang et al. (2012) reported that overexpression of a MYB gene (OsMYB2) in rice resulted in increased soluble sugars and proline accumulation owing to upregulation of proline synthesis and transport related genes, and less accumulation of H2O2 and MDA under salt stress. Our study clearly showed enhanced osmolyte and H2O2 accumulation, as well as less membrane damage in DA plants of wheat genotypes than in their NA3 counterparts, during S2.

Furthermore, the basic leucine zipper (bZIP) family is noted for regulating plant growth and development, crucial for abiotic stress response such as drought, and regulating the expression of stress-related genes in an ABA-dependent manner after binding with the promoter region of specific ABRE (Llorca et al. 2014). A previous study showed that over-expression of a bZIP gene (OsbZIP16) exhibited significantly higher drought tolerance at both the seedling and tillering stages in transgenic rice (Chen et al. 2012). In this study, expression of the bZIP1 gene was upregulated in DA1 plants exposed to initial stress (S1). However, during S2, NA3 plants of wheat genotypes experience a higher expression of this abiotic stress regulatory gene than DA3 plants (Figures 11–13D). Consistent with our results, overexpression of bZIP genes improved tolerance of plants against drought, salt, and freezing in Arabidopsis (Zhang et al. 2015). Collectively, our results confirmed the possible involvement of bZIP TFs in drought acclimation, which can be harnessed for developing better genotypes endowed with drought tolerance.

The basic helix-loop-helix (bHLH) transcription factors are involved in regulation of plant growth and development, and abiotic stress responses (Gangappa and Kumar 2017); (Seo et al. 2011). In this study, the expression of the bHLH gene (TabHLH1) increased under initial and subsequent drought stress in DA1 plants of wheat genotypes, except for PL 371, which showed an expression level of TabHLH1 similar to control plant levels (Figures 11–13E). Furthermore, comparison between DA3 and NA3, in relation to the expression pattern of TabHLH1, showed a higher expression of bHLH1 gene in both DA3 and NA3 plants. Nevertheless, the magnitude of expression of this drought inducible protein was relatively greater in NA3 plants of wheat genotypes than that in DA3 plants (Figures 11–13E). Consistent with our results, other researchers reported that overexpressing of AtbHLH68 in Arabidopsis resulted in enhanced drought tolerance associated with an enhanced sensitivity or increased in ABA content (Le Hir et al. 2017). This indicated the possible role of bHLH TF’s, in relation to drought acclimation.

5. Conclusion

The study investigated the contribution of physiological, biochemical, and molecular determinants resulting in drought acclimation in four wheat genotypes (KG, PL 337, PL 371, and PL 257). All wheat genotypes exhibited marked differences in physiological and biochemical response to drought acclimation, with PL 337 and PL 371 showing a better tolerance than KG and PL 257. Expression of five drought responsive genes were greatly induced in PL 337, indicating the genotypic differences in drought tolerance could be, at least in part, attributed to the ability of plants to acclimate and induce antioxidant defense, enhance growth and water relation, reduced membrane damage, improved photosynthetic activity, and lower gene expression patterns under severe water stress. Exposure of wheat seedlings to initial stress and subsequent drought stress treatment, resulted in altered metabolic functions of wheat seedlings at a greater magnitude than the metabolic changes under subsequent (second) stress. In addition, the non-acclimated plants showed higher water loss, greater H2O2 and osmolyte accumulation, higher membrane damage and decline in photosynthetic pigment, and higher antioxidant defense system and expression of drought responsive genes under subsequent water stress than drought-acclimated plants. The findings from this study will be valuable for initiating a breeding program towards developing wheat cultivars with enhanced tolerance against drought.

Disclosure statement

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