Genome-Wide Identification of Ascorbate Peroxidase Gene Family in Two Contrasting Barley (Hordeum vulgare L.) Cultivars: Essential Roles in Various Abiotic Stress Tolerance

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Abstract

Plants with their antioxidant defense systems evolved under stress conditions and detoxify and remove the levels of reactive oxygen species (ROS). The survey of ascorbate peroxidase (APX) gene families in barley identified eight APX genes. The comprehensive analysis of HvAPX genes in barley has not yet been described. In this study, 8 members of the barley APX family were characterized for phylogenetic tree, conserved motifs, gene ontology, correlation between traits and gene expression, prediction of cis-elements, and gene expression in APX under abiotic stress conditions. In addition, analysis of physiological traits was performed on two contrasting Iranian barley cultivars namely Sahra (drought tolerant) and Nobahar (drought susceptible) under abiotic stress (PEG, heat, ABA, and salt) conditions. Gene expression analysis revealed that 8 HvAPX genes were accumulated in the leaf and root tissues at 24 and 48 hours after abiotic stresses. Furthermore, the gene expression analysis of the HvAPX genes revealed genes increase and decrease in response to PEG, ABA, salt, and heat stresses in the leaf and root tissues. The phylogenetic analysis of the HvAPX proteins sequences in barley were grouped into three clusters. The HvAPX7 and HvAPX8 genes had the highest number of cis-elements in their promoter regions, indicating that they might be stimulated by a plethora of environmental stresses. The HvAPX genes had GT1-motif, STRE, CAAT-box, MYB, and MYC in their promoter regions, playing a key role in response to abiotic stresses. Our findings provide new insights into APX genes and provide a basis for next investigations of APX genes in plant improvement (breeding) programs.

Introduction

Under adverse environmental conditions, a large number of oxygen free radicals accumulate in plant cells and cause oxidative stress and ultimately lead to reduced growth and even plant death in severe cases. Against these conditions, plants can destroy ROS by two enzymatic and non-enzymatic systems (Hasanuzzaman et al., 2017). Non-enzymatic systems include antioxidants such as glutathione (GSH), flavonoids and (ascorbic acid) ASA, and the enzymatic system includes antioxidant enzymes such as ascorbic acid peroxidase (APX), catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD). Hydrogen peroxide is naturally produced from the photosynthetic electron transport chain and some enzymatic reactions in the chloroplast of plants (Liu et al., 2013).

APX genes are known to regulate plant growth processes and response to various stresses by controlling H2O2 signal transduction, which was seen in Arabidopsis thaliana cAPX1 mutation with reduced photosynthesis and delayed flowering compared to the wild type (Szarka et al., 2012). In addition, AtAPX6 protects A. thaliana seeds against oxidative stress during drying, maturation, and germination (Davey et al., 2000). The effect of the OsAPX1 gene on seed growth was reported in rice, where mutant lines showed reduced spike size and weight and produced 58% aborted seeds. In addition, the rate of growth and development before flowering and the number of seeds per spike were similar to the wild type (Mellidou et al., 2012). OsAPX2 mutation decreases pleiotropic effects on growth and tolerance to abiotic stress (Cronje et al., 2012). A high activity of APX was reported in the tolerant genotype of sweet potato at 24 and 48 hours after application of salt stress (Hasanuzzaman et al., 2017). A previous study showed
that increased expression of AtAPX2 was reported under high temperature stress (Davey et al., 2000). As previously reported, peroxisomal Populus APX, OsAPX1, OsAPX2, and OsAPX5-OsAPX7 in O. sativa, and cytosolic APX2 in Vigna unguiculata showed high expression during drought stress (Hasanuzzaman et al., 2017). Overexpression of Solanum lycopersicum tAPX increased salt tolerance and osmotic stress in transgenic tobacco plants (Hasanuzzaman et al., 2017).

Abiotic stresses such as drought and salinity increase respiration rate and as a result of electron leakage and production of ROS including superoxide (2O), hydrogen peroxide (2O2H), and hydroxyl radical (OH) lead to higher concentration of these free radicals, causing toxicity or oxidative damage to various components of living cells, including fats, proteins and nucleic acids (Abogadallah, 2010). The activation of the plant’s antioxidant defense system under oxidative stress (including drought) has been reported in various reports (Soltis and Soltis, 1990; Singh et al., 2014). Under environmental stress conditions, redox homeostasis in plants is possible through the antioxidant system. Generally, the detoxification of free radicals against stresses in plants are classified into two enzymatic and non-enzymatic systems (Foyer et al., 1995). The APX is a highly active class of antioxidants and is a type I carrier peroxidase involved in the ascorbate-glutathione pathway to remove excess H2O2 in plants under normal and stressed conditions (Cronje et al., 2012). The number of APX family genes varies depending on the species. So far, 9 APX genes have been identified in Arabidopsis (Zechmann, 2014), 8 APX genes in Oryza sativa (Szarka et al., 2012), 7 APX genes in L. esculentum (Tripathi et al., 2009), and 26 APX genes in G. hirsutum (Davey et al., 2000). Based on plant genomic information, APX family members have already been reported in several plants including Arabidopsis, rice, tomato, sorghum and cotton (Szarka et al., 2012; Cronje et al., 2012).

Barley performance is severely reduced by environmental stresses such as drought, heat and salinity. Some researchers proved that heat and drought stress have severe effects on growth, yield and quality of barley (Aleem et al., 2022; Wang et al., 2018; Hajibarat and Saidi, 2022). Despite the important role of these genes, systematic characterization of HvAPXs genes in major barley crop has not been done. In the present study, we performed a genomic analysis of HvAPX genes in barley. The identified genes and proteins were studied to a systematic bioinformatics description to investigate their physicochemical characteristics. The function of these genes in abiotic stress responses was analyzed using transcription profiling. Furthermore, the expression of selected genes among drought tolerant and sensitive cultivars was confirmed by qRT-PCR. To confirm their response to abiotic stresses, the APX, POD, CAT content were also measured after ABA, heat, drought, and salt treatments at 24 and 48 hour after stress application.

Material and methods

Identification of HvAPX genes in barley

To investigate genome wide analysis, two techniques were used to identify HvAPX genes in barley. In the first method, the homology of proteins was identified with APX proteins from Arabidopsis and rice. The second technique was to retrieve the APX protein sequence using Hidden Markov Model (HMM) analysis,
Pfam number PF00141 from the Pfam HMM library. Arabidopsis and rice protein sequences were obtained from TAIR and RAP-DB databases, respectively. Known Arabidopsis APX protein sequences were retrieved from TAIR, and used as search sequences for the tBLASTn program in barley to search for similar protein sequences. All putative sequences were checked against SMART and interproscan databases. The remaining 8 non-redundant candidates were identified as HvAPX proteins.

**Phylogenetic analysis, conserved motifs, subcellular localization, gene structure analysis of HvAPX genes**

Alignment of protein sequences of Arabidopsis, maize, Brachypodium distachyon, and barley was performed using ClustalW method. Phylogenetic tree drawing was done using MEGA 7.0 software and NJ algorithm. MEME database was used to identify HvAPX conserved motifs (http://meme.sdsc.edu/meme/meme.html). ExPASy server (http://web.expasy.org/computepl/) was used to predict the theoretical isoelectric point (pI) and molecular weight (kDa) of APX proteins. Subcellular localization of HvAPX proteins was performed using CELLO v.2.5. All APX genes are listed in Table 1 along with gene names, gene details, amino acid length and amino acid isoelectric point.

**Prediction of cis-elements and functional annotation of HvAPX genes**

The PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to predict the cis-elements and the heatmap was displayed using TBtools software. Further, 2000 bp upstream of the promoter region of HvAPX genes was surveyed.

**Plant growth and abiotic induced expression profiles of HvAPX genes**

To investigate the expression pattern of APX gene under abiotic stress, 10 seeds of two contrasting Iranian barley cultivars were grown in pots filled with soil and kept under greenhouse conditions: day/night temperature at 25°C, with 16 hours of light and 8 hours dark period was maintained. In this study, the Sahra and Nobahar cultivars were used as two contrasting cultivars in response to abiotic stresses. The Sahra cultivar, a high-yielding drought tolerant cultivar, is cultivated in most regions of Iran. In contrast, the Nobahar cultivar is sensitive to drought. To evaluate the expression of HvAPXs under abiotic stress, two week-old seedlings were treated with 20% PEG 6000, salt (200mM), and heat (42°C) stresses. Leaves and root from plants were harvested at 24 and 48 hours after abiotic stresses and immediately stored at −80°C for further analysis. The expression profiles of 8 HvAPX genes were analyzed in two barley cultivars based on the qRT-PCR data with three replication. For phyto-hormone treatments, leaves and root were sprayed with 100 µM ABA under normal and stress conditions. Barley cultivars that grew normally were used as normal replicates. We used two time points of 24 and 48 hours after heat, salt, ABA, and PEG treatments to investigate their gene expression under these abiotic stresses.

**RNA extraction and quantitative real-time PCR (qRT-PCR) analysis**

Total RNA was extracted from leaves and roots of barley under normal and stress conditions using the RNX-Plus kit according to the manufacturer's instructions. RNA was extracted from leaves and roots
collected from 2-week-old seedlings after 24 hours and 48 hours of abiotic stress treatment. The purity and concentration of RNA was determined by nanodrop and its quality was confirmed using 1% agarose gel analysis. Then cDNA was prepared according to the instructions of the Easy cDNA Synthesis Kit. For the analysis of each gene, three repetitions were performed, and the actin gene was used as a reference gene. QRT-PCR was performed on an ABI 7500 using SYBR Green Supermix as described in the manufacture's instructions. Relative expression was determined through the $2^{-\Delta\Delta Ct}$ technique after normalizing the Ct value for individual genes against Actin, the most suitable reference gene for leaf and root samples under different stress conditions (Yu et al., 2019). In the current study, due to its stable expression under different environmental stresses, the Actin gene was utilized as the reference gene. The expression profile for the 8 HvAPX genes using leaves under PEG, ABA, heat, and salt stresses were determined using qRT-PCR. Duncan's test was used to compare the means of treatments at $P<0.05$ and the three independent biological replicates. Gene primer sequences for the RT-qPCR are listed in the Table 1.
Table 1
Primers and sequences of *HvAPX* genes used in this study.

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HvAPX7F</td>
<td>GAGGACCCAGCATTCAAGG</td>
</tr>
<tr>
<td>HvAPX7R</td>
<td>GCTTCAAGGGTTCTCTGGC</td>
</tr>
<tr>
<td>HvAPX8F</td>
<td>GCCCCAGCGGATGCTACTA</td>
</tr>
<tr>
<td>HvAPX8R</td>
<td>CACCGATCTTAGTTGCCG</td>
</tr>
<tr>
<td>HvAPX1F</td>
<td>GGTGTCACAAGGAGAGGTC</td>
</tr>
<tr>
<td>HvAPX1R</td>
<td>GCAGTAGTACACACGCTC</td>
</tr>
<tr>
<td>HvAPX3F</td>
<td>CAGGGACTATGCAAGAGGC</td>
</tr>
<tr>
<td>HvAPX3R</td>
<td>GTTCTTCAAGACCATTAC</td>
</tr>
<tr>
<td>HvAPX2F</td>
<td>GACCAGGACATTGTGTGCTC</td>
</tr>
<tr>
<td>HvAPX2R</td>
<td>CGATCTTGAATCAGCAGC</td>
</tr>
<tr>
<td>HvAPX4F</td>
<td>TCGACGCTATGTGGAGCT</td>
</tr>
<tr>
<td>HvAPX4R</td>
<td>GTCATCCAGAAACTCAGAAGC</td>
</tr>
<tr>
<td>HvAPX6F</td>
<td>CTGCCATCTGATGCTGTGCT</td>
</tr>
<tr>
<td>HvAPX6R</td>
<td>TGCAGTTCATCGAAGCA</td>
</tr>
<tr>
<td>HvAPX1.1F</td>
<td>CTGGGAAGGTGTCACAAGG</td>
</tr>
<tr>
<td>HvAPX1.1R</td>
<td>GCAGCAGTACACGACTCAGC</td>
</tr>
<tr>
<td>Actin F</td>
<td>GGTCCATCCTAGCCTACTC</td>
</tr>
<tr>
<td>Actin R</td>
<td>GATAACAGCAGTGAGCCTG</td>
</tr>
</tbody>
</table>

Characterization of physiological indexes

To investigate physiological traits, normal and stressed barley leaves and root were collected at the seedling stage. The fresh leaf samples were washed by distilled water in the laboratory. Then, they were left to dry at room temperature (18°C) to be analyzed for the determination of chlorophylls (a and b) and carotenoids contents. The catalase, ascorbate peroxidase, and peroxidase activity were determined by Li et al., (2012). Following the addition of H\textsubscript{2}O\textsubscript{2}, the absorbance changes for 240 nm, 290 nm, and 470 nm were analyzed to measure catalase, ascorbate peroxidase, and peroxidase activities, respectively.

Statistical analysis
Statistical analysis was performed using SPSS. Differences across tissues were analyzed using one-way ANOVA test. Duncan's test was used to compare the treatment means at $P < 0.05$. Values represent the means of three replications per treatment.

**Result and Discussion**

In the present study, bioinformatics study of *HvAPX* genes was performed using various tools. The *HvAPX* gene names and chemical characteristics are listed in the Table 2. The alignment results of proteins sequences were determined using MEGA7 software and the phylogenetic tree was plotted using the NJ algorithm, according to which the genes were divided into three clusters (Fig. 1). Bioinformatics analysis such as phylogenetic relationships, and conserved motifs were performed. According to the intracellular location of APX, APXs can be grouped into cytoplasmic APX, peroxisome APX, chloroplast APX and chloroplast/mitochondrial APX, but the localization and quantity of APXs are different in cells of different species. Eight genes of the HvAPX gene family have been recognized in barley, including two in the cytoplasm (APX1 and APX2), two in the peroxisome (HvAPX3 and HvAPX4), and three in the chloroplast (HvAPX6, HvAPX7 and HvAPX8). Our results agreed with other researchers (Panchuk et al., 2002; Chew et al., 2003) in that the *HvAPX6*, *HvAPX7* and *HvAPX8* genes play a key role in response to abiotic stresses. Expression profiles and physiological traits of *HvAPXs* genes were analyzed under abiotic stress. The *HvAPX* genes ranged from 250 to 453 amino acids, with a predicted isoelectric point (pI) varying from 4.38 to 8.57 with a molecular weight of 24.46 to 48.91 kDa (Table 2).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein length</th>
<th>Molecular weight (KD)</th>
<th>Isoelectric point (pI)</th>
<th>Subcellular Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>HvAPX1</td>
<td>250</td>
<td>27.46</td>
<td>5.85</td>
<td>cytosolic</td>
</tr>
<tr>
<td>HvAPX1.1</td>
<td>375</td>
<td>42.04</td>
<td>4.38</td>
<td>chloroplast/mitochondria</td>
</tr>
<tr>
<td>HvAPX2</td>
<td>256</td>
<td>26.64</td>
<td>5.10</td>
<td>cytosolic</td>
</tr>
<tr>
<td>HvAPX3</td>
<td>295</td>
<td>32.22</td>
<td>6.72</td>
<td>peroxisome</td>
</tr>
<tr>
<td>HvAPX4</td>
<td>291</td>
<td>31.67</td>
<td>7.76</td>
<td>peroxisome</td>
</tr>
<tr>
<td>HvAPX6</td>
<td>315</td>
<td>34.40</td>
<td>6.76</td>
<td>chloroplast</td>
</tr>
<tr>
<td>HvAPX7</td>
<td>348</td>
<td>37.56</td>
<td>8.57</td>
<td>chloroplast</td>
</tr>
<tr>
<td>HvAPX8</td>
<td>453</td>
<td>48.91</td>
<td>5.72</td>
<td>chloroplast</td>
</tr>
</tbody>
</table>

Plants can respond to drought stress with different morphological, physiological, biochemical or molecular mechanisms. These mechanisms lead to diverse responses from either enabling plants to become more tolerant or avoid facing the stress (Ibrahim et al., 2015). In some of these responses, a
significant increase in the amount of reactive oxygen species (ROS) is observed, which increases the activity of some antioxidant enzymes and antioxidant compounds. Among these antioxidant enzymes, peroxidases, catalase and glutathione reductase can be mentioned (Aghaei et al., 2009).

To identify conserved motifs, MEME database was used using APX protein sequence. Among APX proteins, three various conserved motifs were identified. The presence of conserved motifs in the same group probably indicates the related function of the proteins. In these motifs, the amino acid length varied from 31 to 50. The number of conserved domains among the three APX groups is given in (Fig. 2a).

**Prediction of cis-elements in HvAPX genes**

To investigate regulatory elements within the promoter region of *HvAPX* genes associated with abiotic stresses, the PlantCARE database was utilized to predict the transcription factor binding sites in the promoter region up to 2000bp upstream of the ATG of *HvAPX* genes. The results of promoter region analysis in *HvAPX* genes indicated that there are different cis-elements responsive to light, hormonal and abiotic stresses. The *HvAPX6*, *HvAPX7*, and *HvAPX8* genes had the highest number of cis-elements in the promoter regions (Fig. 3). Further, most of the cis-elements in the upstream region of the genes are related to cis-elements responding to abiotic stress. In this study, elements such as GT-1 motif, MYB, MYC, STRE, and light responsive elements (Sp1, A-box, Box4, and box s) were identified in response to various stresses. Also, cis-elements responsive to different hormones such as SA-responsive elements (TCA-element, as-1), Methyl jasmonate elements (CGTCA-motif and TGACG-motif), ABA-responsive elements (ABRE), Gibberellin-responsive element (TGA-element), and Auxin responsive elements (AuxRR-core) were present in the promoter region of *HvAPX* genes. Some cis-elements were expressed in the promoters of *HvAPXs* genes including low temperature stress responsive (Myb, G-box and LTR) and high temperature stress responsive (WRE3) elements. Cross-talk among hormones through cis-elements related to auxin, gibberellins (GA), Abscisic acid (ABA), and Salicylic acid (SA) play crucial roles at different abiotic stresses in plants. With the presence of different cis-elements in the *HvAPX* genes, plants adapt to increased gene expression during its growth in response to various environmental stresses (30 Saidi and Hajibarat, 2020). ABA, a major phyto-hormone regulating stress responses, interacts with the JA and SA signaling pathways to respond to abiotic stresses. The *HvAPX* genes have regulatory systems that function in their promoters, like DRE, ABRE, and MYB. These signaling factors might be implicated in the amplification of stress signals in different plant cells under abiotic stresses. The ABRE and DRE cis-elements can interact under environmental stresses. The promoter sequences of *HvAPX* genes contain several cis-elements, such as ABRE, G-box, W-box, AuXRE, and DRE, associated with stress responses indicating the potential interaction of abiotic stress responsive TFs with cis-elements 32. According to the study conducted on pepper, most of the APX genes have cis-elements responsive to light and abiotic stresses (Pang et al., 2023).

**Gene ontology of HvAPX proteins**
The gene ontology of HvAPXs showed that the majority of the HvAPX proteins were involved in oxidative stress, response to stress, and cellular process in the biological process. In cellular component, cellular anatomical entity, membrane, and cytoplasm are involved (Fig. 4). Most of the HvAPX are involved in peroxidase activity, tetrapyrrole binding, and antioxidant activity in molecular function. Different kinds of HvAPX are involved in peroxidase activity and response to stress, implying that the APXs might play major roles in detoxification of cytoplasm during abiotic stresses.

**Physiological traits in response to abiotic stresses**

The comparison of average leaf APX content in response to abiotic stresses indicated that the Sahra cultivar's APX content was significantly increased in response to salinity stress at 24 and 48 hours after stress application. Further, as compared to Nobahar cultivar, under the PEG and heat treatments, the leaf APX content of Sahra was significantly increased at 48 hours and 24 hours, respectively. On the other hand, the Sahra cultivar did not show any significant differences for the leaf APX content at both time points in response to ABA stress. Based on the analysis of the catalase content in the leaves, the Sahra cultivar showed a higher significant increase in response to salt, PEG, and heat stresses at 24 and 48 hours, as compared to Nobahar cultivar. However, at 24 and 48 hours of ABA stress, there was no significant difference in catalase content between Sahra and Nobahar cultivars. The examination of the POD content of leaves in the two cultivars showed that Sahra cultivar showed a significant increase in response to salinity stress at 48 hours and PEG at 24 and 48 hours and ABA at 24 hours as compared to Nobahar cultivar. The Nobahar cultivar showed a significant increase in salinity treatment at 24 hours and heat at 24 and 48 hours as compared to Sahra cultivar (Fig. 5).

The mean comparison of the root APX content in response to abiotic stresses revealed that the Sahra cultivar showed a significant increase in response to all stresses at 24 and 48 hours as compared to the sensitive cultivar. Based on the analysis of the catalase content in the root, the Sahara cultivar revealed a significant increase in response to the stresses of PEG at 24 hours, heat at 24 and 48 hours, ABA at 24 and 48 hours as compared to the Nobahar cultivar. However, at 24 and 48 hours of salinity stress, Sahra cultivar did not show any significant difference with Nobahar cultivar. The examination of the POD content of leaves in the two cultivars showed that Sahra cultivar showed a significant increase in response to salt stress at 24 hours, PEG at 48 hours and heat at 48 hours as compared to Nobahar cultivar. Compared to Sahra cultivar, the Nobahar cultivar showed a significant decrease in POD content at 24 hours after heat treatment (Fig. 6).

Plants show a group of morphological, physiological, and biochemical adaptations in response to water stress, among which includes changes in some enzymes such as peroxidase. ROS cause membrane damage and lead to a rapid cellular response to initiate plant defense signaling. The activities of catalase (CAT) and peroxidase (POD) enzymes are increased during biotic and abiotic stresses to protect cells from the potentially dangerous effects of ROS (Farag et al., 2019). APX proteins act as efficient ROS scavengers against various abiotic stresses. Previous studies reported that APX genes have regulatory
roles with respect to tolerance to several stresses, such as salt, heat, drought, and oxidative stress (Aleem et al., 2022; Tao et al., 2018; Leng et al., 2021).

The effects of reactive oxygen species (ROS) on the destruction of nuclear DNA include shape change, oxidation of oxyribose, and DNA strand breakage and mutation. Among the various types of ROS compounds, hydroxyl radicals play more important roles in abiotic stresses. Oxygen free radicals cause oxidative destruction of proteins. It has been reported that the destruction occurs in a specific place of amino acids in the protein (Saavedra et al., 2006).

Correlation between physiological traits and gene expression 24 and 48 hours after different stresses

HvAPX4l had a positive correlation with HvAPX8l. HvAPX6l had a positive correlation with HvAPX1r. HvAPX3r had a positive correlation with HvAPX1r and HvAPX8r. HvAPX1r had a positive correlation with HvAPX8r. HvAPX8r had a negative correlation with HvAPX1br (Fig. 7). APXl had a positive correlation with APXr.

Gene expression analysis of HvAPX genes in barley cultivars under abiotic stresses

In Sahra and Nobahar cultivars, the expression profiles of 8 HvAPX genes were determined under abiotic stress conditions using reverse transcription-PCR (qRT-PCR) quantitative analysis in leaves and root. Evaluation of genes expression in the Sahra cultivar in response to abiotic stresses indicated that most of the genes showed an increased expression under ABA and PEG treatments. In the case of the Nobahar cultivar, genes increased expression under salt and ABA stresses. To display the expression of HvAPX in plant leaf tissues in response to abiotic stresses, their expression levels were analyzed using heatmap (Fig. 8a and 8b). The data showed that the gene expression levels of the HvAPX involved in the growth and development of barley were significantly different in the leaves and root. Based on the results of gene expression, the Sahra cultivar showed a higher expression level of APX genes in Sahra cultivar as compared to Nobahar cultivar. In addition, the number of APX genes was higher in Sahra cultivar as compared to Nobahar cultivar.

In the Sahra cultivar, the expression of HvAPX genes (HvAPX1, HvAPX2, HvAPX1b and HvAPX7) in the root was higher at 48 hours than at 24 hours after applying ABA stress. Also, the HvAPX8 and HvAPX4 genes were significantly increased at 24 hours as compared to 48 hours after applying the ABA stress. The analysis of the expression of HvAPX genes in the leaves in response to ABA stress in Sahra cultivar showed that HvAPX gene was more expressed at 48 hours as compared to 24 hours after applying ABA stress (Fig. 7a). Also, the HvAPX3 gene showed a significant increase in expression at 24 hours after the application of ABA stress as compared to 48 hours after the application of stress. A pervious study showed that most of the ClAPX genes significantly increased expression in watermelon under drought stress (Malambane et al., 2018). These findings can help be provide our perception of APX genes under different stress conditions, especially drought.
The analysis of the expression of HvAPX genes in the leaves in response to heat stress in the Sahra cultivar showed that HvAPX1 and HvAPX2 showed a high expression increase at 24 hours as compared to 48 hours after applying the heat stress. Also, the HvAPX2 and HvAPX3 showed a significant increase in expression at 48 hours as compared to at 24 hours after application of heat stress in leaves. Analysis of the expression of HvAPX genes in roots in response to heat stress in the desert cultivar showed that HvAPX4 showed a high expression increase at 24 hours as compared to 48 hours after applying heat stress. Also, the HvAPX1b and HvAPX7 showed a significant increase in expression at 48 hours after applying heat stress compared to 24 hours after applying heat stress in leaves. Examining the expression of HvAPX genes in the roots of Sahra cultivar in response to PEG stress, it was observed that the HvAPX4 gene showed a high expression increase at 24 hours as compared to 48 hours whereas, the HvAPX1 gene showed a high expression increase at 48 hours as compared to 24 hours after stress application. Also, the HvAPX3 gene showed a significant increase in expression at 24 and 48 hours after stress application (Fig. 8b).

The expression analysis of HvAPX genes in the roots of Sahara cultivar in response to salt stress showed that the HvAPX2 and HvAPX1b genes had high expression increase at 24 and 48 hours, and HvAPX4 and HvAPX7 genes showed a high expression increase at 24 hours as compared to 48 hours after applying the tension. Also, the HvAPX3 gene showed a significant increase in expression in leaves and 48 hours after salt stress application, however, the HvAPX8 showed a high expression increase at 24 hours as compared to 48 hours after applying salt stress. In addition, the HvAPX2 gene showed an increased expression in leaves at 48 hours as compared to 24 hours after stress application. A previous study reported that chloroplast APXs are mostly used to protect the photosynthetic system, while mitochondrial APXs have a positive role in removing hydrogen peroxide produced by fatty acid oxidation (Renu et al., 2011; Andrea et al., 2014). Cytoplasmic OsAPX2 gene plays an effective role in maintaining H2O2 homeostasis (Wu et al., 2018).

Examining the expression of HvAPX genes in the roots of Nobahar cultivar in response to ABA stress, it was observed that the HvAPX1 gene showed a high expression increase at 24 and 48 hours after stress application. On the other hand, the HvAPX3 and HvAPX4 genes showed a high expression increase at 24 hours as compared to 48 hours after stress application. Also, the HvAPX1b gene showed a significant increase in expression in the root at 48 hours as compared to 24 hours after stress application. The HvAPX2 and HvAPX8 genes in the leaf showed a high expression increase at 48 hours as compared to 24 hours after ABA stress application.

Examining the expression of HvAPX genes in the roots of Nobahar cultivar in response to heat stress, it was observed that the HvAPX3 gene showed a high expression increase at 24 and 48 hours, whereas, the HvAPX1 gene showed a high expression increase at 24 hours as compared to 48 hours after applying the stress. Also, the HvAPX6 and HvAPX8 genes showed a significant increase in the roots at 48 hours as compared to 24 hours after applying stress. In the leaf, the HvAPX1b, HvAPX3, HvAPX4, and HvAPX6 genes showed a high expression increase in the leaves at 24 hours as compared to 48 hours after
applying ABA stress. The \textit{HvAPX2} and \textit{HvAPX8} genes showed a high expression increase at 24 and 48 hours after stress application (Fig. 9a).

In response to PEG stress in the roots of Nobahar cultivar, it was observed that the \textit{HvAPX3} gene showed a high expression increase at both 24 and 48 hours after stress application. Whereas, the \textit{HvAPX1} gene showed a high expression increase at 24 hours as compared to 48 hours after stress application. In the leaves, the \textit{HvAPX8} gene showed a high expression increase at 24 and 48 hours after stress application. However, the \textit{HvAPX3} gene showed a high expression increase in the leaf at 48 hours as compared to 24 hours after applying PEG stress. In response to salinity stress in the roots of Nobahar cultivar, it was observed that the \textit{HvAPX7} gene showed a high expression increase at 24 hours as compared to 48 hours after stress application whereas, the \textit{HvAPX1b} gene showed a high expression increase at 48 hours as compared to 24 hours after stress application. In the leaves, the \textit{HvAPX8} gene showed a high expression increase at 24 and 48 hours after stress application. In the leaves, the \textit{HvAPX2} and \textit{HvAPX6} genes showed a high expression increase at 24 hours as compared to 48 hours after applying salt stress. Our findings showed that in the Sahra cultivar, most of the APX genes showed increased expression in response to ABA stress. But in the Nobahar cultivar, most of the genes showed increased expression in response to heat stress (Fig. 9b). The \textit{CaAPX4} showed higher expression at high temperature, suggesting that \textit{CaAPXs} probably played an important role in the abiotic stress response of pepper, which is consistent with our findings (Sharma and Dubey, 2005; Koswitzki et al., 2008).

**Conclusion**

Ascorbate peroxidase (APX) are an important antioxidant enzyme and important role in ROS remove by catalyzing the reduction of $\text{H}_2\text{O}_2$ under different stresses. Some candidate genes such as \textit{HvAPX3/4/7} and \textit{HvAPX8} can be utilized to aid barley breeding programs designed for developing cultivars tolerant to abiotic stresses. In our paper, the \textit{HvAPX7} and \textit{HvAPX8} revealed higher expressions under most stress treatments, suggesting that these genes are involved in several biological processes. The expression levels of \textit{HvAPX1}, \textit{HvAPX3}, \textit{HvAPX7}, and \textit{HvAPX1b} genes exhibited very high levels during abiotic stresses of Sahra barley. The expression profiles of \textit{HvAPX1}, \textit{HvAPX2}, \textit{HvAPX3}, and \textit{HvAPX8} genes exhibited high levels during abiotic stresses and development of Nobahar barley. These results indicate that the APX genes may play critical roles in the normal growth of the plants and during abiotic stress conditions. Based on the physiological traits and gene expression profiles, the Sahra can be considered as a tolerant cultivar to PEG, heat, ABA, and salt stresses.

**Declarations**

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Contributions

Z.H. H.G.H and A.S. wrote the main manuscript text and Z.H. prepared figures 1-7. All authors reviewed the manuscript.

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References


Figures
Figure 1

Phylogenetic tree of *APX* genes created by the neighbor-joining (NJ) method in MEGA7.0 software in *Arabidopsis*, rice, maize, and barley.
Figure 2

Conserved APX protein motifs in barley, as recognized by MEME database. Motifs 1-3 are identified by different colors (a) and consensus sequence for putative motifs (b).
Figure 3

*Cis*-element analysis of 8 *HvAPX* genes from the upstream 2000 bp sequence to the transcription start site.
Figure 4

Gene ontology of HvAPX proteins using Blast2GO.

Figure 5

The effects of abiotic stresses on Sahra and Nobahar barley cultivars for APX (a), CAT (b), and POD (c) at 24 and 48 h after abiotic stresses in leaf. Values represent the means of three replications per treatment.
Different letters demonstrate significant differences between treatments ($P < 0.05$, Duncan's Multiple Range Test).

**Figure 6**

The effects of abiotic stresses on Sahra and Nobahar barley cultivars for APX (a), CAT (b), and POD (c) at 24 and 48 h after abiotic stresses in root. Values represent the means of three replications per treatment.
Different letters demonstrate significant differences between treatments (P < 0.05, Duncan's Multiple Range Test).

Figure 7

The correlation coefficients of physiological and gene expression 24 and 48 hours after different stresses. Leaf Catalase (CATl), Leaf Peroxidase (PODl), Leaf Ascorbate peroxidase (APXl), Root Catalase (CATr), Root Peroxidase (PODr), Root Ascorbate peroxidase (APXr), Leaf HvAPXs (HvAPXI) and Root HvAPXs (HvAPXr).
Figure 8

Heatmaps representing the expression profiles of leaf *HvAPX* genes in Sahra in response to heat, ABA, salt, and PEG stresses, and their gene expression at 24 and 48 hour after stress for leaf (a) and root (b). The heat map was generated using TBtools.
Figure 9

Heatmaps representing the expression profiles of leaf *HvAPX* genes in Nobahar in response to heat, ABA, salt, and PEG stresses, and their gene expression at 24 and 48 hour after stress for leaf (a) and root (b). The heat map was generated using TBtools.