Introduction

Abiotic stresses especially water deficit and salinity are the leading factors affecting plant growth and development and thus reduction in crop productivity especially in rain-fed areas.1–3 These factors are the major causes of osmotic stress, which results in turgor loss due to low water availability and accumulation of excess Na and Cl ions.4 Turgor pressure is maintained through osmotic regulation, and this is important for plant growth by cell expansion.5,6 Significant changes in water potentials in the environment can impose osmotic stress to plants, resulting in various physiological changes, such as excessive production of reactive oxygen species (ROS), which further causes oxidative damage to cells, loss of membrane function, enzyme inactivation, DNA and protein denaturation, as well as ionic and nutrient imbalance.7 Plants have evolved several mechanisms to respond to osmotic stress, and these include changes in their life cycle, adjustment of ion transport, synthesis of compatible solutes, and the detoxification of ROS through the anti-oxidative system.8 The anti-oxidative system is divided into the non-enzymatic and enzymatic components, in which the latter is more effective consisting of enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and glutathione reductase (GR).9,10 In addition, the heme oxygenase-1 (HO1) enzyme system has also gained more attention due to its anti-oxidative properties.11–15 Heme oxygenase (EC 1.14.99.3) catalyzes the formation of biliverdin-IXα (BV), carbon monoxide (CO), and free iron (Fe2+) through the oxidation of heme.16,17 Biliverdin-IXα is further converted into bilirubin (BR) by biliverdin reductase.18 Biliverdin-IXα and BR possess strong anti-oxidant properties in mammals19,20 and plants,16 whereas CO is well recognized as a strong anti-oxidant that regulates ROS homeostasis in animals.14 These statements support the idea that HO1 may play a protective role against oxidative damage.

Plant genomes encode 4 HO genes, namely, HO1, HO2, HO3, and HO4, which were initially identified in the model species, Arabidopsis thaliana. Heme oxygenases include a small gene family with 2 main sub-families including the HO1 sub-family which consists of 3 members, namely, HO1, HO3, and HO4, whereas the HO2 sub-family has only 1 member, HO2.21,22 Members are differentially expressed with HO1 representing the most highly expressed, followed by HO2, while both HO3 and HO4 are expressed at low levels.21 In general, HO1 is known to provide all the anti-oxidative protective effects that are associated with HO. It is induced by various stimuli, including heavy metals,23–26 NO27,28 glutathione depletions,29 UV radiation,30 heat, paraquat,31 H2O2,32,33 and salinity.15,16,34–36 HO2 is also induced by NO, H2O2,32,33 hemin, paraquat, and salinity.31,32,36 The expression and induction of HO genes in response to stress represent their role in mediating a defense mechanism against various stresses.

To date, plant HO genes have only been identified and characterized in a few species including A. thaliana (AtHO1-AtHO4),37 Oryza sativa (OsHO1 and OsHO2),36,38 Medicago sativa (MsHO1 and MsHO2)
and *MsHO2)*, *Brassica oleracea* (*Bo*-*HO1)*, *Triticum aestivum* (*Ta*-*HO1)*, *Brassica napus* (*Bn*-*HO1* and *Bn*-*HO3)*, *Zea mays* (*Zm*-*HO1)*, *Nicotiana tabacum* (*Nt*-*HO1)*, and *Glycine max* (*Gm*-*HO1* and *Gm*-*HO3)*. Some of the challenges in the identification and characterization of HO genes in plants has been attributed to the lack of the publicly available genomic sequences, for example, in wheat. Sorghum (*Sorghum bicolor*) is one of the most important cereal crops worldwide and is considered to be moderately tolerant to drought and salinity. In sorghum, the genome sequence is available and HO genes (Sb-*HO1* and Sb-*HO2*) were previously sequenced while the other two putative members, Sb-*HO3* and Sb-*HO4*, are only annotated. In this study, bioinformatic approaches were employed to identify and characterize HO genes in *Sorghum bicolor*. The sequence parameters of all four sorghum HO genes (Sb-*HO1*-Sb-*HO4*) including gene structure, physicochemical properties, subcellular localization, signature motifs, and evolutionary relationship were analyzed and compared with other HOs from different plant species. Finally, their gene expression profiles in response to osmotic stress were also elucidated using quantitative real-time polymerase chain reaction (qRT-PCR). Our results provide novel insights into the structure, evolution, and the expression profiles of sorghum HOs, particularly in response to osmotic stress.

**Materials and Methods**

**Plant growth and treatment**

Red sorghum (*Sorghum bicolor*) seeds purchased from Agricol, Brackenfell, South Africa, were germinated as described previously with slight modification. Briefly, plant tissue culture vessels containing half strength Murashige and Skoog (MS) media (2.2 g/L MS; 1% [w/v] sucrose; 5 mM MES; and 0.4% [w/v] agar; pH 5.8) and 4 seeds per vessel were incubated at 25°C under a 16-h light/8 h dark regime for 14 days prior to mannitol treatment. For stress treatments, seedlings were carefully transferred to half strength MS media supplemented with 250 mM mannitol and incubated for 3, 12, and 24 h under the same growth conditions. Untreated seedlings were used for the control samples (0h). After stress treatments, tissues that were separated into leaves, stems, and roots were immediately frozen in liquid nitrogen and stored at −80°C until further use.

**Sequence retrieval**

Four *Sorghum bicolor* HO sequences representing HO1 (Sb-*HO1*; AAK63010.1), HO2 (Sb-*HO2*; AAK63011.1), HO3 (Sb-*HO3*; XP_002438642.1), and HO4 (Sb-*HO4*; XP_021304790.1) were obtained in FASTA format from the National Center for Biotechnology Information (NCBI) protein database (http://www.ncbi.nlm.nih.gov/). For comparative purposes, sequences of orthologous genes were obtained from the Phytozome10.3 and NCBI databases to represent a proteome dataset of 32 selected plant species. These include the following: *Asparagus officinalis*, *Aegilops tauschii*, *Amborella trichopoda*, *A. thaliana*, *Brachypodium distachyon*, *Brassica juncea*, *Brassica napus*, *Chenopodium quinoa*, *Cucumis sativus*, *Cucurbita maxima*, *Cucurbita moschata*, *Dendrobium catenatum*, *Elaeis guineensis*, *Glycine max*, *Gossypium hirsutum*, *Hevea brasiliensis*, *Hordeum vulgare*, *Jatropha curcas*, *Manihot esculenta*, *Medicago sativa*, *Nicotiana tabacum*, *Oryza sativa*, *Phalaenopsis equestris*, *Sesamum indicum*, *Setaria italica*, *Solanum lycopersicum*, *Solanum tuberosum*, *Sorghum bicolor*, *Spinacia oleracea*, *Triticum aestivum*, *Zea mays*, and *Ziziphus jujuba*.

**Prediction of gene structure and physicochemical parameters**

The Gene Structure Display Server online tool (GSDS 2.0) was used to analyze the exon-intron structure of HO genes by comparing with the CDS sequences and genomic DNA sequences. Properties such as protein length, molecular weight (Mw) and theoretical isoelectric point (pI) were computed using the ExpASy Proteomic server. Subcellular localization of the different proteins was predicted by CELLO.

**Prediction of conserved domains and signature motifs**

Protein sequences were aligned using the ClustalW2 program of the European Bioinformatics Institute. To deduce the protein family and explore the domain arrangement within proteins, sequences were analyzed using the Conserved Domain Database (CDD) while prediction of conserved motifs was performed using the Multiple Expectation Maximization for Motif Elicitation (MEME 5.0.2) online tools. Parameter settings for MEME were as follows: maximum number of motifs to find: 5; minimum width of motif: 6; and maximum width of motif: 50.

**Phylogenetic analysis**

To determine the evolutionary relationships, phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis version 7.0 (MEGA 7) for bigger database. The program was used to generate a boot-strapped dataset of 1000 replicates. The pair-wise deletion and p-distance model by neighbor-joining (NJ) methods were used.

**Total RNA extraction and reverse transcriptions**

Total RNA was extracted from 100 mg of 2-week-old sorghum seedlings (leaves, stems, and roots) using the Favorgen plant mini RNA extraction kit (Favorgen Biotech Corp., Ping-Tung, Taiwan) according to the manufacturer’s instructions. The RNA was treated with the RNase-free DNase reaction set (New England Biolabs, Massachusetts) to remove genomic DNA and its quality was determined by analyzing on a 1% agarose gel. Concentration and purity were determined using a
Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to analyze the tissue-specific expression profiles of \( Sb\) HO genes. The reaction mixture contained 1 µL template cDNA, 5 µL 2× SYBR Green I Master Mix (Roche Applied Science, Germany), varying concentrations of each primer and ddH\(_2\)O added to a final volume of 10 µL. The reactions were subjected to 95°C for 10 min, 45 cycles at 95°C for 10 s, 55°C for 10 s, and 72°C for 20 s. A melting curve analysis was also performed using default parameters on the LightCycler\(^{®}\) 480 instrument (Roche Applied Science, Germany). The primer information of the target genes \( (Sb\) HO genes) and the reference genes (ubiquitin \( [UBQ]\) and phosphoenolpyruvate carboxylase \( [PEPC]\) ) is shown in Table 1. The expression levels of the target genes were normalized to the reference genes (ubiquitin \( [UBQ]\) and phosphoenolpyruvate carboxylase \( [PEPC]\) ).

NanoDrop spectrophotometer (Thermo Scientific, USA). About 1 µg of the total RNA was used for first-strand cDNA synthesis using the SuperScript\(^{TM}\) III First-Strand Synthesis kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer’s instructions.

Results and Discussion

Sequence comparison and analysis of phyicochemical properties

Plant HOs are mainly known for their involvement in the biosynthetic pathway for the production of phytochrome chromophores that is important for photomorphogenesis.\(^{37}\) HOs also participate in plant growth and development,\(^{41,57,58}\) and their role in protecting cells against oxidative stress is well documented.\(^{37,24,59}\) Heme oxygenases have been annotated in many plant species but their functional characterization is still limited. This study was undertaken to characterize the HO genes from sorghum in comparison with other plant species using bioinformatics tools and analyze their gene expression profiles using qRT-PCR toward identifying their potential biological function. Heme oxygenase members were identified by searching the NCBI database using the BLAST tool and a total of 43 HO orthologs from 32 plant species were obtained. Among the 43 HO orthologs, 4 belonged to sorghum and these include the \( Sb\) HO1 (accession numbers: AAK63010.1, AF320026.1), \( Sb\) HO2 (accession numbers: AAK63011.1, AF320027.1), HO1, chloroplastic Sorghum bicolor (accession numbers: XP_002438642.1, XM_002438597.2; gene ID: Loc8065066, Sobic.3010G184600), and HO1, chloroplastic Sorghum bicolor (accession numbers: XP_02134970.1, XM_021449115.1; Gene ID: Loc8065071, Sobic.3010G184800). The two putative HO1 candidate genes will be referred to as \( Sb\) HO3 and \( Sb\) HO4, respectively, according to their position on chromosome 10. Heme oxygenase genes were further confirmed for the presence of the conserved heme oxygenase domain (PF01126) using the Pfam database and the CDD tool indicating that the sorghum HO proteins are likely to perform the same function as other known and characterized plant HOs.

Characteristics of HOs, including the gene ID, physicochemical parameters, and localization are shown in Table 2. To understand the gene structure of sorghum HOs, genomic sequences and their corresponding CDS sequences were retrieved and analyzed using the GSDS online tool. The structural diversity of different plant HO genes was obtained, analyzed, and showed that 36 HO orthologs have 3 to 5 exons, with most HOs (28 in total) having 4 exons; 5 HOs have 5 exons, and 3 HOs have 3 exons (Figure 1). To be more specific, the first 2 exons of \( Sb\) HO1, 2 and 3, are interrupted by long introns. \( Sb\) HO4 displayed a unique gene structure with the presence of the conserved heme oxygenase domain (PF01126) using the Pfam database and the CDD tool indicating that the sorghum HO proteins are likely to perform the same function as other known and characterized plant HOs.

| GENE NAME | FORWARD PRIMER | REVERSE PRIMER | ACCESSION NUMBER |
|-----------|----------------|----------------|------------------|
| \( Sb\) HO1 | 5′-TTCCAGACGCTGCAAGACAT-3′ | 5′-CCTGGGGATCCTTCTGAGAC-3′ | AF320026.1 |
| \( Sb\) HO2 | 5′-GGAAAAGTGGTTGGAAGCGT-3′ | 5′-AACCTCAGCTCCCTTCTTC-3′ | AF320027.1 |
| \( Sb\) HO3 | 5′-TTCCAGACGCTGCAAGACAT-3′ | 5′-CCTGGGGATCCTTCTGAGAC-3′ | XM_002438597.2 |
| \( Sb\) HO4 | 5′-TTCCCTGCTGATAGCAAGCT-3′ | 5′-TTCCAGACGCTCCTCCAG-3′ | XM_021449115.1 |
| UBQ | 5′-GCC AAG ATT CAT GAT AAG-3′ | 5′-TTG TAA TCA GCC AAT GTG-3′ | XM_002452660 |
| PEPC | 5′-GAA GAA TAT CGG CAT CAA T-3′ | 5′-CTA TGT AAT TGG TAA CTT TC-3′ | XM_002438476 |
Table 2. List of heme oxygenase homologs from 32 plant species and their physiochemical parameters.

| SPECIES NAME | PHYTOZOME GENE ID | CDS (BP) | EXON NO. | PROTEIN LENGTH (AA) | MW (KDA) | PI | LOCALIZATION |
|--------------|-------------------|----------|----------|---------------------|----------|----|--------------|
| Sorghum bicolor HO1 | Sobic.3010G184600 | 557      | 4        | 184                 | 21.3     | 5.59 | Cyto         |
| Sorghum bicolor HO2 | Sobic.3010G347800 | 987      | 4        | 328                 | 36.43    | 5.39 | Nucl/Chlo    |
| Sorghum bicolor HO4 | Sobic.3010G184800 | 1017     | 5        | 338                 | 37.06    | 8.58 | Chlo         |
| Sorghum bicolor HO3 | Sobic.3010G184600 | 867      | 4        | 288                 | 31.88    | 6.42 | Chlo         |
| Aegilops tauschii HO1 | LOC109775269 | 849      | 4        | 288                 | 31.63    | 6.21 | Chlo         |
| Amborella trichopoda HO1 | AMTR_s00078p00062760 | 876    | 4        | 305                 | 34.89    | 9.19 | Mito/Chlo    |
| Arabidopsis thaliana HO1 | AT2G26670 | 849      | 3        | 282                 | 32.69    | 6.50 | Cyto/Chlo    |
| Arabidopsis thaliana HO2 | AT2G26550 | 900      | 5        | 299                 | 34.90    | 5.80 | Nucl         |
| Arabidopsis thaliana HO3 | AT1G69720 | 684      | 5        | 227                 | 25.60    | 8.24 | Mito         |
| Arabidopsis thaliana HO4 | AT1G58300 | 852      | 4        | 283                 | 32.95    | 6.98 | Chlo         |
| Asparagopsis officinalis HO1 | LOC109819496 | 846    | 4        | 272                 | 31.11    | 8.88 | Mito/Cyto    |
| Brassica juncea HO1 | Bradi1g36640 | 870      | 4        | 279                 | 31.08    | 7.03 | Chlo/Mito    |
| Brassica juncea HO2 | LOC105040158 | 876      | 4        | 282                 | 32.19    | 8.95 | Cyto         |
| Brassica juncea HO3 | LOC111500260 | 819      | 4        | 297                 | 33.31    | 7.67 | Chlo         |
| Cucumis sativus HO1 | LOC111500260 | 819      | 4        | 297                 | 33.92    | 8.30 | Chlo         |
| Cucurbita moschata HO1 | LOC111441000 | 918      | 4        | 297                 | 34.02    | 8.61 | Chlo         |
| Dendrobium catenatum HO1 | LOC1104187 | 867      | 5        | 285                 | 32.41    | 8.74 | Mito/Cyto    |
| Elaeis guineensis HO1 | LOC105040158 | 876      | 4        | 282                 | 32.19    | 8.95 | Cyto         |
| Glycine max HO1 | GLYMA_04GG147700 | 282    | 4        | 282                 | 32.11    | 8.63 | Cyto         |
| Glycine max HO3 | GLYMA_06GG21900 | 282      | 4        | 282                 | 32.07    | 8.83 | Cyto         |
| Gossypium hirsutum HO1 | LOC107962617 | 858      | 4        | 285                 | 32.96    | 7.71 | Mito/Cyto    |
| Hevea brasiliensis HO1 | LOC110648797 | 837      | 5        | 291                 | 33.62    | 8.44 | Cyto/Mito    |
| Hordeum vulgare HO1 | LOC105639364 | 837      | 4        | 291                 | 33.29    | 7.70 | Chlo         |
| Jatropha curcas HO1 | LOC14G132400 | 858      | 4        | 289                 | 33.30    | 8.72 | Cyto/Cyto    |
| Nicotiana tabacum HO1 | LOC107818591 | 819      | 4        | 278                 | 32.27    | 8.50 | Mito/Cyto    |
| Oryza sativa HO1 | LOC102600014 | 849      | 4        | 278                 | 32.00    | 8.61 | Cyto/Mito    |
| Oryza sativa HO2 | LOC102600014 | 849      | 4        | 278                 | 32.00    | 8.61 | Cyto/Mito    |
Table 2. (Continued)

| SPECIES NAME     | PHYTOZOME GENE ID       | CDS (BP) | EXON NO. | PROTEIN LENGTH (AA) | MW (KDA) | pI     | LOCALIZATION |
|------------------|-------------------------|----------|----------|---------------------|----------|--------|--------------|
| Triticum aestivum HO1 | –                      | 867      | –        | 288                 | 31.65    | 6.41   | Chlo         |
| Zea mays HO1     | GRMZM2G101004           | 1262     | 4        | 285                 | 31.60    | 6.63   | Chlo         |
| Ziziphus jujuba HO1 | LOC107434821           | 849      | 4        | 293                 | 33.43    | 8.81   | Mito/Cyto    |

Abbreviation: MW, molecular weight. “–” indicates that the genomic sequence and/or gene ID was not available.

Figure 1. Exon-intron organization of heme oxygenase genes from different plant species. Genes analyzed are those from the species listed in Table 2. Species used for this analysis include the following: Asparagus officinalis (AoHO1), Arabidopsis thaliana (AtHO1, AtHO2, AtHO3, AtHO4), Aegilops tauschii (AtaHO1), Amborella trichopoda (AtrHO1), Brachypodium distachyon (BdHO1), Brassica napus (BnHO1), Cucurbita maxima (CmHO1), Cucurbita moschata (CmoHO1), Chenopodium quinoa (CqHO1), Dendrobium catenatum (DcHO1), Elaeis guineensis (EgHO1), Glycine max (GmHO1, GmHO3), Gossypium hirsutum (GhHO1), Hevea brasiliensis (HbHO1), Jatropha curcas (JcHO1), Manihot esculenta (MeHO1), Nicotiana tabacum (NtHO1), Oryza sativa (OsHO1, OsHO2), Phalaenopsis equestris (PeHO1), Sorghum bicolor (SbHO1, SbHO2, SbHO3, SbHO4), Setaria italica (SiHO1), Sesamum indicum (SinHO1), Solanum lycopersicum (SlHO1), Spinacia oleracea (SoHO1), Solanum tuberosum (StHO1), Ziziphus jujuba (ZjHO1), and Cucumis sativus (CsHO1).
The weight ($M_w$) of the different polypeptides ranged from 21.3 to 37.06 kDa with $pI$ values of 5.39 to 9.19. Three $SbHO$ proteins, namely, $SbHO1$, $SbHO2$, and $SbHO3$, are characterized by a $pI$ that is less than 7 suggesting that they are acidic, while $SbHO4$ ($pI = 8.58$) is basic. Another difference is that the $SbHO4$ gene encodes a longer polypeptide with a $M_w$ (37.6 kDa) that is slightly higher than the other $SbHO$ proteins. These data suggest that $SbHO4$ is structurally diverse and might perform a unique function. Subcellular localization of the HO proteins was predicted to be in the chloroplast, nucleus, mitochondria, and cytoplasm (Table 2), and these data correlate with previous studies. These results indicate that sorghum HO candidates share some similarities with other plant HOs.

**Prediction of signature and conserved motifs**

Sequence signature motifs are generally conserved in a protein family since they perform similar structural and functional roles, thus it is important to consider them while grouping novel proteins within specific families. The presence of the HO signature motif was analyzed by multiple sequence alignment using the ClustalX software based on the amino acid sequences of each HO protein. Based on the alignment, almost all amino acid sequences of the HO proteins were conserved in the HO signature sequence (QAFICHFYNI/V), which is important for heme binding (Figure 2). Slight sequence variations within the signature motif were observed within some HO proteins, including $AtHO4$ (PAFICHFYNIY), $SbHO4$ (PAFVCHLYNV).

![Figure 2. Multiple sequence alignment of $SbHO$ genes and other HO orthologs in plant species. The conserved HO amino acids are emphasized with a red block. The black arrow pointing downward indicates the conserved histidine residue for protein stability. Species included in this analysis are the same as those analyzed in the gene structure with addition to Medicago sativa ($MsHO1$ and $MsHO2$), Brassica juncea ($BjHO1$, $BjHO2$, and $BjHO3$), Triticum aestivum ($TaHO1$), and Hordeum vulgare ($HvHO1$).]
and BnHO1 (PSFICHFYNI), SbHO2 and OsHO2 share an identical signature sequence (PAFLSHYYNI), which is different from AtHO2 (PLFLSHFYSFIYF). SbHO1, SbHO2, SbHO3, and SbHO4 share 72%, 53%, 70%, and 51% amino acid sequence identity with AtHO1, AtHO2, AtHO3, and AtHO4, respectively. In addition, SbHOS share above 90% sequence identity with HOs from cereals suggesting a high level of functional similarities.

Conserved HO motifs were predicted using the online MEME tool to better understand the protein’s evolution and function. A total of 5 motifs were predicted and their sequence verified using BLAST (Figure 3). Only motif 1 and motif 2 encoded the HO super-family and are present in all the HO orthologs except for AtHO3 and BnHO2. Motifs 3, 4, and 5 did not encode any conserved domain. Since motif 1 and 2 were found in all HO proteins including the 4 sorghum HOs, the results provide confidence of their identification as bona fide HO encoding genes and infer a functional similarity with other HOs.

**Phylogenetic analysis**

Phylogenetic relationships of HOs from sorghum were compared with other known and well-characterized HO members.
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from other plant species. The phylogenetic tree was constructed using the neighbor joining method, based on the sequence alignment of 43 full-length HO amino acid sequences from 32 plant species to examine the conservation and diversity of the HO domain region (Figure 4). The tree comprises 2 main classes (class I and II); both of which are divided into 2 main groups with several branches. Class I is the biggest class comprising all HO1 members including the sorghum HO1 candidates, whereas class II comprises only HO2s from sorghum, rice, mustard, alfalfa, and Arabidopsis (Figure 4). Based on the phylogenetic analysis, SbHO1, SbHO3, and SbHO4 can be grouped together as belonging to the HO1 sub-family since they fall in class I. These results are consistent with previous reports, and they indicate that the HO family is highly conserved across plant species, with close sequence correlation of SbHOs observed with other cereals.

Analysis of the expression profiles of SbHO genes

To analyze the expression profiles of SbHO genes (SbHO1, SbHO2, SbHO3, and SbHO4), qRT-PCR was performed on different tissues of sorghum seedlings. SbHO transcripts were expressed in all tissues including leaves, stems, and roots but their level of expression was different under normal conditions (Figure 5A). SbHO genes displayed the same pattern of transcript levels in all tissues with the highest observed in the stems, followed by leaves and roots. The SbHO4 transcript was more expressed in the leaves as compared with the other SbHO members. Based on these expression profiles, all 4 SbHO genes are constitutively expressed in all tissues, which suggest that they might be required for growth and development of plants under normal conditions.
The expression of SbHO genes was analyzed in sorghum seedlings treated with 250 mM mannitol to induce osmotic and hence oxidative stress at different time points of 3, 12, and 24 h using qRT-PCR. The analysis indicated that SbHO transcripts were differentially expressed in all tissues and at different time-points (Figure 5B-D). A significant ($P \leq 0.01$) increase in transcript level was observed for SbHO1 in the leaves at 3 h showing a 100-fold increase compared to the control (Figure 5B). While no significant increase was observed in the stem (Figure 5C), a slight increase in the roots (Figure 5D) at 3 and 12 h was observed as compared with the control. Overall, a significantly high SbHO1 transcript level was observed in the leaves upon stress treatment. Upon stress treatment, SbHO2 transcripts were increased in the leaves at 3 and 24 h compared to the control (0 h), showing a 3- and 6-fold increase, respectively. While not much change was observed in the stem, SbHO2 transcripts significantly ($P \leq 0.01$) increased in the roots at 12 h showing ~2-fold increase compared with the control. Similar to the expression pattern of OsHO2, our results indicated that SbHO2 was also significantly induced by osmotic and oxidative stress. The SbHO3 transcript was slightly downregulated in the leaves and stem but a significant ($P \leq .01$) increase in the roots at 12 h was observed showing a 12-fold increase as compared with the control (Figure 5D). The SbHO4 transcript was slightly induced in the leaves at 24 h, followed by a 3-fold increase in the stem at 3 h and a slight increase was observed in the roots at 12 and 24 h.

In comparison, significant expression levels were observed for SbHO1 transcript in the leaves followed by SbHO2, SbHO4, and SbHO3, while in the roots, SbHO2 is the most expressed followed by SbHO3 and SbHO1. These results are consistent with previously published data on A. thaliana HO members which suggest that HO genes are differentially expressed and their expression is dependent on the induction by oxidative stress. The model plant "A. thaliana" is the only plant species where all four HO members have been identified and characterized. These results revealed that the expression of SbHO genes is differentially regulated by osmotic and oxidative stress in different tissues and at different time-points, indicating that individual members have specific spatial and temporal functions. Since SbHO1 and SbHO2 were previously sequenced, this study provided evidence that the two additional putative HO candidates (SbHO3 and SbHO4) exist and that all four members potentially might play a protective role in sorghum against oxidative stress.

**Conclusion**

This study identified and characterized all 4 HO genes in sorghum and their expression in response to osmotic stress analyzed. Results revealed that based on gene structure, subcellular localization, signature motifs, and phylogenetic analysis of HO family members are highly conserved across all plant species analyzed. The data indicated that SbHO genes are transcriptionally expressed in all tissues tested, and expression analysis confirmed that they are inducible by osmotic stress. For future research, it will be interesting and valuable to generate transgenic crops overexpressing sorghum HOIs toward understanding their biological role under different stresses. Thus, the study...
has added new information regarding the possible role of sorghum HOs as part of the defense systems against osmotic and oxidative stress and these data might be useful in the development of stress-tolerant crops.

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Author Contributions

TMM designed the experiments, conducted quantitative real-time PCR, analyzed all the experimental data, and wrote the article; VI conducted in silico characterization and drafted the section on in silico analysis; MM assisted in conducting qRT-PCR experiments and data analysis; AF isolated the genes and assisted in analyzing the results; TMM, CAG, and EI jointly developed the structure and arguments of the manuscript and made critical revisions. All authors reviewed, edited, and approved the final version of the manuscript.

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