

## Identification and expression profiling of heteroglycan glucosidase 1 enzyme of *Cenchrus americanus*

Zainab M. ALMUTAIRI\*

Prince Sattam bin Abdulaziz University, College of Science and Humanities in Al-Kharj, Department of Biology, P.O. Box: 83, Al-kharj 11942, Saudi Arabia; z.almutairi@psau.edu.sa (\*corresponding author)

### Abstract

Maltose metabolism is a critical process during plant growth, which provides energy during development and reproduction. To investigate maltose metabolism in  $C_4$  plants, we identified and analyzed the expression of the heteroglycan glucosidase 1 (HGL1) enzyme from *Cenchrus americanus* (L.) Morrone. The sequenced cDNA of *CaHGL1* (3469 bp) encoded for a deduced protein of 1047 aa. Transmembrane topology revealed that CaHGL1 is a membrane-bound protein that comprises a signal peptide and a transmembrane helix. The promoter of CaHGL1 contains *cis*-elements related to the responses to light, abiotic stress and phytohormones. Real-time PCR revealed high expression in inflorescence and roots during the vegetative stage. Moreover, phytohormone treatments caused an activation of *CaHGL1* expression in the seedling root and shoot by ABA, cytokinin, and BL, and an inhibition by JA in the seedling root and shoot. However, treatment with GA and IAA caused an activation in the *CaHGL1* expression only in the shoot. Stress treatment induced the expression of *CaHGL1* under drought, cold, and salt stress. The results of the current study give insight into the activity of the HGL1 enzyme in maltose metabolism under abiotic stress, which can aid in understanding the different metabolic pathways in *Cenchrus americanus* under stress.

**Keywords:** abiotic stress; *Cenchrus americanus*; cDNA; *Cis*-element; gene expression; heteroglycan glucosidase 1; *in silico* characterization; phytohormones

### Introduction

Starch, a complex polysaccharide that consists of glucose polymers, is considered a primary storage form of carbohydrates in plants. It is synthesized in the photosynthetic organs of the plant, where the final product of photosynthesis, i.e., sucrose, is converted into starch. The latter is then accumulated in plant storage organs (e.g., roots, tubers, seeds, and cotyledons) (James *et al.*, 2003). Starch degradation occurs in photosynthetic plant organs or storage organs that provide sugar as the primary source of energy required for plant growth (Sun and Henson, 1991).

The enzymes required for starch degradation, therefore, play a critical role during the development by providing the plant with the required energy to allow for seed differentiation and reproduction. Several enzymes, including at least four  $\alpha$ -glucosidases, take part in the starch-to-sugar conversions in plants and may

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form part of multiple starch degradation pathways (Lu *et al.*, 2006; Stanley *et al.*, 2011). It is however, unclear which enzymes are critical during the germination stage, and the relevant degradation mechanisms are still poorly understood. One known pathway is that of maltose degradation in the cytosol, where  $\beta$ -amylase breaks down transitory starch in the chloroplast. The resulting maltose is then transported to the cytosol and sequentially degraded into heteroglycan by disproportionating enzyme 2 (DPE2) (Lu and Sharkey, 2006). The enzyme that converts cytosolic heteroglycan into glucose is one of the  $\alpha$ -glucosidase isoforms called heteroglycan glucosidase 1 (HGL1; Lu *et al.*, 2006). Cytosolic heteroglycan is mainly described as being comprised of arabinose, glucose, and galactose (Fettke *et al.*, 2005), and can also be broken down (under different conditions) by cytosolic  $\alpha$ -glucan phosphorylase (PHS2) to produce glucose-1-phosphate (Lu and Sharkey, 2006). Metabolism of maltose is regulated by temperature and day length. Additionally, it has been shown that abiotic stress may increase maltose content and the activity of genes responsible for maltose degradation (Kaplan and Guy, 2004). This suggests that maltose metabolism may form part of the plant's response when experiencing environmental stress. Yet, alteration of the maltose degradation mechanisms has not been unraveled, and to better understand factors that affect plant growth, investigations assessing environmental effects on maltose metabolism are needed.

Cereal crops are considered the main source of dietary energy for humans and animals (Sarwar *et al.*, 2013). These crops include C3 and C4 plants, which have different carbon-fixing mechanisms during photosynthesis. However, C4 cereals are more efficient in photosynthesis and resource usage than C3 cereals. Due to the differences in the physiology between C3 and C4 cereals, the metabolism process under stress is expected to be different between these two classes. For instance, pearl millet is a C4 cereal that is known to grow in hot and dry environments, indicating a distinct physiological mechanism in growth regulation and stress response, including different mechanisms for starch degradation (Treves *et al.*, 2022). The differences in cellular processes between C3 and C4 cereals have been reported as high variation in gene expression of orthologs genes of rice and sorghum during seed germination (Davidson *et al.*, 2012). Thus, this study aims to uncover the role of cytosolic maltose degradation enzyme, HGL1, in pearl millet (*Cenchrus americanus* (L.) Morrone) by analyzing its expression during the development under different abiotic stress and phytohormone treatments.

## Materials and Methods

### Primer design

The rice HGL1 protein sequences (Os07g0420700) were downloaded from Ensembl genome browser 110 (Cunningham *et al.*, 2022) and used as queries in the Whole Genome Shotgun database in the NCBI in the *C. Americanus* genome to find the hypothetical sequence of *HGL1* in the *C. americanus* genome using tBLASTn tool. Primer3 tool was used to design the PCR primers (<https://primer3.ut.ee/>) from the genomic region on chromosome 7 of *C. americanus* (#LKME02052033), which was highly conserved with the query protein. The primer pairs successfully used to amplify the cDNA of *CaHGL1* are listed in Table S1.

### cDNA sequencing and characterization of *CaHGL1*

The plant material used in this study is *C. americanus* variety #1316 in the Saudi Centre of Genetic Resources. Total RNA was extracted from 3-day-old seedlings by PureLink™ Plant RNA reagent (Invitrogen) following the manufacturer protocol. The *CaHGL1* cDNAs were amplified using iScript™ cDNA Synthesis Kit (BIO-RAD). The PCR master mix from Promega was used in the PCR reactions with cycling at 98 °C for 3 min; followed by 32 cycles each of 20s at 95 °C, 25s at 56 °C, and 46s at 72 °C, then, finally, 12 min at 72 °C. Sequencing of the amplified fragments with BigDye Terminator v3.1 was conducted using the protocol described by Sanger (Sanger *et al.*, 1977).

The obtained cDNA sequence was converted into a protein sequence using an ORF finder (<https://www.ncbi.nlm.nih.gov/orffinder/>), and protein domains were predicted using the PFAM server (<http://pfam.xfam.org/>). The Compute pI/Mw subsequently predicted the isoelectric point and molecular weight of the CaHGL1 proteins in the ExPasy server, and the exon/ intron structure of the cDNA was illustrated via Gene Structure Display Server (Hu *et al.*, 2015). The secondary structure of the CaHGL1 protein was generated by the PSIPRED server (Nugent and Jones, 2013), whereas the SWISS-MODEL library (<https://swissmodel.expasy.org/>) was used to model the tertiary structure. Subcellular localization of the translated protein was found by the *DeepLoc-1.0* ([dtu.dk](http://dtu.dk)). The position of the signal peptide and the transmembrane topology was predicted using the MEMSAT-SVM on the PSIPRED server (Nugent and Jones, 2013). Gene ontology prediction for CaHGL1 protein was performed by the FFPred 3 tool (<http://bioinf.cs.ucl.ac.uk/psipred/>). The putative promoter sequences for CaHGL1 were screened for *cis*-elements by retrieving the sequence 2 kb upstream from the gene using the PlantCARE server (Lescot *et al.*, 2002).

#### *Stress treatments and gene expression analysis*

The expression profile of *CaHGL1* in pearl millet tissues were analyzed, during various developmental stages and in response to abiotic stress and phytohormones. To examine the expression of *CaHGL1* during developmental stages, seeds were grown in the field, and then, the leaf, root, and stem of the 28-day-old plant, inflorescence during heading, and spikelet after ripening were collected. An additional group of seeds was grown in Petri dish for 48h to analyze the expression of *CaHGL1* during germination.

The expression of *CaHGL1* during germination was examined for four abiotic stresses (cold, drought, heat, and salt). Stressors to 5-day-old seedlings were applied as follows: treatment with 47 °C for 6h (heat stress), treatment with 180 mM NaCl for 6h (salt stress), treatment with 8 °C for 6h (cold stress), and drought treatment by an airstream for 13 min and keeping the seedlings in open dishes for 18h (in a humidity of 16%). To examine the expression of *CaHGL1* in response to phytohormones, seven groups of 5-day-old seedlings were treated separately for 6h with 1.5 mg/ml of one type of phytohormones including: gibberellic acid (GA), auxin (IAA), abscisic acid (ABA), cytokinin, jasmonic acid (JA), and brassinolide (BL). The seventh group of seedlings was kept with no treatment as control. Roots and shoots for treated and untreated seedlings were analyzed separately for *CaHGL1* expression and then subjected to RNA extraction. Biological replicates were made up of tissues from three different plants.

RNA concentration was measured and equalized of all cDNA samples using the Qubit® 2 fluorometer (Invitrogen) for real-time time-PCR analysis. The delta-delta Ct (ddCt) value was calculated by normalization of *CaHGL1* cycles to the threshold (Ct) values to the reference gene, Elongation factor 1  $\alpha$  Ct values. The primer pairs used for gene expression are listed in Table S1. The qRT-PCR reaction was conducted with KAPA SYBR® FAST qPCR Master Mix from KAPA Biosystems following the manufacturer's instructions. Each reaction was repeated at least three times using the different biological replicates. The PCR program began at 94 °C for 14 min, followed by 50 cycles each of 18s at 94 °C, 32s at 60 °C, and 32s at 72 °C. Thereafter,  $2^{-\text{ddCt}}$  was calculated as fold-change for *CaHGL1* expression according to Livak and Schmittgen's (Livak and Schmittgen, 2001) method.

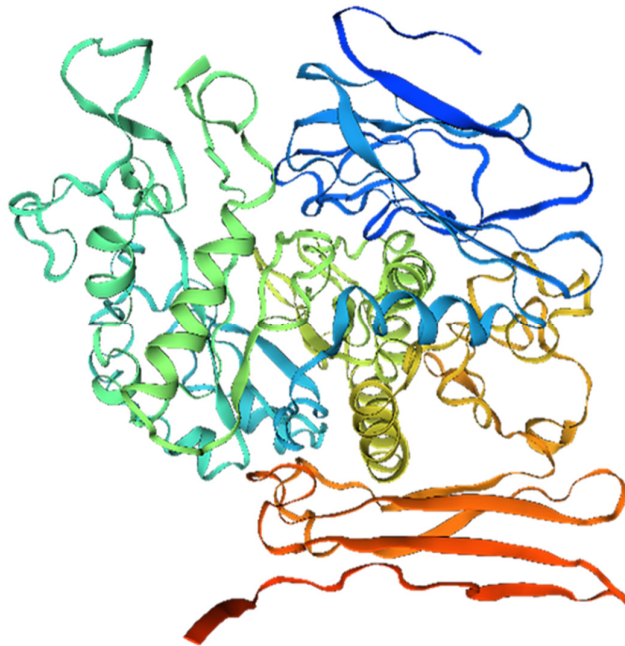
## Results

### *CaHGL1 cDNA characterization*

The cDNA of *CaHGL1* consisted of 3469 bp, with an open reading frame of 3147 bp, and was deposited in the NCBI GenBank under number OR536313. The deduced CaHGL1 protein consisted of 1048 aa and contained a galactose mutarotase-like domain #PF13802 (spanning 223-290 aa), the glycosyl hydrolases family 31 domains #PF01055 (spanning 307-744 aa), and a domain of unknown function #PF17137 (spanning 761-832 aa). The molecular weight of the protein was determined as 114.9 kDa, and the isoelectric point as 5.52. The BLAST search for cDNA of *CaHGL1* revealed an identical genomic region on chromosome 7 #LKME02052033 of *C. americanus*, which spanned from nucleotides #115335617 to #115351345 and shared 81-100% identity with the *CaHGL1* cDNA. Alignment of the *CaHGL1* cDNA with this region revealed 25 exons in *CaHGL1*. Exon lengths ranged from 59-194 bp, whereas intron lengths ranged from 129-2918 bp (Figure S1).

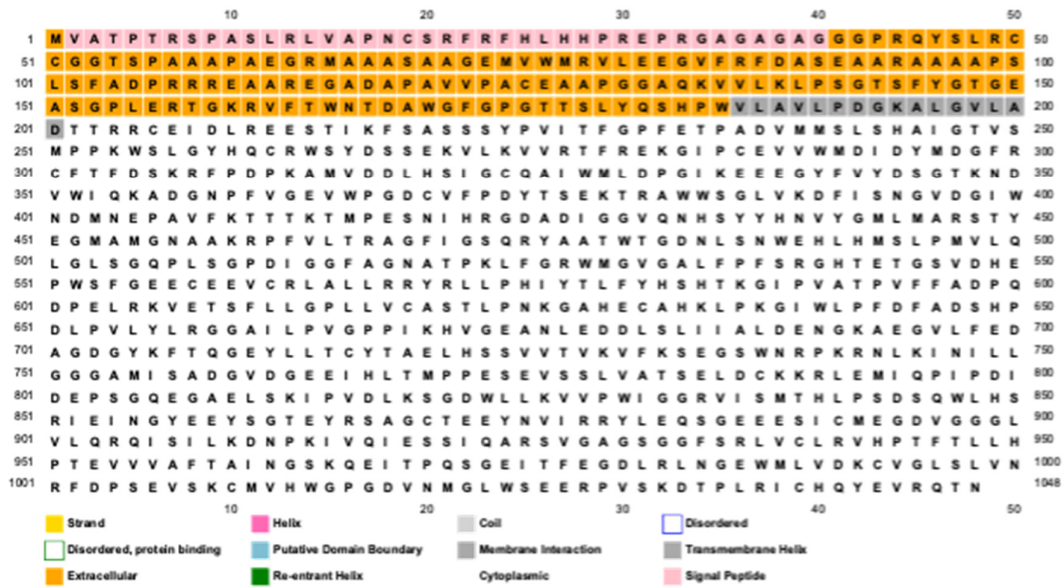
### *CaHGL1 deduced protein characterization*

The secondary structure of the CaHGL1 protein showed 19  $\alpha$ -helices and 62  $\beta$ -strands (Figure S2). The tertiary structure of the  $\alpha$ -glucosidase II (#5djl.1.A) in the SWISS-MODEL template library represented the most similar template to the CaHGL1 deduced protein (sharing 45.96% sequence similarity). This template was subsequently used to build the tertiary structure for the CaHGL1 protein (Figure 1).



**Figure 1.** The tertiary structure of the CaHGL1 protein is based on a similar template (5djl.1) from the Swiss Model database. The N-terminus is indicated in blue, while the C-terminus is indicated in red.

Subcellular localization showed that the CaHGL1 protein may exist in the cytoplasm (with 43% probability) and the chloroplast (with 25% probability) (Figure S3). Transmembrane topology revealed that CaHGL1 comprises a signal peptide (1-38 aa) and a transmembrane helix (187-202 aa). The consensus topology predicted by MEMSAT-SVM indicated that the N-terminus of the CaHGL1 protein may be oriented outside the organelle, whereas the C-terminus is oriented inside the organelle (Figure 2).



**Figure 2.** The topology of the CaHGL1 transmembrane protein (as predicted by MEMSAT-SVM through in server). Amino acids located outside the membrane (orange) or inside the membrane (non-highlighted) are provided. Amino acids that form part of a signal peptide are indicated in pink whereas those that form part of the transmembrane helix are indicated in grey

The biological process prediction by FFPred indicated that CaHGL1 may be involved in cellular response to stimulus (GO:0051716), RNA splicing (GO:0008380), cellular metabolic process (GO:0044237), and gene expression (GO:0010467). In addition, the predicted molecular function showed the involvement of CaHGL1 in catalytic activity (GO:0003824), and RNA binding (GO:0044822) (Tables S2, S3, and S4).

*Distribution of cis-elements in the promoter of the CaHGL1 gene*

The screening of *cis*-elements in the 2 kb putative promoters of *the CaHGL1* gene reveals that the putative promoter of in *CaHGL1* gene includes binding sites for several stress-responsive factors and plant hormones (Figure 3; Table S5). These regulatory elements include the core promoter elements; TATA-box, the common element in promoter and enhancer regions; CAAT-box, drought-inducibility elements; MYB, Myc, and MYC, and unnamed\_4 element (might be responsible for tissue-specific expression). Moreover, various *cis*-elements related to the responses of six kinds of plant hormones are found in *CaHGL1* promoter, including GA (TATC-box), ABA (ABRE), IAA (AAGAA-motifm and TGA-element), JA (CGTCA-motif and TGACG-motif), cytokinin (as-1), and salicylic acid (TCA-element). Moreover, the analyzed promoter contains *cis*-elements related to the responses to stress, such as drought-responsive elements (MYB, Myc, and MYC), low-temperature-responsive elements, LTR, and biotic and abiotic stress responses elements; Unnamed\_\_1. Likewise, four elements related to light response are found in *the CaHGL1* promoter: G-box, box 4, chs-CMA1a, and AE-box, in addition to zein metabolism regulation element; O2-site, and Unnamed\_4 (responsible for tissue-specific expression).

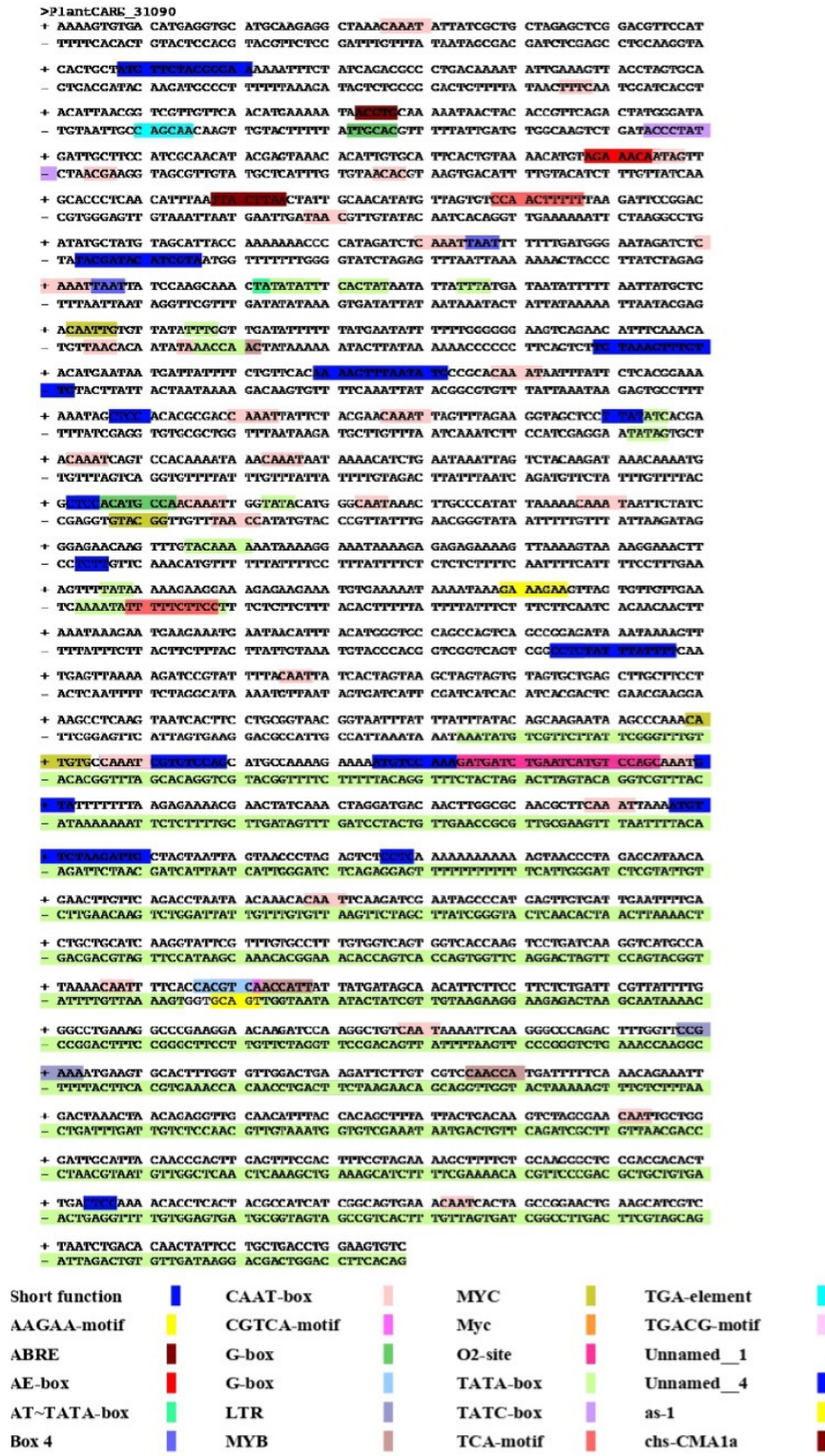
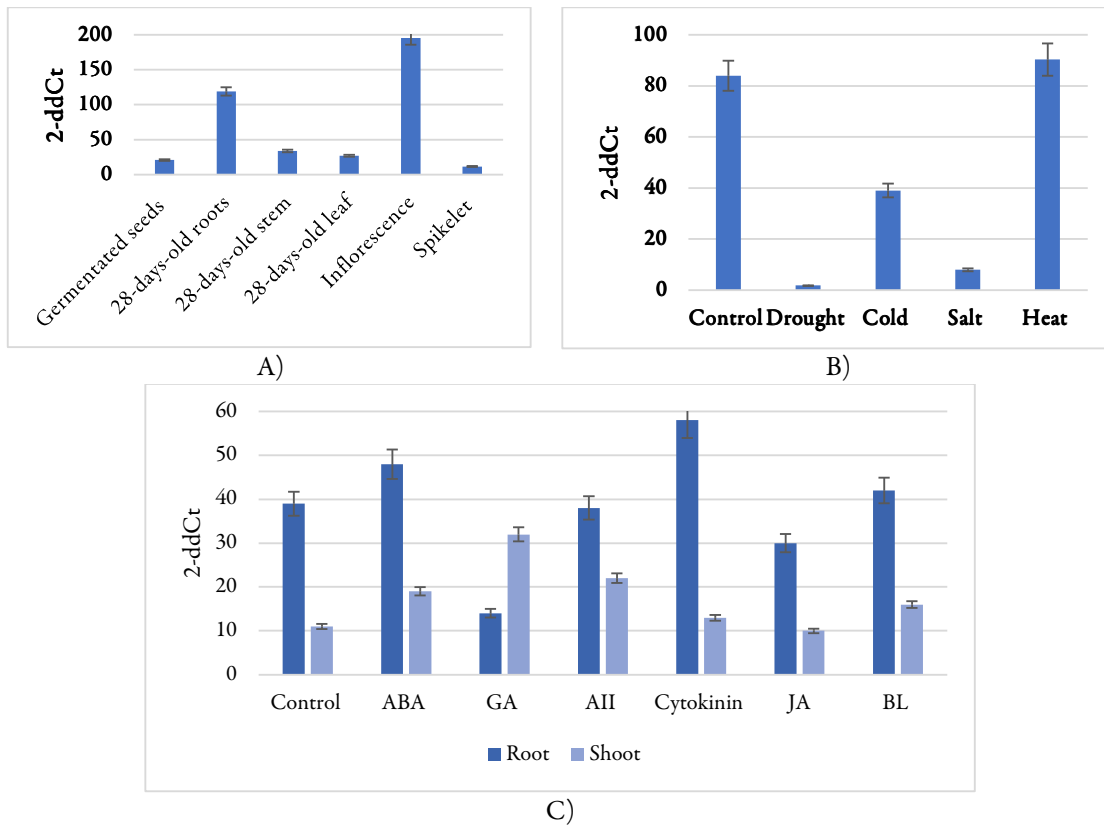


Figure 3. The *cis*-regulatory elements in the putative promoter of the CaHGL1 are predicted by the PlantCARE database. The retrieved promoter sequence was 2 kb upstream of the start codon

*CaHGL1* expression patterns

Expression profiles of the *CaHGL1* gene from *C. americanus* tissues during germination, vegetative, and reproductive stages revealed high expression (between 11 and 195-fold), with the highest expression level in inflorescence (195-fold) and the lowest expression level seen in spikelet (11-fold). The expression in 28-day-old plants revealed high expression in root tissue (119-fold) in comparison with stem and leaf (34-fold, and 27-fold, respectively; Figure 4A). Expression profiles in seedlings under stress revealed a decrease in *CaHGL1* expression level in response to drought, cold, and salt (1.8, 39, and 8-fold, respectively) in comparison with the control (84-fold). The gene expression linked to heat was higher than the control (90-fold; Figure 4B).

The expression of *CaHGL1* responded to phytohormone treatments by activation or repression in comparison with the expression in untreated seedlings. Generally, treatment with ABA, cytokinin, and BL caused an increase in *CaHGL1* expression in the seedling root (42-58-fold) and shoot (13-19-fold) in comparison with the expression in the control (untreated seedling root was 39-fold, and shoot was 11-fold). Likewise, treatment with IAA induced an increase in expression in the seedling shoot (22-fold), whereas the expression in the root (38-fold) was close to the control level. In contrast, treatment with JA caused an inhibition in the expression in the seedling root and shoot (30-fold and 10-fold, respectively) in comparison to control. However, treatment with GA caused an increase in *CaHGL1* expression in the shoot (32-fold) and a decrease in the root (14-fold) (Figure 4C).



**Figure 4.** Expression of *CaHGL1* in *C. americanus* as examined by real-time PCR. A) The expression of *CaHGL1* in seeds during developmental stages including 48h germinated seeds, leaf, root, and stem of 28-days-old plant, inflorescence during heading, and spikelet after ripening. B) The expression of *CaHGL1* in 5-day-old seedlings stressed with drought, cold, salt, and heat. C) The expression of *CaHGL1* in 5-day-old seedling roots and shoots after 6h treatment with phytohormones; ABA, GA, IAA, cytokinin, JA, and BL. The  $2^{-ddCt}$  values represent the mean of three biological replicates.

## Discussion

Heteroglycan, the product of maltose degradation, can be broken down in the cytosol via two routes; the HGL1 enzyme (resulting in glucose) or the PHS2 enzyme (resulting in glucose-1-phosphate). Maltose degradation, essential for plant growth and differentiation (Lu and Sharkey, 2006). is regulated by environmental stress (Kaplan and Guy, 2004). Yet, the effects of stress on maltose degradation pathways remain unclear. This study characterized and analyzed the expression of the *CaHGL1* gene (that encodes the HGL1 enzyme in the pearl millet) during developmental stages and in response to induced stresses and phytohormones.

Prediction of subcellular localization of CaHGL1 protein showed that it may exist in the cytoplasm or the chloroplast, which supports the role of HGL1 protein in degrading cytosolic heteroglycan (Lu and Sharkey, 2006). This prediction agrees with the localization of Arabidopsis HGL1 (AT3G23640) is known to be localized in the chloroplast and nucleus (Zybilov *et al.*, 2008). Prediction of the biological process indicated that CaHGL1 may be involved in RNA splicing and cellular response to the stress stimulus, whereas the predicted molecular function suggested involvement in catalytic activity and RNA binding. This result indicated a possible role of CaHGL1 in the regulation of gene transcription. The CaHGL1 protein also comprises a signal peptide sequence and transmembrane helix, which suggests that the newly synthesized CaHGL1 may be a membrane bound-protein with a role in the translocation of proteins to their cellular destinations (such as the chloroplast).

By measuring the transcript levels in the seedling's tissues, we illustrated that *CaHGL1* was expressed in all tissues during germination, vegetative, and reproductive stages, with high expression shown in inflorescence and roots. This was expected as it is known that maltose is carried from leaves to roots and reproductive organs, as evidenced by the activity of maltose-metabolizing enzymes in plant organs (Lu *et al.*, 2006). Comparison with expression patterns for the rice *HGL1* gene (Os07g0420700) showed expression patterns similar to *CaHGL1* during developmental stages (Wang *et al.*, 2015). The role of the HGL1 protein in enhancing water uptake in plant roots is reported in maize (Song *et al.*, 2022).

The high expression of *CaHGL1* during flowering indicates the critical role of the HGL1 enzyme in providing the energy source, glucose, by degrading maltose during flowering. However, HGL1 has an additional role as a signaling molecule in response to stimulus and gene expression regulation besides its catalytic activity in maltose degradation, as evidenced by gene ontology and transmembrane topology prediction.

Our results further showed that the expression of *CaHGL1* was decreased by drought, cold, and salt stress, while the expression in response to heat stress was close to that in the control. However, the rice *HGL1* is inhibited by heat stress, which shows distinct expression patterns, as shown by our result (Zhao *et al.*, 2017). This might be due to the heat tolerance of the investigated pearl millet variety, which grows at 36-42 °C during the summer season in the western region of Saudi Arabia. Contrastingly, the *HGL1* gene of the C3 plant Arabidopsis is activated in response to 6h treatment of drought, salt, and heat stress and shows similar expression levels under cold and in the control plants (Choi *et al.*, 2022). Decreasing the expression of *CaHGL1* under drought, cold, and salt stress indicates that maltose might be metabolized by PHS2 or DPE2 enzymes (Fettke *et al.*, 2005). This suggestion is confirmed by the evidenced role of PHS2 in the tolerance of abiotic stress, which is active under low-light and when carbohydrate levels are unbalanced (Zeeman *et al.*, 2004). Our result supports the reported fluctuation in maltose concentrations in plants under cold stress (Kaplan and Guy, 2004). Similarly, differential expression of *HGL1* in maize genotypes has been reported under salt stress (Soares *et al.*, 2018). Our results showed that the expression of *CaHGL1* is activated in seedling shoots by ABA, GA, IAA cytokinin, and BL, and activated in seedling roots by ABA, cytokinin, and BL, while JA caused an inhibition in the expression in the seedling root and shoot. Moreover, the metabolism of maltose is regulated by day length, temperature, and circadian (Lu and Sharkey, 2006). Expression patterns of *CaHGL1* are

consistent with *the* expression of its homolog from rice in response to phytohormones (Davidson *et al.*, 2012), suggesting similar hormone regulation mechanisms between rice and pearl millet. Additionally, the screening of *cis*-elements in the *CaHGL* putative promoter reveals binding sites for abiotic stress-responsive factors and several plant hormones, confirming the regulation of CaHGL1 enzyme by stress and phytohormones.

## Conclusions

In conclusion, expression analysis of pearl millet Heteroglycan Glucosidase 1 Enzyme; *CaHGL1*, revealed expression patterns like its ortholog from rice during all developmental stages and in response to phytohormone. However, the *CaHGL1* expression patterns under stress varied from the available data about the orthologs from the C3 plants, Arabidopsis, and rice, suggesting possible variation in the cytosolic maltose metabolism in C4 plants under stress. The combined results of subcellular localization, transmembrane topology, and prediction of molecular function and biological process suggested that, in addition to its known role in heteroglycan breakdown, CaHGL1 may have an additional role in cell signaling.

## Authors' Contributions

The author read and approved the final manuscript.

## Ethical approval (for researches involving animals or humans)

Not applicable.

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## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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