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Original article

Expression profiling and characterization of a G-Box binding protein, *B12Dg*, from pearl millet

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ABSTRACT

Objectives: This study aims to characterize the *B12Dg* from pearl millet and assess its expression in response to abiotic stress and plant hormones during germination.
Methods: cDNA of *B12Dg* has been sequenced, the deduced protein has been characterized through *in silico* tools, and *B12Dg* expression has been examined after treatment by the plant hormones and under abiotic stresses.
Results: The sequenced cDNA for *PgB12Dg* consisted of 314 base pairs and had an open reading frame of 261 base pairs encoded for 87 amino acids. The deduced PgB12Dg protein comprised a B12D domain from 7 to 74 amino acids and a transmembrane helix. Strong conservation was observed among the PgB12Dg protein and its orthologues from monocots. Transmembrane topology prediction and subcellular localization suggested that the deduced PgB12Dg protein accommodates transmembrane helix spanning from 10 to 32 amino acids embedded in the inner mitochondrion membrane. Expression analysis by real-time PCR indicated that *PgB12Dg* was upregulated in roots but downregulated in seeds from 3 day-old-seedlings under drought and cold stress. At the same time, no significant up or downregulation was shown under salt stress. Treatment by plant hormones showed upregulation by gibberellic acid and downregulation by abscisic acid in one-day-old germinated seeds.
Conclusions: The regulation of *PgB12Dg* by plant hormones and abiotic stress in pearl millet during germination can help to understand the transcriptional regulation mechanisms of the PgB12Dg during plant growth under stress.

1. Introduction

The coordination of plant growth and reproductive processes is complicated and involves many regulatory transcription factors. Of these regulatory proteins, G-box transcription factors regulate the transcription of certain genes in plants by recognizing a G-box motif, a *cis*-acting regulatory sequence (CACGTG or CANNTG) in promoters of the target genes (Giuliano et al., 1988). The members of G-box binding proteins are known to be involved in various cellular processes (Schindler et al., 1992). One of the G-box binding proteins is B12Dg, a member of the B12D gene family, which plays an essential role in the regulation of cellular transport and metabolism during various aspects of plant development and stress response (Aalen et al., 2001; He et al., 2014; Steinum et al., 1998; Zhou et al., 2015). B12D proteins appear to be small transmembrane proteins containing the conserved B12D domain involved in critical cellular processes such as transport and ATP biosynthesis (He et al. 2014).

The B12Dg protein was first identified by screening the transcriptome of barley aleurone and embryo (Aalen, 1995). The promotor of barley B12Dg contains a gibberellic acid (GA) recognition element (Steinum et al., 1998). Although it is expressed mainly during germination, its expression is stable during the following developmental stages (Aalen et al., 2001). The homolog of the barley B12Dg protein from sweet potato is expressed differently in senescing leaves (Huang et al., 2001). The B12Dg from Arabidopsis #AT3G48140 is expressed during germination,

Abbreviations: B12Dg, B12D G-box binding protein; cDNA, Complementary deoxyribonucleic acid; *P. glaucum*, *Pennisetum glaucum*; *O. sativa*, *Oryza sativa*; PgB12Dg, B12Dg gene of *Pennisetum glaucum*; BLAST, Basic Local Alignment Search Tool; GA, Gibberellic acid; ABA, Abscisic acid; Ct, Cycles to threshold.

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shoot and root growth, flowering, and silique development (Zhu et al., 2001).

Previous studies have proven that *B12Dg* is regulated in response to various environmental stresses. *Rehmannia glutinosa B12Dg* is upregulated by chromium, GA, and salt stress (Zhou et al., 2015). Wheat *B12Dg* is downregulated under drought stress (Ergen et al., 2009). *B12Dg* from *Beta vulgaris* is differentially expressed in response to salt stress (Wang et al., 2019). *B12Dg* is among the upregulated transcription factors in response to submergence in five rice genotypes. Additionally, promoters of genes upregulated in response to submergence in rice possess G-box binding sites (Mohanty, 2021).

Pearl millet is a cereal crop that can grow in an arid environment (Vadez et al., 2012). More recently, many efforts have been performed to reveal the pearl millet defense mechanisms against environmental stress. Transcriptome analysis of pearl millet genes that are differentially expressed under stress reveals that pearl millet responds to drought through plant hormone signal transduction and MAPK signaling (James et al., 2015; Dudhate et al., 2018). Additionally, plants have several B12Ds regulated by abiotic stress and expressed in various plant tissues (Almutairi, 2022). However, the molecular function of each of these B12Ds has yet to be clearly described. The *MLRQ* gene, a member of the B12D family known to be involved in electron transport in the mitochondrial respiratory chain, has been recently characterized in pearl millet. Expression analysis indicated that the *PgMLRQ* is overexpressed in the roots of seedlings under salt stress and short exposure to cold (Almutairi, 2021).

The present study aims to characterize the *B12Dg* from pearl millet by sequencing its cDNA, characterizing the deduced protein through *in silico* tools, and examining gene expression after treatment by the plant hormones, abscisic acid (ABA) and GA and under drought as well as salt and cold stresses. This study provides insight into the role of the G-box binding protein from the B12D family, B12Dg, during plant germination under stress and in treatment with plant hormones in pearl millet.

2. Materials and methods

2.1. Pearl millet genome retrieval and primer designing

The rice G-box binding protein containing the B12D domain from Oryza sativa #OsJ_20800 in the STRING database (https://string-db.org/) was used as a query in the NCBI tBLASTn (BLAST: Basic Local Alignment Search Tool (nih.gov) against the Whole Genome Shotgun Contigs database, limited by the *Pennisetum glaucum* genome. The *PgB12Dg* primers were designed based on the similarity between the genomic region of *P. glaucum* from chromosome 2 (#LKME02052028.1) from nucleotides 114,581,452 to 114,582,550 and mRNA sequence #CP050798.1 from *Setaria viridis*. Of the tested primer pairs, the primer pair F: 5′-CAGCAGCACAA GATCGAAAA-3′ and R: 5′- CTCGGAGAAGAAGCGGTTGAGG-3′ were found to amplify the cDNA of *PgB12Dg* successfully.

2.2. cDNA amplification and sequencing

The local pearl millet (*P. glaucum* subsp. monodii (Maire) Brunken) variety used in this study is under registration #1316 in the Centre of Genetic Resource in Saudi Arabia. This local variety is grown during the summer season in Saudi Arabia and is known to be tolerant of extreme heat. Total RNA was extracted using TRI reagent (Sigma) on seedlings. The cDNA was synthesized with SensiFAST cDNA Synthesis (Bioline). PCR was performed using High-fidelity PCR Master (Sigma), 0.3 µmol of the designed primers, and 4 µL of cDNA in a total reaction of 20 µL. PCR program started with denaturation at 94 °C for 2 min; then 30 cycles of 94 °C for 15 s, 57 °C for 25 s, and 72 °C for 30 s; and final elongation at 72 °C for 5 min. The PCR product was sequenced by the Sanger method (Sanger et al., 1977) using a BigDye Terminator v3.1. Resulted sequences were assembled using MUSCULE (EMBL-EBI), and the consensus sequence was submitted to *in silico* investigation.

2.3. In silico analysis of the deduced protein

Translation of the open reading frame in the sequence of cDNA and prediction of the isoelectric point (PI) and molecular weight were performed by ExPASy (https://www.expasy.org/). Multiple sequence alignment was conducted for the deduced protein with its orthologues from monocots imported from the NCBI database using MUSCULE (EMBL-EBI). Accession numbers for the aligned proteins are as follows: *Saccharum* (AFO59572.1), *Panicum hallii* (XP_025813718.1), *Zea mays* (NP_001148351.1), *Eragrostis curvula* (TVU11840.1), *Panicum virgatum* (XP_039842942.1), *Setaria italica* (XP_004965125.1), *Digitaria exilis* (KAF8641711.1), *Sorghum bicolor* (XP_002438146.1), *Setaria viridis* (XP_034589112.1), *O. sativa* (XP_015643421.1), *Oryza brachyantha* (XP_006655963.1), *Oryza meyeriana* (KAF0903233.1), *Brachypodium distachyon* (XP_003564009.1), *Aegilops tauschii* (XP_020187542.1), and *Hordeum vulgare* (KAE8785662.1).

Tools from the PSIPRED website (Nugent and Jones, 2013) were used to predict the secondary and tertiary structures of the deduced protein. The molecular function and cellular components of the deduced proteins were predicted by FFPred 3, and the MEMEMBED algorithm predicted the topology of the transmembrane helices. Domains in PgB12Dg were predicted by InterPro (https://www.ebi.ac.uk/interpro/). Subcellular localization of the deduced protein was conducted using the LocTree2 server https://rostlab.org/services/loctree2/ (Goldberg et al., 2012). Functional protein association for the deduced PgB12Dg protein was predicted by the STRING database using the ortholog protein from *O. sativa.*

2.4. Plant treatments and gene expression analysis

Germinated seeds were treated by plant hormones and three types of abiotic stress to examine *PgB12Dg* expression using real-time PCR. The expression of PgB12Dg was measured in the three-day-old seedlings' root, shoot, and seeds under three types of stress; drought, salt, and cold. Pearl millet seeds were surface-sterilized by 5 % sodium hypochlorite for 30 min and washed thrice before germinating. Seven groups of seeds were germinated in Petri dishes at room temperature.; four groups for stress treatment and their control and three other groups for hormones treatment with their control. Their replicates were germinated in each group for real-time PCR biological replicates. The conditions of the stress treatments have been determined based on an initial gradient experiment for each stress type. The tested temperatures for cold stress were 2, 5, 8, and 12 °C. The tested sodium chloride concentrations for salt stress were 100, 150, 200, and 250 mM. These gradient conditions were applied to 3-day-old seedlings for 24 h. The stress conditions affecting growth below optimum levels were selected for plant treatments. Stress treatment was performed by exposing seedlings separately to each type of stress as follows; drought stress was induced by withholding water and leaving the plants in open dishes for 24 h in a relative humidity of 13 %. Seedlings were treated with 200 mM sodium chloride for 6 h to induce salt stress. Another group of seedlings were incubated in the dark at 10 °C for 6 h to induce cold stress. The expression of *PgB12Dg* in untreated seedlings was used as a control for stressed seedlings. The expression of *PgB12Dg* was analyzed in separated tissues of the seedlings: root, shoot, and seed. For the hormones'

treatment, the seeds were soaked in H2O for 24 h and then divided into three groups. Two groups were soaked for 6 h in 1 mg/ml of ABA and GA, respectively, while the third group of seeds was left in H2O as a control group.

Total RNA was extracted from the treated samples and controls, and then cDNAs were amplified using SensiFAST cDNA Synthesis (Bioline). *PgB12Dg*-specific primers were designed to span the second and the third exons (F: 5′- CAAGCGACCGGACGCCGA-3′, R: 5′-TCAACAGCCAAGCAAAATGC-3′). Expression of the control gene *Elongation factor-1 alpha* (primers F: 5′-GTTACAACCCAGACAA GATTGC-3′ and R: 5′-TGGACCTCTCAATCGTGTTG-3′) was used to normalize the *PgB12Dg* expression in all samples. A real-time PCR reaction was performed with 12.5 µL of Power SYBR Green Master Mix from Thermo Fisher Scientific, 2 µL of a cDNA template, and 0.1 µM of each primer in a reaction of 25 µL. The initial cycle was 50 °C for 2 min, then 95 °C for 10 min. The 55 synthesis cycles were 15 s at 95 °C and 1 min at 60 °C. For expression assessment, cycles to threshold (Ct) values were calculated according to Livak and Schmittgen (2001). The Ct values of *PgB12Dg* in untreated tissues were used as calibrators to obtain ddCt and 2^{-ddCt} values.

3. Results

3.1. PgB12Dg cDNA sequencing and characterization

The obtained cDNA sequence of *PgB12Dg* consisted of 314 base pairs and had an open reading frame of 261 base pairs encoded for 87 amino acids. The cDNA sequence of *PgB12Dg* is available in NCBI GenBank under accession numbers MW092093 and QQO98583 for the cDNA sequence and the deduced protein, respectively. The

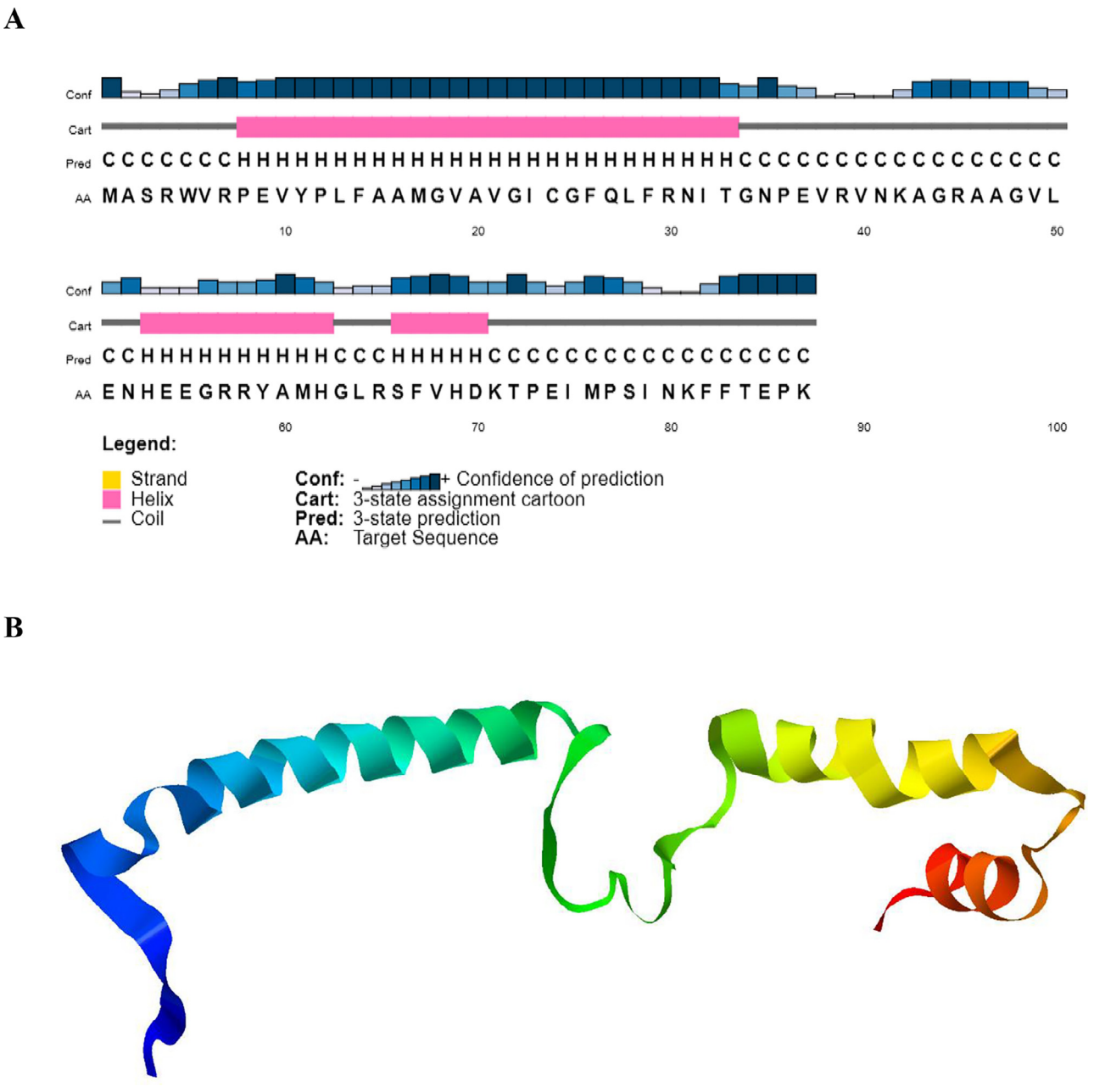

Fig. 1. **(A)** Secondary structure of the deduced PgB12Dg protein predicted by the PSIPRED website. Helices are shown in pink, and the confidence of prediction is indicated by the degree of color of the 'Conf' line. The dark color indicates high confidence, while the light color indicates the low confidence **(B)** The predicted tertiary structure of the PgB12Dg protein were generated by the DMPfold tool on the PSIPRED website. The *N*-terminal is on the blue side and the C-terminal is on the red side.

molecular weight for the deduced protein was 9.8 K_D, and the PI was 9.74. An NCBI BLASTn search against Whole Genome Shotgun Contig revealed a single hit in chromosome 2 (#LKME02052028.1) from nucleotides 114,581,452 to 114,582,550, which shared 92 to 100 % identity with the *PgB12Dg* cDNA. Three exons were detected in the *PgB12Dg* cDNA by aligning it with the sequence of the genomic region from 114,581,417 to 114,582,817 of chromosome 2. The length of the three exons were 52, 87, and 175 base pairs, respectively, whereas the introns were 232 and 865 base pairs, respectively (Supplementary Fig. S1).

3.2. The deduced PgB12Dg in silico analysis

The secondary and tertiary structures of the deduced PgB12Dg protein showed three alpha-helices, as illustrated in Fig. 1. InterPro domain prediction indicated the B12D domain #IPR010530 from 7 to 74 amino acids. Based on Panther prediction, the deduced protein appeared as B12D and G-box binding protein #PTHR33417:SF21. The transmembrane region embedded in the membrane started from 10 to 32 amino acids, based on TMHMM prediction. Additional non-cytoplasmic region (outside of the membrane) from 1 to 11 amino acids and cytoplasmic region (inside the membrane) from 30 to 87 were shown, based on Phobius prediction (Fig. 2A). High conservation was shown in the entire amino acids sequence of PgB12Dg protein. Its orthologues from monocots ranged from 86 to 89 amino acids in length (Fig. 2B). Only the homologous proteins that shared identity above 80 % were selected for multiple sequence alignment to avoid aligning other members from the B12D family.

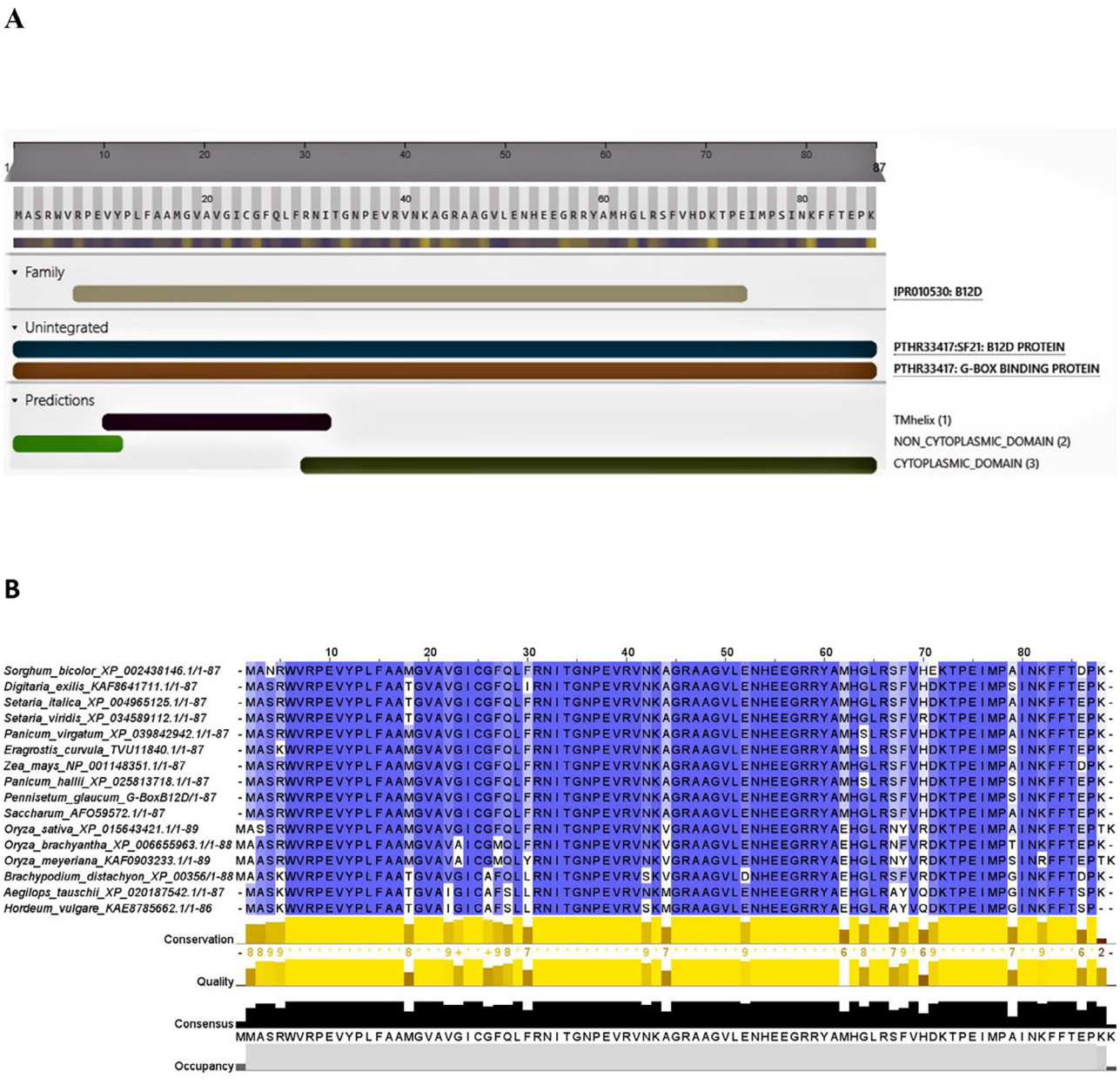

Fig. 2. **(A)** Domains in the deduced PgB12Dg protein as predicted by the InterPro. **(B)** Multiple sequence alignment for the PgB12Dg protein with 15 homologous proteins forms monocots from the NCBI. Only hits that shared more than 80% identity were selected for alignment. Accessions were designated with their scientific names, followed by their NCBI accession numbers. Alignment was conducted by MUSCULE and visualized by Jalview software.

In silico subcellular localization indicated that the deduced PgB12Dg protein is a mitochondrial protein with high probability (99 %, Fig. 3A). The MEMEMBED topology indicated that the transmembrane helix in PgB12Dg protein spanned from 14 to 30 amino acids. As predicted by the three *in silico* tools together, LocTree2, InterPro, and MEMEMBED, the sequence from 10 to 32 amino acids of the deduced PgB12Dg protein appeared to be a transmembrane helix embedded in the inner mitochondrial membrane. Transmembrane helix topology showed that the *N*-terminus of the PgB12Dg protein was in the interspace between the double mitochondrial membranes and that the C-terminus was inside the mitochondrion (Fig. 3B).

Regarding the cellular components prediction of the deduced proteins, PgGB-B12D by FFPred 3 indicated that PgB12Dg was an integral component of the mitochondrial inner membrane, indicated by gene ontology numbers GO:0005743 and GO:0016021 with probability 0.963 and 0.995, respectively. The biological process prediction showed that PgB12Dg was involved in ion transmembrane transport and in the regulation of the metabolic process (GO:0006810, GO: 0034220, and GO:0019222) with high reliability (0.899–0.851 probability value). Molecular function prediction indicated that PgB12Dg has transferase activity transferring glycosyl groups (GO: 0016757) with a probability of 0.922 and transmembrane transporter activity (GO:0022857) probability of

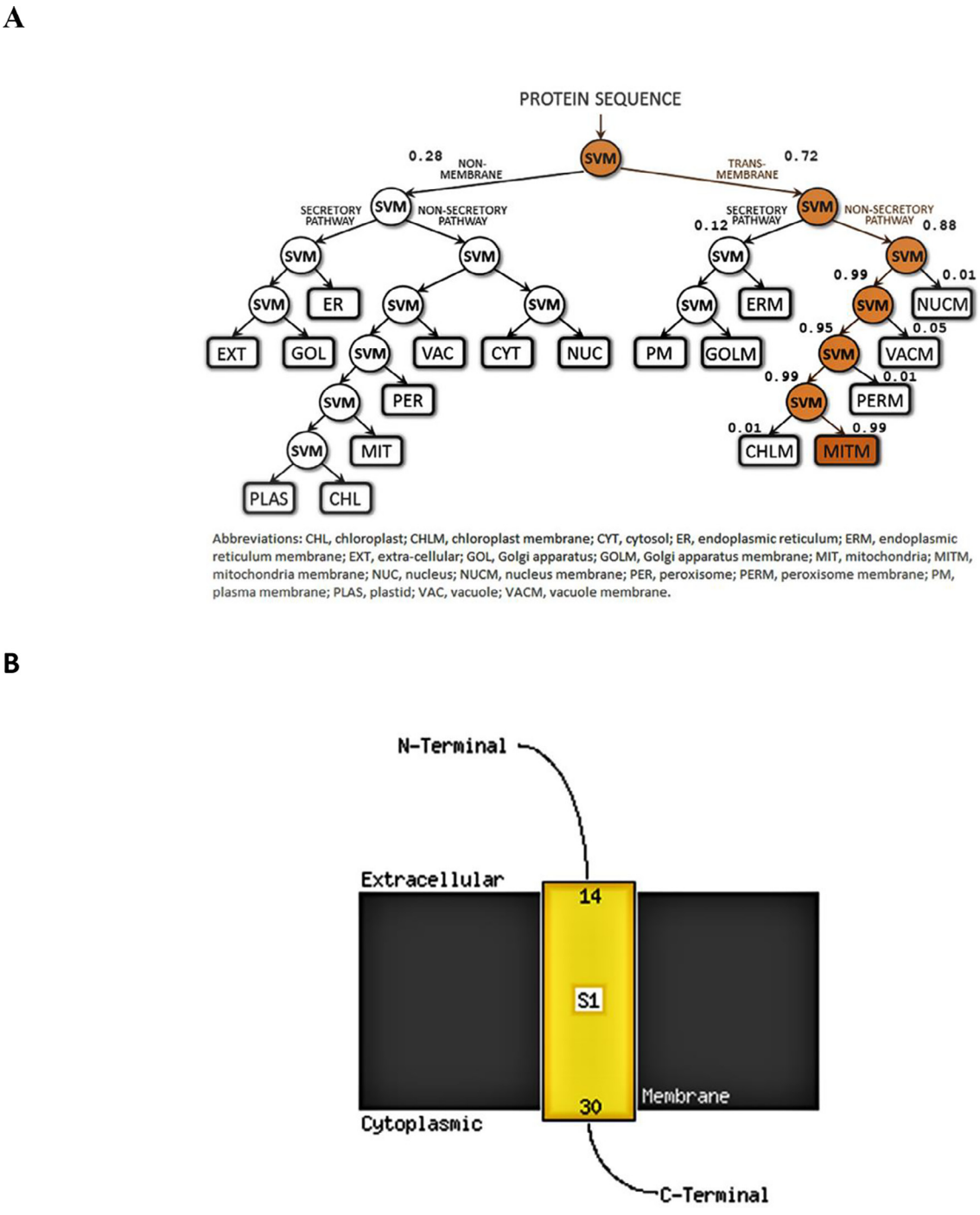

Fig. 3. **(A)** Subcellular localization of the deduced PgB12Dg protein as predicted by the LocTree2 server. **(B)** Topology of the PgB12Dg transmembrane protein in the membrane as predicted by the MEMEMBED algorithm.

value 0.879. Functional protein association for the deduced PgB12Dg protein was predicted by STRING, using the ortholog protein from *O. sativa*, the B12D-G-box binding protein #OsJ_20800 which was found to share 91.8 % similarity with the PgB12Dg protein. Seven sugar transmembrane transporters proteins: SWEET1A, SWEET1B, SWEET2A, SWEET2B, SWEET3A, SWEET3B, and SWEET4, were associated with PgB12Dg homologs in rice (Supplementary Fig. S2).

3.3. PgB12Dg expression profiling

Gene expression profiling revealed that *PgB12Dg* was downregulated (<0.5 folds to untreated seedlings) in shoots and seeds of seedlings stressed with drought, salt, and cold, except seeds under drought, which is considered non-significant downregulation (0.63 folds, Fig. 4). Under the same stresses, roots showed upregulation (greater than2 folds) except for roots under salt stress, which exhibited non-significant upregulation (1.81 folds). The highest expression for *PgB12Dg* was observed in roots under drought (3.33 folds), followed by roots under cold (2.08). Treatment of soaked seeds with plant hormones showed downregulation with ABA (0 folds) and upregulation with GA (2.35 folds).

4. Discussion

Here we sequenced and examined the expression of the G-box binding protein, *PgB12Dg*, from pearl millet. *PgB12Dg* appeared to be a single copy in the pearl millet genome, as it has a single ortholog in rice genome #Os06t0246000, as shown in the rice annotation project database (https://rapdb.dna.affrc.go.jp/, Sakai et al., 2013). Based on *in silico* investigation, the PgB12Dg deduced protein appeared to be embedded in the inner mitochondrial membrane and involved in the transport and regulation of the metabolic process. The transferase activity of glycosyl groups and the associations with sugar bidirectional transporters proteins, SWEETs, suggested the role of the PgB12Dg deduced protein in transporting sugar across the membranes (Chen et al., 2012). This function is consistent with the upregulation of *B12Dg* in Populus tree stems, which results in the accumulation of polysaccharides in the secondary wall in the xylem tissues of the tree stems (Obudulu et al., 2018). The association of *B12Dg* with sugar transporters is confirmed by the post-harvest upregulation of *B12Dg* in sugar beet roots after 46 days of storage, which is required for sugar transport across plasma membrane of storage parenchyma (Chen et al., 2012; Rotthues et al., 2008).

Subcellular localization suggested that the deduced PgB12Dg protein is a mitochondrial membrane-bound transcription factor. These transcription factors are anchored in mitochondrial membranes under normal conditions and are translocated to the nucleus by external or internal signals to regulate their target genes to induce plant responses to these signals (Rhoads and Subbaiah 2007). The homolog of deduced PgB12Dg protein from Arabidopsis #AT3G48140 is localized in the mitochondrion (Senkler et al., 2017), plasma membrane (McLoughlin et al., 2013) and peroxisome (Reumann et al., 2009). However, the accurate localization of PgB12Dg in the cellular compartments of pearl millet needs to be proved by experimental evidence.

Expression profiling of *PgB12Dg* revealed upregulation in the roots and downregulation in the shoots and seeds of seedlings stressed with drought and cold. Similarly, the B12D gene, *MLRQ*, is upregulated in response to drought and 2 h of cold and salt treatment in seedling shoots and roots in pearl millet (Almutairi, 2021). Additionally, the *PgB12Dg* ortholog from *Zea mays* is found to be upregulated under drought (Jiang et al., 2019). In contrast, barley *B12Dg*1 was downregulated by drought in leaves (Talamè et al., 2007). Seedlings under salt stress revealed downregulation in seeds and non-significant upregulation of *PgB12Dg* in roots and shoots. *B12Dg* homologs are upregulated by salt in *Rehmannia glutinosa* roots and leaves (Zhou et al., 2015) and *Beta vulgaris* leaves but not in roots (Li et al., 2020). However, the *B12Dg* homologs of barley and rice are downregulated under salt stress (Aalen et al., 2001; Mizuno et al., 2010). Plant hormones showed downregulation for *PgB12Dg* with ABA and upregulation with GA. This result is consistent with the upregulation of *B12Dg* from *Rehmannia glutinosa* by GA (Zhou et al., 2015) and the inhibition by ABA in barley seedlings (Aalen et al., 2001) and sunflower roots and leaves (Xia et al., 2018).

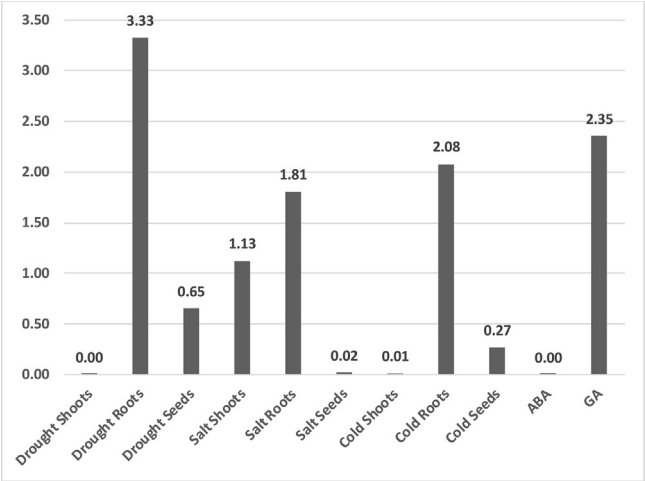

Fig. 4. The expression of the *PgB12Dg* in *P. glaucum* germinated seeds examined by real-time PCR**.** The expression levels of *PgB12Dg* in root, shoot, and seeds of the three-day-old germinated seedlings treated with drought, salt, and cold. RNA was extracted after 24 h of water withholding (drought) and after 6 h of treatment by cold and salt. The expressions of *PgB12Dg* in one-day-old seedlings after being treated for 6 h with ABA and GA are shown in the right of the chart. *Elongation factor-1 alpha* was used as reference gene in real-time PCR analysis. The expression level is indicated by the 2^{-ddCt} value along the y-axis.

5. Conclusion

The *PgB12Dg* gene, a G-box binding protein belonging to the B12D family, is involved in sugar transport and transcriptional regulation. Here, we identified *PgB12Dg* from *P. glaucum* and examined its expression under abiotic stress and in response to plant hormones during germination. *In silico* analysis revealed that the deduced PgB12Dg protein has a transmembrane helix embedded in its mitochondrial membrane. Expression analysis revealed the regulation of the *PgB12Dg* expression under abiotic stress and plant hormones GA and ABA. This study contributes to knowledge about the role of the PgB12Dg during plant growth under stress. In conclusion, the G-box binding protein, B12Dg, is a transmembrane protein that plays an essential role in sugar transportation and appears to respond to stress during the early stage of plant development.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jksus.2022.102448.

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