https://doi.org/10.1590/1678-4499.20230270

BASIC AREAS
Article

Morphological screening and expression of drought-related genes P5SC1 and DREB1A in water-stressed pearl millet (*Pennisetum glaucum*) at the pre-fruiting stage

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Received: Nov. 27, 2023 | **Accepted:** Mar. 21, 2024

Section Editor: Carlos Alberto Scapim

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How to cite: Animasaun, D. A., Mustapha, K. A., Akinbobola, A. M., Bakare, A. T., Ogunjobi, J. T., Adedoyin, K. A. and Awujoola, K. F. (2024). Morphological screening and expression of drought-related genes P5SC1 and DREB1A in water-stressed pearl millet (*Pennisetum glaucum*) at the pre-fruiting stage. Bragantia, 83, e20230270. https://doi.org/10.1590/1678-4499.20230270

ABSTRACT: This study characterized 33 pearl millet accessions for drought tolerance and expression patterns of drought-related genes (*P5CS* and *DREB1A*) at the pre-fruiting stage. The accessions were evaluated for variability and screened for drought tolerance. Five weeks after sowing, we screened the pearl millet accessions for morpho-agronomic variability and to determine their tolerance to water stress over 14 days. From these screenings, we identified the most and least tolerant accessions (NGB00886 and NGB00885, respectively) for gene expression studies. To determine the expression pattern of the *DREB1A* and *P5CS* genes, RNA was extracted from leaf samples and converted to cDNA for analysis via quantitative real-time polymerase chain reaction. The study found significant variation ($p > 0.05$) in morphological traits among the pearl millet accessions. The principal component analysis showed three components accounted for over 90% of the variance. Additionally, there were significant ($p < 0.01$, $p > 0.05$) correlations among the plant growth attributes of the accessions. At genetic index 15, the accessions were grouped into five distinct clusters corresponding to their genetic similarities. Gene expression studies showed differences in the Ct values of the reference gene (*ACT1*) against the target genes. The expression pattern showed that *DREB1A* was up-regulated in the most tolerant and down-regulated in the least tolerant, while *P5CS* was up-regulated in both accessions. The study concluded that there are morphological variations among the accessions in response to water stress at the pre-fruiting stage, and the up- and down-regulation of *DREB1A* and *P5CS* genes are involved in the mechanism of regulation of water stress modulations in pearl millet.

Key words: germplasm, drought tolerance, pearl millet, quantitative real-time polymerase chain reaction, reference genes.

INTRODUCTION

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is a significant cultivated species of the genus *Pennisetum*, in the family Poaceae. The *Pennisetum* genus encompasses numerous species, of which pearl millet is one of the most economically important. It is a crucial crop grown in the less fertile, semi-arid agricultural regions of Africa and Southeast Asia. Pearl millet provides a reliable source of nutritious food and fodder for millions of people living in regions that are too dry for most other cereals to grow and flourish (Animasaun et al. 2017). The crop plays a critical role in tropical agriculture, and an increased understanding of its genetics, diversity, and gene functionality has the potential to open up greater opportunities for its sustainable use. However, one of the major constraints of millet production in the growing areas is drought.

Drought is a climatic hazard that occurs when there is no or very little rainfall for a prolonged period, resulting in moisture reduction in the soil and a decrease in water potential in plant tissues. Drought is often accompanied by elevated

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temperatures, which accelerate evapotranspiration and disrupt photosynthetic kinetics, exacerbating the effects of drought and further reducing crop yields (Mir et al. 2012) and causing significant economic losses (Fahad et al. 2017). Plants use three main techniques (drought escape, drought prevention, and drought tolerance) for adaptation to drought. Plants can reprogram a wide range of responses at the molecular, biochemical, and physiological levels (Fadoul et al. 2021). These changes often occur rapidly and with specific characteristics depending on tissue type, developmental stage, and stress. According to climate change projections, there will be a dramatic increase in drought (Dai 2013). Conversely, areas where pearl millet is grown are vulnerable to drought and declining productivity. Therefore, improved pearl millet cultivars with higher drought tolerance would be the crop of choice under such geoclimatic conditions.

The genetic variability among genotypes could be used as an important source for drought stress screening. Plant breeders have traditionally addressed the issue of environmental stress by selecting for performance adaptability to a range of environmental conditions using rigorous testing and biometric methods (Blum 1988, Meena et al. 2014, Lafta et al. 2020, Roeber et al. 2020, Formisano et al. 2021). However, the genetic pathways that enable these traits are largely unknown because drought tolerance is the result of a complex interaction of morphological, physiological, and biochemical traits, and these traits could be used to screen for acceptable plant ideotypes. Plant osmotic potential, stomatal conductance, carboxylation efficiency, photosynthetic rate, pressure potential, and transpiration rates are all affected by drought (Farooq et al. 2009). These changes often occur rapidly and with specific characteristics depending on tissue type, developmental stage, and stress duration. Thus, understanding the genetic elements that influence plant responses to drought stress will provide an excellent basis for breeding drought-tolerant varieties. The genetic variability among genotypes could be used as an important source for drought stress screening and the identification of desirable plant ideotypes.

The genetic pathways that enable these traits are not well understood, but there is an understanding that response to drought stress results in variable expression of several metabolic pathways at the cellular level. The analysis of gene expression during different developmental stages and environmental conditions is crucial for the elucidation of the molecular mechanisms underlying various biological processes (Trijatmiko et al. 2016). The quantitative real-time polymerase chain reaction (qRT-PCR) is the best technique even for low-abundance mRNA transcripts and has become one of the most widely used techniques for gene expression analysis in recent years (Nolan et al. 2006, Zhang et al. 2016, Yi et al. 2022).

For gene expression and quantification studies using RT-PCR, the use of reference genes or internal standards is crucial. Reference genes are housekeeping genes, which have essential functions in the maintenance of basic cellular metabolism and are independent of physiological conditions (Kozera and Rapacz 2013). Consequently, a number of reference genes has been documented for the normalization of the expression data. This includes *ACT1*, which encodes the single essential actin gene, a highly conserved protein that is involved in various types of cellular activities and is ubiquitously expressed in all eukaryotic cells.

Furthermore, the role of genes and transcriptomic factors in drought tolerance and regulation of water deficiencies in plants have been elucidated (Benny et al. 2019, He et al. 2020, Wang, J. et al. 2021). Among them, there are the pyrroline-5-carboxylate synthase (*P5CS*), and a dehydration response element *B1A* (*DREBIA*). *P5CS* is a bifunctional enzyme with glutamate kinase and γ-glutamyl phosphate reductase (GPR) activity. It responds to osmotic stress and increases the proline content of plants to improve osmotic resistance (Yang et al. 2021). *DREB1A*, on the other hand, encodes a member of the DREB subfamily A-1 of the ERF/AP2 transcription factor family (*CBF3*), which specifically interacts with the dehydration-responsive element (*DRE/CRT*) and induces expression of genes involved in environmental stress tolerance in Arabidopsis.

Understanding the minor changes in gene expression among genotypes allows for the identification of drought-related genes that could be used in the selection of drought-tolerant traits. To this end, the objectives of the present study were:

- To determine the extent of genetic diversity among 33 pearl millet accessions collected from different locations;
- To utilize multivariate analysis to determine the variation in the accessions' agronomic traits and to elucidate the existing correlation among the traits;
- To screen the accessions for drought tolerance to identify the most and the least tolerant accessions;
- To determine the expression pattern and the role of drought-related genes (*DREB1A* and *P5CS*) in drought resistance at the pre-fruiting stage using qRT-PCR.

MATERIALS AND METHODS

Plant materials

A total of 33 pearl millet accessions was used for the study. Twelve accessions from the germplasm were maintained in the gene bank of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. Twenty-one additional accessions from germplasm were maintained by the National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Oyo State, Nigeria (Suppl. Table 1).

Morpho-agronomic characterization of accessions

The morpho-agronomic characterization of the 33 accessions of the pearl millet for drought resistance was carried out in a greenhouse facility at the Faculty of Agriculture, University of Ilorin, Ilorin, Kwara State, Nigeria. The geographical and ecological details of the location are already described (Animasaun et al. 2022). The growth conditions in the greenhouse were 80 $\mu mol \cdot m^{-2} \cdot s^{-2}$ light intensity at 23–28°C with 16 hours light and 8 hours dark photoperiods. Seeds of each accession were sown directly into labelled pots (25 × 20 cm) filled with 10 kg of loose garden soil in Oct–Dec 2019 and repeated from Jan–Mar 2020. The experiment was designed as a completely randomized design in five replications with 0.5 m between the rows and between the pots. Watering was done every other day with 0.75 L of water, and weeding and cultural practices were carried out as usual, but no fertilizers or additives were used.

Morpho-agronomic traits were assessed at two, three and five weeks after sowing (WAS), as described by a standard descriptor (IBPGR & ICRISAT 1993). Quantitative morpho-agronomic traits such as plant height, number of leaves, leaf length, leaf width, and stem girth were assessed at two, three and five WAS. Other traits assessed included days to germination, pubescence, and stem/culm color. Plant height, leaf length, and leaf width were measured using a tape rule. Stem girth was measured using a digital vernier calliper (ATD-8656). Days to germination and the number of leaves per plant were counted, while pubescence and stem color were recorded by physical observation.

Analysis of variance was performed using Statistical Package for the Social Sciences version 19.0 (IBM Corp., Armonk, NY, United States of America) on the pooled data. Means that were significantly different from each other at $p > 0.05$ were separated using the new Duncan multiple range test. The trait correlation matrix and the biplot analysis at $p > 0.05$ and $p < 0.01$ were generated using GenStat 19.1 Edition (VSNI, United Kingdom), as described by Payne et al. (2007). Multivariate analysis was performed, and the hierarchical clustering was constructed based on the unweighted pair group method with arithmetic mean (UPGMA) method. The genetic relationship of the accessions was presented graphically as a dendrogram.

Sample preparation for gene expression study

Fruiting in pearl millet begins at approximately seven WAS (Animasaun et al. 2017). Therefore, at five WAS, water stress was induced in the treated plants by withholding water for 14 days, while irrigation continued every other day for the control plants. The plants were observed daily for drought symptoms, such as leaf rolling, stunning plant, leaf scorching, browning of the leaf, and wilting (Bukhari et al. 2019), to identify the most tolerant and the most susceptible accessions based on the morphological changes dynamics. On day 14, accession NGB00886, which still had some green leaves, less stunning, and standing plants, was adjudged the most tolerant, while NGB00885 showed physical evidence of the highest susceptibility to drought and was recorded as the most susceptible accession.

After 14 days of water stress, leaf tissues were collected from the control and the water-stressed plants of the observed most, and least tolerant accessions (NGB00886 and NGB00885, respectively) for RNA extraction and gene expression studies. With the aid of clean sterile scalpels, fresh leaf samples were collected into separate Ziplock bags for each of the two water stress-contrasting accessions and their control plants. Total RNA was extracted from the samples using a Quick-RNATM Plant Miniprep Kit (R2024) (Zymo Research Corp., United States of America) according to the manufacturer's

instructions available at Zymo Research (2024). The RNA concentration was determined by NanoDrop spectrometry (Thermo Scientific, United States of America). cDNA synthesis was performed using the LunaScript® RT SuperMix Kit (E3010) (New England Biolabs), according to the manufacturer's instructions (protocol available at New England Biolabs, 2018), and the synthesized cDNA was stored at -20°C until use.

Selection of targeted and reference genes and primer design

Based on previous reports on the use of reference genes for studies in plant species (Brunner et al. 2004, Zhang et al. 2016, Yi et al. 2022), for this study, we selected *ACT1* as the standard. Two drought-related genes (*DREB1A* and *P5CS*) were used for the expression study (Xu et al. 2017, Yang et al. 2021). *ACT1* encodes the single essential gene, the ubiquitous, conserved cytoskeletal element critical for many cellular processes. *DREB1A*, also known as *CBF3* (AT4G25480), encodes stress-inducible transcription factors associated with stress adaptation by networking with the dehydration-responsive element (*DRE/CRT*) to promote the expression of genes involved in environmental stress modulation such as drought tolerance. The *P5CS1* (AT2G39800) is a delta 1-pyrroline-5-carboxylate synthase gene that catalyzes the rate-limiting enzymes in the biosynthesis of proline. Detailed descriptions and other attributes of these genes are available at the National Center for Biotechnology Information (NCBI) repository.

The nucleotide sequences of these genes were downloaded from the NCBI, and primers for qRT-PCR were designed using GenScript software to investigate the expression of drought-related genes in the two pearl millet accessions with contrasting characteristics: NGB00886 being the most tolerant, and NGB00885 being the least tolerant. The oligonucleotides were selected based on quality scores, melting temperature, sequence length, and amplicon size. The oligonucleotides were synthesized by a commercial company Microgen, Germany; and they included *ACT1*, *DREB1A*, and *P5CS*. The selected oligonucleotides and their properties are listed in Suppl. Table 2.

qRT-PCR procedure

qRT-PCR was performed using the Luna Universal qPCR Master Mix Kit (NEB M3003S, New England Biolabs, United Kingdom). The SYBER Green Master Mix chemistry was used as the reporter for the reactions. The PCR reaction mixtures were as follows: 4 µL of Luna Universal qPCR Master Mix Kit (NEB M3003S), 4 µL of cDNA, 0.5 µL of each primer (forward and reverse), and 11 µL of nuclease-free water. The Sybergreen Master Mix chemistry was used as the reporter for the reactions. The PCR program was performed on a Bio-Rad CFX Deep Well Real-Time System (C1000 Touch Thermal Cycler, Bio-Rad, United States of America).

The cycling conditions consisted of one cycle of preincubation: 95°C for 30 s; 40 cycles of amplification: 95°C for 10 s, 60°C for 30 s; melting-curve one cycle: 95°C for 15 s, 60°C for 60 s, 95°C for 15 s; and cooling one cycle: 40°C for 30 s. Each qRT-PCR analysis was performed with three technical replicates. The qRT-PCR efficiency was determined for the candidate reference gene and the two target genes based on the slope of a linear regression model (Livak and Schmittgen 2001, Pfaffl 2001). Data were analyzed using Bio-Rad CFX Manager™ software.

RESULTS AND DISCUSSION

Morphological variation and diversity

The results of morpho-agronomic characters of eight descriptors considered showed variation and diversity, which can be exploited for selection, improvement, and germplasm conservation. Hairiness of the ligule, leaf sheath, and leaf blade varied among the accessions (data not shown). While a few accessions were densely pubescent, others were either glabrous or with few trichomes. The presence of trichomes (hairiness) in plants is of evolutionary importance as the trichomes could serve as a protective barrier against natural hazards, such as herbivores, ultraviolet irradiation, pathogen attacks, excessive transpiration, biosynthesis of specific metabolites, seed spread, metal detoxification, seed protection, among others (Wang, X. et al. 2021, Li et al. 2023). However, densely hairy plants as observed in some accessions are

less desired by ruminants, making them constraints as forage grass. Some accessions had deep purple or magenta stem color, which gradually changed to green with the growth period. The change in stem base pigmentation at the seedling stage in some of the accessions may be due to the presence of anthocyanin or mutant chlorophyll, which eventually normalised during growth. Similar observations have been reported previously in pearl millet (Animasaun et al. 2017).

Growth and vegetative characteristics of the pearl millet accessions varied (Table 1). In terms of plant height, Akz-Nr PEN5, Akz-Nr PEN558, and Akz-Nr PEN4 are significantly taller than others. These are IPK accessions, which are likely to combine both genetic and environmental responses resulting in tall plants. The highest number of leaves per plant was recorded in NGB00938, and the lowest in NGB00905. In terms of leaf length, NGB01009 produced the longest leaves, and the shortest one was found in NGB00974. Accession NGB00948 recorded the widest leaves. In contrast, the leaves obtained in NGB00893 are narrow. Meanwhile, all the accessions are similar in terms of stem girth. The observed variations in the growth attributes of the pearl millet accessions are likely due to the interplay of genetic action and environmental influences which according to Burson et al. (2015) could have a significant impact on the vegetative and yield performance of cereals. Because of these interactions, certain genotypes may perform best in a given environment (Nwofia et al. 2014).

Table 1. Morpho-agronomic traits of 33 accessions of pearl millet at five weeks after sowing*.

Accession	PH (cm)	NL	LL (cm)	LW (cm)	SG (cm)
Akz-Nr PEN1030	16.50^{abc}	9.33^{bcde}	63.83^{fghijk}	1.80^{bcdefg}	0.70^{abcde}
Akz-Nr PEN1040	24.07^{bc}	9.67^{bcde}	66.23^{hijk}	1.93^{defghij}	0.73^{bcdef}
Akz-Nr PEN1052	14.07^{ab}	11.00^{def}	58.63^{defgh}	1.60^{bcde}	0.83^{defg}
Akz-Nr PEN1257	26.50^{c}	9.00^{abcde}	65.73^{ghijk}	2.20^{fghijklm}	0.70^{abcde}
Akz-Nr PEN3	18.47^{abc}	9.00^{abcde}	58.37^{defgh}	2.03^{efghijk}	0.73^{bcdef}
Akz-Nr PEN4	35.27^{d}	10.00^{bcde}	58.87^{defgh}	2.03^{efghijk}	0.53^{ab}
Akz-Nr PEN5	38.53^{d}	9.33^{bcde}	55.50^{bcdefg}	2.07^{efghijk}	0.63^{abcde}
Akz-Nr PEN558	35.63^{d}	8.67^{abcde}	57.73^{cdefgh}	2.23^{ghijklm}	0.70^{abcde}
Akz-Nr PEN687	17.07^{abc}	10.67^{def}	65.80^{ghijk}	2.53^{klm}	1.13^{ij}
Akz-Nr PEN705	16.40^{abc}	10.33^{cde}	62.90^{fghijk}	2.17^{fghijkl}	0.87^{efgh}
Akz-Nr PEN711	19.23^{abc}	9.33^{bcde}	59.10^{defghi}	1.87^{defghi}	0.97^{fghi}
Akz-Nr PEN837	22.23^{abc}	10.33^{cde}	65.80^{ghijk}	2.60^{lm}	1.00^{ghi}
NGB00885	18.37^{abc}	9.00^{abcde}	58.80^{defgh}	1.83^{cdefgh}	0.63^{abcde}
NGB00886	16.13^{ab}	10.67^{def}	60.63^{efghij}	2.17^{fghijkl}	0.97^{fghi}
NGB00893	13.03^{a}	7.33^{ab}	48.37^{abc}	1.03^{a}	0.47^{a}
NGB00903	22.67^{abc}	8.33^{abcd}	55.80^{bcdefg}	1.50^{abcd}	0.63^{abcde}
NGB00905	14.20^{ab}	6.33^{a}	51.03^{abcde}	1.30^{ab}	0.57^{abc}
NGB00915	15.83^{ab}	9.00^{abcde}	49.30^{abcd}	1.63^{bcde}	0.60^{abcd}
NGB00931	13.37^{a}	8.33^{abcd}	54.40^{abcdef}	1.70^{bcdef}	0.60^{abcd}
NGB00938	17.27^{abc}	13.00^{f}	65.80^{ghijk}	2.03^{efghijk}	0.77^{bcdefg}
NGB00948	19.10^{abc}	11.33^{ef}	66.30^{hijk}	2.70^{m}	1.10^{hij}
NGB00974	16.70^{abc}	8.67^{abcde}	44.80^{a}	1.57^{bcde}	0.70^{abcde}
NGB00978	15.67^{ab}	9.00a^{bcde}	46.10^{ab}	1.63^{bcde}	0.57^{abc}
NGB00998	13.40^{a}	7.67^{abc}	49.67^{abcd}	1.33^{abc}	0.67^{abcde}
NGB01009	15.70^{ab}	9.00^{abcde}	71.57^{k}	2.37^{hijklm}	0.67^{abcde}
NGB01023	15.67^{ab}	9.33^{bcde}	64.67^{ghijk}	2.07^{efghijk}	0.87^{efgh}
NGB01057	15.60^{ab}	8.67^{abcde}	69.13^{ijk}	2.30^{ghijklm}	0.70^{abcde}
NGB01059	15.33^{ab}	10.67^{def}	62.13^{fghijk}	2.40^{ijklm}	0.87^{efgh}
NGB01060	18.00^{abc}	10.33^{cde}	65.17^{ghijk}	2.43^{jklm}	1.27^{j}
NGB01061	16.50^{abc}	9.00^{abcde}	64.07^{fghijk}	2.30^{ghijklm}	0.80^{cdefg}
NGB01062	14.50^{ab}	7.67^{abc}	63.40^{fghijk}	2.30^{ghijklm}	0.87^{efgh}
NGB01064	16.53^{abc}	8.33^{abcd}	69.63^{ijk}	2.20^{fghijklm}	0.87^{efgh}
NGB01066	16.67^{abc}	8.67^{abcde}	57.50^{cdefgh}	2.17^{fghijkl}	0.63^{abcde}
Mean	18.92	9.30	59.90	2.00	0.77
Max. Value	38.53	13.00	71.57	2.70	1.27
Min. Value	13.03	6.33	44.80	1.03	0.47

*The values are the means of three replicates, the means in the same column having the same superscript are not significantly different from each other at $p \leq 0.05$; PH: plant height; NL: number of leaves; LL: leaf length; LW: leaf width; SG: stem girth.

The observed variation in the morpho-agronomic traits (for both quantitative and qualitative attributes) of the 33 pearl millet accessions may be crucial for selecting desirable accessions for further pearl millet breeding. For instance, if taller plants with high stover yield are desired, any of the three accessions Akz-Nr PEN5, Akz-Nr PEN558, and Akz-Nr PEN4 could serve as potential parent materials. However, if consideration were for high-forage purposes, the NGB00938 and NGB01009 would be most promising. Meanwhile, semi-arid zones are characterised by high wind and stormy rain. Therefore, tall millet varieties will be prone to logging, which will be of economic loss. Thus, characterising accessions/germplasm for diversity is essential for germplasm utilization. This further reiterates that morpho-agronomic characterization and phenotyping, though basic, are pivotal for trait selection for crop improvement, in particular for stress tolerance (Lafta et al. 2020, Formisano et al. 2021).

Correlation of the morpho-agronomic characters of the pearl millet accessions is shown in Fig. 1. There are significant positive correlations at $p > 0.05$ between leaf length and stem girth, leaf width and stem girth, and the number of leaves and stem girth. As expected, there is a strong positive correlation ($p < 0.01$, $r = 0.7612$) between leaf length and leaf width. In contrast, stem girth and plant height had a weak negative correlation. The significant positive correlations of vegetative characters obtained here showed that the traits are associated, possibly due to gene linkage and/or pleiotropy. Linked characters may allow simultaneous improvement through correlated responses to selection in the segregating progeny (Bello and Olawuyi 2015). This finding is consistent with positively significant correlations of characters obtained in sesame (Azeez et al. 2017), pearl millet (Animasaun et al. 2017), and groundnut (Olorunmaiye et al. 2019). The performance of the 33 accessions of pearl millet evaluated showed that the leaf length is the attribute as a morphological marker to discriminate the accessions (Suppl. Fig. 1). It is important to notice that traits that are negatively correlated are mostly not genetically linked, which means that they cannot be bred simultaneously and, therefore, need to be improved independently (Malek et al. 2014).

Principal component analysis for the distribution of variability in pearl millet accessions revealed that three principal components accounted for 99.93% of the total observed variation, grouping the accessions into four quadrants and five major groups (Table 2). The first principal component (PC-1) accounted for 54.57% of the variation, with leaf length contributing the most (0.96). The PC-2, which accounted for 43.79% of the total variation, had plant height contributed the most (0.965), while the PC-3 (1.55%) was dominated by the number of leaves (0.993). The higher eigenvalues for vegetative traits such as leaf length and plant height by PC-1 showed their importance and significance in vegetative differentiation among the accessions (Azeez et al. 2017). The plot of PC-1 vs. PC-2 divided the 33 accessions into major groups (Fig. 2), in which a group consists of only IPK accessions, and other groups are mixtures of IPK and NACGRAB accessions. The dendrogram constructed for vegetative morpho-agronomic traits of the accessions using minimum dissimilarity distance and UPGMA clustering method divided the accessions into two major groups (A and B) at about 46% similarity index (Fig. 3). The cut-off for clustering was set at a genetic distance index of 15, which divided the accessions into five major clusters. Group A was divided into two subgroups AI and AII, with a distance scale of 17 (about 83% similarity index) with each of the subgroups having either IPK or NACGRAB accession or a mixture of the two. Likewise, group B was further divided into sub-clusters.

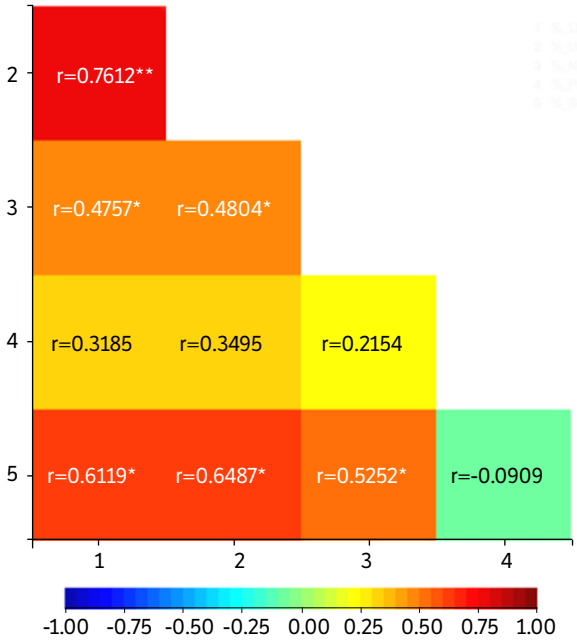
Table 2. Eigenvectors and percentage variation explained by the first three principal components of the related traits of 33 accessions of pearl millet based on morpho-agronomic traits five weeks after sowing.

Components	PC-1	PC-2	PC-3
Eigenvalue variance	50.3916	40.4398	1.43382
Individual percentage	54.576	43.798	1.5529
Cumulative percentage (%)	54.576	98.374	99.9269
Eigenvectors	PH (0.2631)	PH (0.9645)	PH (-0.01825)
	NL (0.08376)	NL (-0.0045)	NL (0.9925)
	LL (0.96)	LL (-0.2638)	LL (-0.08551)
	LW (0.04508)	LW (-0.0007204)	LW (0.06872)
	SG (0.01298)	SG (-0.008551)	SG (0.05136)

PH: plant height; NL: number of leaves; LL: leaf length; LW: leaf width; SG: stem girth.

The pattern of clustering showed that, although some accessions are closely related, there is genetic variability between the accessions. This is consistent with the findings of Burson et al. (2015), who noticed that millet accessions do not necessarily cluster in the same group based on their geographical distribution, but accessions in the same cluster are genetically related. The

dendrogram constructed based on vegetative characters showed IPK and NACGRAB accessions in a cluster suggesting a common ancestor regardless of their sources (Jauhar 1981, Animasaun et al. 2017). The similarity of AKz-Nr PEN3 and NGB00885, with a genetic distance index of less than 3 showed the accessions are closely related, although some variation is possible.



1: Leaf length; 2: leaf width; 3: number of leaves; 4: plant height; 5: stem girth; **correlation is significant at 0.01 level (2-tailed); *correlation is significant at 0.05 level (2-tailed).

Figure 1. Correlation coefficients of morpho-agronomic traits of 33 pearl millet accessions evaluated five weeks after sowing showing two-tail correlations of traits. Deep blue signifies strong negative correlation, blue negative correlation, cyan no correlation, yellow weak positive correlation, magenta significant correlation at $p < 0.05$ (0.35–0.75 scale), and deep brown characters that correlated at $p < 0.01$ (≥ 0.75 scales).

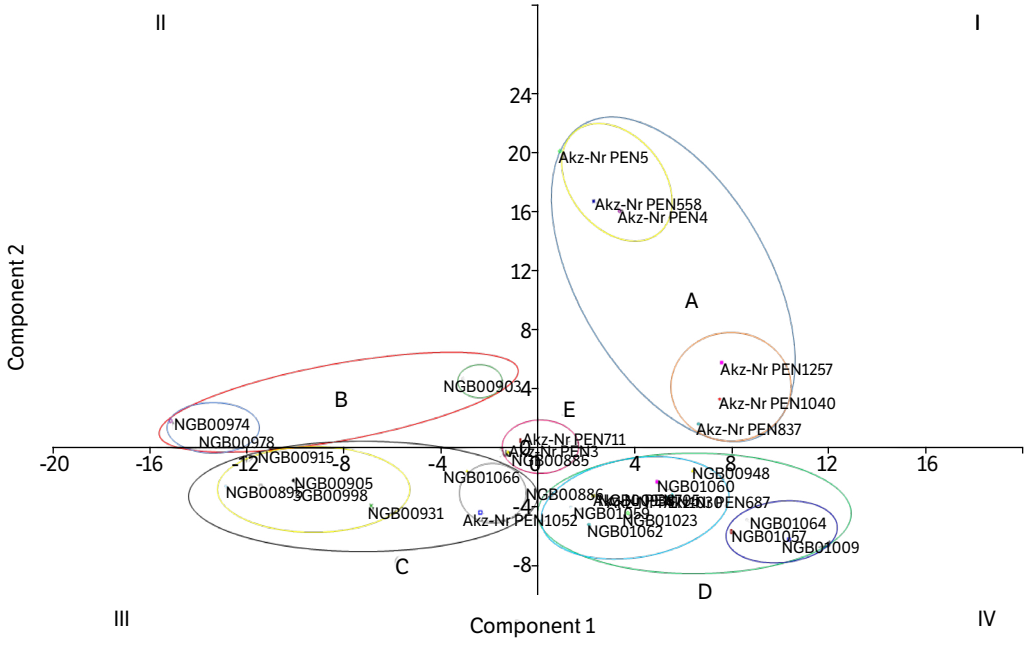


Figure 2. The ordination of 33 accessions of pearl millet on principal component axis 1 versus 2 based on cluster analysis of morpho-agronomic traits. Group A consists of six accessions in two subclusters of three accessions each. Group B had three accessions in two clusters, while group C comprises six accessions in two clusters of four and two accessions, respectively. Group D has the highest number of accessions, 13, with a subgroup of 10 mixed accessions from Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) and National Centre for Genetic Resources and Biotechnology (NACGRAB), and a small cluster of three all NACGRAB accessions, while group E consistes of three closely related IPK accessions. The accessions with the Prefix "Akz-Nr" are from IPK Germany and those with the "NGB" prefix are from NACGRAB, Nigeria.

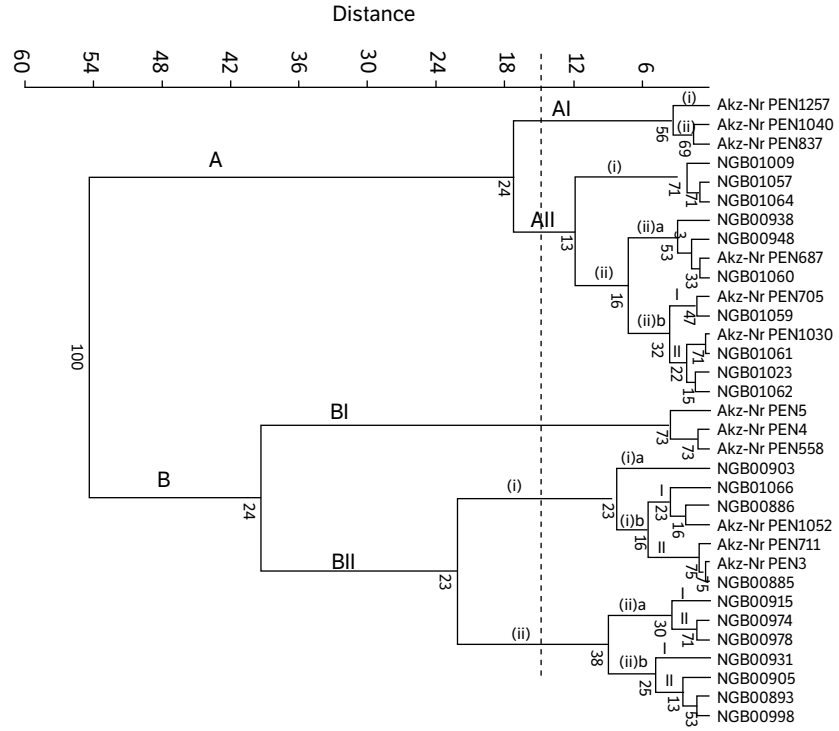


Figure 3. Dendrogram generated using minimum dissimilarity distance based on unweighted pair group method with arithmetic mean Ward's clustering method (bootstrapped at 95%) showing the genetic relationship among 33 accessions of pearl millet based on vegetative morpho-agronomic traits.

Selection of tolerant and water-stressed susceptible accessions

Food insecurity is a major global concern, and the improvement of small millets of the arid and semi-arid regions could mitigate the menace of hunger. However, drought is a critical factor for yield decline in millet production in those regions, and the condition may be exacerbated by the effects of global climate change, as water deficit induces plant morphological, physiological, biochemical, and molecular changes in plants that ultimately affect crop performance and yield (Zlatev et al. 2006, Anjum et al. 2017, Fadoul et al. 2021). The pearl millet accessions evaluated in this study responded differently to induced water stress at the pre-fruiting stage. Accessions tolerant and susceptible to water stress were selected based on physical observations, such as wilting, leaf color change, and morphological data. In this case, accession NGB00886 was the most tolerant to water stress, followed by accession NGB00974. Both accessions were still standing firm and erect with green leaves after 14 days of water stress. On the other hand, accession NGB00885 was the least tolerant to water stress, followed by accession AKz-Nr PEN1257. Thus, accessions NGB00886 and NGB00885 were selected as the most tolerant and the most susceptible, respectively.

The ability of the accessions to survive the induced water deficit regime suggests they have inert water deficit adaptive traits that could be further screened and selected for the development of high drought tolerance cultivars. During the first seven days of induced drought, most of the accessions withstood the condition, but, as time progressed, the susceptible accessions wilted and died. This happened because a decline in soil moisture and prolonged water-stress period impact negatively on the plant performance. Different genotypes of a species may show differential gene expression under biotic and abiotic stresses. As observed in the current study, NGB00886 was the most resilient with the highest tolerance to water stress, while NGB00885 was the most susceptible. NGB00886 had green, non-wilted leaves even after 14 days without a water supply. To achieve this, the accession may have introduced some morphological and physiological changes with adaptive traits (Chaves and Oliveira 2004, Fadoul et al. 2021).

Furthermore, plant performance during water deficit periods may be influenced by genotype, stress intensity, duration, and recovery effectiveness (Laxa et al. 2019). These factors may elicit numerous metabolic and molecular changes resulting in phenotypic feedback of plants to the water stress (Vergara-Diaz et al., 2020). Drought at the seedling or pre-fruiting stage can be devastating as it affects growth (Medina et al. 2017), tillering (van Oosterom et al. 2006), and flowering (Vadez et al. 2012), as well as other plant physiological changes. Therefore, the identification of natural drought-tolerant accessions at this stage of development is crucial for breeding drought-tolerant varieties.

Gene expression studies

The cDNA converted from RNA extracted from leaves of the most tolerant accession (NGB00886) and the most susceptible accession (NGB00885) and their controls were used for the gene expression studies. Two drought-related genes, *DREB1A* and *P5CS*, modelled from *Arabidopsis thaliana*, were used with *ACT1* as the reference. The melting temperature of the samples ranged from 78 to 82.5°C with unimodal curves for the primers (Suppl. Figs. 2 and 3). The cycle threshold (Ct), which is the number of cycles required for the fluorescence signal to cross the threshold, for the samples, ranged from 23.56 to 35.91. The specific primers used in this study generated sufficient copies of DNA from which the functional genome was determined. The singleplex analysis showed similar baseline values for all samples due to their similar genetic composition, although a primer pair can produce non-specific products when applied to mixed, heterogeneous samples with high diversity.

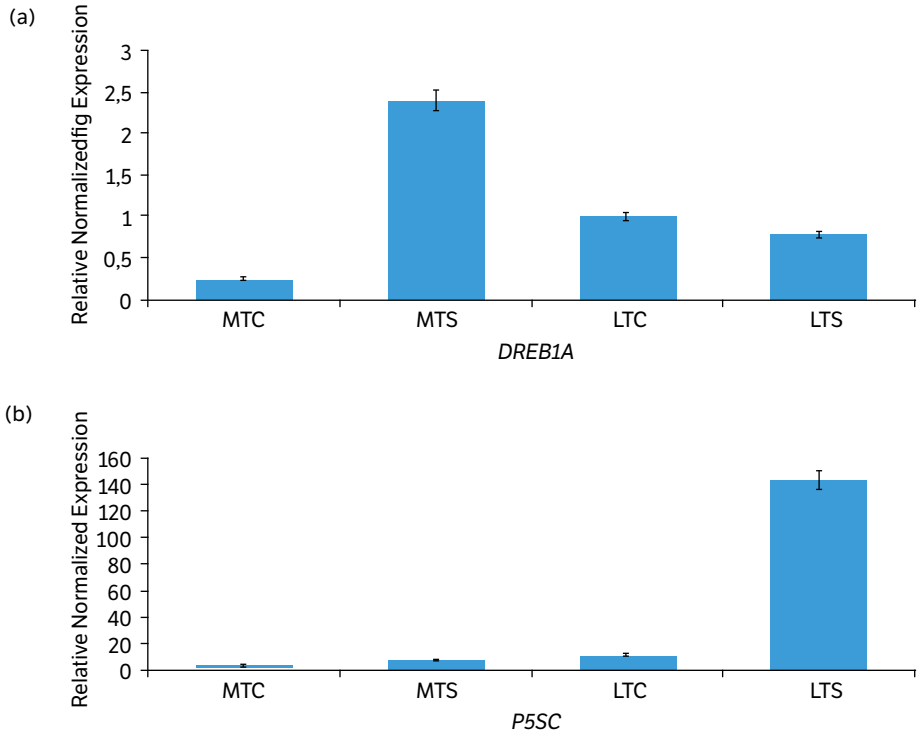
The Ct values of the samples were used to calculate the fold expression difference given by the relationship ΔCt = Ct gene – Ct reference gene; ΔΔCt = ΔCt stressed – ΔCt control; $2^{-\Delta\Delta Ct}$ = fold expression difference (Table 3). The expression of the *DREB1A* gene in the most tolerant and susceptible accessions with *ACT1* as a reference gene is shown in Fig. 4a. The relative expression of *DREB1A* in the most tolerant accession (NGB00886) is 15.327, which means that the gene in the water-stressed sample is 15.327 times more than in the control. This indicates a higher copy number of *DREB1A* in the stressed accession, conferring water stress tolerance. On the other hand, the relative expression of *DREB1A* in the least tolerant accession (NGB00885) is 0.755 in comparison with its control. Thus, the stressed plants have a lower copy number of the *DREB1A* gene and are therefore susceptible to water stress. This agrees with Xu et al. (2017) on the overexpression of the *DREB1A* gene in transgenic Poa pratensis. *DREB1A* is an important elixir of the gene modulation system and may play a crucial role when a plant is under water-stress conditions. They are among the frontline transcription factors responsible for gene regulation to combat water deficit under drought conditions (Agarwal et al. 2006, Xu et al. 2017).

Table 3. The fold expression of the internal control *ACT1* and two drought-related genes (*DREB1A* and *P5CS*) used for the expression studies of pearl millet accessions under the water-stressed condition at the pre-fruiting stage of growth*.

Sample	ACT1 Ct	DREB1A Ct	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	P5CS Ct	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
MTC	34.21428	34.37535	0.161063	-3.93804	15.327	25.37518	-8.8391	-1.66802	3.177
MTS	35.91536	32.13839	-3.77697			25.40824	-10.5071		
LTC	33.73643	31.23004	-2.50639	0.40375	0.755	25.43995	-8.29648	-2.51144	5.701
LTS	34.37498	32.27234	-2.10264			23.56706	-10.8079		

MTC: most tolerant control; MTS: most tolerant stressed; LTC: least tolerant control; LTS: least tolerant stressed; Ct: cycle threshold; ΔCt: Ct gene – Ct reference gene; ΔΔCt: ΔCt stressed – ΔCt control; $2^{-\Delta\Delta Ct}$: fold expression difference; *the accession NGB00886 is the most tolerant, while NGB00885 is the most susceptible accession to drought.

The expression pattern of the *P5CS* gene is shown in Fig. 4b. The *P5CS* gene is expressed in the stressed accession at 3.177-fold of the control for the tolerant accession (NGB00885). This signifies a low copy number of *P5CS* in the water-stressed plant, which possibly is required to confer water stress tolerance. In contrast, the drought susceptible accession had 5.701, which shows a higher copy number of *P5CS* in the stressed accession with respect to the control plant. It is most likely that the high copy number of *P5CS* genes is associated with accession susceptible to water stress.



MTC: Most tolerant control; MTS: most tolerant stressed; LTC: least tolerant control; LTS: least tolerant stressed.

Figure 4. The relative expression is based on the expression ratio of a target gene versus a reference gene and is adequate for most purposes to investigate physiological changes in gene expression levels. (a) Relative normalization expression of *DREB1A* using *ACT1* as the internal control. (b) Relative normalization expression of *P5CS* using *ACT1* as reference gene. The accession NGB00886 is the most tolerant, while NGB00885 is the most susceptible accession to drought.

The mechanism by which the gene regulates drought resistance may involve osmotic adjustment to increase water use efficiency (Wu et al. 2014), which promotes the synthesis of osmoregulatory substances such as proline, water-soluble carbohydrates, and soluble proteins. Thus, the upregulation of *DREB1A* expression in NGB00886 may enhance the ability of the accession for osmotic adaptation, coupled with other water use efficiency mechanisms, that promote drought modulation and water-stress resistance in the accession.

DREB1A was expressed as a downstream gene in the least tolerant accession (NGB00885) with a few copies, suggesting that more copies of *DREB1A* genes are required to confer water-stress tolerance. The current result is consistent with Saha and Blumwald's report (2014), which demonstrated that copy numbers are directly linked with the expression pattern. *P5CS* was expressed as an upstream gene in both the most and least tolerant accessions, but the copies were different. Apparently, a small amount of the *P5CS* gene is required for drought tolerance; too many copies could make the plant becomes susceptible. Muzammil et al. (2018) showed that moderate upregulation of *P5CS1* enhanced proline accumulation and drought adaptation in barley, although both P5CS1 and P5CS2, the two variants of the *P5CS* gene, are synergistically involved in the conversion of glutamate to proline (Funck et al. 2020). The low copy number of *P5CS1* obtained in this study for the drought susceptible accession (NGB00885) is likely below the threshold of expression, making it unable to modulate significant tolerance to water deficit, resulting in most plants dying after a few days of water stress.

CONCLUSION

Genetic diversity is essential for selection and crop improvement. The morpho-agronomic variability revealed in the pearl millet accessions utilized in this study can be used to guide selection for a breeding program targeting a specific

attribute. The degree to which water stress affects the pearl millet accessions tested differs, demonstrating that accessions have a natural propensity to respond differently to stresses, particularly drought. Meanwhile, accession NGB00886, which has the highest tolerance to water stress, can be used to develop drought-tolerant cultivars. The expression patterns of the drought-related genes *DREB1A* and *P5CS* revealed that water-stress tolerance in pearl millet requires a high copy number of *DREB1A* and a low number of *P5CS* genes. This may create fresh opportunities to relate it to the pattern of diversity for other traits of interest.

CONFLICT OF INTEREST

Nothing to declare.

AUTHORS' CONTRIBUTION

Conceptualization: Animasaun, D. A. and Mustapha, K. A.; **Methodology:** Animasaun, D. A., Mustapha, K. A., Akinbobola, A. M., Bakare, A. T., Ogunjobi, J. T., Adedoyin, K. A. and Awujoola, K. F.; **Investigation:** Mustapha, K. A. and Akinbobola, A. M.; **Writing – Original Draft:** Animasaun, D. A. and Mustapha, K. A.; **Writing – Review and Editing:** Animasaun, D. A., Mustapha, K. A., Akinbobola, A. M., Bakare, A. T., Ogunjobi, J. T., Adedoyin, K. A. and Awujoola, K. F.; **Supervision:** Animasaun, D. A.

DATA AVAILABILITY STATEMENT

Data and supplementary files are available with the corresponding author upon request.

FUNDING

No funding was received for this study.

ACKNOWLEDGMENTS

The authors are thankful to Dr. Andreas Boerner, from the Plant Genetic Resources Unit IPK, Gaterslebeen, Germany, and the Genebank Unit, National Centre for Genetic Resources and Biotechnology, Ibadan, Nigeria, for providing the pearl millet accessions for the study.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1. List of thirty-three pearl millet accessions were used for the drought resistance screening.

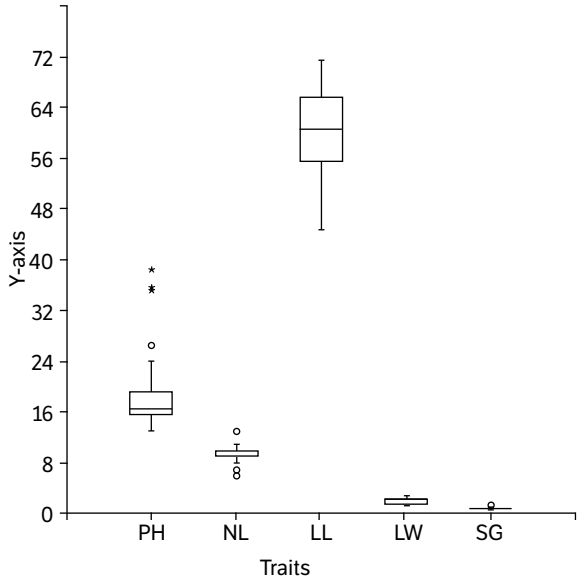
Accession	Source
Akz-Nr PEN1030	IPK
Akz-Nr PEN1040	IPK
Akz-Nr PEN1052	IPK
Akz-Nr PEN1257	IPK
Akz-Nr PEN3	IPK
Akz-Nr PEN4	IPK
Akz-Nr PEN5	IPK
Akz-Nr PEN558	IPK
Akz-Nr PEN687	IPK
Akz-Nr PEN705	IPK
Akz-Nr PEN711	IPK
Akz-Nr PEN837	IPK
NGB00885	NACGRAB
NGB00886	NACGRAB
NGB00893	NACGRAB
NGB00903	NACGRAB
NGB00905	NACGRAB
NGB00915	NACGRAB
NGB00931	NACGRAB
NGB00938	NACGRAB
NGB00948	NACGRAB
NGB00974	NACGRAB
NGB00978	NACGRAB
NGB00998	NACGRAB
NGB01009	NACGRAB
NGB01023	NACGRAB
NGB01057	NACGRAB
NGB01059	NACGRAB
NGB01060	NACGRAB
NGB01061	NACGRAB
NGB01062	NACGRAB
NGB01064	NACGRAB
NGB01066	NACGRAB

Twelve accessions from the germplasm maintained in the gene bank of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. Twenty-one additional accessions from germplasm maintained by the National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Oyo State, Nigeria

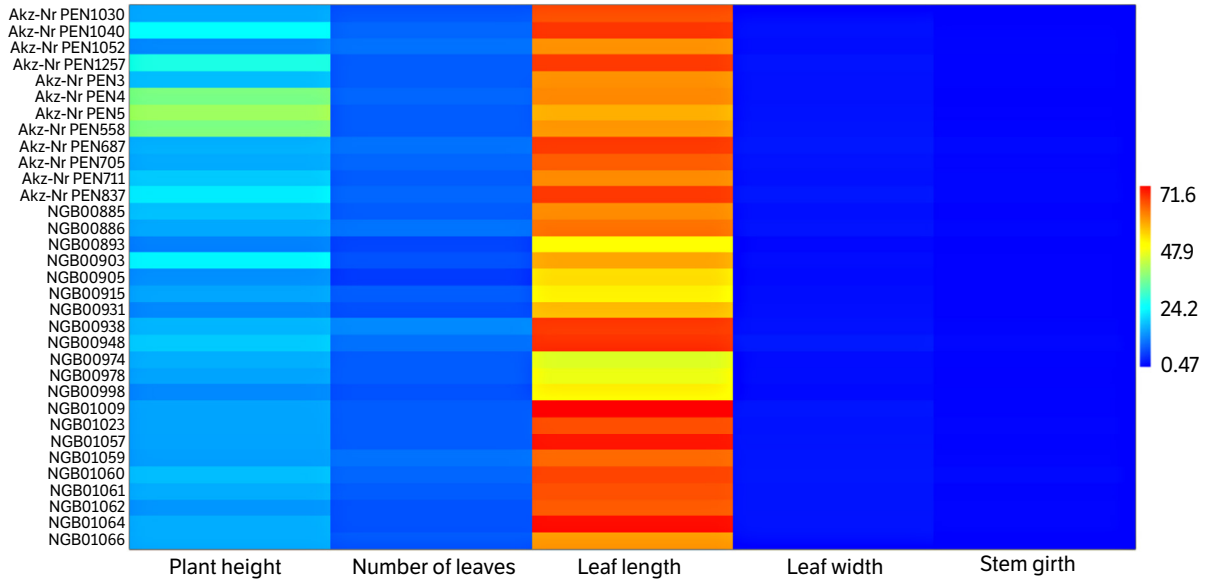
Supplementary Table 2. The name, sequences, and properties of the selected genes for expression study in pearl millet under water stress conditions at the pre-fruiting stage.

Name	Sequence 5'- 3'	Length	GC %	Tm	OD	n/moles	MW
ACT1 F	TTTCCCCCAAACGTGACCCG	20	60	55.88	10.49	52.46	6078.57
ACT1 R	CTAGCGAGCCGAAGCGGAAA	20	60	55.88	11.59	57.95	6265.74
DREB1A F	TCTCCGGCTTCTCACCGGAA	20	60	55.88	13.43	67.14	6109.58
DREB1A R	CCCCATGGCCGTCGTCTTAC	20	65	57.93	12.1	60.52	6085.55
P5CS F	CTGCTGTGGTCACTCGCCAA	20	60	55.88	11.93	59.63	6149.61
P5CS R	CCAAGGCCAACAGCACCTGA	20	60	55.88	10.48	52.42	6145.66

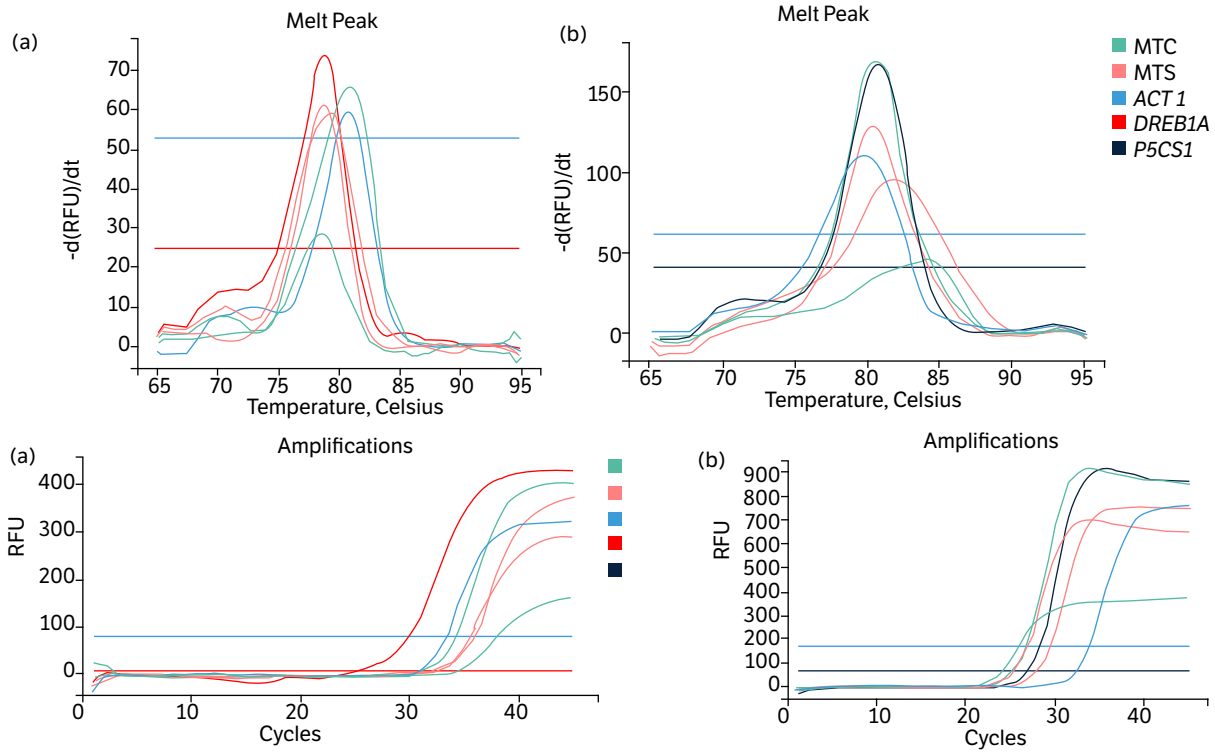
Legends: F: Forward; R: Reverse; GC%: Percentage guanine-cytosine composition of oligonucleotides; Tm: Melting temperature; OD: Optical density; n/moles: oligonucleotide concentration; MW: Molecular weight of the primer. All the primers are from the 5' strand sense to 3'.



Supplementary Figure 1. Box plot of morpho-agronomic traits of thirty-three accessions of pearl millet at 5 weeks after sowing. The y-axis represents the mean values of the evaluated traits while the x-axis contains the traits. Key: PH: Plant height; NL: Number of leaves; LL: Leaf length; LW: Leaf width; SG: Stem girth.



Supplementary Figure 2. Matrix of performance of thirty-three accessions of pearl millet evaluated at 5WAS. Deep blue signifies poor contribution, light blue very minimal contribution, cyan for fair contribution, yellow for moderate contribution, orange shades for high contribution, and red for very strong significant contribution to the observed variations.



Supplementary Figure 3. Melting temperature curves for (a) *ACT1* and *DREB1A* and (b) *ACT1* and *P5CS* used in the qRT-PCR genome quantification of accession. Primary Amplification curves for (a) *ACT1* and *DREB1A* and (b) ACT1 and *P5CS* used in the qRT-PCR genome quantification of accession NGB00886. MTC: Stress-free (control) sample; MTS: Water-stressed sample.