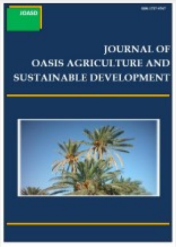

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Agro-morphological characterization and qRT-PCR validation of SNAC1 and DREB1A drought tolerance genes expression in Okra (*Abelmoschus esculentus* L. Moench) seedlings

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Abstract

Okra (*Abelmoschus esculentus* L. Moench) belongs to the family Malvaceae, it is widely grown in most parts of the world. Its ubiquitous presence in African households significantly contributes to both the economic and nutritional well-being of communities. However, the escalating challenges posed by climate change, characterized by irregular rainfall and drought like conditions threatens Okra production. To address this growing concern, the development of stress-resistant Okra varieties has become necessary. This goal cannot be achieved without Identifying and validating Okra assessions with drought resistance genes. It is in this light that this study sought to validate and determine the expression of 2 drought resistance related genes *DREB1A* and *SNAC1*, using *ERF4* as the reference gene in the most tolerant and least tolerant accessions of Okra, employing the qRT-PCR technique for gene expression analysis. Morphological parameters such as Plant height, Leaf number, length and width were recorded for the four Okra accessions (NGB 00332, NGB 00466, NGB 00349, and NGB 00373) to assess the impact of drought on the accessions to identify the least and most tolerant accessions used for molecular studies. NGB 00332 and NGB 00466 emerged as the most drought-tolerant and least drought-tolerant accession respectively. Findings from the gene expression studies revealed upregulation of *DREB1A* and *SNAC1* with fold expression values of 70.03 and 2.95 respectively in the stressed samples of the most tolerant accession, while the least tolerant accession exhibits substantially lower gene expression levels with expression values 0.57 and 0.47 respectively, underscoring its vulnerability to drought stress. This study provides plant breeders with valuable insights for the selection of drought resilient Okra accession and development of superior Okra varieties capable of withstanding the looming threats posed by climate change, thereby enhancing food security.

1. INTRODUCTION

Okra (*Abelmoschus esculentus* (L.), Moench) Okra is a commercial vegetable crop with considerable area under cultivation in Africa and Asia. Okra belongs to the family Malvaceae. It probably originated in Ethiopia and is widely spread all over the world's tropical, subtropical and warm temperate regions (Benchasri, 2012). It is cultivated for its tender fruits and the dried seed of okra has been found to be a good source of protein, fat and antioxidant activity (Adelakun and Oyelade, 2011). Thus, okra seed is an increasingly highly valued crop in the human diet. Okra exhibits a certain level of drought tolerance (Mkhabela et al., 2022) due to its ability to withstand periods of reduced water availability. However, the molecular mechanism behind this observed tolerance has not been fully explored. Molecular processes including gene validation and gene expression studies can be adopted to underscore the mechanism. Gene validation refers to the process of confirming the function or significance of a specific gene in a biological context. This is crucial because not all genes identified through genomic analysis have known functions. Validating a gene involves experiments and analyses that provide evidence supporting the gene's role, whether in a specific biological process, a disease pathway, or other cellular functions (Xiao *et al.*, 2016).

Gene expression (GE) is the synthesis of a functional gene products such as proteins or functional RNA molecules using the information provided by deoxyribonucleic acid (DNA) (Perdew et al., 2006). The two main stages of gene expression are transcription (the synthesis of RNA from DNA) and translation (the synthesis of proteins from RNA). Gene expression is tightly regulated and can be influenced by a range of factors, including developmental stages, environmental conditions, and cellular needs. It plays a critical role in determining an organism's traits, functions, and responses to external stimuli. Techniques used to study gene expression include Reverse Transcription Polymerase Chain Reaction (RT-PCR) which allows quantification of specific RNA molecules to assess gene expression levels. Due to features such as cost effectiveness, specificity, and sensitivity, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is the most extensively used technology for analysing gene expression. However, normalization procedures must be used to control sample to sample variation and experimental error to produce accurate and dependable results. The use of reference genes (RGs) also known as control genes or housekeeping genes as internal controls is the most popular and effective strategy for RT-qPCR normalization (Su *et al.*, 2019).

Currently, high-throughput sequencing has been widely used in plant transcriptomics to mine functional genes and to elucidate the genetic/evolutionary mechanisms in plant drought responses. For example, Shi *et al.* (2019) compared the transcriptomes of okra seedling leaves under different degrees of drought stress and found that the number of differentially expressed genes was positively associated with the severity of drought stress. As expected, drought caused reprogramming of the gene expression in multiple metabolic pathways. Remarkably, many transcription factor (TF) genes including *bHLH*, *MYB*, *C2H2,* and *bZIP* were significantly up-regulated in okra seedlings under drought stress (Shi *et al.*, 2019). Further investigation on these *TFs* may promise the generation of okra plants with enhanced drought resistance. However, it is the aim of this study to validate and determine the expression of such transcription factor genes namely DREB1A (a member of the Dehydration-Responsive-Element-Binding) family of genes, SNAC1; stress-responsive NAC1 a member of the NAC (NAM, ATAF1/2 and CUC2) and ERF4 (Ethylene-Response Factor) as the reference gene modelled from model plant *Arabidopsis thaliana*.

The study of abiotic stress tolerance mechanisms is one of the most active lines of research in plant biology, given its undoubted academic interest and practical implications in agriculture (Gull *et al.*, 2019). In recent years, the increasing negative effects of climate change on agricultural yield has led to a global food shortage. Drought stress is a significant factor that can have a substantial impact on agricultural output worldwide. Drought stress is the absence or limited amount of rainfall for a period especially during crop growing season and that causes moisture-depletion in soil and followed by a decrease of water potential in plant tissues (Temesgen, 2020). Today, drought and salt stresses are the global issues which must be controlled to ensure rapid and sustainable food production through producing adaptive and stable agricultural crops (Jaleel *et al.*, 2007).

Crop plants show various adaptive and acclimatization strategies to drought stress, which range from seemingly simple morphological or physiological traits (Bhargava & Sawant, 2012). However, a plant may exhibit more than one strategy to cope with drought stress. Previous research activities have dwelt much on the physiological mechanisms and responses of plants to drought stress conditions. The molecular machinery involved in drought stress perception, signalling and regulation of gene expression has been fairly well understood. However, there are lacunae in our understanding of how it correlates with phenotypic alterations in the plant (Blum, 2011) and these studies will further provide the necessary information for development and improvement of stress-tolerant and resistant varieties as the use of well adapted and high-yielding varieties with resistance to drought stress is important to reach maximum yield potential as long as possible through minimizing the risk of climate change (Temesgen, 2020)

To address this impending situation, there is urgent need to improve our understanding on the genetic mechanisms of drought tolerance in okra as varied reactions to water stress result in differential expression of many metabolic pathways at the molecular level. As a result, examining differences in gene expression in okra accessions makes it easier to find drought-related genes that could be utilized to select drought-tolerance features in different accessions. This will aid in providing relevant information in developing more resilient varieties to drought stress.

2. MATERIAL AND METHODS

This study was conducted in two major phases: the screen house characterization of okra accessions and laboratory experiments comprising RNA extraction and gene expression studies.

2.1. Materials Collection

A total of 4 accessions of Okra seeds; NGB 00332, NGB 00466, NGB 00349 and NGB 00373 were obtained from the National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Oyo State, Nigeria.

2.2. Screen House Location

Morpho-agronomic characterization of the 4 accessions of Okra was conducted in a screen house in University of Ilorin Province, Ilorin, Kwara state, Nigeria between July 2023 to August, 2023. The screen house is located between 8.4799°N and longitude 4.5418 °E. The average temperature of the screen house ranges from 33°C to 35°. Relative humidity ranges from 75% to 88%

2.3. Experimental Treatments and Design

2.3.1. Screen house characterization of the accessions under study

Dry seeds of Okra accessions were grown for morphological characters and diversity assessment in the screen house. Planting bags (25cm X 20cm) with perforations to drain excess water were filled with 5kg of fertile clayey-loamy soil were used for planting. Planting bags were appropriately labelled corresponding to each accession planted in them. The experiment was laid out in an arranged manner in order of the accessions with 0.2m X 0.2m space in-between planting pots in two treatments for each accession. The treatments were labelled thus; control (C) and stress (S). Seeds of each accession of okra were sown in separate planting bags. Watering was done daily with 30cl of water. Weeding was done by hand-picking throughout the period of evaluation. Thinning was done after seedling emergence to reduce the plant density to 3 plant stands per planting bag to prevent overcrowding and unnecessary competition for space, air and nutrients. At 2 weeks after planting (2WAP) watering was stopped for the bags labelled stress (S) for the drought study while watering continued for the control (S), morphological data were recorded to compare the effect of drought on the morphology of okra accessions.

Data were recorded for quantitative characters such as Plant height (PH), Leaf Length (LL), Leaf Width (LW) and Leaf number (LN) from three randomly selected and tagged plants at from 3-6 weeks after planting (WAP) for both the control and stress treatment. Recorded data were subjected to two-way Analysis of Variance (ANOVA) using Statistical Packages for Social Sciences (SPSS) version 20. The mean values were separated by Duncan Multi-Range Test (DMRT) at P<0.05 and post hoc tests (Tukey HSD) were conducted.

2.3.2. Molecular Studies

Based on previous reports on the effective application of reference genes used in the study of various plants species (Czechowski et al., 2005; Nicot et al., 2005; Jain et al., 2008; Tong et al., 2009; Lin et al., 2010). Primers from a candidate reference gene ERF4 and two drought resistance related genes; DREB1A and SNAC1 were designed for qRT-PCR analysis using gene script software to investigate the expression of drought related genes in Okra. The sequences of the primers with melting temperatures of 55-61°C, primer lengths of 20bp, 60-65% GC content, and TM (melting temperature) are shown in Table 1. At 6 WAP, green leaves samples were harvested from both the control (C) and stressed (S) of the observed most tolerant (MT) and least tolerant (LT) accessions

Table 1. Primer sequences used and their properties

Name	Sequence 5' 3'	Length	GC%	Melting Temperature
SNAC1F	AAGGCGCTCGTCTTCTACGC	20	60	55.88
SNAC1R	GATCCCTTCTTGGAGGCGGC	20	65	57.93
DREB1A F	GTGGCCGATCAGCCTGTCTC	20	65	57.93
DREB1A R	CGGAAGCGGCAAAAGCATCC	20	60	55.88
ERF4 F	TATGCCGCCCAGATCCGAGA	20	60	55.88
ERF4 R	ATTCCCAGCCGTCGCTGAAG	20	60	55.88

to drought stress respectively. Genomic RNA was extracted from the leaf samples using Qiagen RNeasy® Plant Mini Kit subsequently, qRT-PCR was carried out for gene expression using Luna Universal One-step RT-qPCR kit (NEB 3005S). Cq (quantification cycle) values obtained from the qRT-PCR were used to calculate the fold expression of the drought-related genes (Table 7) using the formula below :

$\Delta Cq = Cq\ Gene - Cq\ Reference$

$\Delta\Delta Cq = \Delta\ Cq\ Stress - \Delta Cq\ Control$

$2\text{-}\Delta\Delta Cq = $ Fold Expression.

3. RESULTS

3.1. Water Stress Studies

The various Okra accessions in this study exhibited distinct responses to the water stress conditions initiated at 3 Weeks After Planting (WAP). Their ability to withstand or succumb to water stress was assessed based on observable physical indicators such as leaf wilting and alterations in leaf color. Among the accessions, NGB 00332 stood out as the most resilient in the face of water stress. Even under the stress conditions, it maintained a healthier appearance with vibrant green leaves. In contrast, Accession NGB 00466 was observed to be the least resilient, showing more pronounced signs of distress and a lower tolerance to the water stress conditions.

3.2. Growth Parameters Studies

The mean ± SD plant height, Leaf number, Leaf width and Leaf Length for four Okra accessions (NGB 00332, NGB 00466, NGB 00349, and NGB 00373) were presented (Tables 2-5) under two treatments, namely Control and Stress, at specific time points; 3 WAP (Weeks After Planting), 4 WAP, 5 WAP, and 6 WAP.

3.3. Plant Height (PH)

The mean±SD recorded for four Okra accessions (NGB 00332, NGB 00466, NGB 00349, and NGB 00373) was presented (Table 2). Plant height varied significantly among the accessions of Okra used at a confidence level of P< 0.05. At 3 WAP, accession NGB00332 had the highest mean±SD height (19.37±0.32 and 23.50±0.28) for the control and stress treatments respectively and accession NGB 00349 had the lowest mean height of (14.17±2.74 and 14.35±1.20) for the control and stress treatment. Both accessions maintained this trend till 6 WAP. significant differences were recorded in plant height between the Control and Stress treatments at each time point in accession NGB 00332 and NGB 00373 with a *p value of 0.002 and 0.001 respectively*. However, in accession NGB 00466 only at 6 WAP did the values between the treatments (control and stress) varied significantly with a *p value of 0.038* while NGB 00349 had no significant difference in plant height between the treatments.

3.4. Leaf Number (LN)

As regards the leaf number (LN) (measured in whole number), the mean±SD leaf count. Notably, there were significant differences in leaf number among the Okra accessions, with statistical significance at a confidence level of P< 0.05. At the 5th WAP (Table 3), Accession NGB 00373 exhibited the highest mean leaf count (7.67±0.58 for Control and 6.00±0.00 for Stress), while Accession NGB 00349 displayed the lowest mean leaf count (3.67±0.57 for Control and 5.5±0.71 for Stress). It is noteworthy that the stressed group consistently demonstrated a higher mean leaf count compared to the control group, and this trend persisted until the 6th WAP. Significant differences in leaf numbers existed between the Control and Stress

Table 2. Mean plant height (PH) of Okra accessions at different weeks of planting

Accessions	Treatments	3	4	5	6
NGB 00332	**Ctrl**	19.37±0.32^{e}	26.43±1.21^{c}	29.83±2.25^{b}	32.97±1.75^{a}
	Strs	23.50±0.28^{d}	31.17±0.38^{ab}	31.43±0.51^{ab}	31.53±0.67^{ab}
NGB 00466	**Ctrl**	17.00±0.61^{bcd}	18.77±0.38^{bc}	20.90±0.70^{ab}	23.67±1.36^{a}
	Strs	13.80±3.96^{d}	13.73±3.00^{d}	17.23±3.69^{bcd}	15.77±1.50^{cd}
NGB 00349	**Ctrl**	14.17±2.74^{a}	14.47±2.97^{a}	16.67±3.04^{a}	16.60±3.83^{a}
	Strs	14.35±1.20^{a}	16.57±2.97^{a}	18.23±3.78^{a}	17.27±3.32^{a}
NGB 00373	**Ctrl**	16.53±0.85^{d}	21.00±0.00^{c}	27.20±0.00^{b}	32.50±0.00^{a}
	Strs	19.95±0.35^{c}	22.50±1.00^{c}	25.20±3.60^{b}	26.83±1.44^{b}

The values are the mean of the three replicates, the means in the same column having the same superscript are not significantly different from each other at P≤0.05. Control, Strs: Stress.

Table 3. Mean and standard deviation of leaf number (LN) of Okra accessions at different weeks after planting.

Accessions	Treatments	Weeks After Planting (WAP) 3	4	5	6
NGB 00332	**Ctrl**	5.00±0.00^{abc}	6.00±0.00^{a}	6.33±1.16^{a}	6.33±1.16^{a}
	Strs	5.00±0.00^{abc}	5.50±0.71^{ab}	4.50±0.71^{bc}	4.00±0.00^{c}
NGB 00466	**Ctrl**	4.00±0.00^{b}	3.67±0.58^{b}	3.67±0.58^{b}	3.67±0.58^{b}
	Strs	4.00±0.00^{b}	4.00±0.00^{b}	5.5±0.71^{a}	6.00±0.00^{a}
NGB 00349	**Ctrl**	4.00±0.00^{b}	4.67±0.58^{ab}	5.33±0.58^{a}	5.33±0.58^{a}
	Strs	4.00±0.00^{b}	5.00±0.00^{a}	5.50±0.71^{a}	5.50±0.71^{a}
NGB 00373	**Ctrl**	4.00±0.00^{c}	5.00±0.00^{b}	7.67±0.58^{a}	8.00±1.00^{a}
	Strs	4.33±1.16^{c}	4.00±0.00^{c}	6.00±0.00^{b}	5.00±1.41^{b}

The values are the mean of the three replicates, the means in the same column having the same superscript are not significantly different from each other at P≤0.05. Ctrl: Control, Strs: Stress.

treatments at each time point for the four Okra accessions, specifically in Accession NGB 00349 and NGB 00373 at 5 and 6 weeks after planting, with corresponding *p-values of 0.02 each* for the 5th WAP and (p=*0.001 and 0.002)* for the 6th WAP, respectively. However, Accession NGB 00466 and NGB 00332 did not exhibit significant variation between the treatments concerning the 3-6 weeks after planting.

3.5. Leaf Length (LL)

Leaf length for the Okra accessions exhibited noteworthy differences in mean±SD values between the control and stress groups as well as among the accessions at 3 to 6 weeks after planting (WAP). In the third week, Accession NGB 00332 displayed the longest range of leaf length, with mean values of (6.67±0.29), (6.40±0.28) for the control and stress treatments respectively (Table 4). In contrast, Accession NGB 00349 exhibited the shortest leaf length, with mean values of (2.93±0.21), (2.85±0.21) for the control and stress group respectively during the same period. Remarkably, Accession NGB 00349 maintained its position with the shortest leaf length (LL) until the 6th WAP, where it had mean values of (6.17±0.25), (6.85±0.85) for the control and stress groups respectively. In contrast, Accession NGB 00373 emerged as the accession with the longest LL at the 6th WAP, with mean values of (11.33±0.57), (5.30±0.00) for the stress control and control group respectively. The two-way ANOVA outcome revealed the presence of significant differences in leaf length between the control and stress treatment groups, but this was observed in only two out of the four accessions, specifically Accession NGB 00332 and NGB 00373. The corresponding *p-values were 0.007 and p=0.009* respectively. Significant differences in leaf length were observed in Accession NGB 00332 from 3 to 6 WAP between the control and stress groups. However, in the case of Accession NGB 00373 and NGB 00349, the treatment differences were significant only in weeks 5 and 6 after planting (WAP). However, it's worth noting that Accession NGB 00466 exhibited significant differences between the treatment groups at 6 WAP, with a *p-value of 0.017*.

3.6. Leaf Width (LW)

The mean width ± standard deviation (SD)

Table 4. Mean leaf length (LL) of Okra at different weeks of planting

Accessions	Treatments	3	4	5	6
NGB 00332	**Ctrl**	6.67±0.29^{b}	8.30±0.26^{ab}	9.80±1.34^{a}	10.03±1.58^{a}
	Strs	6.40±0.28^{b}	8.20±0.23^{ab}	7.55±0.71^{b}	6.75±1.59^{b}
NGB 00466	**Ctrl**	5.13±0.85^{abc}	5.00±0.17^{abc}	5.13±0.40^{abc}	6.17±0.25^{ab}
	Strs	3.55±1.20^{bc}	4.60±1.56^{bc}	5.05±1.35^{abc}	6.85±0.85^{a}
NGB 00349	**Ctrl**	2.93±0.21^{cd}	3.73±1.50^{bcd}	4.83±1.50^{bc}	5.70±0.27^{a}
	Strs	2.85±0.21^{d}	3.55±0.07^{bcd}	4.40±0.14^{bcd}	5.15±0.21^{ab}
NGB 00373	**Ctrl**	3.90±0.52^{e}	6.03±1.11^{d}	9.47±1.10^{b}	11.33±0.57^{a}
	Strs	5.55±0.21^{de}	5.60±0.85^{de}	7.20±1.27^{cd}	8.00±0.00^{bc}

The values are the mean of the three replicates, the means in the same column having the same superscript are not significantly different from each other at P≤0.05. Ctrl: Control, Strs: Stress.

measurements for the four Okra accessions revealed that NGB 00349 consistently displayed the smallest mean width from 3 to 6 WAP, both for the control and stress groups, with values of (2.93±0.21, 2.85±0.21) at 3 WAP and (6.03±0.55, 6.20±0.60) at 6 WAP. In contrast, NGB 00332 consistently had the widest mean leaf width from 3 to 6 WAP, as at 3 WAP, it recorded a mean leaf width of (6.40±0.61 and 6.70±0.00) for the control and stress treatments respectively while at 6 WAP, it exhibited a mean leaf width of (10.87±0.15 and 7.77±0.15) for the control and stress treatments, respectively. It is worth noting that both NGB 00466 and NGB 00349 showed a slightly higher mean width for the stress group compared to their respective control groups at 6 WAP, with values of (6.67±0.68, 6.85±0.85) and (6.03±0.55, 6.20±0.60), respectively (Table 5). Statistical analysis of variance revealed the presence of significant disparities in leaf width between the control and stress treatment groups, but these differences were observed only at specific time points. Regarding, accession NGB 00332, significant differences in leaf width were found solely at the 5th WAP, with a corresponding p-value of 0.002. However, accession NGB 00466 exhibited significant differences in leaf width at the 6th WAP, with a p-value of 0.001, while accession NGB 00349 showed significant differences at the same time point with a p-value of 0.002 while accession NGB 00373 displayed significant differences in leaf width at both the 5th and 6th WAP, with corresponding p-values of 0.001 and 0.000 respectively.

3.7. Water Stress Studies

The various Okra accessions in our study exhibited distinct responses to the water stress conditions initiated at 3 Weeks After Planting (WAP). The ability to withstand or succumb to water stress observed based on observable physical indicators such as leaf wilting and alterations in leaf color. NGB 00332 stood out as the most resilient as it maintained a relatively healthy appearance with vibrant green leaves. while Accession NGB 00466 was observed to be the least resilient, showing more pronounced signs of distress and a lower tolerance to the water stress conditions.

3.8. Molecular Studies

The primary amplification curve and temperature melting curve of ERF4, SNAC1 and DREB1A genes for accessions NGB 00332 and NGB 00466 respectively (shown in supplementary Figs. 2, 4 and 1, 3 respectively. Table 6 presents melting temperatures (Tm) and

Table 5. Mean leaf width (LW) of Okra at different weeks after planting

Accessions	Treatments	3	4	5	6
NGB 00332	**Ctrl**	6.40±0.61^{cd}	8.27±0.58^{bc}	8.67±0.15^{b}	10.87±0.15^{a}
	Strs	6.70±0.00^{bcd}	8.15±0.07^{bc}	7.60±0.58^{b}	7.77±0.15^{b}
NGB 00466	**Ctrl**	4.23±0.21^{c}	4.43±0.49^{c}	4.93±0.65^{c}	6.67±0.68^{ab}
	Strs	3.55±1.49^{c}	4.30±1.27^{c}	5.25±1.06^{bc}	6.85±0.85^{ab}
NGB 00349	**Ctrl**	2.93±0.21^{c}	3.17±1.53^{bc}	4.73±1.46^{abc}	6.03±0.55^{a}
	Strs	2.85±0.21^{c}	3.15±0.50^{bc}	4.45±0.50^{abc}	5.30±1.27^{ab}
NGB 00373	**Ctrl**	3.30±0.10^{d}	5.27±0.86^{cd}	8.13±1.70^{ab}	10.33±1.16^{a}
	Strs	5.05±0.78^{cd}	6.40±1.56^{bc}	8.30±1.70^{ab}	9.00±0.00^{a}

The values are the mean of the three replicates, the means in the same column having the same superscript are not significantly different from each other at P≤0.05. Ctrl: Control, Strs: Stress

Table 6. Gene expression analysis: Tm and Cq values of most and least tolerant accessions of Okra using qRT - PCR

Sample	Tm°C	Cq Value
***DREB1A* LTC**	79.50	37.61
***DREB1A* LTS**	82.00	40.86
***DREB1A* MTC**	73.00	32.30
***DREB1A* MTS**	79.50	31.57
***SNAC1* LTC**	77.00	34.10
***SNAC1* LTS**	76.00	35.24
***SNAC1* MTC**	79.00	29.19
***SNAC1* MTS**	77.50	26.35
***ERF4* LTC**	85.50	35.69
***ERF4* LTS**	84.50	36.04
***ERF4* MTC**	82.00	34.83
***ERF4* MTS**	82.00	38.12

Tm- melting temperature MTC- most tolerant control; MTS- most tolerant stressed; LTC- least tolerant control; LTS- least tolerant stressed.

quantification cycle (Cq) values of drought resistant genes SNAC1, DREB1A and reference gene ERF4 of Most and least tolerant accessions of Okra, melting temperatures ranged from 76^{o}C – 85^{o}C. In the least tolerant accession NGB 00349, drought resistant gene DREB1A had a Tm of 79.50 and 82.00 for control and stress sample respectively while a Tm of 73.00 and 79.50 was recorded for control and stress of the most tolerant accession. With respect to the SNAC1, the control and stress sample of the least tolerant accession had a Tm of 77.00 and 76.00 respectively while 79.00 and 77.50 were recorded respectively for the control and stress sample of the most tolerant accession. The reference gene ERF4 had a Tm of 85.50 and 84.50 respectively for the control and stress of the least tolerant accession and a Tm of 82.00 each for control and stress of the most tolerant accession.

Table 7 shows the fold expression difference of DREB1A and SNAC1 with respect to the stress and control samples of the most and least tolerant accessions, with ERF4 employed as the reference gene. These expression patterns shed light on the genetic response to water stress conditions in these accessions. Results revealed SNAC1, the most tolerant accession demonstrated a considerably higher fold difference of 70.03. This signifies that under water stress, the SNAC1 gene exhibits a remarkable 70.03 fold upregulation (Table 7) in the stress treatment compared to control. However, the least tolerant accession exhibits a modest fold difference of 0.57 for SNAC1, indicating that the SNAC1 gene is expressed in the stressed plants at only a 0.57-fold increment compared to the control group.

With respect to DREB1A gene, the most tolerant accession displays a fold expression difference of 2.95. This suggests that, in response to water stress, the DREB1A gene is expressed at a robust 2.95-fold higher level than in the control group. the least tolerant accession presents a significantly lower fold difference of 0.47 for DREB1A. This implies that the DREB1A gene is expressed in the stressed plants at 0.47 fold increment compared to the control group. This lower fold difference indicates a diminished abundance of DREB1A genes in the stressed accession, which infers its vulnerability to drought stress. Table 8 shows the expression pattern of DREB1A and SNAC1 genes in the most and least tolerant accessions NGB 00332 and NGB 00466 respectively. In the most tolerant accessions, the 2 drought resistance genes were up regulated i.e (fold expression value >1) and were down regulated in the least tolerant accession i.e (fold expression value <1).

4. DISCUSSION

4.1. Morpho-agronomic traits of accessions used for the study.

Four distinct accessions of Okra, namely NGB 00332, NGB 00349, NGB 00349, and NGB 00373, were subjected to morphological examination to investigate how these accessions respond to different treatments, specifically Control and Stress, over a span of 4 weeks (3 to 6 WAP). Morphological traits, such as plant height, leaf number, leaf length, and leaf width were studied. Among the four studied accessions, NGB 00332 emerged as the accession displaying the highest degree of tolerance to drought stress conditions, while NGB 00466 exhibited the least tolerance. A comprehensive overview of the mean morphological characteristics of these accessions, with a particular focus on their responses to water stress. Notably, the findings as regards to Plant height revealed that, in most instances, the mean plant height of the control

Table 7. Fold expression difference of DREB1A and SNAC1 using ERF4 as reference gene.

	ERF4 Cq	SNAC1 Cq	ΔCq	ΔΔ Cq	$2^{-\Delta\Delta Cq}$	DREB1A	ΔCq	ΔΔ Cq	$2^{-\Delta\Delta Cq}$
MTC	34.83	29.19	-5.64	-6.13	**70.03**	32.30	-2.53	-1.56	**2.95**
MTS	38.12	26.35	-11.77			31.57	-6.55		
LTC	35.69	34.10	-1.59	0.79	**0.57**	37.61	1.92	1.09	**0.47**
LTS	36.04	35.24	-0.80			40.86	-4.82		

KEY: MTC- Most Tolerant Control; MTS- Most Tolerant Stressed; LTC- Least Tolerant Control; LTS- Least Tolerant Stressed

Table 8. Expression pattern of DREB1A and SNAC1 genes in accession NGB 00332 and NGB 00466.

	DREB1A EXPRESSION PATH	SNAC1 EXPRESSION PATH
NGB 00332	↑	↑
NGB 00466	↓	↓

group surpassed that of the stressed group. This observation aligns with our understanding that drought stress tends to impede normal plant growth and according to Brodersen et al., (2019), about 80–95% of the fresh biomass of the plant body is comprised of water, which plays a vital role in various physiological processes including many aspects of plant growth, development, and metabolism. However, there was a noteworthy exception in the case of accession NGB 00349, where the plant height of the stressed group consistently exceeded that of the control group throughout the drought period. Findings revealed notable variations in plant height among the Okra accessions, with statistically significant differences. At 3 WAP, Accession NGB 00332 exhibited the highest mean height in both control and stress conditions, whereas accession NGB 00349 displayed the lowest mean height. This trend persisted throughout the study period, indicating consistent differences in height among the accessions. Notably, drought stress tended to inhibit plant growth, as evidenced by the generally taller plants in the control group compared to the stressed group. The results obtained are consistent with the study of Khalid *et al*., (2023) on wheat where he reported that reduction in plant height is generally associated with an increase in lodging resistance, drought tolerance and grain yield of wheat. However, Accession NGB 00349 exhibited a unique response, with stressed plants surpassing control plants in height, signifying a distinctive adaptability to stress conditions.

Furthermore, the analysis of the number of leaves (NL) showed a consistent increase in the control group. In contrast, the stressed group exhibited a limited increase in leaf count. This phenomenon can be attributed to the physiological impact of drought on various metabolic processes, particularly those involved in regulating leaf production. As elucidated by Seleiman *et al*. (2021), drought stress commonly triggers a range of observable symptoms in plants, including increased leaf senescence, wilting, drooping, scorching, brittleness, leaf rolling, closed flowers, etiolation, and premature leaf fall, among others. The variations observed among the accessions and over the course of the drought treatment weeks underscore the presence of genetic and physiological diversity in how these Okra accessions respond to drought stress. This observation agrees with the perspectives of Nyabundi *et al*. (2016) and Zakir, (2018) emphasizing the significant influence of both genetic factors and environmental conditions on vegetative growth and yield performance in plants. It is worth noting that the interaction between genotype and environment (often denoted as G x E) plays a pivotal role in the expression of quantitative traits, a concept that has been well-established in scientific literature.

Leaf length and width analyses further illuminated the impact of drought stress on morphological traits. Notably, Accession NGB 00332 consistently exhibited the longest and widest leaves, while Accession NGB 00349 consistently had the shortest and narrowest leaves. These differences suggest genetic variations in leaf morphology among the accessions. Additionally, the response of leaf width to stress conditions showed significant differences at specific time points, further highlighting the nuanced effects of stress on leaf morphology.

4.2. Gene Expression studies

The use of known reference genes is considered a standard validation method in the adoption of reference Gene. As suitable reference genes must be stably expressed under different experimental conditions to obtain accurate and reproducible data for relative quantification (Zhang et al., 2020). However, there is an inadequacy of a systematic validation of RGs in Okra, available literature on validation of reliable reference genes for qRT-PCR analysis in okra was a study carried out by Zhang *et al*., 2022, validating 11 reference genes excluding ERF4. ERF4 is a widely used reference gene present in the model plant *Arabidopsis thaliana* but has not been validated in Okra to the best of our knowledge prior to this study, therefore ERF4 was adopted and validated by this study. Also, the role of 2 candidate genes *SNAC1 and DREB1A* used in this study have not been previously studied in Okra (*Abelmoschus esculentus)*. The present work represents a pioneering effort to study the expression of genes *SNAC1 and DREB1A* in tolerant and sensitive Okra cultivars in response to abiotic stress (Drought) using qRT-PCR. The Reference Gene (ERF4) may be also useful in the qPCR analysis of gene expression studies in other closely related species.

At the end of this study, the expression of *SNAC1* in the most tolerant and least tolerant accessions with *ERF4* as a reference gene was recorded. The fold difference of *SNAC1* in the most tolerant accession is 70.03 implying that *SNAC1* gene was expressed in stressed plant 70.03 folds of the control. This indicated that the high expression of *SNAC1* gene in the most tolerant accession conferred drought tolerance, this finding is consistent with the research of several scientists such as Liu *et al.* (2014) where it was reported that overexpression of *SNAC1* improved drought and salt tolerance in rice. Also, in another study as reported by Li *et al.* (2019), using RNA-Seq genome-wide identification of *SNAC1*-targeted genes, 93 *SNAC1*-targeted genes were reported to be involved in drought responses in rice. The fold difference of *SNAC1* in the least tolerant accession is 0.79 inferring *SNAC1* gene was minimally expressed in the accession, a value of 0.79 indicates lower copies of *SNAC1* genes and infers the accession's sensitivity to water stress thereby making it emerge as the least tolerant accession. The fold difference of *DREB1A* in the most tolerant accession was 2.95 which indicated that *DREB1A* gene was expressed in stressed plant 2.95 folds of the control. This shows a significant expression of *DREB1A* gene in the tolerant accession inferring its tolerability. The fold difference of *DREB1A* in least tolerant accession was 0.47 significantly lower expression of *DREB1A* genes which infers the accession sensitivity and vulnerability to water stress. Conferment of drought tolerance observed by *DREB1A* was consistent with the work of Kasuga *et al.* (2004) where he reported that the overexpression of *DREB1A* improved drought and low temperature stress in tobacco.

5. CONCLUSION

Overexpression of Drought resistant genes SNAC1 and DREB1A were inferred to confer drought tolerance in the most tolerant Okra accession NGB 00332 when subjected to drought stress in contrast there was low expression of these genes in the least tolerant accession NGB 00466. ERF4 as a candidate gene was also validated in Okra suggesting its adoption for other studies relating to okra or related species. Also, the agro-morphological studies revealed accession NGB 00332 to possess higher mean growth parameters across the weeks of planting. With the growing global population, global warming and effects of climate change affecting the cultivation and effective production of crops like okra, the results of this study can aid geneticists and plant breeders in selecting and developing resistant varieties thereby improving global food security.

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