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Differential Expression of *VrNAC* Genes in Shoot and Root Tissues of Mung Bean Grown Under Drought Condition

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Keywords: Mung bean; V. radiata L. var. BARI Mung-6; VrNAC gene; Drought stress; qPCR .

Abstract

Background: Mung bean (*Vigna radiata* L.) is an economically, nutritionally and ecologically important legume crop, cultivated in East, Southeast and South Asian countries. Drought affects the production of mung bean by altering its growth at various stages of its lifecycle.

Objective: This study attempted to explore the drought tolerance ability of *V. radiata* L. var. BARI Mung-6 by analyzing growth parameters and the expression profiles of stress response genes *VrNAC*.

Method: The experiment was conducted under controlled (greenhouse) conditions with two groups of treatments: the experimental group (drought treatments: watering in 2, 5, 10 and 15 days of interval) and the control group (well-watered) at the vegetative stage. Various morphological traits were observed and expressions of four drought-responsive NAC genes (*NAC011*, *NAC085*, *NAC092* and *NAC109*) were assessed in leaves and roots by quantitative real-time PCR (qPCR).

Results: After 30 days, significant reductions were observed in the growth parameters of highly stressed plants compared to the control e.g., shoot length (36.1%), shoot fresh weight (62.86%), dry weight (66.67%) and turgid weight (57.14%). However, the root length (9.48 cm) and lateral root numbers (31) were significantly greater in highly water-deficit conditions. Relative expression analysis revealed the alteration of *VrNAC* genes in stressed leaves and roots under drought stress e.g., *VrNAC085* and *VrNAC092* expressions varied from 9.80 to 312.85 fold and 9.03 to 133.76 fold respectively in highly stressed leaves.

Conclusion: This investigation provided a foundation for further research into the molecular mechanism of drought tolerance in mung beans aiming to improve stress-tolerant mung bean varieties.

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Introduction

Abiotic stress owing to global climate change has created enormous concerns for the agriculture and hence global food security and sustainability. Drought stress is an important constraint that limits growth, development and yield of crop plants [1-4]. Several studies have reported detrimental effects of drought on legume crops e.g., *Glycine max*, *Phaseolus vulgaris*, *Cicer arietinum* and *Lens culinaris* [5-7]. An enhanced understanding of the effects of drought on physiological and molecular attributes of legume crops is relevant for better management of agricultural lands and the improvement of crops in the context of global climate change.

Mung bean (*Vigna radiata* L.) is one of the most important legume crops, contributing to 90% of the world's current crop production. It is also beneficial to human health due to its high nutritional and medicinal value antioxidant, anti-inflammatory and antitumor properties [8-10] along with other multiple uses in food, feed and industrial application [11]. It is also a potential crop for sustainable agriculture because it can fix nitrogen, improve soil health and fertility and reduce greenhouse gas emissions [12].

Despite being an economically and ecologically important crop, the productivity of mung bean is stagnant due to erratic weather conditions coupled with various biotic and abiotic stresses [13,14]. The loss of crop yield is reported to exceed 50% due to both biotic and abiotic stresses, pests and diseases [15,16]. Among the abiotic stresses, drought is the most limiting factor for mung bean cultivation that hampers the growth and yield [17]. Drought stress affects various morpho-physiological processes associated with growth and molecular functions, which lead to poor grain yield [18-21]. Observed 51% to 85.50% yield reduction due to drought stress in the mung bean [22].

Plants encode a wide range of stress-responsive genes to overcome stress conditions [23]. Transcription factors (TFs) are such genes that act as pivotal regulators of plant responses to a number of abiotic stresses including drought [24]. Among these transcription factors NAC (NAM, no apical meristem; ATAF1-2, *Arabidopsis thaliana* activating factor and CUC2, cup-shaped cotyledon) family performs numerous functions in plants i.e., the formation of plant shoots apical meristems [25], nutrient transfer, control of the cell cycle in the senescence process [26], plant stress response [25], regulation of plant innate immunity [27] and hormone signaling [28]. A number of NAC proteins may positively regulate plant defense responses by activating pathogenesis-related genes, inducing genes involved in mediating the hypersensitive response and programmed cell death at the infection site in resistant plant species [29-32]. NAC genes play an important role in plant abiotic stress response e.g., overexpression of *OsNAC6* resulted in increased tolerance to blast disease in rice [33], *GmNAC20* may regulate stress tolerance through activation of the *DREB/CBF-COR* pathway and may control lateral root development by altering auxin signaling related genes [34]. Numerous NAC genes have been identified to be involved in plant response to drought and salinity stresses e.g., *OsNAC2*, *OsNAC6*, *OsNAC10* in rice [35]; *TaNAC69* and *TaNAC6* in wheat [36,37] and *CarNAC3* in chickpea [38]. NAC has been functionally characterized in common bean [39], chickpea [40] and soybean [41] and its expression enhances plant abiotic stresses and defense responses e.g., wound, cold and drought. Soybean leaves showed various expression profiles for 139 *GmNAC* genes during drought stress [42]. Though NAC TFs play a key role in growth, development and stress tolerance in plants, such genes have not yet been well studied in mung bean. Understanding the morpho-physiological and molecular responses of plant part is pertinent for the improvement of crops in the context of stress condition. The objective of the present study was therefore to investigate the physiological changes and the expression patterns of some *VrNAC* genes in root and shoot tissues of mung bean under drought stress conditions. The findings of the study will provide a foundation for an improved understanding of *VrNAC* genes for further improvement of the cultivation of mung bean varieties.

Materials and methods

Sample collection and preparation

Seeds of the possible drought tolerant variety of mung bean BARI Mung-6 were collected from Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur. Seeds were sterilized with 3% sodium hypochlorite for 10 minutes and washed five times with sterile distilled water. The sterilized seeds were germinated with water on moist absorbent two layers of filter papers in petri dishes and kept in a dark place. The germinated seeds were grown in plastic pots in greenhouse at 17-19ºC temperature with 50-53% relative humidity and 12 hours light/dark cycles. Each pot contains five seeds with three replicates. There were four treatments including a control group. The experiment was conducted under controlled drought stress with 2 days (Control), 5 days (Treatment 1), 10 days (Treatment 2) and 15 days (Treatment 3) irrigation gap (Figure 1).

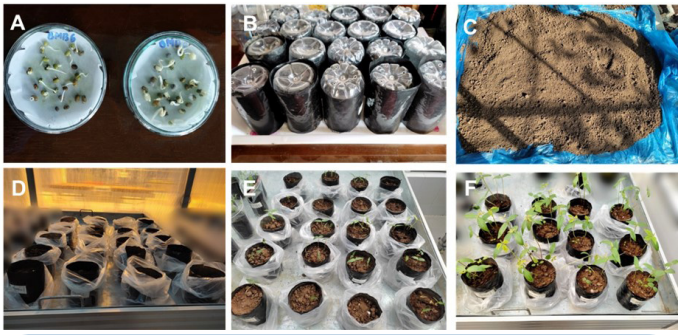

Figure 1: Different stages of plant samples preparation: **(A)** Seed germination on Petri plates; **(B)** Pot preparation; **(C)** Soil preparation; **(D)** Seed plantation and placement in the greenhouse; **(E)** Germination on 3rd weak; **(F)** Germination on 5th weak.

Analysis of functional parameters

After 30 days of plantation, morphological parameters e.g., shoot length (cm), root length (cm), fresh and dry weight of shoot and root (gm), turgid weight of shoot and root (gm), lateral root number from each replicate of each treatment were recorded at the vegetative stage and compared the treatment group with well-watered healthy plants.

The upper part of the plants from each pot were harvested and recorded the Fresh Weight (FW); and soaked in distilled water at room temperature for 24 h. The samples were weighed after removing excess water by gently wiping them with a paper towel, which is called Turgid Weight (TW). The Dry Weight (DW) of the samples were taken after drying the samples in the oven at 95˚C for 24h. The estimated fresh, dry and turgid weights of the upper portion of plants were used to calculate Relative Water Content (RWC) [43] Water Saturation Deficit (WSD) [44], Water Retention Capacity (WRC) [45] and Water Uptake Capacity (WUC) [46]. The formula of the all the parameters was presented below.

$$\text{Relative water content (RWC)} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100$$

$$\text{Water saturation deficit} = 100 - \text{RWC}$$

$$\text{Water retention capacity (WRC)} = \frac{\text{Turgid weight}}{\text{Dry weight}}$$

$$\text{Water uptake capacity (WUC)} = \frac{\text{Turgid weight} - \text{Fresh weight}}{\text{Dry weight}}$$

Analysis of NAC gene expression

The tissue-specific sequences of all NAC genes were downloaded from the National Centre for Biotechnology Information (NCBI) database by the Basic Local Alignment Search Tool (BLAST). To analyze the expression of drought-responsive NAC genes in *V. radiate* L. var. BARI Mung-6, sample plants were prepared in such a way that the control group remains in well-watered condition (watered in 2 days intervals) and the treatment group is stressed in drought (kept in 15 days without watering). Total RNA was extracted from fresh leaf and root tissues individually (100 mg) using Trizol reagent. The concentration of RNA was quantified by Nanodrop spectrophotometer (Tharmo Scientific Nano Drop 1000, USA). Quality was confirmed using values of 260/280≥1.8, 260/230≥2.0, 28S/18S≥1.8, and RIN≥7.5, and checked in 1.5% formaldehyde denaturing agarose gel. Total RNA was reverse transcribed into cDNA using 'VERSO cDNA synthesis kit'. This cDNA was used as the template for amplification.

qPCR was conducted with a power-up SYBR-green master mix using 'TUB' (tubulin) as the reference gene. Primers of selected genes for qPCR were designed by 'sequence manager' and 'oligo analyzer' tools and the specificity of each primer was guaranteed by melting peaks and dissociation curves (Table 1). The thermal cycler conditions were fixed at 95 ºC for 2 min and 30 cycles of 95 ºC for 30 s, 57-60 ºC for 20 s, and 72 ºC for 30 s. The resulting data was calculated by the 'Livak method' or, $2^{-\Delta\Delta CT}$ method, which is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments [47]. Statistical significance was calculated using a student's t-test with JMP software. All data were obtained in triplicate.

Table 1: Primers used in gene expression analysis.

Primer names	Sequences (5'___3')
Vig011f	ACGCTCCAACTTTCAAACGC
Vig011r	CGGATACTTTCGGTCCCTCG
Vig085f	GTTCAAGAGAGAGACCCTC
Vig085r	TCAGTTCCCGTCGCTTTCC
Vig092f	GTCTACCCCCTGGTTTTCGG
Vig092r	TGGCTTTCCAATACCCCGAC
Vig109f	GCCGCCACCGCATAATTCTC
Vig109r	CCGGTTGCTTTCCAGTACC
VigTUBf	CTGGTTTTGCATTGACTTGC
VigTUBr	CTGCAGCTGGAAACACTTTG

Statistical analysis

The data was analyzed using the one-way Analysis of Variance (ANOVA) performed using values of individual parameters to determine the mean, Standard Deviation (SD) and standard Error of Mean (SEM). Treatment means were compared by the Least Significant Difference (LSD) test at a 0.05 probability level. Graphical presentation of data was done by MS Excel and 'JMP 4.0 software (SAS institute, Cany, NC, USA).

Results

Morphological changes of mung bean

The ANOVA analysis revealed that the effect of drought was significant at $p<0.05$ among the treatments than the control. The plants that were in stressed condition showed a gradual decrease in shoot length (18.7 to 11.95 cm) following the shoot fresh weight (0.70 to 0.26 gm), dry weight (0.12 to 0.04gm) and turgid weight (0.84 to 0.36gm) from minimal stress to higher stress compared to control. In contrast, the stressed plants showed the highest root length (4.37 to 9.48 cm) and lateral root number (15 to 31) (Figure 2, Figure 3) while the lowest values were observed in the root fresh weight of stressed plants but the change in dry weight and turgid weight of the root was insignificant.

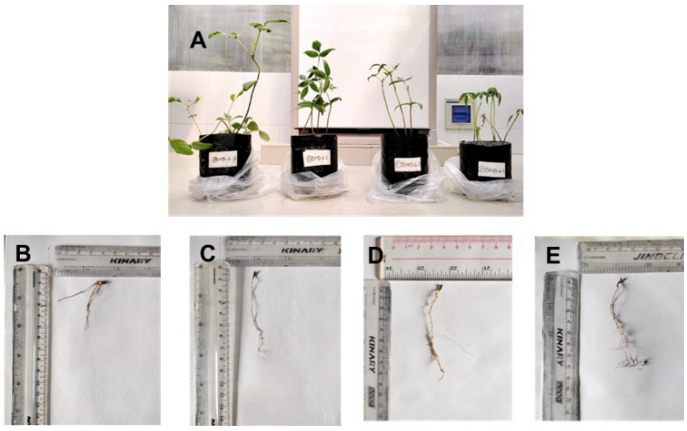

Figure 2: Shoot and root samples of four watering conditions at the time of harvesting. (A) Young mung bean plants, watered with an interval of 2 days, 5 days, 10 days and 15 days respectively (from left to right); (B, C, D, E): Root samples of plants watered with an interval of 2, 5, 10 and 15 days, respectively.

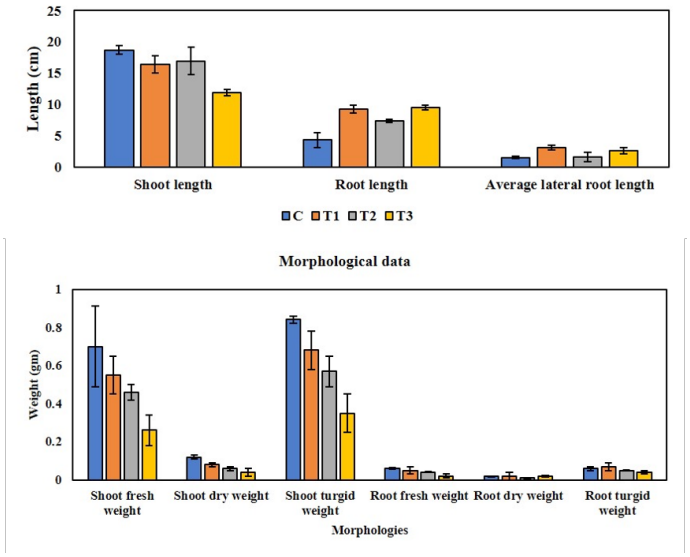

Figure 3: Mean values of the effects of drought on the morphological parameters of *Vigna radiata* var. BARI Mung-6 at four different levels of water regimes. Control (watering every 2 days interval); T1, minimal stress (watering every 5 days interval); T2, moderate stress (watering every 10 days interval) and T3, high stress (watering every 15 days of interval).

To determine the plant water relationships, Relative Water Content (RWC), Water Saturation Deficit (WSD), Water Retention Capacity (WRC) and Water Uptake Capacity (WUC) of the individuals were observed in different drought stress conditions (Table 2). The RWC varied from 68.75 to 80.56% under Well-Watered (WW) and Water Deficit (WD) conditions respectively. The highest RWC was found in well-watered Condition (C) and the lowest was observed in highly stressed (T3) condition. In the case of reduction percentage over control, minimum reduction was observed in T2 (2.64%), whereas the maximum reduction (14.66%) was in T3 group.

The values of WSD ranged from 19.44 to 31.25% under WW and WD conditions. The higher WSD was obtained in T3 (31.25%) plants and lower obtained in Control (19.44%) among the treatments. In WD condition the WSD increased from 10.96 to 60.75% over WW and the minimum increase was observed in T2 (10.96%) and higher in T3 (60.75%).

WUC quantifies the capacity of plants to reach turgid condition by absorbing water per unit of dry weight. The WUC in studied mung bean were greatly influenced by WD condition and it increased under stress condition. Results also showed that 1.17% WUC was recorded in WW and 1.63 to 2.3% ranged under WD conditions. The findings revealed that a sufficient supply of water (WW) exhibited the lowest value of WUC whereas in WD conditions T3 showed the higher WUC (2.3%) and T1 demonstrated the least (1.63%). The WUC highly increased 96.58% in T3 when the least in T1 (39.32%) under WD condition as compared to WW condition. The WRC showed significant variation among the treatment groups of WD conditions. The increment of WRC was higher in plants raised in WD conditions than the plants raised in adequate supply of water. Here the WRC increased from 21.43 to 35.71% among the mung bean plants in WD as compared to their WW condition. However, T1 plants gave the minimum relative increment (21.43%) whereas T2 gave the maximum relative increment (35.71%) (Table 2).

Table 2: Relative Water Content (RWC), Water Saturation Deficit (WSD), Water Uptake Capacity (WUC) and Water Retention Capacity (WRC) at the vegetative stage of mung bean under control and water deficit condition.

RWC			WSD			WUC			WRC		
WW condition	WD condition	% decrease over WW condition	WW condition	WD condition	% increase over WW condition	WW condition	WD condition	% increase over WW condition	WW condition	WD condition	% increase over WW condition
80.56	78.33	2.77	19.44	21.67	11.4	1.17	1.63	39.32	7.0	8.5	21.43
	78.43	2.64		21.57	10.96		1.83	56.41		9.5	35.71
	68.75	14.66		31.25	60.75		2.30	96.58		9.0	28.57

Note: WW: Well Watered; WD: Water Deficit.

Expression analysis of NAC genes

The results suggested the alteration of four *VrNAC*s expression in drought following the application of stress, but *VrNAC085* in leaf and *VrNAC092* in both leaves and roots showed significant differences in their expression level. The average expression level of *NAC085* in the stressed leaf samples was found to be 9.80 to 312.86 fold and *NAC092* was found to be 9.03 to 133.76 fold. The expression pattern of each *VrNAC* gene varied in different tissues under different levels of drought stress indicating their potential roles in influencing drought tolerance in these tissues. Moreover, *NAC085* and *NAC092* were more highly expressed in leaves than in roots, indicating their critical roles in leaves.

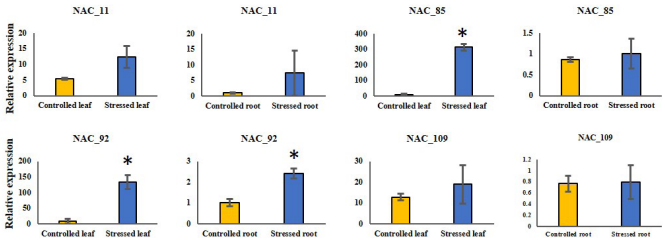

Figure 4: Mean values of the relative expression of the *VrNAC* genes in leaf and root tissues of mung bean (*Vigna radiata* L. var. BARI Mung-6) under well-watered (control) and stressed (watering in 15 days of interval). Each sample was analyzed using three biological replicated and normalized to a TUB (tubulin) expressing gene and * is significantly different at $p<0.05$ compared with the relative control.

In contrast, *NAC011* and *NAC109* were expressed at low levels in both leaf and root. Based on the observed expression patterns of the *VrNAC* genes, it could be concluded that *VrNAC* genes played significant roles in inducing drought tolerance under drought stresses through different pathways.

Discussion

Increased intensity of droughts owing to climate change has created serious concerns to crop production worldwide [48]. Crop production is being hampered by altering biological attributes of plants along with soil fertility and cropping patterns. Particularly it severely affects the morpho-physiological, biochemical, and molecular functioning of the short duration plants like mung bean [17]. Detailed understanding about the defense mechanisms of plants against drought stress is relevant for better management of such stress condition.

In this study, the effect of drought stresses has been observed in plants grown under different moisture regimes in the greenhouse condition. Shoot height was adversely affected by the severe stress (watering at 15 days interval) applied in terms of various watering conditions. The highest shoot length was found in well-watered plants and the height decreases as the stress increases respectively. According to, drought reduced the height of mung bean plants in both the vegetative and reproductive stages [2]. BARI Mung-6 is able to maintain higher turgidity due to its higher water uptake capacity and xylem exudation rate, which ultimately helped it to have higher plant height [49].

Watering at different time intervals significantly affected the shoot fresh weight and dry weight of plants. Shoot fresh weight was found higher in minimal stressed condition and respectively decreased in highly stressed condition. In case of turgid weight, the highest weight was recorded in well-watered plants and gradually decreased in the stressed plants. In case of dry weight, the pattern of reduction was also the same. Other studies also found a reduction in the dry weight of plants un-

der water stress conditions [50,51]. However, BARI Mung-6 was reported to be the most competent to accumulate dry matter under drought stress condition [49].

Drought stress generally affects both the root and shoot growth of plants which results the reduction of plant growth. Plant roots serve as primary sensory organs, detecting alterations in soil conditions and playing a crucial role in confronting water stress [52]. There is a significant reduction in shoot and root length under drought condition. Root length was reduced uniformly under water stress [53]. The decrease in root length might be due to decrease in cell elongation resulting from loss of water by drought stress. However, in this study, we found the highest root length in severely stressed condition and there was a gradual increase in root length from well-watered to highly stressed plants. Not only the root length but also the number of lateral roots were also found higher in stressed plants than in the control. It can be said that, when there was unavailability of water, roots tended to go deeper into soil in search of it like *Cunninghamia lanceolate*. *Cunninghamia lanceolate* can increase root complexity and elongation, reduce root branching angles, leading to steeper and deeper roots system to adapt to drought stress [54].

The RWC is an effective measurement tool of the water status in plants that reflects the metabolic activity in plants and is used as a most evocative indicator for stress tolerance [55]. Moreover, when plants were fallen water stress, leaves demonstrate a great reduction in RWC and water potential [56]. In this experiment, the plants have the highest relative reduction of RWC in higher drought over control. In case of WSD, the higher value indicates that the plants are subjected to a greater degree of water shortage [57]. Here the result exhibited the highest value of WSD in highly stressed plants over the control. This result means that the plants that were watered every 15 days of interval have severely been suffered due to water deficit stress compared to control. The maximum WUC in a plant is subjected to a greater degree of water deficit [57]. It represents the efficiency of a plant to uptake water per unit of dry weight at a particular stage [57]. Data of this study depicted an increment of WUC in the WD condition. The highest percentage of increment over control indicated that the highly stressed plants suffered more than the well-watered plants. The WRC interprets the water-holding capacity of leaves under particular conditions [58]. The plant rising under an optimal supply of water preserves a higher WRC, and it might be due to the lesser demolition of plant tissues under Drought Stress (DS) [59]. In this present study, plants raised under drought stress condition had maximum ratio than that of the plants raised under well-watered condition. In contrast to the general expectation our study observed a significant increase in WRC in plants subjected to drought stress which possess adaptive strategies enhancing water retention under stress.

The BARI Mung-6 variety used in this experiment is thought to be tolerant to drought. To assess the effects of abiotic stress, plants were exposed to drought stress. In this study, presence of several *GmNAC* candidate genes [60] were identified in *V. radiata* and their expression were found to be altered in drought stress condition. The expression pattern of four different NAC genes in mung bean e.g., *VrNAC011*, *VrNAC085*, *VrNAC092* and *VrNAC109* were evaluated under drought stress by real time quantitative PCR (qRT-PCR). The expression of *VrNAC011* enhanced from 5.46 - 12.31 fold in stressed leaf and 1.03-7.37 fold in stressed root samples. *NAC011* is a very attractive candidate gene as its overexpression in *Arabidopsis* was shown to enhance salt tolerance and improve lateral root development [34]. It is also found upregulated in drought stressed roots of soybean [60]. *VrNAC085* expression enhanced from 9.80 - 312.86 fold in stressed leaf and 0.86 - 1.01 fold in stressed root. *NAC085* is a particular interest, since it shares 39% identity at protein level with *SNAC1*, a rice NAC gene, whose overexpression enhanced drought tolerance of transgenic rice plants under field conditions [61]. *VrNAC092* increased from 9.03 - 133.76 fold in stressed leaf and from 1.02 - 2.42 fold in stressed root. This *NAC092* was upregulated in drought stressed roots of soybean [60] and together with *ANAC019*, *ANAC055* and *ANAC072* were demonstrated to enhance drought tolerance when overexpressed in transgenic *Arabidopsis* plants [62]. *VrNAC109* expressed from 12.88 to 18.96 fold higher in stressed leaf and from 0.76 to 0.79 fold in stressed root samples. So, the *VrNAC085* and *VrNAC092* are two upregulated genes in the leaf of mung bean among these four NAC genes in drought stress.

Conclusion

Drought adversely affects the growth of plants. The expression analysis revealed the presence of NAC genes and their differential expression in drought conditions in root and shoot tissues of mung bean. An increase in the frequency of drought severity is expected in the coming years due to global climate change. Therefore, there is a need to develop a favorable mung bean variety that performs better in drought environments. However, much work remains to fully understand the mechanism of *VrNAC* genes in drought stress response. Our research provides understanding for future studies to overcome the drought stress in mung bean. Having a detailed knowledge of stress responsive *VrNAC*s in mung bean will be highly valuable for future food legumes to improve mung bean in diverse environmental conditions.

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Declaration of Competing Interest: The authors declare no competing interests.

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