Identification and characterization of CAT1 gene during drought stress in moth bean [Vigna aconitifolia (Jacq.) Marechal]

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Summary
Moth bean [Vigna aconitifolia (Jacq.) Marechal] is a drought-hardy orphan pulse crop. However, the information related to the identification and characterization of drought stress tolerance genes is very limited. Therefore, the present investigation was formulated to identify and characterized drought-tolerant gene(s) from moth bean. Five genes were selected from available expression studies of moth bean and their expression pattern was assessed during a time course experiment of drought stress in moth bean. During the time-course drought stress experiment in moth bean, the catalase1 (CAT1) gene was exponentially expressed and up-regulated. Thus, CAT1 gene of the moth bean was identified as a potential candidate gene and validated through Sanger’s sequencing. The genomic sequence CAT1 gene was named VacoCAT1 and was further characterized using various bioinformatics tools. The VacoCAT1 showed an incomplete ORF with a length of 213 bp, which encoded 71 amino acids. The coding sequence of VacoCAT1 gene was shown as a single exon due to the incomplete nature of genomic sequences. The multiple sequence alignment of VacoCAT1 revealed the highly conserved region at the 3’ site of the gene as compared to CAT1 gene of other crop species, including legumes. The phylogenetic analysis of VacoCAT1 and CAT1 genes of other crop species, including legumes, revealed three clusters. The cluster VacoCAT1 gene showed proximity with V. radiata CAT1 in cluster one of a phylogenetic tree. The identified and characterized VacoCAT1 gene can be utilized as a genomic resource for enhancing drought tolerance in susceptible pulses and other crops.

INTRODUCTION
Moth bean [Vigna aconitifolia (Jacq.) Marechal] is an annual legume crop which belongs to the subfamily Papilionaceae of Fabaceae. It indicates the wide social acceptance and geographic adaptation and is considered as the native pulse of India. It is, however, the most crucial pulse in arid zone perspective. During the extremity of drought stress, this is the only crop, cultivated for grain and fodder with minimum water, tillage and other agronomic inputs. In order to combat the adverse effects of drought, it is very essential to develop water-deficit stress-tolerant genotypes in crop plants. To achieve this, a better understanding of the stress-induced responses and the inter-relationships of physiological and biochemical traits in drought tolerant crops needed to be studied. Since, moth bean is a drought-hardy pulse thus it can prove the usefulness of drought stress tolerance mechanisms. Additionally, the moth bean is a native crop of hot and dry habitats of northern and western parts of India. In arid and semi-arid regions drought as abiotic stress, is one of the major factors limiting plant growth at various stages of their life. Water stress in plants reduces the water potential and turgor and increases the concentration of solutes in the cytosol. Consequently, cell enlargement, gas exchange, transpiration, plant nutrients uptake and transport are decreased. A major effect of drought is a reduction in photosynthesis, which arises from a decrease in leaf expansion, impaired photosynthetic machinery, premature leaf senescence and associated reduction in food production. Acclimation of plants to water deficit is the result of different events, which lead to adaptive changes in plant growth and physio-biochemical processes, such as changes in plant structure, growth rate, tissue osmotic potential and antioxidant defences. It has been recognized that plants exhibit several adaptations to survive under stress conditions. Reduced leaf area, stomatal closure to prevent transpirational water loss, decreased stomatal conductance, limited internal CO2 concentration, reduced photosynthesis are very vital. Water-deficit stress tolerance is thus the result of coordination of
physiological and biochemical alterations at the organ, cellular and molecular levels. Although these physiological and biochemical mechanisms of the stress response are common to all plants, major differences exist in terms of strategies adopted by different crop species to cope with drought stress. Drought-tolerant and sensitive varieties differed in their protein profiling with newer genes showing their expression in tolerant ones. Higher basal activity of enzymes and total soluble sugar during water deficit in drought-tolerant varieties was reported by Soni et al. (2011).

Since the moth bean is known as stress hardy orphan pulse, however, expression studies to understand stress tolerant mechanism at the molecular level is very limited. Few studies have been brought to understand the drought tolerant mechanism in moth bean. However, molecular cloning and characterization of stress-responsive genes have not been reported in moth bean. Thus, in order to combat the adverse effects of drought, it is very essential to develop water-deficit stress-tolerant genotypes. Thus, a better understanding of the stress-induced responses and the inter-relationships of physiological and biochemical traits in drought tolerant crops such as moth bean can prove to be very useful. Therefore, few drought stress-responsive genes were profiled at expression level during drought stress in moth bean to identify the best drought-tolerant gene.

Materials and Methods

Plant material and gene-specific oligos
Seeds of moth bean cv. RMO-40 were collected from Agricultural Research Station (ARS), Swami Keshwanand Rajasthan Agricultural University, Bikaner. Five up-regulated genes from publicly available expression studies of moth bean (Table 1) were selected and genomic sequences pertaining to selected genes were identified in EST database of moth bean (https://www.ncbi.nlm.nih.gov/nuccore) and used for designing gene-specific primers using PrimerQuest software (https://www.idtdna.com/pages/tools/primerquest). The gene-specific primers were synthesized from GCC Biotech Pvt. Ltd, India.

### Table 1: Selected genes and gene-specific oligos used in this study

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer Sequence (5'-3')</th>
<th>Tm</th>
<th>Reference</th>
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<tbody>
<tr>
<td>CAT1</td>
<td>F – CTCTCGGAGGATTATCATCT&lt;br&gt;R – AAGGAGAACATGTGAAGGCT</td>
<td>55 °C</td>
<td>Tiwari et al. (2018)</td>
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<tr>
<td>HSP70</td>
<td>F – ATGACAGTGTGATTCGAG&lt;br&gt;R – TCTTCATCCTCTGCTTGT</td>
<td>55 °C</td>
<td>Tiwari et al. (2018)</td>
</tr>
<tr>
<td>HSP90</td>
<td>F – GACGATGTAATCAACTGCT&lt;br&gt;R – CTATATAGGATCACCAGCA</td>
<td>55 °C</td>
<td>Tiwari et al. (2018)</td>
</tr>
<tr>
<td>P450</td>
<td>F – GAGAAGACATGGTGATGTG&lt;br&gt;R – GAAGCATATGCATCTGTC</td>
<td>55 °C</td>
<td>Tiwari et al. (2018)</td>
</tr>
<tr>
<td>ACTIN</td>
<td>F – AGCCACTGGAATTATGAAACG&lt;br&gt;R – TGCTGCTTGGCTAGGACT</td>
<td>55 °C</td>
<td>Rampuria et al. (2012)</td>
</tr>
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Growing of plants and drought stress treatment
The seeds of RMO-40 were grown in 8-inch plastic pots pre-filled with a 1:1 ratio of field soil and perlite. The seedling was raised in a plant growth chamber (Cole-Parmer, India) by providing controlled environmental conditions (30 °C temperature, 60% relative humidity and 16/8 light-dark cycle). The seedlings were irrigated thrice a week with Hoagland solution (Hi-media, India). After 15 days, only one healthy seedling per pot was retained by uprooting of other comparatively less vigoured seedlings. Two sets of seedlings were raised for control and drought stress treatment, respectively. For creating drought stress, fifteen days old seedlings were subjected to drought and heat stress by the withholding of water and at 35 °C, respectively. The control samples were maintained by irrigation with Hoagland solution parallelly (Fig 1). The leaf samples were collected from drought stress-treated seedlings along with the control sample (seedling without stress) at 0, 3, 6, 24 and 72 hours of post-stress. The experiment was conducted in three subsequent biological replication and the statistical significance of the replication was assessed using a student’s t-test at p-value <0.05.
RNA isolation and cDNA synthesis
The total RNA was isolated from 100 mg of moth bean leaf samples using an RNeasy plant mini kit (Qiagen, Germany) according to the manufacturer’s instructions. The genomic contamination from total RNA was removed by treating the solution with RNase-free DNasel (Invitrogen, USA). The quality and quantity of total RNA were assessed electrophoretically on 2% agarose gel and spectrophotometry (Shimadzu, Japan), respectively. The RNA quality was assured on the agarose gel by visualizing it under UV-transilluminator. The quantities of the RNA samples were determined at 260 nm absorbance and a ratio from 1.9 to 2.0 was considered as good quality RNA. The 3 μg of total RNA was used to reverse transcribe into cDNA as per the manufacturer’s instructions (Takara Bio. Inc., Japan). The uniformity and quality of cDNA was assessed by amplification of actin gene of moth bean.

Semi-quantitative reverse transcriptase PCR analysis
The gene-specific primers were validated in drought stress-treated samples of moth bean using semi-quantitative reverse transcriptase PCR (semi-qRT PCR) analysis. The PCR reaction was performed in 20 μl reaction volume containing 10 μl of Dream Taq master mixture (Genetix Biotech Asia Pvt. Ltd., India), 1.0 μl of each forward and reverse primers (10 mM), 1 μl MgCl2 (10 mM), 2 μl of a template (cDNA) and nuclease-free water (Hi-media, India) for volume make-up. The PCR was performed in a thermal cycler machine (Bio-Rad, USA) with the following thermos cycling conditions: one step of initial denaturation at 94 ºC for 5 min, followed by 28-30 cycles of denaturation at 94 ºC for 30 s, primer annealing at 55 ºC for 30 s and primer extension at 72 ºC for 2 min. followed by one step of final extension at 72 ºC for 10 min. The PCR products were visualized electrophoretically on 2.0% agarose gel and documented in gel documentation system (SynGene, USA). The house-keeping gene (actin) of moth bean was used as an internal control.172

Sequence characterization
The exponentially expressed catalase gene in terms of up-regulated manner during the time course experiment of drought stress was selected for Sanger’s sequencing to determine the nucleotide sequences. The catalase gene was amplified on cDNA of moth bean. The PCR product was purified using QIAquick PCR purification kit (Qiagen, Germany) and validated through sequencing from GCC Biotech Pvt Ltd., India. The resultant nucleotide sequence of the catalase gene was validated through a homology search in NCBI Blast analysis (https://blast.ncbi.nlm.nih.gov) and the catalase gene of moth bean was nomenclature as VacoCAT1 gene. The open reading frame was predicted from the resultant genomic sequence of VacoCAT1 gene using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/).

Gene structure and conserved motifs analysis
Full-length gene sequences and corresponding CDS of catalase gene were downloaded from the Legume Information System database of legume crops (https://www.legumeinfo.org/). The retrieved genomic information of catalase gene of legume crops was compared with VacoCAT1 gene for prediction of exon-intron distribution using PIECE2 software (http://probes.pw.usda.gov/piece or http://aegilops.wheat.ucdavis.edu/piece). The conserved motifs in VacoCAT1 were examined in MEME suite (https://meme-suite.org/meme/) with 20 numbers of motifs as parameters. Multiple sequence alignment and phylogenetic analysis
For multiple sequence alignment analysis, nucleotide sequence encoded amino acid sequences of VacoCAT1 were aligned with catalase genes of related legumes using
Clustal omega online tool (https://www.ebi.ac.uk/Tools/msa/clustalo). The amino acid sequence alignment was graphically represented by ESPript 3.0 online software (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). To reveal the phylogenetic relationship between the VacoCAT1 and catalase genes of related legumes and Arabidopsis, multiple sequence alignment of amino acid sequences were established further and alignment was utilized for the phylogenetic tree construction using poison model and Neighbor-joining method with 1000 bootstrap replicates in MEGA 6.0 software (www.megasoftware.net).

Results and Discussion

Assessment of drought stress-responsive expression of moth bean genes
Moth bean has peculiar traits like a tap root system, the spreading nature of plants which make it stressful hardy legume. Moth bean possesses resistance to extreme temperature regimes. Our experiment conducted on moth bean during withholding of water and grown at 35 °C has shown that these seedlings can efficiently live and perform their metabolic activity. Thus, owing to resistance against heat and drought stress, it is quite imperative to identify and characterize genes related to drought stress tolerance mechanisms at the molecular level. Thus, in the present investigation, the physiological adoption of the moth bean was tried to understand at the molecular level. It was aimed to achieve five reported up-regulated genes during drought and heat stress in moth bean. The expression of genes for abiotic stress responsiveness was carried out by various gene expression studies in legumes. Similarly, in the present study, the expression characterization of selected genes was done by semi-qRT-PCR analysis. This was done to select the gene which shows the exponential up-regulation during the time course experiment of drought in moth bean.

To validate the expression of five drought-tolerant genes of moth bean during drought stress, a semi-qRT-PCR analysis was done using gene-specific primers. The semi-qRT-PCR assay (Fig 2) revealed that the CAT1 gene expressed exponentially in the up-regulated manner in a time course study from 0 to 72 h of drought stress. Exceptionally, this gene was observed down-regulated only at a single time point (24 h) as compared to the control. Such type of interruption in the exponential expression of CAT1 gene was suggested that the stability of the encoded protein product to give tolerance against drought stress is up to 24 h or less. However, this gene was expressed at later time points (72 h) which also suggested the requirement of encoded protein against drought stress in moth bean. The HSP70 gene was significantly up-regulated at 6 h; whereas, it was significantly down-regulated as compared to the control at 24 and 72 h of drought stress. The HSP90 gene was down-regulated at all-time points of drought stress but it was significantly further down-regulated at 72 h. Similarly, the P450 gene was down-regulated at 3 and 72 h of drought stress, whereas it was significantly up-regulated at 6 and 24 h of drought stress as compared to the control. This gene showed a circadian type of expression pattern during drought stress experiments in moth bean. The P5CS gene was not shown a significant expression in the time course experiment of drought stress in moth bean.

Heat-shock proteins including HSP70, HSP90 also identified as heat stress tolerance genes in crop plant. In the present study, the HSP70 and HSP90 genes were also analyzed during drought stress conditions in moth bean. However, HSP70 and HSP90 genes were showed circadian type of gene expression with down-regulation. Similarly, the P450 gene was showed up-regulation during drought stress in moth bean gene expression studies. However, it was expressed contradictorily in the present investigation. It also showed the circadian type of gene expression with down-regulation. The P5CS gene also showed potential expression during stress conditions in moth bean.
present study, *P5CS* gene was significantly not expressed during drought stress in moth bean. The contradictory issues related to the expression of HSP70, HSP90, *P450* and *P5CS* genes during abiotic stress conditions in moth bean could be due to the different experimental conditions of reported studies and present investigation.

The *catalase* gene showed up-regulation in the gene expression study of moth bean and other various crop plants.28,29 Similarly, the *catalase* gene was also found to be the most up-regulated gene as compared to the other four genes during drought stress conations in RMO-40 in our study. Thus, *catalase* gene was selected as a potential drought-tolerant candidate gene of moth bean for sequencing and further characterization by bioinformatics means.

Selection and sequencing of potential drought tolerant gene, *VacoCAT1*

Based on the expression pattern during drought stress experiment in moth bean, the selected and characterized five drought tolerant genes were categorized into three main groups such as (i) the exponentially up-regulated with sustainable expression – *CAT1* gene; (ii) genes with circadian and down-regulation in expression – HSP70, HSP90 and *P450* gene; and (iii) significantly not expressive gene – *P5CS* gene. Thus, based on the sustainable expression during the course time course experiment of drought stress in moth bean, the *CAT1* gene was considered as a potential candidate as the drought-tolerant gene of moth bean. Identifying genes of interest is immensely needed in sequence analysis and functional annotation with existing known genes.30 Therefore, the *CAT1* gene was amplified at cDNA level and it was validated through Sanger’s sequencing. A good-quality nucleotide sequence of the partially amplified *CAT1* gene of moth bean was obtained from the Sanger’s sequencing and the gene was named as *VacoCAT1*. The *VacoCAT1* gene showed high sequence similarities with *catalase1* gene of *V. radiata*.

Structural analysis of *CAT1* gene

To predict the structure of *VacoCAT1* gene of moth bean in terms of exon-intron distribution, number and length of open reading frame (ORF), peptide length, etc., the nucleotide sequences obtained from Sanger’s sequencing were analyzed using various online softwares. The exon-intron distribution of genes is revealing structural organization and evolutionary events of the genes.31 Thus, for exon-intron distribution, the *VacoCAT1* gene was compared with *CAT1* gene of *Vigna radiata* in Gene Structure Display Server 2.0 software. The analysis revealed only one exon of around 650 bp length in *VacoCAT1* gene. No introns were predicted by the software due to the incomplete nature (partial) of nucleotide sequences of *VacoCAT1* gene (Fig 3a). The ORF finder revealed the four ORF in *VacoCAT1* gene. Out of four ORF predicted, the ORF1 was found to be the longest (213 bp) which encoded 71 amino acids (Fig 3b).

Multiple sequences alignment and prediction of conserved motifs

The multiple sequence alignments in amino acid sequences of genes reveal the sequence similarity and conservation among the sequences. The multiple sequence alignment was done in amino acid sequences of *CAT* genes of various crop plants including legume crops.23,24,25 Similarly, the nucleotide sequence of *CAT1* gene of moth bean was aligned and compared with *CAT1* genes of *V. radiata*, *V. angularis*, *V. unguiculata* and *Arabidopsis thaliana*. The MSA analysis revealed a significant sequence homology with conservation among the nucleotide sequences of *CAT1* gene of moth bean along with *CAT1* gene of *V. radiata*, *V. angularis*, *V. unguiculata* and *A. thaliana* (Fig 4). The sequence similarity analysis showed close proximity of *CAT1* gene of moth bean with *CAT1* gene of *V. radiata*. The analysis also revealed the highly conserved region among the polypeptide of *CAT1* genes was amino acids at 3’ region of the gene. For the prediction of the conserved motif present in *CAT1* gene of the moth bean, the peptide sequence of *CAT1* gene was analyzed in MEME suite software. The analysis revealed 6 motifs presented in the *VacoCAT1* gene as compared to the *CAT1* gene of *V. radiata*. The discrepancy in the conserved motif was found due to the partial nature of gene sequences.
Fig 4: Multiple sequence alignment analysis of VacoCAT1 gene with catalase gene of other plant species.
Phylogenetic analysis

The nucleotide sequence of VacoCAT1 gene was compared along with CAT1 genes of related legume and A. thaliana during phylogenetic analysis. The phylogenetic tree revealed 3 clusters of CAT1 genes (Fig. 5). The cluster I was further divided into two sub-groups representing one group with CAT1 genes of legume crops and another with A. thaliana, Z. mays, wheat and rice. The phylogenetic analysis also showed the closed proximity of VacoCAT1 gene with CAT1 gene of V. radiata as shown in the MSA analysis. The phylogenetic analysis in this study showed the CAT1 genes of legumes in the same phylogenetic clade. The analysis suggested that the CAT1 gene is evolutionarily conserved in legume crops. The phylogenetic conservation of catalase genes was also found in many organisms including crop plants.

Conclusion

In conclusion, the five drought responsive genes were expressed differentially during drought stress in moth bean. Out of five expressed genes, catalase1 gene was expressed exponentially in up-regulated manner as compared to control. Thus, based on up-regulation during drought conditions in moth bean, the catalase1 gene was selected as drought tolerant candidate gene of moth bean. The catalase1 gene of moth bean was validated through sequencing and characterized based on various bioinformatics tools and software. The VacoCAT1 gene showed significant sequence homology with CAT1 gene of V. radiata and proximity with CAT1 gene of legume crops during phylogenetic analysis. The molecular cloning of VacoCAT1 gene at the full-length level and functional validation of the gene is the future area of this study. The VacoCAT1 gene can be utilized as a heterologous candidate gene for the development of abiotic stress tolerance in legume crops.
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Declaration of interests
The authors have no conflict of interest to declare.

Data sharing
All relevant data are within the manuscript.

References


