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Identification and characterization of catalase genes in *Eleusine coracana* under abiotic stresses

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Abstract

Reactive oxygen species (ROS) are byproducts of metabolic processes such as respiration and photosynthesis in plants. Production of ROS leads to rapid cell damage, and plants developed a complex system of enzymatic and non-enzymatic antioxidants to scavenge these ROS. Catalase is an important enzyme, which plays a key role in elimination of toxic effects of hydrogen peroxide and plays a major role as an antioxidant. When characterizing heat responsive genes in finger millet (*Eleusine coracana* L.) using a suppression subtractive hybridization (*SSH*) library, we isolated two catalase genes and named them as *EcCATA1* and *EcCATB1*. The lengths of the *EcCATA1* and *EcCATB1* open reading frames were 1 482 and 1 426 bp, respectively. We characterized these genes under different abiotic stresses and in different tissues. The tissue wise expression revealed that *EcCATA1* expression was higher in leaves whereas *EcCATB1* expression was higher in roots than in other organs. Under stress conditions, the expression of *EcCATA1* was highest under salt stress followed by mannitol treatment. In the case of *EcCATB1,* the highest expression was observed under mannitol treatment followed by cold and dehydration. We also studied expression of both the genes under heat stress in different finger millet genotypes and observed that expressions of these genes can be correlated with heat tolerance. For both the genes, a detailed computational investigation was also performed for understanding their structural properties and physicochemical characteristics. Overall, this is the first study to identify and characterize catalase genes from climate resilient finger millet crop.

Additional key words: finger millet, gene expression and cloning, heat, mannitol, reactive oxygen species, salinity.

Introduction

Finger millet [*Eleusine coracana* (L.) Gaertn.] (2n=4×=36) is a minor millet grown in the broad range of environmental conditions. This crop comprises 12 % of the worldwide millet area. It is cultivated in over 25 countries of Asia and Africa as one of the major food sources (Gowda *et al*. 2000, Adhikari 2012) and it is an excellent resource of mineral nutrients like calcium, iron, phosphorus, and amino acids like methionine and lysine (Dida *et al*. 2008). Finger millet encompasses numerous salient characteristics such as an inbreeding nature, short life cycle, and climate resilience (Kumar *et al*. 2016).

Aerobic metabolism generates reactive oxygen species (ROS) as a by-product in plant cells. Their production increases when plants are subjected to biotic and abiotic stresses resulting into cell injury, DNA and RNA damage, lipid peroxidation, protein oxidation, and enzyme inhibition (Scandalios 1993, Miller *et al*. 2008). Oxygen toxicity is an intrinsic characteristics of aerobic life, as it has been supposed that 1 % of the oxygen used by plants is altered to generate ROS (Asada 1992). Under control conditions, production and scavenging ROS remain in equilibrium. Increased activities of ROS scavenging enzymes have a high potential of plant metabolic regulations in a stressful environment (Chaitanya *et al*. 2002, Foyer and Noctor 2005). Plants have developed a complex antioxidant defense system, which is made of enzymes such as catalase, glutathione peroxidase, ascorbate peroxidase, superoxide dismutase, glutathione reductase, and peroxiredoxins. Among the ROS, H_2O_2 is a signaling molecule, but disproportionate formation of H_2O_2 has been shown to

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Abbreviations: *EcCATA1* and *EcCATB1* - *Eleusine coracana catalase A1* and *B1*; GRAVY - grand average of hydropathicity; qPCR - quantitative polymerase chain reaction; ROS - reactive oxygen species; SSH - suppression subtractive hybridization.
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be cytotoxic outcoming in hypersensitive cell death. A low production of H_2O_2 obstructs the progression of cell cycle and acts as a developmental sign for the beginning of secondary wall differentiation. Hence, the production of H_2O_2 must be specifically controlled by plants (Ushio-Fukai 2006). Certainly, antioxidant activity regulation in the plant is a key for adaption to environmental stresses, as well as a crucial aspect in regulating cellular signal transduction (Veal *et al*. 2007). Characterization of the antioxidant reaction may provide better understanding the nature of photo-oxidative processes activated by a specific stress.

Catalase (1.11.1.6) was the first antioxidant enzyme identified and characterized. Catalase is a heme containing tetrameric ubiquitous enzyme, which catalyzes the division of two H_2O_2 molecules into oxygen and water. There are many different types of enzymes present in plants which degrade H_2O_2, still, catalases are unique because they do not need cellular reducing equivalent (Scandalios *et al*. 1997). Three catalase genes are identified in most angiosperm species, *e.g*., in tobacco Class I catalases are expressed in photosynthetic tissues and are controlled by irradiance; Class II catalases are expressed mostly in vascular tissues whereas Class III catalases are abundant in young seedlings and seeds (Willekens *et al*. 1995). In general, the importance of catalase can be either in its straight antioxidant activity or in its capability to trigger signal transduction pathways that require H_2O_2 as a messenger (Willekens *et al*. 1994a, Anderson *et al*. 1998). Earlier, an increased expression of wheat catalase in rice shows an enhanced tolerance to low temperature stress in transgenic rice (Matsumura *et al*. 2002). Catalase from *Suaeda salsa* provides resistance against oxidative stress in transgenic rice (Zhao and Zhang 2006).

A high temperature triggers a broad range of plant reactions along with an increase in ROS and up-regulation of their scavengers. Gene expression study in response to heat stress provides a definite appraise of antioxidant gene activation beside enzyme activity. Therefore, the aim of the present study was to characterize highly upregulated catalase genes identified in our heat stress suppression subtractive hybridization (SSH) library. In this study, we isolated and characterized two catalase genes *EcCATA1* and *EcCATB1* with respect to high-temperature treatment and also under other abiotic stresses to better utilize in the crop improvement.

Materials and methods

Plants and treatments: The finger millet [*Eleusine coracana* (L.) Gaertn.] cultivar MR1 was used in this study. Along with it, 16 other genotypes were also used for expression analysis of *EcCAT* genes under heat stress. Seeds of all the genotypes were sown in *Soilrite*. Seedlings were grown under a 24 ± 1 °C temperature, a 60 % relative humidity, a 16-h photoperiod, and an irradiance of 510 μmol m^{-2} s^{-1}. The seedlings (14-d-old) were exposed to 42 °C and collected after 2, 4, and 24 h of heat treatment. Seedlings grown at 24 ± 1 °C were used as a control. Three biological replicates were used for all the experiments. Further, MR1 seedlings were exposed to the following abiotic stresses: seedlings were placed in Petri plates having filter papers saturated with 10 cm^3 of 200 mM NaCl, 10 % (m/v) polyethylene glycol (PEG_{6000}), or 200 mM mannitol. For heat and cold treatments, seedlings were subjected to incubation at 42 °C and 4 °C, respectively. Samples were collected after different time intervals as described Chopperla *et al*. (2017). For each time point, around 10 seedlings were harvested, pooled, and frozen immediately in liquid nitrogen and placed at -80 °C until further study. For tissue expression analysis, leaves, roots, stems, panicles, and germinated seeds were collected along with whole seedlings.

Isolation of RNA and cDNA preparation: Total RNA was extracted from finger millet seedlings using a *Spectrum*™ plant total RNA kit (*Sigma Aldrich*, St. Louis, USA) according to the user manual. Genomic DNA contamination was eliminated using *On column* DNase digestion (*Sigma Aldrich*). Concentration and quality of total RNA preparation was determined with a spectrophotometer *ND-1000* (*Thermo Fisher Scientific*, San Jose, USA) and 1 % (m/v) agarose/formaldehyde gel electrophoresis. The cDNA was synthesized using 1 μg of total RNA isolated with a *High Capacity cDNA RT* kit (*Applied Biosystems*, Foster City, USA) according to the user manual. Random hexamer and oligo(dT) primers were used in the reverse transcription reactions. The resultant cDNAs were diluted 5-fold and from this dilution, 1 mm^3 was used as a template for quatitative polymerase chain (qPCR) reaction.

Sequence analysis: We designed full-length gene specific primers using a *Primer3* online tool (http://bioinfo.ut.ee/primer3-0.4.0/) to amplify *EcCAT* genes using heat treated cDNA (Table 1 Suppl.). After PCR, the amplified products were electrophoretically separated on an agarose gel (1.2 %) and eluted with an *QIAquick* gel extraction kit (*Qiagen*, Valencia, USA). The extracted products were cloned into a pGEM-T easy vector (*Promega*, Madison, USA). Positive colonies were screened through gene specific colony PCR and subjected to plasmid isolation. The *Eco*RI was used to release the insert, and these clones were confirmed by Sanger sequencing. As the information of finger millet catalase protein is unavailable, we calculated its physiochemical properties using a *ProtParam* tool of the *ExPASy* server (Wilkins *et al*. 1999). For multiple sequence alignment analysis, amino acid sequences of EcCAT proteins with other homolog catalases from different plant species were aligned using the *ClustalX2.1* software. A phylogenetic tree was generated using the *MEGA 7.0* software (http://www.megasoftware.net/).

Protein modeling and structure validation: The protein 3D structure of EcCAT proteins was modeled through the *I-TASSER* server (http://zhang.bioinformatics.ku.edu/I-TASSER/; Jianyi *et al*. 2015). Structural models were constructed using an appropriate Protein database bank through an iterative fragment assembly method followed

by a functional prediction with known proteins from the functional databases. The generated models were further assessed using the volume area dihedral angle reporter (*VADAR*; Willard *et al*. 2003) and the structure analysis and verification server (*SAVES*; http://nihserver.mbi.ucla.edu/SAVES/), which harbors different analysis tools like *PROCHECK* and *ProFunc* (Laskowski *et al*. 1993, 2005), *ERRAT* (Colovos and Yeates 1993) and *Verify3D* (Bowie *et al*. 1991). The *VADAR 1.5* program (http://vadar.wishartlab.com/) gives qualitative information on modeled protein, which includes mean hydrogen bond energies and hydrogen bonding distance, accessible surface area, steric quality, and excluded volume including solvation free energy. *ProSA* was used for the analysis of *Z* scores and energy plots (Sippl 1993).

Expression analysis: The qPCR was carried out with primers of *EcCATA1* and *EcCATB1* (Table 1 Suppl.) using the diluted cDNA as a template. The PCR was carried out with a 1× *SYBR* mix (*Stratagene*, California, USA) using *Light cycler 480II* (*Roche*, Indiana, USA) with preheating at 95 °C for 5 min proceeded with 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. A PCR amplification specificity was analyzed at the end of amplification by melt curve analysis. To normalize the quantity of cDNA in each reaction, the *EcUbiquitin* gene from finger millet was amplified as an internal reference gene. The amplified PCR products were analyzed on a 1 % (m/v) agarose gel to confirm an expected size. Three replicates were used for each cDNA sample, and the data were analyzed for relative fold expression as per Kanakachari *et al*. (2016).

Statistical analysis: For each experiment, three biological replicates, each containing at least three technical replicates, were used. Data sets were calculated as means ± SEs and one-way analysis of variance was used to determine the significance of differences between the samples. For comparison of different means, the Tukey honestly significat difference (HSD) test was used at $\alpha = 0.05$.

Results

Two cDNA clones of catalase, highly represented in the SSH cDNA library generated by us from heat treated finger millet seedlings, were used in this analysis. Gene-specific primers were designed and PCR was carried out. The products were further cloned and sequenced. On the basis of their homology with respective rice catalases, names of the genes were designated as *EcCATA1* and *EcCATB1*. Finally, both the sequences were submitted to *NCBI* (an accession number of *EcCATA1*: MH822444 and of *EcCATB1*: MH822445).

Physicochemical characterizations of EcCATA1 and EcCATB1 are extremely important to show properties like

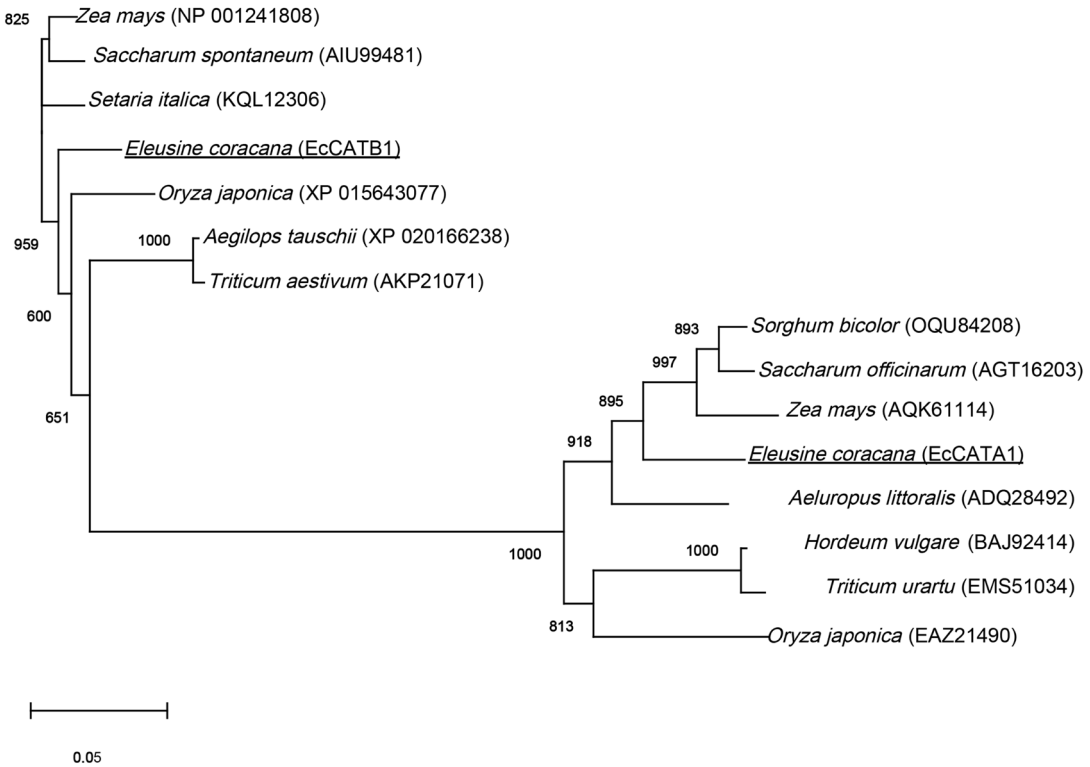

Fig. 1 Phylogenetic analysis of EcCATA1 and EcCATB1 with catalase proteins from some related species. All proteins were initially aligned using *ClustalW* and were then used for phylogenetic analysis using the *MEGA 7.0* software (http://www.megasoftware.net). A phylogenetic tree was constructed using the neighbor-joining method. Acession numbers generated in the study are underlined. The numbers on the individual node signify bootstrap values from 1000 replicates. A multiple alignment used for phylogeny of EcCATA1 and EcCATB1 is given in Fig. 1 Suppl.

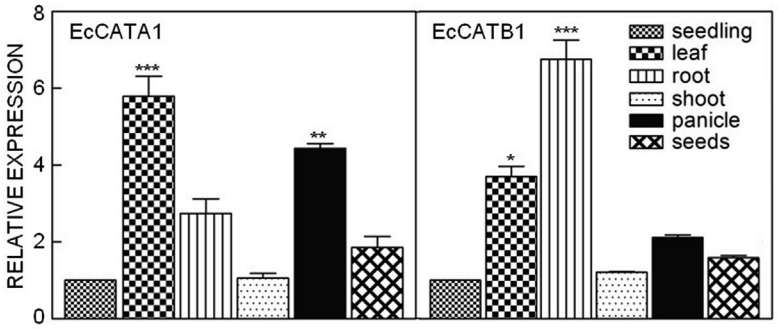

Fig. 2. Expression analysis of *EcCATA1* and *EcCATB1* in different tissues of finger millet determined by quantitative PCR analysis of 14-d-old seedlings. Means ± SEs, $n = 3$, *asterisks* indicate significant differences at $P < 0.05$ (the Student *t*-test).

the number of amino acid residues, theoretical isoelectric points, the number of atoms, coefficients of absorption, instability indices, molecular mass, aliphatic indices, atomic composition, and grand average of hydropathicity (GRAVY) (Table 2 Suppl.). The total numbers of amino acid residues in EcCATA1 and EcCATB1 were 493 and 472, respectively. Putative molecular masses of EcCATA1 and EcCATB1 determined *in silico* were 56.67 and 54.49 kDa, and isoelectric points 6.54 and 6.91, respectively. Similarly, predictive subcellular localizations of these proteins were examined. Analysis with the *WoLF PSORT* program indicates that EcCATA1 was localized in cytoplasm and EcCATB1 was localized in peroxisomes. Aliphatic indices for these catalases were 69.61 (EcCATA1) and 74.77 (EcCATB1) suggesting a higher thermostability of these proteins. Charges at pH 7 for EcCATA1 and EcCATB1 were -3.01 and -0.3, respectively, which means both proteins were acidic in nature. Stability indices for EcCATA1 and EcCATB1 were 35.16 and 27.83, respectively. As values of instability index for both proteins were lower than 40, these are stable proteins. The GRAVY value of EcCATA1 was -0.550 and of EcCATB1 -0.480. This lower GRAVY values show the establishment of a better interaction between the protein and water.

The two *EcCAT* genes had a significant sequence identity between themselves at the nucleic acid level (70 %) with a 75 % query coverage, and at the deduced amino acid level 70 % with 100 % query coverage. The pairwise sequence alignments of EcCATA1 and EcCATB1 protein sequences with other catalases show the presence of conserved active site residues (Fig. 1 Suppl.). The *BLASTP* searches against the *GenBank* database indicate that EcCATA1 shared a 93 % similarity with catalase from *Sorghum bicolor* (accession number OQU_84207), 92 % with *Saccharum* cultivars (accession number AGT_16203), 91 % with *Aeluropus littoralis* (accession number ADQ_28492), and 90 % with *Zea mays* (accession number AQK_61114). The EcCATB1 shared a 92 % similarity with *Setaria italica* (accession number KQL_12306), *Zea mays* (accession number NP_001241808), and *Sacchrum spontaneum* (accession number AIU_99481). When a phylogenetic tree was prepared to compare the *EcCAT* genes with foxtail millet, sugarcane, rice, maize, and wheat catalase genes, two clear clusters of EcCATA1 and EcCATB1 were observed (Fig. 1). Furthermore, a conserved domain database was used for identification of conserved domains in both the proteins. The EcCATA1 protein contained four domains: *1*) catalase (18 - 398 amino acids), *2*) catalase-rel (catalase-related immuno-responsive; 78 - 114 amino acids), *3*) tRNA (Ile) lysidine synthetase - Tils (Tils substrate binding domain; 440 - 484 amino acids), and *4*) histone-like nucleoid structuring dependent expression A (425 - 487 amino acids) whereas EcCATB1 contained only two domains: *1*) catalase (18 - 386 amino acids) and *2*) catalase-rel (403 - 466 amino acids). A detailed description of the different domains and the structures of EcCATA1 and EcCATB1 are shown in Fig. 1 Suppl.

A graphical representation of the percentages of sheets, helices, strands, and turns of both EcCATA1 and EcCATB1 is given in Fig. 2 Suppl. EcCATA1 showed 43 β-turns compared with 47 in EcCATB1. EcCATA1 had 3 β-sheets, 12 strands, 18 γ-turns, and 20 helices whereas EcCATB1 had 2 sheets, 11 strands, 13-γ turns, and 18 helices identified by *ProFunc*. The alignment of EcCATA1 and EcCATB1 sequences with selected templates having different query coverage scores, identity scores, and Z-scores were predicted in Figs. 3 and 4 Suppl., respectively. Out of three models predicted, one best model of EcCATA1 (C-Score = 1.79) and EcCATB1 (C-Score = 1.89) (Fig. 3 and 4 Suppl.) were selected to validate the structure and further consideration. The Ramachandran plot was generated *via* the *PROCHECK* server to calculate the existence of residues present in different regions. The allowed regions for molecular fragment replacement of EcCATA1 and EcCATB1 demonstrated 76.8 and 78.0 % residues, respectively whereas 2.1 and 0.5 % residues were presented in disallowed regions (Table 3 and 4 Suppl.). Evaluation of EcCATA1 and EcCATB1 with *ProSA-Web* revealed the Z-score values as -8.52 and -8.12, respectively, which suggests that both structures had native features and near to a crystal structure (Fig. 3 and 4 Suppl.). *ERRAT* calculated overall quality factor scores for non-bonded atomic interaction and scored to the tune of 87.474 and 92.358 in EcCATA1 and EcCATB1, respectively. The mean residue volume of the EcCATA1 and EcCATB1 models were 1.414 and 1.426 μm, respectively, which shows a good packing density of these proteins.

Results of qPCR with gene-specific primers shows a relative transcript abundance of *EcCATA1* in finger millet

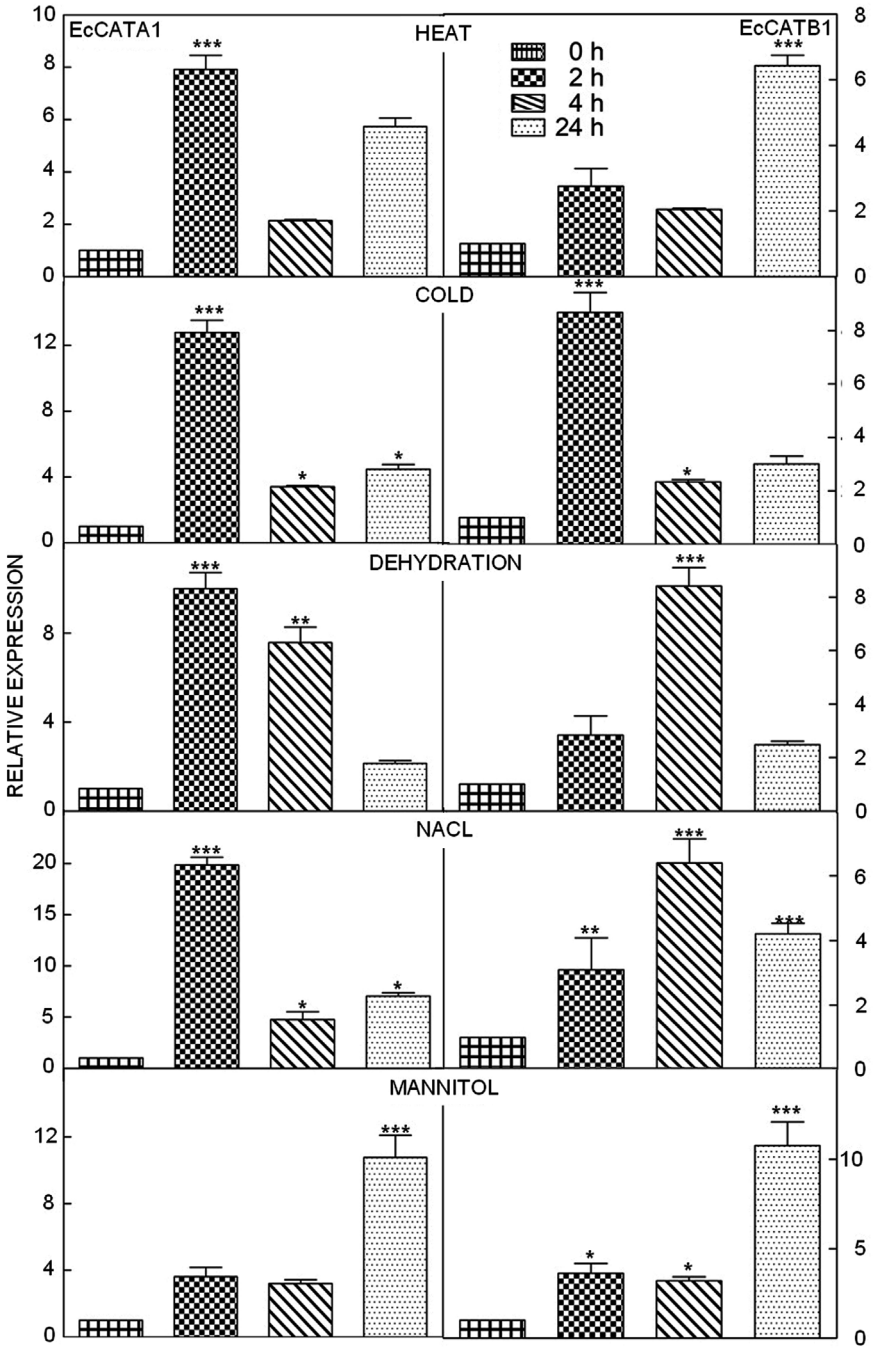

Fig. 3. Expressions of *EcCATA1* and *EcCATB1* determined by quantitative PCR in finger millet under different abiotic stresses in 14-d-old seedlings after 2, 4, and 24 h. Means ± SEs, $n = 3$, *asterisks* indicate significant differences at $P < 0.05$ (the Student *t*-test).

leaves and panicles whereas in the case of *EcCATB1*, a maximum relative expression was observed in roots followed by leaves (Fig. 2). Under the abiotic stresses, the maximum expression of *EcCATA1* was observed under NaCl followed by cold, PEG, and heat at 2 h (Fig. 3). Only under the mannitol stress, the maximum abundance was observed after 24 h. For *EcCATB1* a relative abundance of the transcript increased after 2 h of the cold stress, after 4 h of the PEG and NaCl stresses and after 24 h under the mannitol and heat stresses (Fig. 3).

Expression analysis of *EcCATA1* and *EcCATB1* in 16 finger millet genotypes was carried out using real-time PCR. Expression of *EcCATA1* changed from 0.28- to 10-folds under the heat stress in the studied cultivars (Fig. 4). The results show that expression of *EcCATA1* increased mostly at 2 h under the stress conditions in all the cultivars. Along with up-regulation at 2 h, the maximum expression of *EcCATA1* was observed at 24 h in the PES110 and PR202 genotypes whereas the lowest expression was observed in KOPN330 and GN5. Transcription of *EcCATB1* changed from 0.18- to 8-folds in the studied cultivars (Fig. 5). The results show that expression of *EcCATB1* was maximum

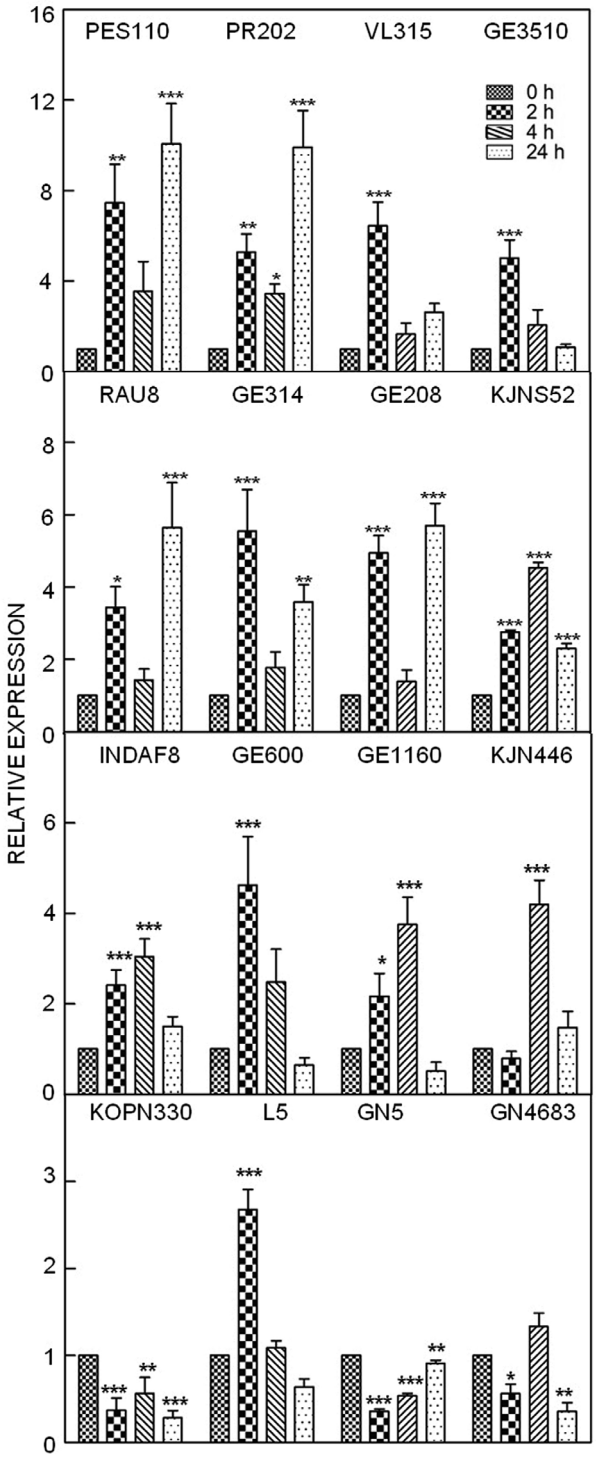

Fig. 4. Expression of *EcCATA1* determined by quantitative PCR analysis in 16 finger millet genotypes after heat stress at 42 °C for different time periods in 14-d-old seedlings. Means ± SEs, $n = 3$, *asterisks* indicate significant differences at $P < 0.05$ (the Student *t*-test).

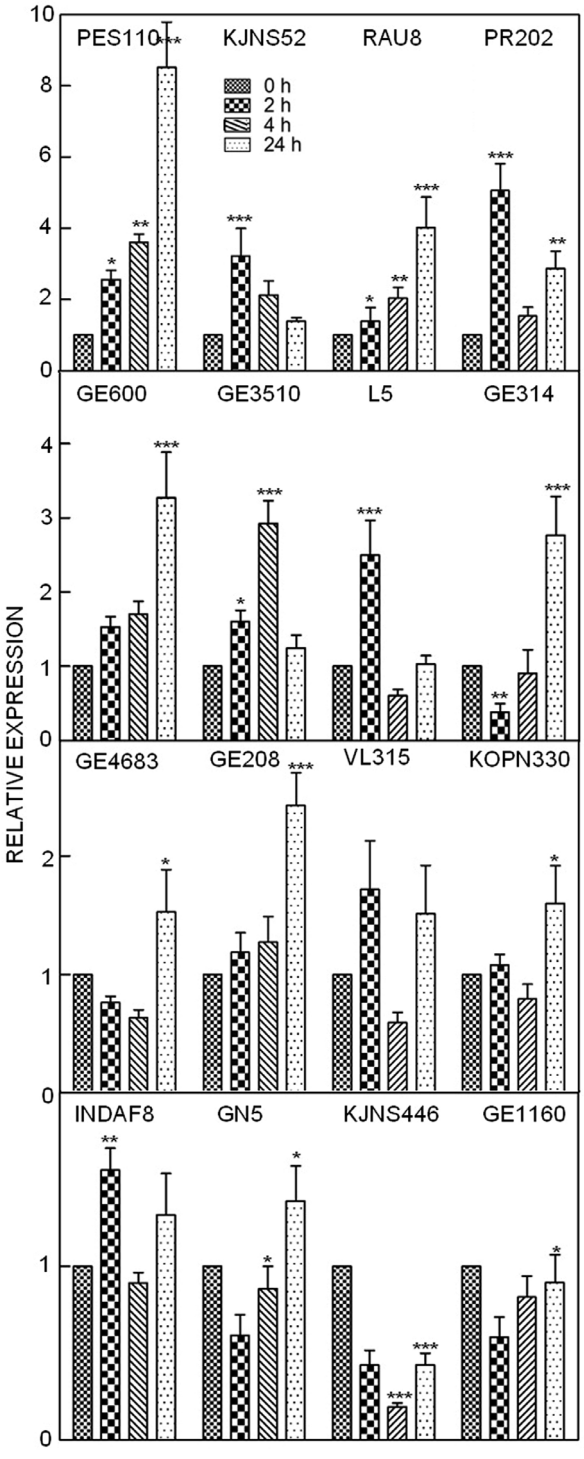

Fig. 5. Expression of *EcCATB1* determined by quantitative PCR analysis in 16 finger millet genotypes after heat stress at 42 °C for different time periods in 14- d-old seedlings. Means ± SEs, $n = 3$, *asterisks* indicate significant differences at $P < 0.05$ (the Student *t*-test).

at 24 h in most of the genotypes. In few genotypes, the maximum expression was observed at 2 h of the heat stress. The highest expression was detected in the PES110 genotype with an 8.5-fold increase at 24 h whereas the least expression was observed in the genotype KJNS446 at 4 h of the heat stress.

Discussion

The principal propose of this study was to isolate and characterize the essential ROS scavenging catalase genes (*EcCATA1* and *EcCATB1*) from finger millet and to analyze the expression pattern of these genes under different abiotic stresses and plant developmental stages to confirm their role in abiotic stress tolerance of finger millet. We obtained two full-length catalase genes from the SSH library prepared from the heat-treated finger millet seedlings. We observed that catalase members of finger millet contained distinctive domains specific to the catalase and catalase immune responsive family (Cat-rel), which is the major class of ROS scavenging antioxidants (Mhamdi *et al*. 2010). Based on the crystal structure of catalase, a successful 3D structural model was constructed for both the EcCATA1 and EcCATB1 proteins by using homology modeling method. In our study, with the aid of molecular dynamics and mechanics methods, we achieved the final protein model of EcCATA1 and EcCATB1, and further these models were evaluated by standard programs, like *SAVES* and *VADAR*, which proved that the final refined protein models are reliable.

The expression analysis revealed that *EcCATA1* was abundant in leaves as compared with panicles and roots whereas negligible in shoots and seeds. *EcCATB1* was equally expressed in panicles, shoots, and seeds whereas the maximum expression was observed in roots and leaves. Similarly, a higher expression of the *CAT2* gene was observed in root tissue of cucurbit species under heat stress (Ara *et al*. 2013). Several previous studies are in agreement with our findings that both *EcCATA1* and *EcCATB1* mRNAs are highly abundant in leaves (Willekens *et al*. 1994b, Esaka *et al*. 1997). Therefore, it is indicative that *EcCATA1* and *EcCATB1* are involved in scavenging photorespiratory H_2O_2 as suggested by Willekens *et al*. (1994b).

Catalase genes are differently expressed not only in developmental stages but also are responsive to numerous environmental signals, *e.g.*, in brocoli, rice, and chickpea (Lin *et al*. 2010, Nahakpam and Shah 2011, Kaur *et al*. 2013). The activity and expression of catalases also trigger in various stress conditions, for example at NaCl, drought, chilling, osmotic stress, UV-B, O_3, and SO_2 (Mittler *et al*. 1994, Willekens *et al*. 1994a, Guan and Scandalios 2000, Luna *et al*. 2005, Yong *et al*. 2017). Similarly, in the present study, both *EcCATA1* and *EcCATB1* showed a high mRNA abundance under the NaCl, mannitol, dehydration, and cold stresses. Several studies reported expression of catalase genes in response to heat stress in maize, *Arabidopsis*, tobacco, mustard, and cabbage (Willekens *et al*. 1994a, McClung 1997, Scandalios *et al*. 1997, 2000, Dat *et al*. 1998, Lin *et al*. 2010). Herein, we report the response of the finger millet catalase gene expression to heat stress and show the differential expression profile: an initial expression of *EcCATA1* restored within 2 h of the stress, further reduction was observed at 4 h, and again expression increased at 24 h in the heat tolerant genotypes. In contrast, *EcCATA1* expression did not increase in the susceptible genotypes, or in some genotypes, it increased only slightly. This may be a result of changes in photorespiration rate, which has been noticed at temperature stress (Berry and Bjorkman 1980). It is possible that the initial heat shock led to a rapid decline in *EcCATB1* transcripts in the heat sensitive finger millet genotypes similarly to *N. plumbaginifolia Cat2* transcripts, which decreases during heat stress (Willekens *et al*. 1994b). This is the first study of isolation and characterization of finger millet catalase genes under different abiotic stress conditions.

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