Isolation and characterization of drought responsive EcDehydrin7 gene from finger millet (Eleusine coracana (L.) Gaertn.)

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Abstract

Finger millet is known for its drought tolerance ability. The present investigation was carried out to explore drought responsive genes from finger millet. To generate more genetic and genomic information, a highly normalized cDNA library was prepared from 12 days old stress imposed seedlings and utilized it for identification of drought responsive genes using cDNA macroarray. Differentially expressing six genes (Dehydrin7, Hypothetical protein1, Chloroplast envelope membrane protein, Hypothetical protein2, S-adenosyl methionine decarboxylase2 and unknown gene) were selected for qRT-PCR analysis under different stress conditions. Gene EcDehydrin7 (Accession No. KM096446) showed high expression under drought and heat stress. Functional validation of this gene can provide more information about drought tolerance mechanism in crop plants.

Key words: Finger millet, cDNA, EcDehydrin7, drought and heat stress

Introduction

Due to ever increasing urbanization, industrialization and desertification, feeding burgeoning population of billions with ever shrinking non renewal resources, poses huge challenge to come up with expectation. So, to manage the effect of desertification and drought, it is centrally importance to promote the effective and efficient use of plant genetic resource. An abiotic stress remains the big threat to crop production. Globally, it has been estimated that approximately 70 % of yield reduction is due to cause of abiotic stresses [1]. It hampers plant growth and productivity by inhibiting photosynthesis [2].

Finger millet (Eleusine coracana (L.) Gaertn.) is a hardy cereal known for its great level of tolerance against drought, salinity and diseases. Finger millet crop is tagged as orphan or understudied crop because of its meager genetic and genomic resource available till date due to negligence of research, preference of particular food, less interest in scientific community and government policies [3-5]. It is necessary to dissect the transcriptome information under different stress conditions to prospect novel genes. Hence, in our study we developed normalized library to get the maximum number of transcript information of finger millet under different abiotic stresses.

Several approaches have been reported to generate the normalized cDNA libraries. Broadly two approaches based on physical isolation of all transcripts and denaturation-hybridization of double-stranded cDNA molecules has been fully utilized [6]. Based on the experimental condition every approach has its own weakness and strength. Problems associated with physical isolation method for normalization are as it is complex procedure, requires large amount of starting material, escape of rare transcripts, non reproducible and non reliable [7-11]. PCR base suppression and selective amplification of normalized cDNA library is used for fragmented cDNA libraries preparation and this approach too is having weakness to prevent the loss of rare transcripts [12]. Hybridization based normalization by using specific enzyme is the most simple, reproducible and robust method for normalization. In current scenario, a new enzyme-duplex-specific nuclease (DSN) based
normalization method is reported with their simple and effective means to get full-length-enriched normalized cDNA prior to library cloning [13-17]. Construction of normalized cDNA library and their confirmation by dot blot hybridization followed sequencing has been carried out [18].

DNA macroarray can be a useful tool for simultaneously monitoring the mRNA expression of different samples under different conditions. Differential expression profile of 830 unigenes in drought stress were reported using macro-array (dot blots) followed by validation of few selected genes by northern blotting and quantitative real-time PCR assay [19-22].

In the present study, we prepared normalized cDNA library of finger millet under different abiotic stresses. Expressions of different transcripts were analyzed using dot blot and real time PCR. Results confirmed that EcDehydrin7 (EcDHN7) is highly expressing under drought and heat stress conditions. This transcript information under abiotic stress conditions warrants further studies to validate functionally by developing transgenics.

Material and methods

Plant sample, growth environments and types of stress imposed

Finger millet cv MR1 was chosen as an experimental material for analysis. Seeds were obtained from the University of Agricultural Science, Bangalore, India. Seeds were washed thrice with sterilized double distilled water and were kept on tissue paper to remove the excess moisture for 10 minutes. Seeds were sown in pots filled with autoclaved composite soilrite and kept in tissue culture growth chamber with a photoperiod of 16 h light/ 8 h dark, temperatures at 28°C and with relative humidity of 80%. Normally grown 12 days old seedling were harvested and treated as control.

Seven different kinds of stress treatment were imposed on normally grown seedlings. For drought stress, water was withdrawn from pots on 4th day up to 11th day and samples were harvested on 12th days. Heat stress, normally grown seedlings were kept in oven at 42°C for 8 hour and kept at room temperature for one hour recovery and samples were harvested. Drought and heat stress, drought treated sample was kept at 42°C for 1 hour and then kept at room temperature for one hour recovery and samples were harvested. For, salt stress, normally grown seedlings were irrigated from 4th to 11th days after germination with 200 mM NaCl solution and samples were harvested on 12th days. For cold stress, normally grown seedlings were incubated in growth chamber at 4°C for overnight and kept at room temperature for 2 hours recovery and samples were harvested. For UV stress, normally grown seedlings were incubated under UV (UV C) light for 1 hour and allowed to recuperate for 1 hour at natural light and samples were harvested. Wound stress, normally grown seedlings were injured by tearing and crushing with forcep and needle and seedlings were permitted recovery of 1 hour and samples were harvested. While harvesting, stress forced samples were frozen in liquid nitrogen and finally kept at -70°C for further use.

RNA isolation, quality measures and cDNA synthesis

Samples were taken from –70°C and ground to fine powder using pre-chilled mortar and pestle using liquid nitrogen. Total RNA was isolated by Spectrum™ Plant Total RNA kit (Sigma, USA) and then sample were treated with DNase I (Sigma, USA) as per the manufacturer's instructions to get rid of genomic DNA traces. The quality and quantity was monitored on 1.2% denatured agarose gel and Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA). All samples were mix equally (1 µg) to make a pool prior to cDNA synthesis. 3 µg of pooled total RNA was reverse transcribed using the SMART cDNA synthesis kit (Clontech, USA). Resulted cDNA was used for normalization.

Normalization of cDNA

Normalization of cDNA was done by using TRIMMER cDNA Normalization Kit (Evrogen). cDNA was denatured and allowed to reassociate and treated with Duplex Specific Nuclease (DSN). Degradation of double stranded fraction formed during reassociation of cDNA occurred to equalization of ss cDNA fraction. First PCR amplification was done to amplify the normalized ss_DNA fraction. Second PCR amplification was done to regulate the average length of the PCR product. Finally normalization efficiency test was done prior to cDNA library preparation. Normalized cDNA were cloned in pGEM-T Easy vector. Transformation was done via electroporation (Bio-Rad USA). Colony PCR and sequencing of 96 randomly selected positive clones were carried out to check the efficiency of normalized cDNA library.
Preparation of Dot Blot or cDNA macroarray

Colony PCR of 2500 randomly picked clones was done using T7 and SP6 primers. Out of 2500 clones, 1000 PCR clones showing intact single band were selected for cDNA macroarray preparation. PCR was done for finally differential selected positive clones. Two replicas were prepared by spotting 3 µl (1.2µg)/spot purified PCR product with the help dot-blot apparatus in 96 formats (Biometra, Dot blot 96) assisted by vacuum pump (Today’s Rocker 300). Blots were treated in denaturation solution (1M NaCl, 0.5 M NaOH) for 5 minutes followed by neutralization solution (1.5 M NaCl, 0.5 M Tris HCl pH 7) for 5 minutes. Blots were allowed to dry at room temperature and finally UV cross-linking was done.

Preparation of specific probe, hybridization and screening of differential expressed genes

Probes were prepared from control and drought treated RNA samples. Probe labeling was done during first-strand cDNA synthesis. Total RNA (4 µg) was used for reverse transcribing, using AffinityScript QPCR cDNA Synthesis Kit (Stratagene, Agilent Technologies, USA) in the presence of γ-[32P] dCTP. The blots membranes were prehybridized in hybridization buffer at 42°C for 6 hours. The denatured probe was added, and allowed hybridization for 18 hours. Blots were washed and exposed to X-ray film (Kodak) and film cassettes were incubated at –70°C for 7 days and finally films were developed. On the basis of intensity of actin1a gene blots were normalized and other transcripts were analyzed for their differential expression.

Expression profile of selected genes in Finger millet

Real-time amplification reactions were performed using SYBR Green detection chemistry and run on 96-wells plates with the MX-3000 Real time PCR (Stratagene). Two biological replicates for each of the 8 samples of finger millet were used for real-time PCR analysis, and three technical replicates were analyzed for each biological replicate. A no-template control (NTC) was also included in each run for each gene. Each reaction contained 1 µl 4-fold diluted cDNA template, 0.4µm of each primer, and 1× SYBR Green Master Mix (USB Stratagene), in a final volume of 25 µl was subjected to the following conditions: 50°C for 3 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s in 96-well optical reaction plates (Bio-Rad, Foster City, CA, USA). The melting curves were analyzed at 60-95°C after 40 cycles. The close range of efficiencies between the targets and controls allowed for a ∆∆CT analysis using Actin 1a as calibrator. Error was calculated as Standard Error of the Mean (SEM).

Results and discussion

Number of genes express in eukaryotic cells and their corresponding transcripts abundance varies by several magnitudes. As for as cellular transcriptome is concern, the number of mRNA copies per gene may varies by several orders. The cDNA library proceed from the non normalized will have unnecessary sequencing burden, difficulty in isolation of rarely expressed gene and unfit for functional screening. Normalization method allowed equalizing copies number of genes in library which may provide opportunity to identify and clone gene(s) transcribing even at relatively low level or for purposefully screenings [23].

cDNA normalization using Duplex Specific Nuclease (DSN) treatment

There was no much marked phenotypic difference in stress treated samples except drought and heat samples than the control plant (Fig. 1A). Separately total RNA was isolated prior to make pooled total RNA (Fig. 1B). cDNA was prepared from pooled total RNA (Fig. 2A). This experiment tells the clue regarding exact dilution of enzyme to be used in the normalization of cDNA library. Here, ours results showed DSN1 and DSN1/4 cDNA samples were over and under digested, respectively. So, were unfit for normalization. The DSN1/2 cDNA sample showed optimum and best dilution of DSN enzyme, and therefore was used in the normalization of cDNA library. Here, ours results showed DSN1 and DSN1/4 cDNA samples were over and under digested, respectively. So, were unfit for normalization. The DSN1/2 cDNA sample showed optimum and best dilution of DSN enzyme, and therefore was used in the normalization of cDNA library. Here, ours results showed DSN1 and DSN1/4 cDNA samples were over and under digested, respectively. So, were unfit for normalization. The DSN1/2 cDNA sample showed optimum and best dilution of DSN enzyme, and therefore was used in the normalization of cDNA library. Here, ours results showed DSN1 and DSN1/4 cDNA samples were over and under digested, respectively. So, were unfit for normalization. The DSN1/2 cDNA sample showed optimum and best dilution of DSN enzyme, and therefore was used in the normalization of cDNA library. Here, ours results showed DSN1 and DSN1/4 cDNA samples were over and under digested, respectively. So, were unfit for normalization. The DSN1/2 cDNA sample showed optimum and best dilution of DSN enzyme, and therefore was used in the normalization of cDNA library. Here, ours results showed DSN1 and DSN1/4 cDNA samples were over and under digested, respectively. So, were unfit for normalization. The DSN1/2 cDNA sample showed optimum and best dilution of DSN enzyme, and therefore was used in the normalization of cDNA library. Here, ours results showed DSN1 and DSN1/4 cDNA samples were over and under digested, respectively. So, were unfit for normalization. The DSN1/2 cDNA sample showed optimum and best dilution of DSN enzyme, and therefore was used in the normalization of cDNA library.
construction. Result showed that DSN1/2 is optimal and best normalized cDNA under studied samples. Products of DSN1 and DSN1/4 of cDNA showed incomplete normalization (Fig. 2D). Normalization efficiency was calculated based on the results obtained by colony PCR and sequencing of selected clones (Fig. 3A, B). Results showed a highly non redundant library with normalization efficiency of 97.6 %.

Fig. 1. (A) Different stress imposed 12 days old ragi seedlings. (B) Total RNA from stressed samples

Fig. 2. cDNA normalization. (A) cDNA from pooled total RNA. (B) DSN treatment. lane 2 DSN1, lane 3 DSN1/2, lane 4 DSN1/4. DSN1/2 showed best dilution of enzyme. (C) Selection of optimal number of cycles, 12 cycles taken optimal for secondary PCR (D) Lane 2, control. Lane 3-5 showed normalized product in different dilution, DSN1/2 showed best normalized product

(A) Library clones in Blue/white screening plate (B) Colony PCR of positive clones

Screening and selection of differential expressed genes from dot blot

1000 colonies were picked randomly for PCR amplification. PCR products after confirmation were blotted on macroarray and total 10 blots with two replicates were prepared. One replicate was used for hybridization against drought specific probe and another one against control sample. On the basis of intensity of actin1a gene blots were normalized and other transcripts were analyzed for their differential
expression. Out of these, 6 differentially up regulated genes were selected for qRT-PCR analysis (Fig. 4). These genes were EcDehydrin7 (EcDHN7), Hypothetical protein1, Chloroplast envelope membrane protein, Hypothetical protein2, S-adenosyl methionine decarboxylase and Novel gene. Further full length EcDehydrin7 gene was cloned, sequenced and submitted to NCBI (Accession No. KM096446).

**Relative expression profile of selected genes by qRT PCR**

Differentially up and down regulated genes were characterized by quantitative real time PCR (qRT PCR). Bar graph shows the relative expression of genes compare to control sample. EcDehydrin7 (EcDHN7), Hypothetical protein1, Chloroplast envelope membrane protein, Hypothetical protein2, S-adenosyl methionine decarboxylase2 and Novel gene showed differential expression under different stress condition (Fig. 5). Dehydrin or RAB family proteins are small group of GTP-binding proteins play role in intracellular trafficking. They also play critical roles in several important plant development processes. Plants having a unique endosomal trafficking network provide the first report of a functional link between a specific RAB and a specific SNARE complex [24, 25]. The chloroplast envelope related protein, caleosin isoform (Clo-3), is having high and up regulated expression upon exposure to abiotic stresses, like salt and drought, and to biotic stress such as pathogen infection. It has been reported that Clo-3 is part of an oxylipin pathway, expressed by multiple stresses and may also produced fatty acid derive anti-fungal compounds which strengthening plant defense [26]. In Arabidopsis, constitutively expressed S-adenosylmethionine decarboxylase gene showed increases in drought tolerance by inhibiting the ROS-induced transcription of the metacaspase II [27, 28]. Blastx results of three genes showed two are hypothetical protein and one is unknown protein.

**Fig. 4. Differential screening results.** Blots showed relative expression of genes, hybridized with normal and drought specific probes, EcDehydrin7 (EcDHN7) (E10 spot). Actin1a and water are used as a control.

**Fig. 5. Quantitative expression of genes in response to a abiotic stresses.** (A) EcDehydrin7 (DHN7) (B) Hypothetical protein1 (C) Chloroplast envelope membrane protein (D) Hypothetical protein2 (E) S-adenosyl methionine decarboxylase2 (F) Novel gene. Accumulation of genes transcripts were determined by qRT-PCR with 1 µg of total RNA. The Actin (1a) gene was used as normalizer.
The *EcDehydrin7* showed more relative expression in drought, drought+heat and heat compare to control sample. This gene expression in cold, salt and UV is down regulated (Fig. 5A). The *hypothetical protein1* showed more expression in drought condition compare to control (Fig. 5B). The expression of *Chloroplast envelope membrane protein* was more in heat stress than control (Fig. 5C). The *hypothetical protein2* exhibited more expression in drought, drought+heat and heat stress condition compare to control (Fig. 5D). Under heat stress, the expression of *S-adenosyl methionine decarboxylase2* was noted to be higher than the expression level in non-stressed plants (Fig. 5E). Unknown/novel gene showed more expression in drought condition compare to control (Fig. 5F).

In conclusions we have generated normalized cDNA library from finger millet seedlings exposed to different abiotic stress conditions to maximize representation of each transcripts. Further using dot blot analysis, we identified drought responsive genes. Among these genes *EcDehydrin7* (*EcDHN7*) showed high expression under drought and heat stress and may proved to be a potential candidate gene after validation to develop drought resistant crops.

References


