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BIOTECHNOLOGY

Expression of ECMYB Transcription Factor Gene Under Different Abiotic Stress Conditions in *Eleusine coracana*

Priya Jadhav, Prafull Salvi, Megha Bhatt and Pushpa Lohani*

Department of Molecular Biology & Genetic Engineering, GB Pant University of Agriculture & Technology, Pantnagar, Uttarakhand, India

*Corresponding author: pushpa.lohani@yahoo.com (**ORCID ID:** 0000-0002-2425-190X)

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ABSTRACT

Plants are exposed to various abiotic stress conditions during their lifespan. Abiotic stresses such as drought, salinity, extreme temperature, ROS etc. affect crop yield to great extent. Global warming may worsen the situation in the years to come in most agricultural regions. Therefore, it is critical to understand the mechanisms that enable plants to cope with water deficit. Strategies involving genetic engineering show great promise. Coordinated expression of stress responsive genes is very important for the survival of plant under stress conditions and the regulation is brought about by transcription factors (TF). Myb is a family of transcription factor comprising of a few drought responsive TF. The expression of the TF may be regulated with the onset of drought or other abiotic stress conditions. *Eleusine coracana* being a rainfed crop could be a good source to fish out drought responsive myb gene. The study was carried out to demonstrate the expression of Ecmyb gene in sensitive (PES-400) and tolerant (PRM6107) genotype of *E. coracana* under drought, cold, ROS and salt stress. Drought stress was provided by withholding water for 11 days whereas cold stress was provided by incubating the plants in BOD incubator at 4^0C for different time periods. Similarly salt stress was given by watering the plants with sodium chloride and ROS was created by spraying paraquat on the plants. RT-PCR was carried out to study the expression of Ecmyb gene in different stress conditions. The gene was expressed in the tolerant genotype in all the stress conditions except cold. However, no expression was observed in sensitive genotype under stress condition. Both the sensitive as well as tolerant genotypes did not show Ecmyb gene expression under unstressed condition. The study concludes that the expression of Ecmyb gene was induced with the onset of drought, ROS and salt stress. Cold stress had no effect on the expression of the gene. The transcript was sequenced and submitted to NCBI database (Accession No. JN107890). In-silico analysis showed maximum similarity with drought responsive genes of rice and maize. Future prospects include full length cloning and functional validation of the gene.

Highlights

- The expression of Ecmyb gene was observed in drought tolerant genotype only.
- The expression of Ecmyb gene took place under drought and other abiotic stress conditions like, salinity and ROS. Cold stress did not induce the gene expression.
- The expression of Ecmyb gene may be related to drought (abiotic stress) tolerance.

Keywords: myb gene, transcription factor, drought, salinity, ROS, *Eleusine coracana*

Plants are exposed to various biotic and abiotic stresses during their lifecycle which adversely affect their growth, development and productivity. Abiotic stresses such as drought, Golldack (2014), salinity Gupta (2014), ROS (reactive oxygen species) Luis (2105), metal toxicity, nutritional deficiency, cold, and heat stresses have major impacts on crop productivity. Among them, drought is the major abiotic stress, causing significant reduction of crop yields worldwide (Boyer 1982; Bray *et al*. 2000), Aiguo. (2011) The population of the world is increasing exponentially and it is the challenge to feed the growing population

with shrinking agricultural land. Our ability to meet the challenge rests on our ability to produce adequate crop yields in less than ideal environments. Therefore, there is an urgent need of bio-prospecting of genes and germplasms that is tolerant to abiotic stresses.

Finger millet is a hardy crop and can be grown in arid regions where rainfall is less. It grows best in moist climates in almost any type of soil Baker *et al.* 2004, Das (2014). It is rich in minerals and vitamins and has high fiber content in its seed, which are generally less or deficient in the Indian diet. It is also a good source of many essential amino acids. Due to its high nutritious quality and its ability to cope up with abiotic stress conditions, finger millet can be considered a good source to fish out genes that may provide tolerance to abiotic stress in plants. Breeding of crop plants for various abiotic stresses have shown promising results but are still limiting and have been shown to have inverse relationship with yield. Molecular approaches for engineering plants against various abiotic stresses have sprung up as an important tool in this regard. Stress tolerance mechanism is a complex process that triggers an array of physiological and biochemical processes in plants. Many signaling pathways are involved simultaneously that regulate the response and there is an overlap between the patterns of expression of the genes induced in response to different stress factors (Seki *et al*. 2001), Parihar (2015), Bartwal (2013). Thus, the focus is shifted in isolating genes specifically transcription factor genes that work as master regulator and regulate expression of a whole range of transcripts expressed to overcome the deleterious effects of stress conditions.

Transcription factors (TFs) are master regulators that control expression of gene clusters. A single transcription factor can control the expression of many target genes through specific binding of the TF to the *cis* – acting element in the promoters of respective genes. This type of transcriptional regulatory system is called regulon. The regulons like the NAC, MYB (myeloblastosis), bZIP (basic leucine zipper) and DREB (dehydration responsive element binding proteins) etc are shown to be involved in abiotic stress –responsive gene expression. The MYB family represents one of the large, functionally diverse classes of proteins, found in all eukaryotes. In general, most of the MYB proteins function as transcription factors and are characterized by the presence of variable numbers of N-terminus conserved MYB repeats (R), mainly associated with DNA-binding and protein-protein interactions. The variable C-terminal region is responsible for modulating the regulatory activity of the protein. Several members of this family have been identified in Arabidopsis, rice, maize, and soybean and shown to be involved in regulating various cellular processes, including cell cycle and cell morphogenesis, biotic and abiotic stress responses (Jin and Martin 1999). Over expression of various Myb transcription factors like Myb15, AtMyb41 etc confer improved tolerance to drought and salt stresses (Ding *et al.* 2009; Lippold *et al.* 2009).

The present study was carried to study the expression of myb gene in *Eleucine coracana* under influence of various abiotic stress like drought, salinity, cold and ROS. Expression profiling of the myb gene in tolerant and sensitive genotype of finger millet could be a promising strategy for developing stress tolerant genotypes of important commercial crops.

MATERIALS AND METHODS

In the present study, seeds of two genotypes of finger millet namely PES -400 (drought sensitive) & PRM -6107 (drought resistant) were used which were obtained from MBGE department, GBPUAT Pantnagar. Seeds of both the genotypes of finger millet were sown in trays filled with soil, peat moss and vermiculite in 3:1:1 proportion. Small plants of nearly 10 cm height were transferred to pots in polyhouse.

Abiotic Stress Treatment

Forty five days old plants having nearly same height of both the genotypes of finger millet i.e. PRM -6107 and PES- 400 were kept in BOD incubator at 4°C for 24 h, 48 h and 72 h for providing cold stress. Similarly, for providing salinity stress, Forty five days old plants having nearly same height of both the genotypes of finger millet i.e. PRM -6107 and PES- 400 were watered with 100 mM, 200 mM and 300 mM NaCl solution. Drought stress was imposed on both the genotypes of finger millet of same age and height by withholding watering for 11 days. Oxidative stress was created by spraying the finger millet genotypes with solution of 10 μM, 20 μM and 30 μM paraquat and sample were harvested after 7 daysAppropriate controls were taken for each

treatment. All the experiments were performed in triplicate and results were recorded based on the mean value of the observations.

Morpho-Physiological Studies

Plant Height: A meter scale was used to record plant height from soil to the base of flag leaf of each genotype. Measurement was performed before and after stress treatment for control and stress treated plant. Plant height was expressed in centimeters.

Root Length: Plants from both control as well as stress treatment were uprooted and length of the longest root was measured using a meter scale. Root length was also expressed in centimet.

Photosynthetic Rate: Photosynthetic rate was measured by Infra Red Gas Analyser (TPS-2PP system, USA) in the morning time between 10:00am to 11:00am. Measurements were taken before and after stress treatment. It is a unit less quantitity.

Relative Water Content: The relative water content of the leaves was estimated following the method of Weatherly (1950). Leaves of both genotypes under stress condition as well as control were taken, washed with distilled water, blotted for removal of the surface water and fresh weight was recorded. The leaves were then immersed in distilled water for 4 hours, blotted again and the turgid weights were taken. For dry weight measurement the same leaves were oven dried at 80 °C till completely dried.

Relative water content was calculated according to the formula:

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

Primer Designing: Since no information was available regarding Myb genes or other abiotic responsive transcription factor of finger millets at National Centre for Biotechnological Information (NCBI), so sequences were downloaded from other crops like *Zea mays, Oryza sativa, Sorghum bicolor, Triticum asetivum and Hordeum vulgare* whose sequences were available at NCBI. These sequences were aligned using off-line bioinformatics tool CLUSTALX2. Based on homology and conserved region, different degree of degenerate primers were designed using the online tool primer 3. Synthesis of primers was done by Sigma.

Table 1: List of degenerate primers

Code	Primer sequence (5′-3′)	Tm(°C)
Myb2L	5′ CTCTGGAACTCGTGCATCAA 3′	59.98
Myb2R	5′ TGTGCTCTGTGGCTCAAATC 3′	59.99
Myb4L	5′ GCCCAAGAATGCAGGTAAC 3′	59.97
Myb4R	5′ CATACATGGTCCCTCACAATTT 3′	58.70

RNA Isolation

The quality of the RNA directly influence the amount of sequence information that can be converted into cDNA. So, it is important to optimize the isolation of RNA (Sambrook 1989). RNA was extracted from leaves of both genotypes after stress treatment by the guanidine isothiocyanate/acid-phenol method (TRIZOL), originally described by Chomzynski and Sacchi (Chomczynski and Sacchi 1987).

Expression Profiling: To check the expression of MYB transcription factor gene reverse transcriptase PCR was performed by using one step RT –PCR kit of Qiagen and PCR product was checked by electrophoresis method using 1.2% agarose gel. Gel elution of desired band from gel was performed by Qiagen gel extraction kit.

RESULTS AND DISCUSSION

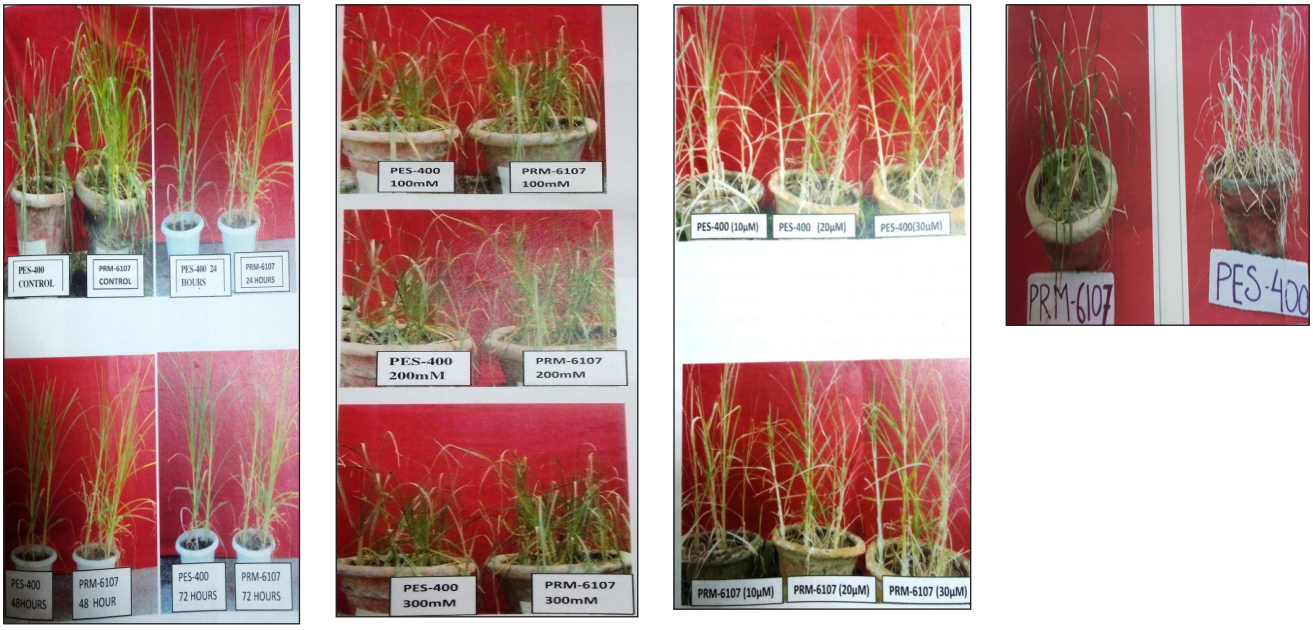
Stress Treatment: It was observed that PRM-6107 the stress tolerant genotype survived 11 days of drought treatment. It also endured 25 days of salinity stress and ROS stress created by spraying different conc. of 10μM, 20μM, 30μM of paraquat. PES-400 was not able to survive the stress treatment. Cold stress did not have any significant affect on the plants of both the genotype. (Fig. 1A, B, C, D).

Morpho-Physioloical Parameters

(a) Plant Height

Plant height was significantly lowered with increase in stress condition. However, the decline of plant height in PRM -6107 was less as compared to PES -400 Fig. 2(A) and (B).

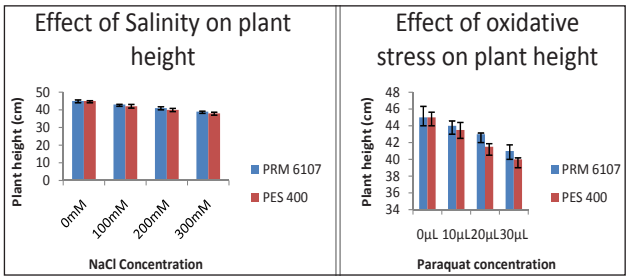
At salt conc. 0.1mM, 200mM and 300mM, plant height reduced (42cm, 40 cm, 38cm) respectively in PES-400 as compared to control (45cm). In case of PRM-6107 plant height was also reduced but reduction was less than PES-400. Plant height of both genotype were significantly lowered with increase in oxidative stress conditions. In case



(A) **(B)** **(C)** **(d)**

Fig. 1: (A) Cold Stress Treatment for 3 Days **(B)** Salinity Stress Treatment for 25 Days **(C)** Oxidative Stress Treatment for 7 Days **(D)** Drought stress for 7 days

of PRM-6107 plant height decrease was less as compared to PES -400. No significant changes were observed in cold stress. Kramer(1969) reported that growth and development in morphological traits is severly hampered as a result of abiotic stress. Inamullah *et al.* (1999) also reported about significant reduction in plant height under stress conditions. The decrease in plant height under stress conditions may be due to inhibition of cell division or cell expansion.

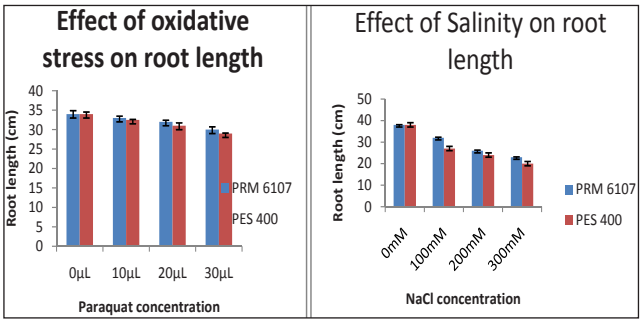


(A) **(B)**

Fig. 2: (A) Effect of Salinity on Plant Height **(B)** Effects of Oxidative Stress on Plant Height

(b) Root Length

Salinity stress affects plant growth by weakening the plant's root ability to absorb water from the soil. As a result, most of the plants become weak and in some cases end up with dying. At salt conc. 0.1mM, 200mM, 300mM, plant root length was reduced (28cm, 25 cm, 20cm) respectively in PES-400 as compared to control (34cm). In case of PRM-6107 root length was also reduced (31cm, 27cm, 23cm) but reduction was less than PES-400 (Fig. 3 A). Root length also decreased significantly in oxidative stress (Fig. 3B). However, no significant changes were observed in cold stress.



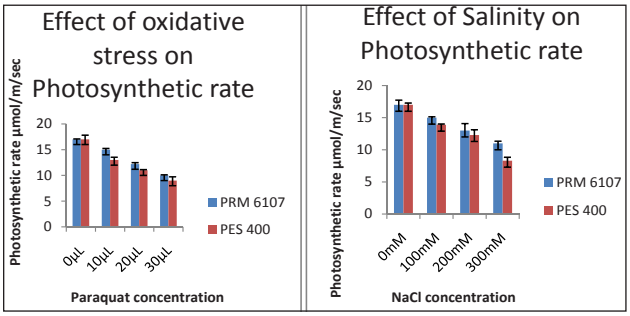
(A) **(B)**

Fig. 3: (A) Effects of salinity on root length **(B)** Effects of Oxidative stress on root length

(c) Photosynthesis Rate

It is observed that photosynthesis rate was decreased after giving salt stress treatment. The percentage decrease in photosynthesis rate was

found to be more in case of PES-400 than PRM-6107 in vegetative state (Fig. 4A). Oxidative stress also decreased the photosynthesis rate, in PES-400 as compared to control (Fig. 4B). However, the decrease in photosynthetic rate was less in PRM6107. No significant changes were observed in cold stress.



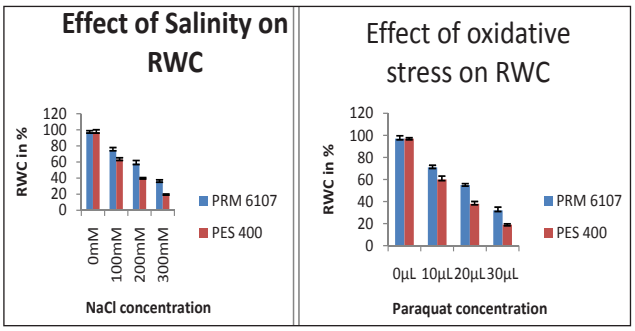
(A) **(B)**

Fig. 4: (A) Effects of Salinity on Photosynthetic rate **(B)** Effects of Oxidative Stress on Photosynthetic rate

Changes in leaf contents of chlorophyll of seedlings, showed a gross decline with increasing level of abiotic stress. Stress affects photosynthesis due to decreasing chlorophyll content. It reduces the chlorophyll a and chlorophyll b content with increasing stress in both varieties compared to control plants. Decline in leaf chlorophyll and as a consequence of stress is a very common observation (Chakraborty *et al.* 2001; Kar 2002). ROS causes chlorophyll degradation and membrane lipid peroxidation, reducing memberane fluidity and selectivity. Water stress-induced chlorophyll loss is ascribed mainly to degradation, although a retardation of synthesis also may be equally important. Among both investigated varieties, PRM-6107 showed lesser decline in the chlorophyll level than PES-400. The reduction of chlorophyll pigment in the present study might have been degradation of chlorophyll by chlorophyllase and reactive oxygen species generated during photorespiration under stress. It is attributed to a salt-induced weakening of protein-pigment-lipid complex and due to the suppression of the specific enzyme which is responsible for synthesis of green pigments (Souza *et al.* 2004) or increases chlorophyllase enzyme activity (Sreenivasulu *et al.* 1999). In our study also, there is a gradual decline in chlorophyll content in both varieties with increasing severity of stress. However there is comparatively less decrease in PRM-6107 variety. Hence it can be considered as more stress tolerant variety.

(d) Relative Water Content (RWC)

It was observed that water status of both the genotypes significantly decreases under stress conditions. There was a decline in RWC with salinity, in PRM-6107. RWC percentage decreased (72%, 55%, 35%) and in PES-400 (65%, 40%, 21%) as compared to control (98%) (Fig. 5A). Under oxidative stress, RWC values indicates a high extend of water loss in both the genotypes. However, decline of RWC in PRM -6107 was less (75%, 55%, 30%) as compared to PES-400 (70%, 40%, 20%) (Fig. 5B). No significant changes was observed in cold stress.



(A) **(B)**

Fig. 5: (A) Effects of Salinity on Relative Water Content **(B)** Effects of Oxidative Stress on Relative Water Content

Relative water content (RWC) of any plant tissue expresses the existing water status of the tissue in relation to its maximum water holding capacity. So, it is an important parameter in water stress experiments since it reflects the cellular capacity to maintain the water status under stress. The water status of both genotype PES-400 & PRM-6107 were significantly lowered with increase in stress condition. Reduced RWC values indicate a high extent of water loss under stress treatment. However, the decline of RWC in PRM-6107 was less as compared to PES-400. This shows that there is a better maintenance of water status in this variety indicating stress tolerance through osmoregulation. Osmotic adjustment is a powerful mechanism for conserving cellular hydration under drought stress and can be accounted for measuring leaf RWC. Osmotic adjustment commonly occurs in plant roots and leaves in response to stress. (Suriya *et al.* 2004)

Isolation of Total RNA

The RNA obtained was free from contamination

of DNA and other impurities since the DNase treatment was given to each sample (Fig. 6).

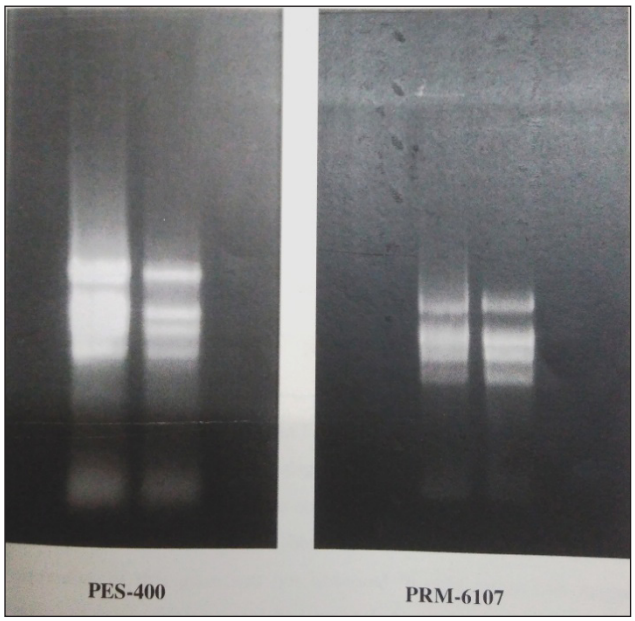
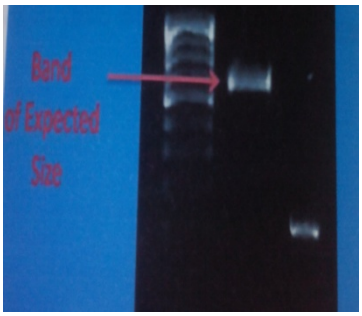


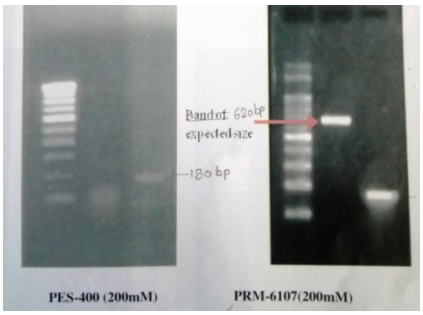
Fig. 6: RNA isolation from PES -400 & PRM-6107

Expression Profiling

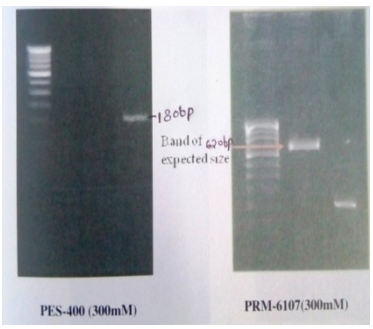
Expression profiling of myb gene under different abiotic stress conditions like drought, salinity, oxidative and cold stress using different degenerate primers produced a single band of 620bp with only one set of primer. The band was present only in the tolerant genotype i.e. PRM-6107 in all the conditions except cold stress. The expression of myb gene was not observed in sensitive genotype PES-400. Under the influence of drought, expression of myb gene was observed in tolerant genotype (Fig. 7A). The expression further increased with the severity of the drought. When salinity stress was provided, the expression of myb gene was observed at higher concentration of salt i.e. 200 mM (Fig. 7B1) and 300mM (Fig. 7B2). The expression of myb gene was also recorded under oxidative stress condition when the tolerant genotype was exposed to 30 µM of paraquat (Fig. 7C). The gene did not show any expression when the plants were exposed to cold stress. These observations suggest that the myb gene expressed in finger rmillet is drought, salt and oxidative stress responsive gene. Expression of the gene takes place in tolerant genotype only under stress condition and not in normal condition. Moreover, the expression of gene does not take place in sensitive genotype too. This shows that the expression of the gene is not a constitutive expression but a condition dependent expression where the gene expresses itself only in response to abiotic stress conditions. The expression of the



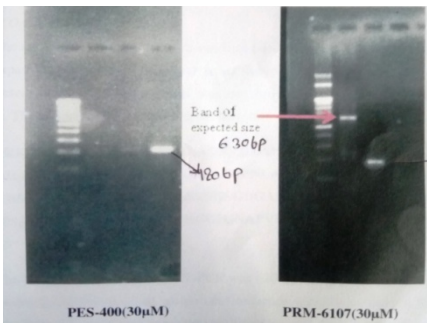
(A)



(B1)



(B2)



(C)

Fig. 7: Expression Profiling of myb gene under influence of **(A)** drought, **(B1)** salt 200mM, **(B2)** salt 300mM and **(C)** oxidative stress

myb gene in tolerant genotype may be upregulating the expression of many downstream genes whose products may be providing stress tolerance.

Many earlier reports indicate that Myb transcription factor is related to stress. In a study by Cominelte *et al.* 2005, a Myb transcription factor AtMyb60 was reported to be involved in stomata movement and drought stress in Arabidopsis. AtMyb96 has also been shown to play similar role by Seo *et al.* 2009. *AtMyb70, AtMyb73, AtMyb13, AtMyb15, AtMyb33, AtMyb41* and many others found to be associated with stress responses. (Jung *et al.* 2008 & Reyes & Chua 2007). Studies on Arabidopsis transgenic plants over expressing OsMyb 3R-2 gene showed increased tolerance to cold, drought and salt stress (Xiaoyan Dai *et al*. 2007). These reports indicate that transcription factor Myb acts as a master switch in drought stress tolerance.

***In-Silico* Studies:** The 620bp band was gel eluted and sequenced. The sequence was submitted in NCBI database (Accession No. JN107890). The sequence was subjected to multiple sequence alignment and the phylogenetic tree that was produced (Fig. 8). myb gene fragment which was designated as EcMyb1 showed 87% homology with *Zea mays* MYB transcription factor mRNA which is a partial cds, 87% with *Saccharum officinarum* MYB18 gene complete cds, *Oryza sativa* Japonica Group mRNA for MYB16 protein, *Oryza sativa* Japonica Group MYB transcription factor mRNA etc.

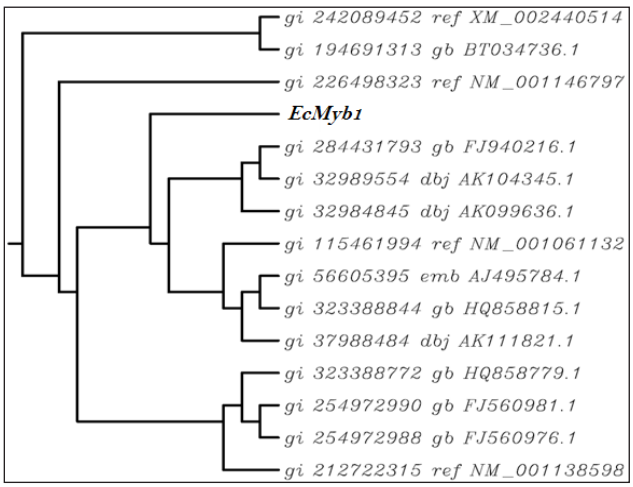


Fig. 8: Phylogenetic tree of sequences obtained from nucleotide blast of EcMyb1 gene

CONCLUSION

The tolerant genotype of Eleusine coracana showed the expression of abiotic stress tolerant MYB gene designated as *EcMyb1* gene which is expressed in severe drought, salinity and ROS condition, but does not express in low temperature (4 ˚C). Expression of *EcMyb1* may be involved in regulating the expression of downstream genes which causes stress resistance to the plants . Study of EcMyb 1 will help us in understanding stress tolerant strategies at molecular level in *Eleusine coracana*. Therefore, full length cloning and functional validation of *EcMyb1* will be helpful to carry out future work.

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