

Gateway cloning and *in-planta* transformation of drought stress responsive Ecmvb1 gene isolated from Eleusine coracana var.PRM 6107

Bhatt, M. ¹, Salvi, P. ², Lohani, P. ¹

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Abstract

Drought is one of the key abiotic stress that critically influences the crops by restraining their growth and yield potential. Being sessile, plants tackle the detrimental effects of drought stress via modulating the cellular state by changing the gene expression. Such alteration of gene expression is essentially driven by the transcriptional syndicate. Transcription factors (TF) are the key regulatory protein that controls the expression of their target gene by binding to the cis-regulatory elements present in the promoter region. Myb-TFs ubiquitously present in all eukaryotes belong to one of the largest TF family, and play wide array of biological functions in plants including anthocyanin biosynthesis, vasculature system, cell signaling, seed maturation and abiotc stress responses. In the present study the full length Myb TF from *Eleusine* corocana was subcloned using Gateway cloning system and further transformed into Arabidopsis thaliana through floral dip method. Transgenic Arabidopsis thaliana plants harbouring Ecmyb1 gene were screened and grown in transgenic glasshouse under controlled conditions.

Key words: Abiotic stress, Transcriptional regulation, MY-transcription factor, Gateway cloning, Finger millet

Introduction

Global change in climatic conditions, population order to overcome such problem an important and environmental degradation has led to extreme depletion of water resources thus causing widespread drought. Greenhouse emission rise has led to erratic rainfall, increase in parched land area, desertification and ultimately reduction in crop productivity. Drought is a major abiotic stress that is a cause of severe agony to humankind. It is generally defined as protracted time period of scarce precipitation compared to the statistical cumulative average for a region that results in water shortage for some activity, group or environmental (Leng and Hall, 2019, Jie et al, 2020, southasiadisasters.net March 2015). It is a major abiotic stress that culminates in extreme loss of yield to crops and has proven to be more scourging than any other abiotic hazard. Since 1900, a causality of more than 11 million people has been logged and approximately 2 billion have been influenced by drought conditions (Boyer, 2013). In

Author's Address

¹Department of Molecular Biology and genetic Engineering, CBSH, GBPUniversity of Ag. & Tech. Pantnagar, India ²Agriculture Biotechnology Department National Agriculture Biotechnology Institute, Mohali, Punjab, India **E-mail.:** *megha4bhatt@gmail.com*

approach is to generate crops with enhanced drought tolerance, by utilizing biotechnology and molecular biology techniques. This strategy requires the identification of traits relating to drought tolerance and then the genes that determine these traits. Plants often carry genes that enable them to maintain their performance despite severe moisture stress conditions. Such plants are referred to as drought tolerant and for tolerance several genes have been recognized and characterized.

Further, it is necessary to explore the molecular and genetic bases behind drought tolerance and genes that are responsible for coding the major transcription factor and their functional proteins (Kapoor, et al., 2020). These stress-inducible genes can be classified into two groups. The first group includes proteins that function in abiotic stress tolerance.

These constitute of molecules such as late embryogenesis abundant (LEA) proteins, chaperones, antifreeze proteins, mRNA-binding proteins, osmotin, key enzymes for osmolyte biosynthesis, sugar, water channel proteins and proline transporters, detoxification enzymes and various proteases. The second group comprises of



regulatory proteins. These include various transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules (Ciarmiello and Woodrow, 2011). Thus, drought tolerance is a multigenic quantitative trait involving complex genetic control.

Till date researchers have not been completely successful in developing drought tolerant cultivars by targeting a single gene (Kaur et al., 2015). Therefore, research on the master regulators that modulate expression of all such genes gained limelight. The master regulators are the transcription factors that are induced at the onset of drought conditions (Salvi et al., 2016). They bind specifically to the promoters of downstream genes associated with drought tolerance and upregulate their expression. For abiotic stress response mechanism various transcription factors have been known so far (Negi et al., 2017). An important transcription factor amongst these is the MYB transcription factor. The MYB proteins are characterized by a MYB DNA binding domain in their N-terminus that is composed of one or more imperfect tandem repeat(s).

MYB with the R2R3 type repeat is found in plants and have been reported to be responsible for influencing the expression of ABA regulated drought tolerance related genes (Saxena *et al.*, 2013, 2020).The expression of MYB transcription factor gene has been studied in finger millet. The expression of the myb gene is conditional and takes place only with the onset of drought (Salvi *et al.*, 2012). The expression level increases many folds with the increase in the severity of drought (Jadhav *et. al.*, 2018).

Finger millet a member of poeceae, is a hardy crop and shows tolerance towards abiotic stresses specifically drought. Finger millet can survive in harsh environmental stress conditions due to their potent alleles which show drought resistant characteristics. Such crops will be of great significance in meeting challenges due to water shortage (Gull *et al.*, 2014). Therefore, in present investigation cloned full length sequence of drought stress inducible *Ecmyb1* gene was first subcloned through Gateway vector system and further transformed into *Arabidopasis thaliana* through floral dip method.

Materials and Methods

Isolation and cloning of full-length gene encoding *Ecmyb*1

In our previous study we have identified and isolated a partial gene (CDS) encoding MYB-TF from *Eleusine coracana* which was found to induced after drought stress exposure. To further understand the molecular role and regulation of the *Ecmyb1*, we sought out to clone full length sequence of the gene. In order to clone the full-length gene, we isolated the RNA from the *Eleusine coracana* leaves and reverse transcribed it to cDNA. The cDNA was used to amplify the full-length gene amplicon of *Ecmyb1*, and subsequently cloned in pGEM-T vector system according to manual instruction. The cloned amplicon was confirmed by sequencing and subsequently submitted to NCBI GenBank.

Primers used for full length sequence were 5'TCAACTAATGGTAGCCCTTCCCTCT-3' (sense) and 5'- GATATTCTCAAAAGACAGTTGCATTCT-3' (antisense). For designing the primer sequences, partial sequence of Ecmyb1 gene which has been submitted with Genbank accession number JN107890 was nBLAST with *Eleusine coracana* whole genome sequence (GenBank: LXGH01099917.1).The sequence that showed similarity was considered for ORF searched using ORF finder tool (NCBI). The lateral flanking sequence of ORF was used for primer designing.

Gateway cloning of full length Ecmyb1 gene for functional analysis and expression analysis

The Gateway Technology utilizes the site specific recombination system of lambda. The principle behind this process is to enable the incorporation of lambda genome into E.coli chromosome and consequently this integration leads to transition between lytic and lysogenic cycle.For Gateway system first step is to generate an entry clone in which our gene of interest will be present and is flanked by modified att sites. Entry clone was generated using TOPO cloning method as described in manual (Ptashne, 1992). Single colony was taken from positive selected transformed cells after colony PCR selection and PCR was carried out using primers sense (5'CACCATGGTAGCCCTTCCCTCTGCGATG3') and antisense (5'GATATTCTCAAAAGACAGTTGC3'). These primers were same as gene specific primers with 4 base pair sequence CACC which was



necessary for directional cloning on the 5' end of flowering stage in a greenhouse with temperatures the forward primer.Further transformation was carried out using competent E. coli cell. Colony PCR was performed to check the trans-formants of the desired fragments. The recombination reaction of an *attL*substrate is indicated as entry clone which fuses with an attR substrate known as destination vector. This reaction generates an attB containing the expression clone in which our gene of interest will be present along with the by product containing attP sequence. This process is known as LR reaction. Single positive colony was taken from the bacterial plate containing Ecmyb1 gene: E.coli DH5a: TOPO vector (entry clone) and PCR reaction was carried out using M13 forward and M13 reverse primers sense (5'GTAAAACGACGGCCAGT 3') and antisense (5'AACAGCTATGACCATGT Further. 31). colony-PCR was carried out to check the transformants of the desired fragments using Ecmyb1 gene specific primers.

Agrobacterium tumefaciens mediated gene transformation in *Ecmvb1* gene

Primary culture was prepared by inoculating a single colony of Agrobacterium tumefaciens strain EHA105 in 5 ml of LB medium with rifampicin (10 mg/l) and incubated at 28°C with overnight shaking at 200 rpm. The primary culture (2 ml) was transferred to 50 ml LB broth containing rifampicin (10 mg/l) antibiotic and grown till an optical density of 0.5 is achieved. Cells were pelleted by centrifugation at 5,000g for 5 minutes at 4°C and supernatant was discarded. The pellet was resuspended in 10 ml of pre-chilled 0.5M NaCl and was again centrifuged at 5,000g for 5 minutes at 4°C. Finally, the pellet was resuspended in 1.0 ml of ice-cold 20 mM CaCl₂ and transformed by freeze thaw method.

Showing of *Arabidopsis* thaliana ecotype **Columbia seeds**

Seeds were washed properly and kept in dark at 4⁰ C for stratification process for 2-3 days. Seeds were then inoculated in Jam bottles containing half strength of MS media for germination. After ten to fifteen day seedlings were transferred to pots that contained autoclaved soil (soil vermiculite in ratio of 3:10). Arabidopsis plants were grown till

24°C day/20°C night.

Floral Dip Method for gene transformation

Positive single colony was taken from the bacterial plate generated after transformation with Agrobacterium (EHA105:pMDC84:Ecmyb1) and inoculated in YEP media for 48 hours at 28°C with 200 rpm in a shaker in the presence of antibiotic rifampicin (10 mg/l) and kanamycin (50 mg/l). After 48 hours when the OD_{600} of the suspension culture reached to 0.8, culture was then centrifuged at 4000g for 10 minutes at room temperature. Harvested cells were gently re-suspended in 250 ml of freshly made 5% (w/v) sucrose solution. Surfactant Silwet L-77 was added to this solution at a concentration of 0.02% (v/v) and stirred uniformly. The aerial parts of the plant (at 50% flowers open stage) were dipped in this solution for about 5-10 seconds, with gentle agitation. The pots containing inoculated plants were covered with perforated plastic polybag for 16-24 hours to maintain high humidity. The treated plants were allowed to grow to full maturity. After the plants reached complete maturity and dried, the seeds were carefully harvested and screened for transformants.

Screening of primary transformants

For screening of primary transformants, T₀ seedling of Arabidopsis thaliana was used. Seeds were surface sterilized by treating them with 70% ethanol for 1 min followed by treatment with 1 % bleach solution for 1-2 min. Seed were then rinsed three times with sterile water till the water appeared clear without any yellow color. Presence of yellow color indicates that residual bleach is still present in the wash. Sterilized seeds were kept for stratification process of 2-3 days. Seeds were then inoculated in jam bottles containing half strength of MS media with antibiotic hygromycin 25mg/l for selection of transformants and placed in a tissue culture room under continuous light (21500 lux photon) conditions. Transformed plantlets appeared from the seeds after 7-10 days.

PCR confirmation of transformed seedlings of Arabidopsis thaliana using hypromycin primers The positively selected transformed plants were transferred from MS medium to soil under shaded



green house until flowering stage. Further, these seedlings were used for DNA isolation and PCR reaction was executed using hygromycin primers sense (5'TTCAGCTTCGATGTAGGAGGG3') and antisense (5'ATGTTGGCGACCTCGTATTGG 3').

Results and Discussion

Full length Ecmyb1 gene was amplified using gene the specific primers. After PCR the product was cloned in pGEM-T easy vector and further sequenced. The Ecmyb1 gene contained 1053 nucleotides that were translated to 350 amino acid sequence using EXPASY tool (Fig 1). Complete sequence of Ecmyb1 gene was submitted to NCBI with accession number MT312253. Additionally, Ecmyb1 gene was subcloned into plant expression vector pMDC84 using Gateway system. The confirmed cloned were transformed in Agrobacterium tumefaciens strain EHA105.



Figure 1: L1-1 kb plus DNA Ladder, L-2 1053 bp fragment of *Ecmyb1* gene after colony PCR

Transformation of EcMyb1 gene into *Arabidopsis thaliana* through floral dip method

In-planta transformation protocol in Arabidopsis thaliana and others plant systems through Agrobacterium mediated method has made many successful attempts (Zia et al., 2011). In Arabidopsis thaliana transformation through Inplanta methods early success was achieved by the Feldmann et al., 1987 work. Later Bechtold et al., (1993) reported that in Arabidopsis thaliana the transformation rate enhanced if plants were inoculated at flowering stage(Supartana et al., 2006). In floral dip method Agrobacterium

containing *Ecmyb1* gene (Fig 2) was grown in YEP medium supplemented with antibiotic (kanamycin and hygromycin) at 28°C, 250 rpm for 48 hours(TianZi *et al.*, 2010). After 48 hours cells were harvested by centrifugation and suspended in 250 ml of freshly made 5% (w/v) sucrose and 0.02 % solution of surfactant Silwet L-77. In the next step aerial parts of *Arabidopsis* plants were inverted and gently stirred in the suspension for 5to 10 sec. Sucrose and silvet L-77 are very important for *Agrobacterium* infection. Sucrose helps in activation of *vir* genes by providing a signal whereas Silwet L-77 increases the entry of *Agrobacterium* by reducing the surface tension (Clough *et al.*, 2000).

Subsequently, pots were covered with plastic bag to create humidity for maintaining adequate environment for *Agrobacterium* infection. *Arabidopsis thaliana* plants were grown for transformation until maturity after which seeds were collected and used for selection of *Ecmyb1* gene.



Figure 2: Transformed colonies of PMDC84 : Ecmyb1 gene Selection was carried out by using kanamycin (25mg/L) and rifampicin (10mg/l)

Hygromycin based selection of transgenic *Arabidopsis thaliana* plants

The Agrobacterium infected Arabidopsis thaliana plants were further selected on hygromycin. For hygromycin selection seeds were grown on half strength of MS media with 25mg/l antibiotic hygromycin. Only transformed seeds were able to



grow on hygromycin medium (Fig3). The possible reason behind this selection is hygromycin which interferes with protein synthesis process of non transformed cells. Hygromycin competes with the binding site between elongation factor FE-2 and ribosome. This interference destroys the function of ribosome which ultimately leads to inhibition in protein synthesis process and makes plants sensitive to hygromycin (Duan *et al.*, 2011).



Figure 3: Tansgenic seedlings transformed with pMDC84:*Ecmyb1* gene selected on 25mg/l hygromycin, non transformed seeds were unable to grow in hygromycin.

PCR confirmation of transformed seedlings of *Arabidopsis thaliana* using hygromycin primers

Seedlings of Arabidopsis thaliana which was selected through hygromycin selection were further processed for PCR to confirm the T-DNA inserted containing *Ecmyb1* gene. The positively selected transformed plants were transferred from MS medium to soil under shaded green house until flowering stage. Further, genomic DNA was isolated from these seedlings and PCR reaction was performed using hygromycin primers. PCR generates amplicon size of 587bp that corresponds to size of hygromycin sequence present in vector (Fig 4). Transformation efficiency for floral dip method in this study was 1% as out of 700 seeds 7 transgenic plants were screened in hygromycin selection (Fig 4). This result obtained in the present study were in the acceptable range (0.5-3%) as established by the study of Clough and Bent 1998; 2000).



Figure 4: PCR confirmation of transgenic Arabidosis thaliana seedlings containing *Ecmyb1* gene with primer specific to hygromycin gene using genomic DNA as template. L- 1000bp ladder, W-wild type *Arabidopsis* plants (no band), L- 1,2,3,5,6,7 transformed plants showing amplicon size of 587bp

Conclusion

Drought is a foremost abiotic stresses that harmfully influences the crops by restraining their growth and immensely reducing the yield capacity. Henceforth, it is necessary to comprehend the mechanisms that support plants to handle the deficiency of water. Plants often carry drought tolerance genes that enable them to maintain their performance despite of severe moisture stress conditions, such plants are referred to as drought tolerant plants. Transcription factors (TF) are mainly responsible for creating molecular level changes in plants during drought stress condition. They build strong association with general transcription factor at promoter of target gene and regulate their expression. Association of transcription factors with each other developed in response to intracellular signals which leads to activation or repression of target gene. An important transcription factor amongst these is the transcription factor. The specialized MYB characteristic of MYB protein is that they contain MYB DNA binding domain in their N-terminus which is composed of one or more imperfect tandem repeat(s). In this study, we have subcloned a full-length coding sequence of a drought responsive gene that encodes MYB- transcription factor which was already submitted to NCBI (Accession number MT312253) through Gateway system. Transgenic Arabidopsis thaliana plants harbouring Ecmyb1 gene were produced in the



present study. The transgenic plants will be maintained and screened for two generations to attain homozygosity. After functional validation study of the gene in Arabidopsis, it could be recommended for transfer in other plants like rice or maize to produce drought tolerant crops.

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