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# MOLECULAR CLONING OF *BEMYB140* FROM *BAMBUSA EMINENCIES*, CANVASSING TRANSACTIVATION AND EXPRESSION PROFILING FOR ITS DYNAMIC CHUNK AGAINST ABIOTIC STRESSES

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**Abstract**

The MYB is a major family of transcriptional factor's (TFs), which significantly regulate several physiological processes in plants, i.e. growth, metabolism and development on one side and defenses against abiotic and biotic stresses on other. In this research a novel gene *"BeMYB140"* (GenBank accession number MG763923) encoded for TF was cloned and characterized from *Bambusa emeiensis*. The isolated gene *BeMYB140* has an open reading frame (ORF) of 723bp which encoded a protein of 240 amino acids with a predicted relative molecular mass of 27.34346 kDa. Multiple proteins sequence alignment and phylogenetic analysis revealed that this protein contains a typical MYB domain and showed high homology with *HvMYB1*, *ZmMYB38* and *AtMYB4*. Meanwhile, transactivation analysis and sub-cellular localization assay unveiled that the *BeMYB140* protein is a transcriptional activator and localized in the nucleus. Moreover, *BeMYB140* exhibited a dynamic expression under different abiotic stressors, such as, in response to ABA and $Na_2SO_4$ treatments down regulation was followed by a significant up-regulation; whereas the opposite results were observed during PEG treatment. The highly significant difference and maximum fold changes were observed in PEG, NaCl and $H_2O_2$ treatments. In ABA and NaCl treatments the peak expression level were observed during 3hrs, meanwhile, under PEG and $H_2O_2$ treatments *BeMYB140* was strongly induced during later stages i.e. 24hrs. It is concluded from the results, that *BeMYB140* involved in broad range of abiotic stresses and mediates stress tolerance in *Bambusa emeiensis.*

**Abbreviations: ABA=** Abscisic acid, **GFP=** Green fluorescent protein, **PEG=** Polyethylene glycol, **Y1H=** Yeast one way Hybridization, **NAM=** No apical meristem, **SD=** Synthetic dropout, **ORF=** Open reading frame, **PCR=** Polymerize chain reaction.

**Key words:** Abiotic stress, *Bambusa emeiensis*, Bioinformatics, Transactivation, Localization.

## Introduction

Transcription factors (TFs) play a key role in plants phenotypic and genetic retorts as to counter abiotic and biotic factors (Wei *et al*., 2017). Among TFs; MYB is a major family, which play a key role in plants response to various stresses (Li *et al*., 2015). From diverse plants species several MYB TFs have been isolated; such as *Arabidopsis thaliana* > 198 (Yanhui *et al*., 2006), cotton ≈ 200 (Cedroni *et al*., 2003), and soybean > 156 (Ambawat *et al*., 2013) etc. In MYB family "R2R3-MYB" is the largest subfamily which displays a major role in plants metabolism and development of particular morphogenesis as to enhance the plant tolerance to different abiotic stresses (Liu *et al*., 2017). These stresses include; ABA, cold, NaCl, $Na_2So_4$, $H_2O_2$, and PEG etc. which severely damage both the somatic and propagative growth of the plants (Gao *et al*., 2008; Neocleous *et al*., 2011). MYB transcriptional factors play very important part in the plants morphogenesis; such as specialized cells, organ and leaf development by enhancing the metabolism of plants against abiotic stresses (Zhang *et al*., 2012). The expression of *MYB12* regulate evapotranspiration (Nakabayashi *et al*., 2014). While *MYB60* regulates the movement of stomata as to counter drought stress (Oh *et al*., 2011). *AtMYB15* and *GbMYB5* have very positive role in the systematic defence against drought tolerance by triggering different receptive gene (Baldoni *et al*., 2015). Whereas Different TFs such as *OsMYB48-1, AtMYB41*, *AtMYB96 and AmMYB1 etc.* have very effective response against salinity. While *OsMYB2, OsMYB48-1 and OsMYB55* etc. plays very important role in plants response against cold (Xiong *et al*., 2014). With the development of modern research ample efforts have been carried in the identification of R2R3-MYB TFs against different abiotic stresses. There is no previous work on *BeMYB140* from *Bambusa emeiensis;* therefore the current research work on *BeMYB140* is novel. The aim and objective of current research is to highlight the role of *BeMYB140* for future use in transgenic plants. In current research a wide investigation was carried on bioinformatics analysis, the phylogenetic relationship, subcellular localization, Y1H and expression profiling against chunk of different abiotic-stresses.

## Materials and Methods

**Cloning of *BeMYB140* from *Bambusa emeiensis*:** While adopting bioinformatics approach a novel gene *BeMYB140* was identified and cloned from *Bambusa emeiensis*. The α-*amino* sequences of *HvMYB1* was the basic structure for the query probe at the transcriptome database of *Bambusa emeiensis* and a full-length sequence was designated and named as *BeMYB140*. Gene specific primers were designed while using the DNA sequence (Table 1). For the full length of *BeMYB140* amplification, the tissues from *Bambusa emeiensis* root, shoot, and leaf were collected. The total RNA was extracted from the root, stem and leaf tissues by the total RNA extraction kit (Omega BIO-TE, US). The primeScript$^{tm}$ RT reagent kit (RR047A, TaKaRa Dalian,

China) and oligo T (18) primer were used to prepare cDNA. 25µL PCR protocol was followed for the amplification of the full length *BeMYB140*. The reaction mixture containing 12.5µL of 2XGC PCR buffer (TaKaRa Dalian, China), 0.2µL of polymerase (5U/µl) (TaKaRa Dalian, China), 4µL of dNTPs (2.5mM each), 1µL of each forward and reverse primers (10µM), 5µL of cDNA as a template and dd$H_2O$ was added to maintain the volume of 25µL. The PCR conditions were adjusted as; the initial denaturation achieved at 95ºC for 3 min, followed by 34 cycles of 95ºC for 30 sec, 58ºC for 30 sec, 72ºC for 1 min and 72ºC was used for 10 min as final extension. The amplified PCR products were separated on 1% agarose gel stained with gold-view. Desired PCR products were extracted from agarose gel using universal DNA purification kit (TaKaRa Dalian, China) and cloned into the *pMD19-T* vector (TaKaRa Dalian, China). The *pMD19-T* easy vector system allows the selection of positive recombinant plasmids by an easy blue-white screening of positive recombinant plasmids. For further confirmation gene was sequenced.

**Bioinformatics analysis:** The cloned gene was designated as *BeMYB140*. The conserved MYB domain was searched using MEME (http://memesuite.org/tools/meme). Alignment of relevant sequences was performed using DNAMAN (version.7) and the phylogenetic tree was constructed using MEGA (version.7) (Kumar *et al*., 2016). The domain structure modal was constructed using phyre2 online software.

**Table 1. Primers used for *BeMYB140* TF for various experiments.**

| ***BEMYB140*** | **Primers direction** | **Various sequences of primers used** | **Enzyme site** |
|---|---|---|---|
| Cloning primers | F | GCTCTAGAGCATGGGGAGGTCGCCGTGCTGCG | Xba-I |
| | R | GGGGTACCGCTCATTTCATTTCAAGGCTTCTG | Kpn-I |
| Sub-cellular localization primers | F | CGGGGTACCCCGATGGGGAGGTCGCCGTGCTGCG | Kpn-I |
| | R | CCGGAATTCCGGTTTCATTTCAAGGCTTCTGAAG | EcoR-I |
| Yeast experiment primers | F | CGGAATTCCGATGGGGAGGTCGCCGTGCTGCG | EcoR-I |
| | R | CCGCTCGAGCGGTCATTTCATTTCAAGGCTTCTG | Xho-I |
| Tubulin Primers | F | GCCGTGAATCTCATCCCCTT | |
| | R | TTGTTCTTGGCATCCCACAT | |
| RT-PCR primers | F | TCAAGTGCCCAGACCTCAAC | |
| | R | TCATTTCATTTCAAGGCTTC | |

**Y1H screening of the *BeMYB140:*** The Y1H screening was performed using yeast strain *EGY48* and *pEG202*. The transcriptional activity of *BeMYB140* was assessed in *pEG202* (*HIS3, 2 µm, Ap$^r$, ADH constitutive promoter, LexA DNA-binding domain*) and the yeast strain *EGY48* (*MATatrp1his3ura3leu2:6lexAop-lacZ*). Through forward/ reverse primers the template of the *BeMYB140* was amplified by PCR containing E.coRI and XhoI restriction sites (Table 1). The PCR products were ligated into pEG202 vector. The recombinant vector *pEG202-BeMYB140* and the empty *pEG202* vector were introduced into the yeast strain *EGY48* via the PEG/LiAc method. The transformed yeast was confirmed by the PCR (Lopato *et al*., 2006). The yeast strain was streaked on synthetic dropout (SD) plates (SD /- Ura), (SD /- Ura-His) and (SD /- Ura-His- X-Gal) incubated at 30ºC for 3 days.

**Subcellular localization of *BeMYB140*:** The *BeMYB140* amplified via PCR with specific primers of *BeMYB140*- Kpnl -F *and BeMYB140*- EcoRI-R (Table 1). The PCR product was ligated with *pTEX-GFP* vector to generate a *BeMYB140::GFP* in-frame fusion protein. After that, the onion (*Allium cepa*) epidermal cells were incubated for 4 hours in dark; while using MS media containing mannitol (0.2mol/L) and sorbitol (0.2mol/L). *BeMYB140::GFP* and *GFP* empty vector were hosted into onion epidermal cells using the biolistic transformation system (PDS-1000/He, Bio-Rad, and Hercules, USA). Each plate was bombarded twice at 9 cm distance. For each bombardment 80 µg of gold was used and carried out with a pressure of 1350 psi. Afterward, the onion tissue was incubated into the dark for 16 hours and fluorescence was observed under Leica DFC 500 fluorescent microscope (Duan *et al*., 2014).

**Plant material and stress treatment:** 6 months old plants of *Bambusa emeiensis* were used for the experiment. The plants were treated with different abiotic stresses with variable concentrations i.e., ABA: 250µM, PEG: 20%, NaCl: 250mM, $H_2O_2$: 15mM and $Na_2SO_4$: 50Mm. Three biological repeats from each treated samples were collected at different interval i.e., 0, 3, 6, 12 and 24 hrs. The samples were straight away iced up in liquid nitrogen and kept at -80ºC till the total RNA extraction.

***BeMYB140* expression analysis through qRT-PCR:** The stored samples were used for total RNA extraction and cDNA synthesis. The qRT-PCR primers were designed as given in Table 1. qRT-PCR was carried out for the analysis of *BeYMY140* expression in response to stress treatments. The cDNA was used as the template for amplification. The qRT-PCR was performed using SuperReal PreMix Plus (FP205-02, SYBR Green, and Tiangen) on a CFX Connect ™Optics Module (Bio-Rad) Real-Time PCR System. The PCR conditions were used as previously described by (Wang *et al*., 2015) Tubulin gene expression was used as internal control. The experiments were performed in triplicate. $2^{-\Delta\Delta Ct}$ method was used for the analysis of gene expression (Livak & Schmittgen, 2001).

**Statistical analysis**

In the present study, three biological replicates of each experiment for abiotic stresses were performed, and the means were calculated as the average of three replicates, the data are shown here are the means ± SDs. The SPSS (Chicago, IL, US) was used for statistical analyses. The t-test was performed to analyze the significant difference. Asterisks represent significant difference of expression level after the application of treatments at different time interval (*$p$<0.05; **$p$<0.01; ****$p$<0.001).

**Results**

**Cloning and bioinformatics analysis:** Through PCR amplification *BeMYB140* was clones from *Bambusa emeiensis* by means of forward/reverse primers. The PCR product was isolated on the gel electrophoresis as to confirm the correct band of *BeMYB140* (Fig. 1a).

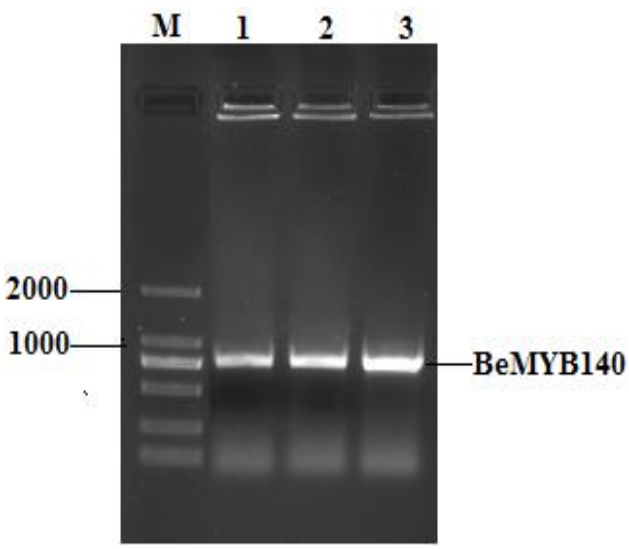


Fig. 1a. The PCR amplification and analysis on the Gel Electrophoresis of *BeMYB140*.

*BeMYB140* is having full-length open reading frame (ORF) of 723bp. It translates into a protein with a predicted molecular mass of 27.34346kDa (https://web.expasy.org/cgi-bin/compute_pi/pi_tool). Multiple Sequence alignment (DNAman software version 7.0) shown that the *BeMYB140* had a representative MYB domain structure. The *BeMYB140* has a typical MYB domain structure contained 6 α-helix (Fig. 1c). The *BeMYB140* protein sequence from *Bambusa emeiensis*; organized together with particular MYB protein sequences belonging to other plant species, were aligned in MEGA7 (Kumar *et al*., 2016). The alignment was used to calculate distance matrices for the neighbor-joining method. Bootstrap analysis with 1,000 replicates was performed to test the robustness of the internal branches. The phylogenetic investigation revealed that *BeMYB140* belongs to the R2R3-MYB family from *Bambusa emeiensis* as shown in (Fig. 1d). This novel gene BeMYB140 has high similarity with *HvMYB1* (89%) Accession: P20026; *AtMYB4* (86%) Accession: XM-006660837.2; *ZmMYB38* (86%) Accession: GRMZM2G084583; as shown in (Fig. 1b). *BeMYB140* have high homology with transcriptional factors having role in abiotic stresses, reduced the flavonol biosynthesis, charge particles and UV-Protection (Fornalé *et al*., 2014; Franken *et al*., 1994).

**Yeast one-hybrid screening of the *BeMYB140*:** The positive transformation of *BeMYB140* with *pEG202* was analyzed by the PCR (Fig. 2a). The transformed yeast (*EYG-48-pEG202-BeMYB140*) successfully grows on all three kinds of medium and stained blue in the presence of X-gal. The yeast strain (*EYG-48*) only grows on (SD /-Ura) medium; meanwhile on other two mediums growth were not observed. The *pEG202* empty vector (*EYG-48-pEG202*) showed the normal growth but the blue staining was not observed (Fig. 2b). These results indicate that *BeMYB140* is a transactional activator.

**Subcellular localization of *BeMYB140*:** The ligated *GFP-BeMYB140* was transformed into the onion epidermal cells thought the particle delivery system of the gene gun. The expression of the *GFP::BeMYB140* and *GFP* were examined under the fluorescent microscope. The results suggested that the *BeMYB140* is localized in the nucleus of the cell (Fig. 3).

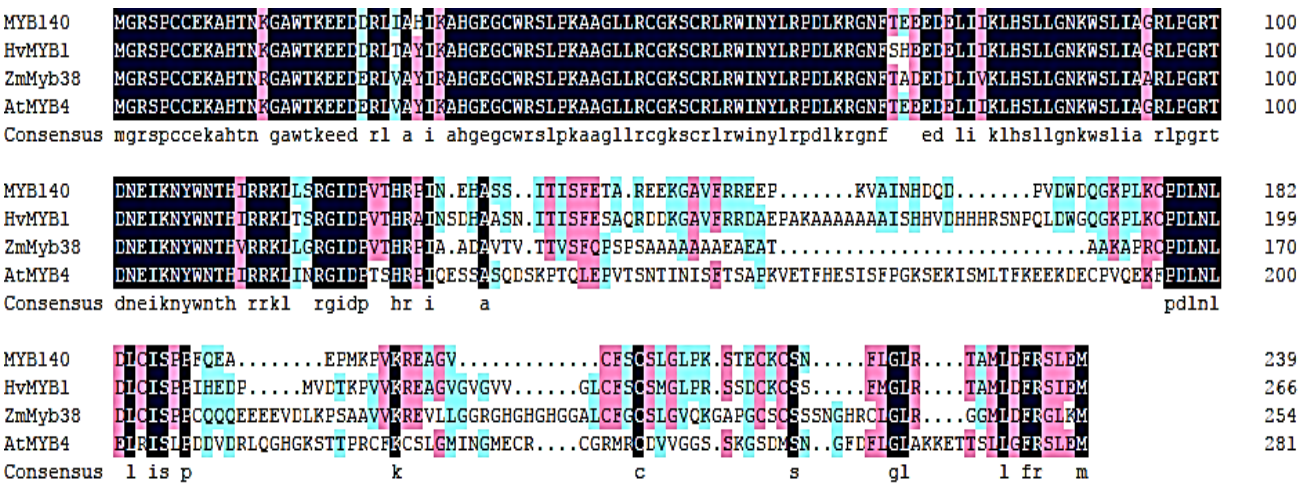


Fig. 1b. Multiple sequence alignment for homologous proteins of *BeMYB140* with *Arabidopsis thaliana* (*AtMYB1, AtMYB4*) and Zia maze (*ZmMYB38*).

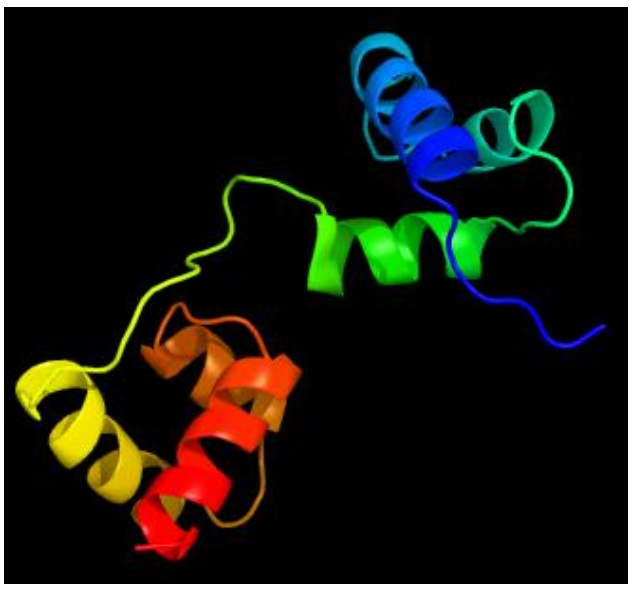

Fig. 1c. The domain structure of *BeMYB140* having 6 α-helix without β-sheets.

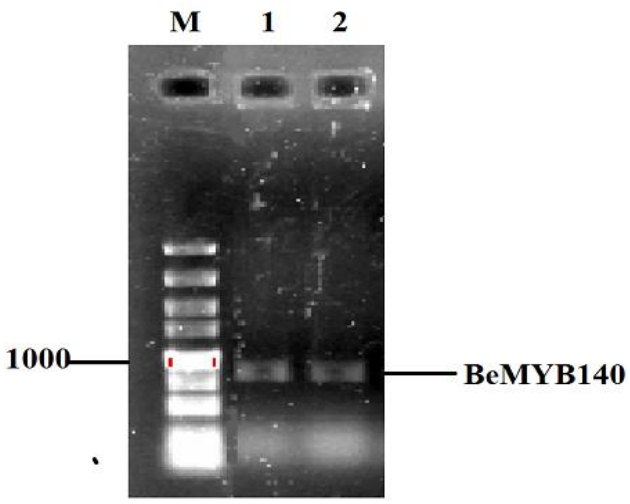


Fig. 2a. PCR confirmation of *BeMYB140* from yeast *EYG-48.*

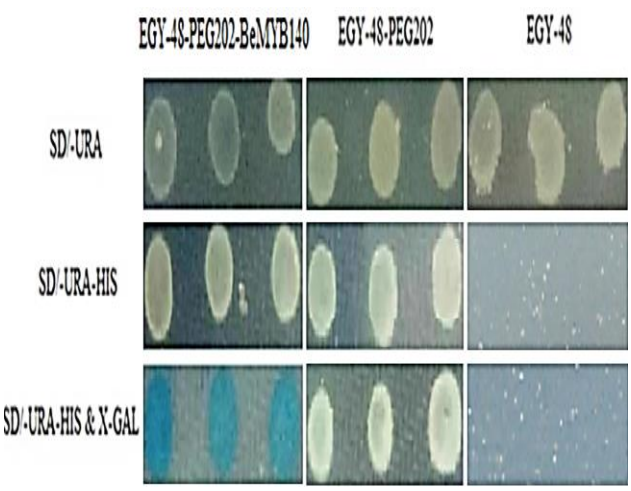


Fig. 2b. Transactivation assay of *BeMYB140*. The blue staining indicates the transactivation of *EYG-48-pEG202-BeMYB140* in comparison with *EYG-48-pEG202.*

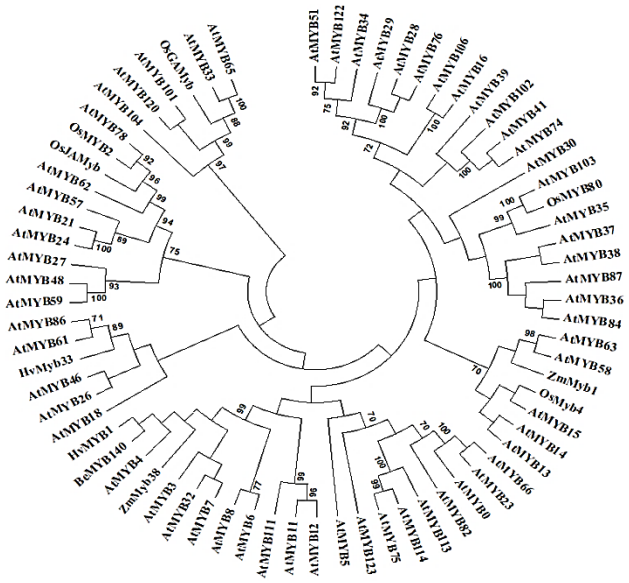


Fig. 1d. The phylogenetic tree analysis of different MYB faimly related TFs protein sequence from diverse plant species while using MEGA7 software.

**The expression profiling of *BeMYB140*:** The *BeMYB140* TF expression profiling and role against abiotic stress was carried out by applying different stresses inducers. The 6 months old plants of *Bambusa emeiensis* were treated with different concentrations of ABA, PEG, NaCl, $H_2O_2$, and $Na_2SO_4$. The abiotic stress inducers were analyzed through different time intervals i.e. 0hr, 3hrs, 6hrs, 12hrs, and 24hrs. The *BeMYB140* at a different level of stresses at different time interval were confirmed through qRT- PCR. From the outcome we can determine that the *BeMYB140* is having both up and downward trends in its expression. In response to the ABA treatment the down regulation was followed by up-regulation at 24 hrs (Fig. 4a). mRNA accumulation of the *BeMYB140* in response to PEG and $H_2O_2$ changed up to the 3hrs; during 6hrs, 12hrs and 24hrs. The maximum up-regulation was observed at 24hrs (Figs. 4b and 4d). In response to salinity i.e., Nacl and $Na_2SO_4$ the maximum fold change was seen at 6 hrs. Conversely, the mRNA accumulation declined slowly with a time interval i.e., at 24hrs the expression level goes down abruptly to very lower level (Fig. 4c and 4e). The *BeMYB140* is up-regulated significantly when treated with ABA, PEG, NaCl, and $H_2O_2$ on one hand while it is down-regulated significantly when treated with $Na_2SO_4$ on the other hand.

## Discussion

The family of MYB TFs have been comprehensively studies regarding biotic and abiotic stresses (Liu *et al*., 2017; Mohamed *et al*., 2017), Drought (Cominelli *et al*., 2005), fibers (Zhou *et al*., 2009) and cell-wall development etc. (Zhong *et al*., 2008). In the current research article *BeMYB140* was broadly investigated as to highlight its role in abiotic stresses. Through comparative analysis we recognized 3 very similar TFs i.e. *HvMYB1, ZmMYB38* and *AtMYB4*.

These TFs have played key role against different stresses; such as drought, salinity, stress-induced phenylpropanoid metabolism, lignin and charge particles. (Fornalé *et al*., 2014; Franken *et al*., 1994; Wissenbach *et al*., 1993). The similar TFs have a role in abiotic stresses on one side, while on other side it is having a very important role in flavonol biosynthesis and Stress-Induced Phenylpropanoid Metabolism (Cavallini *et al*., 2015). Through comparative analysis it is evident that the *BeMYB140* is having very important role in abiotic stresses as extensively studies in our research work. From current research it is evident that *BeMYB140* is significantly expressed when treated with different abiotic stresses (ABA, PEG, NaCl, $H_2O_2$, and $Na_2So_4$). The MYB family has always been well known for abiotic stresses.

From sub-cellular localization experiment of *BeMYB140-GFP* we can clearly observe the fluorescence in the nucleus of the onion epidermal cells. (Fig. 4) our results are consistent with previous studies of subcellular localization (Mofatteh and Bullock 2017). The method of Y1H screenings allows the transfer of full length *BeMYB140- pEG202* for its transactivation analysis. From Y1H method we attained a blue color because of protein-protein interaction. The results were obtained which are very much in line with the results previously achieved by (Breton *et al*., 2016). The over expression of *BeMYB140* against different chunks of abiotic stress clearly highlights its role against abiotic stresses. The outcome of salt induced stress proposed that the *BeMYB140* is involved in retorts to salinity, which is in covenant to the networks of transcription regarding abiotic stress responses in Arabidopsis, (Duan *et al*., 2014). *AtMYB77* is having low expression against ABA (Jaradat *et al*., 2013); the same results has been achieved in our research i.e. the down-regulation with high significance of *BeMYB140* to ABA with time interval. While with *BeMYB140* is positively regulated when treated with $H_2O_2$ as already been described by (Borevitz *et al*., 2000). *TaMyb5* is greatly involved against PEG (Chen *et al*., 2005) as described in our research work. Form our research it is obvious that *BeMYB140* is a key TF; which have the potential to counter abiotic stresses.

## Conclision

*BeMYB140* was cloned from *Bambusa emeiensis*; after that, it was sent to company for sequencing and confirmation. Latterly the sequence was registered in NCBI database with GenBank accession number MG763923. The trans-activational analysis confirms the expression of *BeMYB140* in the nucleus of onion cells and blue colour from Y1H experiment confirms the activation of *BeMYB140*. From comparative analysis we can suggest that *BeMYB140* will have a major role in defense against abiotic stresses, fibre quality improvement and plants metabolism. From the application of different stresses; it is observed that the *BeMYB140* is up-regulated i.e. PEG, NaCl and $H_2O_2$, while on the application of ABA and $Na_2SO_4$ the down-regulation was followed by up-regulation. The current research offers an innovative understanding about the role of *BeMYB140* in abiotic stresses; especially the transactivation analysis shows its active role in transgenic plants.

## Acknowledgments

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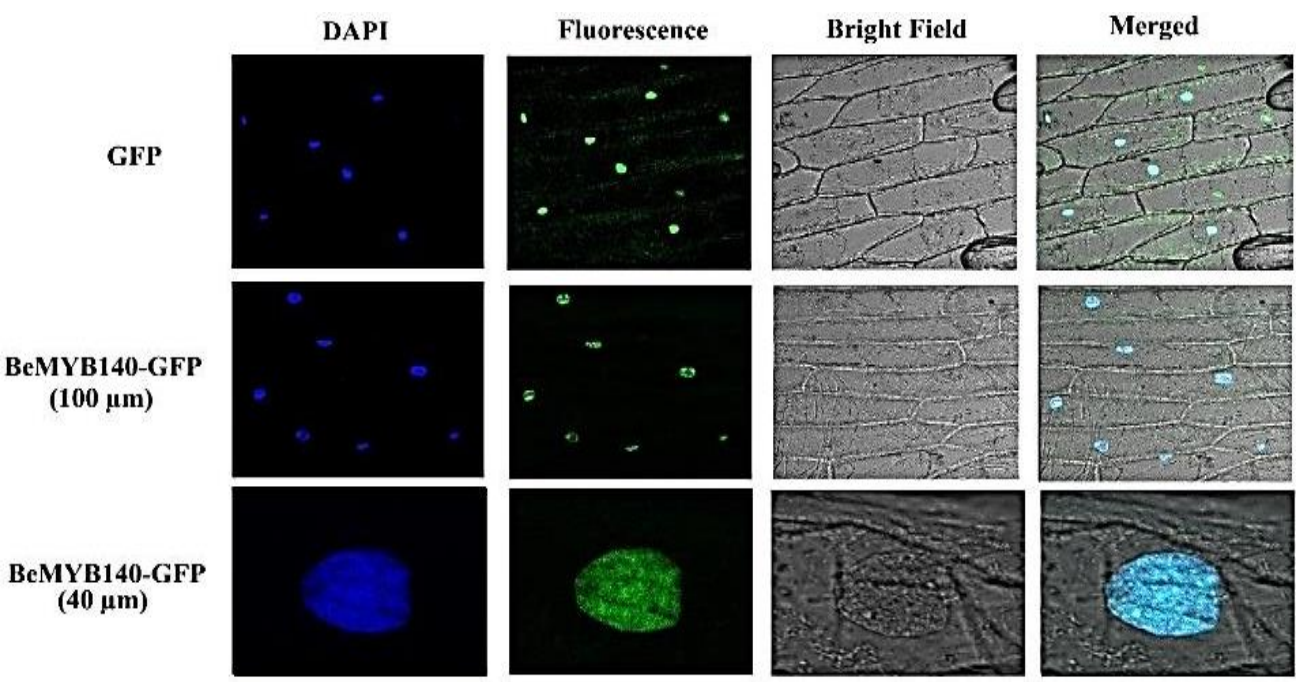


Fig. 3. For subcellular localization onion epidermal cells were used for the *pTEX-BeMYB140–GFP* through bombardment. The *pTEX-BeMYB140-GFP* and empty vector *pTEX-GFP* were applied to onion epidermal cells; which were afterward analyzed through a fluorescent microscope.

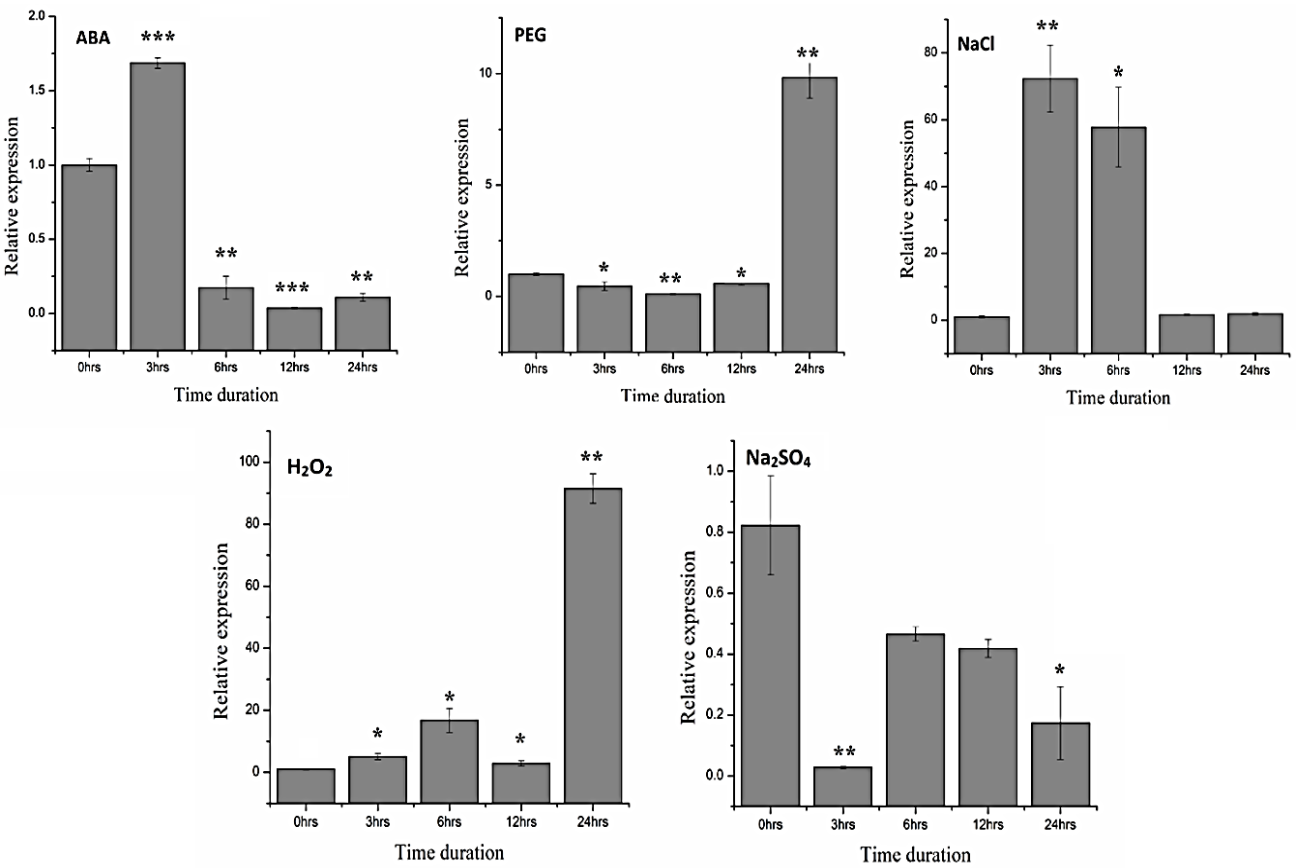


Fig. 4. The expression profiling of *BeMYB140* under different abiotic stresses i.e. ABA, PEG, NaCl, $H_2O_2$, and $Na_2SO_4$. The analysis was carried through qRT-PCR. Data are means ± SD calculated from three replicates. The bar indicates standard deviation. Asterisks indicate the significance level after abiotic stresses were applied at different time interval. (**$p$<0.05; ***$p$<0.01; ****$p$<0.001).

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