



Isolation and functional validation of stress tolerant *EaMYB18* gene and its comparative physio-biochemical analysis with transgenic tobacco plants overexpressing *SoMYB18* and *SsMYB18*

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Abstract

MYB transcription factors are one of the most important mediators for the survival of plants under multiple stress responses. In the present study, *EaMYB18*, encoding a single R3 repeat MYB DNA binding domain was isolated from stress-tolerant wild relative species of sugarcane *Erianthus arundinaceus*. In silico analysis of 948 bp coding mRNA sequence of *EaMYB18* exhibited the presence of four exons and three introns. Further, the *EaMYB18* gene was transformed in tobacco and its stable inheritance was confirmed through antibiotic resistance screening, PCR amplification and Southern hybridization blotting. Results of the estimation of MDA, proline, total chlorophyll and antioxidant activities of *EaMYB18* transgenic tobacco lines exhibited least oxidative damage under drought and cold stress over the untransformed ones, the over-expression of *EaMYB18* has improved drought and cold stress tolerance ability in tobacco. The comparative physiological and biochemical analysis of transgenic tobacco plants overexpressing *SoMYB18*, *SsMYB18* and *EaMYB18*, revealed that the *EaMYB18* and *SsMYB18* transgenic plants demonstrated effective tolerance to drought and cold stresses, while *SoMYB18* showed improved tolerance to salt stress alone. Amongst these three genes, *EaMYB18* displayed the highest potential for drought and cold stress tolerances as compared to *SoMYB18* and *SsMYB18* genes.

Keywords Abiotic stress · MYB · Sugarcane · Transcription factor · Tobacco

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Introduction

Abiotic stress affects plants at different levels by reducing the CO₂ assimilation rate, hampering stomatal opening, water potential, transpiration rate, plant growth rate, and leaf cell size (Zhu 2002). Abiotic stresses (drought, soil salinity, and temperature extremes) that cause the depletion of cellular water content, are the major factors limiting the productivity of plants (Hare et al. 1998). Upon exposure to different stresses, many plant species protect themselves through dynamic physiological, biochemical, and molecular response mechanisms. This comprises of expression of stress-specific genes, enabling their survival under erratic environmental circumstances (Hare et al. 1998; Prabu et al. 2011; Pagariya et al. 2012; Agarwal et al. 2013; Chen et al. 2015). Numerous studies have reported the overproduction of reactive oxygen species (ROS) and hydrogen peroxide in response to drought and salt stress (Pagariya et al. 2012; Shingote et al. 2015; Mahajan and Yadav 2014; Movahedi et al. 2014). To prevent the cell injuries induced by such

ROS, cells are equipped with enzymatic scavenging systems such as ascorbate peroxidase (APX), glutathione reductase (GR), glutathione-S transferase (GST) and catalase (CAT) (Mahajan and Yadav 2014; Movahedi et al. 2014; Chen et al. 2015). A close relationship between the rate and extent of antioxidant activity concerning plant stress tolerance has been reported in many plant species (Sairam et al. 2005). Therefore, acquisitive stress tolerance in plants can be achieved through the improvement of its antioxidant system. The plant genotype plays a crucial role in these responses to environmental cues. The key regulatory genes, particularly transcription factors act as a nodal point in interweaving a complicated signal transduction network in plant adaptation to abiotic stresses (Rahaie et al. 2010; Sairam et al. 2005; Baldoni et al. 2013; Ambawat et al. 2013; Agarwal et al. 2013; Li et al. 2014a, b).

Previously, abiotic stress response studies in different plant systems have not only testified the expression of transcription factor and stress-responsive genes but also ingrained their role in protecting the plant cells from stress responses (Seo et al. 2009; Zhang et al. 2011; Ambawat et al. 2013; Agarwal et al. 2013; Li et al. 2014a, b; Chen et al. 2015).

Erianthus arundinaceus (*E. arundinaceus*) is a sugarcane wild relative species with excellent vigour and adaptability to water deficits and other environmental aberrations (Piperidis et al. 2000; Augustine et al. 2014). For accomplishing sustainable plant stress tolerance through genetic engineering, the identification, isolation and characterization of the novel stress-responsive genes from the wild gene pool are necessary. The MYB proteins belong to a large family of transcription factors and are functionally diverse in all eukaryotes. Copious MYB proteins have been previously reported in the sugarcane, *Arabidopsis*, rice, maize and cotton genomes (Rahaie et al. 2010; Baldoni et al. 2013; Agarwal et al. 2013; Chen et al. 2015), and most of these functions as transcription factors with varying numbers of MYB-DNA binding domain repeats (Dubos et al. 2010).

Drought and salt are the primary causes of lower cane yield in rain-fed and saline soil confined areas of India as well as other countries across the globe. *Erianthus* sp. has the mechanisms to tolerate extreme drought conditions and they continue to their normal growth during the summer season, where the growth of commercial sugarcane cultivars ceases. In our previous studies, we have successfully isolated and functionally characterized the transgenic tobacco plants over-expressing orthologous MYB18 genes from sugarcane variety Co740 and *Saccharum spontaneum*, a wild relative species of sugarcane. Physiological and biochemical estimations of stable transgenic tobacco plants over-expressing *SoMYB18* and *SsMYB18* genes have exhibited high tolerance to salt and drought stress respectively (Shingote et al. 2015, 2016). In expectancy, to find a more effective multiple

stress tolerance gene from the C4 plant species, an ortholog MYB18 transcription factor gene of *E. arundinaceus* was isolated and functionally characterized in transgenic tobacco plants accompanied by its comparative physio-biochemical analysis with formerly reported MYB18 genes.

Materials and methods

Sugarcane wild relative species *E. arundinaceus* was collected from Vasantdada Sugar Institute, Manjari (Bk), Pune, Maharashtra, India for the experimental work, and used to isolate and clone the genomic *EaMYB18* gene. Tobacco (*Nicotiana tabacum*) was used to generate *EaMYB18*-transgenic plants. All the reagents and chemicals used in the study were of high purity, analytical grade and procured from reliable sources.

Cloning and sequence analysis of *EaMYB18* family member

The genomic DNA from sugarcane species *E. arundinaceus* was isolated by the CTAB method (Kale et al. 2017). A pair of primers reported in our previous study MYB18 FP and MYB18 RP were used to amplify full-length MYB18 gene (Shingote et al. 2015; Table S1). The obtained *EaMYB18* sequence was analyzed using different in silico tools to explore conserved regions in the gene sequence. The open reading frame (ORF) was defined by FGENISH software and amino acid sequence alignment was obtained with the Clustal W2 program, then based on homologies the consensus R2R3 MYB DNA-binding domain were orderly grouped. The phylogeny was obtained using the Clustal W program and the neighbour-joining method (1000 bootstraps).

Generation of *EaMYB18* over-expressing transgenic tobacco plants

The full-length genomic sequence encoding *EaMYB18* gene was cloned into binary vector pBinAR between the restriction sites *KpnI* and *BamHI* under the control of CaMV 35S promoter with OCS terminator. The MYB18pBINKpnI_FP and MYB18pBINBamHI_RP primers were used for *EaMYB18* amplification (Table S1). In vitro grown tobacco plants (1-month-old) were used for transformation using *Agrobacterium tumefaciens* (LBA4404) containing pBinAR::*EaMYB18* as per standard procedure (Horsch et al. 1985).

Screening of *EaMYB18* putative transformed plants

The transformed plants were selected on MS (Murashige and Skoog 1962) medium containing 50 mg L⁻¹ of Kanamycin.

After rooting and acclimatization, the regenerated plants were grown in a greenhouse to set seeds by self-pollination. The seeds of transgenic T₁ plant lines were selected on MS medium containing 50 mg L⁻¹ Kanamycin. Resistant plants were transferred to a close greenhouse, allowed to produce seeds and used in further analyses. For polymerase chain reaction (PCR) studies, genomic DNA was isolated from in vitro developed plantlets of both control (untransformed) as well as *EaMYB18* transgenic lines. Integration of the transgene was screened by PCR, and Southern blotting using *EaMYB18* gene-specific primers (Table S1). The transgene expression of Southern positive *EaMYB18* transgenic lines was confirmed through reverse transcription PCR (RT-PCR) analysis as per the method given by Shingote et al. (2016). The *EaMYB18* primers were designed according to the FGESH predicted mRNA sequences of the *EaMYB18* gene to encompass the ORF from start to stop codon. The PCR products were separated on 1% (w/v) agarose gel.

Abiotic stress treatments of untransformed and *EaMYB18* transgenic tobacco

Seeds of *EaMYB18* expressing transgenic and untransformed tobacco plants of T₂ generation were sterilized and germinated on medium containing 50 mg L⁻¹ kanamycin. For the salt and drought treatments 2–3-week-old plants were treated with 200 mM NaCl and 4% polyethylene glycol (PEG-8000) incubated at 25 ± 2 °C under 16 h light/8 h dark, respectively. Leaf tissues were collected at 0, 6, 12, 24 and 48 h sampling points. For the cold treatment, transferred 2–3-week-old plants from 22 °C to 4 °C and harvested the samples at 0, 6, 12, 24 and 48 h sampling points. Untransformed plants were kept under the ambient conditions. All the treatment experiments were performed in triplicates.

Measurement of the malondialdehyde (MDA), proline and total chlorophyll contents

Following the treatment, the seedlings were harvested for the measurements of physiological parameters. All the measurements were repeated three times. The proline and MDA contents were estimated as described by Shingote et al. (2015). Chlorophyll content was measured spectrophotometrically according to the method of Liu et al. (2011).

Enzyme extraction

Enzyme extraction was carried out with few modifications in Mahajan and Yadav's (2014) protocol. Total protein concentrations were measured by the spectrophotometric method previously described by Bradford (1976) using BSA as standard. The crude supernatant was then immediately used for CAT, peroxidase (POX) and superoxide dismutase

(SOD) assays according to the method given by Mahajan and Yadav (2014) and Liu et al. (2011). The CAT, POX and SOD activities were determined spectrophotometrically and were expressed in U mg⁻¹ protein min⁻¹. All the measurements were made in triplicate until and unless stated, and values are expressed as a mean ± SEM.

Results and discussion

PCR amplification, cloning, and sequencing of *EaMYB18*

The genomic *EaMYB18* gene was PCR amplified from the genomic DNA of *E. arundinaceus* using MYB18 primers (Table S1). The reaction conditions were optimized to obtain a single band of 1652 bp length (Fig. S1). The amplified fragment was then cloned in pGEMTM-Easy vector (Promega) and sequenced. The full-length *EaMYB18* sequence was subjected to different analyses using bioinformatics tools to explore conserved regions within the gene sequence.

Phylogenetic and in silico characteristics of *EaMYB18*

BLAST results indicated that the isolated sequence belongs to MYB genes showing homologies with the existing *MYB18* sequences. The full-length genomic *EaMYB18* (Accession no. MN907120) was 1466 bp in length from start to stop codon with 62 bp 5'UTR and 124 bp 3'UTR region, respectively. This genomic sequence of 1466 bp consisted of four exons which were further separated by three introns (Fig. 1). SWI3, ADA2, N-CoR, and TFIIIB DNA binding domains spanned exons 1, 2 and 3, comprised a single MYB repeat domain. The FGESH predicted mRNA sequence of 948 bp encoded 315 amino acid proteins (Fig. 1b). The predicted amino acid sequence BLAST has showed 93% similarity with the available full-length MYB sequences from *Oryza brachyantha* [XP_006644076.1], *O. sativa* [NP_001054597.1], *Zea mays* [NP_001132070.1, ACF79741.1] and *Sorghum bicolor* [XP_002440559.1] (Fig. 2). *EaMYB18* gene has the characteristics of transcriptional activators; implying transcription, RNA processing, modification, cell division and chromosome partitioning. The known and predicted DNA binding residues and the SANT domains are important for the DNA binding activity of the *EaMYB18* protein. The highly conserved tryptophan (W) residues involved in the folding of the DNA binding domain, are also signified in the *EaMYB18* sequence (Fig. 3). An alignment analysis of conserved SANT domain using Clustal W program and neighbour-joining method exhibited the highest percent similarity coverage within the *Saccharum* complex *MYB18* group.

Fig. 1 Depiction of mRNA transcript architecture of *EaMYB18* gene. **(a)** Genomic DNA containing three introns (I) and four exons (E). **(b)** FGENESH predicted mRNA transcript

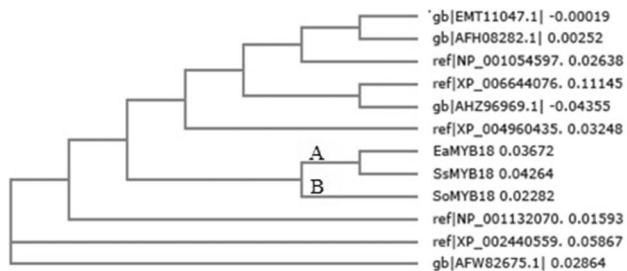
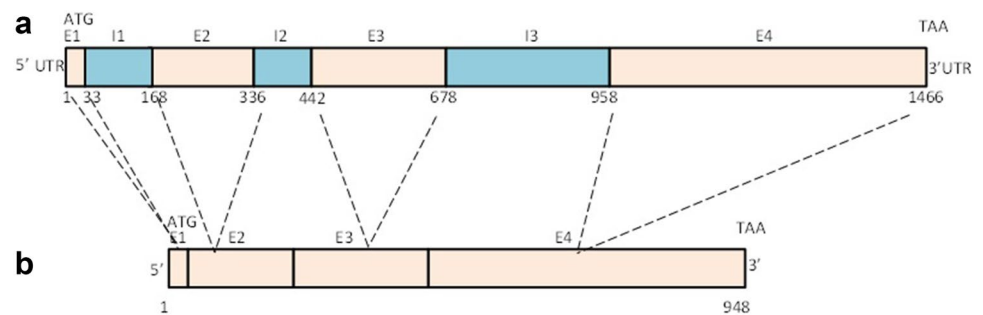


Fig. 2 Neighbour-joining tree of predicted MYB proteins. (The phylogeny was based on an alignment derived using the Clustal W program and the Neighbour-joining method (1000 bootstraps). The phylogeny with respect to the conserved SANT could be segregate the sugarcane MYB18 genes into 2 groups viz; A and B

In silico analysis of the *EaMYB18* sequence revealed a phylogenetic tree branched into a distinct monocot clade, which advocated that the sequence of *EaMYB18* belongs to monocot MYB transcription factors. The sequence information decodes that *SoMYB18* has 'type 2' while *SsMYB18* and *EaMYB18* have 'type1' MYB DNA binding domain. Being prime wild species of the *Saccharum* complex, *SsMYB18* and *EaMYB18* are evolutionary close to each other and are hence placed in the same clade. However, *SoMYB18* is placed slightly distant as it is a commercial sugarcane hybrid developed through the conventional breeding process and recombination events that might have also occurred during the breeding process (Figs. 1 and 3). The single-repeat MYB domain of *EaMYB18* gene is comprised of 42 amino acids and is similar as reported in *SsMYB18*. Furthermore, single repeat MYB proteins were also reported from other plant species signifying their importance in cellular processes (Baranowskij et al. 1994; Ganesan et al. 2012). The highest percent similarity coverage was illustrated amongst the *EaMYB18*, *SsMYB18*, and *SoMYB18* gene sequences, which revealed that they might have evolved from the same MYB family ancestor.

Development of transgenic tobacco lines over-expressing the *EaMYB18* gene

EaMYB18 gene was cloned in the pBinAR binary vector and the construct was confirmed by PCR, restriction

digestion and sequencing (Fig. S2). The *EaMYB18* recombinant pBinAR vector was transformed through *Agrobacterium*-mediated method and the positive plants were selected on kanamycin (50 mg L^{-1}) selection medium (Fig. S3). Out of the 48 putative transgenic tobacco plants, 45 plants were found to be PCR positive (Fig. S4). The Southern analysis confirmed the integration of the *EaMYB18* gene into the tobacco genome, where, T₀-6, T₀-12 and T₀-32 showed single gene integration while T₀-36 possessed double integration (Fig. S5). Three transgenic tobacco lines (T₀-6, T₀-12 and T₀-32) were hardened under greenhouse conditions. Based on kanamycin resistance, homozygous transgenic lines were selected for further analysis (Fig. S6). The T₂ generation *EaMYB18* expressing transgenic plants of T₀-6, T₀-12 and T₀-32 derived lines were used further for the functional analyses.

Performance of *EaMYB18* transgenic tobacco plants under drought, salt, and cold stresses

To confirm the role of *EaMYB18* in the regulation of stress tolerance, transgenic lines over-expressing *EaMYB18* were obtained in T₂ generation. Three single event transgenic tobacco lines T₂-6, T₂-12, and T₂-32 were further studied for the physiological and biochemical parameters under the stress conditions. The effect of *EaMYB18* transgenic plants was correlated with the changes in proline, MDA, and chlorophyll contents with respective untransformed plants under the stress conditions (Fig. 4, Table S2). Further, the activation of specific enzymes (SOD, POX and CAT) was determined for ROS. Amongst the three events, T₂-6 event has shown significant results and therefore comparative results of this event with untransformed plants were described and discussed in detail (Table S2, S3, and S5). The transgene expression of southern positive transgenic lines (T₂-6) was confirmed with the RT-PCR analysis. All the three lines showed expression of transgene (*EaMYB18*) under control, drought, salt, and cold stress conditions, whereas the expression was not detected in non-transformed tobacco plants (Fig. S7).

Fig. 4 Stress induction experiments of T₂ generation single copy *EaMYB18* transgenic tobacco plants (TT) and respective untransformed tobacco plants (UT). 4% PEG stress, 200 mM NaCl stress and 4 °C cold stress at 6 h, 12 h, 24 h and 48 h intervals

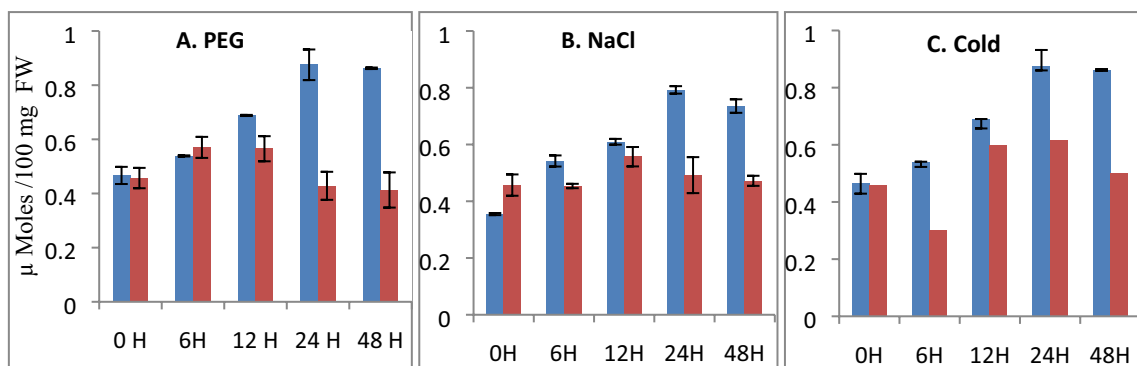
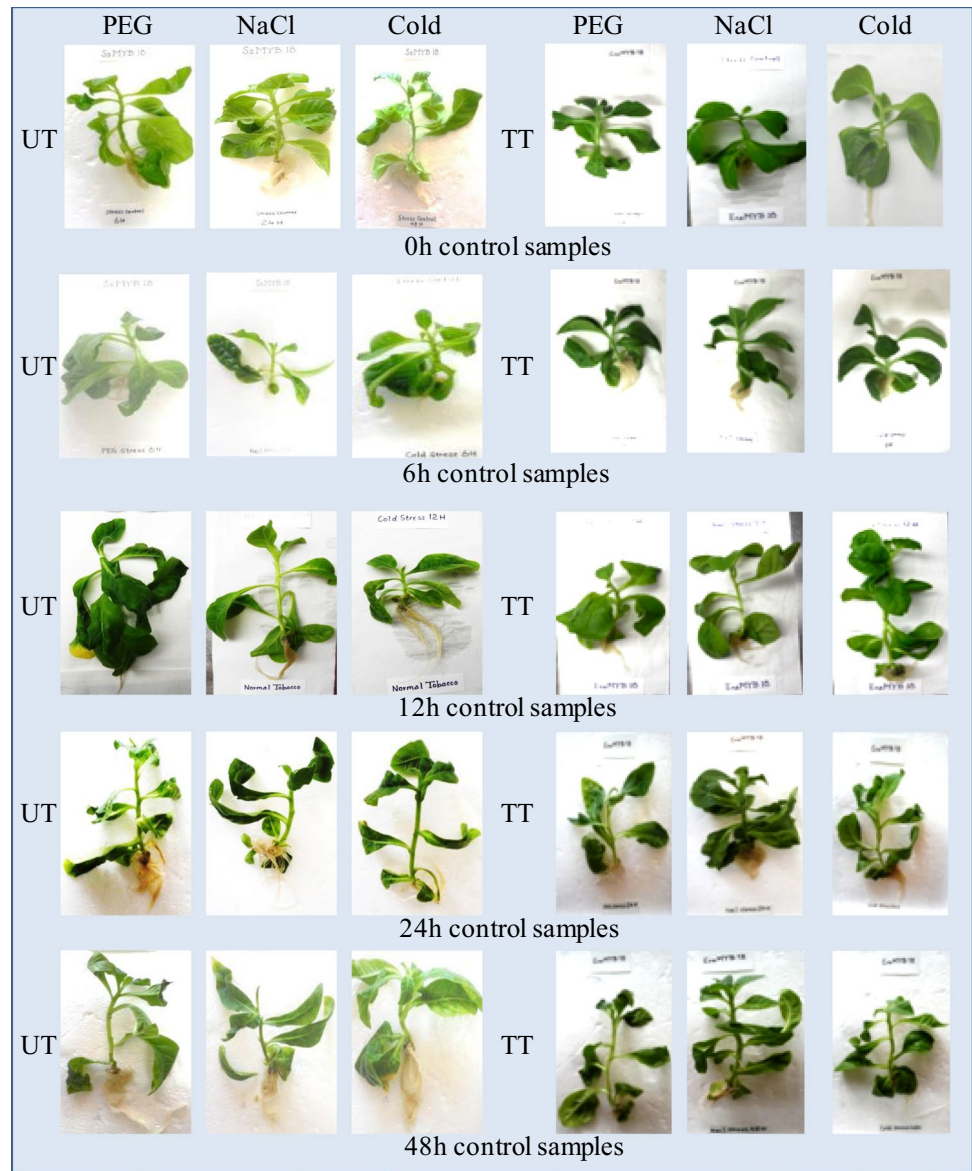


Fig. 5 MDA content of un-transformed tobacco (UT) versus *EaMYB18* over-expressing transgenic tobacco

plants (6 h and 12 h) as compared to untransformed ones (Fig. 6b). The proline content got abruptly increased in the untransformed tobacco at 24 h followed by a decline at 48 h than the transformed one, while the proline accumulation remained steady at 48 h under the NaCl stress. Upon exposure to cold stress, the proline content was higher in transgenic tobacco plants over the untransformed ones except at 12 h (decreased by 0.8 folds; Fig. 6c). In transgenic tobacco plants, the relative proline content showed an increment by 10 log folds at 6 h.

Total chlorophyll of *EaMYB18* over-expressing transgenic tobacco plants

During the initial exposure of PEG stress, the total chlorophyll content of transformed tobacco plants at 0 h and 6 h was 2% and 52% higher than that of respective untransformed plants, respectively. The relative difference in total chlorophyll content of *EaMYB18* transformed against the untransformed plants was not noteworthy for the rest PEG stress period (Fig. 7a). Upon exposure to salt stress, the total chlorophyll content of both transformed and untransformed

plants increased at 6 h (1.74 and 1.66 mg g⁻¹ fresh weight) and further declined by 18% at 12 h in transformed plants over the untransformed plants. Further, the chlorophyll content got reduced in untransformed tobacco plants at 24 h and 48 h, while it was 40% and 27% higher in *EaMYB18* over-expressing plants, respectively (Fig. 7b). Alike the salt stress, a similar trend was visualized under the cold stress. The total chlorophyll content increased in untransformed tobacco. The total chlorophyll content was higher at 0 h, 12 h, 24 h and 48 h under cold stress, while a 27% decrease was observed at 6 h with respective untransformed plants (Fig. 7c).

Over-expression of the MYB transcription factor gene in the heterologous plant system induces stress tolerance. Hence, a few MYB TFs are being used to transform drought and salt-sensitive crops to improve these traits (Agarwal et al. 2013; Li et al. 2014a, b). As indicative, biochemical determinants were analyzed in *EaMYB18* transgenic tobacco plants for examining the level of tolerance to drought, salt and cold stresses. MDA is widely recognized as a key biochemical component for lipid peroxidation (Muley et al. 2019) and hence a key major to gauge plant responses to abiotic stress (RoyChoudhury et al. 2007). The results of

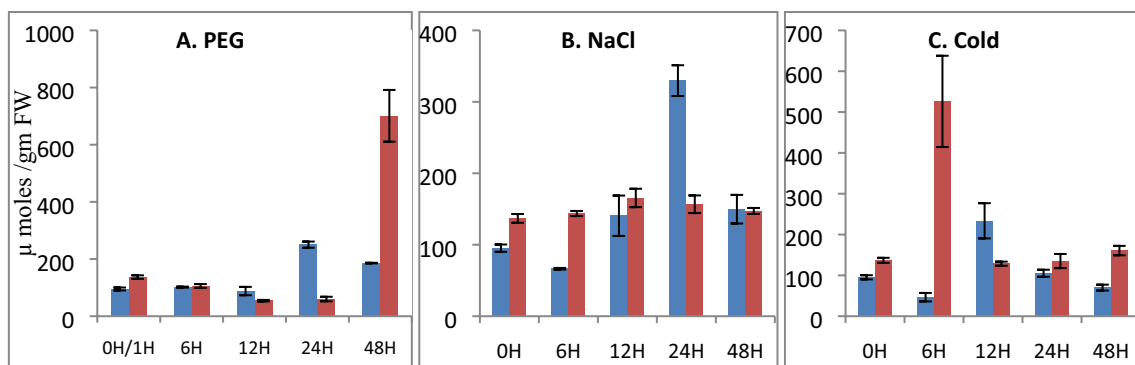


Fig. 6 Proline content of un-transformed tobacco (UT) versus *EaMYB18* over-expressing transgenic tobacco

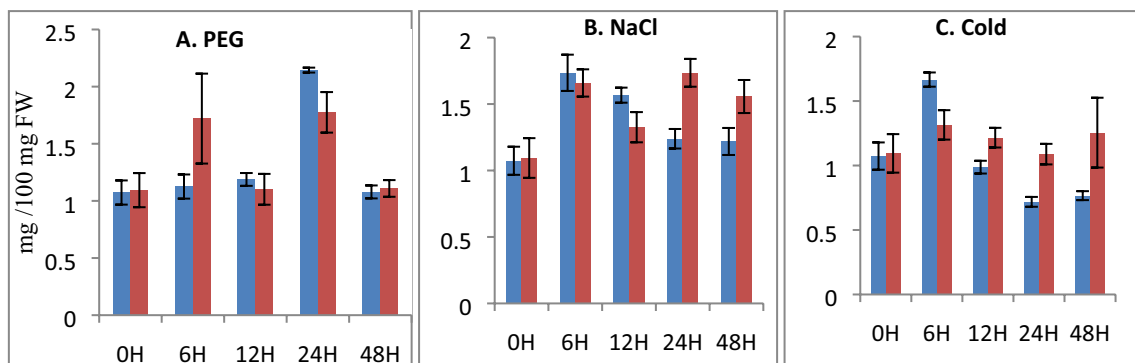


Fig. 7 Total chlorophyll content of un-transformed tobacco (UT) versus *EaMYB18* over-expressing transgenic tobacco. Bars represent mean values \pm SE ($n=9$). Bars represent mean values \pm SE ($n=9$).

Note: blue colour indicates untransformed tobacco, while red indicates transformed tobacco plants over-expressing *EaMYB18* gene

our study displayed a steady increase in the MDA content of untransformed tobacco plants, while it was comparatively steady in the transformed tobacco plants for the management of all three stresses. Moreover, analogous results have been reported in transgenic plants expressing stress tolerant genes in tobacco under abiotic stress (RoyChudhury et al. 2007; Wang et al. 2014; Mahajan and Yadav 2014). Initially, the MDA content increased in both *EaMYB18* transgenic as well as untransformed tobacco (6 h and 12 h) but it was remarkably stable in transgenic for further stress treatments (24 h and 48 h). The reason for this might be the plants were suddenly exposed to stress but over-expression of *EaMYB18* might have helped the transgenic tobacco in tolerating the auxiliary stress. In conclusion, less MDA accumulation under prolonged stress in *EaMYB18* transgenic tobacco plants exhibited less oxidative damage under the severe drought, salt, and cold stress conditions.

Accumulation of proline to counteract the effects of osmotic stress is one of the adaptive mechanisms for plant responses to stress conditions (Yang et al. 2012). In our studies, under PEG stress initially, the proline content was relatively less (6 h, 12 h, and 24 h) while the proline accumulation reached the highest peak at 48 h to 701.1 $\mu\text{mole g}^{-1}$ fresh weight (Table S2). Thus, supporting the osmotic adjustments of *EaMYB18* transformed tobacco plants at the utmost stress treatment. Other researchers have also reported a similar trend under different abiotic stresses by over-expressing *AtMYB2A* in *Arabidopsis* (Mao et al. 2011), *OsMYB3R2*, *OsMYB2* and *OsMYB4* in rice (Ma et al. 2009; El-Kereamy et al. 2012; Xiong et al. 2014). Under NaCl stress conditions the proline content was initially higher 116% and 17% in *EaMYB18* transgenic plants (6 h and 12 h) as compared to the untransformed. It was lesser in transgenic at 24 h and almost equal at 48 h (Table S2). Upon exposure to cold stress, the proline content was higher in transgenic tobacco plants than the untransformed plants except at 12 h (decreased by onefold; Table S2). A sudden increase in logarithmic proline content was observed in transgenic tobacco plants (1029%) at 6 h, and this might be an instantaneous response of the plant to unexpected cold shock. Nonetheless, it got declined upon further exposure to cold stress (81%). The highest proline accumulation was reported in transgenic plants at 48 h under PEG stress, and this entails that *EaMYB18* might have played a crucial role in regulating proline biosynthesis genes under drought stress. However, the decreased osmotic potential in stressed *EaMYB18* transgenic was not completely consistent with their free proline contents. It may be due to (1) proline may act as an initial (salt) and late (PEG) signaling/regulatory molecule able to activate multiple responses, through the components of the adaptation process (Pagariya et al. 2012), (2) *EaMYB18* could be partially held accountable for the osmotic potential reduction through proline accumulation

(Mao et al. 2011), (3) The *EaMYB18* over-expression might have incited the cross-talk between different gene cascades of tolerance, resulting in less proline accumulation at early stress treatments.

Photosynthesis is a key energy source for plant metabolism is the primary processes to be challenged by biotic and abiotic stresses in plants (Liu et al. 2011; Muley et al. 2019). Analysis of photosynthetic performance suggested a crucial role of *EaMYB18* in scaling up pigment synthesis at the initial stress period but incapable to do so under prolonged drought stress (Fig. 7a). Upon exposure to salt and cold stress, the total chlorophyll content of transformed plants was higher at 24 h and 48 h whereas a relative decline was reported in untransformed plants (Fig. 7b). Several researchers have previously reported a clear effect on leaf pigment contents, normal physiology and entire metabolic balance of plant under the stress conditions which were pertinent to our findings as well (Liu et al. 2011; Mao et al. 2011; Ganesan et al. 2012). The *EaMYB18* could have contributed significantly towards the inhibition of the chlorophyll degradation under salt and cold but not under the drought stress.

Effect of *EaMYB18* over-expression on the antioxidant system of transgenic tobacco plants

It has been reported that plants have an array of antioxidant defence enzymes to scavenge excess ROS and maintain cellular ROS homeostasis (Muley et al. 2019). We have evaluated CAT, POX and SOD activities of transgenic tobacco plants exposed to PEG, NaCl and cold stress to ascertain the role of *EaMYB18* in escalating the activities of antioxidant enzymes.

Under normal conditions, the activity of CAT was lower in transformed plants than the untransformed tobacco plants (3 folds). As shown in Fig. 8a, the activity of CAT increased profoundly in *EaMYB18* transgenic tobacco plants (160% higher) at 6 h PEG stress, while it slightly decreased to 38% at 12 h further followed by an increment of 177% and 185% at 24 h and 48 h than the respective untransformed plants (Table S3). Similar to PEG stress, under the NaCl stress, the CAT activity increased radically at 6 h (306%) in *EaMYB18* transformed tobacco, and it was higher at 12 h and 24 h by 25% and 9% than the untransformed tobacco, respectively, but it decreased slightly at 48 h (Fig. 8b). Under cold stress, the CAT activity in *EaMYB18* transformed tobacco plants were consistently higher than the respective untransformed plants by 212%, 205%, 80%, and 228% at 6 h, 12 h, 24 h, and 48 h, respectively (Table S3; Fig. 8c). Under all three stresses, the activity of CAT in the transgenic tobacco plants was highest at the initial stress (6 h) period proposing that *EaMYB18* could have played a central role in mitigating the

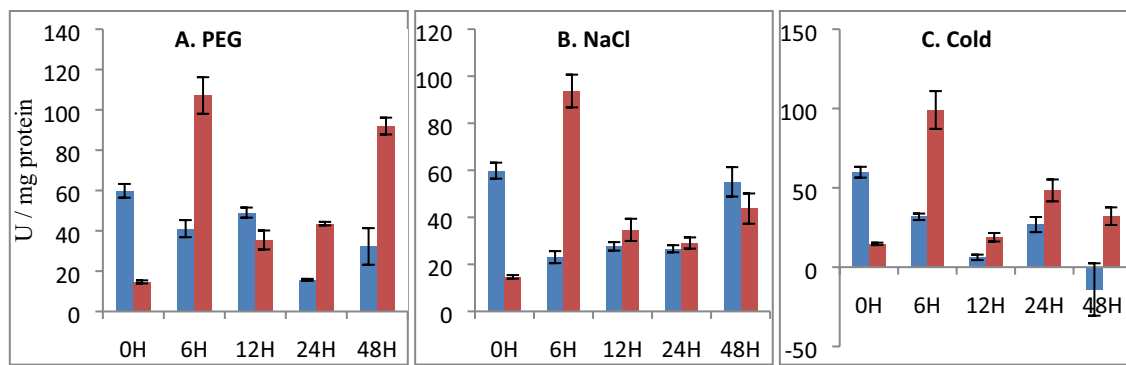


Fig. 8 CAT activity of un-transformed tobacco (UT) versus *EaMYB18* over-expressing transgenic tobacco

stress by regulating the ROS scavenging mechanism of the plant.

A noticeable elevation in the activity of POX was recorded in all the stress samples with a slight decline at 24 h, under all the PEG stress treatments (Fig. 9a). The increase was 415% at 6 h, 152% at 12 h and 147% at 48 h, while 26% reduction at 24 h was witnessed in *EaMYB18* transformants concerning untransformed plants (Table S3). Under NaCl stress, the POX activity was 69% higher at 12 h as compared with the untransformed plants, but it got diminished in other samples (Fig. 9b). An identical trend was recorded for POX activity during the cold stress. The relative POX activity was 1.2 folds higher at 12 h, while it got reduced by almost 30% in all other transgenic tobacco plants (Fig. 9c).

Initially, the SOD activity was considerably higher (18 folds) in transgenic tobacco plants as compared to the untransformed ones (Fig. 10). At 6 h PEG stress, the SOD activity of transgenic plants was 4.3 folds higher whilst a trivial relative difference was observed during the further sampling points (0.7, 0.08 and 0.6 folds). Upon exposure to the salt stress, the percent relative SOD activity was significantly elevated by 620%, 83%, and 308% at 6 h, 12 h

and 48 h in transformed than the untransformed plants, respectively, however a decline of 17% was observed at 24 h (Table S3, Fig. 10b). SOD activity during cold stress followed an identical trend like drought stress. The general trend inferred that *EaMYB18* over-expressing plants possessed relatively higher SOD activity (121%) at 6 h, while a sharp decline by 248%, 226%, and 9% at 12 h, 24 h, and 48 h, respectively (Fig. 10c) over the untransformed plants.

In consensus to our studies, tobacco plants over-expressing mangrove species R3-MYB transcription factor exhibited enhanced tolerance to abiotic stress intermediated through the elevated levels of ROS-scavenging enzyme activities was reported by previous studies (Ganesan et al. 2012). An impulsive increase in CAT activity at 6 h under drought conditions in our studies can be allocated to the plant's spontaneous confront to unexpected stress, while a further decline at 12 h can be ascribed to the failure of first-line defence of plants system to cope up with the stress. Further, an increase in the CAT activity at later stages of drought stress (24 h and 48 h) and higher CAT activity at all the stages except at 48 h under salt stress perhaps was the consequence of *EaMYB18* mediated activation of second-line of defence. In our studies, the CAT activity of *EaMYB18*

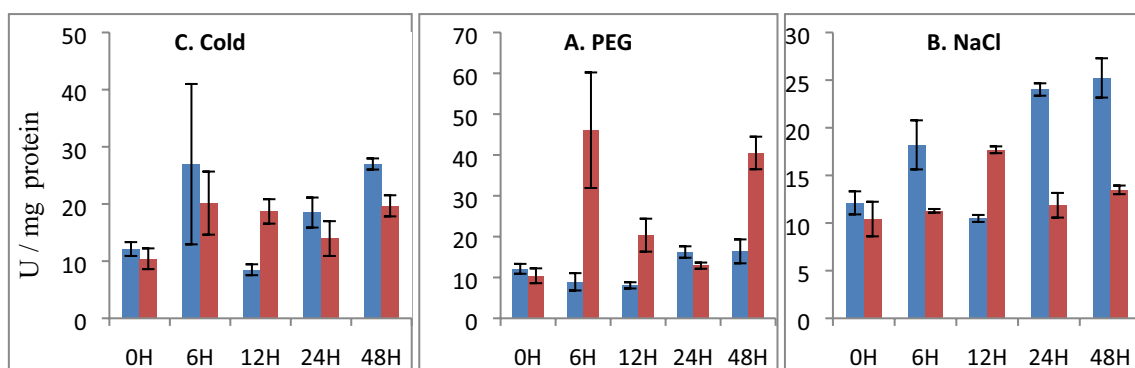


Fig. 9 POX activity of un-transformed tobacco (UT) versus *EaMYB18* over-expressing transgenic tobacco

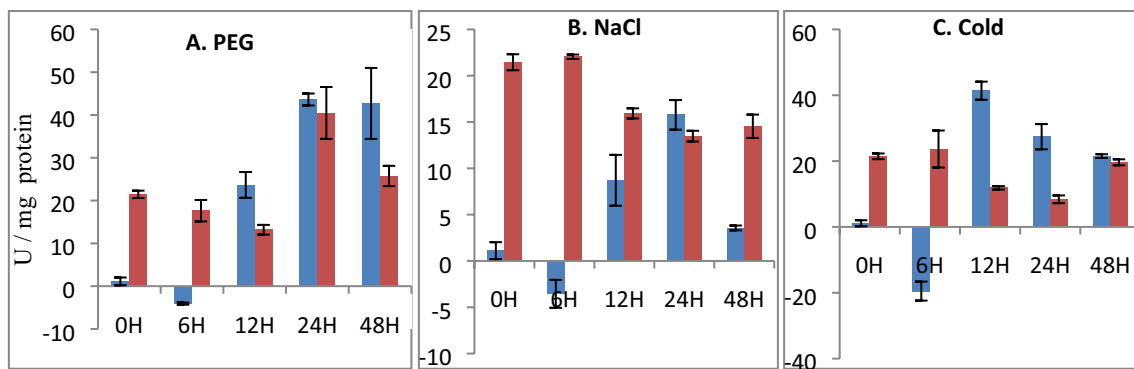


Fig. 10 SOD activity of un-transformed tobacco (UT) versus *EaMYB18* over-expressing transgenic tobacco. Bars represent mean values \pm SE ($n=9$). Note: blue colour indicates untransformed tobacco, while red indicates transformed tobacco plants over-expressing *EaMYB18* gene

transgenic plants was significantly higher than the respective untransformed plants under drought, salt and cold stresses (Fig. 8). Similar findings were reported by Yang et al. (2012) and Movahedi et al. (2014) in rice and poplar, respectively. Besides, a few recent studies also reported enhanced CAT activity in transgenic tobacco expressing rice *Rab16A* and *Zea mays* *ZmMPK5* genes (RoyChoudhury et al. 2007; Zhang et al. 2014a, b).

POX plays an essential role in scavenging ROS and protecting cells in higher plants, by consuming H_2O_2 (Gill and Tuteja, 2010). The higher POX activity in PEG stressed transgenic lines could be due to the over-expression of *EaMYB18* triggered up-regulation of defence responsive genes. Akin results were reported by Chen et al. (2015) and Li et al. (2015) while expressing *GbMYB5* and *SpWRKY1* genes, respectively, in tobacco plants under the drought stress. The POX activity was higher in transgenic lines under both salt and cold stress at 12 h only delineating that *EaMYB18* may not have a significant role in regulating the POX activity at other stress intervals. The SOD activity in both drought and cold stress was 18 folds higher at 0 h and 6 h in *EaMYB18* transgenic tobacco plants while the relative difference in further sampling points was nonsignificant (Fig. 10). Upon exposure to salt stress, the relative percent SOD activity of transformed plants was significantly elevated in all sampling points except at 24 h (Fig. 10B). Thus, hinting that *EaMYB18* has a role in upregulating the SOD activity under prolonged salt stress, whilst it has an initial contribution only at early stress regulation in drought and cold stresses.

POX and CAT functions in the elimination of H_2O_2 and converts it to water, while SOD is an important enzyme in catalyzing the ROS (Muley et al. 2019). In our results, it was found that the *EaMYB18* over-expressing plants showed tolerance to all three stresses through the collective activities of these enzymes. Although a relative percentage of CAT and POX activities of *EaMYB18* transgenic were noted, a

variable decline concerning an increase of stress period, while a notable increase was observed in the SOD activity in comparison with the respective control plants (Table S3).

In conformity, the results concluded that the joint activity and inter-correlation between these enzymes might have an important role in preventing the formation of ROS and achieving stress tolerance. The balanced regulation in the activities of these enzymes can be induced by ROS directly or indirectly (Pagariya et al. 2012). In contrast, few researchers have stated that ROS enzyme activities were closely related to stress tolerance of many transgenic tobacco and *Arabidopsis* plants (Ramegowda et al. 2012; Mahajan and Yadav 2014; Zhang et al. 2014a, b). These results anticipated that *EaMYB18* over-expression has played an important role in balancing the combined effects of CAT, POX, and SOD to combat or alleviate the stress damage in plants.

Taken together, our results suggested that over-expression of *EaMYB18* in tobacco enhances the osmotic tolerance by decreasing the lipid peroxidation under PEG, salt and cold stress conditions. The *EaMYB18* over expression might have triggered the stress tolerance through a different cascade of genes that resulted in relatively less proline accumulation at initial stress treatments while higher accumulation upon extended drought and cold stress (48 h). The findings suggested the participatory role of *EaMYB18* through increased synthesis of pigments at the initial drought stress period. This hinted the effective role of MYB TFs in the maintenance of plant metabolism thereby preventing chloroplast deformation due to salt and cold stress, respectively. Enzyme assay results projected that the over-expression of *EaMYB18* could have collectively regulated the CAT, POX and SOD activities and thus resulted in attaining stress tolerance of transgenic tobacco plants. Chen et al. (2015) reported akin results in transgenic tobacco over-expressing *GbMYB5* and reported enhanced accumulations of CAT, POX and CAT enzymes with reduced production of MDA in transgenic tobacco under drought stress.

Comparative effect of *EaMYB18*, *SsMYB18* and *SoMYB18* during salt, drought and cold stresses

A detailed characterization of *SoMYB18* and *SsMYB18* transcription factors were reported in our previous findings (Shingote et al. 2015, 2016). For evaluation of the best potential MYB18 ortholog, we have compared the performance of these three transcription factors under different stress conditions. The sequence analysis showed that *SoMYB18* was 1557 bp member of the R2R3-MYB subfamily containing two MYB DNA binding domains (R2, R3) and a SANT/MYB DNA binding domain, whereas *SsMYB18* and *EaMYB18* contain single-repeat R3 MYB domain with 1470 and 1466 bp in length, respectively (Table S4). The *EaMYB18* and *SsMYB18* configuration were similar to that of other R3 MYBs formerly reported by Baranowskij et al. (1994) and Ganesan et al. (2012). Besides, all three genes have variable numbers and sizes of introns and exons with a significant difference at the mRNA level. The DNA binding domains of all three transcription factors were as: *SoMYB18* had two SANT (R2-R3), CARD, ARM and CLECT; *SsMYB18* comprised of single SANT (R3), REB1 and PLN03091; and *EaMYB18* contained a single SANT (R3) and PLN03091, respectively (Table S4). Thus, there was a predictable difference in all three *MYB18* gene sequences at genomic, mRNA and protein/domain level isolated from sugarcane and its wild relative species. This intimated that there could be a difference in their DNA binding ability to different regulatory elements entailing a diverse ability to combat drought, salt, and cold stresses, respectively.

We evaluated the best-performing transgenic events each of *EaMYB18*, *SsMYB18* and *SoMYB18* through a relative percentage change in the biochemical parameter of transgenic over the respective transformed tobacco plants as represented. The improved stress tolerance of all three *MYB18* transgenic plants was correlated with changes in the MDA, proline, and total chlorophyll contents (Figs. S8–S10). Although these changes are not the only way through which plants cope with the environmental abrasions but these physiological changes are considered as important indicators of plant tolerances to stress.

MDA is an important parameter related to responses of plants to abiotic stress and widely recognized as a parameter for membrane stability index (RoyChoudhury et al. 2007). Under drought stress, the relative percentage of MDA content was trivial but with an increase in stress period, there was a slight increase in *EaMYB18* and *SoMYB18* transgenic plants at 6 h followed by a significant decline at 12 h (Fig. S8). The comparative decrease in the relative percent of MDA content was most significant in *EaMYB18* (51% and 52%) at 24 h and 48 h, respectively (Fig. S8). The relative percentage of MDA got reduced in the *SsMYB18* transgenic and found to be stable throughout the sampling period.

Under salt stress regime relative MDA percentage in all the three *MYB18* transgenics got significantly declined till 24 h of the stress period. It was almost the same in all three transgenics, and an approximate decline of around 37% whilst the highest decline of 51% was noted at 48 h in *SsMYB18* transgenic lines than the un-transformed plants, respectively. Upon exposure to cold stress, the relative percent MDA of all three transgenic lines got decreased and it was most significant in *EaMYB18* transgenic (– 41%) at 48 h (Fig. S8). These results inferred that *EaMYB18* and *SsMYB18* transgenic lines exhibited the least oxidative damage under the severe drought and cold stress conditions than the *SoMYB18* transgenic lines. Whereas, *SoMYB18* has shown significantly less oxidative damage under only salt stress and moderate under drought and cold stress.

Proline is one of the most common osmolytes in plants and its accumulation is associated with responses of plants to the external stress conditions (Zhang et al. 2014a, b). When the plants were exposed to PEG stress, at the initial period (6 h) the relative percentage of proline content was significantly higher in the *SoMYB18* transgenic plants. Under the salt and cold stresses, it was considerably higher in *EaMYB18* transgenic plants (Fig. S9). During the extended stress regime, it was higher in *EaMYB18* transgenic under PEG and cold stress whilst it was highly significant (almost 600%) in *SoMYB18* under salt stress. Consequently, *EaMYB18* transgenic showed an accumulation of more amount of proline under the prolonged drought and cold stress, while *SoMYB18* in salt stress only (Fig. S9).

Adaptations in total chlorophyll content are also reported as important cues of plant response to its growth and stress situations (Li et al. 2014a, b; Muley et al. 2019). After exposure to drought stress the *EaMYB18* transgenic demonstrated a lesser amount relative percentage of chlorophyll content than the *SsMYB18* and *SoMYB18*, respectively (Fig. S10). The relative percent of chlorophyll content was most significant in *SsMYB18* transgenic under salt and cold stress, whereas for *SoMYB18* it was significant under the salt stress only. These results endorsed the vital role of *EaMYB18* and *SoMYB18* in up-regulating the synthesis of pigments only at the initial period but unable to do so under prolonged drought stress. On the other hand, *SoMYB18* regulated it effectively throughout the regime, *EaMYB18* regulated it moderately higher upon prolonged stress exposure (24 h and 48 h). Thus, *SsMYB18* most predominantly prevented the degradation of chlorophyll under drought and cold stress while *SoMYB18* under salt stress only (Fig. S10).

The relative percentage of CAT activity under all three stresses increased noticeably in *EaMYB18* and *SsMYB18* lines. Particularly at extreme drought stress period, it was highest in *EaMYB18*, while it was most significant in *SoMYB18* under salt and *SsMYB18* under cold stress, respectively. These results suggested that *EaMYB18* has a

significant role in the upregulation of CAT activity under drought, while *SoMYB18* and *SsMYB18* regulate CAT activity under cold stress, respectively (Fig. S11). Thus, it can be said that *EaMYB18* and *SsMYB18* could have probably played a role in developing drought and cold stress tolerance, and a moderate salt stresses tolerance by enhancing the CAT activity. Furthermore, *EaMYB18* was also seemed to be more effective in managing extended drought stress. *SoMYB18* upregulated the CAT activity under salt stress only and appeared as a promising candidate to manage salt stress (Fig. S11). The relative percentage of POX activity under drought stress was higher in *SsMYB18* and *EaMYB18* transgenic at all sampling points (except *EaMYB18* at 24 h). It was more significant in *SsMYB18* transgenic, and moderately higher in *EaMYB18* at the initial sampling point, while it was non-significant during all sampling points in *SoMYB18* transgenic plants. These results inferred that *EaMYB18* could have an involvement in the upregulation of the POX activity, but lesser as compared with *SsMYB18* under drought, salt, and cold stress, respectively, while under the same circumstances *SoMYB18* might not have significant role in up-regulating the POX activity (Fig. S12). Upon exposure to PEG, salt and cold stress, the relative percentage of SOD activity was higher in *SoMYB18* transgenic plants when compared with the *SsMYB18* and *EaMYB18* plants (Fig. S13). *SsMYB18* and *EaMYB18* transgenic plants have significant SOD activity only under salt stress while it was non-significant under drought and cold stresses (Fig. S13).

Conclusion

Taken together, overexpression of *EaMYB18* transcription factors might lead the changes in CAT, POX and SOD activities as revealed from the enzyme assay data. These metabolic changes can be assigned to over-expression of *EaMYB18* transcription factor which might have allowed plants for effective osmoregulation, less oxidative damage and combined balanced activity of ROS scavenging enzymes under stress conditions. The comparative physiological and biochemical analysis of transgenic tobacco plants overexpressing *SoMYB18*, *SsMYB18* and *EaMYB18*, revealed that *EaMYB18* and *SsMYB18* transgenic plants demonstrated effective tolerance to drought and cold stresses, while *SoMYB18* shown improved tolerance to salt stress alone. Amongst these three genes, *EaMYB18* seems to have more potential for drought and cold stress tolerances than *SoMYB18* and *SsMYB18* genes. Further identification of target pathway genes and unraveling signaling networks through which *EaMYB18* interacts to alleviate or combat the environmental stresses. It will provide further cues to improve the *EaMYB18* based stress tolerance of C3 and C4 crop species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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