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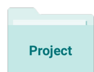

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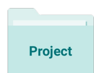

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RESEARCH ARTICLE

Ectopic Expression of *SsMYB18*, a Novel MYB Transcription Factor from *Saccharum spontaneum* Augments Salt and Cold Tolerance in Tobacco

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Abstract MYB transcription factors play imperative role in developmental and numerous defense processes in plants. In this study, functional characterization of *SsMYB18*, a single-repeat MYB transcription factor isolated from *Saccharum spontaneum*, a stress-tolerant wild relative species of sugarcane is reported. *SsMYB18* gDNA is 1470 bp in length with an open reading frame of 1074 bp having four introns and five exons, encoding 357 amino acids. The deduced amino acid sequence showed similarities to MYB proteins reported in other plants, including the conserved MYB-binding domain. *SsMYB18* is a new member of the MYB transcription factor subfamily. The *SsMYB18* gene was transferred in to *Nicotiana tabacum* by A*grobacterium*-mediated transformation. Subsequent screening through PCR and southern, transgenic tobacco lines harboring *SsMYB18* were identified and assessed for drought, salt and cold tolerance. Compared to non-transformed tobacco host plants, *SsMYB18* overexpressing plants demonstrated appreciably augmented tolerances to salt, cold and drought stresses. In these transgenic lines, the activities of antioxidant enzymes (SOD, POX and CAT) were notably increased virtually to non-transformed ones. The MDA content was less, and levels of chlorophyll and proline were also markedly high in *SsMYB18* overexpressed plants than that of non-transformants. The results suggested that transgenic tobacco plants constitutively expressing the single-repeat *SsMYB18* transcription factor showed high tolerance to salt and cold, while moderate to drought stresses as compared to controls. Besides *SsMYB18* transgenic tobacco plants showed advantage over its homolog *SoMYB18* by having additional high tolerance to cold stress rather than only to salt stress.

Keywords Single-repeat MYB TFs · SANT DNA domain · *Saccharum* complex · Abiotic stress

Abbreviations

CaMV 35S	Cauliflower mosaic virus promoter
CAT	Catalase
MS medium	Murashige and Skoog medium
NaCl	Sodium chloride
PEG	Polyethylene glycol
POX	Peroxidase
SOD	Superoxide dismutase

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Introduction

Abiotic stress like drought, high salinity and freezing adversely affects the plants growth and development, causing more than 50 % yield loss in major crops (Bray et al. 2000). As sessile organisms, plants had to adapt to these environmental aberrations through various

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biochemical and physiological mechanisms. In sugarcane, it is complicate to transfer the stress tolerance traits from wild relative species to cultivable through conventional breeding due to incompatibility of seed production and restrictions of inter species crossing. To overcome this, biotechnological tools are becoming convenient, as these allow incorporation of only the specific genes into an organism while restricting the transfer of undesirable genes from donor organism. Through transgenic, pyramiding of genes with similar effects can also be accomplished. Thus, detection and characterization of such genes that facilitate tolerance to multiple abiotic stresses provide a powerful method to engineer plants with enhanced tolerance to abiotic stress (Valliyodan and Nguyen 2006).

To cope with abiotic stresses, plants have developed complex regulatory mechanisms, which include the expression of stress-related genes. The teamwork among transcription factors and/with *cis*-acting elements is requisite for up-regulation of stress-related genes to combat or adapt stress conditions in plants (Grotewold 2008). In anticipation, much attention is focused on transcription factors, key components playing substantial role in regulation of physio-biochemical pathways to combat with different stresses (Du et al. 2012; Ambawat et al. 2013). Previous studies shown that copious plant MYB genes are occupied in response to diverse environmental stresses. Plenteous plant MYB proteins involved in responses to biotic and abiotic stresses have been portrayed having role in controlling of plant-specific processes using biotechnological approaches (Ambawat et al. 2013; Dubos et al. 2010). It has been reported that an Arabidopsis MYB protein up-regulated on exposure to environmental stresses (Mengiste et al. 2003). A definite role of TaPIMP1 as transcriptional activator during biotic and abiotic stresses has been validated (Liu et al. 2011). In transgenic, potato overexpressing StMYB1R-1 revealed improved drought tolerance through activation of HB-7, RD28, ALDH22a1 and ERD1-like drought combative genes (Shin et al. 2011). OsMYB2 is customary in imparting salt, low-temperature and water-deficit stress tolerance via regulation of downstream LEA3, Rab16A and DREB2A genes in rice (Yang et al.2012). OsMYB3R-2 overexpression in rice enhances cold tolerance and in Arabidopsis confers resistance to multiple abiotic stresses (Ma et al. 2009). In wheat, TaMYB1 and TaMYB2 plays active proline retort to abiotic stresses, involving TaMYB1 for salt and ABA stresses (Lee et al. 2007). A Wheat MYB TF, TaMYB2A, had reported enhanced tolerance to abiotic stresses in transgenic Arabidopsis (Mao et al. 2011). Zhang et al. (2012) validated salt stress tolerance of *A. thaliana* overexpressing TaMYB. AtMYB60 and AtMYB96 acted through the ABA signaling cascade to regulate drought stress tolerance (Cominelli et al. 2005; Seo et al. 2009).

Saccharum spontaneum, a sugarcane wild relative species possess tolerance to abiotic stresses (Grivet and Arruda 2001). In recent, past efforts were made to explore the molecular adaptation mechanisms to biotic and abiotic stresses and to reinforce stress tolerance in sugarcane (Gupta et al. 2009; Prabu et al. 2010; Prabu and Prasad 2011; Pagariya et al. 2011, 2012). Of late, 78 MYB transcription factor genes in sugarcane were identified by computational searches (Geethalakshmi et al. 2014). The sugarcane MYB transcription factor gene, ScMYB26/AS1, that was reported up-regulated in response to abiotic stress (Prabu and Prasad 2011). There are numerous studies of MYBs and promoters overexpression through agroinfiltration of tobacco demonstrating its potential for future advancement of stress tolerance trait through plant transformation (Prabu and Prasad 2011; Geethalakshmi et al. 2014; Kharte et al. 2016). In our recent study, a stress-inducible *SoMYB18* have been functionally characterized from stress-tolerant sugarcane var. Co740. Its role was characterized by physiological and biochemical estimations in stable transgenic tobacco plants, deciphered the higher tolerance to salt stress alone (Shingote et al. 2015). Thus, in higher plants, MYB gene family is of enormous importance in transcriptional control, since number of genes involved controls the plant-specific processes. However, MYB genes are meticulously studied in other crop species such as Arabidopsis, rice and maize, but studies in non-model plant, sugarcane and its wild relative species so far limited to transient agroinfiltration studies (Geethalakshmi et al. 2014). At this instant, analyzing the function of orthologs in wild relative species which are more stress tolerant is crucial for further understanding of the molecular insights in governing plant stress responses and in developing improved stress tolerance in sugarcane through genetic engineering. The aim of this study solely lies on functional portrayal of the novel *MYB18* gene isolated from stress-tolerant wild relative species of sugarcane in relation to abiotic stresses.

Materials and Methods

Plant Material, Isolation and Sequence Analysis of the *SsMYB18* gene

Saccharum spontaneum accession IS-76-192 was collected from VSI fields for the experimental work. A pair of primers MYB18pBIN*Kpn*I FP and MYB18pBIN*Bam*HI RP from our previous study of SoMYB18 was used to isolate full length of the MYB18 gene from *S. spotaneum* genomic DNA (Table 1). Genomic DNA was extracted, amplified and sequenced as per Shingote et al. (2015). Different bioinformatics tools were used to explore the full-length

Table 1 Primers used in this study

Sr no.	Primer name	Sequence 5′–3′	Expected size (bp)
1.	MYB18pBIN *Kpn*I FP	5′-CGG**GGTACC**CCGGCTTCGTGCTACT-3′	1657
	MYB18pBIN *Bam*HI RP	5′-CGC**GGATCC**GCGGATTTCTGTATCAACTTAATG-3′	
2.	35 S promoter sequencing primer	5′-CTATCCTTCGCAAGACCCTTC-3′	
3.	SsMYB18 S_F (southern)	5′-GATGCCCGTGTATGACAAGA-3′	457
	SsMYB18 S_R (short length)	5′-CTCGTGCTCTGTGGTTCAAA-3′	
4.	SsMYB18 FP (start codon)	5′-ATGGCTGCTGGAGTTCTGTTC-3′	1470
	SsMYB18 RP (stop codon)	5′-CTAGATATTCTCAAAAGACAGTTGCAT-3′	

sequence and swot their conserved regions. The ORF was delineated by FGENESH software. The sequence homolog explored using NCBI Blastn with default parameters (http://www.ncbi.nlm.nih.gov/blast/). Sequences alignment of MYB proteins was achieved using ClustalW program (http://www.ebi.ac.uk/clustalw/). Phylogenetic tree of MYB protein sequences for constructing phylogenetic tree was retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al. 2013).

Construction of Plant Transformation Vectors

PCR was performed with MYB18pBIN*Kpn*I FP and MYB18pBIN*Bam*HI RP primers (Table 1). The amplified fragments were cloned into pBinAR vector under control of a *Cauliflower mosaic virus* (CaMV) 35S promoter. The recombinant *SsMYB18::pBinAR* plasmid was confirmed through colony PCR, restriction digestion and CaMV35S primer sequencing. The resulting constructs were recombined into *Agrobacterium tumefaciens* strain LBA4404 by freeze–thaw method (Holsters et al. 1978) and then transformed in tobacco using the method reported in Mahajan and Yadav (2014). The transgenic plants were screened on MS medium containing 50 mg L^{-1} kanamycin.

Screening of Transgenics Through PCR and Southern

A pair of gene-specific primers SsMYB18FP-RP, enclosing start and stop codons (Table 1), was designed and used to screen the integration of the transgene in plant genome. For PCR studies, genomic DNA was isolated from control and *SsMYB18* transgenic lines. The PCR product was then separated on 0.8 % agarose gel.

The southern blot technique was used to determine the copy number of corresponding *SsMYB18* gene transformed into each transgenic line. The selected T_0 generation PCR positive transgenic tobacco plants were used for southern analysis. Un-transformed tobacco plants were used as controls. DNA gel blot analysis was performed according to the protocol described previously (Shingote et al. 2015). The SsMYB18 S (southern) short-length primers were used for probe synthesis (Table 1).

RT-PCR Confirmation of Transgene Expression in Transgenics Plants

The transgene expression of southern positive plants was carried out by reverse transcription (RT) PCR under salt, drought and cold stress conditions. For this, total RNA was extracted from the leaf of control and *SsMYB18* expressing transgenic tobacco lines using RNeasy plant mini kit (Qiagen). First-strand cDNA was synthesized from 1 µg of total RNA using SMARTScribe™ reverse transcriptase (Clontech) according to the supplier's instructions. This cDNA was used as a template for PCR amplification through *SsMYB18* gene-specific primers (start and stop codon).

Hardening and Development of Stable Transgenic Tobacco Plants Overexpressing *SsMYB18*

The single event southern positive *SsMYB18* overexpressing transgenic tobacco (T_0) lines were hardened and were grown in a greenhouse to set seeds by self-pollination. The collected T_1–T_2 seeds were germinated on 1/2 MS medium containing 50 mg L^{-1} kanamycin and screened through *SsMYB18* gene-specific PCR. Non-transformed tobacco plants were used as controls.

Tolerance of Transgenic Tobacco Plants to Drought, Salt and Cold

Seeds of T_2 *SsMYB18* southern positive single copy independent transgenic plants (TT) were sterilized and germinated on medium containing 50 mg L^{-1} kanamycin and non-transformed tobacco (NT) without kanamycin. The 30-day culture-grown whole transgenic plants and controls

Table 2 Homologous sequences to SsMYB18 obtained after BLAST

Sr no.	Homologous sequences	Query cover (%)	Ident (%)	Accession
1.	*Saccharum arundinaceum* myb18 gene for MYB18 transcription factor, exons 1–3	98	97	HF546406.1
2.	*Saccharum barberi* myb18 gene for MYB18 transcription factor, exons 1–4	98	95	HF546403.1
3.	*Saccharum elegans* myb18 gene for MYB18 transcription factor, exons 1–4	98	95	HF546405.1
4.	*Saccharum officinarum* myb18 gene for MYB18 transcription factor, exons 1–4	98	95	HF546401.1
5.	*Saccharum robustum* myb18 gene for MYB18 transcription factor, exons 1–4	98	95	HF546402.1
6.	*Saccharum bengalense* myb18 gene for MYB18 transcription factor, exons 1–3	98	94	HF546404.1
7.	*Saccharum* sp. GP-2012 myb18 gene for MYB18 transcription factor, exons 1–4	98	94	HF546407.1
8.	*Saccharum officinarum* cultivar Co740 MYB18 gene, complete cds	98	94	FJ560976.1

were conceded to stress in liquid MS media for drought (MS + 4 % PEG), salt (MS + 200 mM NaCl) and cold stress (held in reserve at 4 °C). During the course of stress treatments, both transgenic and control plants were retained under the identical condition at 21 ± 2 °C and 16-h light/8-h dark. Leaf samples were withdrawn for physiological and biochemical analysis at 0-, 6-, 12-, 24- and 48-h intervals from each set of stress treatment.

Determination of MDA, Proline and Chlorophyll Content

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) as a biomarker for oxidative stress. The amount of MDA present was calculated from the extinction coefficient of 15.5 mM^{-1} cm^{-1} using thiobarbituric acid (TBA) method. Proline and chlorophyll pigments of the leaf samples were assessed spectrophotometrically as described by Shingote et al. (2015).

Enzyme Extraction and Activity Assays

Enzyme extraction was carried out as described by Mahajan and Yadav (2014) protocol. The amount of total proteins was calculated by Bradford (1976) method. The unfinished supernatant was instantaneously used for SOD (EC 1.15.1.1) assays as described by Gay and Tuzun (2000) with minor alterations, and activity was expressed as U mg^{-1} $protein^{-1}$ min^{-1}. CAT (EC 1.11.1.6) activity was estimated spectrophotometrically by the subsequent H_2O_2 consumption at 240 nm as per Pagariya et al. (2012). POX (EC 1.11.1.7) activity was measured as explained by Movahedi et al. (2014).

Statistical Analysis

All estimates were made in triplicate, and data analyzed using SPSS statistical package. One-way ANOVA was accomplished, and values expressed as a mean ± SEM. The significant treatment effects of ANOVA were compared with means of Duncan's multiple range tests at $p < 0.05$.

Results

Cloning and Sequencing of *SsMYB18*

The genomic DNA of *S. spontaneum* was amplified to obtain a single band of 1657 bp using MYB18pBIN *Kpn*I FP and *Bam*HI RP primers (Table 1; Fig. S1). The amplified fragments were a-tailed, cloned in pGEM-T™ Easy vector and transformed into *E. coli*. The transformed recombinant colonies were confirmed through colony PCR and sequencing. The full-length *SsMYB18* sequence was characterized for conserved domains through different bioinformatics tools.

SsMYB18 is a Single-Repeat MYB Transcription Factor in *S. spontaneum*

The sequence BLAST with non-redundant nucleotide sequences over the NCBI database implicated that isolated gene belongs to MYB family viz., *SsMYB18* (accession no. KU578002; Table 2; Fig. S2). The amplified genomic *SsMYB18* was 1657 bp in length including 63 bp 5′UTR and 124 bp 3′UTR region (Fig. 1). Based on start and stop codons, the full-length *SsMYB18* genomic ORF was of 1470 bp in length. In the full-length genomic sequence of 1470 bp, there are four introns, viz. I1: 134 bp (34–167); I2: 105 bp (337–441); I3: 44 bp (808–851); and I4: 112 bp (928–1040), and five exons E1: 33 bp (1–33); E2: 169 bp (168–336); E3: 366 bp (442–807); E4: 76 bp (852–927); and E5: 430 bp (1041–1470), to form the coding mRNA of 1074 bp as predicted by FGENISH (Fig. 1). *SsMYB18* protein contained a putative nuclear localization signal and highly conserved tryptophan residues separated by 19–31 amino acids (Fig. 2; Table S2). The predicted mRNA sequence of 1074 bp encoded

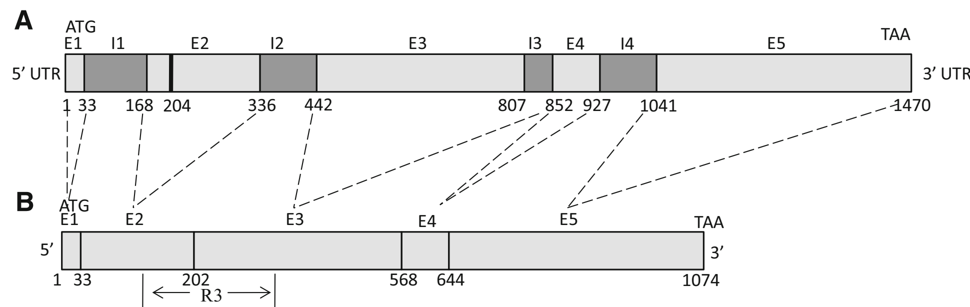

Fig. 1 Depiction of mRNA transcript architecture of *SsMYB18* gene. **a** Genomic DNA containing four introns (Intron I1: 134 bp; Intron I2: 105 bp; Intron I3: 44 bp; and Intron I4: 112 bp) and five exons (Exon E1: 33 bp; Exon E2: 169 bp; Exon E3: 366 bp; Exon E4: 76 bp; and Exon E5: 430 bp). mRNA sequence with marked coding region is shown. Exons are shown as (*light gray*) while introns are shown as (*dark gray*) *boxes*. The putative open reading frame starts from the start codon (ATG 1st bp), stop codon (TAA, 1470 bp). **b** FGENESH-predicted mRNA transcript of 1074 bp includes five exons Exon E1: 33 bp; Exon E2: 169 bp; Exon E3: 366 bp; Exon E4: 76 bp; and Exon E5: 430 bp. A single MYB domain repeat is indicated with sets of *arrows*

357 aa protein having calculated molecular weight of 39.85 kDa (http://pir.georgetown.edu/cgi-bin/comp_mw.pl). The *SsMYB18* (KU578002) protein sequence showed bootstrap value of 82 with *SoMYB18* and that of with *S. bicolor* (XP_002457686.1), *S. officinarum* (AFN08700.1), *B. distachyon* (XP_003567480.1), *A. tauschii* (EMT11047.1), *T. aestivum* (AFH08282.1) was 99, *B. distachyon* (XP_003568972.1), and that of *O. sativa* (NP_001054597.1) and *O. brachyantha* (XP_006654025.1) was 97. NCBI blast tree of full-length MYB proteins demonstrated that *SsMYB18* clusters with monocot MYB family proteins (Fig. 3).

Construction of Plant Expression Vectors

The *SsMYB18* gene was cloned in plant transformation vector pBinAR. The recombinant *pBinAR::SsMYB18* was reconfirmed by PCR, restriction digestion (Fig. 4a, b) and sequencing through 35S promoter, found to be in correct orientation (Table 1). The binary vector overexpressing *SsMYB18* gene under the influence of CaMV35S promoter (Fig. 5) was introduced into tobacco using *Agrobacterium*-mediated leaf disk transformation protocol. Non-transformed tobacco was used as a control. The putative transformants were primarily screened on MS medium supplemented with kanamycin 50 mg L^{-1} (Fig. S3) and later by PCR using a pair of specific primers derived from *SsMYB18* start and stop codon regions (Table 1). Out of 24 putative transgenic tobacco plants screened by gene-specific PCR, 19 plants were positive (Fig. S4). These T_0 generation PCR positive lines of tobaccos were analyzed for accessing the copy number through southern hybridization. The southern blot analysis confirmed that three transgenic tobacco lines (R1, R2 and R3) have single copy integration of the *SsMYB18* gene in tobacco genome while other found negative (Fig. S5). The transgene expression of southern positive transgenic lines (R1, R2 and R3) was confirmed through RT-PCR analysis. All three lines shown the expression of transgene (*SsMYB18*) under drought, salt and clod stress conditions, while expression was not detected in non-transformed tobacco plants (Fig. S6 and S7). Further these three lines were subjected to hardening and analyzed for further inheritance studies. The homozygous single event transgenic tobacco lines were obtained through PCR and kanamycin screening (Fig. S8). The T_2 generation *SsMYB18* expressing transgenic plants derived from R1, R2 and R3 were used for further functional analysis.

Expression of *SsMYB18* in Tobacco Confers Tolerance to Drought, Salt and Cold Stresses

To characterize the role of *SsMYB18* in regulating plant stress responses, T_2 generation homozygous tobacco plants derived from R1, R2 and R3 lines each were subjected to drought, salt and cold stress. After treatments, the *SsMYB18* transgenic plants exhibited enhanced tolerance to all three stresses, respectively, relative to non-transformed tobacco (Fig. S9). The relative amount of proline, lipid peroxidation, chlorophyll content and accumulation of ROS was measured in control and *SsMYB18* overexpressing transgenic lines (R1, R2 and R3). Most of the physio-biochemical analysis revealed that R3 line was significant over the R1 and R2. Thus, most of the results described for R3 lines and details of all three lines are summarized in Table S1.

The MDA content under PEG stress was 3.8, 4.8, 5.3 and 3.8 % lower in R3 transgenic line than R1, R2 and the respective control plants at 6, 12, 24 and 48 h (Fig. 6a). Under salt stress at initial period, MDA content in transformed plants was inordinate than the non-transformed ones (Fig. 6b). With increasing stress period, a continuing

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SsMYB18                          ---------MAMAAGVLFPN---SLFNYLNSRRR-------IVAGLQRCG 31
SoMYB18                          MGRHSCCYKQKLRKGLWSPEEDEKLMNHITKHGHGCWSSVPKLAGLQRCG 50
gi|115461995|ref|NP_001054597.   MGRHSCCYKQKLRKGLWSPEEDEKLMNHITKHGHGCWSSVPKLAGLQRCG 50
gi|573943302|ref|XP_006654025.   MGRHSCCYKQKLRKGLWSPEEDEKLMNHITKHGHGCWSSVPKLAGLQRCG 50
gi|475548293|gb|EMT11047.1|      MGRHSCCYKQKLRKGLWSPEEDEKLMNHITKHGHGCWSSVPKLAGLQRCG 50
gi|383302750|gb|AFH08282.1|      MGRHSCCYKQKLRKGLWSPEEDEKLMNHITKHGHGCWSSVPKLAGLQRCS 50
gi|357134737|ref|XP_003568972.   MGRHSCCYKQKLRKGLWSPEEDEKLMNHITKHGHGCWSSVPKLAGLQRCG 50
gi|242057081|ref|XP_002457686.   MGRHSCCYKQKLRKGLWSPEEDEKLMNHITKHGHGCWSTVPKLAGLQRCG 50
gi|393395657|gb|AFN08700.1|      MGRHSCCYKQKLRKGLWSPEEDEKLMNHITKHGHGCWSTVPKLAGLQRCG 50
gi|357131715|ref|XP_003567480.   MGRHSCCYKQKLRKGLWSPEEDEKLLNHINKHGHGCWSTVPKLAGLQRCG 50
                                           :  *:  *:   .*:*::..: :        :******.

SsMYB18                          KSCRLRWINYLRPDLKRGAFSQDEEDLIIELHAVLGNRWSQIAAQLPGRT 81
SoMYB18                          KSCRLRWINYLRPDLKRGAFSQDEEDLIIELHAVLGNRWSQIAAQLPGRT 100
gi|115461995|ref|NP_001054597.   KSCRLRWINYLRPDLKRGAFSQEEEDLIIELHAVLGNRWSQIAAQLPGRT 100
gi|573943302|ref|XP_006654025.   KSCRLRWINYLRPDLKRGAFSQEEEDLIIELHAVLGNRWSQIAAQLPGRT 100
gi|475548293|gb|EMT11047.1|      KSCRLRWINYLRPDLKRGAFAQDEEDLIVELHAVLGNRWSQIAAQMPGRT 100
gi|383302750|gb|AFH08282.1|      KSCRLRWINYLRPDLKRGAFAQDEEDLIVELHAVLGNRWSQIAAQMPGRT 100
gi|357134737|ref|XP_003568972.   KSCRLRWINYLRPDLKRGAFAQDEEDLIIELHAVLGNRWSQIAAQMPGRT 100
gi|242057081|ref|XP_002457686.   KSCRLRWINYLRPDLKRGAFSQEEEDLIIELHAVLGNRWSQIATRLPGRT 100
gi|393395657|gb|AFN08700.1|      KSCRLRWINYLRPDLKRGAFSQEEEDLIIELHAVLGNRWSQIATRLPGRT 100
gi|357131715|ref|XP_003567480.   KSCRLRWMNYLRPDLKRGAFEQEEEDLIIELHAVLGNKWSQIATRLAGRT 100
                                 *******:************ *:*****:********:*****:::.***

SsMYB18                          DNEIKNLWNSCIKKKLRQKGIDPNTHKPLT-EADRGGAAPTISTERTSRS 130
SoMYB18                          DNEIKNLWNSCIKKKLRQKGIDPNTHKPLT-EADRSGVAPTISTERTSGS 149
gi|115461995|ref|NP_001054597.   DNEIKNLWNSCIKKKLRQKGIDPNTHKPLT-EADRRGAAPTVSTERTSGS 149
gi|573943302|ref|XP_006654025.   DNEIKNLWNSCIKKKLRQKGIDPNTHKPLS-DADRRGAAPTISTERTSGS 149
gi|475548293|gb|EMT11047.1|      DNEIKNLWNSSLKKKLRQKGIDPNTHKPLT-EADRSGAAPTISTERTSGS 149
gi|383302750|gb|AFH08282.1|      DNEIKNLWNSSLKKKLRQKGIDPNTHKPLT-EADRSGAAPTISTERTSGS 149
gi|357134737|ref|XP_003568972.   DNEIKNLWNSSLKKKLRQKGIDPNTHKPLT-EVDRSGAAPTISTERTSGS 149
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gi|393395657|gb|AFN08700.1|      DNEIKNLWNSSIKKKLRQKGIDPNTHKPLA-EVEHSKAAPTISTERTSES 149
gi|357131715|ref|XP_003567480.   DNEIKNLWNSCIKKKLRQKGLDPNTHKPLADEADRRKVAPTMSTERTSES 150
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Fig. 2 Sequence alignment analysis of *SsMYB18* amino acids. Amino acid sequence alignment was obtained with ClustalW2 and then orderly grouped on the basis of homologies to the consensus MYB superfamily proteins, including transcription factors and mRNA splicing factors (*right left arrow*), the SANT/MYB DNA-binding region (*dash line*) and the single MYB domain is covered by *box*, *star* indicates identical amino acid residues and dots are the conserved or semi-conserved substitutions observed and dashes indicate gaps. The numbers on the *right* indicate the amino acid position relative from translation start codon. The names on the *left* indicate the accession numbers of MYBs found in different plants. The *arrows* represent the conserved tryptophan residues; SoMYB18 S*accharum officinarum* MYB18 gene, SsMYB18 S*accharum spontaneum* MYB18 protein

decrease was evidenced with respect to control non-transformed plants. At 24 and 48 h, we noticed 36 and 51 %decrease in the MDA contents in *SsMYB18* plants (R3 line) than that of respective non-transformed plants under salt stress analogous inclination was followed in R1 and R2 lines (Fig. 6b; Table S1). A progressive increase in lipid peroxidation was observed in transgenic and non-transgenic plants under cold stress. Accumulation of MDA was less in transgenic tobacco as compared to non-transgenics with least in R3 line under all stresses, i.e., 1.1-, 1.2-, 1.5- and 1.0-fold lesser at 6, 12, 24 and 48 h, respectively, than the non-transformed plants (Fig. 6c).

High level of salt and cold stress induced a decrease (except 6 h) in the pigment fractions in non-transformed tobacco as compared to transformed tobacco lines under similar conditions. Average increase in chlorophyll of R3 line was 45, 30, 37, 28 and 45 % higher than that of non-transformed ones during PEG stress with respective stress period and highest at 12-h PEG stress 2.50 mg gm^{-1} of tissue FW (Fig. 7a). The chlorophyll pigments was highest in NaCl-stressed R3 transgenic plants and improved by 1.8-, 1.4-, 1.6-, 1.4- and 1.8-fold at 0, 6, 12, 24 and 48 h with respective non-transformed plants, showing highest rise at initiation and culmination period of stress (Fig. 7b). In cold

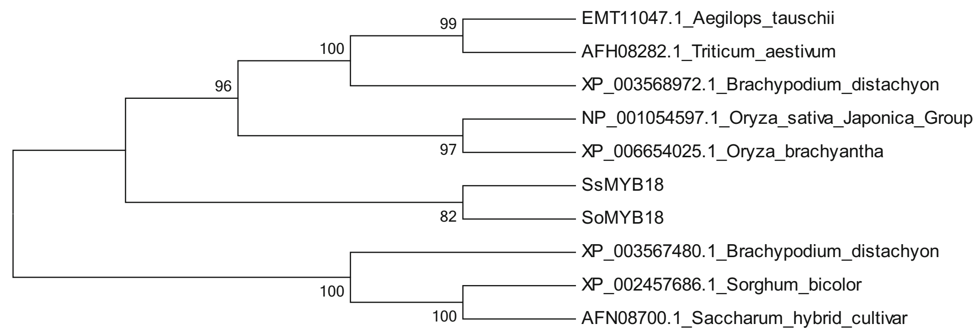

Fig. 3 Neighbor-joining tree of predicted MYB proteins. The phylogeny was based on an alignment derived using the MEGA6 program and the Neighbor-joining method (1000 bootstraps). The phylogeny with respect to the conserved SANT could be segregated into 2 groups. The GenBank accession numbers with respective MYBs XP_002457686.1—Sorghum bicolor, AFN08700.1 *Sccharum officinarum* cultivar, XP_003567480.1—*Brachypodium distachyon*, EMT11047.1—*Aegilops tauschii*, AFH08282.1—*Triticum aestivum*, XP_003568972.1—*Brachypodium distachyon*, NP_001054597.1—*Oryza sativa* Japonica Group, XP_006654025.1—*Oryza brachyantha*

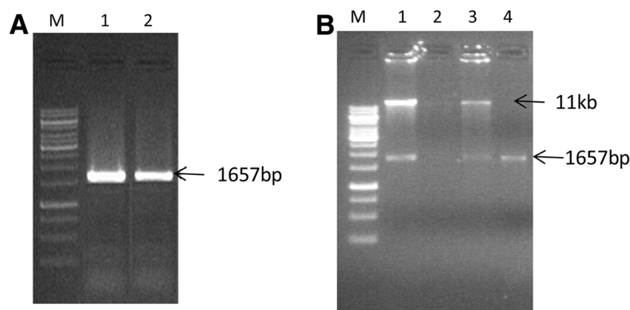

Fig. 4 PCR and restriction digestion confirmation of *SsMYB18* gene cloning in pBinAR vector. **a** Colony PCR confirmation of pBinAR SsMYB18 clones. M-1.0 kb ladder, *Lane 1* and *2 white colonies*, **b** reconfirmation of gene cloned in pBinAR vector by restricts digestion using *Kpn*I and *Bam*HI enzymes. M-1.0 kb ladder, *Lane 1*, *2* and *3* plasmid of +ve clones restricted by *Kpn*I and *Bam*HI, *Lane 4* *Kpn*I- and *Bam*HI-restricted *SoMYB18* gene used for ligation

stress, the chlorophyll content of *SsMYB18* overexpressing plants was higher initially 45 % (at 0 h), a slight decrease observed at 6 h but again recorded an average increase by 56, 60 and 55 % at 12, 24 and 48 h, respectively, (R3), than control plants (Fig. 7c).

Initially proline content was higher in control plants compared to transgenics. Under PEG stress, proline accumulation was less in transgenic plants (R1, R2 and R3) than the respective non-transformed plants except 5 % higher at 48 h (Fig. 8a). Under salt stress, the proline accumulation in *SsMYB18* plants was onefold (R3) higher at 6 h than the non-transformed ones as compared to initial and later decrease (Fig. 8b). A quantum leap of threefold rise in proline content was observed under cold stress at 6 h (R3 line); after stress induction further a decline of 36 % (12 h) and a rise of twofold were observed at 24 h (Fig. 8c). Inline trend was observed in R1 and R2 lines but not more than the R3 line (Fig. 8c).

Effect of *SsMYB18* Overexpression on Antioxidant System of Transgenic Plants

The general trend showed that *SsMYB18* overexpressing all three lines (R1, R2 and R3) have higher SOD activities than non-transformants during initial stress induction period (0 and 6 h) underneath all three stresses. Under PEG stress, the initial rise in SOD activity by threefold and twofold was observed at 0 and 6 h in R3 transgenic line, while a declining trend was observed in all three lines for rest of the stress period with respect to control plants (Fig. 9a). The SOD activity under salt and cold stress was increased in leaves of *SsMYB18* transgenics compared to respective controls (except 6-h cold) and remained all time higher in R3 line under respective conditions (Fig. 9b, c). Under salt stress, transgenic tobacco plants have noted a sharp rise by more than ten-fold in R3 lines at 6 h which was at its highest and an another sharp rise by 7.5-fold at the culmination stress period than the non-transformed plants (Fig. 9b). Whereas under cold stress during the initial period of stress (0 and 6 h), SOD activity has noted a quick rise by threefold and twofold followed by a sharp decrease at 12 h (48 %) with a further study increase by 1.3-fold toward the culmination period (Fig. 9c).

POX activity was found elevated in all transgenic lines than the non-transformed ones over entire sampling time under PEG, salt and cold stresses. The POX activity during PEG stress in R3 transgenic lines recorded a progressive rise from 1.5- to 14-fold during stress period and was at its highest at the time of termination of stress respective to control plants (Fig. 10a). The POX activity in R3 transgenic plants under salt stress was higher than the respective non-transformants which was 1.5-, 1.2-, 5.1-, 3.1- and 1.9-fold higher at 0, 6, 12, 24 and 48 h, the highest more than 410 % rise in POX activity was observed at 12 h

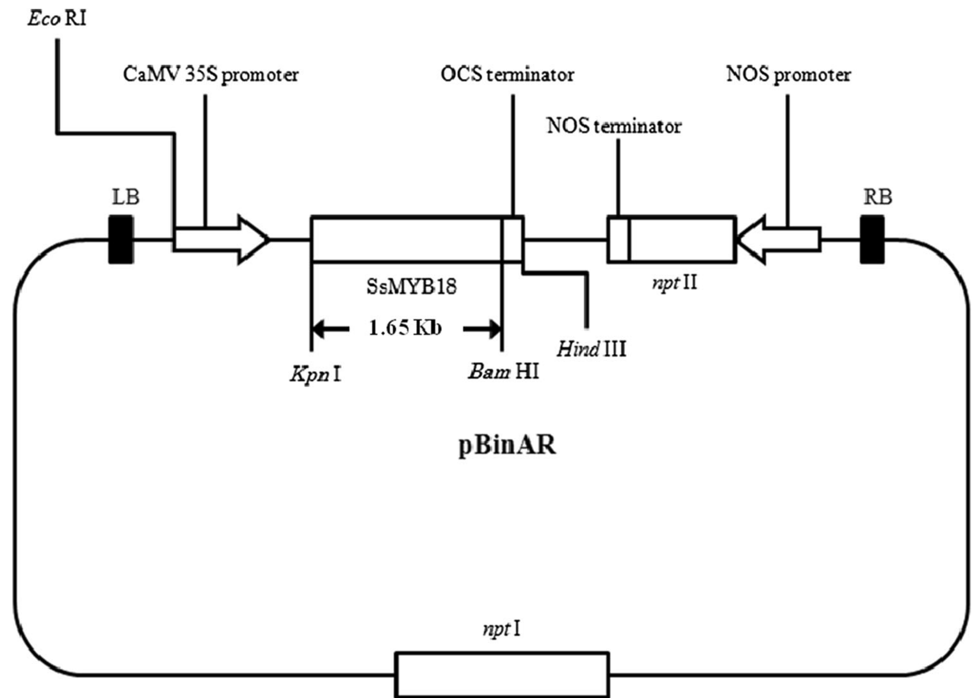

Fig. 5 pBinAR::SsMYB18 gene construct driven by CaMV35S promoter. The pBinAR::SsMYB18 construct was transformed in tobacco plants by *Agrobacterium*-mediated transformation method

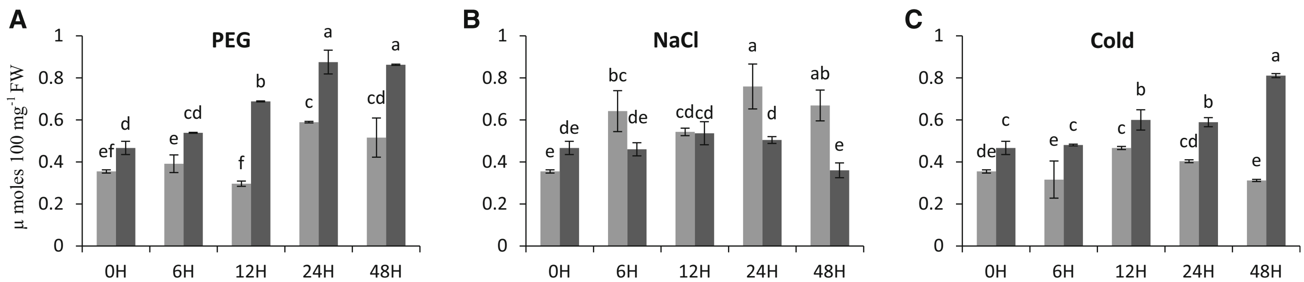

Fig. 6 MDA content of *SsMYB18* overexpressing transgenic tobacco. *Bars* represent mean values ±SE ($n = 9$). Means followed by the *same letter* are not significantly different at 0.05 % level based on Duncan's test. *Light gray* indicates un-transformed tobacco, *dark gray* indicates transformed tobacco line R3

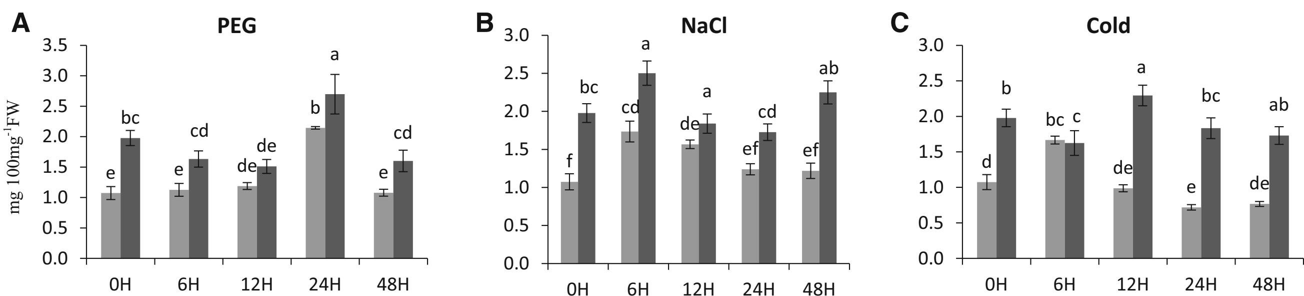

Fig. 7 Total chlorophyll content of *SsMYB18* overexpressing transgenic tobacco. *Bars* represent mean values ±SE ($n = 9$). Means followed by the *same letter* are not significantly different at 0.05 % level based on Duncan's test. *Light gray* indicates un-transformed tobacco, and *dark gray* indicates transformed tobacco line R3

(Fig. 10b). Similar trend was observed under cold stress, the POX activity delineated a sharp increase from 1.5-fold at 0 h to 8.8-fold at 12 h of stress. An increase of 1.4- and 1.2-fold at 24 and 48 h was observed though it was less in comparison with rise observed in initial period of stress (Fig. 10c). The highest specific enzyme activity noted was

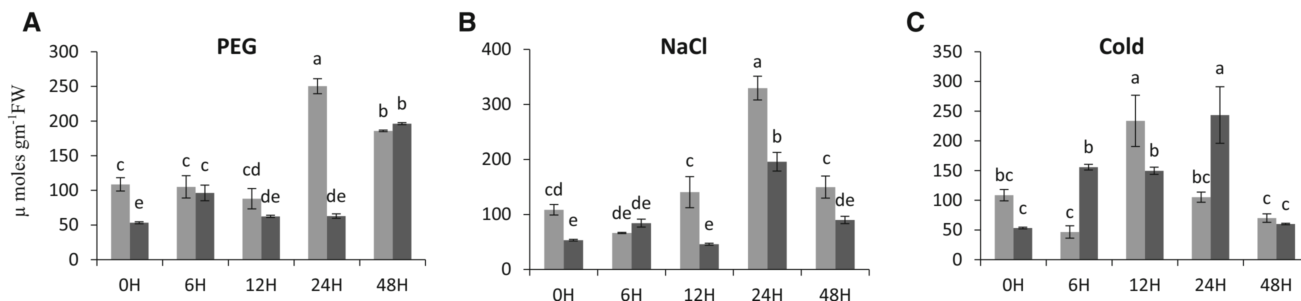

Fig. 8 Proline content of *SsMYB18* overexpressing transgenic tobacco. *Bars* represent mean values ±SE ($n = 9$). Means followed by the *same letter* are not significantly different at 0.05 % level based on Duncan's test. *Light gray* indicates un-transformed tobacco, and *dark gray* indicates transformed tobacco line R3

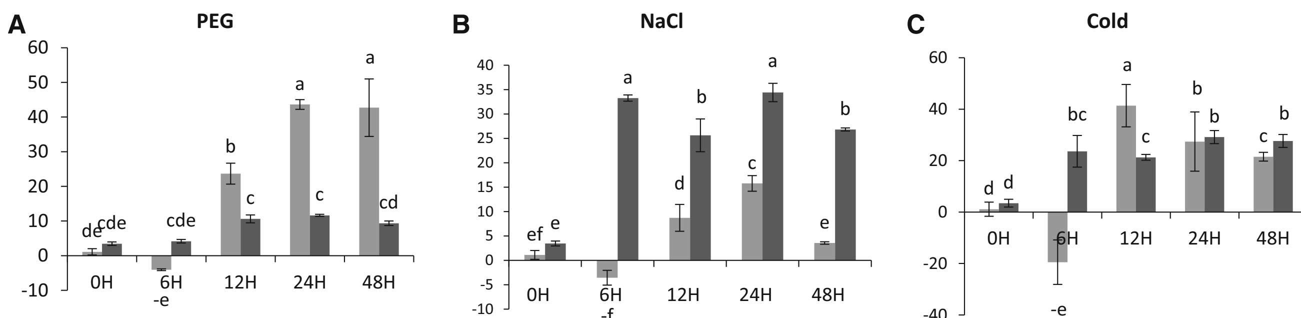

Fig. 9 SOD activity of *SsMYB18* overexpressing transgenic tobacco. *Bars* represent mean values ±SE ($n = 9$). Means followed by the *same letter* are not significantly different at 0.05 % level based on Duncan's test. *Light gray* indicates un-transformed tobacco, and *dark gray* indicates transformed tobacco line R3

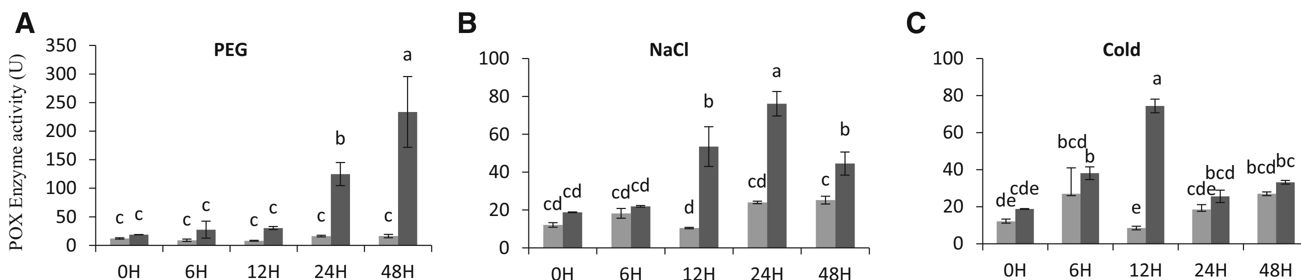

Fig. 10 POX activity of *SsMYB18* overexpressing transgenic tobacco. *Bars* represent mean values ±SE ($n = 9$). Means followed by the *same letter* are not significantly different at 0.05 % level based on Duncan's test. *Light gray* indicates un-transformed tobacco, and *dark gray* indicates transformed tobacco line R3

233.64 U at 48 h, 76.12 U at 24 h and 74.42 U at 12 h, respectively, in PEG, salt and cold stress (Table S1).

With the progression of stress, elevation in CAT activity in different time periods was evidenced in comparison with the controls (except PEG 12 and 48 h). Under PEG stress, the CAT activity in R3 transgenic tobacco plants was increased up to 26 % at 6 h followed by 21 % decrease at 12 h than the respective non-transformed plants. Under PEG stress, a drastic decrease in CAT activity was observed in non-transgenic plants during stress period, while transgenic lines showed intermittent increase (at 6 and 24 h), and was at its highest in R3 at 24 h (208 %) than the controls with a trivial decline during late stress period (Fig. 11a). In NaCl stress, CAT activity of R3 transgenic line was 2.5-, 2.2- and 2.3-fold higher than the respective control plants at 6, 12 and 24 h while declining by 1.1-fold at 48 h (Fig. 11b). In cold stress, the enzyme activity in R3 lines evidenced a sudden increase from 1.4-fold (0 h) to 2.6-fold (6 h), 8.8-fold (12 h), 2.3-fold (24 h) and 5.7-fold (48 h) over non-transformed plants (Fig. 11c). Aligned increase was observed in R1 and R2 lines but not as high as R3 (Fig. 11c).

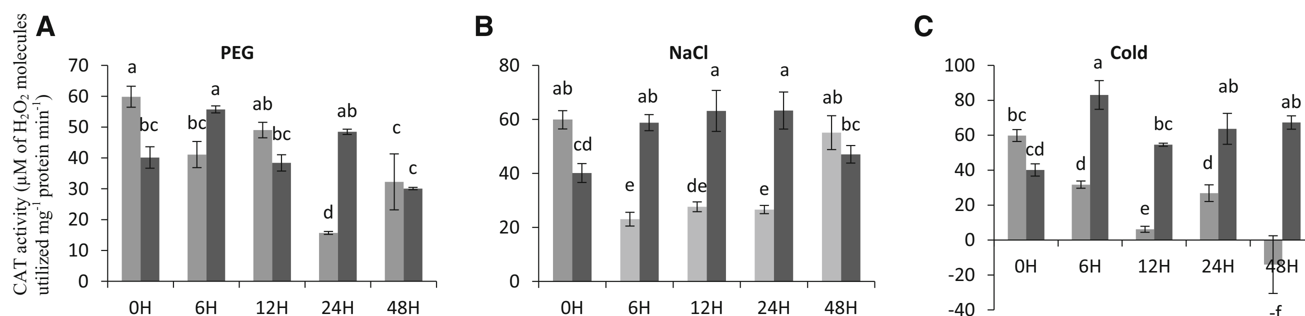

Fig. 11 CAT activity of *SsMYB18* overexpressing transgenic tobacco. *Bars* represent mean values ±SE ($n = 9$). Means followed by the *same letter* are not significantly different at 0.05 % level based on Duncan's test. *Light gray* indicates un-transformed tobacco, and *dark gray* indicates transformed tobacco line R3

Discussion

Saccharum spontaneum is a wild species of sugarcane showing high level of tolerance to abiotic and biotic stresses (Grivet and Arruda 2001). Isolation of MYB orthologs from stress-tolerant wild species is of prime importance for establishing a better understanding of regulatory mechanisms. Thus, deploying them in sugarcane and other crop plants is very crucial for modern agriculture.

In our previous study, we had functionally characterized stress-inducible R2–R3 *SoMYB18* from sugarcane. The *SoMYB18* transgenic tobacco biochemical analysis inferred that the *SoMYB18* overexpression resulted in salt and dehydration stress tolerance (Shingote et al. 2015). In present study, anortholog *SsMYB18* was isolated from *S. spontaneum* and a series of experiments were conducted to explore the function of this gene in abiotic stress tolerance. Phylogenetic analysis of the *SsMYB18* sequence revealed a phylogenetic tree that branched into monocot clades, which suggests that the sequence of *SsMYB18* belongs to monocots MYB transcription factors and placed closely to *SoMYB18*. *S. spontaneum SsMYB18* sequence is lacking the characteristic plant MYB domain without R2-R3 MYB repeats in the N-terminal region, representing that *SsMYB18* differs from *SoMYB18*. Highly conserved amino acid residues present within the *SsMYB18* single repeat are in agreement with similar MYB proteins reported from other plants and suggesting its importance in maintaining the MYB repeat structure (Ganesan et al. 2012). To date, many reports have been published on the functions of transcription factors in model plants (Olsen et al. 2005), but a functional analysis of MYB transcription factors from wild *S. spontaneum* is lacking. To investigate the function of this gene, transgenic plants were produced that overexpressed the *SsMYB18* gene in tobacco. The transgene integration and expression were successfully confirmed through PCR, southern blotting and RT-PCR. It was observed that R3 transgenic event was at par significant over the R1 and R2 lines; consequently, most of the results were discussed by comparing this event with controls.

MDA, a fatty acid produced by lipid peroxidation, can be a worthy indicator of stress in plants. Based on our results, *SsMYB18* expressing tobacco plants evinced a gradual reduction in MDA contents than that of respective non-transformed plants under drought, salt and cold stress with a significant twofold decrease at late salt stress period (Fig. 6a–c), indicating that transgenics suffered much lesser lipid peroxidation injury than non-transformed ones. Our results are in agreement with our previous findings where *SsMYB18* plants have shown lesser MDA levels during all three stresses in contrast to *SoMYB18* plants, which have shown consistent reduction only during salt stress (Shingote et al. 2015). Comparable results have been reported in transgenic plants expressing stress-tolerant genes in tobacco as well in *A. thaliana* under abiotic stress (Wang et al. 2012; Mahajan and Yadav 2014; RoyChoudhury et al. 2007; Gao et al. 2011). Furthermore, under all stresses, less MDA accumulation in transgenic plants signifies that lipid peroxidation was reduced in *SsMYB18* transgenic plants as compared to non-transformed ones and *SoMYB18* transgenic plants.

It was noted that through stress stimulus chlorophyll content decreased along the period of time in non-transformed tobacco plants under salt and cold stress except at 6 h, while it was remarkably higher in *SsMYB18* overexpressing plants (Fig. 7). Thus, proving the stress induced a significant decrease in the contents of pigment fractions, inline results were previously reported by earlier researchers through MYB tobacco transgenic (Ganesan et al. 2012, Mahajan and Yadav 2014, Liu et al. 2011). These results are almost facsimile of previous finding in relation to *SoMYB18*, in fact *SsMYB18* expressing transgenic lines have higher pigment levels (1.2-fold higher at 24 h) during late PEG stress than *SoMYB18* (Shingote et al. 2015). The results indicated that *SsMYB18* overexpression more profoundly responds through pigment level increase

to all three stresses as against *SoMYB18* which only responds to salt and cold stress.

Although proline accumulation in plants is an important factor in determining plant osmotic adjustment under abiotic stress (Bartels and Sunkar 2005). In our studies, in contrast decrease in proline accumulation was observed at initiation of stress period. One fold increase in the proline content was noticed at 48 h in PEG, 1.2-fold at 6 h in salt stress, while a quantum leap of 33- and 23-fold rises at 6 and 24 h, respectively, in cold stress (Fig. 8). At this moment, in case of *SsMYB18,* it holds true that proline might act as early (salt), early and mid-late (cold) and late (PEG) signaling molecule which activate multiple responses to encounter the stress (Pagariya et al. 2012). However, the decreased proline levels in PEG and salt stress imply that free proline might only moderately responsible for the osmotic adjustment as formerly reported (Mao et al. 2011:Shingote et al. 2015). In comparison with *SoMYB18*, *SsMYB18* have shown high proline levels during mid- and prelate cold stress, implying that this may be a potential suitable candidate for improving cold stress tolerance in plants.

The activities of plant anti-oxidative enzymes such as CAT, POX and SOD have been studied to assess the impact of different stresses (Pagariya et al. 2012; Apel and Hirt 2004). We observed that SOD and POX activities increased significantly during PEG, salt and cold stresses in both transformed and non-transformed plants. Also, under normal conditions, the transgenic plants had higher SOD and POX levels than the non-transgenic, akin results were reported in poplar by Movahedi et al. (2014). Furthermore, the impulsive increase in SOD, POX and CAT activity observed in *SsMYB18* transgenics on exposure to 6-h stress indicating a high ROS accumulation (in all stresses), in which the plant antioxidant system could deal with moderately effectively (Fridivich 1989). The SOD activity was progressively increased in salt- and cold-stressed plants than the respective control plants, hinting overexpression of *SsMYB18* in tobacco resulted in up-regulation of SOD activity. Further it was observed that, during salt and cold stress, there was higher amount of SOD activity in *SsMYB18* overexpressing tobacco plants from initiation to culmination period than the PEG stress which was higher at initiation period only. Analogous results were also reported in transgenic tobacco (Shingote et al. 2015; Liu et al. 2011) in CcCDR transgenic Arabidopsis (Tamirisa et al. 2014).

The POX activity increases logarithmically during PEG stress (threefold to 14-fold) and at the same time SOD activity was declined with the respective non-transformed plants during later period. Similar correlation was observed and supported by Pagariya et al. (2012) and Liu et al. (2014). But in contrast to *SoMYB18*, POX and SOD activities were found significantly elevated under salt and cold stress in this study. These results deciphering that *SsMYB18* has better ability to balance the ROS regulation compared to *SoMYB18* in reducing oxidative damage caused during these stresses. Thus, SOD is an important enzyme in catalyzing the reactive oxygen species, whereas POX and CAT functions to eliminate H_2O_2. Initially at 0 h, the CAT activity was lesser in transgenic than the non-transgenic plants under all stresses, while SOD and POX activities were high, implying that they have interrelation and compensate each other (Liu et al. 2014). Moreover, a rise and fall were noticed in CAT activity under PEG stress as compared to respective controls. It was higher in NaCl and cold stress except mere decline at 48 h. In conformity with the results concluded, we found joint activity and interrelation between these enzymes which collectively preventing the formation of ROS and achieving better salt and cold tolerance. Thus, *SsMYB18* might be triggering the cascades for controlling the ROS through increasing the CAT activity in transgenics. Inline results were reported in transgenic tobacco expressing Rab16A gene under salt stress (RoyChoudhury et al. 2007), in rice overexpressing OsMYB2 under salt, cold and dehydration stress (Yang et al. 2012). We had observed contrasting results in *SoMYB18* transgenic tobacco lines where the CAT activity was lesser in transgenic tobacco throughout all three stresses (Shingote et al. 2015).

Overall these studies delineates that ectopic overexpression of *SsMYB18* in tobacco tolerates salt and cold stress through synergistic role of CAT, SOD and POX. Increased SOD, POX and CAT activities are closely related to abiotic stress tolerance of many plants as reported in previous researches (Yang et al. 2012, Parvaiz and Satyawati 2008) as observed in our studies as well. One significant finding is that the *SsMYB18* transgenic plants exhibiting greater biochemical and antioxidant activities under salt and cold stress exhibited enhanced tolerance to both these stresses. *SsMYB18* plants showed high tolerance to salt and cold stress, whereas moderate tolerance to drought stress. In summary, single-repeat MYB transcription factor isolated from *S. spontaneum*, *SsMYB18* seems to be more promising candidate than the R2-R3-*SoMYB18* gene as it is providing high level of tolerance to salt and cold stress and moderate to drought.

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Author contributions P.G.K. and M.C.P. designed the experiments. P.R.S., M.C.P., P.R.R. and P.G.K. performed experiments. P.R.S., M.C.P., S.B.K. and P.G.K. analyzed and interpreted the data. P.R.S., M.C.P. and P.G.K. wrote the manuscript with input from all authors.

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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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