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ORIGINAL ARTICLE

Indexing heat stress induced changes in transgenic tobacco by overexpressing membrane-tethered transcription factor from *Sorghum bicolor bZIP17*

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Abstract

Basic leucine zipper (bZIP) TF family is key regulator of diverse biological functions, including heat stress tolerance. However, its role in response to heat stress is unexplored in *Sorghum bicolor*, an abiotic stress-tolerant cereal plant. Bio-prospecting of genes for abiotic stress tolerance from elite natural stress-tolerant species is a promising approach for development of abiotic stress-tolerant crops. Therefore, qRT-PCR analysed for heat stress induced *bZIP17* gene from *S. bicolor*, showed 14.7 and 17.3-fold expression at 4 h and 6 h of heat stress respectively at one-month old seedling stage. Further its cDNA sequence was cloned (named as *SbbZIP17*) for further functional validation in model plant system. *In silico* analysis showed *SbbZIP17* encodes for bZIP polypeptide, an endoplasmic reticulum (ER) type II membrane-tethered transcription factor (type II MMTF), highly conserved, having a single ligand-binding site, interacting with heat stress-responsive proteins (HSP70, NF-Ys), expressed in different tissues and organs. Over-expression of *SbbZIP17* in independent events of transgenic tobacco (*Nicotiana tabacum*) lines (T_1) is responsible for activation of genes involved in unfolded protein response (UPR) pathway under heat stress. Transgenic tobacco lines showed enhancement in hydration status, antioxidant activity, reduction in chlorophyll loss, and membrane damage. Our analysis demonstrated that *SbbZIP17* plays an important role in regulating heat stress tolerance in plants.

Key message

SbbZIP17 (encoding membrane-tethered transcription factor) from *Sorghum bicolor* overexpressed in tobacco plants showed heat stress tolerance.

Keywords Endoplasmic reticulum stres · UPR Pathway · Membrane-tethered TF · Gene expression · *In silico* analysis · ROS

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Introduction

Rapidly changing global climate conditions result in different abiotic stresses, especially heat stress which is a major constraint for future food security. Heat stress hampers physiological and biochemical processes by negatively affecting crop growth, development, and ultimately yield (Saeed et al. 2023). Plants have evolved a variety of tolerance mechanism in response to heat stress conditions and undergo various physiological, biochemical, and molecular modifications for adaptation (Janni et al. 2020; Saeed et al. 2023). However, many of these modifications are achieved via stress signal transduction and transcriptional modulation of heat stress-responsive genes by different transcription

factors (TFs) (Saeed et al. 2023). TFs families, such as HSF, bHLH, bZIP, WRKY ERF, and MYB are well known as crucial parts of transcriptional regulatory network of stress-responsive genes to mitigate heat stress conditions (Janni et al. 2020).

Basic leucine zipper (bZIP) is the most important and largest TF family; there are 75 bZIPs in *(A) thaliana*, 89 in *P. trichocarpa*, 89 in *O. sativa*, 92 in *S. bicolor*, 96 in *(B) distachyon*, 121 in *M. acuminata*, 77 in *M. esculenta*, and 187 in *T. aestivum* (Agarwal et al. 2019). Preceding studies have explained that bZIP TFs are key regulators of fundamental biological processes, such as pathogen resistance, hormone signaling, developmental processes, heat, cold, salt, and drought stress tolerance (Sornaraj et al. 2016; Banerjee et al. 2017). The bZIPs protein comprises a conserved bZIP domain having binding affinity to specific DNA sequence along with a less conserved leucine zipper domain for dimerization after binding to dsDNA (preferably to ACGT sequence). The bZIP region contains 40–80 amino acid residues and the basic region consists of sixteen amino acid residues with a nuclear localization signal (NLS) (Sornaraj et al. 2016; Banerjee et al. 2017). The bZIPs are classified as ten different types (Type-I to Type-X) of group in *O. sativa* (89) and *A. thaliana* (75) (Nijhawan et al. 2008). Among them, Type-II bZIP membrane-tethered transcription factors (MTTFs) take part in unfolded protein response (UPR) pathway also referred as endoplasmic reticulum (ER) stress response (Howell 2013). The UPR triggers when improperly assembled proteins start accumulating in the ER under abiotic stress conditions (Howell 2013). Type-II bZIP protein consists of site 1 protease (S1P) cleavage position and a transmembrane domain (Sornaraj et al. 2016). ER is the site of biosynthesis for Type-II bZIP MTTFs (Membrane-tethered transcription factor), for instance AtbZIP60, AtbZIP49, AtbZIP28, and AtbZIP17 in *A. thaliana*. Under normal growth conditions, MTTFs are situated in ER membrane, while during stress conditions they move to Golgi body for proteolytic cleavage accompanied by Golgi-specific proteases i.e., S1P and S2P. The cleaved part of MTTFs is transported to the nucleus and activates stress-responsive genes of UPR pathway including *Calreticulin* (*CRT1*), *Calnexin* (*CNX*), protein disulphide isomerase (PDIL), and binding protein (BiP) (Liu and Howell 2010). Recent analysis exposed that plant UPR affect cellular processes in many ways and would be one of the reasons for providing abiotic stress tolerance (Hayashi et al. 2013).

In *A. thaliana, AtbZIP17* and *AtbZIP24* TFs are functionally validated for their role in salt stress tolerance (Liu et al. 2008; Yang et al. 2009). Similarly, overexpression of *bZIP* gene from *O. Sativa, T. aestivum, P. trifoliata*, *M. hupehensis*, and *C. annum* improved abiotic stress tolerance ability via modulating the genes involved in UPR pathway (Huang et al. 2010; Zhang et al. 2012; Yang et al. 2019; Agarwal et al. 2019; Gai et al. 2020). To date, several genes encode TFs have been cloned from naturally stress-tolerant crop plant species and characterized for enhancement of abiotc stress tolerance (Agarwal et al. 2013; Gupta et al. 2014) but limited studies are available in *S. bicolor*. *Sorghum* (*S. bicolor* L. Moench) is a prominent cereal crop (fodder and grain) having outstanding ability to tolerate drought, heat, and waterlogging conditions. Therefore, it is an appropriate model crop to study the biochemical and molecular mechanism underlying abiotic stress tolerance (Mutava et al. 2011; Tari et al. 2013; Baillo et al. 2020). Even though, *S. bicolor* is considered as a source of genes liable for abiotic stress tolerance, just few of them (*Hsp70, SbP5CS1*, and *SbP5CS2*) are functionally characterized (Su et al. 2011; Mulaudzi-Masuku et al. 2015; Halder et al. 2016).

The genome of *S. bicolor* encodes 92 *bZIP* genes, but only a single gene has been cloned i.e., Opaque2 (Z. *mays*) homologous gene (Vettore et al. 1998). However functional role of *bZIP* gene family in terms of abiotic stress tolerance is still unexplored in *S. bicolor* (Wang et al. 2011). Here, we analysed expression of *SbbZIP17* under heat stress and after cloning analysed using *in-silico* based bioinformatic approach. Furthermore, tobacco plants were over-expressed with *SbbZIP17* to study its role under heat stress conditions.

Materials and methods

Plant growth conditions and heat stress for *S. bicolor*

Seeds of *S. bicolor* genotype "Swati" was surface sterilized using 70% (v/v) alcohol for 1.5 min afterwards treated with 12% sodium hypochlorite (NaOCl) solution for 15 min. Sterilized seeds were sown in a pot containing soilrite under growth chamber condition at SDMVMs College of Agricultural Biotechnology, Aurangabad, India. Conditions of growth chamber were maintained for seed germination and establishment as 16 h light/8 h dark photoperiod, temperature, and light intensity of 24 ± 2 °C and 100 µmol $m^{-2}s^{-1}$ respectively. One-month-old seedlings were subjected to heat stress conditions for 0 h, 2 h, 4 h, and 6 h at 42 °C inside a growth chamber in a sinusoidal manner; the temperature stress was increased from 25 °C (ambient) to 42 °C with 1 °C/5 min (Ngara et al. 2012; Mulaudzi-Masuku et al. 2015; Goswami et al. 2016). The heat stress exposed and control leaf tissues were collected in three replicates for RNA isolation, instantly exposed to liquid nitrogen before storage in deep freezer (-80 °C).

Expression analysis of *SbbZIP17*

Relative expression level of *SbbZIP17* under heat stress conditions was analyzed using qRT-PCR. Isolation of total RNA was carried out from heat stress (2 h, 4 h, and 6 h) and control leaf samples (0 h) using MagMAX™ Plant RNA Isolation Kit (Applied Biosystems, USA). The cDNA was synthesized from total RNA (2 µg) using SuperScript™ III first-strand cDNA synthesis kit (Invitrogen, USA). Integrated DNA Technology (IDT) software (www.idtdna.com) was used to design the primers specific to genes and got synthesized from IDT (Table S1). The level of gene expression was normalized using internal reference *S. bicolor GAPDH* gene (Glyceraldehyde 3 phosphate dehydrogenase) (Table S1) (Baillo et al. 2020). The total reaction mixture volume of 20 µl contained 10 µl of KAPA SYBR® FAST qPCR Master Mix (KAPA Biosystems, USA), 2 µl of cDNA (200 ng), 0.5 µl each of forward and reverse primers (400 nM of each primer). Nuclease-free water was added to make up the total volume of 20 µl. The qRT-PCR reactions were carried out in biological triplicates for each heat stress (2 h, 4 h, and 6 h) and control leaf samples, and technical triplicates were taken for each biological replicate (Panzade et al. 2021, 2022). Steps of PCR cycles were as follows: 94 °C for 4 min then 32 cycles of 94 °C for 16 s, and 60 °C for 16 s, and 72 °C for 22 s. The $2^{-\Delta\Delta Ct}$ equation was used to evaluate the relative expression level (Livak and Schmittgen 2008). Leaf samples without exposed to heat stress were used as control. The primers and template-specific binding were analyzed by generating the melting curve. Cp values were noted down for each sample to analyze the fold change in gene expression.

Cloning and sequence analysis of *SbbZIP17*

To amplify the CDS (Coding DNA Sequence) of gene *SbbZIP17*, primers were designed according to upstream and downstream sites of *S. bicolor SbbZIP39* from NCBI (Accession No. LOC8085134). For directional cloning of gene *SbbZIP17* in binary vector, restriction sites *Nde*I and *Sal*I were incorporated in forward and reverse primers respectively (Table S1). The PCR cycles were as follows: 94 °C for 2.5 min, 32 cycles of 94 °C for 55s, 61 °C for 35s, 72 °C for 120s. Proofreading Ex Taq DNA polymerase (Takara, Japan) was used to ensure zero error while amplification. The amplified PCR product was analyzed on agarose gel (1.2%) and then eluted from the agarose gel using GenElute™ Gel Extraction Kit (Sigma, USA). The amplicon of gene *SbbZIP17* was ligated to pGEM®-T Easy vector (Promega, USA). The ligation was performed at 4 °C for 16 h and 10 µl of ligated reaction mix was transformed to *E. coli* strain DH5α, the transformed cells were selected for blue/white screening. The PCR positive clones were picked up for sequencing. The sequences were aligned to obtain entire CDS (Coding DNA Sequence) and submitted to NCBI database.

In silico analysis of SbbZIP17

The SbbZIP17 sequence homology analysis was carried out by the BLAST tool available at National Centre for Biotechnology Information (NCBI). The conserved domains in *SbbZIP17* were identified by SMART tool (https://smart.embl-heidelberg.de/). CELLO2GO server (http://cello.life.nctu.edu.tw/cello2go/) was used for the prediction of protein at a subcellular level. The phylogenetic analysis was performed with homologous genes of *SbbZIP17* from several species using MEGA 7.0 software tool (https://www.megasoftware.net/). The physio-biochemical features of protein SbbZIP17 were assessed by Protparam tool (https://web.expasy.org/protparam/). MEME (Multiple Em for Motif Elicitation) server (https://meme-suite.org/meme/tools/meme) was used for the identification of conserved motif domains. YinOYang 1.2 software and NetNGlyc 1.0 software was used to analyse *O*-linked and *N*-linked glycosylation in SbbZIP. The secondary structure was predicted by PSIPRED tool. For protein 3D structure and ligand-binding site prediction Phyre^2 and 3D Ligand binding site software was used respectively. Protein to protein interaction of SbbZIP with closely related proteins was analyzed using the STRING (https://string-db.org/) online tool. Transcript (*SbbZIP1*) abundance was analyzed with the help of Genevestigator tool (https://genevestigator.com/) using the publicly available mRNA Seq *S. bicolor* database (mRNA Seq Gene level *S. bicolor* (ref: Sbicolor v3.1.1) at default parameters. The probeset (Sobic.009G137100) encoding *SbbZIP17* was used for the analysis of spatio-temporal expression level of *SbbZIP17* at various developmental phases and in 32 anatomical organs of *S. bicolor*. Further, we analyzed the similar and correlated genes to *SbbZIP17* using the probeset Sobic.009G137100, and the comparative gene expression was assessed in 32 different organs of *S. bicolor*. The number of target genes were set at 50.

Binary vector construction

The pRI101-AN and pGEM-T-*SbbZIP17* vectors were restricted with *Nde*I and *Sal*I restriction enzymes. The purified CDS of *SbbZIP17* was ligated to linearized pRI101-AN vector having sites overhang, the reaction mix was incubated at 4 °C for 12 h using Rapid DNA Ligation Kit (Invitrogen, USA). The DH5α strain of *E. coli* was transformed with the ligation mix using heat shock method and plated on LB agar plates having kanamycin selection (50 mg L^{-1}).

The positive clones were confirmed by PCR using conditions as mentioned above and the resulting recombinant plasmid was named pRI101-AN-*SbbZIP17*. The pRI101-AN-*SbbZIP17* construct was transformed into EHA105 strain of *A. tumefaciens* competent cells using heat shock (Kaman-Toth et al. 2018).

Development of transgenic tobacco plants

pRI101-AN-*SbbZIP17* vector was transformed into 30 days old healthy tobacco (*Nicotiana tobaccum* cv. 'Benthamiana') leaf discs via *Agrobacterium*-mediated transformation method (Zhou et al. 2011). Cells of *A. tumefaciens* harboring pRI101-AN-*SbbZIP17* were grown in YEM (Yeast Extract Mannitol) broth media containing antibiotics rifampicin (5 mgL^{-1}) and kanamycin (100 mgL^{-1}) at 28 °C till Optical Density (O.D., A_{600}) reached 0.6–0.8. The *A. tumefaciens* cells were pellet down and resuspended in 30 ml ½ strength MS media. Small leaf discs were infected by *A. tumefaciens* cells in liquid MS for 20 min. Afterward, the leaf discs were air-dried using sterile Whatman filter paper and further placed on MS media for co-cultivation for 48 h at 23 ± 2 °C in dark. After 48 h, the leaf discs were shifted to selection MS media (kanamycin 200 mgL^{-1}, Timentin 150 mgL^{-1}, BAP 2 mgL^{-1}, and NAA 0.2 mgL^{-1}) and placed under normal light conditions. The leaf discs were subcultured at a regular interval of 10 d. After 1 month, the developed shoots were placed on rooting media (kanamycin 100 mgL^{-1} and timentin 300 mgL^{-1} in MS medium) for the development of plantlets. Following transformation of tobacco, a total of 11 probable transgenic lines (at T_0 stage) were developed on kanamycin selection media. After the roots became stronger, plantlets were transferred from jam bottles to soil for hardening and kept under controlled conditions in growth chamber, and later shifted to greenhouse till maturity.

Putative tobacco transgenic plants confirmation

After screening on antibiotic selection medium, two months old transgenic tobacco T_0 plants and wild-type plants were taken for genomic DNA isolation using CTAB method. The genomic DNA was checked on 0.8% agarose gel, Nanodrop spectrophotometer (ThermoScientific, USA) was used to quantify the DNA. The existence of transgene was identified by using PCR with *nptII* and *SbbZIP* primers (Table S1).

Southern hybridization of transgenic tobacco *SbbZIP17* plants at T_1 stage

Southern hybridization was performed for *SbbZIP17*-T_1 transgenic lines. The genomic DNA (25 µg) of WT and transgenic lines was digested with *Sal*I enzyme and thereafter resolved on agarose gel (0.8%). Resolved genomic fragments transferred onto a blotting membrane (Merck, USA) using capillary method (Russell and Sambrook 2001). PCR DIG Probe Synthesis kit (Roche, Germany) was used to prepare probe for *SbbZIP17*. Pre-hybridization was performed at 45 °C in a hybridization oven for 1 h subsequently followed by hybridization for overnight (16 h). Washing, blocking, incubation steps were carried out as per protocols given in DIG Luminescent Detection Kit (Roche, Germany). Fluorescent signals from the nitrocellulose membrane were exposed on an X-ray film placed in a cassette.

Expression analysis of *SbbZIP17* and UPR pathway genes in *SbbZIP17*-T_1 transgenic lines

The qRT-PCR was performed to asses expression of *SbbZIP17* transcripts in 30-days-old T_1 tobacco transgenic lines. For expression analysis of UPR pathway genes, 30-days-old seedlings of *SbbZIP17*-T_1 transgenic lines and WT exposed to 42 °C of heat stress for 0 h and 6 h. Total RNA was isolated from transgenic and WT plants. The cDNA (complementary DNA) was synthesized from total RNA (5 µg) using SuperscriptIII First-strand cDNA synthesis kit (Invitrogen, USA). The qRT-PCR reaction steps were as follows: 95 °C for 3 min, followed by 32 cycles of 95 °C for 10 s, and 60 °C for 10 s, and 72 °C for 10 s. All the reactions were performed in three replicates and gene expression was estimated using $2^{-\Delta\Delta Ct}$ equation (Schmittgen and Livak 2008). Reference gene *Ntactin*, was used for normalization (Table S1). The Ct value of transgenic line *SbbZIP17*-2 was observed to be lowest and considered as 1 (1-fold) for comparing gene expression with another transgenic lines (Fig. 4a).

Physio-biochemical analysis of *SbbZIP17*-T_1 transgenic tobacco lines under stress

Wild type (WT) plants and independent single copy containing southern positive *SbbZIP17*-T_1 transgenic lines (*SbbZIP17*-2, *SbbZIP17-3*, and *SbbZIP17*-4) were analyzed for physiological and biochemical indices. After sterilization, seeds of *SbbZIP17*-T_1 transgenic lines were placed on MS medium having 100 mg L^{-1} kanamycin, while seeds of WT plants were placed on plain MS medium under conditions of 16 h light/8 h dark and temperature of 24 ± 1 °C. After 30d of germination, plantlets were exposed to heat stress of 42 °C for 0 h, 2 h, 4 h and 6 h in an incubator chamber, thereafter leaves were collected for physio-biochemical analysis. Following analyses were performed in biological triplicates.

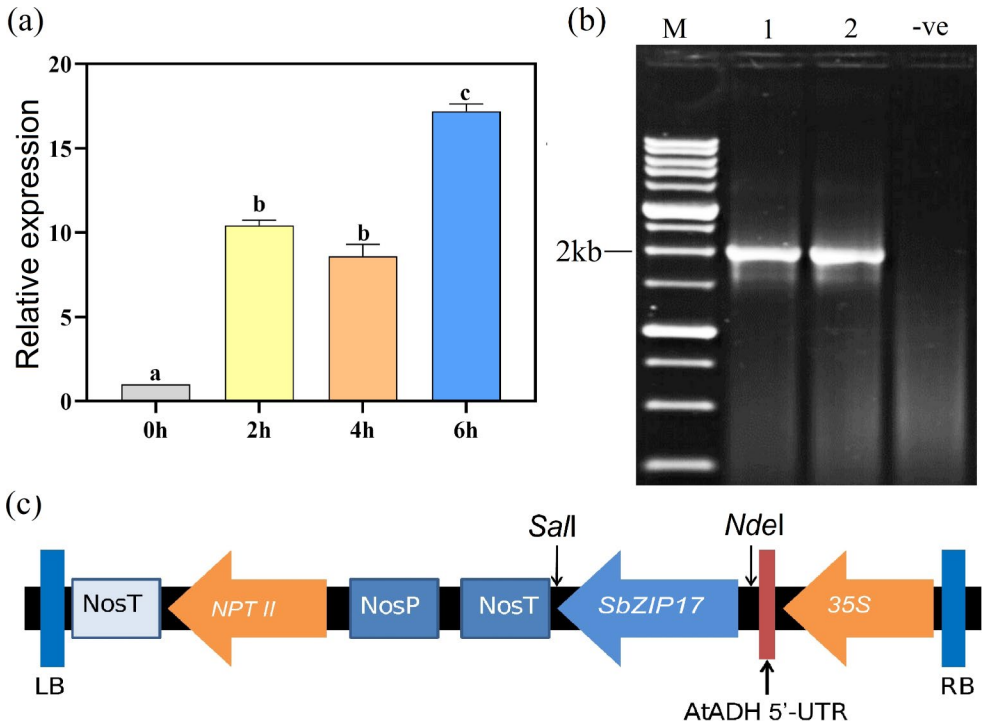

Fig. 1 Schematic representation of qPCR analysis, gene amplification and binary vector construct preparation for *SbbZIP17*. (**a**) Expression pattern of *S. bicolor SbbZIP17* transcript. Statistical significance difference was indicated with an asterisk ($p \leq 0.05$). Error bars represents ± SD from three technical replicates (**b**) PCR amplification of *SbbZIP17* from *S. bicolor* cDNA. Lane 1 and 2 correspond to *SbbZIP17* fragments,–ve corresponds to the negative control. M refers to the molecular marker (1Kb DNA ladder RTU #BM101-R500). (**c**) T-DNA region of binary vector pRI101-AN carrying *SbbZIP17* gene under 35 S enhancer promoter. *nptII* (Neomycin Phosphotransferase) gene for Kan resistance, NOS (nopaline synthase gene terminator), RB-Right border, and LB-Left border

Relative water content (RWC) was estimated as reported by Smart et al. (1973). Extracted and estimated total chlorophyll content as reported by Barnes et al. (1992). The MSI (Membrane Stability Index) was estimated as reported by Sairam et al. (1994) with a conductivity meter. Membrane damage was analyzed by Malondialdehyde (MDA) content, which was estimated by thiobarbituric acid (TBA) reaction as previously reported by Draper et al. (1993). A crude enzyme extracted from leaf tissue was used to assay superoxide dismutase (SOD) activity as reported by Zhang and Kirkham (1994). Total SOD activity was estimated by a capability to hinder reduction of colourless substrate NBT (Nitro Blue Tetrazolium) (Zhang and Kirkham 1995).

Statistical analysis

The estimated data of qRT-PCR and physio-biochemical analysis were applied to a one-way ANOVA through DMRT (Duncan's Multiple Range Test) to estimate the significant difference among the means ($p \leq 0.05$). All given values are means of a minimum of three replicates ± SD. GraphPad Prism 5.0 software was used for statistical analysis. Lower case letters were used to indicate the significant difference.

Results

Expression analysis, cloning and construct preparation for *SbbZIP17*

The qRT-PCR analysis of *S. bicolor SbbZIP17* was evidenced for increased expression level under heat stress. The expression level of *SbbZIP17* was induced rapidly at 2 h (14.7-fold) and reduced at 4 h (8.9-fold), then again increased at 6 h (17.3-fold) of heat stress in comparison to control sample (Fig. 1a). The PCR amplification of *SbbZIP17* with complete ORF carried out using cDNA from total RNA (2 h) (Fig. 1b). Followed by sequencing and annotation of *SbbZIP17*, further it was cloned into shuttle vector pRI101-AN (Fig. 1c).

In silico analysis of SbbZIP17

Sequencing analysis confirmed that the length of *SbbZIP17* CDS was 1971 bp, which was translated into a protein of 656 aa, having a molecular weight of 69.57 kDa. A BRLZ (basic region leucine zipper) domain (177–240 aa) was predicted in SbbZIP17, along with a nuclear localization signal (NLS) (198–207 aa) within BRLZ domain, a TM (Transmembrane) domain (320–342 aa) and a canonical S1P protease site (557–561 aa) (Fig. 2a). Thus, the presence of NLS at N-terminal (towards cytosol) and a TM (Transmembrane) domain at C-terminal (towards ER lumen), is a property of homologous proteins, which led to point out

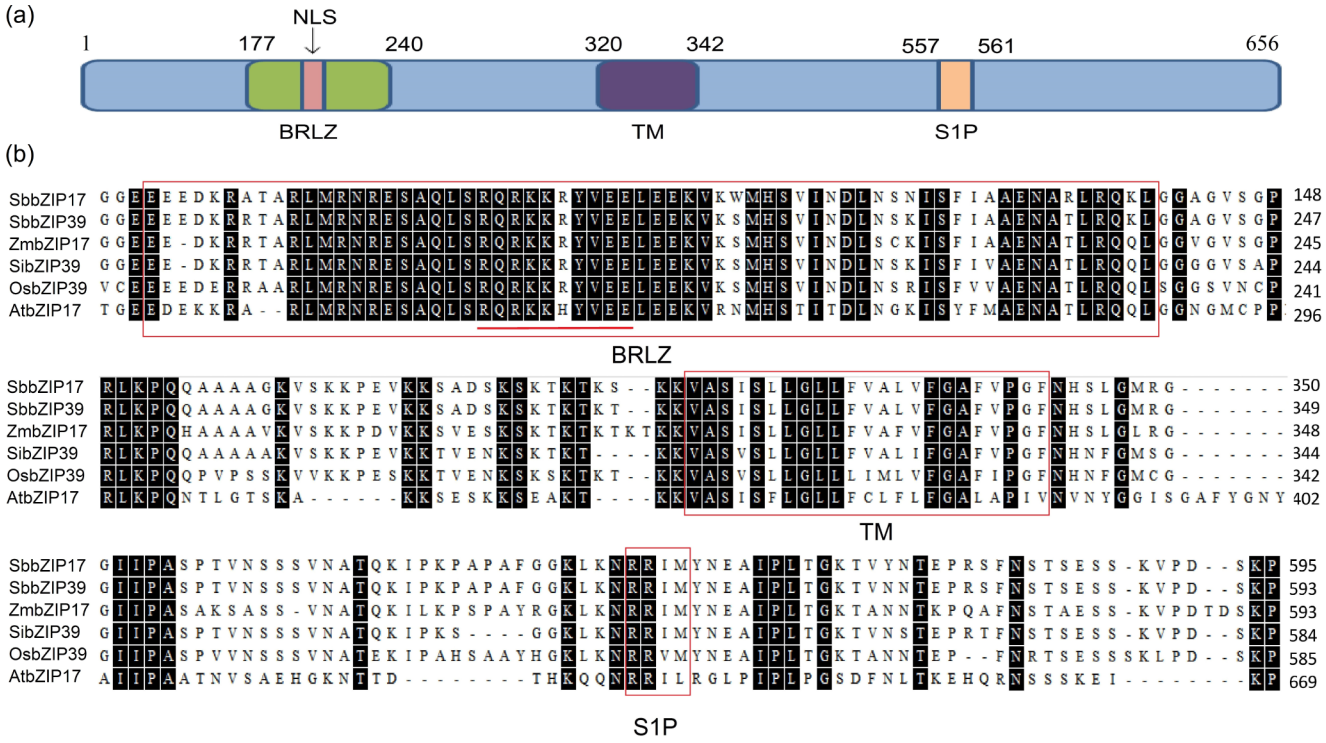

Fig. 2 Domain prediction and multiple sequence analysis of *SbbZIP17* protein. (**a**) Prediction of major domain regions, nuclear localization signal, and S1P protease site in *SbbZIP17* protein (**b**) Multiple sequence alignment of *SbbZIP17* with other bZIP proteins

that SbbZIP17 may be present in the ER membrane. Multiple sequence alignment and motif analysis illustrated that SbbZIP17 is extremely conserved among different plant species (Fig. 2b). SbbZIP17 shared similar motifs while motif 15 present only once in SbbZIP17, while it occurred twice in homologous protein (Fig. S1). Homology analysis of SbbZIP17 revealed 97.71% identity with *S. bicolor* SbbZIP39 (XP_002441096.1), 84.85% identity with the *S. italica* SibZIP39 (XP_004962093.1), 85% with *Z. mays* (NP_001148077.1), 73.52% with *O. sativa* OsbZIP39 (XP_015640085.1), 38.52% with *A. thaliana* (NP_565946.1). Physio-biochemical features of SbbZIP17 are given in Table S2. However, a few additional homologous proteins, HvbZiP17 (KAE8789011.1), AcbZIP39 (XP_020086811.1), MabZIP17 (XP_009384980.1), and SobZIP17 (XP_021840232.1) were used to analyse the evolutionary relationship (Fig. S2). The homologous proteins are well recognized to contribute a significant function in the UPR or the ER stress response under abiotic stress conditions. It is predicted to be localized inside nucleus and gene ontology analysis indicated a role in biological processes including response to stress, signal transduction, and biosynthetic process. Cloned CDS was designated as *SbbZIP17* (accession no: MW532120).

The protein SbbZIP17 was predicted to have a transmembrane helix (TMH) from a region of 320 to 342 amino acids (Fig. S3a). Further, there were 6 *N*-glycosylation sites and 28 *O*-linked glycosylation sites present in the protein (Fig. S3b, c). The secondary structure of SbbZIP17 observed 8 strands and 9 helices (Fig. S4a). 3D ligand binding site was predicted and it showed that only 1 amino acid (i.e., Leucine) was involved in binding located at position 187 (Fig. S4b). Protein-protein interaction showed that SbbZIP17 interacted with stress-responsive proteins and TFs like heat shock 70 (Sb01g010460.1), bZIP transcription factor 60 (Sb02g001970.1), NF-YC4 (Sb07g005060.1), NF-YC3 (Sb07g005540.1) (Fig. S4c). SbbZIP17 also interacted with protease like endoribonuclease IRE1 (Sb02g032960.1), membrane-bound TF site-2 protease (Sb03g029800.1), subtilisin-like protease SBT6.1 (Sb10g004450.1). This analysis further concluded that the SbbZIP17 has a major role under abiotic stress conditions. Based on available mRNA-seq data, we observed expression of *SbbZIP17* was higher at stem elongation, heading, milking, and ripening phase (Fig S5a). Similarly, in almost all other organs expression of *SbbZIP17* was more than 4-fold including leaf, flag leaf while in case of pollen grains it was down-regulated (Fig S5b). The tissue-specific expression analysis of genes homologous to *SbbZIP17* showed diverse differential expression pattern (Fig S6).

Development of tobacco lines overexpressing *SbbZIP17*

The T_0 seeds of all transgenic lines were harvested to obtain T_1 generation lines for further analysis (Fig. S7). Among them, 7 lines were identified as transgenic by using PCR with *nptII* and *SbbZIP17* primers at T_0 stage (Fig. 3a). Southern hybridization at T_1 stage revealed that 6 out of 7 tobacco PCR positive transgenic lines were independent events having 1 to 2 copies of transgene integrations (Fig. 3b). The transgenic lines *SbbZIP17-2, SbbZIP17-3* and *SbbZIP17-4* showed single-copy transgene integration (Fig. 3b), the transgenic line *SbbZIP17-1, SbbZIP17-6*, and *SbbZIP17-7* showed two copies of transgene. However, transgenic line *SbbZIP17-5* failed to show any transgene integration (Fig. 3b). These analyses showed that *SbbZIP17* was integrated into the tobacco genome independent of other lines. Single-copy transgene containing transgenic tobacco lines were used for more analysis.

Expression analysis of *SbbZIP17* and UPR pathway genes in *SbbZIP17*-T_1 transgenic lines

Transgene *SbbZIP17* was expressed 26.4 to 52.6 folds in T_1 transgenic tobacco lines (Fig. 4a). Further to verify the role of *SbbZIP17* in activation of UPR stress responsive genes we performed expression analysis of BiP, PDIL, CNX, and CRT1. Under normal conditions, the genes of UPR pathway expressed approximately at similar level in WT and transgenic lines. However, in response to heat stress all UPR pathway genes were induced in transgenic tobacco than WT plants (Fig. 4b, c, d, e). These analyses showed that *SbbZIP17* expression in transgenic tobacco lines, which involved in activation of genes related to UPR pathway under heat stress conditions.

Physio-biochemical analysis of *SbbZIP17*-T_1 transgenic lines in response to heat stress

Biochemical indexes such as Membrane Stability Index (MSI), Relative Water Content (RWC), and Total chlorophyll content was observed to increase in *SbbZIP17*-T_1 transgenic lines as compared to WT plants under heat stress (Fig. 5). The physiological parameters were positively improved in all tobacco transgenic lines in comparison to WT plants.

Changes in lipid peroxidation activity in *SbbZIP17*-T_1 transgenic lines in response to heat stress were studied. Under controlled conditions, WT and transgenic lines demonstrated nearly similar concentrations of MDA (Fig. 5d). However, under heat stress conditions, the quantity of MDA in transgenic lines and WT were considerably increased, yet the mean increased level of the MDA quantity in tobacco transgenic lines was less in comparison to WT (Fig. 5d). This analysis demonstrated that tobacco transgenic lines with less cell membrane damage as compared to the WT under heat stress. Further, analyzed the quantity of antioxidant enzyme (SOD), between WT and *SbbZIP17*-T_1 transgenic lines under heat stress. It revealed that amount of SOD enzyme in *SbbZIP17*-T_1 transgenic lines significantly enhanced, however less increment was observed in WT plants comparatively.

Discussion

The *bZIP* gene family play important functions in development and adaptation to abiotic stress conditions in plant species. As well, *Sorghum bicolor*, an elite species naturally tolerant to abiotic stresses and has outstanding potential source for bioprospecting of genes liable for abiotic stress tolerance (Mutava et al. 2011; Tari et al. 2013; Baillo et al. 2020). Therefore, present study was performed for functional analysis of the *bZIP* gene from *S. bicolor.*

The qRT-PCR analysis of *SbbZIP17* from *S. bicolor* was showed increased expression level for more than 4-fold under heat stress of 2 h, 4 h and 6 h (Fig. 1a). However, in response to heat stress, genes of UPR pathway (CRT1, BiP, and PDIL) were induced in transgenic tobacco lines overexpressing *SbbZIP17* than WT plants (Fig. 4b, c, d, e). An abiotic stress condition disrupts functional protein assembly and promotes misfolding in the ER leads to turn on UPR pathway (Howell 2013). When build-up of misfolded proteins in ER, moves ER located MTTFs in Golgi body for processing by S1P and S2P protease present in it. DNA binding section of MTTFs transported into nucleus upon S1P intramembrane processing and acts as a TF. In nucleus MTTFs increase expression levels of UPR pathway genes, for instance, CRT1, CNX, BiP (ER chaperone), and PDIL, which assist protein folding in ER lumen thereby provide abiotic stress tolerance (Liu 2012). In *A. thaliana*, four MTTFs AtbZIP members (AtbZIP17, AtbZIP28, AtbZIP49, and AtbZIP60) responsible for the transduction of ER stress signals (Liu and Howell 2010; Bao and Howell 2017).

The cloned *SbbZIP17* encodes a type II transmembrane bZIP MTTF, these TFs usually have a cytosolic facing N-terminus and C-terminus with a canonical S1P cleavage site facing the ER lumen (Howell et al. 2013; Yang et al. 2013). Recently abiotic stress-responsive *bZIP* genes including MTTFs bZIP from different plant species, *T. aestivum*, and *C. annum* were isolated and analyzed based on in *silico* studies (Agarwal et al. 2019; Gai et al. 2020;). TFs reported in *A. thaliana* such as AtbZIP17, AtbZIP28, and AtbZIP49 are type II MTTFs processed with regulated

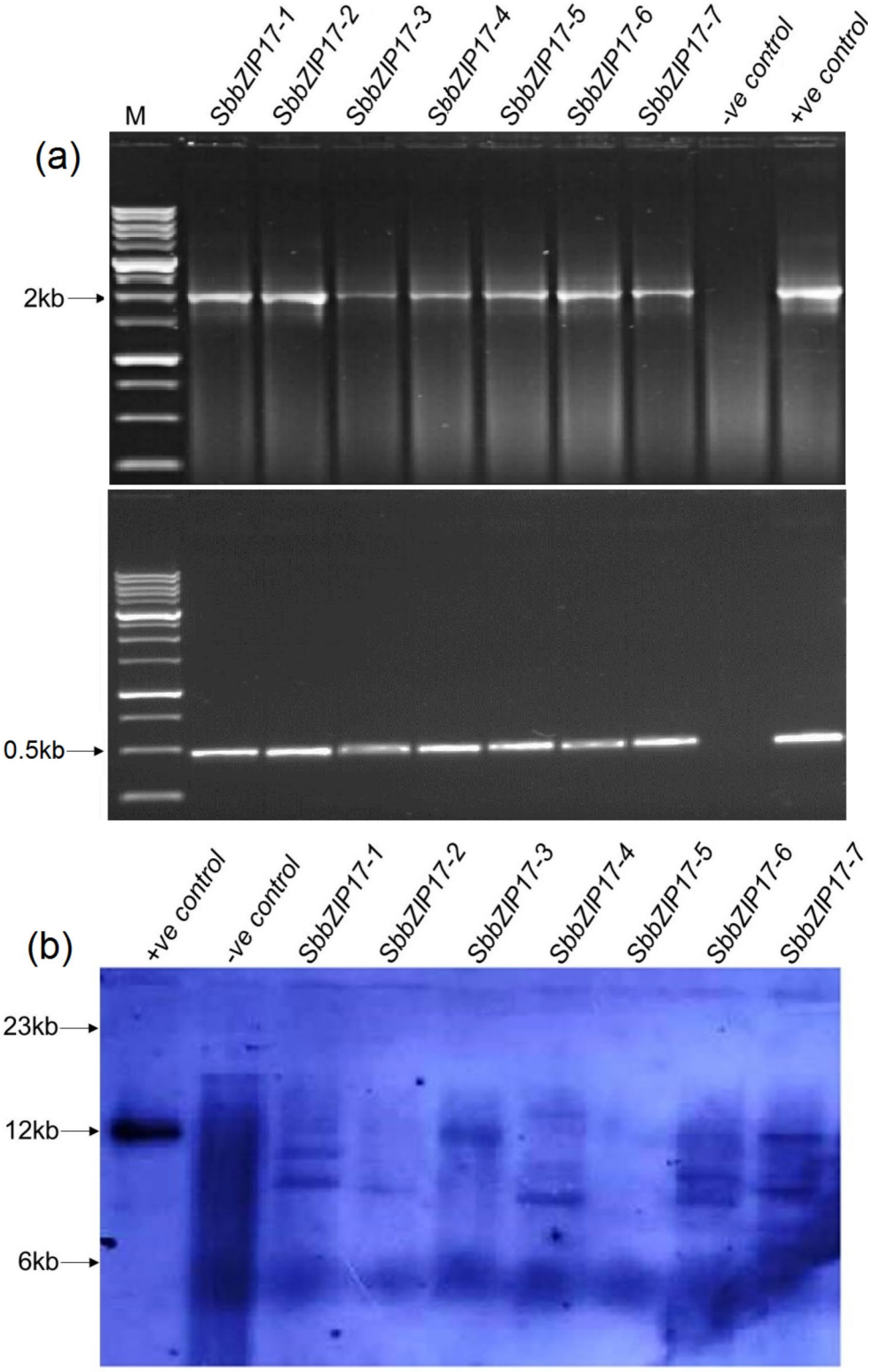

Fig. 3 Putative tobacco transgenic plants confirmation and southern hybridization of transgenic lines. (**a**) PCR analysis of transgenic tobacco lines at $T_{0,}$ using *SbbZIP17* gene (1971 bp) and *nptII* (750 bp) specific primers. '–' sign represents negative control i.e., reaction without any template DNA, '+' sign indicates positive control. (**b**) Southern hybridization of *SbbZIP17*-T_1 transgenic lines using *SbbZIP17* probe., '+' indicates digested and linearized pRI101-AN-*SbbZIP17* vector backbone (~ 10 kb), -ve represent genomic DNA of wild type tobacco plant

intramembrane proteolysis (RIP) and moves to nucleus thereby turn on the UPR pathway. In contrast AtbZIP60 was processed by IRE1-dependent splicing under ER stress (Howell et al. 2013). In *Zea mays*, ZmbZIP60 was identified to be involved in UPR and it belongs to the IRE1 pathway (Howell et al. 2013). Similarly, in our study protein-protein interaction predicted that SbbZIP17 interacted with protease endoribonuclease IRE1 (Sb02g032960.1),

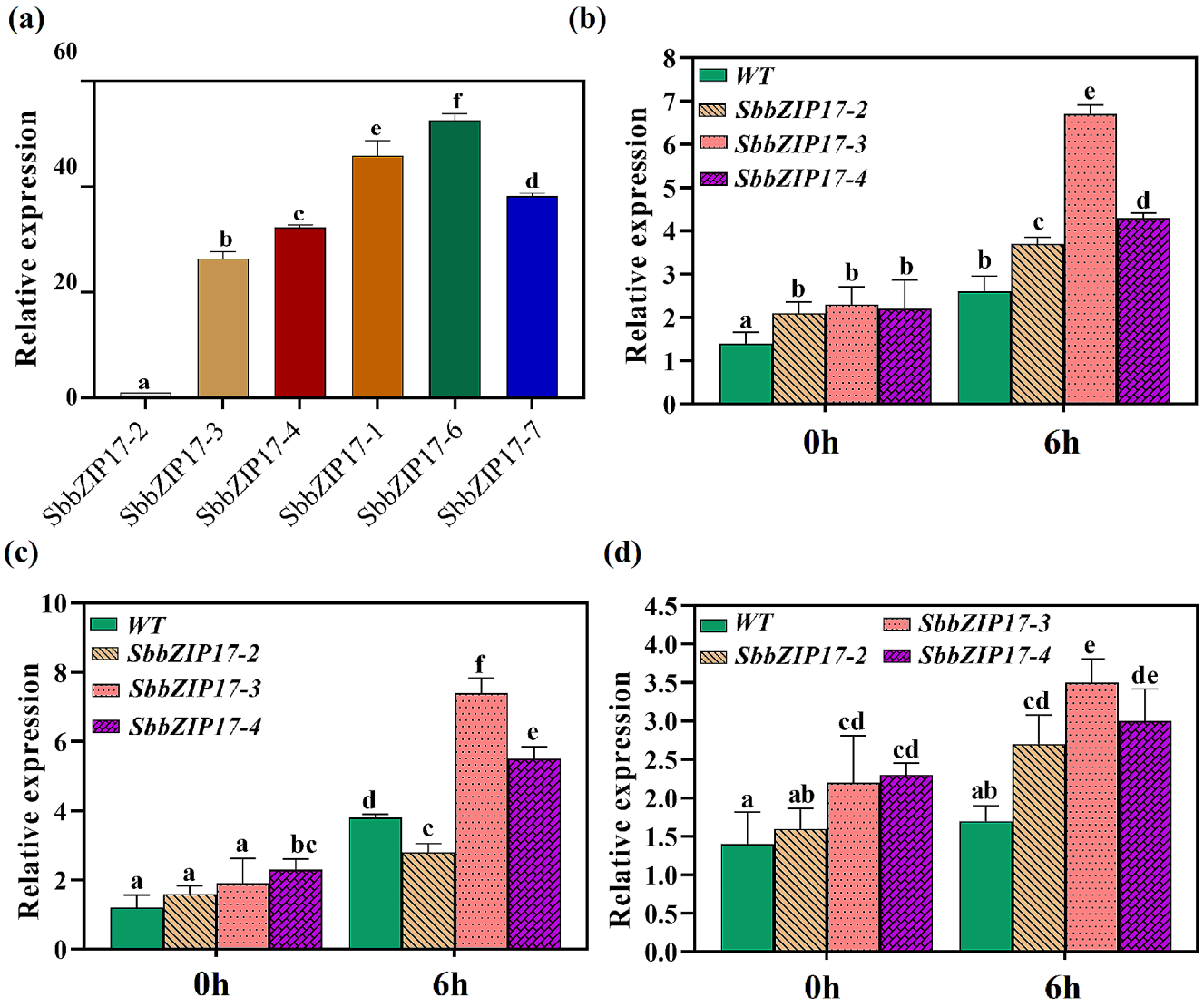

Fig. 4 Expression analysis of *SbbZIP17* and UPR pathway genes in *SbbZIP17*-T_1 transgenic lines. (**a**) Expression analysis of *SbbZIP17* in *SbbZIP17*-T_1 transgenic lines. The Ct value of *SbbZIP17*-2 was the lowest and set as 1-fold for comparison. Expression level of UPR pathway genes (**b**) BiP (**c**) PDIL and (**d**) CRT1 in *SbbZIP17*-T_1 transgenic lines

membrane-bound TF site-2 protease (Sb03g029800.1), and subtilisin-like protease SBT6.1 (Sb10g004450.1). Even under the absence of major abiotic stress conditions, little quantity of bZIPs move from the Golgi body into nucleus because plants are continuously exposed to different small intensity stresses, for instance, temperature variations and irrigation times (Che et al. 2010). Therefore, we observed *in silico* expression of *SbbZIP17* at the stem elongation, heading, milking, and ripening phase and lowest during germination (Fig S5a). Almost in all analysed anatomical organs expression of *SbbZIP17* was observed including flag leaf and other leaves while in pollen grain it was found to be down-regulated (Fig S6b).

Further to analyse the role of *SbbZIP17* in response to heat stress, we developed single-copy transgene integrated tobacco lines overexpressing *SbbZIP17* (Fig. 3b). Several abiotic stress-responsive members of bZIP family from *S. bicolor, P. trifoliate*, *M. hupehensis, T. aestivum*, *O. sativa, P. trifoliata*, and *C. annum* were functionally characterized for drought, heat, osmotic and salinity stress tolerance (Huang et al. 2010; Wang et al. 2011; Zhang et al. 2012; Agarwal et al. 2019; Yang et al. 2019; Gai et al. 2020). Earlier experiments demonstrated that the constitutive expression of bZIP MTTFs such as *AtbZIP60* and *AtbZIP28* in *A. thaliana* offer tolerance to high temperature and salinity stress, correspondingly (Fujita et al. 2007; Gao et al. 2008). *A. thaliana* overexpressing maize *ZmbZIP17* the ortholog of *AtbZIP17* demonstrated that ER acts as stress transducer under stress and optimal growth conditions (Yang et al. 2013). The *ZmbZIP17* was upregulated by ER stress-inducing agents

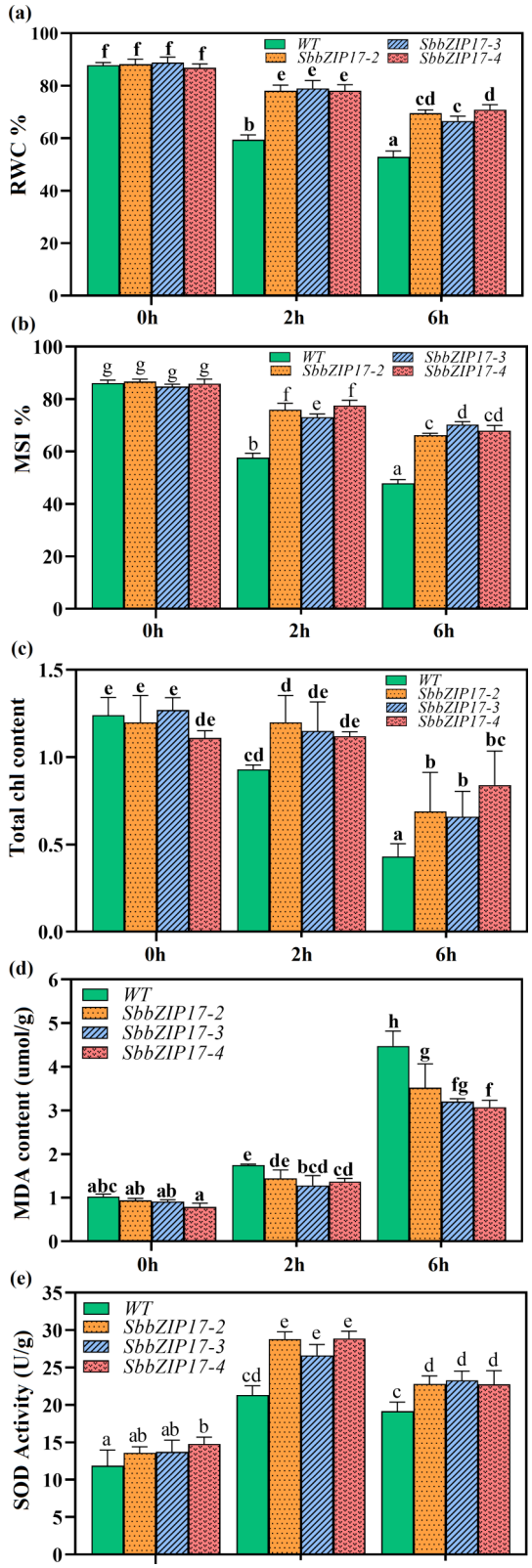

Fig. 5 Biochemical assay of transgenic tobacco lines at T_1 stage under heat stress conditions. (**a**) Relative water content (RWC), (**b**) Membrane stability index (MSI), (**c**) Chlorophyll content, (**d**) MDA (Malondialdehyde) content, (**e**) SOD

and ABA. ZmbZIP17 localized in ER under normal conditions thereafter transported to nucleus due to ER stress-eliciting agents or deletion of transmembrane domain (Yang et al. 2013).

Transgenic tobacco lines overexpressing *SbbZIP17* genes showed enhanced hydration status, antioxidant activities, reduced chlorophyll loss, and membrane damage under heat stress conditions (Fig. 5) The RWC is regarded as an index of dehydration tolerance which points out the metabolic activities and measured the status of water in plant cells (Sinclair and Ludlow 1986). Abiotic stresses such as heat or cold can negatively affect Chl biosynthesis and lead to its degradation, therefore Chl content is considered as a parameter of thermo-tolerance (Rossi et al. 2017). Additionally, heat stress damage the photosynthetic machinery, consequently inhibit plant growth and development. Transgenic tobacco overexpressing *EcbZIP17* from *E. coracana* increased chlorophyll and RWC content in the leaves (Ramakrishna et al. 2018). A few reactive oxygen species (ROS) like hydroxyl radical, hydrogen peroxide, and superoxide, would be produced and build up in plant cells under abiotic stress conditions (Gosavi et al. 2014). The build-up of ROS is cytotoxic to cell and lead to increase in cell membrane permeability, inactivate enzymes, and damage cellular components (Karuppanapandian et al. 2011). To reduce oxidative damages in plant cells, antioxidative enzymes including SOD is playing a fundamental role in scavenging harmful ROS (Gill et al. 2010; Hameed et al. 2012). In *O. sativa* overexpressing *OsbZIP62* gene enhanced the oxidative and drought stress tolerance, whereas *OsbZIP62* mutants showed contrasting phenotype by modulating levels of SOD, MDA, and chlorophyll content (Huang et al. 2010; Yang et al. 2019). Cell membrane injury and destruction of cell structural components are the characteristic attributes related to heat stress damage in plant species (Xu et al. 2014). Transgenic tobacco over-expressing *EcbZIP17* and *PtrABF* bZIP gene from *E. coracana* and *Poncirus trifoliate* respectively strengthen membrane stability (Huang et al. 2010; Ramakrishna et al. 2018). MDA is the ultimate product of membrane peroxidation, higher the peroxidation, the more amount of MDA produced (Hameed et al. 2012). The *OsbZIP62* mutant of *O. sativa* contains significantly greater H_2O_2 and MDA contents than wild type and reduced tolerance to oxidative damage and drought stress (Fig. 5) (Yang et al. 2019). Similarly, *SbbZIP17* expressing transgenic tobacco lines suggested that *SbbZIP17* was involved in enhanced heat stress tolerance. The gene *SbbZIP17* is a potential genomic resource for breeders and researchers for genetic gains in Sorghum crop especially to provide heat stress tolerance under globally changing climatic conditions.

Conclusion

Expression level of *SbbZIP17* from *S. bicolor* was increased under heat stress condition. Bioinformatics analysis of cloned *SbbZIP17* showed that it belongs to type II MTTF of bZIP family. It is highly conserved, having a single ligand-binding site, interacting with heat stress-responsive proteins (HSP70, NF-Ys), expressed in different tissues and organs. Transgenic tobacco lines overexpressing *SbbZIP17* responsible for increased hydration status, SOD, and reduces MDA content, chlorophyll loss, and membrane damage by regulating the UPR pathway genes. The *SbbZIP17* can be used as a potential candidate for the development of climate-resilient crops. Present work has paved way for further study to understand the function of *S. bicolor bZIPs* for abiotic stress tolerance.

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Author contributions **KPP** Conceptualize, designed methodology, conducted experiments, most of formal analysis, data curation, visualization, wrote manuscript and supervise; **HV** conducted part of *in silico* analysis, helped in experiment, edited and reviewed the manuscript; **SPK** help in experiments, edited, and reviewed the manuscript.

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Data availability Gene *SbbZIP17* sequence data was deposited into NCBI database under accession number MW532120.

Declarations

Ethical approval All the authors have been agreed to submit it. This article does not contain any studies with animals performed by any of the authors.

Consent to participate Before the submission of paper, all the author has given the consent to publish.

Consent to Publish All the authors have given the consent to publish.

Conflict of interest The authors declare that they have no conflict of interests to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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