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Full Length Article

Foxtail Millet Stress Associated Protein Gene *SiSAP4* Enhances Drought Stress Tolerance in Transgenic *Arabidopsis*

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Abstract

Abiotic stresses like drought affect plant growth and crop yield with climate change worsening. Stress associated proteins (SAPs), as the zinc finger proteins with A20/AN1 domain, play an important role in regulating abiotic stress response. As a typical summer dryland grain crop in the north of China, foxtail millet has the characteristics of drought resistance, making it a valuable resource for anti-stress gene exploitation and utilization. In this study, *SiSAP4* gene was cloned from foxtail millet variety Yugu 1. Analysis showed that *SiSAP4* gene was expressed in roots, stems and leaves at seedling stage, and the highest expression level was detected in leaves. Expression patterns under different stress conditions showed that expression level of *SiSAP4* gene was significantly up-regulated under drought stress, suggesting it may be involved in drought stress response. Subcellular localization indicated that *SiSAP4* was present in the nucleus and cytoplasm. It was revealed that *SiSAP4* had no function in transcriptional activation in the yeast system. Overexpression of *SiSAP4* in transgenic *Arabidopsis* resulted in enhanced tolerance to drought stress, which was simultaneously demonstrated by increased expression of a broad range of stress response genes. Based on those results, *SiSAP4* has the potential to be used in transgenic breeding to improve drought stress tolerance in other crops.

Keywords: Foxtail millet; *SiSAP4*; Drought tolerance; Transgenic *Arabidopsis*

Introduction

Plants are mostly affected with abiotic stresses such as high temperatures, waterlogging, salt and drought stress. During the process of evolution, plants formed complex regulation network mechanism in response to environmental challenges. Plants can be regulated at molecular and cellular levels to survive adverse environmental events (Hirayama and Shinozaki 2010; Shi *et al*. 2015). This regulatory network relies on transcriptional factors to activate downstream target genes in response to environmental stimulus (Zhang *et al*. 2012; Chakraborty *et al*. 2015). Zinc finger proteins contain numerous functional proteins that play major roles as transcriptional factors, protein modification enzymes and RNA-binding proteins that protect cells against environmental stresses (Zhang *et al*. 2014; Baek *et al*. 2015; Wang *et al*. 2019; Han *et al*. 2020). Stress associated proteins (SAPs) belong to A20/AN1 zinc finger proteins involved in pathways for plant growth and development and in abiotic stress tolerance.

The SAP gene family is broadly present across plant species. Until now, many *SAP* genes have been identified. *OsiSAP1* was the first member among this gene family to be studied (Giri *et al*. 2013). *OsiSAP1* overexpression has been found to enhance the resistance of transgenic tobacco to high salinity, low temperature and drought condition during germination and seedling stages (Mukhopadhyay *et al*. 2004). It has been shown that *OsiSAP8* can be induced by a variety of adverse stresses, namely heat, cold, salt, desiccation, submergence, wounding and heavy metals. Overexpression of *OsiSAP8* showed no significant reduction in rice yield with high salinity and drought stresses at flowering stage (Kanneganti and Gupta 2008). *AtSAP5* can improve the tolerance of transgenic cotton to heat stress by regulating the expression of genes related to water deficit and heat stress protecting the photosystem Ⅱ complex in photosynthesis (Hozain *et al*. 2012). *TaSAP5* can function as E3 ubiquitin ligase, which can ubiquitinize and degrade DRIP protein to increase the accumulation of DREB2A protein, activate the expression of downstream genes, and improve the drought resistance of wheat (Zhang *et al*. 2017). In addition, *SAP* genes have been reported in maize, sorghum, tomato, *medicago*, banana, *aeluropus littoralis* and other crops, where expression responses under stress conditions were determined and functional validation

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using transgenic systems performed (Solanke *et al*. 2009; Saad *et al*. 2010; Xuan *et al*. 2011; Charrier *et al*. 2012; Sreedharan *et al*. 2012; Wang *et al*. 2013).

Foxtail millet (*Setaria italica* L.) originated from northern China and has been an important dryland grain crop. Foxtail millet not only has the characteristics of strong adaptability to drought and infertility, but also has a small genome size, genetic diversity and short life cycle. Therefore, it can be a valuable resource for abiotic stress resistant gene exploration (Muthamilarasan and Prasad 2015; Yang *et al*. 2020). At present, the research on functional genomic data of foxtail millet including the annotation and functional characterization of genes involved in abiotic stress responses has been not analyzed. In this study, *SiSAP4* was cloned from Yugu 1. Expression profile of this gene was analyzed in foxtail millet and the function validated through transfer of the gene to *Arabidopsis*.

Materials and Methods

Plant treatment and growing conditions

Foxtail millet variety "Yugu 1" was used in this study. The millet seeds were planted in vermiculite: nutrient soil 1:1 for three weeks at 23°C with a 16/8 h (light/dark) photoperiod in the chamber. The leaves, stems and roots of the seedlings were taken and utilized to analyze the expression levels of the target gene in different tissues. To analyze the expression levels of target gene in variable stress conditions, millet seedlings with uniform growth at three weeks were selected for stress treatments (Min *et al*. 2013). These millet seedlings were exposed to the following stress treatments: 6% PEG 6000, 100 m*M* NaCl and low temperature (4°C). Leaf samples were collected at 0.5, 1, 3, 6, 9, 12 and 24 h after stress treatment, respectively. All samples were stored at -80°C at once.

Transgenic studies were conducted on *Arabidopsis thaliana* ecotype Col-0 with the chamber condition as: temperature – 23°C; photoperiod – 16/8 h (light/dark) and relative humidity – 65%. To study the expression pattern of water deficit stress response genes both wild type (WT) and transgenic *Arabidopsis* were grown on MS medium supplemented with 250 mM mannitol for 3 h.

Isolation of *SiSAP4* gene and sequence analysis

The full-length cDNA of *SiSAP4* was amplified using primers *SiSAP4* (forward primer, 5´-AGTAGTCATGGAACACAAGG -3´; reverse primer, 5´-CTTGCAGATCACAACCCATC -3´). *pEASY*-Blunt vectors (TransGen, Beijing) were used for PCR product ligation after purification. After successful transformation, the positive clones were picked for sequencing. The amino acid composition, molecular weight and isoelectric point of *SiSAP4* were predicted by online analysis software ProtParam tool (https://web.expasy.org/protparam/). The protein sequence of *SiSAP4* was used as query in a BLASTP program by collecting highly similar sequences to study relationship between *SiSAP4* with other family members from NCBI website. Alignment of sequences was performed by DNAMAN to check similarity and phylogenetic tree was built by neighbor-joining method with 1000 bootstrap replicates using MEGA5.0 (Tamura *et al*. 2011).

Quantitative real-time PCR

Total RNA was isolated from foxtail millet seedlings and *Arabidopsis* plants using RNAprep Pure Plant Kit (Tiangen, Beijing) as manufacturer's instructions. The cDNA was synthesized from RNA template after treatment with DNaseI using Fast Quant RT Kit (Tiangen, Beijing). To check the level of gene expression quantitative real-time PCR (qRT-PCR) was done. The internal control *SiActin* (forward primer, 5´- GGCAAACAGGGAGAAGATGA -3´; reverse primer, 5´- GAGGTTGTCGGTAAGGTCACG -3´) and *AtActin2* (forward primer, 5´-AGCACTTGCACCAAGCAGCATG-3´; reverse primer, 5´-ACGATTCCTGGACCTGCCTCATC-3´) were utilized to determine the relative transcript level of target genes in the *Arabidopsis* and foxtail millet. The qRT-PCR was done in three replicates with an ABI Prism 7500 system consuming the SYBR Green Master Mix kit (TaKaRa, Japan). The relative gene expression levels were calculated by the $2^{-\triangle\triangle CT}$ method (Schmittgen and Livak 2008).

Subcellular localization of *SiSAP4* protein

Agrobacterium mediated transformation was performed in tobacco leaves using strain GV3101 by making gene construct between (pCAMBIA1300- *SiSAP4-GFP*) and control (pCAMBIA1300-GFP). This was kept for incubation at 25°C with a 16/8 h (light/dark) photoperiod for 2 d and fluorescence signals were checked by confocal laser scanning microscope.

Generation of *Arabidopsis* transgenic plants

The full-length cDNA of *SiSAP4* gene was inserted between *Xba*I and *Kpn*I position (forward primer, 5´-TGCTCTAGAATGGAACACAAGGAGGCG -3´; reverse primer, 5´- CGGGGTACCGATCTTGTCGAGCTTCTC -3´, *Xba*I and *Kpn*I sites underlined) of the pCAMBIA1300 vector. Prepared gene construct was inserted into GV3101 strain of *Agrobacterium tumefaciens* and then transformed into *Arabidopsis* using floral infiltration (Clough and Bent 1998). After hygromycin resistance screening and PCR detection, T_3 homozygous transgenic lines were obtained and three lines were randomly selected for subsequent experiments.

Stress tolerance assays of transgenic plants

To observe the effect of osmotic stress on transgenic *Arabidopsis*, 7-d-old seedlings were transplanted to MS medium with 0 or 250 mM mannitol for 10 d and then phenotypes were observed. The primary root length of five transgenic plants and control (WT) were calculated. To further investigate the drought stress tolerance of transgenic *Arabidopsis*, 7-d-old seedlings were grown in plates filled with mixture of soil, well-watered and kept in growth chamber under short day conditions (12/12 light/dark) without watering. After a water-withholding period and then re-watering for 3 d, the survival rate of transgenic plants and control (WT) were calculated. The abiotic stress-related physiological characterization of transgenic *Arabidopsis*, including cell membrane stability (CMS) and water loss rate was measured (Mao *et al*. 2010).

Expression analysis of the stress response genes

The expression level of stress response genes in transgenic *Arabidopsis* was analyzed by qRT-PCR. *Arabidopsis* seedlings were subjected to the MS medium with 250 mM mannitol for 3 h and the tissue samples were harvested. Based on the conserved regions of stress response genes, specific primers were designed to detect the expression level (Supplementary Table S1).

Transcriptional activity assay

For transcriptional activity assays GAL4-based Matchmaker Two-Hybrid System (Clontech) with AH109 strain of *Saccharomyces cerevisiae* were utilized. For cloning purpose, pGBKT7 vector was used by inserting full length ORF of *SiSAP4* and two truncations to make fusion with GAL4-binding domain and then transformed to AH109 yeast strain and kept for culturing until optical density at 600 nm reach to 1.0. Later, the suspension was grown into SD/-Trp and SD/-Trp/-His medium. An empty pGBKT7 vector was used as control.

Statistical analysis

Three replications of each sample were utilized for the experiments. The data represented is the mean ± SD. To study normal and drought stress conditions parameters data were analyzed by two tailed Student's t-test method. The significant differences were represented at the level $P < 0.05$ or $P < 0.01$.

Results

Isolation and sequence analysis of *SiSAP4*

The target gene *SiSAP4* was isolated from foxtail millet. The full length of *SiSAP4* was 516 bp, encoding 171 amino acids. The predicted molecular weight was 18.21 kD, and the isoelectric point was 8.28. Protein structure prediction *SiSAP4* contained an A20 and an AN1 domain. Sequence alignment showed that the amino acid sequences of the two domains of *SiSAP4* were highly similar to those of other species (Fig. 1A). Phylogenetic evolutionary analysis showed that *SiSAP4* can be classified in other monocotyledonous plants, including sorghum, *Zea mays*, rice, wheat and *Brachypodium distachyon* (Fig. 1B).

Expression patterns of *SiSAP4* in various tissues and under abiotic stresses

The expression patterns of *SiSAP4* in various tissues at seedling stage were examined by qRT-PCR. The transcript of *SiSAP4* was identified in leaf, stem and root. The highest expression levels were observed in leaf tissues. The lowest expression was checked in root (Fig. 2A). Hence, the current research mainly focused on the leaf tissues for the consequent analyses.

Expression levels of *SiSAP4* detected under different stress treatments, including PEG, salt and cold showed that its expression was significantly activated by PEG, but relatively slightly by salt and cold (Fig. 2D). Under PEG conditions, the expression levels of *SiSAP4* reached a peak at 1 h and maintained to 3 h, with the corresponding maxima being 4.2 greater than the control. Under high salinity conditions, the expression levels of *SiSAP4* firstly decreased and then increased gradually, reaching a peak at 9 h, 1.5 greater than the control. At low temperature, the expression of *SiSAP4* showed two small peaks at 0.5 h and 12 h, both 1.5 times higher than the control.

Subcellular localization of *SiSAP4*

Subcellular localization of *SiSAP4* was observed in tobacco leaves. The construct *SiSAP4-GFP* fusing protein was driven by the CaMV 35S promoter. The green fluorescence was observed in the cytoplasm, cell membrane and nucleus in tobacco leaf epidermal cells using fluorescence microscopy. Hence, it was found that *SiSAP4-GFP* was located in the cytoplasm, cell membrane and nucleus (Fig. 3).

Overexpression of *SiSAP4* in *Arabidopsis* to enhanced drought stress tolerance

Three transgenic homozygous T3 *Arabidopsis* lines were selected randomly to study the role of *SiSAP4* under abiotic stress. Variable level of expression observed in *SiSAP4* transgenic lines though it was remarkable higher than expression in WT plants (Fig. 4B). The expression level of *SiSAP4* was significantly up-regulated under osmotic stress. The 7-day-old transgenic *Arabidopsis* and WT were placed in MS medium with 250 m*M* mannitol. After 10 days, the phenotype of transgenic *Arabidopsis* and WT was basically the same in MS medium. The growth of transgenic

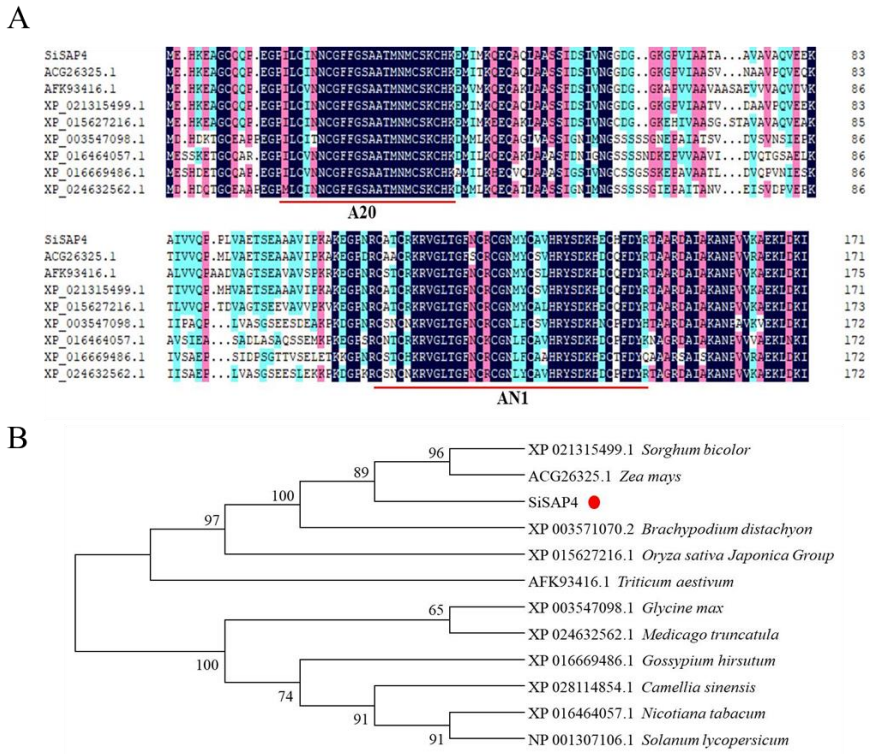

Fig. 1: Sequence alignment of *SiSAP4* and SAPs in various plant species. (**A**) Alignment of SAPs from different plant species. ACG26325.1 from *Zea mays*, AFK93416.1 from *Triticum aestivum*, XP_021315499.1 from *Sorghum bicolor*, XP_015627216.1 from *Oryza sativa Japonica Group*, XP_003547098.1 from *Glycine max*, XP_016464057.1 from *Nicotiana tabacum*, XP_016669486.1 from *Gossypium hirsutum*, XP_024632562.1 from *Medicago truncatula*. Common identical amino acid residues are shaded black. The conserved A20 domain and AN1 domain are marked under the alignment with lines. (**B**) Construction phylogenetic tree of SAPs using neighbor-joining method with 1000 bootstrap replicates by MEGA5.0. *SiSAP4* is marked with red dots

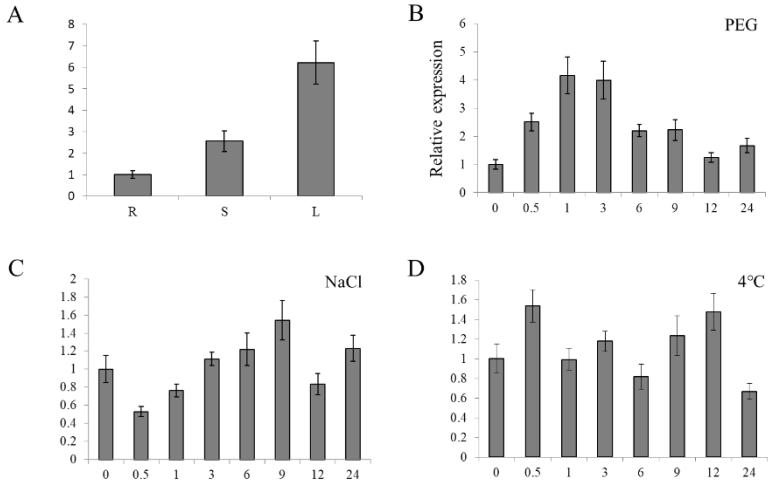

Fig. 2: Expression patterns of *SiSAP4*. (**A**) Tissue expression patterns of *SiSAP4* at seedling stage. L, leaf; R, root; S, stem. (**B**) Relative expression of *SiSAP4* under polyethlene glycol-6000 (PEG) treatment. (**C**) Relative expression of *SiSAP4* under NaCl treatment. (**D**) Relative expression of *SiSAP4* under cold (4°C) treatment. Means were calculated from three independent experiments

Arabidopsis and WT both were inhibited in MS medium with 250 mM mannitol, however, the growth of transgenic *Arabidopsis* was less inhibited (Fig. 4A). The length of primary root of transgenic *Arabidopsis* was significantly higher than WT (Fig. 4C).

To further verify the function of *SiSAP4* under drought stress, the 7-day-old transgenic *Arabidopsis* and WT were planted in nutrient soil for drought stress treatment. Seedling

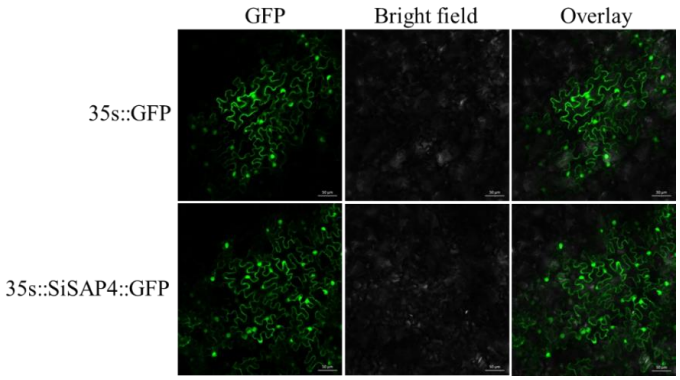

Fig. 3: Subcellular localization of *SiSAP4* in tobacco leaf cells. The fusion construct (35S::*SiSAP4*::GFP) was transiently expressed in tobacco epidermal cells. Empty vector (35S::GFP) was used as control. Scale bar=50 μm

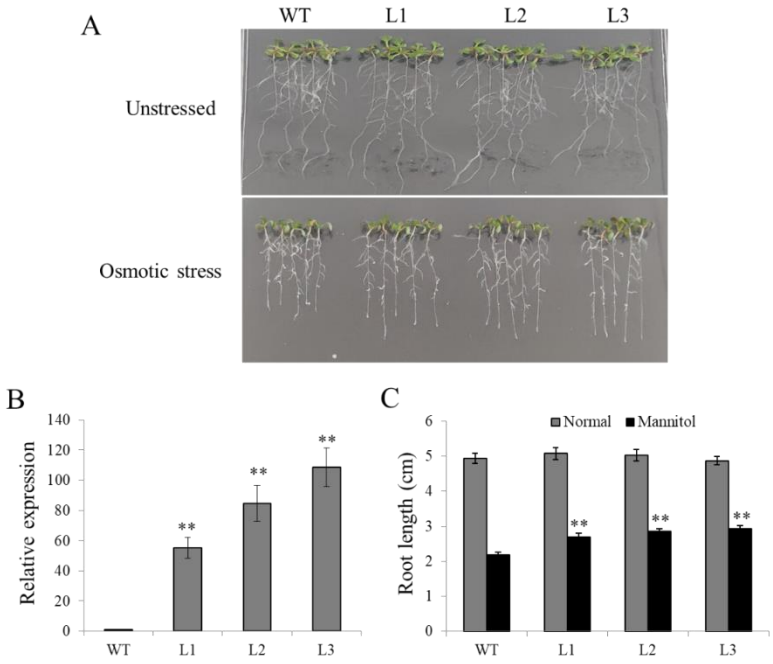

Fig. 4: Overexpression of *SiSAP4* improves osmotic stress tolerance in transgenic *Arabidopsis*. (**A**) Phenotypes of three *SiSAP4* transgenic lines (L1–3) and wild type (WT) under osmotic stress. (**B**) The expression level of *SiSAP4* in three overexpressing *Arabidopsis* lines. (**C**) Comparison of primary root lengths of *SiSAP4* overexpressing *Arabidopsis* lines. **, $P < 0.01$

survival percentage was calculated after rewatering for three days, and it was significantly higher (56–69%) for transgenic lines than WT (17%) (Fig. 5A–B). There are certain physiological changes observed in plants due to drought stress. CMS of *SiSAP4* transgenic plants was observed to be higher than WT (Fig. 5C). Water loss assay was performed in 8-h detached-rosette of 4 weeks old *SiSAP4*-transgenic overexpressed *Arabidopsis* plants and WT. The higher water loss rate was observed in WT than the transgenic plants (Fig. 5D).

SiSAP4 enhances expression of abiotic stress response genes in transgenic plants

Phenotypes assays indicated that *SiSAP4* transgenic lines had enhanced tolerance to drought stress. Plants established intricate cellular signaling mechanism to manage the drought stress. In this study, it was further confirmed that several abiotic stress-responsive genes were up-regulated by *SiSAP4*. The expression level was checked in 8 abiotic stress related genes (*P5CS1*, *RD29A*, *RD29B*, *RD22*, *COR47*, *COR15a*, *RAB18* and *KIN1*) under drought stress and normal environment. It was inferred that the expression level upregulated in *P5CS1*, *RD29A*, *RD29B*, *RD22*, *COR47* and *KIN1* genes under water deficit conditions in transgenic plants by 1.3 to 6.9 fold than WT plants. However, compared to the WT, expression level of transgenic plants in *COR15a and RAB18* genes had no remarkable changes under both water deficit and normal environment (Fig. 6).

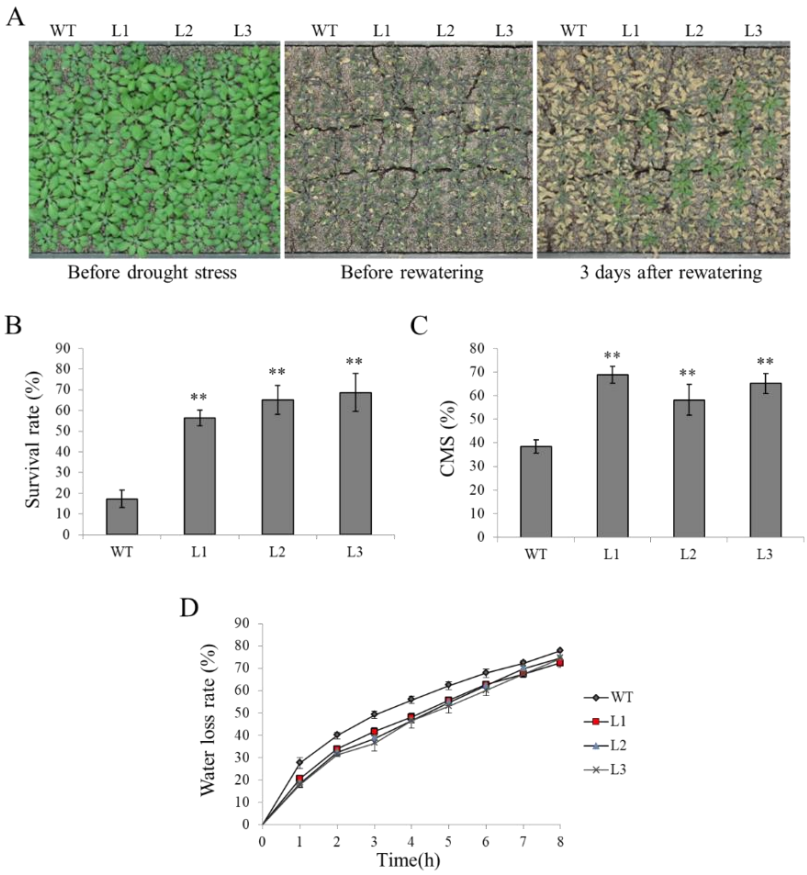

Fig. 5: Overexpression of *SiSAP4* improves drought stress tolerance in transgenic *Arabidopsis*. (**A**) Phenotypes of three *SiSAP4* transgenic lines (L1–3) and wild type (WT) under drought stress. Three transgenic lines and WT were planted in two rows, respectively. (**B**) Comparison of seedling survival rate between transgenic plants and WT. (**C**) Comparison of cell membrane stability (CMS) between transgenic plants and WT. (**D**) Comparison of water loss rates for detached rosettes between transgenic plants and WT. **, $P < 0.01$

SiSAP4 lacks transcriptional activation potential

To detect the transcriptional activation of *SiSAP4*, the full-length ORF of *SiSAP4* and two truncations, according to the two domains (A20 and AN1), were cloned into pGBKT7 to produce in-frame fusions to GAL4-binding domain. The yeast containing two truncations and the full-length ORF of *SiSAP4* can grow in SD-Trp medium, however, they don't grow in SD-Trp/-His medium (Fig. 7). The above results showed that *SiSAP4* and two domains did not have transcriptional activation.

Discussion

There are abundant combinations of the domains of stress associated proteins (*SAPs*) in plants, including A20+AN1, A20, AN1 and 2AN1 *etc*. The most common combination of the domains was A20+AN1 (Vij and Tyagi 2008). In this study, *SiSAP4* included an A20 and an AN1 domain, being a typical domain combination of SAP. In different plants, most members of the SAP gene family had no introns (Jain *et al*. 2008). *SiSAP4* also had no introns, suggesting that it can be rapidly transcripted and expressed to perform biological functions.

Numerous studies show that the plant SAP gene family is constitutively expressed. For example, the transcript of *TaSAP17-D* had been shown to be expression in various tissues at different development stages and higher expression in leaves at seedling stage (Xu *et al*. 2018). In this study, expression analysis in different tissues at seedling stage showed that the expression levels of *SiSAP4* were higher in leaves than in stems and roots, suggesting that it mainly played its biological role in leaves. The active and variable expression levels of *SiSAP4* under different abiotic stresses were assessed. *SiSAP4* was up-regulated in dehydration stress, suggesting that it played a major role in coping with the drought stress rather than salt and cold stresses. Most members of the SAP gene family were reported as positive regulators in the plant drought stress responses, such as *AlSAP*, *MtSAP1* and *MusaSAP1* (Saad *et al.* 2012; Sreedharan *et al.* 2012; Charrier *et al.* 2013). It was observed that *SiSAP4* overexpressing plants possessed longer roots in the osmotic stress. Meanwhile, survival percentage of transgenic plants was improved in drought

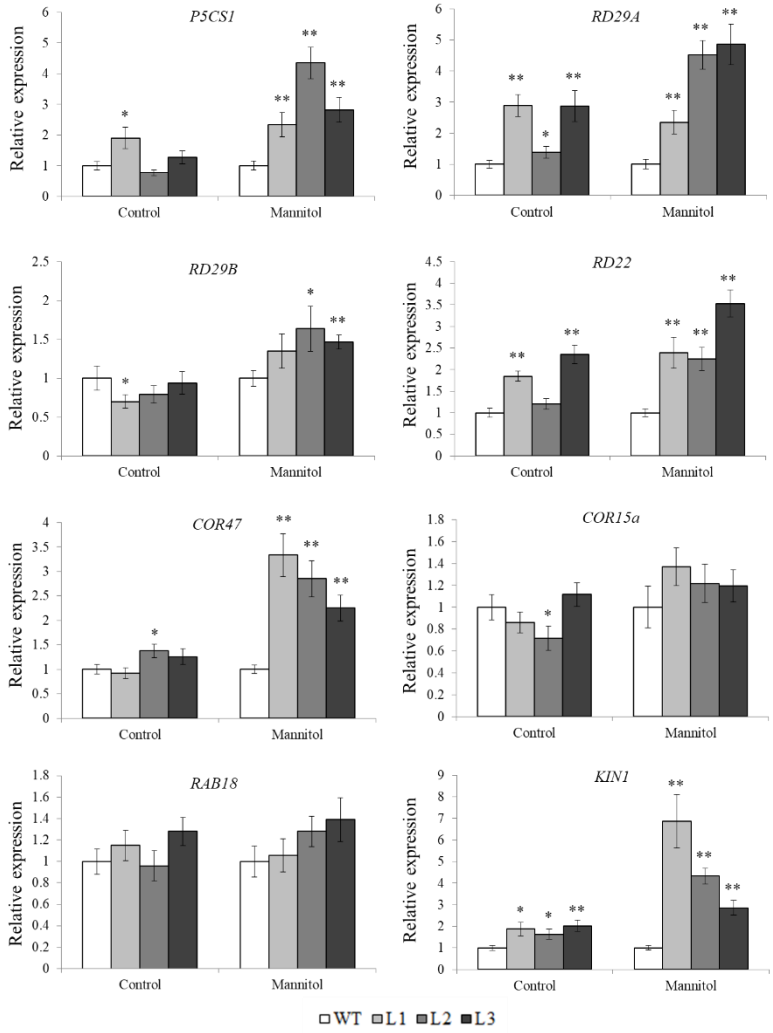

Fig. 6: Effect of overexpression of *SiSAP4* in transgenic *Arabidopsis* on the expression of stress-responsive genes. Relative expression levels of stress-responsive genes were determined by qRT-PCR in transgenic plants and wild type (WT) under normal condition and osmotic stress. *, $P < 0.05$; **, $P < 0.01$

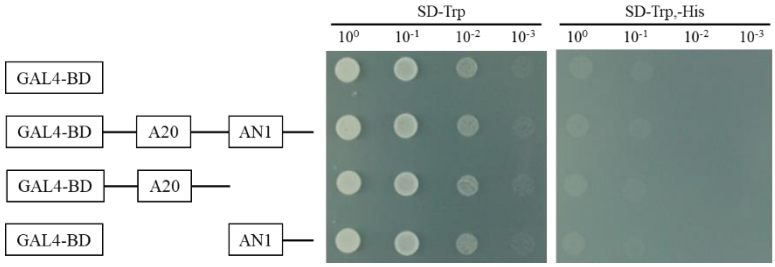

Fig. 7: Transcriptional activation activity assay of *SiSAP4*. According to the amino acid position of the conserved domain, transcriptional activation activity of *SiSAP4* of the full-length and two truncations. A20 domain truncation is 1-111 amino acid residues. AN1 domain truncation is 112-171 amino acid residues

stress contrast to the WT plants. Drought stress induces various biochemical and physiological change. CMS and water loss rate were selected to monitor drought stress tolerance in the present study. Plants with higher CMS often have enhanced tolerance to drought stress (Farooq and Azam 2006). It was inferred from the study that *SiSAP4* transformants showed higher CMS under drought stress than the WT. Detached-leaf water loss rate is suggested as an indicator of water status (Clarke *et al*. 1989; Dhanda and Sethi 1998). In present research work the WT plants showed

higher detached-leaf water loss rate than *SiSAP4* transgenic plants, that strongly shows that transgenic plants had higher water retention capacity.

Plant signaling system during stress response was quite complex and mostly interconnected. Drought is a major abiotic stress to limit crop yields. Drought-related transcription factors can activate the expression of many downstream genes about regulation and signal transduction to improve plant stress resistance (Bartels and Sunkar 2005). The present studies show that SAPs can regulate the expression of downstream genes, especially those involved in stress responses (Wang *et al.* 2016). In this study, the overexpression of *SiSAP4* upregulated a broad range of stress responsive genes. It is inferred that the improved tolerances to drought stress are major attributable to consistently and significantly enhanced expression of stress responsive genes including *P5CS1*, *RD29A*, *RD29B*, *RD22*, *COR47* and *KIN1*. In contrast, no deviation in the expression of *COR15a* and *RAB18* was found in transgenic plants and WT suggesting the two genes are not involved in the drought stress pathway affected by *SiSAP4*. Taken together, *SiSAP4* can be used as a candidate gene to play a role in crop resistance to drought stress. Interestingly, we found that *SiSAP4* lacks transcriptional activation potential. It was speculated that *SiSAP4* could work with other proteins to activate the expression of many downstream genes.

Conclusion

According to our findings, we suggested that the improved tolerance to drought stress by *SiSAP4* overexpressing plants was due to the up-regulation expression of abiotic stress related genes. The *SiSAP4* gene could be instrumental in improving drought stress tolerance in foxtail millet and other plants.

Acknowledgements

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Author Contributions

Wenlu Li and Liguang Zhang designed and conducted the experiments; Faheeda Soomro and Pingyi Guo analyzed the experimental data; Xiangyang Yuan and Yixue Wang helped conceiving the study and participated in manuscript writing.

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