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Research

The *Oryza sativa Stress Associated Protein* (*OsSAP*) Promoter Modulates Gene Expression in Response To Abiotic Stress by Utilizing Cis Regulatory Elements Within The Promoter Region

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ABSTRACT

The occurrence of extreme weather patterns induced by climate change has resulted in abiotic stress problems impacting the growth and productivity of plants. Rice (*Oryza sativa*), a staple food source for most Asians, is similarly affected by these challenges. Previous studies have identified the *Oryza sativa Stress Associated Protein (OsSAP)* genes to play a significant role in responding to abiotic stress. Among the 18 Stress Associated Protein members, *OsSAP4* was highly expressed during drought and salinity conditions. Therefore, further experiments have been conducted, focusing specifically on the promoter region, to comprehend its regulation in response to abiotic stresses. Various types of cis-elements binding sites have been identified within the *OsSAP4* promoter, encompassing MYB, CAMTA, CPP, C3H, HDZIP, bZIP, WRKY, and ERF. However, promoter analysis revealed that the distribution of the Cis-Regulatory elements bound by the Ethylene Response Factor (ERF) was the most prominent in the *OsSAP4* promoter. Consequently, an analysis of promoter regulation was conducted using GUS reporter in *Arabidopsis thaliana* (*A. thaliana)* on two different sizes of *OsSAP4* promoter sequences, each containing different quantities of ERF transcription factor binding sites. A noticeable difference in GUS staining activity was observed between *pOsSAP4(1524 pb)::GUS* and *pOsSAP4(460 pb)::GUS*, where *pOsSAP4(1524 pb)::GUS* exhibited higher GUS staining activity than *pOsSAP4(460 pb)::GUS*. The differences in GUS staining analysis are evident at the vegetative stage (leaf), silique, and inflorescence stages. This implies the participation of various other cis-element binding sites that influence the expression pattern of the *OsSAP4* promoter during abiotic stress.

Key words: Abiotic stress, arabidopsis, β-glucuronidase (GUS), cis-regulatory elements, promoter, rice, *Stress Associated Protein 4*

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INTRODUCTION

Concerns surrounding food security have intensified due to the expanding global population and the growing unpredictability of environmental conditions (Waqas *et al*. 2019). Plant stress, arising from external factors that disrupt growth, development, and productivity, can have profound effects. It can reshape gene expression, alter metabolic processes, impede growth rates, and imperil crop yields (Cohen & Jan 2019; Khairulbahri 2021). Conditions such as cold, heat, drought, flooding, salinity, nutrient deficiency, and fluctuations in light intensity encountered by plants negatively impact their growth and development (Ramakrishna & Ravishankar 2011; Husain *et al*. 2020). These stressors can lead to substantial reductions in plant survival and output, accounting for approximately 70% of crop yield losses (Waqas *et al*. 2019). Understanding how plants react to abiotic stresses is pivotal in mitigating their adverse impact on growth, productivity, and consistent crop yields. Plants employ physiological and biochemical

strategies to counteract abiotic stress (Dos santos *et al*. 2022).

As stationary organisms, plants have developed adaptive mechanisms to endure and acclimatize to environmental stresses. Recent advancements in plant molecular technology have unveiled the complex molecular mechanisms underlying responses to abiotic stresses, shedding light on specific stress-related genes. For instance, in times of stress, genes may be either activated or suppressed via the ubiquitin-proteasome system, ensuring proper regulatory responses to stressful conditions (Gibbs *et al*. 2011; Peleg & Eduardo 2011; Gibbs *et al*. 2014; Lenka & Kailash 2019; Ullah *et al*. 2019).

Amidst numerous discoveries of stress-related genes, the Stress-associated proteins (SAPs) gene family has recently emerged as a significant regulator of stress tolerance, also recognized as zinc finger proteins (ZFPs). The protein is known with an initial A20 domain located at the C-terminal region and an AN1 domain positioned at the N-terminus. An early milestone in transgenic plant research involving SAP genes focused on the introduction of the *SAP8* gene (*OsSAP8*) into tobacco and rice. Overexpression resulted in improved tolerance to salt, drought, and cold stresses during seed germination and seedling stages, evidenced by measures such as germination percentage and fresh weight after stress recovery (Kanneganti & Gupta 2008). According to Roslan *et al*. (2017), regulating *OsSAP8* expression confers tolerance to drought, salinity, and insensitive to ABA during germination, indicating that modulating *OsSAP8* gene expression influences ABA sensitivity and signaling (Roslan *et al*. 2017). Another recent experiment involving transgenic rice overexpressing *OsSAP16* shows that it plays an important role as a regulator of low-temperature germination (Wang *et al*. 2018). Overexpression of *AtSAP5* in cotton plants resulted in improved tolerance to drought and heat stress by regulating genes associated with stress responses (Kang *et al*. 2011). Similarly, the overexpression of Arabidopsis *SAP10* (*AtSAP10*) was found to enhance tolerance to high temperatures and toxic metals (Dixit & Dhankher 2011). Additionally, *AtSAP13* was shown to confer tolerance to drought, salinity, and toxic metals (Dixit *et al*. 2018).

However, no studies have reported on the SAP regulatory mechanisms in response to stress, particularly at the transcriptional level. Understanding promoter regulation is important to allow manipulation and optimization of gene expression for desired traits, such as stress resistance or increased yield. The promoter sequence consists of three sections, namely the core promoter, proximal promoter, and distal promoter (Wang *et al*. 2018). These sections are categorized based on their function and their distance from the 'Transcription Start Site (TSS)'. The core promoter is the segment of the promoter that is adjacent to the TSS (Villao-Uzho *et al*. 2023). The length of the core promoter varies depending on the species but is typically around 250 base pairs. The core promoter contains promoter motifs such as TATA and CAAT boxes, downstream promoter elements, and initiation elements. The proximal promoter is situated next to the core promoter, followed by the distal promoter. There are three main types of promoters in plants: constitutive promoters, inducible promoters, and promoters' specific organs or tissues (Brooks *et al*. 2023).

Constitutive promoters are frequently used in plant transformation processes and are employed to express transgenes for various purposes, including enhancing disease resistance, adapting to environmental changes, producing secondary metabolic products, regulating plant development, and increasing crop yield (Villao-Uzho *et al*. 2023). Constitutive promoters can express certain genes in all organs and tissues throughout the plant developmental process (Melo *et al*. 2021). While constitutive promoter expression typically results in a high and consistent level of expression, it can pose challenges due to its nonspecific expression in plants (Zhao *et al*. 2021). Certain genes are only necessary in plant systems at specific times or under specific environmental conditions. Moreover, nonspecific gene expression can interfere with proper gene expression, leading to growth issues in plant development.

The alteration in gene expression originates from promoter activities and is sometimes guided by specific cis-regulatory elements (CREs), short nucleotide motifs encoding critical non-coding DNA regions for regulating gene transcription (Heidari *et al*. 2015). These CREs interact with transcription factors (TFs), enhancing or suppressing transcription efficiency (Lenka & Kailash 2019). Understanding gene expression coordination and regulation across various levels, from transcriptional to post-translational, through CREs and TFs, holds potential for advancements in genetic engineering.

In this paper, our discussion centered around the distribution of cis-elements with enriched transcription factor binding sites (tfbs) associated with abiotic stress within the promoters of *OsSAP4*. This specific gene was chosen for in-depth analysis due to its elevated expression levels in response to drought and salinity (Roszelin *et al*. 2023), surpassing those of other *OsSAPs*. Examining the crucial role of cis-regulatory elements that are bound by the specific transcription factor is paramount in understanding their contribution to enhancing *OsSAP4* expression during stress conditions and conferring abiotic stress tolerance.

MATERIALS AND METHODS

Retrieval of upstream sequences and promoter analysis of all *OsSAP*s genes

For promoter analysis, a segment of approximately 2 kb upstream from the ATG start codon of all *OsSAPs* was selected. The promoter sequence was obtained from the Ensembl Plants (https://plants.ensembl.org/index.html). The crucial elements of the plant promoter such as CAAT-box, TATA box (core promoter element), and transcription start site (TSS) in the 5′ upstream regions of the gene were analysed using the TSSP/Prediction of PLANT Promoters database (http://www.softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=promoter) and Promoter 2.0- Transcription start sites in vertebrate DNA database (https://services.healthtech.dtu.dk/service.php?Promoter-2.0. The abundance of Cis elements (CE) and transcription factor (TF) elements associated with all *OsSAP* genes were determined in this study with significant value (p<0.05). Transcription Factor Database (TFDB) server (http://plantregmap.gao lab.org/regulation_prediction.php) and PlantPan Database (http://plantpan.itps.ncku.edu.tw/plantpan4/index.html) were used to analyze cis-regulatory elements enriched with TF binding site in all *OsSAPs* gene and specifically in *OsSAP4*.

Cloning of the promoter *OsSAP4*

Two sizes of *OsSAP4* promoter regions were cloned into Gateway vectors using Gateway Cloning Technology which were 1524 bp and 460 bp respectively. During the initial cloning step, the pDONR221 vector functions as an intermediate clone using BP clonase. The promoter regions were cloned into the pDONR221 vector, facilitating heterologous recombination through specific adaptors in both the primers and the intermediate vector. The entry clones were subsequently transformed into competent *E. coli* Top10 using the heat-shock method. Successful transformants were selected based on Kanamycin resistance at 50 µg/mL, and PCR colonies were conducted to identify positive clones. The successfully generated intermediate constructs were then employed in the LR clonase recombination reaction. In this process, the promoter regions of *OsSAP4* were cloned into the final expression vector, pKGWFS7, fused with the GUS reporter gene. Positive colonies were screened on an antibiotic-selective plate with Spectinomycin at 50 µg/mL. Expression clones containing the genes were confirmed through PCR and subsequently sent for sequencing.

Arabidopsis floral dipping transformation

Following the cloning process, the final constructs, designated as *pOsSAP4(1524bp)::GUS,* and *pOsSAP4(460bp)::GUS* respectively were introduced into *Agrobacterium tumefaciens* strains GV3101 for subsequent transformation into Arabidopsis Col-0 using the floral dipping method. A bacterial culture with 0.05% silwet-77 and a 5% sucrose solution was employed. To enhance the transformation efficiency, a second round of floral dipping was conducted after a 7-day interval. For analysis of positive Arabidopsis transformant selection, seeds from T1 were planted into ½ MS Media containing antibiotic Kanamycin 50 µg/mL. The presence of promoter regions in the positive transgenic Arabidopsis was confirmed through PCR using the specific forward primer for *OsSAP4* and reverse primer eGFP gene resulting in a specific-sized product. Seeds from positive T1 plants were then collected and propagated until T3 generation seeds were obtained. T3 seeds were used for GUS expression analysis.

Experimental condition and stresses

Two types of Abiotic stress treatment were applied at two developmental stages (vegetative and inflorescence stages) and the treated samples were utilized for GUS analysis. Three-week-old plants underwent three treatments: control (regular watering), salinity (200 mM NaCl), and drought (withheld watering) over 14 days (Roszelin *et al*. 2023). GUS assay assessments were conducted on plants subjected to the treatments on day 7 and day 14. For inflorescence and siliques data, the same treatments as before were carried out during the inflorescence and siliques production stages for 7 days (45-day-old plant) and the staining was done after that (Kramer *et al*. 2015).

GUS staining analysis

For GUS activity analysis, treated leaves, buds, and siliques were put in 50 mL centrifuge tubes containing 90% acetone for 15 min on ice. Samples were then rinsed with 1× Phosphate buffer before being incubated in the X-Gluc solutions (0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4, 0.1 M K_3 [$Fe(CN)_6$], 0.1 M $K_4[Fe(CN)_6]$, 0.5 M EDTA, pH 7-8, 20% methanol, 1 mM X-Gluc). All samples were incubated in the X-Gluc solution for 16 hr at 37°C in the dark. After incubation, samples were rinsed with 70% ethanol and analyzed.

RESULTS AND DISCUSSION

The abundance of abiotic-stress-related Cis-regulatory elements in all *OsSAPs* promoter regions

Cis-regulatory elements especially enriched with Transcription factors binding sites can regulate gene expression by either activating or repressing gene expression during responses to abiotic, biotic stresses, and plant development. Hence, it is crucial to examine the presence of Cis-regulatory elements within gene promoter regions to comprehend the mechanisms of gene regulation in response to environmental stresses. In this study, the promoter analysis was conducted by examining the presence of Cis-regulatory elements in all *OsSAPs* promoter sequences (Supplementary 1). The analysis from Transcription Factor Database (TFDB) server and PlantPan Database revealed 28 types of cis-elements also with enriched transcription factor binding sites embedded across all 18 *OsSAPs* promoter regions with a significant $p<0.05$ (Figure 1). Each transcription factor plays a distinct role; thus, they are categorized into two main functions: abiotic stresses and plant development, (Supplementary 1). More than half (342 out of 535) of these cis-regulatory elements are associated with abiotic stress-responsive genes (Figure 1) showing the *OsSAP* gene's involvement in abiotic stress responses. Within the abiotic stresses category, cis-elements that are bound by the transcription factor binding sites linked to Ethylene Response Factors (ERF) were the most prevalent across the entire *OsSAP* gene promoters. The APETALA 2/ethylene-responsive factor (AP2/ERF) superfamily, marked by the AP2/ERF conserved domain, encompasses a diverse collection of plant-specific transcription factors with roles ranging from growth and flower development to responses to stresses and hormone signaling (Xie *et al*. 2019; Muller & Munné-Bosch 2015).

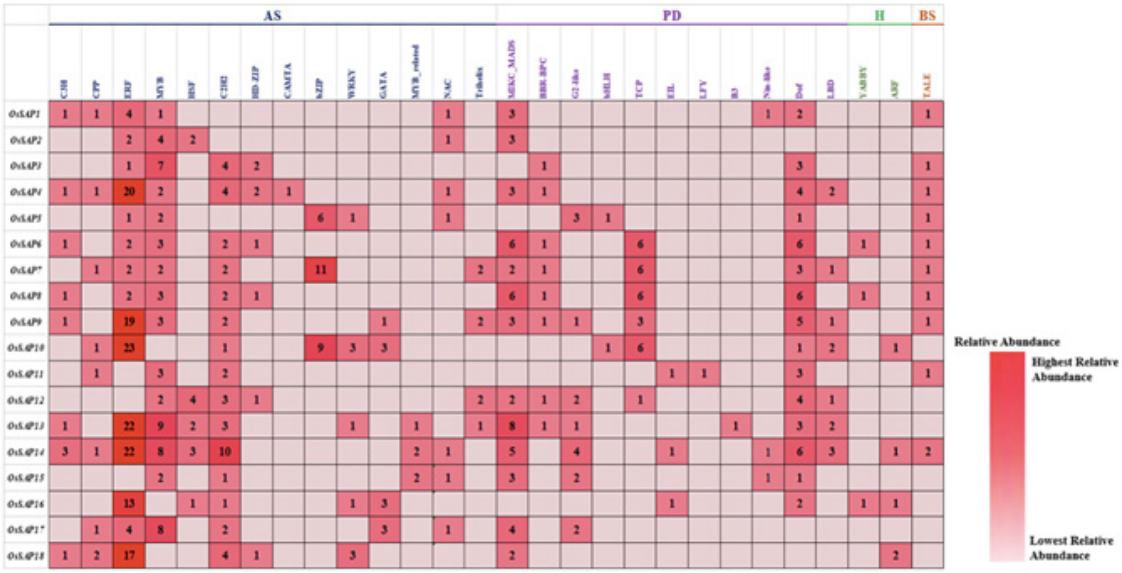

Fig. 1. The distribution of transcription factor binding sites in the promoter of all *OsSAP* genes. AS indicates Abiotic Stress, PD indicates Plant Development, H indicates Hormone, and BS indicates Biotic Stress associated binding sites respectively. The number represents the significant value of Cis-regulatory elements present within the promoters. Significant $p<0.05$.

Among the 28 types of transcription factor binding sites found in *OsSAPs* promoter regions, the predictive cis-regulatory elements bound by ERF transcription factor were highly abundance in most of the *OsSAPs* promoters as follows; *OsSAP10* (23), *OsSAP13* (22), *OsSAP14* (22), *OsSAP4* (20), *OsSAP9* (19), *OsSAP18* (17) and *OsSAP16* (13) respectively (Figure 1). The ERF group is a major group in the response to abiotic stresses with the primary binding motif GCCGCC. Primarily, ERF transcription factors will bind to the GCC cis-element, thereby enhancing the expression of genes involved in the response to salinity stress to increase plant tolerance to salinity and drought stress (Liu *et al*. 2018). In addition to regulating the expression of genes involved in the response to abiotic stress, ERF also serves as a signaling pathway hub for various plant hormones such as Abscisic Acid (ABA) and Ethylene (ET) (Xie *et al*. 2019). The ethylene signal transduction, in turn, aids in the binding of the ERF motif, thereby expressing genes that respond during salinity stress (Kazan 2015; Muller & Munne-Bosch 2015; Tao *et al*. 2015). Therefore, for further experimental design, the focus was on analyzing the significant roles of ERF transcription factor binding sites in one of the Stress Associated Protein genes, namely *OsSAP4*. The selection of *OsSAP4* was based on previous analyses by Roszelin *et al*. (2023), which demonstrated high expression of the *OsSAP4* gene during salinity and drought. Thus,

understanding whether the inducible expression of the *OsSAP4* gene is attributed to the distribution of ERF transcription factor binding sites is crucial. This exploration may aid in elucidating the hypothetical model of *OsSAP4* promoter regulation during stress response about ERF transcription factor binding sites.

The high abundance of Ethylene Response Factor transcription factor binding sites in the promoter region of *OsSAP4*

Building upon prior in silico investigations, subsequent analysis was conducted on the positioning of transcription binding sites within the *OsSAP4* promoter region, with a specific emphasis on the ERF transcription factor binding sites. Two fragments of the *OsSAP4* promoter regions were selected for further GUS analysis: a 1524 bp fragment containing 13 types of Cis-regulatory elements and a 460 bp fragment containing only 3 types of Cis-regulatory elements (Figure 2). Among the 13 identified Cis-regulatory elements in the longer fragment (1524 bp), 9 are associated with responding to abiotic stress, 3 are involved in plant development, and 1 is related to biotic stress responses. Meanwhile, all three identified cis-regulatory elements in the shorter fragment (460 bp) are involved in abiotic stress response in plants. (Supplementary 1).

Further analysis focusing on the cis-regulatory elements bound by the *ERF* identified 20 predictive *ERF* transcription factor binding sites within the 1524 bp *OsSAP4* promoter fragment whereas only 4 sites were found within the 460 bp fragment (Figure 3). Previous studies have shown that overexpressing *ERF* transcription factors boosts plant resilience against salinity and drought stress. *ERF* transcription factors play a crucial role in the Abscisic Acid (ABA) hormone pathway, thereby stimulating the expression of various genes during abiotic stresses (Lata & Prasad 2011). ABA hormones collaborate with *ERF* transcription factors to amplify abiotic stress signal transduction in plants. Due to the different number of *ERF* transcription binding sites found within the short and long *OsSAP4* promoter segments, this study employs the two promoter sizes to investigate whether the disparity in the number of *ERF* binding sites influences the plant's resilience to abiotic stress.

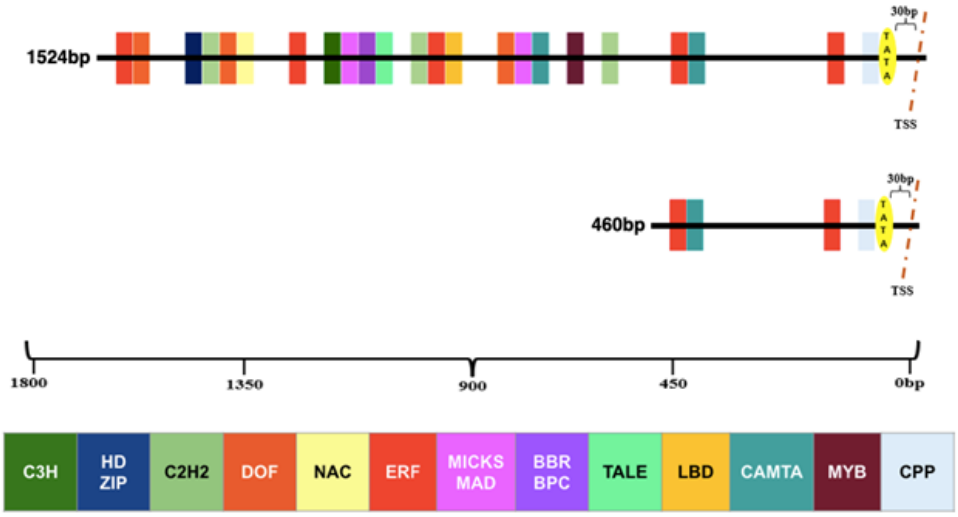

Fig. 2. The numbers of different types of Cis-regulatory elements present in the promoter fragments of *OsSAP4* (1524 bp) and *OsSAP4* (460 bp).

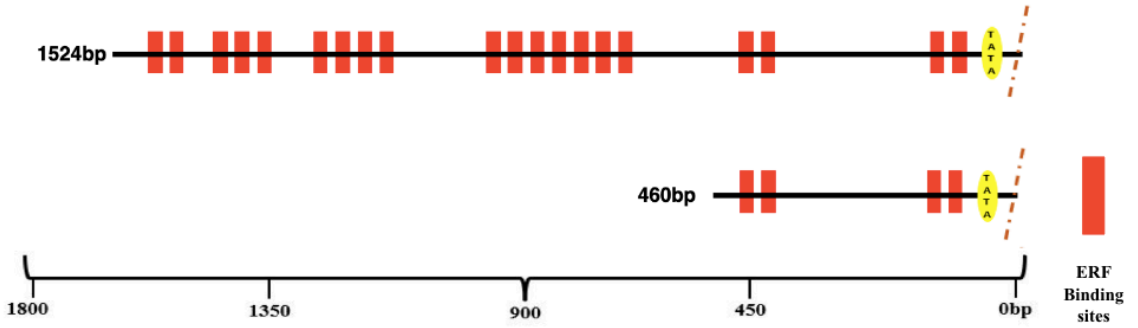

Fig. 3. The predictive distribution of ERF transcription factor binding sites in the promoter fragments of *OsSAP4* (1524 bp) and *OsSAP4* (460 bp). Significant cut-off $p<0.05$. Red boxes indicate the abundance of ERF within the promoter.

Analysis of GUS expression under the *OsSAP4* promoter revealed significant inducibility during stress conditions, correlating with the abundance of *ERF* and other abiotic stress-related transcription factor binding sites

GUS expression analysis was conducted on transgenic *A. thaliana* plants harboring *pOsSAP4(1524 bp)::GUS* and *pOsSAP4(460 bp)::GUS* constructs. This analysis aimed to compare and analyze the expression patterns of GUS regulated by promoter fragments with two distinct sizes under salinity and drought stress conditions. Two phases of developmental stages were selected for GUS expression analysis, which are the vegetative and inflorescence stages of the *A. thaliana* as well as in the matured siliques.

GUS staining was apparent in *A. thaliana* transgenic leaves carrying the p*OsSAP4*(1524bp)::GUS construct under salinity and drought stress treatments compared to the short promoter fragment transgenic leaves (p*OsSAP4*(460)::GUS) (Figure 4). The presence of GUS activity in these tissues during salinity and drought stress compared to the control shows the presence of the *OsSAP4* promoter regulatory activity. This regulatory activity suggests that the distribution of transcription factor binding site elements found in the *OsSAP4* promoter is induced under salinity and drought conditions. Assessing the Cis-regulatory elements enriched with transcription factor binding site elements in the *OsSAP4* promoter enhances our understanding of the promoter's regulatory response to abiotic stress. The p*OsSAP4*(1524bp)::GUS construct contains 13 types of Cis-regulatory elements: C3H, HD-ZIP, C2H2, DOF, NAC, ERF, MICKS MAD, BBR-BPC, TALE, LBD, CAMTA, MYB, and CPP. In silico, promoter analysis revealed that seven of these Cis-regulatory elements, particularly in *Oryza sativa*, play a role in abiotic stress responses (Supplementary Table 1).

The presence of the Cis-regulatory elements bound by the ERF transcription factor in the p*OsSAP4*(1524 bp)::GUS construct indirectly indicates its importance. Other than the 13 types of Cis-regulatory elements found in the *OsSAP4* promoter, further analysis specifically on the ERF binding sites showed 20 locations were embedded in the 1524 bp *OsSAP4* promoter. These ERF binding sites are distributed throughout the promoter region, including core, proximal, and distal promoter regions. Notably, the distal promoter region has a significantly higher distribution of ERF transcription factor binding sites compared to other regions. The ERF group is particularly significant in abiotic stress response, as ERF transcription factors bind to the GCC cis-box element, thereby enhancing the expression of genes involved in salinity stress response and increasing plant tolerance to salinity and drought stress (Liu *et al*. 2018).

Among the 14 ERF group transcription factor binding sites, the Cytokinin Response Factor (CRF) is a member of the ERF subgroup. The expression of the Cytokinin Response Factor (CRF) is regulated by the presence of the plant hormone cytokinin and abiotic stress, particularly freezing and osmotic stress (Zwack & Rashotte 2015). CRF expression, which depends on cytokinin presence, regulates oxidative reactions and reduces plant damage caused by increased hydrogen peroxide levels (H_2O_2).

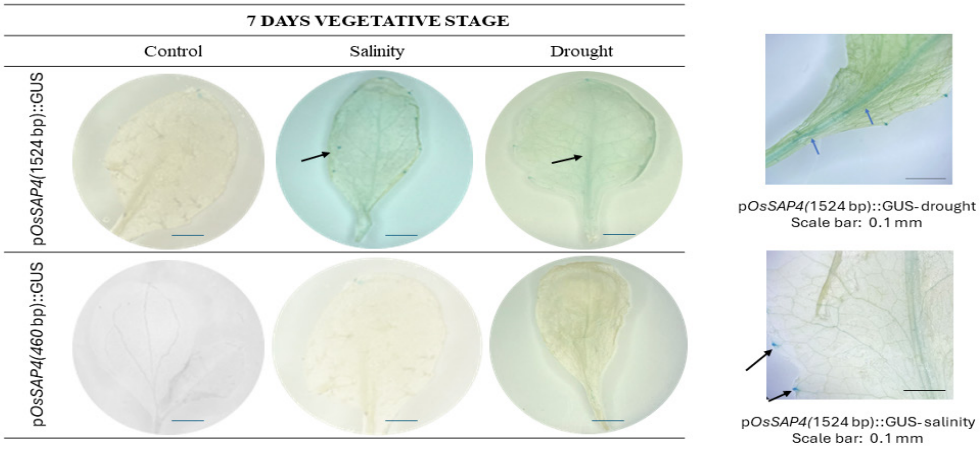

Fig. 4. GUS staining analysis of transgenic *Arabidopsis thaliana* carrying the constructs *pOsSAP4*(1524 bp)::GUS and *pOsSAP4*(460 bp)::GUS was conducted during the vegetative phase. The 28 old-day plants (after 7 days of treatment) with salinity stress (200 mM NaCl) and drought stress (no watering) were used for staining. Scale bar: 0.3 mm. The arrow indicates GUS staining activity.

The pattern of GUS activity on leaves is also different during drought stress compared to salinity during 7 and 14 days of treatments (Figure 4 & Figure 5). During drought stress, GUS expression is widespread across the entire leaf surface (Figure 4), while during salinity stress, it is mostly confined to the leaf tip (Figure 4). However, no GUS activities were observed in *pOsSAP4(460 bp)::GUS* transgenic lines. This could be attributed to the fact that although the *OsSAP4* promoter fragment (460 bp) contains the ERF transcription factor binding site, the ERF regulatory network analysis in abiotic stress responses indicates the involvement of other transcription factor binding site elements as well. For instance, during salinity stress, the ERF transcription factor's expression has been found to activate the HD-ZIP transcription factor element and NAC (Liu *et al*. 2007). Additionally, signal transduction in the ABA hormone pathway, which is regulated by the ERF transcription factor, suggests the participation of the MYB transcription factor in the drought stress response (Liu *et al*. 2018). Hence, the lack of specific transcription factor binding site elements in the shorter promoter (p*OsSAP4*(460 bp)::GUS) likely resulted in the reduced GUS expression, leading to the absence of observable GUS activity in the transgenic *A. thaliana* plants carrying the p*OsSAP4*(460 bp)::GUS construct.

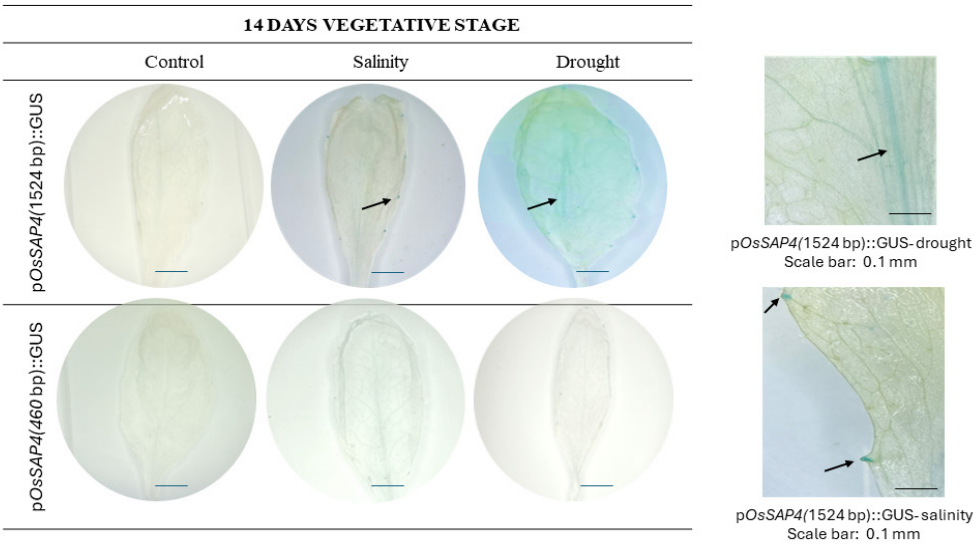

Fig. 5. GUS staining analysis of transgenic *Arabidopsis thaliana* carrying the constructs *pOsSAP4*(1524 bp)::GUS and *pOsSAP4*(460 bp)::GUS was conducted during the vegetative phase. The 35 old-day plants (after 14 days of treatment) with salinity stress (200mM NaCl) and drought stress (no watering) were used for staining. Scale bar: 0.3 mm. The arrow indicates GUS staining activity.

In both transgenic *A. thaliana* plants carrying the p*OsSAP4*(1524 pb)::GUS and p*OsSAP4*(460 pb)::GUS constructs, GUS activities were observed in the inflorescence section under saline and drought conditions (Figure 6). However, GUS activities were more present in *A. thaliana* transgenic plants harboring the p*OsSAP4*(1524pb)::GUS construct (Figure 6) compared to those with the p*OsSAP4*(460 pb)::GUS construct. This discrepancy is attributed to the higher regulatory activity of the long promoter, resulting in increased GUS expression, as previously reported by Nanjareddy *et al*. (2017).

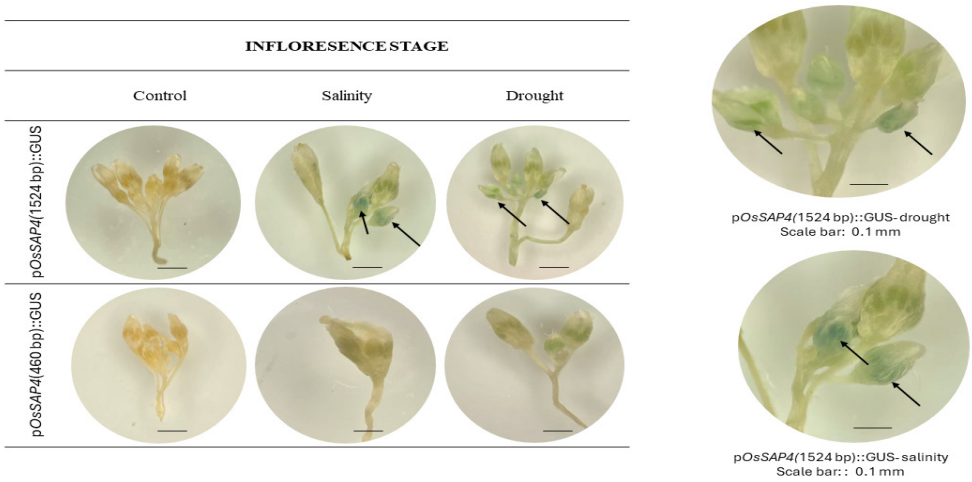

Fig. 6. GUS expression analysis of transgenic *Arabidopsis thaliana* plants in the inflorescence region carrying the constructs *pOsSAP4*(1524 bp)::GUS and *pOsSAP4*(460 bp)::GUS was performed, specifically at 45 days old plants after 7 days of treatment with salinity stress (200 mM) and drought stress (no watering). Scale bar: 0.5 mm. The arrow indicates GUS staining activity.

As previously shown, GUS activity was not detected in the vegetative phase leaves carrying *pOsSAP4(460 bp)::GUS* promoter under saline or drought conditions whereas the same promoter induced GUS activity at the inflorescence stage. The presence of GUS staining activity in the inflorescence section may be attributed to the presence of the CAMTA transcription factor binding site in the p*OsSAP4*(460 pb)::GUS construct. This is supported by the highest expression of the CAMTA transcription factor being recorded in the flower part during the flowering phase, as demonstrated by Fang *et al*. (2022). Consequently, it is plausible that the CAMTA transcription factor interacts with the short promoter fragment to stimulate GUS expression.

The GUS staining activity in silique regions was observed in transgenic *A. thaliana* plants carrying the *pOsSAP4(1524 bp)::GUS* construct and was highly induced during drought stress treatments (Figure 7). The increase in GUS activity under drought stress in siliques of transgenic *pOsSAP4(1524 bp)::GUS* compared to the control indicates an upregulation of *OsSAP4* promoter activity. This suggests that the transcription factor binding sites present in the 1524 bp *OsSAP4* promoter play a role in responding to drought stress compared to salinity.

Evaluating the transcription factor binding sites in the *OsSAP4* promoter aids in understanding the regulation of response to abiotic stress. There are 13 types of transcription factor binding sites identified in the *pOsSAP4*(1524 bp)::GUS construct, which was C3H, HD-ZIP, C2H2, DOF, NAC, ERF, MICKS MAD, BBR-BPC, TALE, LBD, CAMTA, MYB, and CPP. In silico promoter analysis reveals these transcription factors play a role in the response to abiotic stress, particularly in *Oryza sativa*. In contrast to *pOsSAP4(1524 bp)::GUS,* the short promoter fragment transgenic *pOsSAP4(460 bp)::GUS* in the vegetative stage did not show any GUS staining under salinity and drought comparable to the control sample. This might be due to the absence of certain Cis-regulatory elements related to abiotic stresses in the shorter region (460 bp). It suggests that, for increased expression of *OsSAP4* under stress conditions, a longer promoter region containing additional transcription factor binding sites is necessary. Furthermore, the results indicate that the longer *OsSAP4* promoter (1524 bp) can serve as an inducible promoter in genetic modification strategies for crop improvement, as it specifically responds to salinity and drought signals.

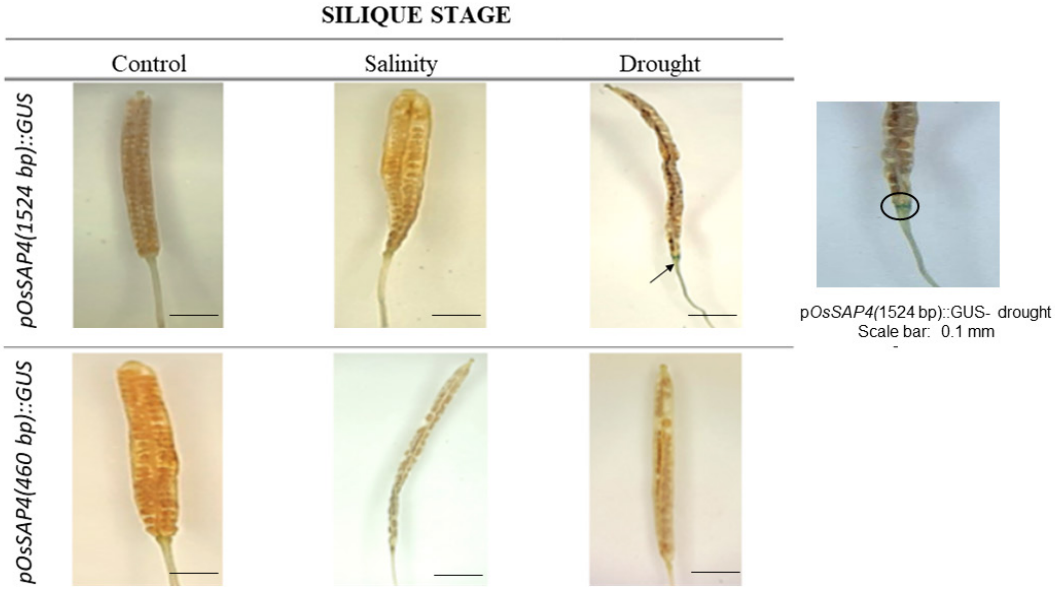

Fig. 7. GUS staining analysis of transgenic *Arabidopsis thaliana* plants carrying the constructs *pOsSAP4*(1524 bp)::GUS and *pOsSAP4*(460 bp)::GUS was conducted during the siliques production phase, specifically at 45 days old plants after 7 days of treatment with salinity stress (200mM NaCl) and drought stress (no watering). Scale bar: 1 mm. The arrow indicates GUS staining activity.

CONCLUSION

The in-silico analysis of *OsSAP* gene regulatory components has uncovered various transcription factor binding sites with the potential to drive *OsSAP* gene expression during abiotic stress. Based on the previous expression analysis of *OsSAP* genes, *OsSAP4* has been selected for further study due to its high gene expression levels under salinity and drought stress, as well as the discovery of 20 ERF transcription factor binding sites in the promoter fragment along with other stress-related transcription factor binding sites. GUS staining analysis has been conducted to understand the regulation of *OsSAP4*, using two different sizes of promoter fragments, each with different distributions of Cis-regulatory elements. The results of the GUS staining analysis indicate that transgenic *A. thaliana* plants carrying the *pOsSAP4(1524 bp)::GUS* construct exhibit intense GUS staining compared to transgenic plants carrying the *pOsSAP4(460 bp)::GUS* construct. This suggests that the expression pattern of the *OsSAP4* regulator during abiotic stress is not only influenced by the presence of ERF transcription factors alone but also by numerous other transcription factor elements that might be involved. In conclusion, this study has provided insights and information regarding the regulatory system and gene expression of the *OsSAP4* promoter. The *OsSAP4* promoter holds potential use as an inducible promoter in generating transgenic rice resistant to salinity and drought stress.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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