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Research Article

Regulation of *OsSAP8* Promoter in Response to Abiotic Stresses

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ABSTRACT

Abiotic stresses such as drought, salinity, and extreme temperatures pose significant challenges to crop production, particularly impacting rice yield and quality. These stresses are exacerbated by climate change and the escalation of the human population. Plant adaptation to abiotic stresses involves intricate molecular mechanisms, including gene expression alterations, metabolic adjustments, and stress-responsive gene activation. Phytohormones play a pivotal role in regulating these adaptive responses by playing a central role in regulating plant growth and enhancing resilience to stress. Previous studies have shown that *Oryza sativa* Stress-associated protein 8 (*OsSAP8*) enhanced plant tolerance to drought and salinity stresses throughout the growth and developmental stages. In this study, we focused on the *OsSAP8* promoter, especially the phytohormone-responsive *Cis*-Regulatory Elements (CREs), to deepen our understanding of its regulation under abiotic stress conditions. Promoter analysis identified several CREs associated with Abscisic Acid (ABA), Gibberellic Acid (GA), and Methyl-Jasmonate (MeJA) phytohormones. Subsequently, promoter deletion was performed using two different lengths of *OsSAP8* promoter fragments, comprising different sets of phytohormone CREs. Promoter-β-glucuronidase (GUS) fusion constructs in transgenic Arabidopsis plants revealed that the truncated promoter fragment of p*OsSAP8*(934 bp)::GUS exhibited stronger GUS activity compared to the full-length promoter, p*OsSAP8*(1801 bp)::GUS under drought and salinity stresses. This suggests that the CREs responsible for *OsSAP8* expression under stress conditions are located within this shorter promoter region. These findings underscore the importance of *OsSAP8* in plant stress responses and provide a foundation for future research on enhancing agricultural sustainability amid changing environmental conditions.

Key words: Abiotic stress, β-glucuronidase (GUS), cis-regulatory elements, phytohormone, promoter, Stress-associated Protein 8

INTRODUCTION

Abiotic stressors such as drought, salinity, flooding, and extreme temperatures are major constraints on crop production, significantly impacting both rice yield quality and quantity (Martin *et al*., 2012; Roslan *et al*., 2017; Cohen & Leach, 2019; Boon Teck *et al*., 2021; Gopalakrishnan & Kumar, 2021; Iqbal *et al*., 2023). These issues are expected to worsen with climate change and the increasing demands of a growing human population that recently surpassed 8.1 billion. According to the latest United Nations forecast, the human population is expected to reach around 8.5 billion by 2030 and 9.7 billion by 2050 (United Nations, 2022). However, climate change has led to abiotic stress, which induces alterations of plant gene expression, affecting cell metabolism, growth rate, crop yield, and even plant death. By 2050, it is projected that salinity will cause over 20-50% of agricultural lands to become saline (Jafar *et al*., 2022; Roșca *et al*., 2023). Moreover, soil salinity issues contribute to annual agricultural production losses amounting to USD 27.3 billion (FAO, 2022). Drought is often accompanied by salinity in coastal, arid, and semi-arid regions. As soil water evaporates, salts become concentrated in the soil, creating a combined effect of drought and salinity stresses (Hailu & Mehari, 2021).

As plants are sessile, they have mechanisms against abiotic stresses, such as complex interactions between signaling molecules and pathways (Kim *et al*., 2011; Martin *et al*., 2012), regulation of ion homeostasis, accumulation of antioxidants, and phytohormone adjustment often involving *Cis*-Regulatory Elements and Transcription Factors (TFs). Other than that, plants also induce stress neutralization for damage repair mechanisms (Bhoi *et al*., 2022) and activate genes involved in stress response (Marothia *et al*., 2021; Wang *et al*., 2022). Genomics, transcriptomics, proteomics, and metabolomics are holistic approaches to understanding how plants respond to abiotic stresses. These methods aid researchers in gaining insights into the mechanisms governing plant stress responses and revealing the functions of each component in the signaling pathways. Furthermore, enhancing agricultural productivity can be accomplished through genetic engineering techniques to develop plants resilient to both abiotic and biotic stressors (Roslan *et al*., 2017).

According to prior studies, Stress-associated proteins (SAPs) play a crucial role in plant responses to abiotic stresses. The SAP gene family is characterized by the presence of the A20/AN1 domain within its coding sequence (Kanneganti & Gupta, 2008; Vij & Tyagi, 2008; Li *et al*., 2022). There are 14 SAP genes in *Arabidopsis thaliana* and 18 SAPs in *Oryza sativa*. A previous

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study has reported enhanced numbers of the inflorescence stem and flower with higher relative chlorophyll content in transgenic Arabidopsis overexpressing *Oryza sativa SAP8* (*OsSAP8*) gene compared to *sap2* Arabidopsis mutant when subjected to abiotic stress treatments during vegetative and inflorescence stages (Roszelin *et al*., 2023). This indicates that *OsSAP8* provides tolerance to various abiotic stresses, especially in drought and salinity conditions (Kanneganti & Gupta, 2008; Roslan *et al*., 2017; Li *et al*., 2022). However, the mechanisms behind OsSAP8 regulation in response to these abiotic stresses (drought and salinity), particularly at the transcriptional level, remain unclear.

Phytohormones are natural bioactive compounds produced within plants that have a crucial role in plant growth, plant development, and stress responses. Abscisic Acid (ABA), Gibberellic Acid (GA), Auxin (IAA), Brassinosteroid (BR), Ethylene (ET), Cytokinin (CK), Methyl Jasmonate (MeJA) and Salicylic Acid (SA) are well-known phytohormones that act as plant growth regulators, aiding plants in adapting to different stresses by mediating a wide range of adaptive responses (Tuteja & Sopory, 2008; Davies, 2010; Santner & Estelle, 2010; Sabagh *et al*., 2022; Wahab *et al*., 2022). Basically, phytohormones rapidly alter gene expression by activation or repression of transcriptional regulators, often via the ubiquitin-proteasome system (UPS) (Peleg & Blumwald, 2011; Lenka & Bansal, 2019; Asad *et al*., 2019). ABA and GA often act antagonistically during the regulation of several plant growth and developmental responses, such as seed dormancy, seed germination and maturation, root growth, and flowering time control (Weiss & Ori, 2007; Liu & Hou, 2018; Shu *et al*., 2016, 2018). This crosstalk ensures a balanced allocation of resources, allowing plants to adapt efficiently to abiotic stresses while maintaining optimal growth and development (Cutler *et al*., 2010; Quesada, 2022; Shu *et al*., 2018; Singh & Roychoudhury, 2023).

Promoters play an important role in controlling gene expression (Hernandez-Garcia & Finer, 2014). Hence, our promoter study aimed to gain a deeper understanding of the *OsSAP8* gene regulation by focusing on the *Cis*-Regulatory Elements (CRE) associated with phytohormone-responsive elements. CRE is a short regulatory motif (5–20 bp) present in a gene promoter region. CRE is important in transcriptional gene regulation by mediating various stress responses (Biłas *et al*., 2016; Ishibashi *et al*., 2016). The comprehension of *cis*-elements enables the modulation of gene expression patterns that offer opportunities for plant genetic engineering and crop protection against abiotic stresses. Therefore, phytohormonal CREs have emerged as a focal point of research as *OsSAP8* showed ABA and GA interplay in response to abiotic stresses throughout the plant growth and developmental stages (Zhang *et al*., 2016; Roslan *et al*., 2017; Sahid *et al*., 2020; Li *et al*., 2022; Roszelin *et al*., 2023). Promoter deletion was conducted to investigate the effectiveness and function of the CRE element in facilitating the transcription process, aiming to comprehend the regulation of the *OsSAP8* gene under abiotic stress responses.

In this study, we examined the transcriptional activities of *OsSAP8* through promoter deletion by measuring β-glucuronidase (GUS) activity. The full-length (1801 bp) and truncated (934 bp) OsSAP8 promoter fragments differ in their composition of phytohormone-responsive cis-regulatory elements (CREs), with the truncated fragment (934 bp) containing fewer ABA- and GA-related CREs compared to the full-length promoter, which harbors a broader range of these elements, including for developmental purposes. Both promoters were fused with the GUS reporter gene in a destination vector for Agrobacterium-mediated transformation of Arabidopsis plants. Histochemical analysis showed that the truncated promoter showed greater GUS activity compared to the full-length promoter construct. Both transgenic lines were induced with drought and salinity treatments. The findings from this study improve our comprehension of *OsSAP8* regulation and offer valuable insights for developing strategies to enhance crop resistance to abiotic stresses.

MATERIALS AND METHODS

In silico OsSAP8 promoter analysis

A 2 kb upstream sequence from the ATG start codon of *OsSAP8* was chosen for promoter analysis. The promoter sequences were retrieved from Ensembl Plants (https://plants.ensembl.org/index.html; (Bolser *et al*., 2017). The crucial elements of plant promoters, such as CAAT-box, TATA box (core promoter element), and transcription start site (TSS) in the 5′ upstream regions of the gene were analyzed using TSSP/Prediction of PLANT Promoters (http://www.softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=promoter; Shahmuradov *et al*., 2017) and Promoter - 2.0 Transcription start sites in vertebrate DNA database (https://services.healthtech.dtu.dk/service.php?Promoter-2.0; Knudsen, 1999). The abundance of *cis*-regulatory elements (CREs) of *OsSAPs* genes associated with phytohormone-responsive elements was determined using the PlantCARE tool (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lescot *et al*., 2002) and the PLACE tool (https://www.dna.affrc.go.jp/PLACE; Higo *et al*., 1999).

Construction of *OsSAP8* promoter deletion fragments

To generate promoter deletion constructs, two *OsSAP8* promoter fragments (1801 bp & 934 bp) were obtained through two-step PCR and amplified using the KOD Fx Neo kit (Toyobo). The primers used in the first step PCR were gene-specific primers attached to 12 attB sites as follows: attb::*OsSAP8*(934 bp)_F-5'- AAAAAGCAGGCTTCGTGCTCCTCCCTGGCCGATC-3', attb::*OsSAP8* (1801 bp)_F-5'-AAAAAGCAGGCTTCTTCCAACTACTAAACTAA–3' and attb::*OsSAP8*(934 bp&1801 bp)_R-5'-AGAAAGCTGGGTCGGCTTCCTTTTAACTATAAA–3'. Meanwhile, for the second step PCR, both promoter fragments were fused with universal adapter gateway primer containing complete attB sequences; *OsSAP8*_F-5'- GGGGACAAGTTGTACAAAAAAGCAGGCTTC-3' and *OsSAP8*_R-5'- GGGGACCACTTTGTACAAGAAAGCTGGGTC-3'. The underlined sections in the primer sequences denote adaptor sequences used for the Gateway cloning system (Invitrogen).

Construction of *OsSAP8* cloning and expression vectors

Both promoter fragments were cloned into Gateway vectors using BP and LR clonase (Gateway Cloning Technology, Invitrogen), as described by the manufacturer. By using BP clonase, the donor vector, pDONR221, was used to produce entry clones that harbor both *OsSAP8* promoter fragments. The entry clones were transformed into competent *E. coli* Top10 using the heat-shock method. Then, selection was carried out using kanamycin (50 µg/mL), and colony PCR was done to select positive

colonies. Successful intermediate constructs were used in subsequent cloning steps involving LR clonase. In this reaction, both *OsSAP8* promoter fragments were cloned into the destination vector, pKGWFS7, to generate promoter fusions of a GUS reporter gene: p*OsSAP8*(934 bp)*::*GUS and p*OsSAP8*(1801 bp)*::*GUS. The expression clones were transformed into *E. coli* Top 10 using the heat-shock method and selected using spectinomycin (75 µg/mL). Verification was carried out through colony PCR using a universal adapter gateway primer, followed by Sanger sequencing.

Agrobacterium-mediated transformation in Arabidopsis

Expression clones harboring p*OsSAP8*(934 bp)::GUS and p*OsSAP8*(1801 bp)::GUS were transformed into *Agrobacterium tumefaciens* GV3101 for floral dip transformation (Clough & Bent, 1998) of Arabidopsis Columbia-0 (Col-0) (Liu *et al*., 2020; Zhang *et al*., 2006). Bacterial culture was supplemented with 0.05% silwet-77 and 5% sucrose solution. Floral dipping was performed twice to enhance the transformation rate (Davis *et al*., 2009). The T1 transformants were screened on half Murashige and Skoog (MS) agar plate supplemented with kanamycin (50 µg/mL). The presence of a promoter transgene was confirmed through PCR using specific forward primers of *OsSAP8* 934 bp and 1801 bp promoter and a GUS-specific reverse primer, eGFP (5'- CATGGTCCTGCTGGAGTTCGTG- 3'). PCR was performed using the MyTaq Red Mix kit (Meridian Bioscience). Seeds from positive T1 plants were collected and sown on soil and propagated to obtain T2 and T3 seeds. The T3 seeds were then planted for GUS functional studies.

Abiotic stress treatments

T3-generation plants were treated with drought and salinity treatments at two developmental stages, namely the seedling stage and the vegetative to inflorescence stage. T3 Arabidopsis seeds were soaked in distilled water at 4°C (chiller) for 2 days to initiate germination before being cultivated on half-strength MS agar plates. Subsequently, the plates were wrapped with aluminum foil and kept at 4°C for another 2 days before being transferred to a suitable growth environment (under 150 µmoL m^{-2} s^{-1} continuous light at 22°C) (Rivero *et al*. 2014; Shaw 2021). The Arabidopsis plants achieved the seedling stage around 11 days after sowing and continued until 25 days old (Boyes *et al*., 2001; Krämer, 2015). For the seedling stage, the plants were exposed to a 3-hour drought (20% PEG) and salinity treatments (200 mM NaCl). While for the vegetative to inflorescence stage, the 3 weeks-old seedlings were subjected to drought (withheld watering) and salinity treatment (200 mL of 200 mM NaCl) for 3 weeks (seven days of treatment; 28-old- day plant, 14 days of treatment; 35-day-old plant, and 21 days of treatment; 42-day-old plant) as described by Roszelin *et al*. (2023). Control plants were grown under non-stress conditions with regular watering without adding NaCl.

Histochemical GUS Assays and Microscopy

Histochemical staining for GUS activity was performed on both transgenic lines using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) as a substrate (Jefferson *et al*., 1987). Tissues were stained overnight at 37°C in X-Gluc reaction buffer (50 mM pH 7 sodium phosphate, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 2 mM X-Gluc). Then, the samples were cleared with 70% ethanol to remove chlorophyll before being observed under a stereo microscope. All tissue samples were observed and photographed using an AmScope FMA037 microscope.

RESULTS

Identification of CRE in the *OsSAP8* promoter region

Sequences (~2 kb) of the upstream regions of 18 *OsSAP* genes were retrieved from Ensembl Plants for analysis using PlantCARE and PLACE tools to identify putative *cis*-regulatory elements involved in hormonal regulation (Table 1). Analysis of the full-length *OsSAP8* promoter revealed 13 CREs influenced by phytohormone-responsive elements. Among these, seven are associated with ABA, four with GA, and two with MeJA (Table 1).

For all identified pytohormone-related *OsSAP8* CREs*,* the domain sequences with their predicted functions are listed in Table 2 and visualized in Figure 1a. *OsSAP8* also possesses common motifs such as TATA-box and CAAT-box located near various transcription start sites (TSS). TATA-box is crucial to initiate transcription and act as a core promoter element, while CAAT-box controls transcription initiation and efficiency (Misra & Ganesan, 2021; Murray *et al*., 2022; Schmitz *et al*., 2022).

OsSAP8 promoter deletion analysis

To understand the regulation of *OsSAP8* promoter activity, 5' promoter deletion was performed to yield a 934 bp truncated *OsSAP8* promoter fragment (Figure 1b). Both promoter sequences were cloned through the Gateway Cloning System. Notably, both promoter fragments contain distinct sets of hormone-responsive CREs within the *OsSAP8* promoter region (Figure 1). Analysis of the 1801 bp full-length *OsSAP8* promoter revealed a total of 13 hormone-responsive CREs, including seven ABA-responsive elements, comprising five ABREs and two G-boxes located at the same positions as the ABREs; four GA-responsive elements, which consist of two P-boxes, and two GARE-motifs; and two MeJA-responsive elements, containing a TGACG-motif and a CGTCA-motif (Figure 1a). In contrast, the 934 bp promoter fragment of *OsSAP8* contains only five hormone-responsive CREs, including two ABA-responsive elements of one ABRE and one G-box situated together at +390 with three GA responsive elements of one P-box at +256 and two GARE motifs at +514 and +608 (Figure 1b).

Table 1. Phytohormone-responsive elements in promoter regions of all *OsSAP*s.

	ABA responsive elements					GA responsive elements		MeJA responsive elements		Ethylene responsive elements	Salicylic acid responsive elements		Auxin responsive elements		
	ABRE	ABRE2	ABRE3a	ABRE4	G-box	GARE-motif	P-box	TGACG-motif	CGTCA-motif	TGA-element	AuxRR-core	ERE	TCA	TCA-element	TGA-box
OsSAP1	13	0	2	2	9	0	0	5	6	0	0	3	2	1	0
OsSAP2	2	0	0	0	5	0	0	5	5	1	1	0	1	0	0
OsSAP3	6	0	1	1	6	0	0	1	1	0	0	0	0	1	0
OsSAP4	9	1	0	0	10	0	0	1	1	2	0	2	0	1	0
OsSAP5	8	0	3	3	7	0	0	5	5	1	2	0	1	1	0
OsSAP6	1	0	0	0	1	1	1	0	0	1	0	1	0	0	0
OsSAP7	6	1	2	2	9	1	0	2	2	0	0	1	1	0	0
OsSAP8	3	0	1	1	2	2	2	1	1	0	0	0	0	0	0
OsSAP9	5	0	1	1	7	0	1	1	1	0	0	2	1	0	0
OsSAP10	2	0	0	0	2	1	0	8	8	1	0	1	0	0	0
OsSAP11	4	0	1	1	3	0	0	3	3	1	0	1	0	1	0
OsSAP12	6	0	2	2	7	0	0	3	3	1	0	0	1	1	0
OsSAP13	7	0	1	1	8	0	0	1	1	0	0	1	0	0	0
OsSAP14	1	0	0	0	2	0	1	6	6	0	0	2	0	0	1
OsSAP15	3	0	0	0	5	1	0	0	0	0	0	2	0	2	0
OsSAP16	1	0	0	0	1	0	0	3	3	0	1	0	0	0	0
OsSAP17	2	0	0	0	3	0	0	6	6	1	0	0	1	1	0
OsSAP18	1	0	0	0	3	0	0	1	1	0	0	0	0	1	0

Footnote: The numerical value in the boxes indicates the number of CRE copies within a 2 kbp promoter sequence across all *OsSAPS*. Colour in the box indicates high abundance (red) to low (orange-yellow), or not present (green).

Table 2. List of hormone-related CREs in *OsSAP8* promoter regions

Plant hormonal regulation	*Cis*-regulatory elements (CRE)	Domain sequence	Function
ABA responsive element	ABRE	ACGTG, CACGTG, TACGTGTC	Involved in ABA responsiveness (Shokri-Gharelo *et al*., 2017; Su et al., 2021; Zhou *et al*., 2020) – Regulate dehydration and salinity response.
	ABRE3a	TACGTG	Integrate ethylene or ABA hormones and stresses to photoperiod responses (Mongkolsiriwatana *et al*., 2009) Act as a binding site (Maqsood *et al*., 2022)
	ABRE4	CACGTA	
	G-box	TACGTG, CACGTG, CACGTC	– Involved in ABA, MeJA, anaerobiosis, and light intensity – Involved in inducing ethylene and seed-specific expression – Known as ABRE motif (Kaur *et al*., 2017)
GA responsive element	GARE-motif	TCTGTTG	– Gibberellin-responsive element (Chao *et al*., 2020; Kaur *et al*., 2017)
	P-box	CCTTTTG	– Gibberellin-responsive element (Chao *et al*., 2020; Kaur *et al*., 2017)
MeJA responsive element	TGACG-motif	TGACG	– Involved in MeJA and SA responsiveness (Ma *et al*., 2018; Zhang *et al*., 2020, 2019)
	CGTCA-motif	CGTCA	– Involved in MeJA and stress responses (Kaur *et al*., 2017; Mongkolsiriwatana *et al*., 2009; Zhang *et al*., 2019)

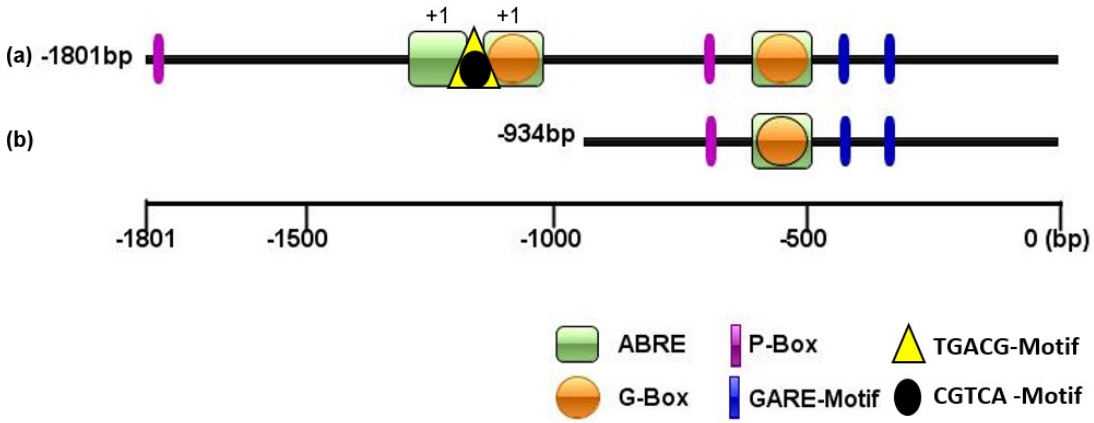

Fig. 1. The distribution of CREs related to ABA, GA, and MeJA phytohormones embedded in (a) full-length (1801 bp) and (b) truncated (934 bp) *OsSAP8* promoter sequence. Different colors and shapes indicate different *cis*-elements. +1 indicates an additional ABRE element is present in multiple repetitive copies at the same position as other CREs in the *OsSAP8* region.

OsSAP8 GUS expression analysis

To evaluate *OsSAP8* expression pattern during transcriptional regulation in response to drought and salinity, we generated a p*OsSAP8*::GUS fusion gene with two distinct entry clones, namely p*OsSAP8*(934 bp)::GUS and *pOsSAP8*(1801 bp)::GUS (Figure 2). Then, these fusion constructs were introduced into *A. thaliana* Col-0 through the floral dipping method, and homozygous transgenic lines were screened using an antibiotic. At least six independent transgenic lines for each construct were qualitatively analyzed for GUS expression under abiotic stresses of drought and salinity during plant growth (seedling stage) and developmental (vegetative to inflorescence) stages.

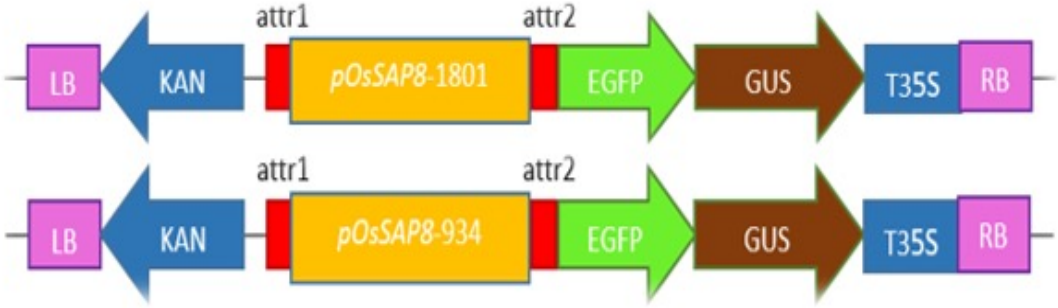

Fig. 2. Schematic illustration of *OsSAP8* full-length (1801 bp) and truncated (934 bp) promoter fragments fused to a GUS reporter gene.

OsSAP8 GUS activity in response to abiotic stresses during the seedling stage

GUS enzyme activities are observable in multiple seedling organs, such as in young leaves, stipules, and roots, when subjected to various stress conditions (Figure 3). During drought treatment, the expression of GUS activity was highly intense in the young leaf, stipule, and root of transgenic *A. thaliana* plants harboring the p*OsSAP8*(934 bp)::GUS construct. In contrast, the GUS activity of the plant carrying the p*OsSAP8*(1801 bp)::GUS construct was expressed in the young leaf and stipule but absent in the root. During salinity treatment, both promoter constructs exhibited GUS activity exclusively in the young leaf and stipule organ only. Nevertheless, the *pOsSAP8(934 bp)::GUS* construct exhibited stronger GUS activity, as indicated by more intense blue staining in both organs, compared to the *pOsSAP8(1801 bp)::GUS* construct. Under normal growth conditions (control), no GUS activity was detected for either promoter construct.

OsSAP8 GUS activity in response to abiotic stresses during the vegetative stage

Throughout the vegetative stage, both promoter constructs showed a consistent GUS activity pattern in the leaf area of transgenic plants that were 28 days old (7 days of stress treatments) and 35 days old (14 days of stress treatments) (Figures 4 & 5, respectively). The GUS activity primarily occurred in the leaves, with localization primarily at the tips of the leaves, called hydathodes and leaf veins, under both drought and salinity stress conditions (Figures 4 & 5). Under normal growth conditions (control), no detectable GUS activity was observed in either the pOsSAP8(934 bp)::GUS or pOsSAP8(1801 bp)::GUS constructs.

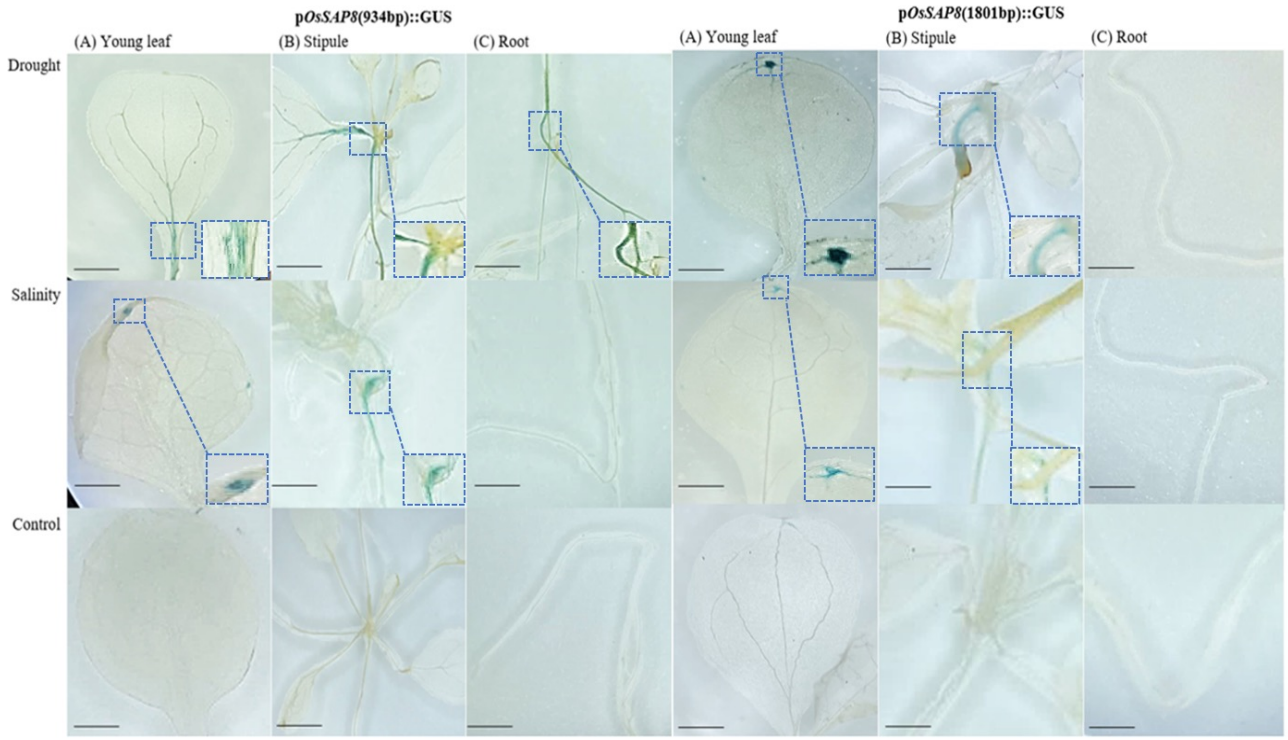

Fig. 3. Expression of GUS activity of transgenic *A. thaliana* carrying p*OsSAP8*(934 bp)::GUS and p*OsSAP8*(1801 bp)::GUS constructs during the seedling stage. The 20-day-old seedlings, which had undergone 3 hours of drought (20% PEG) and salinity (200 mM NaCl) treatments, were analyzed for GUS staining as indicated by the blue color. The squared boxes represent a magnified view of the GUS staining activity. Scale bar = 1 mm

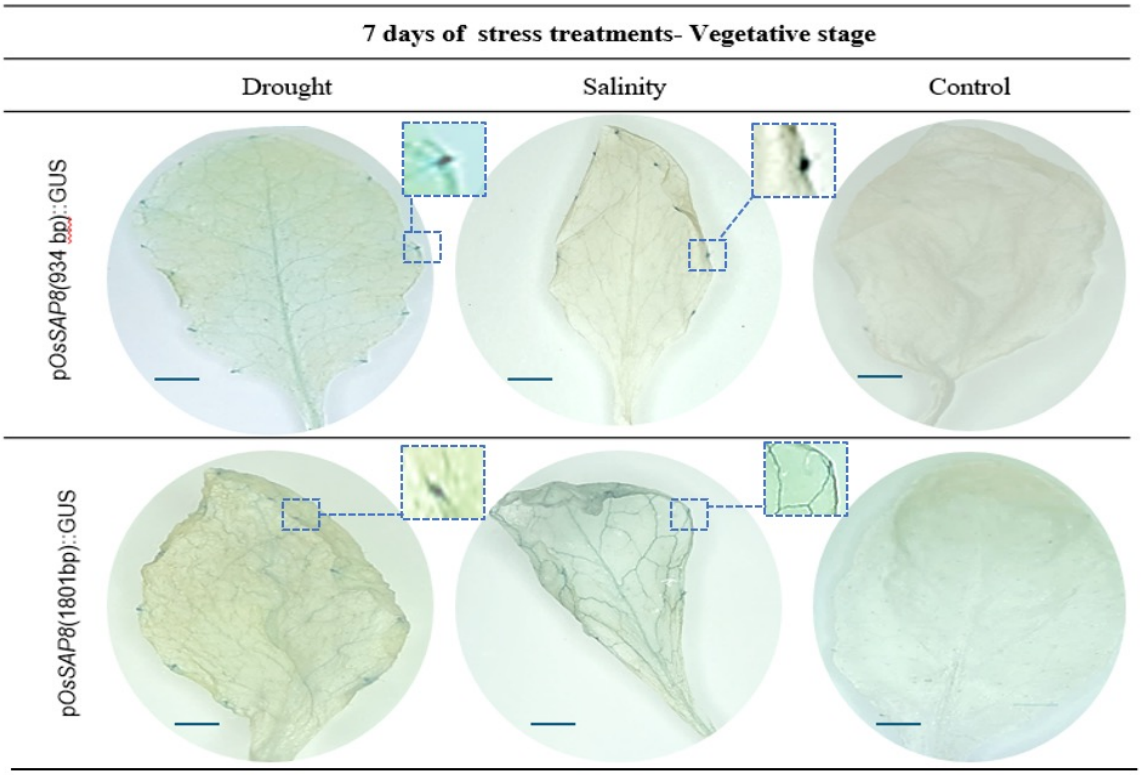

Fig. 4. Expression of GUS activity of transgenic *A. thaliana* carrying p*OsSAP8*(934 bp)::GUS and p*OsSAP8*(1801 bp)::GUS constructs during the vegetative stage. The 28-day-old plants, which had undergone 7 days of drought (withheld watering) and salinity (200 mM NaCl) treatments, were analyzed for GUS staining as indicated by the blue color. The squared boxes represent a magnified view of the GUS staining activity. Scale bar = 3 mm.

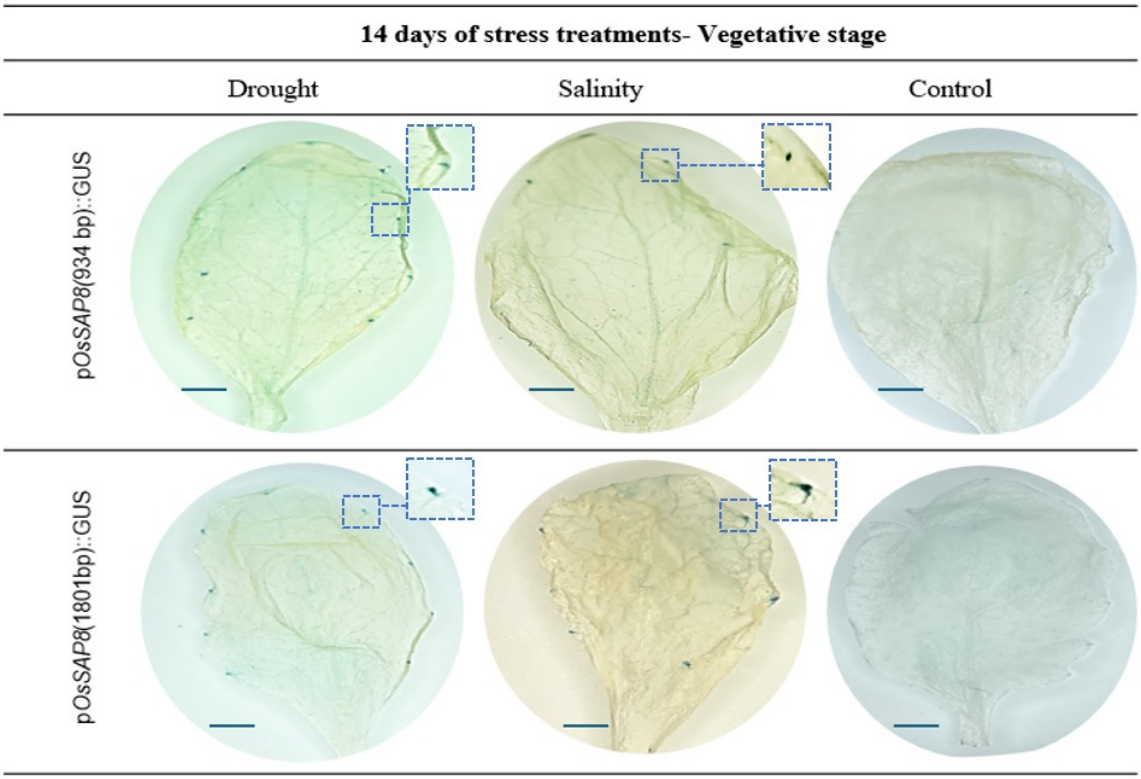

Fig. 5. Expression of GUS activity of transgenic *A. thaliana* carrying p*OsSAP8*(934 bp)::GUS and p*OsSAP8*(1801 bp)::GUS constructs during the vegetative stage. The 35-day-old plants, which had undergone 14 days of drought (withheld watering) and salinity (200 mM NaCl) treatments, were analyzed for GUS staining as indicated by the blue color. The squared boxes represent a magnified view of the GUS staining activity. Scale bar = 5 mm.

OsSAP8 GUS activity in response to abiotic stresses during the inflorescence stage. During the inflorescence stage, both pOsSAP8(934 bp)::GUS and pOsSAP8 (1801 bp)::GUS lines exhibited GUS staining in the flower organs under drought and salinity treatments (Figure 6). However, the pOsSAP8(1801 bp)::GUS construct displayed weaker GUS activity when subjected to both stress treatments, as evidenced by a lighter staining compared to the pOsSAP8(934 bp)::GUS. Under standard growth conditions (control), neither the pOsSAP8(934 bp)::GUS nor the pOsSAP8(1801 bp)::GUS constructs showed detectable GUS activity.

DISCUSSION

Overexpression of *OsSAP8* displayed high tolerance when subjected to abiotic stresses, especially in drought and salinity stress (Kanneganti & Gupta, 2008; Zhang *et al*., 2016; Roslan *et al*., 2017; Sahid *et al*., 2020). Moreover, the *OsSAP8* plant demonstrated the ability to produce inflorescence stems, generate more flowers, and maintain higher relative chlorophyll content under abiotic stress conditions throughout both vegetative and inflorescence stages (Roszelin *et al*., 2023). However, the specific role and regulatory mechanisms of the *OsSAP8* gene in response to abiotic stresses have not been comprehensively studied yet. Therefore, identifying the CREs embedded in the *OsSAP8* promoter analysis provides insights into how its gene expression is regulated. This study aimed to clone and characterize the *OsSAP8* promoter. We specifically focused on the CRE-associated phytohormone responses, as phytohormones enhance a plant's ability to adapt to changing environmental conditions and regulate plant growth and development.

The *in silico* analysis of the *OsSAP8* promoter revealed several potential CREs related phytohormones phytohormone-responsive elements, scattered within the 1801 bp promoter region. Among the identified phytohormone CREs, ABA, GA, and MeJA responsive elements were enriched in the *OsSAP8* promoter sequence. Prior research has indicated that the *OsSAP8* gene was regulated through ABA and GA signaling pathways (Giri *et al*., 2011; Sahid *et al*., 2020; Li *et al*., 2022; Roszelin *et al*., 2023).

The ABA Responsive Element (ABRE) is a hormonal *cis*-acting element primarily involved in ABA signaling in response to abiotic stresses such as drought, high salinity, and low temperature (Magwanga *et al*., 2018; Misra & Ganesan, 2021; Maqsood *et al*., 2022; Hazbir *et al*., 2024). ABRE was originally discovered in the EmBP-1b promoter of wheat (*Triticum aestivum*) as an 8 bp sequence (CACGTGGC) with a core element ACGT (Singh & Laxmi, 2015; Lenka & Bansal, 2019). ACGT core embedded in the ABRE2, ABRE3a, and ABRE4 motifs acts as a binding site for bZIP family transcription factors governing transcriptional regulation of ABRE (Verma *et al*., 2016; Maqsood *et al*., 2022). ABA is crucial for seed development, including inducing seed dormancy, promoting the accumulation of storage products, controlling the formation of root and seedling growth, and regulating adaptive responses to abiotic and biotic stresses (Danquah *et al*., 2014; Ishibashi *et al*., 2016; Brookbank *et al*., 2021). Under osmotic conditions such as drought and high salinity, ABA is known to trigger short-term responses such as stomatal closure to maintain water balance (Zhang *et al*., 1987), as well as long-term growth responses by regulating stress-responsive genes (Verma, Ravindran & Kumar, 2016). Osmotic stresses induce the upregulation of ABA biosynthesis genes such as the

Zeaxanthin epoxidase gene (*ZEP*), *Aldehyde oxidase* gene (*AAO3*), *9-cis-Epoxycarotenoid dioxygenase* gene (*NCED3*), and the *Molybdenum cofactor sulfurase* gene (*MCSU*), resulting in ABA accumulation (Zhu, 2002). Furthermore, ABA-responsive gene expression requires multiple copies of ABRE or another specific *cis*-acting element to operate as an active CRE.

The G-box (CACGTG) element, also referred to as ABRE, plays a role in responding to ABA, MeJA, light, and anaerobiosis. Additionally, it plays roles in ethylene induction, acts in defense against pathogen attack, and is involved in seed-specific expression (Kong *et al*., 2018). The G-box can be bound by bZIP proteins like G/HBF-1, or by basic/helix-loop-helix (bHLH) proteins and bHLH-leucine zipper MYC proteins, to initiate the plant defense response against pathogens (Kong *et al*., 2018). Usually, G-box plays roles by merging with promoters of ABA-responsive genes or with other CREs. This can be seen on Figure 1a and Figure 1b, where the G-box is located at the same location within the ABRE motif. However, there is limited information on its function and role as it has not been thoroughly studied.

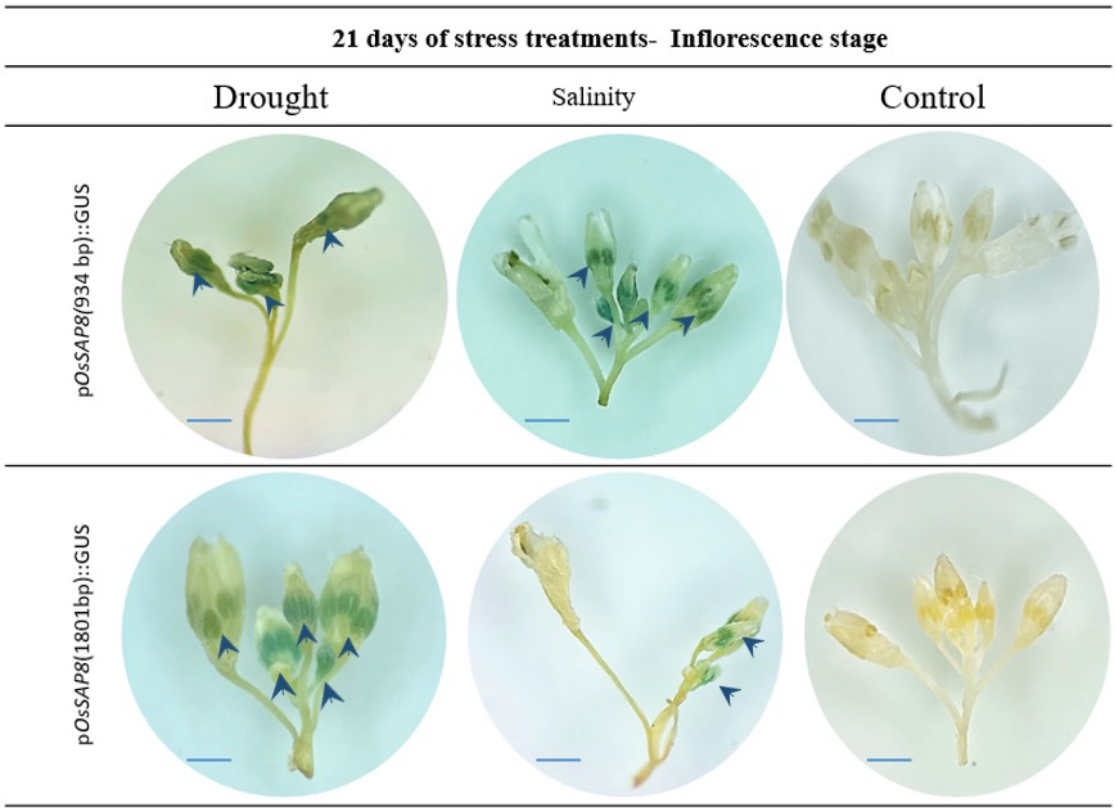

Fig. 6. Expression of GUS activity of transgenic *A. thaliana* carrying p*OsSAP*8(934 bp)::GUS and p*OsSAP8*(1801 bp)::GUS constructs during the inflorescence stage, focusing on the flower organ. The 42-day-old plants, which had undergone 7 days of drought (withheld watering) and salinity (200 mM NaCl) treatments, were analyzed for GUS staining as indicated by the blue color and the arrowhead. Scale bar = 3 mm.

The P-box, or known as the prolamin box (CCTTTTG) and GARE (TCTGTTG) motifs are important regulatory element involved in the gibberellic acid (GA) signaling pathway (Ramkumar *et al*., 2014; Tao *et al*., 2015; Hou *et al*., 2016; Lai *et al*., 2019; Misra & Ganesan, 2021; Bao *et al*., 2024). In some promoters, a single GARE motif can significantly enhance hormonal transcription by synergizing with other *cis*-acting elements (Heidari *et al*., 2015).

The TGACG-motif and CGTCA-motif have been reported to play roles in environmental stress responses and MeJA responsiveness (Wang *et al*., 2011; Zhang *et al*., 2019; Maqsood *et al*., 2022). MeJA, a derivative of jasmonic acid (JA), regulates various functions in plant growth, development, and stress responses, particularly by bolstering plant defenses against necrotrophic pathogens and insects (Verma *et al*., 2016; Venegas-Molina *et al*., 2020; Wang *et al*., 2021). The TGACG-motif, known as the 'as-1 element,' enhances gene expression specifically in response to wounding stress (Srivastava *et al*., 2014). The TGACG-motif was initially discovered in viral and bacterial T-DNA promoters (Bouchez *et al*., 1989; Lam *et al*., 1989; Kong *et al*., 2018). The transcriptional activation of pathogenesis-related genes is facilitated by the TGACG-motif through the binding of BZIP TGA transcription factors to this motif (Wang *et al*., 2013).

In this study, we evaluated the drought and salinity stress responses of two promoter sequences, consisting of a full-length (p*OsSAP8*(1801 bp)::GUS) and a truncated (p*OsSAP8*(934 bp)::GUS) promoter construct during the seedling and vegetative to inflorescence stages through histochemical staining. The GUS activity of the two promoters was examined in transgenic Arabidopsis. Notably, the study revealed that GUS expression activity in p*OsSAP8*(934 bp)::GUS was consistently higher than in p*OsSAP8*(1801 bp)::GUS during both developmental stages.

During drought and salinity treatment in the seedling stage, GUS expression activity was detected in all organs for p*OsSAP8*(934 bp)::GUS, whereas for p*OsSAP8*(1801 bp)::GUS, GUS activity was absent in the roots (Figure 3). The absence of GUS activity in the roots of p*OsSAP8*(1801 bp)::GUS under drought and salinity is likely due to the regulation of ABA induced by stress. Abscisic acid (ABA) and specific cis-regulatory elements (CREs) can inhibit the expression of stress-associated genes under certain conditions. While ABA often activates stress-responsive genes, it can also repress gene expression, depending on the promoter architecture and interacting transcription factors. If the promoter contains repressor-type elements (like negative

ABRE variants or binding sites for repressive TFs), ABA may suppress its expression. ABA can also activate transcriptional repressors that bind to the promoter, indirectly reducing expression. In this study, *in silico* promoter analysis also supports this statement, showing that the 1801 bp promoter construct contains more ABA-responsive elements compared to the 934 bp promoter construct, which may contribute to the negative regulation of *OsSAP8* expression in the longer promoter compared to the truncated one.

Meanwhile, during the vegetative (28-day-old plant with 7 days of stress treatments) to inflorescence stage (35-day-old plant with 14 days of stress treatments), both promoter constructs showed increased GUS activity primarily in hydathodes, followed by leaf veins when subjected to drought and salinity stress (Figures 4 & 5). This finding implies that the *OsSAP8* promoter regulatory activity was elevated under abiotic stress treatments. Hydathodes are specialized plant structures located at the tips of the leaf serrations and margins of the leaves, facilitating water excretion through guttation (Figueroa-Balderas *et al*., 2006; Yagi *et al*., 2021a, 2021b; Bellenot *et al*., 2022). These structures play a crucial role in maintaining water balance and also serve as entry points for pathogens (Yagi *et al*., 2021a; Bellenot *et al*., 2022). Hydathodes consist of three main elements, which are water pores, xylem ends, and small cells known as epithem (E) cells (Kawamura *et al*., 2010; Yagi *et al*., 2021a).

The elevated GUS activity observed in hydathodes is likely due to the abundance of E cells. Previous studies have shown that these cells exhibit prominent green fluorescent protein (GFP) fluorescence within the leaf area, corroborating our findings (Yagi *et al*., 2021a). Additionally, as transgenic *OsSAP8* plants in this study were subjected to drought and salinity, ABA was accumulated as a response to combat these stresses (Tuteja, 2007; Yang *et al*., 2022). ABA accumulation triggers high expression of *OsSAP8* (Sahid *et al*., 2020). Arabidopsis hydathodes water pores that act similarly to stomata, exhibit responsiveness to abiotic stresses, such as ABA and light conditions (Cerutti *et al*., 2017). This suggests that stomata and hydathode pores shared a common regulatory mechanism in responding to environmental changes. Therefore, throughout the vegetative stage, both promoter constructs displayed intense GUS activity in the leaves (hydathode and vein) due to the accumulation of ABA, hydathode pore responses to ABA, and the presence of ABA-responsive elements within both constructs.

The p*OsSAP8*(934 bp)::GUS demonstrated stronger GUS activity in the flower organ during the inflorescence stage compared to p*OsSAP8*(1801 bp)::GUS constructs (Figure 6). This may be due to the absence of MeJA phytohormones in the 934 bp promoter fragment. MeJA phytohormone has been reported to potentially inhibit flowering in Arabidopsis (Huang *et al*., 2017; Wasternack & Song, 2017). Besides, sequence analysis revealed that there were three CREs related to GA responsive elements and only two related to ABA-responsive elements in the 934 bp *OsSAP8* promoter fragment. We hypothesized that these GA responsive elements played an important role in increasing GUS activity in the flower organ. This is supported by a previous study that stated *OsSAP8* gene involvement in GA biosynthesis (Li *et al*., 2022; Roszelin *et al*., 2023).

In summary, the *OsSAP8* promoter exhibited inducible expression in response to various stress stimuli such as drought and salinity (Figure 3-6). The use of an inducible promoter has become a standard practice for creating transgenic plants with enhanced resilience to diverse abiotic stresses (Saad *et al*., 2010; Rerksiri *et al*., 2013; Hou *et al*., 2016; Misra & Ganesan, 2021). Moreover, the GUS expression activity observed in this study indicates the *OsSAP8* gene to be particularly responsive to drought and salinity stresses. Notably, throughout the growth and developmental stages of the *OsSAP8* plant, the truncated promoter construct showed greater GUS activity compared to the full-length constructs. Thus, we concluded that the truncated promoter is sufficient for driving the transcription and gene expression in response to abiotic stresses. According to Bao *et al*. (2024) and Chen *et al*. (2023), shorter promoters generally produce more robust GUS expression than the longer ones. Sometimes, elements within the promoter function as repressors, leading to a decrease in GUS expression in longer promoters due to negative feedback regulation. Therefore, the truncated *OsSAP8* promoter represents a promising candidate for genetic manipulation for rice improvement programs, by developing crops that are tolerant to abiotic stresses.

CONCLUSION

In silico analysis of *OsSAP* genes has identified *cis*-regulatory elements (CREs) linked to phytohormone-responsiveness, which may regulate *OsSAP* gene expression under abiotic stress. *OsSAP8* was chosen in this study due to its proven resilience to salinity and drought stress, demonstrated by its ability to maintain higher relative chlorophyll content, produce robust inflorescence stems, and increase flower production. Analysis of the *OsSAP8* promoter fragment revealed CREs related to ABA and GA, phytohormones. The GUS staining analysis showed that transgenic *A. thaliana* plants carrying the p*OsSAP8*(934 bp)::GUS construct exhibited significantly stronger GUS activity compared to the p*OsSAP8*(1801 bp)::GUS construct. This suggests that the truncated promoter is more efficient in driving *OsSAP8* gene expression under abiotic stress, possibly due to the lack of other CREs and fewer ABA-related CREs. In conclusion, this study provides valuable insights into the regulation of the *OsSAP8* gene expression, which can be used as a stress-inducible promoter for enhancing plant resilience to salinity and drought stresses, particularly in rice. Additionally, future research could investigate the interactions between *OsSAP8* and the potential CREs through electrophoretic mobility shift assays (EMSA) to further elucidate their binding dynamics.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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