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Research Article

Arabidopsis thaliana Stress Associated Protein 2 (*AtSAP2*) Expression in Response to Salinity and Drought is Regulated by the RAP2.4 Transcription Factor

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ABSTRACT

Plants cannot move to shelter themselves and need to adapt to environmental changes through complex regulatory mechanisms. These adaptations commonly involve the role of stress-related transcription factors (TF). This study explores the role of Ethylene Response Factor (ERF) transcription factor binding sites by examining their distribution within the promoter region of the *Arabidopsis thaliana Stress Associated Protein 2 (AtSAP2).* Previous studies have highlighted the importance of *AtSAP2* in conferring tolerance to plants under abiotic stress, and its ortholog in rice (*OsSAP4*) has been shown to exhibit increased expression due to ERF binding sites. Thus, understanding the connection between specific ERF sites and the regulation of *AtSAP2* is crucial. Analysis of the 2 kb region upstream of the *AtSAP2* promoter revealed several Transcription Factor Binding Sites (TFBS), including bZIP, C2H2, CPP, ERF, HD-ZIP, G2-like, MYB, and WRKY. Among these, the Ethylene Response Factor (ERF) binding sites, along with C2H2, are the most abundant in the *AtSAP2* promoter. Notably, *RAP2.4*, an ERF, is significantly predicted to be present in the *AtSAP2* promoter ($p<0.05$). To investigate the relationship between *AtSAP2* and *RAP2.4* regulation, a promoter study was conducted using Arabidopsis. Two different constructs were generated and transformed into Arabidopsis: Col-0 (*pAtSAP2::Col-0*) and the *rap2.4* mutant (*pAtSAP2::rap2.4*). The *rap2.4* mutant was used to determine whether a mutation in *RAP2.4*, an ERF, affects *AtSAP2* expression through the *RAP2.4* binding site. A clear difference in GUS staining activity was observed between *pAtSAP2::Col-0* and *pAtSAP2::rap2.4*. The *pAtSAP2::Col-0* plants exhibited blue stain, indicating GUS activity, while no GUS activity was detected in *pAtSAP2::rap2.4* plants at either the vegetative (leaf) or inflorescence stage. This suggests that the *RAP2.4* protein plays a crucial role in regulating *AtSAP2* gene expression through its binding site during abiotic stress.

Key words: Arabidopsis, β-glucuronidase (GUS), drought, ERF, salinity, stress-associated protein

INTRODUCTION

Crop productivity and yield are seriously threatened by global climate change because it accelerates and intensifies both biotic and abiotic environmental pressures. Abiotic stresses are one of the environmental factors that pose a serious threat to plant growth, development, and productivity (Raza *et al*., 2019). According to meta-analysis studies, about 90% of the world's arable lands are affected by one or more of the abiotic stresses. This poses a serious problem for sustainable agriculture in the future because it can result in a 70% reduction in the major food crops' output yearly (Waqas *et al*., 2019).

Plants have evolved intricate regulatory mechanisms to adapt and respond to changing environmental conditions. One of the regulatory mechanisms is the transcription factor, a protein that controls the transcription of a certain gene by either directly activating the gene or preventing its expression (Zhang *et al*., 2023). Therefore, various stress-related transcription factors have been identified and studied to produce plants that are resistant and tolerant towards abiotic stress through modern breeding and genetic engineering (Muthuramalingam *et al*., 2021).

One class of genes that are identified during stress response is the Stress Associated Proteins (SAPs), which have a signature of AN1 and A20 zinc-finger domains located at the N and C-terminal protein. In *Oryza sativa*, 18 SAP genes (*OsSAP1–18*) have been identified, while in *Arabidopsis thaliana*, 14 SAP genes (*AtSAP1–14*) have been reported (Dixit & Dhankher, 2011).In plants, the first SAP protein containing A20/AN1 domains was identified as *OsiSAP1* in indica rice, and its expression is reportedly induced by various environmental stresses such as cold, salinity, drought, submergence, wounding, and heavy metal exposure. (Mukhopadhyay *et al*., 2004). This discovery led to the identification of other *SAP* genes, especially their role in responding to abiotic stress. One of the discoveries is *ZmSAP8* from maize, where the overexpression of *ZmSAP8* in *A. thaliana* showed an improved resistance towards drought stress, as seen by the increase in seed germination and root length at the germination stage and seedling stage (Su *et al*., 2022).

The expression of these genes is also reported to be regulated by several families of transcription factors, and one of

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them is the family of *Ethylene Responsive Factor (ERF)* (Hazbir *et al*., 2024). The *ERF* family has been reportedly playing an important function in the regulation of transcription of various biological processes, including response to environmental stimuli. Previous studies on the *AtSAP13* gene using yeast one-hybrid analysis have identified several AP2/EREBP family transcription factors that interact with the *AtSAP13* promoter under drought stress conditions (Dixit *et al*., 2018). The *RAP2.4,* a member of the ERF family, has been identified as a transcriptional activator. Microarray analysis from previous studies revealed that the *RAP2.4* downregulated four zinc finger genes while upregulating one during drought stress. Indirectly, this indicates that *ERF* transcription factors may potentially regulate the *SAP* gene expression during abiotic stresses (Rae *et al*., 2011).

Therefore, this study aimed to investigate the role of the *RAP2.4* gene, a member of the *ERF* family, in regulating *AtSAP2* expression by targeting *RAP2.4* binding sites within the *AtSAP2* promoter under drought and salinity stress conditions. To study this promoter activity, the *AtSAP2::GUS* promoter construct was generated and transformed into two different Arabidopsis genotypes, which are the Col-0 and *rap2.4* mutants, to produce the *AtSAP2::GUS* (*pAtSAP2::Col-0*) and (*pAtSAP2::rap2.4*), respectively. These constructs were designed for promoter transcriptional regulation analysis, where the promoter was fused to a GUS reporter gene to assess promoter activity. The Arabidopsis lines *pAtSAP2::Col-0* and *pAtSAP2::rap2.4* were exposed to drought and salinity stress during the vegetative and inflorescence stages. Promoter activity was assessed through GUS staining, as indicated by the presence of blue pigmentation representing GUS expression.

MATERIALS AND METHODS

In silico analysis of all *Arabidopsis thaliana Stress Associated Protein (AtSAPs)* promoters

For promoter analysis, a segment of approximately 2 kb upstream from the ATG start codon of all *AtSAPs* was selected. The promoter sequence was obtained from the Ensembl Plants database (https://plants.ensembl.org/index.html). The crucial elements of the plant promoter, such as the 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 program of the PLANT Promoters database to predict TATA box and other cis-elements binding factors. The 2 kb upstream promoter region of *AtSAP2* (At1g51200) was subsequently analysed using PlantCare and PlantRegMap to predict the distribution of ERF binding sites potentially involved in the regulation of *AtSAP2* gene expression, with the statistically significant cut-off value set at $p<0.05$ (Chen *et al*., 2017; Tian *et al*., 2021). Other Cis-Elements (CEs) and transcription factor family within the *AtSAP2* 2 kb upstream promoter sequence were also determined in this study using a *p*-value of $p<0.05$.

Cloning of the *AtSAP2* promoter

Synthetic DNA templates with a size of 1284 base pairs were amplified using Polymerase Chain Reaction (PCR) and cloned according to the Gateway cloning system using the BP clonase reaction. The amplified PCR product was cloned into pDONR221 via heterologous recombination through specific adaptors in both the primers and intermediate vector. The entry clones were then introduced into competent *E. coli* Top10 cells via the heat-shock method. Transformants were selected by their resistance to kanamycin (50 µg/ml), and colony PCR was performed to identify positive clones (Reece-Hoyes & Walhout, 2018). Successfully generated entry clones were then inserted into the final vector, pKGWFS7, fused with the GUS reporter gene, using the LR Clonase recombination reaction. Positive transformants were selected on LB agar plates containing spectinomycin (50 µg/mL), and colony PCR was performed to confirm the presence of the desired clones (Karimi *et al*., 2002). The positive clones were sent for DNA sequencing to verify the sequence accuracy.

Arabidopsis floral dipping transformation

After the cloning process, the final *AtSAP2::GUS* constructs were introduced into *Agrobacterium tumefaciens* strain 0 (GV3101). These constructs were then used to transform *Arabidopsis thaliana* Col-hereafter referred to as *pAtSAP2::Col-0*) and the *rap2.4* mutant (hereafter referred to as *pAtSAP2::rap2.4*) via the floral dipping method. A bacterial culture containing 0.05% Silwet-77 and a 5% sucrose solution was used for the transformation. To improve transformation efficiency, a second round of floral dipping was performed after a 7-day interval. To select positive Arabidopsis transformants, seeds from the T1 generation were planted on ½ MS media supplemented with 50 µg/mL kanamycin (Murashige & Skoog,1962; Clough & Bent, 1998; Zhang *et al*., 2006; Weise, 2017). Polymerase Chain Reaction (PCR) was used to confirm the presence of the promoter regions in the positive transgenic Arabidopsis plants, employing a specific forward primer for *AtSAP2* and a reverse primer for the eGFP gene in the vector, which generated a product of the expected size. Seeds from the positive T1 plants were harvested and grown through successive generations until T3 seeds were produced. Subsequently, the T3 seeds were grown, and the plants were subjected to stress treatments to evaluate GUS activity.

Experimental condition and stresses

Abiotic stress treatment was carried out at the vegetative and inflorescence phases. First, *pAtSAP2::Col-0* and *pAtSAP2::rap2.4* seeds were plated and germinated on ~~the~~ ½ MS media for 7-14 days before soil transfer. Then, salinity and drought stress treatments were applied at two developmental stages (vegetative & inflorescence stages). Three weeks after transplanting, the plants were exposed to three different treatments over a period of 14 days: control (regular watering), salinity (150 mM NaCl), and drought (withheld watering) (Hazbir *et al*., 2024 & Roszelin *et al*., 2023). GUS assays were conducted on plants at 7 and 14 days post-treatment. For the inflorescence and silique data, identical treatments were applied during the inflorescence and silique developmental stages for 7 days up to 45 days old, followed by staining (Ma *et al*., 2019; Hazbir *et al*., 2024).

GUS staining analysis

For GUS activity analysis, treated leaves, buds, and siliques were placed in 50 mL centrifuge tubes containing X-Gluc solution composed of (0.2 M Na2HPO4, 0.2 M NaH2PO4, 0.1 M K3[Fe(CN)6], 0.1 M K4[Fe(CN)6], 0.5 M EDTA, pH 7-8, 20% methanol, & 1 mM X-Gluc). The samples were incubated in the X-Gluc solution for 16 hr at 37°C in the dark. After incubation, the samples were washed with 70% ethanol for 3 hr to decolourize the chlorophyll and then analyzed under the stereo microscope (Jefferson *et al*.,1987; Basu *et al*., 2004; Dedow *et al*., 2022).

RESULTS AND DISCUSSION

Abiotic stress-related transcription factor binding sites are common in all *AtSAP* promoters.

Cis elements are DNA sequences that act as binding sites for transcription factors, regulating the expression of a target gene by either activating or repressing its transcription. The precise and accurate regulation of genes by transcription factors is crucial for proper cellular function, plant growth, development, and response to environmental stimuli. Therefore, identifying the distribution of transcription factor binding sites in the promoter region is essential for understanding the complex mechanisms behind gene regulation in response to abiotic stress. For this study, an in silico analysis of the promoter was conducted to examine the distribution of transcription factor binding sites across all *AtSAP* promoter sequences. PlantRegMap analysis (p<0.05) revealed 26 transcription factor binding site families spread across the 14 *AtSAP* promoter regions. Each transcription factor plays a unique and crucial role in plants, primarily in two main categories: response to abiotic stresses and regulation of plant development in terms of hormones. In the hormonal regulation, Auxin Response Factors (ARFs), the family of plant-specific transcription factors that play a central role in regulating gene expression in response to the plant hormone auxin, have been identified. Auxin itself is crucial for various aspects of plant growth and development, including embryogenesis, root and shoot development, and flower and fruit formation (Shani *et al*., 2006; Li *et al*., 2016). In addition, auxin signaling, mediated by ARFs, is also involved in plant responses to both biotic and abiotic stresses. For instance, *ARF2* and *ARF7* have been shown to participate in stomatal regulation and the modification of root architecture under drought conditions. Furthermore, ARFs contribute to the coordination of biotic stress responses by mediating the crosstalk between auxin and other hormonal pathways, such as salicylic acid and jasmonic acid (Li *et al*., 2016; Zhang *et al*., 2022). Thus, the presence of ARF binding sites in the promoter regions of *SAP* genes may suggest a regulatory role for auxin-responsive transcription factors in modulating *SAP* gene expression under stress conditions.

Approximately 60.35% of the binding sites are linked to the transcription factors involved in regulating plant responses to abiotic stresses, highlighting the critical role of *AtSAP* genes in stress response (Figure 1). The *ERF* transcription factors show a particularly high prevalence of binding sites within the *AtSAP* promoter region under the abiotic stress response category. The AP2/ERF superfamily, characterized by the conserved AP2/ERF domain, plays a key role in mediating plant responses to abiotic stresses. Their roles encompass regulating growth, flower development, and hormone signalling pathways, which help plants adapt to abiotic stresses. Among the 26 types of transcription factors found across all *AtSAP* promoter regions, ERF transcription factor binding sites are notably abundant. The distribution of these binding sites is as follows: *AtSAP11* and *AtSAP12* (67), *AtSAP13* (62), *AtSAP4* (25), *AtSAP8* (15), *AtSAP6* (14), and *AtSAP2* (7). This suggests that ERF transcription factors may play a critical regulatory role in the plant's adaptation to abiotic stresses.

	AS														PD											H
	bZIP	CAMTA	CPP	C2H2	C3H	ERF	HD-ZIP	HSF	GATA	MYB	MYB_related	NAC	Trihelix	WRKY	BBR-BPC	bHLH	B3	Dof	G2-like	EIL	LBD	MIKC_MADS	nin-like	RAV	TCP	ARF
AtSAP1			1	17	1	1	3	5	2	11		22	2	14	3	6	2	5	1			1				
AtSAP2	2		1	5		7	1	4		3	2	3	1	2	8	3	2	32	2			17	3	1	1	2
AtSAP3	11			1		11		15	13	17	6	8	37	3	3	29	6	12	1	2	6	7		1	2	1
AtSAP4	2			14		25	5		27	12	19	5	1	1	27	5	3	21	4	1	2	3				3
AtSAP5	3	5	1		1	2	1	4	5	7	2	3	7	1	3	4	5	74	3		6	33	1	1	6	4
AtSAP6	4		3	27	1	14	3	3	8	18	1	7	1	3	12	5	2	7	2		6	66		2		
AtSAP7	2	1	5	1	2	4	5	3	1	11	18	12	1		2	1	7	28	6		5	8	1			5
AtSAP8	2			12		15	2	8	1	3	4	5	2	16	1	4	7			2	5	5	1			1
AtSAP9	16	2	6	12	1	2	9		11	25	5	33	2		5	7	4		9		2	9				3
AtSAP10	2	2	1	21	1	6			4	6	3	27	16	24	25	4	1	2	4		2	24	1	3	4	3
AtSAP11	1	1	1	14		67		7	7	4	1	12	3	16	4	7	1	15	5	2	4	3	1	3	11	1
AtSAP12	1	1	1			67		7	7	4	1	12	3		16	8	1	15	5	2		3	1	3	11	2
AtSAP13	1			2		62	2		1	8	1	2	2	15	3	1		4	1		6	1				1
AtSAP14	3		6	4		10	7	3	5	4	2	17		4	3	3	5	3	1			2		1	3	

Fig. 1. The distribution of transcription factor binding sites within the promoter regions of all *AtSAP* genes is shown. "AS" represents Abiotic Stress, "PD" stands for Plant Development, and "H" denotes Hormones. The numbers indicate the significance of the presence of binding sites within the promoter regions. The numbers present show a significant prediction of cis-elements distribution across all *AtSAPs*.

ERF site abundance in *AtSAP2* promoter guides RAP2.4 selection for regulatory study

Among the 14 *AtSAPs* analyzed, the *AtSAP2* promoter was selected for further investigation. This is because *AtSAP2* is the ortholog of *OsSAP8* and *OsSAP4* in rice (Roszelin *et al*., 2023; Hazbir *et al*., 2024). Studies on both *OsSAP8* and *OsSAP4* in our laboratory have shown that these genes increased in expression during stress, with GUS staining revealing that ERF cis-elements in the *OsSAP4* promoter enhance expression under salinity and drought conditions (Hazbir *et al*., 2024). Analysis of the *AtSAP2* mutant Arabidopsis also showed that the mutation is susceptible to salinity and drought compared to the Col-0 wild type and overexpression versions (Roslan *et al*., 2017). Therefore, further analysis was conducted to examine the distribution of transcription factor binding sites within the *AtSAP2* promoter region, with a particular focus on ERF transcription factor binding sites. This analysis, which concentrated on the 2 kb promoter sequence of the *AtSAP2* gene, identified a total of 14 families of

TF binding sites (Figure 2).

Among the 14 families identified, 9 are associated with plant responses to abiotic stress (bHLH, bZIP, C2H2, ERF, NAC, HD-ZIP, MYB, WRKY, and ZF-HD). In silico analysis revealed a significantly higher abundance of ERF binding sites in the *AtSAP2* promoter compared to other binding sites linked to abiotic stress response. The distribution of these binding sites was as follows: ERF (7), C2H2 (5), MYB (3), bZIP (2), and WRKY (2) (Figure 2). This suggests a potential key regulatory role for ERF in regulating *AtSAP2* expression in response to abiotic stress. Specifically, 7 ERF binding sites were identified in the promoter region: *ERF027, ERF037, ORA47, RAP2.4, DREB2E, DREB2C,* and *EREBP* (Table 1). Of these 7 ERF binding sites, *ORA47, RAP2.4, DREB2E, DREB2C,* and *EREBP* have been previously reported to play a role in plant responses to various abiotic stresses (Table 1) (Lin *et al*., 2008; Chen *et al*., 2010, 2016; Rae *et al*., 2011; Yang *et al*., 2020; Jarambasa *et al*., 2023; Li *et al*., 2024).

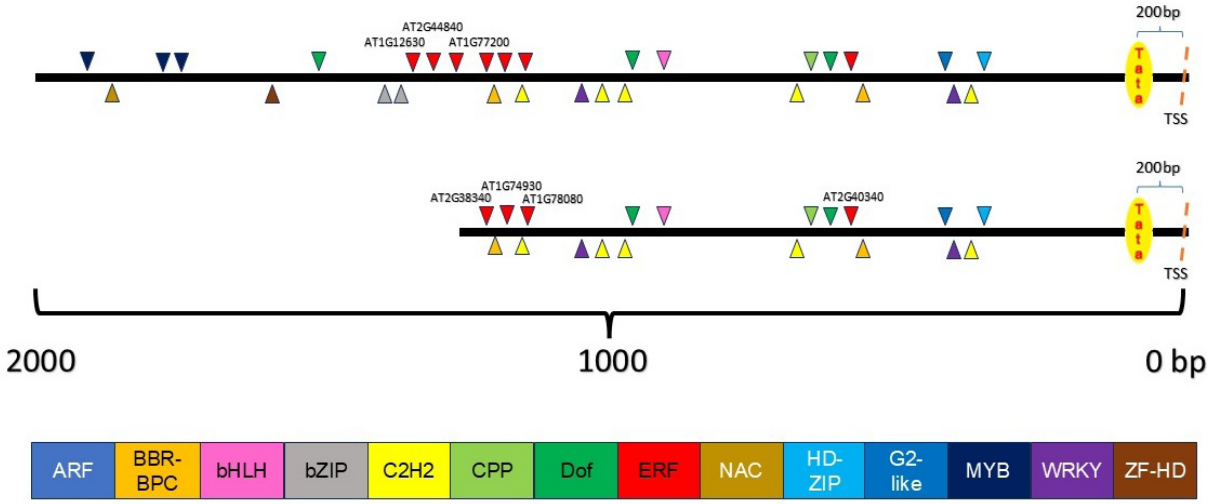


Fig. 2. The distribution of transcription factor binding site cis-elements within the promoter fragments of a) the 2 kb *AtSAP2* sequence and b) the 1258 bp *AtSAP2* sequence, with a significance threshold of $p<0.05$. At2G38340 [*DREB2E*], At1G74930 [*ORA47*] and At2g40340 [*DREB2C*] and At1G78080 [*RAP2.4*].

Table 1. Displays the identification of key ERF cis-element binding sites in the *AtSAP2* promoter, along with their corresponding AGI codes.

Agi code	Common name#	Group	Description
AT1G12630	*ERF027*	III	N/A
AT1G74930	*ERF018, ORA47*	II	involved in regulating JA and ABA biosynthesis during wounding (Chen *et al*., 2016)
AT1G77200	*ERF037*	III	N/A
AT1G78080	*AtWIND1, ERF059, RAP2.4*	I	RAP2.4 provides tolerance to drought stress through the activation of cuticular wax biosynthesis (Rae *et al*., 2011; Yang *et al*., 2020)
AT2G38340	*ERF46, REB19, DRE2E*	IV	Previous study reported that DREB2E was induced in leaves during drought stress through genome-wide analysis (Jarambasa *et al*., 2023)
AT2G40340	*AtERF48, DREB2C*	IV	DREB2C is involved in the heat stress response through the activation of Heat Shock Factor A3 (HsfA3) and has been reported to increase tolerance to oxidative stress (Chen *et al*., 2010).
AT2G44840	*AtERF13, EREBP*	II	*AtERF13* acts as a negative regulator of plant growth and tolerance to the toxic metal cadmium (Chen *et al*., 2024).

RAP2.4 was selected for further analysis of *AtSAP2*. As a member of the *ERF* gene family, *RAP2.4* belongs to the Ethylene Response Factor group of transcription factors and has been shown to play an important role in plant responses to abiotic stress (Nakano *et al*., 2006). *RAP2.4* has also been demonstrated to increase in transcript levels during stress response and plays a crucial role in activating cuticular wax biosynthesis, which helps reduce water loss and enhances drought tolerance (Yang *et al*.,

2020). Further analysis of microarray experiments revealed that RAP2.4 regulates several genes, including those encoding zinc finger proteins, which are involved in various stress responses and developmental processes. Our in silico analysis identified the presence of an RAP2.4 binding site in the *AtSAP2* promoter region, suggesting that RAP2.4 may regulate the expression of *AtSAP2* during abiotic stress. To investigate this, a synthetic DNA template comprising 1,284 base pairs (~1.2 kb) was designed, incorporating the RAP2.4 binding site while intentionally minimizing the inclusion of other abiotic stress-related binding elements. The 1,284 bp promoter region also contains binding sites for other ERF transcription factors, including At2G38340 (*DREB2E*), At1G74930 (*ORA47*), and At2G40340 (*DREB2C*) (Figure 2). However, the use of the *rap2.4* mutant specifically allows verification of the role of RAP2.4 in regulating *AtSAP2*, in comparison to the wild-type Col-0. This study used two transgenic Arabidopsis lines carrying the 1284 base pair *AtSAP2* promoter construct—one in the wild-type Col-0 background and the other in the *rap2.4* mutant background. The *rap2.4* mutant was included to investigate whether the mutation in *rap2.4* still allows the enhancement of *AtSAP2* expression via the ERF binding site during salinity and drought stresses.

***AtSAP2* promoter-driven GUS expression is induced by stress under RAP2.4 regulation**

The transcription level and expression pattern of a gene are controlled by its promoter. The promoter is an upstream DNA sequence that regulates gene expression by enabling the binding of proteins, such as RNA polymerase and transcription factors, to specific sequences within the promoter region, thereby initiating gene expression (Cui *et al*., 2023). One method to study and analyse promoter activity during abiotic stress (such as drought and salinity) is through GUS staining (β-glucuronidase staining). This histochemical technique utilizes a reporter gene to visualize and analyse the blue pigment that appears in tissues and cells (Lee & Schoffl, 1997; Dedow *et al*., 2022).

In this study, GUS localization was analyzed in transgenic *Arabidopsis thaliana* plants containing constructs of *pAtSAP2::Col-0* and *pAtSAP2::rap2.4,* respectively. The use of two different genotypes allows for a comparison to assess whether mutations in *rap2.4* (an *ERF*) can enhance *AtSAP2* gene expression through the ERF binding site. During the first 7 days of drought and salinity treatments, neither genotype displayed any blue pigment from the GUS staining (Figure 3). At the vegetative stage, blue pigments were observed in the *pAtSAP2::Col-0* leaves on day 14 of drought stress (primary vein) and salinity stress (secondary vein) (Figure 4). However, no blue pigment was observed in the *pAtSAP2::rap2.4* leaves on day 14 of either stress (Figure 4). The presence of blue pigment from GUS activity in the leaves of *pAtSAP2::Col-0* during both drought and salinity stress on day 14 suggests that *AtSAP2* promoter activity is regulated by the RAP2.4 binding site in the promoter region. Gene expression is regulated in a spatio-temporal manner, with certain genes being highly expressed at specific times and developmental stages (Dutt *et al*., 2014). This may explain why no GUS staining was observed after 7 days of salinity and drought treatment, while GUS activity was specifically detected in the leaf veins of the *pAtSAP2::Col-0* plants after 14 days of stress treatment.

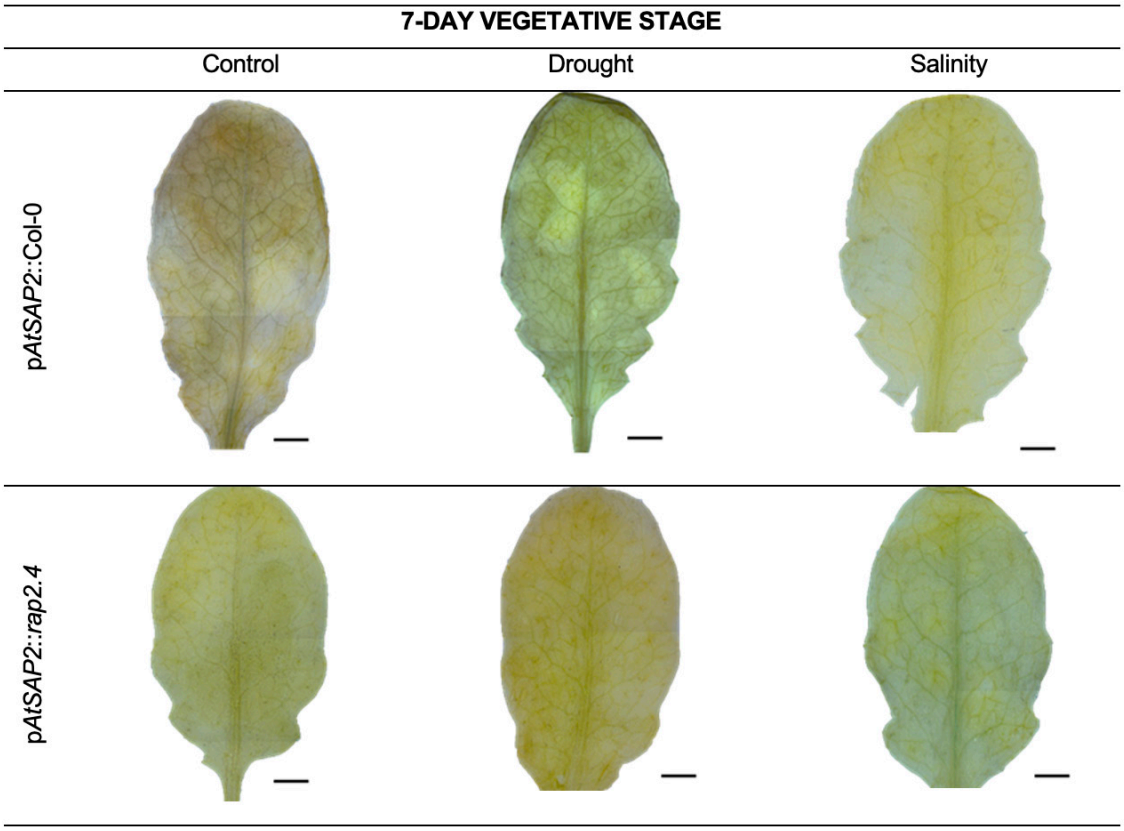


Fig. 3. GUS staining analysis was performed on the Arabidopsis *pAtSAP2::Col-0* plant and *pAtSAP2::rap2.4* mutant during the vegetative stage. The GUS staining was carried out over 7 days of drought (no watering) and salinity stress (150 mM NaCl). Scale bar = 1 cm.

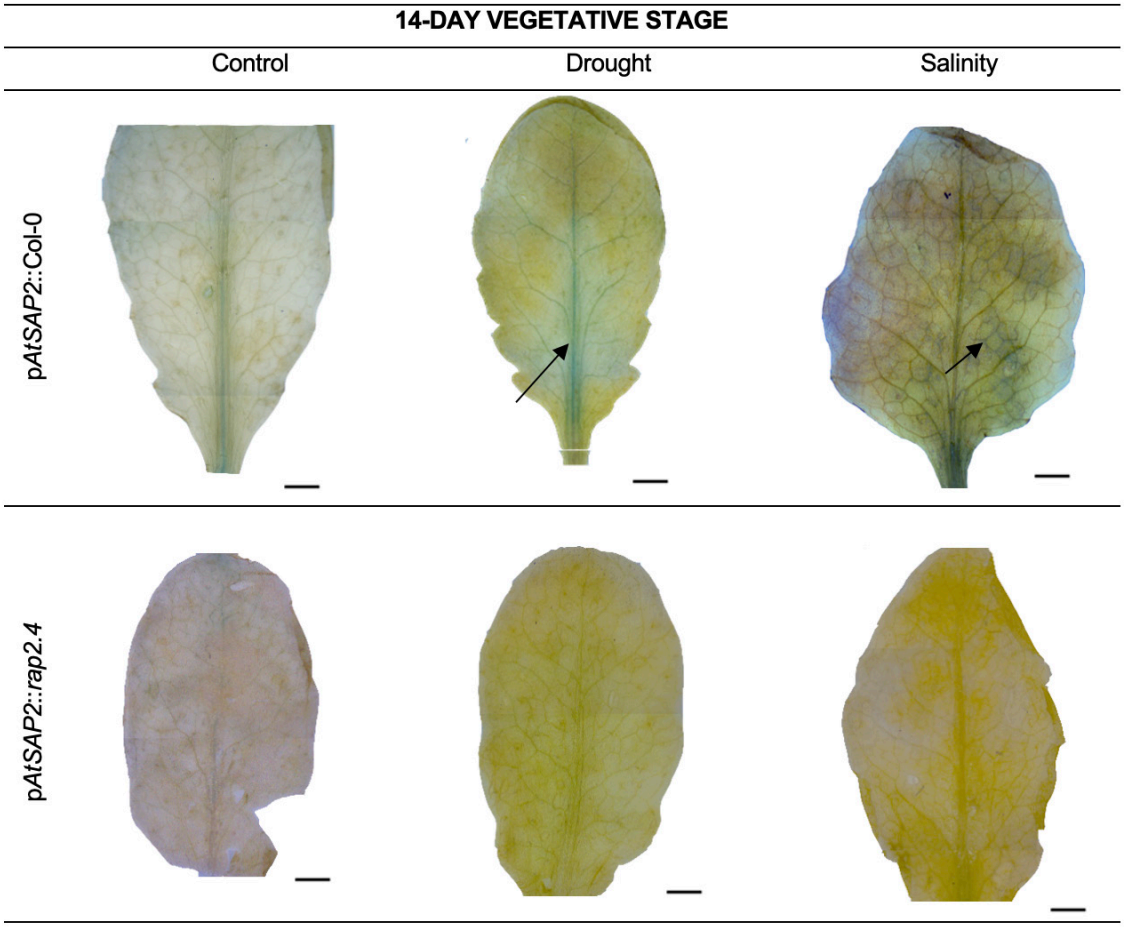


Fig. 4. GUS staining analysis was performed on an Arabidopsis *pAtSAP2::Col-0* plant and *pAtSAP2::rap2.4* mutant during the vegetative stage. The GUS staining was carried out over 14 days of drought (no watering) and salinity stress (150 mM NaCl). The arrow indicates blue staining, representing GUS activity. Scale bars = 1 cm.

Additionally, the presence of the blue pigments in the leaf veins suggests that *AtSAP2* promoter activity is particularly active in this area, playing a crucial role in processes occurring in the leaf veins during drought and salinity stress. Recent research indicated that the *NAC* transcription factor gene *GhNAC4* exhibited intense GUS staining in the vascular bundles and guard cells during drought stress, suggesting its significant role in the water and nutrient transport regions involved in drought stress response (Trishla *et al*., 2020). This finding implies that the regulation of the *AtSAP2* promoter by RAP2.4 at its binding site in the promoter region during drought and salinity stress is significant. The strong blue pigment observed in the leaf veins indicates that *AtSAP2* likely plays an important role in drought and salinity responses through effective water, nutrient, and hormone transport. This also demonstrates that, despite the presence of many other cis-elements in the *AtSAP2* promoter, the expression of *AtSAP2* during drought and salinity stress cannot be enhanced in the *rap2.4* mutant due to the absence of the RAP2.4 protein. This highlights the crucial role of the RAP2.4 binding element in boosting *AtSAP2* gene expression in response to stress. Additionally, this cis-element is part of the ERF group, and the ERF is an important cis-element in enhancing *OsSAP4* expression as previously reported (Hazbir *et al*., 2024).

During the inflorescence and silique stage, GUS staining analysis of the *pAtSAP2::Col-0* flowers under drought stress revealed a positive blue pigment in the flowers but not in the siliques (Figure 5). This suggests that RAP2.4 is active in driving *AtSAP2* gene expression under drought conditions and may contribute to the early flowering phenotype as an adaptive mechanism during drought stress. Early flowering under drought stress is considered a drought escape strategy, where plants accelerate their life cycle to complete reproduction before severe drought occurs (Ilyas *et al*., 2021). These findings indicate that RAP2.4 regulation of *AtSAP2* expression may be associated with the early flowering phenotype observed under drought stress.

Based on this finding, a hypothetical model was proposed to better understand the role of RAP2.4 in *AtSAP2* expression (Figure 6). In the wild-type Col-0, the RAP2.4 protein is present during the stress response, and its accumulation in the cell enables it to bind to the *AtSAP2* promoter, thereby enhancing its expression. In contrast, in the *rap2.4* mutant, the RAP2.4 protein is absent, even under stress conditions, preventing it from binding to the *AtSAP2* promoter site and resulting in a reduced or absent enhancement of *AtSAP2* expression. However, to further validate this model, additional analysis, such as the Electrophoretic Mobility Shift Assay (EMSA), would be necessary to observe the binding of RAP2.4 protein to the *AtSAP2* promoter directly.

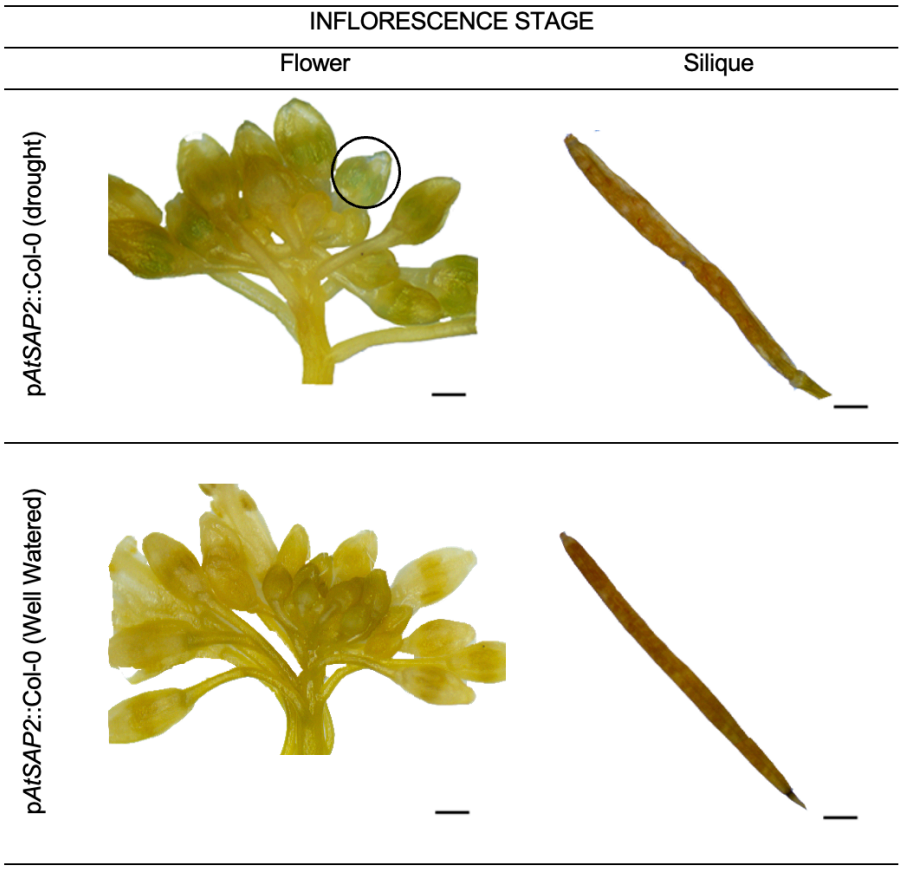
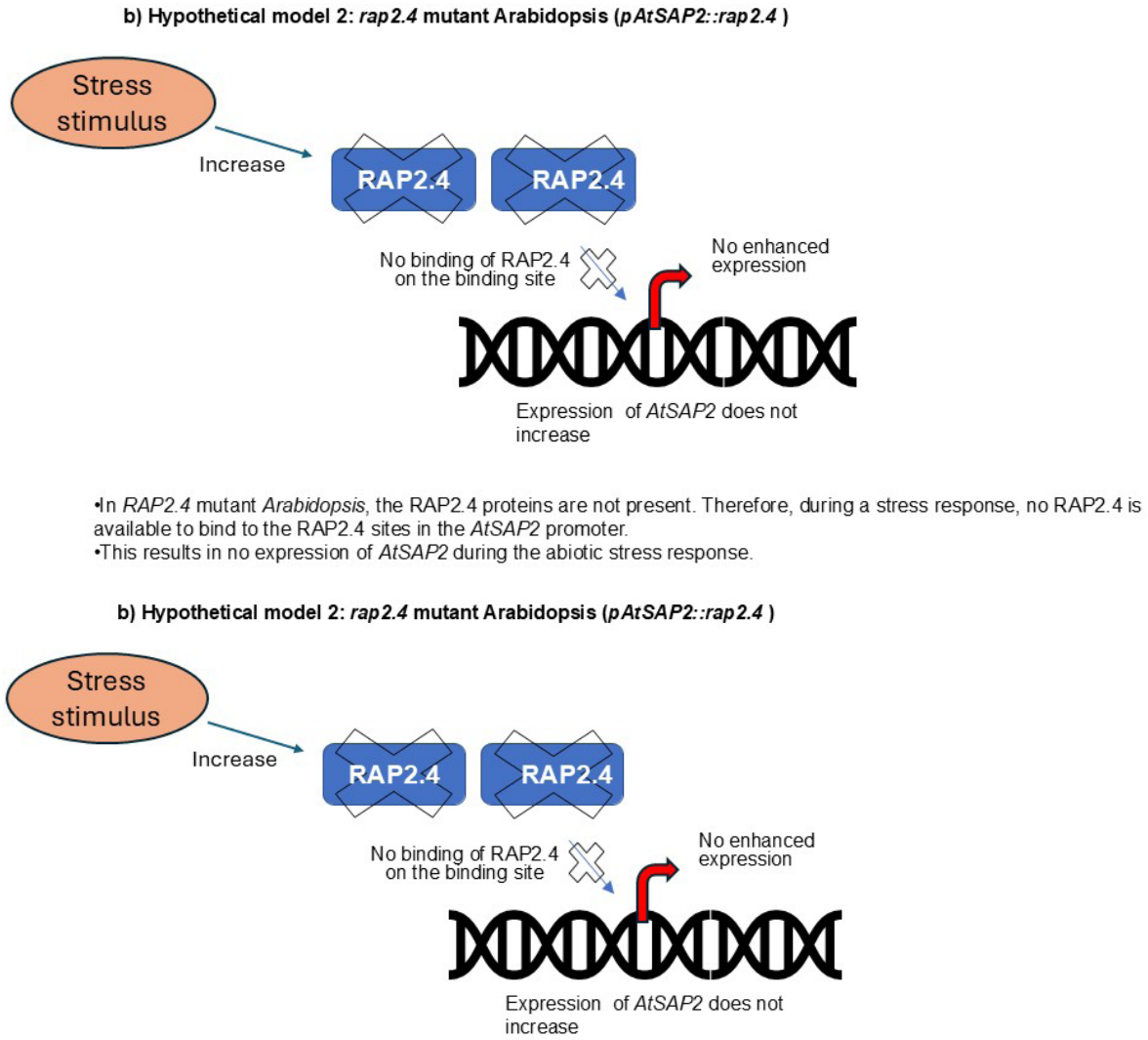


Fig. 5. GUS staining analysis was performed on Arabidopsis plants carrying *pAtSAP2::Col-0* and *pAtSAP2::rap2.4* constructs during the inflorescence and silique stages. The staining was conducted after 21 days of drought (no watering). The circle indicates the blue staining representing GUS activity. Scale bars = 1 cm.



b) Hypothetical model 2: rap2.4 mutant Arabidopsis (pAtSAP2::rap2.4)
Stress stimulus
Increase
RAP2.4
RAP2.4
No binding of RAP2.4 on the binding site
No enhanced expression
Expression of AtSAP2 does not increase
•In RAP2.4 mutant Arabidopsis, the RAP2.4 proteins are not present. Therefore, during a stress response, no RAP2.4 is available to bind to the RAP2.4 sites in the AtSAP2 promoter.
•This results in no expression of AtSAP2 during the abiotic stress response.

Fig. 6. (a) During abiotic stress, the expression of the *RAP2.4* gene increases, leading to higher protein abundance in the cell. These proteins bind to the RAP2.4 binding sites in the *AtSAP2* promoter, resulting in enhanced expression of the *AtSAP2* gene in response to abiotic stress. **(b)** In the *rap2.4* mutant Arabidopsis, RAP2.4 proteins are absent. Therefore, during stress, no RAP2.4 binds to the RAP2.4 sites in the *AtSAP2* promoter, resulting in no *AtSAP2* expression in response to abiotic stress.

CONCLUSION

The *in-silico* analysis of *AtSAP2* promoter cis-regulatory elements identified several transcription factor binding sites that could potentially enhance *AtSAP2* gene expression under abiotic stress conditions. Previous studies showed that *OsSAP4*, the orthologue of *AtSAP2* in rice, has enhanced expression through the ERF cis-element binding site. Building on this, we focus on how *AtSAP2* is regulated via a specific ERF binding site, namely *RAP2.4*.To investigate this regulation, GUS staining analysis was conducted by generating two transcriptional fusion plants: *pAtSAP2::Col-0* and *pAtSAP2::rap2.4*. Based on the GUS staining analysis, *pAtSAP2::Col-0* plants exhibited blue pigment in the leaf veins, indicating promoter activity, while the *pAtSAP2::rap2.4* mutant showed no blue pigment, suggesting an absence of promoter activity during Arabidopsis response to drought and salinity at the vegetative stage. This strongly indicates that RAP2.4 may regulate the expression of the *AtSAP2* gene during drought and salinity stress through its binding site in the promoter. Although other cis-elements are present in the promoter region, the absence of RAP2.4 in the mutant did not lead to a noticeable increase in GUS activity. This research provides the first evidence connecting the regulatory mechanism of the *AtSAP2* promoter and its modulation by RAP2.4. The *AtSAP2* promoter shows potential as an inducible promoter for developing transgenic plants. It can play a key role in plant improvement programs by enabling the creation of plants that are tolerant and resistant to osmotic and salinity stress through the manipulation of the *AtSAP2* gene and the use of environment-specific stress promoters.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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