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Research

Characterization And Functional Study Of Stress-Associated Protein In Rice And Arabidopsis

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ABSTRACT

Environmental stress can hinder the growth and development of crops, thereby reducing productivity. Plants can adapt to changing environments through various morpho-physiological changes, transcriptome regulation, signaling, translational and post-translational modifications. Stress Associated Proteins (*SAP*s) have been shown to play a crucial role in plant adaptation to biotic and abiotic stressors. They are encoded by a family of genes that produce a zinc finger protein with A20 and/or AN1 domains at either their N or C-terminal ends. Therefore, this study focused on understanding the role of the *Oryza sativa SAP* gene family (*OsSAP*s) in response to drought and salinity stress. *In-silico* analysis revealed that most of the *OsSAP* family members were upregulated by stress; two highly inducible *OsSAP* genes were also upregulated in response to stress under a rice-specific background. To study gene function, an *Arabidopsis* transformation system was employed using three genotypes: *Col-0* (wild type), overexpressed transgenic *OsSAP8*, and *atsap2* T-DNA knockout mutant. Arabidopsis *AtSAP2* gene, which is homologous to rice *OsSAP8*, was used as a comparison to the loss of function mutation in Arabidopsis. Morpho-physiological analysis showed that the *atsap2* mutant displayed a sensitive phenotype to drought and salinity stress through low relative chlorophyll content and delayed inflorescence development and flowering as compared to *Col-0* and transgenic *OsSAP8*. This suggests that the abolished *atsap2* gene may contribute to reduced stress tolerance in plants. In contrast, transgenic *OsSAP8* overexpression demonstrated tolerance to drought and salinity stress by maintaining relative chlorophyll content under both stress conditions, indirectly reflecting sustained photosynthetic machinery and stable photosynthetic rate. Further investigation, such as measuring the photosynthesis rate, is required to establish the correlation between chlorophyll data and photosynthesis activity.

Key words: Abiotic stress, arabidopsis, A20/AN1 domains, rice, Stress Associated Protein, zinc finger

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INTRODUCTION

By 2050, it is expected that the global human population will be over 9 billion which prompts for aggressive production in agricultural production. Recent climate change has raised concerns about agricultural production. Every year, tons of potential crop yields are lost due to exposure to devastating conditions that are caused by abiotic stress (Toulette, 2022). Current uncertain climate change has an impact on agriculture growth and development such as the appearance of heat waves, heavy rainfall, elevated CO_2 concentration, and extreme temperatures which therefore becomes a limiting factor for the growth and development of crop production (Martin *et al*., 2012; dos Santos *et al*., 2022; Chaudhry & Sidhu, 2022). This phenomenon retards growth, impairs photosynthesis, and reduces physiological responses in crops (Sen *et al*., 2020). Significant amounts of crop yields are lost annually due to exposure to various climate hazards that are caused by climate change (Ali *et al*., 2017).

Rice (*Oryza sativa)* is one of the staple crop species that is consumed by 3.5 billion people which is more than half of the world's population (Narsai *et al*., 2013; Qin *et al*., 2020). It is an important source of fiber, energy, minerals, vitamins, and other biomolecules (Sen *et al*., 2020) and

provides diverse antioxidant molecules that play an essential role in health promotion. A significant increase of up to 35% in rice crop yield is needed to meet the demand for rice by 2030. However, recent climate change has resulted in abiotic stresses such as salinity, drought, and cold stress in rice crop production. Among stresses, generally, salinity is the most challenging (He *et al*., 2018) and has been implicated in a loss of 20% in agricultural production (Zhao *et al*., 2021). The sudden increase in the amount of salt is due to the rising sea level as an effect of glaciers and ice sheets melting (Ullah *et al*., 2021). To make matters worse, the wastewater drilling contributed by the oil and gas extraction further increases the rise in salt concentration of seawater (Zhang *et al*., 2020). Salt stress is troublesome as it hinders seed germination, growth, and development as well as flowering and fruiting. Heavily accumulated salt in soil constricts water and nutrient absorption in plants thus resulting in water deficiency and nutritional imbalance that can lead to secondary stresses such as osmotic stress and ionic stress.

Meanwhile, other than salinity, drought which refers to the low availability of water in soil is another environmental stress that impairs plant growth and development (Hossain *et al*., 2016). However, plants will only be considered to experience drought stress when there is a higher transpiration rate from the leaf surfaces as compared to the water uptake in roots (Lisar *et al*., 2012). Global climate changes through fluctuating rainfall patterns have caused severe water deficiency in some areas. Besides, the elevated evaporation rate of water in the soil that is caused by the high temperature, high light intensity, and dry wind can also lead to drought (Trenberth *et al*., 2014). Drought stress can lead to disruption of homeostasis at both the cellular level and the whole plant levels (Zhu 2001). Molecular damage, growth arrest, and even death may arise from extreme changes in ion and water homeostasis (Serrano & Rodriguez-Navarro 2001; Zhu 2001; Wang *et al*., 2016). Hence, in this study, we will be focusing on abiotic stress in salinity and drought conditions since both of them are the most common and serious environmental hazards to agricultural production.

Plants can sense stressful environmental conditions because they have developed the mechanisms and complex interactions between the signaling molecules and pathways to encounter various stress conditions (Martin *et al*., 2012; Iqbal *et al*., 2021). Genome sequencing, transcriptomic, proteomic, and metabolomics are methods to examine how plants respond to abiotic stresses which allows researchers to understand the mechanism of plant responses and acknowledge the components that are involved in signaling pathways. Plants have two survival response strategies in salinity stress, which are the activation of the MAPK pathway to tackle subsequent osmotic stress and the activation of the oxidative stress pathway to handle large increases in reactive oxygen species (ROS).

Based on previous studies, Stress Associated Protein (*SAP*) plays an important role in various abiotic stresses plant responses where this gene can be determined by the presence of the A20/AN1 domain that is contained in the coding sequence which A20 domain located at N-terminal while AN1 domain located at C-terminal (Kanneganti & Gupta 2008; Hedayati *et al*., 2015; Pungging *et al*., 2017; Lai *et al*., 2020; Sahid *et al*., 2020). A. *thaliana* genome comprises of 14 *SAP* gene members while *Oryza sativa* comprises 18 *SAP* gene members (Vij & Tyagi 2006; Li *et al*., 2022b). *Arabidopsis thaliana (A. thaliana)* is a flowering plant known as a model organism extensively utilized to comprehend and investigate developmental processes and plant resistance mechanisms against abiotic and biotic stressors. Previous studies have focused on the effect of *OsSAP8* overexpression towards multiple stresses but none reported on the effect of gene mutation in response to stress. In this study, we also reported the effect of gene mutation using the *OsSAP8* orthologue in Arabidopsis which is the *AtSAP2*. The *AtSAP2* has been previously analyzed and showed high similarity in protein sequence to *OsSAP8* (Roslan *et al*., 2017). High similarity in protein sequence may indicate that these two genes have similar functions in both plant species respectively. The use of *OsSAP8* overexpression and mutant *atsap2* is to compare differences in morpho-physiological changes between two transgenics to understand the genes' regulation. Despite an established understanding of the stress-associated genes and transcription factors, insufficient evidence on the signaling and regulatory mechanisms has influenced the development of crop improvement programs. Therefore, a deeper knowledge of stress physiology and its regulatory mechanisms is required to strategize for its tolerant crop development.

In this study, we provide preliminary data on the *in silico* expression pattern of 18 *Oryza sativa* Stress Associated Proteins *(OsSAP)* in abiotic stress as well as densitometry analysis on the two highest expression *OsSAP* genes in response to drought and salinity stress. Besides, the effect of different abiotic stress treatments is also analyzed during the vegetative and inflorescence stage on *atsap2* mutant and overexpression transgenic line *OsSAP8.* Understanding stress tolerance mechanisms and the role of specific family genes using multidisciplinary approaches can help researchers understand plant adaptation to environmental stresses and open new opportunities for agricultural applications.

MATERIALS AND METHODS

Expression pattern analysis

Expression level data analysis was done using the Rice EFP Browser (http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi). The threshold value was set at the lowest stress expression level 990 to

standardize the expression analysis and the probe set. Rice stress mas were used as the data source to analyze the abiotic stress expression level. The data was tabulated using a heat mapper to provide a simple visualization of the expression pattern data analysis result.

Rice genotypes and stress conditions

Three biological replicates from each rice genotype which are MR219, *OsABP57* (rice overexpression transgenic of Auxin Binding Protein57), and Pokkali were used in this study (Tan *et al*., 2018). The *Oryza sativa* MR219 was obtained from the Malaysian Agricultural Research and Development Institute (MARDI), while the *OsABP57* was from the generosity of Prof. Zamri Zainal's transgenic seed collections and Pokkali from Bangladesh (Table 1). Surface sterilization of seeds was done using 0.1% $HgCl_2$ for 10 min and thorough washing with distilled water. The seeds were soaked in distilled water and allowed to germinate in the dark for about three to two days. These seeds were grown in distilled water in a culture room at 28 °C ± 1 °C with a daily photoperiodic cycle of 14-h light and 10-h dark. Two different abiotic stresses (salinity and PEG stress respectively) were given at 14 days old seedling. For salinity stress, 14-day-old seedlings were transferred to a beaker containing 200 mm NaCl solution for 3 h while for osmotic stress using PEG, 14-day-old seedlings were transferred to a beaker containing 20% PEG solution for 3 h. The 14-day-old seedlings that were kept in water for 3 h, at 28 °C ± 1 °C were served as control. Three biological replicates for each rice genotype treated and untreated were collected and frozen using liquid nitrogen for RNA extraction purposes.

Table 1. The rice variety was chosen for Semi-quantitative reverse transcription PCR

Rice	Description
MR219	Malaysian Rice Cultivar
OsABP57	Drought Tolerant Rice transgenic
Pokkali	Salinity Tolerant Rice Variety

RNA extraction and semi-quantitative reverse transcription Polymerase Chain Reaction (PCR)

Fourteen-day-old rice-treated and untreated seedling samples were subjected to RNA extraction. Shoot RNA extraction was performed according to the TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Quantification of total RNA was checked using a Nanodrop ND1000 spectrophotometer (Nanodrop, Wilmington, USA), and the ratio of absorbance at 260 nm and 280 nm was recorded. The quality of total RNA was then visualized using 1% agarose gel electrophoresis with 0.1 mg/mL ethidium bromide per 100 mL 1× TBE buffer. TURBO DNase Kit was used to purify and remove any genomic DNA. 3000 ug of each DNase-free RNA sample were converted to cDNA using Superscript III Reverse transcriptase (Invitrogen, Paisley, UK), and the cDNAs were subjected to semi-quantitative PCR. OneTaq® Quick-Load® 2× Master Mix with Standard Buffer was selected as the Taq polymerase due to its high yields across a wide range of AT- and GC- content. The *OsU6* gene was selected as the internal constitutive control to normalize the expression result. PCR for *Oryza sativa* Stress Associated Protein 8 gene (*OsSAP8*), *Oryza sativa* Stress Associated Protein 4 gene (*OsSAP4*), and *OsU6* gene were carried out as follows: initial denaturation (2 min, 94 °C), denaturation (30 s, 94 °C), annealing (1 min, (OsSAP8: 52 °C) (OsSAP4: 53 °C) (OsU6: 56 °C), elongation (20 s, 68 °C) and final elongation (5 min, 68 °C). Primer used for *OsSAP8* Forward 5'CAAGTCGAGGTGAAGACGCT3', Reverse 5'TCCGGTAAGACCAACCCTCT3', for *OsSAP4* Forward 5'TGACGGTGGGAAGGAGCATA3', Reverse 5' TGACCTTGGGGACTACAGCA3' and for *OsU6* Forward 5'TACAGATAAGATTAGCATGGCCCC3', Reverse 5'GGACCATTTCTCGATTTGTACGTG3'.

Densitometry analysis

The OsU6 gene was selected as the internal constitutive control. To calculate the intensity of the band expression, densitometry analysis using Image J was performed. Image J allows the measurement of density profiles, peak heights as well and peak intensity of the band of the expected molecular weight. Gene expression is relative to the loading control gene from the control plant (without treatment) and normalized to the internal constitutive gene (target gene × (loading gene (control)/internal constitutive gene)) (Zambrose *et al*., 2020). The fold change value was generated from the normalized value. The normalized samples were divided by the value of the loading gene (control).

Arabidopsis *OsSAP8* overexpression transgenic analysis

Three ecotypes of *Arabidopsis thaliana* were used in this study, which is wild type *Col-0* that acts as a control, transgenic overexpressed 35s::*OsSAP8* and T-DNA insertional mutant *atsap2*. Seeds of *A. thaliana* ecotype *atsap2* were obtained from Nottingham Arabidopsis Stock Centre (NASC), UK.

Arabidopsis growth condition and stress treatments

The Arabidopsis seeds were immersed in distilled water for 2 days at 4 °C to allow germination to occur before being sown on soil and vermiculite (2:1 v/v) (Nelson *et al*., 1984; Zhang *et al*., 2019; Wang *et al*., 2021; Li *et al*., 2023). Plants were grown in a growth chamber at 22°C with 65% relative humidity and 16 h light/8 h dark photo cycle (He *et al*., 2019; Yang *et al*., 2019). After vegetative stress arose, 3 weeks-old seedlings were treated to control treatment (maintained without adding NaCl and consecutively watering), salinity treatment (200 mL of 200 mM NaCl), and drought treatment (withhold watering) for 3 weeks (seven days treatment, 14 days treatment & 21 days treatment) (Ströher *et al*., 2009; Roslan *et al*., 2017; Nutan *et al*., 2019). Both stresses have six individual plants for biological replicates.

Measurement of relative chlorophyll content (RCL) and morphological analysis

Relative chlorophyll content has been measured from 6 biological replicates for each Arabidopsis genotype respectively. Measurement was performed on fresh leaves of 3-week-old seedlings at 14 and 21 days of treatment. The chlorophyll was measured at three different rosette leaves in one plant by using SPAD METER 502. Overexpression *OsSAP8*, mutant *atsap2,* and *Col-0* plants that have been treated with different stress treatments have been analyzed. This function is to analyze the effect of abiotic stress on mutant *atsap2* plants and transgenic overexpression *OsSAP8* plants during the vegetative and inflorescence stages. The ability of the plants to produce inflorescence stems and flowering after stress treatment was observed in this experiment. To determine the effect of abiotic stress treatments during the vegetative to flowering stage, the plants were maintained in standard growth conditions for three weeks until they reached the vegetative stage. Then, three-week-old seedlings were subjected to control treatment, salinity treatment (200 mL of 200 mM NaCl), and drought treatment (withholding water) for three weeks. Data were taken on days 7, 14, and 21 of salt and drought treatments. The emergence of inflorescence, flowers, and siliques on treated plants was observed throughout the treatment periods.

Statistical analysis

Statistical Analysis System (SAS) software was used to determine the level of variation of all varieties, stress treatments, and treatment periods by using analysis of variance (ANOVA). The mean and coefficient of variation (CV) were calculated for each variation. Duncan's Multiple Range Test (DNMRT) was used to reveal significant differences among variation means. The CORR procedure of SAS was used to evaluate the correlations among the traits. All experiments in the study were repeated at least twice and the data explained are the mean ± SE of independent experiments. The letters above the column of figures indicated a significant difference at $p<0.05$.

RESULTS

Expression pattern analysis *in silico*

In this study, *in silico* analysis of the expression patterns of all 18 *OsSAP* genes under salinity, drought, and cold stress on shoot and root was carried out using a publicly available database, (eFP Browser). This analysis was done to understand the potential function of the *OsSAP* family gene in response to multiple abiotic stress. The threshold value setting was adjusted to 990, the lowest expression level in response to stress recorded by the *OsSAP3*, this threshold value was used as a comparison, to observe the gene expression level from lowest to highest value. Overall, the results showed that most of the *OsSAP* genes were induced by abiotic stress. *OsSAP8* recorded the highest expression level for all three different abiotic stresses, while the *OsSAP4, OsSAP1, OsSAP9,* and *OsSAP16* also showed highly expressed in response to drought and salt. Meanwhile, *OsSAP3* recorded the lowest expression level for all three abiotic stress responses with the cut-off value of 990 and it was shown that the *OsSAP3* expression level was not detected in response to drought at this cut-off (Figure 1). After *OsSAP8*, the *OsSAP4* recorded the second highest expression level but was highly inducible by salinity and drought stress only. The *OsSAP11* for example is highly expressed in shoot and root under cold environments (Figure 1). The result may suggest that each of the *OsSAPs* genes is involved in diverse stress-related regulatory networks (Zhang *et al*. 2019a). Based on this analysis, *OsSAP8* and *OsSAP4* were selected for further experimental analysis since both show highly inducible expression triggered by salt and drought. Furthermore, both genes were grouped in the same clade based on previous phylogenetic analysis (Zhang *et al*., 2019b; Sun *et al*., 2022).

Semi-quantitative Reverse Transcription PCR Analysis

To further confirm the expression levels of two *OsSAP* genes in response to abiotic stresses, we examined their fold change value in response to salinity and drought stress by semi-quantitative reverse transcription PCR. In this study, the transcript profiles of the *OsSAP8* and *OsSAP4* genes in response to salinity and drought stress in three different rice genotypes were analyzed. The three different rice

genotypes were chosen because each of the rice genotypes has a different tolerance to certain types of environmental stress conditions. For example, pokkali has a high tolerance to salinity, while the *OsABP57* overexpression transgenic rice has a high tolerance to drought and MR219 is a commercial rice planted in Malaysia (Tan *et al*., 2018; Goswami *et al*., 2020).

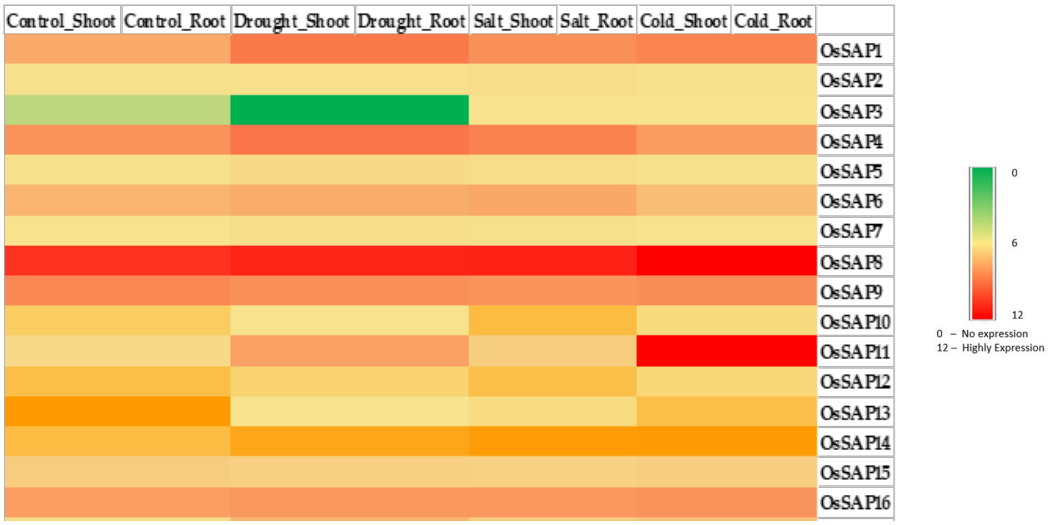

Fig. 1. Expression pattern of all 18 Oryza sativa Stress Associated Protein (*OsSAP*) in response to drought, salt, and cold treatments. The threshold value was set at 990, the lowest stress expression level exhibited by *OsSAP3*. Source of database analysis from eFP browser.

Densitometry analysis showed that there is an increase in the fold change value for both salinity and drought stress (Figure 2). Densitometry analysis showed higher fold change value across both salinity and drought stress in all three genotypes for both genes. *OsSAP8* was highly up-regulated by 2.4-fold during salinity stress in Pokkali and 2.5-fold during drought stress in the *OsABP57* transgenic line. Nevertheless, a small difference was recorded between the expression pattern in *OsSAP*8 and *OsSAP4*. *OsSAP4* exhibits a 2.1-fold change value under salinity stress in Pokkali and 2.2-fold under drought stress in the *OsABP57* transgenic line (Figure 2). As a conclusion, the expression patterns of both *OsSAP* genes can be seen highly inducible in response to salinity stress in Pokkali rice compared to the other two rice genotypes (MR219 & *OsABP57* transgenic), while for drought inducible, both *OsSAP* genes can be seen highly expressed in *OsABP57* transgenic line compared to MR219 and Pokkali. This also validated the hypotheses that, Pokkali in origin is the salinity stress tolerance rice variety while the *OsABP57* transgenic line was designed to be highly tolerant to drought conditions. The expression patterns of both genes were paralleled to the rice genotypes tested. Nevertheless, the MR219 was a Malaysian commercial rice variety, planted in the paddy field showing high yield but susceptible to both abiotic and biotic stresses.

Effect of drought and salinity stress on relative chlorophyll content (RCL)

The combined analysis of variance (ANOVA) for chlorophyll content data is shown in Table 2. The data shows a significant difference (p<0.05) for genotypes (G), day of treatment (D), interaction between genotypes and day of treatment (G × D), and interaction between day of treatment and stress treatment (D × T). Throughout the treatment period, drought and salinity treatment significantly reduced the chlorophyll content of the *atsap2* plant compared to *Col-0* and *OsSAP8* plants. Both treatment periods clearly show that mutant *atsap2* had the lowest relative chlorophyll content while overexpression transgenic of *OsSAP8* had the highest value in relative chlorophyll content under both stress treatments at 14 and 21 days (Figure 3). However, the comparison of relative chlorophyll content between *OsSAP8* and Col-0 under both treatment periods (14 & 21 days) for salinity and drought has no significant difference. The only significant differences in chlorophyll content were recorded in *atsap2* under both stresses' treatment at day 21 in comparison to both *OsSAP8* and Col-0 (Figure 3). During drought treatment, the chlorophyll content of the *atsap2* plant was significantly reduced to 33% of chlorophyll content, followed by *Col-0* plant (10%) and *OsSAP8* plant (6%) at 14 to 21 days of treatment. Salinity also reduced the chlorophyll content for all genotypes. *atsap2* plant had the greatest reduction in relative chlorophyll content (22%) compared to *Col-0* (10%) and *OsSAP8* (4%). However, for control treatment, all plants showed a similar trend of relative chlorophyll content throughout the treatment period where relative chlorophyll content was increased up to 14% (*OsSAP8* & *Col-0* plants) and 15% (*atSAP2*), respectively as the plant growing.

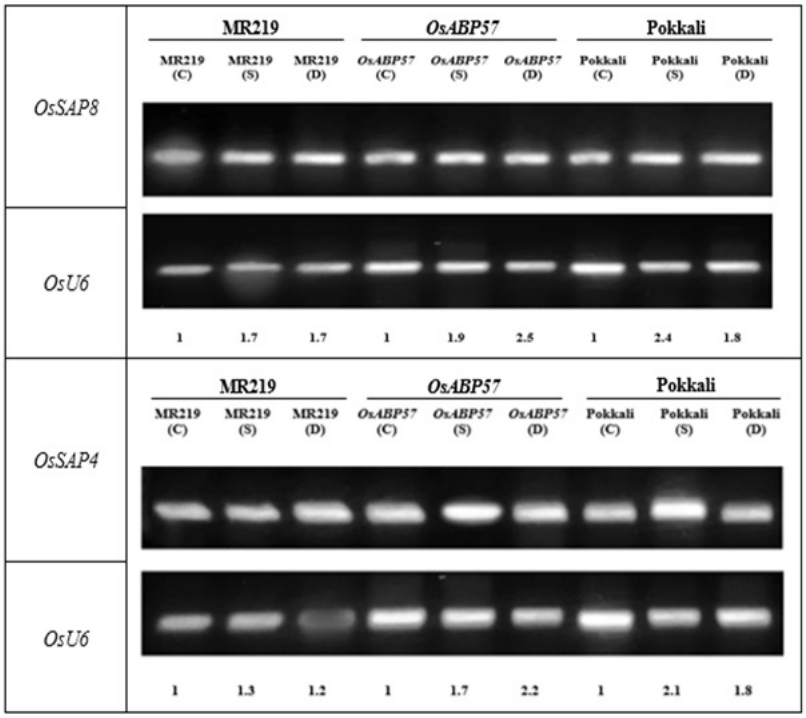

Fig. 2. Densitometry analysis using Image J for both *OsSAP8* and *OsSAP4* on three different rice genotypes. The number indicates the fold change value normalized to the internal constitutive gene (*OsU6*) and compared to the control samples. Label indicator: Control (C), Salinity stress (S), Drought stress (D).

Table 2. Analysis of variance for chlorophyll content

		Mean square
Variables	df	Chlorophyll content (nmol chl/cm^2)
Replicates (R)	5	4.348
Genotype (G)	2	114.390*
Day of Treatment (D)	4	125.257*
Stress Treatment (T)	3	2.737
G × D	8	61.487*
D × T	12	34.353*
G ×T	6	0.951
G × D × T	24	8.936
Error	295	4.08

*The mean difference is significant at the 0.05 level

The emergence of inflorescence on the treated plant throughout the treatment period

According to the findings, during the vegetative stage (seven days of treatment period), all treated plants have not produced any primary inflorescence yet (first flower buds) during treatments (Figure 4). After 14 days of treatment, *Col-0* showed better flower development compared to *OsSAP8* and *atsap2* mutant even under prolonged drought and salt stress (Figure 5). During 21 days of treatment, *OsSAP8* and *Col-0* under salinity stress plant produced more flowers and complete inflorescence compared to mutant *atSAP2*. *atSAP2* plant has shown retarded inflorescence stem development and is unable to produce complete inflorescence during salinity treatment. However, under drought treatment all three genotypes were able to produce flowers and siliques after 21 days of exposure to drought conditions and the results were the same as in the control environment (Figure 6).

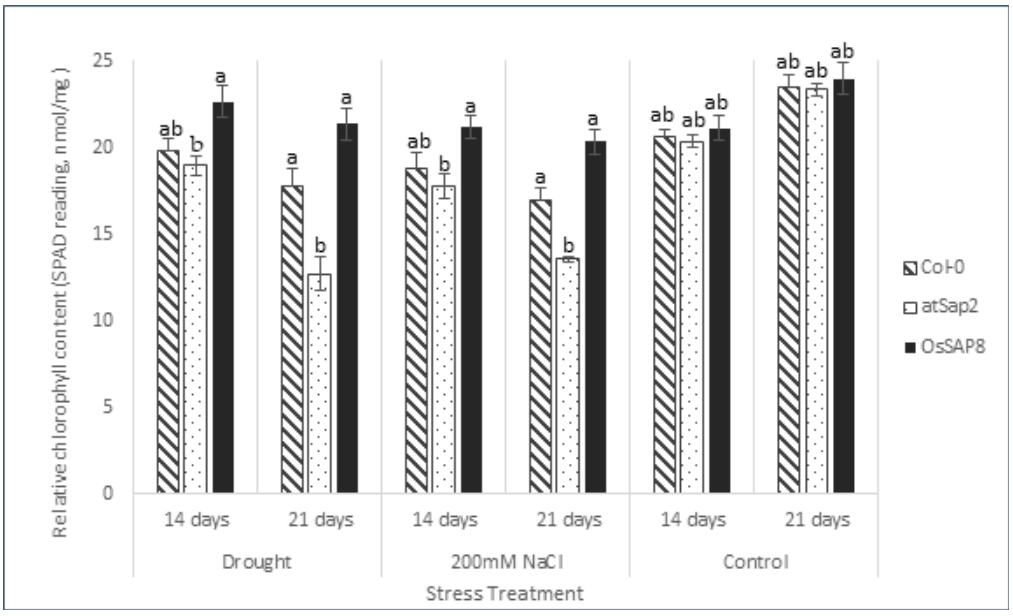

Fig. 3. Effect of abiotic stress treatments on leaf chlorophyll content of treated plants at 14 and 21 days. Vertical bars represent ± standard error. The small letter at the top of the bar indicates significant differences between varieties at the same time point using the DNMRT with a *p*-value of 0.05.

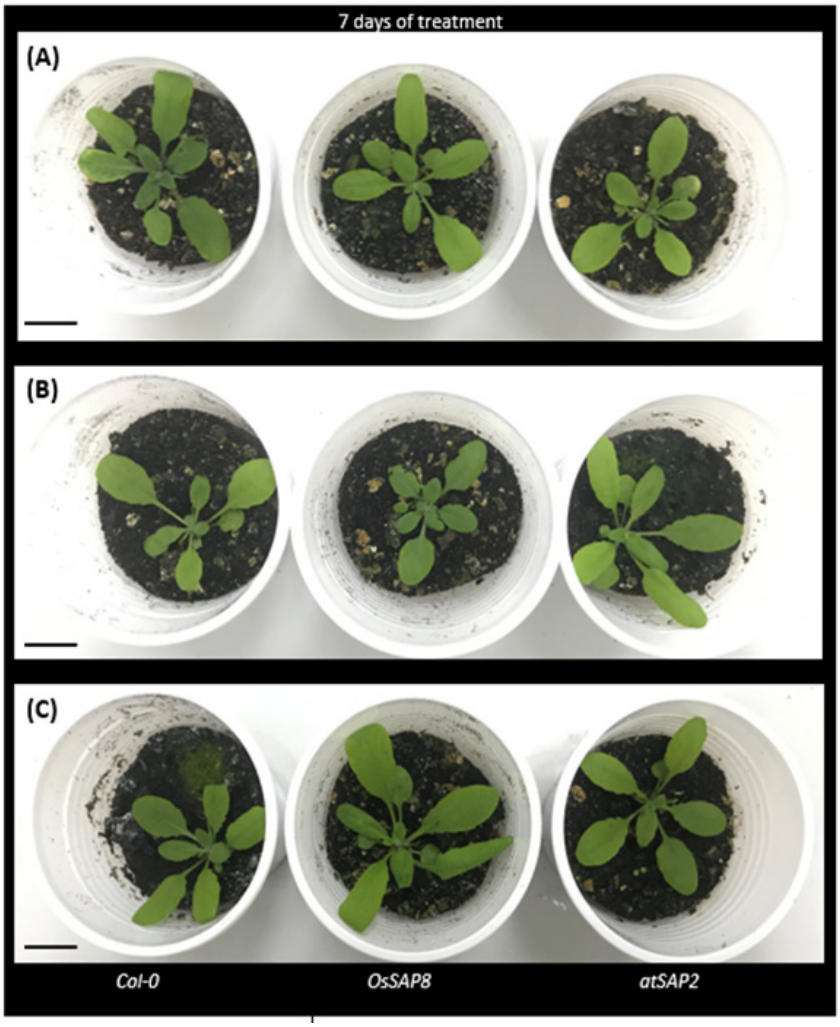

Fig. 4. Morphological and growth phenotypes of 7-day-old treated plants during stress treatments of wild type (Col-0), transgenic overexpression *OsSAP8*, and mutant *atsap2*. (A) Drought stress treatment. (B) Salinity stress treatment (200mM NaCl). (C) Control treatment. Scale bar = 3 cm.

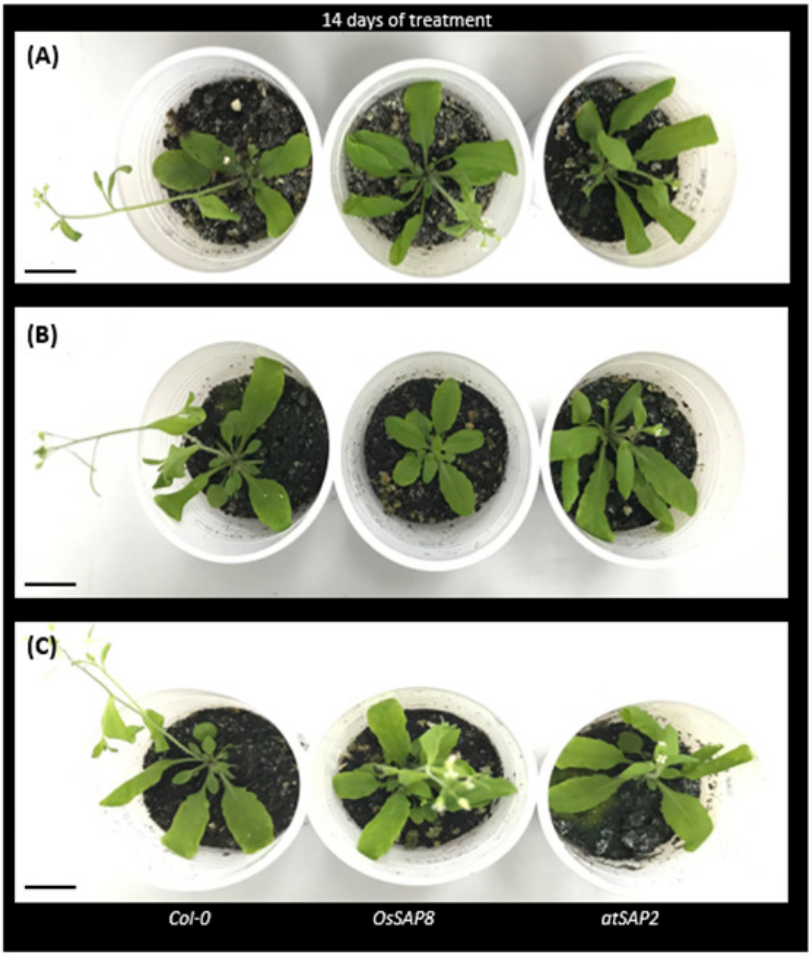

Fig. 5. Morphological and growth phenotypes of 14-day-old treated plants during stress treatments of wild type (*Col-0*), transgenic overexpression *OsSAP8,* and mutant *atsap2*. (A) Drought stress treatment. (B) Salinity stress treatment (200mM NaCl). (C) Control treatment.

DISCUSSION

In this study, the importance of the *Oryza sativa Stress Associated Protein* (*OsSAP)* family in response to salt and drought has been investigated. *In-silico* gene expression pattern analysis for all *OsSAP* returns with the result that almost all *OsSAP* genes are inducible with *OsSAP3 being* the lowest responsive genes to abiotic stress. Meanwhile, *OsSAP11* responded specifically to cold stress as compared to the rest. On the other hand, *OsSAP8* is the most responsive gene towards stress and *OsSAP4* and *OsSAP1* were more sensitive to drought compared to *OsSAP9*. These preliminary bioinformatics data showed that the family of *OsSAP* genes are responsive to multiple stresses and some of the genes are specific towards certain environmental stresses, similar to other research species for *SAP* genes. For example, genome-wide analysis of *Gossypium hirsutum* also known as cotton shows that nearly all *SAP* genes responded to these challenging growth conditions, particularly salt stress (Gao *et al*., 2016) including *SAP* genes of *Populus euphratica* (Jia *et al*., 2016), soybean (Zhang *et al*., 2019a), including *Cucumis sativus SAP* (Lai *et al*., 2020). Among these *OsSAPs, OsSAP8* and *OsSAP4* are significantly upregulated as compared to other *OsSAPs* genes during salinity and drought stress. These suggested that *OsSAP8* and *OsSAP4* might confer saline and drought tolerance in *O. sativa* better than the other members. This is most probably due to the same clade clustering of both *OsSAP8* and *OsSAP4* in the phylogenetic analysis (Zhang *et al*., 2019a; Sun *et al*., 2022) thus their similar function in response to specific stresses. *In-silico* analysis has also predicted that *OsSAP* genes may have a role in regulating other plant hormones such as abscisic acid (ABA), jasmonic acid (JA), auxin (Aux), brassinosteroid (br), gibberellins and cytokinin in shoot and roots of the rice (Muthuramalingam *et al*., 2021). Previous research on SAPs has widely reported its involvement in various stress responses such as in regulating Gibberellin (GA) biosynthesis by modulating transcription factors *OsbZIP58* and *OsKO* (Zhang *et al*., 2016). Also, the *OsSAP4* gene which has high similarity in sequence with *OsSAP8*, plays roles in modulating GA and ABA biosynthesis and increasing the sensitivity towards abiotic stress (Waadt *et al*., 2022). Hence, the overexpression of the *OsSAP8* gene regulates the plant phytohormone that helps confer drought stress tolerance.

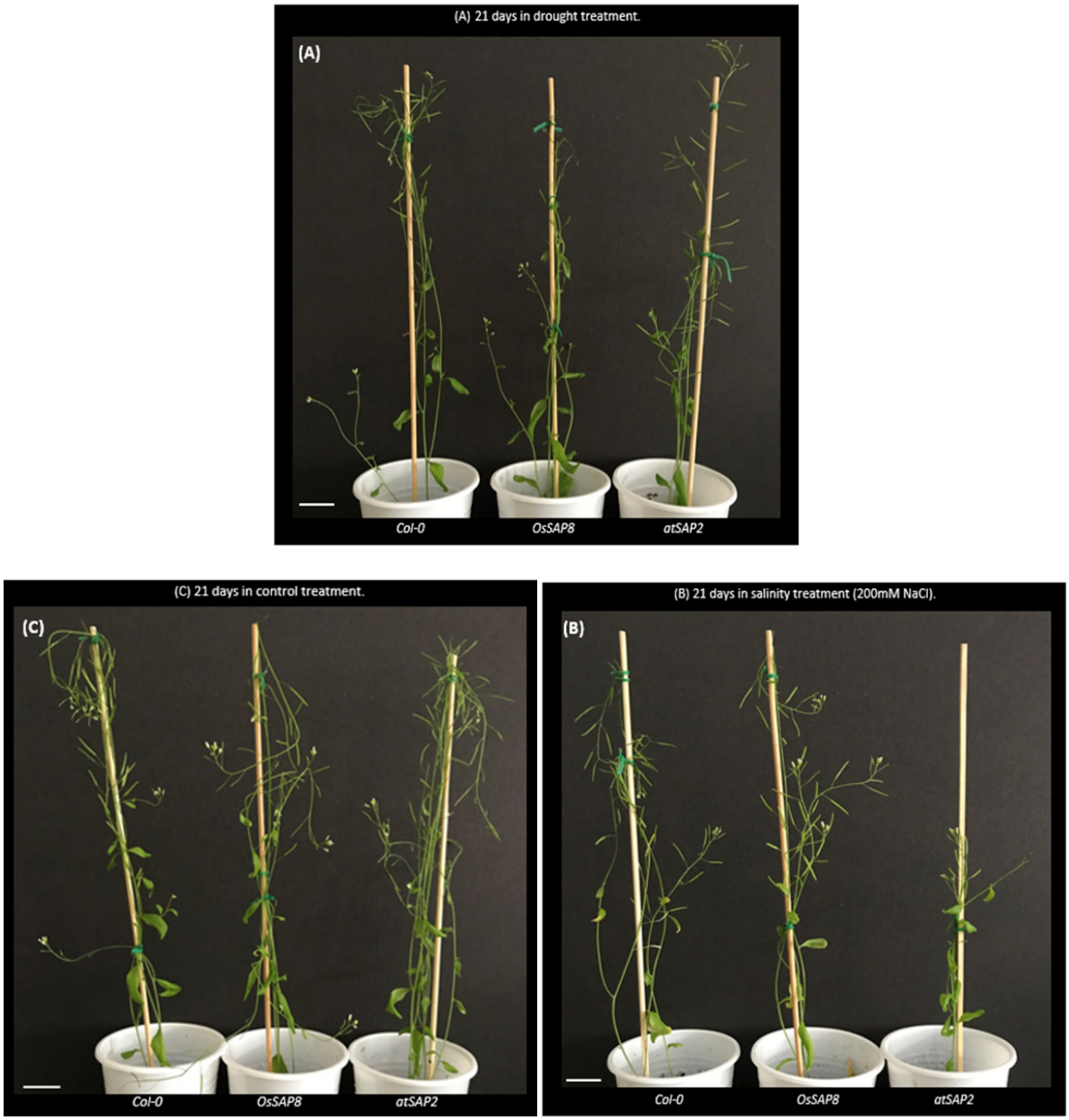

Fig. 6. Morphological and growth phenotypes of 21-day-old treated plants during stress treatments of wild type (*Col-0*), transgenic overexpression *OsSAP8* and mutant *atSAP2*. (A) Drought stress treatment. (B) Salinity stress treatment (200 mM NaCl). (C) Control treatment (water). The picture showed under drought treatment all three genotypes were able to produce flowers and siliques after 21 days of exposure to drought conditions and the results were the same as in the control environment (water). However, differences can be observed in salinity stress treatment (200 mM NaCl) where *atsap2* growth was retarded in response to salinity compared to the other two genotypes (*OsSAP8* and Col-0).

Based on the *in-silico* finding, further analysis of the *OsSAP8* and *OsSAP4* expression pattern through semi-quantitative PCR in three different rice genotypes was carried out. It was observed that there were differences in the level of expression for each of the genes when challenged with stress. *OsSAP8* exhibits slightly higher expression than *OsSAP4* which validates previous findings similar to the Pokkali rice genotype which is a traditional salt-tolerant variety cultivated in parts of coastal Kerala, India (El-Shabrawi *et al*., 2010). Their ability to withstand saline waters has brought about scientists' interest. The endophytes living in the Pokkali rice were found to have developed strategies to activate responses in their host plant (Sampangi-Ramaiah *et al*., 2020) along with intricate regulation of different cellular components induced by a variety of miRNAs that allow it to grow under saline conditions (Goswami *et al*., 2020). Besides, its transcriptomic study also indicated higher expression of *OsSAP* genes therefore contributing to higher salinity tolerance (Parihar *et al*., 2015). Through semi-quantitative PCR it was observed that *OsSAP8* expression level was slightly higher compared to *OsSAP4* specifically in Pokkali

compared to the other two rice backgrounds. This supported the hypothesis that Pokkali is a tolerant rice variety specific to high salt response and may trigger the expression of stress genes associated with salt tolerance.

Unlike Pokkali, the expression level of *OsSAP8* was slightly higher in response to drought under the *OsABP57* transgenic line compared to the other rice genotypes. The *OsABP57* is the overexpression transgenic line overexpressing the *ABP57* gene. Previous studies have shown that Auxin and plasma membrane H+-ATPase are involved in the plant abiotic stress which influences the drought tolerance mechanisms in plants (Xu *et al*., 2010; Tan *et al*., 2018). *Auxin-binding protein, ABP57*, is responsible for activating the plasma membrane H+-ATPase which is important for cellular expansion, intracellular pH regulation, secondary transport, stomata aperture, and osmoregulation. Hence, the overexpression of the *Auxin-binding protein, ABP57* in *O. sativa* results in the development of lateral root, which confers drought stress tolerance (Kamarudin *et al*., 2020). Therefore, the result obtained from this study parallels the earlier hypotheses that both *OsSAP8* and *OsSAP4* might be highly induced and expressed in the *OsABP57* transgenic line background compared to Pokkali and MR219 because *OsABP57* is the rice genotype design and tested to be drought tolerance based on Auxin-binding protein overexpression.

Stresses also induce a negative impact on plants' photosynthetic efficiency and reduce chlorophyll content (Hamani *et al*., 2020; Sharma *et al*., 2020b, 2020a; Sherin *et al*., 2022). Thus, we used the Arabidopsis plant model to evaluate the *OsSAP8* gene's function in response to drought and salinity due to its short generating time for the transgene lines (Zhai *et al*., 2021) in addition to its reliable and effective result comparable to other plants with longer transformation and regeneration time (Zhai *et al*., 2021). This work attempts to deduce *OsSAP8* gene function in Arabidopsis and comparatively relate the phenotypic changes with T-DNA knockout mutant line *atsap2*. Throughout the treatment periods, we found out overexpression transgenic *OsSAP8* can tolerate better to drought and salinity conditions based on its relative chlorophyll content and capability to develop an early inflorescence stem at 14 days of treatment. The transition from vegetative to flowering stages is important as it indicates the ability of the plant to develop and complete its life cycle by producing new seeds or grains thus ensuring its survival (Yang *et al*., 2015; Takeno, 2016). Most flowering plants such as A. *thaliana* produce multiple flowers in sequence from a reproductive shoot apex to form a flower spike (inflorescence). Other than that, the flowering event is also regulated by many factors such as photoperiodic, vernalization as well and environmental stress (Takeno, 2016). The mutant *atsap2* showed the least developed flowering at salinity stress treatment. This may show that the absence of the *AtSAP2* gene contributes to less tolerance towards salinity thus affecting its flower development. However different results were gathered in the *Col-0* wild type plant, where there was better inflorescence compared to *OsSAP8* at 14 days of drought and salinity treatments. This may indicate that stress somehow triggered early flowering in plants (Takeno, 2016). Since *OsSAP8* also showed slow progress in producing inflorescence stem as compared to Col-0, it may also indicate gene alteration since the use of 35S promoter results in constitutive gene expression throughout their developmental stages and in all tissues or organs could interrupt other spatial-temporal gene function (Zhang *et al*., 2017; Jiang *et al*., 2018). The best way to evaluate the gene function is using the inducible promoter which can enhance the gene expression, particularly in stress response (Misra & Ganesan, 2021).

OsSAP8 and *atsap2* gene comprises both A20 and AN1 zinc finger domains. Generally, these domains are mainly involved in the regulation of plant response towards abiotic stresses by modulating the ABA and GA contents and their signal transduction pathway (Zhang *et al*., 2016). In general, prolonged abiotic stress may cause overproduction of ROS such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), singlet oxygen (O_2), and hydroxyl ions (OH^-) and lead to inhibition of RUBISCO activity. This activity will cause an impediment to vital processes like the photosynthesis of plants by declining photosynthetic efficiency as well as lead to incomplete inflorescence (Boyes *et al*., 2001; Zhang *et al*., 2022). The reduction of relative chlorophyll content in *atsap2* was recorded and this is possibly associated with the loss-of-function of the gene and contributes to the rapid oxidative damage in the chloroplast and lipid membrane peroxidation of thylakoids (Kaur *et al*., 2019; Sharma *et al*., 2020b; Shakri *et al*., 2022). However, unlike *OsSAP8*, the overexpression of the gene results in a maintained level of relative chlorophyll content in response to both stresses. This is one of the ways plants adapt to stress by maintaining their chlorophyll content which can reflect the sustaining of photosynthetic machinery and photosynthesis rate (Zhang *et al*., 2022). This indicates that *OsSAP8* possibly plays a role in maintaining relative chlorophyll content indirectly or directly through the balance of ROS production or by the production of an antioxidant to sustain its biochemical activity.

Salt stress also affects the allocation of photosynthetic products within plants, which influences plant growth and maintenance under stress. Drought and salt stress lead to hyperosmotic signals and the accumulation of ABA which then triggers various adaptive responses in plants. This can be seen in the Roslan *et al*. (2017) study, where *OsSAP8* has a higher tolerance towards a high concentration of ABA by showing an insensitive germination phenotype while *atsap2* showed a sensitive phenotype when applied with an even lower concentration of ABA. *OsSAP8* is also a cytoplasmic protein, located in the nucleus and cytoplasm; and consists of both A20 and AN1 finger domains, which are essential in plant stress responses. These factors could have contributed to the survival of the overexpression

transgenic *OsSAP8* during stress treatments. This observation shows an agreement with a previous study that stated that *OsSAP8* confers drought and salinity tolerance (Kanneganti & Gupta, 2008; Vij & Tyagi, 2008; Zhang *et al*., 2016; Roslan *et al*., 2017; Sahid *et al*., 2020; Li *et al*., 2022). Besides, ABA and GA signaling pathways are regulated during stress exposure to modulate *the OsSAP8 gene*; and it may interact with other SAPs via regulating the intricate signaling mechanism to adapt to the stresses (Giri *et al*., 2011; Sahid *et al*., 2020; Li *et al*., 2022). The survival of overexpression *OsSAP8* may be due to high levels of ABA content that significantly provide tolerance against stresses by controlling stomatal aperture and preventing excessive water loss (Sherin *et al*., 2022). This mechanism ensures that plants have adequate water for survival during drought stress. Contrary to salinity stress environments, ABA has ionic and osmotic homeostasis, which will enhance plant photosynthetic rate throughout the condition (Sagervanshi *et al*., 2021).

CONCLUSION

Abiotic stress has been proven to negatively affect plant growth and development and yield production. In this study, we have characterized the *Oryza sativa* Stress Associated Protein (*OsSAP*) gene family *in silico* and validated two of the highly inducible genes through semi-quantitative PCR. The analysis demonstrated the important function of the *OsSAP* genes family in regulating abiotic stress response specifically for two of the gene members (*OsSAP8* and *OsSAP4*) which showed higher expression triggered by stress under specific rice genotypes. Other than that, more physiological experiment also shows during the vegetative stage, *OsSAP8* plants still can produce inflorescence stem, produce more flowers, and has higher relative chlorophyll content followed by wild type, *Col-0,* and its T-DNA mutant plants *atSAP2* whereas *atSAP2* plant was *unable* to produce inflorescence stem and has lowest relative chlorophyll content. It is important to understand how 35S::*OsSAP8* can survive due to abiotic stress conditions at the transition window from the vegetative to inflorescence stage. Understanding gene regulation may facilitate researchers to make targeted and specific changes to the DNA, enabling them to modify or delete specific genes with high accuracy through current gene editing technology. Overall results from semi-quantitative-PCR and morphological analysis reveal that *Oryza sativa Stress Associated Protein8* (Os*SAP*8) has great potential to be used in genetic manipulation programs for crop improvement.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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