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Expressing *OsiSAP8*, a Zinc-Finger Associated Protein Gene, Mitigates Stress Dynamics in Existing Elite Rice Varieties of the 'Green Revolution'

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Citation: Krishnan, S.R.; Muthuramalingam, P.; Priya, A.M.; Prasanth, M.I.; Gopinath, K.; Mohan, C.; Muthusamy, K.; Balamurugan, K.; Gupta, A.K.; Ramesh, M. Expressing *OsiSAP8*, a Zinc-Finger Associated Protein Gene, Mitigates Stress Dynamics in Existing Elite Rice Varieties of the 'Green Revolution'. *Sustainability* **2022**, *14*, 10174. https://doi.org/10.3390/su141610174

Academic Editor: Francesco Paolo Fanizzi

Received: 22 May 2022
Accepted: 12 August 2022
Published: 16 August 2022

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Abstract: Key message: Overexpression of *OsiSAP8* driven by *Port Ubi2.3* from *Porteresia coarctata* imparts drought and salinity stress tolerance in transgenic rice. Stress associated proteins (SAPs) possess the zinc-finger domains that are wildly evolving functional and conserved regions/factors in plants to combat abiotic stresses. In this study, the promoter region of *OsiSAP8*, an intron-less, multiple stress inducible gene, was compared in silico with a strong constitutive promoter, *Port Ubi2.3*. This resulted in developing rice, resistant to drought and salinity expressing *OsiSAP8* promoted by *Port Ubi2.3*. (*Porteresia coarctata*), through *Agrobacterium*-mediated transformation in the popular rice varieties, IR36 and IR64. Southern blot hybridization confirmed the integration of *OsiSAP8*, and the T0 transgenic lines of IR36 and IR64 were evaluated for their drought and salinity tolerance. The IR36-T1 progenies showed an enhanced tolerance to water withhold stress compared to wild type and IR64-T1 progenies. Physiological parameters, such as the panicle weight, number of panicles, leaf wilting, and TBARS assay, showed the transgenic IR36 to be superior. The transgenic lines performed better with higher 80–95% relative leaf water content when subjected to drought for 14 days. Gene expression analysis of *OsiSAP8* in IR36 T1 showed a 1.5-fold upregulation under mannitol stress. However, IR64 T1 showed a two-fold upregulation in NaCl stress. An enhanced drought and salinity stress tolerance in the transgenic IR36 cultivar through overexpression of *OsiSAP8* was observed as it had a native copy of *OsiSAP8*. This is perhaps the first study using a novel ubiquitin promoter (*Port Ubi2.3*) to generate drought and salinity stress-tolerant transgenic rice. Thus, we report the overexpression of a rice gene (*OsiSAP8*) by a rice promoter (*Port Ubi2.3*) in rice (IR36) to resist drought and salinity.

Keywords: abiotic stress; IR36; *OsiSAP8*; *Port Ubi2.3*; promoter; zinc-finger protein

1. Introduction

Rice (*Oryza sativa* L.), being the most popular and domesticated *kharif* crop, is the staple source of carbohydrate to nearly half of the world's population [1–4]. The sessile and

acquiescent nature of higher plants makes them prone to various biotic and abiotic cues. Such environmental stresses constrain their growth, resulting in a reduced yield and quality. Drought and salinity are the major stresses that affect almost 50% of the crop production worldwide [5,6]. Since the 1960s, the rice trait/breed perfection has been performed by the incorporation of different candid genes. With the advent of the green revolution and the development of the semi-dwarf rice IR8 using the *sd-1* gene, there was a substantial increase in the rice grain yield leading to new horizons in rice research [7,8]. During the second segment of the green revolution, elite rice varieties, such as IR36 and IR64, were developed. IR36, the most cultivated rice variety (11 million hectares/year), is a semi dwarf type containing the defective semi-dwarfing allele (*sd-1*) [9] with a faulty gibberellin 20-oxidase gene which might be due to gene introgression [10] and led to the drought susceptibility [11]. Certain key elements, such as DRE/CRT-binding transcription factors (TF) (DREBs/CBFs), mitogen-activated protein kinases (MAPKs), and heat shock factor/proteins (HSF/HSPs), are highly conserved in plants with respect to abiotic stresses [12–14]. Many vital proteins are produced that are regulated by key TF that contemplate stress tolerance [15]. Such proteins have been reported to act on other systems, e.g., bacteria and animals, and play a key role in the immune response [16–18].

Dixit et al. (1990) first reported the A20 zinc-finger domain and multiple cys2/cys2 motifs from human endothelial cells at the C-terminus part of TNFα [19]. The International Rice Genome Project [20] and the genome-wide analysis of these proteins report the presence of 18 members of the A20/AN1 -type zinc finger domain proteins [20,21]. These proteins were categorized for their protagonist role in the immune response and hence associated with multiple abiotic stresses [20,22–24]. *OSiSAP1* was classified to be a multiple stress responsive gene coding Stress Associated Protein (SAP) [25,26]. Transgenic tobacco plants expressing *OSiSAP1* were tolerant against dehydration, salinity, and cold stress. Jin et al. (2007) [27] reported that a large number of these genes are involved in the abiotic stress response, thus signifying the importance of the A20/AN1-type zinc-finger proteins in combating stress. Although the abiotic stress tolerance mechanism is well known, their induction by multiple stresses is poorly understood [28]. Sharma et al. (2015) studied the expression of *OSiSAP7* on *Arabidopsis thaliana* with respect to abscisic acid (ABA) stress and their twin localization was observed in the nucleus and subcellular granules. The involvement of the E3 ubiquitin ligase activity was observed on YFP-tagged *OSiSAP7* overexpression which led to ABA insensitivity on water deficit stress [21]. Kanneganti and Gupta (2008) reported *OsiSAP8* from IR50 to be a single copy intronless gene conferring tolerance to numerous abiotic stresses such as salinity, heat, cold, dryness, submergence, wounding, abscisic acid, and some heavy metals, e.g., zinc, copper, cadmium, and lithium. Salination of soil affects about half of the global crop production [29]. The use of an appropriate promoter is a prerequisite for an efficient gene expression [28]. CaMV 35S promoter, though routinely used for heterologous transformation [30], confers a lower expression in monocots [31]. Rice *Act1* [32,33] and maize *Ubi1* [34] are the predominantly used monocot promoters. Port *Ubi2.* promoter derived from *P. coarctata* is a salt secreting halophytic wild rice that thrives in the coastal-riverine interface [35,36]. Recent reports on overexpressing DNA helicase (PDH45), co-transformation of DREB (*EaDREB2* from *Erianthus arundinaceus*) with DNA helicase or expressing heat shock protein (EaHSP70 from *E. arundinaceus*) driven by *Port Ubi2.3* have been successfully validated in sugarcane, which gave the maximum tolerance against salt and drought stresses [37–39].

In this study, we report the overexpression of *OsiSAP8* gene in IR36 and its superior variety IR64, driven by a ubiquitin promoter from *Porteresia coarctata*, a stress-tolerant wild rice. *Port Ubi2.3* was reported to promote strong *GUS* expression in rice (monocot), nearly sevenfold higher than the CaMV 35S promoter [40]. We also compared the expression of *OsiSAP8* and correlated the resistance to drought and salinity between wild type (WT) and transgenic rice (IR36 and IR64), through various physiological and molecular parameters.

2. Materials and Methods

2.1. Identification of OsiSAP8 Gene and Their Attributes in Rice

OsiSAP8 gene was subjected to *Oryazabase* to fetch the RAP ID, then RAP ID were loaded onto RiceNetDB to retrieve the CDS coordinates, nucleotide and amino acid length, chromosome number, molecular weight, probeset ID, Pfam ID, isoelectric point (pI), subcellular localization, UniProtKB, KEGG, Ensemble, EMBL, Regulatory genes.

2.2. Gene Expression Analysis

OsiSAP gene RAP ID were loaded onto plant developmental and plant hormonal datasets which are included in RiceXPro database [41] for analyzing the spatio-temporal and plant hormonal expression patterns throughout growth in the natural field condition.

2.3. Protein-Protein Interaction of OsiSAP8

Molecular cross-talks of *OsiSAP8* gene were analyzed by STRING v10.5 and the cross-talks were used to understand the functional and physical associations of the candid gene involved [42].

2.4. In Silico Analysis of OsiSAP8 Regulatory Region and Its Comparison with Port Ubi2.3 Promoter

Phytozome (http://phytozome.jgi.doe.gov/pz/portal.html# accessed on 10 May 2022) was used to retrieve the putative regulatory region of *OsiSAP8* and the cis acting regulatory elements were predicted by using PLACE database [43]. The transcription start site was assigned based on the position of the predicted TATA box and the arrangement of *cis*-regulatory elements was plotted using Cytoscape V 3.2.1 software [44]. In addition, the cis regulatory elements pertaining to abiotic stresses and expression enhancement, present in both *OsiSAP8* and *Port Ubi2.3* promoters were compared to obtain a better understanding of their role in gene regulation.

2.5. Explant Source and Tissue Culture Processing

O. sativa L. ssp. *Indica* seeds of IR64 and IR36 were procured from the Tamil Nadu Rice Research Station, Aduthurai, Tamil Nadu, India. The mature embryos of IR64 and IR36 were processed and inoculated in the respective callus induction medium [45,46]. The cultures were incubated in dark (25 $\pm$ 2 °C) for 4 weeks and subsequently transferred to a proliferation medium for 3 weeks. Friable, nodular, and embryogenic calli (4–5 mm diameter) were used for the transformation experiments.

2.6. Vector Construction and Generation of Transgenic Rice

pCAMBIA1305.1 with the *Port Ubi2.3* promoter was restricted with *Spe*I and *Pml*I to replace the *GUS* gene with the *OsiSAP8* gene (Genbank Acc. No. AY345599) flanked with *Spe*I and *Pml*I (5′ and 3′ ends respectively) synthesized in Bio Basic Inc. (Markham, ON, Canada). The presence of the gene was checked using gene-specific PCR and confirmed by restriction analysis. The plasmid construct was named as pCAM-Port2.3-*OsiSAP8* and transferred to LBA4404 [47] by triparental mating [48] for *Agrobacterium*-mediated transformation.

2.7. Generation of Rice Transgenics

The super virulent strain LBA4404 with the binary vector pCAM-Port2.3-*OsiSAP8* was infiltrated in the mature seed derived nodular, friable, and embryogenic callus of IR36 [45] and IR64 [46]; subsequently they were subjected to hygromycin (40 mg L^{-1}) selection. After two rounds of hygromycin selection, actively proliferating hygromycin resistant calli were subjected to regeneration. The putative transgenic plantlets were then transferred to a versatile plant growth chamber (SANYO) in Soilrite (Kel Tech Energies, Pvt. Ltd. Bengaluru). Six-week-old WT and the transgenic rice (IR36 and IR64) were later transferred to D-262 H AXIVA-plantation pots (Axiva Sichem Biotech, NSP, Delhi, India) with garden soil:soilrite:farmyard manure (1:1:1) and established in a greenhouse. The

OsiSAP8 gene integration was confirmed by DNA blot. The pots were watered until seed setting and maturation. Later, the T_1 seeds were harvested and analyzed for the presence of transgene and subsequently tested for drought and salinity.

2.8. Molecular Analysis of Transgenic Plants and Events Selection

The genomic DNA of WT and the transgenic lines were isolated from the leaves of the putative transgenics by CTAB [49] method and the integration of T-DNA in the putatively transformed plantlets were determined by polymerase chain reaction (PCR). Gene-specific primers for *OsiSAP8* and *hpt* genes (Supplementary Table S1) were used for PCR amplification in a programmable thermal cycler (Mastercycler gradient, Eppendorf, Germany) under the following conditions. PCR was performed with a reaction mixture containing 50 ng of DNA template, 2.5 µL of 10X PCR buffer, 1.5 µL of $MgCl_2$ (1.5 mM), 2 µL (20 pmol) of each primer, 0.625 µL of dNTPs mix (0.25 mM), 1.0 U of *Taq* polymerase and made upto 25 µL with sterile nuclease free water. The PCR products (5 µL) were resolved on an ethidium-bromide-stained 0.8% agarose gel and were visualized and photographed using Gel Doc XR + (BIO RAD, Quarry Bay, Hong Kong).

2.9. Southern Blot Hybridization

To check the *OsiSAP8* gene integration and the copy number, Southern blot hybridization was performed. Twenty micrograms of genomic DNA from PCR-positive transgenic IR36 and IR64 were digested with *Pml*I and *Bam*HI respectively, resolved on 1% agarose gel electrophoresis and blotted on BioBond™ Nylon transfer membrane (Sigma Aldrich, Bangalore, India). The *OsiSAP8* gene was amplified from pCAMBIA1305.1 and used as probe to check the integration in transformed IR36 and IR64 lines. AlkPhos direct labelling (Amersham™, Citiva, New York, NY, USA) was used to label the probe as described by Sambrook et al. [50] After hybridization for 16 h at 65 °C, the membrane was processed with posthybridization buffers. The membrane was air dried, and the detection reagent (Amersham™ CDP-Star™, Citiva) was run over the membrane. The air-dried membrane was kept for exposure on Amersham Hyperfilm™ (Citiva) ECL in Amersham Hypercassette Autoradiography Cassette. The film was developed using KODAK GBX Fixer and Replenisher.

2.10. Segregation Analysis of T_1 Progeny

Hundred seeds from the southern blot-positive T_0 transformants from IR36 ($T_{0–1}$ to $T_{0–5}$) and IR64 ($T_{0–1}$ to $T_{0–6}$) were germinated on half-strength MS medium incorporated with 40 mg L^{-1} of hygromycin for 2 weeks. The data was observed and analyzed for Mendelian style of inheritance. Further, segregation analyses on the hygromycin resistance for T_1 progeny (100 seeds) were done in accordance with the expected and observed frequency and χ^2 test was performed to confirm the Mendelian Law of segregation (3:1) in all the five transgenics (T_0).

2.11. Analysis of Abiotic Stress Tolerance in WT and T_1 Lines

The T_1 seeds of confirmed transgenic lines (T_0) with a single copy transgene were used for physiological and qPCR analysis.

2.12. Relative Leaf Water Content (RWLC)

The RLWC was calculated following the protocol described by [51]. Fresh weights of the fully expanded leaves were noted and immersed in distilled water in petri dishes and left for 2 h; later the leaves were blot dried and the turgid weight was noted. The samples were then dried in an oven (70 °C) to a constant weight. Subsequently, RLW was calculated using the following formula:

$$RLWC\% = [(FW - DW)/(TW - DW)] \times 100$$

where FW is the fresh weight; DW is the dry weight; and TW is the turgid weight.

2.13. Visual Scoring on Stress Resistance

In the first set of experiments, the seeds (WT and T_1) were sown in 7 cm (diameter) × 9 cm (height) transparent plastic cups filled with 125 g of soilrite (pH 6 ± 0.5) with 75% humidity and an average irradiance of 50 mmol m^{-2} s^{-1} was maintained in a versatile plant growth chamber. It was ensured that the physiological conditions were the same for the experimental set up. After germination and acclimatization for 45 days (d), the plantlets were subjected to drought stress by withholding water and the day was noted as day 0. Through a modified approach of [52], the leaves were visually scored for drought stress tolerance by witnessing leaf wilting on the 7th and 14th days. Leaf wilting was scored with gauge (1 = no wilting; 2 = marginal wilting; 3 = wilting, shown in leaves during hot hours which later recovered; and 4 = chronic wilting, where leaves became pale and severe wilting was observed). The stress assay was stopped, plants were irrigated after 14 days of water being withheld, and the physiological parameters were observed.

2.14. Leaf Disc Bioassay

The leaf chlorophyll withholding capacity on IR36 and IR64 (WT and T_1 lines) were performed according to [53]. One cm leaf was cut from the second leaf and immersed in half-strength MS medium supplemented with various concentrations of NaCl (100, 200, 300, 400, and 500 mM) for 7 days and the leaf bleaching was observed [54].

2.15. Effect of Mannitol on Plantlet Growth

Four-days-old, germinated seeds of transformed (T_1 lines) IR36 and IR64 were transferred to half-strength MS medium with a varied concentration of mannitol (100, 200, 300, 400, and 500 µM). Later, the cultures were kept in vitro at 25 ± 2 °C in long daylight conditions (16/8 h day/night photoperiod), with an average irradiance of 50 mmol m^{-2} s^{-1}. The physiological parameters were observed and recorded.

2.16. Thiobarbituric Acid Reactive Substances (TBARS) Assay

Leaf tissues (100 mg) were homogenized by adding 0.5 mL 0.1% (*w*/*v*) TCA and centrifuged for 10 min (15,000× *g*, 4 °C). The supernatant was collected and 0.5 mL of the supernatant was mixed with 1.5 mL 0.5% TBA diluted in 20% TCA. The mixture was incubated in a water bath at 95 °C for 25 min and the reaction was stopped by incubating on ice. The absorbance was measured at 532 nm and the results were expressed as µmol MDA 100 mg^{-1} FW [55].

2.17. OsiSAP8 Gene Expression Analysis Using qPCR

The total RNA was extracted from the second leaf (100 mg), of 15 days old drought and salinity treated transgenic (T_1 lines) and non-transgenic (control) rice lines of IR36 and IR64, using TRI Reagent® (SIGMA-ALDRICH) and was treated with DNAse and subsequently quantified using BioSpec-Nano. The RNA was then reverse transcribed with 10× RT Buffer, 25× dNTPs Mix (100 mM), 10× oligodT primer and MultiScribe™ Reverse Transcriptase, RNase inhibitor as per the manufacturer's instructions by using High-Capacity cDNA Reverse Transcription Kit (Applied-Biosystems, Waltham, MA, USA). For qPCR analysis, *OsiSAP8* gene-specific primers (Table S1) and housekeeping primer, 25SrRNA were used [56]. The fold difference in the expression ($2^{-\Delta\Delta ct}$) was calculated by comparative Ct value analysis with 25SrRNA normalization. [57].

2.18. Statistical Analysis

The Relative water content, Lipid peroxidation, Chlorophyll content, Mannitol stress, and qPCR were done in biological and experimental triplicates, which were subjected to one way analysis of variance (ANOVA) using IBM SPSS 20.0. Chi-square (χ^2) test was done manually to study the segregation ratio in T_1 progeny (Pagano and Gauvreau 2004). Values at $p < 0.05$ level of significance were used for mean comparison.

3. Results

3.1. OsiSAP8 Gene Attributes

OsiSAP8 gene with their corresponding RAP-ID, CDS coordinates, nucleotide and amino acid length, chromosome number, molecular weight, probeset ID, Pfam ID, pI, subcellular localization, UniProtKB, KEGG, Ensemble, EMBL, Regulatory genes were retrieved from *Oryzabase* and RiceNetDB (Table 1).

Table 1. *OsiSAP8* gene and their molecular attributes.

Gene	***OsSAP8***
Gene Synonym	*OsiSAP8; Osi-SAP8*
CGSNL Gene Name	Stress Associated Protein 8
MSU ID	LOC_Os06g41010.1
RAP ID	Os06g0612800
Chromosome Number	06
CDS Coordinates (5′-3′)	24491979–24494238
Nucleotide Length	516
Amino acid Length	172
Molecular Weight	18,402.1
Isoelectric Point	8.0476
Subcellular Localization	Nucleus
Probeset ID	Os.28384.1.S1_at
Pfam ID	PF01754, PF01428
UniprotKB	A3BDI8
Ensembl	LOC_Os06g41010.1 LOC_Os06g41010.3 LOC_Os06g41010.4
Refseq	NP_001058066.1
EMBL	AP003626, AP003711, AP008212, CM000143
KEGG	Osa: 4341520; dosa:Os06t0612800-01
	Regulatory genes
Upstream genes	LOC_Os04g46390; LOC_Os05g38550; LOC_Os01g22010; LOC_Os05g28290; LOC_Os03g18180; LOC_Os02g06640; LOC_Os05g01970; LOC_Os06g04030; LOC_Os03g27310; LOC_Os03g01910; LOC_Os04g33830; LOC_Os05g40820; LOC_Os01g59990; LOC_Os06g01210; LOC_Os06g05550; LOC_Os04g57220; LOC_Os01g38620; LOC_Os04g46820; LOC_Os04g46810; LOC_Os04g44830; LOC_Os05g07070.
Downstream genes	LOC_Os07g42300; LOC_Os12g02340; LOC_Os12g02310; LOC_Os11g02424; LOC_Os11g02369; LOC_Os11g02400; LOC_Os06g46770; LOC_Os09g30438; LOC_Os09g30418; LOC_Os09g30412; LOC_Os07g47640; LOC_Os12g02330; LOC_Os10g34180; LOC_Os11g13850; LOC_Os07g33240; LOC_Os11g47760; LOC_Os12g37650; LOC_Os11g47460; LOC_Os10g41410; LOC_Os09g30340; LOC_Os07g12200; LOC_Os07g12170; LOC_Os08g44680; LOC_Os12g05430.

3.2. Spatio-Temporal and Plant Hormone Expression Analysis of OsiSAP8

OsiSAP8 and their developmental (Spatio-temporal) tissues specific expression signature was noted for 48 tissues and/or organs at various growth stages (Figure 1). *OsiSAP8* gene revealed higher expression signature in multiple tissues and/or organs such as leaf blade (reproductive—00:00), leaf sheath (reproductive—12:00; 00:00), Root (reproductive—12:00; 00:00), stem (reproductive—00:00, ripening—00:00), embryo (14, 28, 42 DAF) and endosperm (07, 10, 14, 28 and 42 DAF) (Figure 1) as imputed by RiceXPro. In addition, this gene showed the hormonal signatures in various time intervals such as 1, 3, 6, 12 h

and 15, 30 min, 1, 3, 6 h on shoot and root, respectively (Figures 2 and 3). Phyto-hormonal expression signature of *OsiSAP8* gene was expressed under field conditions. In shoot, candidate gene showed an increased level of abscisic acid (ABA) (6 and 12 h) and negligible levels of expression were observed in jasmonic acid (JA) (6 and 12 h) and auxin (3 and 6 h) (Figure 2). In root, a negligible level of expression was observed in ABA (30 min), gibberellic acid (6 h), and auxin (1 h) (Figure 3).

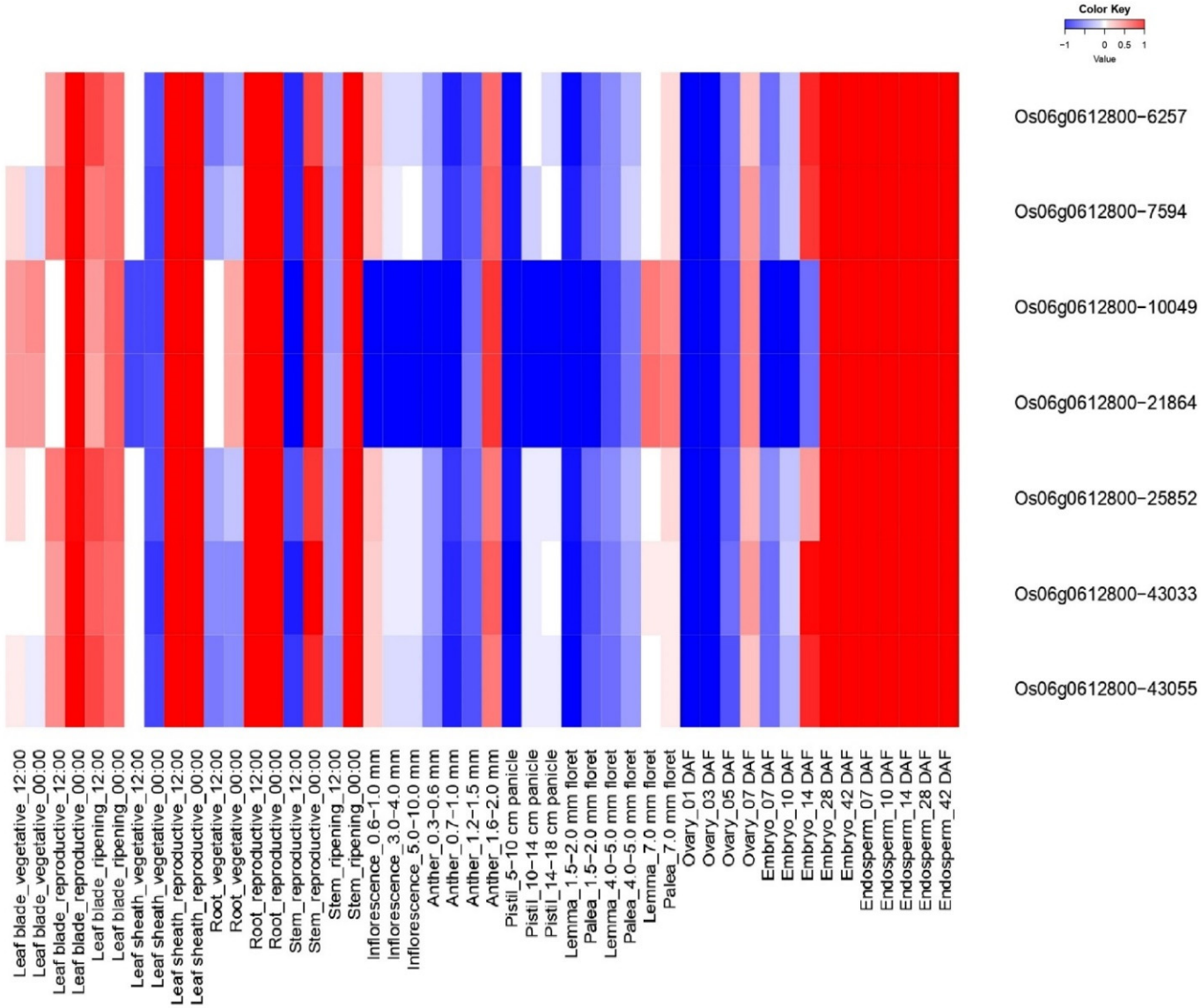

Figure 1. Heatmap represent the spatio—temporal expression profiling *OsiSAP8* gene in the tissues/organs at various developmental stages in field conditions. Blue color—down regulation; Red color—up regulation; White color—unchanged. The colored scale bar at right side top indicates relative expression value, where −1 and 1 represent down and up regulation of AbS responsible *OsSAP* genes respectively.

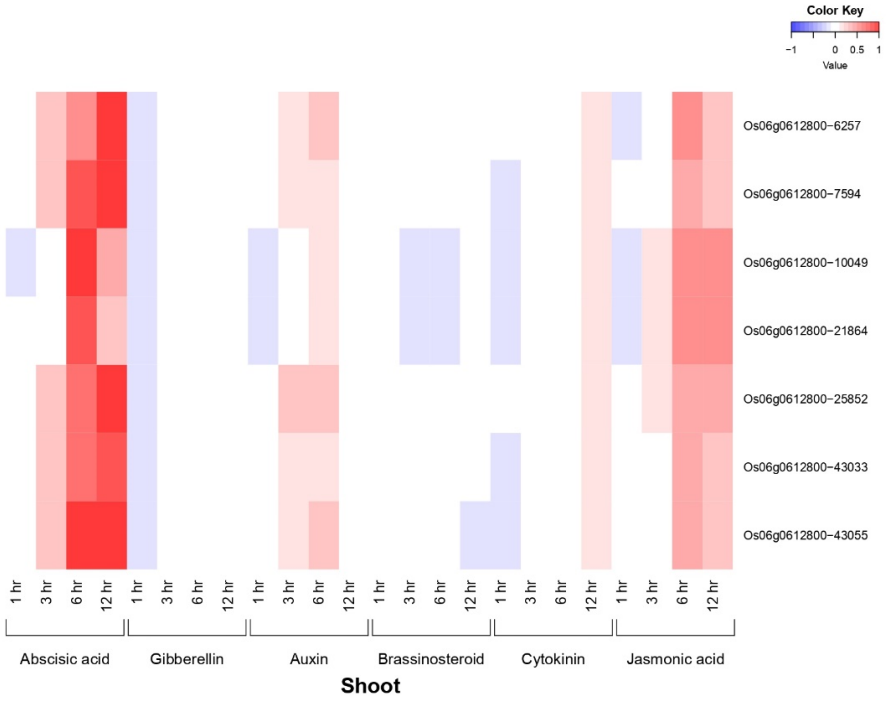

Figure 2. Heatmap denotes the *OsiSAP8* gene and their plant hormonal expression pattern in shoot which are differentially regulated across the throughout growth in the natural field conditions. Blue color—down regulation; Red color—up regulation; White color—unchanged. The colored scale bar at right side top indicates relative expression value, where −1 and 1 represent down and up regulation of AbS responsible *OsSAP* genes respectively.

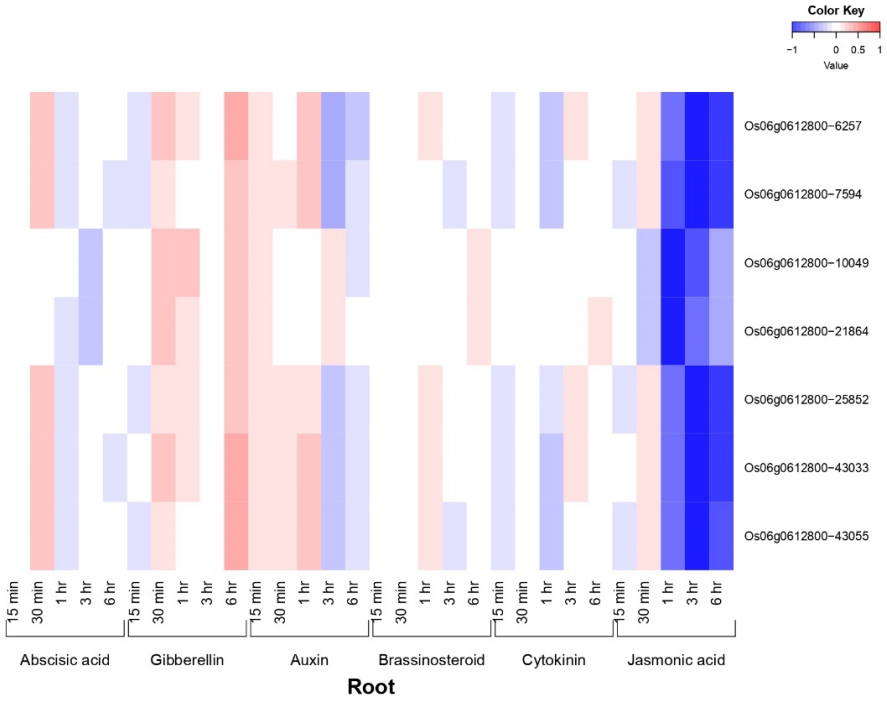

Figure 3. Heatmap denotes the *OsiSAP8* gene and their plant hormonal expression pattern in root which are differentially regulated across the throughout growth in the natural field conditions. Blue color—down regulation; Red color—up regulation; White color—unchanged. The colored scale bar at right side top indicates relative expression value, where −1 and 1 represent down and up regulation of AbS responsible *OsSAP* genes respectively.

3.3. Signalome of OsiSAP8 Gene

OsiSAP8 seed proteins were copied from *O. sativa* ssp. *indica* for signalome analysis. Figure 4 revealed that *OsiSAP8* gene had complexity and its proving quantitative and multigenic nature.

Figure 4. *OsiSAP8* gene and their interaction network. *Indica* rice *OsiSAP* gene interaction showing inter-connected functional components. Colored lines between the proteins encode various types of interaction signals. Black color, co-expression; green color, gene neighbourhood; blue color, gene co-occurrence; pink color, experimentally determined. Protein nodes filled with ribbon like structure indicates the availability of protein 3D structural information is predicted or known.

3.4. Comparative In Silico Analysis between Port Ubi2.3 and OsiSAP8 Regulatory Region

The regulatory elements of *OsiSAP8* were grouped as tissue specific, biotic, and abiotic stress and were plotted using Cytoscape V 3.2.1 (Figure 5). The presumed promoter region of *OsiSAP8* had putative core element TATA box, scattered all along the promoter region at 7 places under bZIP protein binding site, light and anaerobic specific elements. A large portion of these elements are involved in root and seed-specific expression. MYCCONSEN-

SUSAT regions were found profoundly along a wide range in cold, drought, seed, and osmotic regulatory regions that codes for protein binding factors like CBF1 and CBF3. PRECONSCRHSP70A and heat shock factors (CCAAT box)/SE (heat shock element) regulate the proteins required for developmental processes. The predicted elements were compared with *Port Ubi2.3* promoter wherein the CAATBOX (CAAT) functions as a quantitative element. Apart to it presence of ARR1AT (NGATT) for salinity stress at 15 locations and ACGTATERD (ACGT) for bZIP protein binding site at 4 locations are present along the positive strand at both sides of TSS. Light regulative genes GT1CONSENSUS (GRWAAW) elements of *OsiSAP8* were predicted at 12 sites that have GT1 regulatory genes. Root-specific elements, RAV1AAT, were predicted at three regions and these encode a novel DNA-binding protein specific to the root and shoot system in higher plants. The *OsiSAP8* regulatory region is devoid of MART Box and has only five NGATT regions that act as enhancers as predicted by PLACE. Four drought specific *cis*-elements WAACCA are present in the *Port Ubi2.3* promoter as compared with only one element present on *OsiSAP8* regulatory region. IBOX elements that are regulated by light and tissue specific responses are scattered over four regions along the promoter region. In addition, TTWTWTTWTT (MART Box) and NGATT that act as enhancers of transgene expression were present in three and 32 numbers respectively [58]. The dehydration stress specific regions MYB (CNGTTR) and MYC (CANNTG) were predicted to be present at 18 and eight sites respectively thus making it superior in promoting expression during abiotic stresses [40]. Hence, for the present study, we used *Port Ubi2.3* promoter to drive *OsiSAP8* gene. A comparison between *OsiSAP8* and *Port Ubi2.3* promoter core putative *cis*-elements is depicted in Table 2.

Table 2. A comparison of *cis*-acting elements identified using PLACE program in *Port Ubi2.3* and *OsiSAP8* promoters.

Putative *cis*-Elements/Consensus		Function/Response	No. of Positions		References
			Port Ubi2.3	*OSiSAP8*	
ARR1AT (NGATT)	Enhancers	Salinity stress	15	5	[59,60]
MARTBOX (GATAA)	Enhancers	Scaffold attachment	3	0	
ACGTATERD (ACGT)	Abiotic stress	bZIP protein binding site	4	2	[61]
CAATBOX (CAAT)	Abiotic stress	Quantitative element	4	12	[62]
GATABOX (GATA)	Abiotic stress	Light responsive	8	4	[63]
GT1 (GRWAAW)	Abiotic stress	Light responsive/cell type specific	9	12	[64]
IBOX	Abiotic stress	Light regulated and tissue specific	4	4	[58]
MYB (CNGTTR)	Abiotic stress	Dehydration stress	18	1	[65]
MYC (CANNTG)	Abiotic stress	Dehydration stress	8	53	[66]
DOFCOREZM (AAAG)	Tissue specific	Signal responsive and/or tissue specific gene expression	4	0	[67]
CACTFTPPCA (YACT)	Tissue specific	Mesophyll specific	17	10	[68]
WBOX (TGACT)	Tissue specific	Wound responsive	2	0	[69]
GTGANTG (GTGA)	Tissue specific	Pollen specific	5	6	[70]
WRKY (TGAC)	Biotic Stress	Pathogen responsive	2	9	[71]
EBOX (CANNTG)	Biotic Stress	Plant pathogen interaction	1	43	[72]

Equal | Greater | Lesser

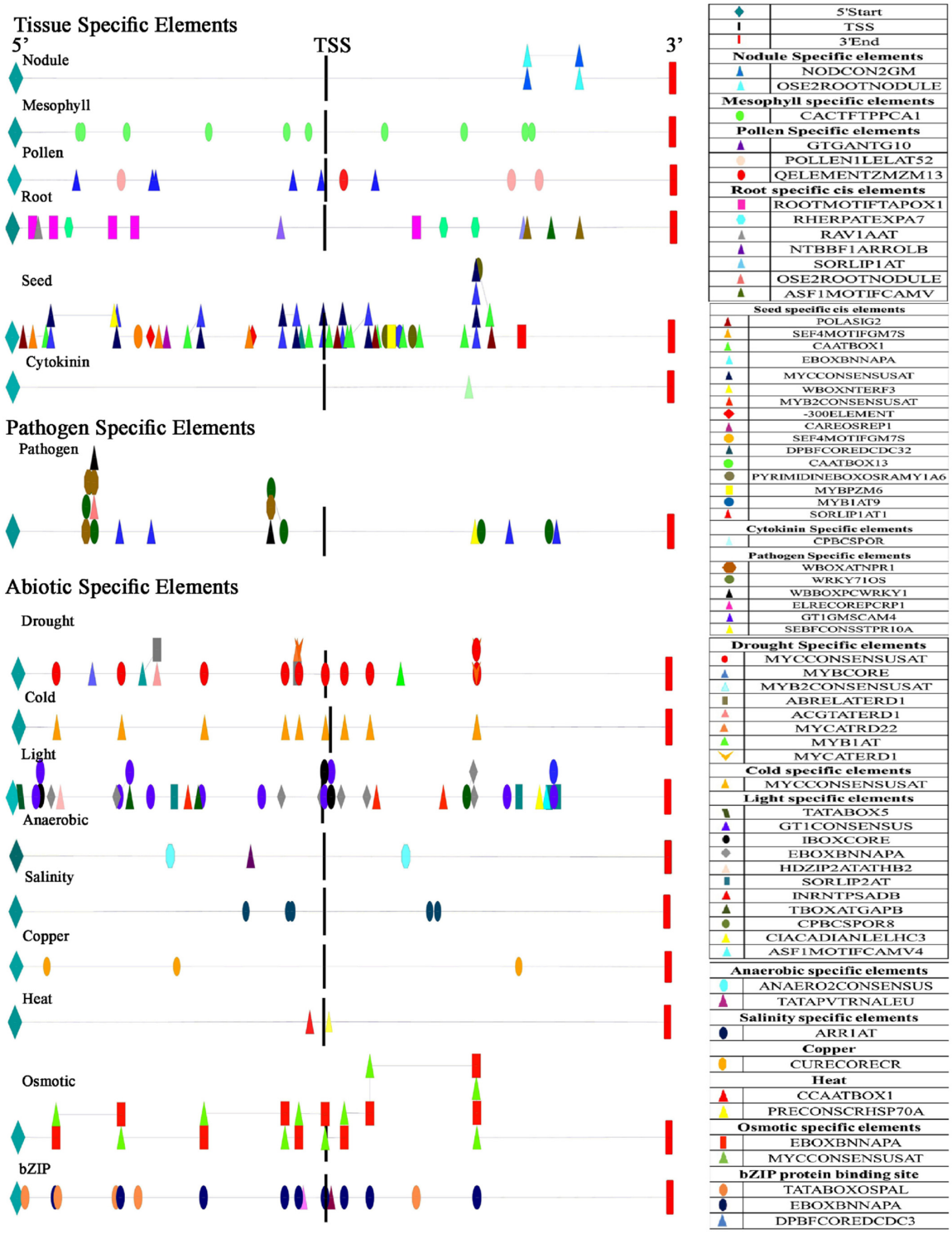

Figure 5. *Cis*-acting elements distribution pattern in predicted promoter of *OsiSAP8*. Divided as three major groups. Abiotic, biotic, and tissue-specific elements. Each line represents *OsiSAP8* promoter sequence and shapes correspond to predicted *cis*-elements in the positive strand. TSS-transcription start site.

3.5. Genetic Transformation with Port Ubi2.3-OsiSAP8 Gene and Molecular Analysis of Transgenic Plants

The *OsiSAP8* gene was sub-cloned in the T-DNA region of pCAMBIA 1305.1 driven by the Port *Ubi* promoter (2.3 kb) using *Spe*I and *Pml*I devoid of *GUS* reporter gene (Figure 6).

The hygromycin resistant calli (Figure 7a,f) were efficiently regenerated on a half-strength MS [73] medium (Figure 7b,g) and shoots developed efficiently on the shoot induction medium (Figure 7c,h). The transgenic lines appeared normal with well-acclimatized roots and shoots (Figure 7d,i). Further, the gene-specific PCR confirmed the presence of the transgene and *hpt*. The positive transformants were hardened to get T_1 seeds of IR36 and IR64 (Figures 7e,j and 8a,b).

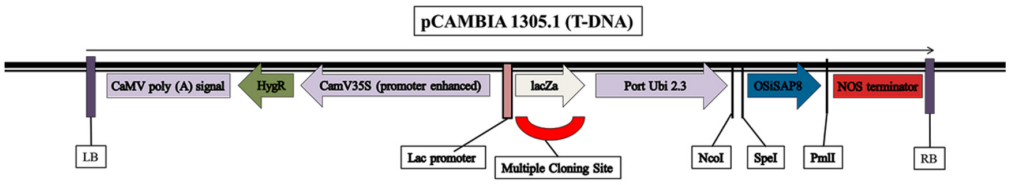

Figure 6. Vector construct in pCAMBIA1305.1. Vector construct of *OsiSAP8* gene in the T-DNA region of pCAMBIA 1305.1 driven by Port Ubi promoter (2.3 kb).

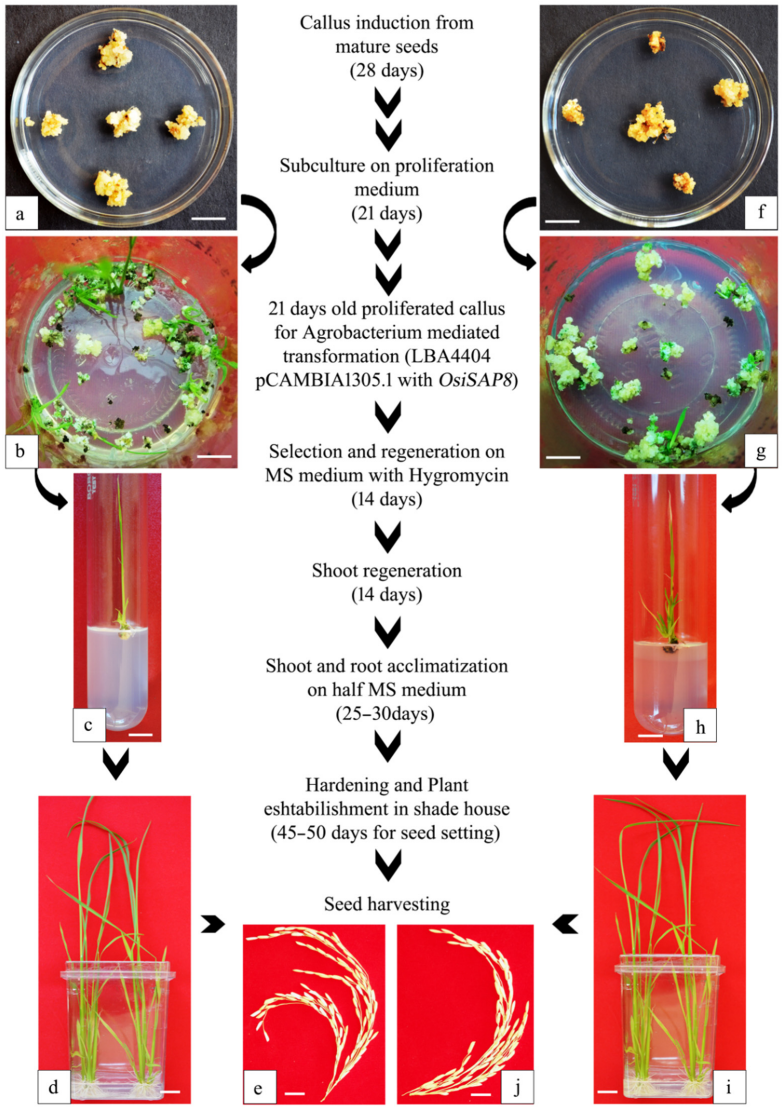

Figure 7. Overall workflow: Generation of *OsiSAP8* transgenic rice. Gene transfer process in (**a**–**e**): IR36 and (**f**–**i**): IR64. (**a**,**f**) Transformed callus kept for secondary selection in hygromycin medium, (**b**,**g**) efficiently regenerated callus with shoot primordia, (**c**,**h**) shoot development in half MS medium, (**d**,**i**) well developed plantlet with acclimatized roots and shoots, (**e**,**j**) harvested seeds from hardened transgenic rice (T_0) established in shade house.

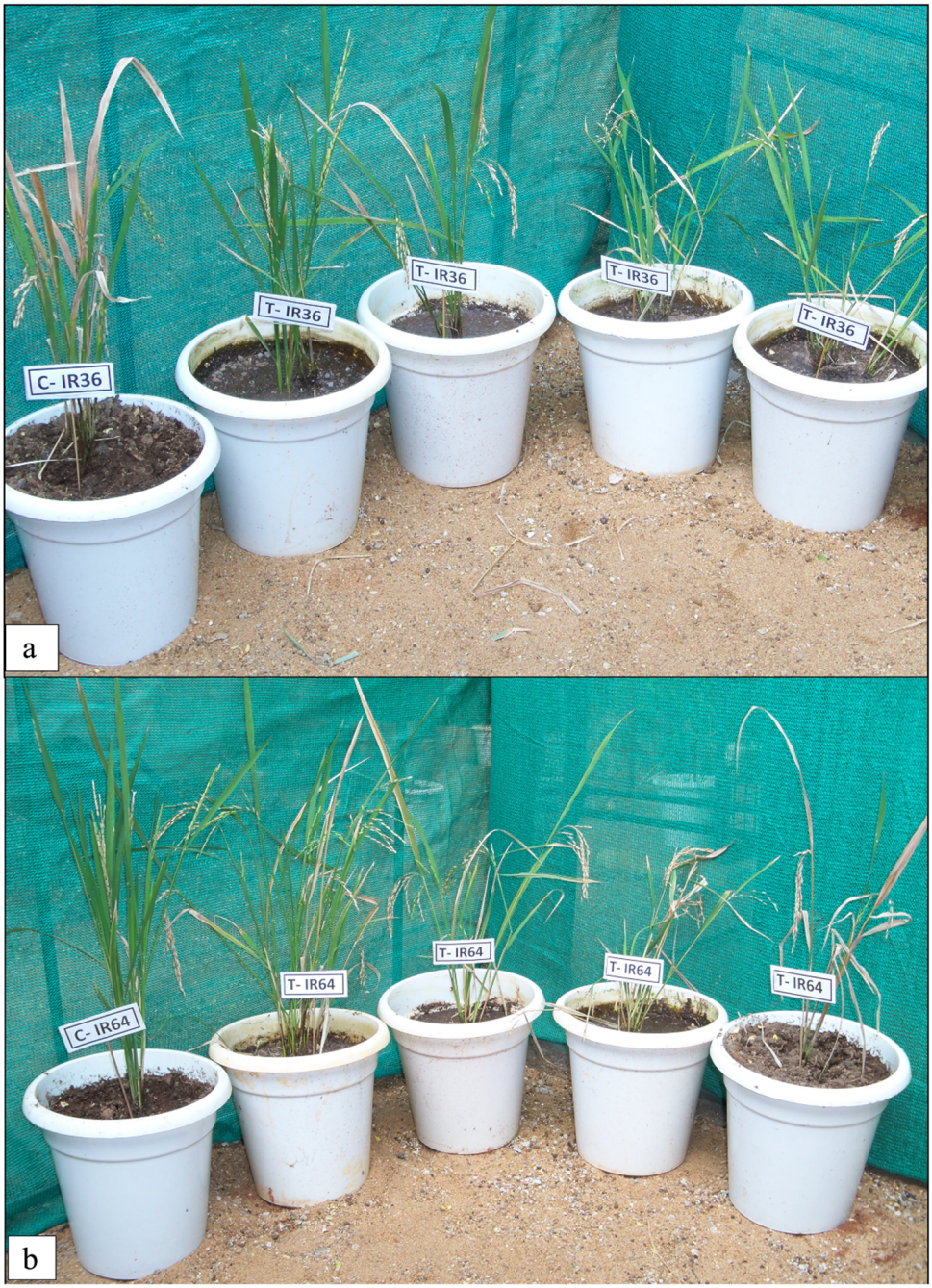

Figure 8. Transgenic rice. 145-days-old transgenic and WT rice. (**a**) IR36. (**b**) IR64. The transformed plantlets were transferred to the plantation pots.

3.6. Southern Blot Hybridization and Segregation Analysis of T_1 Progeny Plants

The stable integration of the *OsiSAP8* genes in randomly selected putatively transformed $T_{0–1}$ to $T_{0–5}$ (IR36) and $T_{0–1}$ to $T_{0–6}$ (IR64) plants were confirmed by Southern blot analysis. Molecular evidence and the number of copies of gene integration were shown in Figure 9a,b. The T_1 progenies were used to check the hygromycin resistance to discriminate the transgene carrier and a non-carrier. The observed Chi-square (χ^2) value correlated with Mendelian segregation (3:1) on the basis of the expected and observed frequencies (Table 3). The resistant T_1 seeds grew normally when compared with the non-transgenic, which stopped germinating after the coleoptile growth or did not germinate and resulted in the browning of the embryo. The observed segregation ratio was reliable in three of the six transgenic lines with a single T-DNA insertion locus in IR64 T_1, whereas the other two

progenies of IR36 T_1 showed the expected monogenic segregation ratio of 3:1 (Table 3). The χ^2 values of all that lines which had a two copy of transgene were found non-significant ($p < 0.5$), indicating a variation between the observed and the expected frequencies. However, the IR36 T_1 that had more than two transgene loci showed a ratio of 3:1.

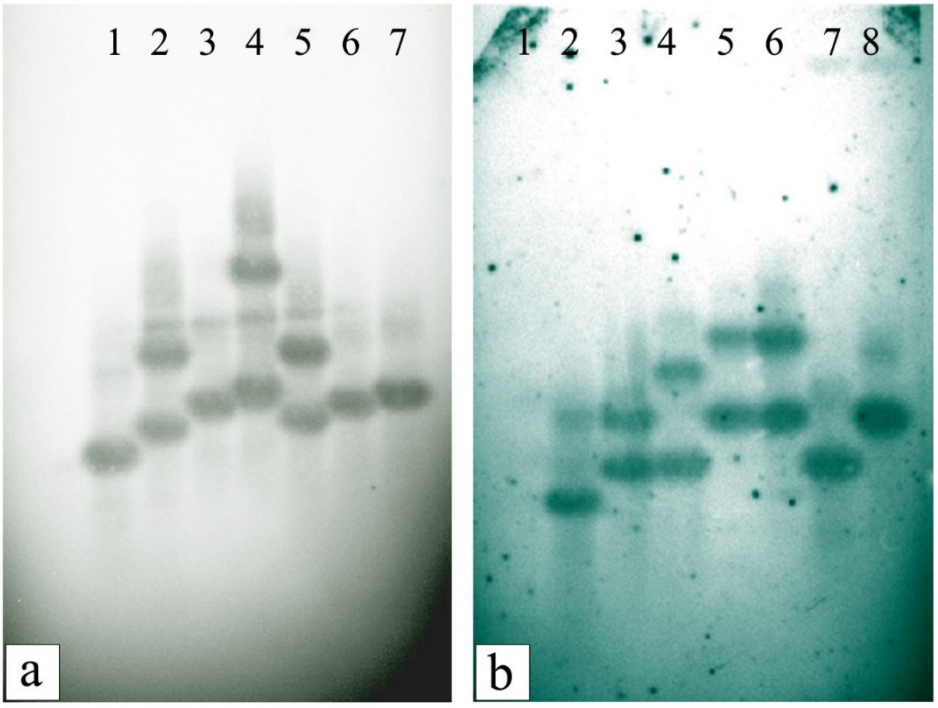

Figure 9. DNA blot analysis: Validating *OsiSAP8* gene integration. (**a**) Lane 1. Plasmid control-pCAM-Port2.3-*OsiSAP8*, Lanes 2–6. IR36 T_0 lines, Lane 7. WT IR36; (**b**) Lane 1. WT IR64, Lane 2. Plasmid control- pCAM-Port2.3-*OsiSAP8*, Lanes 3–8. IR64 T_0 lines.

Table 3. Segregation analysis of T_1 progeny plants.

T_0 Plants	T_1 Progeny	No. of Copies	Hygromycin Resistant	Hygromycin Susceptible	χ^2sum	Likelihood of *p* According to χ^2 Test	Segregation Ratio
IR36-T_0 Lines	T_{0-1}	2	78	24	0.16	>0.75	3.2:1
	T_{0-2}	1	79	21	0.853333333	>0.5	3.8:1
	T_{0-3}	2	79	21	0.853333333	>0.5	3.8:1
	T_{0-4}	2	83	17	3.413333333	<0.05	4.9:1
	T_{0-5}	1	76	24	0.053333333	>0.90	3.2:1
IR64-T_0 Lines	T_{0-1}	2	75	25	0	0.99	3.0:1
	T_{0-2}	2	80	20	1.333333333	>0.25	4.0:1
	T_{0-3}	2	80	20	1.333333333	>0.25	4.0:1
	T_{0-4}	2	79	21	0.853333333	>0.5	3.8:1
	T_{0-5}	1	77	23	0.213333333	>0.75	3.3:1
	T_{0-6}	1	76	24	0.053333333	>0.90	3.2:1

Deviation from monogenic segregation (as shown by χ^2 test) was only significant among all the progeny. Deviation from monogenic segregation (as shown by χ^2 test) was only significant among all the progeny. (df = 1, $p = 0.05$).

3.7. Relative Leaf Water Content

The IR36 T_1 lines could maintain 76–92% RLWC on day 7 (Figure 10), which was superior to IR64 (T_1 lines) that had just 40% of events and could maintain a level of 75–85% RLWC. As the stress increased, a change in the RLWC value was observed. IR64 (T_1 lines) showed a decline in RLWC in 46% of events tested but only 26% of IR36 (T_1) showed reduction in RLWC. Eighty percent of the IR36 T_1 events had above 80–95% RLWC, which

declined up to 60–75% on the 14th day of stress. IR36 (T_1 lines) reasonably maintained a higher RLWC. The factors observed were correlated with leaf wilting (Table 3).

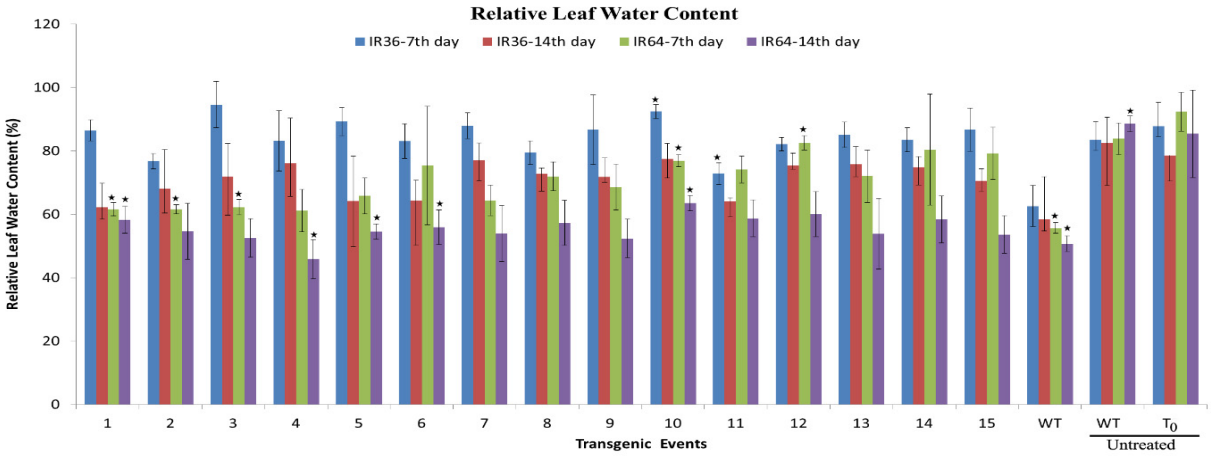

Figure 10. Relative leaf water content. Relative leaf water content in 15 transgenic events and WT of IR36 and IR64 after water withholds. Comparison with the untreated wildtype and transgenic. * indicates significance at the level of $p < 0.5$.

3.8. Visual Scoring on Stress Resistance

Fifteen plantlets were randomly used for this experiment of which the IR64 WT (4, 10, and 11) succumbed to death (Table 4). The IR36 T_1 lines were stable or slightly wilted during the second week of water-drought stress. Other features such as the number of tillers, panicles, and the average total grain weight per tiller were also assessed (Figures 11 and 12). By the 14th day, 40% of IR36 showed severe wilting and 20% of IR64 succumbed to death; there was a corresponding variation in the IR36 and IR64 transgenic events. Eighty percent of the IR36 T_1 transgenics showed slight wilting, with a corresponding good tiller setting in them after 14 days of water withholding and yielded 13 g/tiller as total grain weight. However, the IR64 lines had only 66% of events that were slightly wilted after which the tillers were set and thus resulted with only 8 g/tiller.

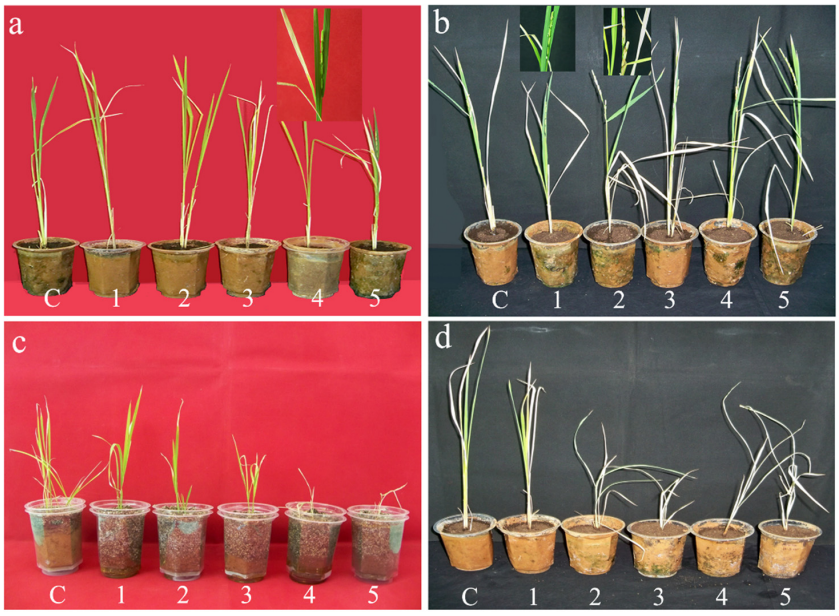

Figure 11. Water withhold stress on wildtype and transgenic (T_1) plants. The 45-days-old mature plantlets were analyzed for water deficit for 14 days. (**a**,**b**) Transgenic (T_1) IR36 and IR64, respectively. (a5,b1,b2) showing the panicles set for seed filling, (**c**,**d**) WT IR36 and IR64 respectively.

Table 4. Visual scoring of *OsiSAP8* transgenic events upon water deficit stress.

Grouping	Wilting Phase	Distinctive Visual Features
1	Normal (Not Wilted)	No signs of wilting or drought stress
2	Slightly Wilted	Slight leaf angle changes but no folding, rolling or changes in leaf surface structure
3	Wilted	Strong leaf angle change but no cell death
4	Severely Wilted	Very strong change of leaf angle or protrusion of veins on the leaf surface with beginning necrosis

Events	IR36				IR64			
	Wild type		T_0		Wild type		T_0	
	7th Day	14th Day	7th Day	14th Day	7th Day	14th Day	7th Day	14th Day
1	1	3	1	2	2	4	1	2
2	1	3	1	2	1	3	1	2
3	1	3	1	2	1	3	1	2
4	2	4	1	2	2	-	1	3
5	2	4	1	2	2	4	1	2
6	2	4	1	2	1	3	1	2
7	1	3	1	1	1	3	1	3
8	1	3	1	2	2	4	1	2
9	2	4	1	1	1	3	1	2
10	1	3	1	2	2	-	1	3
11	1	3	1	2	2	-	1	2
12	2	4	1	2	1	3	1	2
13	2	4	1	1	1	3	1	1
14	1	3	1	2	1	3	1	3
15	1	3	1	2	1	3	1	2

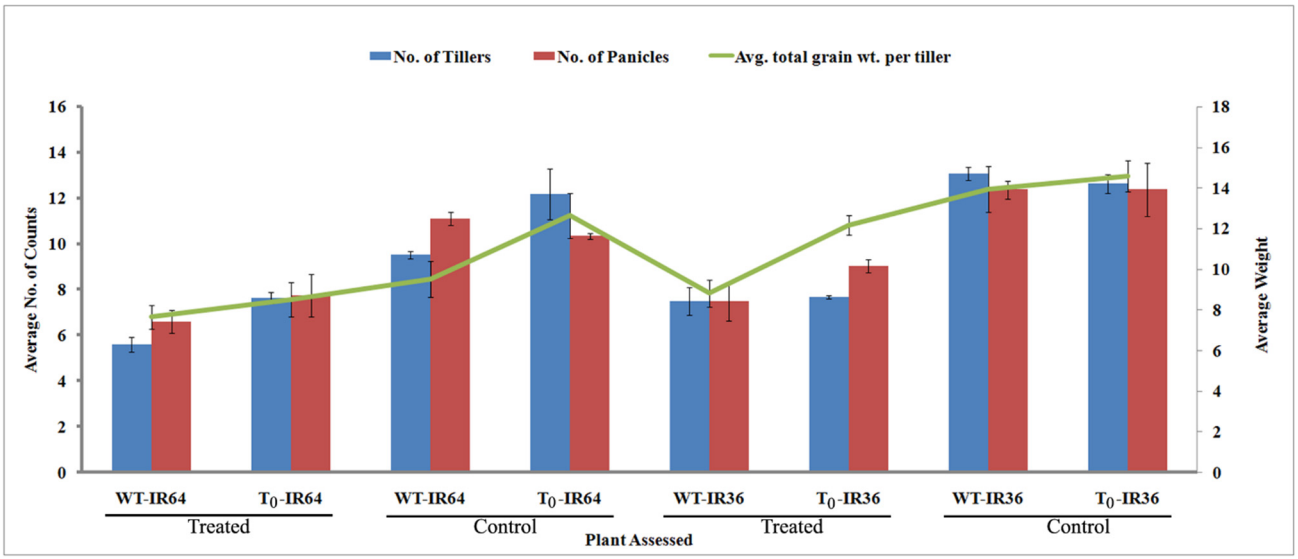

Figure 12. Assessment of tillers. Graph depicting the number of tillers, no. of panicles and average number of tiller per plant observed after the water deficit stress. The treated and control represent plants under water withhold and normal watering respectively. Values represent mean ± Standard deviation; with significance between control and stress treated at the level of $p < 0.05$.

3.9. Evaluating Dehydration Stress by Mannitol on Plantlet Growth

The drought response due to mannitol stress was observed on the germinated seeds. No significant difference was observed on germination by T_1 progeny with increase in mannitol stress. Coleoptile formation was delayed as the mannitol stress peaked. At 100 mM, coleoptile formed by sixth day and was delayed by 5 days at 500 mM in the IR36 T_1 progeny. The superior variety IR64 (T_1 progeny) produced coleoptile on the

13th day when exposed to 500 mM mannitol. The mat root and the seminal root system were predominantly formed till 200 mM mannitol stress. A better root formation and biomass was observed in the IR36 T_1 progeny (Figures 13 and 14). The transgenic lines showed resistance at 400 and 500 mM mannitol for 15 days, after which they succumbed to chlorophyll pigment retardation and eventually to leaf browning. Comparatively, IR64 (T_1 progeny) sustained up to 200 mM mannitol stress and later succumbed to drought when exposed to a 300 mM mannitol stress. The seminal and nodal root length was greater in IR36 (T_1 progeny) compared with IR64 (T_1). A greater number of mat roots formed in IR36 (T_1 lines) as the mannitol concentration increased, while IR64 (T_1 progeny) showed increase in the seminal root length (Figure 14b5) with a gradual decline in the shoot height.

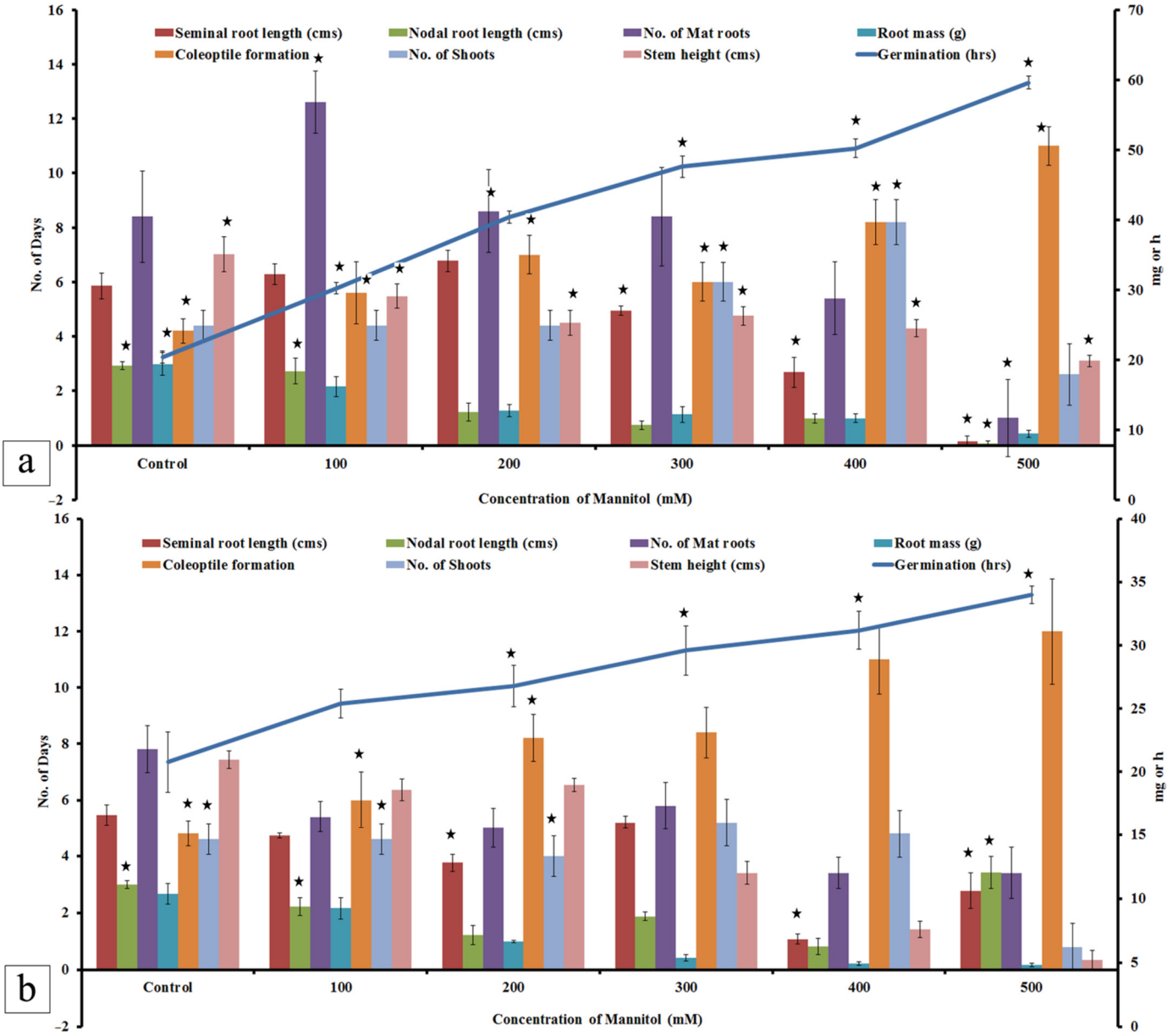

Figure 13. Analysis of physiological parameters during mannitol stress. Effect of mannitol stress on (**a**) IR36 T_1 and (**b**) IR64 T_1 lines. Values represent mean ± Standard deviation; * indicates the significance between control and stress treated at the level of $p < 0.05$.

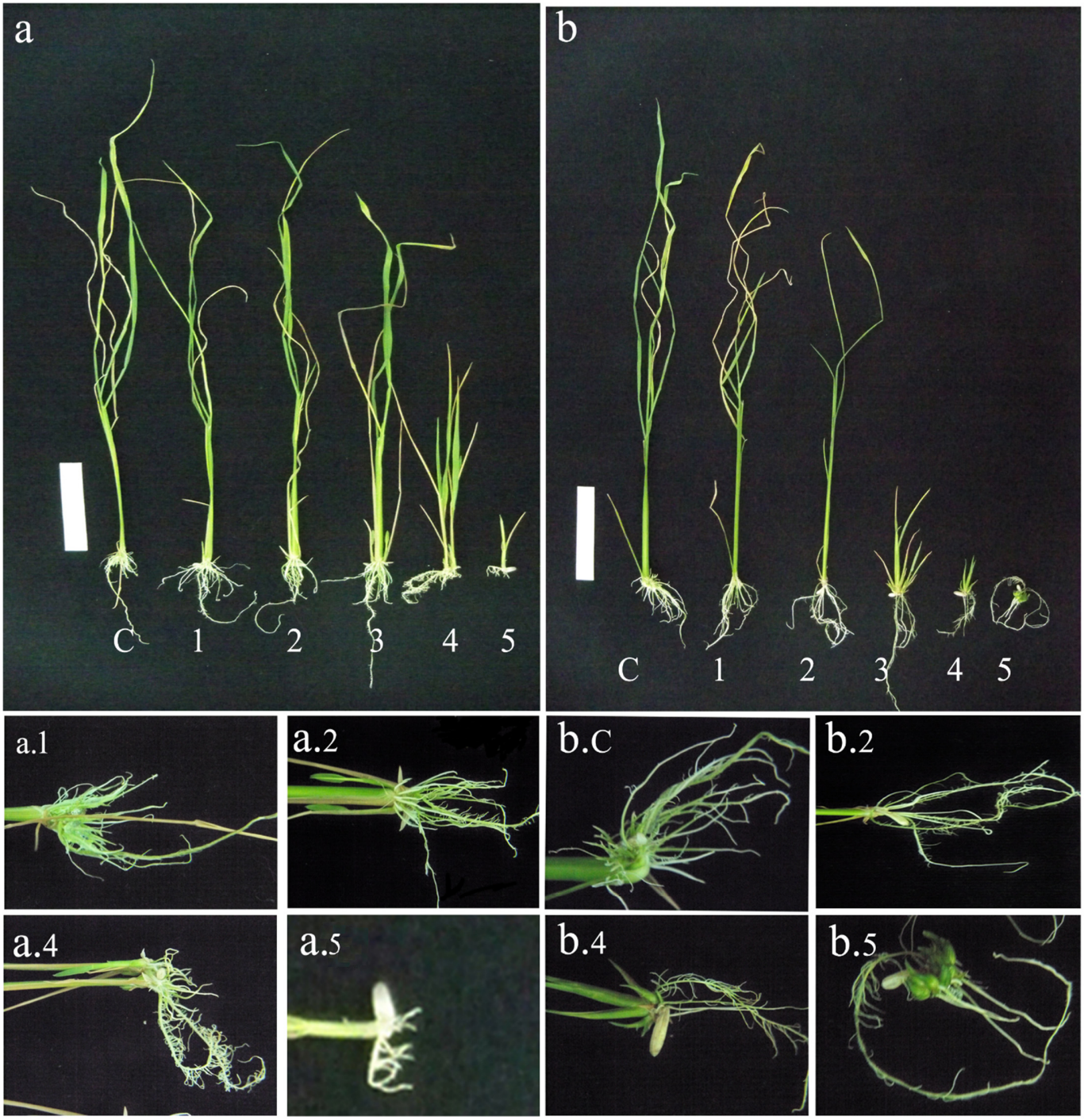

Figure 14. Drought assay on transgenic seeds of IR36 and IR64. Drought assay using mannitol in MS medium with a concentration 100–500 mM corresponding to 1–5, respectively, and C represents control. (**a**) IR36 T_1 and (**b**) IR64 T_1 seed derived plantlets. White line: Scale bar. 5 cm.

3.10. Leaf Disc Bioassay

A standard assay for the chlorophyll withholding capacity was performed. The transgenic leaf segments retained the chlorophyll to a maximum of 4 days and thereafter the retention increased gradually from 400 to 500 mM NaCl stress compared with that of WT that could withstand only 48 h with retention from a 200–500 mM concentration. The wound triggered by stress was significantly simulated in the level of whitening in the leaf tissue after 72 h. It was noted that the salinity-induced loss of chlorophyll was lower in the

T_1 progeny (IR36), compared with IR64 (Figure 15 and Table S2). The study revealed that the transgenic rice overexpressing *OsiSAP8* has a healthier ability to resist salinity stress than WT.

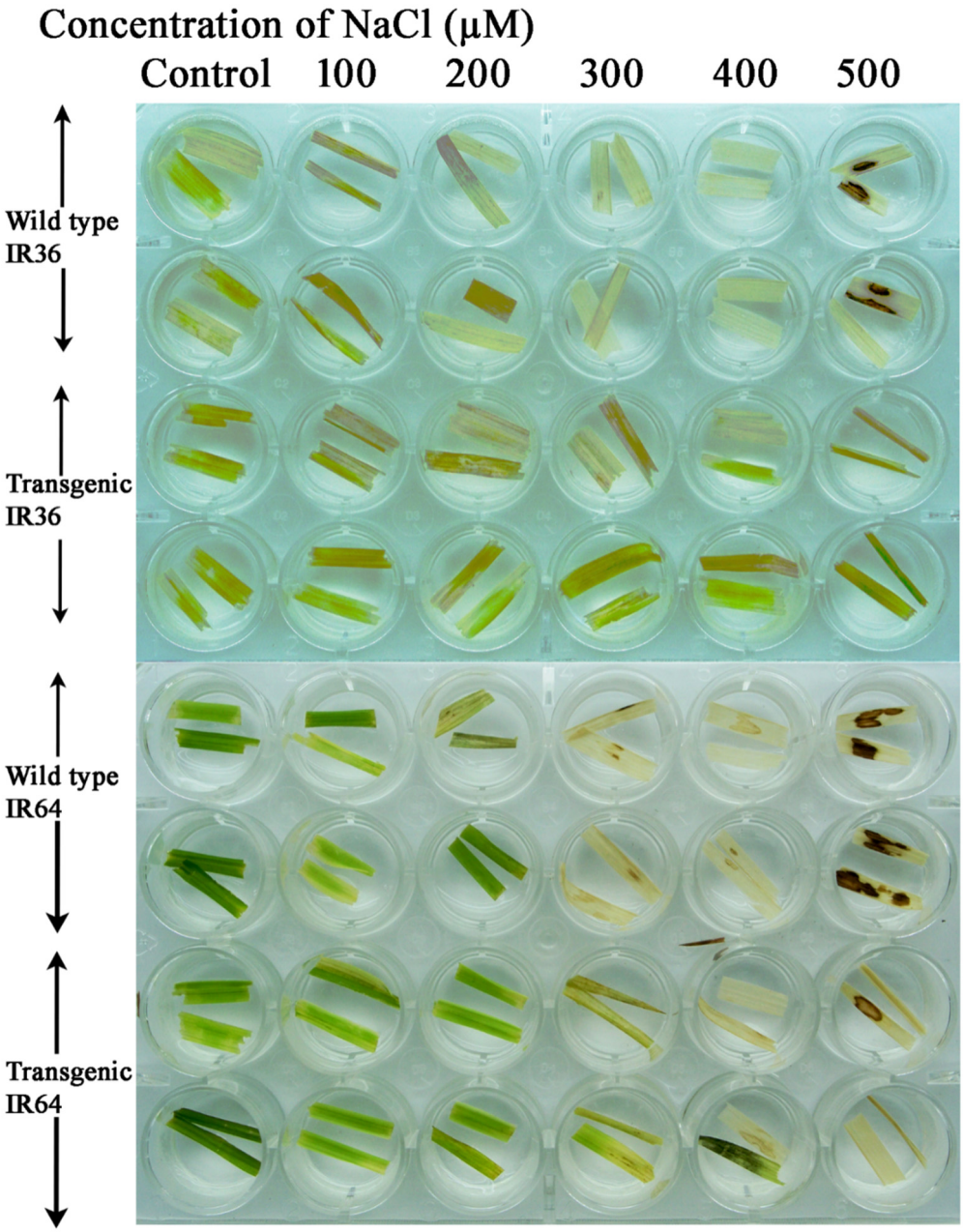

Figure 15. Leaf disc assay of IR36 and IR64 germinated plants. Increase in chlorophyll retention as the NaCl concentration (100–500 mM) increases. The chlorophyll retention and leaf wilting speeds are more in comparison with WT.

3.11. Thiobarbituric Acid Reactive Substances (TBARS) Assay

There was no significant difference in the level of lipid peroxidation between the WT and 100 mM treated T_1 (IR36 and IR64) progeny that had 0.35 ± 0.001 nmol/g F.Wt. of malondialdehyde (MDA). The MDA content showed a gradual increase from 0.4 to 0.6 ± 0.003 (Table 5) as the concentration of NaCl was increased, but the T_1 lines maintained their MDA value (0.36 to 0.41 ± 0.001). Correspondingly the observations correlated with the leaf disc assay, wherein bleaching of leaves occurred from an NaCl concentration 300 mM (Figure 15). IR64 (T_1 lines) showed a stable increase in the MDA content with

maximum wilting at 600–700 mM as the stress level was raised, while the WT showed leaf wilting and retention in chlorophyll withholding (Figure 15). IR36 (T_1 lines) were recessive till 500 mM stress while IR64 could withstand a stress of about 300 mM.

Table 5. TBARS assay.

NaCl Concentration (mM)	TBARS (nmol/g F.Wt.)			
	Wild Type (WT) IR36	Transgenic IR36 (T_0)	Wild Type (WT) IR64	Transgenic IR64 (T_0)
Control	0.4 ± 0.0	0.35 ± 0.001	0.5 ± 0.002	0.034 ± 0.016
100	0.4 ± 0.001	0.36 ± 0.001	8.9 ± 1.455	0.035 ± 0.016
200	0.4 ± 0.00 *	0.37 ± 0.001	0.7 ± 0.002	0.035 ± 0.016
300	0.5 ± 0.001 *	0.38 ± 0.0	7.7 ± 1.227	0.035 ± 0.016
400	0.5 ± 0.002 *	0.39 ± 0.001 *	0.6 ± 0.004	0.036 ± 0.016
500	0.5 ± 0.002 *	0.42 ± 0.002	8.2 ± 0.003	0.036 ± 0.016
600	0.6 ± 0.005	0.41 ± 0.001 *	0.7 ± 0.002	0.036 ± 0.017
700	0.6 ± 0.004	0.41 ± 0.0 *	6.7 ± 1.044	0.037 ± 0.017
800	0.6 ± 0.003 *	0.41 ± 0.001 *	0.8 ± 0.002	0.040 ± 0.018

Values represent mean ± Standard deviation; * indicates significance between control and stress treated column wise at the level of $p < 0.05$.

3.12. *OsiSAP8* Gene Expression Analysis

At a lower concentration of mannitol (100–200 mM) stress down regulation of *OsiSAP8* was observed by onefold in both IR64 T_1 and IR36 T_1 progenies. The transcript level was upregulated to 1.5-fold (IR36 T_1) at 300 mM and it peaked to 2 folds when treated at 400 mM mannitol dearth. IR64 T_1 progeny showed a fluctuating fold change at higher concentrations. At a higher concentration (500 mM) of mannitol, the transcript level downregulated by twofold (Figure 16a). A significant increase in the *OsiSAP8* transcript level of IR64 T_1 progeny was observed from one-fold to 2.5-fold when exposed to NaCl (100–300 mM). Down regulation (0.5-fold) of *OsiSAP8* transcript was observed in the IR36 T_1 progeny at 100 mM which gradually peaked by threefold at 300 mM salinity stress (Figure 16b). Both transgenic progenies (IR64 and IR36) showed a dearth (two-fold) in their *OsiSAP8* transcript level at 500 mM NaCl stress.

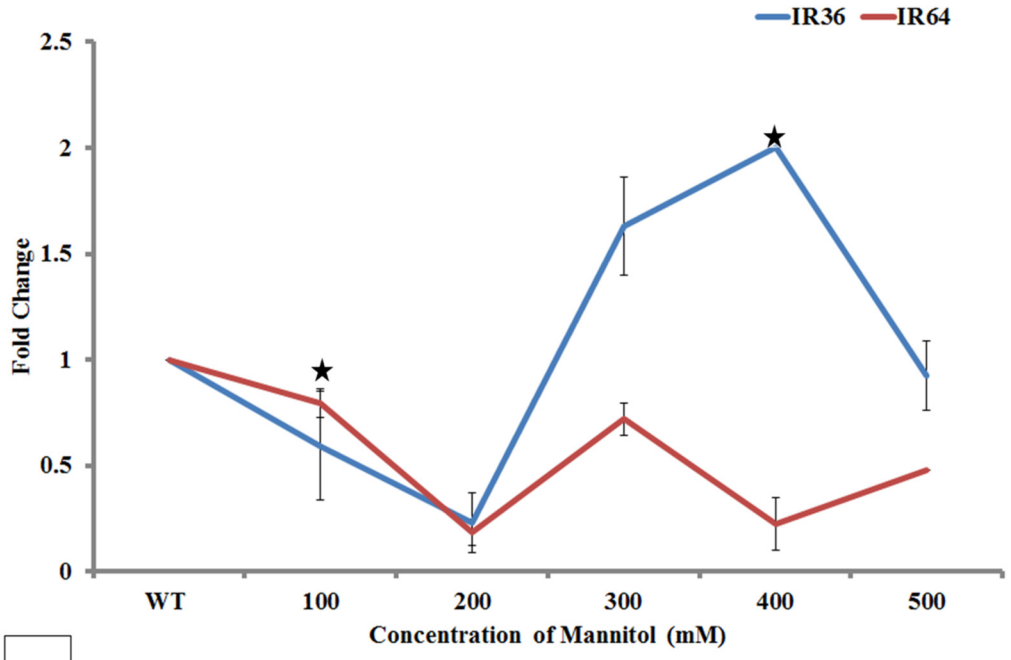

Figure 16. *Cont.*

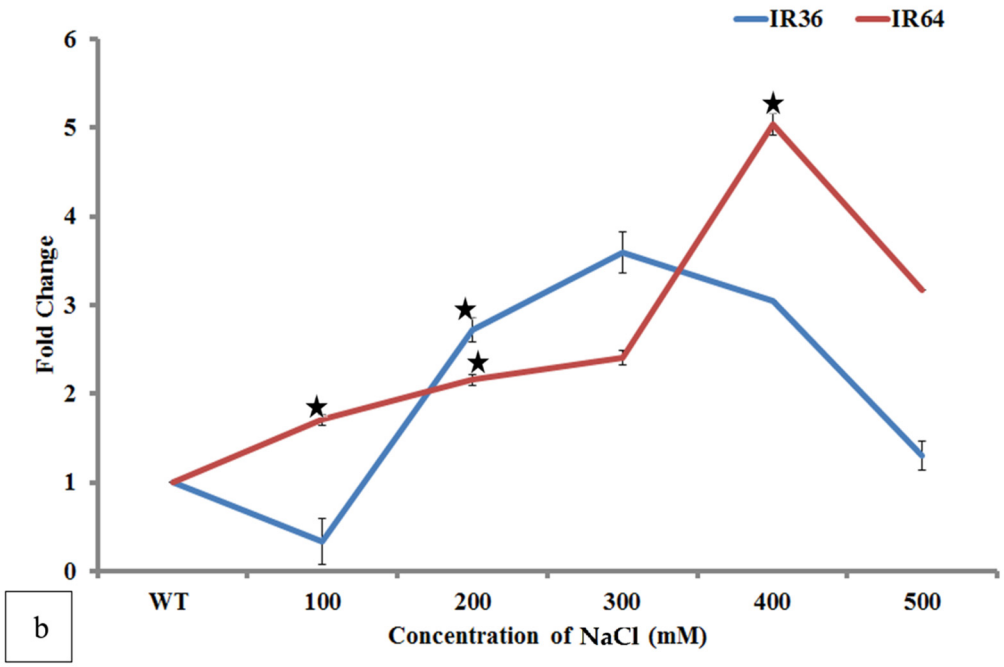

Figure 16. qPCR analysis for drought and salinity tolerance. (**a**) Gene expression analysis for drought tolerance. (**b**) Gene expression analysis for salinity tolerance. * Indicates significance at the level of $p < 0.5$.

4. Discussion

In silico prediction of OsiSAP8 upstream regulatory region revealed superiority of Port Ubi2.3 promoter as an abiotic stress regulator. In this study, a constitutive ubiquitin promoter *(Port Ubi2.3*) was used to drive the *OsiSAP8* gene. So as to have a comparative knowledge on the regulatory cis-elements, the SAP promoter was analyzed. In silico analysis of the 2.0 kb 5′ regulatory region revealed multiple cis-acting elements involved in the regulation of the *OsiSAP8* gene. The transcription start site (TSS) was predicted using PLACE and was assigned +1 site based on the TATA box position. Putative cis-elements like ARR1AT [59,74,75], which are most common for salinity stress regulation, were observed at 15 sites (in *Port Ubi2.3*) when compared to five sites in the *OsiSAP8* regulatory region. The MARTBOX elements were most predominant in *Port Ubi2.3* regulating scaffold attachment [60] and plays a major role as an enhancer and OsiSAP8 is devoid of it. The *OsiSAP8* promoter has WRKY71OS and GT1CONSENSUS elements that encode for WRKY, which are the largest families of TFs in higher plants, and WRKY genes are crucial protagonists in plant growth and react to environmental stresses [76–78]. These are DNA binding proteins that are present in a scattered style all over the *OsiSAP8* promoter regulatory region (Figure 1). Numerous annotated rice genome projects paved the way for the identification of members of diverse gene families that code different proteins and have been linked with the Rice Genome Annotation Project (RGAP) [2]. Pathogenesis-related cis-regulatory elements, SEBFCONSSTPR10A is present at the 524th region from the TSS encoding for pathogenesis-related gene, PR-10a. In plants overexpressing OsWRKY IIa, the PR10a gene is upregulated [79], which enhances the basal defense against *Xanthomonas oryzae* pv. Oryzae (Xoo). ROOTMOTIFTAPOX1 (ATATT) and RAV1AAT (CAACA) were prominent among the root specific cis-acting elements, reported to be an important factor in the root specific expression of PZmCBF3 [80], an AP2/ERF transcription factor family. The MYCCONSENSUSAT element is present in a wide range that combats multiple abiotic stresses and code for CBF/DREB1, originally identified in Arabidopsis, which plays a significant role in combating drought and salinity stress. The stress elements also have homologs, e.g., MYB and MYC, to combat abiotic stresses [81]. Heat shock factors (CCAAT box)/SE (Heat shock element) play a vital role in the regulation of proteins responsible for developmental processes [18,62] like flowering and is proximal to PRECONSCRHSP70A

that induces HSP70A gene. Heat induces the heat shock factors to respond and the heat shock elements do not have any organ specificity [82]. Conversely, the bZIP protein binding site has DPBFCOREDCDC3, a unique class of TF with DPBF-1 and 2 (Dc3 promoter-binding factor-1 and 2) binding core sequence expression that are normally embryo specific and can also be induced by ABA. The Port Ubi2.3 promoter has four bZIP protein binding sites under abiotic stress [61]. In addition to these regulatory elements, a large number of regulons are involved in pathogen-related stresses, such as WBOXATNPR1 (TTGAC) as DNA binding proteins and WRKY71OS, which are TGAC-containing W box elements within the pathogenesis-related class10 (PR-10) genes and regulate the gibberellin signaling pathway. GT1CONSENSUS (GRWAAW) regulons are reported to be found in *Pisum sativum*, *Avena sativa*, *O. sativa*, *Nicotiana tabacum*, *Arabidopsis thaliana*, and *Spinacia oleraceaare* putative *cis-acting* elements that control the GT-1 binding site in many light-regulated genes. GT1CONSENSUS and WRKY have been studied [78,83,84] to combat stress by binding of GT-1-like factors to the PR-1a promoter, influencing the level of salicylic acid (SA)-inducible gene expression. The *OsiSAP8* promoter has more light regulated and tissue specific *cis*-elements as compared with *Port Ubi2.3* with GT1 (GRWAAW) and GATABOX [63,64]. Similarly, tissue specific cis-elements DOFCOREZM (AAAG), CACTFTPPCA (YACT), and WBOX (TGACT) were recorded at numerous positions and they are responsible for signal responsive and/or tissue specific gene expression [67–69]. A total of 52 biotic stress (Plant pathogen responsive/interaction) *cis*-elements were predicted on *OsiSAP8* regulatory region with WRKY (TGAC) and EBOX (CANNTG) [71,72]. On the other side, Port Ubi2.3 promoter 16 cis-elements (CANNTG) specific to seed/stress, 10 water stress cis-elements (CNGTTR), and 12 TGAC elements act as good defense regulators [65,66,72]. Added to this, the presence of a whooping 32 NGATT enhancer elements makes it a better and more unique promoter then the others being used for monocot specific expression research. Ubiquitin promoters are known to enhance the transgene expression upon exposure to stresses [85–87]. Augustine et al. (2015a) reported that use of the *Port Ubi2.3* promoter could enhance the tolerance levels of transgenic sugarcane on drought and salinity stresses to a greater extent. This may be attributed to the high number of stress responsive cis-acting regulatory elements present throughout the sequence [40]. In addition, they also reported an enhanced *GUS* expression upon drought stress when *Port Ubi2.3* promoter was used [40].

OsiSAP8 is a member of the SAP gene family, characterized by the presence of the A20/AN1 domains and a class of the zinc-finger protein [28,88]. The inherent *OsiSAP8* in IR36 outwits IR64. It is a single-copy gene in WT IR36 which was overexpressed to resist abiotic stresses, drought, and salinity, thus validating the report by Kanneganti and Gupta (2008) [29]. *OsiSAP8* was absent in WT IR64, whereas the T_1 lines had one or two homologous copies. The hygromycin sensitivity assay of the T_1 lines reiterates the Mendelian segregation. The T_1 seeds (IR36 and IR64) were tested with hygromycin; those carrying a functional copy of *hpt* remained green, while those lacking the transgene or a functional form became bleached. The lines with more than one copy number of the *OsiSAP8* gene showed a slightly higher ratio (3 > :1) but were up to the expected χ^2 value.

Sharma et al. (2015) studied rice *OSiSAP7* in *A. thaliana* and inferred downregulation during abscisic acid stress and also sensitivity to water dearth [21]. In this study, *OsiSAP8* overexpression is observed to be resistant to water deficit and a saline environment. A 2.5-fold upregulation was shown by the transgenic IR36, which possesses the native *OsiSAP8* and also the transgene. Hence, comparatively, it had greater tolerance than the transgenic IR64. Earlier reports on the transcript level were performed on eight-day-old seedlings [28] within 2 h of salt and drought stress. To face the extreme saline environment, plants maintain ionic and osmotic balance, which could inhibit the normal metabolism of a plant and its productivity [89]. Our studies on the physiological parameters in 40-day-old rice plants correlated with that of Jongdee et al. (2002) [90]. Drought was imposed on both four-day old germinated seeds and 45-day-old acclimatized plants and the tolerance level was measured based on the visual scoring of leaf damage [51,52,91,92]. Visual scoring revealed that 40% of both IR36 and IR64 WT showed immediate leaf wilting symptoms.

Transpiration and the leaf surface texture play a candid role in the metabolism of a plant. The IR64 T_1 lines could withstand water deficit for two weeks and tillers were set on all the plants. IR36 WT could resist for up to a week upon drought after which leaf rolling and wilting appeared. The wildtype and T_1 transgenic lines showed a similar number of tillers and panicles, but the treated T_1 lines showed significant tiller setting, producing more panicles compared with WT. The stressed T_1 lines of IR36 had a greater average weight of the tiller per panicle. The lower the RLWC, the higher the transpiration and susceptibility to stress. The RLWC content in 80% of IR36 transgenic showed a tolerance to withhold stress up to the 7th day after water withhold; later 86% of the events had an average of 60–75% RLWC content, which were comparatively more that IR64 transgenic events. This validates the present data on the water withhold stress. The water deficit significantly reduced the leaf biomass in transgenic events, which was lower than WT [93,94]. A superior recovering ability was observed with plants having a larger leaf length at the end of the drought stress [93,94]. Moreover, biomass under stress correlates with the yield [95] and was used as an index for drought tolerance. The water withholding capacity and the total grain per tiller (13 g) indicates significantly higher stress tolerated by the IR36 T_1 lines. Thus, T1 progenies showed enhanced stress tolerance compared with WT These results show that *OsiSAP8* overexpression in transgenic rice have a profound increase in the abiotic stress tolerance compared with WT rice.

Thiobarbituric acid reactive substances are naturally existent in organic specimens and include lipid hydroperoxides and aldehydes, which increase in concentration as a response to oxidative stress [96]. Once lipid peroxidation is enhanced during salinity stress, the inhibition of plant growth is initiated. TBARS assay values are usually reported in malonaldehyde (MDA) equivalents, a compound that results from the decomposition of polyunsaturated fatty acid lipid peroxides [97]. A comparison of the lipid peroxidation levels during NaCl stress revealed that with increasing concentrations of NaCl, the MDA content increased. However, the IR64 (WT) showed a fluctuating MDA value and succumbed to leaf wilting. The salt stress tolerance of transgenic plants of T_1 generation in comparison with the WT plants was manifested by an increase in the shoot height, biomass production (data not shown), higher chlorophyll retention and a lower rate of lipid peroxidation on exposure to salinity.

Gene expression analysis is the next mandatory step to know the expression pattern under varied stresses which revealed that transgenic IR36 was better than IR64 during drought and salinity conditions [98]. Both the cultivars withstood a maximum of 400 mM (mannitol or NaCl) but the yield and the panicles count were comparatively more in IR36. Transcription factors (TFs) are dynamic elements to transduce any signal in a living body. Apart from the recognized zinc fingers, several new types of Zn fingers that are governed by key TFs [99,100] and are expressed differentially during reproductive, development and abiotic stresses were identified. Three housekeeping genes were used in this study. To our surprise, the elongation factor (eEF-1α), which is normally used as an internal housekeeping gene [56,101,102], varied during the gene expression analysis (data not shown) in the overexpressed IR36 but was absent in transgenic IR64 and wildtype. Wu et al. (2005) [103] reported that the subtraction library obtained from drought/cold stress-treated upland rice yielded a number of regulatory proteins [104]. eEF-1α was reported to provide significant catalytic activity in plants during abiotic stress [104]. In plants, the eEF-1α is induced by salinity, wounding, and low temperature [105–107], wherein α subunit of eEF-1α, plays a candidate role in the polypeptide chain elongation to deter abiotic stress. Many transcripts homologous with ribosomal proteins were upregulated in rice (IR29 and Pokkali) upon salt treatment for just 1 h [108]. Salinity tolerant [109] IR36, was more resistant and productive than IR64, which is salinity and drought sensitive [110]. Pokkali being resistant to salinity stress had a time precise synthesis of transcripts, whereas the salinity liable IR29 did not show any tolerance and it was inferred that the production of ribosomal proteins, and eEF-1α could have a role in reorganizing the protein synthesis

mechanism [111,112]. Despite being a house keeping gene, it also is reported to express constitutively during abiotic stresses like drought and salinity [29].

Crop plants unceasingly feel and react to their surrounding environment so as to protect themselves from abiotic and biotic abnormalities. Their stress response signaling involves numerous key promoters that are highly conserved and have evolved as promising targets to combat abiotic stress in a variety of crop plants [12–14]. During dehydration assays, the transgenic IR36 and IR64 had molecular changes but physiological changes such as leaf wilting were observed at a later stage compared with that of the wildtype correspondingly the eEF-1α varied in, gene expression. Drought induces dehydration, which instigates pathways related to desiccation. Griffiths et al. (2014) [113] reported a high level of eEF-1α expression in *Sporobolus stapfianus* leaves that were fully hydrated [114]. Rapid growth of the coleoptile is a prerequisite for successful establishment in a plant to access oxygen. The WT IR36 and IR64 formed coleoptile within 4 to 5 days after germination (Figure 4) when compared with the stress-exposed transgenic seeds on mannitol deprived of sucrose source. There was a significant difference between the transgenic IR36 and IR64. IR36, which has a native gene (*OsiSAP8*), was comparatively fastidious in developing a coleoptile and the same was validated by gene expression analysis. The coleoptile length and the gene expression have been correlated with stress tolerance.

To study the multiple stress-inducing characters of SAPs, transgenic plants that resist stress and yield more could be studied for the drought yield index (DYI) so as to screen the highly tolerant events. The DYI and mean yield index (MYI) has been studied from a group of 129 advanced rice yield lines, and 39 genomes (including IR64 and IR36) were tested [114]. This could lead to the selection of the superior cultivar and edify the signaling mechanism of abiotic stress resistance by the A20/AN1 zinc-finger domains of SAP proteins.

5. Conclusions

In order to achieve crop improvement, genetic transformation plays a pivotal role in the development of genetically engineered crops with desirable traits. Despite considerable efforts, the genetic transformation of rice remains a demanding process due to the variation in its reproducibility. Moreover, rice is highly susceptible to drought and salinity stress. In the present study, rice (var. IR36 and IR64) was transformed with the *OsiSAP8* gene driven by *Port Ubi2.3* promoter. Overexpression of *OsiSAP8* in IR36 delayed leaf wilting by maintaining RLWC during drought stress over its superior variety, IR64. The transgenic rice overexpressing zinc-finger protein driven by *Port Ubi2.3* showed reliable resistance and tiller setting than the WT. This study reiterates that *OsiSAP8* acts as key abiotic stress regulator in plants. Our investigation also provides a platform to unravel the *OsiSAP8* regulatory region, which will manifest an altered relationship between stress tolerance and its multiple stress inducible nature.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su141610174/s1. References [45,49,115–118] are cited in the supplementary materials.

Author Contributions: Conceptualization: S.R.K., A.M.P., A.K.G. and M.R.; data curation: S.R.K., P.M., A.M.P. and K.G.; formal analysis: S.R.K., P.M., M.I.P., K.G., C.M., K.B. and M.R.; funding acquisition: S.R.K., A.M.P. and M.R.; investigation: S.R.K., M.I.P., K.G., C.M. and M.R.; methodology: S.R.K., P.M., A.M.P., M.I.P., C.M. and K.B.; project administration; S.R.K., A.M.P., A.K.G. and M.R; resources: S.R.K., M.I.P., K.G., C.M., K.M. and M.R.; software: S.R.K., P.M., K.G. and K.M.; supervision: S.R.K. and M.R.; validation: S.R.K. and K.G.; visualization: S.R.K.; writing—original draft: S.R.K. and P.M.; writing—review & editing: S.R.K., P.M., K.G. and M.R. All authors have read and agreed to the published version of the manuscript.

Funding: Council of Scientific and Industrial Research (CSIR), New Delhi Grant No. 38(1315)/12/EMR-II dt 03/04/2012].

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Genbank Submission: NCBI Accession No. MN256433.1.

Acknowledgments: The authors gratefully acknowledge the financial assistance rendered by Council of Scientific and Industrial Research (CSIR), New Delhi The strains DH5α harboring the binary plasmid pCAMBIA 1305.1 with Port Ubi promoter were kindly granted by N. Subramonian, Emeritus Scientist, ICAR-Sugarcane Breeding Institute, Coimbatore. The strain LBA 4404 was kindly granted by P. Balasubramanian, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The authors are very much grateful to A. Ganapathi, (Former position—UGC-BSR Emeritus Faculty, Department of Biotechnology and Genetic Engineering, Bharathidasan University) for helping us in standardizing the Southern blot procedures to check the copy numbers of the transgene integrated. We are also thankful for the support rendered by R. Sathish Kumar (Department of Biotechnology, Bharathiar University, Tamil Nadu, India) for providing the Greenhouse facility for analyzing transgenic rice lines.

Conflicts of Interest: The authors declare that they have no conflict of interest.

Abbreviations

cDNA	Complementary DNA;
CTAB	Cetyl trimethyl ammonium bromide;
DW	Dry weight;
FW	Fresh weight;
MDA	Malondialdehyde;
RLWC	Relative leaf water content;
TBA	Thiobarbituric acid;
TW	Turgid weight;
WT	Wild type

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