Enhanced tolerance to salinity stress and ABA is regulated by *Oryza sativa STRESS ASSOCIATED PROTEIN 8 (OsSAP8)*

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Abstract

Increased tolerance to multiple abiotic stresses in plants is a key target for food security. *OsSAP8* is a member of the stress-associated protein (SAP) gene family in rice and is characterized by the presence of two zinc finger domains, A20 and AN1. In this research, we aim to understand the regulatory mechanism of *OsSAP8* in response to multiple stresses for rice survival. We used the Arabidopsis transformation system to understand the gene's function. Two Arabidopsis lines were used; *OsSAP8* overexpression line and Arabidopsis *atsap2* knockout loss-of-function mutation in comparison to wild type Columbia-0 (Col-0). The protein sequence of Arabidopsis *atsap2* has been shown to be highly identical to the *OsSAP8*. An *atsap2* knockout showed a sensitive phenotype to high concentration of NaCl and PEG with reduced germination percentage. *OsSAP8* overexpression showed a tolerance phenotype to 150 mM NaCl and 20% PEG compared to *atsap2*. *OsSAP8* also showed an insensitive germination phenotype to high concentration of ABA compared to Col-0 and *atsap2*. The insensitive phenotype of *OsSAP8* on the high concentration of ABA (12 μM) and the sensitive phenotype of its orthologue *atsap2* in Arabidopsis suggests that *OsSAP8* may have a role in part through ABA signaling as a putative transcriptional regulator for ABA during stress response.

Keywords: abiotic stress, Arabidopsis, *atsap2* gene, *Oryza sativa*, *OsSAP8* gene, seed germination.
Abbreviations: ABA_Abscisic acid; NaCl_Sodium chloride; PEG_Polyethylene glycol.

Introduction

Agricultural productivity is a major challenge due to the increasing world population and the associated high demand for food production (Godfray et al., 2010). Abiotic stresses have been identified as a major threat to the farmer as a result of reduced crop yields. These stresses include drought, salinity, flood, heat and cold, and are mainly related to global climate change and adversely affect crop productivity. High salinity in soil negatively impacts crop growth, development, yield quality and quantity due to an excess of Na^+ and Cl^- ions (Deinlein et al., 2014; Munns and Tester 2008; Munns, 2002). This occurs by impairing metabolic processes, osmotic content, nutritional balance in plants and decreasing photosynthetic efficiency (Tester and Davenport, 2003). Plants have specific mechanisms to retain resources for growth and development in response to environmental stress (Atkinson et al., 2015).

Genetic engineering has been used for agricultural improvement by producing crops that are resistance to stress. Transgenic approaches have been used widely in order to understand the mechanism of plant responses to abiotic stress (Vij and Tyagi, 2007; Cattivelli et al., 2008). This study has employed the genetic engineering approach to understand the molecular mechanism underlying abiotic stresses. The Stress Associated Protein (SAP) family, which is known to have an important role in multiple abiotic stress response in plants, comprises 18 and 14 gene members that have been identified in *Oryza sativa* and *Arabidopsis thaliana* genomes, respectively. One of the members from the rice SAP gene family, *OsSAP8* has been identified to be differentially up-regulated in our salt-stress subtractive hybridization (SSH) library (Hedayati et al., 2015). *OsSAP8* is well known as having multiple stress inducible genes. These stresses include heat, cold, salt, desiccation, submergence, wounding and heavy metals (Kanneganti and Gupta, 2008). *OsSAP8* is characterized by the presence of two types of zinc finger domains, A20 and AN1. The A20 domain is embedded in the N-terminal of the coding region whilst the AN1 domain is embedded near the C-terminal of the coding sequence (Martin et al., 2012).

Abscisic acid (ABA) is a major plant phytohormone involved in stress response. Previous studies have dissected the core signaling pathway for ABA including the receptors and all other components (Nambara and Marion-Poll, 2005; Nambara et al., 2010). Several different classes of ABA receptors have been reported. PYRABACTIN RESISTANCE 1 (PYR1) was identified as an ABA receptor through a chemical genetic approach (Park et al., 2009). The Arabidopsis genome encodes 13 genes that have similarity to

PYR1, which were named PYR1 LIKE-13 (PYL1-13). These genes are alternatively named REGULATORY COMPONENTS OF ABA RECEPTOR (RCARs) (Ma et al., 2009). All PYR1/PYL1-PYL13/RCARs belong to the same gene family in Arabidopsis (Umezawa et al., 2010; Park et al., 2009; Ma et al., 2009). Protein phosphatase 2C (PP2C) is a member of the protein serine/threonine phosphatases. PP2C has been shown to have important roles in ABA signaling, acting as a negative regulator. ABSCISIC ACID INSENSITIVE1, 2 (ABI1, ABI2), which were identified based on their insensitivity to ABA have been shown to encode paralogous PP2Cs (Leung et al., 1997; Meyer et al., 1994; Leung et al., 1994). Positive regulators of ABA signaling which act downstream of the PP2Cs have been identified as SNF-1 related protein kinase 2s (SnRK2s) (Nambara et al., 2010 & Umezawa et al., 2010). There are 10 SnRK protein kinases in Arabidopsis, divided into 3 subclasses (Kobayashi et al., 2004). Each subclass has a different response to a different abiotic stress. Subclass I SnRK2s for example are strongly activated by an osmotic stress but they are not activated by ABA, whilst Subclass II and III are activated by both osmotic stress and ABA and only Subclass III has a strong response to ABA (Umezawa et al., 2010). This finding has encouraged many researchers to explore more the relationship between ABA and other potential transcriptional regulators in response to environmental stresses.

In this study, we provide an understanding of *OsSAP8* regulation under different stress conditions including ABA. Overexpression of *OsSAP8* resulted in tolerance to high concentrations of NaCl compared to its knockout mutant *atsap2*; this result was observed through its high germination score, assessed on day 7. Interestingly, *OsSAP8* showed an insensitive phenotype to high concentrations of ABA up to 12 μM compared to the knockout mutant *atsap2* and wild type Col-0. This suggests that *OsSAP8* may play a role as a putative transcriptional regulator of ABA during plant response to abiotic stress. However, more studies need to be performed to determine the relationship between *OsSAP8* and ABA signaling.

Results

Analysis of OsSAP8 positive transformants and atsap2 mutants

Seeds harvested from transformed plants were screened on 150 mg/ml BASTA. Surviving plants were positive transformants as they carry the pB2GW7-*OsSAP8* construct. PCR on the genomic DNA showed fragments with a size of ~519 bp which indicates the presence of *OsSAP8* in two out of four transformed lines (Fig. 1). Verification of *atsap2* knock-out mutant line was confirmed through semi-quantitative RT-PCR. We verified that this homozygous knock-out mutant has a null transcription by semi-quantitative RT-PCR. The semi-quantitative RT-PCR result showed that there is no transcript expression of *atsap2* identified in all the *atsap2* knock-out mutant plants (Fig. 2), while only Col-0 showed the expression of *atsap2* with a size of 288bp.

Germination of OsSAP8 overexpression seeds is more tolerant to NaCl

In order to understand the role of *OsSAP8* in regulating salt-stress, germination analysis of *OsSAP8* at different NaCl concentrations was carried out. Seeds used in this experiment

were after-ripened. A controlled experiment was performed on water agar media to observe seed viability for both seeds used in NaCl and PEG tests (Figs. 3 and 5). Germination response revealed that *OsSAP8* seeds were tolerant to NaCl up to 150 mM, with 98% of seeds being germinated compared to 83% for Col-0 (Fig. 3). In fact, *OsSAP8* showed rapid germination on all media supplemented by NaCl. This can be observed on day 1, where seeds of *OsSAP8* germinated almost 90% on media supplemented by up to 75 mM NaCl, whilst a fast germination rate can be observed on day 2 for media supplemented with 150 mM NaCl compared to *atsap2* and Col-0 (Fig. 3). Instead of *OsSAP8*, we also carried out a germination test on *atsap2* T-DNA knockout seeds. *atsap2* is the orthologue gene of *OsSAP8* in Arabidopsis (Fig. 4). Both *OsSAP8* and *atsap2* consist of the same zinc finger domain A20 and AN1. To investigate whether the presence of Stress Associated Protein is important during salt-stress, we carried out the same germination test on *atsap2* knockout mutants. Results showed that *atsap2* germination is impaired on media supplemented with 150 mM NaCl, with 47% germination score compared to wild type Col-0, 83%. This indicates that in the knockout *atsap2*, the expression of Stress Associated Protein2 is completely abolished.

Other than salt-stress, we also carried out research on PEG osmotic stress. This was performed using various PEG-6000 concentrations (10%, 20%, 40%, 50%) and water agar media as control experiment. Both *OsSAP8* and *atsap2* seeds were plated onto various concentrations of PEG, with Col-0 acting as control. Germination curves showed that *OsSAP8* seeds were germinated more rapidly than *atsap2* and Col-0 on 10% and 20% PEG-6000, which can be observed on day two (Fig. 5). Overall, *OsSAP8* showed rapid germination but as the imbibition day prolonged to day seven, germination percentage for both *OsSAP8* and Col-0 did not significantly differ (Fig. 5). However, germination percentage for *OsSAP8* was much higher compared to *atsap2* in all media supplemented with PEG (Fig. 5). Both results from NaCl and PEG indicate that overexpression of *OsSAP8* enhanced seeds' tolerance to salt but not osmotic stress, whilst the knockout mutant seed of *atsap2* showed a susceptible phenotype to both stresses.

Germination of OsSAP8 overexpression seeds is ABA insensitive

To investigate whether ABA affected germination potential in *OsSAP8* and *atsap2*, a germination assay was carried out at different ABA concentrations over seven days. *atsap2* and Col-0 germination were relatively sensitive at 12 μM ABA with 16% and 30% seed germination, respectively, while *OsSAP8* showed an insensitive germination phenotype on 12 μM ABA with 84% seed germination (Fig. 6). Besides, *OsSAP8* showed a faster germination rate compared to *atsap2* and Col-0 on all media supplemented with different ABA concentrations (2 μM, 5 μM, 7 μM, 10 μM and 12 μM) (Fig. 6). This indicates that the overexpression of *OsSAP8* gene in Arabidopsis made the seed insensitive to high concentrations of ABA, while the knockout version of *atsap2* showed a sensitive germination phenotype to ABA (Fig. 6).

Rice abiotic stress co-expression network for OsSAP8

Further understanding in terms of how *OsSAP8* relates to other abiotic stress genes is important. This allows us to interpret the regulatory mechanism of *OsSAP8* in relation to other genes during stress situations. A co-expressed network

Table 1. List of Co-expressed genes, Table representing six genes co-expressed with *OsSAP8*. Detail of the Rice ID is shown in the table with the gene annotation for each.

Rice ID	Annotation
LOC_Os05g48800	drought induced 19 protein, putative, expressed
LOC_Os04g57220	ubiquitin-conjugating enzyme, putative, expressed
LOC_Os11g43900	translationally-controlled tumor protein, putative, expressed
LOC_Os07g34589	translation initiation factor SUI1, putative, expressed
LOC_Os12g43600	RNA recognition motif containing protein, expressed
LOC_Os03g46770	RNA recognition motif containing protein, expressed

Fig 1. 35S::*OsSAP8* positive transformants were screened via PCR. Agarose gel electrophoresis of polymerase chain reaction (PCR) shows the presence of full length sequence of *OsSAP8* (~519 bp) in overexpression *Arabidopsis thaliana* transgenic plants (lane OE2 and OE4). Ladder indicates 100bp ladder, -VE indicates negative control, WT indicates genomic DNA of wild type (Col-0) and OE indicates overexpression lines.

Fig 2. Verification of *atsap2* expression in T-DNA mutant lines. Expression of *atsap2* was confirmed by semi-quantitative reverse transcriptase PCR (RT-PCR). Reverse-transcription PCR result shows that *atsap2* only expressed in Col-0 indicated by one single band with a size of 288bp, but not in all T-DNA knockout mutant lines. M indicates 100bp marker, WT indicates wild-type Col-0 and KO indicates T-DNA knockout mutants (KO3, KO4, KO7, KO10).
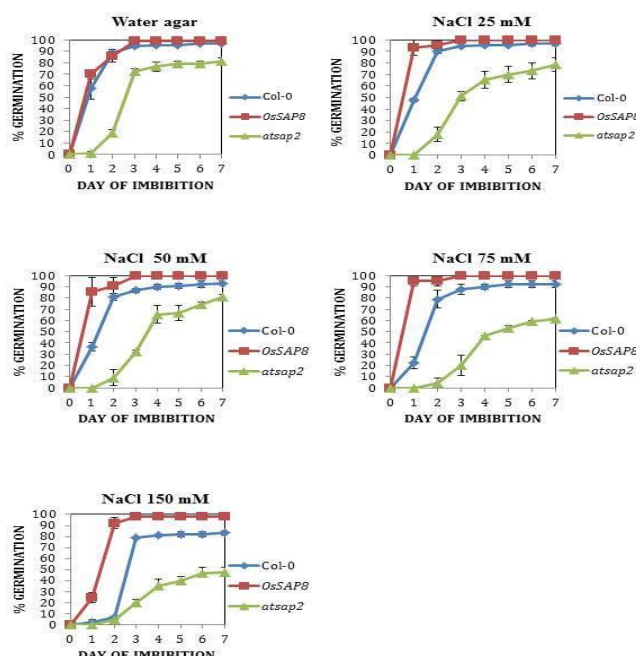

Fig 3. Germination analysis was carried out for Col-0, *OsSAP8* and *atsap2* in response to different concentrations of NaCl. The graph shows the effect of different NaCl concentrations (25 mM, 50 mM, 75 mM and 100 mM) on the germination of Col-0, *OsSAP8* and *atsap2* seeds over seven days. Water agar was used as a control experiment to observed seed viability. *atsap2* showed a sensitive germination phenotype to high concentrations of NaCl up to 150 mM compared to Col-0 and *OsSAP8*. *OsSAP8* showed a tolerant phenotype to high concentrations of NaCl and rapid germination rate on day 1 in media supplemented with 25 mM, 50 mM and 75 mM compared to *atsap2* and Col-0.

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OsSAP8    MEHKETGCQQ-PEGPILCINNCGFFGSAATMNMCSKCHKEMIMKQEQAKLAASSIDSIVN     59
atsap2    MDHDKTGCQSPPEGPKLCTNNCGFFGSAATMNMCSKCHKDMLFQQEQGAKFASAVSGTSS     60
          *:*.:****. **** ** ********************:*::***.   **::..  .


OsSAP8    GGDSGKEPIIAGHAEVAVAQVEVKTLVAQPAEIAGPSEGV-TVNPKGREGPNRCSTCRKR     118
atsap2    SSNIIKETFTAALVDIETKSVEPMTVSVQPSSVQVVAEVVAPEEAAKPKGPSRCTTCNKR     120
          ..:   ** : *. .:: . .**   *: .**:.:   :* *   :   :**.**:**.**


OsSAP8    VGLTGFNCRCGNLYCAMHRYSDKHDCQFDYRTAARDAIAKANPVVKAEKLDKI     171
atsap2    VGLTGFKCRCGSLFCGTHRYADVHDCSFNYHAAAQEAIAKANPVVKAEKLDKI     173
          ******:****.*:*. ***:* ***.*:*::**::*****************
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Fig 4. Protein sequence alignment for both *OsSAP8* and *atsap2* was carried out using Clustal Omega software. Highlighted in yellow is the A20 domain, while highlighted in blue is the AN1 domain. These two domains are highly conserved in both *OsSAP8* and *atsap2* protein sequences. These two zinc-finger domains were identified to be involved in stress response.
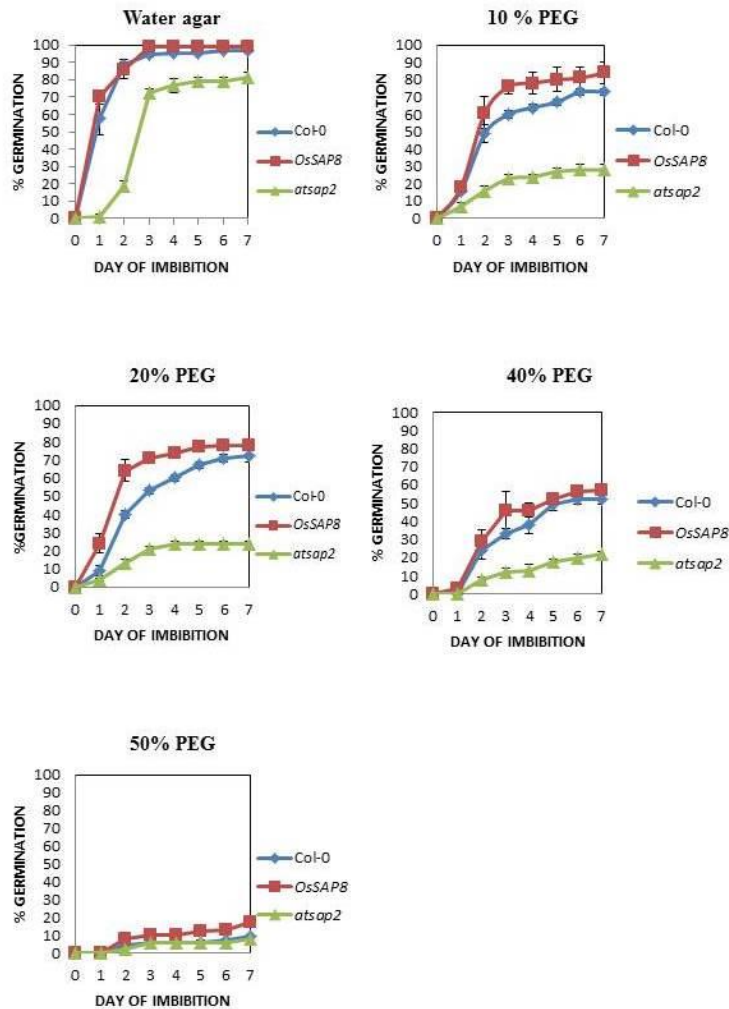

Fig 5. Germination analysis was carried out for Col-0, *OsSAP8* and *atsap2* in response to different concentrations of PEG. The graph shows the effect of different concentrations of PEG (10 %, 20 %, 40 %, 50 %) on the germination of Col-0, *OsSAP8* and *atsap2* seeds. Water agar was used as a control experiment to observed seed viability (the same seed batch as used in the NaCl experiment). *atsap2* showed a sensitive germination phenotype to all concentrations of PEG (10 %, 20 %, 40 %,50 %) compared to Col-0 and *OsSAP8*. *OsSAP8* showed a tolerant phenotype to 10 %, 20 %, 40 % concentrations of PEG, though this was not significantly different to Col-0.
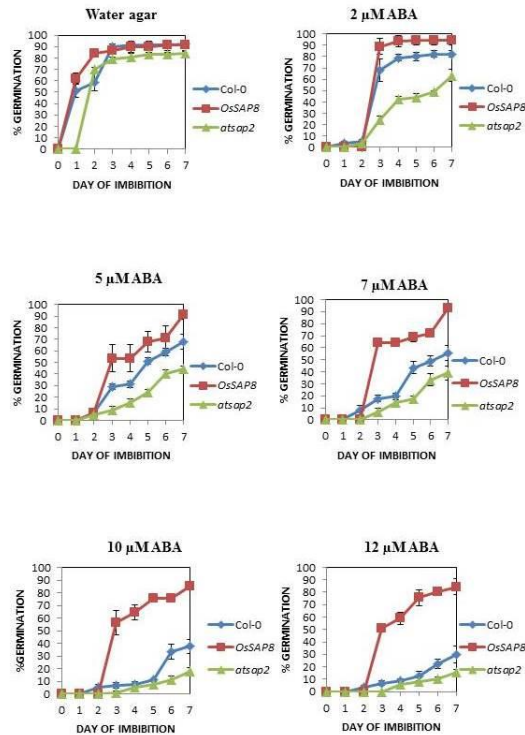
Fig 6. Germination analysis was carried out for Col-0, *OsSAP8* and *atsap2* in response to different concentrations of ABA. The graph shows the effect of different concentrations of ABA (2 µM, 5 µM , 7 µM , 10 µM, 12 µM) on the germination of Col-0, *OsSAP8* and *atsap2* seeds. OsSAP8 showed an insensitive phenotype to ABA up to 12 µM concentration, whilst the *atsap2* showed a sensitive phenotype to ABA as low as 2 µM ABA. Water agar was used as a control experiment to observe seed viability. *OsSAP8* showed an insensitive phenotype to high concentrations of ABA up to 12 µM compared to Col-0 and *atsap2*.
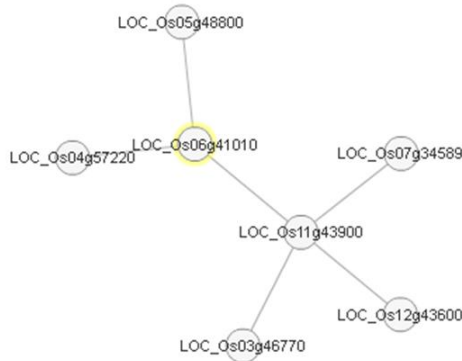

Fig 7. Rice Abiotic Stress Co-expression Network. The co-expression network was carried out using the Rice Oligonucleotide Array Database with the correlation coefficient cutoff at 0.8 and the depth to search co-expressed genes set at 2. The co-expression network result shows 7 nodes with 6 internodes associated with *OsSAP8*. LOC_Os06g41010 is the *OsSAP8* Rice gene Identification (ID).

was built using the Rice Oligonucleotide Array Database available online (Cao et al., 2012). There were six genes that were found to be differentially co-expressed with *OsSAP8* during abiotic stress (Fig. 7, Table 1). These genes were divided into two distinct clusters, with one cluster directly co-expressed with *OsSAP8* and another co-expressed through one node represented by gene encoded translationally-controlled tumor protein, putative (LOC_Os11g43900). Among these, one of the genes was drought induced 19 proteins, putative (LOC_Os05g48800). Ontological analysis showed that drought induced 19 proteins; putative plays a role in response to stress and abiotic stimuli. Another gene that was directly co-expressed with *OsSAP8* during stress was the ubiquitin-conjugating enzyme, putative

(LOC_osg0457220). This gene is known as E2 enzyme, and plays a role as a catalyst in the covalent attachment of ubiquitin to target proteins. Another gene that was connected to *OsSAP8* encodes translationally-controlled tumor protein (TCTP), which is co-expressed together with three other genes: translation initiation factor SUI1, putative, expressed (LOC_Os07g34589) and RNA recognition motif containing protein, expressed encoded by two different gene IDs (LOC_Os12g43600 and LOC_Os03g46770).

Discussion

In this paper, we describe the role of *OsSAP8* in response to multiple abiotic stresses (NaCl and PEG) including ABA. We

show that the overexpression of *OsSAP8* enhanced tolerance to salinity stress stimulus (NaCl) compared to its knockout version *atsap2*. *OsSAP8* also showed rapid germination rate compared to Col-0 and *atsap2* on media supplemented with different concentrations of NaCl. However, as the imbibition day prolonged to day seven, germination percentage for both *OsSAP8* and Col-0 did not significantly differ. This may be due to the plant recovery process and stress condition adaptation. Plant abiotic stress mechanisms consist of very complex processes, which include the plant structure itself, post-transcriptional and post-translational modifications, epigenetic regulation, metabolic processes and functional proteins such as molecular chaperons and heat-shock proteins (Hirayama and Shinozaki, 2010; Ruibal et al., 2013). Exposing the plant to a very long stress condition may somehow activate different stress regulatory mechanisms to cope with the situation. In contrast to NaCl, analysis on germination potential of *OsSAP8* in response to PEG showed a similar germination curve to Col-0. This may indicate that the *OsSAP8* function is more specific during salinity stress compared to osmotic stress. This result also supports our previous salt-stress subtractive hybridization (SSH) library (Hedayati et al., 2015), which shows that this gene is differentially up-regulated in response to salinity stress stimuli. However, this observation is different in *atsap2*, where an increase in the concentration of NaCl and PEG resulted in reduced germination. In the absence of *atsap2* function, the seed was totally impaired in response to stress, showing that the SAP family gene is important in the regulation of abiotic stresses. This might be due to the presence of two zinc finger domains, A20 and AN1. *In-silico* analysis showed that both *OsSAP8* in rice and *atsap2* in Arabidopsis consist of two zinc finger domains A20 and AN1, respectively. Both domains have been shown to be important during abiotic stresses in several plant species (Paul and Kumar, 2015; Zhang et al., 2015; Giri et al., 2011). Interestingly, these domains also have been shown to be involved in the regulation of plant response to abiotic stresses by modulating the Abscisic acid (ABA) and Giberellin (GA) contents and their signal transduction pathway (Zhang et al., 2015). In the case of abiotic stress in plants, endogenous ABA level will increase to provide a tolerance mechanism against stress. For example, elevated ABA during stress is purported to control stomatal aperture and prevent excessive water loss. This mechanism ensures that plants have adequate water for survival, especially during drought. Ethylene is also another plant hormone that regulates plant response to abiotic stress, besides having a role in the regulation of seed germination in conjunction with ABA (Jun Tao et al., 2015; Linkies et al., 2009).

The co-expression network also showed that several genes connected to *OsSAP8* are important for allowing plants to cope with abiotic stresses. One such gene is the ubiquitin-conjugating enzyme, putative (LOC_osg0457220). Previous studies have shown that the ubiquitin pathway also has a role in the regulation of plant response to stress (Stone, 2014). The proteolysis event is part of the mechanism that regulates the expression of certain genes or protein activity which are involved in plant stress response. Other than that, the gene encodes for translationally-controlled tumor protein (TCTP), which is also found in the network. This gene has been identified to be involved in calcium binding and microtubule stabilization. Previous studies have shown that calcium ions (Ca2+) have an important role during abiotic stress. The Ca2+ has been shown to be involved in the abiotic stress signaling cascade as a secondary messenger. The presence of calcium enhanced plant response to stress stimuli therefore

triggering the activation of transduction pathways and downstream target genes (Das and Pandey, 2010; Tuteja and Sopory, 2008).

An interesting finding involved germination media supplemented with different concentrations of ABA. The overexpression of *OsSAP8* showed an insensitive germination phenotype to high concentrations of ABA, while the *atsap2* loss-of-function mutant showed a sensitive phenotype to ABA. This sensitive phenotype can be observed on media supplemented with ABA concentration as low as 2 μM. A previous study has shown that ABA plays an important role to prevent seed germination and maintain seeds in a dormant state, while GA acts to antagonize ABA in the regulation of seed dormancy to germination (Bewley, 1997; Kucera et al., 2005). The ABA signaling pathway components have been identified via several experiments. One of the components is a negative regulator of ABA signaling which is PROTEIN PHOSPHATASE 2C (PP2C). This component acts by inhibiting the activity of protein kinase (SnRK2) (Nambara et al., 2010). PP2C is a negative regulator of ABA signaling. In the absence of ABA, this component acts by inhibiting the downstream target, therefore inactivating the ABA response. However, in the presence of ABA, the PP2C becomes inactive and releases the SnRK2 to phosphorylate the downstream target for ABA activation genes to be functional. Loss-of-function mutation of PP2C showed a sensitive phenotype to high concentration of ABA. Another report has also shown that overexpression of PP2C like protein in rice, *OSPP108* had an insensitive phenotype to ABA (Singh et al., 2015). This insensitive phenotype in the overexpression line is similar to that of our overexpression *OsSAP8* phenotypically. However, the sequence comparison between *OsSAP8* and PP2C like protein in rice, *OSPP108* does not share any significant similarity in the protein sequences. However, the result of the ABA sensitivity test shows that the *OsSAP8* and its orthologues *atsap2* may have a role in ABA signaling during germination and also in response to stress. More experiments are required to confirm the relationship between *OsSAP8* and ABA.

Materials and methods

Bioinformatics analysis

The co-expression network analysis of *OsSAP8* was performed using the Rice Oligonucleotide Array Database. Gene ontology enrichment analysis was carried out via the Gene Ontology Consortium. Protein sequence alignment was performed using Clustal Omega software, available at EBI-EMBL.

Plant material and stress treatment

Oryza sativa MR219 were obtained from the Malaysian Agricultural Research and Development Institute (MARDI). The rice seeds were immersed in water for two days before being sown on soil and grown in a glasshouse for two weeks to reach the seedling stage. The rice seedlings were then treated with 150 mM NaCl to mimic salinity stress in soil. This amount of NaCl is enough to induce stress in MR219 and to enhance the expression of *OsSAP8* in MR219 rice samples for gene isolation (Hedayati et al., 2015). Three replicate samples were collected and immediately frozen in liquid nitrogen for RNA extraction preparation. The gene ID for the *OsSAP8* gene in the rice genome database is LOC_Os06g41010.

Another plant used in this experiment was the T-DNA mutant Arabidopsis line. The gene mutation in Arabidopsis is an orthologue to *OsSAP8*, which is *atsap2*. *atsap2* T-DNA knockout seeds (SALK_008677C) from homozygous lines were ordered from Nottingham Arabidopsis Stock Centre (NASC). The AGI code for the *AtSAP2* gene in Arabidopsis is AT1G51200.

RNA extraction, DNase digestion, and cDNA synthesis

RNA was extracted using TRIzol reagent as described by the manufacturer (Life Technologies). Ethanol precipitation was carried out to further purify the RNA. DNase digestion was carried out using a TURBO DNase Kit (Ambion). RNA was converted to cDNA using a First Strand cDNA Synthesis Kit (Maxima). cDNA were then stored at -80°C.

Amplification of OsSAP8 and cloning

PCR was performed with Q5 High Fidelity DNA Polymerase using the cDNA template. The following primer was used for PCR:
*OsSAP8*_F:5'-GGGGACAAGTTTGTACAAAAAAGCAGG CTTCATGGAGCACAAGGAGACT-3' and *OsSAP8*_R: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT CCTAAATTTTGTCAAGTTTCTCC-3'.
The full sequence of *OsSAP8* was cloned into Gateway vectors using BP and LR Clonase (Gateway Cloning Technology), as described by the manufacturer. Using BP clonase, donor vector, pDONR221 was used to generate entry clone harbouring *OsSAP8*. Entry clone was transformed into competent *E.coli* Top10 using the heat-shock method. Selection was carried out using 50 μg/ml Kanamycin and colony PCR. The next cloning step was carried out using the LR clonase, plasmid DNA from positively transformed *E. Coli* were cloned into the destination vector, pB2GW7 under 35S CaMV promoter (35S::*OsSAP8)* (Liang et al., 2013). The expression clone was transformed into *E.coli* Top 10 using the heat-shock method. Selection was carried out using 75 μg/ml Spectinomycin. Expression clones harbouring *OsSAP8* were confirmed using PCR and sent for sequencing. Expression clones harbouring *OsSAP8* were then transformed into *Agrobacterium tumefaciens* strains GV3101 prior to Arabidopsis transformation. Underlined in the primer sequences were gateway adaptor sequences for the gateway cloning system.

Agrobacterium-mediated transformation in Arabidopsis

Agrobacterium tumefaciens harbouring the 35S::*OsSAP8* construct were transformed into *Columbia-0* via the floral-dip method (Zhang et al. 2006). Bacterial culture was supplemented with 5% sucrose and 0.05% silwet-77 prior to inflorescence dipping.

Screening of OsSAP8 positive transformant

T_1 seeds were screened for positive transformants with 150 mg/ml BASTA. Genomic DNA was extracted from positive transformants (T_1) and a PCR reaction was performed to validate the presence of *OsSAP8*. The following primer was used for the PCR: 35S_F: 5'- TCCCACTATCCT TCGCAAGACC -3' and *OsSAP8*_R: 5'-CTAAATTTTGTC AAGTTTCTCC-3'. Seeds from T_1 plants were then collected and sown on soil until T_3 seeds were obtained. T_3 seeds were used for functional studies.

Semi-quantitative RT-PCR of atsap2 mutants

Validations of *atsap2* expression were confirmed by semi-quantitative RT-PCR. Total RNA was extracted using the QIAGEN RNeasy Plant Mini Kit and converted into cDNA using the First Strand cDNA Synthesis Kit Maxima. Reverse Transcriptase PCR (RT-PCR) was done by using these primers; *atsap2*_F: 5'-CTGCGGTTTCTTTGGAAGCG-3', *atsap2*_R: 5'-CTTGGTCCCTTTGGTTTCGC-3'.

Germination test

Seeds of *OsSAP8*, *atsap2* and wild-type were plated onto water agar media (for control) and for each of the treatments (NaCl, PEG, ABA). These ½ MS media contained 0.4 g MES, 2.2 g Murashige and Skoog, 5 g sucrose and 3 g gelrite and were adjusted in a range of pH 5.7 to 5.8. The seeds were incubated in the dark at 4^0C for 4 days. After incubation, seeds were kept at 25^0C and germination rates were assessed for 7 days. The rupture of the micropylar endosperm is considered as germination of *Arabidopsis thaliana* seeds.

Conclusion

Here, we have shown that regulating the expression of rice *STRESS ASSOCIATED PROTEIN 8 (OsSAP8)* can alter tolerance to multiple abiotic stresses. Impairment in the gene's expression led to an increased susceptibility of the plant to multiple abiotic stresses including ABA. This might be related to the two zinc finger domains embedded in the *OsSAP8* sequence and its orthologue, *atsap2*. These two domains have been characterized and shown to regulate plant abiotic stress response. We also showed that enhanced expression of *OsSAP8* gene increased seed insensitivity towards ABA and vice versa. It is interesting to look at the relationship between *OsSAP8* and ABA in the regulation of environmental stress, as ABA is a major plant hormone involved in abiotic stress response.

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