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Research Article

SALT AND ABA RESPONSE ERF1 improves seed germination and salt tolerance by repressing ABA signaling in rice

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Abstract

Rice (*Oryza sativa*) germination and seedling establishment, particularly in increasingly saline soils, are critical to ensure successful crop yields. Seed vigor, which determines germination and seedling growth, is a complex trait affected by exogenous (environmental) and endogenous (hormonal) factors. Here, we used genetic and biochemical analyses to uncover the role of an APETALA2-type transcription factor, SALT AND ABA RESPONSE ERF1 (OsSAE1), as a positive regulator of seed germination and salt tolerance in rice by repressing the expression of *ABSCISIC ACID-INSENSITIVE5* (*OsABI5*). *ossae1* knockout lines exhibited delayed seed germination, enhanced sensitivity to abscisic acid (ABA) during germination and in early seedling growth, and reduced seedling salt tolerance. *OsSAE1* overexpression lines exhibited the converse phenotype, with increased seed germination and salt tolerance. In vivo and in vitro assays indicated that OsSAE1 binds directly to the promoter of *OsABI5*, a major downstream component of the ABA signaling pathway and acts as a major regulator of seed germination and stress response. Genetic analyses revealed that *OsABI5*-mediated ABA signaling functions downstream of *OsSAE1*. This study provides important insights into OsSAE1 regulation of seed vigor and salt tolerance and facilitates the practical use of OsSAE1 in breeding salt-tolerant varieties suitable for direct seeding cultivation.

Introduction

Rice (*Oryza sativa*) is a staple crop for half of the world's population, and rice production is critical for ensuring global food security. To ensure grain yield and quality, rice seeds must be vigorous (Gommers and Monte, 2018). In the agricultural production, seeds with high vigor will behave the characteristics of speed and uniform germination and vigorous seedling growth (Chen et al., 2019). As a crucial developmental step for continuing the plant life cycle, seed germination, and seedling establishment are complex processes precisely regulated by environmental stimuli and phytohormones (He et al., 2019, 2020; Xu et al., 2020; Wang

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et al., 2020a, 2020b). Dissecting the key genes involved in seed vigor and elucidating the underlying mechanism will facilitate rice breeding.

The phytohormone abscisic acid (ABA), which is considered a stress hormone, plays pivotal roles in abiotic stress tolerance and seed germination in plants (Tang et al., 2016; Ma et al., 2018, 2019; Zhao et al., 2020a, 2020b). Mutation or overexpression (OE) of genes involved in ABA biosynthesis or signaling pathway often results in abnormal germination and stress-tolerant phenotypes (Tang et al., 2012; Liu et al., 2014; Zong et al., 2016; Han et al., 2017). Numerous studies in Arabidopsis (*Arabidopsis thaliana*) have determined a core ABA signaling pathway: ABA is perceived by the PYRABACTIN-RESISTANCE PROTEINS/PYR-LIKE PROTEINS/REGULA TORY COMPONENTS OF ABA RECEPTOR (PYR/PYL/RCAR) receptors, which interact with the subclass A type 2C protein phosphatases (PP2Cs), thus blocking the dephosphorylation of SNF1-RELATED PROTEIN KINASE 2 (SnRK2s). As a result, SnRK2s phosphorylate and activate basic leucine zipper (bZIP) transcription factors to regulate the expression of ABA-responsive genes (Cutler et al., 2010; Raghavendra et al., 2010; Umezawa et al., 2010). ABSCISIC ACID-INSENSITIVE 5 (ABI5), a bZIP transcription factor functioning in ABA signaling, has been reported as a major regulator of seed germination and stress responses (Zou et al., 2008; Kong et al., 2013; Skubacz et al., 2016; Chang et al., 2019).

APETALA2/ethylene response factors (AP2/ERFs) are a large super-family of transcription factors found mainly in plants (Okamuro et al., 1997; Magnani et al., 2004; Nakano et al., 2006). AP2/ERF proteins contain one or two AP2/ERF domain, which consists of ~60–70 amino acids and is important for DNA binding. The AP2/ERF family can be divided into four subfamilies: ERF, AP2, related to ABI3/VIVIPAROUS 1 (VP1) (RAV), and dehydration-responsive element-binding protein (DREB) (Nakano et al., 2006; Sharoni et al., 2011). Numerous AP2 subfamily genes have been identified and investigated in plants, and shown to regulate various developmental processes, including responses to hormones and stress (Zhang et al., 2013; Zhao et al., 2015; Cheng et al., 2018; Xie et al., 2019; Feng et al., 2020). For example, OsERF3 negatively regulates drought tolerance in rice by repressing ethylene biosynthesis (Zhang et al., 2013); RICE STARCH REGULATOR1 (OsRSR1) negatively regulates starch biosynthesis (Fu and Xue, 2010); SHATTERING ABORTION1 (OsSHAT1) is required for seed shattering through specifying abscission zone development in rice (Zhou et al., 2012); SUPERNUMERARY BRACT (OsSNB) controls rice seed shattering and seed size, and regulates the transition from spikelet to floral meristem development (Lee and An, 2012; Jiang et al., 2019); and INDETERMINATE SPIKELET1 (OsIDS1) regulates salt tolerance, inflorescence architecture, and floral meristem establishment in rice (Lee and An, 2012; Cheng et al., 2018). However, the function of AP2 proteins in seed germination is largely unclear.

Previously, via evaluating the survival rates and yield traits in saline and alkali soil, we identified 20 genes' transgenic plants that generated by artificial microRNA interfering ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif-containing transcription factor, including OsERF3 and OsIDS1 (Zhang et al., 2013; Cheng et al., 2018). Here, we have functionally characterized SALT AND ABA RESPONSE ERF 1 (OsSAE1), a member of the AP2 subfamily. Using genetic and biochemical analyses, we reveal it to be a positive regulator of seed germination, seedling growth, and salt tolerance via direct repression of *OsABI5*. These results deepen our understanding of the regulatory function of AP2 proteins involved in ABA signaling and stress response, and provide avenues to improve seed vigor and seedling tolerance via molecular breeding.

Results

OsSAE1 is a transcriptional repressor and can bind the GCC-box motif

Previously, we identified 20 EAR-motif-containing transcription factors that are associated with salt tolerance in rice, and OsSAE1 is one of them (Cheng et al., 2018). OsSAE1 encodes 440 amino acids and belongs to AP2 subfamily. Sequence alignments showed that OsSAE1 contains two typical AP2 domains and an EAR motif (LELSL; Supplemental Figure S1, a and b). Phylogenetic tree analysis showed that OsSAE1 is closely related to OsSHAT1, OsRSR1, OsSNB, and OsIDS1 (Supplemental Figure S1c). These proteins function as transcriptional repressors that modulate processes such as seed size and shattering, spikelet and floral organ establishment, and stress tolerance (Fu and Xue, 2010; Lee and An, 2012; Zhou et al., 2012; Cheng et al., 2018; Jiang et al., 2019), and are all targets of miR172 (Zhu et al., 2009; Lee et al., 2014). OsSAE1 similarity with these proteins suggests that OsSAE1 may also be a transcriptional repressor with roles in reproductive development and/or stress response.

To examine whether OsSAE1 possesses transcriptional repression activity, we performed a transcription activity assay using an effector-reporter system by comparing luciferase (LUC) expression levels after the accumulation of GAL4 DNA binding domain (BD), BD-OsSAE1 fusion protein, and BD-OsSAE1 with a mutated EAR motif (BD-OsSAE1-EARm; Figure 1A), and a Renilla (REN) LUC reporter driven by the *35S* promoter was used as an internal control. As shown in Figure 1B, LUC activity was significantly repressed in BD-OsSAE1 compared with that in BD, whereas the LUC activity in BD-OsSAE1-EARm showed no obvious difference with that in BD (Figure 1B), indicating that OsSAE1 has intrinsic transcriptional repression activity in vivo, and the transcriptional repression activity of OsSAE1 is largely dependent on the EAR motif. To further confirm the repression activity of OsSAE1, the coding region of OsSAE1 was fused with the activator VP16 to generate VP16-OsSAE1. According to our results, VP16-OsSAE1 exhibited much lower relative LUC activity compared to the control VP16 (Figure 1B). These

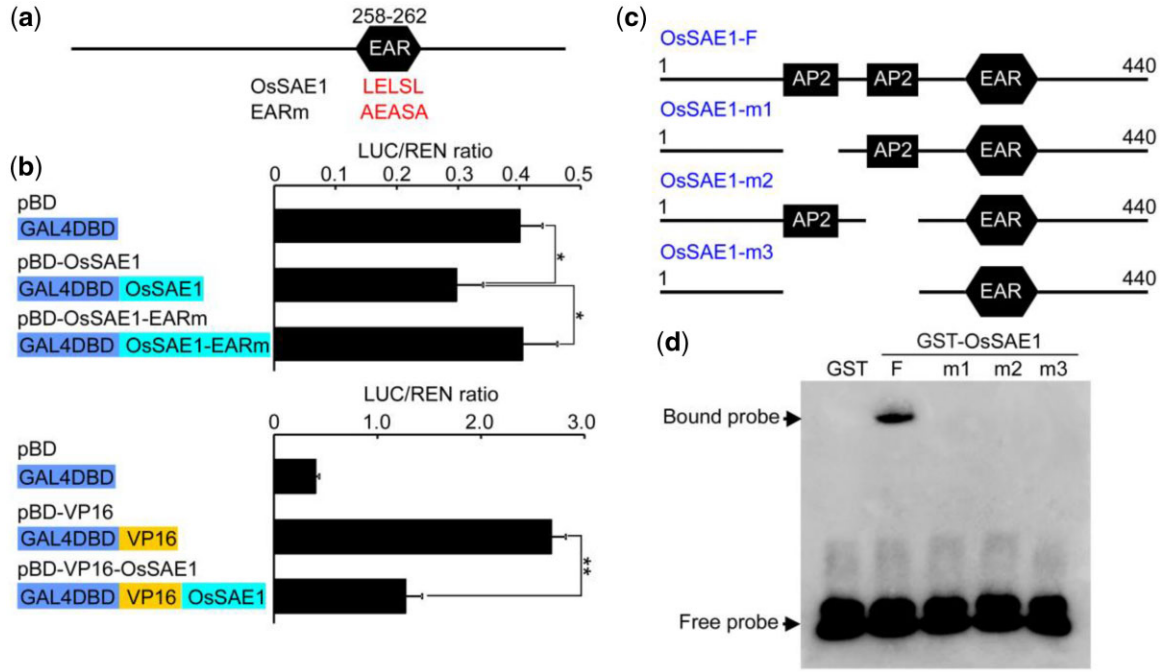

Figure 1 OsSAE1 confers transcriptional repression activity and DNA-binding activity to the GCC-box element. A, Intact and mutated amino acid sequence of the OsSAE1 EAR motif. B, Transient dual-LUC expression assays illustrating the transcriptional repression activity of OsSAE1. The reporters LUC and REN and the effectors (pBD, pBD-OsSAE1, pBD-OsSAE1-EARm, pBD-VP16, and pBD-OsSAE1-VP16) were separately constructed as shown on the left. The activities of LUC and REN were determined 16 h after transformation, and the relative LUC/REN ratios represent the transcriptional activation activities. Error bars represent STDEVs (SDS) among three independent replicates. Asterisks indicate significant differences between the compared two samples at $^{*}P < 0.05$ and $^{**}P < 0.01$ using a Student's *t* test. C, Schematic diagrams of wild-type and deletion mutants of OsSAE1 cloned into the pGEX-6p-1 vector. D, EMSA using GCC-box probe with GST-tagged OsSAE1 (GST-OsSAE1-F) or mutant OsSAE1 (GST-OsSAE1-m1/m2/m3). GST-tag was used in place of GST-OsSAE1 for no-protein controls. Three biological replicates were performed, with similar results.

results suggested that OsSAE1 may function as a transcriptional repressor.

Previous studies have shown that AP2/ERF superfamily transcription factors preferentially bind a GCC-box upstream of target genes to regulate downstream gene expression (Cai et al., 2014; Mao et al., 2016). To examine the DNA-binding properties of OsSAE1, we conducted an electrophoretic mobility shift assay (EMSA) with the biotin-labeled DNA probes that contained the GCC-box motif (5′-GCCGCC-3′). Our results showed that the intact OsSAE1 protein was able to bind the probe containing the GCC-box motif, whereas no binding was observed for OsSAE1 protein lacking one or two AP2 domains (Figure 1, C and D), indicating that both AP2 domains are involved in DNA binding.

Expression of *OsSAE1* in rice tissues and in response to various treatments

To speculate on the function of OsSAE1, we checked the expression profile of *OsSAE1* in different tissues by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The results showed that *OsSAE1* is constitutively expressed in young and mature plant tissues (Figure 2A). In seedling 10 d after imbibition (DAI), *OsSAE1* was expressed at similar levels in root and shoot (Figure 2A). In mature plants, its expression was detected in all organs from the vegetative to reproductive stages and was preferentially expressed in leaves and internodes (Figure 2A), suggesting the possible role of *OsSAE1* throughout the development process.

Next, we investigated the expression pattern of *OsSAE1* in response to various abiotic stresses and hormone treatments. Our results showed that the expression of *OsSAE1* was substantially induced by salt (NaCl), osmotic stress (PEG), ABA and 1-aminocyclopropane-1-carboxylate (an ethylene precursor), and reached peak at 8 or 10 h after treatment (Figure 2B). These results suggest that OsSAE1 might be associated with ethylene- and ABA-related environmental stimuli in rice.

OsSAE1 negatively regulates ABA sensitivity in seed germination and postgermination growth

To facilitate our analysis of OsSAE1 function, we generated OsSAE1 knockout mutants (*ossae1-1* and *ossae1-2*) using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9 system. The *ossae1-1* mutant contained 2-bp insertion in the coding region of the target gene, resulting in an early generation of termination codon, whereas the *ossae1-2* mutant contained 5-bp deletion in the coding region of the target gene, leading to a frame shift in the open reading frame and the

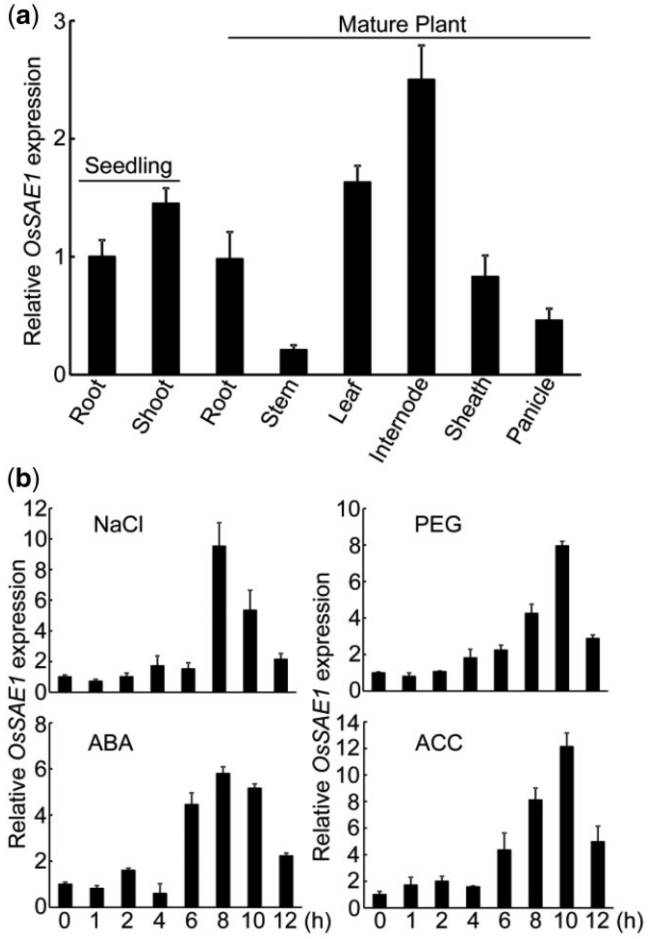

Figure 2 *OsSAE1* expression in different plant tissues and under stress and hormone treatments. A, *OsSAE1* expression in different seedling and mature rice tissues. B, Expression of *OsSAE1* in response to salt (NaCl), PEG, ABA, and ACC. The 10 DAI Nip seedlings were treated with 150-mM NaCl, 10% (w/v) PEG6000, 10-μM ABA, or 10-μM ACC for 0, 1, 2, 4, 6, 8, 10, and 12 h. RNA was isolated from shoots and used for RT-qPCR. *Actin1* was used as an internal control. The relative expression levels were represented by fold change relative to the expression levels of root (A) or 0 h (B). The error bars indicate the SDS based on three independent replicates.

generation of a premature stop codon (Supplemental Figure S2, a and b). We also generated the OE lines by overexpressing the coding region of *OsSAE1* under the control of the *Ubiquitin* promoter, the increased expression of the *OsSAE1* was confirmed by RT-qPCR (Supplemental Figure S2c).

The finding that exogenous ABA treatment induces the expression of *OsSAE1* enlightens us that OsSAE1 might be involved in ABA signaling in rice. To confirm this, we assessed the ABA sensitivity of transgenic plants at the germination stage. Seeds of wild-type Nipponbare (Nip), *OsSAE1* OE (*OsSAE1-OE*) lines, and *ossae1* mutants were imbibed in water for 48 h at 4°C, and then transferred to ABA solution to germinate at 28°C. Under control conditions, the germination rate of *OsSAE1-OE* lines reached 65%–70% at 3 DAI and 95% at 4 DAI, compared with the Nip germination rate of 53% at 3 DAI and 80% at 4 DAI. The germination rate of *ossae1* mutants was lower than that of Nip, 45% at 3 DAI and 65% at 4 DAI (Figure 3, A–C), indicating that OsSAE1 positively regulates rice seed germination. Under ABA treatments, seeds of all the three genotypes exhibited some delay in germination, with *OsSAE1-OE* seeds being less sensitive to increasing concentrations of ABA than Nip seeds, which in turn were less sensitive than *ossae1* seeds (Figure 3, A–C), indicating that OsSAE1 negatively regulates ABA sensitivity during rice seed germination.

Nip, *OsSAE1-OE*, and *ossae1* seedlings were also assessed after 10 d of ABA treatment. Without ABA, root and shoot lengths of *OsSAE1-OE* seedlings were significantly longer than those of Nip seedlings, whereas root and shoot lengths of *ossae1* seedlings were identical to Nip seedlings (Figure 3, D–F). Further analysis of fresh weight of shoots showed that the fresh weight of *ossae1* shoots was slightly lower than Nip and *OsSAE1-OE*, which were similar to each other (Figure 3G). ABA treatment significantly reduced the root length, shoot length, and fresh weight of shoots in Nip seedlings, with a milder phenotype in *OsSAE1-OE* seedlings and a more pronounced phenotype in *ossae1* seedlings (Figure 3, D–G). We further checked the expression level of *RAB21*, a commonly used marker gene in ABA response (Tang et al., 2012). The expression level of *RAB21* was lower in *OsSAE1-OE* plants and higher in *ossae1* mutants compared with that in Nip under normal growth conditions (Figure 3H). ABA treatment significantly induced the expression of *RAB21* in Nip seedlings, and this tendency was weakened in *OsSAE1-OE* plants, but enhanced in *ossae1* mutants (Figure 3H). These results indicate that OsSAE1 may negatively regulate ABA signaling in rice during germination and postgermination growth.

At maturation stage, the plant height and panicle length of the *OsSAE1-OE* plants were shorter than Nip and *ossae1* mutants (Supplemental Figure S2, d–f). Tiller number was not significantly affected in the three genotypes (Supplemental Figure S2g). The grain size was further examined in *ossae1* mutants and *OsSAE1-OE* plants using well-filled grains. In *ossae1* mutants, grain length and grain width were slightly increased compared to those of Nip, whereas grain length and grain width of *OsSAE1-OE* plants were identical to Nip plants (Supplemental Figure S2, h–i). Measurement of 1000-grain weight for well-filled grains showed that *OsSAE1-OE* plants had significant reductions, whereas *ossae1* mutants had slight increases in this parameter (Supplemental Figure S2j). These results indicate that OsSAE1 negatively regulates yield-related traits in rice.

OsSAE1 positively regulates salt tolerance in rice seedlings

Regulators of ABA signaling have also been shown to contribute to plant tolerance to abiotic stress (Xiang et al., 2008; Joo et al., 2019; Liu et al., 2019). As OsSAE1 was obtained by salt tolerance analysis of rice ethylene-responsive element binding factor-associated amphiphilic repression (EAR)-containing transcription factors and salt

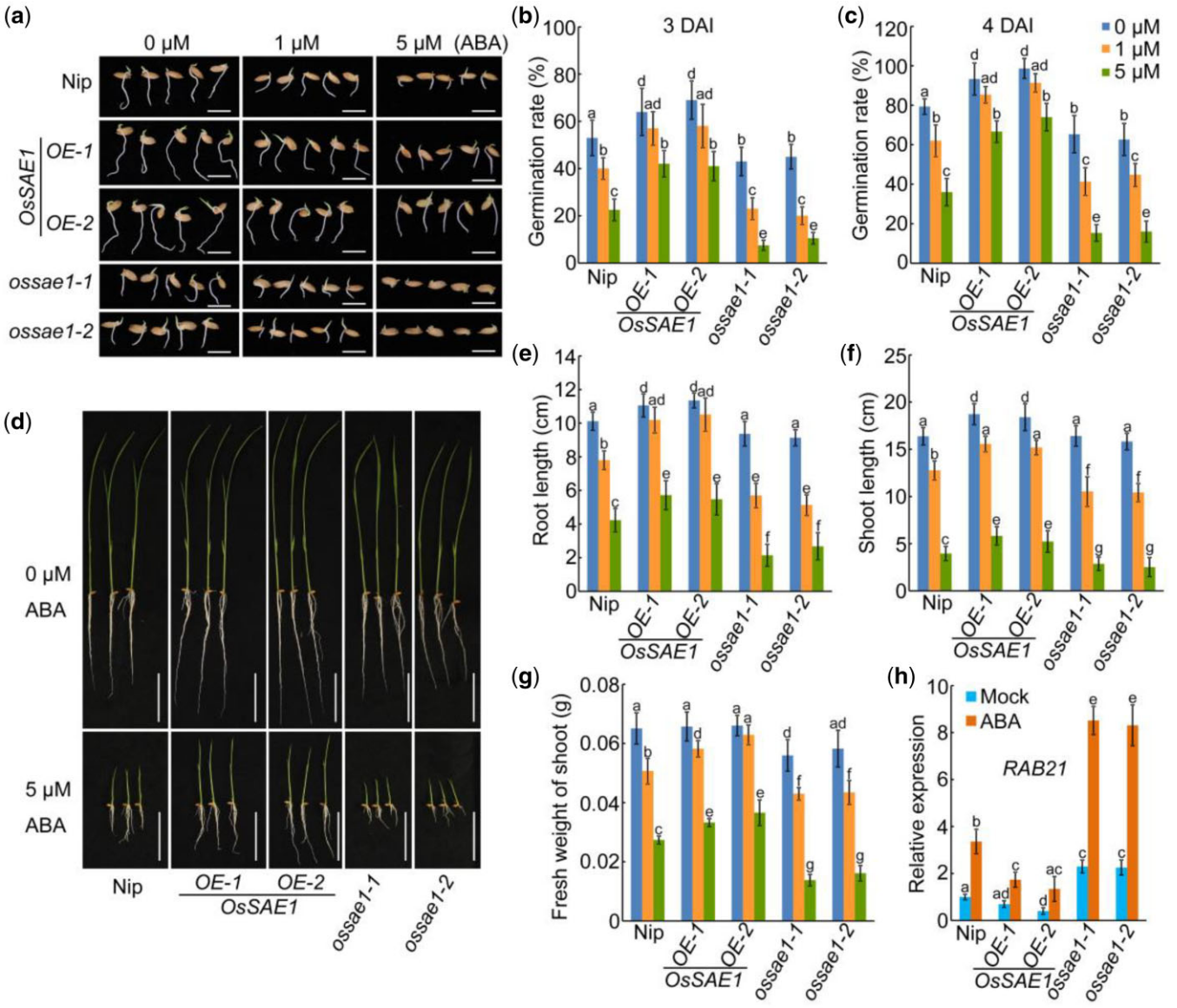

Figure 3 OsSAE1 negatively regulates ABA sensitivity at germination and in seedlings. A, Germination performance of wild-type (Nip), *OsSAE1-OE*, and *OsSAE1* knockout (*ossae1*) seeds under 0, 1, or 5 μM ABA treatment at 3 DAI. Bars = 1 cm. B and C, Germination rate of seeds in (A) at 3 DAI (B) and 4 DAI (C). The error bars indicate the SDS based on three independent replicates. D, Growth of the 10 DAI Nip, *OsSAE1-OE* and *ossae1* seedlings under 0- or 5-μM ABA treatment. Bars = 5 cm. The images of the plants were digitally extracted for comparison. E–G, Root length (E), shoot length (F), and fresh weight of shoots (G) under 0, 1, or 5 μM ABA treatment of 10 DAI Nip, *OsSAE1-OE*, and *ossae1* seedlings. Error bars indicate SDS, $n \geqslant 10$. H, Expression of *RAB21* in Nip, *OsSAE1-OE*, and *ossae1* seedlings with or without ABA treatment. The 10 DAI seedlings were treated with or without 1-μM ABA for 6 h. RNA was isolated from shoots and used for RT-qPCR. *Actin1* was used as an internal control. The relative expression levels were represented by fold change relative to the expression levels of Nip-mock. The error bars indicate the SDS based on three independent replicates. For (B, C, E, F, G, and H), different letters indicate significant differences ($P < 0.05$, one-way ANOVA with Tukey's test).

induces *OsSAE1* expression, we hypothesized that OsSAE1 is involved in regulating salt tolerance in rice. To verify this, we treated Nip, *OsSAE1-OE*, and *ossae1* seedlings with NaCl. After 7 d of treatment with 150-mM NaCl, *OsSAE1-OE* seedlings were obviously less affected than *ossae1* and Nip seedlings by salt stress (Figure 4A). After recovery for 7 d in nonsalt conditions, the survival rate of *ossae1* seedlings was significantly lower, and that of *OsSAE1-OE* seedlings was significantly higher, than that of the Nip seedlings (Figure 4B). The expression of salt stress-responsive marker genes, *SALT OVERLY SENSITIVE 1* (*SOS1*), and *LATE EMBRYOGENESIS ABUNDANT PROTEIN 3* (*LEA3*; Cheng et al., 2018), was further checked. The results showed that salt treatment significantly induced the expression of *SOS1* and *LEA3* in Nip seedlings, this tendency was weakened in *ossae1* mutants, but enhanced in *OsSAE1-OE* lines (Figure 4, C and D). These results indicate that OsSAE1 positively regulates the response of rice to salt stress at seedling stage.

Salt stress leads to accumulation of reactive oxygen species (ROS) and malondialdehyde (MDA), which have detrimental

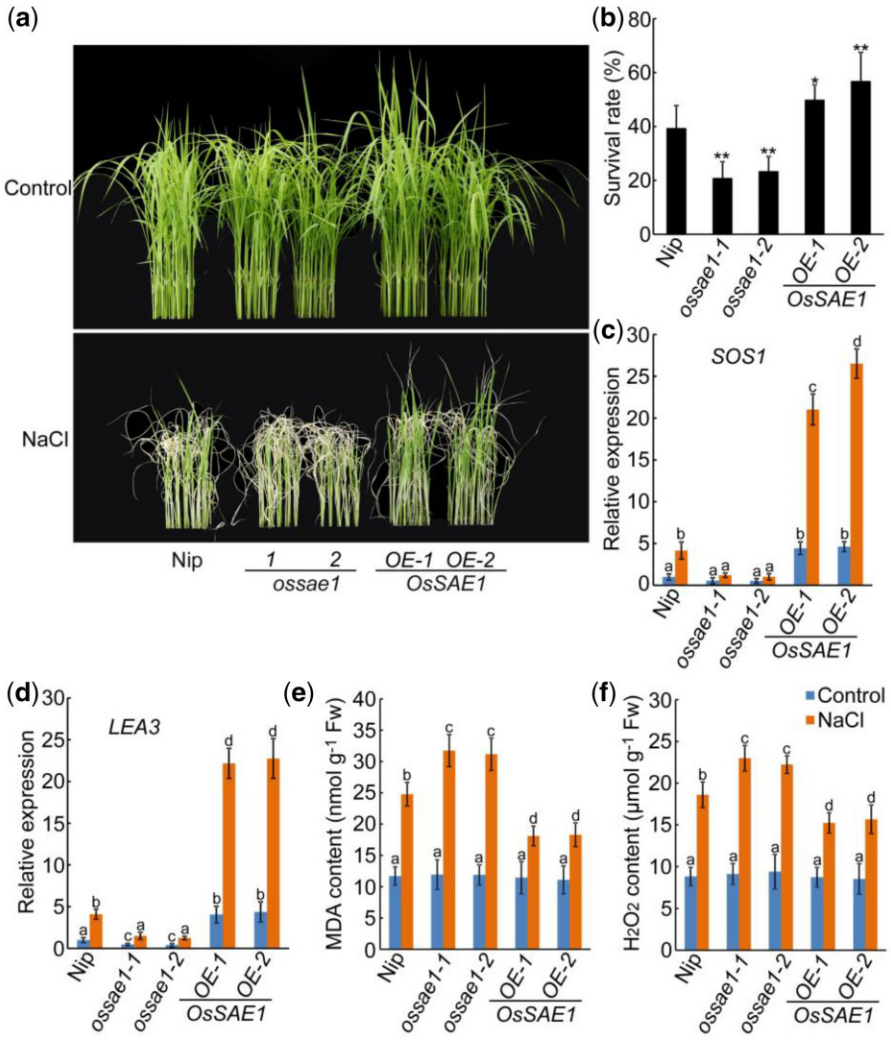

Figure 4 OsSAE1 positively regulates salt tolerance in rice seedlings. A, Phenotypes of Nip, *OsSAE1-OE*, and *OsSAE1* knockout (*ossae1*) seedlings under salt stress. The images of the plants were digitally extracted for comparison. B, Survival rates of the plants shown in (A), 7 d after stopping salt stress. Approximately 50–60 seedlings were used per experiment. Data show mean ± SD of three independent replicates. Asterisks indicate significant differences compared to Nip at $^{*}P < 0.05$ and $^{**}P < 0.01$ by Student's *t* test. C and D, Expression of *SOS1* (C) and *LEA3* (D) in Nip, *OsSAE1-OE*, and *ossae1* seedlings with or without salt treatment. Ten DAI seedlings were treated with or without 150-mM NaCl for 6 h. RNA was isolated from shoots and used for RT-qPCR. *Actin1* was used as an internal control. The relative expression levels were represented by fold change relative to the expression levels of Nip-control. E and F, MDA (E) and H_2O_2 (F) contents in leaves of 10 DAI seedlings with or without 150-mM NaCl treatment for 3 d. For (C, D, E, and F), the error bars indicate the SDs based on three independent replicates, different letters indicate significant differences ($P < 0.05$, one-way ANOVA with Tukey's test).

effects on plant tissues (Zhao et al., 2020a, 2020b). Thus, both ROS and MDA contents serve as important indicators to evaluate abiotic stress tolerance. Although the MDA and hydrogen peroxide (H_2O_2) contents increased in all genotypes following a 3-d salt treatment, these indicators were significantly lower in *OsSAE1-OE* plants compared to the Nip and *ossae1* mutants (Figure 4, E and F). These results suggest that OsSAE1 positively regulates salt tolerance in rice by reducing the accumulation of MDA and H_2O_2 under salt stress.

OsSAE1 directly binds to the promoter of *OsABI5* and represses its expression

To elucidate the molecular network underlying OsSAE1-regulated seed germination and salt tolerance, we examined changes in gene expression in Nip, *OsSAE1-OE*, and *ossae1* plants using transcriptome deep sequencing (RNA-seq). We identified 3,002 differentially expressed genes (DEGs; 1,611 upregulated and 1,391 downregulated) in *ossae1* plants compared to the Nip plants, and 4,266 DEGs (2,475 upregulated and 1,791 downregulated) in *OsSAE1-OE* plants compared to the Nip plants (Figure 5A; Supplemental Dataset 1). Venn diagram analysis showed that 96 genes were upregulated in *OsSAE1-OE* and downregulated in *ossae1*, and 16 genes were downregulated in *OsSAE1-OE* and upregulated in *ossae1* (Figure 5A; Supplemental Dataset 1). Gene ontology (GO) enrichment analysis showed that these genes from the intersection of the Venn diagram were involved in cellular metabolic process, regulation of biological process, regulation of

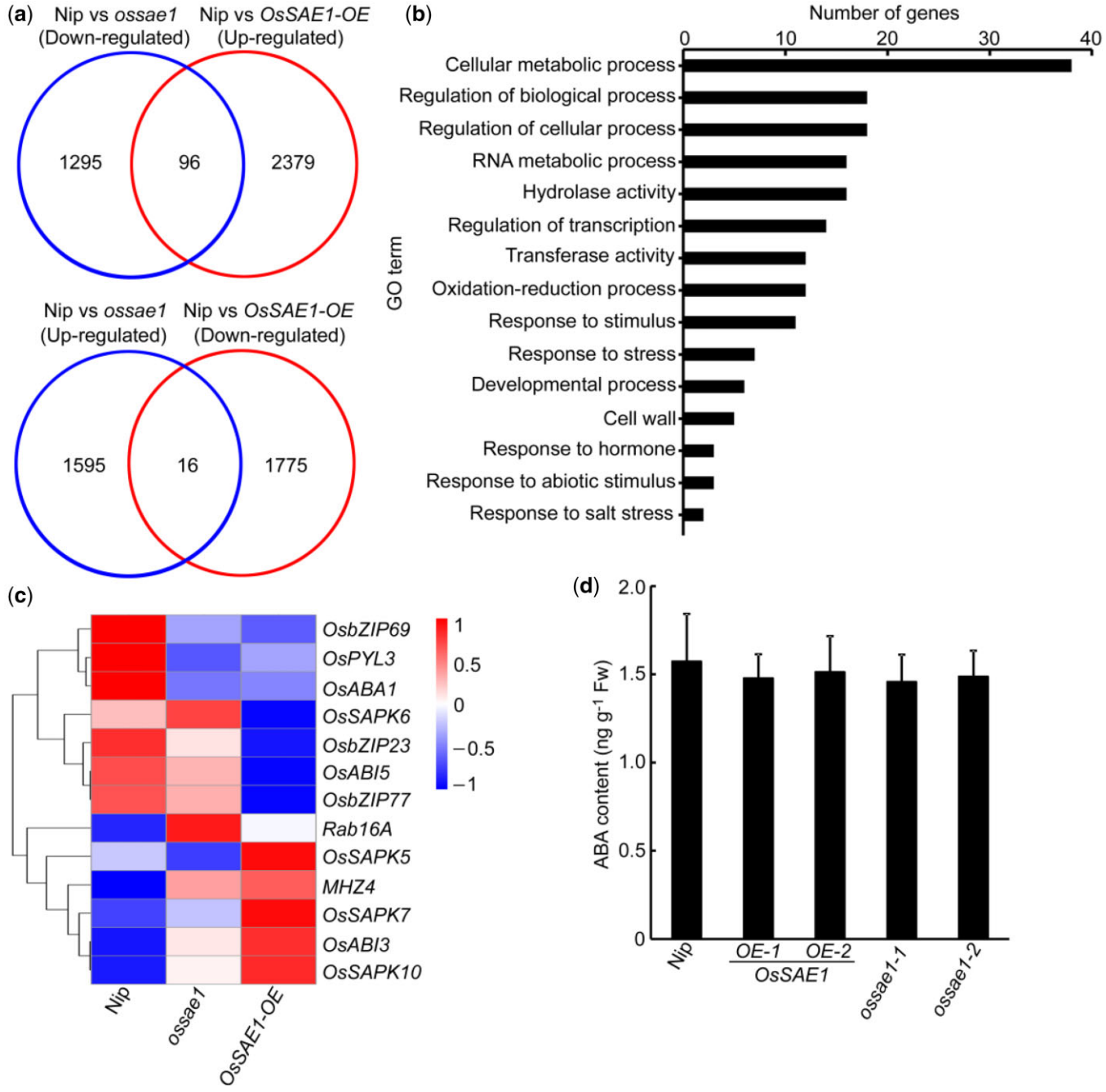

Figure 5 Transcriptome analysis of DEGs regulated by OsSAE1. A, Venn diagrams of the DEGs upregulated and downregulated in *OsSAE1-OE* and *OsSAE1* knockout (*ossae1*) seedlings compared with Nip. B, GO term analysis of OsSAE1-regulated genes. C, Heat map of microarray expression profiles for ABA biosynthesis and signaling-related genes. D, ABA contents in 5 DAI Nip, *OsSAE1-OE*, and *ossae1* seedlings. The error bars indicate the SDs based on three independent replicates. Asterisks indicate significant differences compared to Nip at $^{*}P < 0.05$ and $^{**}P < 0.01$ by Student's *t* test.

cellular process, hydrolase activity, oxidation–reduction process, and stress response (Figure 5B). These results indicate that OsSAE1 is involved in a diverse range of biological processes and molecular functions, including those related to abiotic stress responses.

Considering that OsSAE1 negatively regulates ABA sensitivity during rice seed germination and postgermination growth. We analyzed genes associated with ABA signal transduction in the transcriptome data and found that several genes in the ABA signaling core (*OsSAPKs*, *OsbZIPs*, *OsABIs*, and *OsPYL*) were regulated by OsSAE1 (Figure 5C). Further determination of ABA content showed that no significant differences in ABA content between wild-type, *ossae1* mutants and *OsSAE1-OE* plants (Figure 5D), indicating that disrupting OsSAE1 does not affect ABA biosynthesis, and implying that OsSAE1 might inhibit ABA signaling

to reduce ABA sensitivity during rice seed germination and postgermination growth.

ABIs are downstream factors in ABA signaling pathways, and play key roles in seed germination and stress response (Skubacz et al., 2016; Lin et al., 2020; Zhao et al., 2020a, 2020b). Among the genes involved in the ABA signaling pathway regulated by OsSAE1, we found that *OsABI5* was significantly downregulated in *OsSAE1-OE* plant (Figure 5C). Previous studies have shown that *OsABI5* is involved in stress response and ABA signal transduction (Zou et al., 2007, 2008). RT-qPCR analysis showed that the expression of *OsSAE1* significantly increased, while the expression of *OsABI5* significantly decreased, during wild-type seed germination (Figure 6A), suggesting that OsSAE1 and OsABI5 play opposite roles during seed germination. Further analysis in *OsSAE1-OE* lines and *ossae1* mutants revealed that *OsABI5* expression was significantly lower in *OsSAE1-OE* lines, but higher in *ossae1* mutants, compared with Nip (Figure 6B), suggesting that OsSAE1 negatively regulates *OsABI5* expression and *OsABI5* is a potential target of OsSAE1.

To determine whether OsSAE1 can bind to the *OsABI5* promoter and inhibit the promoter activity, we analyzed the promoter sequence of *OsABI5* and identified one GCC-box, approximately 1.25-kb upstream of the transcriptional start, in the *OsABI5* promoter (Figure 6C). Hence, we performed a transient, dual-LUC expression assay using the 2-kb *OsABI5* promoter to direct expression of the *LUC* reporter gene (*OsABI5p:LUC*) in rice protoplasts cotransfected with or without the effector plasmid (*35S:OsSAE1*). The presence of OsSAE1 significantly decreased LUC activity driven by the *OsABI5* promoter (Figure 6D). Subsequently, we conducted an EMSA using the GST-OsSAE1 fusion protein, which was able to bind directly to DNA probes containing the 5′-GCCGCC-3′ motif in the *OsABI5* promoter (Figure 6E). The binding was specific, as demonstrated by competition using unlabeled (competitor) and mutant probes (Figure 6E). These results indicate that OsSAE1 binds directly to the *OsABI5* promoter to repress its expression.

OsABI5 is a negative regulator of seed germination and salt tolerance

OsABI5 is a member of the third subfamily of bZIP transcription factors and exhibits a high sequence similarity to OsbZIP23 and OsbZIP46, two transcriptional factors that have been characterized as having an essential role in ABA signaling and stress tolerance in rice (Xiang et al., 2008; Tang et al., 2012). Previous studies have shown that the *OsABI5* gene presents only one copy in the rice genome, which contains two splicing variants, *OsABI5-1* and *OsABI5-2*. Both OsABI5-1 and OsABI5-2 had transactivation capacity, but only OsABI5-2 could specifically bind to G-box element (5′-CACGTG-3′), and constitutive expression of *OsABI5-1* and *OsABI5-2* can rescue ABA-insensitivity of *abi5-1* and confer ABA hypersensitivity to wild-type transgenic plants in Arabidopsis (Zou et al., 2007, 2008), suggesting that OsABI5-1 and OsABI5-2 have similar functions to AtABI5. Studies show that OE of *OsABI5-2* confers ABA hypersensitivity and salt sensitivity, and the gene has been designated as OsABI5 (Zou et al., 2007, 2008). To investigate the role of OsABI5 in seed germination and salt tolerance, we generated *OsABI5* OE lines (*OsABI5-OE-1* and *OsABI5-OE-2*) and *OsABI5* loss-of-function mutants (*osabi5-1* and *osabi5-2*). The increased expression of the target gene was confirmed by RT-qPCR and mutant form was identified by nucleotide sequences (Supplemental Figure S3, a–c). The *osabi5-1* and *osabi5-2* mutants harbor a 77-bp and 8-bp deletion in the coding region, respectively, resulting in the early generation of stop codons (Supplemental Figure S3, a and b), indicating that the *osabi5-1* and *osabi5-2* mutants lacked OsABI5. The progeny of these homozygous mutants were used in subsequent experiments.

Next, we investigated the seed germination of Nip, *OsABI5-OE* lines, and *osabi5* mutants. Our results showed that the germination rate of *OsABI5-OE* seeds under normal conditions was significantly lower than that of Nip, whereas the *osabi5* seeds exhibited similar germination rate to Nip seeds (Figure 7, A–C). When subject to ABA treatment, the *OsABI5-OE* seeds were more sensitive, and *osabi5* seeds less sensitive, to ABA compared with Nip seeds (Figure 7, A–C). Consistent with ABA sensitivity during germination, the root length, shoot length, and fresh weight of shoots of *OsABI5-OE* seedlings was significantly lower, whereas *osabi5* seedlings was significantly higher, than those of Nip seedlings in the presence of ABA (Figure 7, D–G). Under normal growth conditions, the expression level of *RAB21* was higher in *OsABI5-OE* plants than this in Nip, whereas no obvious difference between *osabi5* mutants and Nip (Figure 7H). ABA treatment significantly induced the expression of *RAB21* in Nip seedlings, and this tendency was enhanced in *OsABI5-OE* plants, but weakened in *osabi5* mutants (Figure 7h). These results indicate that OsABI5 is a positive regulator of ABA signaling, and plays a negative role in seed germination and postgermination growth.

Previous studies showed that OsABI5 is involved in the stress tolerance of rice and acts as a negative regulator (Zou et al., 2008). To further identify the function of OsABI5 in salt tolerance, we examined the salt tolerance of *OsABI5-OE* lines and *osabi5* mutants at seedling stage. Ten DAI seedlings were treated with 150-mM NaCl for 7 d until the phenotype was observed. Then the plants were transferred to water without salt for additional 7 d before the survival rate was investigated. The results showed that survival rate of *osabi5* mutants (∼45%–55%) were significantly higher than that of wild-type (∼38%) and *OsABI5* OE lines (∼20%–25%; Supplemental Figure S3, d and e). Further detection of the expression of *SOS1* and *LEA3* showed that salt-induced expression of *SOS1* and *LEA3* was weakened in *OsABI5-OE* plants, whereas enhanced in *osabi5* mutants, compared to that in Nip plants (Supplemental Figure S3, f and g). Correspondingly, *OsABI5-OE* plants accumulated more, and *osabi5* mutants accumulated less, MDA and H_2O_2 under salt stress (Supplemental Figure S3, h and i). All these results

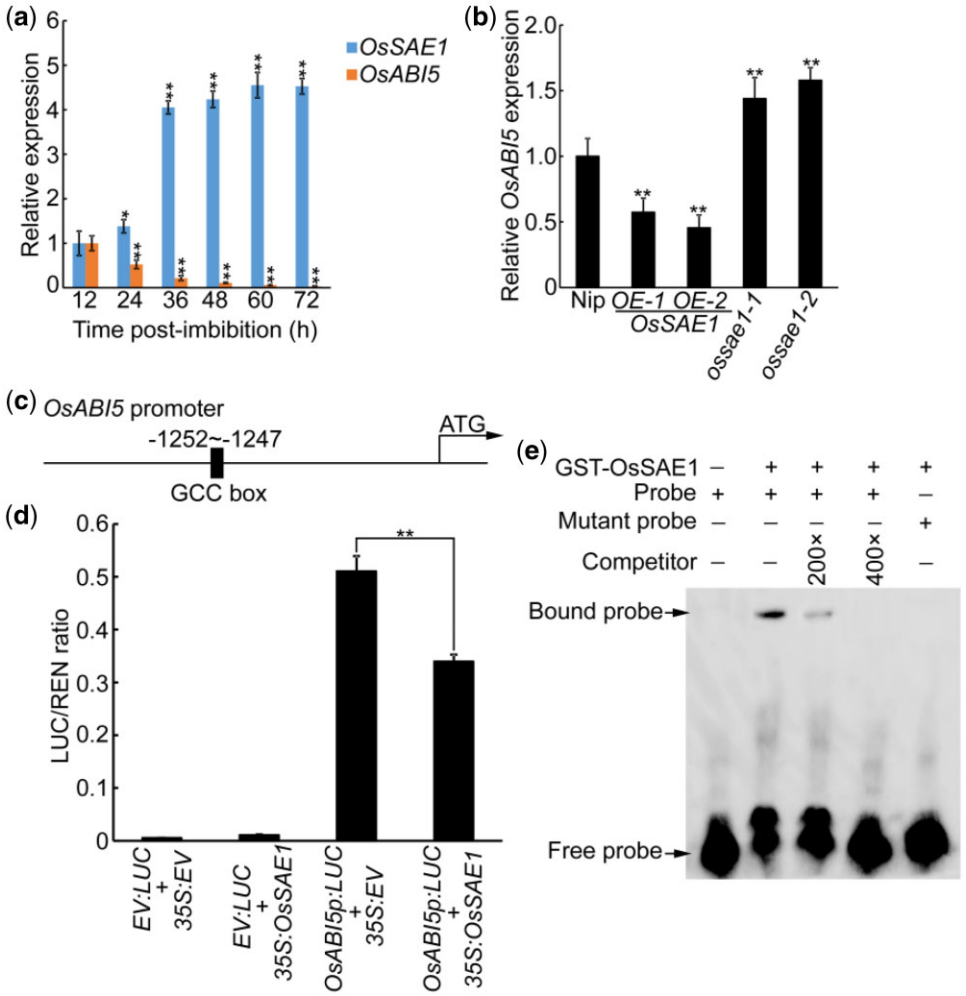

Figure 6 OsSAE1 directly binds to the *OsABI5* promoter to repress its expression. A, Expression of *OsSAE1* and *OsABI5* in germinating Nip seeds. *Actin1* was used as an internal control. The relative expression levels were represented by fold change relative to the expression levels of 12 h. B, Expression of *OsABI5* in Nip, *OsSAE1-OE*, and *OsSAE1* knockout (*ossae1*) seeds at 24-h postimbibition. *Actin1* was used as an internal control. The relative expression levels were represented by fold change relative to the expression levels of Nip. For (A and B), the error bars indicate the SDs based on three independent replicates. Asterisks indicate significant differences from control values (12 h and Nip, respectively) using a Student's *t* test at $^{*}P < 0.05$; $^{**}P < 0.01$. C, Schematic diagram of the *OsABI5* promoter, showing the location of the GCC-box (GCCGCC). D, Dual-LUC assay in rice protoplasts using constructs constitutively expressing *OsSAE1* and/or the LUC reporter gene under control of the *OsABI5* promoter. The error bars indicate the SDs based on three independent replicates. Asterisks indicate significant differences between the compared two samples using a Student's *t* test at $^{**}P < 0.01$. E, EMSA using normal (GCCGCC) and mutated (AAAAAA) *OsABI5* promoter GCC-box probes with GST-tagged OsSAE1 (GST-OsSAE1). GST-tag was used in place of GST-OsSAE1 for no-protein controls. Protein was incubated with biotin-labeled DNA fragments (Probe), tested for competition by adding an excess of unlabeled probe (Competitor), and for specificity with labeled mutant probe. Three biological replicates were performed, with similar results.

suggest that OsABI5 plays a negative role in salt tolerance in rice.

OsABI5 acts downstream of *OsSAE1* to regulate seed germination and salt tolerance

To examine the genetic relationship between *OsABI5* and *OsSAE1*, we generated double mutant lines, *sae1abi5-1* and *sae1abi5-2*. These lines contained a 2-bp insertion and a 5-bp deletion in *OsSAE1*, respectively, and both contained a 1-bp deletion in *OsABI5*; all mutations generated premature stop codons in the protein sequence (Supplemental Figure S4). The germination rates of *sae1abi5-1* and *sae1abi5-2* seeds (~68%) were slightly higher than that of *ossae1-1* (~60%) but lower than that of *osabi5-1* (~77%) and Nip (~74%) seeds at 3 DAI under normal conditions (Figure 8, A and B). In the presence of ABA, seeds of all genotypes exhibited some delay in germination, but *sae1abi5-1* and *sae1abi5-2* seeds were similar to *osabi5* seeds, and less sensitive than Nip and *ossae1* seeds (Figure 8, A–C). Similarly, root length, shoot length, and fresh weight of shoots showed no significant difference between the *sae1abi5-1*, *sae1abi5-2*, and *osabi5* seedlings under ABA treatment, whereas root length, shoot length, and fresh weight of shoots of Nip and *ossae1* seedlings were significantly lower

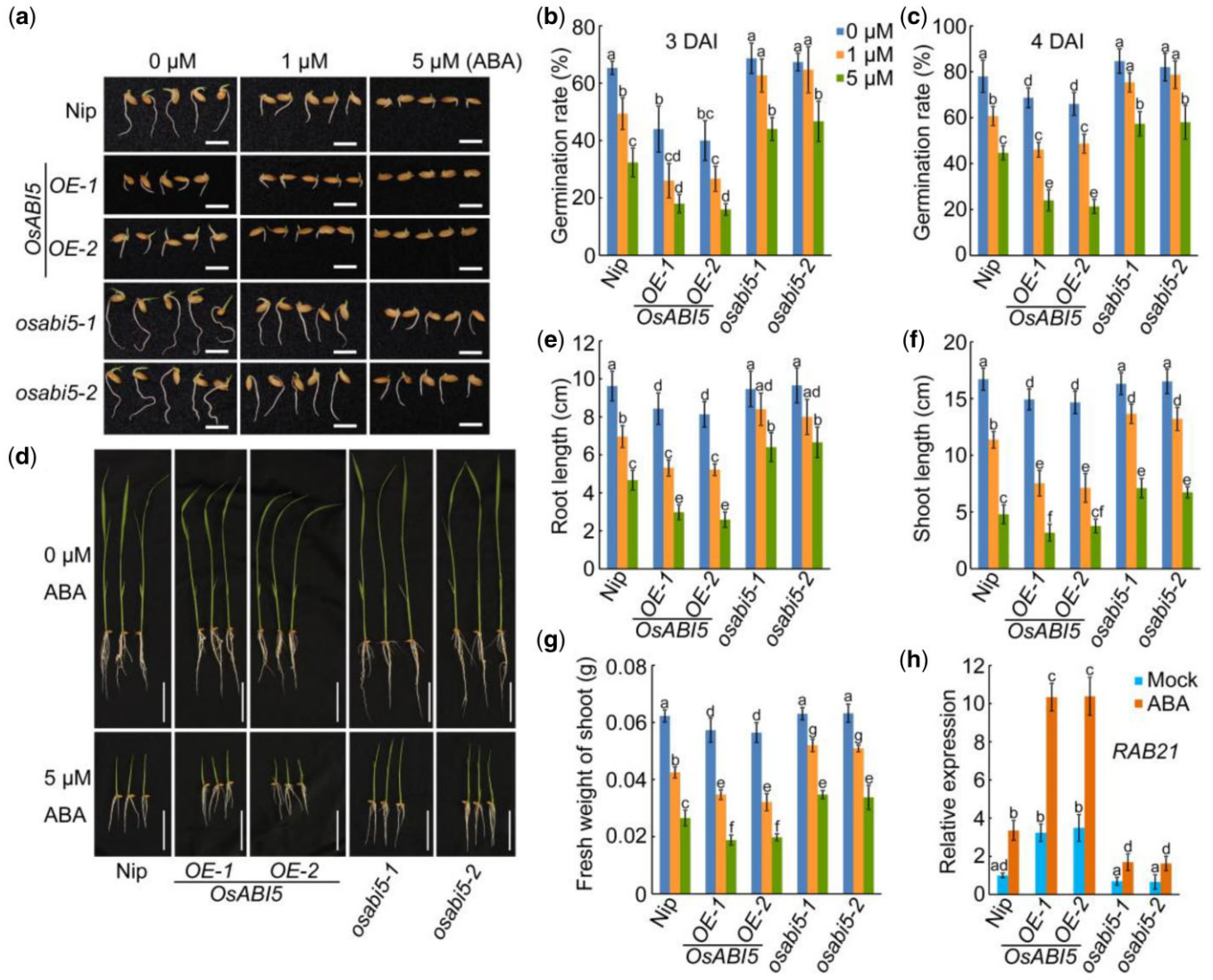

Figure 7 OsABI5 positively regulates ABA sensitivity at germination and in seedlings. a, Germination performance of wild-type (Nip), *OsABI5* OE (*OsABI5-OE*), and *OsABI5* knockout (*osabi5*) seeds under 0-, 1-, or 5-μM ABA treatment at 3 DAI. Bars = 1 cm. B and C, Germination rate of seeds in (A) at 3 DAI (B) and 4 DAI (C). The error bars indicate the SDS based on three independent replicates. D, Growth of 10 DAI Nip, *OsABI5-OE*, and *osabi5* seedlings under 0- or 5-μM ABA treatment. Bars = 5 cm. The images of the plants were digitally extracted for comparison. E–G, Root length (E), shoot length (F), and fresh weight of shoots (G) under 0-, 1-, or 5-μM ABA treatment of 10 DAI Nip, *OsABI5-OE*, and *osabi5* seedlings. Error bars indicate SDS, $n \geqslant 10$. H, Expression of *RAB21* in Nip, *OsABI5-OE*, and *osabi5* seedlings with or without ABA treatment. Ten DAI seedlings were treated with or without 1-μM ABA for 6 h. RNA was isolated from shoots and used for RT-qPCR. *Actin1* was used as an internal control. The relative expression levels were represented by fold change relative to the expression levels of Nip-mock. The error bars indicate the SDS based on three independent replicates. For (B, C, E, F, G, and H), different letters indicate significant differences ($P < 0.05$, one-way ANOVA with Tukey's test).

than *osabi5* seedlings (Figure 8, D–G). Analysis of the expression of *RAB21* showed that the expression level of *RAB21* in *sae1abi5* plants was similar to that in *osabi5* mutant, which was significantly lower than that in *ossae1* mutant in the presence or absence of ABA treatment (Figure 8H). These results indicate that *OsABI5* acts downstream of *OsSAE1* to regulate ABA sensitivity during seed germination and post-germination growth.

To investigate the above genetic relationship in salt tolerance, we subject 10 DAI seedlings to salt stress (150-mM NaCl). After exposure to NaCl for 7 d, the *sae1abi5-1*, *sae1abi5-2*, and *osabi5* seedlings showed increased tolerance to salt stress compared to Nip seedlings, which in turn was higher than *ossae1* seedlings (Figure 9A). After recovery for 7 d, the survival rate of *ossae1* seedlings was significantly lower, and that of *sae1abi5-1*, *sae1abi5-2*, and *osabi5* seedlings significantly higher, than that of Nip seedlings (Figure 9B). The expression levels of *SOS1* and *LEA3* in *sae1abi5-1*, *sae1abi5-2*, and *osabi5* seedlings were significantly higher than that of *ossae1* and Nip seedlings in the presence or absence of salt treatment (Figure 9, C and D). Determination of MDA and H_2O_2 content showed that the

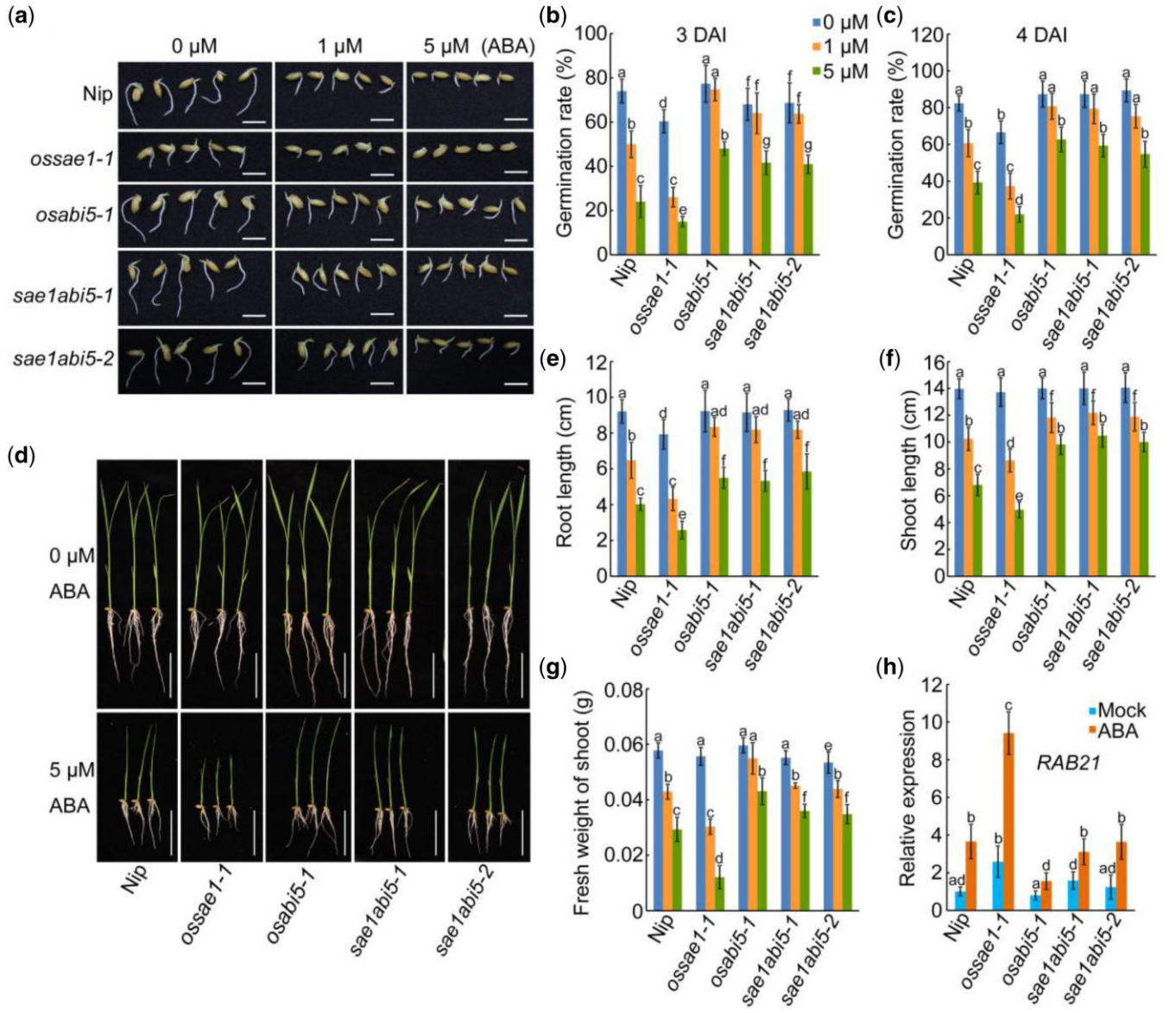

Figure 8 OsABI5 functions downstream of OsSAE1 to regulate ABA sensitivity at germination and in seedlings. A, Germination performance of Nip, *ossae1*, *osabi5*, and *sae1abi5* double mutant seeds under 0-, 1-, or 5-μM ABA treatment at 3 DAI. Bars = 1 cm. B and C, Germination rate of seeds in (A) at 3 DAI (B) and 4 DAI (C). The error bars indicate the SDs based on three independent replicates. D, Growth of 10 DAI Nip, *ossae1*, *osabi5*, and *sae1abi5* double mutant seedlings under 0- or 5-μM ABA treatment. Bars = 5 cm. The images of the plants were digitally extracted for comparison. E–G, Root length (E), shoot length (F), and fresh weight of shoots (G) under 0-, 1-, or 5-μM ABA treatment of 10 DAI Nip, *ossae1*, *osabi5*, and *sae1abi5* double mutant seedlings. Error bars indicate SDs, $n \geqslant 10$. H, Expression of *RAB21* in Nip, *ossae1*, *osabi5*, and *sae1abi5* double mutant seedlings with or without ABA treatment. Ten DAI seedlings were treated with or without 1-μM ABA for 6 h. RNA was isolated from shoots and used for RT-qPCR. *Actin1* was used as an internal control. The relative expression levels were represented by fold change relative to the expression levels of Nip-mock. The error bars indicate the SDs based on three independent replicates. For (B, C, E, F, G, and H), different letters indicate significant differences ($P < 0.05$, one-way ANOVA with Tukey's test).

sae1abi5-1, *sae1abi5-2*, and *osabi5* seedlings accumulated similar levels of MDA and H_2O_2 under salt stress, which were significantly lower than that of *ossae1* and Nip seedlings (Figure 9, E and F). These results indicate that *OsABI5*-mediated pathway is required for OsSAE1 to regulate salt tolerance in rice seedlings.

To further elucidate the relationship between *OsSAE1*- and *OsABI5*-mediated pathways, we compared the downstream DEGs regulated by OsSAE1 or OsABI5 in rice seedlings with or without salt treatment through transcriptome analysis. In the absence of salt stress, 34% (1,004) of DEGs in *ossae1* mutant existed in *OsABI5-OE* plant, and 36% (1,561) of DEGs in *OsSAE1-OE* plant existed in *osabi5* mutant (Supplemental Figure S5a; Supplemental Dataset 1). Further analysis of these DEGs showed that many genes involved in ROS-related pathway were regulated by OsABI5 or OsSAE1 (Supplemental Figure S5b). After exposure to salt treatment, 96 overlapping DEGs were identified in *OsSAE1-*

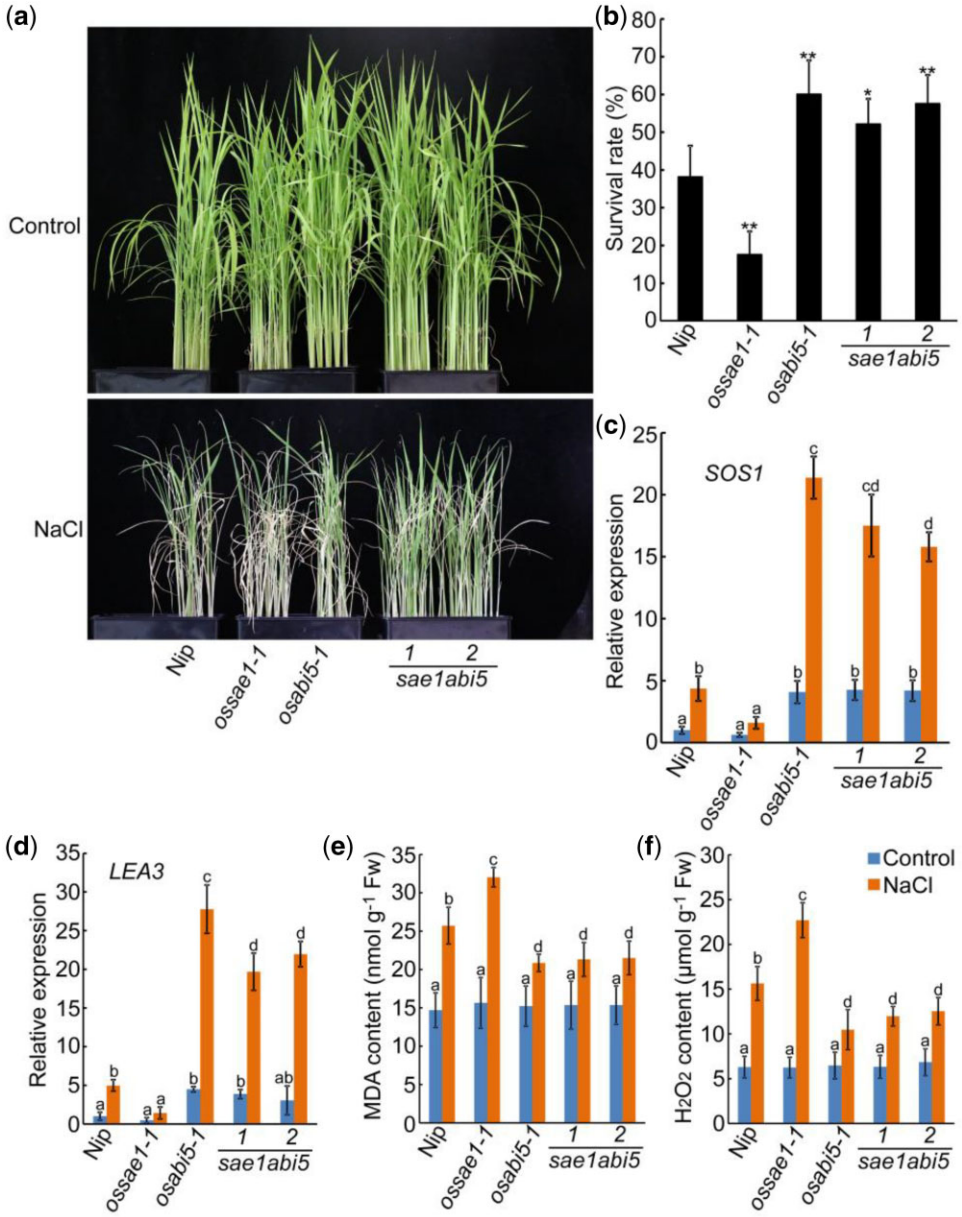

Figure 9 *OsABI5* acts downstream of *OsSAE1* to regulate salt tolerance in rice seedlings. A, Phenotypes of Nip, *ossae1*, *osabi5*, and *sae1abi5* double mutants under salt stress. The images of the plants were digitally extracted for comparison. B, Survival rates of the plants shown in (A), 7 d after stopping salt stress. Approximately 50–60 seedlings were used per experiment. Data show mean ± SD of three independent replicates. Asterisks indicate significant differences compared to Nip at $^{*}P < 0.05$ and $^{**}P < 0.01$ by Student's *t* test. C and D, Expression of *SOS1* (C) and *LEA3* (D) in Nip, *ossae1*, *osabi5*, and *sae1abi5* double mutant seedlings with or without salt treatment. Ten DAI seedlings were treated with or without 150-mM NaCl for 6 h. RNA was isolated from shoots and used for RT-qPCR. *Actin1* was used as an internal control. The relative expression levels were represented by fold change relative to the expression levels of Nip-control. E and F, MDA (E) and H_2O_2 (F) contents in leaves of 10 DAI seedlings with or without 150-mM NaCl treatment for 3 d. For (C, D, E, and F), the error bars indicate the SDs based on three independent replicates, different letters indicate significant differences ($P < 0.05$, one-way ANOVA with Tukey's test).

OE plant (3,561 DEGs) and *osabi5* mutant (140 DEGs), and 423 overlapping DEGs were identified in *ossae1* mutant (3,650 DEGs) and *OsABI5-OE* plant (838 DEGs; Supplemental Figure S5c; Supplemental Dataset 2). These results suggest that OsSAE1 and OsABI5 are differential in terms of gene expression, and *OsABI5* plays a partial role downstream of *OsSAE1*.

Discussion

Seed germination is a complex process, controlled by multiple genes and easily affected by environmental cues (Han and Yang, 2015; Penfield and MacGregor, 2017). Multiple phytohormones are involved in regulating seed germination; ABA has been recognized as a key inhibitor of seed germination (Han and Yang, 2015; Shu et al., 2016). ABA sensitivity

is an important aspect of ABA signaling-dependent regulation. In this study, we identified an AP2 transcription factor, OsSAE1, which functions as a positive regulator of seed germination and salt tolerance in rice. *OsSAE1-OE* promotes seed germination and reduces ABA sensitivity, whereas *OsSAE1* knockout retards seed germination and enhances ABA sensitivity, demonstrating that OsSAE1 is an important regulator in ABA signaling-dependent germination. Moreover, OsSAE1 directly binds the *OsABI5* promoter to repress gene transcription during seed germination, resulting in a decrease in ABA sensitivity in seed germination and postgermination growth. The *OsABI5*-mediated pathway is required for *OsSAE1*-regulated seed germination and ABA sensitivity, as *OsABI5* mutation rescues the seed germination and ABA sensitivity in *ossae1* lines, revealing that OsSAE1 is a key factor involved in seed vigor that might be useful to genetic improvements of seed vigor for direct seeding of rice.

AP2/ERF transcription factors are a large group of factors that are known to regulate diverse processes of plant development and play very important roles in hormonal regulation and stress responses (Licausi et al., 2013; Xie et al., 2019; Feng et al., 2020). OsSAE1 encodes an AP2-type transcription factor and exhibits a close evolutionary relationship with OsSHAT1, OsRSR1, OsSNB, and OsIDS1. These four proteins are known to function as transcriptional repressors and, together with OsSAE1, are miR172 target genes (Zhu et al., 2009; Lee et al., 2014); miR172 has been shown to play a role in floral and seed developmental and salt tolerance in rice (Zhu et al., 2009; Cheng et al., 2021). This study deepens our understanding of the role of AP2 transcription factors, by determining that OsSAE1 acts as a transcriptional repressor involved in regulating seed germination and ABA sensitivity during seed germination and in young seedlings, through ABA signaling pathways mediated by OsABI5. Further investigation will be required to determine how miR172 may be involved in coordinating these developmental processes via OsSAE1 and OsABI5.

ABA-inhibited seed germination and postgermination growth is regulated by a core regulatory mechanism involving the following core components: PYR1/PYL/RCAR receptors, PP2C co-receptors, and SnRK2 kinases (Ma et al., 2009; Park et al., 2009; Nishimura et al., 2010; Soon et al., 2012). ABA binds to PYR1/PYL/RCAR to enable the interaction between PYR1/PYL/RCAR and PP2Cs, thus leading to the activation of SnRK2 protein kinases from inhibition by the PP2Cs. Activated SnRK2 kinases thereby phosphorylate downstream transcription factors and activate the expression of ABA-responsive genes (Ma et al., 2009; Park et al., 2009). In this study, we show that OsSAE1, a negative regulator of ABA signaling pathway, negatively regulates ABA-inhibited seed germination and postgermination growth by repressing the expression of *OsABI5*. Transcriptome analysis showed that several genes in the ABA signaling core were obviously regulated by OsSAE1, such as *OsSAPKs*, *OsbZIPs*, *OsABIs*, and *OsPYL*, indicating that the effect of OsSAE1 on seed germination and postgermination growth occurs through multiple pathways, of which the *OsABI5*-mediated pathway is one. Further studies should focus on the interaction of OsSAE1 with the upstream components of the ABA signaling pathway.

ABA-responsive element (ABRE) binding factor/ABRE binding protein/ABI5 is a small subfamily of bZIP transcription factors that function as key regulators of ABA and abiotic stress responses of plants. Previous reports indicate that OsABI5 possesses transactivation activity and the N-terminal 9-amino acids transactivation domain is critical for transactivation (Zou et al., 2007, 2008; Hong et al., 2011). Moreover, OsABI5 acts as a negative regulator of salt stress response and can be phosphorylated by SnRK2 family member OSRK1 (Chae et al., 2007; Zou et al., 2008), together with PYL5, PP2C30, and SAPK2, constitutes the minimal ABA signaling unit that modulates seed germination and early seedling growth (Kim et al., 2012). Constitutive expression of *OsABI5* can rescue ABA-insensitivity of *abi5-1* and confer ABA hypersensitivity to wild-type transgenic plants in Arabidopsis (Zou et al., 2007), suggesting that OsABI5 has similar functions as AtABI5, and OsABI5 is involved in ABA signal transduction. In this study, we indicate that OsABI5 is a positive regulator of ABA signaling and negatively regulates seed germination, postgermination growth, and salt tolerance in rice. Further investigations showed that *OsABI5* is a downstream target of OsSAE1. Thus, our results enrich the regulatory network of OsABI5. Several studies have shown that OsABI5 could interact with other factors to regulate plant growth and response to external stimuli (Zou et al., 2007; Kim et al., 2012; Chen et al., 2018), it will be interesting to know whether the OsSAE1 and OsABI5 could interact physically in the future.

Salinity is a major environmental stress that constraints rice cultivation worldwide. Improving salt tolerance in rice is considered to be the most economic and effective options to ensure food security and ameliorate the constraints of increasingly saline soils in rice-cultivating regions. Rice is highly susceptible to salinity during early seedling growth stage (Nam et al., 2015), so improving the salt tolerance of rice seedlings is critical for seedling establishment and yield under salinity stress. ABA is a major abiotic stress-responsive hormone and plays pivotal roles in the stress responses by modulating the gene expression and cellular processes (Tang et al., 2016; Xu et al., 2018). The role of ABA in drought tolerance has been extensively investigated and it is generally believed that enhancing ABA biosynthesis or signaling transduction could improve the drought tolerance of plants (Sah et al., 2016; Zhao et al., 2016; Chang et al., 2017). Literature focusing on ABA-mediated responses under salt stress is less consistent, and both positive and negative effects of ABA on salt tolerance have been reported (Xiang et al., 2008; Liu et al., 2014; Sah et al., 2016; Kakan et al., 2021). In this study, we have shown that OsSAE1 positively regulates salt tolerance in rice seedlings by repressing the expression of *OsABI5*, a positive regulator of ABA signaling that increases

susceptibility of rice to salt stress. OsABI5 exhibits high sequence similarity to OsbZIP23 and OsbZIP46 (Tang et al., 2012), which have both been characterized with essential roles in ABA signaling and stress tolerance in rice (Xiang et al., 2008; Tang et al., 2012).

Taken together, our results demonstrate that OsSAE1 may exert its functions through repression of *ABI5*-mediated ABA signaling for seed germination and salt stress tolerance. Thus, our research presents insights into the roles of OsSAE1 in improving seed vigor and salt tolerance in rice, and provides further evidence for the role of ABA signaling via OsABI5 in driving the rice salt response. These results offer targets and strategies for the breeding and development of new, direct seeding rice varieties suitable for saline–alkali land.

Materials and methods

Plant materials and grown conditions

Rice (*O. sativa*) variety Nip (*O. sativa* ssp. *japonica*) was used as a wild-type plant and recipient for genetic transformation in this study. Rice plants were cultivated at the Experimental Station of the Chinese Academy of Agricultural Sciences in Beijing during the natural growing season. In general, germinated seeds were sown in seed beds over the period 25–30 April, and planted in the fields 20–30 d later.

Generation of transgenic plants

Knockout *ossae1* mutants, *osabi5* mutants, and *sae1abi5* double mutants were generated using CRISPR/Cas9 (Xu et al., 2017), in which the target regions 5′-AAGGGTCTGGGAGGGTCTTT-3′ of *OsSAE1* and 5′-CAGGAGGAAGCGGTTTATGT-3′ of *OsABI5* were introduced into the pHUN4c12 vector backbone, and the recombinant vector was transformed into *Agrobacterium* strain EHA105-pSOUP for rice transformation. To generate OE lines, the *OsSAE1* or *OsABI5* coding sequence was amplified from Nip cDNA and cloned into the binary vector pCAMBIA3301 (Wang et al., 2020a, 2020b) under control of the *Ubiquitin* promoter using the PstI and BamHI sites. The resulting plasmids were introduced separately into Nip via *Agrobacterium*-mediated transformation. Primers used for gene editing and plasmid construction are listed in Supplemental Table S1.

ABA and salt treatments

The effect of ABA on seed germination was assessed in triplicate as previously described (Song et al., 2020) with minor modifications. Briefly, 50 seeds per replicate of Nip and transgenic lines were imbibed in Petri dishes with 10-mL sterile distill water at 4°C for 48 h. Water was drained and replaced with ABA solution (0, 1, and 5 μM), and seeds were transferred to a growth chamber under a 14-h light/10-h dark cycle at 28°C. Germination was defined as the emergence of the radical, and the number of germinated seeds was counted every 24 h. The germination rate was calculated as the number of germinated seeds divided by 50 (total seeds assayed).

To determine the ABA sensitivity of postgermination growth, germinated rice seeds (∼30 seeds) were placed on a stainless steel sieve, which was placed in an air-tight 10-L plastic box. Seeds were treated with 6 L of Yoshida's culture solution (Cui et al., 2015) containing 0, 1, or 5 μM ABA. The seedlings were grown under a 14-h light/10-h dark cycle at 28°C for 10 d. Root and shoot lengths were measured via ImageJ software; shoot weight was assessed using the whole shoot. Three replications were performed.

To asses salt-tolerance of rice seedlings, ∼30 germinated seeds (per replicate) were sown in a bottomless 96-well plate in a container of Yoshida's culture solution (Cui et al., 2015) and grown under a 14-h light/10-h dark cycle at 28°C for 10 d. Treated plants were then transferred to Yoshida's culture solution with 150- mM NaCl for another 7 d, then back to salt-free solution for 7 d of recovery, after which surviving seedlings were counted. Experiments were repeated at least 3 times with similar results.

RT-qPCR

Total RNA was extracted from seeds using a Plant Total RNA Purification Kit (GeneMark, Taichung, Taiwan) or from other tissues using an Ultrapure RNA Kit (CWBIO, Jiangsu, China), according to manufacturer's instructions. Approximately 2-μg total RNA was reverse transcribed to cDNA with HiScript II Q RT SuperMix (Vazyme, Nanjing, China) according to manufacturer's instructions. RT-qPCR was performed as previously described (Zhang et al., 2012), using the rice *Actin1* gene as an internal standard to normalize gene expression. Primers used for RT-qPCR are listed in Supplemental Table S1.

Measurement of H_2O_2 and MDA contents

H_2O_2 content was determined using the method described previously (Wang et al., 2019). Briefly, 0.1 g of normal or salt-stressed rice leaves were harvested and homogenized in 1-mL cold acetone. Then, H_2O_2 content was determined using an H_2O_2 assay kit (Solarbio, Beijing, China) according to the manufacturer's instructions.

To measure MDA, 0.1 g of normal or salt-stressed rice leaves was homogenized in 1 mL 0.1% (w/v) trichloroacetic acid (TCA) followed by centrifugation at 8,000*g* for 10 min at 4°C. Four volumes of 0.5% (w/v) thiobarbituric acid in 20% (w/v) TCA were added to one volume of supernatant; the mixture was incubated at 100°C for 1 h. The reaction was terminated by incubating the mixture on ice for 15 min, after which the absorbance was measured spectrophotometrically at 450, 532, and 600 nm. The amount of MDA was calculated from the formula provided with the MDA assay kit (Solarbio, Beijing, China).

Transcriptome analysis

Total RNA was isolated in triplicate from 5 DAI Nip, *OsSAE1-OE*, *OsSAE1* knockout (*ossae1*), *OsABI5* OE (*OsABI5-OE*), and *OsABI5* knockout (*osabi5*) seedlings treated with

150-mM NaCl for 0 h (as control) and 3 h. Whole seedling was collected, immediately frozen in liquid nitrogen, and stored at −80°C. Total mRNA was isolated using the TRIzol method (TIANGEN BIOTECH, Beijing, China), quality checked on a Bioanalyzer (Agilent 2100), and used for RNA-seq library construction with the NEBNext Ultra RNA Library Prep Kilt for Illumina (NEB, Ipswich, MA, USA), according to manufacturer's instructions. Multiplex paired-end adapters were used for multiplex libraries, which were sequenced (paired-end, 150 bp each) by the Illumina genome analyzer (Hiseq TM2500/4000). After removing adaptor and low-quality reads, clean reads were mapped to rice genome MSU version 7.0 using Tophat2, and analyzed using Cufflinks (Trapnell et al., 2012). The Poisson-dispersion model of fragments was used to conduct statistical analysis (false discovery rate < 0.05) and gene expression levels were estimated by fragments per kilobase of transcript per million fragments mapped. Differential expression analysis of two samples was performed using the DESeq R package and genes with an adjusted $P < 0.05$ found by DESeq were assigned as DEGs (Anders and Huber, 2010). Repeatability and correlation were evaluated by Pearson's correlation coefficient (Schulze et al., 2012). GO enrichment analysis of the DEGs was implemented by the GOseq R packages based Wallenius noncentral hyper-geometric distribution (Young et al., 2010).

LUC transient expression assay

The transient dual-LUC expression assays were performed using rice protoplasts. To test the transcriptional repression activity of OsSAE1, a dual-LUC reporter assay was conducted. For the effectors generation, OsSAE1 coding region was fused with the GAL4 DBD to generate pBD-OsSAE1. OsSAE1 containing a transactivating domain VP16 in its N terminus was also fused with the GAL4 DBD to generate a pBD-VP16-OsSAE1 effector. The GAL4 DBD with or without VP16 (pBD and pBD-VP16) was used as the positive and the negative controls, respectively.

To evaluate the transcriptional regulatory activity of OsSAE1 on the OsABI5 promoter, the reporter (*OsABI5p:LUC*) and effector (*35S:OsSAE1*) plasmids or empty vector (EV) controls were transformed into rice protoplasts via polyethylene glycol-mediated transfection as previously described (Bart et al., 2006).

Firefly LUC and REN activities were measured with a dual-LUC reporting assay kit (Promega, Madison, WI, USA), according to manufacturer's instructions. For each replication, three independent transformations were conducted. Primers used for plasmid construction are listed in Supplemental Table S1.

EMSA

Glutathione S-transferase-tagged OsSAE1 fusion protein (GST-OsSAE1) was expressed in *Escherichia coli* BL21 (DE3) and purified with *ProteinIso* GST Resin (Transgen, Beijing, China), according to manufacturer's instructions. Oligonucleotides were synthesized and labeled with biotin. Unlabeled oligonucleotides were used as competitors. To generate double-stranded probes, an equal quantity of the complementary single-strand oligos was mixed, heated to 95°C for 2 min, and annealed by gradually cooling to 25°C. Probe sequences are given in Supplemental Table S1.

EMSA was performed using a LightShift Chemiluminescent EMSA Kit (Thermo Fisher, Waltham, MA, USA) according to manufacturer's instructions. Reaction solutions were incubated for 20 min at room temperature. The protein–probe mixture was separated on a 5% (v/v) native polyacrylamide gel and transferred to a nylon membrane (GE, UK). Following crosslinking under UV light, DNA on the membrane was detected using a Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher, Waltham, MA, USA), according to manufacturer's instructions.

Phylogenetic analysis

All sequences of the AP2/ERF proteins were available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). The nucleotide sequences and deduced amino acid sequences were aligned using the Multiple Sequence Alignment program of the DNAMAN software (version 7.0, LynnonBiosoft, Quebec, Canada). A phylogenetic tree was also constructed from the alignment these sequences using DNAMAN software.

Statistical analysis

Student's *t* test was used for significant difference analysis between two samples. One-way analysis of variance (ANOVA) followed with Tukey's test ($P < 0.05$) was used for pairwise multiple comparisons. All the analyses were performed with GraphPad Prism version 5 software.

Accession numbers

Sequence data from this article can be found in the Rice Genome Annotation Project website (http://rice.plantbiology.msu.edu/) under the following accession numbers: *OsActin1*, LOC_Os03g50885; *OsSAE1*, LOC_Os06g43220; *OsABI5*, LOC_Os01g64000; *RAB21*, LOC_Os11g26790; *SOS1*, LOC_Os12g44360; *LEA3*, LOC_Os05g46480. The transcriptome data from this study can be found in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) under the accession number PRJNA793282.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Sequence and phylogenetic analysis of OsSAE1.

Supplemental Figure S2. Agronomic traits of *OsSAE1* knockout and OE lines.

Supplemental Figure S3. OsABI5 negatively regulates salt tolerance in rice seedlings.

Supplemental Figure S4. Identification of *sae1abi5* double mutants.

Supplemental Figure S5. DEGs in *OsSAE1-OE*, *ossae1*, *OsABI5-OE*, and *osabi5* seedlings with or without salt treatment.

Supplemental Table S1. Primers used in this study.

Supplemental Dataset 1. DEGs in *OsSAE1-OE*, *ossae1*, *OsABI5-OE*, and *osabi5* seedlings under normal conditions.

Supplemental Dataset 2. DEGs in response to NaCl in Nip, *OsSAE1-OE*, *ossae1*, *OsABI5-OE*, and *osabi5* seedlings.

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Conflict of interest statement. None declared.

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