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# Nitrate-responsive *OsMADS27* promotes salt tolerance in rice

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## ABSTRACT

**Salt stress is a major constraint on plant growth and yield. Nitrogen (N) fertilizers are known to alleviate salt stress. However, the underlying molecular mechanisms remain unclear. Here, we show that nitrate-dependent salt tolerance is mediated by OsMADS27 in rice. The expression of *OsMADS27* is specifically induced by nitrate. The salt-inducible expression of *OsMADS27* is also nitrate dependent. *OsMADS27* knockout mutants are more sensitive to salt stress than the wild type, whereas *OsMADS27* overexpression lines are more tolerant. Transcriptomic analyses revealed that OsMADS27 upregulates the expression of a number of known stress-responsive genes as well as those involved in ion homeostasis and antioxidation. We demonstrate that OsMADS27 directly binds to the promoters of *OsHKT1.1* and *OsSPL7* to regulate their expression. Notably, *OsMADS27*-mediated salt tolerance is nitrate dependent and positively correlated with nitrate concentration. Our results reveal the role of nitrate-responsive *OsMADS27* and its downstream target genes in salt tolerance, providing a molecular mechanism for the enhancement of salt tolerance by nitrogen fertilizers in rice. *OsMADS27* overexpression increased grain yield under salt stress in the presence of sufficient nitrate, suggesting that *OsMADS27* is a promising candidate for the improvement of salt tolerance in rice.**

**Key words:** OsMADS27, nitrate-dependent salt tolerance, salt stress, grain yield

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## INTRODUCTION

Salinity is among the critical agricultural crises around the globe, and the majority of food crops are sensitive to salinity (Qadir et al., 2014). Elevated soil salinity not only causes ion toxicity and osmotic stress but also results in severe nutrient deficiency in plants (Munns and Tester, 2008). Plants have evolved various strategies to cope with salinity-triggered damage, depending on their habitat and the severity of the stress (Ashraf et al., 2008; Adem et al., 2014; Bose et al., 2014; Chakraborty et al., 2016). Among these numerous strategies, appropriate acquisition of mineral nutrients is undoubtedly an effective way to improve salinity tolerance, growth, and yield under salt stress (Kaya et al., 2007; Gao et al., 2016; Guo et al., 2017). Therefore, it is important to understand the mechanisms by which nutrients alleviate plant salt stress in order to breed robust salt-tolerant crop varieties.

Potassium, a vital nutrient for plant growth and development, is well known for its role in balancing sodium concentrations in plants (Clarkson and Hanson, 1980; Raddatz et al., 2020; Wu et al., 2018; Zorb et al., 2014). Under salt stress, accumulation of sodium ions ($Na^+$) in the cytoplasm leads to membrane depolarization and promotes leakage of potassium ions ($K^+$) out of the cell. Therefore, to survive in saline soil, it is crucial for plants to maintain an appropriate $K^+/Na^+$ ratio in the cytoplasm, which depends on the operation of $Na^+/K^+$ transporters (Wu et al., 2018). The rice potassium transporter OsHAK1 promotes $K^+$ uptake and the $K^+/Na^+$ ratio under both low and high potassium conditions, which is essential for maintaining potassium-mediated growth and salt tolerance (Chen et al., 2015). The rice shaker $K^+$ channel OsAKT2 mediates $K^+$ recirculation from shoots to roots to maintain $Na^+/K^+$ homeostasis and improve salt tolerance (Tian et al., 2021). Moreover, high-affinity $K^+$ transporters such as HKTs also grant salinity tolerance to rice (Hamamoto et al., 2015; Rosas-Santiago et al., 2015; Wang et al., 2015; Suzuki et al., 2016a). Calcium ($Ca^{2+}$) can regulate the perception, uptake, and transport of various ions through

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the SOS (salt overly sensitive) pathway (Lin et al., 2009; Qiu et al., 2002; Yang and Guo, 2018a; b; Zhu et al., 1998), thereby coordinating $Na^+/K^+$ homeostasis in plants (Asano et al., 2012; Campo et al., 2014; Manishankar et al., 2018). The $Na^+/H^+$ antiporter SOS1 in the cell membrane is associated with $Na^+$ extrusion via roots in a saline environment and confers salinity tolerance to rice (Martínez-Atienza et al., 2007). *SOS2* and *SOS3*, encoding a protein kinase and $Ca^{2+}$-binding protein, respectively, are required for salinity tolerance in rice because they perceive the change in $Ca^{2+}$ in the cytosol under salinity and activate the signaling cascade (Kumar et al., 2013).

Apart from potassium, few mineral nutrients have been studied for their roles in salt tolerance. Sulfur nutrients have been found to improve plant photosynthesis and growth under salt stress by increasing glutathione production and abscisic acid (ABA) accumulation (Cao et al., 2014; Fatma et al., 2014, 2021; Chen et al., 2019). Nitrogen (N), an essential plant macronutrient, has been shown to improve salt tolerance (Mansour, 2000) through stimulation of antioxidation (Rais et al., 2013), osmotic adjustment (Nasab et al., 2014), maintenance of ion balance (Khan et al., 2016b), mitigation of ionic toxicity (Iqbal et al., 2015), and activation of numerous enzymes (Aragao et al., 2012). However, the underlying molecular mechanisms of N-improved salt tolerance in plants are currently unclear.

Transcription factors (TFs) play essential roles in the transcriptional control of stress-associated genes and are thus of utmost importance for breeding stress-tolerant crops (Zhang et al., 2017; Ahammed et al., 2020). MADS (MCM1, AG, DEFA, and SRF) family TFs control important growth and developmental processes such as seed germination and flowering time (Moyle et al., 2005; Chen et al., 2016; Wu et al., 2017; Yu et al., 2017; Yin et al., 2019). MADS-box TFs are also involved in the response to various abiotic stresses. For example, *OsMADS26* is a negative regulator of drought stress tolerance in rice (Khong et al., 2015). *OsMADS57*, in concert with *OsTB1*, mediates the transcription of *OsWRKY94* to confer cold tolerance in rice (Chen et al., 2018b). *OsMADS25*, *OsMADS27*, and *OsMADS57* are involved in the response to nutrient deficiency in rice (Yu et al., 2015; Chen et al., 2018a; Huang et al., 2019), and *OsMADS25* improves rice salinity tolerance when overexpressed (Wu et al., 2020).

We previously reported the *Arabidopsis* MADS-box TF AtAGL16 as a negative regulator of salt and drought tolerance (Zhao et al., 2020, 2021). To extend our work to rice, we have identified *OsMADS27*, which has the highest sequence similarity to *AtAGL16*. *OsMADS27* is induced by nitrate ($NO_3^-$) and ABA and acts as a target gene of miR444 to control root development in a $NO_3^-$-dependent manner (Puig et al., 2013; Yu et al., 2014; Chen et al., 2018a; Pachamuthu et al., 2022). When overexpressed, *OsMADS27* confers enhanced salt tolerance in transgenic seedlings (Chen et al., 2018a). However, the molecular mechanism underlying *OsMADS27*-mediated salt tolerance remains unclear. Likewise, the relationship of *OsMADS27*-mediated salt tolerance to N nutrients has not been investigated in rice. In this study, we reveal the molecular mechanism of *OsMADS27*-mediated salt tolerance and the $NO_3^-$ dependence of *OsMADS27*-mediated salt tolerance in rice, which can be exploited for the improvement of crop salinity tolerance.

## RESULTS

### Expression of *OsMADS27* is specifically induced by nitrate, and NaCl-induced expression of *OsMADS27* is nitrate dependent

To characterize the detailed expression pattern of *OsMADS27*, we examined its spatiotemporal expression by quantitative RT–PCR (qRT–PCR) analyses at three rice developmental stages: seedling, vegetative, and premature stages. *OsMADS27* was expressed in all tissues examined but was expressed at much higher levels in roots, leaves, and sheaths (Supplemental Figure 1A). The tissue-specific expression patterns of *OsMADS27* were also examined in *OsMADS27pro*::*GUS* transgenic plants (Supplemental Figure 1B) and were consistent with our qRT–PCR results and previous reports (Puig et al., 2013; Yu et al., 2014; Chen et al., 2018a; Pachamuthu et al., 2022). Notably, a strong GUS signal was detected in the root stele (Supplemental Figure 1B).

To examine the response of *OsMADS27* to nutrient and salt stress, we grew wild-type (WT) seedlings under normal conditions, transferred 7-day-old seedlings to hydroponic medium without N for 48 h, and then transferred the seedlings to hydroponic medium supplemented with 2 mM $KNO_3$, 2 mM $NH_4Cl$, 2 mM KCl, or 150 mM NaCl. Surprisingly, NaCl did not induce the expression of *OsMADS27* under our conditions, nor did $NH_4Cl$ or KCl. Only $KNO_3$ induced the expression of *OsMADS27*, which plateaued at 12 h with an approximately five-fold increase (Figure 1A). In addition, when the seedlings were transferred to N-free medium, the $KNO_3$-induced expression of *OsMADS27* gradually decreased (Figure 1B). These results clearly show that the expression of *OsMADS27* is specifically responsive to $KNO_3$.

To show the nitrate dependence of NaCl-induced *OsMAD27* expression, we treated N-starved seedlings under 0 mM $KNO_3$ conditions with 150 mM NaCl for 3 h and then added 2 mM $KNO_3$ for another 3 h. The qRT–PCR results clearly showed that NaCl was unable to induce the expression of *OsMADS27* in the absence of $KNO_3$. NaCl stimulated the expression of *OsMADS27* only in the presence of $KNO_3$ (Figure 1C). This was further confirmed with *OsMADS27pro:GUS* transgenic rice, in which the GUS signal exhibited a similar response. No change in GUS activity was observed under treatment with KCl plus NaCl, whereas a strong induction of GUS was seen in roots treated with $KNO_3$ plus NaCl (Figure 1D and 1E).

To determine whether there was a synergistic effect of nitrate plus NaCl or ABA, we treated seedlings with KCl as a control, nitrate, NaCl, ABA, and $KNO_3$ plus NaCl or ABA. The $KNO_3$ plus NaCl and $KNO_3$ plus ABA treatments significantly increased *OsMADS27* transcript levels compared with the $KNO_3$ treatment, indicating that *OsMADS27* expression was synergistically induced by $KNO_3$ plus NaCl and $KNO_3$ plus ABA. By contrast, NaCl and ABA alone did not significantly induce *OsMADS27* expression compared with the KCl treatment (Figure 1F). We also quantified OsMADS27 protein levels in *OsMADS27pro:OsMADS27-GFP* plants by western blotting with anti-GFP antibodies under low, normal, and high concentrations of $KNO_3$ (0.02 mM, 0.2 mM, and 2 mM) with or without 100 mM NaCl for 10 days. The results in Figure 1G show that OsMADS27 protein level was positively correlated with $KNO_3$ concentration and enhanced by NaCl treatment.

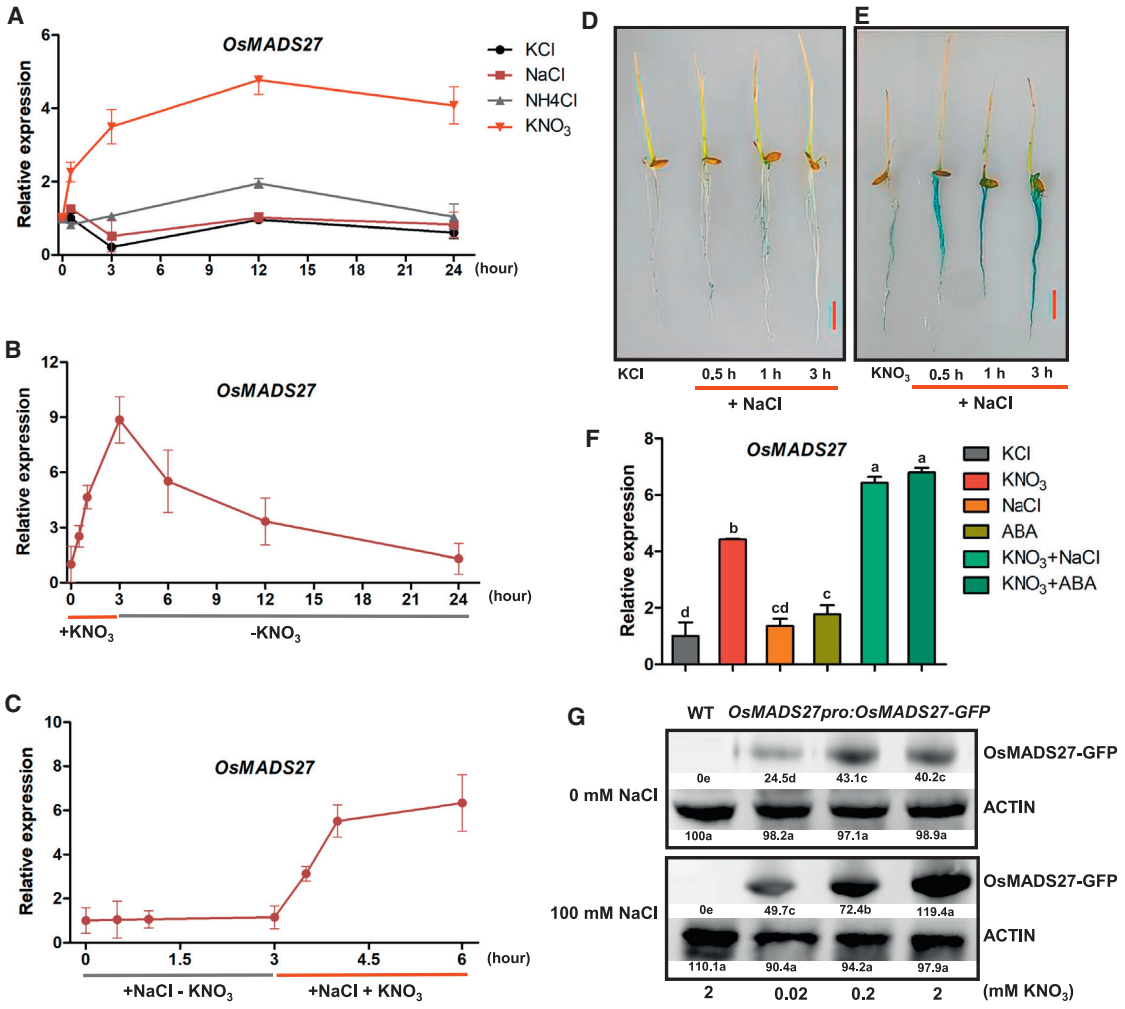


**Figure 1. *OsMADS27* is specifically responsive to nitrate.**

**(A)** Time-course analyses of *OsMADS27* expression in response to N and salt stress. Seven-day-old wild-type plants grown on hydroponic medium with 1.5 mM $KNO_3$ were transferred to hydroponic medium without N for 2 days and then transferred to hydroponic medium with 2 mM $KNO_3$, 2 mM $NH_4Cl$, 140 mM NaCl, or 2 mM KCl for 0, 0.5, 3, 12, or 24 h. RNA was extracted from whole seedlings for qRT–PCR analyses as described in the methods. Values are the mean ± SD (n = 3).

**(B)** Time-course analyses of *OsMADS27* expression in response to $KNO_3$ depletion. Seven-day-old wild-type plants grown on hydroponic medium with 1.5 mM $KNO_3$ were treated with 2 mM $KNO_3$ for 0, 0.5, 1, or 3 h and then transferred to hydroponic medium without $KNO_3$ for 3, 6, 12, or 24 h. RNA was extracted from whole seedlings for qRT–PCR analyses as described in the methods. Values are the mean ± SD (n = 3).

**(C)** $KNO_3$-dependent induction of *OsMADS27* expression by NaCl. Wild-type seedlings grown hydroponically on N-free medium for 7 days were treated with 140 mM NaCl for 0, 0.5, 1, or 3 h and then transferred to hydroponic medium with 140 mM NaCl + 2 mM $KNO_3$ for 0.5, 1, or 3 h. RNA was extracted from whole seedlings for qRT–PCR analyses as described in the methods. Values are the mean ± SD (n = 3).

**(D and E)** The response of *OsMADS27pro*:*GUS* to NaCl. Seven-day-old *OsMADS27pro*:*GUS* lines grown on N-free medium with 2 mM KCl (**D**) or 2 mM $KNO_3$ (**E**) were treated with 140 mM NaCl for 0.5, 1, or 3 h. Seedlings were incubated in GUS buffer for 3 h before photographs were taken. Bar represents 1 cm.

**(F)** Nitrate plus NaCl or ABA synergistically enhances the expression of *OsMADS27*. Seven-day-old wild-type plants grown on hydroponic medium with 1.5 mM $KNO_3$ were transferred to hydroponic medium without N for 2 days and then transferred to hydroponic medium with 2 mM KCl, 2 mM $KNO_3$, 140 mM NaCl, 10 μM ABA, 2 mM $KNO_3$ plus 140 mM NaCl, or 2 mM $KNO_3$ plus 10 μM ABA for 3 h. Total RNA was extracted from whole seedlings for qRT–PCR analyses as described in the methods. Values are the mean ± SD (n = 3). Different letters denote significant differences ($P < 0.05$) from Duncan's multiple range tests.

**(G)** OsMADS27 protein level in *OsMADS27pro:OsMADS27-GFP* plants. Two-week-old *OsMADS27pro:OsMADS27-GFP* seedlings grown hydroponically on medium containing different N concentrations (0.02 mM, 0.2 mM, and 2 mM $KNO_3$) without (control) or with 100 mM NaCl were used for the analysis of OsMADS27 protein levels by western blotting with anti-GFP antibodies. ZH11 (WT) grown on medium with 2 mM $KNO_3$ served as a control. The intensity of OsMADS27-GFP bands was quantified with ImageJ from three replicates, and the statistical results are shown below the gel blots. All band intensities were normalized to that of actin in WT plants grown on medium with 2 mM $KNO_3$ and 0 mM NaCl. Values are the mean (n = 3). Different letters denote significant differences ($P < 0.05$) from Duncan's multiple range tests.

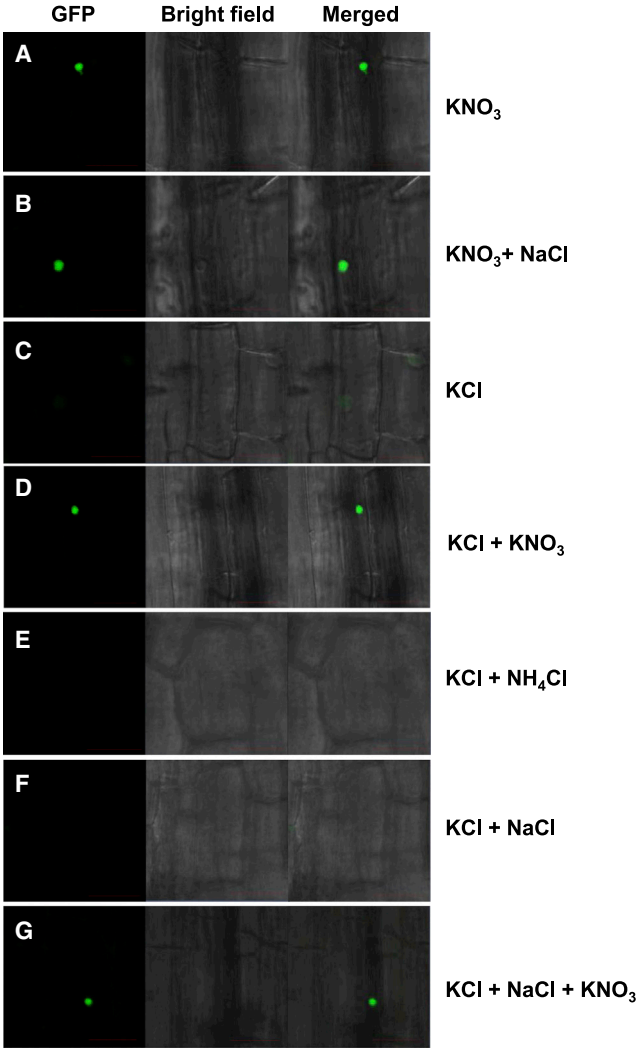


**Figure 2. Nuclear localization of OsMADS27 was observed only in the presence of nitrate.**

*OsMADS27pro:OsMADS27-GFP* plants were grown on N-free MS medium supplied with 2 mM $KNO_3$ (**A**) or 2 mM KCl (**C**) for 10 days. The seedlings in (**A**) were treated with 150 mM NaCl (**B**) for 60 min before green fluorescence observation. The seedlings in (**C**) were treated with 2 mM $KNO_3$ (**D**), 2 mM $NH_4Cl$ (**E**), 150 mM NaCl (**F**), and 150 mM NaCl + 2 mM $KNO_3$ (**G**) for 60 min before GFP observation. The green fluorescence was observed with a Zeiss 880 microscope. Scale bars represent 20 μm.

Taken together, our results clearly show that the expression of *OsMADS27* is specifically induced by $NO_3^-$ and synergistically promoted by $NO_3^-$ plus NaCl or ABA. NaCl- and ABA-induced expression of *OsMADS27* is dependent on $NO_3^-$.

## Nuclear localization of OsMADS27 is responsive to nitrate

To reveal the subcellular localization of the OsMADS27 protein and its response to nutrients, we generated *OsMADS27pro:OsMADS27-GFP* transgenic lines. The transgenic plants were grown on N-free Murashige and Skoog (MS) medium supplemented with 2 mM $KNO_3$ (Figure 2A) or 2 mM KCl (Figure 2C) for 10 days. Then the seedlings receiving $KNO_3$ were treated with 150 mM NaCl (Figure 2B), and the seedlings receiving KCl were treated with 2 mM $KNO_3$ (Figure 2D), 2 mM $NH_4Cl$ (Figure 2E), 150 mM NaCl (Figure 2F), or 150 mM NaCl plus 2 mM $KNO_3$ (Figure 2G) for 60 min before confocal laser-scanning microscopy observation. GFP signals were detected in the nucleus whenever $KNO_3$ was included in the medium, regardless of the presence of other supplements (Figure 2A, 2B, 2D, and 2G). No GFP signals were detected in the presence of KCl (Figure 2C), KCl plus $NH_4Cl$ (Figure 2E), or KCl plus NaCl (Figure 2F). These results clearly show that the nuclear accumulation of OsMADS27 is specifically responsive to nitrate and appears to be correlated with *OsMADS27* transcript level.

## *OsMADS27* is a positive regulator of salt tolerance in rice

To explore the ability of *OsMADS27* to confer salt tolerance to rice, we generated two independent loss-of-function mutant lines of *OsMADS27* (*osmads27-1* and *osmads27-2*) by CRISPR–Cas9-based editing. Protein sequence alignment showed that mutations in both mutants resulted in a premature stop codon that interrupted the open reading frame of *OsMADS27* (Supplemental Figure 2A–2D). We also generated two independent overexpression (OE) lines of *OsMADS27* (OE7 and OE8) driven by the *OsACTIN1* promoter (Supplemental Figure 2E and 2F).

To evaluate the role of *OsMADS27* in salt stress tolerance of rice, we germinated seeds of OE7, OE8, *osmads27-1*, *osmads27-2*, and WT in soil in the presence of 0 mM or 150 mM NaCl. Under 0 mM NaCl conditions, there was no difference in germination rate among the genotypes (Supplemental Figure 3A). However, under 150 mM salt stress, OE lines displayed a germination rate of 80% at day 6 compared with WT and *osmads27* mutants, which exhibited germination rates of 55% and 30%, respectively (Supplemental Figure 3B). We also conducted a salt tolerance assay with soil-grown seedlings (Supplemental Figure 3C). Upon treatment of 20-day-old soil-grown seedlings with 150 mM NaCl for 15 days, 80% of the OE plants survived. By contrast, WT and *osmads27* mutants had survival rates of 43% and 12%, respectively, and all genotypes displayed 100% survival in the 0 mM NaCl control treatment (Supplemental Figure 3D). Together, these results clearly demonstrate that *OsMADS27* is a positive regulator of salt tolerance in rice.

## *OsMADS27*-mediated salt tolerance in rice is nitrate dependent

The $NO_3^-$ dependence of NaCl-induced expression of *OsMADS27* prompted us to test whether *OsMADS27*-mediated salt tolerance was nitrate dependent. Thus, we further explored the salt tolerance of different *OsMADS27* genotypes under different $NO_3^-$ concentrations. We grew seedlings in modified hydroponic culture with different $NO_3^-$ concentrations for 10 days and then supplemented them with or without 140 mM NaCl in the hydroponic culture and allowed the seedlings to grow for another week. Under 0 mM NaCl conditions, the seedling survival rate was 100% for all genotypes under all three $NO_3^-$ concentrations (Figure 3A and 3C). Under 140 mM NaCl conditions, the seedling survival rate of all three genotypes was less than 20% under low $NO_3^-$ conditions (0.02 mM, LN). However, under

normal $NO_3^-$ conditions (0.2 mM, NN), the increased $NO_3^-$ alleviated salt stress, as reflected in the seedling survival rates of *osmads27* mutants (20%), WT (42%), and OE lines (55%) compared with those under LN conditions (Figure 3B and 3D). Under high $NO_3^-$ conditions (2 mM, HN), salt stress was further alleviated, as evidenced by the increased seedling survival rates of *osmads27* mutants (30%), WT (70%), and OE lines (80%). We performed similar hydroponic culture experiments using $NH_4^+$ as the sole N source and found that $NH_4^+$ did not confer salt tolerance in any genotype (Supplemental Figure 4), further supporting the nitrate-dependence of *OsMADS27-mediated* salt tolerance. Together, these results demonstrate that the salt tolerance mediated by *OsMADS27* is dependent on $NO_3^-$ but not on $NH_4^+$.

We next measured the $Na^+$ and $K^+$ contents of hydroponically cultured seedlings under 0.2 mM $NO_3^-$ conditions. In the 0 mM NaCl treatment, $Na^+$ levels were low in the roots and shoots of all three genotypes; by contrast, in the 140 mM NaCl treatment, the $Na^+$ level increased significantly in the roots and shoots of all three genotypes. However, the $Na^+$ content was higher in the *osmads27-1* mutant than in the WT, whereas it was lower in the OE plants (Figure 3E). The $K^+$ content was higher in OE roots and shoots under both 0 and 140 mM NaCl treatment, whereas it was lower in the *osmads27-1* mutant under salt stress than in the WT (Figure 3F). These results indicate that OsMAD27 may regulate the expression of genes involved in ion homeostasis to enhance salt tolerance.

## RNA sequencing reveals *OsMADS27*-regulated genes involved in stress tolerance

To determine the global network of genes regulated by *OsMADS27*, we performed transcriptomic analyses of WT, *osmads27-1*, and OE plants subjected to 0 mM or 100 mM NaCl for 3 consecutive days to identify DEGs (differentially expressed genes). The number of DEGs differed significantly among WT, *osmads27-1*, and OE plants under saline and normal conditions, revealing that *OsMADS27* widely regulates the transcriptome in response to salt stress (Figure 4A and 4B).

In-depth information about the DEGs was obtained by KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway and GO (Gene Ontology) analyses to detect significantly expressed DEGs in *osmads27-1* vs. WT and OE vs. WT under control and salt conditions (Figure 4C and Supplemental Figure 5). Genes involved in the salt response were highly enriched in the DEGs, indicating that *OsMADS27* may coordinately regulate key salt tolerance genes (Figure 4C). The heatmap demonstrates that the transcript levels of an ethylene response factor (*OsWR2*), a salinity-responsive MYB transcription factor (*OsMPS*), an A-type response regulator (*OsRR2*), a rice cyclin gene (*OsCycB1;3*), an oxidative stress 3 homolog (*OsO3L2*), and a heat shock transcription factor (*OsSPL7*) were higher in the OE plants under salt stress. In addition to salt-responsive genes, key genes involved in ion transport, such as $K^+$ transporters (*OsHKT1.1*, *OsHKT2.3*), a $K^+$ channel (*OsKAT3*), a salt-inducible calmodulin gene (*OsCAM1*), and an aluminum-activated malate transporter (*OsALMT4*), were significantly downregulated in the *osmads27-1* mutant but upregulated in the OE line under salt stress. *OsMADS27* also positively regulated the expression of prominent ABA-responsive genes such as *OsNCED1*, *OsRAB16*, and *OsGLP1*, which were expressed at higher levels in OE plants. Moreover, genes encoding peroxidases that function in antioxidation, including *OsPRX29*, *OsPRX27*, *OsPRX74*, *OsGPX*, and *OsPRX132*, were significantly upregulated in OE vs. WT (salt group). *OsMADS27* also positively regulated the expression of N-responsive genes, as the expression levels of *OsNRT2.4*, *OsNAR2.1*, *OsNPF5.16*, *OsNPF2.2/OsPTR2*, and *OsNLA1* were predominantly enhanced in the WT vs. OE group after salt treatment (Figure 4C). In addition, GO enrichment analyses showed that *OsMADS27* also affected the expression of some genes involved in the oxidation–reduction process, regulation of transcription, defense response, and protein phosphorylation under normal conditions (Supplemental Figure 5A and 5B), whereas genes related to hydrogen peroxide catabolic process, flavonoid biosynthesis, ABA catabolism, defense response, and tyrosine catabolism were also regulated by *OsMADS27* under salt stress conditions (Supplemental Figure 5C and 5D).

The expression patterns of genes involved in salt response and ion transport were verified by qRT–PCR, which was largely in agreement with the RNA-seq data (Figure 4D). Taken together, our RNA-seq data suggest that *OsMADS27* confers salt tolerance in rice by regulating salt-responsive genes, maintaining ion balance, and enhancing reactive oxygen species (ROS) scavenging ability.

## OsMADS27 transcriptionally activates *OsHKT1.1* and *OsSPL7*

To demonstrate the ability of OsMADS27 to regulate its target genes, we generated transgenic rice plants expressing *OsMADS27pro:OsMADS27-GFP* for ChIP assays. The *cis*1 region of the *OsHKT1.1* promoter and *cis*2 and *cis*3 regions of the *OsSPL7* promoter were found to be enriched in the transgenic rice plants, as demonstrated by qPCR analyses (Figure 5A and 5B). We also performed transactivation assays using 35S-*OsMADS27* as the effector and *OsHKT1.1* and *OsSPL7* promoter-driven *LUC* (luciferase) as reporters. When reporter and effector were co-injected into tobacco leaves, we observed that OsMADS27 activated the expression of *LUC* genes linked to the promoters of *OsHKT1.1* and *OsSPL7* (Figure 5C and 5D). These results demonstrate that OsMADS27 binds to *cis* elements in the promoters of *OsHKT1.1* and *OsSPL7* and activates their expression.

## *OsMADS27* is a positive regulator of grain yield

To confirm the hydroponic culture results above, we grew plants of the three genotypes in potted vermiculite and fed them with nutrient solutions containing different concentrations of $NO_3^-$ (1.5 mM LN, 2.5 mM NN, 5 mM HN) with or without 65 mM NaCl as described in the methods. Yield-related agronomic trait data were collected for statistical analyses (Supplemental Figure 6A). Supplemental Figure 6B shows the grain yield per plant of the three genotypes under three N levels without salt stress. The OE line exhibited significantly higher yield than the WT at all three N levels, whereas *osmads27-1* showed lower yield than the WT. The OE line exhibited grain yield increases of 29%, 38%, and 25% relative to the WT under LN, NN, and HN conditions, respectively, but *osmads27-1* displayed yield decreases of 20%, 22%, and

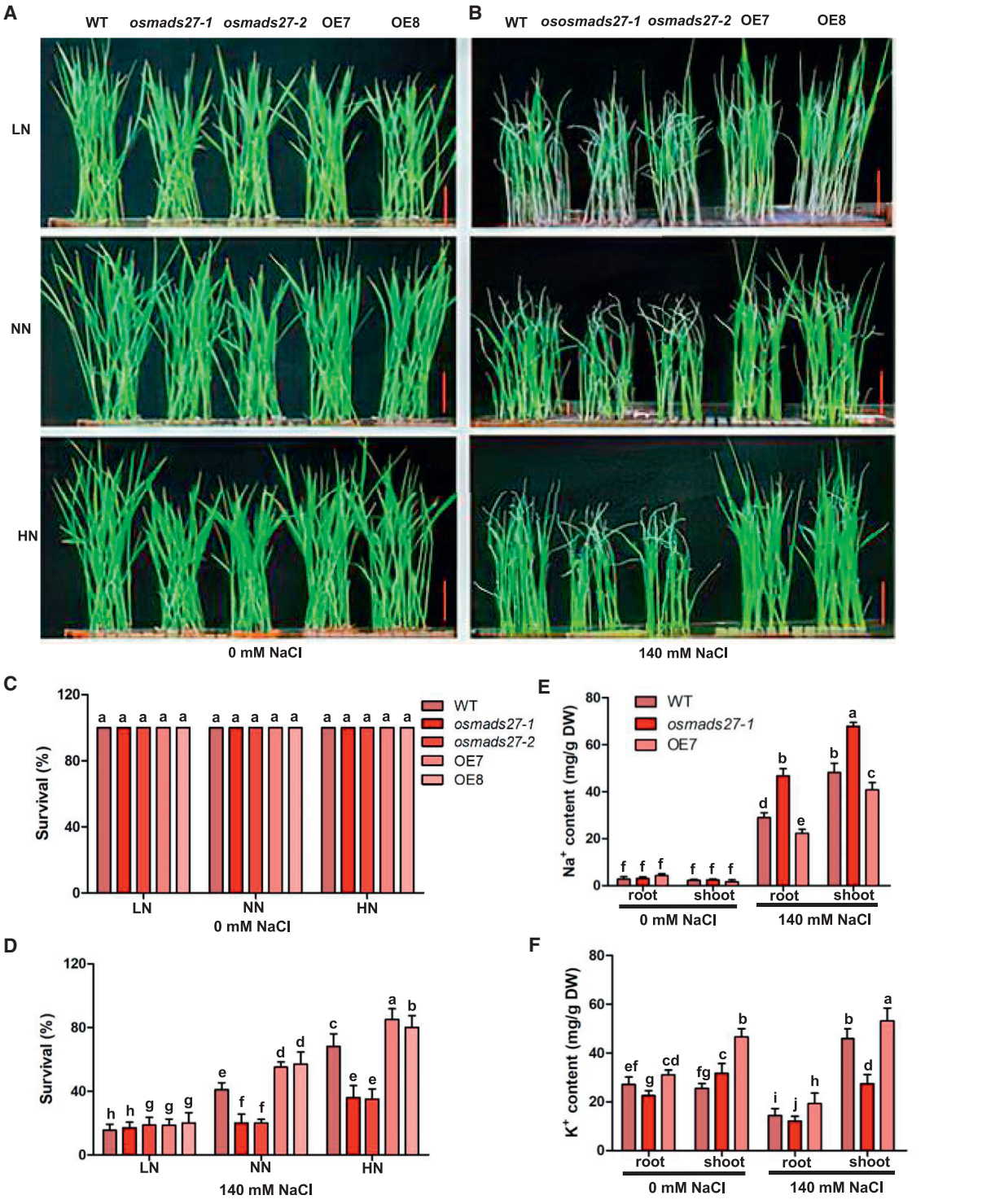


**Figure 3. Nitrate-dependent salt tolerance of seedlings.**

**(A–D)** Hydroponic salt tolerance assay. Seeds of the wild type (WT), *OsMADS27* knockout mutants (*osmads27-1* and *osmads27-2*), and overexpression lines (OE7 and OE8) were germinated at 37°C for 4 days and transferred to modified hydroponic medium containing different N concentrations (0.02 mM, 0.2 mM, 2 mM $KNO_3$) for 7 days followed by application of 0 mM or 140 mM NaCl for 7 days before photographs were taken (**A–B**), and the survival rate was calculated (**C–D**). Values are the mean ± SD (n = 3 replicates, 32 seedlings per replicate).

**(E and F)** Sodium ($Na^+$) and potassium ($K^+$) contents in the roots and shoots of the wild type (WT), *OsMADS27* knockout mutant (*osmads27-1*), and overexpression line (OE7). Seeds were germinated at 37°C for 4 days, transferred to modified hydroponic medium containing 0.2 mM $KNO_3$ for 7 days, and then treated with 140 mM or 0 mM NaCl for 5 days. $Na^+$ and $K^+$ contents were quantified in roots and shoots (**E–F**) as described in the methods. Values are the mean ± SD (n = 3 replicates, 30 seedlings per replicate). Different letters denote significant differences ($P < 0.05$) from Duncan's multiple range tests.

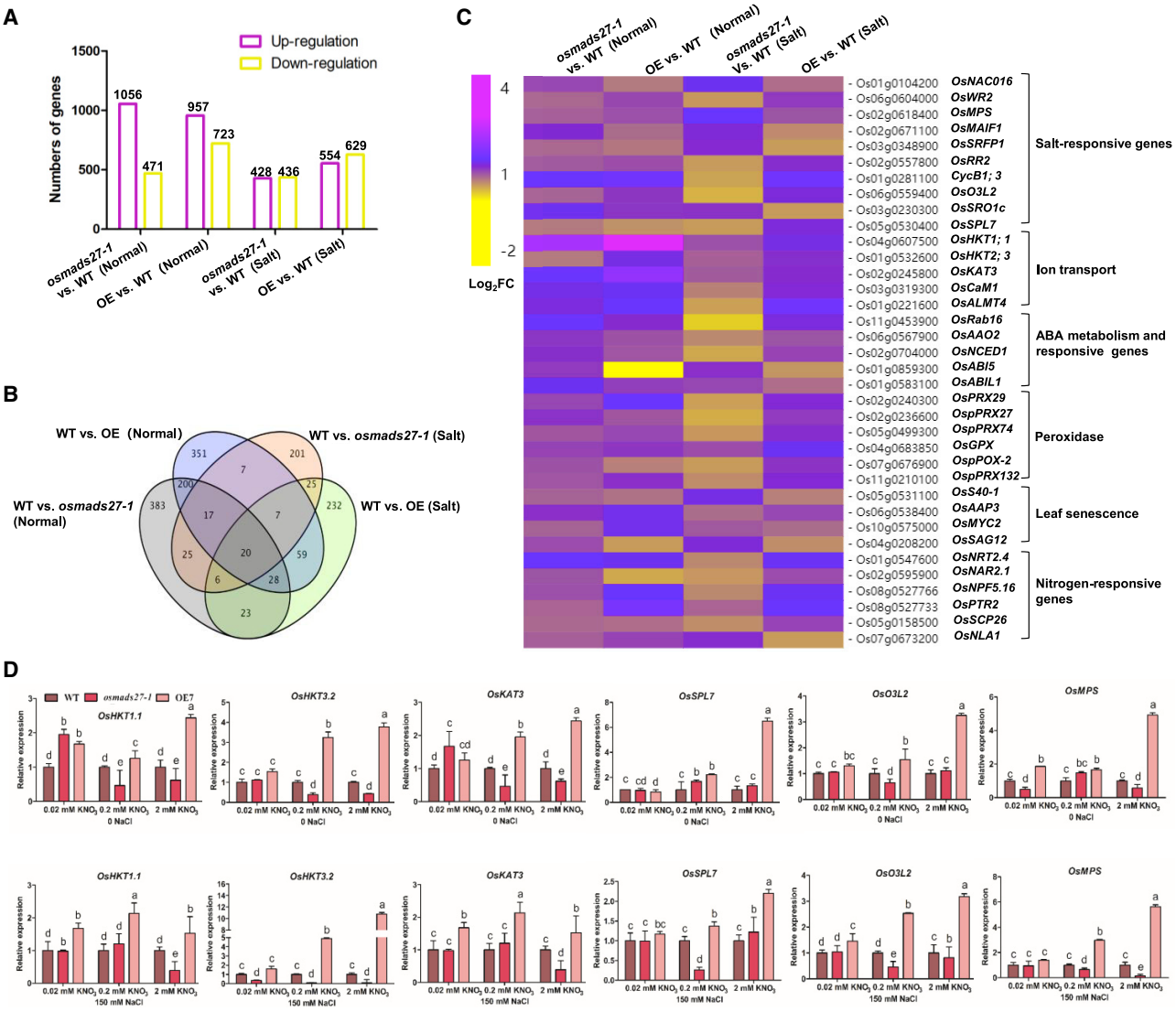


**Figure 4. Transcriptomic analysis of differentially expressed genes (DEGs) affected by OsMADS27.**

**(A)** Number of DEGs. Statistical data of DEGs in the (KO vs. WT)-control, (OE vs. WT)-control, (KO vs. WT)-salt, and (OE vs. WT)-salt comparisons.

**(B)** Venn diagram of DEGs in the (KO vs. WT)-control, (OE vs. WT)-control, (KO vs. WT)-salt, and (OE vs. WT)-salt comparisons. The numbers represent the total numbers of DEGs in different comparison groups.

**(C)** Hierarchical clustering analysis of N- and salt stress-related genes affected by OsMADS27 among the DEGs. The heatmap shows fold changes in the abundance of gene transcripts in different comparison groups.

**(D)** OsMADS27 broadly regulates genes involved in salt tolerance. Seven-day-old plants grown hydroponically on medium containing different N concentrations (0.02, 0.2, and 2 mM $KNO_3$) supplemented with either 0 mM NaCl or 150 mM NaCl were harvested for qRT–PCR analyses of the indicated genes. *Actin* was used as an internal control. Different letters denote significant differences ($P < 0.05$) from Duncan's multiple range tests.

25%. Yield was positively correlated with N level, tiller number per plant (Supplemental Figure 6C), and panicle number (Supplemental Figure 6D). Both tiller and panicle numbers displayed patterns of genotype and N-level effects similar to those of grain yield. Under salt stress, the OE line exhibited grain yield increases of 66%, 40%, and 28% relative to the WT under LN, NN, and HN conditions, respectively, whereas *osmads27-1* displayed yield decreases of 33%, 40%, and 28% under the same conditions (Supplemental Figure 6E). Tiller and panicle numbers displayed trends similar to that of grain yield (Supplemental Figure 6F and 6G). These results suggest that *OsMADS27* is a positive regulator of grain yield and further support the notion that *OsMADS27* positively regulates salt tolerance in a $NO_3^-$-dependent manner in rice.

We also conducted field trials to examine the yields of the three *OsMADS27* genotypes in the field under varying N supply and found that agronomic traits, including nitrogen use efficiency (NUE), actual yield per plot, grain yield per plant, panicle number per plant, number of seeds per plant, and primary branch number per panicle, were significantly increased in OE plants under normal and high N availability but reduced in *osmads27-1* plants compared with the WT (Figure 6). The field trial data further confirm that *OsMADS27* acts as a

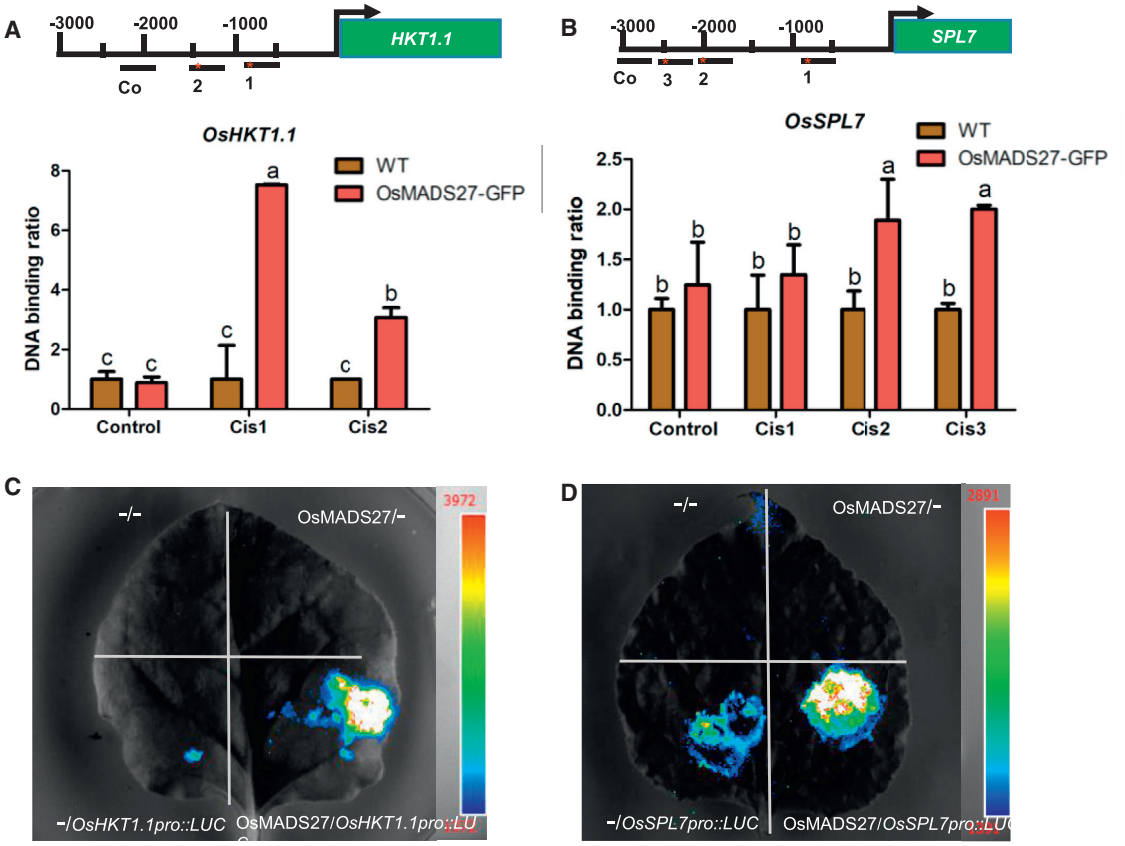


**Figure 5. OsMADS27 activates *OsHKT1.1* and *OsSPL7* by binding to the CArG motif in their promoters.**
**(A and B)** ChIP–qPCR assay. Enrichment of fragments containing CArG motifs (marked with asterisks) in the promoters of *OsHKT1.1* and *OsSPL7* was examined in *OsMADS27pro:OsMADS27-GFP* and wild-type plants. Approximately 200-bp fragments *cis*1 and *cis*2 of the *OsHKT1.1* promoter (**A**) and *cis*2 and *cis*3 of the *OsSPL7* promoter (**B**) were enriched in *OsMADS27pro:OsMADS27-GFP* plants by anti-GFP antibodies, as shown in qRT–PCR analyses. Values are the mean ± SD (n = 3 replicates). Different letters denote significant differences ($P < 0.05$) from Duncan's multiple range tests.
**(C and D)** Luciferase activity assay. pRI101-*OsMADS27* acted as an effector. pGreenII0800-*OsHKT1.1pro::LUC*/*OsSPL7pro::LUC* functioned as reporters. -/- represents the empty pRI101 and pGreenII 0800 plasmids. -/-, *OsMADS27*/-, -/*OsHKT1.1pro::LUC*, and -/*OsSPL7pro::LUC* served as negative controls; *OsMADS27*/*OsHKT1.1pro::LUC* (**E**) and *OsMADS27*/*OsSPL7pro::LUC* (**F**) were experimental groups. Different constructs were separately co-infiltrated into 4-week-old tobacco leaves, and luciferase activity was detected by the luciferase assay system.

positive regulator of grain yield, which is positively correlated with $NO_3^-$ availability.

## DISCUSSION

In addition to being an essential nutrient, $NO_3^-$ acts as a signaling molecule involved in controlling multiple metabolic processes in plants (Crawford, 1995). Importantly, nitrate is also a major factor affecting the salt tolerance of crops. $NO_3^-$ application can promote the growth and yield of rice, wheat, canola, citrus, strawberry, pepper, allium, and other plants under salt stress (Kaya et al., 2003; Kaya and Higgs, 2003; Domingo et al., 2004; Zheng et al., 2008; Gao et al., 2016; Çavuşoğlu et al., 2017). However, the intrinsic molecular mechanism of $NO_3^-$-mediated alleviation of salt stress has not been reported to date. In this study, we revealed that OsMADS27-mediated salt tolerance is nitrate dependent in rice. We demonstrated that the expression of *OsMADS27* was specifically induced by $NO_3^-$ but not by KCl, $NH_4Cl$, NaCl, or ABA alone (Figure 1A and 1F). The responsiveness of *OsMADS27* to nitrate is consistent with previous reports (Puig et al., 2013; Yu et al., 2014; Chen et al., 2018a; Pachamuthu et al., 2022). Furthermore, we showed that NaCl- and ABA-induced expression of *OsMADS27* was also dependent on nitrate, and nitrate plus NaCl or ABA synergistically enhanced *OsMADS27* expression (Figure 1C and 1F). OsMADS27 protein level appeared to be positively correlated with *OsMADS27* transcript level (Figure 1F and 1G). Likewise, the nuclear accumulation of OsMADS27 appeared to be correlated with its transcript level as well (Figure 2A, 2B, 2D, and 2G). OsMADS27 activates an array of stress tolerance-related genes, as revealed by RNA-seq analyses (Figure 4), by directly binding to their promoters, as demonstrated for *OsHKT1.1* and *OsSPL7* (Figure 5), thereby enhancing growth and grain yield under salt stress in rice (Figure 3 and Supplemental Figure 6). However, in the absence of $NO_3^-$, the expression of *OsMADS27* was low, which was insufficient to confer salt tolerance in rice. A working model is proposed for nitrate-responsive *OsMADS27*-promoted salt tolerance (Figure 7). Our study revealed a novel mechanism of $NO_3^-$-dependent salt tolerance mediated by *OsMADS27* that may be exploited for the improvement of rice salt tolerance and grain yield.

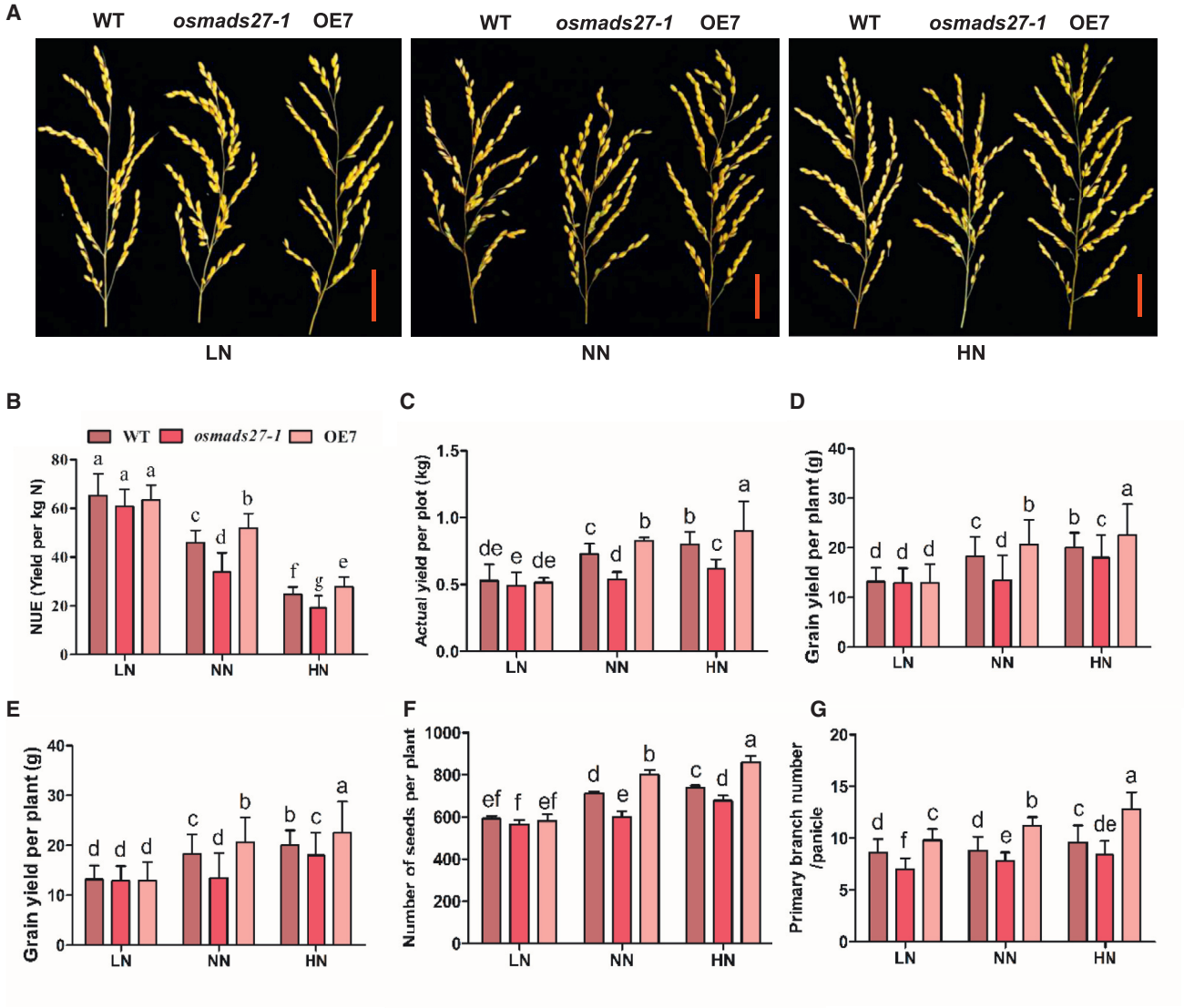


**Figure 6. *OsMADS27* improves NUE and grain yield in the field under different nitrogen concentrations.**

**(A)** The panicles of wild-type ZH11 (WT), *osmads27-1*, and *OsMADS27*-OE7 plants. Scale bar represents 4 cm.

**(B–G)** Nitrogen use efficiency (NUE) **(B)**, actual yield per plot **(C)**, grain yield per plant **(D)**, panicle number per plant **(E)**, number of seeds per plant **(F)**, and primary branch number per panicle **(G)** were calculated. Values are the mean ± SD (n = 3 replicates). Different letters denote significant differences ($P < 0.05$) from Duncan's multiple range tests.

## Mechanisms of *OsMADS27*-conferred salt tolerance

TFs regulate the expression of various stress-related genes by binding with regulatory motifs in their promoters in response to stresses (Yamaguchi-Shinozaki and Shinozaki, 2006). Probably benefiting from the simultaneous coordination of the expression of salt-responsive genes (Figure 4), overexpression of the MADS-box TF *OsMADS27* increased the transcript levels of regulators such as ethylene response factor *OsWR2* (Zhou et al., 2013), salt stress response MYB transcription factor *OsMPS* (Schmidt et al., 2013), A-type response regulator *OsRR2* (Ito and Kurata, 2006), and rice cyclin gene *OsCycB1;3* (La et al., 2006), resulting in significantly improved salt tolerance during the germination, seedling, and reproductive phases of rice (Figure 3, Supplemental Figures 3 and 6).

Salt tolerance is highly dependent on intracellular ion homeostasis to maintain cell turgor and membrane potential (Bargmann et al., 2009). In our transcriptomic data, the expression of $K^+$ transporters such as *OsHKT1.1* (Imran et al., 2020) and *OsHKT2.3* (Zhang et al., 2018), the $K^+$ channel *OsKAT3* (Hwang et al., 2013), and the $Ca^{2+}$ sensor *OsCAM1.1*, which positively regulates salt tolerance in rice (Saeng-ngam et al., 2012), was significantly enhanced in OE plants compared with WT plants under salt stress (Figure 4C), a finding supported by reduced $Na^+$ and increased $K^+$ levels in the OE plants (Figure 3E and 3F). We found that OsMADS27 directly binds and transcriptionally activates *OsHKT1.1*, which encodes a membrane-localized high-affinity $K^+$ transporter (Figure 5). Rice *oshkt1.1* knockout mutants are salt sensitive, highlighting the function of OsHKT1.1 in $Na^+$ retrieval from leaf blades (Wang et al., 2015).

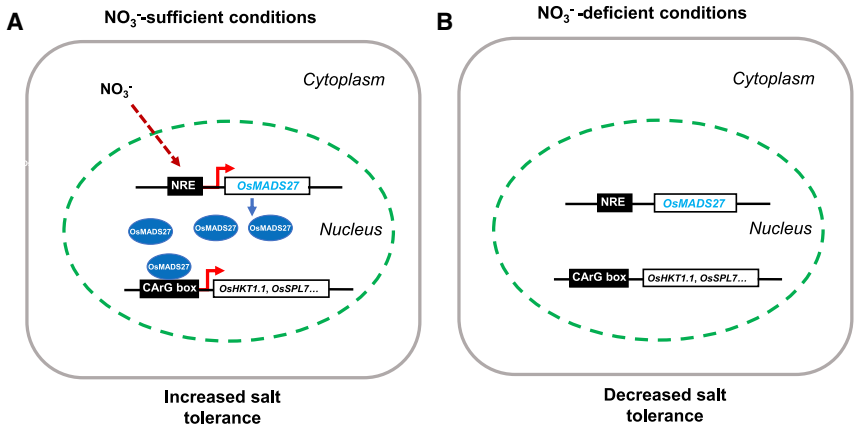


**Figure 7. A working model for nitrate-responsive *OsMADS27*-promoted salt tolerance.**

**(A)** Under nitrate-sufficient conditions, nitrate induces the expression of *OsMADS27*, leading to a high level of OsMADS27 that directly binds to the promoters of its target genes such as *OsHKT1.1* and *OsSPL7*, significantly enhancing their expression and improving the salt tolerance of rice.

**(B)** Under nitrate-deficient conditions, the expression of *OsMADS27* is not induced, thereby attenuating the expression of downstream salt tolerance-related genes.

These results demonstrate that *OsMADS27* positively regulates salt tolerance in rice by maintaining ion homeostasis.

Salinity leads to the accumulation of ROS in plants (Luo et al., 2021), and increased ROS production leads to oxidative burden, damaging cellular membranes as well as macromolecules (Lin et al., 2020). As a target of OsMADS27 (Figure 5), the heat shock transcription factor gene *OsSPL7* plays an important role in maintaining ROS homeostasis in rice. The *spl7* mutant lost regulation of nicotinamide adenine dinucleotide oxidase, resulting in greater accumulation of $H_2O_2$ in cells (Hoang et al., 2019). Our RNA-seq results indicate that *OsMADS27*-overexpressing plants are likely to have improved resistance against oxidative stress (Figure 4C). The upregulation of a number of peroxidases (*OsPRX29*, *OsPRX27*, *OsPRX74*, *OsGPX*, and *OsPRX132*) in OE plants demonstrated that overexpression of *OsMADS27* could ameliorate salt-generated oxidative stress.

The stress hormone ABA plays an important role in the response of plants to salt (Duan et al., 2013; Suzuki et al., 2016b). The enrichment of genes involved in ABA synthesis, such as *OsAAO2* and *OsNCED1* (Huang et al., 2021), and ABA-responsive genes, such as *OsABI5* and *OsRAB16* (Zou et al., 2008; Jiang et al., 2019), in the OE vs. WT comparison under salt stress (Figure 4C) implied that *OsMADS27* might also be involved in ABA signaling. *OsMADS27* controls $NO_3^-$-dependent root growth via the ABA pathway (Chen et al., 2018a). The possible crosstalk between *OsMADS27*, ABA signaling, and salt stress tolerance requires further attention. Taken together, our results suggest that *OsMADS27* mediates salt tolerance in rice mainly by balancing ion homeostasis, enhancing ROS scavenging ability, and regulating stress-responsive regulators and the ABA signaling pathway.

Surprisingly, OsMADS27, which had the highest sequence homology to *Arabidopsis* AtAGL16, has functionally diverged in a manner opposite to AtAGL16. AtAGL16 is a negative regulator of the stress response in *Arabidopsis* (Zhao et al., 2020, 2021), whereas OsMADS27 is a positive regulator of salt tolerance in rice. It is currently unknown how these two genes functionally evolved and diverged in dicots versus monocots, but the study of this interesting phenomenon would be quite appealing to evolutionary biologists.

## What controls the nitrate dependence of *OsMADS27*-mediated salt tolerance?

Multiple members of the MADS-box TF family are involved in the regulation of $NO_3^-$ responses. *Arabidopsis* nitrate regulated1 (AtANR1) is the first $NO_3^-$ regulator found to be involved in the regulation of lateral root developmental plasticity in response to $NO_3^-$ (Zhang and Forde, 1998). The *ANR1*-like gene *OsMADS25* is a positive regulator that controls the development of primary and lateral roots of rice by affecting $NO_3^-$ accumulation (Yu et al., 2015). *OsMADS27* is preferentially expressed in roots, and $NO_3^-$ could significantly induce its expression (Yu et al., 2014). We also found that *OsMADS27* specifically responded to $NO_3^-$ rather than $NH_4^+$ (Figure 1A). The specific $NO_3^-$ responsiveness of *OsMADS27* suggests that a $NO_3^-$-responsive upstream regulator is likely to modulate *OsMADS27*. Recently, $NO_3^-$ restriction was reported to increase the abundance of miR444, thereby inhibiting the expression of *OsMADS27* and thus regulating rice root development (Pachamuthu et al., 2022). These results indicate that there are multiple ways for $NO_3^-$ signaling to regulate *OsMADS27* expression.

## *OsMADS27* is a positive regulator of grain yield

N uptake and assimilation are closely related to crop yield (Daniel-Vedele et al., 1998; Makino, 2011; Hu et al., 2015; Chen et al., 2020). In addition to controlling salt tolerance in rice, OsMADS27 may also positively regulate grain yield by modulating N metabolism and utilization. In our transcriptomic data, a number of $NO_3^-$ transporters were upregulated in OE compared with WT under salt stress conditions; these included the dual-affinity $NO_3^-$ transporter *OsNRT2.4* (Wei et al., 2018), *OsNAR2.1*, which is required by some members of the NRT2 family for $NO_3^-$ transport (Chen et al., 2020), *OsNP5.16*, a positive regulator of grain yield and tiller number (Wang et al., 2021), and the low-affinity $NO_3^-$ transporter *OsPTR2* (Li et al., 2015) (Figure 4C). The significant upregulation of genes encoding these N transporters and the helper protein *OsNAR2.1* correlates with improved yield in transgenic plants

under variable N conditions (Figure 6), suggesting that *OsMADS27* is a positive regulator of rice grain yield.

In conclusion, OsMADS27 positively regulates salt tolerance in rice in a $NO_3^-$-dependent manner by controlling salt-responsive genes, balancing ion homeostasis, and enhancing ROS scavenging. *OsMADS27* also acts as an important determinant of rice yield by modulating the expression of genes related to N uptake and assimilation. Hence, our study fills the gap in the molecular mechanism of $NO_3^-$-dependent salt tolerance and provides a promising candidate for the development of salt-tolerant crops.

## METHODS

### Plant material and culture conditions

The loss-of-function mutants *osmads27-1* and *osmads27-2* in the ZH11 background were generated by Hangzhou Biogle (Hangzhou, China) (http://www.biogle.cn/) using CRISPR–Cas9 technology according to a previously described protocol (Lu et al., 2017). The mutants were selected on the basis of their corresponding resistance to hygromycin B. The *ACTIN1*:*OsMADS27* overexpression construct was made by inserting the coding region of *OsMADS27* into pCB2006 via the GATEWAY cloning system (Lei et al., 2007). The binary vector was transferred into *Agrobacterium tumefaciens* (EHA105) for rice transformation. Homozygous lines ($T_3$ generation) were selected using glufosinate, and expression was confirmed by RT–PCR and qRT–PCR. These homozygous lines were propagated to obtain the $T_4$ generation, which was used for further experimental analyses.

A modified Kimura B solution was used for hydroponic culture of rice seedlings in a growth chamber with a controlled climate as described previously (Wu et al., 2021). Growth conditions were maintained at 28°C with a photoperiod of 16 h light/8 h dark, 70% relative humidity, and a light intensity of 250 mmol $m^{-2}$ $s^{-1}$.

### Salt tolerance assay: Seed germination

Seeds of the WT, *osmads27-1* and *osmads27-2* mutants, OE7, and OE8 were washed with distilled water and incubated at 37°C for 7 days. To analyze seed germination, 60–80 seeds (three replicates per genotype) were randomly placed in petri dishes containing either water or water plus 150 mM NaCl. The seeds were considered to have germinated when their radicle or germ length reached approximately 1 mm. Seed germination was observed daily to calculate the germination percentage.

### Salt tolerance assay: Seedlings in hydroponic culture

Seeds of WT, *osmads27-1*, *osmads27-2*, OE7, and OE8 were washed with distilled water and incubated at 37°C for 3 days. Germinated seeds were transferred to Hoagland solutions (pH 6.0) with different N concentrations (0.02 mM, 0.2 mM, 2 mM $KNO_3$ or $NH_4Cl$) to grow for 7 days, followed by the addition of 140 mM NaCl to the culture medium and treatment for 7 days. The growth conditions were maintained at a 14-h light/10-h dark cycle at 28°C.

### Salt tolerance assay: Seedlings in soil

For the salt treatment in soil, 30 seedlings each of the WT, *osmads27-1*, *osmads27-2*, OE7, and OE8 were grown directly in a soil-filled pot ($5 \times 5 \times 12$ $cm^3$, five plants per pot). After growth for 4 weeks in soil under greenhouse conditions of 16 h light/8 h dark at 30°C, plants were irrigated with either 0 mM or 150 mM NaCl solution for 6–8 days before the seedling survival rate was determined.

### Salt tolerance assay: Long-term salt treatment

Seeds of the WT, *osmads27-1*, and OE7 were germinated in plates for 4 days and then transferred to pots similar to those used previously for salt treatment of 4-week-old seedlings. The plants were grown in pots filled with vermiculite and fed with different N concentrations (1.5 mM, 2.5 mM, or 5 mM $KNO_3$) for 3 weeks, followed by 65 mM NaCl as a salt treatment (or no NaCl as a control) for approximately 10–12 weeks. Every treatment contained eight trays with two pots per genotype, and each pot held a single plant. The plants were grown to maturity under greenhouse conditions, and yield data were collected.

### $Na^+$ and $K^+$ quantification

Seeds of WT, *osmads27-1*, and OE7 were germinated at 37°C for 4 days and transferred to modified hydroponic medium containing 0.2 mM $KNO_3$ for 7 days. The seedlings were treated with or without 140 mM NaCl for 5 days, and then roots and shoots were detached from intact seedlings and dried in an oven at 80°C for 4 days. Dry samples were weighed and extracted with 10 mL nitric acid at 120°C for 1 h. Sodium ($Na^+$) and potassium ($K^+$) contents were measured using inductively coupled plasma-atomic emission spectrometry (ICP–AES, Thermo Fisher, Waltham, USA).

### RNA extraction and qRT–PCR

Total cellular RNA was extracted from rice tissues (0.08–0.1 g) via the TRIzol method (Invitrogen, Carlsbad, USA), 1 μg of which was used for cDNA synthesis. The synthesized cDNA was used for qRT–PCR with TaKaRa SYBR Premix Ex-Taq II kit reagents. The primers used are listed in Supplemental Table 1. At least three biological replicates were used for each experiment.

### GUS analyses

A 2.0-kb promoter region of *OsMADS27* was amplified from rice genomic DNA (ZH11) and cloned into pCB308R (Xiang et al., 1999; Lei et al., 2007). The recombinant *OsMADS27pro:GUS* vector was then transformed into ZH11 to generate *OsMADS27pro:GUS* transgenic plants. For GUS staining, *OsMADS27pro:GUS* transgenic seedlings were incubated in staining solution for 3 h at 37°C and dehydrated in an ethanol series (70%, 80%, 90%, and 100%). The stained tissues were monitored under a HiROX MX5040RZ digital optical microscope (Quester China Limited) and then photographed with a Nikon D700 digital camera.

### Subcellular localization analyses of OsMADS27

The fusion vector *OsMADS27pro*:*OsMADS27-GFP* was created by cloning the 2.0-kb promoter and the full-length coding sequence of *OsMADS27* into the binary vector pCAMBIA1300. The gene insertion was confirmed by nucleotide sequencing, and the resulting vector was transformed into *Agrobacterium tumefaciens* (EH105). Rice callus was transformed by *Agrobacterium*-based transformation, and positive seedlings were selected by culturing them in medium that contained hygromycin B. To investigate the nuclear-cytoplasmic shuttling of OsMADS27, positive seedlings were grown on modified Kimura B solution with 2 mM $KNO_3$ or without N for 10 days. Subsequently, seedlings on N-free medium were treated with either 2 mM $KNO_3$, 2 mM $NH_4Cl$, or 150 mM NaCl for 60 min and returned to the N-free medium. In addition, seedlings on 2 mM $KNO_3$ medium were treated with 150 mM NaCl for 60 min and returned to N-free medium. Confocal microscopy was performed using a Zeiss 710 microscope with an argon laser (488 nm for GFP excitation).

### Western blot analysis

Proteins were extracted using RIPA lysis buffer (strong) (Beyotime, China) from 2-week-old seedlings grown hydroponically on medium containing different N concentrations of 0.02 mM, 0.2 mM, and 2 mM $KNO_3$ without salt as a control or with 100 mM NaCl. For western blot analysis, proteins were electroblotted from a 10% acrylamide gel to a nitrocellulose membrane (Immobilon-P, Millipore, Bedford, MA, USA) after SDS–PAGE separation. The antibodies used in western blotting were as follows: anti-GFP antibody (M20004, Mouse mAb, Abmart, Shanghai, China), 1:1000; anti-ACTIN antibody (M20009, Mouse mAb, Abmart, Shanghai, China), 1:1000; and goat anti-mouse IgG-HRP (M21001, Abmart, Shanghai, China), 1:5000. An Image Quant LAS 4000 (GE, USA) CCD camera

system was used to quantify band intensity with the Super Signal West Femto Trial Kit (Thermo, Rockford, IL, USA).

### Transient transactivation assays in tobacco leaves

A transient transactivation assay in tobacco leaves was performed as previously described (Lim et al., 2017). The coding sequences of *OsMADS27*/*OsNLP4* were cloned into the pRI101 vector as effectors. Approximately 2500-bp promoters of *OsHKT1.1*, *OsSPL7*, and *OsMADS27* were cloned into the pGreenII 0800 vector as reporters. These constructs were electroporated into the *Agrobacterium* GV3101 strain, which was then cultured in LB medium at 28°C for 2 days. The precipitate was collected by centrifugation at 5000 rpm for 5 min, resuspended in infiltration buffer (10 mM MES, 10 mM $MgCl_2$, 150 mM acetosyringone, pH 5.6), and incubated at room temperature for 2 h before co-injection into *Nicotiana benthamiana* leaves. Three days after injection, tobacco leaves were sprayed with LUC substrates (1 mM XenoLight D-luciferin potassium salt). At least three biological replicates were used for each experiment.

### RNA sequencing analysis

Each genotype had approximately 100 seedlings (ZH11 background) in every treatment, which were grown hydroponically in a growth chamber under the conditions described above. The seedlings were cultured in modified Kimura B solution with 1.5 mM $KNO_3$ for 12 days and treated with 100 mM NaCl or without NaCl as a control for another 3 days. Fifteen-day-old seedlings (whole plants) were sampled for RNA sequencing. For each treatment, 20 seedlings were collected as a sample, and three independent biological replicates were performed. RNA library construction and sequence analysis were conducted as described previously (Khan et al., 2016a).

### Chromatin immunoprecipitation–quantitative PCR assay

A chromatin immunoprecipitation (ChIP) assay was carried out according to the protocol described previously (O'Geen et al., 2010) with minor modifications. Transgenic rice (*OsMADS27pro:OsMADS27-GFP*) seedlings were grown under high nitrogen (2 mM) conditions for 2 weeks. Approximately 2.0 g of seedlings was placed in 1% formaldehyde (v/v) at 20°C–25°C under vacuum for 15 min and then homogenized in liquid nitrogen. Chromatin from lysed nuclei was fragmented ultrasonically to achieve an average length of 500 bp. The anti-GFP antibodies (Sigma, F1804) were immunoprecipitated overnight at 4°C. The immunoprecipitated DNA fragments were dissolved in water and kept at −80°C before use. The precipitated fragments were used as templates for quantitative PCR (qPCR).

### Field trial of rice

For the field test of the *osmads27-1* mutant and *OsMADS27*-overexpressing line (OE7) (all in the ZH11 background), T3 generation plants were grown in Chang Xing, Zhejiang, in 2021 (April to September). The plant density was six rows. Twenty plants per row were used for each plot, and four replicates were used for each N condition. Urea was used as the N fertilizer at rates of 94 kg N $hm^{-2}$ for low N (LN), 184 kg N $hm^{-2}$ for normal N (NN), and 375 kg N $hm^{-2}$ for high N (HN). To reduce variability in the field test, the fertilizers were used evenly in each plot for the N application level. Plants at the edge of each plot were excluded from data collection to avoid margin effects.

### Agronomic trait analyses

Individual tiller number, panicle number, and grain yield per plant were measured according to a protocol documented previously (Hu et al., 2015).

## ACCESSION NUMBERS

Sequence data from this article can be found at the Rice Genome Annotation Project (https://rice.plantbiology.msu.edu/) under the following accession numbers: *OsMADS27*, *LOC_Os02g36924*; *OsHKT1.1*, LOC_Os04g51820; *OsNLP4*, LOC_Os09g37710; *OsSPL7*, *LOC_Os05g45410*; *OsHKT2.3*, *LOC_Os01g34850*; *OsKAT3*, *LOC_Os02g14840*; *OsO3L2*, *LOC_Os06g36390*; *OsMPS*, *LOC_Os02g40530*.

## SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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## AUTHOR CONTRIBUTIONS

C.B.X., A.A., P.X.Z., and J.W. designed the experiments. A.A., T.N., J.Z., J.W., Y.S., P.X.Z., and S.U.J. performed the experiments and data analyses. J.W., Z.S.Z., J.Q.X., and Z.Y.Z. performed field trials and data analyses. A.A. and J.W. wrote the manuscript. C.B.X., P.X.Z., and J.W. revised the manuscript. C.B.X. supervised the project.

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