Nitrate-dependent salt tolerance mediated by OsNLP4-OsMADS27 module

Alamin Alfatih, Jing Zhang, Ying Song, Sami Ullah Jan, Zi-Sheng Zhang, Jing-Qiu Xia, Zheng-Yi Zhang, Tahmina Nazish, Jie Wu, Ping-Xia Zhao, and Cheng-Bin Xiang

Division of Life Sciences and Medicine; Division of Molecular & Cell Biophysics, Hefei National Science Center for Physical Sciences at the Microscale; MOE Key Laboratory for Membraneless Organelles and Cellular Dynamics; University of Science and Technology of China, The Innovation Academy of Seed Design, Chinese Academy of Sciences, Hefei, Anhui Province 230027, China

These authors contributed equally.

Correspondence:
Cheng-Bin Xiang (email: xiangcb@ustc.edu.cn, 86-55163600429)
Ping-Xia Zhao (email: zhaopingxia2008@163.com)
Jie Wu (email: wujie199104@163.com)
Abstract

Salt stress is a major constraint of plant growth and yield. Nitrogen (N) fertilizers are known to alleviate salt stress. However, the underlying molecular mechanisms remain unclear. Here we show that OsNLP4-OsMADS27 module controls nitrate-dependent salt tolerance in rice. The expression of OsMADS27 is specifically induced by nitrate. The OsMADS27 knockout mutants are more sensitive to salt stress than the wild type, whereas the OsMADS27 overexpression lines are more tolerant. Transcriptomic analyses revealed that OsMADS27 controls the expression of a number of known stress-responsive genes as well as those involved in ion homeostasis and antioxidation. We demonstrated that OsMADS27 directly binds to the promoter of OsHKT1.1 and OsSPL7 to regulate their expression. Notably, OsMADS27-mediated salt tolerance is nitrate-dependent and positively correlated with nitrate concentration. We further showed that OsNLP4, a nitrate-responsive key regulator in N metabolism and N use efficiency, positively regulates the expression of OsMADS27 by directly binding to the nitrate-responsive cis-element in its promoter, thereby transmitting the nitrate signal to OsMADS27 and conferring its nitrate dependence. Our results reveal the role of nitrate-responsive OsNLP4-OsMADS27 module and its downstream target genes in salt tolerance, filling the gap in the molecular mechanism of nitrate-dependent salt tolerance of rice. Moreover, OsMADS27 overexpression increased grain yield under salt stress in presence of sufficient nitrate, indicating that OsMADS27 is a promising candidate for the improvement of salt tolerance in rice.

Keywords: OsMADS27, OsNLP4, nitrate-dependent salt tolerance, salt stress, grain yield
Introduction

Salinity lies among critical crises in agriculture around the globe and the majority of the food crops are salinity-sensitive (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). To cope with the salinity-triggered damages, plants have evolved various strategies on the bases of their habitat and severity of stress (Adem et al., 2014; Ashraf et al., 2008; Bose et al., 2014; Chakraborty et al., 2016). Among numerous strategies, appropriate acquisition of the mineral nutrients is undoubtedly an effective way to improve salinity tolerance, growth and yield under salt stress (Gao et al., 2016; Guo et al., 2017; Kaya et al., 2007). Therefore, it is important to understand the mechanisms by which nutrients alleviate salt stress in plants for breeding robust salt-tolerant crop varieties.

Potassium, a vital nutrient for plant growth and development, is well known for its role in balancing sodium concentration in plants (Clarkson and Hanson, 1980; Raddatz et al., 2020; Wu et al., 2018; Zorb et al., 2014). Under salt stress, the accumulation of sodium ions (Na⁺) in the cytoplasm leads to membrane depolarization and promotes potassium ions (K⁺) leakage out of the cell. Therefore, it is crucial for plants to maintain an appropriate K⁺/Na⁺ ratio in the cytoplasm to survive in saline soil, which depends on the operation of Na⁺/K⁺ transporters (Wu et al., 2018). Rice potassium transporter OsHAK1 promotes K⁺ uptake and K⁺/Na⁺ ratio in both low and high potassium conditions, which is essential for maintaining potassium-mediated growth and salt tolerance (Chen et al., 2015). 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, the members of high-affinity K⁺ transporters like HKTs also grant salinity tolerance to rice (Hamamoto et al., 2015; Rosas-Santiago et al., 2015; Suzuki et al., 2016a; Wang et al., 2015). Calcium (Ca²⁺) can regulate the perception, uptake, and transport of various ions through the SOS (salt overly sensitive) pathway (Lin et al., 2009; Qiu et al., 2002; Yang and Guo, 2018a; Yang and Guo, 2018b; 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 cell membrane is associated with Na⁺ extrusion via roots under saline environment and confers salinity tolerance to rice (Martínez-Atienza et al., 2007). SOS2 and SOS3, encoding protein kinase and Ca²⁺-binding protein respectively, are required for salinity
tolerance in rice because they perceive the change of Ca$^{2+}$ in cytosol under salinity and activate several downstream genes to start signaling cascade (Kumar et al., 2013).

Apart from potassium, few mineral nutrients have been studied for their roles in salt tolerance. Sulfur nutrient has been found to improve plant photosynthesis and growth under salt stress by increasing glutathione production and abscisic acid (ABA) accumulation (Cao et al., 2014; Chen et al., 2019; Fatma et al., 2014; Fatma et al., 2021). Nitrogen (N), an essential macronutrient for plants, has been shown to improve salt tolerance (Mansour, 2000) via its participation in stimulation of the 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 the activation of numerous enzymes (Aragao et al., 2012). However, the underlying molecular mechanisms of N-improved salt tolerance in plants remain unclear to date.

Transcription factors (TFs) play essential roles in transcriptional control of the stress-associated genes and hence are of utmost importance for breeding stress-tolerant crops (Ahammed et al., 2020; Zhang et al., 2017). The MADS family TFs control important growth and developmental processes such as seed germination and flowering time (Chen et al., 2016; Moyle et al., 2005; Wu et al., 2017; Yin et al., 2019; Yu et al., 2017). MADS-box TFs are also involved in the response to various abiotic stress. 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). Moreover, OsMADS25, OsMADS27 and OsMADS57 are involved in the response to nutrient deficiency in rice (Chen et al., 2018a; Huang et al., 2019; Yu et al., 2015). The overexpression of OsMADS25 improved the salinity tolerance of rice (Wu et al., 2020).

We previously reported Arabidopsis MADS-box TF AtAGL16 as a negative regulator of salt and drought tolerance (Zhao et al., 2020; Zhao et al., 2021). To extend our work to rice, we identified OsMADS27 as the most homologous gene of AtAGL16. OsMADS27 is induced by nitrate ($\text{NO}_3^-$) and ABA, and acts as a target gene of miR444 to control root development in a $\text{NO}_3^-$-dependent manner (Chen et al., 2018a; Pachamuthu et al., 2022; Puig et al., 2013; Yu et al., 2014). 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 relation of OsMADS27-mediated salt tolerance to N
nutrient has not been investigated in rice. In this study, we discovered the NO₃⁻ dependence of OsMADS27-mediated salt tolerance and unveiled the underlying molecular mechanism. Our results demonstrate that OsMADS27-mediated salt tolerance is NO₃⁻-dependent. The OsNLP4-OsMADS27 module plays a key role in the NO₃⁻-dependent salt tolerance, where OsNLP4 senses NO₃⁻ signaling, translocates to the nucleus (Konishi and Yanagisawa, 2010; Wu et al., 2021), and transcriptionally upregulates OsMADS27. Consequently, OsMADS27 directly regulates the expression of stress-responsive genes in rice. Therefore, our findings revealed a novel mechanism of NO₃⁻-dependent salt tolerance, which can be exploited for the improvement of salinity tolerance in crops.

**Results**

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

To gain a detailed expression pattern of OsMADS27, we examined its spatiotemporal expression by quantitative real time PCR (qRT-PCR) at three developmental stages of rice plants: seedling, vegetative, and pre-mature stage. Our results demonstrated that OsMADS27 was expressed in all the tissues examined but with much higher levels in roots, leaves, and sheath (Fig. S1A). In addition, tissue expression pattern of OsMADS27 was revealed in the OsMADS27pro::GUS transgenic plants (Fig. S1B), which was reconcilable with our qRT-PCR results and previous reports (Chen et al., 2018a; Pachamuthu et al., 2022; Puig et al., 2013; Yu et al., 2014). Notably, strong GUS signal was detected in the stelle of the root (Fig. S1B).

To check the response of OsMADS27 to nutrients and salt stress, we grew wild type (WT) seedlings under normal conditions and then transferred 7-day-old seedlings to hydroponic medium without N for 48 hours, then transferred the seedlings to hydroponic medium supplemented with 2 mM KNO₃, 2 mM NH₄Cl, 2 mM KCl, or 150 mM NaCl, respectively. Surprisingly, NaCl did not induce the expression of OsMADS27 under our conditions, neither did NH₄Cl or KCl. Only KNO₃ induced the expression of OsMADS27 that plateaued at 12 hours with about 5 folds increase (Fig. 1A). In addition, we showed that when the seedlings were transferred into N-free medium, the KNO₃-induced expression of OsMADS27 was gradually
decreased (Fig. 1B). These results clearly show that the expression of OsMADS27 is specifically responsive to KNO₃.

Meanwhile, we showed that under normal growth conditions, NaCl and ABA induced the expression of OsMADS27 (Fig. S1C-E), which was inconsistent with the results of NaCl treatment in Fig. 1A. The only difference of these experiments lies in whether nitrate is present in the NaCl treatment, which likely counts for this observed difference of OsMADS27 expression. To confirm this, we treated seedling (N starved) under 150 mM NaCl with 0 mM KNO₃ for 3 hours, then added 2 mM KNO₃ for another 3 hour. The qRT-PCR results clearly show that in the absence of KNO₃, NaCl was unable to induce the expression of OsMADS27. Only in the presence of KNO₃, NaCl stimulated the expression of OsMADS27 (Fig. 1C). This was further confirmed with OsMADS27pro:GUS transgenic rice in which GUS signal exhibited a similar response. No change in GUS activity was observed under the treatment of KCl plus NaCl, while a strong induction of GUS was seen in roots treated with KNO₃ plus NaCl (Fig. 1D and E).

We also quantified the protein level of OsMADS27 in the OsMADS27pro:OsMADS27-GFP plants by western blot using anti-GFP antibodies under low, normal, and high concentration of KNO₃ (0.02 mM, 0.2 mM, and 2mM) with or without 100 mM of NaCl for 10 days. The results in Fig. 1F show that the OsMADS27 protein level is positively correlated with KNO₃ concentration and enhanced by NaCl treatment (Fig. 1F).

Taken together, our results clearly show that the expression of OsMADS27 is specifically induced by NO₃⁻ and NaCl-induced expression of OsMADS27 is NO₃⁻-dependent.

**Nuclear localization of OsMADS27 is responsive to nitrate**

To reveal the subcellular localization of OsMADS27 protein and its response to nutrients, we generated OsMADS27pro:OsMADS27-GFP transgenic lines. The transgenic plants were grown on N-free MS medium supplied with 2 mM KNO₃ (Fig. 2A) or 2 mM KCl (Fig. 2C) for 10 days. Then seedlings receiving KNO₃ were treated with 150 mM NaCl (Fig. 2B), and the seedlings receiving KCl were treated with 2 mM KNO₃ (Fig. 2D), 2 mM NH₄Cl (Fig. 2E), 150 mM NaCl (Fig. 2F), and 150 mM NaCl plus 2 mM KNO₃ (Fig. 2G) respectively for 60 min before confocal laser-scanning microscopic observation. GFP signals were detected in the
nucleus whenever KNO₃ was included in the medium regardless of the presence of other supplements (Fig. 2A, B, D and G). No GFP signals were detected in the presence of KCl (Fig. 2C), KCl plus NH₄Cl (Fig. 2E), or KCl plus NaCl (Fig. 2F). These results indicate that the nuclear accumulation of OsMADS27 is specifically responsive to nitrate, in accordance with that of OsNLP4 (Wu et al., 2021).

OsMADS27-mediated salt tolerance in rice is nitrate-dependent

To explore the capability of OsMADS27 in conferring salt tolerance to rice, we generated two independent loss-of-function mutant lines of OsMADS27 (KO1 and KO2) by using CRISPR/CAS9-based editing. Protein sequence alignment depicted that mutations in both mutants resulted in premature stop codon, hence interrupting the open reading frame (ORF) of OsMADS27 (Fig. S2A-D). Additionally, we generated two independent overexpression (OE) lines of OsMADS27 (OE7 and OE8) driven by OsACTIN1 promoter (Fig. S2E-F).

To evaluate the role of OsMADS27 in salt stress tolerance of rice, we germinated the seeds of OE7, OE8, KO1, KO2, 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 all the genotypes (Fig. S3A). However, under 150 mM salt stress, OE lines displayed a germination rate of 80% at day 6 compared with WT and KO mutants which exhibited a germination rate of 55% and 30% respectively (Fig. S3B). Moreover, we conducted salt tolerance assay on soil-grown seedlings (Fig. S3C). Upon treatment of 20-day-old soil-grown seedlings with 150 mM NaCl for 15 days, 80% of the OE plants survived compared with WT and KO mutants with a survival ratio of 43% and 12% respectively, whereas under the 0 mM NaCl control treatment all genotypes displayed 100% survival (Fig. S3D). Together these results clearly demonstrate that OsMADS27 is a positive regulator of salt tolerance in rice.

The NO₃⁻ dependence of NaCl-induced expression of OsMADS27 prompted us to ask whether OsMADS27-mediated salt tolerance is nitrate-dependent. Thus we further explored the salt tolerance of different OsMADS27 genotypes under different NO₃⁻ concentrations. We grew seedlings in modified hydroponic culture with different NO₃⁻ concentrations for 10 days, then supplemented with or without 140 mM NaCl in the hydroponic culture and allowed seedlings to grow for another week. Under 0 mM NaCl conditions, the seedling survival rate was 100% for
all the genotypes under all three concentrations of NO$_3^-$ (Fig. 3A and C). Under 140 mM NaCl conditions, the seedling survival rate of all the three genotypes was similarly 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 the salt stress as reflected by the seedling survival rate of KO mutants (20%), WT (42%) and OE lines (55%) compared with those under LN conditions (Fig. 3B and D). Under high NO$_3^-$ conditions (2 mM, HN), salt stress was further alleviated as evidenced by the increased seedling survival rate in KO mutants (30%), WT (70%), and OE lines (80%). These results demonstrate that the salt tolerance mediated by OsMADS27 is NO$_3^-$ dependent.

To confirm the above hydroponic culture results, we grew the plants of the three genotypes in potted vermiculite and fed 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 Methods (Fig. S4A). The data of yield-related agronomic traits were collected for statistical analyses. Fig. S4B 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 WT at all three N levels, while the KO showed lower yield than WT. The OE line exhibited grain yield increase by 29%, 38%, and 25% relative to the WT under LN, NN, and HN conditions respectively, while the KO displayed yield decrease by 20%, 22%, and 25%. The yield was positively correlated with N level, tiller number per plant (Fig. S4C), and panicle number (Fig. S4D). Both tiller and panicle number displayed similar pattern of genotype and N level effects as grain yield did. Under salt stress, The OE line exhibited grain yield increase by 66%, 40%, and 28% relative to the WT under LN, NN, and HN conditions respectively, while the KO displayed yield decrease by 33%, 40%, and 28% under the same conditions (Fig. S4E). Tiller and panicle number displayed a similar trend as grain yield did (Fig. S4F and G). These results suggest that OsMADS27 is a positive regulator of grain yield and further support that OsMADS27 positively regulates salt tolerance in a NO$_3^-$ dependent manner in rice.

We also conducted field trials to examine the yield of three OsMADS27 genotypes in the field of varying N supply and found that agronomic traits including nitrogen use efficiency (NUE), actual yield per plot, grain yield per plant, panicles number per plant, number of seeds per plant, and primary branch number per panicle were significantly improved in OE plants.
under normal and high N availability, whereas reduced in KO plants compared with the wild type (Fig. S5). The field trial data further support that OsMADS27 is a positive regulator of grain yield, which is positively correlated with NO$_3^-$ availability.

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

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

The in-depth information about DEGs was obtained by KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway and GO (Gene Ontology) analyses to detect significantly expressed DEGs in KO vs WT and OE vs WT under control and salt conditions (Fig. 4C and S6). Remarkably, the genes involved in salt response were highly enriched in DEGs, indicating that OsMADS27 may coordinately regulate the key genes in salt tolerance (Fig. 4C). The heatmap demonstrates that the transcript level of ethylene response factor (OsWR2), salinity-responsive MYB transcription factor (OsMPS), A-type response regulator (OsRR2), rice cyclin gene (OsCycB1;3), oxidative stress 3 (OsO3L2), and a heat shock transcription factor (OsSPL7) was higher in the OE plants under salt stress. In addition to the salt-responsive genes, key genes involved in ion transport, such as K$^+$ transporters (OsHKT1.1, OsHKT2.3), K$^+$ channel (OsKAT3), salt-inducible calmodulin gene (OsCAM1), and aluminum-activated transporter of malate (OsALMT4) were significantly down-regulated in KO mutant while up-regulated in the OE line under salt stress. OsMADS27 also positively regulates the expression of prominent ABA-responsive genes such as OsNCED1, OsRAB16, and OsGLP1, which were expressed at higher levels in OE plants. Moreover, the genes of peroxidases in antioxidation including OsPRX29, OsPRX27, OsPRX74, OsGPX, OsPRX132 were significantly upregulated in OE vs WT (salt group). Furthermore, OsMADS27 positively regulates the expression of N-responsive genes as the expression level of OsNRT2.4, OsNAR2.1, OsNPF5.16, OsNPF2.2/OsPTR2 and OsNLAI1 was predominantly enhanced in WT vs OE group after salt treatment (Fig. 4C). In addition, GO enrichment analyses show that OsMADS27 also affected the expression of some
genes involved in oxidation-reduction process, regulation of transcription, defense response and protein phosphorylation under normal conditions (Fig. S6A and B), whereas hydrogen peroxide catabolic process, flavonoid biosynthesis, abscisic acid catabolism, defense response and tyrosine catabolism related genes were also regulated by OsMADS27 under salt stress conditions (Fig. S6C and D).

The expression pattern of the genes involved in salt response and ion transport was verified by RT-qPCR, which was largely in agreement with the RNA-seq data (Fig. S7). Taken together, our RNA-seq data suggest that OsMADS27 confers salt tolerance in rice by regulating salt-responsive genes, maintaining ion balance, and enhancing ROS scavenging.

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

To demonstrate the capability of OsMADS27 to regulate its target genes, we generated the transgenic rice plants expressing OsMADS27pro:OsMADS27-GFP for ChIP (chromatin immunoprecipitation) assay. The cis1 region of OsHKT1.1 promoter and cis2 and cis3 regions of OsSPL7 promoter were found to be enriched in the transgenic rice plants as demonstrated by qRT-PCR (Fig. 5A and B). Furthermore, we 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-transfected into the tobacco leaves, we observed that OsMADS27 activated the expression of LUC genes linked to the promoters of OsHKT1.1 and OsSPL7 (Fig. 5C and D). Taken together, these results demonstrate that OsMADS27 binds the cis elements in the promoter of OsHKT1.1 and OsSPL7 and activates their expression.

**Nitrate-responsive OsNLP4 upregulates OsMADS27 and confers its nitrate dependence**

To explore the mechanism by which NO$_3^-$ specifically induces the expression of OsMADS27, we performed cis elements search in the promoter of OsMADS27 and found that the promoter of OsMADS27 harbors multiple nitrate-responsive cis-elements (NREs), the binding site for nin-like protein (NLP) transcription factors (Konishi and Yanagisawa, 2010; Wu et al., 2021). Further exploration revealed that the expression level of OsMADS27 was significantly up-regulated in OsNLP4 overexpression plants and down-regulated in the knockout mutants (Fig. 6A). Subsequent ChIP assay showed that the cis1 portion of the OsMADS27 promoter harboring NRE was significantly enriched, confirming that OsNLP4 binds to the OsMADS27 promoter in...
vivo (Fig. 6B). The binding was further confirmed by electrophoretic mobility shift assay (EMSA) (Fig. 6C). Furthermore, we conducted a dual-luciferase reporter assay to further verify the transcription activation of OsMADS27 by OsNLP4 in tobacco leaves. Strong fluorescent signals were shown when the effector construct 35S-OsNLP4 was co-transfected with the reporter construct OsMADS27pro::LUC (Fig. 6D), indicating that OsNLP4 transcriptionally activates the expression of OsMADS27.

**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 (Çavuçoğlu et al., 2017; Domingo et al., 2004; Gao et al., 2016; Kaya et al., 2003; Kaya and Higgs, 2003; Zheng et al., 2008). However, the intrinsic molecular mechanism of NO$_3^-$-mediated alleviation of salt stress has not been reported so far. In this study, we unraveled the OsNLP4-OsMADS27 module that is crucial for coupling NO$_3^-$ signaling and salt tolerance in rice. We demonstrated that NO$_3^-$ not only induced the expression of OsMADS27 as described previously (Chen et al., 2018a; Pachamuthu et al., 2022; Puig et al., 2013; Yu et al., 2014), but also promoted the nuclear localization of OsMADS27 (Figs. 1 and 2). OsNLP4, a NO$_3^-$-responsive TF translocating into the nucleus in the presence of NO$_3^-$ (Wu et al., 2021), transcriptionally activates the expression of OsMADS27 (Fig. 6). Then OsMADS27 activates an array of stress tolerance-related genes as revealed by RNA-seq analyses (Fig. 4) by directly binding to their promoters as demonstrated for OsHKT1.1 and OsSPL7 (Fig. 5), thereby enhancing growth and grain yield under salt stress in rice (Figs. 3 and S4). However, in the absence of NO$_3^-$, OsNLP4 was mainly localized in the cytoplasm, resulting in very low expression of OsMADS27, which was insufficient to confer salt tolerance in rice as illustrated in the working model (Fig. 8). Our study revealed a novel mechanism of NO$_3^-$-dependent salt tolerance-mediated by OsMADS27, which may be exploited for the improvement of salt tolerance and grain yield in rice.

**Mechanisms of OsMADS27-conferred salt tolerance**
TFs regulate the expression of various stress-related genes by binding with regulatory motifs in the promoters of these genes in response to stresses (Yamaguchi-Shinozaki and Shinozaki, 2006). Likely benefiting from the simultaneously coordinating the expression of salt-responsive genes (Fig. 4), MADS-box TF OsMADS27 overexpression increased the transcriptional 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 in germination, seedlings, and reproductive phase of rice (Figs. 3, S3 and S4).

Salt tolerance is highly dependent on intracellular ion homeostasis in order to maintain the turgidity of cell and membrane potential (Bargmann et al., 2009). In our transcriptomic data, the expression of K+ transporters such as OsHKT1.1 (Imran et al., 2020), OsHKT2.3 (Zhang et al., 2018), K+ channel OsKAT3 (Hwang et al., 2013), and Ca2+ sensor OsCAM1.1 that positively regulates salt tolerance in rice (Saeng-ngam et al., 2012) was significantly enhanced in the OE plants compared with WT under salt stress (Fig. 4C). We found that OsMADS27 directly binds and transcriptionally activates OsHKT1.1, which encodes a membrane-localized high-affinity K+ transporter (Fig. 5). The oshkt1.1 knockout mutant rice plants are salt-sensitive depicting its function in the Na+ retrieval from leaf blades (Wang et al., 2015). These results demonstrate that OsMADS27 positively regulates salt tolerance in rice via maintaining ion homeostasis.

In addition, salinity leads to the accumulation of reactive oxygen species (ROS) in plants (Luo et al., 2021), the increased production of which leads to oxidative burden hence being a havoc to cellular membranes as well as macromolecules (Lin et al., 2020). As a target gene of OsMADS27 (Fig. 5), the heat shock transcription factor OsSPL7 plays an important role in maintaining ROS homeostasis in rice. The spl7 mutant lost regulation of nicotinamide adenine dinucleotide oxidase, resulting in the accumulation of more H2O2 in the cells (Hoang et al., 2019). Consistently, our OsMADS27 overexpression plants exhibited improved resistance against oxidative burden as depicted by our RNA-seq results (Fig. 4C). The upregulation of a number of peroxidases (OsPRX29, OsPRX27, OsPRX74, OsGPX, and OsPRX132) in OE plants demonstrated that the overexpression of OsMADS27 ameliorated salt-generated oxidative stress.
ABA, as a stress hormone, 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 (Jiang et al., 2019; Zou et al., 2008) in the OE vs WT group under salt stress (Fig. 4C) implied the possibility that OsMADS27 may also be involved in ABA signaling. OsMADS27 has been reported to control NO₃⁻-dependent root growth via ABA pathway (Chen et al., 2018a). The possible crosstalk between OsMADS27, ABA signaling, and salt stress tolerance needs future attention. Taken together, the salt tolerance mediated by OsMADS27 in rice is mainly by regulating stress-responsive regulators, balancing ion homeostasis, enhancing ROS scavenging ability, and involving in ABA signaling pathway.

**OsNLP4-OsMADS27 module controls the nitrate dependence of OsMADS27-mediated salt tolerance**

It is known that multiple members of the MADS-box TF family are involved in the regulation of NO₃⁻ responses. *Arabidopsis* nitrate regulated1 (AtANR1) is the first NO₃⁻ regulator found to be involved in the regulation of lateral root developmental plasticity in response to NO₃⁻ (Zhang and Forde, 1998). ANR1-like gene OsMADS25 is a positive regulator controlling the development of primary and lateral roots of rice by affecting NO₃⁻ accumulation (Yu et al., 2015). OsMADS27 is preferentially expressed in roots, and NO₃⁻ could significantly induce its expression (Yu et al., 2014). We also found that OsMADS27 specifically responded to NO₃⁻ rather than ammonium (Fig. 1A, B and 2). The specific NO₃⁻ responsiveness of OsMADS27 suggests that a likely NO₃⁻-responding upstream regulator modulates OsMADS27. Indeed, we found that the early NO₃⁻ response TF OsNLP4 directly binds to the OsMADS27 promoter and upregulates its expression (Fig. 6). OsNLP4 is a key TF for NO₃⁻ signaling through nuclear retention mechanisms. Under NO₃⁻ starvation, OsNLP4 proteins are almost exclusively localized in the cytosol (Wu et al., 2021), hence unable to activate the transcription of OsMADS27. However, after NO₃⁻ was resupplied, OsNLP4 proteins were quickly and predominantly accumulated in the nucleus, resulting in a strong activation of OsMADS27 (Fig. 1A, B and 6). This OsNLP4-OsMADS27 regulatory module promptly controls OsMADS27-mediated salt tolerance in a NO₃⁻-dependent manner. Recently, it was reported that NO₃⁻ restriction increased 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 NO₃⁻ signaling to regulate OsMADS27 expression.

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

N uptake and assimilation is closely related to crop yield (Chen et al., 2020; Daniel-Vedele et al., 1998; Hu et al., 2015; Makino, 2011). 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 NO₃⁻ transporters were upregulated in OE compared to WT under salt stress conditions, such as dual affinity NO₃⁻ transporter OsNRT2.4 (Wei et al., 2018), OsNAR2.1 required by some members of NRT2 family for NO₃⁻ transport (Chen et al., 2020), OsNP5.16, a positive regulator of grain yield and tiller number (Wang et al., 2021), and low-affinity NO₃⁻ transporter OsPTR2 (Li et al., 2015) (Fig. 4C). The significant upregulation of these N transporters and helper protein OsNAR2.1 correlates with improved yield in transgenic plants under variable N conditions (Fig. S5), suggesting that OsMADS27 is a positive regulator of rice grain yield.

In conclusion, OsNLP4-OsMADS27 module positively regulates the salt tolerance in rice in a NO₃⁻-dependent manner by controlling salt-responsive genes, balancing ion homeostasis, and enhancing ROS scavenging. OsMADS27 is also an important determinant of yield in rice by modulating the expression of N uptake and assimilation-related genes. Hence, our study fills the gap in the molecular mechanism of NO₃⁻-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 KO1 and KO2 with ZH11 background were generated by Hangzhou Biogle Co., Ltd (Hangzhou, China) (http://www.biogle.cn/), using CRISPR-CAS9 technology, according to the protocol previously described (Lu et al., 2017). The mutants were selected on the bases of their corresponding resistance to hygromycin B. The **ACTIN1**:OsMADS27 overexpression construct was made by inserting the coding region of OsMADS27 into pCB2006 via GATEWAY cloning system (Lei et al., 2007). The binary vector was transferred into Agrobacterium tumefaciens (EHA105) for rice transformation. Homozygous
lines (T₃ generation) were selected using glufosinate and expression was confirmed by RT-PCR and quantitative RT-PCR. These homozygous lines were propagated for obtaining T₄ generation which was used for further experimental analyses.

A modified Kimura B solution was used for hydroponic culture of rice seedlings in the growth chamber with controlled climate as described (Wu et al., 2021). Growth conditions were maintained at 28°C temperature, photo-regime of 16 hours light / 8 hours dark, 70% relative humidity, and light intensity at 250 mmol m⁻² s⁻¹.

**Salt tolerance assays**

**Seed germination.** Seeds of wild type, KO1 and KO2 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 a 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.

**Seedlings in hydroponic culture.** Seeds of wild type, KO1, KO2, 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 KNO₃) to grow for 7 days, followed by addition of 140 mM NaCl to the culture medium and treated for 7 days. The provided growth conditions were kept at 14-h-light/10-h-dark cycle at 28°C.

**Seedlings in soil.** For the salt treatment in soil, 30 seedlings from each of the wild type, KO1, KO2, OE7, and OE8 were directly grown on soil pot (the pot dimensions were 5×5×12 cm³, and five plants were grown per pot). After grown for 4 weeks in soil under greenhouse conditions of 16 h light/8 h dark at 30°C, plants were either irrigated with 0 mM or 150 mM NaCl solution for 6-8 days before seedling survival rate was counted.

**Long-term salt treatment** Seeds of WT, KO1, and OE7 were germinated in plates for 4 days and then transferred to similar pots as previously used for salt treatment of 4-week-old seedlings. The plants were grown in the pots filled with vermiculite and fed with different N concentrations (1.5 mM, 2.5 mM, 5 mM KNO₃) for 3 weeks, then followed by 65 mM NaCl as salt treatment or without NaCl as control for about 10-12 weeks. Every treatment contains 8 trays
with two pots for each genotype and every pot has a single plant. The plants were grown to mature under the greenhouse conditions and yield data were collected.

**RNA extraction and quantitative real-time PCR (qRT-PCR)**

Total cellular RNA was extracted from rice tissues (0.08-0.1 g) via Trizol method (Invitrogen, Carlsbad, USA), 1 μg of which was used for cDNA synthesis. The synthesized cDNA was used for qRT-PCR using TaKaRa SYBR Pre-mix Ex-TaqII kit reagents. The primers used are listed in the Table S1. 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) followed by its cloning in pCB308R (Lei et al., 2007; Xiang et al., 1999), then recombinant *OsMADS27/promotor:GUS* vector was transformed into ZH11 to generate the *OsMADS27:GUS* transgenic plants. For the purpose of GUS staining, the seedlings of *OsMADS27/prom:GUS* transgenic plants were incubated in staining solution for 3 hours at 37°C and dehydrated in a series of ethanol (70, 80, 90, and 100%). The dyed tissues were monitored under HiROX MX5040RZ digital optical microscope (Quester China Limited) and then photographed by Nikon D700 digital camera.

**Subcellular localization analyses**

The fusion vector *OsMADS27/prom:OsMADS27-GFP* were created by cloning 2.0-kb promoter and the full length coding sequence of OsMADS27 in the binary vector pUC19. The gene insertion was confirmed by nucleotide sequencing and the ultimate vector was transformed into *Agrobacterium tumefaciens* (EH105). The rice callus was transformed by *Agrobacterium*-based transformation and the selection of positive seedlings was performed by culturing them in hygromycin B containing medium. To investigate the nuclear-cytoplasmic shuttling of OsMADS27, the positive seedlings were grown on the modified Kimura B solution with 2 mM KNO₃ or without N for 10 days. Subsequently, N-free medium treated with either 2 mM KNO₃ or 2 mM NH₄Cl or 150 mM NaCl for 60 min, and returned to the N-free medium. Additionally, 2 mM KNO₃ medium treated with 150 mM NaCl for 60 min, and returned to N-free medium. The confocal microscopy was performed by Zeiss 710 microscope having argon laser (488 nm for GFP excitation).
Western blot analysis

Proteins were extracted from the 2-week-old seedlings grown hydroponically on medium containing different N concentrations 0.02 mM, 0.2 mM and 2 mM KNO₃ without salt as control or with 100 mM NaCl using the RIPA lysis buffer (strong) (Beyotime, China). For western blot analysis, proteins were electroblotted from 10% acrylamide gel to nitrocellulose membrane (Immobilon-P, MILLIPORE Corporation, Bedford, MA, USA) after the separation of SDS-PAGE. Antibodies used in western blot were as follows: anti-GFP antibody (M20004, Mouse mAb, Abmart, Shanghai, China), 1:1000 for western blot; anti-ACTIN antibody (M20009, Mouse mAb, Abmart, Shanghai, China), 1:1000 for western blot and goat anti-mouse IgG-HRP (M21001, Abmart, Shanghai, China), 1:5000 for western blot. Image Quant LAS 4000 (GE, USA), as the CCD camera system, was used for the band intensity quantification with SuperSignal West Femto Trial Kit (Thermo, Rockford, IL, USA).

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA) was conducted as previously described (Hellman and Fried, 2007). The coding sequence of OsNLP4 was cloned into pMAL c2x vector and MBP-NLP4 fusion protein was expressed in E. coli Rosseta2 strain. Biotin-labelled DNA that 45 bp fragment containing CArG motif and unlabelled competitor DNA were synthesized by Sangon Biotech Co., Ltd (Shang Hai, China). DNA probes were generated by cooling down the mixtures of complementary oligonucleotides from 95°C to room temperature. EMSA assay was performed using a LightShift™ EMSA Optimization and Control Kit (20148×) (Thermo Fisher Scientific, Waltham, USA). The reaction mixtures were loaded on 6% polyacrylamide gel in 0.5 × TBE buffer and electrophoresed at 4°C. These results were detected by a CCD camera system (Image Quant LAS 4000).

Transient transactivation assays in tobacco leaf

Transient transactivation assay in tobacco leaf was performed as previously described (Lim et al., 2017). The coding sequences of OsMADS27/OsNLP4 were constructed into pRII101 vector as reporters. About 2500 bp promoters of OsHKT1.1, OsSPL7, OsMADS27 were respectively cloned into pGreenII 0800 vector as reporters. These constructs were electroporated into Agrobacterium GV3101 strain, then cultured in LB medium at 28°C for 2 days. The precipitate
was collected by centrifugation at 5000 rpm for 5 min, resuspended with infiltration buffer (10 mM MES, 10 mM MgCl₂, 150 mM acetosyringone, pH 5.6), and incubated at room temperature for 2 hours before co-injecting into *Nicotiana benthamiana* leaves. 3 days after injection, tobacco leaves were sprayed using LUC substrates (1 mM Xeno lightTM D-luciferin potassium salt). At least three biological replicates were used for each experiment.

**RNA-sequencing analysis**

Each genotype has about 100 seedlings (ZH11 background) of every treatment was grown hydroponically in a growth chamber with the condition described above. The seedlings were cultured in modified Kimura B solution with 1.5 mM KNO₃ for 12 days and treated with 100 mM NaCl or without NaCl as a control for another three days. 15-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 conducted. RNA library construction and sequence analysis were conducted as described previously (Khan et al., 2016a).

**Yeast one-hybrid assay**

The protein putative binding sites were cloned into BD vector (pHIS2), and the coding sequences of *OsMADS27/OsNLP4* were cloned into AD vector (pAD-GAL4-2.1), respectively. A yeast one-hybrid (Y1H) assay was conducted according to the procedure described previously (Mao et al., 2016).

**Chromatin immunoprecipitation–quantitative PCR assay**

A chromatin immunoprecipitation (ChIP) assay was carried out according to the protocol described before (O’Geen et al., 2010) with minor modifications. Transgenic rice (*OsMADS27pro:OsMADS27-GFP*) seedlings were grown under high nitrogen (2 mM) condition for 2 weeks. About 2.0 g of seedlings were placed in 1% formaldehyde (v/v) at 20-25 °C in vacuum for 15 min and then homogenized within 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 immuno-precipitated DNA fragments were dissolved in water and kept at −80 °C before use. The precipitated fragments were used as template for quantitative PCR (qPCR).
Field trial of rice

For the field test of KO1 mutant and OsMADS27-overexpressing (OE7) (all with ZH11 background), T3 generation plants were grown in Chang Xing, Zhejiang in 2021 (April to September). The plant density was 6 rows. 20 plants per row for each plot, and four replicates were used for each N condition. Urea was used as the N fertilizer at 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 the variability in the field test, the fertilizers were used evenly in each plot for N application level. The plants at the edge were excluded from data collection in each plot in order to avoid margin effects.

Agronomic trait analyses

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

Accession numbers

Sequence data from this article can be found in 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 Data

Fig. S1. Expression pattern of OsMADS27.

Fig. S2. Verification of the CRISPR/Cas9-edited mutations in OsMADS27 and overexpression lines of OsMADS27.

Fig. S3. OsMADS27 positively affects salt tolerance in germination and seedling growth.

Fig. S4. OsMADS7 positively affects grain yield in a N-dependent manner under normal and salt stress conditions.
Fig. S5. *OsMADS27* improves NUE and grain yield in the field of different nitrogen concentrations.

Fig. S6. The gene ontology (GO)-based enrichment analysis of DEGs.

Fig. S7. *OsMADS27* broadly regulates the genes involved in salt tolerance.

Table S1. List of primers used in this study.

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**Author Contributions**


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**References:**


Figure Legends

Figure 1. OsMADS27 is specifically responsive to nitrate.

A. Time-course analyses of OsMADS27 expression in response to N and salt stress. 7-day-old wild type plants grown on hydroponic medium with 1.5 mM KNO₃ were transferred to hydroponic medium without N for 2 days, and then transferred to hydroponic medium with 2 mM KNO₃, 2 mM NH₄Cl, 140 mM NaCl or 2 mM KCl for 0, 0.5, 3, 12, 24 hours. RNA was extracted from the whole seedlings for qRT-PCR analyses as described in the Materials and method. Values are mean ± SD (n = 3).

B. Time-course analyses of OsMADS27 expression in response to KNO₃ depletion. 7-day-old wild type plants grown under on hydroponic medium with 1.5 mM KNO₃ were treated with 2 mM KNO₃ for 0, 0.5, 1, 3 hours, then transferred to hydroponic medium without KNO₃ for 3, 6, 12, 24 hours. RNA was extracted from the whole seedlings for qRT-PCR analyses as described in the Materials and method. Values are mean ± SD (n = 3).

C. KNO₃-dependent induction of OsMADS27 expression by NaCl. Wild type seedlings hydroponically grown on N-free medium for 7 days were treated with 140 mM NaCl for 0, 0.5, 1, 3 hours, and then transferred to hydroponic medium with 140 mM NaCl + 2 mM KNO₃ for 0.5, 1, 3 hours. RNA was extracted from the whole seedlings for qRT-PCR analyses as described in the Materials and method. Values are mean ± SD (n = 3).

D-E. The response of OsMADS27pro:GUS to NaCl. 7-day-old OsMADS27pro:GUS lines grown on N-free medium with 2 mM KCl (D) or 2 mM KNO₃ (E) were treated with 140 mM NaCl for 0.5, 1, 3 hours, respectively. Seedlings were incubated in GUS buffer for 3 hours before photographs were taken. Bar = 1 cm

F. OsMADS27 protein level in OsMADS27pro:OsMADS27-GFP plants. 2-week-old OsMADS27pro:OsMADS27-GFP seedlings grown hydroponically on medium contains different N concentrations (0.02 mM, 0.2 mM and 2 mM KNO₃) without (control) or with 100 mM NaCl were used for the analysis of OsMADS27 protein level by western blot with anti-GFP antibodies. ZH11 (WT) grown on medium with 2 mM KNO₃ served as a control.

Figure 2. Nitrate-responsive nuclear localization of OsMADS27.
OsMADS27 pro:OsMADS27-GFP plants were grown on N-free MS medium supplied with 2 mM KNO₃ (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 KNO₃ (D), 2 mM NH₄Cl (E), 150 mM NaCl (F), and 150 mM NaCl + 2 mM KNO₃ (G) respectively for 60 min before GFP observation. The green fluorescence was observed on the Zeiss 880 microscope. Scale bars = 20 μm.

Figure 3. Nitrate-dependent salt tolerance of seedlings

A-D. Hydroponic salt tolerance assay. Seeds of WT (ZH11), KO1, KO2 mutants, OE7 and OE8 lines were germinated at 37 °C for 4 days and transferred to modified hydroponic medium containing different N concentration (0.02 mM, 0.2 mM, 2 mM KNO₃) for 7 days followed by application of 0 mM, 140 mM NaCl for 7 days before photographs were taken (A-B), and survival rate was calculated (C-D). Values are mean ± SD (n=3 replicates, 32 seedlings per replicate).

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

A. The number of differentially expressed genes (DEGs). The statistics data of differentially expressed genes in (KO vs WT)-control, (OE vs WT)-control, (KO vs WT)-salt, and (OE vs WT)-salt groups.

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C-D. Luciferase activity assay. pRI101- OsMADS27 acts as effector. pGreenII0800-OsHKT1.1pro::LUC / OsSPL7pro::LUC function as reporters. “/-” represents pRI101 and pGreenII 0800 empty plasmids. “/-”, “OsMADS27/-”, “/OsHKT1.1pro::LUC”, “/OsSPL7pro::LUC” as negative controls; “OsMADS27/OsHKT1.1pro::LUC” (E), “OsMADS27/ OsSPL7pro::LUC” (F) as experimental groups. Different constructs were separately coinfiltrated into 4-week-old tobacco leaves, then the luciferase activity was detected by the luciferase assay system.

Figure 6. OsNLP4 binds to the promoter of OsMADS27 and activates its expression.

A. qRT-PCR analysis of OsMADS27 expression in wild type, nlp4 mutants and OsNLP4-OE lines. Seeds were germinated and grown in the hydroponic medium for 16 days before RNA isolation from the whole seedling. Values are mean ± SD (n=3 replicates). Different letters denote significant differences (P < 0.05) from Duncan’s multiple range tests.

B. ChIP-qPCR assay. The enrichment of the fragments containing NRE motif (marked with asterisks) in promoters of OsMADS27 was checked in OsACTIN1pro:OsNLP4-GFP plants and wild type. About 200 bp cis1 and cis3 of OsMADS27 promoter were enriched in OsACTIN1pro:OsNLP4-GFP plants by anti-GFP antibodies as shown in qRT-PCR analyses. Values are mean ± SD (n=3 replicates). Different letters denote significant differences (P < 0.05) from Duncan’s multiple range tests.

C. EMSA assay. Recombinant MBP-NLP4 protein was purified from E. coli cells and used for DNA binding assays with the promoter of OsMADS27. Competition for OsNLP4 binding was conducted with 5×, 10×, 50× unlabeled OsMADS27 probes. Shifted bands are indicated by asterisk.
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**Figure 7. A working model of nitrate-dependent salt tolerance mediated by OsNLP4-OsMADS27 module.**

A. Under nitrate sufficient condition, nitrate triggers OsNLP4 production and nuclear localization, consequently activating the expression of *OsMADS27*, leading to 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 condition, less OsNLP4 protein is produced and the vast majority of OsNLP4 protein is localized in the cytoplasm, resulting in a relatively low expression of *OsMADS27*, thereby attenuating the downstream salt tolerance-related genes.
Figure 1. *OsMADS27* is specifically responsive to nitrate.

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