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Targeted Mutagenesis of NAC Transcription Factor Gene, *OsNAC041*, Leading to Salt Sensitivity in Rice

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Abstract: Salinity is a major abiotic stress factor that seriously affects plant growth. Many genes are involved in the response to salt stress with various metabolism pathways. A number of plant transcription factor family genes have been found to be involved in the salt stress response, and NAM, ATAF and CUC (NAC) transcription factors are thought to act as active regulators during abiotic stress, especially salt stress. In this study, we detected a rice NAC transcription factor coding gene, *OsNAC041*, and confirmed that it influenced the germination of seeds under salt stress and salt tolerance of plants. *OsNAC041* was primarily expressed in the leaves and located in the nucleus. Furthermore, the CRISPR/Cas9 method was used to obtain a targeted *osnac041* mutant, of which the plant height was higher than that of the wild-type, showing increased salt sensitivity. Moreover, RNA-seq analysis revealed a number of differentially expressed genes (DEGs) involved in several important signaling pathways in the *osnac041* mutant. Subsequently, Kyoto Encyclopedia of Genes and Genomes annotation also revealed differential expression of DEGs associated with mitogen-activated protein kinase signaling, peroxisome, eukaryotic-type ABC transporters, photosynthesis and plant hormones, which are involved in stress-related signaling pathways. Overall, our study suggested that *OsNAC041* was involved in the salt stress response in rice. These findings not only provide empirical evidence of *OsNAC041* function, but also provide new insight into its potential application in rice resistance breeding.

Key words: rice; NAC transcription factor; CRISPR; Cas9; RNA-seq; salinity

Salt stress is one of the most important factors affecting plant cultivation and crop productivity (Hayashi et al, 1998; Kou et al, 2018). High salt concentrations can cause plant wilting and slow growth, and often result in death. In rice, salinity induces pollen sterility, threatening survival and causing large losses in yield (Dolferus, 2014). Salt stress prevents plants from reaching their full potential via ion toxicity, disruption of protein synthesis and interference with normal enzyme activity. Importantly, salt stress can also cause a reduction in the rate and efficiency of photosynthesis, resulting in wilting and programmed cell death (Ma et al, 2017).

Various factors, for example, kinases, phosphatases, plant hormones and transcription factors, play a pivotal role in salt stress responses such as the salt overly sensitive (SOS) pathway (Lata and Prasad, 2011). The plant transcription factor family, including myeloblastosis (MYB), basic leucine zipper (bZIP), WRKY, dehydration responsive element binding (DREB) and NAC (NAM, ATAF1/2 and CUC2), are now considered important components of stress tolerance studies (Zhu, 2002; Duan et al, 2017; Tian et al, 2017). For example, over-expression of *OsWRKY30* increases drought resistance, while over-expression and knockout

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of *OsWRKY47* was participated in the response to water stress (Shen et al, 2012). Moreover, combined over-expression of DREB and phytochrome interacting factor transcription factors improves drought stress tolerance and cell elongation in transgenic plants (Kudo et al, 2017). Transcription factors have also been shown to have multiple functions during plant growth and development (Petricka et al, 2012), from organogenesis and hormonal signal responses (Nuruzzaman et al, 2012) to abiotic stress responses (Zheng et al, 2009).

The NAC family is plant-specific transcription factor that plays important roles in a number of biological metabolic pathways. The first reported NAC gene is isolated in petunias and plays a role in shoot apical meristem development (Souer et al, 1996). NAC genes found in *Arabidopsis* (ATAF1/2, CUC1) reveal a conserved N-terminal region (Aida et al, 1997). Protein motif is also highly conserved, while the N-terminal end contains five subdomains and the C-terminal end is highly variable (Ernst et al, 2004). Accordingly, the unique structure is related to specific biotic functions during biotic and abiotic stress responses. Meanwhile, *AtNAC096* is thought to form a synergistic relationship with a subset of abscisic acid (ABA)-responsive-element binding factors (ABFs), triggering transcriptional activation of ABA-inducible genes in response to dehydration and osmotic stress (Xu et al, 2013).

A total of 158 NAC transcription factors have been identified in rice and subsequently listed in the Plant Transcription Factor Database (PlantTFDB v4.0). Studies on their functions in rice have since been carried out. The rice transcription factor *OsNAC6* is involved in numerous molecular mechanisms related to drought tolerance such as root structural adaptions and nicotianamine biosynthesis (Lee et al, 2017). Salinity and drought both can induce *OsNAP* transcription, suggesting induction of an abiotic stress response via the ABA pathway (Chen et al, 2014). Meanwhile, *OsNAC2* overexpression lines possess increased resistance to high salt and drought conditions, while RNAi induces an increase in tolerance at both the vegetative and reproductive stages (Shen et al, 2017).

In the study, we detected a rice NAC transcription factor coding gene, *OsNAC041* (LOC_Os03g013300). Initial salt treatment of wild-type plants confirmed that *OsNAC041* is induced by salt stress. Mutants were then created based on the CRISPR/Cas9 method (Tang et al, 2016; Zhou et al, 2017; Zhong et al, 2018) to further determine the specific function of *OsNAC041* under salt treatment. The global transcriptome profile of *OsNAC041* knockout plants was also determined to explore the major molecular function of *OsNAC041* under salt stress conditions.

MATERIALS AND METHODS

Plant materials and culture conditions

Wild-type (Nipponbare, *Oryza sativa* L. *japonica*) and *osnac041* mutant plants were grown in a light incubator at 28 ºC under a 16 h light (3000 Lux)/8 h dark cycle. To induce salt stress, seedlings at the 4-week stage were irrigated with solution consisting of 200 mmol/L NaCl. To examine growth performance, they were then grown on 1/2 strength Murashige and Skoog (MS) medium, which was supplemented with 150 mmol/L NaCl.

OsNAC041 expression profile analysis

Root, stem and leaf samples from 18-day-old rice seedlings were used to isolate total RNA using the modified Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was then performed to determine *OsNAC041* expression in wild-type plants using the primers listed in Supplemental Table 1, with an *Actin* gene as an internal control.

Targeted mutagenesis of *OsNAC041*

To create targeted *OsNAC041* mutants, the sgRNA oligonucleotide pair was annealed (Supplemental Table 1), then cloned into the CRISPR-Cas9 backbone vector, pZHY988 (Tang et al, 2016; Zhou et al, 2017; Zhong et al, 2018). *Agrobacterium tumefaciens* strain EHA105 was used to carry the vector and infected rice calli (Toki et al, 2006). Single-strand conformation polymorphism (SSCP) and Sanger sequencing were carried out for mutant identification (Zheng et al, 2016; Zhou et al, 2017).

Subcellular localization confirmation

NLS_Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) (Kosugi et al, 2009) was used to predict the possible location of *OsNAC041*, and the pZmUbi::OsNAC041-eGFP::HspT vector was constructed to confirm its location *in vivo*. Transformation of rice protoplasts was conducted by Tang et al (2017).

Physiological measurements

Four-week-old seedlings were irrigated with 200 mmol/L NaCl solution. Detached leaf samples were then incubated overnight in 0.5 mg/mL nitro-blue

tetrazolium (NBT) or 5 mg/mL diaminobenzidine (DAB) (pH 3.8) in the dark. Production of O_2^- and the H_2O_2 content were then quantified according to Rai et al (2013). Malondialdehyde (MDA) concentration was also determined according to the thiobarbituric acid method (Wu et al, 2014). Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities were determined based on the methods of Wu et al (2014), Munoz-Munoz et al (2009) and Sousa et al (2015), respectively. Each experiment was carried out in triplicate.

RNA-seq and data analysis

RNA-seq analysis was carried out using wild-type and *osnac041* (A/A) mutant plants grown for 18 d under normal conditions or for 15 d under normal conditions and 3 d under salt treatment. Three *osnac041* (A/A) T_1 seedlings were selected and combined to form a mixed sample. Total RNA was then isolated from the mixed sample using the modified Trizol method. RNA-seq was carried out by Beijing Genomics Institute (Shenzhen, China). To confirm the accuracy of the RNA-seq expression profiles, 10 genes with significantly different expression profiles were randomly selected for qRT-PCR. Expression levels were then quantified by normalization against *Actin*. All assays were carried out in triplicate under identical conditions.

RESULTS

Expression profile of *OsNAC041*

One NAC transcription factor coding gene in rice was cloned and named as *OsNAC041* according to database serial number (PlantTFDB v4.0). qRT-PCR revealed that the highest expression level was observed in the rice leaves (Fig. 1-A). Salt treatment was then applied to determine whether *OsNAC041* expression was induced by salt stress. Accordingly, after salt treatment (Na^+) for 3 d, *OsNAC041* expression level increased by two-fold (Fig. 1-B). Using nuclear localization signal (NLS) Mapper, candidate nuclear localization signal elements of *OsNAC041* were revealed (Supplemental Table 1). The pZmUbi::OsNAC041-eGFP::HspT expression vector was subsequently constructed to confirm *OsNAC041* localization in rice protoplast. As a result, the *OsNAC041*-eGFP fusion protein was concentrated in the nucleus, similar to NLS-eGFP (Fig. 1-C).

OsNAC041 targeted mutagenesis

The designed sgRNA oligonucleotide pair (Supplemental Table 1) was designed into the *OsNAC041* exons (Fig. 2-A). A total of 27 stable transgenic T_0 lines were obtained via *Agrobacterium*-medicated rice calli transformation. SSCP was then used to screen the mutant seedlings for the positive transgene, and Sanger sequencing was used to further confirm the mutation genotype (Fig. 2-B and -C). Of the 27 *OsNAC041*-sgRNA01 T_0 lines, 26 were confirmed as containing mutations (96.3%), and seven of these were biallelic (26.9%). Moreover, the height of the four-week mutant seedlings in T_1 generation increased significantly compared with the wild-type (Fig. 2-D).

osnac041 mutants possess a salt-sensitive phenotype

To determine the response of *OsNAC041* to stress, *osnac041* mutants were treated with 150 mmol/L NaCl. Compared with the wild-type plants, seven days of seed germination and subsequent growth were

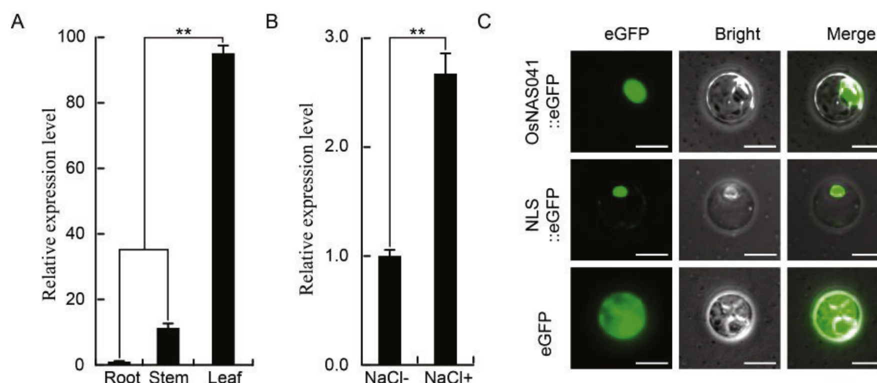

Fig. 1. Expression profile and subcellular location analysis of *OsNAC041*.

A, Quantitative real-time PCR (qRT-PCR) analysis of *OsNAC041* expression in samples of root, stem and leaf from two-week-old wild-type (WT) plants. B, qRT-PCR analysis of *OsNAC041* expression patterns in WT plants before (NaCl-) and after (NaCl+) salt stress. C, Nuclear localization of OsNAC041 protein in the rice protoplast. NLS, Nuclear localization signal. Scale bar = 20 µm.

Data represent Mean ± SE (n = 3). **, Significance at the 0.01 level.

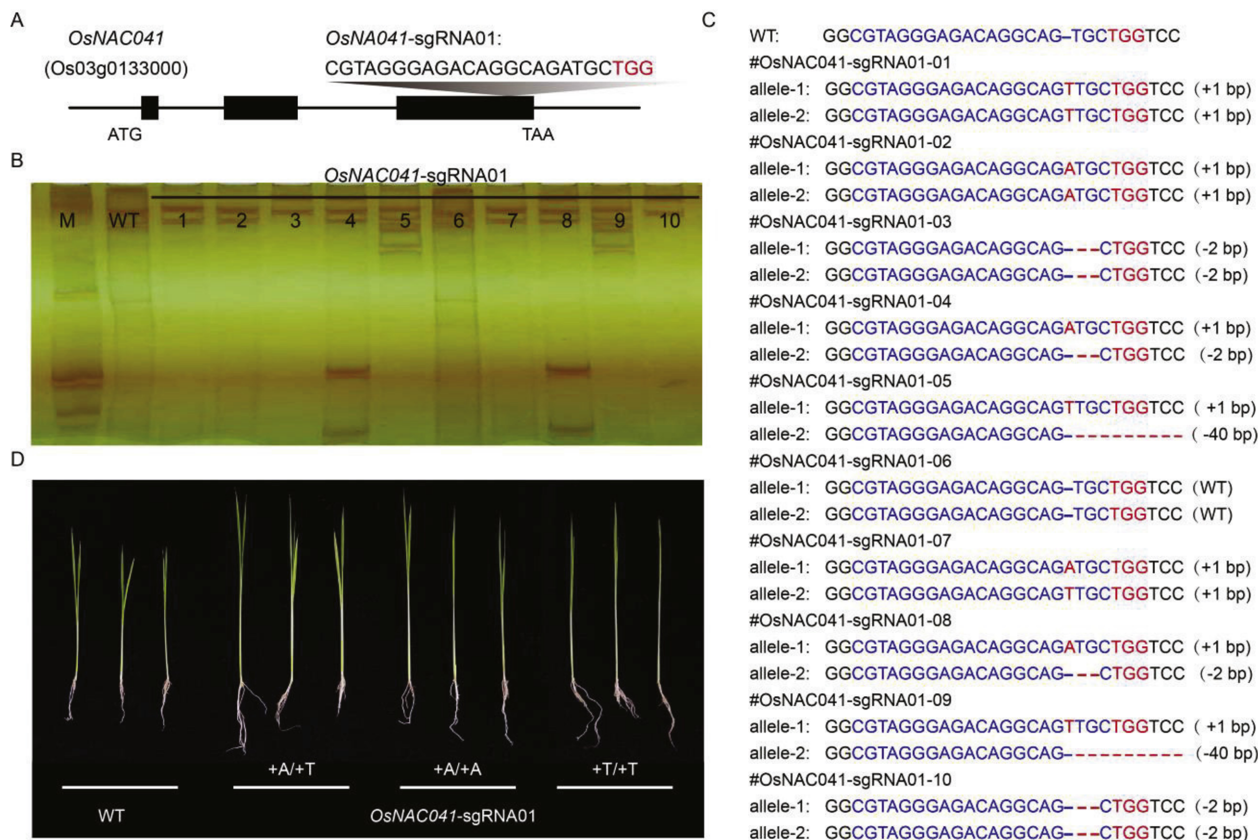

Fig. 2. Targeted mutagenesis of the *OsNAC041* locus using CRISPR-Cas9 system.

A, Design of sgRNA sites for the *OsNAC041* exons. B, Single-strand conformation polymorphism analysis of 10 independent *OsNAC041*-sgRNA01 T_0 lines. M, Marker; WT, Wild-type. C, Sanger sequencing of the target site in the *OsNAC041*-sgRNA01 T_0 lines. D, Phenotypic analysis of *OsNAC041* T_1 mutant lines under normal condition.

inhibited under 150 mmol/L NaCl treatment. Moreover, shoots of the wild-type seedlings were longer than those of the mutants under salt stress (Fig. 3-A). The *osnac041* mutants also showed a salt-sensitive phenotype at the vegetative growth stage. Moreover, after treatment with 150 mmol/L NaCl for 15 d, the wild-type seedlings remained alive, whereas almost all of the mutant seedlings died (Fig. 3-B and -C).

Reactive oxygen species (ROS) accumulation in the mutant plants was subsequently observed under salt stress. O_2^- and H_2O_2 accumulation were examined using NBT and DAB staining, respectively. As a result, NBT staining was detected in all the three mutant lines, *osnac041* (A/T), *osnac041* (A/A) and *osnac041* (T/T), compared with the wild-type. DAB staining subsequently revealed increased levels of H_2O_2 in the mutants compared with the wild-type (Fig. 3-F). Quantitative analysis of the O_2^- and H_2O_2 levels also revealed a significant increase in ROS accumulation in the mutants compared with the wild-type (Fig. 3-F and -G), which was consistent with the results of DAB and NBT staining.

MDA content, which reflects lipid peroxidation, was also measured in wild-type and *osnac041* mutant leaves. After 15-day salt treatment, a significant increase in MDA content was observed in the *osnac041* mutants compared with the wild-type (Fig. 3-H). In addition, SOD, POD and CAT activities were also determined to examine the response of the membrane protection system, which are involved in scavenging harmful ROS during stress. Overall, activities of these antioxidant enzymes were lower in the wild-type than the mutants (Fig. 3-I to -K), suggesting that knockout of *OsNAC041* affected the membrane protection system, causing an increase in toxic substances, and thereby weakening salt tolerance.

Targeted knockout of *OsNAC041* results in global gene expression changes in rice

To understand how *OsNAC041* responds to salt and regulates certain pathways, we examined global transcriptome changes following *OsNAC041* knockout.

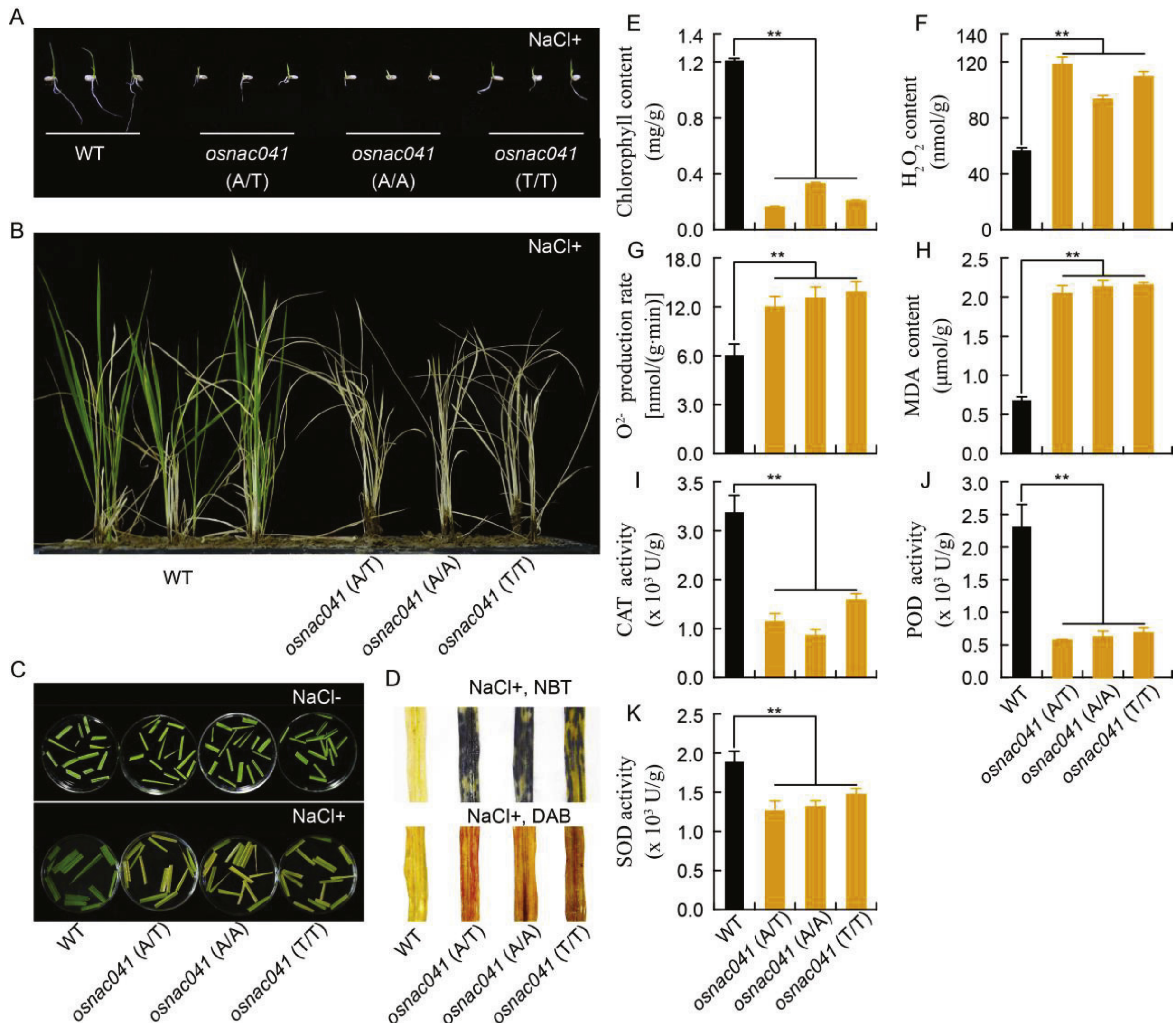

Fig. 3. *OsNAC041* targeted mutants showed the salt-sensitive phenotype.

A, Germination of *OsNAC041* T_1 mutant and wild-type (WT) seeds grown on 1/2 MS medium supplemented with NaCl (150 mmol/L). B, Phenotypic analysis of *OsNAC041* T_1 mutant lines under salt stress. C, Growth of *OsNAC041* T_1 mutant lines in plugs. D, Levels of O_2^- and H_2O_2 in WT and *OsNAC041* T_1 mutant lines subjected to salt stress. Salt-stressed leaf samples were incubated in nitro-blue tetrazolium (NBT) or diaminobenzidine (DAB) solution. E, Chlorophyll content after 15-day salt stress. F, H_2O_2 content after 15-day salt stress. G, O_2^- production rate after 15-day salt stress. H, Malondialdehyde (MDA) content after 15-day salt stress. I, Catalase (CAT) activity after 15-day salt stress. J, Peroxidase (POD) activity after 15-day salt stress. K, Superoxide dismutase (SOD) activity after 15-day salt stress.

Data represent Mean ± SE (n = 3). **, Significant at the 0.01 level.

To do so, RNA-seq assay of wild-type and *osnac041* (A/A) plants was carried out under normal and salt stress conditions (Fig. 4-A). The average mapping ratio of the reference genome was 97.22%. Meanwhile, the average mapping ratio at the gene level was 89.06%, with a total of 26 575 genes detected.

Under normal conditions, a total of 575 genes were up-regulated, while 1 960 were down-regulated in WT compared with mutant without NaCl treatment. Genes with fold change of the expression level ≥ 2.00 with the FDR (false discovery rate) ≤ 0.001 are differentially expressed genes (DEGs). Meanwhile, more than half of the rice genes (52.78%, 13 494) were differentially expressed under salt stress conditions: 8.35% (2 218) were up-regulated and 44.43% (11 276) were down-regulated. The number of DEGs increased 5.3-fold under salt stress compared to normal conditions (Fig. 4-B). In Venn diagram analysis, 1 447 genes were co-expressed in the WT/*osnac041*::NaCl+ comparison and WT/*osnac041*::NaCl- comparison, while 12 047 genes were expressed only in the WT/*osnac041*:: NaCl+ comparison and 1 088 genes only in the WT/*osnac041*::NaCl- comparison (Supplemental Fig. 1). To validate the results of RNA-seq, qRT-PCR was

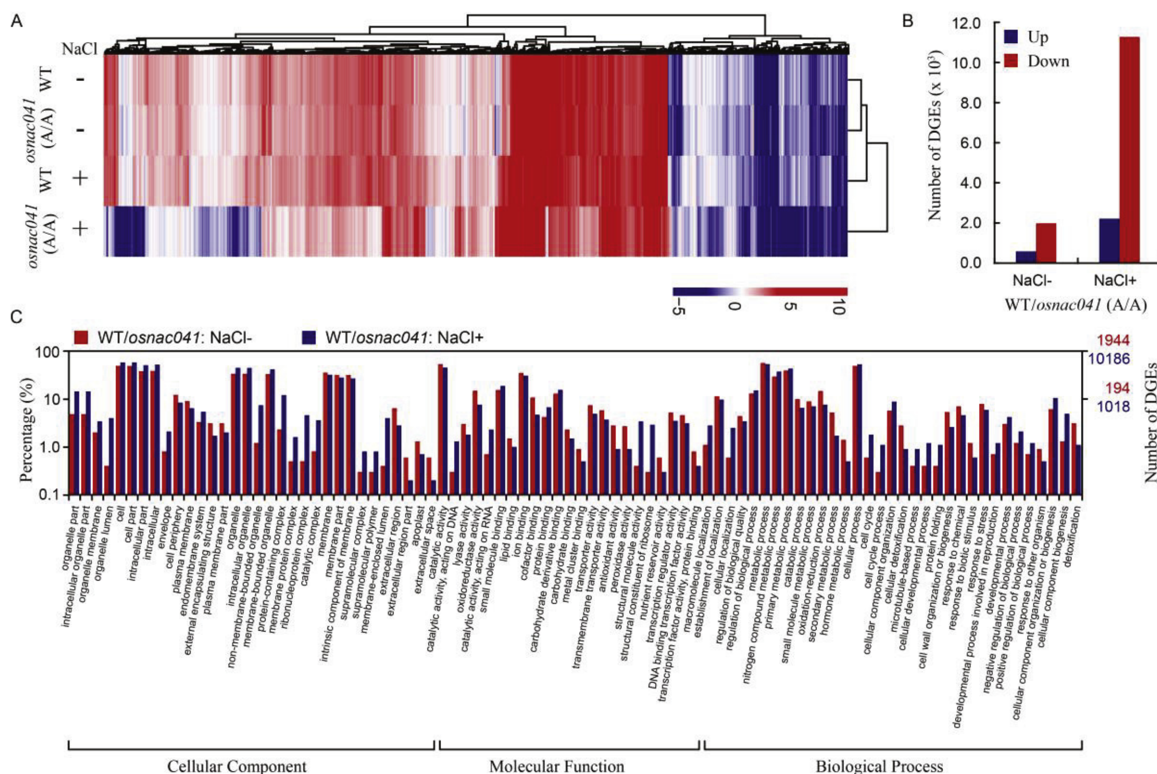

Fig. 4. Targeted knockout of *OsNAC041* resulted in global gene expression changes in rice.

A, Number of differentially expressed genes (DEGs) in the wild-type (WT) and *osnac041* (A/A) T_1 mutant lines, based on expression profiles obtained by RNA-Seq. B, Clustering analysis of DEGs in WT and *osnac041* T_1 mutant lines. Targeted knockout of *osnac041* resulted in global changes compared with the WT both with and without salt stress. The color scale corresponds to $\log_2$(FPKM) values of the genes. C, Gene ontology classification of DEGs in the following two comparisons: WT and *osnac041* T_1 mutant lines under normal and salt stress conditions. x-axis shows user selected GO terms; y-axis shows the percentages of genes (number of a particular gene divided by total gene number).

carried out using 10 genes selected randomly from significantly up-regulated genes in *osnac041* (A/A) compared with wild-type plants following salt treatment (Supplemental Fig. 2). As a result, a high correlation was found between the RNA-seq and qRT-PCR results, confirming the accuracy of the RNA-seq data.

Gene oncology (GO) classifications further revealed that these DEGs were enriched during ion binding and metabolic processes under normal and salt stress conditions (Fig. 4-C). A total of 1 697 significant DEGs were related to metabolic processes, while 1 539 DEGs were related to cellular processes, suggesting that salt stress affected biological processes in the mutants. In the cellular component category, organelle (1 184 DEGs), membrane (996 DEGs) and extracellular region (183 DEGs) GO terms were significantly enriched, suggesting that salt stress also affected the membrane and cell parts in the mutant plants. Moreover, 1 493 DEGs associated with binding, 1 489 DEGs associated with catalytic activity, and 192 DEGs associated with transporter activity were also revealed, suggesting that salt stress also influenced molecular function in the mutants.

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was also carried out to predict the biochemical pathways associated with the DEGs (Supplemental Fig. 5). Interestingly, a large number of differential genes were concentrated in several signaling pathways associated with salt stress. DEGs identified in the WT/*osnac041* (A/A) comparison were associated with mitogen-activated protein kinase (MAPK) signaling (632, 3.06%), plant hormone signal transduction (804, 3.90%), peroxisome (178, 0.86%), eukaryotic-type ABC transporters (188, 0.91%) and photosynthesis (125, 0.60%).

Differential expression analysis revealed potential transcriptional responses to salt stress

Of the major signaling pathways, photosynthesis is most important, converting light energy into organic matter and releasing oxygen. In the mutants, most photosynthesis-related genes were down-regulated, consistent with the salt-sensitive phenotype (Fig. 5-A). Similar to photosynthesis, peroxisomes produce important

signals during stress responses. *PEX1*, *PEX2*, *PEX3* and *PEX7* are key genes encoding peroxisome membrane proteins, and all are up-regulated during peroxidase synthesis (Supplemental Fig. 6).

It is well known that when plants are dehydrated, they are more susceptible to injury and death. In this study, GO analysis identified up-regulation of at least 12 DEGs related to water deprivation in mutant *osnac041* (A/A) under salt stress, which may have accelerated dehydration (Fig. 5-C). This finding suggests that the mutants are more likely to lose water under salt stress. Heat-map analysis revealed that the MAPK pathway expression was down-regulated under salt stress compared to normal conditions, suggesting a decrease in the ability of the mutants to respond to salt stress (Fig. 5-D). The ABC superfamily is an ancient large family of ATP-driven pumps. As the gatekeeper of cells, ABC transporters preserve nutrients within the cell and expel toxins. *ABCA3* in the ABCA subfamily, *ABCB10* in the ABCB subfamily, and *ABCC1* and *ABCC10* in the ABCC subfamily were significantly up-regulated, while more than 20 ABC members were significantly down-regulated in the *osnac041* mutant, suggesting that differential expression of ABC family genes also affected the stress response (Fig. 5-E). Moreover, metabolic activities

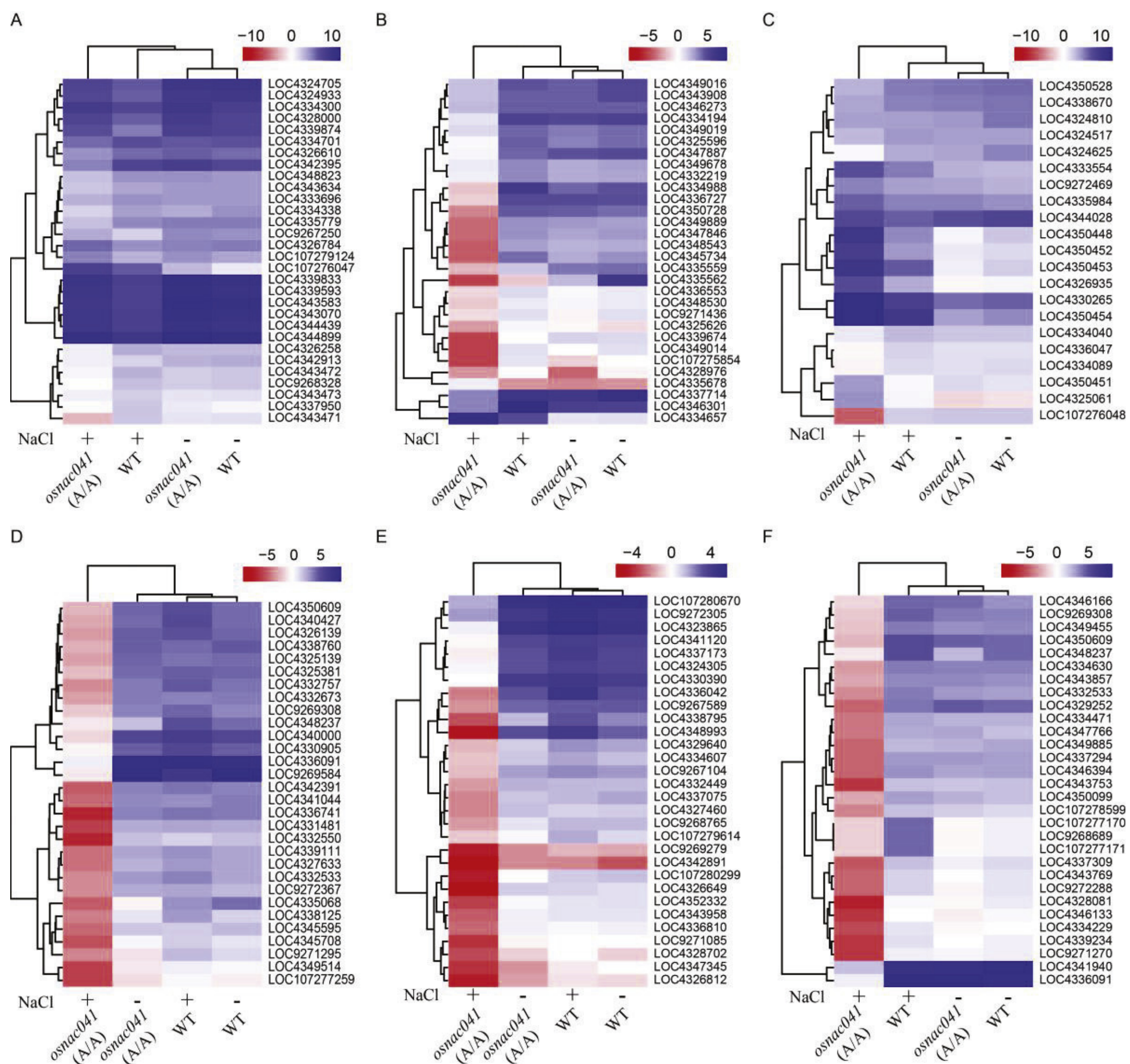

Fig. 5. Transcriptome analysis of genes systemically regulated in the wild-type (WT) and *osnac041* T_1 mutant lines in response to salt stress.

A, Photosynthesis-related genes. B, Peroxisome-related genes. C, Water deprivation-related genes. D, Mitogen-activated protein kinase signal-related genes. E, Eukaryotic-type ABC transporter-related genes. F, Plant hormone regulatory pathway-related genes.

Log_2(Fold change, FC) of differentially expressed genes in WT and *osnac041* T_1 mutant lines before (NaCl-) and after (NaCl+) salt stress is defined as [-1.5 > log(FC) > 1.5] with false discovery rate (FDR) < 0.05, and the top 30 most significant differences are shown in the heat-map.

of the auxin, cytokinin, salicylic acid, jasmonic acid, ethylene and brassinosteroid biosynthetic pathways were also seriously impacted in the mutant plants, suggesting that various pathways associated with plant hormones were also involved in *OsNAC041*-mediated salt tolerance (Fig. 5-F).

DISCUSSION

It was previously revealed that a number of NAC transcription factors are associated with stress. For example, in rice, NAC transcription factors *SNAC1* (Hu et al, 2006; Redillas et al, 2012), *SNAC2* (Hu et al, 2008), *OsNAC5* (Jeong et al, 2013), *OsNAP* (Liang et al, 2014) and *OsNAC2* (Kunieda et al, 2008) all affect the abiotic stress pathway. Over-expression of *SNAC1* in rice also enhances drought resistance and salt tolerance (Hu et al, 2006), while over-expression of *SNAC2* improves salt tolerance (Hu et al, 2008). Meanwhile, over-expression of the homologous gene in *Arabidopsis* (Supplemental Fig. 3), *AtNAC036*, results in a dwarf phenotype (Kato et al, 2010). In this study, the amino acid sequence of *AtNAC036* has 52% identity with *OsNAC041* as well as a clear NAC domain (Supplemental Fig. 4). The transgenic *AtNAC036* over-expression line also shows relatively slow vegetative growth (Kato et al, 2010). These findings suggest that the phenotype of gain-function of *OsNAC041* correspond with the loss of function observed in this study.

In addition to great changes in photosynthesis (Supplemental Fig. 5), ROS over-accumulation in plants occurs under diverse abiotic stresses including high salinity, and often causes damage (Miller et al, 2010). Many studies have also shown that the plant response to abiotic stresses occurs via regulation of ROS metabolism (Schmidt et al, 2013; Fang et al, 2015). For example, over-expression of *SERF1* improves salinity tolerance via regulation of ROS-dependent signaling in rice (Schmidt et al, 2013). Meanwhile, *OsLG3*-overexpressing plants show improved drought tolerance as a result efficient ROS scavenging, which reduces membrane lipid peroxidation (Xiong et al, 2018). In this study, the *OsNAC041* knockout plants were salt sensitive (Fig. 3-A), and presented with yellow leaves after salt treatment (Fig. 3-B). Moreover, more H_2O_2 and MDA accumulated in the leaves of *OsNAC041* knockout plants compared with the wild-type (Fig. 3-F and -H), suggesting that the reduction in salt tolerance was the result of reduced ROS scavenging and increased levels of MDA. Plants have developed a complex antioxidant system, which includes enzymatic antioxidants (Miller et al, 2010). Under salt stress, activities of SOD, POD and CAT, which can scavenge toxic ROS, were all lower in *osnac041* mutant plants compared with the wild-type. These findings suggest that *OsNAC041* is associated with the ROS system and membrane protection, thereby regulating salt sensitivity.

Plants rapidly detect environmental changes and, in response, initiate related intercellular and intracellular signal transduction pathways (Hu and Xiong, 2014). In this study, *OsNAC041* mutation caused global changes in rice transcriptome (Fig. 5). Furthermore, six enriched pathways related to the salt stress phenotype were observed in the *osnac041* mutant plants including MAPK signaling, plant hormone signal transduction, peroxisome, eukaryotic-type ABC transporters and photosynthesis (Fig. 5). Among these, targeted mutagenesis of *OsNAC041* had the largest impact on the MAPK signaling pathway and ABC transporter pathway (Fig. 5-D and -E).

MAPKs are conserved eukaryote protein kinases that are involved in signaling under environmental stress (Doczi et al, 2012). Plants overexpressing *ZAT6*, an *Arabidopsis* C_2H_2-type zinc finger protein, show improved seed germination under salt stress conditions via interaction with phosphorylation by MAPK6 at two sites (Liu et al, 2013). Moreover, *AZI1*, a lipid transfer protein-related hybrid proline-rich protein involved in salt stress, is also phosphorylated by MAPK3 *in vitro* (Pitzschke et al, 2014). Up- and down-regulation of core components of the MAPK signaling pathway in the *OsNAC041* knockout plants therefore suggests flexibility in adapting to salt stress, although the exact mechanisms remain unclear (Supplemental Fig. 7).

ABC transporter proteins contain an ATP binding domain and are grouped into eight subfamilies, ABCA to ABCI. Plants reportedly contain twice as many as ABC transporters as animals (Theodoulou and Kerr, 2015). Since plants are stationary, they have to deal with changing environments (Lefevre et al, 2015). ABC transporters in plants are involved in a number of functions such as secondary metabolite transport (Francisco et al, 2013), heavy metal detoxification (Archer, 2014), antibiotic transport (Mentewab and Stewart, 2005) and phytohormone transport (Hu and Xiong, 2014). The changes to the ABC subfamily observed in the *OsNAC041* knockout plants therefore suggest that *OsNAC041* also affects ABC transporters, regulating

growth and development in rice (Supplemental Fig. 8).

Plant hormones are critical for adaptation to environmental changes. ABA is a stress-responsive hormone, and along with other phytohormones such as brassinosteroids, ethylene, salicylic acid and jasmonic acid, its role in environmental stress responses is becoming increasingly well understood (Peleg and Blumwald, 2011; Cabello et al, 2014; Zhou et al, 2014). Recent studies suggest that plant hormones are involved in multiple signaling pathways, allowing plants to cope with biotic stress (Depuydt and Hardtke, 2011; Shi et al, 2015). Based on our RNA-seq data, various pathways are thought to be involved in *OsNAC041*-mediated salt sensitive in *OsNAC041* knockout plants. For example, biosynthesis of auxin, cytokinin, gibberellin, ethylene, brassinosteroid, jasmonic acid and salicylic acid were all affected (Supplemental Fig. 9). These findings suggest that knockout of *OsNAC041* alters plant hormone homeostasis, which may be the main cause of the salt sensitive phenotype.

In conclusion, this study presented evidence that *OsNAC041* plays important roles in salt resistance in rice. Induction of *OsNAC041* by salt stress was confirmed along with localization in the cell nucleus. Transcriptome analysis of *OsNAC041* knockout plants showed changes in the expression level of genes involved in MAPK signaling, plant hormone signal transduction, peroxisome, eukaryotic-type ABC transporter and photosynthesis pathways. The salt sensitive phenotype of the *OsNAC041* knockout plants was thought to be the result of changes to ROS-related genes, while differential expression analysis revealed potential transcriptional responses to salt stress. These findings provided a theoretical basis for further molecular studies of salt stress in rice and subsequent salt tolerance breeding programs.

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SUPPLEMENTAL DATA

The following materials are available in the online version of this article at http://www.sciencedirect.com/science/journal/16726308; http://www.ricescience.org.

Supplemental Table 1. Result of the nuclear localization signals-mapper predictions of the location of *OsNAC041* expression.

Supplemental Table 2. Oligonucleotides used in this study.

Supplemental Fig. 1. Validation of the RNA-seq results by quantitative real-time PCR.

Supplemental Fig. 2. Venn diagram analysis of the differentially expressed genes.

Supplemental Fig. 3. Alignment between *OsNAC041* and *AtNAC036*.

Supplemental Fig. 4. Phylogenetic tree analysis of *OsNAC041*.

Supplemental Fig. 5. Analysis of photosynthesis-related differentially expressed genes and photosynthesis-antenna proteins.

Supplemental Fig. 6. Analysis of peroxisome-related differentially expressed genes.

Supplemental Fig. 7. Analysis of MAPK signaling-related differentially expressed genes.

Supplemental Fig. 8. Analysis of eukaryotic-type ABC transporter-related differentially expressed genes.

Supplemental Fig. 9. Analysis of plant hormone signal transduction-related differentially expressed genes.

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