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Transcription Factor *OsDOF18* Controls Ammonium Uptake by Inducing Ammonium Transporters in Rice Roots

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Nitrogen is one of the most important mineral elements for plant growth. We studied the functional roles of *Oryza sativa DNA BINDING WITH ONE FINGER 18* (*OsDOF18*) in controlling ammonium uptake. The growth of null mutants of *OsDOF18* was retarded in a medium containing ammonium as the sole nitrogen source. In contrast, those mutants grew normally in a medium with nitrate as the sole nitrogen source. The gene expression was induced by ammonium but not by nitrate. Uptake of ammonium was lower in *osdof18* mutants than in the wild type, while that of nitrate was not affected by the mutation. This indicated that OsDOF18 is involved in regulating ammonium transport. Among the 10 ammonium transporter genes examined here, expression of *OsAMT1;1*, *OsAMT1;3*, *OsAMT2;1*, and *OsAMT4;1* was reduced in *osdof18* mutants, demonstrating that the ammonium transporter genes function downstream of *OsDOF18*. Genes for nitrogen assimilation were also affected in the mutants. These results provide evidence that *OsDOF18* mediates ammonium transport and nitrogen distribution, which then affects nitrogen use efficiency.

Keywords: ammonium, ammonium transporters, nitrate, rice, transcription factor DOF

INTRODUCTION

Nitrogen (N) is an essential component for plant growth and development (Sonoda et al., 2003; Tabuchi et al., 2007). The major sources of inorganic N ions are ammonium (NH_4^+) and nitrate (NO_3^-), which can be absorbed and used by paddy crops in submerged soil (Tabuchi et al., 2007). As a N source, ammonium is preferable to nitrate for root uptake because less energy is needed for assimilation in the plants (Bloom et al., 1992; Gu et al., 2013; Masumoto et al., 2010).

Ammonium is mobilized by ammonium transporter (AMT). Rice (*Oryza sativa*) contains 10 members of the AMT family: OsAMT1;1, OsAMT1;2, OsAMT1;3, OsAMT2;1, OsAMT2;2, OsAMT2;3, OsAMT3;1, OsAMT3;2, OsAMT3;3, and OsAMT4;1. Whereas the OsAMT1 members are characterized as high-affinity transporters, the other three family members are considered low-affinity transporters (Loqué and von Wirén, 2004; Sonoda et al., 2003; Suenaga et al., 2003). Three *OsAMT1* genes show distinct expression patterns: *OsAMT1;1* is constitutively expressed in shoots while *OsAMT1;2* and *OsAMT1;3* are expressed specifically in roots (Sonoda et al., 2003). *OsAMT1;1* and *OsAMT1;2* are up-regulated in plants following exposure to ammonium, whereas *OsAMT1;3* is up-regulated by N-deprivation (Kumar et al., 2003; Sonoda et al., 2003; Suenaga et al., 2003; Xuan et al., 2013). Overexpression of *OsAMT1;1* results in higher production of biomass with increased amounts of ammonium and glutamine (Ranathunge et al., 2014). However,

overexpression of *OsAMT1;3* causes growth retardation (Bao et al., 2015). OsAMT2;1 shares only 20 to 25% sequence identity with proteins in the AMT1 family, and is more closely related to the yeast METHYLAMINE PERMEASE (MEP) transporter sequence (Suenaga et al., 2003).

Up to 40% of the total N is taken up in the form of nitrate by NITRATE TRANSPORTER (NRT) in paddy (Kirk and Kronzucker 2005). In rice, there are four high affinity NTR2; OsNRT2;1, OsNRT2;2, OsNRT2;3, and OsNRT2;4 (Feng et al., 2011). The coding region sequences of *OsNRT2.1* and *OsNRT2.2* are identical although their untranscribed regions are different. These NRT2 genes are highly homologous to other monocotyledons, while *OsNRT2.3* and *OsNRT2.4* are closely related to Arabidopsis NRT2 (Cai et al. 2008).

Ammonium is first assimilated by glutamine synthetase (GS) to yield the amino group of glutamine that serves as a major nitrogen source transported from root to shoot in rice (Kiyomiya et al. 2001). Glutamine synthetase is coupled with glutamate synthase (GOGAT) in the GS/GOGAT cycle. *OsNADH-GOGAT1* is predominantly expressed at root tips, leaves and seeds, while *OsNADH-GOGAT2* is highly expressed in mature leaves (Tamura et al., 2011). Phosphoenolpyruvate carboxylase (PEPC) plays an important role in carboxylation of phosphoenolpyruvate to form oxaloacetate. NADP-malate dehydrogenase (MDH) converts the reaction between oxaloacetate and malate. OsPEPC4 and OsMDH play crucial roles in ammonium assimilation (Kurai et al., 2011; Masumoto et al., 2010).

DNA-binding with one finger (DOF) transcription factors participate in various biological processes, including tissue differentiation and hormone signaling (Noguero et al., 2013). *Zea mays DOF1* (*ZmDOF1*) enhances the C4 pathway genes *PEPC* and *cytosolic orthophosphate dikinase* (*cyPPDK*) by binding to their promoters. *Zea mays DOF2* (*ZmDOF2*) represses the promoter activity of *ZmPEPC* and *ZmPPDK* by blocking transactivation of *ZmDOF1* (Yanagisawa and Izui, 1993; Zhang et al., 1995). Overexpression of *ZmDOF1* increases the nitrogen content in transgenic *Arabidopsis* plants (Yanagisawa et al., 2004) and results in better growth of transgenic rice plants under low-N conditions (Kurai et al., 2011).

Rice *OsDOF18*, also named *OsDOF24* or *OsDOF25*, is most homologous to *ZmDOF1* (Kushwaha et al., 2010; Lijavetzky et al., 2003). Its heterologous expression in *Arabidopsis* alters carbon and nitrogen metabolism (Santos et al., 2012). In addition, *OsDOF18* appears to have a function in carbohydrate metabolism by controlling *OsPPDK* (Zhang et al., 2015). Here, we demonstrated that *OsDOF18* modulates ammonium uptake by inducing ammonium transporter genes.

MATERIALS AND METHODS

Plant materials and growth conditions
Japonica rice (*Oryza sativa* cv. Dongjin) plants were grown in controlled environment rooms as previously described (Ryu et al., 2009). Seeds were germinated either on an MS medium containing 3% sucrose or in soil, as previously reported (Yi and An, 2013). The T2 progeny of *osdof18* knockout mutants were grown on a 1/2 MS medium containing 50 µg

mL^{-1} hygromycin. For ammonium uptake experiment, plants were grown hydroponically on Yoshida medium containing 1.44 mM NH_4NO_3, 0.3 mM NaH_2PO_4, 0.5 mM K_2SO_4, 1.0 mM $CaCl_2$, 1.6 mM $MgSO_4$, 0.075 µM $(NH_4)_6Mo_7O_{24}$, 18.8 µM H_3BO_3, 9.5 µM $MnCl_2$, 0.16 µM $CuSO_4$, 0.15 µM $ZnSO_4$, 35.6 µM $FeCl_3$, and 74.4 µM citric acid, pH5.5 (Yoshida et al., 1976).

Isolation of *osdof18* mutants
A T-DNA-tagged *osdof18-1* mutant (Line number 3A-16330) was identified from the rice T-DNA insertion sequence database (An et al., 2005a; 2005b; Jeong et al., 2006). Homozygous mutants were confirmed by PCR, using genomic DNA. The Ds-tagged *osdof18-2* mutant (Line number Ds-17925), generated in 'Dongjin', was obtained from the Rice Division of Yeongnam Agricultural Research Institute, National Institute of Crop Science, Korea. Gene-specific primers are listed in Supplemental Table S1.

Analyses of nitrogen induction
Plants were grown in a chamber under the following conditions: 200 µmol m^{-2}s^{-1} photosynthetic photon flux, 12-h photoperiod, 70% relative humidity, and 28°C/24°C (day/ night). Seeds were first treated with 2% sodium hypochlorite for 30 min, then washed with distilled water three to five times, and placed in a glass bottle containing distilled water. At 14 days after germination (DAG), the seedlings were transferred to a glass tube containing 4 ml of Yoshida medium (Yoshida et al., 1976) that contains both ammonium and nitrate as the N source. Medium was harvested at 2-d intervals during the nitrogen uptake experiments, and ammonium and nitrate levels were determined with a UV-1800 spectrometer (Shimadzu, Japan) at OD$_{625}$ and OD$_{220}$, respectively (Martín-Rodríguez et al., 2015; Weatherb, 1967; Wu et al., 2015).
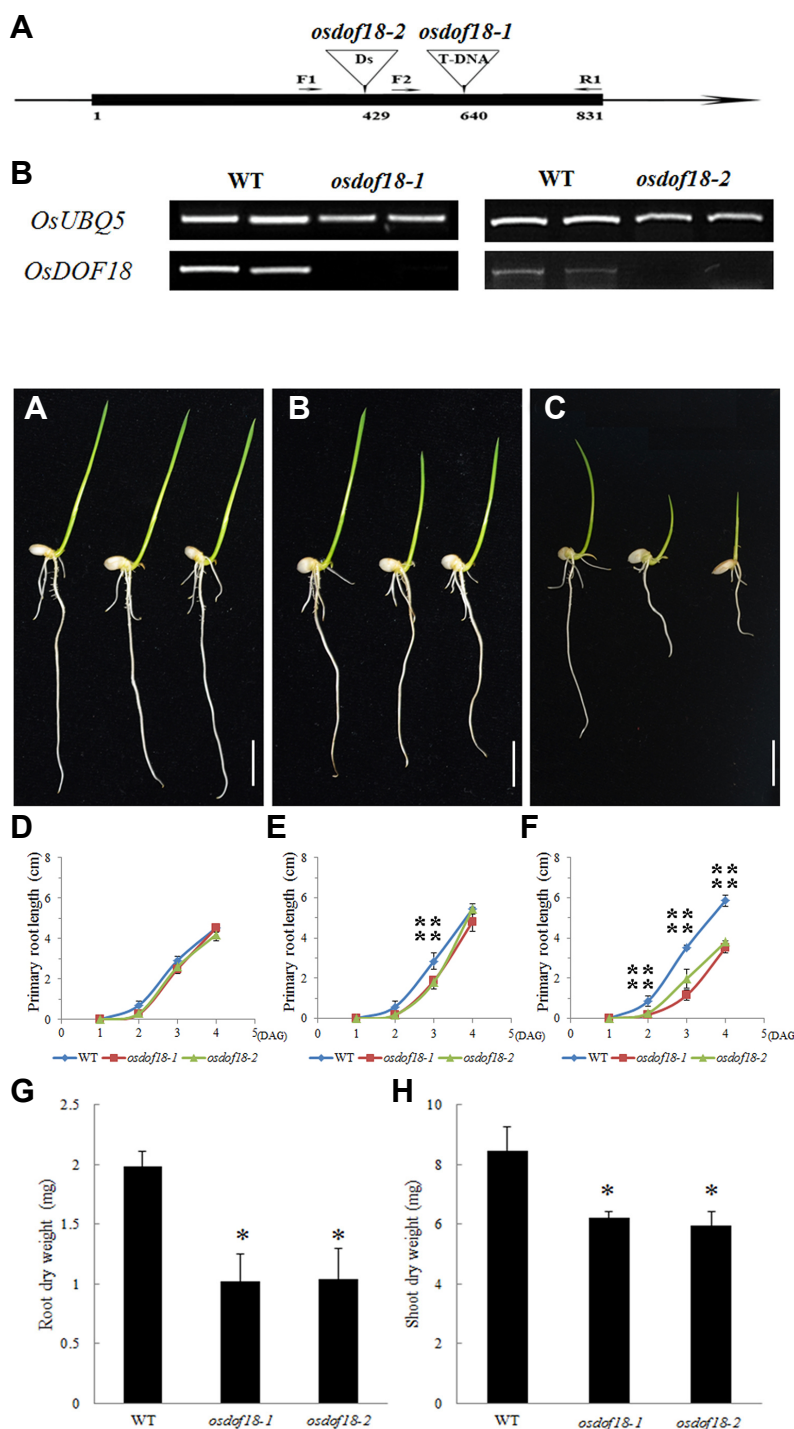
RT-PCR analyses
Total RNA was isolated from seedling roots at 4 DAG using RNAiso Plus (TaKaRa, Shiga, Japan; http://www.takarabio. com). The cDNAs were synthesized and quantitative real-time RT-PCR was performed as previously described (Cho et al., 2016; Ryu et al., 2009; Yang et al., 2013; 2014). Expression levels were normalized with rice *UBQ5* (*LOC_Os01g 22490*). All experiments were conducted at least three times, with three or more samples taken at each point. To ensure primer specificity, we performed the experiments when the melting curve showed a single sharp peak. The PCR products were sequenced to verify the specificity of the reaction (Ryu et al., 2009; Yang et al., 2013; 2014). All primers are listed in Supplemental Table S1.

RESULTS

Identification of knockout mutants in *OsDOF18*
The T-DNA insertion mutant *osdof18-1* was isolated from our T-DNA tagging population (An et al., 2003; 2005a; Jeon et al., 2000; Jeong et al., 2002; 2006; Ryu et al., 2004) and another allele, *osdof18-2*, was identified from Ds insertion lines (Chin et al., 1999; Kim et al., 2004) (Fig. 1A). In both

Fig.1. Structure of *OsDOF18* and position of T-DNA/Ds insertion. (A) Positions of insertion mutants. Black boxes, exons; lines between boxes, introns. Arrows indicate primers for genotyping. (B) RT-PCR analyses of *OsDOF18* expression levels.
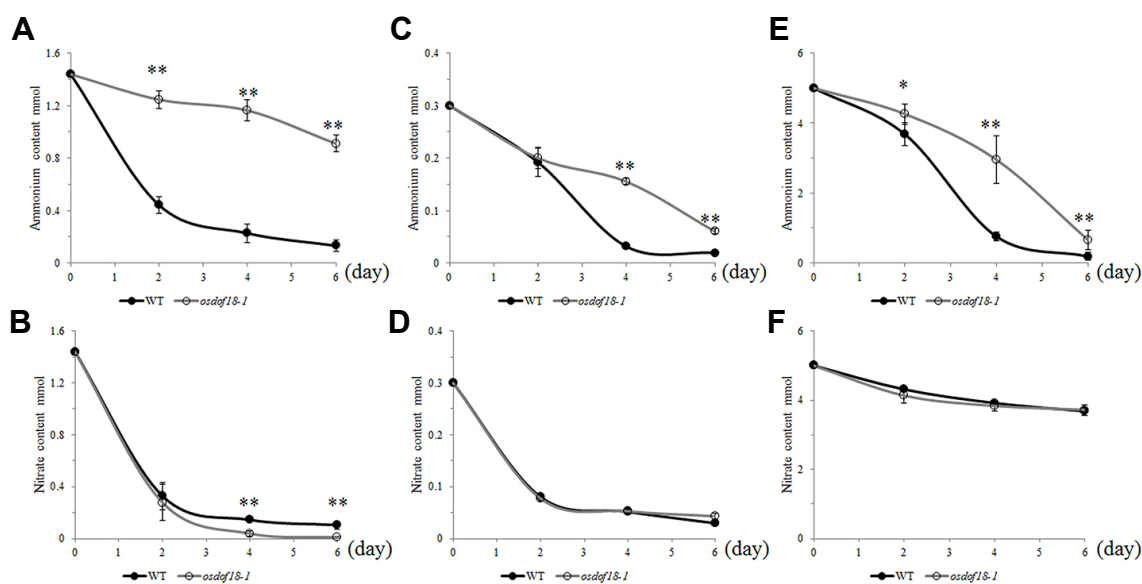

Fig. 2. Phenotypes of *osdof18* mutants. (A-C) Plant phenotypes in Yoshida medium (A), Yoshida medium with nitrate as sole N source (B), or Yoshida medium with ammonium as sole N source (C). (D-F) Primary root lengths in Yoshida medium (D), Yoshida medium with nitrate as sole N source (E), or Yoshida medium with ammonium as sole N source (F). Blue line, WT; red line, *osdof18-1*; green line, *osdof18-2*. Root (G) and shoot (H) dry weights of *osdof18* mutants and WT at 7 DAG grown in Yoshida medium with ammonium as a sole N source. Error bars represent standard error (SE) for at least 5 samples. *, $P < 0.05$; **, $P < 0.01$.

lines, insertions occurred within the coding region, and *OsDOF18* transcript was not detectable in the plants (Fig. 1B). This indicated that both are null mutants.

Mutations in *OsDOF18* cause growth retardation when ammonium is the sole N source

The *osdof18* mutants grew normally in Yoshida medium containing both ammonium and nitrate (Figs. 2A and 2D).

Shoot growth was slightly reduced in the mutant compared to WT when they were grown on Yoshida medium with nitrate as the sole N source (Figs. 2B and 2E). However, when placed on the medium where ammonium was the sole N source, growth of the mutants was significantly retarded (Figs. 2C and 2F). At 4 DAG, primary root lengths from the mutant seedlings were 60% of that measured from the WT. Dry weights for roots and shoots from the

Fig. 3. Uptake activity of ammonium (A-C) and nitrate (D-F) in *osdof18* mutants and WT. A-B; Plants were grown on Yoshida medium with 1.44 mM NH_4NO_3 as sole N source; C-D; Plants were grown on Yoshida medium with 0.3 mM NH_4NO_3 as sole N source; E-F; Plants were grown on Yoshida medium with 5 mM NH_4NO_3 as sole N source. Closed circles, WT; open circles, *osdof18-1*. Error bars represent SE for at least 3 plants. $^*P < 0.05$; $^{**}P < 0.01$.

mutants at 7 DAG were 60% and 73%, respectively, of values recorded for the WT (Figs. 2G and 2H). The defect was likely due to their diminished capability in taking up or assimilating ammonium.

Ammonium uptake level is slow in *osdof18* mutants

To study ammonium uptake level, we grew mutant and WT seedlings in water until 14 DAG to deplete the N stored in the seeds. The plants were then transferred to the Yoshida medium and uptake levels were estimated by measuring the amount of ammonium that remained in the medium. The initial concentration of ammonium was 1.44 mM. For WT plants, that concentration was rapidly reduced in the first 2 d, with approximately 69.3% of the ammonium being removed (Fig. 3A). The concentration was further reduced as the plants grew, with only 9.1% of the ammonium remaining at Day 6. By comparison, the amount of ammonium in the medium where *osdof18* mutants were grown was reduced by only 13.5% at 2 d and 36.8% at 6 d (Fig. 3A).

We also examined the ammonium uptake level at 5 mM and 0.3 mM concentrations. The uptake level in the mutants was slower compared to WT at both reduced (Fig. 3C) and increased (Fig. 3E) concentrations of ammonium. These experiments indicate that *OsDOF18* affects long term ammonium uptake at both low and high concentration. In contrast, nitrate uptake levels were similar between the WT and mutant plants (Figs. 3B, 3D and 3F). These experiments supported our conclusion that *OsDOF18* functions in the uptake of ammonium but not nitrate into the roots.

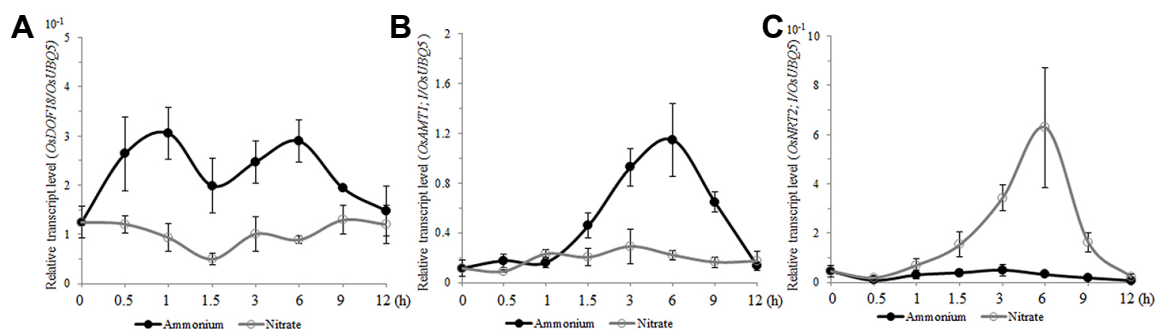### Expression of *OsDOF18* is induced by ammonium

Seedlings were grown in water for 14 d to deplete stored N

in the seeds. After they were transferred to Yoshida medium with ammonium as the sole N source, the level of *OsDOF18* expression increased within 30 min ammonium and peaked at 1 h (Fig. 4A). However, the gene expression was not increased during the 12 h treatment in Yoshida medium with nitrate as the sole N source. Using *OsAMT1;1* and *OsNRT2;1* as controls (Figs. 4B and 4C), we found that the former responded more significantly to ammonium supply than to nitrate while the latter responded mainly to a nitrate supply. These results suggested that *OsDOF18* functions in controlling *OsAMT1;1*.
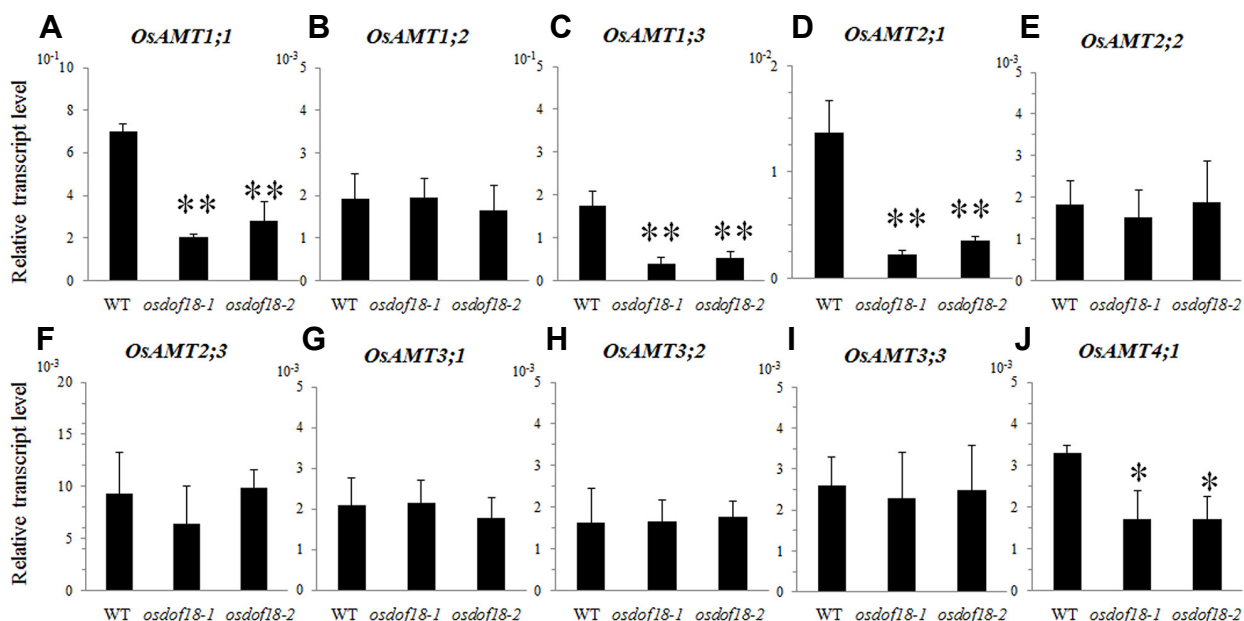
Mutations in *OsDOF18* affect expression of ammonium transporter genes

Transcript levels of 10 ammonium transporter genes were compared between *osdof18* mutants and WT in roots from 4 DAG seedlings grown on the ammonium-supplemented Yoshida medium. When compared with the WT, expression of *OsAMT1;1*, *OsAMT1;3*, *OsAMT2;1*, and *OsAMT4;1* was low in the mutants while that of *OsAMT1;2*, *OsAMT2;2*, *OsAMT2;3*, *OsAMT3;1*, *OsAMT3;2*, and *OsAMT3;3* was not significantly affected by the mutation (Fig. 5). Transcript levels of the nitrate transporter genes, *OsNRT2;1*, *OsNRT2;2* and *OsNRT2;3*, were not changed. We also observed no alteration of the transcript level of *OsIDD10* that acts as an inducer of *OsAMT1;2* (Figs. 6A-6D).

Expression of *OsGOGAT1*, *OsPEPC4*, and *OsNADPH-MDH* was also decreased in the mutants while that of *OsGOGAT2* was unchanged (Figs. 6E-6H). These results suggested that *OsDOF18* functions to affect ammonium uptake as well as the expression of genes involved in ammonium assimilation.

Fig. 4. Expression patterns of *OsDOF18*, *OsAMT1;1*, and *OsNRT2;1* after plants were supplied with ammonium or nitrate. Quantitative RT-PCR analyses of transcript levels of *OsDOF18* (A), *OsAMT1;1* (B), and *OsNRT2;1* (C) after ammonium or nitrate was supplied to 14 DAG seedlings grown in water. Expression levels were normalized to *OsUBQ5*. Closed circles, ammonium treatment; open circles, nitrate treatment. Error bars represent SE for at least 3 samples.
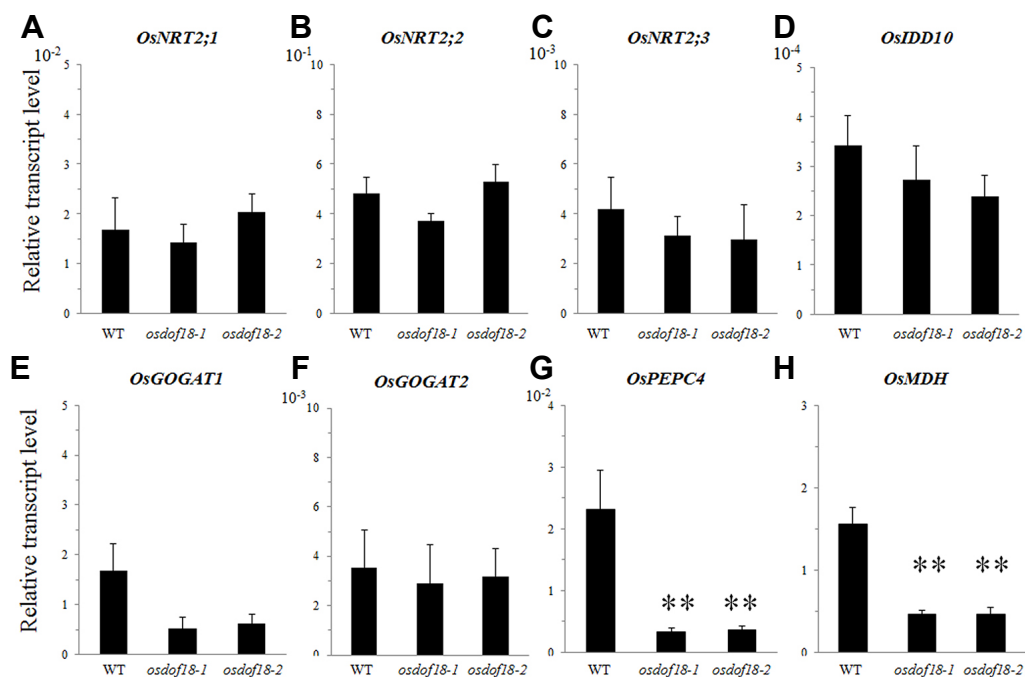

Fig. 5. Expression levels of OsAMT genes. Quantitative RT-PCR analyses of transcript levels of *OsAMT1;1* (A), *OsAMT1;2* (B), *OsAMT1;3* (C), *OsAMT2;1* (D), *OsAMT2;2* (E), *OsAMT2;3* (F), *OsAMT3;1* (G), *OsAMT3;2* (H), *OsAMT3;3* (I), and *OsAMT4;1* (J) in WT and *osdof18* mutants. RNAs were isolated from roots of 4 DAG plants grown in Yoshida medium with ammonium as sole N source. Expression levels were normalized to *OsUBQ5*. Error bars represent SE for at least 3 samples. $^{*}P < 0.05$; $^{**}P < 0.01$.

DISCUSSION

Transcription factors containing a zinc finger motif control ammonium transport

Mutations in *OsDOF18* caused growth retardation when ammonium was the sole N source. The level of ammonium uptake was significantly lower in *osdof18* mutants than in the WT. This ammonium-dependent phenotype was similar to that described for *osidd10*, which displays delayed germination and slower primary root growth when the medium is supplemented only with ammonium (Xuan et al., 2013). However, the molecular mechanisms involved in ammonium uptake are apparently different between OsIDD10 and OsDOF18. Whereas the former enhances the expression of *AMT1;2* by binding to its promoter region, the latter does not affect *AMT1;2* expression. The influence of OsIDD10 on *AMT1;1* is not clear because gene expression is moderately reduced in both *osidd10* knockout mutants and *OsIDD10*-overexpressing plants. The relationship between OsIDD10 and other AMT genes has not yet been examined. Therefore, further study is needed to determine whether *OsAMT2;2*, *OsAMT2;3*, *OsAMT3;1*, *OsAMT3;2*, and *OsAMT3;3*, which are not targets of OsDOF18, are controlled by OsIDD10. Although OsDOF18 and OsIDD10 belong to different gene families, they have zinc finger motifs in common, with the former containing one zinc finger and the latter having four

Fig. 6. Expression levels of OsNRTs, *OsIDD10, OsGOGAT1, OsGOGAT2, OsPEPC4,* and *OsMDH* genes. Quantitative RT-PCR analyses of transcript levels of *OsNRT2;1* (A), *OsNRT2;2* (B), *OsNRT2;3* (C) and *OsIDD10* (D), *OsGOGAT1* (E), *OsGOGAT2* (F), *OsPEPC4* (G), and *OsMDH* (H) in WT and *osdof18* mutants. RNAs were isolated from roots of 4 DAG plants grown in Yoshida medium with ammonium as sole N source. Expression levels were normalized to *OsUBQ5*. Error bars represent SE for at least 3 samples. $^{*}P < 0.05$; $^{**}P < 0.01$.

such motifs.

OsDOF18 expression is induced by ammonium

Transcription levels of *OsDOF18* were induced when ammonium was supplied, but were not significantly altered by nitrate supplementation. Expression of *OsAMT1;1* was also ammonium-dependent, thereby indicating that both genes are associated with ammonium transport. However, *OsDOF18* was induced in response to ammonium, peaking within the first 60 min after application whereas *OsAMT1;1* was slowly induced, reaching the highest level only after 6 h. These differential induction rates demonstrated that *OsDOF18* functions upstream of *OsAMT1;1*.

OsDOF18 regulates ammonium transporter genes

Multiple AMTs function in ammonium uptake by the roots (Kumar et al., 2003; Sonoda et al., 2003; Suenaga et al., 2003). We observed that four AMT genes were affected in *osdof18* mutants. Of these, *OsAMT1;1* and *OsAMT1;3* encode high-affinity ammonium transporters while *OsAMT2;1* and *OsAMT4;1* encode low-affinity transporters. This indicates that *OsDOF18* controls both types. A reduction in *OsAMT1;3* levels in *osdof18* was unexpected because the AMT gene is not induced but, instead, is repressed when plants are supplemented with ammonium (Suenaga et al., 2003). This may result when ammonium accumulates in the growth medium because the mutants are defective in their uptake capacity.

Heterogeneous expression of *OsDOF18* in *Arabidopsis* enhances ammonium uptake by increasing expression of *At-AMT1.1* and *AtAMT2.1*, and promotes ammonium assimilation by elevating transcription of *AtPK1, AtPK2, AtPEPC1, AtPEPC2, AtGS1.1, AtGS1.2, AtGS1.3,* and *AtGS2* (Santos et al., 2012). This indicates that functional role of *OsDOF18* is conserved between *Arabidopsis* and rice. Overexpression of *ZmDOF1*, a maize ortholog of *OsDOF18*, promotes *PEPC* and *PPDK* expression (Yanagisawa et al., 2004). Chromatin immunoprecipitation assays show enrichment of OsDOF18 on the promoter region of *OsPPDK* chromatin (Zhang et al., 2015), suggesting that OsDOF18 may also function in photosynthesis.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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