Minireview

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

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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 (NH₄⁺) and nitrate (NO₃⁻), 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 (Kyomiyi et al., 2001). Glutamine synthetase is coupled with glutamine 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., 2015). Zea mays DOF1 (ZmDOF1) enhances the C4 pathway differentiation and hormone signaling (Noguero et al., 2011). 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 NH₄NO₃, 0.3 mM NaH₂PO₄, 0.5 mM K₂SO₄, 1.0 mM CaCl₂, 1.6 mM MgSO₄, 0.075 μM (NH₄)₂MoO₄·10H₂O, 18.8 μM H₂BO₃, 9.5 μM MnCl₂, 0.16 μM CuSO₄, 0.15 μM ZnSO₄, 35.6 μM FeCl₃, and 74.4 μM citric acid, pH 5.5 (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.

**Analysis 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 OD625 and OD220, respectively (Martin-Rodriguez 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_Os01g22490). 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; Leon 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
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
OsDOF18 Controls Ammonium Uptake by Ammonium Transporters
Yunfei Wu et al.

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 NH4NO3 as sole N source; C-D; Plants were grown on Yoshida medium with 0.3 mM NH4NO3 as sole N source; E-F; Plants were grown on Yoshida medium with 5 mM NH4NO3 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 functions in ammonium uptake and assimilation
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 OsDD10 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.
OsDOF18 Controls Ammonium Uptake by Ammonium Transporters
Yunfei Wu et al.

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...
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 demonstrate 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 AtAMT1.1 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 2mDOF1, 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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