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Overexpression of Nitrate Transporter 1/Peptide Gene OsNPF7.6 Increases Rice Yield and Nitrogen Use Efficiency

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Abstract: Overuse of nitrogen fertilizer in fields has raised production costs, and caused environmental problems. Improving nitrogen use efficiency (NUE) of rice is essential for sustainable agriculture. Here we report the cloning, characterization and roles for rice of OsNPF7.6, a member of the nitrate transporter 1/peptide transporter family (NPF). The OsNPF7.6 protein is located in the plasma membrane, expressed in each tissue at all stages and is significantly regulated by nitrate in rice. Our study shows that the overexpression of OsNPF7.6 can increase the nitrate uptake rate of rice. Additionally, field experiments showed that OsNPF7.6 overexpression increased the total tiller number per plant and the grain weight per panicle, thereby improving grain yield and agronomic NUE in rice. Thus, OsNPF7.6 can be applied to be a novel target gene for breeding rice varieties with high NUE, and provide a reference for breeding higher yielding rice.

Keywords: 15NO3− influx; agronomic nitrogen use efficiency; functional analysis; OsNPF7.6; Oryza sativa

1. Introduction

Nitrogen is one of the most crucial macronutrients for crops and a sufficient nitrogen supply can ensure plant development and high yield [1–4]. Nitrogen fertilization is the key factor to improve crop yield and reduce hunger worldwide [5]. In an effort to boost productivity, farmers typically apply an excessive amount of nitrogen fertilizer, but less than 50% of it is actually absorbed by the plants, and the remnant ends up polluting the environment [6]. Improving rice (Oryza sativa L.) nitrogen use efficiency (NUE) offers an effective and practical way to increase grain yield and solve the environmental issues caused by excessive nitrogen application.

NO3− and NH4+ are two main sources of inorganic nitrogen during crop growth. Rice makes up about 25% of all calories consumed globally [7–9]. Conventionally grown rice is submerged in water, which inhibits nitrification and makes NH4+ the most significant inorganic nitrogen in rhizosphere soil. Based on this, rice is regarded as an ammonium-prefering plant [10]. On the other hand, rice can release some oxygen produced by photosynthesis to the rhizosphere through roots due to its mature aerial tissue [11]. Because oxygen promotes the growth and propagation of nitrifying bacteria, partial NH4+ in the rhizosphere can be converted to NO3− [12]. Consequently, during rice growth, its root system is under the mixed nutrition of NO3− and NH4+ [13].
Previous studies have established that the prominent families of transporters involved in nitrate-nitrogen uptake and transport in plants include nitrate transporter1/peptide transporter family (NPF) and nitrate transporter 2 (NRT2). The NRT2 family encodes high-affinity \( \text{NO}_3^- \) transport proteins, which have essential functions in response to low-nitrogen (\( \text{NO}_3^- \)) environments [14]. Among the members of NRT2s, OsNRT2.1, OsNRT2.2 and OsNRT2.3a require the assistance of OsNAR2.1 to complete the transport of nitrate [15]. The interaction of OsNAR2.1 with OsNIT1 and OsNIT2, affects the uptake of nitrate and ammonium by rice roots [16]. OsNRT2.3 yields two transcripts including OsNRT2.3a and OsNRT2.3b [17]. OsNRT2.3a plays a role in the long-distance transport of nitrate from roots to aerial parts [18], while OsNRT2.3b can elevate the pH buffering ability of rice [19]. Compared with all other NRT2 genes, OsNRT2.4 is a dual-affinity nitrate transporter, which is necessary for nitrate-regulation of root and shoot growth of rice [20].

The NPF family includes two genes, NRT1 (nitrate transporter1) and PTR (peptide transporter). The former is generally believed to encode the low-affinity \( \text{NO}_3^- \) transporter, and the latter encodes the oligopeptide transporter [21]. Due to the high sequence similarity of the two members, in the same branch of evolutionary relationship, the NRT1/PTR gene is uniformly named NPF (NRT1/PTR family) [22]. There are many members of the NPF family, and greater focus and thorough investigation have been given to the NPF genes’ roles. Numerous studies have demonstrated that NPF genes are widely implicated in the uptake and utilization of nitrogen in plants and have essential roles and applications in improving nitrogen utilization and enhancing yield-related traits [23–28]. With the use of the homologous sequence of Arabidopsis AtNRT1.1, OsNRT1.1 has been cloned in rice [29]. Two varying splicing forms including OsNRT1.1a and OsNRT1.1b are shown in OsNRT1.1. OsNRT1.1a only acts under high-nitrogen situations, while OsNRT1.1b is found to facilitate nitrogen uptake under low-nitrogen situations [30]. Moreover, overexpression of OsNRT1.1A could increase NUE [25]. A single-base variation of NRT1.1B explains the difference in NUE between indica and japonica subspecies [26]. The NADH/NADPH-dependent nitrate reductase gene OsNR2 can interact with OsNRT1.1b, and promote the uptake of nitrate in indica rice [31]. OsNPF6.1 serves as a dual-affinity nitrate transporter, the transcription factor OsNAC42 could activate OsNPF6.1, which can thus elevate rice NUE [32].

Here, we report that the nitrate transporter OsNPF7.6 is induced by nitrate, and overexpression of OsNPF7.6 increases nitrate uptake rate, yield, as well as NUE in rice.

2. Materials and Methods

2.1. The Construction of pUbi: OsNPF7.6 Transgenic Rice

The ORF of OsNPF7.6 (LOC_Os04g50930) was amplified from the full-length cDNA of rice japonica cv. ‘Nipponbare’, primer pairs were shown in Supplementary Table S1. PrimeSTAR HS DNA Polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China) was employed during the polymerase chain reaction (PCR). The parameters for PCR were 95 °C for 5 min, 94 °C for 30 s, 56 °C for 1 min (30 cycles), and 72 °C for 10 min. The PCR products were then ligated into the pMD19-T vector independently. The correct sequence fragment was ligated to the expression vector pTCK303 to obtain the pUbi:OsNPF7.6 fusion vector. According to the previous description, the vector was introduced into the Agrobacterium tumefaciens strain, EHA105 via electroporation and subsequently transformed into Zhonghua 11 rice (WT) [3].

2.2. Identification of Positive Seedlings of Transgenic Lines

Through the application of the CATB method, the genomic DNA of T2 generation transgenic and wild-type plants was extracted [3] for PCR amplification of hygromycin fragments to identify positive seedlings, with primer pairs shown in Supplementary Table S2. The parameters for PCR were 95 °C for 3 min, 95 °C for 30 s, 55 °C for 30 s (30 cycles), and 72 °C for 10 min. Gel electrophoresis was used to show PCR products.
2.3. qRT-PCR

Following the previously reported method, plant genomic RNA extraction and gene expression analysis was conducted [3]. The primers for qRT-PCR were demonstrated in Table S3.

2.4. Subcellular Localization of OsNPF7.6

The coding sequence of OsNPF7.6 for subcellular localization was amplified using the pMD19-T vector and ligated into pSAT6A-GFP [33] with the correct direction. The OsNPF7.6-GFP vector was transformed into Arabidopsis mesophyll protoplasts and identified by a laser scanning microscope (LSM410; Carl Zeiss, Jena, Germany). Plasma membrane dye FMTM4-64FX (Invitrogen, Life Technologies, Carlsbad, CA, USA) was used as marker.

2.5. Plant Growth Conditions

The pUbi:OsNPF7.6 transgenic plants and WT were planted in three plots, which had 180 kg N/ha. The plot size was 2 m × 2.5 m, and seedlings were planted in a 10 × 10 array. During the stage of flowering and maturation, the samples of T3 generation were collected for further analysis.

The total nitrogen was analyzed as stated by Chen et al. [3]. According to our previously reported method, total nitrogen accumulation at the anthesis stage, total nitrogen accumulation at maturity stage, grain nitrogen accumulation at maturity, nitrogen translocation, nitrogen translocation efficiency, post-anthesis nitrogen uptake, nitrogen harvest index, as well as agronomic NUE were calculated [3]. Briefly, the calculation formulae are as follows. Nitrogen translocation (g/m2) = total nitrogen accumulation at anthesis − (total nitrogen accumulation at maturity − grain nitrogen accumulation at maturity); nitrogen translocation efficiency (%) = (nitrogen translocation/total nitrogen accumulation at anthesis) × 100%; post-anthesis nitrogen uptake (g/m2) = total nitrogen accumulation at maturity − total nitrogen accumulation at anthesis; the nitrogen harvest index (%) = grain nitrogen accumulation at maturity/total nitrogen accumulation at maturity. In addition, agronomic NUE (g/g) = grain yield/N supply.

2.6. Determination of 15N Influx Rates in Roots

Rice seedlings were planted in 1 mM NH4+ within a period of 3 weeks and subsequently subject to nitrogen starvation for 3 days for the root 15N uptake experiment. Initially, the plants were rinsed with 0.1 mM CaSO4 for 1 min, which were later transferred to either 0.5 mM 15NO3− or 2 mM 15NO3− (atom % 15N: 99%) solution for 5 min. Then rinsed the plants once more with 0.1 mM CaSO4 for 1 min. Based on our previously reported method, 15N influx rates were calculated [3].

2.7. Statistical Analysis

In order to perform statistical analysis, we used the single factor analysis of variance (ANOVA) and Tukey’s test in this study. The IBM SPSS Statistics 20 software (SPSS Inc., Chicago, IL, USA) was adopted for all statistical analysis.

3. Results

3.1. OsNPF7.6 Was Induced to Be Expressed by Nitrate and Localizes in the Plasma Membrane

The OsNPF7.6 was localized on chromosome 4 with six exons and five introns (Figure S1). Under different nitrogen source treatments, the OsNPF7.6 had the highest expression under 2.5 mM NO3− conditions, followed by 0.5 mM NO3− conditions, and the weakest expression under 2.5 mM NH4+ conditions (Figure 1A). The expression pattern of different sites at different fertility stages revealed that OsNPF7.6 was constitutively expressed in rice at all sites at all times (Figure 1B). OsNPF7.6 had 10 transmembrane structures (Figure S2). In order to detect the subcellular localization of the OsNPF7.6 protein, an expression vector containing the OsNPF7.6-GFP fusion gene initiated by cauliflower mosaic virus 35S promoter was
constructed and transformed into *Arabidopsis thaliana* protoplasts. After overnight transformation, the expression of OsNPF7.6-GFP in protoplasts was observed by confocal microscopy. OsNPF7.6-GFP fluorescence was completely merged with plasma membrane dye FM^4-64FX* (Figure 1C), and OsNPF7.6 was identified as a plasma membrane localization protein.

![Figure 1. Expression pattern and subcellular localization of OsNPF7.6. (A) OsNPF7.6 expression levels in roots of rice (cv. Nipponbare) under varying nitrogen supplies. The seedlings were grown in IRRI nutrient solution including 1 mM NH_4^+ for a period of 2 weeks, later moved to N-free solution for 3 d and then resupplied with NH_4^+ or NO_3^- solution for 24 h. LN, 0.5 mmol/L NO_3^-; HN, 2.5 mmol/L NO_3^-; LA, 0.5 mmol/L NH_4^+; HA, 2.5 mmol/L NH_4^+. Values indicated ± SE (n = 3). The varying letters (a, b, c, d) suggest an obvious change under varying nitrogen supplies. (p < 0.05, one-way ANOVA) (B) Expression levels of OsNPF7.6 in different organs at varying growth stages. Samples were taken from rice (cv. Nipponbare) planted in a paddy field. Values mean ± SE (n = 3). (C) OsNPF7.6::GFP vector was transformed into Arabidopsis mesophyll protoplasts and detected by laser scanning microscope (LSM410; Carl Zeiss, Germany). Scale bars = 10 µm.

3.2. Acquisition of OsNPF7.6 Overexpression Lines

The pUBi: OsNPF7.6 fusion vector was introduced into *Agrobacterium tumefaciens* strain EHA105 via electroporation and subsequently transformed into Zhonghua 11. A total of 72 transgenic seedlings from 10 strains were available, and 32 transgenic seedlings from eight strains survived after 7 days of screening with 200 mg/L hygromycin solution. The DNA of these 32 strains was extracted, the hygromycin fragments were amplified by PCR, and the target bands were detectable in all 32 transgenic seedlings. The positive rate of transgenic seedlings was 44.4%.

Three transgenic strains were selected for T2 generation (Figure 2A), and then positive seedlings were verified by PCR amplification of hygromycin fragments (Figure 2B). qRT-PCR revealed that the expression level of OsNPF7.6 increased about 7-fold in the shoot and root of transgenic lines relative to WT (Figure 2C).
3.3. Expression of OsNPF7.6 Increased $^{15}$NO$_3^-$ Influx Rates in Rice

To evaluate the effect of pUbi:OsNPF7.6 expression on root NO$_3^-$ influx into intact plants, seedlings of transgenic lines and WT were exposed to 0.5 mM $^{15}$NO$_3^-$ or 2.5 mM $^{15}$NO$_3^-$ for 5 min for short-term NO$_3^-$ and NH$_4^+$ absorption. The influx rates of $^{15}$NO$_3^-$ of pUbi:OsNPF7.6 transgenic lines increased by 15.4% and 18.3%, respectively, compared with WT under 0.5 mM $^{15}$NO$_3^-$ and 2.5 mM $^{15}$NO$_3^-$ supply (Figure 3A,B).

![Figure 2. Identification of transgenic lines. (A) Phenotype of WT and pUbi:OsNPF7.6 transgenic plants (OE1, OE2 and OE3). (B) The hygromycin fragment was amplified from the genomic DNA of T2 generation transgenic plants and WT. M represents a 2000 base pair (bp) DNA ladder. (C) qRT-PCR analysis the expression of OsNPF7.6. RNA was extracted from shoot and root. Values indicated ± SE (n = 3). The varying letters (a, b, c, d) suggest an obvious change between the transgenic line and the WT. (p < 0.05, one-way ANOVA).](image)

![Figure 3. $^{15}$NO$_3^-$ influx rates were measured with the use of $^{15}$N–enriched sources. WT and transgenic seedlings were grown in 1 mM NH$_4^+$ for 3 weeks and then were treated with nitrogen starvation for 3 days. $^{15}$N influx rates were measured at (A) 0.5 mM $^{15}$NO$_3^-$ or (B) 2.5 mM NO$_3^-$ for 5 min. DW, dry weight. Error bars: SE (n = 4). The varying letters (a, b) suggest an obvious change between the transgenic line and the WT. (p < 0.05, one-way ANOVA).](image)
3.4. Effects on Agronomic Traits of Rice after Overexpression of OsNPF7.6

In order to analyze the effects of OsNPF7.6 overexpression on rice growth and yield, the current work attempted to investigate the agronomic traits of the transgenic strains. The plant height, seed setting rate, and 1000-grain weight of the OsNPF7.6 overexpression strains were not substantially different from WT ($p > 0.05$, Figure 4A,D,F). Compared to WT, the total tiller number per plant, grain weight per panicle, as well as grain number per panicle of OsNPF7.6 overexpression strains increased by 14.8%, 13.3%, and 10.9%, respectively (Figure 4B,C,E). Ultimately, the OsNPF7.6 overexpression strains improved grain production and dry weight by 21.0% and 21.7%, respectively (Figure 4G,H).

![Figure 4. Comparison of agronomic traits between transgenic lines and WT. (A) Plant height, (B) Total tiller number per plant, (C) Grain weight per panicle, (D) Seed setting rate, (E) Grain number per panicle, (F) 1000-grain weight, (G) Grain yield, (H) Dry weight. Statistical analysis of data from T3 generation, $n = 3$. The varying letters (a, b) suggest an obvious change between the transgenic line and the WT. ($p < 0.05$, one-way ANOVA).](image)

3.5. Effects on Nitrogen Utilization in Rice after Expression of OsNPF7.6

The dry matter production in plants per unit of applied nitrogen is also known as “nutrient utilization efficiency” and refers to the transfer of nitrogen to plants organs and yield [5]. In this study, we tested the total nitrogen content of the T3 generation transgenic lines during the anthesis and maturity stages. At the anthesis stage, total nitrogen accumulated mostly in the leaf and culm. Compared with WT, we found that at the anthesis stage, the total nitrogen content of panicles, leaves, and culms in transgenic lines increased by 16.9%, 13.2%, and 16.3%, respectively (Figure 5A). With the reproductive growth, the proportion of nitrogen content in spike to total nitrogen content of plant begins to increase. The results showed that the total nitrogen content of panicles, leaves, and culms in transgenic lines elevated by 18.7%, 21.1%, and 24.1%, separately at maturity stage (Figure 5B).

For the NUE, no obvious difference in nitrogen translocation efficiency between transgenic lines and WT was found (Figure 5C). Compared with WT, the post-anthesis nitrogen uptake of transgenic lines increased by about 59.9% (Figure 5D). There was no evident divergence in the nitrogen harvest index between transgenic lines and WT (Figure 6A). The agronomic NUE of transgenic lines elevated by about 20.9% (Figure 6B).
Rice contains 93 NPF family members [22]. As the necessary macronutrient for plant growth, nitrogen is an important factor affecting the productivity of rice. We overexpressed OsNPF7.6 and OsNPF7.7 in rice through the pUbi:OsNPF gene expression cassette and constructed three transgenic lines (OE1, OE2, OE3). The variation in nitrogen content in different parts of the transgenic lines is shown in Figure 5.

Figure 5. Total nitrogen content in different parts of transgenic lines at the stage of anthesis and maturity. (A) Anthesis stage, (B) Maturity stage. Comparison of (C) nitrogen translocation efficiency and (D) post-anthesis nitrogen uptake between WT and transgenic lines. Statistical analysis of data from T3 generation, n = 3. The varying letters (a, b) suggest an obvious change between the transgenic line and the WT. (p < 0.05, one-way ANOVA).

The agronomic nitrogen use efficiency (NUE) of transgenic lines can be compared with the WT. As shown in Figure 6, the NUE of transgenic lines was significantly higher than that of the WT. (p < 0.05, one-way ANOVA).

Figure 6. Comparison of NUE between WT and transgenic lines. (A) Nitrogen harvest index, (B) Agronomic nitrogen use efficiency. Statistical analysis of data from T3 generation, n = 3. The varying letters (a, b) suggest an obvious change between the transgenic line and the WT. (p < 0.05, one-way ANOVA).
4. Discussion

Rice contains 93 NPF family members [22]. As the necessary macronutrient for plant growth and development, nitrogen can limit crop productivity when it is scarce in the field [5]. Increasing the uptake and accumulation of nitrogen is the main way to improve the NUE and yield of rice.

Previous research has shown that OsNPF2.4 [23], OsNPF5.16 [24], OsNPF6.1 [24], OsNPF6.3 [25], OsNPF6.5 (OsNRT1.1b) [26], OsNPF7.1 [27], OsNPF7.2 [28], OsNPF7.4 [24,27], and OsNPF7.7 [34] are involved in the NO$_3^-$ uptake process. OsNPF4.5 exerts an important function in the acquisition of nitrate by the arbuscular mycorrhizal symbiosis pathway [25]. The OsNRT1.1b (OsNPF6.5) indica variant improves nitrate uptake and could elevate the NUE [26]. OsNPF7.2 is expressed mostly in thick-walled cells of the root elongation and maturation zones, the cortex, and the mid-column. It is involved in NO$_3^-$ partitioning in different sections of the root [28]. OsNPF7.7 contains two variable splices: OsNPF7.7-1 (encoding the longer product) and OsNPF7.7-2 (encoding the shorter product). OsNPF7.7 is located in the cell membrane (OsNPF7.7-1) and vesicle membrane (OsNPF7.7-2), with higher expression levels boosting the uptake of NO$_3^-$ and NH$_4^+$, respectively (Hu et al., 2018). Nitrate promoted the expression of OsNPF7.6 in rice roots (Figure 1A), and subcellular localization demonstrated that OsNPF7.6 was located in the plasma membrane (Figure 1C). Under the supply of 0.5 mM $^{15}$NO$_3^-$ and 2.5 mM $^{15}$NO$_3^-$, the influx rates of $^{15}$NO$_3^-$ of pUbi:OsNPF7.6 transgenic lines increased by 15.4% and 18.3%, respectively, compared with WT (Figure 3A,B). These results illustrate that OsNPF7.6, a member of the NFP7 subfamily, is also involved in nitrate uptake by the rice root system.

Nitrogen is crucial for crop growth and development, notably for the creation of tillers, which belongs to one of the most crucial elements determining grain yield in rice [25]. OsNPF7.1, OsNPF7.2, OsNPF7.3, OsNPF7.4, and OsNPF7.7 are all members of the NFP7 subfamily that contribute to the development of rice tiller buds, which in turn influences rice tillering. OsNPF7.1 and OsNPF7.4 are two of them that, in response to different nitrogen supply levels, exhibit opposing expression patterns in tiller buds and have enhanced and inhibited effects on tiller bud growth and development, respectively [27]. OsNPF7.2 could control tiller bud growth and root development through the regulation of cytokinin and cell cycle in plants [25,35,36]. OsNPF7.3 overexpression promotes growth, tiller number, grain yield, and grain nitrogen content [37,38]. We also found that overexpression of OsNPF7.6 increased the total tiller number per plant in rice (Figure 4B). Compared with WT, the grain weight and grain number of per panicle increased by 13.3% and 10.9%, respectively, in the OsNPF7.6 overexpression strains (Figure 4C,E). Consequently, the grain yield and dry weight of the OsNPF7.6 overexpression strains increased by 21.0% and 21.7%, respectively (Figure 4G,H).

A series of studies have shown that nitrate is necessary for nitrogen nutrition in rice. It is found that nitrate allocation and the divergent nitrate use efficiency between indica and japonica rice can be mediated by the nitrate transporter OsNPF7.9 [39]. OsNPF6.1HapB is the rare natural allele, which can control nitrate uptake and NUE [32]. OsNRT1.1A/OsNPF6.3 exerts the vital function in nitrogen uptake, transport, and assimilation [25]. In addition, the overexpression of OsNAR2.1 is shown to improve rice yield and NUE [2]. Following the introgression of both the indica OsNRT1.1B and OsNRT2 alleles into japonica accessions, the grain yield and NUE could be deeply enhanced [31]. We also found that overexpression of OsNPF7.6 can increase the agronomic NUE in rice (Figure 6B). Although the nitrogen accumulation of OsNPF7.6 overexpression lines increased significantly (Figure 5A,B), there existed no obvious difference in nitrogen translocation efficiency and nitrogen harvest index between transgenic lines and WT (Figures 5C and 6A). Thus, the overexpression of OsNPF7.6 does not affect nitrogen distribution in rice.

In conclusion, OsNPF7.6 is a nitrate transporter located in the plasma membrane. Overexpression of OsNPF7.6 can increase the nitrate uptake rate of rice. Field experiments showed that overexpression of OsNPF7.6 elevated the total tiller number per plant and
the grain weight per panicle, which could thus enhance grain yield and agronomic NUE in rice.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3909/life2121981/s1, Table S1. Primers were used to amplify the OsNPF7.6 open reading frame, Table S2. Primers were used to amplify the hygromycin fragment, Table S3. Primers were used to detect the expression of OsActin, OsNPF7.6, Figure S1. Analysis of OsNPF7.6 gene structure. Checked through the Rice Genome Annotation Project (http://rice.uga.edu/index.shtml (accessed on 10 August 2022)), Figure S2. Analysis of transmembrane domains of OsNPF7.6. Predicted by TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (accessed on 10 August 2022).

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