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Response of nitrate transporters and PM H^+-ATPase expression to nitrogen flush on two upland rice varieties contrasting in nitrate uptake kinetics

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Abstract

The aim of this study was to evaluate the behavior of high-affinity nitrate (NO_3^-) transporters and plasma membrane H^+-ATPase isoforms in rice varieties, contrasting in NO_3^- uptake kinetics parameters. Two varieties of rice were studied: cv. IAC-47 (improved for high N input) and cv. Piauí (a local landrace variety cultivated for low input farmers). We evaluated the expression of two PM H^+-ATPase isoforms *(OsA2* and *OsA7*), two NO_3^- transporters (*OsNRT2.1* and *OsNRT2.2*), and *OsNAR2.1*, the rice regulatory genes for nitrate transport. The results showed that the Piauí variety has higher expression of *OsNRT2.1-2.2*, *OsNAR2.1* and *OsA2* and *7*, compared to IAC-47 variety, mainly after 24h under low N condition. After 24h of 0.2 mM NO_3^--N resupply, the Piauí variety showed a net nitrate uptake and PM-H^+-ATPase activity, 71% and 47% higher than IAC-47, respectively. This behavior of Piauí variety may be a strategy for better harnessing of available N that occurs in the tropical environment in a short period of time, after the onset of the rainy season. Changes in the expression levels of *OsNRT2.1-2.2/OsNAR2.1* and *OsA2* and *7* occur synchronously over time. Our results suggest the synchronized behavior of high affinity nitrate transporters and PM-H^+-ATPases under N flush conditions.

Keywords: *Oryza sativa*; Proton pumps; nitrogen; nitrate transporters; nitrate uptake.
Abbreviations: ATP_adenosine triphosphate; BTP_bis-tris-propane; cDNA_complementary DNA; CO_2_carbon dioxide; DTT_dithiothreitol; EDTA_ethylenediamine tetraacetic acid; EGTA_ethylene glycol tetraacetic acid; H_2SO_4_sulfuric acid; HATS_high affinity transport system; KCl_potassium chloride; K_M_Michaelis-Menten constant; KNO_3_potassium nitrate; LATS_low affinity transport system; $MgCl_2$_magnesium chloride; $MgSO_4$_magnesium sulfate; MOPS_4-morpholinepropanesulfonic acid; $(NH_4)_2MoO_4$_ammonium molybdate; N_2O_nitrous oxide; Na_2MoO_4_sodium molybdate; NaCl_sodium chloride; NaN_3_sodium azide; NAR_nitrate assimilated related; NH_4^+_ammonium; NO_3^-_nitrate; NRT_nitrate transporters; NTES_NaCl/Tris/EDTA/SDS buffer; OsA_*Oryza sativa* H^+-ATPase; Pi_phosphate; PM_plasma membrane; PMSF_phenylmethylsulfonyl fluoride; PVP_Polyvinylpyrrolidone; RNAi_RNA interference; SDS_sodium dodecyl sulfate; TRIS_Tris(hydroxymethyl)aminomethane; V_{max}_maximum influx;

Introduction

Nitrogen (N) is one of the elements that essentially required for plant growth. A shortage in its supply can limit the productivity of crops. Nitrogen fertilization is a common practice that can cause serious environmental damage when performed in a non-sustainable way, mainly because of nitrate (NO_3^-) leaching and subsequent contamination and/or eutrophication of water bodies and watercourses. Emission of nitrous oxide (N_2O) is another potential negative consequence of excessive use of nitrogen fertilizers, because this gas is a potent inducer of global warming and is often more harmful than CO_2. The NO_3^- uptake efficiency by plants may play a key role in environmental sustainability and in improving the economic aspect with regard to use of nitrogen fertilizers (Kant et al., 2011). Rice cultivars with the ability to uptake and assimilate N more efficiently could reduce the need for excessive use of fertilizers without affecting plant productivity (Souza et al., 1998). It is essential to understand the physiological and molecular basis of plant responses to different amounts of N, especially under limiting conditions (Good et al., 2004; Kant et al., 2011). Some rice plants were found to be tolerant to long periods of nitrogen deprivation in studies using local varieties of upland rice (landraces) from the State of Maranhão-Brazil (humid tropics) (Rodrigues et al., 2004; Santos et al., 2007). The Piauí variety stands out among these rice varieties; some previously published results indicated that this variety has developed biochemical and

molecular mechanisms directed toward more efficient use of N, likely because of long years of cultivation in low-fertility soils (Souza et al., 1998). Kinetic studies of NO_3^--N uptake indicate that the Piauí variety presents higher V_{max} values and smaller K_M values when cultivated with low levels of N compared with the improved rice variety IAC-47 (Santos et al., 2011).

Rice is one of most consumed cereals in the world. Since its genome has been fully sequenced, it has been used for studying various physiological phenomena, including those related to N uptake, at the molecular level (Sonoda et al., 2003; Araki and Hasegawa, 2006; Bi et al., 2009; Feng et al., 2011; Yan et al., 2011; Xu et al., 2012). Nitrate is absorbed from the soil by the plant cells via specific transporters located in the plasma membrane (PM). These transporters can possess high affinity (HATS: "high-affinity transport system") and operate at low NO_3^- concentrations or low affinity (LATS: "low-affinity transport system") and operate at elevated NO_3^- concentrations (Aslam et al., 1993). The high- and low-affinity transporters are encoded by the *NRT2* and *NRT1* gene families, respectively. Four high-affinity NO_3^- transporters (OsNRT2.1~2.4) and two NAR proteins (OsNAR2.1-2.2) have been identified in rice. The *OsNRT2.1*, *OsNRT2.2*, and *OsNAR2.1* transporters, induced by NO_3^-, are potential candidates for use in breeding programs aimed at increasing NO_3^- uptake efficiency in rice (Araki and Hasegawa, 2006). *OsNRT2.1*, *OsNRT2.2*, *OsNRT2.3* and *OsNAR2.1* are mainly expressed in the rice root, and *OsNAR2.1* is essential for NO_3^- uptake by *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a* (Feng et al., 2011). OsNRT2.3a also requires OsNAR2.1 for nitrate transport, but has a 10-fold lower affinity for nitrate than OsNRT2.1 and OsNRT2.2 (Yan et al., 2011). In rice, it has been shown that over-expression of *OsNRT2.1* gene alone did not increase nitrate uptake (Katayama et al., 2009). Therefore, Yan el al. (2011) provided an explanation for this data and showed that the over-expression of *OsNRT2.1*, without *OsNAR2.1*, is unlikely to increase nitrate uptake in rice. Thus, NO_3^- uptake is highly depends either to the interaction of OsNRT2.1, OsNRT2.2 and OsNRT2.3a with OsNAR2.1 (Yan et al., 2011) or PM H^+-ATPase activity (Santi et al., 2003). The PM H^+-ATPase plays a fundamental role in the uptake of nutrients because its activity maintains the membrane potential ($\Delta\psi$) and generates the proton-motive force (Δp) required for many ions transport (Sondergaard et al., 2004). Uptake of NO_3^- occurs via symport with two hydrogen ions (H^+) (Glass et al., 1992). Therefore, the PM H^+-ATPases are fundamental in this process (Santi et al., 1995). The PM H^+-ATPases are encoded by a gene family with different regulatory properties (Baxter et al., 2003). Some of the characterized isoforms are involved in the uptake of nutrients (Quaggiotti et al., 2003; Santi et al., 2003; Santi et al., 2009, Sperandio et al., 2011). These proteins are divided into five subfamilies; the members of subfamilies I and II are expressed at high levels, while those of subfamilies, III, IV, and V are expressed at low levels or in specific tissues (Arango et al., 2003). In maize, PM H^+-ATPase isoforms *MHA3* and *MHA4* (subfamily II) showed enhanced expression after NO_3^- supply (Sorgonà et al., 2011). Sperandio et al. (2011) observed that, among the 10 isoforms of PM H^+-ATPase in rice, *OsA2* and *OsA7* were the most induced by the NO_3^--N resupply and can be involved in its uptake efficiency. The *OsA2* isoform belongs to subfamily I, while *OsA7* belongs to subfamily II, and both are expressed at high levels. The use of rice varieties that naturally exhibit adaptation under low NO_3^- levels can be an alternative to evaluate the mechanisms involved in NO_3^- uptake efficiency in rice (Hirel et al., 2007). In this study, rice plants with contrasting NO_3^- uptake kinetics were evaluated. The Piauí variety is adapted to naturally low-fertility soils and the seasonal flow of N that is common in the humid tropics. The IAC-47 variety was developed for cultivation in soil receiving a significant contribution of nutrients via the addition of fertilizers (Souza et al., 1998; Santos et al., 2011). Since NO_3^- uptake depends on the H^+ ($\Delta\mu H^+$) gradient generated by the PM H^+-ATPases, it is reasonable to propose that there is a synchronism in the activities of the NO_3^- transporters and PM H^+-ATPases, in particular, at low levels of NO_3^--N.

Using varieties contrasting in N uptake kinetics, the aim of the present study was to evaluate the expression of the *OsA2* and *OsA7* PM H^+-ATPase isoforms and critical high-affinity NO_3^- transporters (*OsNRT2.1-2.2* and *OsNAR2.1*) in rice under distinct regimens of NO_3^- nutrition. Correlations were established among *OsA2*, *OsA7*, *OsNRT2.1*, *OsNRT2.2*, and *OsNAR2.1* expression levels. Net nitrate uptake, nitrate content and PM-H^+-ATPase activity were also performed.

Results

Expression of the OsA2 and OsA7 isoforms and level of PM H^+-ATPase activity in the roots of the IAC-47 and Piauí rice varieties

At a constant supply of 2.0 mM NO_3^--N, the Piauí variety at 9 h displayed higher *OsA2* and *OsA7* expression in the roots, compared to IAC-47 (3- and 5-times greater, respectively) (Fig. 1a). The expression of *OsA2* and *OsA7* increased 3-fold at 24 h in both varieties when treated with a constant supply of 2.0 mM NO_3^--N (Fig. 1a). No difference was observed in the expression of *OsA2* and *OsA7* in roots of both varieties under N-deficient conditions (Fig. 1b). Three hours after resupply with 0.2 mM NO_3^--N, 10-fold and 5-fold increases were observed in *OsA2* expression in the Piauí variety and IAC-47, respectively. Furthermore, *OsA7* expression was induced 7-fold in the Piauí and 3-fold in the IAC-47 compared to the groups receiving a constant supply of 2.0 mM NO_3^--N (Fig. 1 c). At 9 h and 24 h, the varieties showed more striking differences with regard to PM H^+-ATPase expression. At 24 h, the Piauí variety showed an expression of 4-fold and 2-fold higher in *OsA2* and *OsA7* genes, respectively, compared to IAC-47 variety (Fig. 1c). In both varieties, a 8-fold increase in *OsA2* expression was observed 3 h after resupplying with 5.0 mM NO_3^--N, followed by a decrease in expression at 6 h, 9 h, and 24 h (Fig. 1 d). Despite the drop in expression levels at 24 h, the Piauí variety presented an *OsA2* expression level, 4-times higher than IAC-47 (Fig. 1d).

Table 1. Net nitrate uptake and PM H^+-ATPase activity 24 h after treatment addition.

	Net NO_3^- uptake µmol NO_3^--N g^{-1} root FW h^{-1}			PM H^+-ATPase activity µmol Pi mg^{-1} ptn h^{-1}		
	IAC-47	Piauí	Rate of change (%)	IAC-47	Piauí	Rate of change (%)
Constant	15.57 Ba*	15.99 Ba	+ 2.70	13.70 Ca	16.11 Ca	+ 8.59
Starvation	ns	ns		23.66 Aa	22.25 Ba	- 5.95
R0,2	1.60 Cb	2.75 Ca	+ 71.87	17.69 Bb	26.14 Aa	+ 47.76
R5,0	23.14 Ab	27.34 Aa	+ 18.15	21.28 Aa	20.72 Ba	- 2.63

* averages followed by the same uppercase letter in the columns did not differ significantly ($p < 0.05$), and averages followed by the same lowercase letter in the line did not differ significantly ($p < 0.05$).

In the Piauí variety, the resupply of 5.0 mM NO_3^--N led to a 7-fold increase in *OsA7* gene expression at 3 h, compared to IAC-47 (Fig. 1d). At 6 h, 9 h, and 24 h, a decrease in *OsA7* expression was observed in both varieties, similar to what occurred with the *OsA2* gene (Fig. 1d). At a constant supply of 2.0 mM NO_3^--N, the PM-H^+-ATPase activity was lower than at starvation or nitrate resupply (Table 1).

The Piauí variety showed an increased NRT2 and OsA expression, accompanied by higher net nitrate uptake and PM-H^+-ATPase activity at 24h after 0.2 mM NO_3^--N nitrate resupply, compared to IAC-47 (Fig.1c, Fig.2c and Table 1).

Expression of the NO_3^- transporters (OsNRT2.1 and OsNRT2.2) and OsNAR2.1

Plants with a constant supply of 2.0 mM NO_3^--N showed low expression of *OsNRT2.1*, *OsNRT2.2*, and *OsNAR2.1* at 3 h and 6 h. However, an increase in expression was observed at 24 h for both varieties (Fig. 2a). With the lower expression levels, the Piauí variety showed higher *OsNRT2.1* and *OsNAR2.1* at 9 h with continuous 2.0 mM NO_3^--N treatment (Fig. 2a). The expression of *OsNRT2.1*, *OsNRT2.2*, and *OsNAR2.1* in plants under NO_3^-N-deficient conditions was low, and varied throughout the day (Fig. 2b).

At 3 h, the expression of *OsNRT2.1* and *OsNRT2.2* with a resupply of 0.2 mM NO_3^--N was 78-fold in the Piauí variety and 50-fold in the IAC-47 variety (Fig. 2c). After the high expression levels at 3h, there was a decrease of *OsNRT2.1-2.2* and *OsNAR2.1* expression at 6 h and 9 h in both the varieties, without differences between varieties (Fig. 2c).

At 24 h, the Piauí variety showed increased expression of *OsNRT2.1* and *OsNRT2*.2 by 125-fold and 185-fold, respectively, while the IAC-47 variety presented only 21-fold and 11-fold increases in the expression of *OsNRT2.1* and *OsNRT2.2*, respectively (Fig. 2c). The expression pattern for *OsNAR2.1* was similar to that of *OsNRT2.1* and *OsNRT2.2* with the 0.2 mM NO_3^--N resupply, with higher expression levels at 3 h and 24 h in the Piauí variety compared to IAC-47 (Fig. 2c).

Three hours after resupply of 5.0 mM NO_3^--N, expression of *OsNRT2.1* showed a 48-fold increase in the IAC-47 variety and a 30-fold increase in the Piauí variety, while *OsNRT2.2* expression displayed a 13-fold increase in both varieties (Fig. 2d). Contrary to observations with the 0.2 mM NO_3^--N resupply, there was no increase in the expression levels of *OsNRT2.1-2.2/OsNAR2.1* after 24 h of resupply with 5.0 mM NO_3^-N, compared to 6 h and 9 h (Fig. 2d). Correlation coefficients of 0.84 to 0.94 were found between the expression values of *OsNRT2.1*, *OsNRT2.2*, *OsNAR2.1*, *OsA2*, and *OsA7* in both varieties, which suggested a joint and synchronized involvement of NO_3^- transporters and PM-H^+-ATPase in NO_3^- uptake (Table 2).

Net NO_3^- uptake and NO_3^--N content in tissue from the IAC-47 and Piauí rice varieties

Resupply with 5.0 mM of NO_3^--N resulted in higher net NO_3^- uptake, and the Piauí variety showed higher net NO_3^- uptake relative to IAC-47 at 24 h after treatment (Table 1). Following resupply with 0.2 mM of NO_3^--N, the Piauí variety also showed higher net NO_3^- uptake than IAC-47 (Table 1). On the other hand, under constant supply with 2.0 mM NO_3^- N, there was little difference in net NO_3^- uptake between the IAC-47 and Piauí varieties. The Piauí variety showed increased levels of NO_3^--N in the tissues, especially in the plants treated with a resupply of 5.0 mM NO_3^--N (Fig. 3).

Plants from the Piauí variety, cultivated constantly with 2.0 mM NO_3^-N, accumulated more NO_3^--N in the sheaths, compared with IAC-47 variety (Fig. 3a). There were no differences in root or shoot weight between varieties under constant supply of 2.0mM NO_3^--N (Table 3). Deficiency in NO_3^--N for 3 days led to the consumption of the NO_3^- accumulated in the roots, while in the leaves, the contents were still above 25 µmoles NO_3^--N g·FW^{-1} (Fig. 3b). Despite of increase in root nitrate reductase activity at 6 and 9h (Fig. S2), the Piauí variety showed the highest NO_3^--N content in all examined tissues with the resupply of 0.2 mM NO_3^--N (Fig. 3c) and no differences were observed in root or shoot weight in this treatment (Table 3). The Piauí variety showed higher levels of NO_3^--N with the resupply of 5.0 mM NO_3^--N at 24 h in all examined tissues (Fig. 3d).

Starvation and resupply with high N level resulted in lower Piauí plants weight (Table 3). Thus, the higher nitrate content was observed in Piauí plants, when submitted to high nitrate resupply (Fig. 3d), which might be due to the concentration effect.

Discussion

In plants, the transcriptional response to differential NO_3^- availability are specific via the regulation of genes for nutrient uptake, such as the NO_3^- transporters and PM H^+-ATPases. This could be a key mechanism for survival in environments, where temporary nutrient limitations arise. Under low NO_3^--N levels, Santos et al. (2011) identified higher V_{max} and low K_M in the Piauí rice variety, compared to IAC-47. These features of the Piauí variety would be useful in environments with low N levels. In our experiment, the Piauí variety also showed higher net NO_3^- uptake compared to IAC-47 under 0.2 mM of NO_3^--N (Table 1). It is important to note that no differences in root or shoot weight between varieties were observed in this treatment (Table 3). In addition, nitrate reductase activity was slightly higher at 6h and 9h in the roots of Piauí variety (Fig. S2).

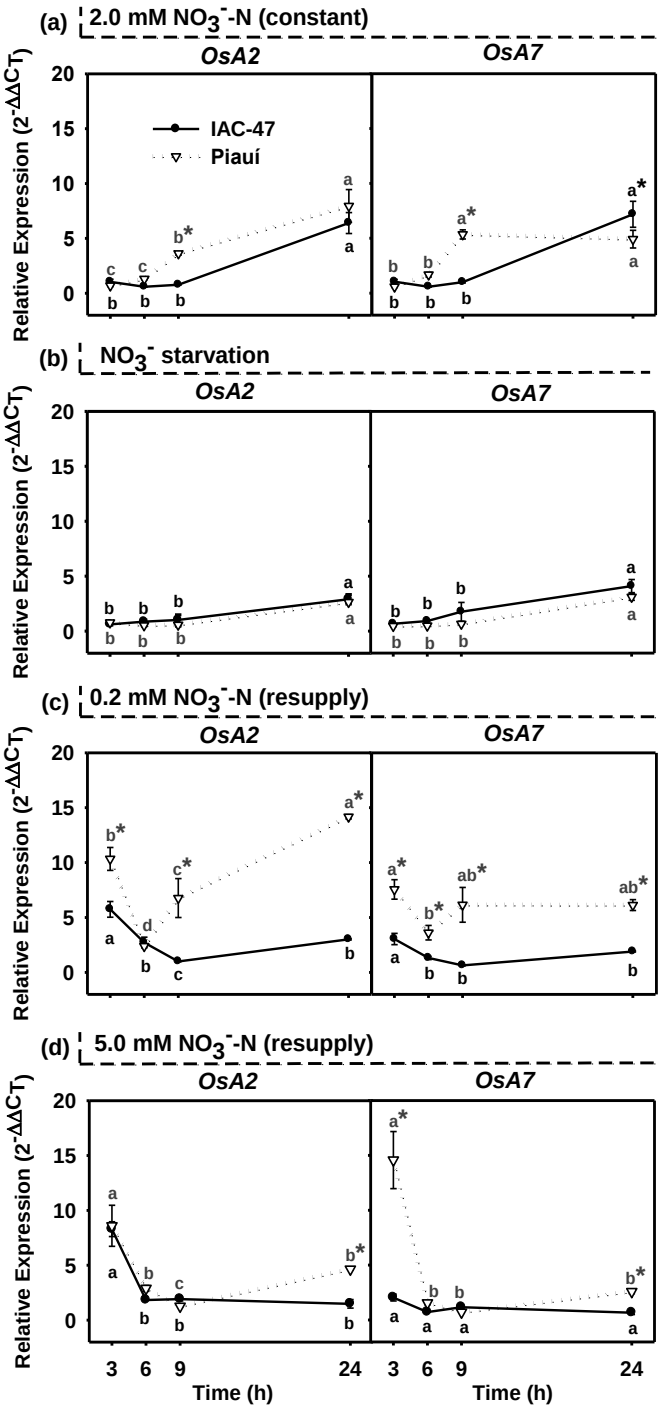

Fig 1. Expression of PM H^+-ATPase *OsA2* and *OsA7* isoforms in the roots of IAC-47 (improved) and Piauí (landrace) rice varieties with a continuous supply of 2.0 mM NO_3^--N (a), N-deficiency for 3 days (b), resupply of 0.2 mM NO_3^--N (c), and resupply of 5.0 mM NO_3^--N (d). Evaluations were performed at 3 h, 6 h, 9 h, and 24 h after the start of treatment. To calculate relative gene expression, a value of 1.0 was assigned to the IAC-47 variety in the treatment group receiving a continuous supply of 2.0 mM NO_3^--N for 3 h. Same letters following data for same line do not differ significantly (Tukey's test, $P < 0.05$). *represents significant differences between the IAC-47 and Piauí rice varieties (Tukey's test, $P < 0.05$). The bars represent the standard error of the mean of four biological replicates.

In addition to the increased expression levels of *OsNRT2.1-2.2*, Piauí variety also showed higher expression of *OsNAR2.1* under low N supply at 3h and 24h (Fig. 2c). In rice, it has been shown that over-expression of *OsNRT2.1* gene alone do not have any effect on nitrate uptake (Katayama et al., 2009). Yan el al. (2011) provide an explanation for this data and showed that increased OsNRT2.1 expression without OsNAR2.1 do not increase nitrate uptake by rice. In rice, knockdown of *OsNAR2.1* by RNAi, suppressed the expressions of *OsNRT2.1*, *OsNRT2.2* and *OsNRT2.3a,* with negative effect on the high and low affinity nitrate transport (Yan et al., 2011).

Expression of *OsNAR2.1* displayed the same pattern of expression that was observed for *OsNRT2.1* and *OsNRT2.2* (Fig. 2c). Despite the similar expression patterns between *NRT2* and *NAR2*, the expression amplitudes were distinct and always higher for *NRT2* (Fig. 2c and d). Wirth et al. (2007) demonstrated that NAR2.1 is essential for the activity of NRT2.1 in the plasma membrane of *Arabidopsis*, while Yan et al. (2011) showed that OsNAR2.1 is essential for the activities of OsNRT2.1, OsNRT2.2, and OsNRT2.3a in rice. Therefore, the higher *OsNAR2.1* expression in the Piauí variety may have contributed to higher net NO_3^- uptake with the resupply of 0.2 mM and 5mM NO_3^--N compared with IAC-47 (Table 1).

Besides the presence of nitrate transporters in the plasma membrane, the PM H^+-ATPases are necessary to maintain the membrane potential ($\Delta\psi$) and H^+ gradient ($\Delta^+\mu H$) during nitrate uptake (Palmgren and Harper, 1999). In rice, *OsA2* and *OsA7* are the most induced PM H^+-ATPase isoforms in the roots, following the resupply of NO_3^--N (Sperandio et al., 2011). The Piauí variety showed higher expression of *OsA2* and *OsA7* compared to IAC-47, especially after resupply with 0.2 mM of NO_3^--N (Fig 1c). At 24h after treatments, Piauí variety also showed higher PM-H^+-ATPase activity under low N supply, compared to IAC-47 (Table 1). Sperandio et al. (2011) had already demonstrated an increase in the expression and activity of PM-H^+-ATPases after nitrate and ammonium provision in rice.

High correlations among the expression of *OsNRT2.1*, *OsNRT2.2*, *OsNAR2.1*, *OsA2*, and *OsA7* (Table 2), in both rice varieties, indicate the joint involvement of the PM H^+-ATPases and *OsNAR2.1/OsNRT2.1-2.2* in the NO_3^- uptake. Using corn plants, Sorgonà et al. (2011) verified that the induction of PM H^+-ATPases and NRT2 occurs at 4 h after the first exposure to NO_3^--N (50 µM KNO_3), and then drops after 24 h of exposure. These results obtained by Sorgonà et al. (2011) are similar to our present results for the IAC-47 variety. However, the Piauí variety (more efficient in NO_3^- uptake) showed a distinct behavior, with increased *OsA2*, *OsA7*, *OsNRT2.1-2.2*, and *OsNAR2.1* expression, even at 24 h after the resupply of 0.2 mM NO_3^--N (Fig. 1 and 2).

The results observed for the Piauí variety are in accordance with the place of origin of this variety, where soils are poor in nutrients, and there is a seasonal flow of N (Souza et al., 1998; Santos et al., 2011). These conditions were reproduced in this study by producing NO_3^- deficiency and then offering NO_3^- resupply. It is likely that the highest NO_3^- uptake capacity in seasonal flow conditions is related to increased expression of NO_3^- transporters and PM H^+-ATPases in the roots. Kant et al. (2008) also found that *Thellungiella halophila* (adapted for low N supply) displayed greater *AtNRT2.1* expression under conditions of low NO_3^- supply when compared to the *Arabidopsis* Columbia ecotype.

Table 2. Pearson correlation coefficients between gene expression values for *OsNRT2.1*, *OsNRT2.2*, *OsNAR2.1*, *OsA2*, and *OsA7* in roots from IAC-47 (improved) and Piauí (landrace) rice varieties.

	IAC-47 variety			
Genes	*OsNRT2.1*	*OsNRT2.2*	*OsNAR2.1*	*OsA2*
OsNRT2.2	0.9423**			
OsNAR2.1	0.9030**	0.8665**		
OsA2	0.8410**	0.8484**	0.8414**	
OsA7	0.8894**	0.9012**	0.8941**	0.9406**
Piauí variety				
OsNRT2.2	0.9385**			
OsNAR2.1	0.9358**	0.8727**		
OsA2	0.9027**	0.8943**	0.8532**	
OsA7	0.9178**	0.8592**	0.8675**	0.9137**

** values significant at $p < 0.01$.

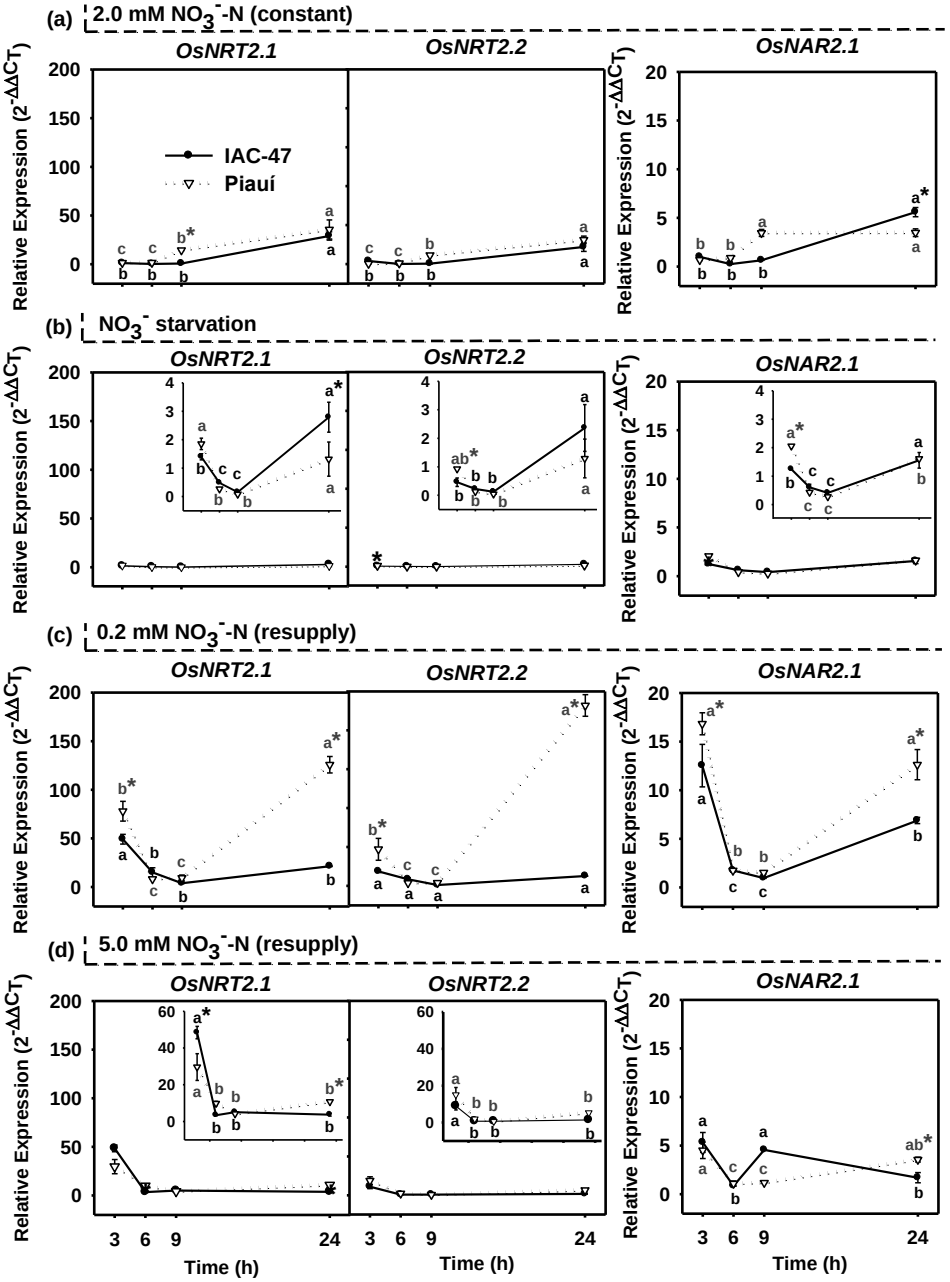

Fig 2. Expression of *OsNRT2.1, OsNRT2.2,* and *OsNAR 2.1* NO_3^- transporters in rice roots from the IAC-47 (improved) and Piauí (landrace) varieties with a continuous supply of 2.0 mM NO_3^--N (a), N deficiency for 3 days (b), resupply of 0.2 mM NO_3^--N (c), and resupply of 5.0 mM NO_3^--N (d). To calculate relative gene expression, a value of 1.0 was assigned to the IAC-47 variety in the treatment group receiving a continuous supply of 2.0 mM NO_3^--N for 3 h. Same letters following data for same line do not differ significantly (Tukey's test, $P < 0.05$). *represents significant differences between the IAC-47 and Piauí rice varieties (Tukey's test, $P < 0.05$). The bars represent the standard error of the mean of four biological replicates.

Table 3. Fresh weigh and root:shoot ratio of the IAC-47 (improved) and Piauí (landrace) rice varieties with continuous supply of 2.0 mM NO_3^--N, N deficiency, resupply of 0.2 mM NO_3^--N, and resupply of 5.0 mM NO_3^--N.

	Total fresh weight (g. pot^{-1})		Shoot fresh weight (g. pot^{-1})		Root fresh weight (g. pot^{-1})		Root:Shoot ratio	
	IAC-47	Piauí	IAC-47	Piauí	IAC-47	Piauí	IAC-47	Piauí
Constant	8.05aA*	8.75aA	5.02aB	5.49aA	3.18aA	3.25aA	0.60aC	0.59aC
Starvation	7.46aA	6.71aB	4.29aB	3.85aB	3.78aA	2.91bA	0.73aA	0.74aA
R 0.2	7.55aA	7.65aB	4.45aB	4.53aB	3.10aA	3.11aA	0.69aB	0.69aB
R 5.0	7.80aA	6.96aB	5.63aA	4.12bB	3.79aA	3.05bA	0.63bC	0.68aB

*Averages followed by the same uppercase letter in the columns did not differ significantly (Tukey test, $p < 0.05$), and averages followed by the same lowercase letter in the line did not differ significantly (Tukey test, $p < 0.05$) in the rice varieties in the same part of the plant.

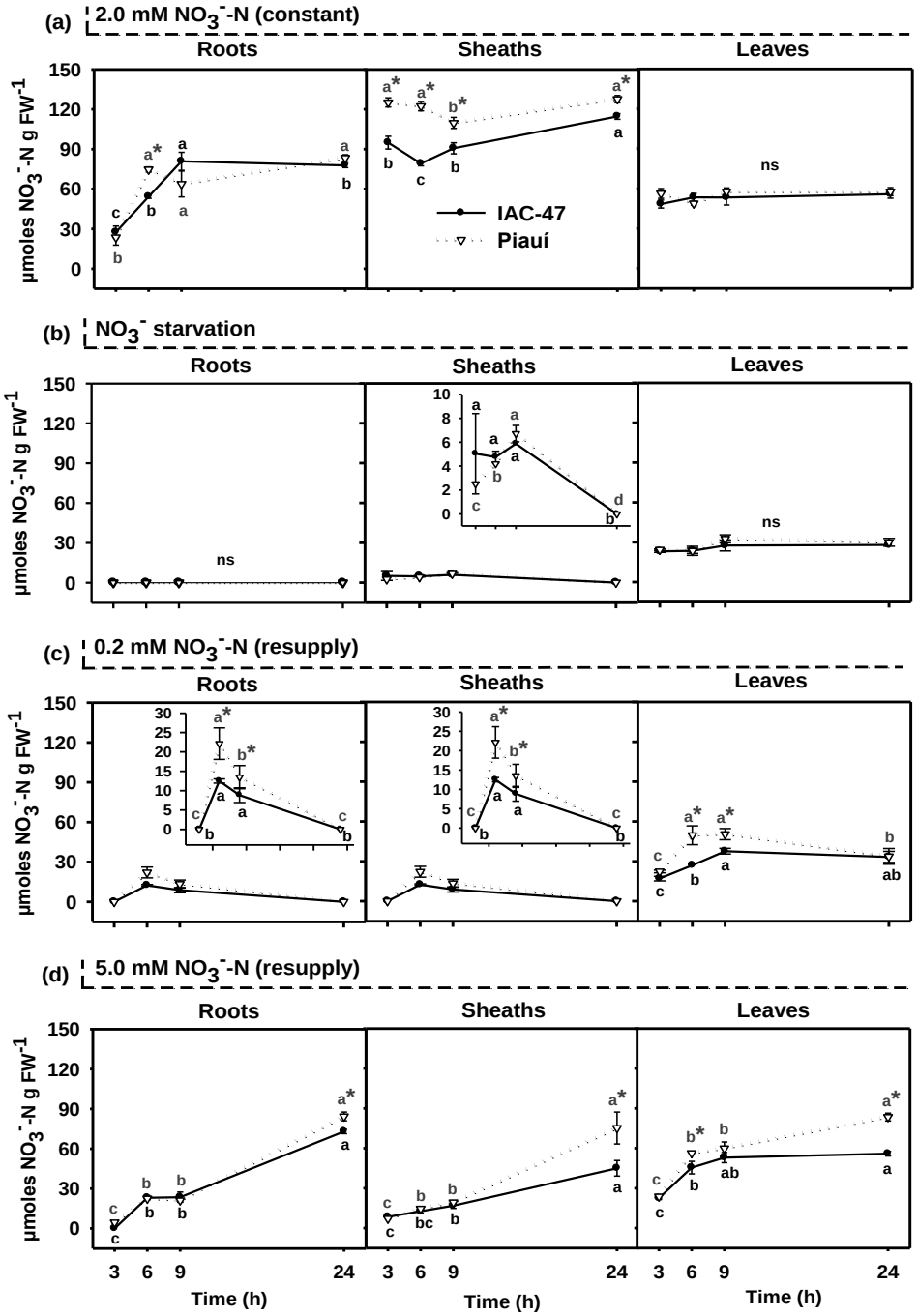

Fig 3. Levels of NO_3^--N contents in the roots, sheaths, and leaves of the IAC-47 (improved) and Piauí (landrace) rice varieties with constant supply of 2.0 mM NO_3^-N (a), N deficiency for 3 days (b), resupply of 0.2 mM NO_3^--N (c), and resupply of 5.0 mM NO_3^--N (d). Evaluations were performed at 3 h, 6 h, 9 h, and 24 h of initiating the treatments. Same letters following data for same line do not differ significantly (Tukey's test, $P < 0.05$). *represents significant differences between the IAC-47 and Piauí rice varieties (Tukey's test, $P < 0.05$). The bars represent the standard error of the mean of four biological replicates.

The highest influx and accumulation of NO_3^--N observed in the Piauí variety when compared to the IAC-47. It may be an evidence of its adaptation to low fertility soils, and could also be essential for its survival in tropical environments, where seasonal flow of N occurs (Santos et al., 2009). Souza et al. (1998) suggest that under N-limiting conditions, NO_3^- accumulation is an essential characteristic during the plant initial growth period. This is strategic and crucial for optimal plant yields during the reproductive stage. At high doses, such as with the resupply of 5.0 mM NO_3^--N and constant supply of 2.0 mM NO_3^-N, part of the NO_3^- supplied may accumulate in plant cell vacuoles. This phenomenon was observed in both varieties studied. However, the Piauí variety showed greater NO_3^- accumulation (Fig. 3). Because the sheaths represent the main site of NO_3^- accumulation with continuous supply of NO_3^-N, and the highest levels of this nutrient are observed in the leaves after 3 days of N-deficiency. It is likely that NO_3^--N transfer occurs from the sheaths to the leaves with the onset of the deficiency period.

Overall, the results indicated that under nitrate resupply, the higher expression levels of high affinity nitrate transporters (*OsNAR2.1*/*OsNRT2.1-2.2*) and PM H^+-ATPases (*OsA2* and *OsA7*) is an adaptive response of Piauí variety to low fertility and the seasonal flush of nitrogen common in the tropical humid environments. In such an environment, the seasonal flush of N occurs at the beginning of rainy season with rapid organic matter degradation, and then, the N availability drops to very low levels (Santos et al., 2007). The expression levels of *OsNRT2.1-2.*2, *OsNAR2.1*, *OsA2* and *OsA7*, occur synchronously over time for all NO_3^- treatment groups.

Materials and Methods

Plant materials

The experiment was conducted in a growth chamber for a 14-h photoperiod, at a photosynthetic photon flux of 500 µm mol m^{-2} s^{-1}, relative humidity of 70%, and day/night temperature of 28°C/24 °C. A randomized complete block design with four replications was used.

Rice seeds from the IAC-47 (high N input variety) and Piauí (low N input local variety) varieties were disinfected in 2% sodium hypochlorite solution for 10 min, rinsed several times in distilled water, and transferred to pots containing additional distilled water. Six days after germination, four seedlings, chosen according to their uniformity, were transferred to 0.7-L pots containing one-fourth of the total ionic strength of Hoagland and Arnon's modified solution (Hoagland and Arnon, 1950) at pH 5.5, with 2.0 mM NO_3^--N as the only source of N. After 3 days, the plants received half of the total ionic strength of Hoagland's solution with 2.0 mM NO_3^--N. This solution was refreshed every 3 days.

Eighteen days after germination, one group of plants received Hoagland's solution without NO_3^--N, while another group continued with the Hoagland solution containing 2.0 mM NO_3^--N. At 21 days, the treatments were applied as follow: one-third of the plants cultivated without NO_3^--N received Hoagland's solution with 0.2 mM NO_3^--N (resupply), one-third received this solution with 5.0 mM NO_3^--N (resupply), and one-third continued to receive the solution without NO_3^--N (N deficiency). The group with constant 2.0 mM NO_3^--N supply was kept as a control. Therefore, four treatments were applied: plants continually cultivated with 2.0 mM NO_3^--N (control), plants submitted to resupply of 0.2 mM NO_3^--N (low levels of N), plants submitted to resupply of 5.0 mM NO_3^--N (high levels of N), and plants cultivated under N-deficient conditions (Fig. S1). The NO_3^- treatments has started three hours after onset of the light period.

After the treatments, plants were harvested at 3 h, 6 h, 9 h, and 24 h. One gram each of roots, sheaths and leaves were kept in ethanol (80%) and after partition with chloroform, water extract was used for determination of nitrate-N (Cataldo et al., 1975). Additional root samples were collected for total RNA extraction and plasma membrane H^+-ATPase activity.

Net NO_3^- uptake measurement and nitrate reductase activity

In the same experiment described above, 1mL solution was taken up at 3h, 6h, 9h and 24h after transferring plants to the experimental solution. Nitrate concentration was measured according to Cataldo et al. (1975). The amount of nitrate taken up from the experimental solution was evaluated based on the NO_3^- uptake index as NO_3^- uptake from the solution per root fresh weight of four independent pots with four plants each. Net NO_3^- uptake was calculated based in the depletion of NO_3^- at time of harvests. In every time described above, plants were harvested and nitrate reductase activity measured in the roots, sheaths and leaves (Jaworski, 1971).

Analysis of NO_3^--N content in the plant tissue

Samples comprising 0.5 g of leaves, sheaths, or roots were homogenized in 80% ethanol, and after chloroform partition (Fernandes, 1983), the soluble fraction was used to determine the NO_3^--N content (Cataldo et al., 1975).

Extraction of total RNA, cDNA synthesis, and real-time PCR

Total RNA was extracted according to the method of Gao et al. (2001), using NTES buffer (0.2 M Tris-Cl pH 8.0, 25 mM EDTA, 0.3 M NaCl, and 2% SDS). The total extracted RNA was treated with DNase I (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Thereafter, 1 µg of treated RNA was used for cDNA synthesis using a High-Capacity RNA to cDNA Master Kit (Life Technologies), according to the manufacturer's instructions.

Real-time PCR reactions were performed using a Power SYBR® Green PCR Master Mix kit (Life Technologies), according to the manufacturer's instructions. The PCR cycling conditions were 10 min at 95 °C, 40 amplification cycles at 95 °C for 15 s, 60 °C for 1 min (annealing, extension, and detection of fluorescence), and finally, generation of a dissociation curve with a temperature rise of 0.3 °C from 60 °C to 95 °C for to verify reaction specificity. Primers sequence for *OsNRT2.1*, *OsNRT2.2*, *OsNAR2.1*, *OsA2* and *OsA7* used in Real Time PCR are the same used by Sperandio et al. (2011). The actin 1 gene (NM_001057621.1) was used as the reaction's endogenous control (Jain et al. 2006). Calculations for gene expression were made according to Livak and Schmittgen (2001). To compare the Piauí and IAC-47 varieties, all gene expression calculations used the sample of IAC-47 with constant supply of 2.0 mM NO_3^--N at 3 h as a control; therefore, there is no zero time point to compare the varieties.

Preparation of microsomal fraction and assessment of PM H^+-ATPase activity

Plasma membrane vesicle extraction and assessment of PM H^+-ATPase activity were performed according to the methods of Façanha and De Meis (1995) and Santos et al. (2009), respectively. Root samples harvested at 24h were homogenized in cold extraction buffer (50 mM Tris-HCl pH 8.0, 250 mM sucrose, 100 mL L^{-1} glycerol, 150 mM KI, 100 mM choline chloride, 2 mM EGTA, 2 mM EDTA, 10 g L^{-1} polyvinylpyrrolidone (PVP), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT), 5 mmol L^{-1} mercaptoethanol, and 5 g L^{-1} albumin). The homogenates were filtered 4 times through cheesecloth, and then centrifuged at 3,600 × *g* for 10 min. The resultant supernatant was collected and centrifuged at 8,000 × *g* for 10 min. The supernatant was again collected and re-centrifuged at 105,000 × *g* for 1 h. The precipitate (microsomal fraction) was dissolved in 2.0 mL of solubilizing buffer (30 mM Tris-HCl pH 7.5, 150 mL·L^{-1}glycerol, 1 mM EGTA, 1 mM EDTA, 2 mM $MgCl_2$, 2 mM DTT, and 1 mM PMSF), frozen in liquid N_2, and stored at -80 °C. The protein content was quantified using the Bradford (1976) assay.

PM H^+-ATPase activity was measured according to Santos et al. (2009), using a reaction medium composed of 30 mM MOPS-BTP (pH 6.5), 5 mM $MgSO_4$, 50 mM KCl, 1 mM Na_2MoO_4, 0.02 % of Triton X-100, 50 mM KNO_3, 1 mM NaN_3, and 5 mM ATP. The reactions for each sample were performed with and without 0.2 mM vanadate (PM H^+-ATPases activity inhibitor) at 30 °C, and initiated with the addition of 15 µg of protein. One milliliter of stop solution (2% v/v H_2SO_4, 5% p/v SDS, and 0.7% p/v $(NH_4)_2MoO_4$) and 50 µL of 10% ascorbic acid was added to each tube after 1-h incubation. After 10 min, 1.45 mL of medium containing citrate was added (4% sodium citrate and 2% glacial acetic acid) to avoid further color development. The absorbance was read at 820 nm. The activity of PM H^+-ATPases was calculated as the difference in the amounts of Pi released in the reaction with and without vanadate.

Statistical analysis

The experiments were conducted in a completely random design with four replications. The averages of the treatments were compared using the least significant difference (LSD) and Tukey's test ($P < 0.05$), after identifying significant differences by variance analysis (ANOVA). Moreover, Pearson correlation values were calculated among *OsNRT2.1*, *OsNRT2.2*, *OsNAR2.1*, *OsA2*, and *OsA7* gene expression.

Conclusion

The higher NO_3^- influx in the Piauí variety under low N resupply condition was supported by higher *NRT2* expression and PM-H^+-ATPase expression and activity, an adaptive response to low fertility soil and/or low input crops. These results establish synchronism efforts of high affinity nitrate transporters and PM-H^+-ATPases under low N flush condition.

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