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Rice Varieties Tonoplast and Plasma Membrane H⁺-ATPases Differential Activities in Response to Nitrate Pulses

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Abstract: In a growth chamber experiment, the response of two rice varieties (Piauí and IAC-47) to nitrate suppression (24 h) and pulses (72 h) was studied. Nitrate suppression in nutrient solution increased plasma membrane H⁺-ATPase activity in Piauí plants previously grown under 0.5 mM and IAC-47 under 5.0 mM NO₃⁻-N. Under NO₃⁻ pulses, IAC-47 presented highest H⁺-ATPase activity in its plasma membrane, while Piauí showed increases in tonoplast V-H⁺-ATPase activity. These results suggested that under nitrate pulses IAC-47 increased its ability to take up NO₃⁻ from nutrient solution. Piauí plants preferentially retrieves nitrate stored from the vacuoles. Plasmalemm protein content in Piauí tonoplast was enhanced particularly in -N plants previously grown under 5.0 mM NO₃⁻-N. We suggest that these enzymes (H⁺-ATPases) could be monitored as markers in nitrogen use efficiency study in rice plants.

Key words: Tropical agriculture, N-nutrition, H⁺ pumping

INTRODUCTION

Rice plants can take up N either as NO₃⁻ or as NH₄⁺ (Fernandes, 1990; Aslam *et al.*, 1997). Nitrate uptake is an active process, made through co-transport with two H⁺. Two transport systems have already been described for NO₃⁻ (Forde, 2000; Loqué *et al.*, 2003; Okamoto *et al.*, 2006), one of them work at low NO₃⁻ concentrations in the environment High Affinity Transport System (HATS), while the other operates at high NO₃⁻ concentrations (low affinity system -LATS). Both transport systems use protonmotive force (Δp) at proton gradients ($\Delta \mu_{H^+}$) plants H⁺-ATPases. High affinity systems can be constitutive or inducible, 1 mM is suggested as the limit between the two systems (Forde, 2000; von Wirén *et al.*, 1997).

N-NO₃⁻ taken up by plants is transported to shoots, reduced to NO₂⁻ in the cytosol or transported to the vacuoles through anion channels. NO₃⁻ movement from cytosol to vacuoles can be very fast and it results in NO₃⁻ depletion from cytosol. Even though, total NO₃⁻ content of cells remains high specially in some genotypes with enhanced grain yield and grain nitrogen content (Quaggiotti *et al.*, 2003). De Angeli *et al.* (2006) described AtCLCa as a transporter able to mediate nitrate influx into the vacuole. Vacuoles are thus

turned into substrate pools (Vidmar *et al.*, 2000; Miller *et al.*, 2001; Fan *et al.*, 2006).

NO₃⁻ at vacuoles can be transported back to the cytoplasm thanks to a symport system (NO₃⁻/1 H⁺) (Cookson *et al.*, 2005). Once back to the cytosol, NO₃⁻ can then be reduced to NO₂⁻ by Nitrate Reductase (NR) enzyme. As NR is an inducible enzyme with a very short half life, its depletion from the cytosol leads to a reduction in the Nitrate Reductase Activity (NRA) (Debouba *et al.*, 2007). Vacuoles (substrate pools) are thus an important system, part of a cellular network that enables a plant to store nutrients and to maintain optimal metabolic conditions in the cytosol (Martinoia *et al.*, 2007).

Hirel *et al.* (2001) suggested that NO₃⁻-N accumulation corn plants leaves in its early growth stages is a good evidence of its high capacity to grain production due to its N storage. These authors suggest the formation of these reserve pools to be under genetic control and to be of great importance in later stages of N-assimilation and grain development in corn. The authors conclude that to select plants for higher N-use efficiency one should pick up plants with high capacity to store N in the shoots at early growth stages. During its vegetative phase, plants have had lower NRA, so that shoots stored NO₃⁻ could be used later for grain production.

In fact, Brazilian landraces, adapted to low nutrients environments have developed the capacity to accumulate larger N amounts in the shoots (Souza *et al.*, 1999; Rodrigues *et al.*, 2004).

It seems like higher capacity to take up NO_3^- from low-N environments, higher capacity to store NO_3^- in the substrate pools and higher capacity to remobilize stored N to the sites of protein synthesis are important features when breeding plants for high N efficiency use. Fan *et al.* (2007) detected very little depletion of storage pools in whole-tissue analysis after 24 h N supply withdrawal. However, in the more efficient variety, there was a nitrate activity depletion in xylem, but epidermal cell cytosolic nitrate activities were significantly higher when compared to the other variety.

All vacuoles seem to contain the vacuolar-type H^+ -ATPase, H^+ -translocating inorganic pyrophosphatase and TIP-like aquaporins, different in function depending on type of vacuole they belong to (Martinoia *et al.*, 2007). H^+ -ATPases are electrogenic pumps that generate protonmotive force necessary for nutrient uptake against electrochemical gradients. At tonoplast, they could allow NO_3^- store remobilization from the vacuoles (Palmgren, 2001). However, De Angeli *et al.* (2006) found out that a Cl antiporter also mediates NO_3^- storage in plant vacuoles, what is favorable to enhance plants nitrogen use efficiency.

Often, at Humid Tropic there are two main seasons; a rainy winter and a dry summer. At the beginning of the rain season there is a nitrate flush that accumulates at the surface of the soils (Rodrigues *et al.*, 2004; Rodrigues and Garrido, 2005). Nitrate content can go from near zero to about 200 mg kg^{-1} in a short period of time. At the beginning of rainy season, most of this NO_3^- is washed out of soil superior layer and also it remains out of the roots range. In such environment it should be an advantage for plants to have the ability to take up as much NO_3^- as possible. In this way, it would be an advantage store it in the vacuoles and later use this N for the plant's metabolism.

In this study we used a landrace rice from the state of Maranhão-Brazil (Piauí), that was shown to have up to 11% of grain protein (Ferraz-Junior *et al.*, 2001), aluminium tolerance also it was used and an improved variety (IAC-47). Rodrigues *et al.* (2004) found out that Piauí variety is very efficient at N remobilization from shoots to grain, while IAC-47 depended on N immediately assimilated for protein synthesis in the grains.

As the H^+ -ATPases activity may be important decisive for NO_3^- uptake and stock, we also determined the activities of the P and V- H^+ -ATPases and studied the possibilities of using it as a marker for NO_3^- efficient uptake in rice.

MATERIALS AND METHODS

This study was carried on at Universidade Federal Rural do Rio de Janeiro, Seropédica, campus, Brazil, between 2004 and 2005. Two rice (*Oryza sativa* L.) varieties were used: Piauí and IAC-47. Rice plants were grown in a growth chamber, with nutrient solutions until 26 DAG (Days after germination). Average temperature during growth period was 24°C with 12/12 h light/dark periods. A modified Hoagland and Arnon (1950) solution was used at ¼ of its ionic strength. Four Days After Germination (DAG) it was changed to ½ of its ionic strength and kept so up to 8 DAG. After that, plants were grown in a full strength solution up to the final harvest at 26 DAG.

At 20 DAG, nitrate pulses were given in treatments T_1 and T_2 as follows: at 20 DAG, N was suppressed from the nutrient solutions for 72 h. Control plants were grown with 0.5 mM (C_1) or 5 mM (C_2) NO_3^- -N throughout the experimental period. After that NO_3^- nutrition at 0.5 mM (T_1) or 5.0 mM (T_2) was restored. At 21 DAG, 10 replications of each treatment and controls were harvested.

For ATPases activities determination, tonoplast and plasma membrane vesicles were extracted by cell fractionation (Yoshida *et al.*, 1983). Five grams of plant roots were rinsed with deionized water, they were dried and its fresh weight was taken. Ten grams of this material was ground in an ice cold mortar and pestle, with 10 mL of a buffer solution (0.25 M sorbitol; glycerol 10%; 50 mM TRIS-acetate; 1 mM EDTA; 2 mM DTT; 20 μM PMSF; 0.3% BSA; 1% PVP; 0.25 M KI and H_2O ; pH 7.5). The ground material was filtered through 4 layers of cheesecloth. The extract was centrifuged at 5,470 g for five min, at 4°C. The supernatant was taken and centrifuged at 136,000 g for 40 min at 4°C. Pellet was resuspended in 2 mL of 0.25 M sorbitol/TRIS at pH 7.5 and put in sucrose gradient centrifuge tubes. The sucrose gradients were: 0.73 M sucrose/TRIS and 1.31 M sucrose/TRIS. This material was then centrifuged at 136,000 g for 1.5 h, at 40°C.

The tonoplast (top) and plasma membrane (middle) layers were taken out of the centrifuge tubes, centrifuged again for one hour at 136,000 g and the pellet was resuspended in 1 mL of a 0.25 mM sorbitol/TRIS solution and kept at -20°C until used for the H^+ -ATPases assay determination. Enzymes activities were determined in 0.03 mg mL^{-1} of protein; 1 M Mops, pH 6.25; 2 mM KCl; 0.1M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 M ATP. Assays were made under vanadate to check out for P- H^+ -ATPase activities. Free Pi was determined using the Fiske solution (1,5% of the ammonium molybdate in 0,05N H_2SO_4 and ascorbate 2%) after 30 min and read at 700 nm in a spectrophotometer (Fiske and Subarrow, 1925).

To determine the NO₃⁻-N depletion from nutrient solution, Piaui and IAC-47 plants were grown under the same conditions mentioned before.

At 18 DAG, half plants were kept in the 0.5 and 5.0 mM NO₃⁻-N solution while the other half was moved into a -N solution. After this, plants were harvested each half hour up to 6:30 h and NO₃⁻ content was determined in the nutrient solution. After this, two more harvests were made at 24 and 48 h and NO₃⁻ content was determined the same way.

Statistical analysis using Tukey test 5% was carried on with six repetitions from each treatment.

RESULTS AND DISCUSSION

Table 1 showed Piaui and IAC-47 fresh weight at 22 and 26 DAG. At both treatments, as well as in IAC-47 controls had higher fresh weight than Piaui plants. This effect was enhanced by NO₃⁻ pulse (72 h).

In Table 2 it was observed plasma membrane H⁺-ATPase activity from both varieties. After 24 h in the -N solution, Piaui plants previously grown under 0.5 mM NO₃⁻ had higher activity than the controls (+N). The contrary was true for plants that previously received 5.0 mM NO₃⁻-N. On the other hand, IAC-47 cell roots showed the highest ATPase activity in the plasma membrane in

the -N plants previously grown under 5.0 mM NO₃⁻-N. Table 2 also presented Piaui and IAC-47 responses to NO₃⁻-N pulse (72 h). Here, differences were significant: IAC-47 enhanced H⁺-ATPase activity more than it happened in Piaui over all treatments and controls. That behavior may suggest greater response of IAC-47 to nitrate pulse, since a greater H⁺-pumping activity may increase NO₃⁻ uptake by enhancing the proton motive force (Δp). Interestingly, highest H⁺-ATPase activity was shown by IAC-47 plants cultivated under 0.5 mM NO₃⁻-N, after NO₃⁻ pulse.

The V-H⁺-ATPase activities of Piaui and IAC-47 were shown in Table 2. Piaui plants previously grown under 5.0 mM NO₃⁻-N showed high V-H⁺-ATPase activity after 24 h in the -N treatment. That response is totally different from that of IAC-47 plants, where the highest activities are shown in control plants. IAC-47 plants previously submitted to 5 mM NO₃⁻-N had no V-H⁺-ATPase activity in the -N treatment. Also in Table 2 it can be seen NO₃⁻ pulses effects over Piaui and IAC-47 plants V-H⁺-ATPase activities. In both varieties, the highest activities were found in plants previously grown with 0.5 mM NO₃⁻-N. Here, despite of what did happen in the plasma membranes, ATPase activities at the tonoplast of Piaui plants was about 5 times enhanced than that of IAC-47 plants.

Table 1: Rice plants shoots fresh weight (g pot⁻¹) (Piaui and IAC-47) at 22 and 26 days after emergence (DAE). Plants received 0.5 (C1) or 5.0 (C2) mM N-NO₃⁻ until 22 days after emergence, remained 24 h without N in treatments (T₁) and received 72 h N (pulse) (T₂) since 26 days after emergence (DAE)

Parameters	Piaui		IAC-47	
	24 h without N	72 h pulse	24 h without N	72 h pulse
	22 DAE	26 DAE	22 DAE	26 DAE
0.5 mM without N	9.92±0.39 ^b	14.08±1.21 ^a	14.42±1.94 ^b	20.45±0.66 ^a
Control	8.26±0.26 ^b	8.75±0.49 ^b	14.80±0.12 ^b	19.13±0.53 ^a
5.0 mM without N	6.98±0.05 ^c	9.69±0.51 ^b	14.72±0.50 ^b	19.67±0.75 ^a
Control	6.74±0.50 ^c	9.22±0.24 ^b	15.30±0.56 ^b	21.06±0.42 ^a

The same letter(s) show values that do not differ between treatments (5% Tukey). The arrow pointed out roots harvest to tonoplast HATPases

Table 2: Rice root cells plasma membrane and tonoplast H⁺-ATPases activity (Pi μmol protein mg⁻¹ h⁻¹). Plants received 0.5 or 5.0 mM N- NO₃⁻ until 22 days after emergence (DAE), remained 24 h without N in treatments and received 72 h N (pulse) until 26 days after emergence (DAE)

Parameters	Piaui		IAC-47	
	24 h without N	72 h pulse	24 h without N	72 h pulse
	22 DAE	26 DAE	22 DAE	26 DAE
-----Pi μmol protein mg ⁻¹ h ⁻¹ -----				
Plasma membrane				
0.5 mM without N	81.00±4.11 ^a	22.36±0.00 ^b	43.82±21.08 ^b	323.80±12.9 ^a
Control	52.60±8.56 ^b	20.88±1.00 ^b	20.24±1.90 ^b	251.30±0.00 ^b
5.0 mM without N	20.36±2.16 ^c	60.28±3.72 ^a	80.17±8.57 ^a	214.75±0.00 ^d
Control	100.74±12.79 ^a	0.00±0.00 ^c	0.00±0.00 ^c	228.89±1.36 ^c
Tonoplast				
0.5 mM without N	27.96±9.26 ^c	262.78±2.57 ^a	68.43±0.81 ^c	58.46±5.39 ^a
Control	38.75±1.88 ^b	0.00±0.00 ^d	93.43±3.03 ^b	4.56±2.68 ^b
5.0 mM without N	191.11±1.11 ^a	15.68±1.28 ^c	2.19±1.07 ^d	0.00±0.00 ^b
Control	0.00±0.00 ^d	28.32±9.45 ^b	122.60±0.90 ^a	6.55±3.54 ^b

The same letter(s) show values that do not differ between treatments (5% Tukey)

Table 3: Plasma membrane and tonoplast H⁺-ATPases protein (protein mg fresh weight g⁻¹) in vesicles purified from rice root cells

Parameters	Piaui		IAC-47	
	24 h without N	72 h pulse	24 h without N	72 h pulse
	22 DAE	26 DAE	22 DAE	26 DAE
	Protein mg fresh weight g ⁻¹			
Plasma membrane				
0.5 mM without N	0.470±0.015 ^a	0.029±0.011 ^b	0.022±0.004 ^b	0.010±0.003 ^c
Control	0.370±0.000 ^b	0.015±0.001 ^c	0.029±0.005 ^b	0.010±0.001 ^c
5.0 mM without N	0.045±0.000 ^a	0.024±0.009 ^b	0.041±0.009 ^a	0.022±0.003 ^b
Control	0.045±0.002 ^a	0.019±0.002 ^b	0.037±0.001 ^b	0.015±0.005 ^c
Tonoplast				
0.5 mM without N	0.202±0.006 ^a	0.181±0.028 ^b	0.114±0.005 ^b	0.068±0.001 ^c
Control	0.158±0.016 ^a	0.113±0.001 ^b	0.112±0.006 ^b	0.073±0.005 ^c
5.0 mM without N	0.289±0.010 ^a	0.345±0.006 ^a	0.147±0.019 ^b	0.085±0.006 ^b
Control	0.281±0.500 ^a	0.429±0.034 ^a	0.140±0.008 ^b	0.135±0.007 ^b

The same letter(s) show values that do not differ between treatments (Tukey test 5%)

Actually, these results showed that both varieties have quite different behavior in response to NO₃⁻ nutrition and NO₃⁻ pulses. Despite, IAC-47, an improved variety, showed an increase in P-H⁺-ATPases in response to NO₃⁻ pulses. However, Piaui, a landrace variety, exhibited a higher V-H⁺-ATPase activity as a response to NO₃⁻ pulses. In both situations, ATPase activities due to NO₃⁻ pulses are higher for plants previously grown under 0.5 mM than under 5.0 mM NO₃-N. Those results are in agreement with data from Souza *et al.* (1999) when these varieties differed in its ability to take up and remobilize N. IAC-47 depended on newly absorbed N for grain filling, while Piaui plants relied mostly on its stored N remobilization.

Table 3 showed H⁺-ATPases protein content of the plasma membranes of Piaui and IAC-47 plants after 24 h in the -N solution and after 72 h on the NO₃⁻ pulse. For both varieties the -N plants had a higher protein content than the NO₃⁻ pulsed plants. Table 3 showed that there are great differences in protein (V-H⁺-ATPases) accumulation in both varieties tonoplast. Piaui showed higher protein accumulation in tonoplast, especially in -N plants, previously grown with 5.0 mM NO₃⁻-N solution. Also, Piaui plants that received 72 h NO₃⁻-N pulse accumulated more protein in tonoplast (Table 3). IAC-47 plants had also more protein in the tonoplast than in the plasma membrane; however, Piaui plants presented greater ATPase protein.

Although we cannot make a direct connection between tonoplast protein content and the V-H⁺-ATPases hydrolytic activity, it should be noticed that Piaui plants vesicles had more protein, both at the -N and 72 h pulse treatment.

Figure 1 showed NO₃⁻ uptake kinetics in both rice varieties. Plants were grown in a 0.5 mM NO₃⁻-N solution up to 18 DAG. From this day on, half of all plants were put under -N solution. Forty eight h after the beginning of the experimental period, IAC-47 plants had taken up more

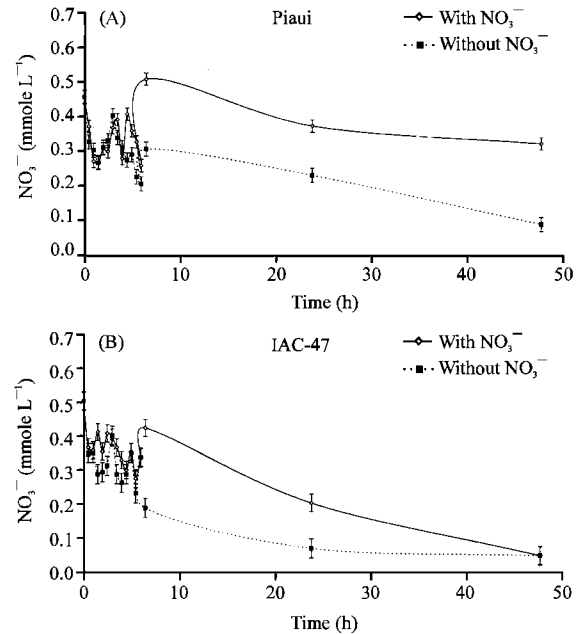


Fig. 1: Nutrient solution NO₃⁻ concentration (NO₃⁻ mmoles L⁻¹) from plants maintained without N or with N during Piaui (A) and IAC-47 (B) harvests. Plants were grown in nutrient solution until 18 days after emergence (DAE), when control plants received N-NO₃⁻ 0.5 mM solution and the treatment was maintained in solution without N. At 18 DAE, NO₃⁻ concentration was analysed at 30 min, 1 h, 1 h 30 min, 2 h, 2 h 30 min, 3 h, 3 h 30 min, 4 h, 4 h 30 min, 5 h, 5 h 30 min, 6 h, 6 h 30 min, 24 h and 48 h

NO₃⁻ than did Piaui plants. This was in agreement with H⁺-ATPase data presented in Table 2, that showed higher H⁺-ATPases activity at IAC-47 plants plasma membrane. Under the -N treatment, IAC-47 plants increased its NO₃⁻

uptake rate, while Piaui plants probably stocked more NO_3^- . V-H⁺-ATPase activities shown in Table 2 were in agreement with this.

Differences in N-uptake and metabolism among these varieties were observed by Souza *et al.* (1999), Rodrigues *et al.* (2004) and Fan *et al.* (2007). Data in Fig. 1 showed that the improved variety (IAC-47) took up and metabolized NO_3^- faster than did Piaui plants, while the latter apparently kept a higher amount of NO_3^- in the storage pool at this stage of growth. To use that NO_3^- from storage pool, plants have to generate a favorable electrochemical gradient, which is achieved through the activation of the H⁺-ATPases at the tonoplast. This tonoplast potential ranges from 0 to 30 mV (Pottosin and Schönknecht, 2007). This would explain differences between these varieties V-H⁺-ATPase activities, as shown in Table 2.

CONCLUSION

Present results showed that the improved variety (IAC-47) answered to N suppression by activating the P-H⁺-ATPases at the plasma membrane, while the landrace (Piaui) one activated preferentially V-H⁺-ATPases at the tonoplast. This confirms varietales differences between improved varieties and landraces through H⁺-pumping enzymes activities. We suggest these mechanisms (H⁺-pumping) can be used as markers in the study of nitrogen use efficiency in rice.

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