Plant Signaling & Behavior 6:1, 160-163; January 2011; © 2011 Landes Bioscience

Further characterization of an aluminum influx transporter in rice

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Nrat1 is a plasma membrane-localized aluminum transporter recently identified in rice, which is a member of Nramp family. Here, we further characterized this transporter in terms of transport substrate specificity. Heterologous assay in yeast showed that Al transport activity by Nrat1 was unaffected by the presence of high concentration of Ca, but significantly inhibited by trivalent ions including Yb and Ga, analogs of Al. Knockout of *Nrat1* did not affect the uptake of Cd and Mn in rice. On the other hand, overexpression of *Nrat1* led to enhanced Al uptake by rice roots compared with wild-type rice, but did not affect Cd uptake. These results provide further evidence that unlike other Nramp members, Nrat1 is an influx transporter for trivalent Al ion.

Key words: aluminum, transporter, rice, substrate specificity

Submitted: 11/30/10

Accepted: 11/30/10

DOI: 10.4161/psb.6.1.14319

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Addendum to: Xia JX, Yamaji N, Kasai T, Ma JF. Plasma membrane-localized transporter for aluminum in rice. Proc Natl Acad Sci USA 2010; 107:18381–5; PMID: 20937890; DOI: 10.1073/pnas.1004949107.

Aluminum ion (mainly Al^{3+}) inhibits root growth and functions, which toxicity is the major limiting factor of crop production on acid soils.[1] However, there is a wide variation in the tolerance to Al toxicity between species and cultivars within a species.[2-4] Species or cultivars with high Al tolerance have evolved various strategies to detoxify Al externally and/or internally.[2-4] Rice has been known as a highly Al-tolerance species. Recent identification of a transcription factor (ART1) for Al tolerance has revealed that multiple genes are involved in high Al tolerance in rice.[5] One of the genes regulated by ART1 is Nrat1.

Nrat1 belongs to Nramp family, but shares low similarity (<60%) with other members.[6] A detailed functional analysis of Nrat1 showed that unlike other Nramp members, which are transporters of divalent metals, Nrat1 is a transporter of trivalent Al ion (Al^{3+}).[6] Nrat1 is localized to the plasma membrane of all root cells. Knockout of Nrat1 resulted in decreased Al uptake, but increased cell wall-binding Al and Al sensitivity.[6] We therefore concluded that Nrat1 is required for prior step of final Al detoxification through sequestration of Al into vacuoles. In this report, we further characterize Nrat1 in terms of transport substrate specificity.

It has been reported that Ca^{2+} can alleviate Al toxicity by decreasing Al accumulation in the roots.[7,8] Therefore, there is a possibility that Ca^{2+} affects Al uptake through Nrat1. To test this possibility, we examined the effect of Ca on Al uptake in yeast expressing *Nrat1* or not. In the presence of Ca up to 10 times of Al, the Al uptake by Nrat1 was unaffected (**Fig. 1A**), indicating that Ca^{2+} does not affect Al uptake mediated by Nrat1 at least in yeast.

Previous results have showed that Nrat1 transports trivalent Al ion, but not other Al forms.[6] Presence of divalent metals including Mn and Cd did not affect the Al uptake by Nrat1 in yeast.[6] To examine whether Nrat1 is able to transport other trivalent ions including Yb^{3+} and Ga^{3+}, analogs of Al, we performed a competition experiment between Al and these two trivalent cations. In the presence of equimolar concentration of Yb or Ga, the Al uptake by yeast expressing *Nrat1* was almost inhibited half (**Fig. 1B**). This result indicates that Nrat1 is also able to transport Yb^{3+} and Ga^{3+}. However, considering that Al is the most abundant metal in the earth's crust, it is likely that Nrat1 mainly functions as an Al transporter.

In the yeast system, Nrat1 did not show transport activity for Fe^{2+}, Cd^{2+} and Mn^{2+}.[6] To confirm this result in rice, we compared the uptake of Cd and Mn between wild-type and two independent

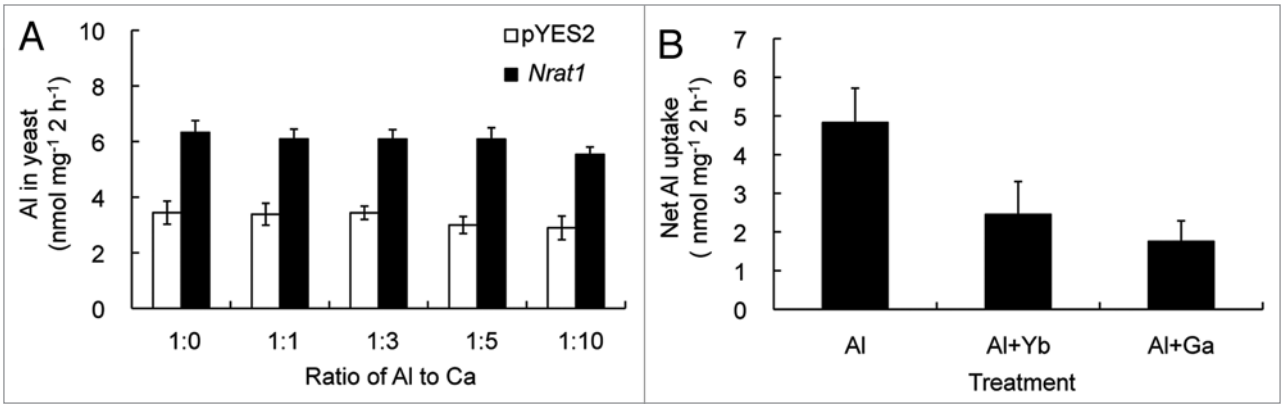

Figure 1. Effect of Ca, Yb and Ga on Al uptake by Nrat1 in yeast. (A) Effect of Ca^{2+} on the transport activity of Al ion by Nrat1. Yeast cells expressing *Nrat1* were exposed for two hours to a solution (pH 4.2) containing 50 μM $AlCl_3$ in the absence or presence of different concentrations of Ca (50, 150, 250 or 500 μM as $CaCl_2$). (B) Effect of Yb and Ga on Al transport activity by Nrat1. Yeast cells expressing Nrat1 were exposed for two hours to a solution containing 50 μM $AlCl_3$ at pH 4.2 in the absence or presence of equal concentration of Yb or Ga. The Al concentration in the yeast was determined by atomic absorption spectrophotometer after digested with 2 N HCl. Data are means ± SD of three biological replicates.

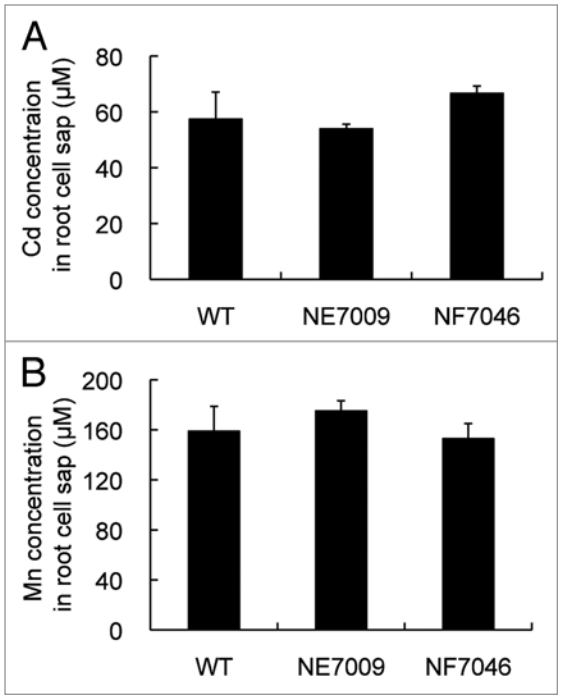

Figure 2. Effect of Nrat1 knockout on transport of Cd and Mn in rice roots. (A and B) Concentration of Cd (A) and Mn (B) in the root cell sap of wild-type rice and two knockout lines of *Nrat1* (NE7009 and NF7046). The roots were exposed to a 0.5 mM $CaCl_2$ solution (pH 4.5) containing 30 μM Cd or 30 μM Mn for eight hours. The concentration of Cd and Mn in the cell sap of root tips (0–1 cm) was determined by atomic absorption spectrophotometer. Data are means ± SD of three biological replicates.

knockout lines of *Nrat1*. Knockout of *Nrat1* significantly resulted in decreased Al uptake into the root cells.[6] In contrast, there were no differences in the concentration of both Cd and Mn in the root-cell sap between wild-type and knockout lines (**Fig. 2A and B**). These results further show that Nrat1 is not capable of transporting divalent metal ions as observed in yeast.

To further confirm that Nrat1 is a transporter of trivalent Al in rice, we overexpressed this gene in rice under control of maize *ubiquitin1* promoter. In two independent transgenic lines, the expression level of *Nrat1* was enhanced by about 9 times under Al treatment condition (**Fig. 3A**). Overexpression of *Nrat1* resulted in increased Al concentration in the root cell sap compared with wild type rice (**Fig. 3B**). However, there was no difference in the Cd concentration of root cell sap between overexpressed and wild-type lines (**Fig. 3C**). These results again indicate that Nrat1 is a transporter for trivalent Al rather than Cd. Overexpression of Nrat1 also resulted in increased Al sensitivity (**Fig. 3D**). Morin staining, an Al specific fluorescent dye, showed that the overexpressed lines exhibited enhanced signal intensity compared with wild-type rice (**Fig. 3E**). Morin can detect Al inside the cells but cannot detect cell wall-bound Al.[9] This result is consistent with Al concentration in the root cell sap of overexpressed lines (**Fig. 3B**). Therefore, increased Al sensitivity in the *Nrat1*-overexpressed lines is likely caused by a high Al concentration in the cytosol due to enhanced Al uptake (**Fig. 3B**). Aluminum entering into the cells may be detoxified by chelation with organic acid anions and/or sequestration into the vacuoles.[4] An ABC transporter ALS1 in Arabidopsis has been suggested to be involved in sequestration of Al into vacuoles.[10] There is a homolog of ALS1 in rice, which also has been suggested to be involved in Al tolerance[5] although the function of these genes have not been characterized in both Arabidopsis and rice. In the overexpressed

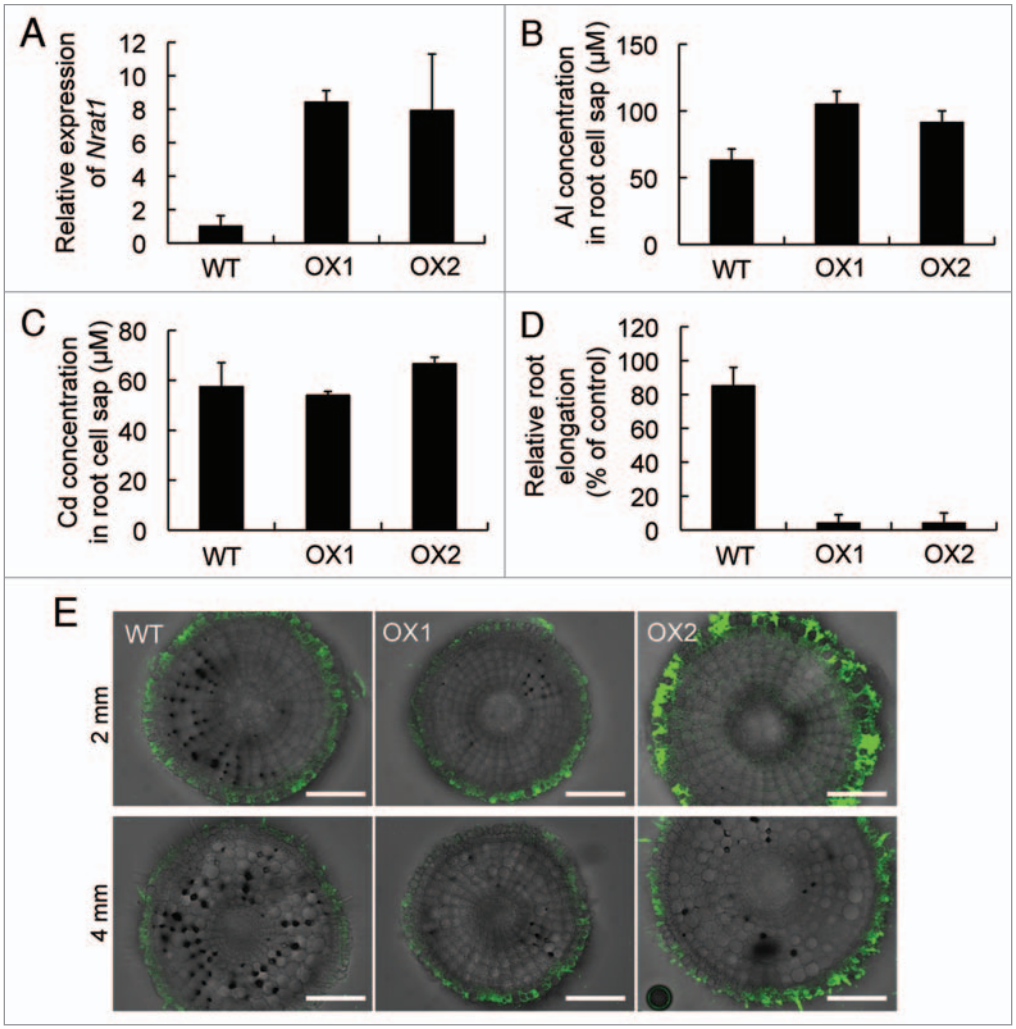

Figure 3. Charaterization of Nrat1-overexpressed lines. (A) Expression of *Nrat1* in two independent *Nrat1* overexpressed lines. Both wild-type rice and overexpressed lines (OX1 and OX2) were exposed to a 0.5 mM $CaCl_2$ solution (pH 4.5) containing 30 μM $AlCl_3$ for six hours. The expression level of *Nrat1* in the roots were determined by quantitative real-time PCR and *Histone H3* was used as an internal standard. Expression relative to the wild-type rice is shown. Data are means ± SD (n = 3). (B and C) Concentration of Al (B) or Cd (C) in the root cell sap of wild-type rice and two Nrat1 overexpressed lines (OX1 and OX2). The roots were exposed to a 0.5 mM $CaCl_2$ solution (pH 4.5) containing 30 μM Al or 30 μM Cd for eight hours. The concentration of Al or Cd in the cell sap of root tips (0–1 cm) was determined by atomic absorption spectrophotometer. Data are means ± SD of three biological replicates. (D) Al sensitivity of Nrat1 overexpressed lines. Seedlings of wild-type rice (WT) and two Nrat1 overexpressed lines (OX1 and OX2) were exposed to a 0.5 mM $CaCl_2$ solution (pH 4.5) containing 30 μM Al for 24 hours. The root length was measured before and after the treatment and the elongation relative to the root growth without Al was shown. Data are means ± SD (n = 8). (E) Morin staining of Nrat1 overexpressed lines. Seedlings of wild-type and the overexpressed lines were exposed to a 0.5 mM $CaCl_2$ solution (pH 4.5) containing 30 μM Al for 24 hours and then stained with 0.1% Morin (green). Roots were cross-sectioned by hands. Scale bar = 100 μm.

line, these capacities for final detoxification were not enhanced simultaneously, resulting in toxic level of Al in the cytosol. It will be interesting to enhance both Nrat1 and internal detoxification capacity in the future.

In conclusion, our findings further demonstrated that Nrat1 is an influx transporter for trivalent Al. It remains to be examined whether similar transporters are present in other plant species and also other organisms.

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