Title page

Identification of structural elements involved in fine-tuning of the transport activity of the rice ammonium transporter *OsAMT1;3*

Dongli Hao^{1,2}, Shunying Yang¹, Yanan Huang^{1,2}, Yanhua Su^{1,*}

1 State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China

2 University of Chinese Academy of Sciences, Beijing 100049, P.R. China

* Corresponding author, Tel: +86 25 86881529, Fax: +86 25 86881000, E-mail: yhsu@issas.ac.cn

Abstract

Ammonium transporters (AMTs) are major routes for plant uptake of the NH_4^+ -form nitrogen. Plant AMTs mediate predominantly the uptake of NH_4^+ and to a lesser extent, its organic analog methylammonium (MeA^+). Mutagenesis studies on potential phosphorylation residues have achieved solid recognition that alteration of the phosphorylation status can result in allosteric regulation and impair the functionality of plant AMTs. However, molecular insights to the fine-tuning of a functional ammonium transporter remain less clear. In this report, we demonstrate that the rice root expressed *OsAMT1;3* (*Oryza sativa* ammonium transporter 1;3) functions as a typical high-affinity NH_4^+ transporter and is weakly permeable to MeA^+ using growth assays in NH_4^+ uptake defective yeast cells and electrophysiological measurements in *Xenopus* oocytes. Upon screening of six point mutations generated with the transporter, we identified two amino acid residues involved in the functional modulation of *OsAMT1;3*. The H199E mutation caused loss of transport activity whereas other five mutations retained the functionality of *OsAMT1;3*. Furthermore, the L56F mutation enabled respectively 5- and 3.5 -fold increased capability for NH_4^+ and MeA^+ uptake with several-fold decreased affinity (K_m) and accelerated V_{max} values. Surprisingly, yeast cells expressing the L56F mutation shown increased Na^+ toxicity leading to a speculation that enhanced Na^+ permeation occurred with this mutation. The phenomenon was further supported by the observation of significant Na^+ uptake current in oocytes. Our results seemingly support a speculation that the L56F mutation of *OsAMT1;3* widens the substrate passage tunnel and allows enhanced permeability to NH_4^+ and larger ions MeA^+ and Na^+ .

Keywords *OsAMT1;3*; functional modulation; point mutations; substrate permeability.

1. Introduction

Plants acquire NH_4^+ through two systems: the high affinity transport system (HATS) and low affinity transport system (LATS) (Wang et al., 1993), respectively according to the concentration range of the

ion available to the plants. The HATS contributes to NH_4^+ uptake under physiological conditions (within the micro-molar range) and are composed of transporters of the AMT/MEP/Rh (Ammonium Transporter/Methylammonium Permease/Rhesus) family (Loque et al., 2009; Ludewig et al., 2007; von Wirén and Merrick et al., 2004). Three AMT1 subfamily ammonium transporters in *Arabidopsis* confer ~ 90% of the total NH_4^+ uptake (Yuan et al., 2007), suggesting a crucial role of this clade in the acquisition of NH_4^+ -form nitrogen. Therefore, the AMTs may be particularly important for plants (for instance, rice) inhabited in NH_4^+ -predominant environment. Plant AMTs are highly selective for NH_4^+ and to a much lesser extent, permeable to its organic analog methylammonium (MeA^+) with larger ionic radius (Ludewig et al., 2002; Neuhauser et al., 2007; Ortiz-Ramirez et al., 2011; Sogaard et al., 2009; Wood et al., 2006; Yang et al., 2015). Considerable permeation to other monovalent ions (e.g. Na^+ , K^+) has been even reported for the human ammonium transporter RhCG. The expression of RhCG in oocytes resulted in significant Na^+/K^+ associated currents, while the other ammonium transporter *Le*AMT1;2 is less permeable to Na^+ . The permeation to Na^+ in RhCG was further confirmed by increased Na^+ toxicity to the alkali metal-sensitive yeast cells (Mayer et al., 2006b).

As demonstrated with the *Arabidopsis* AMT1;1, interactions between the neighboring subunits through the conserved C termini are essential in the formation of a functional trimer (Loque et al., 2007). The transition between the phosphorylation / dephosphorylation status of the conserved T460 may result in an allosteric regulation to the trimer that subsequently leads to the activation / inactivation of the transporter (Loque et al., 2007; Neuhauser et al., 2007). Additionally, mutations to the highly conserved Gly by Asp (e.g. G458D in *Le*AMT1;1, G465D in *Le*AMT1;2, G468D in *At*AMT1;2) impair the transport activity of the resultant AMTs (Ludewig et al., 2003; Neuhauser et al., 2007). The crystal structure of the bacterial AmtB provides a fundamental model for understanding of the mechanisms of substrate transport through AMTs (Javelle et al., 2008; Khademi et al., 2004 ; Zheng et al., 2004). Trimerized AMT subunits form a channel-like structure accommodating the passage of substrates. At the initial step, NH_4^+ ions bind to the outer mouth constructed by W148 and S219, then transport across a hydrophobic filter contributed by F107 and F215. The two histidines, H168 and H318 confine the central section of the pore and allow the passage of permeable substrates (NH_4^+ or its deprotonation species, also includes MeA^+). In case of AmtB, which is thought to be a NH_3 channel, the NH_3 molecules, upon passing through the narrow pore, receive protons (re-protonation, or NH_4^+ “recruitment”) at the cytoplasmic inner face of the entryway (Javelle et al., 2008; Khademi et al., 2004 ; Zheng et al., 2004).

Plant AMTs are thought to engage similar mechanisms as the bacterial AmtB. With many plant AMTs, since NH_4^+ -induced currents are readily observed by electrophysiological measurements, hence they do not likely adopt the transition between NH_4^+ ion and its gas form while passing through the pore, *i.e.* a mechanism of NH_4^+ uniporting (Ludewig et al., 2002; Yang et al., 2015).

In addition to the identification of key residues involved in the formation of a functional AMT, amino acids within the pore regime could contribute to tune the transport activity. In *At*AMT1;1, the Q57H mutation causes ‘channel-like’ uptake of NH_4^+ with a K_m in the millimolar concentration range (approximately 100 folds that of the wild type transporter) and 10-fold accelerated $V_{\max}$ (Loque et al., 2009). However, the uptake of MeA^+ is significantly reduced (Loque et al., 2009). The wheat *Ta*AMT1;1 has been demonstrated to function as an acid-stimulated, NH_3/H^+ co-transporter (Sogaard et al., 2009). In *Ta*AMT1;1, the Leucine56 is reported to be essential for its pH sensitivity (Sogaard et al., 2009). Substitution of this L56 with phenylalanine (L56F) abolishes the pH regulation of *Ta*AMT1;1 and results in 2- to 2.5-fold greater currents induced by NH_4^+ and MeA^+ . Nonetheless, significant

permeability has not been observed for K^+ or Na^+ (Sogaard et al., 2009). Therefore, the effects of L56F in *TaAMT1;1* is supportive for a contribution to the proton-involved (e.g. via recruitment) substrate exit to the cytoplasmic face (Sogaard et al., 2009).

Two histidine residues, for instance H168 and H318 of *EcAmtB* are conceived to confine the narrow section of the pore. Mutation of the first histidine to glutamic acid resulted in accelerated V_{max} and decreased K_m for MeA^+ in *EcAmtB* and *ScMep2* (Boeckstaens et al., 2008). And the H/E mutations at this position of both transporters resulted in more efficient growth restoration of the NH_4^+ uptake deficient yeast strain 31019b under limited NH_4^+ supply, suggesting enhanced ammonium transport by the mutations (Boeckstaens et al., 2008). Similar mutation of the corresponding first histidine to glutamic acid in *PvAMT1;1* caused increased transport activity for NH_4^+ , but abolished transport of MeA^+ (Ortiz-Ramirez et al., 2011). Therefore, the role of the first pore-confining histidine seems divergent among AMTs of different origins.

Rice inhabits in a particular environment where NH_4^+ remains as the predominant form of nitrogen nutrient. In this respect, ammonium transporters are thoughtful critically important for root absorption of nitrogen in rice. Since the functionality of AMTs has been addressed for many plant species, in this report, we investigate the functional modulation of the root-specific *OsAMT1;3* in rice (Bao et al., 2015; Ferreira et al., 2015; Gaur et al., 2012; Sonoda et al., 2003a), with particular interest in the molecular insights that remain less clear. This allows the identification of the indispensable role of H199 (corresponding to the first pore-confining histidine) in the function of *OsAMT1;3*. More interestingly, our results indicate that the L56F mutation causes not only a drastic enhancement of NH_4^+ uptake in *OsAMT1;3*, but also significantly increased permeations to MeA^+ and Na^+ ions of larger hydrated radii. Along the descending order of the diameters of substrate ions (Na^+ , MeA^+ and NH_4^+) in the aqueous solution, the permeability increases accordingly, seemingly supports a “wider” pore may be resulted from the substitution of the L56 by the aromatic amino acid phenylalanine and allows increased passage of less permeable ions. And back to the wildtype *OsAMT1;3*, the conservation of 56L might adopt a restrictive control in rice root to avoid excess acquisition of NH_4^+ , since the uptake of NH_4^+ has been demonstrated to be feedback inhibited by the substrate ions (Sonoda et al., 2003b; Yang et al., 2015).

2. Materials and Methods

2.1. Plasmid Constructs

The open reading frame (ORF) of *OsAMT1;3* (LOC_Os02g40730.1) was amplified from the rice (*cv. Nipponbare*) cDNA library by high fidelity PCR. The primers used were as the following (5' to 3'): GTCGGTACCATGGCAACGTGCGCGGATACC and GTCGCGGCCGCCTAGACATGGCCTCCCATCTC. After verification by sequencing, the ORF was subcloned into the oocyte expression vector pCI and yeast expression vector pYES2, respectively. Point mutations were constructed by overlap extension PCR and subcloned into the oocyte and yeast expression vectors. All PCR products were verified by sequencing.

2.2. Expression in Yeast

Transformation and growth assays of yeast mutant strain 31019b defective in NH_4^+ uptake were

carried out as previously reported (Yang et al., 2015). For growth assays, yeast cells were precultured in liquid YNB medium supplemented with 2% galactose and 2 mM arginine at 30°C until the OD₆₀₀ reached 0.5–0.6. Cells were then harvested and resuspended in sterile water to an OD₆₀₀ of 2.0. Five microliters of cell suspension with serial dilutions (factor of 10) were spotted on the surface of solidified media. For functional complementation and NH₄⁺ toxicity assays, NH₄⁺ (as NH₄Cl or (NH₄)₂SO₄) was used as the sole nitrogen source supplemented to the YNB (without ammonium sulphate) plates at desired concentrations. The media were buffered with 50 mM MES/Tris, pH 4.8 (Loque et al., 2009). A pilot test indicated that the addition of buffering reagent to the media had no effect on yeast growth. For MeA⁺ and Na⁺ toxicity tests, chemicals of desired concentrations were supplemented to YNB media buffered with 50 mM MES/Tris, pH 6.0 or 4.8 (see figure legends). Photos were taken 3 days after spotting.

2.3. Electrophysiological Measurements in Oocytes

Preparation, injection and recordings of oocytes were performed as previous reported (Yang et al., 2015). In brief, oocytes were micro-injected with 59.8 ng of plasmid DNA containing the wildtype *OsAMT1;3* or the L56F point mutation. The recording solution contained (in mM): 100 NaCl, 2 CaCl₂, 2 MgCl₂, 4 Tris (pH adjusted to 7.4 with MES). NH₄⁺ or MeA⁺ (as chloride salt) was added into the solution as indicated. For the evaluation of Na⁺ elicited currents, the major salt component NaCl in the bath solution was replaced by CholineCl, and NaCl was added at desired concentrations. Currents were measured at -70 mV with integrated voltage ramps (-160 mV to + 20 mV in 1.5 seconds, with long holding phase at -70mV) to obtain I-V relationships of peak currents. Upon continuous application of the voltage ramps, currents were measured before, during and upon the withdrawal of the substrate (enabled by perfusion) at the holding epochs (the ‘U’ shaped current traces, see for instance Fig. 4A). This recording strategy is referred as ‘washout’, with which highly convincing current measurements are obtained under visible baseline controls (see Fig. 4A). Instead of a current measurement at a given voltage, the continuous ramp allows the capture of peak and baseline currents at defined range of voltages. In agreement with previous study, oocytes injected with equal amount of water were insensitive to 1 mM NH₄⁺ or 10 mM MeA⁺ (Mayer et al., 2006a; Yang et al., 2015).

2.4. Sequence and Data Analysis

Amino acid sequence alignment and similarity analyses among AMT_S were processed with DNAMAN 6.0. The transmembrane regions prediction was taken with the TMPred (http://www.ch.embnet.org/software/TMPRED_form.html). The yeast growth assays were repeated three times. Electrophysiological data were collected from at least 3 oocytes (in most cases, n>4) from different frogs. Data shown are means ± SE. Graphs and statistical results (t-test) were performed with Sigmaplot 10.0.

3. Results

3.1. *OsAMT1;3* Mediates NH₄⁺ and MeA⁺ Uptake in Yeast Strain 31019b and Oocytes

To obtain the functional properties of rice *OsAMT1;3*, we conducted parallel experiments in both

yeast and *Xenopus* oocyte expression systems. The yeast strain *31019b* is deficient in ammonium uptake on a medium containing less than 5 mM NH_4^+ as sole nitrogen source (Marini et al., 1997). In agreement with this notion, no growth was observed under limited NH_4^+ source with yeast cells transformed with the empty pYES2 plasmid, whereas growth of *OsAMT1;3* transformed cells was restored (Fig. 1a), indicating that *OsAMT1;3* confers a capability of NH_4^+ uptake. Methylammonium (MeA^+) is a widely used analog for NH_4^+ . Addition of 100 mM MeA^+ to the non-selective medium (supported by 2 mM Arg as nitrogen source) did not affect the growth of empty plasmid transformed cells, whereas the growth of *OsAMT1;3*-expressing cells were inhibited (Fig. 1b), implying toxicity by uptake of MeA^+ may occur. The uptake of this toxic analog was further confirmed by the observation of MeA^+ -induced currents in oocytes. Upon perfusion with 1 mM NH_4^+ , significant inward currents were recorded with the *OsAMT1;3* expressing oocytes (Fig. 1c-d). A return to the same concentration of NH_4^+ produced comparable scale of amplitude, indicating the recordings were stable and the NH_4^+ -induced current was reproducible (Fig. 1c, left). With the integrated voltage ramps (-160 to $+20$ mV), the current-voltage relationship could be obtained under the stability control of the “washout” at -70 mV (Fig. 1c, left). NH_4^+ -induced currents were thus obtained at corresponding voltages by subtracting baselines (i) from the peak (ii) currents (Fig. 1c, right). At 1 mM concentrations, the NH_4^+ -induced currents were 5- to 6-fold greater than that induced by MeA^+ (Fig. 1c, left, washout at -70 mV; Fig. 1d, statistical summary of currents measured at -140 mV with integrated voltage ramp). These results were in agreement with most AMTs reported so far, and indicated poor permeability of the larger radius analog MeA^+ at low concentration range.

3.2. Functional Screening of Point Mutations of *OsAMT1;3*

Trimerized AMT subunits form a functional protein (Khademi et al., 2004; Zheng et al., 2004). Within the central regime of the substrate recognition filter along the “channel-like” entryway and the cytoplasmic exit face, AMTs of various origins are normally considered to engage similar conformation as established with the bacterial EcAmtB (Khademi et al., 2004; Zheng et al., 2004). This model greatly facilitates the designing and interpretation of the functional roles of key amino acid residues aligned in the regime. Table S1 summarizes recent progress in the assessments of amino acids contributed to the transport functions of plant and microbial AMTs. Generally, the highly conserved “T” and “G” residues located to the C-termini were assigned as playing dominant roles for the functionality of the transporters, since mutations to these residues caused loss of transport activity (Table S1; Fig. 2a-b). Because the objective of the present work is to explore the molecular elements that tune the transport activity of *OsAMT1;3*, these “dominant” residues were not repeatedly tested.

As illustrated in Fig. 2c, the amino acid “L” (framed) is conserved among plant AMTs reported. This amino acid is predicted to locate to the cytoplasmic exit of the central substrate entryway, taking equivalent position as the 31F of the resolved crystal structure of EcAmtB (Khademi et al., 2004). Furthermore, in the EcAmtB model, two histidine residues, H168 and H318 confine the narrow section of the substrate-entrance pore (Khademi et al., 2004), of which, the first histidine (Fig. 2d, framed) plays a divergent role in AMTs of different origins (Table S1). Therefore, we generated a point mutation to this histidine (H199E) and analyzed its impact to the transport activity of *OsAMT1;3*. Within the non-transmembrane motifs, we also chose other 4 amino acids of *OsAMT1;3* for mutagenesis analyses based on the uniqueness or similarity to other plant AMTs (Fig. 2e, framed). Some of these amino acids were reported to play a role in regulating the Arabidopsis AMT1;2 (Table S1).

The effects of the above six point mutations were tested by growth assays in the ammonium uptake deficient yeast strain 310190b. Previously reported *OsAMT1;1* (Yang et al., 2015) was used as a positive control. Under limited NH_4^+ (3 mM), the R230K, L257T, E384K, R406K and L56F mutations shown growth restoration of the yeast cells comparable to that of the wildtype *OsAMT1;3* (Fig. 3a, left), suggesting the NH_4^+ uptake activity was retained. Whereas the H199E mutation did not support the growth of yeast cells (Fig. 3a, left), leading to a possibility of loss of NH_4^+ uptake activity. This was further supported by the NH_4^+ toxicity tests. Conversely, when extremely high concentration of NH_4^+ (500 mM) was supplied to the yeast growth plates, efficient uptake of NH_4^+ by the expression of the wildtype or mutated *OsAMT1;3* was anticipated to cause toxic effects due to excessive accumulation of NH_4^+ and consequently inhibit the growth of yeast cells. The R230K, L257T, E384K and R406K mutations shown similar growth reduction as the wildtype *OsAMT1;3*, indicating that these mutation did not significantly affect the function of the transporter (Fig. 3a, right). However, cells expressing the H199E mutation shown indistinguishable growth as did the empty vector, further suggesting that the H199E mutation did not confer complementary NH_4^+ uptake capability to the yeast cells. To this end, failure of growth restoration of yeast transformants under limited NH_4^+ and insensitivity to high concentration NH_4^+ toxicity provide that the H199E mutation results in loss of function in *OsAMT1;3*. It should be noted that *OsAMT1;1* transformed cells were less sensitivity to toxic concentration of NH_4^+ (Fig. 3a, right). Our previous work demonstrated that the NH_4^+ uptake activity of *OsAMT1;1* was strongly feedback inhibited by the accumulation of the ion, thus toxic effect could be reduced.

3.3. L56F Mutation in *OsAMT1;3* Increases NH_4^+ and MeA^+ Toxicity in Yeast

Among the five mutations that retained the uptake function of *OsAMT1;3* (Fig. 3a, left), the L56F mutation resulted in an interesting effect. When subjected to toxic concentration of NH_4^+ (500 mM), the growth of yeast cells transformed with the L56F mutation were clearly inhibited as compared with the wildtype *OsAMT1;3* transformed cells (Fig. 3a, right-bottom), providing an implication that enhanced uptake of toxic substrate may occur to the cells. In order to clarify the growth inhibition was caused whether by over-accumulation of NH_4^+ or by the toxicity of Cl^- ions, same concentrations of NH_4^+ (500 mM) were tested as either chloride or sulphate salts. As shown in Fig. 3b, either form of NH_4^+ salt led to similar growth inhibitions, suggesting the inhibitory effect is mainly due to an increased acquisition of NH_4^+ . The L56F mutation markedly strengthened the toxic symptom. Further growth tests indicated that the L56F mutation-expressing cells were more sensitivity to high concentrations of MeA^+ and shown increased extent of growth inhibition (Fig. 3c, in particular, at 50 and 100 mM concentrations), providing another implication that the L56F mutation also facilitated the uptake of this toxic analog. Therefore, further studies were then focused on the L56F mutation.

3.4. The Mutant *OsAMT1;3*-L56F Mediates Increased NH_4^+ and MeA^+ Transport in Oocytes

To confirm the seemingly facilitated uptake of NH_4^+ and MeA^+ with the L56F mutation, we conducted electrophysiological evaluation in another heterologous expression system—the *Xenopus* oocytes. Along the increase of NH_4^+ concentrations, gradually increased inward currents were recorded in *OsAMT1;3* expressing oocytes (Fig. 4a-b). As compared with the wildtype transporter, the current amplitudes recorded in the L56F mutant-injected oocytes were 3- to 5-fold greater under higher concentrations of NH_4^+ (Fig. 4a-b, 400 and 1000 μM). In consistent with the toxic effects observed in

yeast cells, mM ranges of MeA^+ elicited 2- to 3-fold increased currents in the L56F mutant-expressing oocytes (Fig. 4c-d), providing a direct support that significantly enhanced uptake of MeA^+ is associated with the mutant. To this end, markedly enhanced NH_4^+ and MeA^+ uptake activities were observed with the L56F mutation of *OsAMT1;3*.

NH_4^+ -induced currents in both the wildtype *OsAMT1;3* and the L56F mutant showed typical saturable responses to micro-molar concentrations of the substrate (Fig. 5a); whereas, MeA^+ -induced currents were not saturated at the low millimolar concentration ranges (10 mM and below, Fig. 5b). Deduced from the Michaelis-Menten fitting, the L56F mutation strongly accelerated the transport of both substrates (Fig. 5a-b). The maximal currents V_{max} values of NH_4^+ uptake currents were -58.8 ± 0.4 nA and -92.9 ± 0.4 nA calculated respectively at -90 and -140 mV with the wildtype *OsAMT1;3*. The V_{max} of L56F mutation increased to approximately 7-fold values that of the wildtype at either voltage: -430.4 ± 5.7 nA at -90 mV and -648.8 ± 10.4 nA at -140 mV, respectively (Fig. 5a). The V_{max} values of MeA^+ induced currents increased similar-fold with L56F mutant, when compared with those of the wildtype *OsAMT1;3* (Fig. 5b): for the wildtype transporter, the V_{max} were -108.2 ± 2.4 nA and -163.1 ± 3.1 nA, evaluated at -90 mV and -140 mV respectively. These values augmented to -822.7 ± 170.0 nA and -1242.5 ± 44.6 nA respectively for the L56F mutant. Additionally, the K_m values of both NH_4^+ and MeA^+ showed, to certain extents, voltage dependency, with larger values at less negative voltages (Fig. 5c-d). The L56F mutation resulted in 5- to 6-fold K_m values as of the wildtype for NH_4^+ and 2- to 3-fold for MeA^+ induced currents, respectively (Fig. 5c-d).

3.5. The L56F Mutation Enables Increased Permeation to Na^+

The above results demonstrate that strongly enhanced NH_4^+ and MeA^+ permeability is associated with the *OsAMT1;3*-L56F mutation. To further test whether this mutation also facilitates the permeation to other monovalent ions, we re-examined the growth of yeast transformants in the presence of high concentrations of NaCl. On the non-selective medium supported by 2 mM Arg as nitrogen source, the presence of 200 mM NaCl inhibited the growth of all yeast transformants with a more severe symptom with the cells expressing the L56F mutant (Fig. 6a). The Na^+ toxicity was therefore particularly examined with the L56F mutant using gradients of NaCl concentrations. The growth of the L56F transformants was clearly inhibited by 500 mM NaCl under non-restricted nitrogen supply (2 mM Arg, Fig. 6b), speculating a Na^+ toxicity may occur to the cells (since Cl^- was shown not cause a toxic effect on the cells, see Fig. 3b). Under limited nitrogen source (3 mM NH_4Cl), the toxicity effect occurred with lower concentration of NaCl (200 mM, Fig. 6c). This phenomenon led to a speculation that the L56F mutation may facilitate the permeability of Na^+ and attribute to the strengthened Na^+ toxicity to the cells. This speculation was partly supported by the observation of significantly increased Na^+ elicited currents at high concentration range (100 mM) in oocytes (Fig. 6d). In the low concentration range (for instance, 1 mM), currents elicited by Na^+ were below the detection limit (Fig. 6d). Though at 100 mM concentration, considerable Na^+ elicited current could be recorded with the wildtype transporter, the current measured in the L56F-expressing oocytes were approximately 100% greater than that of the wildtype (Fig. 6d), suggesting enhanced permeability to Na^+ .

NH_4^+ was predominantly transported through the wildtype *OsAMT1;3*, whereas MeA^+ and Na^+ weakly induced 'leak-like' currents when higher millimolar concentrations were imposed to the oocytes, indicating a preferentially selective for the transport of NH_4^+ . However, our results indicated that other ions such as MeA^+ and Na^+ were not absolutely restricted from leakage through the channel-like

structure and resulted in small portions of currents (Fig. 1d, Fig. 6d). This leakage became more evident with the L56F mutation. Also, considering the ascending order of the hydrated radii of the ions ($\text{NH}_4^+ < \text{MeA}^+$ and $\text{NH}_4^+ < \text{Na}^+$), the mutation L56F to *OsAMT1;3* was assumed to result in widening of the channel pore that allowed boosted passage of the preferential substrate NH_4^+ and significant permeable to MeA^+ and Na^+ ions (Fig. 6e).

4. Discussion

Rice is cultivated in paddy soils with water submerged during most of its lifespan. Under these circumstances, NH_4^+ remains as the predominant form of nitrogen supporting the growth of the plant. Therefore, efficient uptake of NH_4^+ through root-expressed AMTs is particularly important for rice. Unfortunately, the AMTs from rice have not received sufficient investigations as compared to other plants that are not obligatory to use NH_4^+ , for instance *Arabidopsis* and tomato. Therefore, the present work employs in parallel, the yeast and the *Xenopus* oocyte expression systems to address the functional characteristics of the root-specific *OsAMT1;3* with an emphasis on the molecular elements that modulate its transport activities.

4.1. *OsAMT1;3* Functions as a high-affinity NH_4^+ Transporter

Rice contains at least 10 AMTs in its genome, of which *OsAMT1;2* and *1;3* are predominantly expressed in the root (Bao et al., 2015; Ferreira1 et al., 2015; Gaur et al., 2012; Li et al., 2012; Sonoda et al., 2003a). A previous report has shown that *OsAMT1;3* was capable of restoring the growth of the NH_4^+ uptake deficient yeast cells under limited NH_4^+ conditions (Sonoda et al., 2003a), suggesting a potential role in NH_4^+ uptake. To obtain detailed insights to the function of *OsAMT1;3*, we carried out parallel analyses in both yeast and the *Xenopus* oocyte expression systems. *OsAMT1;3* mediates a typically high affinity (K_m of 24 μM as determined at -140 mV), electrogenic transport of NH_4^+ (Fig. 1c, Fig. 4a). The affinity for *OsAMT1;3* is approximately 5-fold higher than that of previously reported *OsAMT1;1* (in hundred micromolar range, Yang et al., 2015). The order magnitude of affinity difference between *OsAMT1;3* and *OsAMT1;1* might reflect mechanism facilitations in rice root to cope with variations of NH_4^+ availability in soils.

4.2. The Dominant Effect of the H199E Mutation on *OsAMT1;3* Reflects a Diverse Role of the First pore-confining Histidine among AMTs

The crystal structure of *EcAmtB* (Khademi et al., 2004; Zheng et al., 2004) provides a widely used model for the understanding of the substrate transport mechanisms of plant AMTs. Based on this model and sequence alignments, the H199 in *OsAMT1;3* corresponds to the H168 of *EcAmtB* (Fig. 2d), which is one of the two histidines confining the central section of the pore for the passage of permeable substrates (NH_4^+ or its deprotonation species, also includes MeA^+). The H199E mutation in *OsAMT1;3* eventually caused loss of transport activity (Fig. 3a). We assume a collapse of the substrate-passing tunnel might occur with the H199E mutation and prevents the passage of the substrates (Fig. 6e). It has been demonstrated that mutations to the corresponding histidine residue (summarized in Table S1), for instance H168E in bacterial *EcAmtB* (Boeckstaens et al., 2008), H194E in the yeast *ScMep2* (Boeckstaens et al., 2008) and H211E in common bean *PvAMT1;1* (Ortiz-Ramirez et al., 2011), are

fully functional or even with enhanced transport activity. However, an opposite mutation of the E to H (E181H) in the equivalent site causes loss of function with *ScMep1* (Boeckstaens et al., 2008), an effect similar to the H199E mutation in *OsAMT1;3* presented here. These findings indicate a diverse contribution of this histidine to the functions among the AMT/MEP/Rh transporters.

In case of *OsAMT1;3*, mutations to the loop motif residues R230, L257, E384 or R406 do not significantly affect the function of *OsAMT1;3* (Fig. 2e-f, Fig. 3a, Fig. 6a), suggesting that these sites may be not tightly involved in the formation of a functional structure.

4.3. The L56F Mutation is Associated with Enhanced Substrate Permeability in *OsAMT1;3*

The cytoplasmic located F31 in *EcAmtB* is suggested to play a direct role in the exit of transport species (Yang et al., 2007), whereas as the corresponding site in most plant AMTs is a leucine (L) residue (Fig. 2c). In wheat *TaAMT1;1*, mutation of this L to F (L56F) resulted in tripled NH_4^+ -induced currents with a 3-fold higher K_m value. Meanwhile, the uptake of MeA^+ was also promoted (Sogaard et al., 2009). The augmentation of NH_4^+ and MeA^+ uptake was attributed to the facilitation in the removal of protons at the exit face of the entryway by the aromatic phenylalanine residue (Sogaard et al., 2009). Our data demonstrate that the wildtype *OsAMT1;3* predominantly mediates the uptake of NH_4^+ , and to a several-fold lesser extent, is weakly permeable to its organic analog MeA^+ (Fig. 1c-d, Fig. 4c-d). The replacement of L56 by phenylalanine causes strikingly increased uptake of both NH_4^+ (5-fold) and MeA^+ (3.5-fold) in *OsAMT1;3* (Fig. 4, Fig. 5). The changes in current amplitudes are associated with 6-fold accelerated V_{max} and significantly lowered affinities (4- to 5-fold and 1- to 2-fold) greater K_m values for NH_4^+ and MeA^+ , respectively) for both substrates (Fig. 5). The consequence of L56F mutation with *OsAMT1;3* is therefore similar to reported for *TaAMT1;1*, partly implying that the facilitation of NH_4^+ and MeA^+ permeability resulted from substitution of this highly conserved L by F might occur in a broad range of plant AMTs (see consensus in Fig. 2c).

In addition to the significant enhancement of NH_4^+ and MeA^+ transport, yeast transformants expressing the *OsAMT1;3*-L56F are increasingly sensitive to the toxicity of Na^+ . And in oocytes, this mutation elicits fold increased Na^+ -associated current (Fig. 6d). Although this type of Na^+ induced current is also measurable with the wildtype *OsAMT1;3* when high concentration of NaCl is imposed to the oocytes (Fig. 6d). The enhancement of Na^+ toxicity in yeast cells and significantly increased amplitude of Na^+ current in oocytes are suggestive that strengthened permeability / leakage to Na^+ is occurred with the L56F mutation. Such Na^+ permeability has been also reported in *LeAMT1;1*, *LeAMT1;2* and the human RhCG transporters (Mayer et al., 2006b). However, it should be noted that the permeations to high concentrations of Na^+ do not necessarily support that plant AMTs are not selective for NH_4^+ . At physiological concentration range (1 mM or below), uptake of NH_4^+ is dominant whereas Na^+ does not produce detectable current (Fig. 6d). Therefore, physiologically, the permeability to NH_4^+ could be several tens-fold that of Na^+ in *OsAMT1;3*. However, besides NH_4^+ , several-fold increased uptake of MeA^+ and Na^+ enabled by the L56F mutation is supportive for a role of this amino acid in modulating the substrate permeability of *OsAMT1;3*.

Since the MeA^+ is a methylated NH_4^+ ion, the presence of the additional $-\text{CH}_3$ group is assumed to widen the hydrated ionic radius, which may partially support a significantly weaker permeability of MeA^+ as compared with NH_4^+ . The radius of a hydrated Na^+ (3.58 Å) is larger as compared with NH_4^+ ion (3.31 Å) (Nightingale, 1959), is thus assumed to be less permeable. Seemingly, the observations of strongly facilitated permeability to hydrated ions with larger radii (MeA^+ and Na^+) support a

possibility that the L56F mutation may widen the substrate entrance tunnel and enables markedly enhanced transport of NH_4^+ , MeA^+ and / or the leakage of Na^+ (schematically illustrated in Fig. 6e). Other possibilities for instance, partially compromising of the restrictions for the substrate binding might be also associated with the L56F mutation.

In summary, we analyzed the functional properties of the rice root expressed *OsAMT1;3* with particular emphasis on the molecular mechanisms tuning its transport activity. The H199 site is closely associated with the functional determination of *OsAMT1;3*; whereas the L56 is involved in restricting the magnitude of substrate influxes, mutation of this L to F enables enhanced and expanded substrate permeability through the seemingly widened channel-like substrate passage tunnel. Prospectively, this L56 site could be potentially useful as a target for genetic modification to strengthen the NH_4^+ uptake efficiency in rice root.

Acknowledgments This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDB15030202) and the National Science Foundation of China (Grant No. 31501826 and ISSASIP1609).

References

- Bao, A., Liang, Z., Zhao, Z., Cai, H., 2015. Overexpressing of *OsAMT1-3*, a High Affinity Ammonium Transporter Gene, Modifies Rice Growth and Carbon-Nitrogen Metabolic Status. *Int J Mol Sci.* 16, 9037-9063.
- Boeckstaens, M., Andre, B., Marini, A.M., 2008. Distinct transport mechanisms in yeast ammonium transport/sensor proteins of the Mep/Amt/Rh family and impact on filamentation. *J Biol Chem.* 283, 21362-21370.
- Ferreira, L.M., de Souza, V.M., Tavares, O.C.H., Zonta, E., Santa-Catarina, C., de Souza, S.R., Fernandes, M.S., Santos, L.A., 2015. *OsAMT1.3* expression alters rice ammonium uptake kinetics and root morphology. *Plant Biotechnology Reports.* 9, 221-229.
- Fong, R.N., Kim, K.S., Yoshihara, C., Inwood, W.B., Kustu, S., 2007. The W148L substitution in the *Escherichia coli* ammonium channel AmtB increases flux and indicates that the substrate is an ion. *Proc Natl Acad Sci U S A.* 104, 18706-18711.
- Gaur, V.S., Singh, U.S., Gupta, A. K., Kumar, A., 2012. Understanding the differential nitrogen sensing mechanism in rice genotypes through expression analysis of high and low affinity ammonium transporter genes. *Molecular biology reports.* 39, 2233-2241.
- Javelle, A., Lupo, D., Ripoché, P., Fulford, T., Merrick, M., Winkler, F.K., 2008. Substrate binding, deprotonation, and selectivity at the periplasmic entrance of the *Escherichia coli* ammonia channel AmtB. *Proc Natl Acad Sci U S A.* 105, 5040-5045.
- Khademi, S., O'Connell, J., 3rd, Remis, J., Robles-Colmenares, Y., Miercke, L.J., Stroud, R.M., 2004. Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35 Å. *Science.* 305, 1587-1594.

- Li, S.M., Li, B.Z., Shi, W.M., 2012. Expression Patterns of Nine Ammonium Transporters in Rice in Response to N Status. *Pedosphere*. 22, 860-869.
- Loqué, D., Lalonde, S., Looger, L.L., von Wiren, N., Frommer, W.B., 2007. A cytosolic trans-activation domain essential for ammonium uptake. *Nature*. 446, 195-198.
- Loqué, D., Mora, S.I., Andrade, S.L., Pantoja, O., Frommer, W.B., 2009. Pore mutations in ammonium transporter AMT1 with increased electrogenic ammonium transport activity. *J Biol Chem*. 284, 24988-24995.
- Loqué, D., Yuan, L., Kojima, S., Gojon, A., Wirth, J., Gazzarrini, S., Ishiyama, K., Takahashi, H., von Wiren, N., 2006. Additive contribution of AMT1;1 and AMT1;3 to high-affinity ammonium uptake across the plasma membrane of nitrogen-deficient Arabidopsis roots. *Plant J*. 48, 522-534.
- Ludewig, U., Neuhauser, B., Dynowski, M., 2007. Molecular mechanisms of ammonium transport and accumulation in plants. *FEBS Lett*. 581, 2301-2308.
- Ludewig, U., von Wiren, N., Frommer, W.B., 2002. Uniport of NH_4^+ by the root hair plasma membrane ammonium transporter *LeAMT1;1*. *J Biol Chem*. 277, 13548-13555.
- Ludewig, U., Wilken, S., Wu, B., Jost, W., Obrdlik, P., Bakkoury, M.E., Marini, A.M., Andre, B., Hamacher, T., Boles, E., von Wirén, N., Frommer, W.B., 2003. Homo- and hetero-oligomerization of ammonium transporter-1 NH_4^+ uniporters. *J Biol Chem*. 278, 45603-45610.
- Marini, A.M., Soussi-Boudekou, S., Vissers, S., Andre, B., 1997. A family of ammonium transporters in *Saccharomyces cerevisiae*. *Mol Cell Biol*. 17, 4282-4293.
- Mayer, M., Dynowski, M., Ludewig, U., 2006a. Ammonium ion transport by the AMT/Rh homologue *LeAMT1;1*. *Biochem J*. 396, 431-437.
- Mayer, M., Schaaf, G., Mouro, I., Lopez, C., Colin, Y., Neumann, P., Cartron, J.P., Ludewig, U., 2006b. Different Transport Mechanisms in Plant and Human AMT/Rh-type Ammonium Transporters. *J Gen Physiol*. 127, 133-144.
- Neuhauser, B., Dynowski, M., Mayer, M., Ludewig, U., 2007. Regulation of NH_4^+ transport by essential cross talk between AMT monomers through the carboxyl tails. *Plant Physiol*. 143, 1651-1659.
- Neuhauser, B., Ludewig, U., 2014. Uncoupling of ionic currents from substrate transport in the plant ammonium transporter *AtAMT1;2*. *J Biol Chem*. 289, 11650-11655.
- Nightingale, E.R., 1959. Phenomenological theory of ion solvation. Effective radii of hydrated ions. *J Phys Chem*. 63, 1381-1387.
- Ortiz-Ramirez, C., Mora, S.I., Trejo, J., Pantoja, O., 2011. *PvAMT1;1*, a highly selective ammonium transporter that functions as H^+/NH_4^+ symporter. *J Biol Chem*. 286, 31113-31122.
- Sogaard, R., Alsterfjord, M., Macaulay, N., Zeuthen, T., 2009. Ammonium ion transport by the AMT/Rh homolog *TaAMT1;1* is stimulated by acidic pH. *Pflugers Arch*. 458, 733-743.
- Sonoda, Y., Ikeda, A., Saiki, S., von Wirén, N., Yamaya, T., Yamaguchi, J., 2003a. Distinct expression and function of three ammonium transporter genes (*OsAMT1;1-1;3*) in rice. *Plant Cell Physiol*. 44, 726-734.
- Sonoda, Y., Ikeda, A., Saiki, S., Yamaya, T., Yamaguchi, J., 2003b. Feedback regulation of the ammonium transporter gene family AMT1 by glutamine in rice. *Plant Cell Physiol*. 44, 1396-1402.
- von Wirén, N., Merrick, M., 2004. Regulation and function of ammonium carriers in bacteria, fungi, and plants. *Top Curr Genet*. 9, 95-120.
- Wang, M.Y., Siddiqi, M.Y., Ruth, T.J., Glass, A.D.M., 1993. Ammonium Uptake by Rice Roots (II. Kinetics of $^{13}\text{NH}_4^+$ Influx across the Plasmalemma). *Plant Physiol*. 103, 1259-1267.
- Wood, C.C., Poree, F., Dreyer, I., Koehler, G.J., Udvardi, M.K., 2006. Mechanisms of ammonium

- transport, accumulation, and retention in oocytes and yeast cells expressing Arabidopsis *AtAMT1;1*. FEBS Lett. 580, 3931-3936.
- Yang, H., Xu, Y., Zhu, W., Chen, K., Jiang, H., 2007. Detailed mechanism for AmtB conducting $\text{NH}_4^+/\text{NH}_3$: molecular dynamics simulations. Biophys J. 92, 877-885.
- Yang, S., Hao, D., Cong, Y., Jin, M., Su, Y., 2015. The rice *OsAMT1;1* is a proton-independent feedback regulated ammonium transporter. Plant Cell Rep. 34, 321-330.
- Yuan, L., Gu, R., Xuan, Y., Smith-Valle, E., Loqué, D., Frommer, W.B., von Wirén, N., 2013. Allosteric regulation of transport activity by heterotrimerization of Arabidopsis ammonium transporter complexes in vivo. The Plant Cell. 25, 974-984.
- Yuan, L., Loqué, D., Kojima, S., Rauch, S., Ishiyama, K., Inoue, E., Takahashi, H., von Wiren, N., 2007. The organization of high-affinity ammonium uptake in Arabidopsis roots depends on the spatial arrangement and biochemical properties of AMT1-type transporters. Plant Cell. 19, 2636-2652.
- Zheng, L., Kostrewa, D., Berneche, S., Winkler, F.K., Li, X.D., 2004. The mechanism of ammonia transport based on the crystal structure of AmtB of Escherichia coli. Proc Natl Acad Sci U S A. 101, 17090-17095.

Figure legend

Fig. 1 Functional expression of *OsAMT1;3* in yeast strain 31019b and oocytes. Growth of ammonium uptake-deficient yeast 31019b transformed with control plasmid *pYES2*, *OsAMT1;3* on medium with 0.3, 3 mM NH_4^+ (a) or Arg supplemented with 0 or 100 mM MeA^+ (b) as nitrogen source. c, Inward currents (I) activated by 1 mM NH_4^+ or MeA^+ in *OsAMT1;3*- expressing oocytes (left). Right, I-V relationship measured with integrated voltage ramps. The substrate-induced currents were obtained by subtracted i from ii. d, Currents elicited with 1 mM NH_4^+ , 1 mM MeA^+ or return to 1 mM NH_4^+ from oocytes-expressing *OsAMT1;3* at -140 mV. Data shown are means $\pm$ SE (n=4). * Significance at $p < 0.05$ (Student's t-test).

Fig. 2 Amino acid sequence alignments of point mutations documented and involved in the present study. a, positions of functionally dominant T residues documented for Arabidopsis AMT. b, location of another functional controlling G residue among tomato and Arabidopsis AMTs. c, Alignment of amino acid “L/F” located to the cytoplasmic exit of the deduced substrate passage tunnel. In the crystal structure of *EcAmtB*, a “F” amino acid is in the place of “L” and uniquely differed from the rest AMTs reported. d, Alignments shown positions of first “pore-confining” histidines (native E in *ScMEP1* at this position). e, Sequence alignments for choosing R230, L257, E384, R406 for point mutations in *OsAMT1;3*. f, Schematic summary shown positions of point mutations (asterisked) constructed for *OsAMT1;3*. The protein accession numbers are in the following: *TaAMT1;1* (AY525637), *PvAMT1;1* (GQ377869), *OsAMT1;3* (AAL05614.1), *OsAMT1;2* (AAL05613.1), *OsAMT1;1* (AAL05612.1), *LeAMT1;2* (O04161), *LeAMT1;1* (P58905), *AtAMT1;1* (1703292), *AtAMT1;2* (4324714), *AtAMT1;3* (5880355), *AtAMT1;4* (7450345), *AtAMT1;5* (5672513), *EcAmtB* (NP286193), *ScMep1* (P40260), *ScMep2* (P41948).

Fig. 3 Mutation of L56F in *OsAMT1;3* enhances both NH_4^+ and MeA^+ toxicity in yeast mutant 31019b.

a, Growth of yeast cells under 3 or 500 mM NH_4Cl . b-c, Growth of yeast transformants with 500 mM NH_4Cl , 250 mM $(\text{NH}_4)_2\text{SO}_4$ (b) or Arg supplemented with serial concentrations of MeAcl (c). *OsAMT1;1* was used as a positive control (Yang et al., 2015).

Fig. 4 L56F mutation in *OsAMT1;3* enhances uptake for both NH_4^+ and MeA^+ in oocytes. a, Current traces mediated by wild type *OsAMT1;3* or L56F mutant were shown superimposed. Current were recorded at -70 mV with integrated voltage ramps (see “Materials and Methods”) in response to a gradient of NH_4^+ concentrations. b, Summary of NH_4^+ -induced currents in *OsAMT1;3* and L56F. c-d, MeA^+ induced currents from *OsAMT1;3* and L56F expressing oocytes obtained with similar process as a-b. The zero lines indicated the position of 0 nA. * Significance with neighboring data at $p < 0.05$.

Fig. 5 L56F mutation alters transport kinetics of *OsAMT1;3*. a-b, NH_4^+ (a) or MeA^+ (b) transport kinetics for *OsAMT1;3* or L56F analyzed at -90 mV or -140 mV. c-d, Voltage dependence of the K_m values for NH_4^+ (c) or MeA^+ (d). Data are means $\pm$ SE (*OsAMT1;3*, $n=9$; L56F, $n=5$).

Fig. 6 L56F may cause a wider pore that allows enhanced substrate permeability. a, NaCl toxicity test in yeast mutant 31019b. Dilution (1 to 10^{-3}) of yeast transformants were grown under 2 mM Arg or with the presence of 200 mM NaCl. b-c, Dense (dilution factor 1) cells grown under 2 mM Arg (b) or 3 mM NH_4Cl (c) supplemented with series concentrations of NaCl. d, L56F mutation caused significantly increased permeability to Na^+ in oocytes. Inward currents elicited by 1 mM, 100 mM NaCl in L56F and *OsAMT1;3* in the absence of NH_4^+ measured at -140 mV. e, A schematic assumption for the mode-of-action of H199E and L56F. The cartoon was illustrated according to the suggested mode for the *EcAmtB* (Khademi et al., 2004). * Significance between two neighboring data at $p < 0.05$.

Figure

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6