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ORIGINAL ARTICLE

Overexpression of the maize *ZmAMT1;1a* gene enhances root ammonium uptake efficiency under low ammonium nutrition

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Abstract

High-affinity ammonium uptake in maize roots is mainly mediated by AMT1-type ammonium transporters ZmAMT1;1a and ZmAMT1;3, but whether the increased expression of *ZmAMTs* genes is able to enhance ammonium uptake capacity and subsequently improves overall nitrogen use efficiency remains to be elucidated. In this work, *ZmAMT1;1a*-overexpression transgenic maize plants were generated with the elevated levels of transcripts and proteins, and phenotypically analyzed together with wild-type plants grown in nutrient solution under two regimes of ammonium supply. Under low ammonium nutrition (0.04 mM), in relative to wild-type plants, the maize transgenic lines showed an approximately 17% increases in the high-affinity ammonium uptake capacity of roots as revealed by ^{15}N-labeled ammonium influx assay and further contributed to about 7% increases in the total nitrogen uptake at the whole plant level. By contrast, when ammonium was supplied in high amounts (1 mM), wild-type plants expressed higher levels of ZmAMT1;1a, but exhibited a lower ammonium uptake capacity in roots. Furthermore, the transgenic maize line accumulated more amounts of ZmAMT1;1a protein, but did not translate into an enhanced ammonium acquisition, suggesting a possible post-translational down-regulation of ZmAMT1;1a by high ammonium. This study proved the possibility to enhance ammonium acquisition by elevating *ZmAMTs* expression in maize roots and provided an effective transgenic approach on developing high nitrogen use efficient maize cultivars.

Keywords AMT · Ammonium uptake · Nitrogen use efficiency · Transgenic plants · Post-translational regulation · *Zea mays*

Introduction

Nitrogen (N) is quantitatively the most important mineral nutrient for plant growth (Marschner 2012), and N fertilizers are widely used to increase crop production. However, about 50% of the N fertilizer applied to cropping system is not absorbed by plants (Zhang et al. 2013), but lost to the environment contributing to pollution and climate change (Coskun et al. 2017; Gu et al. 2013; Guo et al. 2010). Efforts to reduce fertilizer input and achieve sustainable crop production most recently encompass breeding approaches for N-efficient crop genotypes that can reach higher productivity at reduced N fertilizer input (Fan et al. 2011; Hirel et al. 2007). Breeding of high N use efficient variety is particularly important for the production of maize which, as one of the world's major crops, annually consumes about 16% N fertilizer at the global level (Heffer 2013).

High N uptake efficiency can contribute to overall N use efficiency in plants, and also help prevent N loss to the

Yang Zhao and Zhi Liu contributed equally to this work.

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environment. Plants absorb N from soil mainly in the form of ammonium and nitrate. Although nitrate is dominant in arable land, maize plants can preferentially take up ammonium (Gu et al. 2013), probably due to the faster assimilation of ammonium in roots with better energy conservation. Ammonium nutrition can increase the growth of maize plants in terms of shoot biomass and root branching and extension (Bloom et al. 2002; George et al. 2016; Jing et al. 2012). In addition, root ammonium acquisition can enhance proton extrusion for rhizosphere acidification and subsequently improve phosphorus and iron bioavailability in soils (Hoffmann et al. 2010; Zou et al. 2001). Given the extensive use of ammonium-based fertilizers (urea and anhydrous ammonia) in maize production, planting the proper varieties with improved ammonium uptake capacity is essential for the sustainable agricultural system.

Ammonium uptake in maize root is proposed to be mediated by AMT1-type ammonium transporters ZmAMT1;1a and ZmAMT1;3 that belong to the ammonium transporter/methylammonium permease/rhesus (AMT/MEP/Rh) family (Gu et al. 2013). Both ZmAMTs conferred the high-affinity transport activities for ammonium and localized to the plasma membrane of epidermal cells of maize roots. In addition, their gene expressions are induced by ammonium and revealed high correlations with the high-affinity ammonium influx rates in roots (Gu et al. 2013). Although ZmAMT1;1a and ZmAMT1;3 are most probably the major components for ammonium uptake in the root, their physiological contribution to the overall N uptake and N use efficiency remains a more refined analysis. In rice, considerable attempts have been made to improve ammonium uptake efficiency by overexpression *OsAMT1;1* and *OsAMT1;3* genes, and little successful case was reported (Bao et al. 2015; Ferreira et al. 2015; Hoque et al. 2006; Kosala et al. 2014; Kumar et al. 2006). Whether increasing NUE by genetic modification of AMTs may depend on their elevated expression levels and the transgenic backgrounds or crop species.

In this study, we investigated *ZmAMT1;1a* and *ZmAMT1;3* expression levels in 16 Chinese maize cultivars historically released from the 1930s to the 2000s, and observed the significant increases in new cultivars (hybrids) than old cultivars (OPVs, open-pollination varieties). We then generated the transgenic maize lines overexpressing *ZmAMT1;1a* gene in which the elevated *ZmAMT1;1a* expression could improve ammonium uptake capacity and subsequently contribute to overall N use efficiency under low ammonium condition. Under high ammonium condition, however, higher ZmAMT1;1a protein levels did not result in the enhanced ammonium uptake probably due to the post-translational down-regulation of ZmAMT1;1a. This work provides new insights of ZmAMT1;1a regulatory mechanism as well as the contribution of overall N use efficiency in maize.

Materials and methods

Plant culture

Maize seeds (*Zea Mays* L.), variety B73, and transgenic lines (T_4 homozygous lines) overexpressing the ammonium transporter gene *ZmAMT1;1a* (GRMZM02G175140), driven by a ubiquitin promoter of maize, in variety Hi-II (HA × HB) were used in this study. In addition, 16 maize varieties were selected from Chinese open-pollination varieties (OPVs) and hybrids for evaluation of *ZmAMT1;1a* and *ZmAMT1;3* gene expression. These varieties were historically released from the 1930s to the 2000s in China. Eight OPVs were Dishandahuohuang, Tangshan Bai, Wubaogu, red corn, white teeth (Shandong), golden queen, the British particles, and white teeth (Shanxi). Eight hybrids were Zhongdan 2, Danyu 13, Nongda 60, Nongda 108, Zhengdan 958, P32-22, Xianyu 335, and Zhongnong 99.

Maize seeds were sterilized, using 10% H_2O_2 for 30 min, and germinated on filter paper for hydroponic culture. Seedlings were pre-cultured in deionized water in the dark, and then transferred to modified Murashige and Skoog (MS) medium (Murashige and Skoog 1962), contained: (in mM) 0.1 KH_2PO_4, 0.6 $MgSO_4$, 1 K_2SO_4, 0.5 $CaCl_2$, 0.1 EDTA-Fe, and (in μM) 1.0 H_3BO_3, 0.5 $MnSO_4$, 0.5 $ZnSO_4$, 0.2 $CuSO_4$, and 0.07 Na_2MoO_4. The 4 mM KNO_3 was supplied as the nitrogen source, and for the treatments, 4 mM KNO_3 was replaced by two different concentrations of $(NH_4)_2SO_4$: 0.04 mM (low) and 1 mM (high) according to experimental requirements. The pH of the nutrient solution was adjusted to 5.8, aerated continuously using an electric pump, and renewed every other day. A 14 h/28 °C and 10 h/22 °C day–night rhythm was used in a growth chamber, at a light intensity of 16.7×10^3 lx and 70% humidity.

Quantitative RT-PCR analysis

Total RNA was extracted using Trizol reagent (Takara Bio, Shiga, Japan), and then, 1.5 μg RNA samples were reverse-transcribed into cDNA, with the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio, Shiga, Japan). DNA contamination was verified by PCR (polymerase chain reaction) amplification using the *ZmACT1* intron primers (Supporting Information Table S1). The qPCR (quantitative polymerase chain reaction) reactions were performed using the SYBR Green system (Toyobo, Osaka, Japan). A 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) was used to carry out the three-step PCR procedure. The

primers used in the qPCR analyses are listed in Supporting Information Table S1. To normalize the gene expression, a maize *ZmAlpha-tubulin 4* (*ZmTUB4*) gene was used as the internal control. Primer specificity was confirmed by analysis of the melting curves and the primer efficiencies. Relative expression levels were calculated using mathematical model established by Pfaffl (2001).

Construction of *UBI-ZmAMT1;1a* vector

Transgenic maize lines overexpressed *ZmAMT1;1a* gene were generated by expressing *ZmAMT1;1a* ORF under the control of the *Zea mays* ubiquitin 1 (Ubi) gene promoter (JX947345.1). A modified binary vector *pCAMBIA1301* with the phosphinothricin selection marker was used (An et al. 2014). Using restriction sites *BamH* I and *Spe* I, the DNA fragment containing *CaMV35S* promoter the GUS reporter gene was replaced by the 1497 bp sequence of the *ZmAMT1;1a* ORF (GRMZM2G175140) and 2001 bp sequence of ubiquitin promoter, yielding a destined construct *p1301-UBI-ZmAMT1;1a*.

Maize transformation

The Agrobacterium *tumefaciens* strain EHA105 was transformed with the *p1301-UBI-ZmAMT1;1a* construct using the freeze–thaw method. Single colonies were selected to transform immature embryos of maize hybrid Hi-II using the method adapted from Duncan et al. (1985) and An et al. (2014). The regenerated Hi-II transgenic plants were cross-pollinated with the inbred line B73, and their T_4 homozygote lines or BC_2F_3 lines backcrossed with B73 lines were then selected for the analyses.

Molecular identification of transgenic maize plants

Genomic DNA was isolated from maize plants with Cetyltrimethyl Ammonium Bromide (CTAB) method. The *ZmAMT1;1a* gene fragment in PPT-resistant plants was verified by PCR with *ubi-ZmAMT1;1a*-specific primers (Supplementary Table S1). To analyze the integration of the *ZmAMT1;1a* gene in transgenic plants, southern blot analyses were carried out by the standard procedures as described by Sambrook and Russell (Sambrock and Russel 2001). Total genomic DNA (approximately 100 µg) was digested with *EcoR* I (Takara Bio, Shiga, Japan), purified, and then fractionated on 0.8% agarose gel. After blotted onto a nylon filter (Amersham Biosciences, Buckinghamshire, UK), the membrane was probed with the DIG-11-dUTP labeled *Bar* gene using the Roche™ Labeling System (Roche Molecular Systems, Basel, Swiss Confederation). The *Bar* gene was amplified by PCR using the primers as described in the Supporting Information Table S1. Blots were imaged by exposing to X-ray film (Kodak, Tokyo, Japan).

Protein gel blot analysis

Total microsomal membrane fractions were isolated from maize root tissues as described by Yuan et al. (2007). The tissue of roots was homogenized with a homogenization buffer, and *r* centrifuged at 10,000*g* (Optima L-80 XP Ultracentrifuge, Beckman, CA, USA) for 15 min. The supernatants were filtered through nylon mesh (60 µm) and pelleted by centrifugation at 100,000*g* for 30 min. Microsomal membrane fractions were then suspended in conservation buffer. Bovine serum albumin (BSA) was used as a standard to determine protein concentrations with Bradford protein assay (Bio-Rad, Hercules, CA, USA).

Polyclonal antibodies for ZmAMT1;1a were produced against the peptide sequences at the C terminus of ZmAMT1;1a protein (*n*-EGPRIGRFDHAGRSVALKGHSASLVVLGTFLLWFGWYGFNPGSFTTILKSYGPAGTVHGQWSAVGRTAVTTTLAGSVAALTTLFGKRLQTGHWNVVDVCNGLLGGFAAITAGCSVVEPWAAVICGFVSAWVLIGANALAARFRFDDPLEAAQLHGGCGAWGVLFTGLFARRKYVEEIYGAGRPYGLFMGGGGKLLAAQIIQILVIAGWVSCTMGPLFYALKKLGLLRISADDEMSGMDLTRHGGFAYVYHDEDPGDKAGVGGFMLKSAQNRVEPAAAVAAATSSQV-c) (YouLong Biotech Company, Shanghai, China). Protein of each sample (10 µg) was separated by 10% SDS polyacrylamide gel. After transferred onto PVDF Hybond-P membranes (Amersham, Little Chalfont, UK) by electroblotting (Bio-Rad, Hercules, CA, USA), the membrane was blocked by incubation in TBST with 2% Blocking Reagent (Amersham ECL™ Advance Western Blotting Detection Kit). Incubated the membrane with primary and secondary antibodies, and then detected with ECL detection. To indicate the molecular weights, MagicMark Western Standard (Invitrogen, CA, USA) was used.

^{15}N influx analysis

The influx of ^{15}N-labeled NH_4^+ into roots was measured in 17-day-old hydroponically-grown maize plants which were pre-cultured with 0.04 or 1 mM ammonium as the sole N source. Rinsing the roots of plants in saturated $CaSO_4$ solution for 1 min, followed by incubation of 6 min in the nutrient solution containing 100 µM concentrations of ^{15}N-labeled $(NH_4)_2SO_4$ (99.16 atom% ^{15}N) as the sole N source. Rinsing the roots in saturated $CaSO_4$ solution for another 1 min, roots were harvested and dried. Five mg powder of ground sample was used for ^{15}N analysis by isotope ratio mass spectrometry (*DELTA^{plus} XP*, Finnigan, Thermo, Bremen, Germany).

Results

Expression analysis of *ZmAMT1;1a* and *ZmAMT1;3* in 16 different maize varieties

The previous studies revealed genotypic differences on N use efficiency among maize varieties (Harvey 1939; Mi et al. 2012). The new cultivars generally accumulated more N than the old cultivars, suggesting the improvement of N uptake efficiency by modern breeding processes in USA (Ciampitti and Vyn 2012; Mueller and Vyn 2016). We then examined the expression levels of *ZmAMT1;1a* and *ZmAMT1;3* genes in N-deficient roots of 16 maize varieties historically released from the 1930s to the 2000s in China (Fig. 1). The result showed that gene expression of both *ZmAMT1s* in eight new cultivars (after the 1960s, mainly hybrids) were almost twofolds higher than those in eight old cultivars (before 1960s, mainly OPVs). Given the positive correlation between *ZmAMT1s* gene expression and ammonium uptake capacity in roots (Gu et al. 2013), the elevated *ZmAMT1s* expression seems to likely contribute to the improved N uptake efficiency in modern maize cultivars.

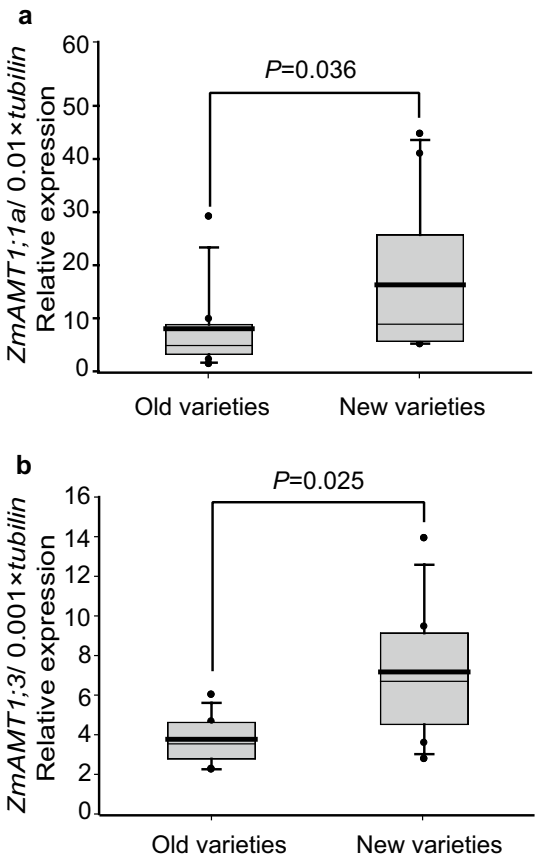

Fig. 1 Higher expression levels of *ZmAMT1;1a* and *ZmAMT1;3* in roots of new maize varieties in relative to old varieties. Sixteen maize varieties released from the 1930s to the 2000s in China were used including eight open-pollination varieties (OPVs) released before the 1960s as old varieties and eight hybrids released after the 1960s as new varieties. Transcripts abundance of *ZmAMT1;1a* (**a**) and *ZmAMT1;3* (**b**) in roots were quantified by qPCR using gene-specific primers. The maize plants were pre-cultured with 2 mM NH_4NO_3 and subject to N starvation for 4 days. The line and square within the box represent the median and mean values of all data, respectively. The bottom and top edges of the box represent 25 and 75 percentiles of all data, respectively. The bottom and top bars represent 5 and 95 percentiles, respectively. *P* values were generated using Student's *t* test to indicate the significant difference between two groups

Generation of transgenic maize plants overexpressing *ZmAMT1;1a*

To verify whether the elevated *ZmAMT1;1a* expression is able to enhance maize N uptake efficiency, the transgenic maize lines overexpressing *ZmAMT1;1a* gene were generated. The transgenic maize lines constitutively expressed *ZmAMT1;1a* ORF under the control of the maize *Ubiquitin* (UBI) promoter (Fig. 2a). After selection with phosphinothricin (PPT), six independent T_0 transgenic events (lines 6-1, -5, -57, -60, -79, and -81) were identified. They were further verified by PCR analysis with an expected amplified DNA fragment of 459 bp, containing the partial *ZmAMT1;1a* ORF (339 bp) and *ubiquitin* promoter sequences (79 bp), and sequence of binary vector *pCAMBIA1301* (41 bp) (Fig. 2b). Southern blot analysis of these lines revealed the visible positive bands corresponding to *Bar* gene fragment with distinct hybridization patterns, indicating their all independent transformation events (Fig. 2c). Four transgenic lines (6-5, -57, -79, and -81) harbored a single copy of T-DNA insertion and other two lines with multiple insertions (6-1 and -60).

Four transgenic lines with single T-DNA insertion were chosen to produce T_4 homozygote progenies and the corresponding wild types (WT). The expression levels of *ZmAMT1;1a* were quantified by real-time PCR in roots of maize plants grown hydroponically under sufficient N condition (4 mM nitrate as N source). Two transgenic lines showed a significantly higher abundance of *ZmAMT1;1a* transcripts compared to WT plants: 14-folds in line 6–79 and 24-folds in line 6–81 (Fig. 3a). To investigate *ZmAMT1;1a* expression at protein level, root microsomal membrane fractions from the same plants were extracted and subjected to protein gel blot analysis using specific antibodies raised against ZmAMT1;1a protein (Fig. 3b). The amounts of ZmAMT1;1a protein in the transgenic lines were remarkably higher than those in WT. In addition, the protein level of ZmAMT1;1a in line 6–81 was about twofolds of that in 6–79, indicating that the overexpressed transcripts were proportionally translated.

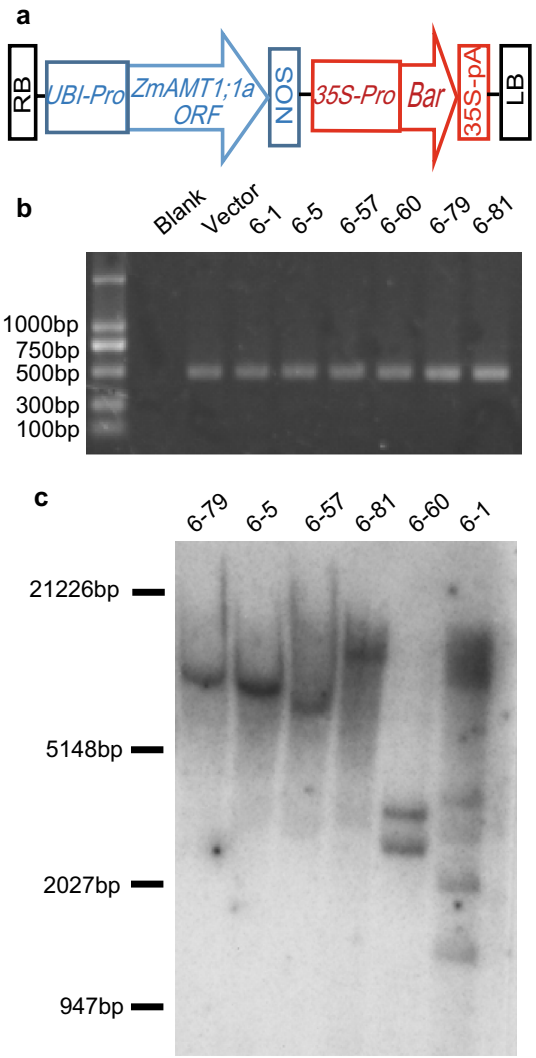

Fig. 2 Generation of transgenic maize lines overexpressing *ZmAMT1;1a* gene. **a** T-DNA structure of the vector *p1301-UBI-ZmAMT1;1a*. *ZmAMT1;1a* gene open reading frame (*ZmAMT1;1a* ORF) and bialaphos resistance gene (*Bar*) were under the control of the ubiquitin (*UBI*) promoter and the *CaMV 35S* promoter, respectively. **b** Screen of putative transgenic maize plants by PCR. Numbers on the left side indicated the length of the amplicon. Blank, negative control; vector, positive control (vector *p1301-UBI-ZmAMT1;1a*); number, each independent transgenic line. **c** Southern blot analysis of transgenic maize lines overexpressing *ZmAMT1;1a*. Genomic DNA was digested with *EcoR*I. Blots were probed with full length of *bar* gene at high stringency. The number on the left side indicated that DNA fraction length revealed by molecular weight markers

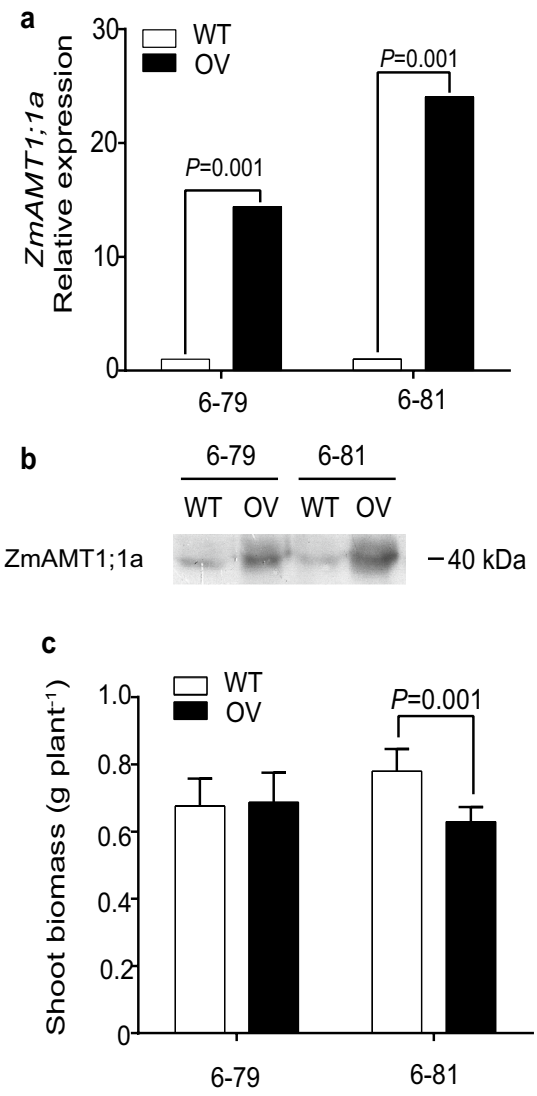

Fig. 3 Elevated expression levels of *ZmAMT1;1a* gene and protein in *ZmAMT1;1a*-overexpression maize lines. Two independent overexpression lines (OV, 6–79 and 6–81) and the corresponding wild types (WT) were cultured hydroponically for 14 days with 4 mM nitrate as sole N source. **a** Transcriptional expression of *ZmAMT1;1a* was quantified by qPCR using gene-specific primers. **b** Protein gel blot analysis of microsomal fractions from maize roots using the specific antibody against ZmAMT1;1a. The detected protein had an apparent size of approximately 40 kD. **c** Shoot biomass of *ZmAMT1;1a*-overexpression lines (OV) and wild types (WT). Bars indicated means ± SD ($n = 9–12$). *P* values were generated using Student's *t* test to indicate the significant differences between WT and OV

However, an unexpected growth depression was observed in line 6–81 as revealed by its significant decrease of shoot biomass than WT (Fig. 3c), probably due to the extremely higher expression level of *ZmAMT1;1a* or the insertion of T-DNA into an essential genomic region. As a result, line 6–79 was chosen for the further study.

Overexpression of *ZmAMT1;1a* enhanced the high-affinity ammonium uptake capacity in roots of transgenic maize under low ammonium supply

As an elevated expression of ZmAMT1;1a in line 6–79, whether the ammonium uptake capacity in roots was

enhanced or not should be valued when ammonium is supplied at low concentrations and represents the major N source. Therefore, we grew line 6–79 together with the corresponding WT plants in nutrient solution supplied with low ammonium (0.04 mM) and high ammonium (1 mM) as sole N source. In WT roots, the expression of *ZmAMT1;1a* was higher (approximately threefolds) under the high ammonium-growing condition at both transcript and protein levels (Fig. 4a, b). Similar to nitrate-grown plants (Fig. 3a), the transcript levels of *ZmAMT1;1a* in line 6–79 were significantly higher (14–25 folds) than those in WT irrespective of ammonium concentrations (Fig. 4a). Again, the overexpressed transcripts were proportionally translated and ZmAMT1;1a protein abundance was much higher in transgenic line 6–79 and even doubled at 1 mM ammonium in comparison with 0.04 mM ammonium supply (Fig. 4b).

To exam potential effects on the ammonium uptake capacity, the same plants were subjected to the short-term influx analysis using ^{15}N-labeled ammonium. Because *ZmAMT1;1a* encodes a high-affinity ammonium transporter, the influx assay was then performed at the external concentration of 200 µM representing the high-affinity transport system for ammonium (Gu et al. 2013). Interestingly, although the less ZmAMT1;1a proteins were expressed in roots of either WT or transgenic plants pre-cultured at 0.04 mM than those at 1 mM ammonium, the high affinity of ammonium uptake capacity almost doubled in roots (Fig. 4c). This may suggest the presence of post-translational down-regulation of ZmAMT1;1a transporter activity by high ammonium. Nevertheless, when plants pre-cultured at low ammonium, the transgenic line 6–79 showed a 17% increase of high-affinity ammonium uptake capacity than WT plants (Fig. 4c). In contrast, no significant difference was detected between two genotypes at high ammonium condition. Thus, we concluded that the elevated expression of *ZmAMT1;1a* could result in an enhanced high-affinity ammonium uptake capacity in roots of maize transgenic plants when ammonium was supplied as the sole N source with a low amount.

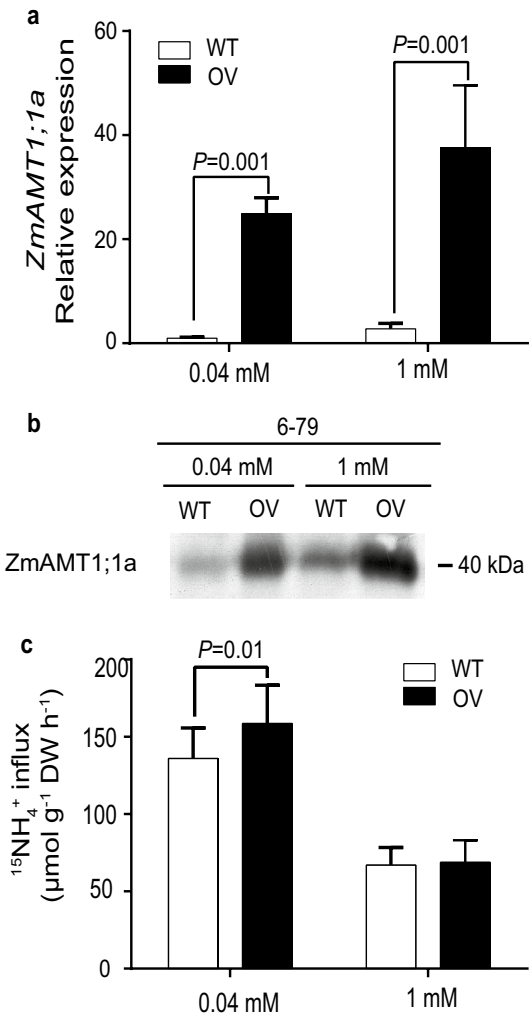

Fig. 4 Enhanced ^{15}N-labelled ammonium influx rates in roots of *ZmAMT1;1a*-overexpression line under low ammonium supply. The overexpression line (OV, 6–79) and the corresponding wild-type (WT) plants were cultured hydroponically for 14 days with 0.04 or 1 mM ammonium as sole N source. **a** Transcriptional expression of *ZmAMT1;1a* was quantified by qPCR using gene-specific primers. **b** Protein gel blot analysis of microsomal fractions from maize roots using the specific antibody against ZmAMT1;1a. The detected protein had an apparent size of approximately 40 kD. **c** Influx of ^{15}N-labeled ammonium into roots. ^{15}N-labeled ammonium was supplied at 200 µM indicative to high-affinity ammonium transport system. Bars indicated means ± SD (n = 9–12). P values were generated using Student's t test to indicate the significant differences between WT and OV

Overexpression of *ZmAMT1;1a* improved growth and N uptake efficiency of transgenic maize under low ammonium supply

To further define the physiological contribution of elevated *ZmAMT1;1a* expression to the overall N use efficiency in transgenic maize, the growth, and N-related phenotype were determined in plants grown under both low and high ammonium supply (0.04 and 1 mM). Under 0.04 mM ammonium condition, the total biomass of transgenic plants was significantly increased about 6% compared to WT plants (Fig. 5e), particularly for root biomass with 15% increase (Fig. 5a) rather than shoot biomass (Fig. 5c). In terms of N uptake efficiency (N accumulation into plants), the transgenic line had higher total N uptake (about 7%) than WT plants (Fig. 5f), which was mainly contributed by the increase (about 33%) of root N uptake (Fig. 5b). By contrast, none of the growth and N-related phenotypic difference was observed between transgenic and WT plants under high ammonium condition (Fig. 5a–f). For *ZmAMT1;1a*-overexpression line, therefore, the enhanced ammonium influx rate

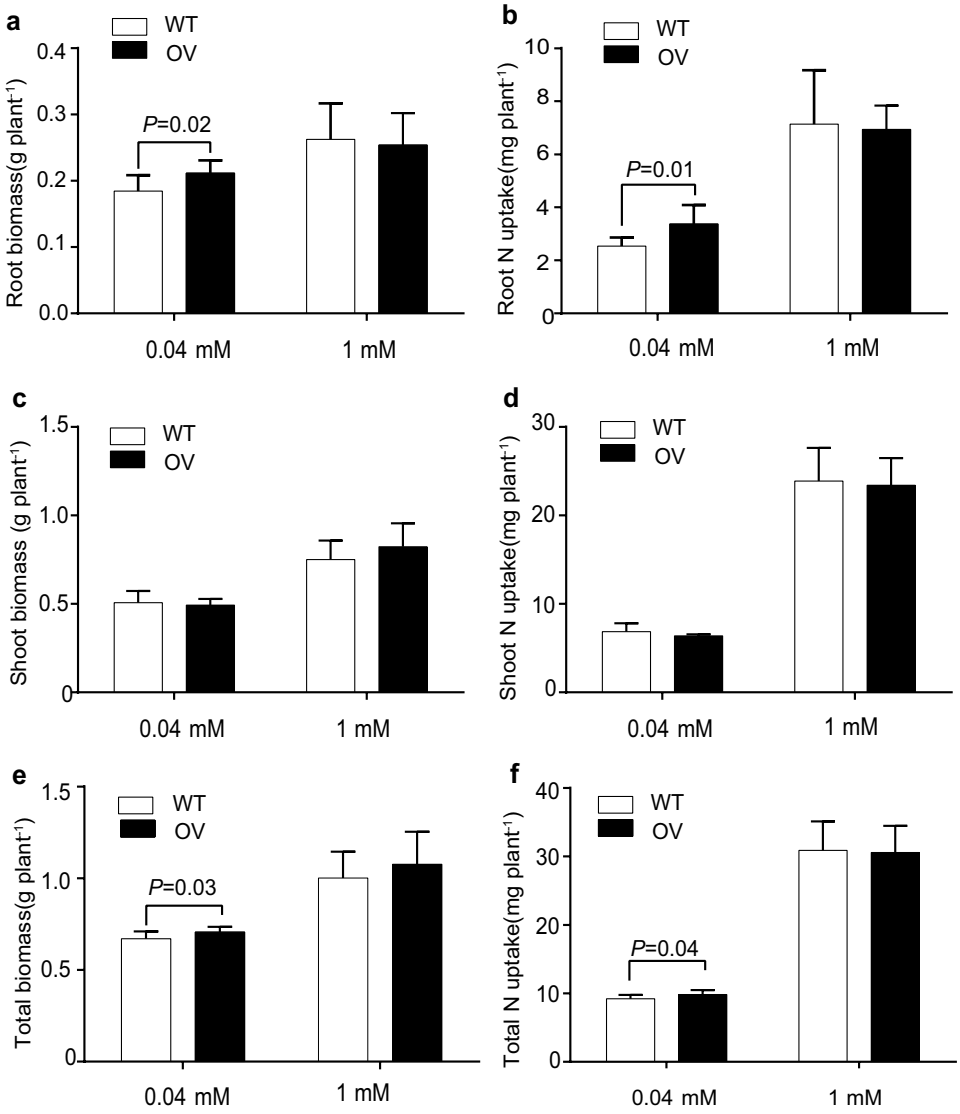

Fig. 5 Enhanced growth and N uptake in roots of *ZmAMT1;1a*-overexpression line under low ammonium supply. The overexpression line (OV, 6–79) and the corresponding wild-type (WT) plants were cultured hydroponically for 14 days with 0.04 or 1 mM ammonium as sole N source. Plant biomass and N uptake of the root (**a, b**), shoot (**c, d**) and whole plant (**e, f**) were measured, respectively. Bars indicated means ± SD ($n = 9$–12). *P* values were generated using Student's *t* test to indicate the significant differences between WT and OV

resulted in a better root N uptake and growth when ammonium was supplied at a low level.

To eliminate the possible influence of genetic background of transgenic plants, we crossed line 6–79 with inbred B73 for two times and generate BC_2F_3 progenies. Compared to non-transformed plant (BC_2F_3 WT) selected from the same BC_2F_3 population, the transgenic line carried *UBI-ZmAMT1;1a* (BC_2F_3 OV) showed approximately 30% increase of total N uptake when plants were grown at 0.04 mM ammonium as the sole N source (Fig. 6). This result confirmed the improved N uptake efficiency in transgenic event 6–79 that can be subsequently used for developing high N use efficient hybrids in the breeding program.

Discussion

N uptake efficiency, referring to the ability of the plant to acquire N from the soil, is one of the two major components of N use efficiency in maize (Moll et al. 1982). N uptake efficient maize genotypes usually have advantages on either root system architecture (Lynch 2013; Mi et al. 2016), or effective N transport rates per unit of root surface (Kamprath et al. 1982), or both. The improvement of N uptake rate can be achieved by enhancing the N-transporter system in plant roots (Glass 2003). In this work, with the transgenic approach, the expression of *ZmAMT1;1a* gene in maize was increased, and the N uptake efficiency of

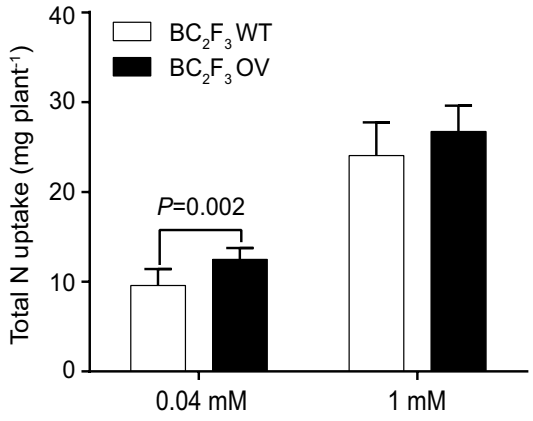

Fig. 6 Improved N uptake efficiency in *ZmAMT1;1a*-overexpression backcross line. The overexpression line 6–79 was backcrossed with B73 to generate BC_2F_3 progenies, in which the overexpression line (BC_2F_3 OV) and the corresponding wild-type (BC_2F_3 WT) plants were selected. Both plants were cultured hydroponically for 14 days with 0.04 or 1 mM ammonium as sole N source. Total N uptake in plants indicative of N uptake efficiency was measured. Bars indicated means ± SD ($n = 9–12$). *P* values were generated using Student's *t* test to indicate the significant differences between BC_2F_3 WT and OV

maize was successfully improved. This provided one of the effective ways to realize N use efficiency in maize through genetic improvement.

The expression level of *AMTs* genes is a key factor limiting the ammonium uptake ability of plant roots. Several lines of evidence indicated that enhancing *AMT* gene expression can subsequently increase the high-affinity ammonium uptake capacity in roots. First, in roots of N-starved or nitrate-pre-cultured maize plants, an external supply of ammonium can induce expression of *ZmAMT1;1a* and *ZmAMT1;3* genes and enhance the high-affinity ammonium uptake capacity accordingly (Gu et al. 2013). Second, the modern maize cultivars performing the higher *ZmAMT1;1a* and *ZmAMT1;3* gene expression in roots may result in more ammonium acquisition for their better N use efficiency than those of old maize cultivars (Fig. 1). Third, *ZmAMT1; 1a* expression enhanced in roots of maize by the transgenic approach could strengthen the ability of root ammonium uptake under low ammonium condition (Fig. 4), and thereby the ability of plants to absorb more N source (Fig. 5). Likewise, overexpression of *OsAMT1;1* in transgenic rice can increase ammonium uptake rates as well as plant growth under low ammonium conditions (Kosala et al. 2014).

An amount of ammonium in the soil (usually less than 100 μM) is beneficial for plant growth, whereas an excess ammonium is toxic (Ludewig et al. 2007). Therefore, the tight regulation of ammonium uptake process in roots is required and mainly mediated by the regulation of AMTs expression and activity. In *Arabidopsis* roots, the high-affinity ammonium uptake capacity rapidly declined when external N sources were resupplied (Gazzarrini et al. 1999; Lanquar et al. 2009; Rawat et al. 1999; Yuan et al. 2007). This can be explained by concomitant changes in cytoplasmic glutamine levels that are supposed to regulate *AtAMT* gene expression by a negative feedback mechanism (Gazzarrini et al. 1999; Rawat et al. 1999). To prevent root cells from ammonium toxicity, AtAMT protein transport activity could further be shut off by allosteric regulation mediated by phosphorylation of a conserved threonine residue in the C-terminal domain via protein kinase CIPK23 (Lanquar et al. 2009; Yuan et al. 2013; Loqué et al. 2007; Neuhauser et al. 2007; Straub et al. 2017). A similar regulatory mechanism can be proposed as the post-translational down-regulation of ZmAMT1;1a transport activity by high ammonium. Under high ammonium roots of maize, wild-type plants revealed more abundance of *ZmAMT1;1a* transcript and protein, but their corresponding high-affinity ammonium influx rates were rather less than those grown under low ammonium (Fig. 4). Furthermore, in the roots of transgenic maize with *UBQ-ZmAMT1;1a*, the amounts of ZmAMT1;1a protein were significantly elevated, but unable to translate into an enhanced ammonium uptake capacity (Fig. 4), probably due to the repression of ZmAMT1;1a under high ammonium at the activity levels. Indeed, a conserved threonine residue in the C-terminal domain of both ZmAMT1;1a and ZmAMT1;3 proteins is also presented (Gu et al. 2013), and this putative phosphorylation site can be targeted to inhibit transport activity when ammonium is in excess. Under N-deficient condition where ammonium is absent, however, the root high-affinity ammonium uptake capacity can enhance without increase of ZmAMTs protein which probably resulted from activating the transport activities via dephosphorylation process (Gu et al. 2013).

By the transgenic approach, the target genes are often driven by constitutive promoters and resulted in the overproduction of the transcripts and proteins in transgenic plants. Despite the presence of down-regulation of ZmAMT1;1a preventing ammonium toxicity, in certain transgenic lines (such as line 6–81), the excessive expression of *ZmAMT1;1a* might exacerbate ammonium influx across the cell membrane that could cause a significant growth depression (Fig. 3). Likewise, the growth depressions also exhibited in the transgenic rice lines overexpressing *OsAMT1;1* (Kumar et al. 2006; Hoque et al. 2006) and *OsAMT1-3* (Bao et al. 2015), which may become a bottleneck in developing crops with genetic modification of *AMTs*. In the further study, weak promoters or organ-specific promoters (e.g., roots) can be used to produce the optimal level of *AMTs* expression in transgenic crops. Alternatively, constructing ammonium assimilation genes *GS*/*GOGAT* together with *AMTs* in a transformed cassette allows the transgenic plants to rapidly convert absorbed ammonium to amino acids for presenting toxicity. Given the complexity of ammonium uptake and

assimilation pathway in plants, identification and further genetic modification of their novel upstream regulators (transcription factors, kinases, etc.) will provide the promising approaches to breed N use efficient crops.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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