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FOCUS ARTICLE

LbAMT3-1, an ammonium transporter induced by arbuscular mycorrhizal in *Lycium barbarum*, confers tobacco with higher mycorrhizal levels and nutrient uptake

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Abstract

Key message **An ammonium transporter LbAMT3-1 overexpression increases the arbuscular abundance of mycorrhizal that opens the possibility of using LbAMT3-1 in breeding programs to improve symbiotic nutrient uptake in Lycium barbarum.**

Abstract Nitrogen (N) is one of the most essential nutrients required by plants and limits net primary production much of the time in most terrestrial ecosystems. Arbuscular mycorrhizal (AM) fungi can enhance plant nutrient uptake and improve plant productivity in nutrient limit ecosystems. Here, we identified an ammonia transporter, *LbAMT3-1*, specifically induced by AM fungi in Lycium barbarum. To understand the expression characteristics and biological functions, *LbAMT3-1* was cloned, characterized, and overexpressed in *Nicotiana tabacum* (tobacco). A BLAST search identified the coding sequence for *LbAMT3-1* with an open-reading frame of 1473 bp. Reverse transcription polymerase chain reaction (RT-PCR) analysis indicated that, besides mycorrhizal roots, *LbAMT3-1* were barely detectable in other tissues, including stems and leaves. Promoter-GUS assay showed that GUS staining was detected in mycorrhizal roots, and GUS activity driven by the *LbAMT3-1* promoter was exclusively confined to root cells containing arbuscules. *LbAMT3-1* functionally complemented the yeast mutant efficiently, and yeast expressing *LbAMT3-1* showed well growth on the agar medium with 0.02, 0.2, and 2 mM NH_4^+ supply. Moreover, overexpression of *LbAMT3-1* in *N. tabacum* resulted a significant increase in arbuscular abundance and enhanced the nutrient acquisition capacity of mycorrhizal plants. Based on the results of our study, we propose that overexpression of *LbAMT3-1* can promote P and N uptake of host plants through the mycorrhizal pathway, and increase the colonization intensity and arbuscular abundance, which opens the possibility of using *LbAMT3-1* in breeding programs.

Keywords Arbuscular mycorrhiza · *Lycium barbarum* L. · Ammonia transporter · Overexpression · Nitrogen

Nitrogen (N) is one of the most essential nutrients required by plants, and is the most closely related nutrient to crop yield. Arbuscular mycorrhizal (AM) fungi can absorb vary forms of N from soil and then the N transferred into the fungal cytoplasm will eventually releases as NH_4^+ into the peri-arbuscular space and passes to the host via plants ammonium transporters (AMTs) (Jin et al. 2005), which indicates the existence of a symbiotic NH_4^+ transport pathway via the interfacial apoplast into plant root cells.

Lycium barbarum L. is an important traditional medicinal plant in Northwest China with high economic value, and establishes well symbiotic relationship with AM fungi. Here, *LbAMT3-1* (Genbank accession number: OK335754), a putative ammonium transporter was isolated and analyzed from *L. barbarum*, and preliminary findings suggested that it is specifically induced by AM fungi. However, the role of *LbAMT3-1* in N uptake via the mycorrhizal pathway and its effect on nutrient exchange between AM fungi and host plants is still unclear. The objectives of the current study were to explore the effect of *LbAMT3-1* overexpression on

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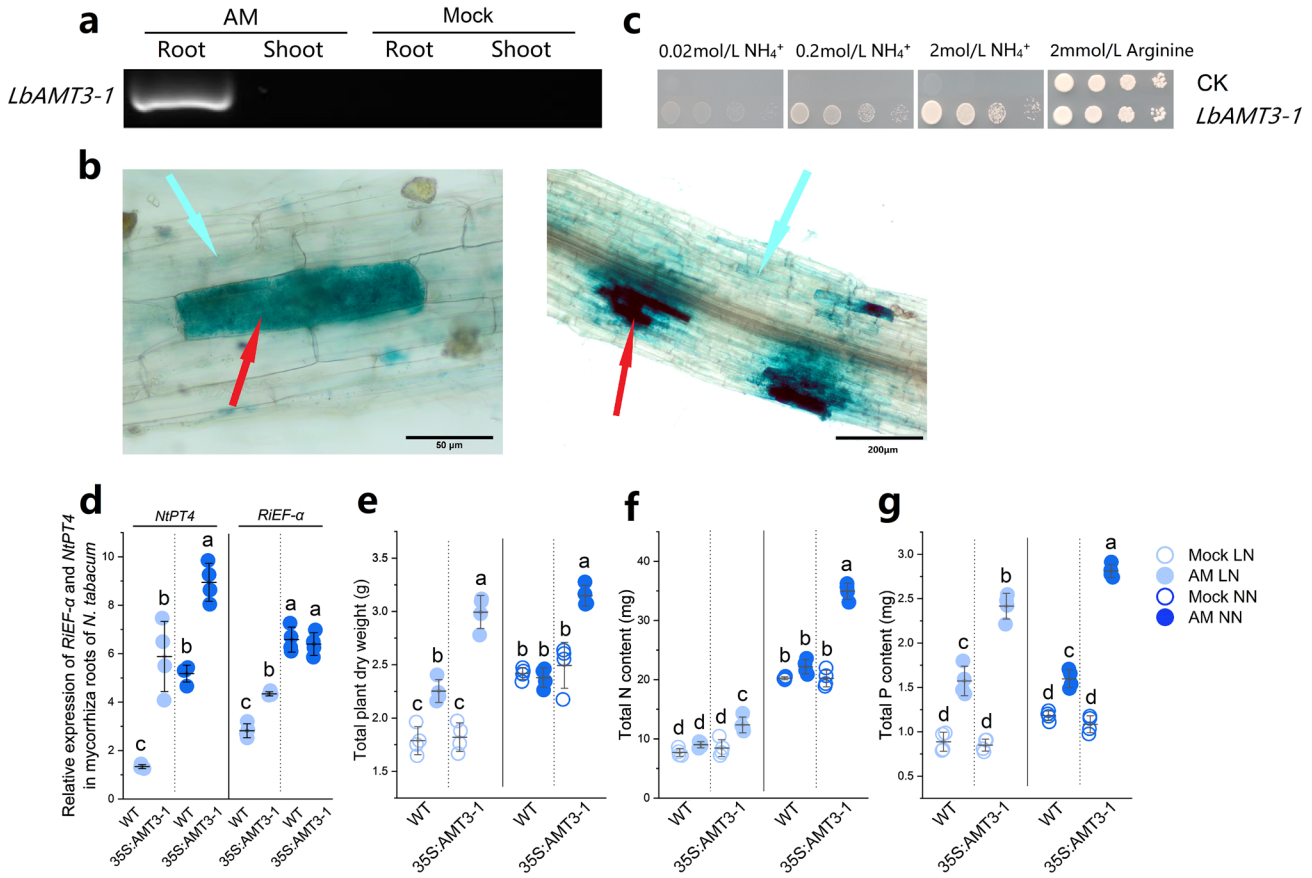

Fig. 1 **a** Reverse transcription-polymerase chain reaction (RT-PCR) from the total RNA of mycorrhizal (AM) and mock-inoculated (Mock) *L. barbarum* root and shoot. **b** Histochemical GUS staining of *N. tabacum* roots at different magnifications. Red arrows indicate arbuscules. Blue arrows denote noncolonized cells in mycorrhizal roots. **c** Expression of *LbAMT3-1* in ammonium uptake deficient mutant yeast. The yeast was grown on the plates at 30 ℃ for 72 h and photographed. The control strain (CK) was transformed with original vector pYES2. **d** Transcripts level of *RiEF1-α* and *NtPT4* in mycorrhizal *N. tabacum* roots with overexpression *LbAMT3-1* lines (35S:AMT3-1) and wild-type (WT) lines under 1.5 mM (limited N, LN) and 15 mM (normal N, NN) NH_4^+ supply. Values are presented as means ± SD ($n = 4$). Means followed by the same letter do not differ significantly at $P < 0.05$ by Tukey' s test. **e** Biomass of inoculated (AM) and mock-inoculated (Mock) *N. tabacum* with overexpression lines (35S:AMT3-1) and wild-type (WT) lines under different N levels (NN, 15 mM NH_4^+; LN, 1.5 mM NH_4^+). **f** and **g** Assay of N and P content in inoculated (AM) and mock-inoculated (Mock) *N. tabacum* with overexpression lines (35S:AMT3-1) and wild-type (WT) lines under different N levels (NN, 15 mM NH_4^+; LN, 1.5 mM NH_4^+). Values are presented as means ± SD ($n = 4$). Means followed by the same letter do not differ significantly at $P < 0.05$ by Tukey' s test

AM colonization and nutrient exchange between AM fungi and host plants.

LbAMT3-1 sequence was obtained from transcriptome sequencing and a BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search identified the coding sequence for *LbAMT3-1* with an ORF of 1473 bp. Reverse transcription-polymerase chain reaction (RT-PCR) analysis indicated that, besides mycorrhizal roots, *LbAMT3-1* were barely detectable in other tissues, including stems and leaves (Fig. 1a). To explore the specific expression pattern of *LbAMT3-1*, we constructed a transcriptional fusion between *LbAMT3-1* 2,043-bp promoter and the coding sequence of the GUS reporter gene and expressed in *Nicotiana tabacum* (tobacco). RT-PCR analysis was used to identify the transgenic lines after AM fungi colonization (S1). Histochemical GUS assays showed GUS staining was detected in mycorrhizal roots and GUS activity driven by the *LbAMT3-1* promoter was exclusively confined to root cells containing arbuscules (Fig. 1b). These results confirm that the expression of *LbAMT3-1* is specific in arbuscule-containing cells. Further studies are required to determine the precise subcellular localization of *LbAMT3-1*.

A yeast mutant complementation test was used to validate the NH_4^+ transport function of LbAMT3-1. The coding sequence of *LbAMT3-1* was inserted into the yeast expression vector pYES2 and expressed in a mutant yeast strain 31019b (Marini et al. 1997), and the control strain (CK) was obtained by transformed original vector pYES2 into the mutant strain. Yeast expressing *LbAMT3-1* showed well growth on the agar medium with 0.02, 0.2, and 2 mM

NH_4^+ supply, whereas no virtually growth of CK strain was observed (Fig. 1c), which showed that *LbAMT3-1* functionally complemented the yeast mutant efficiently. Moreover, growth of the transformed yeast on media with low N concentrations (< 1 mmol/L NH_4^+) indicate that LbAMT3-1 is high-affinity AMT. These results support the hypothesis that a potentially important role of LbAMT3-1 in the transfer of NH_4^+ from the fungus to the plant in the AM symbiosis.

To determine whether *LbAMT3-1* overexpression affects AM symbiosis and nutrient exchange between AM fungi and host plants, *LbAMT3-1* under the control of a CaMV 35S promoter was overexpressed in *N. tabacum*. The transcript expression levels of *LbAMT3-1* in the overexpression lines were confirmed by reverse transcription quantitative real-time PCR (RT-qPCR) (S2). Three transgenic lines 1, 3, and 5 with similar expression level were used as one biological line, overexpressing line (35S: LbAMT3-1), and together with wild-type line (WT) were treated with two AM status (inoculated, AM, or mock-inoculated, Mock, with *Rhizophagus intraradices*) and two N levels (limited N with 1.5 mM NH_4^+, LN, and normal N with 15 mM NH_4^+, NN). After 6 weeks of phosphorus (P) limited (0.2 mM P supply) cultivation, four biological replications of each treatment (total of 32 samples) were harvested and analyzed.

Mycorrhizal colonization was quantified based on the grading assessment method (Trouvelot et al. 1986) after roots were stained with trypan blue. 35S:AMT3-1 plants showed an arbuscular abundance of $19.7 \pm 1.07\%$ and $12.6 \pm 2.10\%$ (a significant increase about 237% and 135% compared with WT plants) and a colonization intensity of $65.4 \pm 2.94\%$ and $51.1 \pm 3.00\%$ (a significant increase about 37% compared with WT plants only after N was limited) under 15 mM and 1.5 mM NH_4^+ supply, respectively. To verify the colonization levels of AM fungi, the relative expression level of *R. intraradices* reference gene *RiEF1-α* (DQ282611.1) was used to verify the colonization intensity, and the expression pattern of *RiEF1-α* correlated well with mycorrhizal colonization intensity (Fig. 1d). *NtPT4* (LOC107766149), the homologous gene of *PT4s* in *Lycopersicon esculentum* and *Solanum tuberosum*, which was previously described as mycorrhiza-specific induced phosphate transporter gene (Nagy et al. 2005), was used as a scale for the arbuscular abundance. There is a significantly higher relative expression level of *NtPT4* in 35S:AMT3-1 plants than WT plants under two N levels (Fig. 1d) meaning a high arbuscular abundance in 35S:AMT3-1 plants. These results indicated that overexpression of *LbAMT3-1* has a considerable positive effect on the establishment of symbiotic system, which may be attributed to the more efficient NH_4^+ transport from peri-arbuscular space to host cells in 35S:AMT3-1 plants to exchange more C that boosting the generation of arbuscular, similar to the exchange of P and C (Kiers et al. 2011). Additionally, it has been shown that P starvation response is actively controlled by N provision, P starvation response-centered network gets activated under more N favorable conditions (Medici et al. 2019)and , thus, the activation of P starvation response in 35S:AMT3-1 plants may profit mycorrhizal infection (Shi et al. 2021).

Higher arbuscular abundance usually means higher nutrient acquisition capacity. 35S:AMT3-1 plants displayed higher biomass and total N content only after AM colonization (Fig. 1e and f), which indicates LbAMT3-1 might function on the peri-arbuscular membrane (Huisman et al. 2020) and did not contribute to the direct N uptake pathway. When total P content was quantified, we found that inoculated WT plants displayed an improved P level in total content, compared to non-inoculated WT plants, while 35S:AMT3-1 plants displayed no less than 29% further increase than inoculated WT plants (Fig. 1g). Koegel et al. (2017) already described the loss of OsAMT3-1 function in rice resulted in a reduction in symbiotic N and P uptake and in a lack of growth stimulation by AM fungi, indicating that AMT3-1 has an appreciable effect on the nutrient fluxes between the AM fungi and the host plants. Moreover, new physiological and molecular evidences show that for P, the mycorrhizal pathway is operational regardless of plant growth responses (Smith et al. 2011); hence, there was an advanced P uptake in mycorrhizal plants, to which the AM fungal colonization contributed, especially in 35S:AMT3-1 plants with a higher arbuscular abundance.

In conclusion, our results indicated that overexpression of *LbAMT3-1* can promote P and N uptake of host plants through the mycorrhizal pathway and increase the colonization intensity and arbuscular abundance that opens the possibility of using *LbAMT3-1* in breeding programs.

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Author contributions statement HZ and MT provided ideas, conceived and designed research. KC conducted experiments and wrote the manuscript. MW and XJ analyzed data. All authors read and approved the final manuscript.

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Declarations

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

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