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NADH-dependent glutamate synthase plays a crucial role in assimilating ammonium in the Arabidopsis root

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Plant roots under nitrogen deficient conditions with access to both ammonium and nitrate ions, will take up ammonium first. This preference for ammonium rather than nitrate emphasizes the importance of ammonium assimilation machinery in roots. Glutamine synthetase (GS) and glutamate synthase (GOGAT) catalyze the conversion of ammonium and 2-oxoglutarate to glutamine and glutamate. Higher plants have two GOGAT species, ferredoxin-dependent glutamate synthase (Fd-GOGAT) and nicotinamide adenine dinucleotide (NADH)-GOGAT. While Fd-GOGAT participates in the assimilation of ammonium, which is derived from photorespiration in leaves, NADH-GOGAT is highly expressed in roots and its importance needs to be elucidated. While ammonium as a minor nitrogen form in most soils is directly taken up, nitrate as the major nitrogen source needs to be converted to ammonium prior to uptake. The aim of this study was to investigate and quantify the contribution of NADH-GOGAT to the ammonium assimilation in Arabidopsis (*Arabidopsis thaliana* Columbia) roots. Quantitative real-time polymerase chain reaction (PCR) and protein gel blot analysis showed an accumulation of NADH-GOGAT in response to ammonium supplied to the roots. In addition the localization of NADH-GOGAT and Fd-GOGAT did not fully overlap. Promoter–β-glucuronidase (GUS) fusion analysis and immunohistochemistry showed that NADH-GOGAT was highly accumulated in non-green tissue like vascular bundles, shoot apical meristem, pollen, stigma and roots. Reverse genetic approaches suggested a reduction in glutamate production and biomass accumulation in NADH-GOGAT transfer DNA (T-DNA) insertion lines under normal CO_2 condition. The data emphasize the importance of NADH-GOGAT in the ammonium assimilation in Arabidopsis roots.

Abbreviations:

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Introduction

Plant ammonium originates not only from photorespiration but also additionally from other endogenous and exogenous sources. Endogenous ammonium is generated by amino acid and protein catabolism, nitrate reduction and phenyl propanoid metabolism (Schjoerring et al., 2002), where as exogenous ammonium derives from nitrogen fertilizers and soil (Britto and Kronzucker, 2002, Lark et al., 2004).

Glutamate synthase (GOGAT) and glutamine synthetase (GS; EC 6.3.1.2) are key enzymes in the assimilation of inorganic nitrogen in all plants (Lam et al., 1996, Ireland and Lea 1999, Funayama et al., 2013, Goodall et al., 2013). GOGAT catalyzes the transfer of the glutamine amid group to 2-oxoglutarate resulting in two glutamate molecules, while GS catalyzes the ATP-dependent amination of glutamate to form glutamine. GOGAT, together with GS, maintains the flow of nitrogen into glutamine and glutamate, therefore the GS / GOGAT cycle represents the major pathway for the assimilation of ammonium in plants (Lea and Miflin, 1974).

There are two types of GOGAT genes in higher plants (Matoh et al., 1980, Oaks and Hirel, 1985). One requires reduced Fd as a reductant (Fd-GOGAT; EC 1.4.7.1), the other NADH (NADH-GOGAT; EC

1.4.1.14). Barley (Kendall et al., 1986) and Arabidopsis (Somerville and Ogren, 1980, Morris et al., 1988) mutants lacking the functional Fd-GOGAT were unable to grow under ambient CO_2 levels, but could grow under elevated CO_2. This suggests that Fd-GOGAT assimilates ammonium derived from photorespiration. Fd-GOGAT defective mutants retained GOGAT activity, and Fd-GOGAT mutants could survive under non-photorespiratory conditions; so it has been assumed that NADH-GOGAT contributes to the ammonium assimilation under non-photorespiratory conditions.

Reverse genetic studies indicate that NADH-GOGAT assimilates non-photorespiratory ammonium (Lancien et al. 2002, Potel et al. 2009) in Arabidopsis. Plant growth and amino acid concentrations were compared between wild-type and *NADH-GOGAT* transfer DNA (T-DNA) insertion lines under high and normal CO_2 conditions (Lancien et al. 2002). The T-DNA insertion lines exhibited reduced biomass accumulation under high CO_2 conditions.

Under exogenous ammonium supply to rice, high NADH-GOGAT levels were detected in roots (Yamaya et al., 1995, Hirose et al., 1997, Tamura et al., 2010). Quantitative real-time PCR revealed that NADH-GOGAT was a major GOGAT in ammonium supplied rice roots. Reverse genetics and (qPCR) pointed the important role of NADH-GOGAT in primary ammonium assimilation in rice roots (Tamura et al., 2010).

Unlike for rice, ammonium is not a major inorganic nitrogen source for other field-grown plant species, since nitrification converts soil ammonium to nitrate (von Wirén et al., 2000). Although ammonium is not a major nitrogen source for Arabidopsis, both NADH-GOGAT and Fd-GOGAT2 were highly expressed in roots (Lancien et al., 2002). Expression analyses indicated that the two GOGATs in Arabidopsis have distinct roles in the ammonium assimilation from the environment. We therefore tested the response and function of NADH-GOGAT

in Arabidopsis grown with ammonium, in order to determine the importance of ammonium assimilation.

The aim of this study was to quantify the contribution of NADH-GOGAT to the ammonium assimilation in Arabidopsis roots. For this purpose, the amino acid composition and growth on ammonium as the sole nitrogen source were determined in wild-type plants and in two independent T-DNA insertion lines, which are defective in NADH-GOGAT gene expression. Furthermore, the localization of the NADH-GOGAT promoter activity in the whole plant was analyzed with an expression of a promoter–β-glucuronidase (GUS) fusion in transgenic plants. Additional methods like gene expression and protein gel blots under different nitrogen conditions as well as immunological approaches were used to determine the localization.

Materials and methods
Plant material and growth analysis

The *nadh-gogat-2* (SALK_033098) and *glt1-T* (Lancien et al. 2002) insertion lines were purchased from the *Arabidopsis thaliana* T-DNA insertion mutant collection Arabidopsis Biological Resource Center (ABRC, Columbus, OH). *Arabidopsis thaliana* Columbia was used as wild-type.

Vertical agar culture

Arabidopsis seeds were surface sterilized and plated onto MGRL (established in Molecular Genetics Research Laboratory) medium (Fujiwara et al. 1992) solidified with Difco agar. The plants were pre-cultured for 2 weeks and transferred to vertical plates containing MGRL salts except nitrogen for 3 days, and then transferred again to vertical plates containing different nitrogen sources at indicated concentrations. Roots and shoots were separately harvested, and frozen in liquid nitrogen immediately after the harvesting. Samples were kept at −80°C till RNA extraction. Plants were cultured

in a growth chamber controlled at 22°C, 60% relative humidity, under 16 h-light/8 h-dark cycles. The light intensity was 40 $\mu mol m^{-2} s^{-1}$.

Hydroponics

Half cut 1.5 ml tubes were filled with moistened rock wool (AO 50/40, Grodan, Roermond, The Netherlands), and three to five Arabidopsis seeds were sown to each tube. The half-cut tubes were placed into the holes of a styrofoam plate (0.24 × 0.36 m, 7 mm thick) with approximately 200 holes, which was floated on 8 l of water in a 15.5 l plastic container (SANBOX#24B, Sanko, Tokyo, Japan). One plant seedling was selected among sown seeds after 3-days of germination in the dark. After this period the water was switched to hydroponic solution (Loque et al. 2006). The hydroponic solution consist of 1mM KH_2PO_4, 1mM $MgSO_4$, 250mM K_2SO_4, 250mM $CaCl_2$, 100mM Na-Fe-EDTA, 50 μM KCl, 50 μM H_3BO_3, 5μM $MnSO_4$, 1μM $ZnSO_4$, 1μM $CuSO_4$, 1μM $NaMoO_4$ and different concentrations of nitrogen. Plants were grown in the hydroponic solution supplemented with 500 μM KNO_3 for the first 4 weeks. Six plants were transferred to a black acrylic resin plate (0.11 × 0.15 m, 5 mm thick) with nine holes. A 0.8 l plastic container (SANBOX#1N, Sanko) was filled with 0.7 l of hydroponic solution and afterwards covered with the resin plate. As the roots passed through the rock wool to the other side at this stage, their tips were able to reach the surface of hydroponic solution in the plastic container. The plants were grown in hydroponic solution containing 1mM NH_4Cl as nitrogen source for 3 weeks. The hydroponic solution was renewed two times during a week. In order to evaluate the acidification in hydroponics, the pH was measured eleven times with a pH meter (Laqua Twin pH meter B-712, Horiba Ltd., Kyoto, Japan) after the transfer to a smaller container. Plants were grown in a climate chamber (Biotron LPH-350S, Nippon Medical and Chemical Instruments Co., Ltd., Tokyo, Japan; 10 h/14 h light/dark, 22°C, 60%

humidity). Each container was connected to the pump for aeration.

Soil material culture

Plants were cultured on verdenite supported with expanded vermiculite/perlite. Pots were filled with the mixture of 14 g expanded vermiculite and 14 g perlite, covered with 3.6 g of verdenite. The medium was moistened with 150 ml hydroponic solution containing 0.5, 1.0 or 5.0mM NH$_4$Cl as the sole nitrogen source. The plant germination was carried out for 11 days on half-strength Murashige and Skoog medium (Murashige and Skoog 1962), afterwards they were grown for 13 days in pots. Soils were moistened three times with hydroponic solution during a period of 13 days.

Quantitative real-time PCR

Total RNA was extracted from liquid nitrogen frozen plant samples with RNeasy plant mini kit (Qiagen, Hilden, Germany). For each point, samples from at least 20 plants were collected as one group. Three independent experiments were carried out. The extraction was performed following manufacturer's instructions. Reverse transcription and DNase-treat were carried out using a PrimeScript® RT reagent Kit with gDNA Eraser (Takara Bio Inc., Otsu, Japan) with 500 ng of total RNA in a 20 μl final volume, according to manufacturer's instructions. After 5 min heat inactivation of the enzyme, the quality of extracted RNA samples were quantified using the NanoDrop 1000 spectrophotometer (NanoDrop, LMS Co., Ltd. Tokyo,

Japan). The A260/280 ratio is generally between 1.9 and 2.0. As amelting curve is performed as standard, a contamination would be visible as additional peak. Aliquots of the resulting reverse transcription (RT) reaction productwere used as template for PCR analysis. cDNA was stored in Eppendorf tubes at −20°C. Quantitative real-time PCR (qRT-PCR) analysis was conducted using gene-specific primers; those were summarized in Table 1. Gene-specific primers for the genes were designed using the DNASIS software (Hitachi Solutions, Ltd., Tokyo, Japan). In silico screens were performed with NCBI BLAST. Multiplex qPCR was not performed. Primers were purchase from Eurofins MWG Operon Inc., Tokyo, Japan. The PCR products were quantified in optical 96-well plates using the Light Cycler® 480 (Roche Diagnostics K.K., Tokyo, Japan) according to the following program: 10 s at 95°C, followed by 50 cycles of 95°C for 5s, 60°C for 34 s. The reaction mixture (in a final volume of 20 μl) contained: 2 μl cDNA sample, 10 μl SYBR® Premix Ex Taq™ II (Takara), 400 μM of each gene-specific primers. Reactions were set up manually. Without a template, no Cq could be determined as it never passed the threshold line. Serial fivefold dilution of cDNAs were used to calculate the standard curve and measure the amplification efficiency for each target and reference gene with the LIGHTCYCLER® 480 Software version 1.2. Specificity of qPCR was confirmed by the sharpness of the peak appeared in dissociation curve analysis. Water was used as non-temperature control, and no significant amplification was observed. Ubiquitin (UBQ2) was used as a reference

gene to standardize the signal intensity, according to the previous article (Ishiyama et al. 2004). In addition, UBQ2 did not show dramatic response to the conditions tested in this work. Results are shown as a mean value of three independent samples, and we confirmed that all results were reproducible.

Protein gel blot analysis

Total protein was extracted and prepared, according to the methods published previously (Ishiyama et al. 1998). Proteins were separated on 7% sodium dodecyl sulfate (SDS)-polyacrylamide gels and trans-blotted onto a polyvinylidene fluoride membrane. Blots were developed by a color precipitation of nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3'-indolylphosphatase p-toluidine salt (BCIP) (Promega KK, Tokyo, Japan), derived from the secondary antibody, goat anti-rabbit immunoglobulin G (IgG) alkaline phosphatase conjugate (Promega). Affinity-purified anti-rice NADH-GOGAT IgG (Hayakawa et al. 1992), anti-rice Fd-GOGAT IgG (Yamaya et al. 1992) and anti-rice GS2 IgG (Kamachi et al. 1991) were diluted in phosphate buffered saline, containing 1% bovine serum albumin (BSA), at 1:100 and used as primary antibodies with secondary antibody at 1:7500. The protein concentration was determined according to Bradford (1976) using BSA as a standard.

Vector construction and plant transformation

The NADH-GOGAT promoter was

Table 1. Gene-specific primers used for quantitative real-time PCR in this study.

Gene	Forward primer	Reverse primer
NADH-GOGAT	AGTTGGGAGAAGGATGAAACCGGGAGG	GTGATAGTGTTGTGTTTCATCTGGTTAAGG
Fd-GOGAT1	CTGGTGGCTGTGTAGTCGTGCTTGGAA	TTCAGCTGCAATTCCCCTGCAGGCGCAGTT
Fd-GOGAT2	ATCATTGCCCCTTTGCTGCTTCCCGTTT	TCGACTGGTTTTCGTCCCGAAGACGCAACT

amplified with the primers 5′-CAA ATA TGA CGA AAC CGC AC-3′ and 5′-ACG GAA TCA GCA GTG AGT GA-3′ using PCR with Arabidopsis genomic DNA as a template. The fragment was first sub-cloned into a pGEM-T easy vector (Promega Corporation), and then the *Eco*RI fragment was sub-cloned into a pBluescript II vector (Stratagene, La Jolla, CA) to attach *Pst*I-*Eco*RV digestion sites. Then, the *Pst*I-*Eco*RV fragment was sub-cloned into the PstI-SmaI position in a pBI221 vector (Clontech, Palo Alto, CA). After the digestion with *Hin*dIII-*Sac*I, the fragment containing about 1 kb of NADH-GOGAT promoter–GUS was sub-cloned into the *Hin*dIII-*Sac*I position of a pBI101 vector (Clontech). The constructs used for these experiments were confirmed by DNA sequencing. The floral dip method (Clough and Bent 1998) was used for plant transformation.

Immunohistochemistry

Plant sections were fixed with 4% (v/v) paraformaldehyde, 0.2% (v/v) glutaraldehyde and 0.01%(w/v) $CaCl_2$ in 0.1*M*sodium cacodylate-HCl (pH 7.4) for 24 h at 4°C. The fixed tissues were sequentially dehydrated, embedded into paraffin, and sliced into 10 μm sections using a microtome (Yamato Kohki Industrial Co., Ltd., PR-50, Saitama, Japan). Then the sections were stretched onto a slide glass. The paraffin was removed with xylene, the sections were hydrated, and incubatedwith antibodies as described previously (Hayakawa et al. 1994). Polyclonal IgG raised against rice-NADH-GOGAT was purified with its corresponding antigen. The antigens were visualized with an amino acid polymer containing anti-rabbit IgG conjugated with horseradish peroxidase (Histofine Simple Stain MAX-PO; Nichirei Co., Tokyo, Japan).

GUS staining

Seeds were vernalized at 4°C for at least 24 h on growth media containing the half concentration of MS salts supplemented with vitamins (Murashige and Skoog 1962), 2% sucrose and 0.8% agar. The promoter dependent GUS activity was visualized according to the protocol in previous work (Kojima et al. 2000).

Amino acid measurement

Plant samples were harvested in 2.0 ml safe lock tube (Eppendorf Co., Ltd., Tokyo, Japan) with zirconia beads and stored in −80°C freezer till amino acid extractions. Plant samples were pre-chilled in liquid nitrogen, and then milled with Tissue Lyser II (Qiagen, K. K., Tokyo, Japan) at 20Hz for 1min. Powdered samples were resolved in 10m*M* HCl, mixed in Tissue Lyser II at 20 Hz for 2 min, and centrifuged at 20 500 g for 15 min at room temperature. The supernatant (approximately 100 μl) was transferred to the prepared Ultra free-MC 5000 NWWL Filter Unit (Millipore, Bedford, MA) on the 1.5ml tube, and centrifuged at 20 500 g for 15 min at room temperature. The flow-through were collected at −30°C freezer till labeling. The AccQ-Tag Ultra Derivatization Kit (Nihon Waters K. K., Tokyo, Japan) was used for derivatization of amino acid and ammonium. First, AccQ-Tag Ultra Reagent Powder was reconstituted. AccQ-Tag Ultra Reagent powder was mixed with AccQ-Tag Ultra Reagent Diluent by using vortex for 10 s, and the powder was resolved at 55°C for 10 min. Second, AccQ-Tag Ultra Borate Buffer (30 μl) and the Amino Acids Mixture Standard Solution, Type H (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or extracted sample (10 μl) were mixed with vortex briefly, and AccQ-Tag Reagent (10 μl)was added and mixed with vortex again for 10 s. After the short incubation at room temperature, samples were heated in block incubator for 10 min at 55°C. AccQ-Tag labeled samples were kept at room temperature and measured with ACQUITY Ultra Performance Liquid Chromatography (UPLC) H-Class (Nihon Waters K. K.).

Statistics

All data setswere analyzed with statistics in the Microsoft Excel add-in software EKUSERU-TOUKEI (Social Survey Research Information Co., Ltd. Tokyo, Japan).

Results

NADH-GOGAT is the major GOGAT form in Arabidopsis roots supplied with ammonium

Time dependent transcriptional changes of GOGAT genes were tested in the roots of either ammonium or nitrate induced nitrogen deficient plants (Fig. 1). The abundance of *Fd-GOGAT1* transcript in nitrogen deficient roots was much lower compared with *NADH-GOGAT* and *Fd-GOGAT2* (Fig. 1A). Neither nitrate nor ammonium supply changed the low expression of *Fd-GOGAT1*. The *Fd-GOGAT2* and *NADH-GOGAT* abundance was relatively high, compared with *Fd-GOGAT1*. However, the response of *GLT1* and *Fd-GOGAT2* to nitrogen supply was different. *NADH-GOGAT* accumulated to almost the double amount after 6 h of ammonium supply, though it did not respond to root nitrate supply (Fig. 1A). Contrariwise, neither ammonium nor nitrate supply significantly changed *Fd-GOGAT2* expression (Fig. 1A). The response of *NADH-GOGAT* to ammonium supply was similar to GS 1;2 (*GLN1;2*) (Fig. 1A), which is known to respond to ammonium supply in Arabidopsis (Ishiyama et al. 2004). Furthermore we tested the ammonium-induced accumulation of NADH-GOGAT in Arabidopsis by protein gel blot analysis, in order to verify if accumulated *NADH-GOGAT* was translated to NADH-GOGAT protein. Anti-rice NADH-GOGAT antibodies (Hayakawa et al. 1994) were able to recognize Arabidopsis NADH-GOGAT, specifically. Protein gel blot analysis showed that NADH-GOGAT protein was highly accumulated in ammonium supplied roots (Fig. 1B). Then, we tested for ammonium concentration-dependent changes of *NADH-GOGAT*
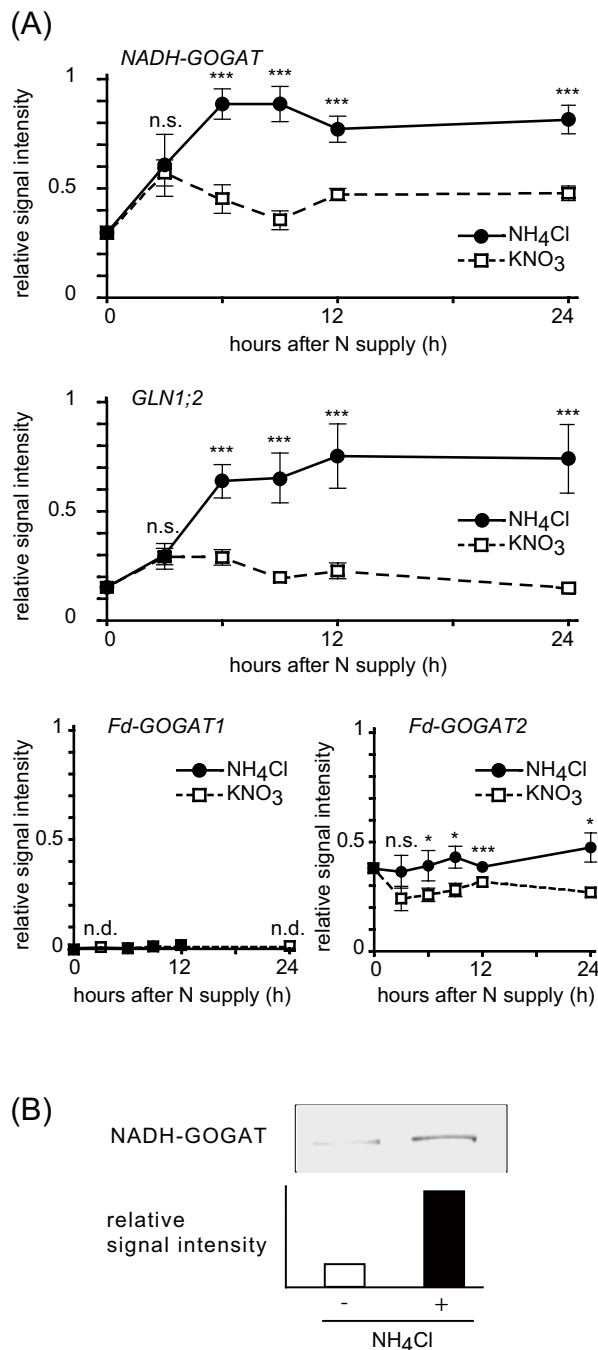
(A)

(B)

Figure 1. NADH-GOGAT highly accumulated in ammonium induced Arabidopsis roots. (A) Quantitative real-time PCR analysis of RNA from wild-type Arabidopsis roots supplemented with either NH_4Cl or KNO_3. Plants were pre-cultured on vertical agar plates containing MGRL salts for 14 days, and then transferred to a plate containing MGRL salts without nitrogen for 3 days, then transferred again to a plate containing MGRL without nitrogen supplemented with either 10mM NH_4Cl or 10mM KNO_3. Gene-specific primers were used to quantify the abundance of GLT1, glutamine synthetase 1;2 (GLN1;2), Fd-GOGAT1 and Fd-GOGAT2, respectively. Signal intensity was standardized by constitutively expressed polyubiquitin (UBQ2). Bars indicate means±SD (n=3 different plant groups), and significant differences at *P <0.05, **P <0.01 and ***P <0.005 are indicated by asterisks. n.s., not significant; n.d., not detected. Differences were analyzed by Student's t test. (B) Protein gel blot analysis of soluble protein in Arabidopsis. Seeds were germinated on MS medium for 14 days, and transferred to filter paper containing MS medium without nitrogen for 3 days. Then, seedlings were supplied or not with 1mM NH_4Cl for 24 h. Soluble protein was separated by SDS-PAGE at 7% (w/v) gel concentration. The affinity-purified anti-rice NADH-GOGAT antibody was used to visualize NADH-GOGAT protein, and signal intensity on the membranes was measured by a densitometer.

expression (Fig. 2) in roots. The accumulation of NADH-GOGAT in roots was significantly increased to almost the double at an ammonium concentration of 0.5mM (Fig. 2). Conversely, *Fd-GOGAT1* and *Fd-GOGAT2* did not respond to ammonium supply. *NADH-GOGAT* was the only form that responded to ammonium supply.

NADH-GOGAT was highly accumulated in non-photosynthetic organs

Fd-GOGAT1 and GS2 are highly accumulated in the chloroplasts of mesophyll cells, where they assimilate the ammonium released by photorespiration. Time dependent changes in *Fd-GOGAT1* and GS 2 (*GLN2*) expressions were compared with those of *NADH-GOGAT* after either nitrate or ammonium supply (Fig. 3A). Shoot *Fd-GOGAT1* sharply decreased for up to 6 h by nitrate or ammonium supply, and then slightly increased up to 12 h. In contrast to this, there was no clear response of *NADH-GOGAT* to nitrogen supply. The changes in *GLN2* expression were similar to those for *Fd-GOGAT1* in shoots. Shoot *NADH-GOGAT* was much lower expressed than shoot *Fd-GOGAT1*; in addition, there was no clear response of shoot *NADH-GOGAT* to nitrogen supply at roots.

The tissue distribution of NADH-GOGAT was compared with the distribution of Fd-GOGAT and GS2 in Arabidopsis organs at the reproductive stage by protein gel blot analysis (Fig. 2B). The accumulation of NADH-GOGAT in the young developing organs, silique and flower, were higher than those in the photosynthetic organs, such as cauline leaves and the shoot. Conversely, GS2 and Fd-GOGAT showed a high accumulation in the cauline leaves and shoot. Fd-GOGAT and GS2 accumulated nearly in the same localization.

Immunohistochemistry and promoter analysis revealed tissue-specific expression of NADH-GOGAT during the Arabidopsis life cycle (Fig. 4). NADH-
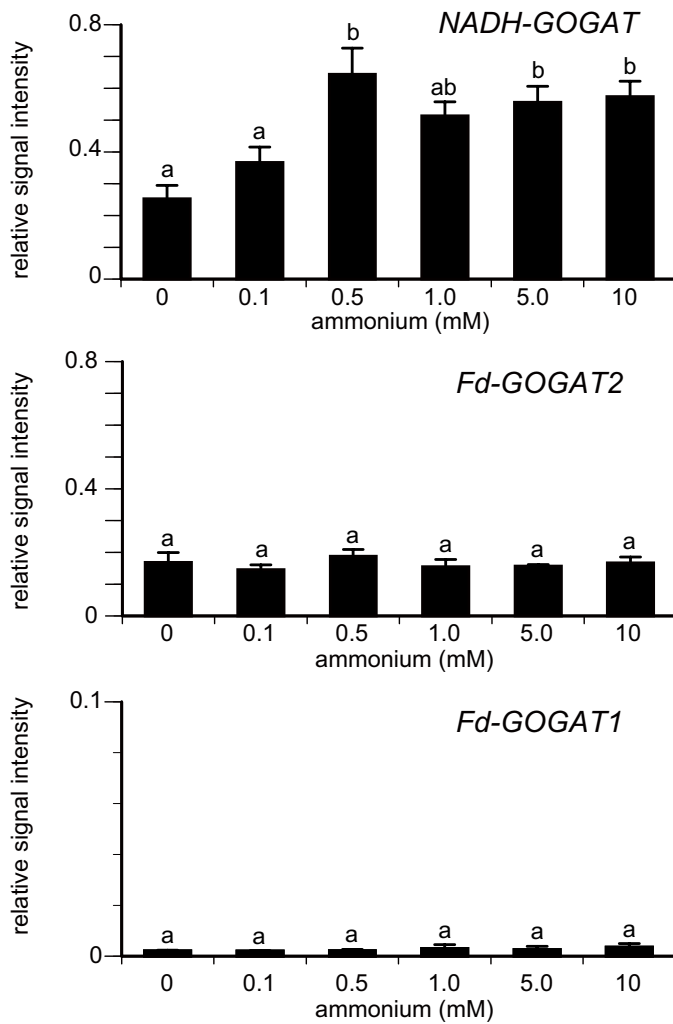
Figure 2. Supplying various concentrations of ammonium changed the expression of NADH-GOGAT in Arabidopsis root. Plants were pre-cultured on vertical agar plates containing MGRL salts for 14 days, and then transferred to a plate containing MGRL salts without nitrogen for 3 days, then transferred again to a plate containing MGRL without nitrogen supplemented with 0, 0.1, 0.5, 1.0, 5.0 and 10mM NH$_4$Cl for 6 h. Gene-specific primers were used to quantify the abundance of NADH-GOGAT, Fd-GOGAT1 and Fd-GOGAT2, respectively. Signal intensity was standardized by constitutively expressed polyubiquitin (UBQ2). Bars indicate means±SD (n=3 different plant groups), and significant differences at P <0.05 within each group are indicated by different letters. One-way ANOVA followed by Bonferroni tests were used to identify significant differences between control and ammonium supplied plants (P <0.05).

GOGAT promoter dependent GUS activity was first detected in parts of the endosperm (Fig. 4A), the tip of leaves (Fig. 4B) and the root (Fig. 4C) at the germination stage. GUS activity was particularly localized in the root tip (Fig. 4D, E) and central cylinder (Fig. 4E, F), but was not detected in the root cortex. Furthermore GUS activity was also detected in shoot (Fig. 4D), especially in hydathode (Fig. 4G) and vascular tissues of rosette leaves (Fig. 4H). Longitudinal sections of rosette leaves showed high GUS accumulation in the vascular bundle tissues (Fig. 4I). Longitudinal sections through 10-day-old GUS-stained transgenic seedlings revealed GUS expression throughout the shoot apical meristem (SAM) and leaf primordia. Furthermore the expression was not restricted to a particular cell layer or region (Fig. 4J). This result is consistent with the histochemical localization of NADH-GOGAT protein in the SAM (Fig. 4K). Almost no signal was present when the NADH-GOGAT antibody was replaced with normal serum (Fig. 4L). Transgenic plants in the reproductive state showed GUS activity in floral buds (Fig. 4M) and in the pollen of anthers (Fig. 4N). GUS activity was also detected in the stigma (Fig. 4O) after flowering. A NADH-GOGAT dependent signal was localized in the cotyledon and root of developing embryos in silique (Fig. 4P). No clear signals were detected when pre-absorbed anti-NADH-GOGAT IgG was used as a primary antibody (Fig. 4Q). Mature embryos showed ubiquitous GUS activity (Fig. 4R).

NADH-GOGAT plays a key role in ammonium assimilation in Arabidopsis roots

NADH-GOGAT T-DNA insertion lines were isolated from the ABRC of the Ohio University (Fig. 5A). Two T-DNA insertion lines were characterized in comparison with their genetic background ecotype, Col. qRT-PCR analysis showed that *NADH-GOGAT* was barely detectable in roots of both T-DNA insertion lines (Fig. 5B). The decrease in functional *NADH-GOGAT* seemed not to lead to an increase in *Fd-GOGAT* (Fig. 5B, C), suggesting only limited compensation ability by Fd-GOGAT for a loss of NADH-GOGAT in roots. Then, we compared the amount of *GLN* mRNA among the plant lines (Fig. 5C, D). Interestingly, the expression levels of *GLN1;1, GLN1;2* and *GLN1;3* in both T-DNA insertion lines were higher than those in wild-type.

The amino acid concentrations were compared in T-DNA insertion lines for *NADH-GOGAT* and their genetic background Col (Figs 6 and 7). We tested three nitrogen conditions, those are nitrogen deficient, ammonium supplied and nitrate supplied conditions. First, total free amino acid concentrations are
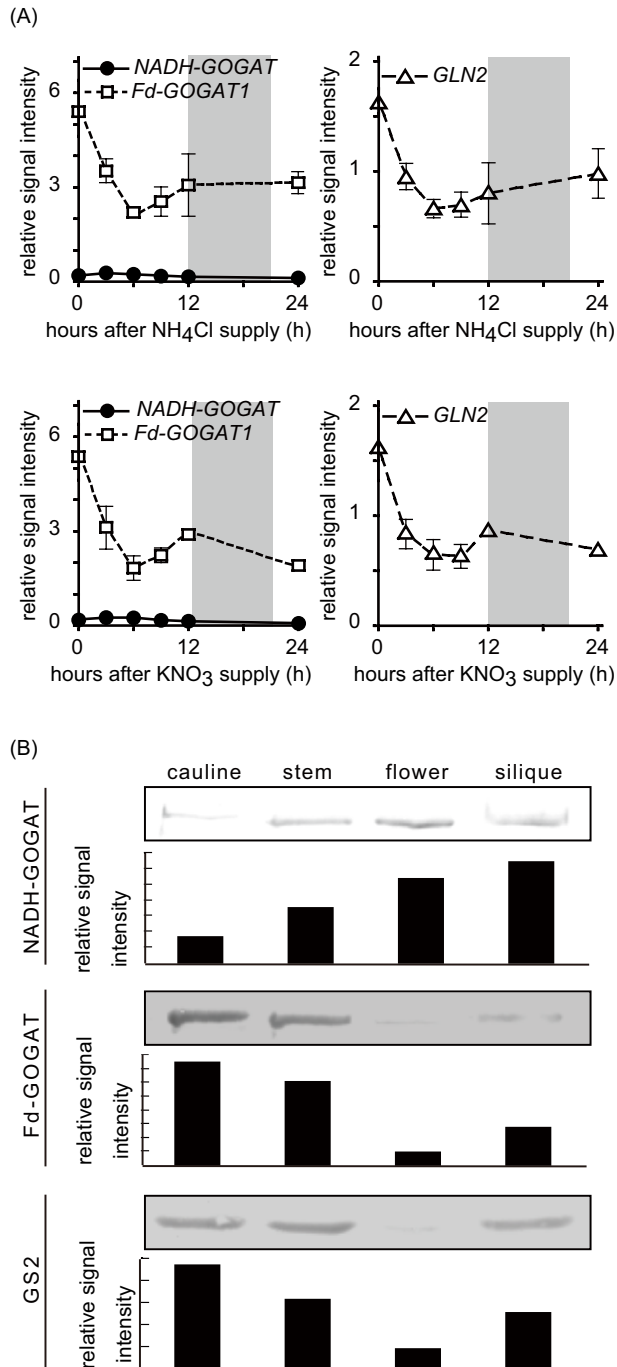
Figure 3. Accumulation of NADH-GOGAT was not correlated with that of Fd-GOGAT1. (A) Quantitative real-time PCR analysis of RNA from wild-type Arabidopsis shoots under either NH₄Cl or KNO₃ supplemented conditions. Plants were pre-cultured on vertical agar plates containing MGRL salts for 14 days, then transferred to a plate containing MGRL salts without nitrogen for 3 days, and transferred again to a plate containing MGRL without nitrogen supplemented with either 10mM NH₄Cl or 10mM KNO₃. Gene-specific primers were used to quantify the abundance of NADH-GOGAT, glutamine synthetase 1;2 (GLN1;2), Fd-GOGAT1 and Fd-GOGAT2, respectively. Signal intensity was standardized by constitutively expressed polyubiquitin (UBQ2). Bars indicate means±SD (n=3 different plant groups). (B) Protein gel blot nalysis of soluble protein in various organs of Arabidopsis. Soluble protein was extracted from 1-month-old Arabidopsis organs and separated by SDS-PAGE at 7% (w/v) gel concentration. Affinity-purified anti-rice NADH-GOGAT, anti-rice Fd-GOGAT and anti-rice GS antibodies were used to visualize signals, respectively, and signal intensity on the membranes was measured by densitometer.

shown (Fig. 6). Second, free amino acid compositions are shown in a diagram (Fig. 7) and in tables (Tables S1, S2, S3, S4 and S5). Plants were grown for 14 days on vertical agar plates supplied with either 10mM nitrate or 10mM ammonium; afterwards the shoots and roots were harvested 6 or 24 h after nitrogen supply. The concentration of total free amino acids changed in neither shoots (Fig. 6A) nor roots (Fig. 6B) after 6 and 24 h of nitrate supply, conversely, it increased in both shoots (Fig. 6C) and roots (Fig. 6D) after 6 and 24 h of ammonium supply in wild-type. As T-DNA insertion in NADH-GOGAT seemed not to change the concentration of total free amino acids after nitrate and ammonium supply (Fig. 6), we compared the free amino acid composition in wild-type and T-DNA insertion lines.

Free amino acids were separated by liquid chromatography, and their concentrations were compared between wild-type and T-DNA insertion lines under three different nitrogen regimes. Excess ammonium supply promoted the formation of amides, such as Gln and Asn in wild-type and T-DNA insertion lines. Glu and Asp concentrations in T-DNA insertion lines were lower than those in wild-type under different nitrogen conditions. T-DNA insertion lines tend to accumulate Gln. The changes of Glu and Gln in T-DNA insertion lines suggest a high contribution of NADH-GOGAT in roots for excess ammonium assimilation.

However, NADH-GOGAT was not able to convert all of the ammonium induced Gln to Glu, thus, we decided to compare the growth of wild-type and T-DNA insertion lines under lower ammonium concentrations in both synthetic soil and hydroponics (Fig. 8).

First, plants were cultured in hydroponics. The NADH-GOGAT T-DNA insertion line plants were smaller than Col. Additionally, the leaves of T-DNA insertion lines showed nitrogen deficient symptoms (Fig. 8A). The shoot biomass of T-DNA insertion lines showed a reduction of 25–30% under hydroponic conditions
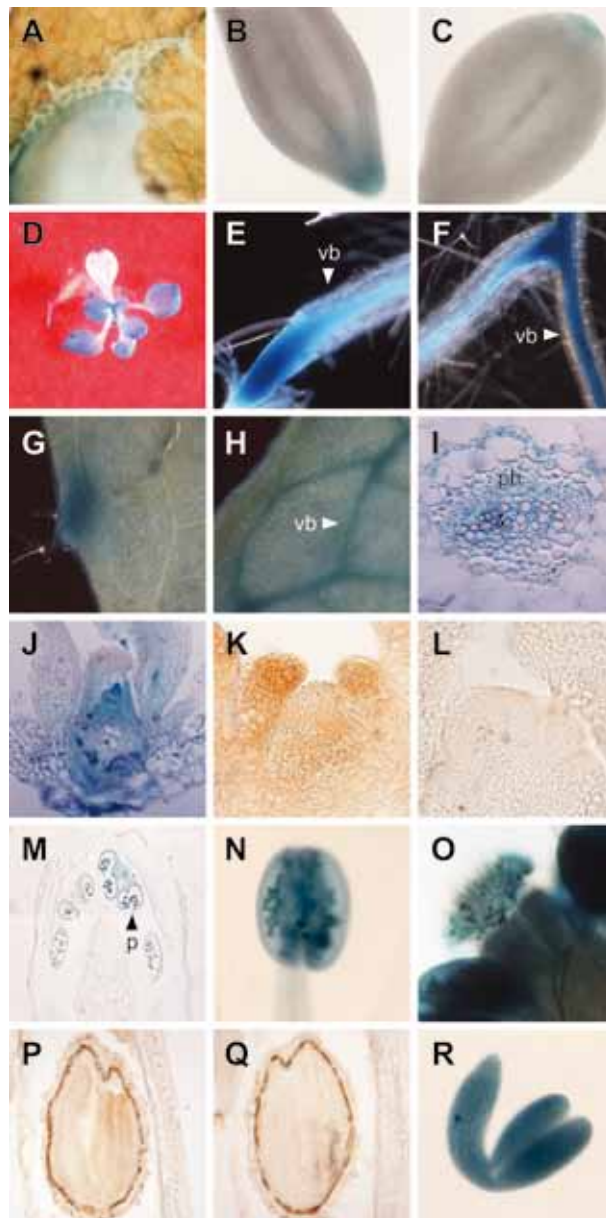
Figure 4. The localization of NADH-GOGAT in various organs of Arabidopsis. NADH-GOGAT promoter dependent GUS activity showed the distribution of NADH-GOGAT promoter activity in various organs (A, B, C, D, E, F, G, H, I, J, M, N, O and R). Samples were stained with affinity-purified NADH-GOGAT IgG pretreated with an excess amount of NADH-GOGAT protein (L and Q), or with affinity-purified NADH-GOGAT IgG as the primary antibody (K and P). Cell type-specific localization of NADH-GOGAT in germinating seeds (A, B and C), in seedlings (D), root tip of the seedlings (E), root branching of the seedlings (F), rosette leaves of the seedling (G, H and I), the shoot apical meristem (J and K), bud (M), anther (N), stigma (O) and developing embryos (P, Q and R) of Arabidopsis.

(Fig. 8B).

Second, plants were cultured on soil material. The shoot dry weight of Col was slightly increased, according to the increase of ammonium concentration in the medium; conversely, the biomass of T-DNA insertion lines showed no dramatically increase (Fig. 8C). Compared with Col, the shoot biomasses of T-DNA insertion lines revealed a decrease of 30% when supplied with 5mM NH4Cl (Fig. 8D).

Discussion

NADH dependent GOGAT was the major GOGAT when the root was supplied with ammonium

In themost aerobic agriculture fields the concentration of ammonium is much lower than that of nitrate (Marschner 1995, von Wirén et al. 2000), thus, the importance of ammonium nutrition in plant root is underestimated (Britto and Kronzucker 2002). However, it is important to understand the ammonium assimilation in roots, as high ammonium levels are able to inhibit plant growth (Britto and Kronzucker 2002, Hachiya et al. 2012). Plants may need to assimilate high exogenous concentrations of ammonium from mainly two resources. One of the resources is the soil, the ammonium concentration ranges from approximately 0.4–4mM in forest-floor soil solution (Vitousek et al. 1982, Britto and Kronzucker 2002). The other one is ammonium from nitrate reduction and/or catabolic reactions.

Both transcripts of Arabidopsis NADH-GOGAT (Fig. 1A) and the corresponding proteins accumulated (Fig. 1B) when the roots were supplied with ammonium, this was not the case under the supply of nitrate (Fig. 1A). An ammonium concentration of $0.5mM$ was sufficient to induce an accumulation of NADH-GOGAT; this range not only occurs in agricultural fields but also in the natural environment (Lark et al. 2004). Neither Fd-GOGAT1 nor Fd-GOGAT2 responded to ammonium supply, suggesting a dominant role of NADH-GOGAT for ammonium assimilation in Arabidopsis roots. Conversely to the roots, Fd-GOGAT1 and GS2 were major GS/GOGAT forms in the shoot (Lam et al. 1995). Fd-GOGAT1 and GS2 are regulated by diurnal rhythm, and they assimilate the ammonium derived from photorespiration (Linka and Weber 2005). The expression of foliar NADH-GOGAT was much lower than that of Fd-GOGAT1 in both light and dark conditions (Fig. 3A), suggesting a limited contribution of NADH-GOGAT to total GOGAT activity
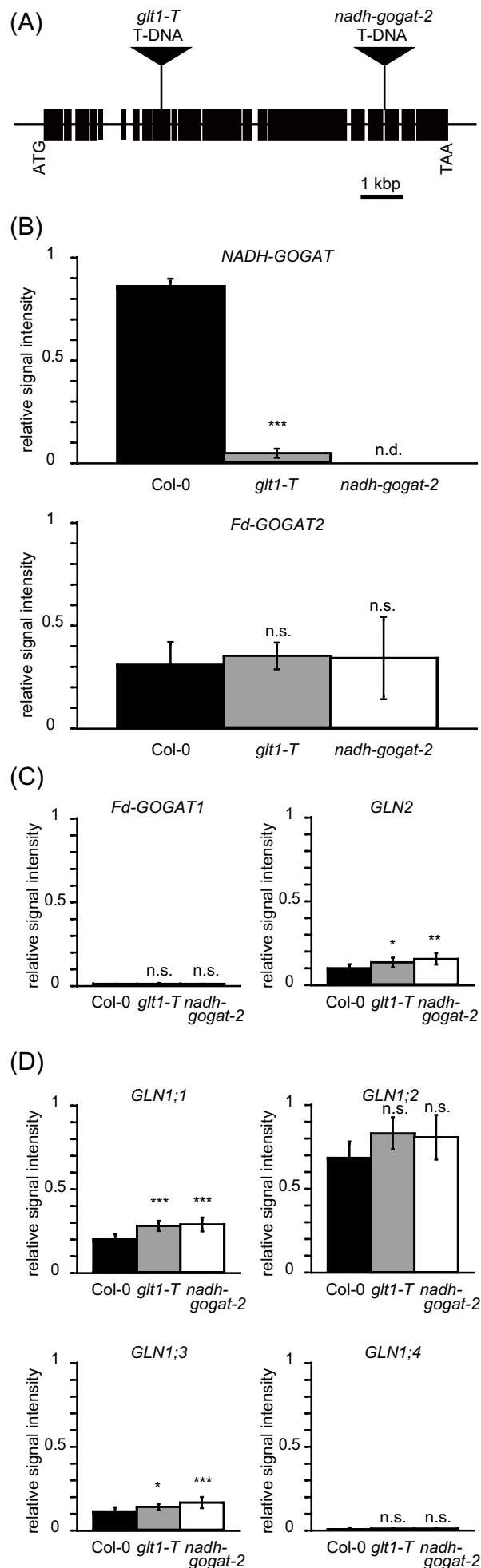
in shoots.

Protein gel blot analysis revealed differences in the organ distribution of Fd-GOGAT1 and NADH-GOGAT (Fig. 3B). Fd-GOGAT and GS2 highly accumulated in organs containing a high number of chloroplasts; conversely, NADH-GOGAT accumulated in non-photosynthetic organs, such as flowers. Promoter analysis and antibody staining revealed that NADH-GOGAT was highly accumulated in organs containing immature chloroplast, such as vascular bundles, the SAM, germinating seeds, flowers and developing seeds (Fig. 4), suggesting non-redundant physiological functions of the two GOGAT molecular species. Previous work also discussed the redundancy of the two GOGAT genes (Lancien et al. 2002, Potel et al. 2009) in shoot. We identified the tissue-specific distribution and the cell-type-specific localization of NADH-GOGAT with qRT-PCR, protein gel blot, promoter analysis and antibody staining. As NADH-GOGAT was increased in roots by ammonium supply, we hypothesized the contribution of NADH-GOGAT in the ammonium assimilation in roots.

Figure 5. Characterization of T-DNA insertion lines for NADH-GOGAT. (A) Positions of T-DNA insertion sites in *NADH-GOGAT* in the lines *glt1-T* and *nadh-gogat-2*. (B, C and D) Quantitative real-time PCR analysis of root RNA from wild-type (Col-0, filled columns), *glt1-T* (gray columns) and *glt1-2* (open columns) plants using gene-specific primers. Root expressed GOGATs (B), chloroplast located Fd-GOGAT/GS2 (C) and root expressed GS1s (D). Plants were pre-cultured on vertical agar lates containing MGRL salts for 14 days, then transferred to a plate containing MGRL salts without nitrogen for 3 days, and transferred again to a plate containing MGRL without nitrogen supplemented with 10mM NH4Cl for 6 h. Bars indicate means±SD (n=3 different plant groups), and significant differences at $*P$ <0.05, $**P$ <0.01 and $***P$ <0.005 are indicated by asterisks. n.s., not significant; n.d., not detected. One-way ANOVA followed by Dunnet tests were used to identify significant differences between wild-type and T-DNA insertion lines.

Our localization study pointed to the importance of NADH-GOGAT in root.

Reverse genetic techniques were used to identify the physiological function of NADH-GOGAT in Arabidopsis (Fig.5). Two NADH-GOGAT T-DNA insertion lines were already isolated and named *glt1-T* (Lancien et al. 2002) and *GLT* (Potel et al. 2009). We isolated a new T-DNA insertion line and named it *nadh-gogat-2* (Fig. 5A), and both *glt1-T* and *nadh-gogat-2* were analyzed in this study.qRT-PCR analysis revealed that neither *Fd-GOGAT2* (Fig. 5B), nor *Fd-GOGAT1* (Fig. 5C) were significantly higher expressed in T-DNA insertion lines, when those transcripts were compared with Col, suggesting that it is not likely that the loss of NADH-GOGAT was compensated
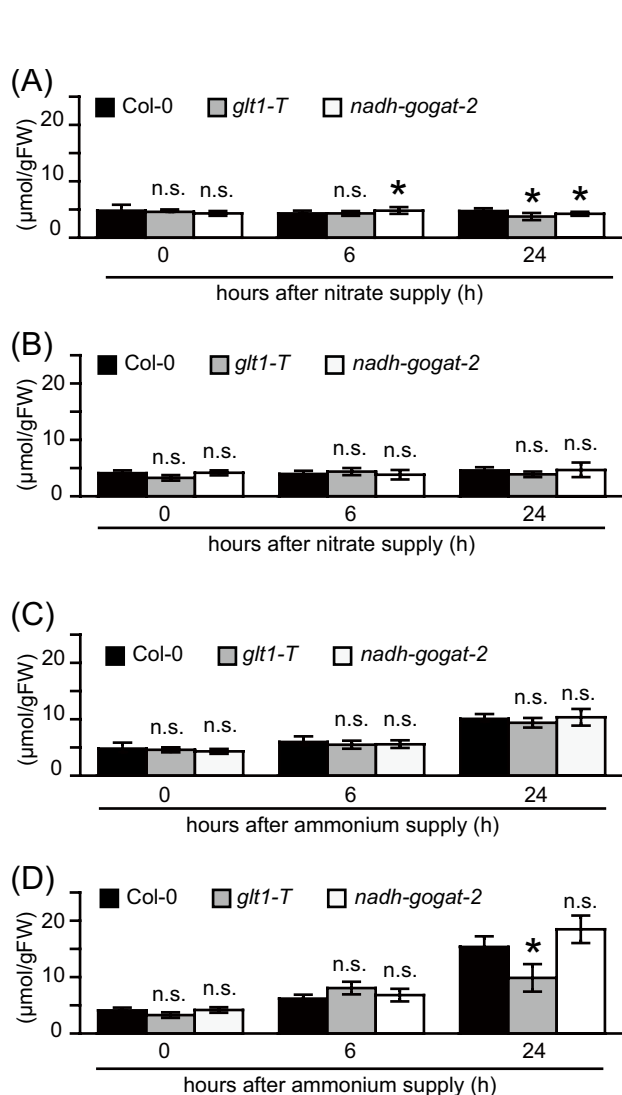

Figure 6. Free amino acid content in plants grown on the vertical agar plate for 14 days.Wild-type (Col-0, filled columns), *glt1-T* (gray columns) and *nadh-gogat-2* (opened columns) were grown on vertical MGRL agar plates containing 7mM nitrate as a nitrogen source for 14 days, and transferred to the MGRL medium without nitrogen, MGRL (−N), for 3 days. After nitrogen starvation, plants were again transferred to MGRL (−N) medium supplemented with either 10mM KNO$_3$ or NH$_4$Cl for 6 or 24 h. Free amino acids in shoot (A, C) and in root (B, D) undereither nitrate (A and B) or ammonium (C and D) supplied conditions. One-way ANOVA followed by Dunnet tests were used to identify significant differences between wild-type and T-DNA insertion lines.
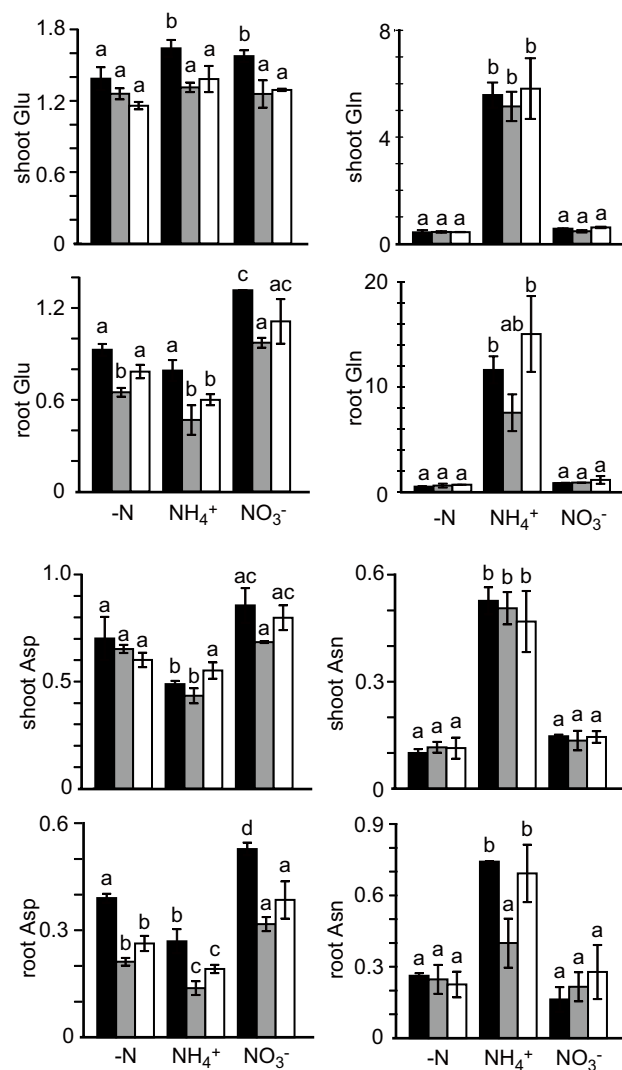
Figure 7. The concentration of Glu, Gln, Asp and Asn in plants grown on the vertical agar plate for 14 days. Wild-type (Col-0, filled columns), *glt1-T* (gray columns) and *nadh-gogat-2* (open columns) were grown on verticalMGRL agar plate containing 7mMnitrate as a nitrogen source for 14 days, and transferred to the MGRL medium without nitrogen, MGRL (−N), for 3 days. After nitrogen starvation, plants were again transferred to MGRL (−N) medium supplemented with 10mM either KNO$_3$ or NH$_4$Cl for 24 h. The concentration of glutamate, glutamine, aspartic acid and asparagine are shown as a column graph. Bars indicated means±SD (n=3 different plant groups), and significant differences at P <0.05 thin each group are indicated by different letters. One-way ANOVA followed by Bonferroni tests were used to identify significant differences between wild-type and T-DNA insertion lines (P <0.05).

by an increase of Fd-GOGAT. *GLN1;1, GLN1;3* (Fig. 5D) and *GLN2* (Fig. 5C) were significantly increased in T-DNA insertion lines. This might be explained by two reasons. First, these three *GLN* isozymes were decreased by glutamate supply (Oliveira and Coruzzi 1999). Upregulation of these three genes may reflect the decrease of glutamate concentration in roots of T-DNA insertion lines (Fig. 7). Second, it is possible to assume that higher 2-OG, a substrate for NADH-GOGAT, might increase transcript abundances in T-DNA insertion lines. Indeed, a previous study showed that 2-OG supply could increase these three GS isozymes, which are also upregulated by light and sugar supply (Oliveira and Coruzzi 1999). These increases in enzyme activity may be related to the fact that ammonium assimilation requires organic molecules as carbon skeleton (Temple et al. 1998) for the production of amino acids. Third, defects in ammonium assimilation may trigger increases in expression. *GLN1;1* and *GLN1;3* were upregulated by ammonium supply in shoots (Potel et al. 2009), and by nitrogen deficiency in root (Ishiyama et al. 2004). T-DNA insertion lines may accumulate excess ammonium and/or cause nitrogen deficiency.

Figure 8. Growth of the T-DNA insertion lines for NADH-GOGAT and wild-type in hydroponics and soil material on different nitrogen sources. Plants were grown in hydroponics (A and B), and in soil material (C and D). Growth of the T-DNA insertion lines for NADH-GOGAT and wild-type in hydroponic culture (A). Shoot dry weights (DW) of the same plants as in A (B). Wild-type (Col-0, filled columns), *glt1-T* (gray columns) and *nadh-gogat-2* (open columns). Bars indicate means±SD (n=3), and significant differences at P <0.05 within each group are indicated by asterisks. Dunnett's tests were used for statistical comparisons. Growth of the T-DNA insertion lines for *NADH-GOGAT* and wild-type on soil material (C). Shoot dry weights (DW) of the same plants as in C (D). Wild-type (Col-0, filled columns), *glt1-T* (gray columns) and *nadh-gogat-2* (open columns). Bars indicate means±SD (n=3–6), and significant differences at P <0.05 within each group are indicated by asterisks. Williams's tests were used for statistical comparisons.

NADH-GOGAT dependent ammonium assimilation in Arabidopsis

The amino acid concentrations were compared in T-DNA insertion lines for NADH-GOGAT and their genetic background Col (Figs 6 and 7). We tested three nitrogen conditions, those are nitrogen deficient, ammonium supplied and nitrate supplied conditions. Neither shoots (Fig. 6A) nor roots (Fig. 6B) showed dramatic changes in total amino acid concentration following the nitrate supply in our culture conditions although nitrate supply is known to activate amino acids and protein synthesis (Stitt 1999). Synthesized amino acids might be quickly used for protein synthesis to recover fromnitrogen deficiency in our condition.
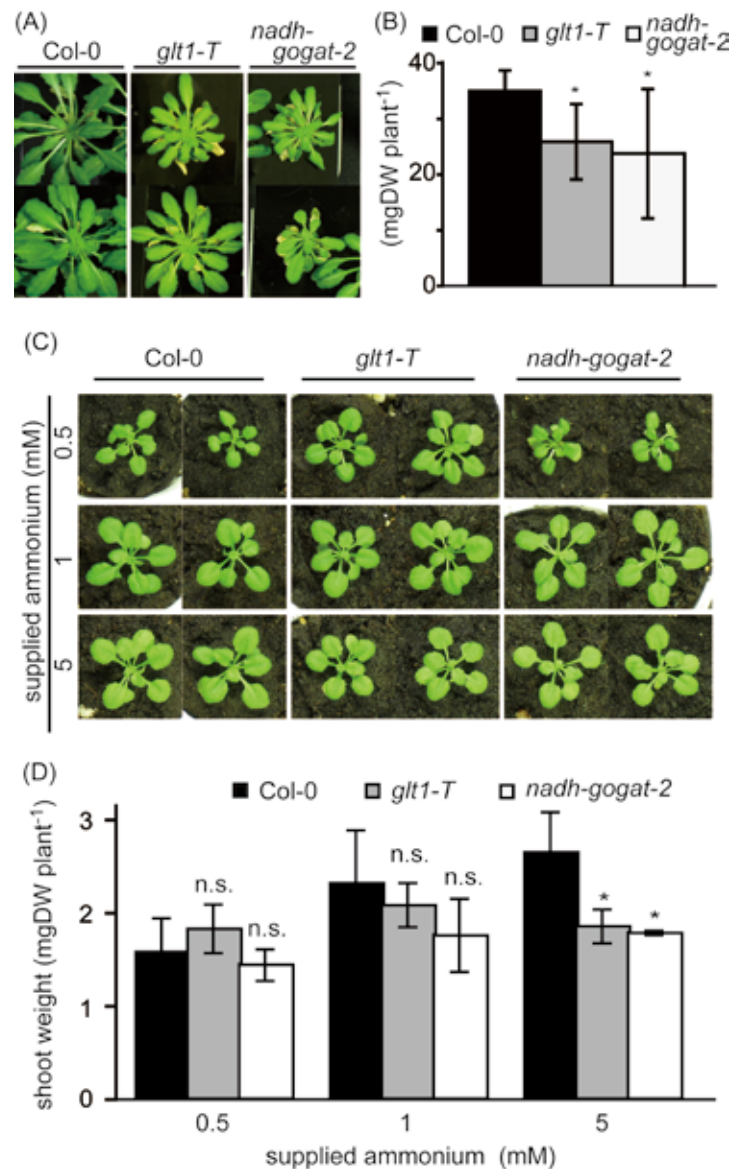
The total amino acid content in both T-DNA insertion lines were significantly lower than that in wild-type 24 h after nitrate supply, suggesting the supportive role of NADH-GOGAT in shoot glutamate synthesis (Lancien et al. 2002, Potel et al. 2009). Contrary to shoots, no significant difference was observed in roots. Most

of nitrate seems to be transported with the transpiration stream via the xylem to the shoot. Ammonium supply rapidly increased the total amino acid contents in either shoots (Fig. 6C) or roots (Fig. 6D) of wild-type and in T-DNA insertions. As the contribution of NADH-GOGAT in the total amino acid synthesis was limited, we next compared the amino acid composition in wild-type and T-DNA insertion lines. The supply of excess ammonium rapidly accumulated amides, such as glutamine and asparagine (Figs 6 and 7). These results fit with previous studies (Clark 1936, Puritch and Barker 1967, Britto and Kronzucker 2002, Forde and Lea 2007). Although NADH-GOGAT was responding to ammonium supply, ammonium induced glutamine and asparagine accumulation was independent of NADH-GOGAT, suggesting the depletion of 2-oxoglutarate levels.

The glutamate concentrations of T-DNA insertion lines were lower than those of wild-type plants under nitrogen deficient and re-supply conditions (Fig. 7), suggesting a major contribution of NADH-GOGAT in root glutamate synthesis. Foliar Fd-GOGAT1 could not maintain the glutamate concentrations under nitrate and ammonium re-supply conditions (Fig. 7). Previous articles discussed the reduction of glutamate levels in T-DNA insertion lines, under high CO_2 conditions (Lancien et al. 2002). We first showed that Fd-GOGAT1 was not able to compensate for a loss of NADH-GOGAT, under not only high CO_2 but also normal air conditions. We also showed that glutamate reduction occurred not only in shoots but also roots under ammonium-supplied condition.

T-DNA insertion lines showed a decrease of aspartate in roots under all tested nitrogen conditions (Fig. 7). Aspartate is formed by the transamination of oxaloacetate (Azevedo et al. 2006). Aspartate aminotransferase (EC 2.6.1.1) catalyzes the conversion of aspartate and 2-oxoglutarate to oxaloacetate and glutamate. Therefore, the decrease of aspartate in T-DNA insertion lines might

be caused by a reduction of glutamate concentration. As glutamate is a pivotal amino acid that is related to the biosynthesis of other amino acids (Stitt et al. 2002), a reduction of glutamate concentration may alter the synthesis of other amino acids.

We next compared the plant growth under various ammonium concentrations (Fig. 8). As amino acid analysis indicated that ammonium supply was able to highlight the physiological role of NADH-GOGAT, we compared the plant growth under normal CO_2 conditions. Plant growth was compared between hydroponics and soil material. In hydroponic solutions, with ammonium as sole nitrogen source, T-DNA insertion leaves were pale green while wild-type leaves were green. It is likely that T-DNA insertion lines suffered from excess ammonium, resulting in a 20% decrease of biomass in T-DNA insertion lines. In soil material, wild-type plants increased the biomass according to the supplied ammonium concentration. Conversely there was no clear statistical difference between shoot dry weights of wild-type and the two T-DNA insertion lines under 0.5 or 1.0mM ammonium supplied conditions. When 5.0mM ammonium was supplied to both T-DNA insertion lines, they showed growth inhibition. As nitrate containing soil material was used in this experiment, a growth inhibition was not as obvious for plants grown in hydroponics. These growth analyses indicated the importance of NADH-GOGAT in ammonium assimilation, and suggesting visible phenotypes under normal CO_2 condition.

Conclusion

When ammonium was supplied to Arabidopsis, the roots accumulated NADH-GOGAT to supply glutamate to GS. This function seems to be unique to NADH-GOGAT because another GOGAT (Fd-GOGAT2) was unable to compensate for the loss of NADH-GOGAT. The loss of functional NADH-GOGAT caused

a biomass reduction when ammonium was served as the major nitrogen source, suggesting a key role for NADH-GOGAT in the ammonium assimilation in roots.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Free amino acid composition under nitrogen deficient conditions.

Table S2. Free amino acid composition in root under nitrate re-supply conditions.

Table S3. Free amino acid composition in shoot under nitrate re-supply conditions.

Table S4. Free amino acid composition in root under ammonium re-supply conditions.

Table S5. Free amino acid composition in shoot under ammonium re-supply conditions.