Progress in Biological Sciences
Vol. 1, No.1, 1-9, Winter/Spring 2011

Effect of *AtNRT2.1* transgene on HATS nitrate uptake in transgenic *Nicotiana plumbaginifolia*

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To investigate the impact of overexpression of *AtNRT2.1* transgene from *Arabidopsis* on nitrate uptake rate and to understand the regulation of endogenous HATS by nitrate and glutamine amino acid (Gln) in tobacco plants, wild-type and transgenic (F line) plants grown on soil for 4 weeks were transferred to hydroponic culture in a controlled-environment with a 16/8h L:D photoperiod at 24° C/20° C, 70% relative humidity and 150 μ mol. m^{-2}. s^{-1} light intensity. Nitrate uptake over time was studied by the ion depletion method. Nitrate uptake in the 2 h exposure to 150 μM nitrate showed an increase in the transgenic line compared to the wild-type plant. Subsequently the uptake trend followed a similar pattern in wild-type plant and transgenic plants. Pretreatment with high concentrations of nitrate (10 mM) increased rate of uptake in both wild-type and transgenic plants. No marked differences between the two genotypes were observed with respect to HATS activity. Glutamine decreased the rate of nitrate uptake in both wild-type and, to a lesser extent, in transgenic plants. Vol. 1, No. 1, 1-9.

KEY WORDS**:** glutamine, iHATS, nitrate transporter, tobacco

INTRODUCTION

Soil solution contains mixtures of inorganic and organic forms of nitrogen. Nitrate is a major source of inorganic nitrogen for most plants and cultivated crops. Plants obtain nitrate from the soil solution and actively absorb it across the plasma membrane of epidermal and cortical cells of the root (Glass et al., 1992; Miller and Smith, 1996). Nitrate is then reduced to nitrite in the cytosol. The sequential reduction of nitrite to ammonium takes place within the plastids. Ammonium is subsequently assimilated into amino acids *via* GOGAT pathways (Forde, 2000). Nitrate levels in the soil solution can vary widely with regional and seasonal conditions (Glass et al., 2002). Therefore, in most natural ecosystems and agricultural systems, plant roots may undergo nitrate deficiency, which retards shoot growth and, in extreme conditions, can lead to general growth inhibition (Orsel et al., 2004). Studies of net nitrate uptake indicate that plants have developed three systems of nitrate transport across plasma membranes of root cells to adjust to variations in nitrate concentration in soil (Tsay et al., 2007). These include a low-affinity transport system (LATS), which shows non-saturable kinetics even at 50 mM external nitrate. The low-affinity transport system is thought to be active for the acquisition of large amounts of nitrate from the soil solution. A low capacity, constitutively expressed, high-affinity transport system (cHATS) is available to transport nitrate at low nitrate concentrations. The third system is stimulated by initial exposure to external nitrate and is presumed to be inducible (iHATS). Although iHATS has a larger capacity than cHATS to transport nitrate, it presents saturable kinetics similar to cHATS.

Molecular studies of root nitrate uptake have led to recognition of two gene families, nitrate transporter1 (*NRT1*) and 2 (*NRT2*), which are presumed to correspond to the components of LATS and HATS, respectively (Crawford and Glass, 1998; Forde, 2000). The first members of

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the *NRT2* gene family were identified and isolated in the fungus *Aspergillus nidulans* (Unkles et al., 1991) and green algae *Chlamydomonas reinhardtii* (Quesada et al., 1994). Most members of the *NRT2* gene family in higher plants such as barley (Trueman et al., 1996; Vidmar et al., 2000a), tobacco (Quesada et al., 1997), soybean (Amarasinghe et al., 1998), wheat (Yin et al., 2007), and *Arabidopsis* (Zhuo et al., 1999; Filleur and Daniel-Vedele, 1999) have been cloned and identified via their sequence homology with *Aspergillus* and *Chlamydomonas NRT2* genes. In the *NRT2* gene family, the *NRT2.1* gene is the best characterized. This gene has been extensively studied in *Arabidopsis thaliana* as a plant model. Evidence found using *atnrt2a* mutants, disrupted in *AtNRT2.1* and *AtNRT2.2* genes, indicates that these genes play an important role in high-affinity nitrate transport (Cerezo et al., 2001; Filleur et al., 2001). Recent findings suggest that the *AtNRT2.1* gene is the major contributor to transport nitrate by iHATS (Li et al., 2007). Moreover, green fluorescent protein imaging has shown that AtNRT2.1 protein is targeted by the root cells plasma membrane (Chopin et al., 2007). AtNRT2.1 protein requires *AtNAR2.1* (recently renamed *AtNRT3.1*) gene expression to be active (Okamoto et al., 2006) and likely interacts with AtNAR2.1 protein to efficiently take up nitrate (Orsel et al., 2006). Nitrate HATS is regulated by many factors, including nitrate itself (Amarasinghe et al., 1998; Krapp et al., 1998; Okamoto et al., 2003), light, sugars (Lejay et al., 1999; Lejay et al., 2003), reduced nitrogen compounds such as ammonium (Amarasinghe et al., 1998; Zhuo et al., 1999; Fraisier et al., 2000; Vidmar et al., 2000 b), and amino acids (Quesada et al., 1997; Krapp et al., 1998; Zhuo et al., 1999; Vidmar et al., 2000 b; Nazoa et al., 2003). Monitoring of nitrate uptake rate using the ion depletion method has indicated that glutamine (Gln) applied to N-deprived tobacco wild-type plants, either as a pretreatment or with NO_3^-, significantly reduces nitrate uptake by HATS compared to control conditions, while low concentrations of nitrate induced nitrate uptake by HATS after a 2 h lag phase (Zoufan and Shariati, 2009). In spite of extensive studies of function and expression of HATS, various nutritional circumstances have used to investigate the effect of nitrate and Gln on nitrate uptake by HATS (Amarasinghe et al., 1998; Krapp et al., 1998; Fraisier et al., 2000; Vidmar et al., 2000 b; Filleur et al., 2001; Nazoa et al., 2003). In some cases, changes in nutritional conditions and experimental protocol have led to differences in results observed between wild type plants and deregulated plants. For example, a study of *NpNRT2.1* gene overexpression in transgenic *Nicotiana plumbaginifolia* revealed no significant differences between wild-type plants and transgenic lines in $^{15}NO_3$ influx, and both genotypes exhibited equivalent accumulations of *NpNRT2.1* transcripts in 1 mM nitrate (Fraisier et al., 2000).

High external concentrations of nitrate decreased both expression of endogenous *NpNRT2.1* and nitrate influx in wild type plants as compared to transgenic tobacco. In the same manner, *atnrt2a* mutants failed to take up concentrations of nitrate up to 0.2 mM (Orsel et al., 2004), while $^{15}NO_3$ influx tests showed that these plants absorbed high levels of nitrate. Therefore, as an alternative approach and in order to better understand the role of *NRT2.1*gene, transgenic *N. plumbaginifolia* transformed with *AtNRT2.1* from *Arabidopsis* was used to examine the effect of deregulation of *NRT2.1* expression on the rate of nitrate uptake under hydroponic conditions. The effect of high concentrations of exogenous nitrate and Gln on the rate of HATS nitrate uptake in wild type and transgenic tobacco plants was investigated using nitrate depletion from nutrition solution.

Material and methods

Plant materials and growth conditions

Nicotiana plumbaginifolia genotypes used in this study were wild type and transformants. Homozygous T2 generation (the third generation) of transgenic tobacco seeds (line F-8) expressing the *AtNRT2.1* coding sequence under control of cauliflower mosaic virus (CaMV) 35S promoter with *AtNAR2.1* (recently designated *AtNRT3.1* by Okamoto et al., 2006) and kanamycin resistance gene marker, originally transformed with *Agrobacterium tumefaciens*

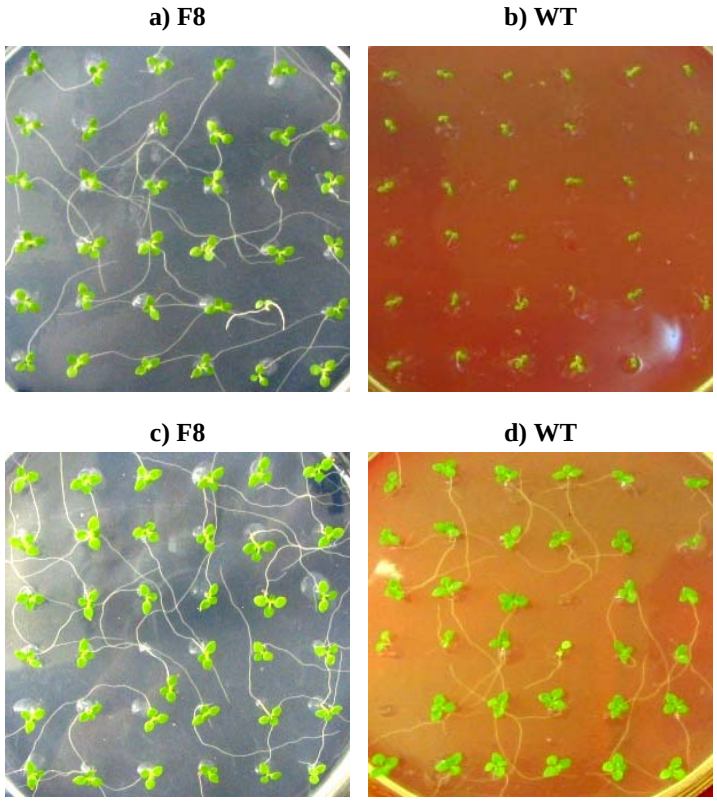

Fig. 1. Confirmation of dominant homozygosity in transformed tobacco *Nicotiana plumbaginifolia* seeds with AtNRT2.1 transgene using MS medium containing kanamycin antibiotic: (a) transgenic tobacco line (F8), (b) wild type (WT); or MS medium without kanamycin antibiotic: (c) tobacco transgenic line (F8), (d) WT.

strain EHA105, was obtained from Dr. A.D.M. Glass, University of British Columbia, Canada. Binary vector of pMOGsNRT was made by introducing gene construct of 35S: *AtNRT2.1* into pMOG402 vector at *Hind*III site in a sense orientation. This vector contains a neomycin phosphotransferase sequence (*NPTII*) coding for kanamycin resistance under control of a nopaline synthase gene promoter (*NosP*) and a terminator sequence of the nopaline synthase gene (*NosT*). A chimeric construct of 35S:: *AtNAT2* (*AtNRT3.1*) cloned in a pRTsNAR2 vector, was released with *Sph*I, and both ends were treated with Klenow enzyme to form sticky ends. This construct was introduced into the *Sma*I site of a pMOGsNRT binary vector in both sense and antisense orientation. The resulting recombinant vector was mobilized into *A. tumefaciens* strain EHA105 to transform leaf disks of *N. plumbaginifolia*. These protocols were conducted at Dr. Glass's laboratory.

For all experiments, the wild type and transgenic seeds were sown on the surface of moistened commercial soil with low release NPK (12.4:5:14.7) fertilizer. The plants were irrigated with tap water three times per week. After 4 weeks, all plants were transferred to hydroponic medium with 0.1 strength modified Johnson's solution (Siddiqi et al., 1990) containing 500 µM $Ca(NO_3)_2$ as a nitrogen source. The pH was adjusted and maintained at 6 ± 0.3 by adding $CaCO_3$ powder. Tubes supporting the plants were placed on floating rafts on the solution surface in 10 l tanks. After one week, the media were replaced with N-free complete nutrient solution. For all steps, the plants were kept in a growth chamber (Conviron E16) with a 16:8 L:D cycle at 24°C/20°C, 70% relative humidity and 150 µmol m^{-2} s^{-1} irradiation. The effect of Gln and high concentration of nitrate on the rate of nitrate uptake in wild type and transgenic tobacco plants was investigated in two stages.

First, to evaluate the effect of high concentrations of nitrate (10 mM nitrate) on HATS activity, 7 days transgenic and wild type N-starved plants were exposed to a complete nutrition solution, either with or without 10 mM nitrate, for 48 h. Subsequently, the roots were rinsed with 0.1 mM $CaSO_4$ for 1 min, and all plants were exposed to 150 µM nitrate. Second, to assess the effect of Gln on HATS activity, seven-day-old transgenic and wild type N-starved plants were treated with 1 mM nitrate. After 3 h, the roots were rinsed with 0.1 mM $CaSO_4$ for 1 min. Plants were then subjected to one of two treatments, either placed in a solution containing 1 mM Gln or remaining in a Gln-free solution. After 3 h, the plants in the Gln-free solution were exposed to either 150 µM nitrate as control or to 1 mM Gln plus 150 µM nitrate. The plants with 3 h pretreatment of Gln were exposed to 150 µM nitrate.

Selection of homozygous generation

Despite confirmation of the above mentioned chimeric gene construct in transgenic tobacco (F line), we conducted it again. The seeds of wild type and transgenic tobacco were surface-sterilized with a 40% bleach solution and sown on 0.1 strength MS medium containing 8% agar and 2% sucrose with or without (as control) 50 mg l^{-1} kanamycin antibiotic. After 14 days, a dominant homozygous generation was selected based on resistance to kanamycin antibiotic by gene marker of *NPTII*.

DNA extraction and PCR conditions

Genomic DNA was extracted from the leaves of a single tobacco plant according to Saghai-Maroof et al. (1984). The chimeric transgene was confirmed by PCR in a Mastercycler gradient Eppendorf thermocycler. Specific primers of *AtNRT2.1* gene were used for cloning of the transgene including forward primer: 5′-GGTCTGAATGGGTCATTCTACTGGTGAG C-3′ and reverse primer: 5′- TGGTCGACAT TGTTGGGTGTGTTCTCAG-3′.Each reaction was performed a total volume of 50 µl. PCR conditions were 95°C 15 sec, 48°C 30 sec, and 72°C 2min for 30 cycles. The PCR products were separated on 0.8% agarose gel.

Nitrate uptake assay

Nitrate uptake rate at 150 µM external nitrate was determined by measuring depletion of nitrate in the roots at 1 h intervals for 8-12 h, using the ion depletion method (Gessler et al., 1998). Samples of the culture medium were taken hourly and stored at -80°C until analysis. For analysis, the aliquots were prepared as described by Zoufan and Shariati (2009). The amount of nitrate in the samples was assayed spectrophotometrically (Shimadzu UV-160A) at 210 nm (Cawse, 1967). At the conclusion of sampling from the incubation solution, the roots were separated from the shoots, dried for 72 h at 70°C, and weighed. Net nitrate uptake rate was calculated based on root dry weight. All experiments were performed in three independent replicates. The data obtained were compared by Duncan's multiple range test at $P \leq 0.05$ confidence level. Statistical analysis was carried out using Sigma Stat2 software.

Results

Confirmation of seed homozygousity and transgene existence in F8 transgenic line

Transgenic seeds grew normally with 4 leaves or more, while kanamycin in the medium repressed growth of wild type seedlings (Fig. 1). In kanamycin-free media, all seeds showed normal growth. Based on these results, the parents of F8 line (T1 generation) were selected as dominant homozygotes. In addition to confirmation of the chimeric gene expression in DNA and mRNA level by Glass' lab (pers. comm.), the presence of the transgene was reconfirmed by PCR and gel electerophoresis (Fig. 2).

Comparison of nitrate uptake rate in wild-type and transgenic tobacco plants

Based on our previous results (Zoufan and Shariati, 2009), a 150 µM concentration of nitrate was selected to determine the induction time of iHATS and the effect of Gln and high concentrations of nitrate on iHATS function. To investigate the consequences of *NRT2.1* over-expression on nitrate uptake of tobacco root, N-

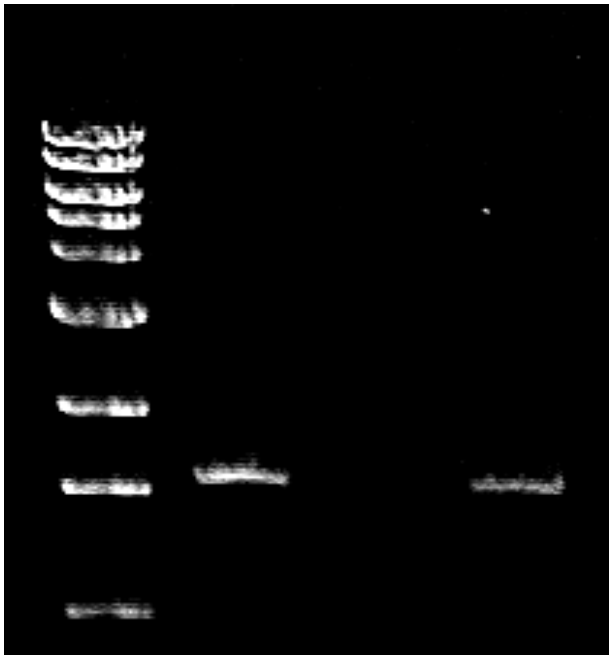

Fig. 2. PCR amplification of transgenic tobacco plants *Nicotiana plumbaginifolia* on 0.8% agarose gel using *AtNRT2.1* gene primers. L 1kb DNA ladder; P plasmid of *Agrobacterium tumefaciens* containing *AtNRT2.1* transgene; WT wild type; F transgenic tobacco plants

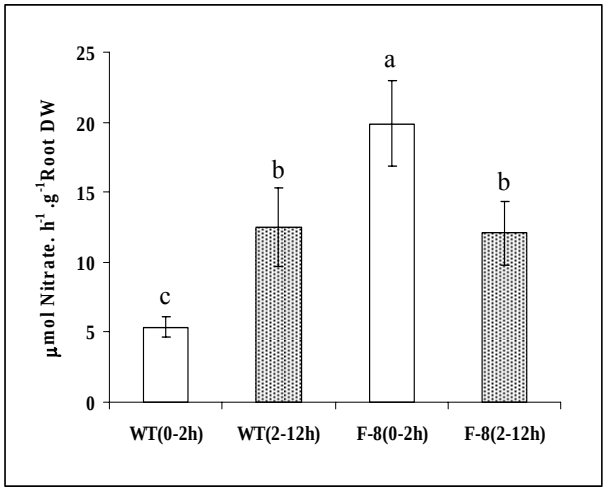

Fig. 3. The rate of uptake of nitrate in wild type and transgenic *Nicotiana plumbaginifolia* in first 2 h and next 2-12 h. 30-day-old plants grown on soil that were transferred to hydroponic media with 500µM nitrate and a 16:8 L:D photoperiod at 24˚C/20˚C, 70% relative humidity and light intensity of 150 µmol m^{-2} s^{-1}. After 7 days, the plants were starved for nitrogen. After 7 days, the plants were transferred to fresh hydroponic media containing 150µM nitrate. The values show means of three independent experiments. Each data point represents the mean ± SD.

starved transgenic and wild type plants were exposed to 150 µM nitrate. The transgenic plants showed a significant increase in rate of uptake nitrate in the first 2 h after nitrate treatment in comparison to wild type plants (Fig. 3). Although the rate of uptake increased in wild type plants after 2 h (2-12 h), a decrease was observed in transgenic plants. No marked difference was detected between wild type and transgenic plants after the initial 2 h.

Effect of 10 mM nitrate on nitrate uptake by HATS:

Pretreatment of N-deprived transgenic plants with 10 mM nitrate for 48 h was associated with an increase in the rate of nitrate uptake in wild type and transgenic plants both within the first 2 h (Fig. 4a) and subsequently (2-12 h) (Fig. 4b). In the first 2 h, this increase was greater than in the following 2-12 h. In general, no significant difference was observed between wild type and transgenic plants. The trend of nitrate depletion in the medium (Fig. 4c) indicated that the plants grown with10 mM nitrate displayed a more rapid increase of nitrate uptake compared to non-treated plants.

Effect of exogenously applied glutamine (Gln) on nitrate uptake by HATS

Fig. 5 shows the comparative effects of Gln treatment and 3 h Gln pretreatment on rate of nitrate uptake in wild type and transgenic plants. Glutamine, whether supplied externally at commencement of experiment or 3 h before as a pretreatment, inhibited the rate of nitrate uptake in both wild type and transgenic plants. Under both conditions the inhibitory effect of Gln in transgenic plants was significantly lower than on wild type plants.

Discussion

The *NpNRT2.1* gene encodes an inducible high affinity nitrate transport system in *Nicotiana plumbaginifolia* (Fraisier et al., 2000). In our previous investigation (Zoufan and Shariati, 2009) characterizing HATS activity in wild type

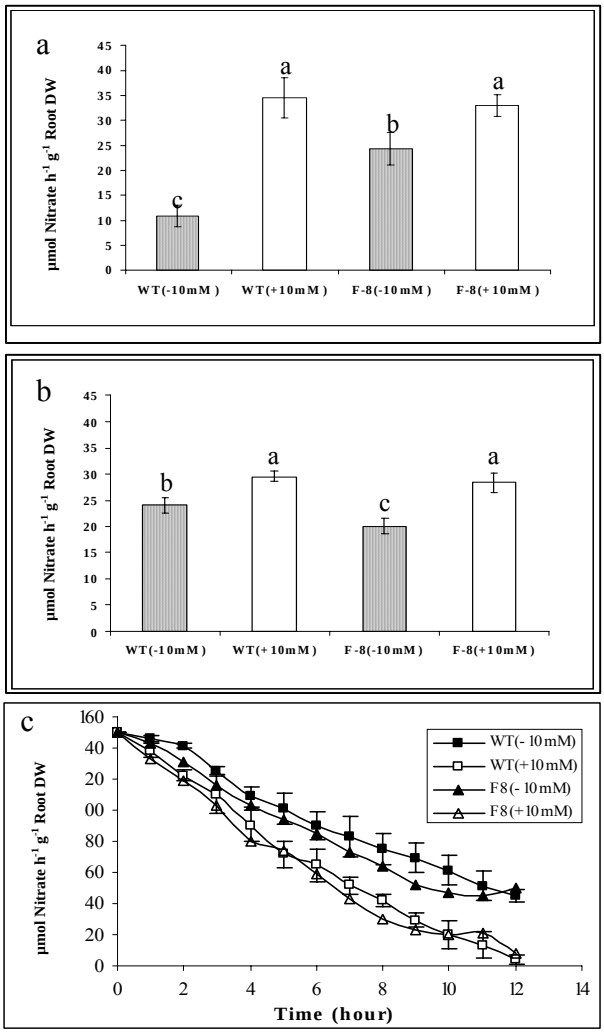

Fig. 4. Effect of 10 mM nitrate on rate of nitrate uptake in wildtype and transgenic *Nicotiana plumbaginifolia,* (a) in first 2 h exposure to 150µM nitrate or (b) next 2-12 h. (c) Time-course of nitrate depletion in the medium in wild type (WT) without (■) or with pretreatment of 10 mM nitrate (□) and transgenic plants (F-8 line) without (▲) or with pretreatment of 10 mM nitrate (Δ) plants. Wild-type (WT) and transgenic (F-8 line) plants were grown hydroponically in N-free media. After 7 days, the plants were transferred to fresh hydroponic media with or without 10 mM nitrate. After 48 h, the roots grown at 10 mM were rinsed with 0.1 mM $CaSO_4$ for 1 min. Then, all plants were supplied with nutrient media containing 150µM nitrate. The values show means of three independent experiments. Each data point represents the mean ± SD.

tobacco plants we showed that HATS activity is induced by nitrate and inhibited by Gln. Nitrate uptake by HATS is a highly regulated process. Mutants and transformants of HATS have been used to better understand and clarify the role of *NRT2* genes in the regulation of nitrate uptake (Fraisier et al., 2000; Cerezo et al., 2001). Quesada et al. (1997) identified and cloned the *Nicotiana plumbaginifolia NpNrt2.1* gene via its sequence homology to the *Aspergillus nidulans crnA* gene family. They showed that the *NRT*2 gene family in *N. plumbaginifolia* contains only 1 or 2 members that are strongly expressed in roots and induced by nitrate.

Several studies have shown that the *NRT2.1* gene is inducible by external nitrate (Trueman et al., 1996; Quesada et al., 1997; Amarasinghe et al., 1998; Krapp et al., 1998; Filluer and Daniel-Vedele 1999; Lejay et al., 1999; Zhou et al., 1999; Vidmar et al., 2000b; Okamoto et al., 2003). A strong correlation has been observed between *NRT2.1* expression level and nitrate uptake by iHATS in higher plants (Amarasinghe et al., 1998; Krapp et al., 1998; Lejay et al., 1999; Zhuo et al., 1999). In spite of the continuous expression of the *AtNRT2.1* transgene in transgenic plants (Glass, pers comm.), no significance difference was observed in the rate of nitrate uptake between transgenic and wild type plants grown with 150 µM nitrate, with the exception the first 2 h of experiment when the greater rate of nitrate uptake was found in the transgenic line. Thus, it is likely that at least 2 h would be sufficient to induce endogenous *NpNRT2.1* gene in wild type plants and increase the rate of uptake to levels observed in transgenic plants. It is thought that constitutive overexpression of the *AtNRT2.1* gene, driven by CaMV 35S virus promoter, in transgenic plants does not increase nitrate depletion from the medium within the first 2 h, but it induces transgenic plants to begin nitrate uptake about 2 h earlier than do wild type. After about 2-3 h, the rate of nitrate uptake in transgenic plants was equivalent to the wild type. The results of 2-12 h exposure are in agreement with previous studies on transgenic tobacco transformed only by the *NpNRT2.1*gene (Fraisier et al., 2000). Similarly, low concentrations of nitrate led to the same responses in $^{15}NO_3$ influxes in transgenic and wild type plants.

It has been reported that high concentrations of nitrate (10 mM) increase nitrate uptake in

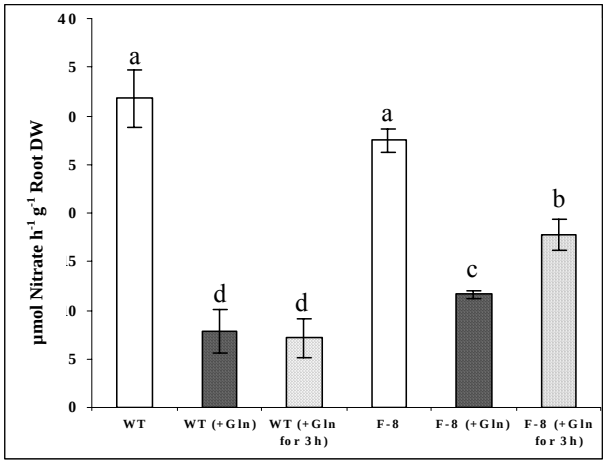

Fig. 5. Effect of Gln on rate of nitrate uptake in wild type (WT) and transgenic *Nicotiana plumbaginifolia* for 8 h. Wild type and transgenic (F-8 line) plants were grown hydroponically in N-free media. After 7 days, the plants were transferred to fresh hydroponic media containing 1 mM nitrate. After 3 h, all roots were rinsed with 0.1 mM $CaSO_4$ for 1 min. Some plants were pretreated with 1 mM Gln (+ Gln for 3 h) and the others remained in N-free media for 3 h. At the same time, the plants grown in N-free media supplied with 1 mM Gln + 150µM nitrate (+ Gln) or only 150 µM nitrate (as control) and the plants pretreated with Gln, exposed with 150µM nitrate. The values show means of three independent experiments. Each data point represents the mean ± SD.

transgenic plants compared to the wild type (Fraisier et al., 2000). However, a closer review of that study shows that 10 mM nitrate had an inhibitory effect on both transgenic and wild type plants, but exerted a lower inhibitory effect on nitrate influx in tobacco plants transformed by the *NpNRT2.1* gene. In the present study, constitutive over-expression of the *AtNRT2.1* gene in transgenic plants pretreated with 10 mM nitrate was not associated with inhibition of nitrate uptake. Wild type and transgenic plants pretreated with 10 mM nitrate displayed a similar increase in rate of nitrate uptake as did untreated plants. It has been reported that inhibition of *AtNRT2.1* gene expression in *Arabiodopsis thaliana* is mediated by AtNRT1.1 transporter at high concentrations of nitrate (Krouk et al., 2006). It seems under our experimental conditions, 48 h treatment of plants with 10 mM nitrate was not sufficient to repress the endogenous *NpNRT2.1* gene in wild type plants.

Glutamine is thought to play an important role in nitrate uptake regulation. Some studies suggest that expression of the *NRT2.1* gene is feedback repressed by amino acids (Quesada et al., 1997; Krapp et al., 1998; Zhou et al., 1999; Vidmar et al., 2000b; Nazoa et al., 2003). Exogenously applied Gln has been shown to reduce nitrate uptake by HATS in *Lolium perenne* (Thornton, 2004), *Arabidopsis* (Nazoa et al. 2003), and barley (Vidmar et al., 2000b; Aslam et al., 2001). Our results indicate that Gln treatment repressed nitrate uptake in both genotypes, when compared to plants grown in Gln-free medium, although Gln had less inhibitory effect on nitrate HATS in transgenic tobacco. Therefore, our results reconfirmed that Gln is able to impair induction of endogenous HATS.

In summary, the data showed that a short duration (48 h) application of a high concentration of nitrate did not repress nitrate uptake and even induces nitrate influx.

In N-starvation conditions with no induction, transgenic tobacco plants do not absorb nitrate at a higher rate than does the wild type, but due to continuous expression of constitutive *AtNRT2.1* trangene, are able to take up the nitrate in the HATS range immediately after exposure to 150 µM nitrate. Indeed, over-expression of constitutive *AtNRT2.1* gene led to greater uptake by transformants in the first 2 h. In general, Gln through inhibition of the endogenous *NpNRT2.1* gene appears to be a suitable indicator for the physiological discrimination of transgenic genotype from the wild type plant. The process of root nitrate uptake has been intensively investigated at the molecular and physiological level. In order to further and better understand the HATS function in higher plants, this genotype should be verified under different nutritional and environmental conditions. Variable conditions possibly led to differences between wild type and transgenic plants phenotypically and physiologically.

Acknowledgments
This work was supported by the Faculty of Postgraduate studies, University of Isfahan, Isfahan, Iran. We also wish to thank Prof. A.D.M Glass, Department of Plant Biology, University of British Columbia, Vancouver, Canada for his comments and suggestions.

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