

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

Parzhak Zoufan² and Mansour Shariati^{1*}

¹Department of Biology, University of Isfahan, Hezarjarib St., Isfahan, Iran

²Department of Biology, University of Shahid Chamran, Ahvaz, Iran

Abstract

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 μ M 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.

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

*Corresponding author: mansour_shariati@yahoo.com
Tel.: +98 31127932472; fax: +98 31127932456

presumed to correspond to the components of LATS and HATS, respectively (Crawford and Glass, 1998; Forde, 2000). The first members of 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; Fraiser 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; Fraiser 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 over-expression in transgenic *Nicotiana plumbaginifolia* revealed no significant differences between wild-type plants and transgenic lines in $^{15}\text{NO}_3$ influx, and both genotypes exhibited equivalent accumulations of *NpNRT2.1* transcripts in 1 mM nitrate (Fraiser 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 $^{15}\text{NO}_3$ 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-

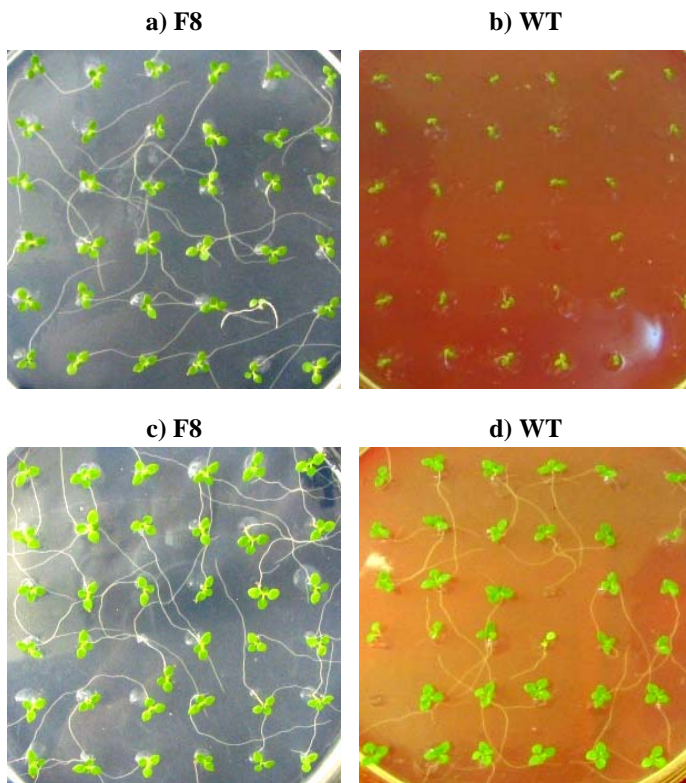


Fig. 1. Confirmation of dominant homozygosity in transformed tobacco *Nicotiana plumbaginifolia* seeds with AtNRT2.1 transgene using MS medium containing kanamycin antibiotic: (a) transgenic tobacco line (F8), (b) wild type (WT); or MS medium without kanamycin antibiotic: (c) tobacco transgenic line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

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* 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 $\text{Ca}(\text{NO}_3)_2$ 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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 CaSO₄ 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 CaSO₄ 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-Marroof 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'-GGTCTAGAATGGGTCATTCTACTGGTGA GC-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 ≤ 0.05 confidence level. Statistical analysis was carried out using Sigma Stat2 software.

Results

Confirmation of seed homozygosity 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 electrophoresis (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-

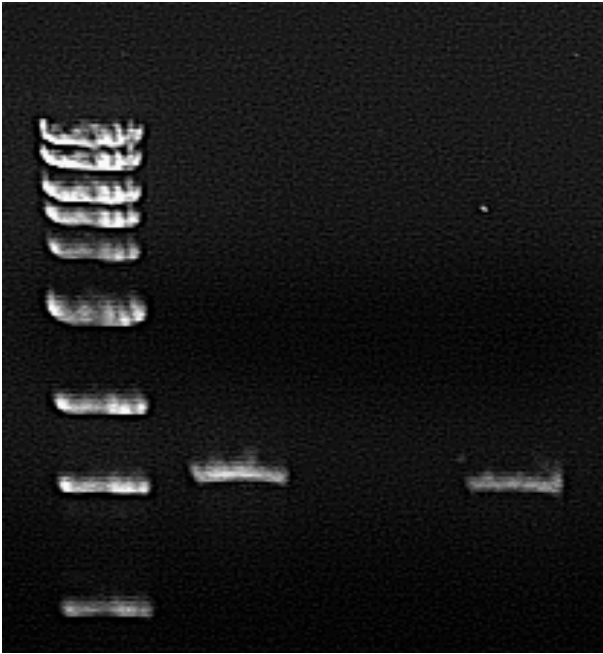


Fig. 2. PCR amplification of transgenic tobacco plants *Nicotiana plumbaginifolia* on 0.8% agarose gel using *AtNRT2.1* gene primers. L 1kb DNA ladder; P plasmid of *Agrobacterium tumefaciens* containing *AtNRT2.1* transgene; WT wild type; F transgenic tobacco plants

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 with 10 mM nitrate displayed a more

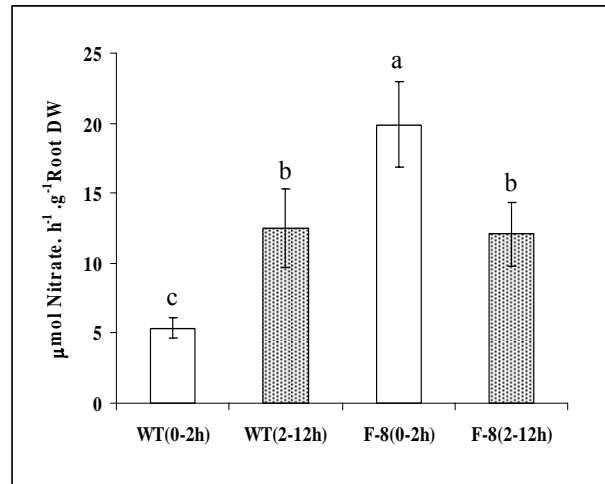


Fig. 3. The rate of uptake of nitrate in wild type and transgenic *Nicotiana plumbaginifolia* in first 2 h and next 2-12 h. 30-day-old plants grown on soil that were transferred to hydroponic media with 500 μM nitrate and a 16:8 L:D photoperiod at 24°C/20°C, 70% relative humidity and light intensity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

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 tobacco plants we showed that HATS activity is induced by nitrate and inhibited by Gln. Nitrate

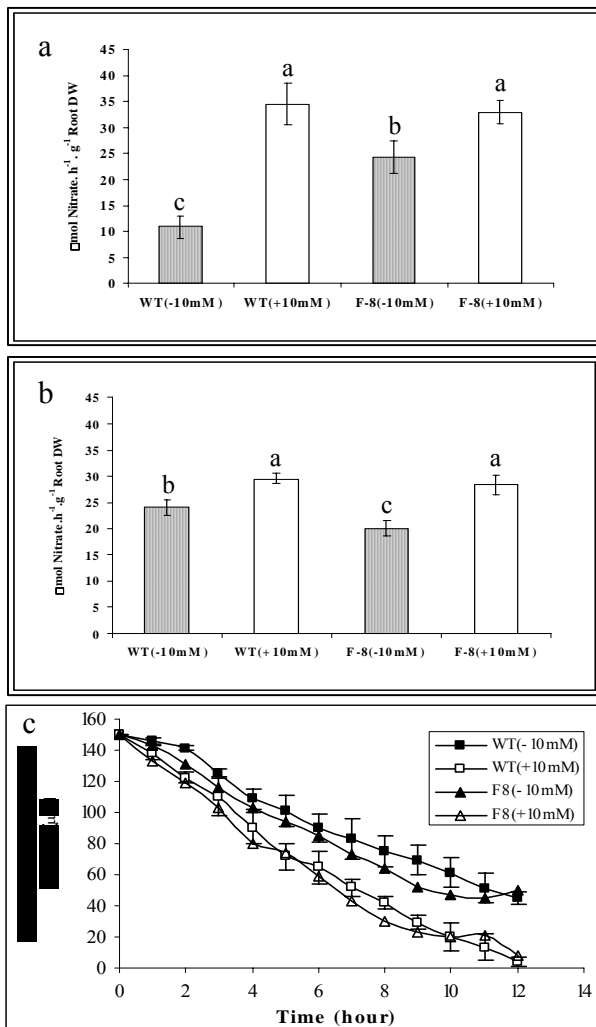


Fig. 4. Effect of 10 mM nitrate on rate of nitrate uptake in wildtype and transgenic *Nicotiana plumbaginifolia*, (a) in first 2 h exposure to 150 μM nitrate or (b) next 2-12 h. (c) Time-course of nitrate depletion in the medium in wild type (WT) without (■) or with pretreatment of 10 mM nitrate (□) and transgenic plants (F-8 line) without (▲) or with pretreatment of 10 mM nitrate (△) 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 CaSO₄ for 1 min. Then, all plants were supplied with nutrient media containing 150 μM nitrate. The values show means of three independent experiments. Each data point represents the mean ± SD.

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 *NRT2* 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 over-expression 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 ¹⁵NO₃ influxes in transgenic and wild type plants.

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

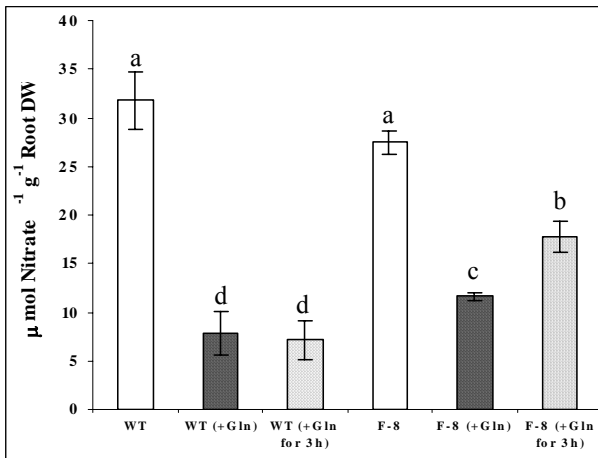


Fig. 5. Effect of Gln on rate of nitrate uptake in wild type (WT) and transgenic *Nicotiana plumbaginifolia* for 8 h. Wild type and transgenic (F-8 line) plants were grown hydroponically in N-free media. After 7 days, the plants were transferred to fresh hydroponic media containing 1 mM nitrate. After 3 h, all roots were rinsed with 0.1 mM CaSO₄ 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 + 150 μM nitrate (+ Gln) or only 150 μM nitrate (as control) and the plants pretreated with Gln, exposed with 150 μM nitrate. The values show means of three independent experiments. Each data point represents the mean ± SD.

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 *Arabidopsis 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; Zhuo 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* transgene, 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.

References

- Amarasinghe, B.H., de Bruxelles, G.L., Braddon, M., Onyeocha, I., Forde, B.G. and Udvardi, M.K. (1998).** Regulation of *GmNRT2* expression and nitrate transport activity in the roots soybean (*Glycine max*). *Planta* 206, 44–52.
- Aslam, M., Travis, R.L. and Rains, D.W. (2001).** Differential effect of amino acids on nitrate uptake and reduction systems in barley roots. *Plant Sci.* 160, 219–228
- Cawse, P.A. (1967). The determination of nitrate in soil solution by ultraviolet spectrophotometry. *Analyst* 92, 311–315.
- Cerezo, M., Tillard, P., Filleur, S., Munos, S., Daniel-Vedele, F. and Gojon, A. (2001).** Major alterations of the regulation of root NO₃⁻ uptake are associated with the mutation of *NRT2.1* and *NRT2.2* genes in *Arabidopsis*. *Plant Physiol.* 127, 262–271.
- Chopin, F., Wirth, J., Dorbe, M.F., Lejay, L., Krapp, A., Gojon, A. and Daniel-Vedele F. (2007).** The *Arabidopsis* nitrate transporter ATNRT2.1 is targeted to the root plasma membrane. *Plant Physiol. Biochem.* 45, 630–635.
- Crawford, N.M. and Glass, A.D.M. (1998).** Molecular and physiological aspects of nitrate uptake in plants. *Trends Plant Sci.* 3, 389–395.
- Filleur, S. and Daniel-Vedele, F. (1999).** Expression analysis of a high-affinity nitrate transporter isolated from *Arabidopsis thaliana* by differential display. *Planta* 207, 461–469.
- Filleur, S., Dorbe, M.F., Cerezo, M., Orsel, M., Granier, F., Gojon, A. and Daniel-Vedele F. (2001).** An *Arabidopsis* T-DNA mutant affected in *NRT2* genes is impaired in nitrate uptake. *FEBS Lett.* 489, 220–224.
- Forde, B.G. (2000). Nitrate transporters in plants: structure, function and regulation. *Biochim. Biophys. Acta.* 1465, 219–235.
- Fraisier, V., Gojon, A., Tillard, P. and Daniel-Vedele, F. (2000).** Constitutive expression of a putative high-affinity nitrate transporter in *Nicotiana plumbaginifolia*: evidence for post-transcriptional regulation by a reduced nitrogen source. *Plant J.* 23, 489–496.
- Gessler, A., Schneider, S., Von Sengbusch, D., Weber, P., Hanemann, U., Huber, C., Rothe, A., Kreutzer, K. and Rennenberg, H. (1998).** Field and laboratory experiments on net uptake of nitrate and ammonium by the roots of spruce (*Picea abies*) and beech (*Fagus sylvatica*) trees. *New Phytol.* 138, 275–285.
- Glass, A.D.M., Shaff, J.E. and Kochian, L.V. (1992).** Studies of nitrate uptake in barley. IV: electrophysiology. *Plant Physiol.* 99, 456–463.
- Glass, A.D.M., Britto, D.T., Kaiser, B.N., Kinghorn, J.R., Kronzucker, H.J., Kumar, A., Okamoto, M., Rawat, S., Siddiqi, M.Y., Unkles, S.H., et al. (2002).** The regulation of nitrate and ammonium transport systems in plants. *J. Exp. Bot.* 53, 856–864.
- Krapp, A., Fraasier, V., Scheible, W., Quesada, A., Gojon, A., Stitt, M., Caboche, M. and Daniel-Vedele, F. (1998).** Expression studies of Nrt2.1:Np, a putative high-affinity nitrate transporter: evidence for its role in nitrate uptake. *Plant J.* 14, 723–731.
- Krouk, G., Tillard, P. and Gojon, A. (2006).** Regulation of the high-affinity NO₃⁻ uptake systems by NRT1.1-mediated NO₃⁻ demand signaling in *Arabidopsis*. *Plant Physiol.* 142, 1075–1086.
- Lejay, L.M., Tillard, P., Lepetit, M., Olive, F.D., Filleur, S., Daniel-Vedele, F. and Gojin, A. (1999).** Molecular and functional regulation of two NO₃⁻ uptake systems by N- and C-status of *Arabidopsis* plants. *Plant J.* 18, 509–519.
- Lejay, L.M., Gansel, X., Cerezo, M., Tillard, P., Muller, C., Krapp, A., Von Wiren, N., Daniel-Vedele, F. and Gojon, A. (2003).** Regulation of Root ion transporters by photosynthesis: functional importance and relation with hexokinase. *Plant Cell* 15, 2218–2232.
- Li, W., Wang, Y., Okamoto, M., Crawford, N.M., Siddiqi, M.Y. and Glass, A.D.M. (2007).** Dissection of the *AtNRT2.1:AtNRT2.2* inducible high-affinity nitrate transporter gene cluster. *Plant Physiol.* 143, 425–433.
- Miller, A.J. and Smith, S.J. (1996). Nitrate transport and compartmentation. *J. Exp. Bot.* 52, 541–549.
- Nazoa, P., Vidmar, J.J., Tranbarger, T.J., Mouline, K., Damiani, L., Tillard, P., Zhuo, D., Glass, A.D. and Touraine, B. (2003).** Regulation of the nitrate transporter gene *AtNRT2.1* in *Arabidopsis thaliana*: responses to nitrate, amino acids and developmental stage. *Plant Mol. Biol.* 52, 689–703.
- Okamoto, M., Vidmar, J.J. and Glass, A.D.M. (2003).** Regulation of *NRT1* and *NRT2* gene families of *Arabidopsis thaliana*: responses to nitrate provision. *Plant Cell Physiol.* 44, 304–317.
- Okamoto, M., Kumar, A., Li, Z., Wang, Y., Siddiqi, M.Y., Crawford, N.M. and Glass, A.D.M. (2006).** High-affinity nitrate transport in roots of *Arabidopsis* depends of expression of the *NAR2*-like gene *AtNRT3.1*. *Plant Physiol.* 140, 1036–1046.
- Orsel, M., Chopin, F., Leleu, O., Smith, S.J., Krapp, A., Daniel-Vedele, F. and Miller, A.J. (2006).** Characterization of a two component high-affinity nitrate uptake in *Arabidopsis*. Physiology and protein-protein interaction. *Plant Physiol.* 142, 1304–1317.
- Orsel, M., Eulenburg, K., Krapp, A. and Daniel-Vedele, F. (2004).** Disruption of the nitrate transporter genes *AtNRT2.1* and *AtNRT2.2* restricts growth at low external nitrate concentration. *Planta* 219, 714–721.
- Quesada, A., Galvan, A. and Fernandez, E. (1994).** Identification of nitrate transporter genes in *Chlamydomonas reinhardtii*. *Plant J.* 5, 407–419.
- Quesada, A., Krapp, A., Trueman, L.J., Daniel-Vedele, F., Fernandez, E., Forde, B.G. and Caboche, M. (1997).** PCR-identification of a *Nicotiana plumbaginifolia* cDNA homologues

of the high-affinity nitrate transporters of the *crnA* family. *Plant Mol. Biol.* *34*, 265-274.

Saghai-Marooif, M.A., Soliman, K., Jorgensen, R.A. and Allard, R.W. (1984). Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location and population dynamics. *PNAS* *81*, 8014-8018.

Siddiqi, M.Y., Glass, A.D.M., Ruth, T.J. and Rufty, T. (1990). Studies of the uptake of nitrate in barley: I. Kinetics of ¹³NO₃ influx. *Plant Physiol.* *93*, 1426-1432.
Thornton, B. (2004). Inhibition of nitrate influx by glutamine in *Lolium perenne* depends upon the contribution of the HATS to the total influx. *J. Exp. Bot.* *55*, 761-769.

Trueman, L.J., Richardson, A. and Forde, B.G. (1996). Molecular cloning of higher plant homologues of the high-affinity nitrate transporters of *Chlamydomonas reinhardtii* and *Aspergillus nidulans*. *Gene* *175*, 223-231.

Tsay, Y.F., Chiu, C.C., Tsai, C.B., Ho, C.H. and Hsu, P.K. (2007). Nitrate transporters and peptide transporters. *FEBS Lett.* *581*, 2290-2300.

Unkles, S.E., Hawker, K.L., Grieve, C., Campbell, E.I., Montague, P. and Kinghorn, J.R., (1991). *crnA* encodes a

nitrate transporter in *Aspergillus nidulans*. *Proc. Nat. Acad. Sci. USA* *88*, 204-208.

Vidmar, J.J., Zhou, D., Siddiqi, M.Y. and Glass, A.D.M. (2000a). Isolation and characterization of *HvNRT2.3* and *HvNRT2.4* cDNA encoding high-affinity nitrate transporters from roots of barley. *Plant Physiol.* *122*, 783-792.

Vidmar, J.J., Zhou, D., Siddiqi, M.Y., Schjoerring, J.K., Touraine, B. and Glass, A.D.M. (2000b). Regulation of high-affinity nitrate transporter genes and high-affinity nitrate influx by nitrogen pools in roots of barley. *Plant Physiol.* *123*, 307-318.

Yin, L.P., Li, P., Wen, B., Taylor, D. and Berry, J.O. (2007). Characterization and expression of a high-affinity nitrate system transporter gene (*TaNRT2.1*) from wheat roots, and its evolutionary relationship to other *NRT2* genes. *Plant Sci.* *172*, 621-631.

Zhuo, D., Okamoto, M., Vidmar, J.J. and Glass, A.D.M. (1999). Regulation of putative high-affinity nitrate transporter (*Nrt2:1At*) in roots of *Arabidopsis thaliana*. *Plant J.* *17*, 563-568.

Zoufan, P. and Shariati, M. (2009). The trend of HATS nitrate uptake in response to nitrate and glutamine in *Nicotiana plumbaginifolia*. *Plant. Res. J. Environ. Sci.* *3*, 163-173.