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The Trend of HATS Nitrate Uptake in Response to Nitrate and Glutamine in *Nicotiana plumbaginifolia* Plant

P. Zoufan and M. Shariati
Department of Biology, University of Isfahan, Hezarjarib Street, Isfahan, Iran

Abstract: Present study was conducted to investigate and characterize the effect of nitrate and Gln (glutamine) on the trend and rate of nitrate uptake by HATS (High-affinity transport system) in *Nicotiana plumbaginifolia* plant under hydroponic system using ion depletion technique. The plants were grown at 16 h light/8h dark at 24/20°C, 70% humidity, 150 μmol m^{-2} sec^{-1} light intensity and on the commercial soil with NPK fertilizer. About 28-day plants were transferred to hydroponic media containing complete nutrition solution with 0.5 mM NO_3^- at the same growth conditions for 1 week. To better understanding of time-course of HATS induction, starved plants of nitrogen for 7 days were supplied with 5 different concentrations including 10, 50, 75, 100 and 150 μM NO_3^-. Monitoring of trend of NO_3^- absorption showed that at least 2 h is adequate to induce HATS activity at 150 μM NO_3^-. A surprising finding was that transfer N-starved plants to media containing high concentration of NO_3^- (10 mM) for 48 h resulted in increasing the rate of NO_3^- uptake by HATS. Amino acid Gln applied to N-deprived plants either as pretreatment or with NO_3^- significantly inhibited nitrate uptake by HATS compared to control conditions. The results collectively indicate that high-affinity nitrate transport is regulated by nitrate itself and the metabolites of its assimilation such as amino acids.

Key words: Nitrate uptake, HATS, *Nicotiana plumbaginifolia*, Gln

INTRODUCTION

Nitrogen is an essential element for all the plants. It is the most abundant mineral element in plant tissues. Plants require nitrogen throughout their development and plant growth and productivity potentially depends on nitrogen content in the soil. Despite severe requirement of plants to nitrogen, only a small proportion of environmental nitrogen is available for plants in the pedosphere. Therefore, application extent of nitrogenous fertilizers particularly in developing countries has increased over the last decade, resulting in accumulation of nitrogenous fertilizers and associated compounds to toxic levels in some conditions, pollution of surface and ground waters and enrichment of atmosphere with NH_3 and N_2O (Miller and Cramer, 2004).

Higher plants obtain nitrogen from the assimilation of nitrate and ammonium reduction. In most soils, nitrate is the primary nitrogen source for many plants and uptake process is of fundamental importance for the N cycle (Tong *et al*., 2005). The plants often grow in the ecosystems which contain very low concentration of nitrate. Moreover, nitrate content of soil solution shows variability associated with season, region and location. However, to adapt and grow, the plants must sense changes of nitrate concentration in environment and adjust their growth to match resource availability (Schachtman and Shin, 2007). Many physiological researches indicate that plants have developed at

Corresponding Author: Mansour Shariati, Department of Biology, University of Isfahan, Hezarjarib Street, Isfahan, Iran
Tel: +98 311 7932472 Fax: +98 311 7932456

least three different uptake systems for nitrate to cope with variations in nitrate concentrations in soils (Siddiqi *et al*., 1990; Crawford, 1995; Crawford and Glass, 1998). High-affinity Transport System (HATS) operates at low external nitrate concentration (≤1 mM). This saturable system has been categorized into two genetically separate transport systems: an inducible HATS (iHATS) by external nitrate which has high-capacity to transport nitrate. A low-capacity HATS which is constitutive (cHATS) and works without previous exposure of the roots to nitrate. Low-affinity Transport System (LATS) displays an important function when external nitrate concentration is above 1 mM and shows a linear kinetics even at nitrate concentration as high as 50 mM. Based on molecular evidence, two types of nitrate transporters NRT1 and NRT2 have been identified in higher plants (Forde, 2000; Orsel *et al*., 2002a; Tsay *et al*., 2007) that are thought to correspond to low- and high- affinity nitrate transporters, respectively. In lower eukaryotic organisms, the *NRT2* gene family of high affinity nitrate transporters was first identified in fungus of *Aspergillus nidulans* (Unkles *et al*., 1991, 2001) and *Chlamydomonas reinhardtii* (Quesada *et al*., 1994). In higher plants, most members of *NRT2* gene family were identified and cloned via their sequence homology with the *CRNA* gene in *A. nidulans* and *CrNRT2.1* gene in *C. reinhardtii*, including barley (Trueman *et al*., 1996; Vidmar *et al*., 2000a), soybean (Amarassinghe *et al*., 1998), tobacco (Quesada *et al*., 1997), wheat (Yin *et al*., 2007) and *Arabidposis* (Filleur and Daniel-Vedele, 1999; Zhuo *et al*., 1999). Among *NRT2* genes, *NRT2.1* gene is the best characterized gene correspond to high affinity transporter in higher plants (Filleur and Daniel-Vedele, 1999; Zhuo *et al*., 1999) which is supposed to be expressed mainly in the roots of *Nicotiana plumbaginifolia* (Quesada *et al*., 1997) and *Arabidopsis thaliana* (Orsel *et al*., 2002b). The expression of nitrate iHATS is induced by external nitrate in nitrate-starved roots and deduced when nitrate supply is maintained (Filleur and Daniel-Vedele, 1999; Zhuo *et al*., 1999). Besides induction by nitrate, reduced-nitrogen metabolites such as NH_4^+, Gln and the other amino acids repress the expression of iHATS as feedback inhibition (Amarasinghe *et al*., 1998; Krapp *et al*., 1998; Vidmar *et al*., 2000b; Nazoa *et al*., 2003).

The species *Nicotiana plumbaginifolia* from Solanaceae family and *Arabidopsis thaliana* from Brassicaceae family have been used extensively as plant model in many physiological and molecular studies of nitrate assimilation (for example; Fraisier *et al*., 2000; Cerezo *et al*., 2001; Li *et al*., 2007). These plants have been much useful to clarify the function of many enzymes and proteins involved in this metabolic pathway. To gain further insights into function and expression regulation of nitrate HATS in planta, extensive physiological and molecular studies have been performed in *Arabidopsis* as a plant model (Filleur and Daniel-Vedele, 1999; Lejay *et al*., 1999; Zhuo *et al*., 1999; Cerezo *et al*., 2001; Filleur *et al*., 2001; Okomato *et al*., 2003; Orsel *et al*., 2002a,b; Nazoa *et al*., 2003; Orsel *et al*., 2004; Okomato *et al*., 2006; Orsel *et al*., 2006; Krouk *et al*., 2006; Remans *et al*., 2006; Li *et al*., 2007). Different nutritional circumstances and experimental plans have been exerted to reveal specific responses of HATS to externally supplied nitrate and Gln (Quesada *et al*., 1997; Amarasinghe *et al*., 1998; Krapp *et al*., 1998; Filleur *et al*., 1999; Fraisier *et al*., 2000; Vidmar *et al*., 2000b; Nazoa *et al*., 2003; Thornton, 2004).

At present, one of the major challenges of modern agriculture is to optimize crop yield while safeguarding the environment from ecological impacts of excessive application of nitrogenous fertilizers. Improving the nitrogen use efficiency will likely contribute to reach this aim. Thus, a further understanding of details of nitrate HATS responses to different nutritional and environmental conditions may permit a more intelligent and effective use of nitrate in agricultural systems. In tobacco, even though some molecular researches illustrated presence and function of a nitrate HATS (Quesada *et al*., 1997; Krapp *et al*., 1998; Fraisier *et al*., 2000), nevertheless, the time-course of HATS nitrate uptake under induced and uninduced conditions has not been well characterized physiologically.

On the other hand most studies on HATS nitrate uptake are included sensitive N labeling methods such as measuring of the accumulation of $^{13}NO_3^-$ and $^{15}NO_3^-$ in plant roots, whereas it seems nitrate depletion method provides more information about trend of nitrate uptake in intact plants during long term of experiment. In this study, as an alternative approach and in order to better realize the trend of nitrate uptake by HATS, *Nicotiana plumbaginifolia* plant was chosen as model to verify the effect of exogenous nitrate and Gln on rate of HATS nitrate uptake using nitrate depletion from nutrition solution under hydroponic conditions. Amid Gln was chosen because of its probable role as primary transducer of plant nitrogen status for regulation of nitrate uptake by HATS (Nazoa *et al*., 2003) and also its previously reported inhibitory effect on the expression of nitrate HATS (Quesada *et al*., 1997; Krapp *et al*., 1998; Zhuo *et al*., 1999; Vidmar *et al*., 2000b; Nazoa *et al*., 2003).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The *Nicotiana plumbaginifolia* seeds used in this study were received from Prof. Glass's lab. UBC, Vancouver, Canada. This work was conducted during 2004-2007. All experiments were carried out in a growth chamber (Conviron E16) with 16h light/8h dark regime at 24/20°C, 70% humidity and a light intensity of 150 μmol m^{-2} sec^{-1}. For all experiments the seeds were sown on the surface of wet sterilized commercial soil with low release NPK (12.4-5-14.7) fertilizer for 4 weeks. The plants were irrigated with tap water. About 28-day-old plants were transferred to hydroponic medium. The tubes supporting the plants were placed on floating rafts, on the surface of 10 L tanks filled with 0.1 strength modified Johnson's solution (Siddiqi *et al*., 1990) containing 500 μM nitrate in form of $Ca(NO_3)_2$ as nitrogen source. The pH was adjusted and maintained at 6±0.3 by adding $CaCO_3$ powder (Siddiqi *et al*., 1989). The tanks were continuously bubbled with air to ensure that the nutrient solution remained aerobic. After one week, the media were replaced with N-free complete nutrient solution to de-induce iHATS and reduce internal nitrogen resources. Prior to nitrate uptake measurements, according to experiment aims, next stages were followed:

- To determine the time course of nitrate uptake induction, after 7 days the nitrogen starved plants were exposed to various concentrations of $Ca(NO_3)_2$ in HATS range including 10, 50, 75, 100 and 150 μM nitrate
- To verify the effect of high concentration of nitrate on HATS activity, deprived plants of nitrogen were treated with complete nutrition solution containing 5 mM $Ca(NO_3)_2$ (10 mM nitrate) or without nitrate for 48 h. After this period, all plants were exposed to 150 μM nitrate
- To evaluate the effect of Gln on HATS activity, N-starved plants for 7 days were supplied with 1 mM nitrate to induce HATS. After 3 h, the roots were rinsed with 0.1 mM $CaSO_4$ for 1 min. Then, some plants were treated with 1mM Gln and the other plants remained in Gln-free solution for 3 h. After 3 h, the plants grown in Gln-free solution were exposed either with 150 μM nitrate as control or with 1mM Gln plus 150 μM nitrate. The plants with 3 h pretreatment of Gln were supplied with 150 μM nitrate as well

Nitrate Uptake Assay

After addition of nitrate in the range of HATS, samples from medium were withdrawn at 1 h intervals up to 8-12 h from commencement of experiment according to the ion depletion method. Aliquots stored at -80°C until analysis. The plants were harvested and fractionated into roots and shoots. These fractions oven-dried at 70°C for 72 h and weighed. Nitrate net uptake rate was calculated

based on nitrate depletion from nutrition solution and related to root dry weight. At the time of analysis, the aliquots were centrifuged (Eppendorf 5415C) at 13000 rpm for 5 min to remove some medium debris. Then, 0.5 mL from supernatant was added to 2 mL from a 5% (vol/vol) perchloric acid solution. The quantity of nitrate in the incubation solution was determined spectrophotometrically (Shimadzu UV-160A) at 210 nm (Cawse, 1967).

RESULTS

Nitrate Uptake by HATS

The rate of nitrate uptake was measured in different concentration 10, 50, 75, 100 and 150 μM of nitrate in tobacco. In first 2 h after addition of nitrate, slow trend of nitrate elimination was observed in media containing 50 to 150 μM (Fig. 1). Two phases of nitrate uptake were observed; a lag phase and an induced phase, 0-2 h and after approximately 2-3 h, respectively. Rate of uptake in first 2 h of exposure of different concentrations of nitrate is postulated to cHATS activity, whereas, 2 h after nitrate treatments, this rate is as a result of combined functions of cHATS plus iHATS. Rate of nitrate uptake by HATS against nitrate different concentrations was increased and displayed a saturable kinetics (Fig. 2). As shown in Fig. 2, rate of nitrate uptake was constant above 75 μM nitrate in the medium. Therefore, all over the experiments, root nitrate uptake rate was measured at 150 μM external concentration.

To investigate induction time of HATS, N-starved plants were supplied with 150 μM nitrate. The respond of plants to 150 μM nitrate showed a significant increase of uptake rate (133%, respectively) 2 h after nitrate treatment in comparison with first 2 h (Fig. 3).

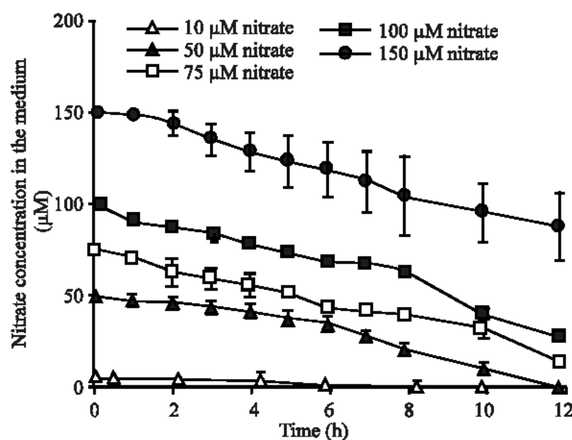

Fig. 1: Time-course of nitrate uptake by HATS in different concentrations of nitrate in the medium during 12 h. About 28-day-old *Nicotiana plumbaginifolia* plants grown on soil were transferred to hydroponics media with 500 μM nitrate. After 1 week, the plants were starved of nitrogen for 7 days. N-starved plants were transferred to hydroponics media supplied with 10, 50, 75, 100 and 150 μM nitrate. Nitrate concentrations in the media determined at the indicated times. The values show means of three independent experiments. Each data point represents the Mean±SD

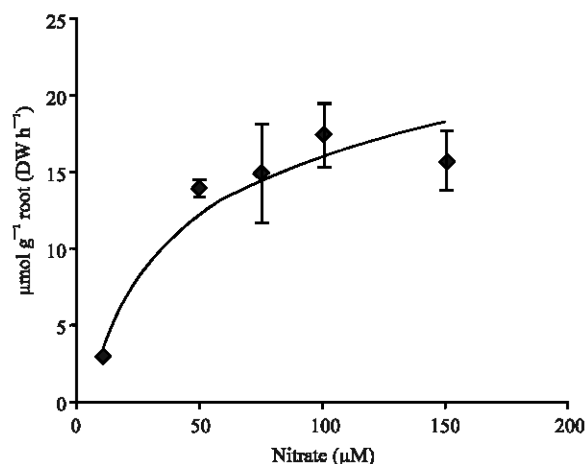

Fig. 2: Kinetic of nitrate uptake rate by *Nicotiana plumbaginifolia* plants. Experimental procedure was the same as described in Fig. 1. N-starved plants for 7 days were transferred to hydroponic media containing 10, 50, 75, 100 or 150 μM nitrate. The values show means of three independent experiments. Each data point represents the Mean±SD

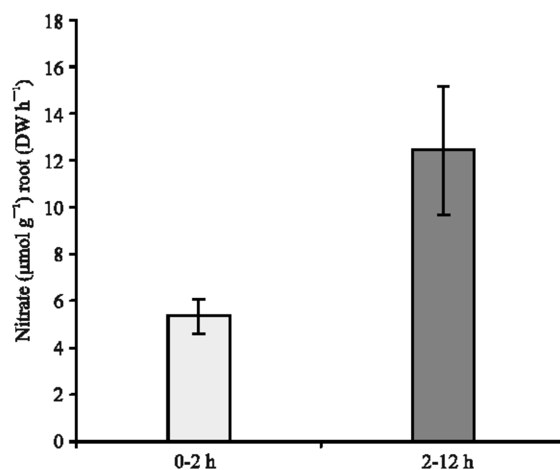

Fig. 3: The rate of uptake nitrate in *N. plumbaginifolia* in first 2 h and next 2-12 h. Experimental procedure was the same as described in Fig. 1. The plants were grown hydroponically in N-free media. After 7 days, the plants were transferred to fresh hydroponic media containing 150 μM nitrate. The values show means of three independent experiments. Each data point represents the Mean±SD

Effect of 10 mM Nitrate on Nitrate Uptake by HATS

Interestingly, the results in present experimental conditions showed an increase in rate of nitrate uptake in plants pretreated with 10 mM nitrate for 48 h in comparison to control conditions either 216% in first 2 h (Fig. 4a) or 22.6% after 2 h (Fig. 4b). As shown in Fig. 4c, the plants grown at 10 mM display more quickly slopes of nitrate uptake compared to non-treated plants.

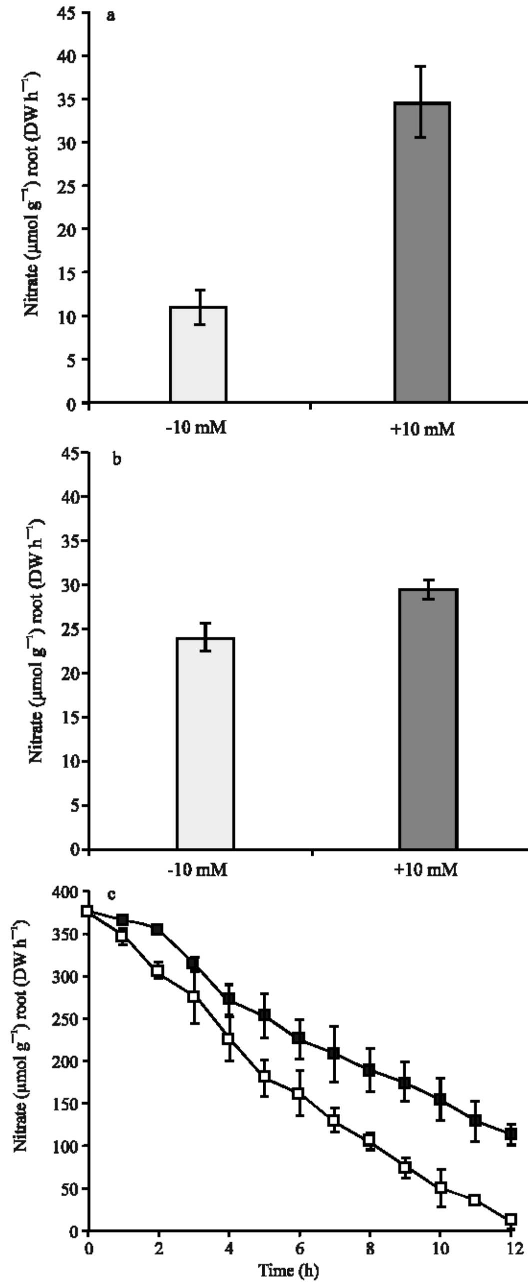

Fig. 4: Effect of 10 mM nitrate on rate of uptake in tobacco plants; (a) in first 2 h exposure to 150 mM nitrate or (b) next 2-12 h. (c) Time-course of rate of nitrate uptake in plants grown without (■) or with pretreatment of 10 mM nitrate (□) during 12 h. The plants were grown hydroponically in N-free 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 150 mM nitrate. The values show means of three independent experiments. Each data point represents the Mean±SD

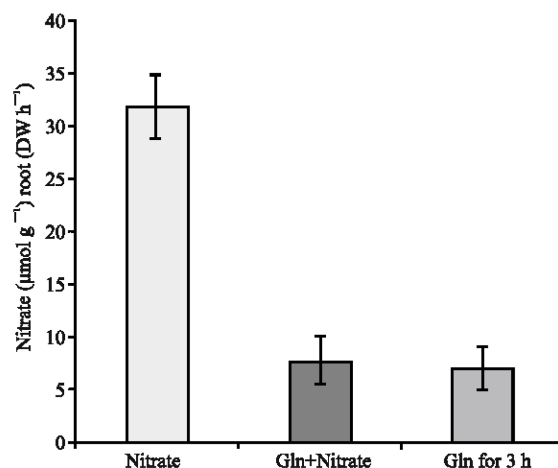

Fig. 5: Effect of Gln on rate of nitrate uptake during 8 h. The plants were grown hydroponically in N-free media. After 7 days, the plants were transferred to fresh hydroponic media containing 1 mM nitrate. After 3 h, all roots were rinsed with 0.1 mM $CaSO_4$ for 1 min. Some plants were pretreated with 1 mM Gln and the other plants 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 mM nitrate or only 150 mM nitrate and the plants pretreated with Gln exposed with 150 mM nitrate. The values show means of three independent experiments. Each data point represents the Mean±SD

Effect of Exogenously Applied Glutamine on Nitrate Uptake by HATS

Figure 5 shows the comparative effects of pretreatment with Gln, Gln plus 150 µM nitrate and only 150 µM nitrate on rate of nitrate uptake in tobacco plants. Gln, whether supplied externally with nitrate or as a pretreatment, inhibited increase in the rate of nitrate uptake by 75 and 77%, respectively.

DISCUSSION

In this study, we decided to use ion depletion technique from incubation solution is used to monitor nitrate uptake by HATS in tobacco intact plants. Molecular studies of iHATS gene family in N. plumbaginifolia (Quesada *et al*., 1997) showed that this gene family are strongly expressed in roots and induced by nitrate. In our experimental conditions, the effect of nitrate level in HATS range (10 to 150 µM nitrate) showed that 10 µM nitrate is rapidly removed from nutrition solution by roots, whereas, the concentrations more than 50 µM, are mostly absorbed approximately after 2 h supplying nitrate and display a lag phase in uptake (Fig. 1). Some nitrate uptake kinetics studies indicates that even though cHATS has higher affinity (km values of 6-20 µM) than iHATS (km values of 13-79 µM) for nitrate (Siddiqi *et al*., 1990), but has much lower capacity to nitrate transport dependence on plant species. In *Arabidopsis*, initial value of $^{13}NO_3$ influx previous to nitrate pretreatment is most likely supposed as a result of cHATS activity (Zhuo *et al*., 1999). In this work, with respect to fast elimination of nitrate from the media including 10 µM nitrate in first 2 h of exposure to nitrate (Fig. 1), it seems that cHATS probably presents a function in nitrate uptake during this time. However, in plants grown at higher concentrations of nitrate such as 50 to 150 µM, it is proposed that enhanced uptake rate after 2 h is possibly mediated through a combination of cHATS and iHATS activities. Indeed, primary entry of nitrate by cHATS induces nitrate subsequent transport

by iHATS. Identical pattern was observed for the plants of barely (Siddiqi *et al*., 1990; Aslam *et al*., 1993; Vidmar *et al*., 2000b) and spruce (Kronzucker *et al*., 1995) and *Arabidopsis* (Zhuo *et al*., 1999; Okamoto *et al*., 2003) grown on N-free nutrient solution for several days when were supplied with nitrate. They found that following exposure to nitrate, rate of uptake by roots was low and increased to a maximum level after 3 h to a few days depending on plant species.

The studies of expression gene in higher plants have been demonstrated that *NRT2.1* gene is induced by external nitrate (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). Induction of *NRT2.1* gene was accompanied by a parallel increase in nitrate uptake by roots of *Arabidopsis* (Lejay *et al*., 1999; Zhuo *et al*., 1999), soybean (Amarasinghe *et al*., 1998), barley (Vidmar *et al*., 2000a) and tobacco (Krapp *et al*., 1998). It suggests that *NRT2.1* is a nitrate-inducible high-affinity transporter which is responsible in nitrate uptake from the soil. On the basis of nitrate uptake kinetic in our experiment, it is suggested that 150 μM nitrate is led to maximal and detectable levels of iHATS activity (Fig. 2). Fallowing exposure to 150 μM nitrate, the plants exhibited a two hours lag phase to uptake nitrate (Fig. 3). After 2 h, the plants showed an elevated HATS activity and the rate of nitrate uptake was increased 57% as compared to first 2 h (Fig. 3). This result is in agreement with previous studies using ^{13}N or ^{15}N methods (Zhuo *et al*., 1999; Okamoto *et al*., 2003; Remans *et al*., 2006), similarly, low concentration of nitrate was led to same responses in nitrate influxes. Thus, in our experimental conditions at least 2 h exposure of tobacco plants to 150 μM nitrate would be sufficient to induce HATS and increase rate of uptake up to the levels observed at Fig. 3. It is speculated that nitrate directly and indirectly via a signal transduction pathway causes to induce transcription of HATS in plants (Amarasinghe *et al*., 1998). Some studies on tobacco (Quesada *et al*., 1997; Krapp *et al*., 1998) and *Arabidopsis* (Filleur and Daniel-Vedele, 1999; Zhuo *et al*., 1999; Okamoto *et al*., 2003) have been shown that in general, maximal levels of HATS expression are observed within 2-4 h of exposure to low nitrate concentrations.

It has been reported that high concentrations of nitrate decreased nitrate uptake in *Arabidopsis* (Zhuo *et al*., 1999) and tobacco (Fraisier *et al*., 2000). In soybean, also reduction of nitrate uptake was observed under high concentration of nitrate (Amarasinghe *et al*., 1998). In present work, the plants pretreated with 10 mM nitrate displayed a significant increase in rate of nitrate uptake comparison to non-treated plants (Fig. 4a-c). It appears that, at least in our experimental conditions 10 mM nitrate has no repressive effect on nitrate uptake by HATS. It supposed that more than 48 h pretreatment of 10 mM nitrate is needed to repress the nitrate iHATS activity in tobacco. Gln repressed nitrate uptake, when compared to the plants grown on nitrate (Fig. 5) that proposes probably Gln has a role in nitrogen signaling responsible for nitrate uptake regulation. This results is in accordance to previous studies in *Lolium perenne* (Thornton, 2004), *Arabidopsis* (Nazoa *et al*., 2003), barely (Vidmar *et al*., 2000b; Aslam *et al*., 2001), when exogenously applied Gln decreased nitrate influxes. The reduction of HATS expression by externally supplied amino acids including Gln (Quesada *et al*., 1997; Krapp *et al*., 1998; Zhuo *et al*., 1999; Vidmar *et al*., 2000b; Nazoa *et al*., 2003) supported this hypothesis that N-status plant, possibly through end products of nitrate assimilation pathway such as amino acids regulates as feedback root nitrate uptake (Lejay *et al*., 1999) and this control is mediated by effects of this products on *NRT2.1* transcription or mRNA stability (Zhuo *et al*., 1999). In soybean, has been supposed that amino acids regulate nitrate influx by post-translational modifications (Amarasinghe *et al*., 1998). However, we can not conclude that Gln *per se* was responsible for negative effect on nitrate uptake. As, some studies indicate addition of Gln is resulted in increasing concentrations of the other amino acids (Nazoa *et al*., 2003; Thornton, 2004). Nevertheless, application of different inhibitors of GOGAT pathway (Zhuo *et al*., 1999; Vidmar *et al*., 2000b) and amino acids analysis (Nazoa *et al*., 2003) showed that probably Gln presents a key role in down-regulation of HATS and decrease of nitrate influx.

CONCLUSION

Induction and inhibition of HATS activity by nitrate and Gln, respectively, suggesting that nitrate uptake by HATS in *N. plumbaginifolia* is regulated by nitrate itself and reduced nitrogen sources such as Gln. This result confirms and completes the previous studies on the function of nitrate HATS in higher plants. Present study is only a preliminary physiological characterization of *N. plumbaginifolia* plants to provide more complementary data about the role and regulation of nitrate HATS in plants. It will be interesting that trend of nitrate uptake by HATS is examined under different experimental and nutritional conditions in tobacco, particularly with various concentrations of nitrogen sources. It is possible that variable conditions allow further elucidating and characterizing nitrate HATS activity physiologically.

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