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ORIGINAL ARTICLE

Ammonium transport and *CitAMT1* expression are regulated by N in *Citrus* plants

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Abstract *Citrus* seedlings (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* Blanco) were used to describe the effects of different N treatments on the NH_4^+ influx mediated by high- and low-affinity transport systems (HATS and LATS, respectively) and *CitAMT1* gene expression. Results show that *Citrus* plants favor NH_4^+ over NO_3^- influx mediated by HATS and LATS when both N sources are present in the nutrient solution and *Citrus* plants display a much higher capacity to take up NH_4^+ than NO_3^-. Furthermore, NH_4^+ exerts a regulatory effect on NH_4^+ HATS activity and *CitAMT1* expression, both are down-regulated by high N status of the plant, but specifically stimulated by NH_4^+ and the balance between these two opposite effects depends on the prior nutrition regime of the plant. On the other hand, supply of NO_3^- inhibits *CitAMT1* expression but doesn't affect NH_4^+ HATS activity on the roots. To explain this discrepancy, it is possible that other CitAMT1 transporters, up-regulated by N limitation, but not repressed by NO_3^- could be involved in the stimulation of NH_4^+ HATS activity under pure NO_3^- nutrition or CitAMT1 transporter could be regulated at the post-transcriptional level.

Keywords Ammonium · Ammonium transporter · *Citrus* · High-affinity transport system · Low-affinity transport system

Abbreviations

HATS or LATS	High-affinity or low-affinity transport systems, respectively
K_m	The external ion concentration giving half of the maximun rate (μM)
V_{max}	The calculated maximun rate of ion influx [μmol $^{15}NH_4^+$ (g root dry weight)$^{-1}$ h^{-1}]

Introduction

Although nitrogen (N) is present in the soil as a complex mixture of organic and inorganic compounds, ammonium (NH_4^+) and nitrate (NO_3^-) are by far the main sources for nutrition of most species of higher plants (Williams and Miller 2001). Nitrogen is often the major limiting macronutrient for plants because the concentrations of these two ions in the soil solution are generally low and fluctuant. Under most conditions, NO_3^- dominates over NH_4^+ with concentrations in the soil solution typically 10 to 1,000 times higher for NO_3^- than for NH_4^+ (up to 10 mM for NO_3^- as compared to below 50 μM for NH_4^+, Marschner 1995; von Wirén et al. 2000a; Miller et al. 2007). However, this difference in soil concentrations does not necessarily reflect the uptake ratio of both N sources by the plants.

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Indeed, the role of NH_4^+ in plant nutrition has probably been underestimated, because most plants display a much higher capacity for root NH_4^+ influx than for root NO_3^- influx when both forms are present in similar concentrations (Serna et al. 1992; Gessler et al. 1998; Gazzarrini et al. 1999). Furthermore, NH_4^+ requires theoretically less energy for uptake and assimilation than NO_3^-, mainly because NO_3^- has first to be reduced to NH_4^+ prior to assimilation, and that the two-step NO_3^- reduction process (catalyzed by nitrate and nitrite reductases) is highly energy-consuming (Bloom et al. 1992). By contrast, at high and exclusive supply, NH_4^+ tends to generate toxicity and to inhibit plant growth as compared to NO_3^- as sole N source (Britto and Kronzucker 2002). Nevertheless, when NO_3^- and NH_4^+ are provided together, growth and yield of plants are often enhanced significantly when compared with either NO_3^- or NH_4^+ alone (Kronzucker et al. 1999; Kirk and Kronzucker 2005; Baozhen et al. 2006).

Citrus fruits are of high economic importance in the Mediterranean, and overfertilization and overirrigation are frequent practises to achieve high production yields. Fertilization takes place in March and May in which N is present as NH_4^+, while N is present as NO_3^- and NH_4^+ in July and August (Reboll et al. 2000). Therefore, it is important to know the regulation of the absorption mechanisms of both ions and the interaction between them to be able to optimize the fertilizing doses and to avoid polluting the aquifer through excessively nitrogenated fertilization.

In *Citrus*, there are three systems for NO_3^-: two high-affinity transport systems (HATS), either constitutive (cHATS), or inducible by NO_3^- (iHATS), respectively; and one low-affinity transport system (LATS) (Cerezo et al. 2000; 2007). For NH_4^+, only two systems were previously characterized: one HATS and one LATS (Cerezo et al. 2001). At the molecular level, several gene families encoding putative NO_3^- and NH_4^+ transporters have been identified in plants. The *NRT1* and *NRT2* families are thought to encode the LATS and HATS for NO_3^-, respectively (Daniel-Vedele et al. 1998; Huang et al. 1999; Forde 2000; Fraisier et al. 2000), while the *AMT1* family includes genes encoding high-affinity transporters participating in the HATS for NH_4^+ (Ninnemann et al. 1994; Gazzarrini et al. 1999; Howitt and Udvardi 2000; von Wirén et al. 2000a; Loqué et al. 2006; Yuan et al. 2007a). The *NRT* and *AMT1* genes have been identified in many species (Loqué et al. 2004; Tsay et al. 2007), but are only very partially characterized in woody plants. We recently cloned the *CitAMT1* cDNA, the first member of this family in *Citrus* (Camañes et al. 2007).

Concerning the regulation of root N uptake, there is a general agreement on the hypothesis that two main mechanisms are involved in the control of NH_4^+ and NO_3^- uptake systems, and more specifically of the HATS. The first one corresponds to the stimulation by photosynthesis (Lejay et al. 2003), which ensures that both NH_4^+ and NO_3^- uptake are controlled by the C status of the plant to coordinate N and C acquisition. This regulation is operative in *Citrus*, where we found a strong correlation between photosynthetic activity in the shoots, NH_4^+ HATS activity and *CitAMT1* expression, suggesting that the variations in production and transport of photosynthates to the roots are responsible for the diurnal changes of both *CitAMT1* expression and NH_4^+ HATS activity (Camañes et al. 2007). The second regulatory mechanism is the repression exerted by endogenous N assimilates, mediating a negative feedback regulation by the N status of the whole plant (Gazzarrini et al. 1999; Rawat et al. 1999; Cerezo et al. 2001; Loqué and von Wirén 2004). This feedback control modulates both NH_4^+ and NO_3^- HATS to match the N demand of the plant, and results in a down- or an up-regulation of the transport systems when the N status is high or low, respectively. Accordingly, several NRT2 or AMT1 transporters in various species were found to be repressed at the mRNA level by N metabolites such as amino acids (Gazzarrini et al. 1999; Lejay et al. 1999; Loqué et al. 2004; Tsay et al. 2007). Concerning more specifically NH_4^+ transport, there is strong correlation in *Arabidopsis* between the increase in *AtAMT1.1* mRNA level in the roots and that of NH_4^+ HATS activity in response to N deprivation (Gazzarrini et al. 1999; Rawat et al. 1999; Gansel et al. 2001). *AtAMT1.3* also displays a higher expression in the roots under nitrogen-limiting conditions (Gazzarrini et al. 1999; Loqué et al. 2006), as it is the case for other *AMT1* genes in other species, e.g., *LeAMT1.1* in *Solanum lycopersicon* (von Wirén et al. 2000b) and *OsAMT1.1* in *Oryza sativa* (Kumar et al. 2003). Conversely, *OsAMT1.1* expression decreases upon NH_4^+ re-supply to nitrogen-starved rice plants (Kumar et al. 2003). Investigation of KO mutants in *Arabidopsis* confirmed that the N-regulated *AMT1* genes (namely, *AtAMT1.1* and *1.3*) are indeed responsible for the up-regulation of the NH_4^+ HATS by N deprivation (Loqué et al. 2006). Recently in *P. trichocarpa,* a woody species, more genes from the AMT family have been found than in *A. thaliana*. These genes are also differentially regulated, and some are also tissue-specific. *PtrAMT1.1* and *1.2* respond to the lack of N in roots, whereas *PtrAMT1.6* is expressed in leaves. Likewise, *PtrAMT1.2* is regulated by light in roots and *PtrAMT1.6* is strongly affected by the diurnal cycle in leaves. Other members of the AMT family are expressed in other organs (*PtrAMT3.1* in senescent leaves, *PtrAMT2.1* in leaves, *PtrAMT2.2* in petioles and *PtrAMT1.5* in stamens (Couturier et al. 2007).

Despite these recent advances, several reports suggest that the overall regulation of NH_4^+ transporters by the N status of the plant may be much more complex, and may involve other regulatory mechanisms than just

down-regulation of *AMT1* gene expression by N metabolites. First, the exact nature of the N compounds acting as regulatory signals for the NH_4^+ HATS is still unclear. On the one hand, *AtAMT1.1* mRNA level is inversely correlated with the concentration of free glutamine in the roots, suggesting a predominant role of this compound as a repressor of *AtAMT1.1* expression (Rawat et al. 1999). On the other hand, *AtAMT1.1* was found to be repressed by short-term NO_3^- supply in a microarray study (Wang et al. 2000), indicating that NO_3^- itself could also be involved in *AtAMT1.1* down-regulation. This is consistent with split-root studies (Gansel et al. 2001), which showed that *AtAMT1.1* expression is repressed by 1 mM KNO_3. Recently, Engineer and Kranz (2007) have even shown that regulation of *AtAMT1.1* gene expression by the N status of the plant differs between roots and shoots, thus suggesting that depending on the gene or the organ, N compounds may either repress or at the opposite stimulate *AMT1* expression. The data obtained with *LeAMT1.1* and *LeAMT1.2* in tomato also agree with this hypothesis (Lauter et al. 1996; von Wirén et al. 2000b; Wang et al. 2001). Moreover, when N-deficient *Arabidopsis* plants were re-supplied with NH_4^+, root high-affinity NH_4^+ influx showed a faster time-dependent repression relative to *AtAMT1.1* mRNA level in roots (Rawat et al. 1999), a discrepancy that could be explained by the occurrence of post-transcriptional control of *AtAMT1.1* (Yuan et al. 2007b). Finally, the regulation of the LATS for NH_4^+ is much less documented and contrasting conclusions are found in the literature. An indication that the LATS is also under feedback repression by N metabolites was provided by a study with spruce seedlings (Kronzucker et al. 1996). However, Mäck and Tischner (1994) found no change in the activity of the NH_4^+ LATS in response to N-starvation in barley. Finally, experiments with rice and *Citrus* indicated that in these species the activity of the NH_4^+ LATS is, on the contrary, stimulated by previous NH_4^+ provision (Wang et al. 1993; Cerezo et al. 2001).

In our previous study, we reported on the identification of *CitAMT1* in *Citrus*, and on the regulation of its expression by photosynthesis (Camañes et al. 2007). In this present study, we show that *CitAMT1* expression in the roots is also regulated by the N source and by the N status of the plant.

Materials and methods

Plant material and growth conditions

Seeds of citrange Troyer (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* Blanco) (Beniplant, Valencia, Spain) were allowed to germinate in vermiculite in a growth chamber under the following environmental conditions: light/dark cycle of 16/8 h, temperature of 20/24°C, light intensity of 200 µmol m^{-2} s^{-1} and an RH of 70%. The seeds were irrigated twice a week with distilled water. After 6 weeks, seedlings were irrigated with Hoagland solution lacking nitrogen (Hoagland and Arnon 1950). The nutrient solution was complemented with 1 mM NH_4NO_3 and an addition of 1.5 mM K_2SO_4 and 3 mM $CaSO_4$ were added to compensate for the absence of K^+ with 3 mM KNO_3 and the absence of Ca^{2+} with 3 mM $Ca(NO_3)_2$ in the solution. The pH of the nutrient solution was adjusted to 6.0 with 1 mM KOH.

Prior to the experiments, 3-month-old plants with a single shoot were selected for uniformity of size, and transferred to an aerated complemented Hoagland solution for 7 days on hydroponic culture devices. Nutrient solutions were renewed twice weekly and on the day of the experiments. All experiments were repeated three times, and typical results are shown.

Measurement of ^{15}N influx

$^{15}NH_4^+$ or $^{15}NO_3^-$ influx by *Citrus* roots was measured at a low (0.2 mM) and high (5 mM) external concentration of either NH_4^+ or $^{15}NO_3^-$, respectively, which is representative of both the high- and low-affinity transport systems (HATS and LATS, respectively). The $^{15}NH_4^+$ or $^{15}NO_3^-$ influx in roots was determined on six plants after transferring to 0.1 mM $CaSO_4$ for 1 min, then to $^{15}NH_4^+$ solution for 5 min, and finally to 0.1 mM $CaSO_4$ for 1 min (Gazzarrini et al. 1999). The $^{15}NH_4^+$ or $^{15}NO_3^-$ solution was the N-free Hoagland nutrient solution, supplemented with 1 mM MES pH 6.0, where N was supplied as either $[^{15}N](NH_4)_2SO_4$ (98 atom % ^{15}N in excess) or $[^{15}N]KNO_3$ (98 atom % ^{15}N in excess) at the indicated concentrations (0.2 and 5 mM). After labelling, the roots were separated from the shoots and dried for 48 h at 65°C, crushed in a hammer, mill and weighed. The ^{15}N analysis was performed using an integrated system for continuous flow isotope ratio mass spectrometry (Euro-EA elemental analyser (EuroVector S.P.A., Milan, Italy) and Isoprime mass spectrometer (GV Instruments, Manchester, UK). The values of the root ^{15}N influx are expressed in µmol ^{15}N (g root DW)$^{-1}$ h^{-1}. The experiments were repeated at least three times and the mean ± SE is shown (n = 18).

Influence of the NH_4^+/NO_3^- ratio in the medium on NH_4^+ influx and *CitAMT1* gene expression

Three-month-old *Citrus* plants were grown on a complete nutrient solution containing 1 mM NH_4NO_3 as an N source and 3 days before the experiment plants were transferred from 1 mM NH_4NO_3 to five different solutions at 1 mM

total N concentration, but with the following $(NH_4)_2SO_4$/KNO_3 ratio 100:0; 75:25; 50:50; 25:75; 0:100. Under these conditions, $^{15}NH_4^+$ and $^{15}NO_3^-$ influxes were measured, and roots of *Citrus* plants were frozen for later studies of *CitAMT1* gene expression.

Influence of nitrogen deficiency

Three-month-old *Citrus* plants were grown hydroponically on 1 mM NH_4NO_3. Before the experiment, one group of plants was kept under the same conditions (control), while another group was transferred to nitrogen-free nutrient solution, and a third group was transferred to 1 mM KNO_3. Root $^{15}NH_4^+$ influx was measured daily for a week, and the roots of *Citrus* plants were frozen for later studies of *CitAMT1* gene expression.

Kinetics of $^{15}NH_4^+$ influx

The kinetics of the $^{15}NH_4^+$ influx as a function of the external NH_4^+ concentration was measured in plants with $[^{15}NH_4^+]_0$ ranging from 20 µM to 30 mM. The double reciprocal plots of the influxes versus substrate concentrations were subjected to linear regression analysis. The Michaelis-Menten kinetic constants (K_m and V_{max}) were calculated from these regression equations at the concentration range of 20 µM to 1 mM. When the concentration exceeded 1 mM $[^{15}NH_4^+]_0$, the measured $^{15}NH_4^+$ influx appeared to result from the participation of two transport systems (HATS and LATS). Thus, the differences between the measured influx at concentrations >1 mM $[^{15}NH_4^+]_0$ and the calculated V_{max} for HATS were taken as the estimates of the influx only due to LATS.

Effect of supplying different concentrations of nitrogen sources under de-repression and de-induction conditions

Three-month-old *Citrus* plants were grown hydroponically on 1 mM NH_4NO_3 and transferred for 3 days (de-repression) or 7 days (de-induction) from 1 mM NH_4NO_3 to nitrogen-free nutrient solution. After starvation, plants were divided into four groups and solutions were supplied with 0.1 mM $(NH_4)_2SO_4$; 0.2 mM KNO_3; 5 mM $(NH_4)_2SO_4$ or 10 mM KNO_3, respectively. Root $^{15}NH_4^+$ influx was measured after 24 h and the roots of *Citrus* plants were frozen for subsequent studies of the *CitAMT1* gene expression.

RNA extraction and real-time PCR analysis

For all mRNA expression analyses, root samples taken from six plants at time points or treatments were ground to powder under liquid nitrogen for total RNA extraction using the Total Quick RNA kit (Talent, Trieste, Italy) according to the manufacturer's instructions. Samples were treated with DNAse I to avoid contaminating the DNA. A total of 1 µg of total RNA was annealed to random hexamers and reverse-transcribed using the Omniscript® Reverse Transcription kit (Qiagen, Izasa, Barcelona, Spain) to obtain cDNA. The sequences of the gene-specific oligonucleotides designed and used for real-time PCR are the following: AMT forward: 5′CCCACCTCCAACTTCGACTA3′ and reverse: 5′CAGAACCAATGGGAGACGAC3′; and *18S* forward: 5′GAACAACTGCGAAAGCATTTGC3′ and reverse: 5′CCTGGTAAGTTTCCCCGTGTTG3′. Real-time PCR was conducted using the QuantiTect™ SYBR Green PCR Kit (Qiagen) and the SmartCycler II instrument (Cepheid, Sunnyvale, USA). Each reaction was set up in two replicates. The PCR conditions to amplify the *CitAMT1* fragment were as follows: 95°C for 15 min and 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Agarose gel electrophoresis and melting curve analysis were performed to confirm the specific gene product formation and did not represent primer dimmer or non-specific products.

Statistical analysis

Statistical analysis was carried out using the Statgraphics software support. The data are expressed as means and SE. Mean values were compared by an LSD (least significant difference) test. Differences were taken into account only when they were significant at the 5% level. All experiments were repeated at least three times.

Results

Effect of the N source on NH_4^+ and NO_3^- uptake systems

To determine how the nature of the N source affected the expression and activity of the root uptake systems for NH_4^+, as compared to those for NO_3^-, plants grown hydroponically on 1 mM NH_4NO_3 were acclimated for 3 days to various N regimes with different NH_4^+/NO_3^- ratio (100:0, 75:25, 50:50, 25:75 and 0:100) prior to the measurements of both HATS and LATS activities for NH_4^+ and NO_3^-, and of *CitAMT1* transcript levels in the roots (Fig. 1). For HATS activity, root $^{15}NH_4^+$ and $^{15}NO_3^-$ influxes were measured at 0.2 mM, and are thus expected to provide a measurement of the maximum capacity (i.e., V_{max}) for the corresponding systems. LATS activity was estimated as the difference between ^{15}N influx at 5 and 0.2 mM external $^{15}NH_4^+$ or $^{15}NO_3^-$ concentrations.

Under all situations investigated, HATS and LATS activities for NH_4^+ far exceeded those measured for NO_3^- (Fig. 1a), showing that *Citrus* plants have a much higher

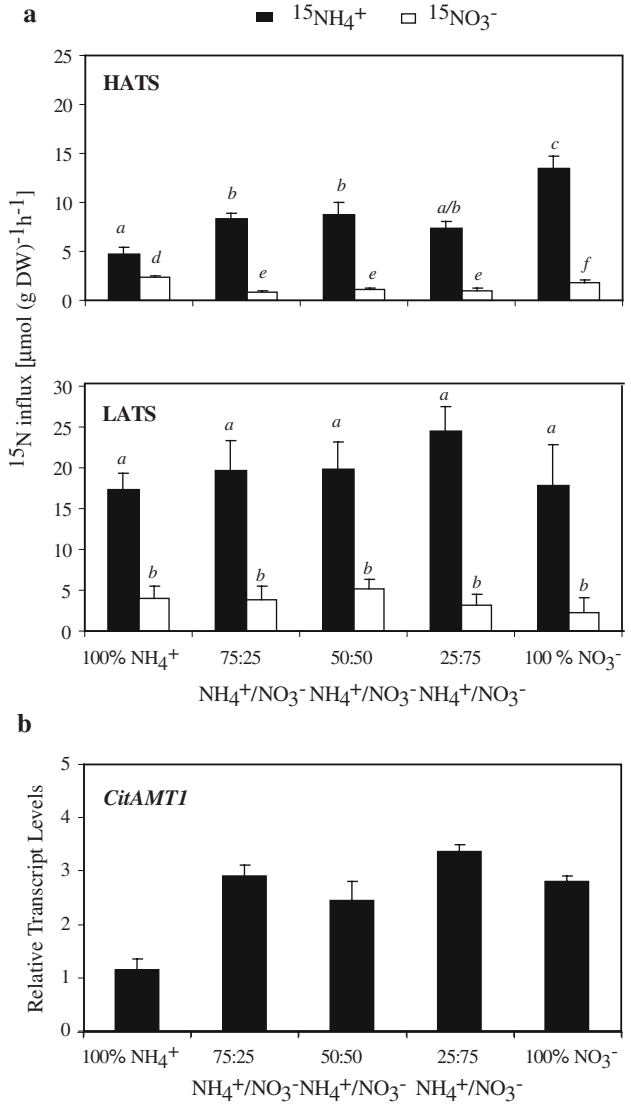

Fig. 1 Effect of pre-treating the *Citrus* plants for 3 days with different NH_4^+/NO_3^- ratios on the $^{15}NH_4^+$ and $^{15}NO_3^-$ influxes and the *CitAMT1* gene expression $^{15}NH_4^+$ influx in *Citrus* roots after transferring the plants grown hydroponically for 3 days from 1 mM NH_4NO_3 to different solutions with $(NH_4)_2SO_4/KNO_3$ ratios (100; 75:25; 50:50; 25:75; 0) to a final N concentration of 1 mM. The HATS-mediated $^{15}NH_4^+$ or $^{15}NO_3^-$ influx was measured in roots at 0.2 mM. The LATS-mediated $^{15}NH_4^+$ or $^{15}NO_3^-$ influx was calculated by subtracting the influx measures at 0.2 mM $^{15}NH_4^+$ or $^{15}NO_3^-$ from that measured at 5 mM. The values shown are the means of 18 replicates ±SE. *Different letters* indicate significant differences ($P < 0.05$). **b** Real-Time PCR analysis of the expression of *CitAMT1*. Plants were taken from the same experiment as those used for measurements of influx activity in **a**. The *CitAMT1* transcript level was normalized to the expression of *18S* rRNA measured in the same samples. *Each bar* represents the average data with standard error bars of two independent experiments ($n = 4$)

capacity for root NH_4^+ uptake than for root NO_3^- uptake. The respective capacities of the NH_4^+ and NO_3^- HATS changed significantly as a function of the N source, with HATS-mediated $^{15}NH_4^+$ influx unexpectedly increasing with decreasing external NH_4^+/NO_3^- ratio, while the reverse was observed with HATS-mediated $^{15}NO_3^-$ influx (Fig. 1a). Thus, highest NH_4^+ HATS capacity was found with NO_3^- as the sole N source, and conversely, highest NO_3^- HATS capacity was found with NH_4^+ as the sole N source. Although surprising, this pattern of NH_4^+ and NO_3^- HATS regulation matched quite well the associated changes in *CitAMT1* expression (Fig. 1b). Accumulation of *CitAMT1* transcript in the roots was minimal under pure NH_4^+ nutrition, and increased nearly 3-fold when NO_3^- was added in the medium, regardless of whether NH_4^+ was also present.

Response of root NH_4^+ uptake systems to N starvation or to specific removal of NH_4^+ from the nutrient solution

To determine how both NH_4^+ HATS and LATS react to the removal of their substrate from the external medium, plant grown on 1 mM NH_4NO_3 were transferred for 7 days either to a N-free solution or to a nutrient solution containing 1 mM NO_3^- as sole N source. Both treatments resulted in a strong but transient up-regulation of NH_4^+ HATS, as shown by the 3 to 4-fold increase in $^{15}NH_4^+$ influx after 2–3 days, followed by an equivalently fast decline until day 7, where NH_4^+ HATS activity was re-established to its initial values (Fig. 2a, b). No such changes were observed for the NH_4^+ LATS, which kept a roughly constant activity whatever the N nutrition regime of the plants (1 mM NH_4NO_3, 1 mM NO_3^- or N-free solution, Fig. 2a, b).

Changes in *CitAMT1* expression in the roots closely paralleled those of NH_4^+ HATS activity in plants subjected to N-deprivation (compare Fig. 2a, c). Indeed, transcript accumulation of *CitAMT1* dramatically increased (up to 20-fold) upon transfer to N-free medium, to peak at day 2 (as it was the case for NH_4^+ HATS) and decline thereafter (Fig. 2c). However, the response pattern of *CitAMT1* expression in the roots of plants transferred to the 1 mM NO_3^- nutrient solution was totally different, since no increase in *CitAMT1* mRNA level was observed (Fig. 2d), despite a marked stimulation of NH_4^+ HATS activity (Fig. 2b). Although surprising, this discrepancy between the responses of *CitAMT1* and of NH_4^+ HATS to the removal of NH_4^+ from the external medium fits with the results of the experiments in Fig. 1, which also show that NH_4^+ HATS activity, but not *CitAMT1* expression, is increased under pure NO_3^- nutrition, as compared to mixed N nutrition (Compare Fig. 1a, b). These data thus suggest that up-regulation of *CitAMT1* expression may be responsible for de-repression of NH_4^+ HATS in response to N starvation, but not in response to the specific absence of the NH_4^+ N source.

One hypothesis would then be that other NH_4^+ transporters than *CitAMT1* account for the up-regulation of the NH_4^+

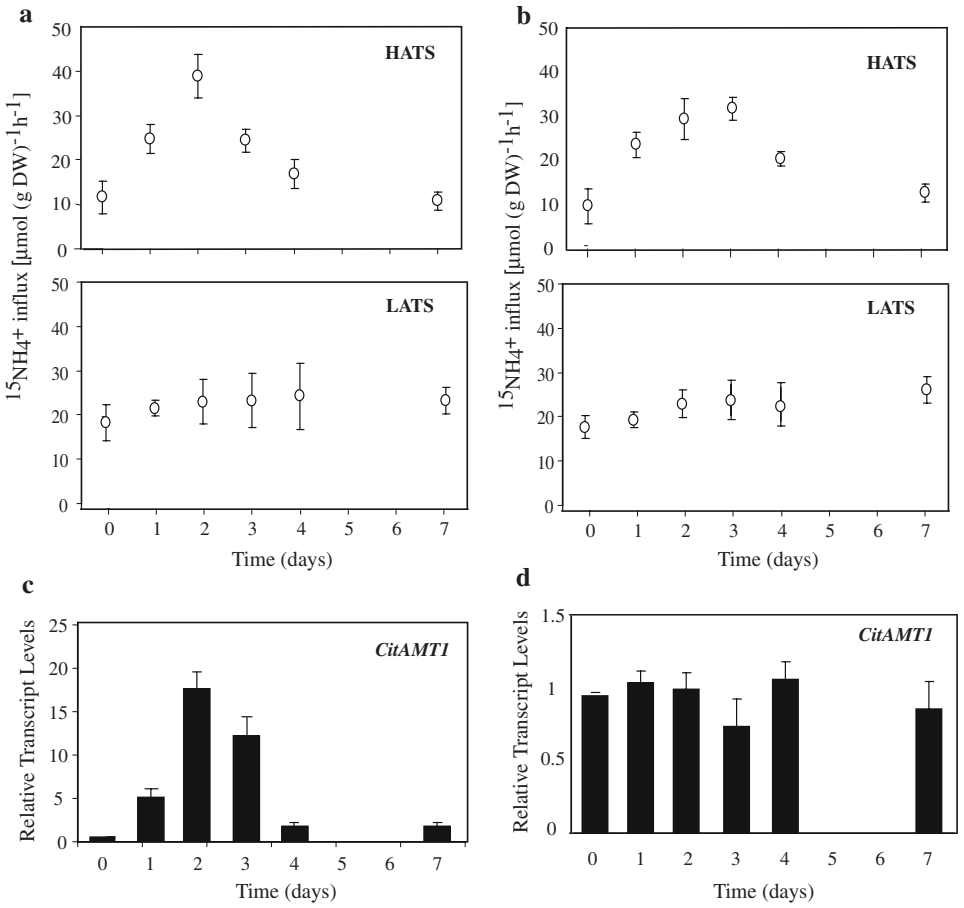

Fig. 2 Correlation between the $^{15}NH_4^+$ influx and the *CitAMT1* gene expression after subjecting roots to either nitrogen deficiency or NO_3^- as the sole nitrogen source $^{15}NH_4^+$ influx in *Citrus* roots after transferring hydroponically grown plants from 1 mM NH_4NO_3 to either a nitrogen-free nutrient solution **a** or 1 mM KNO_3. **b** The $^{15}NH_4^+$ influx was measured daily for a week. The HATS-mediated $^{15}NH_4^+$ influx was measured in roots at 0.2 mM. The LATS-mediated $^{15}NH_4^+$ influx was calculated by subtracting the influx measures at 0.2 mM $^{15}NH_4^+$ from that measured at 5 mM. The values shown are the means of 18 replicates ±SE **c** and **d** Real-Time PCR analysis of expression of *CitAMT1*. Plants were taken from the same experiment as those used for measurements of influx activity in (**a** and **b** respectively). The *CitAMT1* transcript levels were normalized to the expression of *18S* rRNA measured in the same samples. *Each bar* represents the average data with standard error bars of two independent experiments ($n = 4$)

HATS in response to the provision of NO_3^- as sole N source. To address this possibility, we then investigated whether N-starvation or provision of NO_3^- as sole N source have a differential effect on the kinetic parameters of $^{15}NH_4^+$ influx in the roots, that could indicate that NH_4^+ transporters with different functional properties are involved. In all groups of plants (fed with 1 mM NH_4NO_3, transferred to 1 mM NO_3^- or to N-free solution), a biphasic kinetics pattern was observed for root $^{15}NH_4^+$ influx (Fig. 3). At a low $[^{15}NH_4^+]_o$, $^{15}NH_4^+$ influx followed the typical Michaelis-Menten-type kinetics of the saturable HATS systems (Fig. 3a), while at high $[^{15}NH_4^+]_o$, a linear pattern was found for $^{15}NH_4^+$ influx in agreement with the action of a non saturable LATS (Fig. 3b). As expected, both transfer to N-free solution or to 1 mM NO_3^- stimulated HATS, but not LATS activity (Fig. 3a, b). The calculated values of both V_{max} and K_m for the HATS (Table 1) confirmed that the two N treatments led to a very similar increase in the HATS capacity, with a more than 2-fold increase in V_{max}, whereas specific removal of NH_4^+ from the medium resulted in a significantly higher increase in HATS affinity than total N-starvation (decrease in K_m from 86 to 55 µM in $-NH_4^+$ plants, as compared to 71 µM in N-starved plants).

Response of root NH_4^+ uptake systems to re-supply of NH_4^+ or NO_3^- following N starvation

Data of Fig. 2 show that both N starvation and transfer of the plants to NO_3^- as sole N source resulted in a two-phase response of the NH_4^+ HATS, with an initial up-regulation followed by a marked decline. To clarify the mechanisms responsible for this dual pattern of regulation, the effect of NH_4^+ or NO_3^- re-supply was investigated either after 3 days of N starvation (i.e., when the HATS is fully up-regulated) or after 7 days of N starvation (i.e., at the end of the subsequent decline in HATS activity).

As in the experiment of Fig. 2, HATS-mediated $^{15}NH_4^+$ influx was increased nearly 3-fold in plants transferred for 3 days to N-free medium, as compared to the controls (Fig. 4a). Re-supply of NH_4^+ at 0.2 or 10 mM external concentration led after 24 h to a down-regulation of HATS activity, with a decrease in $^{15}NH_4^+$ influx of 25 and 40% with 0.2 or 10 mM external NH_4^+, respectively (Fig. 4a). Unlike NH_4^+, re-supply of NO_3^- for 24 h at the same 0.2 or 10 mM external concentrations had no effect on HATS-mediated $^{15}NH_4^+$ influx, which remained as high as in N-starved plants. On the other hand, neither N-starvation nor NH_4^+ or NO_3^- re-supply resulted in any significant

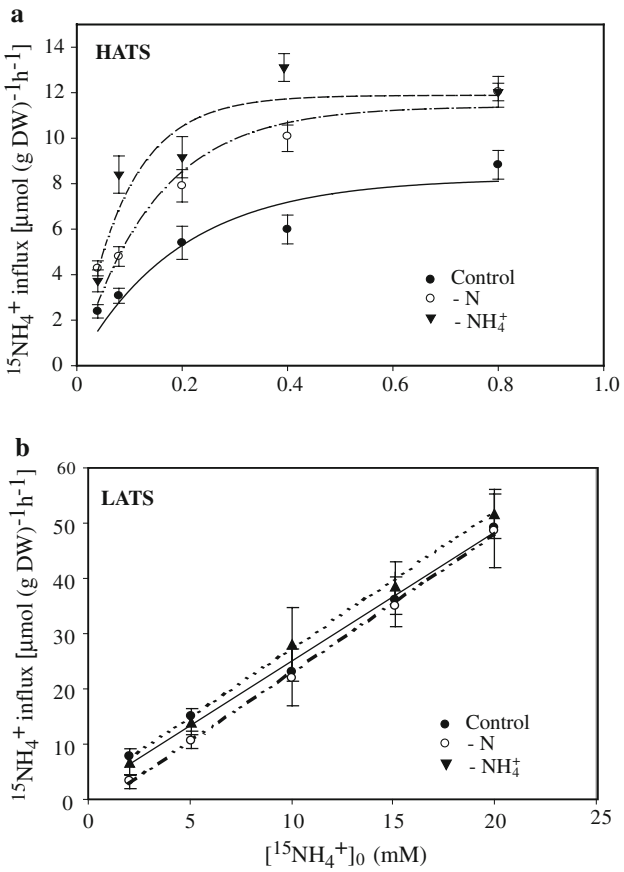

Fig. 3 Kinetics of the $^{15}NH_4^+$ influx in *Citrus* roots in the low **a** and high **b** $^{15}NH_4^+$ concentration range Three-month-old *Citrus* plants were grown hydroponically on 1 mM NH_4NO_3. Three days before the experiment, one group of plants was kept under the same conditions (control), another group was transferred to a nitrogen-free nutrient solution (–N), while a third group of plants was transferred to 1 mM KNO_3 (–NH_4^+). The $^{15}NH_4^+$ influx was measured on the third day at different concentrations of external $^{15}NH_4^+$. All the values are the means of 18 replicates ±SE

change in NH_4^+ LATS activity (Fig. 4a). Also consistent with the data of Fig. 2, *CitAMT1* expression in the roots was strongly stimulated by N starvation (Fig. 4a). Both NH_4^+ or NO_3^- re-supply reversed *CitAMT1* up-regulation, and led to a decrease in *CitAMT1* transcript accumulation that was more pronounced with NH_4^+ than with NO_3^- (Fig. 4b). Taken together with those of Fig. 2, these results are thus consistent with the hypothesis that NH_4^+ HATS activity is repressed by external NH_4^+, but not by external NO_3^-, and that *CitAMT1* expression is down-regulated by both N sources.

When assayed after 7 days of N starvation, HATS-mediated $^{15}NH_4^+$ influx was found unchanged as compared to control plants, but was slightly stimulated after 24 h of either NH_4^+ or NO_3^- re-supply, regardless of the external concentration of these ions (Fig. 5a). As expected from its consistent lack of response to the various changes in N

Table 1 Kinetic parameters for saturable and linear phases of the $^{15}NH_4^+$ influx of three-month-old citrange Troyer (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* Blanco) roots, according to as a function of $[^{15}NH_4^+]_o$

	Parameters	Control	–N	–NH_4^+
HATS	V_{max}	12.6 ± 0.8^a	26.6 ± 1^b	30.4 ± 1.5^b
	K_m	86 ± 6^a	71 ± 5^b	55 ± 4^c
LATS	a	1.8	2.3	2.3
	b	2.3	2.5	2.5
	r^2	0.99	0.99	0.98

Three-month-old *Citrus* plants were grown hydroponically on 1 mM NH_4NO_3. Three days before theexperiment, one group of plants was kept under the same conditions (control), another group wastransferred to a nitrogen-free (–N), while a third group of plants was transferred to 1 mM KNO_3 (–NH_4^+).The $15NH_4^+$ influx was measured on the third day at different concentrations of external $15NH_4^+$. All thevalues are the means of 18 replicates ±SE. *Different letters* indicate significant differences ($P < 0.05$)

nutrition investigated above, LATS-mediated $^{15}NH_4^+$ influx was measured at a quite constant value for all treatments (Fig. 5a). Interestingly, *CitAMT1* expression, which was no more up-regulated at this late stage of N starvation (Fig. 5b, see also Fig. 2c), was strongly stimulated upon NH_4^+, but not NO_3^- re-supply (Fig. 5b). The positive effect of NH_4^+ was similarly recorded at both low and high external concentrations. Collectively, all the data obtained from these N re-supply experiments indicate that exogenous NH_4^+ can have two opposite effects on its own HATS, depending on the prior N nutrition of the plant. Indeed, both NH_4^+ HATS activity and *CitAMT1* expression are repressed by NH_4^+ supply in plants N-starved for 3 days, while they are stimulated by the same NH_4^+ supply in plants N-starved for 7 days.

Differential regulation of *CitAMT1* expression in roots and shoots

CitAMT1 is expressed in all organs of vegetative *Citrus* plants, but predominantly in secondary roots. Although clearly up-regulated by short-term N starvation (i.e., 3 days) in the roots, this gene appears to display the opposite response in the shoot, with a significant decrease in the steady-state transcript level in both stems and leaves of N-starved plants, as compared to control plants (Fig. 6).

Discussion

Citrus plants favour NH_4^+ over NO_3^- uptake when both N sources are present in the medium

Our results confirm that when both sources of N (NH_4^+ and NO_3^-) are present in the nutrient solution, uptake of

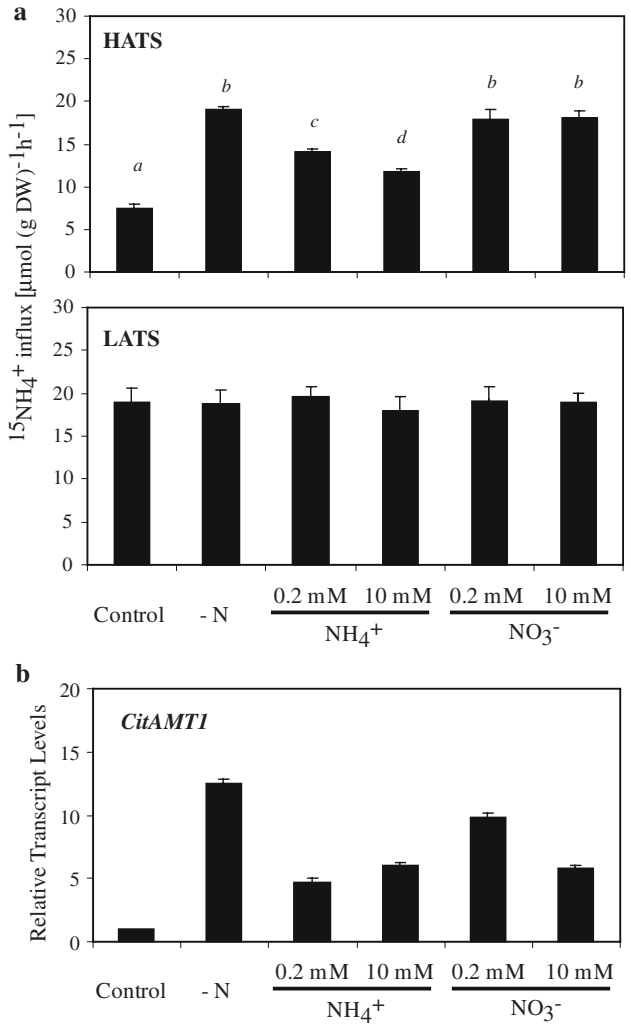

Fig. 4 Influence of a re-supply of different concentrations and sources of nitrogen on both the $^{15}NH_4^+$ influx and the *CitAMT1* gene expression under de-repression conditions. **a** Three-month-old *Citrus* plants were grown hydroponically on 1 mM NH_4NO_3 (control) and transferred for 3 days from 1 mM NH_4NO_3 to nitrogen-free nutrient solution (–N). After starvation, plants were divided into four groups and solutions were supplied with 0.1 mM $(NH_4)_2SO_4$; 0.2 mM KNO_3; 5 mM $(NH_4)_2SO_4$ or 10 mM KNO_3, respectively. The HATS-mediated $^{15}NH_4^+$ influx was measured in roots at 0.2 mM. The LATS-mediated $^{15}NH_4^+$ influx was calculated by subtracting the influx measures at 0.2 mM $^{15}NH_4^+$ from that measured at 5 mM. The values shown are the means of 18 replicates ±SE. *Different letters* indicate significant differences ($P < 0.05$). **b** Real-Time PCR analysis of the expression of *CitAMT1*. Plants were taken from the same experiment as those used for measurements of influx activity in **a**. The *CitAMT1* transcript levels were normalized to the expression of *18S* rRNA measured in the same samples. *Each bar* represents the average data with standard error bars of two independent experiments ($n = 4$)

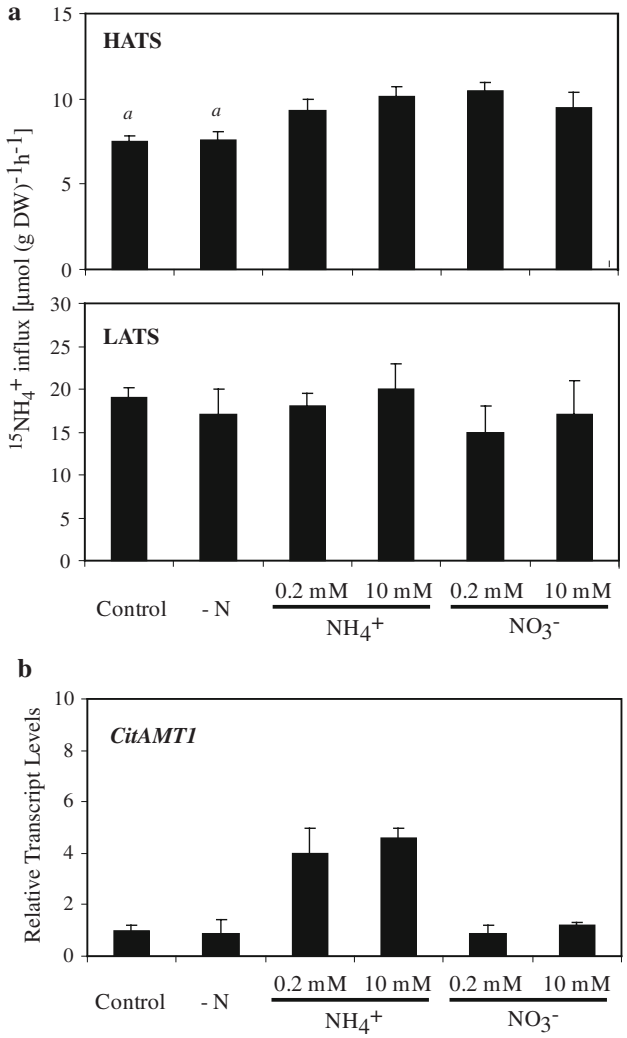

Fig. 5 Influence of a re-supply of different concentrations and sources of nitrogen on both the $^{15}NH_4^+$ influx and the *CitAMT1* gene expression under de-induction conditions. **a** Three-month-old *Citrus* plants were grown hydroponically on 1 mM NH_4NO_3 and transferred for 7 days from 1 mM NH_4NO_3 to a nitrogen-free nutrient solution (–N). After starvation, plants were divided into four groups and solutions were supplied with 0.1 mM $(NH_4)_2SO_4$; 0.2 mM KNO_3; 5 mM $(NH_4)_2SO_4$ or 10 mM KNO_3, respectively. The HATS-mediated $^{15}NH_4^+$ influx was measured in roots at 0.2 mM. The LATS-mediated $^{15}NH_4^+$ influx was calculated by subtracting the influx measures at 0.2 mM $^{15}NH_4^+$ from that measured at 5 mM. The values shown are the means of 18 replicates ±SE. *Different letters* indicate significant differences ($P < 0.05$). **b** Real-Time PCR analysis of the expression of *CitAMT1*. Plants were taken from the same experiment as those used for measurements of influx activity in **a**. The *CitAMT1* transcript levels were normalized to the expression of *18S* rRNA measured in the same samples. *Each bar* represents the average data with standard error bars of two independent experiments ($n = 4$)

NH_4^+, mediated by either transport system (HATS or LATS), is favoured compared to that of NO_3^- (Fig. 1). When both ions are at similar external concentration, a higher influx capacity for root NH_4^+ uptake systems than for root NO_3^- uptake systems is a common observation in many plant species, including *Citrus* (Serna et al. 1992; Gessler et al. 1998; Gazzarrini et al. 1999; Min et al. 2000). This does not always mean that NH_4^+ is the preferred N source under natural conditions since NH_4^+ availability in the soil solution is generally much lower than that of NO_3^-. However, our data provide evidence

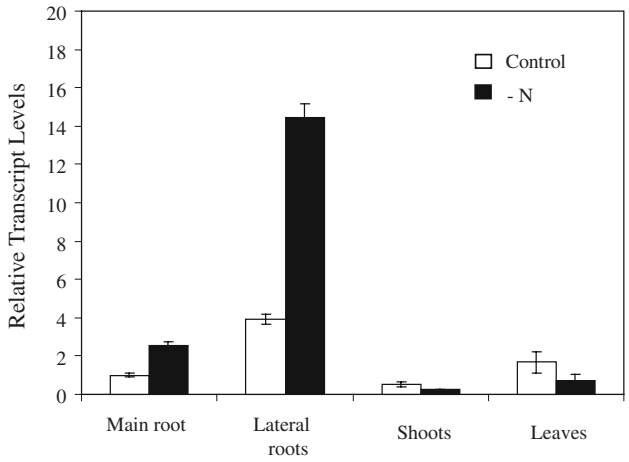

Fig. 6 Organ-dependent expression of *CitAMT1* in the main and lateral roots, stems and leaves of *Citrus* plants Real-Time PCR analysis of the expression of *CitAMT1*. Three-month-old *Citrus* plants were grown hydroponically on 1 mM NH_4NO_3 (control) and transferred for 3 days from 1 mM NH_4NO_3 to a nitrogen-free nutrient solution (–N). The *CitAMT1* transcript levels were normalized to the expression of *18S* rRNA measured in the same samples. *Each bar* represents the average data with standard error bars of two independent experiments ($n = 4$)

that even if NO_3^- is the major N form supplied in the nutrient solution, *Citrus* plants still display a much higher capacity to take up NH_4^+ than NO_3^- (Fig. 1a). This is particularly striking when considering the regulation of both NH_4^+ and NO_3^- HATS by the NH_4^+/NO_3^- external balance. Indeed, the decrease in NH_4^+/NO_3^- ratio in the nutrient solution strongly amplified the difference between NH_4^+ and NO_3^- HATS capacities, suggesting that *Citrus* plants react to the predominance of NO_3^- as a N source by displaying an increased preference for NH_4^+ as the N form taken up. Interestingly, this is not only due to increased NH_4^+ HATS activity but also to decreased NO_3^- HATS activity in response to increased NO_3^- provision (Fig. 1a). This was unexpected for at least two reasons. First, this resulted in an extremely low NO_3^- HATS capacity (around 2 µmol h^{-1} g^{-1} DW) in plants fed with 1 mM NO_3^- as sole N source (see 100% NO_3^- in Fig. 1a), suggesting that the NO_3^- HATS is unable to sustain efficient NO_3^- acquisition in *Citrus* plants. Second, the observation that NO_3^- HATS activity was maximal in the absence of NO_3^- (Fig. 1a) does not fit with the occurrence of a NO_3^-–inducible HATS component (iHATS), which is generally observed in most plant species, including *Citrus* (Aslam et al. 1992; Kronzucker et al. 1995; Cerezo et al. 1997). The LATS-mediated influxes of NH_4^+ and NO_3^- are not altered when plants are exposed to different NH_4^+/NO_3^- ratio (Fig. 1a). This could reveal a lack of control on these systems when the plant N status is adequate (Mäck and Tischner 1994; Wang et al. 1998).

NH_4^+ exerts a dual regulatory effect on both NH_4^+ HATS activity and *CitAMT1* expression

The data from the N deprivation and NH_4^+ re-supply experiments strongly suggest that both NH_4^+ HATS activity and *CitAMT1* expression in the roots are down-regulated by high N status of the plant, but specifically stimulated by NH_4^+. Accordingly, provision of NH_4^+ in the nutrient solution has a dual effect, either repressive through its role as a nutrient promoting high N status (Figs. 2a–4), or stimulatory possibly through its role as an inducer (Figs. 2b and 5). Although counter-intuitive at first glance, this regulatory pattern is well known for many genes involved in NO_3^- transport or assimilation, which are both induced by NO_3^- itself, and repressed by downstream products of NO_3^- assimilation (Wang et al. 2004). The balance between these two opposite effects depends on the prior nutrition regime of the plant. For instance, the temporal pattern of both NH_4^+ HATS and *CitAMT1* expression responses to N deprivation (transient up-regulation followed by down-regulation, Fig. 2a is illustrative of this dual regulatory mechanism. These results coincide with those found by Couturier et al. (2007) for the genes of *Ptr*AMT1.1 and *Ptr*AMT1.2. This pattern closely parallel that observed for NO_3^- HATS and *AtNRT2.1* expression in *Arabidopsis* (Lejay et al. 1999), and is typically explained by the fact that N deprivation first alleviates repression by N status (hence resulting in up-regulation), and only subsequently suppresses induction exerted by the ion itself (thus leading to down-regulation). Accordingly, NH_4^+ supply at early stages of N deprivation (when only repression by N status is relieved) has an inhibitory effect (see Fig. 4), while NH_4^+ supply at later stages of N deprivation (when induction by NH_4^+ is suppressed) has a stimulatory effect (see Fig. 5). Interestingly, after 7 days of N deprivation, *CitAMT1* expression is up-regulated by NH_4^+ but not by NO_3^- (Fig. 5b), suggesting a specific signalling role for NH_4^+ (or a product of its metabolism). Transcriptional repression of individual *AMT* genes by N status of the plant has been widely documented in many species (Gazzarrini et al. 1999; Rawat et al. 1999; von Wirén et al. 2000b; Gansel et al. 2001; Glass et al. 2002; Sonoda et al. 2003; Loqué and von Wirén 2004), but the occurrence of NH_4^+–inducible NH_4^+ transporters is still a matter of debate (Loqué and von Wirén 2004). However, there are several examples of *AMT* genes that respond positively to NH_4^+ supply, such as *LeAMT1.2* in tomato (von Wirén et al. 2000b), and *OsAMT1.1* and *1.2* in rice (Sonoda et al. 2003). The nature of the inducing signal is nevertheless unclear since stimulation of *OsAMT1.1* and *1.2* expression can be obtained with glutamine as well (Sonoda et al. 2003), while *LeAMT1.2* is also responsive to NO_3^-, which is not the case for *CitAMT1*.

As commonly observed in other species, the LATS-mediated influx of NH_4^+ in *Citrus* plants does not respond to the

changes in N availability in the medium (Mäck and Tischner, 1994).

CitAMT1 expression in the roots is repressed by NO_3^-, while NH_4^+ HATS is not

Although NH_4^+ HATS activity and *CitAMT1* expression in the roots appear to be closely co-regulated in response to changes of the plant N status or in response to NH_4^+ supply (see above), this is clearly not the case in response to the supply of NO_3^- as sole N source. Indeed, NO_3^- is unable to repress the NH_4^+ HATS while it seems to be a potent inhibitor of *CitAMT1* expression in the roots. This is evidenced firstly by the strong up-regulation of HATS-mediated NH_4^+ influx, but not of *CitAMT1* transcript accumulation, after transfer of the plants to pure NO_3^- nutrition (Figs. 2b, d, and 3), and secondly by the down-regulation of *CitAMT1* expression, but not of NH_4^+ HATS activity, upon NO_3^- resupply following 3 days of N starvation (Fig. 4). The lack of effect of NO_3^- on NH_4^+ HATS activity most likely results from the fact that NO_3^- does not appear to be an efficient N source for nutrition of the plant. Indeed, under pure NO_3^- nutrition, both HATS and LATS for NO_3^- exhibit a very low influx capacity (Fig. 1a), which is certainly not sufficient to sustain a high N status of the plant. Thus, for *Citrus* seedlings, the supply of NO_3^- as sole N source probably corresponds to an N limiting condition, leading to the relief of the feedback repression exerted on NH_4^+ HATS by the plant N status. Despite *CitAMT1* also appears to be under the control of this feedback repression (see above), its lack of up-regulation under pure NO_3^- nutrition strongly suggests that NO_3^- per se is also a repressor of its expression. This makes a strong parallel with the *AtAMT1.1* gene of *Arabidopsis*, which has been shown to be specifically repressed by NO_3^- (Wang et al. 2000; Gansel et al. 2001), in addition of being down-regulated by high N status of the plant (Gazzarrini et al. 1999; Rawat et al. 1999).

CitAMT1: a key NH_4^+ transporter governing root NH_4^+ uptake in *Citrus* plants?

The central role of AMT transporters in the high-affinity root uptake of NH_4^+ has been recently firmly established in *Arabidopsis*, where disruption of individual *AtAMT1* genes (*AtAMT1.1*, *1.2*, *1.3*) resulted in a deficit of NH_4^+ HATS activity, as compared to the wild-type (Loqué et al. 2006; Yuan et al. 2007a). Furthermore, multiple *amt* mutants display stronger NH_4^+ uptake inhibition than simple mutants (up to >90% in the quadruple *atamt1.1,atamt1.2,atamt1.3, atamt2.1* mutant), indicating that most AMT transporters expressed in the root actually contribute to part of the whole NH_4^+ acquisition, and that their respective contributions are additive (Loqué et al. 2006; Yuan et al. 2007a). It is also noteworthy that a correlation exists between the factors affecting the expression of the *AMT* genes (e.g., N nutrition regime) and those determining the functional importance of the corresponding transporters for the overall NH_4^+ root uptake. For instance, the AtAMT1.1 transporter, which is up-regulated by N deprivation, plays a role in stimulating root NH_4^+ uptake under N deficient conditions (Loqué et al. 2006). Collectively, these considerations suggest that the CitAMT1 transporter plays an important role in governing NH_4^+ acquisition from the external medium in *Citrus* plants. In our previous report, we found that *CitAMT1* expression is regulated as NH_4^+ HATS activity in response to changes in the C status of the plant (Camañes et al. 2007). Here, we show that expression of this gene is also co-regulated with NH_4^+ HATS in response to changes in the N status of the plant. Thus, we hypothesize that CitAMT1 is a key transporter allowing *Citrus* plants to adapt their N acquisition to environmental changes affecting their nutritional status. Nonetheless, our data also indicate that changes in *CitAMT1* mRNA level do not always account for the regulation of the NH_4^+ HATS. Indeed, the repressive action of NO_3^- on *CitAMT1* expression is not associated with a corresponding inhibition of HATS-mediated NH_4^+ uptake. Clearly, other factors have to be considered to explain this discrepancy. It is possible that other CitAMT transporters, up-regulated by N limitation, but not repressed by NO_3^-, are involved in the stimulation of NH_4^+ HATS activity under pure NO_3^- nutrition. The differential changes observed for K_m of the NH_4^+ HATS in response of either N deprivation or transfer to NO_3^- as sole N source may be consistent with this hypothesis (see Fig. 3 and Table 1), which however will require a more complete characterization of the *AMT* gene family in *Citrus*. Alternatively, there is now increasing evidence that AMT transporters are also regulated at the post-transcriptional level (Rawat et al. 1999; Loqué et al. 2006; Yuan et al. 2007a). Hence, it is still conceivable that despite an unchanged *CitAMT1* transcript level, a stimulation of CitAMT1 activity at the protein level can be responsible for the up-regulation of the NH_4^+ HATS under pure NO_3^- nutrition.

Regulation of *CitAMT1* expression is organ-dependent

Expression studies on most the *AMT* genes have been limited to mRNA profiling in roots. However, several of these genes are also expressed in the aerial parts of the plant, such as *AtAMT1.1* in *Arabidopsis* (Ninnemann et al. 1994; Gazzarrini et al. 1999; Kaiser et al. 2002), *LeAMT1.2* and *1.3* in tomato (von Wirén et al. 2000), and *OsAMT1.1* in rice (Sonoda et al. 2003) and *PtrAMT1.5; 1.6; 2.1; 2.2* and *3.1* in *P. trichonocarpa* (Couturier et al. 2007). A possible role for *AMT* transporters in leaves is to prevent loss of

NH_4^+ from cells during photorespiration (Mayer and Ludewig 2006), a function that can also be fulfilled by CitAMT1 in *Citrus*. Moreover, citrus plants are perennial plants, and they need to mobilize nitrogen from different organs, as demonstrated in *Populus trichocarpa* (Couturier et al. 2007). However, differential regulation of the same gene between roots and shoot is generally not recorded, with the exception of *AtAMT1.1* (Engineer et al. 2007; Yuan et al. 2007a). Hence, the regulation observed for *CitAMT1* expression bears strong similarity with that of *AtAMT1.1*, since both genes are expressed in both roots and shoots, and down-regulated by N status of the plant and by NO_3^- (Fig. 6). However, unlike *AtAMT1.1*, *CitAMT1* is in addition inducible by NH_4^+ (or by a product of its assimilation), and by sugars (Camañes et al. 2007), two features which are found for other *AMT* genes. The reason why CitAMT1 exhibits such a complex pattern of regulation by various signals is unknown, but makes this transporter quite unique in possibly allowing *Citrus* plants to adapt their N acquisition to a wide range of environmental factors.

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References

Aslam M, Travis RL, Huffaker RC (1992) Comparative kinetics and reciprocal inhibition of nitrate and nitrite uptake in roots of uninduced and induced barley (*Hordeum vulgare* L.) seedlings. Plant Physiol 99:1124–1133

Baozhen L, Weijie X, Shubin S, Qirong S, Guohua X (2006) Physiological and molecular responses of nitrogen-starved rice plants to re-supply of different nitrogen sources. Plant Soil 287:145–159

Bloom AJ, Sukrapanna SS, Warner RL (1992) Root respiration associated with ammonium and nitrate absorption and assimilation by barley. Plant Physiol 99:1294–1301

Britto DT, Kronzucker HJ (2002) NH_4^+ toxicity in higher plants: a critical review. J Plant Physiol 159:567–584

Camañes G, Cerezo M, Primo-Millo E, Gojon A, García-Agustín P (2007) Ammonium transport and *CitAMT1* expression are regulated by light and sucrose in *Citrus* plants. J Exp Bot 58:2811–2825

Cerezo M, GarciaAgustin P, Serna MD, PrimoMillo E (1997) Kinetics of nitrate uptake by *Citrus* seedlings and inhibitory effects of salinity. Plant Sci 126:105–112

Cerezo M, Flors V, Legaz F, García-Agustín P (2000) Characterization of the low affinity transport system for NO_3^- uptake by *Citrus* roots. Plant Sci 160:95–104

Cerezo M, Tillard P, Gojon A, Primo-Millo E, Garcia-Agustin P (2001) Characterization and regulation of ammonium transport systems in *Citrus* plants. Planta 214:97–105

Cerezo M, Camañes G, Flors V, Primo-Millo E, García-Agustín P (2007) Regulation of nitrate transport in *Citrus* rootstocks depending of nitrogen availability. Plant Signal Behav 2:337–342

Couturier J, Montanini B, Martin F, Brun A, Blaudez D, Chalot M (2007) The expanded family of ammonium transporters in the perennial poplar plant. New Phytol 174:137–150

Daniel-Vedele F, Filleur S, Caboche M (1998) Nitrate transport: a key step in nitrate assimilation. Curr Opin Plant Biol 1:235–239

Engineer CB, Kranz RG (2007) Reciprocal leaf and root expression of *AtAMT1.1* and root architectural changes in response to nitrogen starvation. Plant Physiol 143:236–250

Forde BG (2000) Nitrate transporters in plants: structure, function and regulation. Biochim Biophys Acta 1465:219–235

Fraisier V, Gojon A, Tillard P, Daniel-Vedele F (2000) Constitutive expression of putative high-affinity nitrate transporter in *Nicotiana plumbaginifolia*: evidence for post-transcriptional regulation by a reduced nitrogen source. Plant J 23:489–496

Gansel X, Munos S, Tillard P, Gojon A (2001) Differential regulation of the NO_3^- and NH_4^+ transporter genes *AtNrt2.1* and *AtAmt1.1* in *Arabidopsis*: relation with long-distance and local controls by N status of the plant. Plant J 26:143–155

Gazzarrini S, Lejay T, Gojon A, Ninnemann O, Frommer WB, von Wirén N (1999) Three functional transporters for constitutive, diurnally regulated, and starvation-induced uptake of ammonium into *Arabidopsis* roots. Plant Cell 11:937–947

Gessler A, Schneider S, von Sehgbusch D, Weber P, Hanemann U, Huber C, Rothe A, Kreutzer K, 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 ADM, Britto DT, Kaiser BN, Kinghorn JR, Kronzucker HJ, Kumanr A, Okamoto M, Rawat S, Siddiqi MY, Unkles SE, Vidmar JJ (2002) The regulation of nitrate and ammonium transport systems in plants. J Exp Bot 53:855–864

Hoagland DR, Arnon DJ (1950) The water culture method for growing plants without soil. Calif Agric Exp St Circ 347:1–32

Howitt SM, Udvardi MK (2000) Structure, function and regulation of ammonium transporters in plants. Biochim Biophys Acta 1465:152–170

Huang NC, Liu KH, Lo HJ, Tsay YF (1999) Cloning and functional characterization of an *Arabidopsis* nitrate transporter gene that encodes a constitutive component of low-affinity uptake. Plant Cell 11:1381–1392

Kaiser BN, Rawat SR, Siddiqi MY, Masle J, Glass ADM (2002) Functional analysis of an *Arabidopsis* T-DNA "Knockout" of the high-affinity NH_4^+ transporter *AtAMT1.1*. Plant Physiol 130:1263–1275

Kirk GJD, Kronzucker HJ (2005) The potential for nitrification and nitrate uptake in the rhizosphere of wetland plants: a modelling study. Ann Bot 96:639–646

Kronzucker HJ, Siddiqi MY, Glass ADM (1995) Kinetics of NO_3^- influx in spruce. Plant Physiol 109:319–326

Kronzucker HJ, Siddiqi MY, Glass ADM (1996) Kinetics of NH_4^+ influx in spruce. Plant Physiol 110:773–779

Kronzucker HJ, Siddiqi MY, Glass ADM, Kirk GJD (1999) Nitrate-ammonium synergism in rice: a subcellular flux analysis. Plant Physiol 119:1041–1046

Kumar HJ, Silim SN, Okamoto M, Siddiqi MY, Glass ADM (2003) Differential expression of three members of the *AMT1* gene family encoding putative high-affinity NH_4^+ transporters in roots of *Oryza sativa* subspecies *indica*. Plant Cell Environ 26:907–914

Lauter FR, Ninnemann O, Bucher M, Riesmeier JW, Frommer WB (1996) Preferential expression of an ammonium transporter and of two putative nitrate transporters in root hairs of tomato. Proc Natl Acad Sci USA 93:8139–8144

Lejay L, Gansel X, Cerezo M, Tillard P, Muller C, Krapp A, von Wirén N, Daniel-Vedele F, Gojon A (2003) Regulation of root ion transporters by photosynthesis: functional importance and relation with hexokinase. Plant Cell 15:2218–2232

Lejay L, Tillard P, Lepetit M, Olive FD, Filleur S, Daniel-Vedele F, Gojon A (1999) Molecular and functional regulation of two NO_3^- uptake systems by N- and C-status of *Arabidopsis* plants. Plant J 18:509–519

Loqué D, von Wirén N (2004) Regulatory levels for the transport of ammonium in plant roots. J Exp Bot 55:1293–1305

Loqué D, Yuan L, Kojima S, Gojon A, Wirth J, Gazzarrini S, Ishiyama K, Takahashi H, von Wirén N (2006) Additive contribution of *AtAMT1.1* and *AtAMT1.3* to high-affinity ammonium uptake across the plasma membrane of nitrogen-deficient *Arabidopsis* roots. Plant J 48:522–534

Mäck G, Tischner RJ (1994) Constitutive and inducible net NH_4^+ uptake of barley (*Hordeum vulgare*. L.) seedlings. Plant Physiol 144:351–357

Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic Press, London

Mayer M, Ludewig U (2006) Role of *AMT1.1* in NH_4^+ acquisition in *Arabidopsis thaliana*. Plant Biol 8:522–528

Miller AJ, Fan XR, Orsel M, Smith SJ, Wells DM (2007) Nitrate transport and signalling. J Exp Bot 58:2297–2306

Min X, Siddiqi Y, Guy RD, Glass ADM, Kronzucker HJ (2000) A comparative kinetic analysis of nitrate and ammonium influx in two early-successional tree species of temperate and boreal forest ecosystems. Plant Cell Environ 23:321–328

Ninnemann O, Jauniaux JC, Frommer WB (1994) Identification of a high-affinity NH_4^+ transporter from plants. EMBO J 13:3464–3471

Rawat SR, Silim SN, Kronzucker HJ, Siddiqi MY, Glass ADM (1999) *AtAmt1* gene expression and NH_4^+ uptake in roots of *Arabidopsis thaliana*: evidence for regulation by root glutamine levels. Plant J 19:143–152

Reboll V, Cerezo M, Roig A, Lapeña L, García-Agustín P (2000) Influence of wastewater vs groundwater on young citrus trees. J Sci Food Agric 80:1441–1446

Serna MD, Borras R, Legaz F, Primo-Millo E (1992) The influence of nitrogen concentration and ammonium/nitrate ratio on N-uptake, mineral composition and yield of citrus. Plant Soil 147:13–23

Sonoda Y, Ikeda A, Saiki S, von Wirén N, Yamaya T, Yamaguchi J (2003) Distinct expression and function of three ammonium transporter genes (OsAMT1;1–1;3) in rice. Plant Cell Physiol 44:726–734

Tsay YF, Chiu CC, Tsai CB, Ho CH, Hsu PK (2007) Nitrate transporters and peptide transporters. FEBS Lett 581:2290–2300

von Wirén N, Gazzarrini S, Gojon A, Frommer WB (2000a) The molecular physiology of ammonium uptake and retrieval. Curr Opin Plant Biol 3:254–261

von Wirén N, Lauter FR, Ninnemann O, Gillissen B, Walch-Liu P, Engels C, Jost W, Frommer WB (2000b) Differential regulation of three functional ammonium transporter genes by nitrogen in root hairs and by light in leaves of tomato. Plant J 21:167–175

Wang MY, Siddiqi MY, Ruth TJ, Glass ADM (1993) Ammonium uptake by rice roots.2. Kinetics of NH_4^+-^{13}N- Influx across the plasmalemma. Plant Physiol 103:1259–1267

Wang R, Liu D, Crawford (1998) The Arabidopsis CHL protein plays a major role in high-affinity nitrate uptake. Proc Natl Acad Sci USA 95:15134–15139

Wang R, Guegler K, LaBrie ST, Crawford NM (2000) Genomic analysis of a nutrient response in *Arabidopsis* reveals diverse expression patterns and novel metabolic and potential regulatory genes that are induced by nitrate. Plant Cell 12:1491–1510

Wang Y-H, Garvin DF, Kochian LV (2001) Nitrate-induced genes in tomato roots: array analysis reveals novel genes that may play a role in N nutrition. Plant Physiol 127:345–359

Wang R, Tischner R, Gutierrez R, Hoffman M, Xing X, Chen M, Coruzzi G, Crawford N (2004) Genomic analysis of the nitrate response using a nitrate reductase-null mutant of *Arabidopsis*. Plant Physiol 136:2512–2522

Williams LE, Miller AJ (2001) Transporters responsible for the uptake and partitioning of nitrogenous solutes. Annu Rev Plant Physiol Plant Mol Biol 52:659–688

Yuan L, Loqué D, Kojima S, Rauch S, Ishiyama K, Inoue E, Takahashi H, von Wirén N (2007a) The organization of high-affinity ammonium uptake in *Arabidopsis* roots depends on the spatial arrangement and biochemical properties of AMT1-type transporters. Plant Cell 19:2636–2652

Yuan L, Loqué D, Ye F, Frommer WB, von Wirén (2007b) Nitrogen-dependent posttranscriptional regulation of the ammonium transporter AtAMT1.1. Plant Physiol 143:732–744