

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 maximum rate (μM)
V_{max}	The calculated maximum rate of ion influx [$\mu\text{mol } ^{15}\text{NH}_4^+$ (g root dry weight) $^{-1} \text{h}^{-1}$]

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; Fraiser 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

162	down-regulation of <i>AMT1</i> gene expression by N metabo-	211
163	lites. First, the exact nature of the N compounds acting as	212
164	regulatory signals for the NH_4^+ HATS is still unclear. On	213
165	the one hand, <i>AtAMT1.1</i> mRNA level is inversely corre-	214
166	lated with the concentration of free glutamine in the roots,	215
167	suggesting a predominant role of this compound as a	216
168	repressor of <i>AtAMT1.1</i> expression (Rawat et al. 1999). On	217
169	the other hand, <i>AtAMT1.1</i> was found to be repressed by	218
170	short-term NO_3^- supply in a microarray study (Wang et al.	219
171	2000), indicating that NO_3^- itself could also be involved in	220
172	<i>AtAMT1.1</i> down-regulation. This is consistent with split-	221
173	root studies (Gansel et al. 2001), which showed that	222
174	<i>AtAMT1.1</i> expression is repressed by 1 mM KNO_3 .	223
175	Recently, Engineer and Kranz (2007) have even shown that	224
176	regulation of <i>AtAMT1.1</i> gene expression by the N status of	225
177	the plant differs between roots and shoots, thus suggesting	226
178	that depending on the gene or the organ, N compounds may	227
179	either repress or at the opposite stimulate <i>AMT1</i> expression.	228
180	The data obtained with <i>LeAMT1.1</i> and <i>LeAMT1.2</i> in tomato	229
181	also agree with this hypothesis (Lauter et al. 1996; von	
182	Wirén et al. 2000b; Wang et al. 2001). Moreover, when N-	
183	deficient <i>Arabidopsis</i> plants were re-supplied with NH_4^+ ,	
184	root high-affinity NH_4^+ influx showed a faster time-depen-	
185	dent repression relative to <i>AtAMT1.1</i> mRNA level in roots	
186	(Rawat et al. 1999), a discrepancy that could be explained	
187	by the occurrence of post-transcriptional control of	
188	<i>AtAMT1.1</i> (Yuan et al. 2007b). Finally, the regulation of	
189	the LATS for NH_4^+ is much less documented and contrast-	
190	ing conclusions are found in the literature. An indication	
191	that the LATS is also under feedback repression by N	
192	metabolites was provided by a study with spruce seedlings	
193	(Kronzucker et al. 1996). However, Mäck and Tischner	
194	(1994) found no change in the activity of the NH_4^+ LATS	
195	in response to N-starvation in barley. Finally, experiments	
196	with rice and <i>Citrus</i> indicated that in these species the	
197	activity of the NH_4^+ LATS is, on the contrary, stimulated	
198	by previous NH_4^+ provision (Wang et al. 1993; Cerezo	
199	et al. 2001).	
200	In our previous study, we reported on the identification	
201	of <i>CitAMT1</i> in <i>Citrus</i> , and on the regulation of its expres-	
202	sion by photosynthesis (Camañes et al. 2007). In this pres-	
203	ent study, we show that <i>CitAMT1</i> expression in the roots is	
204	also regulated by the N source and by the N status of the	
205	plant.	
206	Materials and methods	
207	Plant material and growth conditions	
208	Seeds of citrange Troyer (<i>Citrus sinensis</i> L. Osbeck \times <i>Pon-</i>	
209	<i>cirus trifoliata</i> Blanco) (Beniplant, Valencia, Spain) were	
210	allowed to germinate in vermiculite in a growth chamber	
	under the following environmental conditions: light/dark	211
	cycle of 16/8 h, temperature of 20/24°C, light intensity of	212
	200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and an RH of 70%. The seeds were irri-	213
	gated twice a week with distilled water. After 6 weeks,	214
	seedlings were irrigated with Hoagland solution lacking	215
	nitrogen (Hoagland and Arnon 1950). The nutrient solution	216
	was complemented with 1 mM NH_4NO_3 and an addition of	217
	1.5 mM K_2SO_4 and 3 mM CaSO_4 were added to compen-	218
	sate for the absence of K^+ with 3 mM KNO_3 and the	219
	absence of Ca^{2+} with 3 mM $\text{Ca}(\text{NO}_3)_2$ in the solution. The	220
	pH of the nutrient solution was adjusted to 6.0 with 1 mM	221
	KOH.	222
	Prior to the experiments, 3-month-old plants with a sin-	223
	gle shoot were selected for uniformity of size, and trans-	224
	ferred to an aerated complemented Hoagland solution for	225
	7 days on hydroponic culture devices. Nutrient solutions	226
	were renewed twice weekly and on the day of the experi-	227
	ments. All experiments were repeated three times, and typi-	228
	cal results are shown.	229
	Measurement of ^{15}N influx	230
	$^{15}\text{NH}_4^+$ or $^{15}\text{NO}_3^-$ influx by <i>Citrus</i> roots was measured at a	231
	low (0.2 mM) and high (5 mM) external concentration of	232
	either NH_4^+ or $^{15}\text{NO}_3^-$, respectively, which is representa-	233
	tive of both the high- and low-affinity transport systems	234
	(HATS and LATS, respectively). The $^{15}\text{NH}_4^+$ or $^{15}\text{NO}_3^-$	235
	influx in roots was determined on six plants after transfer-	236
	ring to 0.1 mM CaSO_4 for 1 min, then to $^{15}\text{NH}_4^+$ solution for	237
	5 min, and finally to 0.1 mM CaSO_4 for 1 min (Gazzarrini	238
	et al. 1999). The $^{15}\text{NH}_4^+$ or $^{15}\text{NO}_3^-$ solution was the N-free	239
	Hoagland nutrient solution, supplemented with 1 mM MES	240
	pH 6.0, where N was supplied as either $^{15}\text{N}[(\text{NH}_4)_2\text{SO}_4$ (98	241
	atom % ^{15}N in excess) or $^{15}\text{N}[\text{KNO}_3$ (98 atom % ^{15}N in	242
	excess) at the indicated concentrations (0.2 and 5 mM).	243
	After labelling, the roots were separated from the shoots	244
	and dried for 48 h at 65°C, crushed in a hammer, mill and	245
	weighed. The ^{15}N analysis was performed using an inte-	246
	grated system for continuous flow isotope ratio mass spec-	247
	trometry (Euro-EA elemental analyser (EuroVector S.P.A.,	248
	Milan, Italy) and Isoprime mass spectrometer (GV Instru-	249
	ments, Manchester, UK). The values of the root ^{15}N influx	250
	are expressed in $\mu\text{mol } ^{15}\text{N} (\text{g root DW})^{-1} \text{h}^{-1}$. The experi-	251
	ments were repeated at least three times and the mean \pm SE	252
	is shown ($n = 18$).	253
	Influence of the $\text{NH}_4^+/\text{NO}_3^-$ ratio in the medium on NH_4^+	254
	influx and <i>CitAMT1</i> gene expression	255
	Three-month-old <i>Citrus</i> plants were grown on a complete	256
	nutrient solution containing 1 mM NH_4NO_3 as an N source	257
	and 3 days before the experiment plants were transferred	258
	from 1 mM NH_4NO_3 to five different solutions at 1 mM	259

260	total N concentration, but with the following (NH ₄) ₂ SO ₄ /	according to the manufacturer's instructions. Samples were	305
261	KNO ₃ ratio 100:0; 75:25; 50:50; 25:75; 0:100. Under these	treated with DNase I to avoid contaminating the DNA. A	306
262	conditions, ¹⁵ NH ₄ ⁺ and ¹⁵ NO ₃ ⁻ influxes were measured,	total of 1 µg of total RNA was annealed to random hexa-	307
263	and roots of <i>Citrus</i> plants were frozen for later studies of	mers and reverse-transcribed using the Omniscript®	308
264	<i>CitAMT1</i> gene expression.	Reverse Transcription kit (Qiagen, Izasa, Barcelona, Spain)	309
265	Influence of nitrogen deficiency	to obtain cDNA. The sequences of the gene-specific oligo-	310
266	Three-month-old <i>Citrus</i> plants were grown hydroponically	nucleotides designed and used for real-time PCR are the	311
267	on 1 mM NH ₄ NO ₃ . Before the experiment, one group of	following: AMT forward: 5'CCCACCTCCAACCTTCGA	312
268	plants was kept under the same conditions (control), while	CTA3' and reverse: 5'CAGAACCAATGGGAGACGA	313
269	another group was transferred to nitrogen-free nutrient	C3'; and <i>18S</i> forward: 5'GAACAACCTGCGAAAGCATT	314
270	solution, and a third group was transferred to 1 mM KNO ₃ .	TGC3' and reverse: 5'CCTGGTAAGTTTCCCCGTG	315
271	Root ¹⁵ NH ₄ ⁺ influx was measured daily for a week, and the	TTG3'. Real-time PCR was conducted using the Quanti-	316
272	roots of <i>Citrus</i> plants were frozen for later studies of	Tect™ SYBR Green PCR Kit (Qiagen) and the SmartCyc-	317
273	<i>CitAMT1</i> gene expression.	ler II instrument (Cepheid, Sunnyvale, USA). Each	318
274	Kinetics of ¹⁵ NH ₄ ⁺ influx	reaction was set up in two replicates. The PCR conditions	319
275	The kinetics of the ¹⁵ NH ₄ ⁺ influx as a function of the exter-	to amplify the <i>CitAMT1</i> fragment were as follows: 95°C for	320
276	nal NH ₄ ⁺ concentration was measured in plants with	15 min and 40 cycles of 95°C for 15 s, 60°C for 30 s, and	321
277	[¹⁵ NH ₄ ⁺] ₀ ranging from 20 µM to 30 mM. The double	72°C for 30 s. Agarose gel electrophoresis and melting	322
278	reciprocal plots of the influxes versus substrate concentra-	curve analysis were performed to confirm the specific gene	323
279	tions were subjected to linear regression analysis. The	product formation and did not represent primer dimmer or	324
280	Michaelis-Menten kinetic constants (<i>K_m</i> and <i>V_{max}</i>) were	non-specific products.	325
281	calculated from these regression equations at the concentra-	Statistical analysis	326
282	tion range of 20 µM to 1 mM. When the concentration	Statistical analysis was carried out using the Statgraphics	327
283	exceeded 1 mM [¹⁵ NH ₄ ⁺] ₀ , the measured ¹⁵ NH ₄ ⁺ influx	software support. The data are expressed as means and SE.	328
284	appeared to result from the participation of two transport	Mean values were compared by an LSD (least significant	329
285	systems (HATS and LATS). Thus, the differences between	difference) test. Differences were taken into account only	330
286	the measured influx at concentrations >1 mM [¹⁵ NH ₄ ⁺] ₀	when they were significant at the 5% level. All experiments	331
287	and the calculated <i>V_{max}</i> for HATS were taken as the esti-	were repeated at least three times.	332
288	mates of the influx only due to LATS.	Results	333
289	Effect of supplying different concentrations of nitrogen	Effect of the N source on NH ₄ ⁺ and NO ₃ ⁻ uptake systems	334
290	sources under de-repression and de-induction conditions	To determine how the nature of the N source affected the	335
291	Three-month-old <i>Citrus</i> plants were grown hydroponically	expression and activity of the root uptake systems for	336
292	on 1 mM NH ₄ NO ₃ and transferred for 3 days (de-repres-	NH ₄ ⁺ , as compared to those for NO ₃ ⁻ , plants grown hydro-	337
293	sion) or 7 days (de-induction) from 1 mM NH ₄ NO ₃ to	ponically on 1 mM NH ₄ NO ₃ were acclimated for 3 days to	338
294	nitrogen-free nutrient solution. After starvation, plants were	various N regimes with different NH ₄ ⁺ /NO ₃ ⁻ ratio (100:0,	339
295	divided into four groups and solutions were supplied with	75:25, 50:50, 25:75 and 0:100) prior to the measurements	340
296	0.1 mM (NH ₄) ₂ SO ₄ ; 0.2 mM KNO ₃ ; 5 mM (NH ₄) ₂ SO ₄ or	of both HATS and LATS activities for NH ₄ ⁺ and NO ₃ ⁻ ,	341
297	10 mM KNO ₃ , respectively. Root ¹⁵ NH ₄ ⁺ influx was mea-	and of <i>CitAMT1</i> transcript levels in the roots (Fig. 1). For	342
298	sured after 24 h and the roots of <i>Citrus</i> plants were frozen	HATS activity, root ¹⁵ NH ₄ ⁺ and ¹⁵ NO ₃ ⁻ influxes were mea-	343
299	for subsequent studies of the <i>CitAMT1</i> gene expression.	sured at 0.2 mM, and are thus expected to provide a mea-	344
300	RNA extraction and real-time PCR analysis	surement of the maximum capacity (i.e., <i>V_{max}</i>) for the	345
301	For all mRNA expression analyses, root samples taken	corresponding systems. LATS activity was estimated as the	346
302	from six plants at time points or treatments were ground to	difference between ¹⁵ N influx at 5 and 0.2 mM external	347
303	powder under liquid nitrogen for total RNA extraction	¹⁵ NH ₄ ⁺ or ¹⁵ NO ₃ ⁻ concentrations.	348
304	using the Total Quick RNA kit (Talent, Trieste, Italy)	Under all situations investigated, HATS and LATS	349
		activities for NH ₄ ⁺ far exceeded those measured for NO ₃ ⁻	350
		(Fig. 1a), showing that <i>Citrus</i> plants have a much higher	351

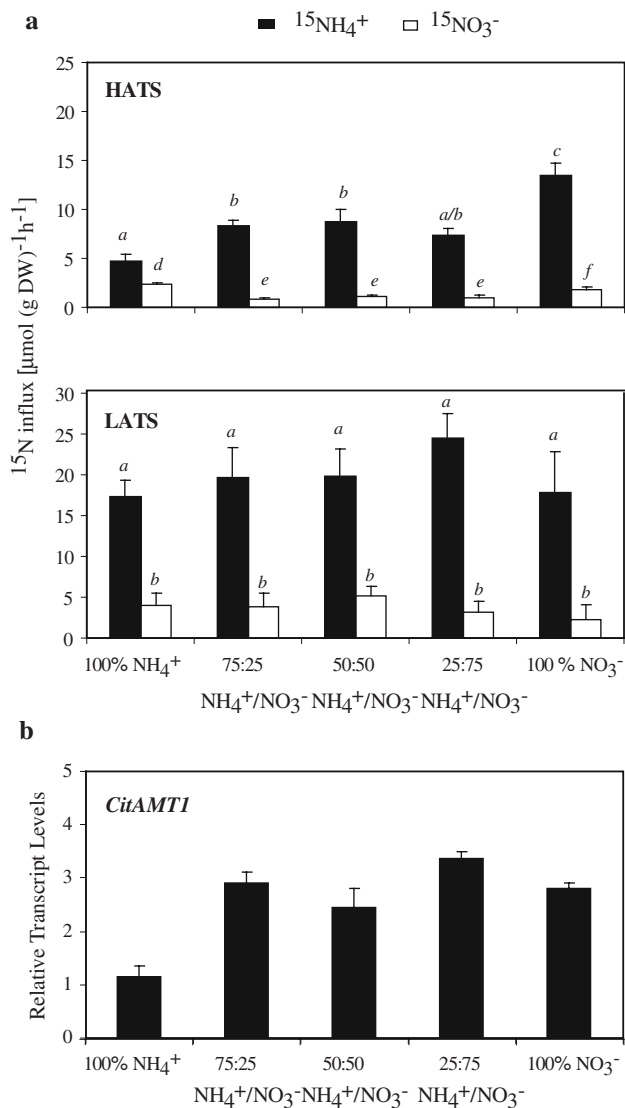


Fig. 1 Effect of pre-treating the *Citrus* plants for 3 days with different NH₄⁺/NO₃⁻ ratios on the ¹⁵NH₄⁺ and ¹⁵NO₃⁻ influxes and the *CitAMT1* gene expression ¹⁵NH₄⁺ influx in *Citrus* roots after transferring the plants grown hydroponically for 3 days from 1 mM NH₄NO₃ to different solutions with (NH₄)₂SO₄/KNO₃ ratios (100; 75:25; 50:50; 25:75; 0) to a final N concentration of 1 mM. The HATS-mediated ¹⁵NH₄⁺ or ¹⁵NO₃⁻ influx was measured in roots at 0.2 mM. The LATS-mediated ¹⁵NH₄⁺ or ¹⁵NO₃⁻ influx was calculated by subtracting the influx measures at 0.2 mM ¹⁵NH₄⁺ or ¹⁵NO₃⁻ 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)

352 capacity for root NH₄⁺ uptake than for root NO₃⁻ uptake.
 353 The respective capacities of the NH₄⁺ and NO₃⁻ HATS
 354 changed significantly as a function of the N source, with
 355 HATS-mediated ¹⁵NH₄⁺ influx unexpectedly increasing

with decreasing external NH₄⁺/NO₃⁻ ratio, while the reverse was observed with HATS-mediated ¹⁵NO₃⁻ influx (Fig. 1a). Thus, highest NH₄⁺ HATS capacity was found with NO₃⁻ as the sole N source, and conversely, highest NO₃⁻ HATS capacity was found with NH₄⁺ as the sole N source. Although surprising, this pattern of NH₄⁺ and NO₃⁻ HATS regulation matched quite well the associated changes in *CitAMT1* expression (Fig. 1b). Accumulation of *CitAMT1* transcript in the roots was minimal under pure NH₄⁺ nutrition, and increased nearly 3-fold when NO₃⁻ was added in the medium, regardless of whether NH₄⁺ was also present.

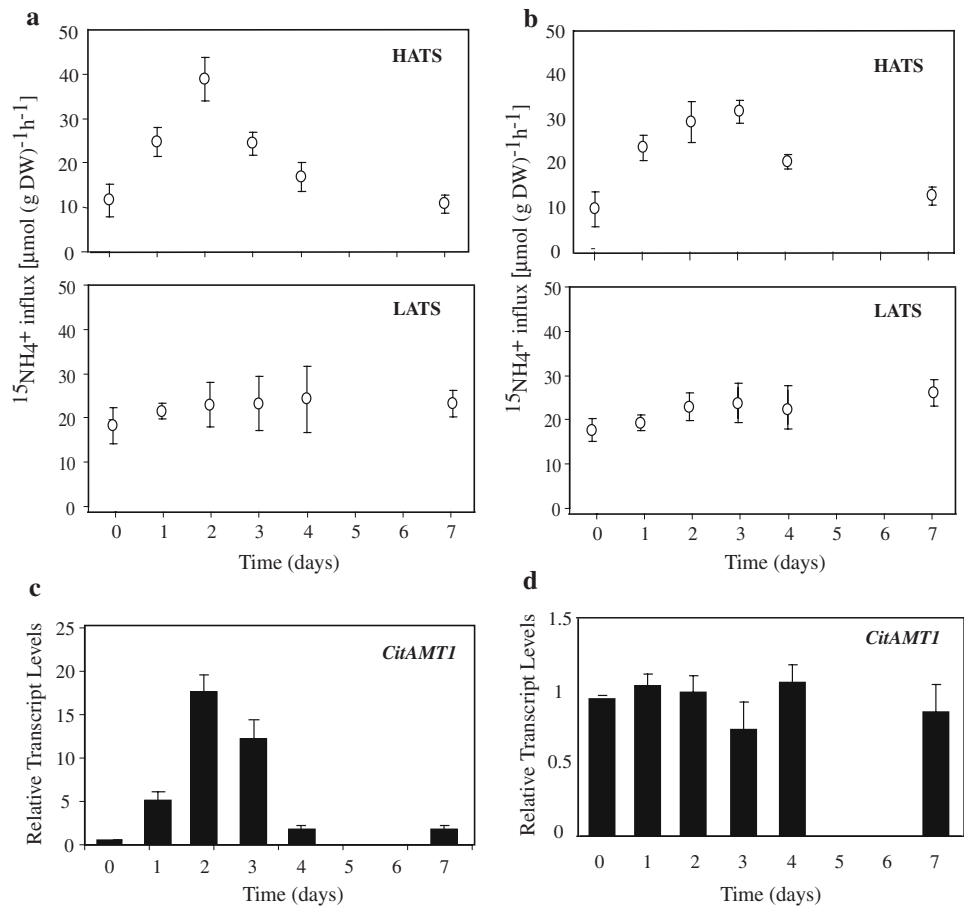
Response of root NH₄⁺ uptake systems to N starvation or to specific removal of NH₄⁺ from the nutrient solution

To determine how both NH₄⁺ HATS and LATS react to the removal of their substrate from the external medium, plant grown on 1 mM NH₄NO₃ were transferred for 7 days either to a N-free solution or to a nutrient solution containing 1 mM NO₃⁻ as sole N source. Both treatments resulted in a strong but transient up-regulation of NH₄⁺ HATS, as shown by the 3 to 4-fold increase in ¹⁵NH₄⁺ influx after 2–3 days, followed by an equivalently fast decline until day 7, where NH₄⁺ HATS activity was re-established to its initial values (Fig. 2a, b). No such changes were observed for the NH₄⁺ LATS, which kept a roughly constant activity whatever the N nutrition regime of the plants (1 mM NH₄NO₃, 1 mM NO₃⁻ or N-free solution, Fig. 2a, b).

Changes in *CitAMT1* expression in the roots closely paralleled those of NH₄⁺ 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 NH₄⁺ HATS) and decline thereafter (Fig. 2c). However, the response pattern of *CitAMT1* expression in the roots of plants transferred to the 1 mM NO₃⁻ nutrient solution was totally different, since no increase in *CitAMT1* mRNA level was observed (Fig. 2d), despite a marked stimulation of NH₄⁺ HATS activity (Fig. 2b). Although surprising, this discrepancy between the responses of *CitAMT1* and of NH₄⁺ HATS to the removal of NH₄⁺ from the external medium fits with the results of the experiments in Fig. 1, which also show that NH₄⁺ HATS activity, but not *CitAMT1* expression, is increased under pure NO₃⁻ 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 NH₄⁺ HATS in response to N starvation, but not in response to the specific absence of the NH₄⁺ N source.

One hypothesis would then be that other NH₄⁺ transporters than *CitAMT1* account for the up-regulation of the NH₄⁺

Fig. 2 Correlation between the $^{15}\text{NH}_4^+$ influx and the *CitAMT1* gene expression after subjecting roots to either nitrogen deficiency or NO_3^- as the sole nitrogen source $^{15}\text{NH}_4^+$ 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 $^{15}\text{NH}_4^+$ influx was measured daily for a week. The HATS-mediated $^{15}\text{NH}_4^+$ influx was measured in roots at 0.2 mM. The LATS-mediated $^{15}\text{NH}_4^+$ influx was calculated by subtracting the influx measures at 0.2 mM $^{15}\text{NH}_4^+$ from that measured at 5 mM. The values shown are the means of 18 replicates \pm 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 ($n = 4$)



407 HATS in response to the provision of NO_3^- as sole N
 408 source. To address this possibility, we then investigated
 409 whether N-starvation or provision of NO_3^- as sole N source
 410 have a differential effect on the kinetic parameters of
 411 $^{15}\text{NH}_4^+$ influx in the roots, that could indicate that NH_4^+
 412 transporters with different functional properties are
 413 involved. In all groups of plants (fed with 1 mM NH_4NO_3 ,
 414 transferred to 1 mM NO_3^- or to N-free solution), a biphasic
 415 kinetics pattern was observed for root $^{15}\text{NH}_4^+$ influx
 416 (Fig. 3). At a low $^{15}\text{NH}_4^+$, $^{15}\text{NH}_4^+$ influx followed the
 417 typical Michaelis-Menten-type kinetics of the saturable
 418 HATS systems (Fig. 3a), while at high $^{15}\text{NH}_4^+$, a linear
 419 pattern was found for $^{15}\text{NH}_4^+$ influx in agreement with the
 420 action of a non saturable LATS (Fig. 3b). As expected, both
 421 transfer to N-free solution or to 1 mM NO_3^- stimulated
 422 HATS, but not LATS activity (Fig. 3a, b). The calculated
 423 values of both V_{max} and K_m for the HATS (Table 1) con-
 424 firmed that the two N treatments led to a very similar
 425 increase in the HATS capacity, with a more than 2-fold
 426 increase in V_{max} , whereas specific removal of NH_4^+ from
 427 the medium resulted in a significantly higher increase in
 428 HATS affinity than total N-starvation (decrease in K_m from
 429 86 to 55 μM in $-\text{NH}_4^+$ plants, as compared to 71 μM in
 430 N-starved plants).

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

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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-reg-
 ulated) 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 $^{15}\text{NH}_4^+$
 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 con-
 centration led after 24 h to a down-regulation of HATS
 activity, with a decrease in $^{15}\text{NH}_4^+$ 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 $^{15}\text{NH}_4^+$ 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

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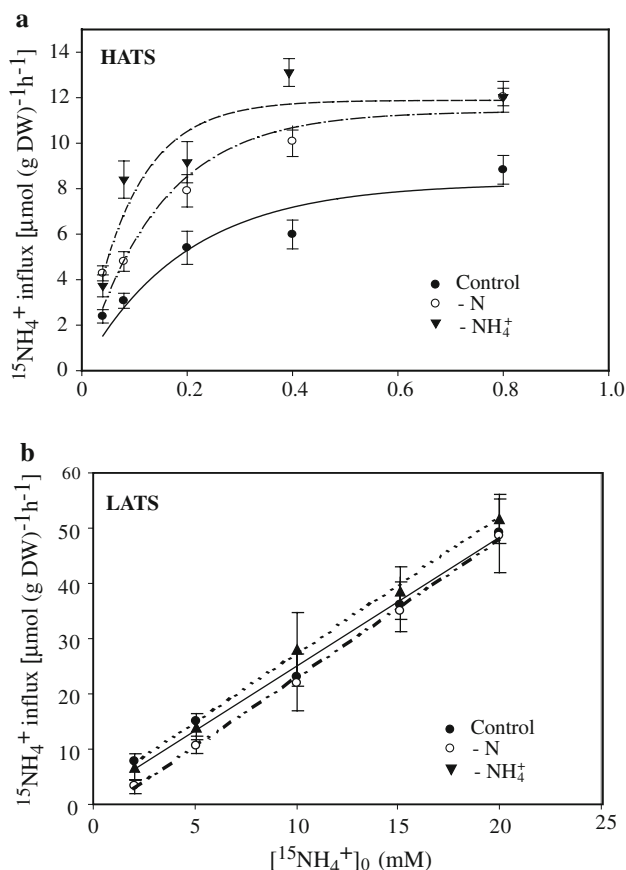


Fig. 3 Kinetics of the $^{15}\text{NH}_4^+$ influx in *Citrus* roots in the low **a** and high **b** $^{15}\text{NH}_4^+$ concentration range. Three-month-old *Citrus* plants were grown hydroponically on 1 mM NH_4NO_3 . Three days before the experiment, one group of plants was kept under the same conditions (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 ($-\text{NH}_4^+$). The $^{15}\text{NH}_4^+$ influx was measured on the third day at different concentrations of external $^{15}\text{NH}_4^+$. All the values are the means of 18 replicates \pm SE.

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

465 When assayed after 7 days of N starvation, HATS-mediated
 466 $^{15}\text{NH}_4^+$ influx was found unchanged as compared to
 467 control plants, but was slightly stimulated after 24 h of
 468 either NH_4^+ or NO_3^- re-supply, regardless of the external
 469 concentration of these ions (Fig. 5a). As expected from its
 470 consistent lack of response to the various changes in N

Table 1 Kinetic parameters for saturable and linear phases of the $^{15}\text{NH}_4^+$ influx of three-month-old citrange Troyer (*Citrus sinensis* L. Osbeck \times *Poncirus trifoliata* Blanco) roots, according to as a function of $[\text{NH}_4^+]_0$.

Parameters		Control	-N	$-\text{NH}_4^+$
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 the experiment, one group of plants was kept under the same conditions (control), another group was transferred to a nitrogen-free (-N), while a third group of plants was transferred to 1 mM KNO_3 ($-\text{NH}_4^+$). The $^{15}\text{NH}_4^+$ influx was measured on the third day at different concentrations of external $^{15}\text{NH}_4^+$. All the values are the means of 18 replicates \pm SE. Different letters indicate significant differences ($P < 0.05$)

nutrition investigated above, LATS-mediated $^{15}\text{NH}_4^+$ 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

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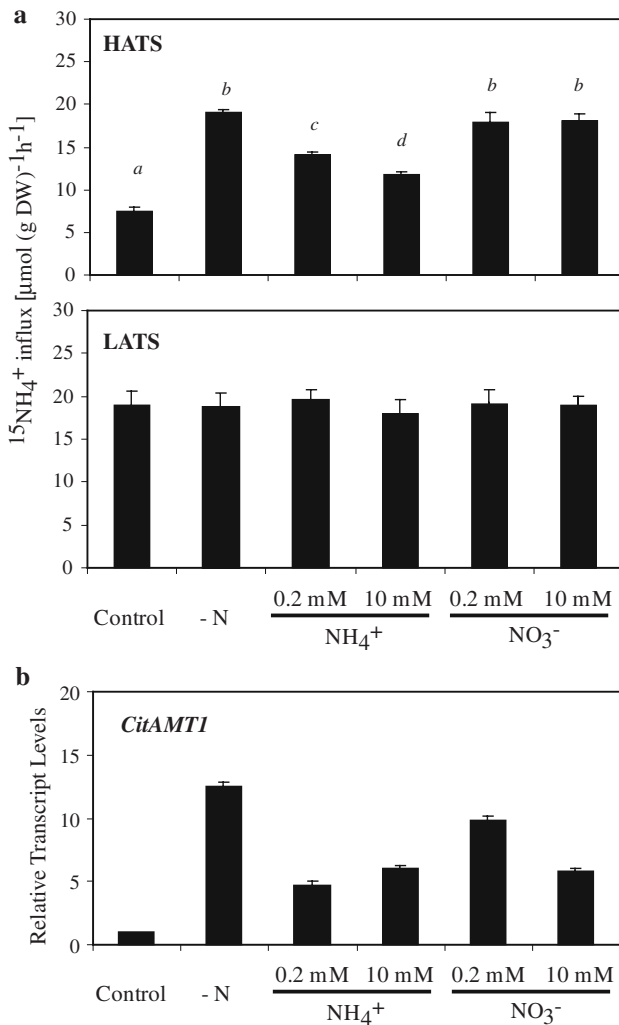


Fig. 4 Influence of a re-supply of different concentrations and sources of nitrogen on both the $^{15}\text{NH}_4^+$ influx and the *CitAMT1* gene expression under de-repression conditions. **a** Three-month-old *Citrus* plants were grown hydroponically on 1 mM NH_4NO_3 (control) and transferred for 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 $(\text{NH}_4)_2\text{SO}_4$; 0.2 mM KNO_3 ; 5 mM $(\text{NH}_4)_2\text{SO}_4$ or 10 mM KNO_3 , respectively. The HATS-mediated $^{15}\text{NH}_4^+$ influx was measured in roots at 0.2 mM. The LATS-mediated $^{15}\text{NH}_4^+$ influx was calculated by subtracting the influx measures at 0.2 mM $^{15}\text{NH}_4^+$ from that measured at 5 mM. The values shown are the means of 18 replicates \pm 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 ($n = 4$)

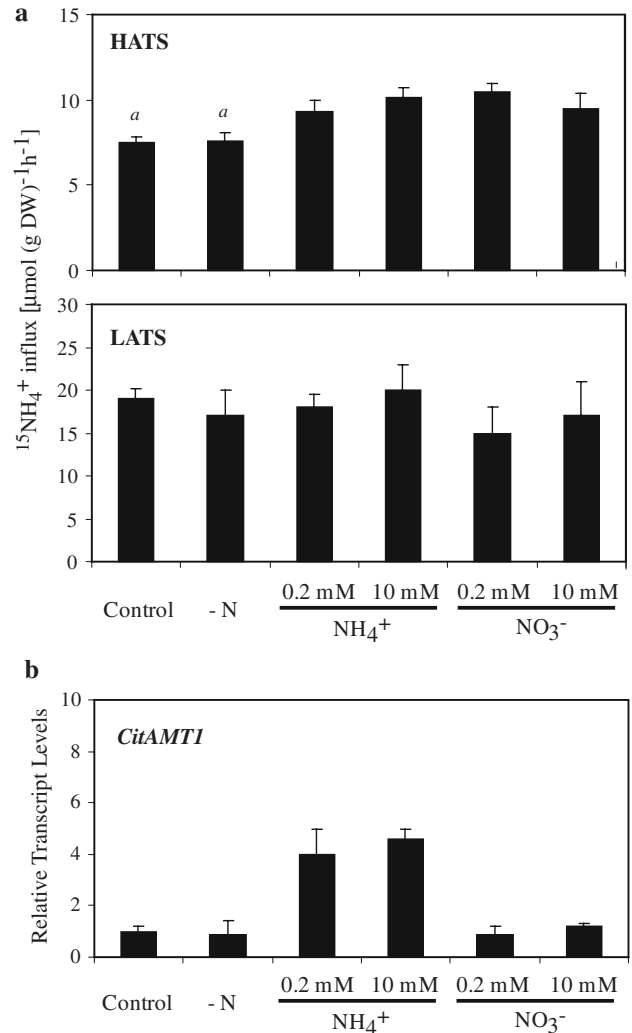


Fig. 5 Influence of a re-supply of different concentrations and sources of nitrogen on both the $^{15}\text{NH}_4^+$ influx and the *CitAMT1* gene expression under de-induction conditions. **a** Three-month-old *Citrus* plants were grown hydroponically on 1 mM NH_4NO_3 and transferred for 7 days from 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 $(\text{NH}_4)_2\text{SO}_4$; 0.2 mM KNO_3 ; 5 mM $(\text{NH}_4)_2\text{SO}_4$ or 10 mM KNO_3 , respectively. The HATS-mediated $^{15}\text{NH}_4^+$ influx was measured in roots at 0.2 mM. The LATS-mediated $^{15}\text{NH}_4^+$ influx was calculated by subtracting the influx measures at 0.2 mM $^{15}\text{NH}_4^+$ from that measured at 5 mM. The values shown are the means of 18 replicates \pm 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 ($n = 4$)

500 NH_4^+ , mediated by either transport system (HATS or
 501 LATS), is favoured compared to that of NO_3^- (Fig. 1).
 502 When both ions are at similar external concentration, a
 503 higher influx capacity for root NH_4^+ uptake systems than
 504 for root NO_3^- uptake systems is a common observation in
 505 many plant species, including *Citrus* (Serna et al. 1992;

Gessler et al. 1998; Gazzarrini et al. 1999; Min et al. 506
 2000). This does not always mean that NH_4^+ is the preferred 507
 N source under natural conditions since NH_4^+ 508
 availability in the soil solution is generally much lower 509
 than that of NO_3^- . However, our data provide evidence 510

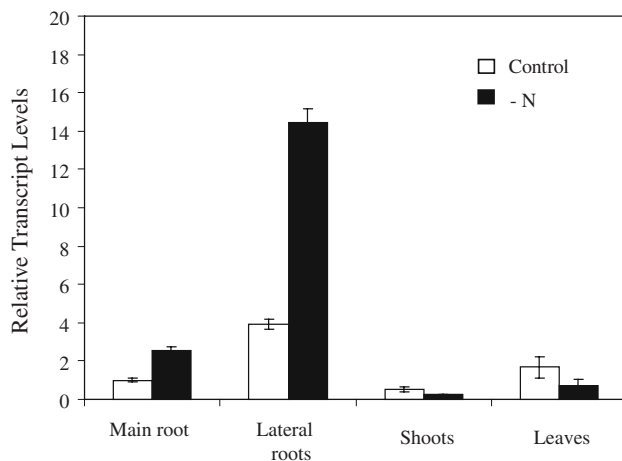


Fig. 6 Organ-dependent expression of *CitAMT1* in the main and lateral roots, stems and leaves of *Citrus* plants. Real-Time PCR analysis of the expression of *CitAMT1*. Three-month-old *Citrus* plants were grown hydroponically on 1 mM NH_4NO_3 (control) and transferred for 3 days from 1 mM NH_4NO_3 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 ($n = 4$)

511 that even if NO_3^- is the major N form supplied in the
 512 nutrient solution, *Citrus* plants still display a much higher
 513 capacity to take up NH_4^+ than NO_3^- (Fig. 1a). This is par-
 514 ticularly striking when considering the regulation of both
 515 NH_4^+ and NO_3^- HATS by the $\text{NH}_4^+/\text{NO}_3^-$ external bal-
 516 ance. Indeed, the decrease in $\text{NH}_4^+/\text{NO}_3^-$ ratio in the
 517 nutrient solution strongly amplified the difference
 518 between NH_4^+ and NO_3^- HATS capacities, suggesting
 519 that *Citrus* plants react to the predominance of NO_3^- as a
 520 N source by displaying an increased preference for NH_4^+
 521 as the N form taken up. Interestingly, this is not only due
 522 to increased NH_4^+ HATS activity but also to decreased
 523 NO_3^- HATS activity in response to increased NO_3^- pro-
 524 vision (Fig. 1a). This was unexpected for at least two rea-
 525 sons. First, this resulted in an extremely low NO_3^- HATS
 526 capacity (around $2 \mu\text{mol h}^{-1} \text{g}^{-1} \text{DW}$) in plants fed with
 527 1 mM NO_3^- as sole N source (see 100% NO_3^- in Fig. 1a),
 528 suggesting that the NO_3^- HATS is unable to sustain
 529 efficient NO_3^- acquisition in *Citrus* plants. Second, the
 530 observation that NO_3^- HATS activity was maximal in the
 531 absence of NO_3^- (Fig. 1a) does not fit with the occurrence
 532 of a NO_3^- -inducible HATS component (iHATS), which
 533 is generally observed in most plant species, including *Cit-*
 534 *rus* (Aslam et al. 1992; Kronzucker et al. 1995; Cerezo
 535 et al. 1997). The LATS-mediated influxes of NH_4^+ and
 536 NO_3^- are not altered when plants are exposed to different
 537 $\text{NH}_4^+/\text{NO}_3^-$ ratio (Fig. 1a). This could reveal a lack of
 538 control on these systems when the plant N status is ade-
 539 quate (Mäck and Tischner 1994; Wang et al. 1998).

NH_4^+ exerts a dual regulatory effect on both NH_4^+ HATS activity and *CitAMT1* expression 540 541

The data from the N deprivation and NH_4^+ re-supply experi- 542
 ments strongly suggest that both NH_4^+ HATS activity and 543
CitAMT1 expression in the roots are down-regulated by high 544
 N status of the plant, but specifically stimulated by NH_4^+ . 545
 Accordingly, provision of NH_4^+ in the nutrient solution has a 546
 dual effect, either repressive through its role as a nutrient pro- 547
 moting high N status (Figs. 2a–4), or stimulatory possibly 548
 through its role as an inducer (Figs. 2b and 5). Although 549
 counter-intuitive at first glance, this regulatory pattern is well 550
 known for many genes involved in NO_3^- transport or assimila- 551
 tion, which are both induced by NO_3^- itself, and repressed 552
 by downstream products of NO_3^- assimilation (Wang et al. 553
 2004). The balance between these two opposite effects 554
 depends on the prior nutrition regime of the plant. For 555
 instance, the temporal pattern of both NH_4^+ HATS and 556
CitAMT1 expression responses to N deprivation (transient 557
 up-regulation followed by down-regulation, Fig. 2a is illus- 558
 trative of this dual regulatory mechanism. These results coin- 559
 cide with those found by Couturier et al. (2007) for the genes 560
 of *PtrAMT1.1* and *PtrAMT1.2*. This pattern closely parallel 561
 that observed for NO_3^- HATS and *AtNRT2.1* expression in 562
Arabidopsis (Lejay et al. 1999), and is typically explained by 563
 the fact that N deprivation first alleviates repression by N sta- 564
 tus (hence resulting in up-regulation), and only subsequently 565
 suppresses induction exerted by the ion itself (thus leading to 566
 down-regulation). Accordingly, NH_4^+ supply at early stages 567
 of N deprivation (when only repression by N status is 568
 relieved) has an inhibitory effect (see Fig. 4), while NH_4^+ 569
 supply at later stages of N deprivation (when induction by 570
 NH_4^+ is suppressed) has a stimulatory effect (see Fig. 5). 571
 Interestingly, after 7 days of N deprivation, *CitAMT1* expres- 572
 sion is up-regulated by NH_4^+ but not by NO_3^- (Fig. 5b), sug- 573
 gesting a specific signalling role for NH_4^+ (or a product of its 574
 metabolism). Transcriptional repression of individual *AMT* 575
 genes by N status of the plant has been widely documented in 576
 many species (Gazzarrini et al. 1999; Rawat et al. 1999; von 577
 Wirén et al. 2000b; Gansel et al. 2001; Glass et al. 2002; 578
 Sonoda et al. 2003; Loqué and von Wirén 2004), but the 579
 occurrence of NH_4^+ -inducible NH_4^+ transporters is still a 580
 matter of debate (Loqué and von Wirén 2004). However, 581
 there are several examples of *AMT* genes that respond posi- 582
 tively to NH_4^+ supply, such as *LeAMT1.2* in tomato (von 583
 Wirén et al. 2000b), and *OsAMT1.1* and *1.2* in rice (Sonoda 584
 et al. 2003). The nature of the inducing signal is nevertheless 585
 unclear since stimulation of *OsAMT1.1* and *1.2* expression 586
 can be obtained with glutamine as well (Sonoda et al. 2003), 587
 while *LeAMT1.2* is also responsive to NO_3^- , which is not the 588
 case for *CitAMT1*. 589

As commonly observed in other species, the LATS-med- 590
 iated influx of NH_4^+ in *Citrus* plants does not respond to the 591

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

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

596 Although NH_4^+ HATS activity and *CitAMT1* expression in
597 the roots appear to be closely co-regulated in response to
598 changes of the plant N status or in response to NH_4^+ supply
599 (see above), this is clearly not the case in response to the
600 supply of NO_3^- as sole N source. Indeed, NO_3^- is unable to
601 repress the NH_4^+ HATS while it seems to be a potent inhib-
602 itor of *CitAMT1* expression in the roots. This is evidenced
603 firstly by the strong up-regulation of HATS-mediated NH_4^+
604 influx, but not of *CitAMT1* transcript accumulation, after
605 transfer of the plants to pure NO_3^- nutrition (Figs. 2b, d,
606 and 3), and secondly by the down-regulation of *CitAMT1*
607 expression, but not of NH_4^+ HATS activity, upon NO_3^- re-
608 supply following 3 days of N starvation (Fig. 4). The lack
609 of effect of NO_3^- on NH_4^+ HATS activity most likely
610 results from the fact that NO_3^- does not appear to be an
611 efficient N source for nutrition of the plant. Indeed, under
612 pure NO_3^- nutrition, both HATS and LATS for NO_3^-
613 exhibit a very low influx capacity (Fig. 1a), which is cer-
614 tainly not sufficient to sustain a high N status of the plant.
615 Thus, for *Citrus* seedlings, the supply of NO_3^- as sole N
616 source probably corresponds to an N limiting condition,
617 leading to the relief of the feedback repression exerted on
618 NH_4^+ HATS by the plant N status. Despite *CitAMT1* also
619 appears to be under the control of this feedback repression
620 (see above), its lack of up-regulation under pure NO_3^-
621 nutrition strongly suggests that NO_3^- per se is also a repres-
622 sor of its expression. This makes a strong parallel with the
623 *AtAMT1.1* gene of *Arabidopsis*, which has been shown to
624 be specifically repressed by NO_3^- (Wang et al. 2000;
625 Gansel et al. 2001), in addition of being down-regulated by
626 high N status of the plant (Gazzarrini et al. 1999; Rawat
627 et al. 1999).

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

630 The central role of AMT transporters in the high-affinity
631 root uptake of NH_4^+ has been recently firmly established in
632 *Arabidopsis*, where disruption of individual *AtAMT1* genes
633 (*AtAMT1.1*, *1.2*, *1.3*) resulted in a deficit of NH_4^+ HATS
634 activity, as compared to the wild-type (Loqué et al. 2006;
635 Yuan et al. 2007a). Furthermore, multiple *amt* mutants dis-
636 play stronger NH_4^+ uptake inhibition than simple mutants
637 (up to >90% in the quadruple *atamt1.1, atamt1.2, atamt1.3,*
638 *atamt2.1* mutant), indicating that most AMT transporters
639 expressed in the root actually contribute to part of the
640 whole NH_4^+ acquisition, and that their respective contribu-

tions are additive (Loqué et al. 2006; Yuan et al. 2007a). It
is also noteworthy that a correlation exists between the fac-
tors 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 sug-
gest 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 affect-
ing their nutritional status. Nonetheless, our data also indi-
cate 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-medi-
ated NH_4^+ uptake. Clearly, other factors have to be consid-
ered 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*. Alterna-
tively, there is now increasing evidence that *AMT* trans-
porters 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* activ-
ity at the protein level can be responsible for the up-regula-
tion 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 lim-
ited 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

692 NH_4^+ from cells during photorespiration (Mayer and Lude-
 693 wig 2006), a function that can also be fulfilled by *CitAMT1*
 694 in *Citrus*. Moreover, citrus plants are perennial plants, and
 695 they need to mobilize nitrogen from different organs, as
 696 demonstrated in *Populus trichocarpa* (Couturier et al.
 697 2007). However, differential regulation of the same gene
 698 between roots and shoot is generally not recorded, with the
 699 exception of *AtAMT1.1* (Engineer et al. 2007; Yuan et al.
 700 2007a). Hence, the regulation observed for *CitAMT1*
 701 expression bears strong similarity with that of *AtAMT1.1*,
 702 since both genes are expressed in both roots and shoots, and
 703 down-regulated by N status of the plant and by NO_3^-
 704 (Fig. 6). However, unlike *AtAMT1.1*, *CitAMT1* is in addition
 705 inducible by NH_4^+ (or by a product of its assimilation),
 706 and by sugars (Camañes et al. 2007), two features which
 707 are found for other *AMT* genes. The reason why *CitAMT1*
 708 exhibits such a complex pattern of regulation by various
 709 signals is unknown, but makes this transporter quite unique
 710 in possibly allowing *Citrus* plants to adapt their N acquisition
 711 to a wide range of environmental factors.

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 718 Jaume I where ^{15}N analysis was performed.

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