Ammonium transport and CitAMT1 expression are regulated by light and sucrose in Citrus plants

Gemma Caman˜es1, Miguel Cerezo 1,*, Eduardo Primo-Millo 2, Alain Gojon 3 and Pilar García-Agustı´n1,∗

1 Área de Fisiologı´a Vegetal, Departamento de Ciencias Agrarias y del Medio Natural, Escuela Superior de Tecnologı´a y Ciencias Experimentales, Universitat Jaume I, E-12071 Castellón de la Plana, Spain
2 Departamento de Citricultura, Instituto Valenciano de Investigaciones Agrarias, Apartado oficial, E46113 Moncada, Valencia, Spain
3 Biochimie et Physiologie Moléculaire des plantes, UMR 5004, Agro-M, CNRS, UM2, Place Viala, F34060 Montpellier, Cedex 1, France

Received 27 February 2007; Revised 23 May 2007; Accepted 23 May 2007

Abstract
Here the isolation and characterization of CitAMT1 cDNA from citrange Troyer (Citrus sinensis L. Osbeck×Poncirus trifoliata Blanco) is reported, suggesting that this belongs to the AMT gene family, which is involved in the high-affinity transport system (HATS). Results show that in Citrus plants, the HATS is much more dependent on the light conditions and C status of the roots than the low-affinity transport system. Most importantly, a strong correlation was found between the regulation of both HATS activity and CitAMT1 expression. CitAMT1 expression is sucrose-stimulated and may account for the regulation of NH4+ HATS. Furthermore, a similar link was also recorded with photosynthetic activity in the shoots, suggesting that the variations in production and transport of photosynthates to the roots are responsible for the diurnal changes of both CitAMT1 expression and NH4+ HATS activity. On the other hand, results indicate that the effect of stimulating light on CitAMT1 expression and NH4+ HATS activity is independent of the circadian rhythm. Finally, CitAMT1 expression seems to be specifically stimulated by sucrose, suggesting that sucrose is a pivotal signal governing both assimilate partitioning from source organs and assimilate utilization in sink organs.

Key words: AMT, Citrus, HATS, LATs, NH4+ uptake, sucrose.

Introduction
Plants can extract and use a wide range of inorganic and organic forms of nitrogen (N) from soils. However, in agricultural systems fertilized with urea, nitrate (NO3–) and ammonium (NH4+) are believed to provide the bulk of the N resource available to the plants. Most studies highlight the predominant role of NO3– but, for several reasons, the importance of NH4+ as a direct source of N for plant growth has probably been substantially underestimated. First, NH4+ availability in soils could be more constant in both time and space than that of NO3–, which can easily be leached following rainfall and is often undetectable in the soil solution (Glass and Siddqi, 1995; Kielland, 1994; Stark and Hart, 1997; Loqué and von Wirén, 2004), although NH4+ may be lost through nitrification (Lewis, 1986). Secondly, NH4+ is used efficiently by plants. It is generally taken up at higher rates than NO3– when both ions are present at similar external concentrations, and its assimilation requires little energy compared with that of NO3– (Sasakawa and Yamamoto, 1978; Passama et al., 1987; Marschner, 1995; Gazzarrini et al., 1999). Thirdly, NH4+ strongly inhibits NO3– uptake (Aslam et al., 1996; Lee and Drew, 1989). Thus, in agricultural soils where both NO3– and NH4+ are present, root NH4+ uptake may be favoured as a result of the specific down-regulation of NO3– uptake systems. Fourthly, combinations of NO3– and NH4+ usually result in greater vegetative growth than when either N form is supplied alone (Schrader et al., 1972; Gashaw and Mugwira, 1981; Edwards and Horton, 1982; Hartman et al., 1986), indicating that even when its contribution to the overall N uptake is not predominant,
NH$_4^+$ still has a significant positive effect on plant N nutrition. Finally, it is well documented that NH$_4^+$ constitutes the preferred N source for many plant species and, in particular, for most tree species. In Citrus plants for instance, when both forms of inorganic N are present, seedlings absorbed NH$_4^+$ at a higher rate than NO$_3^-$ (Serna et al., 1992). Hence, it is particularly relevant to investigate the mechanisms of root NH$_4^+$ uptake in woody species. An additional topic is to try to reduce fertilization because this has an environmental impact. Thus, the uptake of NH$_4^+$, and particularly its regulation at physiological and molecular levels, warrants much greater attention in Citrus trees because they are the main species in the Mediterranean area. A better understanding of plant response to NH$_4^+$ should allow a more rational use of nitrogen fertilizers.

The physiological characterization of the transport systems responsible for NH$_4^+$ uptake in plants relies on an extended series of short-term uptake studies in which roots were supplied with $^{15}$N and $^{15}$N-labelled NH$_4^+$ to assay the NH$_4^+$ influx component specifically. Regarding concentration-dependent uptake kinetics, biphasic patterns have been observed with at least two distinct components in NH$_4^+$ influx: a low-affinity non-saturable (LATS) and a high-affinity saturable (HATS) component (Ullrich et al., 1984; Wang et al., 1993b; Kronzucker et al., 1995; Ludewig et al., 2002). Similar results have been obtained in Citrus plants (Cerezo et al., 2001b). Concerning the regulation of root N uptake, there is general agreement on the hypothesis that two main mechanisms are involved in the control of NH$_4^+$ and NO$_3^-$ uptake systems. The first one is the feedback repression exerted by the nitrogen nutritional status of the plant (Ullrich et al., 1984; Morgan and Jackson, 1988; Clarkson and Lütgge, 1991; Lee et al., 1992; Wang et al., 1993a; Kronzucker et al., 1996; von Wirén et al., 1997, 2000a; Gazzarrini et al., 1999; Cerezo et al., 2001b; Pal’ove-Balog and Mistrík, 2002; Loqué and von Wirén, 2004). The second regulatory mechanism corresponds to the stimulation by photosynthesis, because both root NH$_4^+$ and NO$_3^-$ uptake are increased in response to illumination of the shoot, to an increase in CO$_2$ concentration in the atmosphere, and to the supply of sugars or carboxylic acids to the roots (Aslam et al., 1979; Hänisch Ten Cate and Breteler, 1981; Gastal and Saugier, 1989; Rulft et al., 1989; Touraine et al., 1992; Delhon et al., 1996; Müller et al., 2001; D’Apuzzo et al., 2004).

The molecular characterization of NH$_4^+$ transporters in plants (AMT) has largely confirmed the conclusions coming from the above physiological studies. Genes encoding NH$_4^+$ transporters were first identified in yeast (MEP genes; Marini et al., 1994) and in Arabidopsis (AMT genes; Ninnemann et al., 1994). So far, the various AMT transporters characterized in Arabidopsis (six AMT genes present in the genome), as well as in other species, behave as high-affinity transporters when expressed in yeast, suggesting that they may contribute to the HATS (Howitt and Udvardi, 2000; Loqué and von Wirén, 2004; von Wirén et al., 2000b). The investigation of the role of AMTs in NH$_4^+$ uptake in planta has been limited to AMT1.1 and AMT1.3 in Arabidopsis, showing that both transporters indeed participate in the activity of the NH$_4^+$ HATS (Kaiser et al., 2002; Loqué et al., 2006). Moreover, the occurrence of regulatory mechanisms for root NH$_4^+$ uptake, related to either N or C status of the plants, has also been documented at the molecular level (Loqué and von Wirén, 2004). For instance, mRNA accumulation of AMT1.1, AMT1.2, and AMT1.3 genes has been shown to be differentially regulated by either N or C provision to the roots, thus suggesting that expression of these transport genes may be a key level for the regulation of the NH$_4^+$ HATS in Arabidopsis (Gazzarrini et al., 1999; Lejay et al., 2003; Loqué et al., 2006).

Despite several studies indicating that both the structure and the regulation of root NH$_4^+$ uptake systems in woody species are similar to those in herbaceous plants (Min et al., 1999; Cerezo et al., 2001b), nothing is known at the molecular level concerning AMT transporters in trees. Therefore, the aim of the present work has been to identify AMT genes in Citrus plants, and to determine whether the control of their expression can also be an important step for the regulation of NH$_4^+$ uptake by the roots. The study concentrated on the effects of photosynthesis and sugar supply because there is already some indication that changes in the C status of the plant result in responses in gene expression in Citrus (Li et al., 2003). Furthermore, several studies suggest that the C status of the Citrus tree is a key determinant of fruit set, and in particular that sucrose supplementation produces various physiological effects in Citrus fruits (Abdin et al., 1998; Iglesias et al., 2001). Recently, Iglesias et al. (2003) have concluded that fruit set in Citrus is highly dependent on carbohydrate availability.

This present study reports on the isolation the CitAMT1 cDNA, and it is shown that its expression in the roots is regulated by light and sucrose, and that this regulation strongly parallels that of root NH$_4^+$ influx, photosynthesis rate, and root sugar content.

**Materials and methods**

**Plant material and growth conditions**

Seeds of citrange Troyer (Citrus sinensis L. Osbeck × Poncirus trifoliata Blanco) were allowed to germinate in vermiculite in a culture 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 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$_4$NO$_3$ and addition of 1.5 mM K$_2$SO$_4$ and 3 mM CaSO$_4$ to
compensate for the absence of 3 mM KNO₃ and 3 mM Ca(NO₃)₂ in the solution. The pH of the nutrient solution was adjusted to 6.0 with 1 M KOH.

Prior to the experiments, 3-month-old plants with a single shoot were selected for uniformity of size, and transferred for 7 d to aerated Hoagland solution on hydroponic culture devices. Nutrient solutions were renewed twice a week and on the day of the experiments.

Measurement of ¹⁵NH₄⁺ influx
³¹⁵NH₄⁺ influx by Citrus roots was measured at low (200 µM) and high (5 mM) external NH₄⁺ concentrations, representative of both high- and low-affinity NH₄⁺ transporter systems (HATS and LATS, respectively). ¹⁵NH₄⁺ influx in roots was determined on six plants after transfer to 0.1 mM CaSO₄ for 1 min, then to ¹⁵NH₄⁺ solution for 5 min, and finally to 0.1 mM CaSO₄ for 1 min (Gazzarrini et al., 1999). The ¹⁵NH₄⁺ solution was the N-free Hoagland nutrient solution, supplemented with 1 mM MES pH 6.0, and where N was supplied as ¹⁵N[(NH₄)₂SO₄ (98 atom% ¹⁵N in excess) at the indicated concentrations (0.2 mM 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 ¹⁵N analysis was performed using an integrated system for continuous flow isotope ratio mass spectrometry [Euro-EA elemental analyser (EuroVector S.P.A.) and Isoprime mass spectrometer (GV Instruments)]. The values of root ¹⁵NH₄⁺ influx are expressed in μmol ¹⁵NH₄⁺ (g root DW)⁻¹ h⁻¹. The experiments were repeated at least three times and the mean of the two most representative experiments ± SE are shown (n=12).

Study of the effect of the light/dark cycle and continuous light
Three-month-old Citrus plants were grown on a complete nutrient solution containing 1 mM NH₄NO₃ as an N source under a light/dark cycle of 16/8 h. ¹⁵NH₄⁺ influx for the HATS and the LATS was measured every 2 h for a whole day in a growth chamber under the conditions described with a light/dark cycle of 16/8 h at normal intensity (200 µmol m⁻² s⁻¹). Five days before the experiment one group of plants was illuminated at the normal light intensity, and two other groups were shaded to decrease the light intensity at the canopy height to 120 µmol m⁻² s⁻¹ and 10 µmol m⁻² s⁻¹, respectively. In these conditions, ¹⁵NH₄⁺ influx was measured and roots of Citrus plants were frozen for later studies of CitAMT1 gene expression.

Kinetics of ¹⁵NH₄⁺ influx
The kinetics of ¹⁵NH₄⁺ influx as a function of external NH₄⁺ concentration were measured in plants with ¹⁵NH₄⁺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 (Kₘ and Vₘₐₓ) were calculated from these regression equations in the concentration range of 20 µM to 1 mM (Segel, 1975). Above 1 mM ¹⁵NH₄⁺₀, measured ¹⁵NH₄⁺ 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 ¹⁵NH₄⁺₀ and the calculated Vₘₐₓ for the HATS were taken as estimates of the influx due only to the LATS.

Isolation and characterization of CitAMT1 cDNA
The 3' and 5' ends of CitAMT1 were isolated by rapid amplification of cDNA ends (RACE). The 3' and 5' ends were obtained by RACE by using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) and the CitAMT1 specific primers AMT-F (5'-GTCCGATTTGCACCTTGATGAC-3') and AMT-R (5'-CTTCAATCGTGCCACCTCAACATT-3') to amplify the 3' end and AMT-R (5'-CCTCAAGTTCCCTCACCTCAACATT-3') to amplify the 5' end, following the manufacturer’s instructions. RACE reactions were performed using 1 µg total RNA extracted from citrus roots. The PCR products obtained were sequenced by the Servicio de Secuenciación del Servicio Central de Soporte a la Investigación Experimental (SCSIE) at the University of Valencia (Valencia, Spain). Homology searching was performed using BLAST algorithm (Altschul et al., 1990) and FASTA algorithm (Pearson and Lipman, 1988) and computer translation by using the EMBOSS Transeq by the European Molecular Biology Laboratory. Comparison and alignment of nucleotide or amino acid sequences were conducted using CUSTAL W (Thompson et al., 1994).
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 a powder under liquid nitrogen for extraction of total RNA using the Total Quick RNA kit (TALEN, Italy) according to the manufacturer’s instructions. To avoid contaminating DNA, the samples were treated with DNase I. A total of 1 μg of total RNA was annealed to random hexamers and reverse transcribed using the Omniscript reverse transcription kit (QIAGEN) to obtain cDNA. The sequences of the gene-specific oligonucleotides designed and used for real-time PCR are as follows: AMT forward 5'-CCCACCTCCACACTT-CGACTA-3' and reverse 5'-CAGAACCATTGGGAGACGC-3'; 18S forward 5'-GAACAACTGCGAAAGCATTTGC-3' and reverse 5'-CTCTGGTAGTTCCCGTGTG-3'. Real-time PCR was conducted using the QuantiTect SYBR Green PCR Kit (QIAGEN) and the SmartCycler II instrument (Cepheid). Every reaction was set up with two replicates. The programme used was 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 was used to confirm specific gene product formation and did not represent primer dimer or non-specific products.

The accession number in the database GenBank of CitAMT1 is DQ887678.

Statistical analysis

Statistical analysis was carried out using the Statgraphics software support. Data are expressed as means and standard error. 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

Identification and isolation of CitAMT1 cDNA

The full-length CitAMT1 cDNA (GenBank accession number DQ887678) contains a 1762-nucleotide-long ORF with 72 nucleotides before the putative ATG start codon. The translation product of this ORF was 502 amino acids long, with a predicted molecular weight of 53.5 kDa. CitAMT1 cDNA shows high homology to the functionally characterized NH₄⁺ transporter AMT1 of Arabidopsis thaliana (80% identity), Lotus japonicus (85%), and Camellia sinensis (80.9%).

CitAMT1 expression in the roots is regulated by light and sucrose as is NH₄⁺ HATS activity

To study the regulation of root NH₄⁺ uptake by photosynthesis in Citrus plants, first the time-course of both HATS and LATS activity, and of CitAMT1 mRNA accumulation in the roots during a normal day/night cycle was investigated. Therefore, root ¹⁵NH₄⁺ influx was assayed at two different ¹⁵NH₄⁺ concentrations (0.2 mM and 5 mM), and at various intervals during the 16 h light and 8 h dark periods (Fig. 1A). As observed in many plant species, root ¹⁵NH₄⁺ influx mediated by the HATS (0.2 mM ¹⁵NH₄⁺) displayed pronounced diurnal changes, with a peak [around 20 μmol (g root DW)⁻¹ h⁻¹] in the middle of the light period, and a very low value [<5 μmol (g root DW)⁻¹ h⁻¹] at the end of the day and during the whole night period. By contrast, ¹⁵NH₄⁺ uptake that is attributed to the LATS (difference between root influxes at 5 mM and 0.2 mM) remained at quite a constant rate during the
light/dark cycle. During the same experiments, marked changes in CitAMT1 expression were recorded in the roots (Fig. 1B) that paralleled those of the HATS activity (Fig. 1A). As for root high-affinity $^{15}$NH$_4^+$ influx, CitAMT1 transcript accumulation was highest at midday, and decreased thereafter by 75% until the middle of the night.

The supply of 1% sucrose (w/v) in the nutrient solution at the beginning of the dark period resulted after 7 h in a 3-fold increase in root $^{15}$NH$_4^+$ influx mediated by the HATS, compared with values measured on control plants (Fig. 2A). A similar, although less pronounced, response was observed for CitAMT1 expression (Fig. 2B), but not for the LATS activity, which remained unaffected by the treatment (Fig. 2A).

These results show that, in Citrus plants, the HATS is much more dependent on the light conditions and C status of the roots than the LATS and, most importantly, that a strong correlation is found between regulation of both HATS activity and CitAMT1 expression. Furthermore, a similar correlation was also recorded with photosynthetic activity in the shoots (Fig. 3A), suggesting that the variations in production and transport of photosynthates down to the roots are responsible for the diurnal changes of both HATS activity and CitAMT1 expression. Accordingly, sucrose, glucose, and fructose concentrations in the roots followed a generally similar pattern as photosynthesis (Fig. 3). However, sucrose concentration undergoes a more pronounced decrease during the second half of the light period, when both HATS activity and CitAMT1 transcript accumulation began to be down-regulated.

To rule out the hypothesis that the diurnal changes observed in $^{15}$NH$_4^+$ HATS activity and CitAMT1 gene expression are due to a circadian rhythm, independent from the current photosynthesis of the plant, the effect of continuous light was investigated by keeping the lights on during the normal night. In response to this treatment, a gradual stimulation of the HATS activity occurred from the low level reached at the end of the 16 h previous light period (Fig. 4A). After 24 h of continuous light, $^{15}$NH$_4^+$ influx mediated by HATS at the end of the dark period was more than twice that recorded in plants subjected to the usual day/night cycle (compare Figs 4A and 1A). As expected from its unaffected activity after light to dark transition, the LATS was not modified by continuous light. Again, CitAMT1 expression showed a similar behaviour as the HATS activity, with an increase when light was kept on during the normal dark period (Fig. 4B). On the other hand, continuous light not only prevented the decrease in sucrose concentration in the roots, but even stimulated the accumulation of this compound after the 16 h normal photoperiod (Fig. 5). This was not observed for both glucose and fructose concentrations, which continued to decline during the first 4 h of the extended light period, and only increased thereafter (Fig. 5).
Together with the data obtained during a normal day/night cycle, these results indicate that the stimulating effects of light on NH$_4^+$ HATS activity and CitAMT1 expression are more correlated with the changes in the concentration of sucrose, than with those of glucose or fructose.

Moreover, when Citrus plants remained under continuous light for 3 d prior to the measurements, both diurnal changes of HATS-mediated $^{15}$NH$_4^+$ influx and of sucrose concentration in the roots were abolished (Fig. 6A, B). Interestingly, while the LATS activity remained almost unchanged as compared with control plants kept under a regular day/night cycle (compare Figs 6B and 1B), root $^{15}$NH$_4^+$ influx accounted by the HATS was maintained at a rather constant value [6–8 μmol (g root DW)$^{-1}$ h$^{-1}$] that is intermediate between the extremes recorded during the normal photoperiod (Fig. 1A). The same is true for the endogenous sucrose contents on the roots, which remained steady at 25–30 mg (g root DW)$^{-1}$ (Fig. 6B), i.e. at a value nearly twice that measured at night in control plants, but 30% lower than that measured in these plants at midday (Fig. 3B). This suggests that continuous light did not enhance the overall NH$_4^+$ acquisition by the roots, but simply set the NH$_4^+$ uptake rate at a constant value by preventing not only its decrease at night but also its increase during the day.

The relationship between NH$_4^+$ uptake in the roots and C status of the plant was documented further by the strong dependence of the root $^{15}$NH$_4^+$ influx and of the CitAMT1 transcript accumulation on the light intensity received by
the plant canopy (Fig. 7). When the light intensity was decreased from 200 \( \text{mol} \text{ m}^{-2} \text{s}^{-1} \) to 120 \( \text{mol} \text{ m}^{-2} \text{s}^{-1} \) or to 10 \( \text{mol} \text{ m}^{-2} \text{s}^{-1} \) (continuous light), \( ^{15}\text{NH}_4^+ \) influx by the HATS was reduced by 45\% and 72\%, respectively (Fig. 7A), while that by the LATS was not affected. The \( \text{CitAMT1} \) transcript level in the roots was reduced by the same treatments by 20\% and 80\%, respectively (Fig. 7B). Moreover, the amount of soluble sugars in the roots also diminished when the light intensity decreased to 120 \( \text{mol} \text{ m}^{-2} \text{s}^{-1} \) and 10 \( \text{mol} \text{ m}^{-2} \text{s}^{-1} \) (Fig. 7C).

The kinetics of \( ^{15}\text{NH}_4^+ \) influx of \( \text{Citrus} \) plants in different conditions of light intensity (200, 120, and 10 \( \text{mol} \text{ m}^{-2} \text{s}^{-1} \)) follows a classical biphasic pattern (Fig. 8A, B). In the low concentration range, \( ^{15}\text{NH}_4^+ \) influx showed Michaelis–Menten kinetics up to 0.6 mM \( [^{15}\text{NH}_4^+]_0 \) (Fig. 8A), typical of the activity of a saturable HATS. In the high concentration range (>1 mM), \( ^{15}\text{NH}_4^+ \) influx increased almost linearly with \( [^{15}\text{NH}_4^+]_0 \) (Fig. 8B), representative of the action of a non-saturable LATS.

The kinetic parameters \( V_{\text{max}} \) and \( K_m \) for the HATS were estimated using linear regression analysis (Table 1). The decrease in light intensity (200, 120, and 10 \( \text{mol} \text{ m}^{-2} \text{s}^{-1} \)) triggered a reduction of the \( V_{\text{max}} \) from 15.2 to 9.21 and 6.2 \( \text{mol} \text{ (g root DW)}^{-1} \text{h}^{-1} \), respectively, and a substantial increase in \( K_m \), from 80.5 to 94.9 and 112 \( \mu \text{M} \), indicating a decrease in both HATS activity and affinity at low light intensity.

In the high concentration range, the equations derived from the linear increasing \( ^{15}\text{NH}_4^+ \) influx yielded intercepts of -0.3905, 0.8234, and 1.7541 for (200, 120, and 10 \( \text{mol} \text{ m}^{-2} \text{s}^{-1} \)), respectively (Table 1). The measured fluxes above 1 mM \( [^{15}\text{NH}_4^+]_0 \) result from the combined activities of both HATS and LATS. To evaluate kinetics of the LATS only, the \( V_{\text{max}} \) values for HATS (Table 1) were subtracted from the measured influxes at elevated \( [^{15}\text{NH}_4^+]_0 \) showing the same effect of light intensity on the activity of the LATS (Fig. 8B).

To test the specificity of the action of photosynthates, the effect of an exogenous supply of various carbon metabolites such as sugars like sucrose, glucose, and fructose and...
carboxylic acids was investigated. When 1% (w/v) of fructose or glucose was added to the nutrient solution at the beginning of the dark period, there were no significant differences in the $^{15}$NH$_4^+$ influx (either HATS or LATS) with respect to the control. Nevertheless, when 1% of sucrose (w/v) was supplied, the activity of the HATS was increased 3-fold with respect to the control, while the LATS did not respond (Fig. 9A). The level of CitAMT1 transcript accumulation also increased 2.5-fold with sucrose as compared with the control, but was not modified by either glucose or fructose supply (Fig. 9B).

Fructose accumulated slightly in the roots in response to treatment with exogenous fructose, but not with exogenous glucose and sucrose (Fig. 9C). Glucose concentration increased with exogenous glucose and sucrose. However, sucrose concentration in the roots increased only when sucrose was supplied exogenously (Fig. 9C).

Neither 2-oxoglutarate nor malate was able to mimic the stimulating effect of sucrose on the HATS-mediated $^{15}$NH$_4^+$ influx, and on the expression of the CitAMT1 gene (Fig. 10A, B), despite the fact that the supply of these carboxylic acids triggered an increase in sucrose concentration in the roots (Fig. 10C).

Discussion

Identification and isolation of CitAMT1 cDNA

Here, for first time, a cDNA of the CitAMT1 gene has been isolated which could be involved in the HATS with ammonium and suggesting that these members of the AMT1 gene family may play a role in the influx of NH$_4^+$. An homologous gene has been isolated and characterized before in other plant species, such as Arabidopsis thaliana (AtAMT1;1, AtAMT1;2, AtAMT1;3; Gazzarrini et al., 1999), Lycopersicon esculentum (=Solanum lycopersicum) (LeAMT1;1, LeAMT1;2, LeAMT1;3; Lauter et al., 1996; von Wirén et al., 2000b), Oryza sativa (OsAMT1;1, OsAMT1;2, OsAMT1;3; von Wirén et al., 1997; Saiki et al., 2002; Kumar et al., 2003), Brassica napus (BnAMT1;2; Pearson et al., 2002), and Lotus japonicus

Fig. 7. Effect of light intensity on the correlation of $^{15}$NH$_4^+$ influx, CitAMT1 gene expression, and sugar accumulation in the roots of Citrus plants. (A) Three-month-old Citrus plants were grown hydroponically on 1 mM NH$_4$NO$_3$ under a light/dark cycle of 16/8 h at normal light intensity (200 $\mu$mol m$^{-2}$ s$^{-1}$). Five days before the experiment one group of plants was illuminated at the normal light intensity, and two other groups of plants were shaded to decrease the light intensity at the canopy height to 120 $\mu$mol m$^{-2}$ s$^{-1}$ and 10 $\mu$mol m$^{-2}$ s$^{-1}$, respectively. On the day of the experiment, root $^{15}$NH$_4^+$ influx was measured 8 h after the beginning of the light period. The HATS-mediated $^{15}$NH$_4^+$ influx was measured in the root at 200 $\mu$M. The LATS-mediated $^{15}$NH$_4^+$ influx was calculated by subtracting the influx measured at 0.2 mM $^{15}$NH$_4^+$ from that measured at 5 mM. The values of root $^{15}$NH$_4^+$ are the means of 12 replicates ±SE. Different letters mean statistically significant differences (LSD test; at $P < 0.05$). (B) Real-time PCR analysis of the expression of CitAMT1. The plants were 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 measured in the same samples. Each bar represents average data with standard error bars from two independent experiments ($n=4$). (C) Fructose, glucose, and sucrose concentration in the roots. The results shown are means of three replicates ±SE. Different letters mean statistically significant differences within groups (LSD test; at $P < 0.05$).
Ammonium transport and CitAMT1 expression are regulated

Changes with the highest rates in the middle of the photoperiod, and the lowest rates at night. These results agree with those obtained in other species, and showed that uptake rates of NH$_4^+$, as well as those of several other ions such as NO$_3^-$, SO$_4^{2-}$, or K$, are dependent on light conditions and fluctuate diurnally (Smith and Cheema, 1985; Hatch et al., 1986; Le Bot and Kirkby, 1992; Delhon et al., 1995; Peuke and Jeschke, 1998; Gazzarrini et al., 1999; Lejay et al., 1999; Kumar et al., 2003). Furthermore, as previously reported in herbaceous plants (Hänisch Ten Cate and Breiteler, 1981; Delhon et al., 1996; Lejay et al., 1999, 2003; Matt et al., 2001), several lines of evidence indicate that the diurnal changes in NH$_4^+$ uptake by Citrus plants are not due to light per se, but are illustrative of a regulation exerted by photosynthates on root transport systems. First, the activity of the NH$_4^+$-dependent to photosynthesis. Finally, the HATS activity was found to be markedly reduced by shading, showing that the effect of light is quantitative, as expected for a control by photosynthesis.

Taken together, the present results reveal that regulation of root NH$_4^+$ uptake by light in a woody species like Citrus bears strong similarity with what has been described for

Table 1. Kinetic parameters for saturable and linear phases of $^{15}$NH$_4^+$ influx of 3-month-old clementine (Citrus hystrix L. Osbeck×C. trifoliata Blanco) roots, as a function of $^{15}$NH$_4^+$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Light intensity (µmol m$^{-2}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200</td>
</tr>
<tr>
<td>HATS</td>
<td>V$_{max}$</td>
</tr>
<tr>
<td></td>
<td>K$_m$</td>
</tr>
<tr>
<td>HATS+LATS</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>r$^2$</td>
</tr>
<tr>
<td>LATS</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>r$^2$</td>
</tr>
</tbody>
</table>

Root NH$_4^+$ uptake is regulated by photosynthesis in Citrus plants

The present results demonstrate that root NH$_4^+$ uptake by the HATS in Citrus plants is strongly dependent on the illumination of the shoot, and thus displays marked diurnal

Changes with the highest rates in the middle of the photoperiod, and the lowest rates at night. These results agree with those obtained in other species, and showed that uptake rates of NH$_4^+$, as well as those of several other ions such as NO$_3^-$, SO$_4^{2-}$, or K$, are dependent on light conditions and fluctuate diurnally (Smith and Cheema, 1985; Hatch et al., 1986; Le Bot and Kirkby, 1992; Delhon et al., 1995; Peuke and Jeschke, 1998; Gazzarrini et al., 1999; Lejay et al., 1999; Kumar et al., 2003). Furthermore, as previously reported in herbaceous plants (Hänisch Ten Cate and Breiteler, 1981; Delhon et al., 1996; Lejay et al., 1999, 2003; Matt et al., 2001), several lines of evidence indicate that the diurnal changes in NH$_4^+$ uptake by Citrus plants are not due to light per se, but are illustrative of a regulation exerted by photosynthates on root transport systems. First, the activity of the NH$_4^+$-dependent to photosynthesis. Finally, the HATS activity was found to be markedly reduced by shading, showing that the effect of light is quantitative, as expected for a control by photosynthesis.

Taken together, the present results reveal that regulation of root NH$_4^+$ uptake by light in a woody species like Citrus bears strong similarity with what has been described for

Changes with the highest rates in the middle of the photoperiod, and the lowest rates at night. These results agree with those obtained in other species, and showed that uptake rates of NH$_4^+$, as well as those of several other ions such as NO$_3^-$, SO$_4^{2-}$, or K$, are dependent on light conditions and fluctuate diurnally (Smith and Cheema, 1985; Hatch et al., 1986; Le Bot and Kirkby, 1992; Delhon et al., 1995; Peuke and Jeschke, 1998; Gazzarrini et al., 1999; Lejay et al., 1999; Kumar et al., 2003). Furthermore, as previously reported in herbaceous plants (Hänisch Ten Cate and Breiteler, 1981; Delhon et al., 1996; Lejay et al., 1999, 2003; Matt et al., 2001), several lines of evidence indicate that the diurnal changes in NH$_4^+$ uptake by Citrus plants are not due to light per se, but are illustrative of a regulation exerted by photosynthates on root transport systems. First, the activity of the NH$_4^+$-dependent to photosynthesis. Finally, the HATS activity was found to be markedly reduced by shading, showing that the effect of light is quantitative, as expected for a control by photosynthesis.

Taken together, the present results reveal that regulation of root NH$_4^+$ uptake by light in a woody species like Citrus bears strong similarity with what has been described for

Changes with the highest rates in the middle of the photoperiod, and the lowest rates at night. These results agree with those obtained in other species, and showed that uptake rates of NH$_4^+$, as well as those of several other ions such as NO$_3^-$, SO$_4^{2-}$, or K$, are dependent on light conditions and fluctuate diurnally (Smith and Cheema, 1985; Hatch et al., 1986; Le Bot and Kirkby, 1992; Delhon et al., 1995; Peuke and Jeschke, 1998; Gazzarrini et al., 1999; Lejay et al., 1999; Kumar et al., 2003). Furthermore, as previously reported in herbaceous plants (Hänisch Ten Cate and Breiteler, 1981; Delhon et al., 1996; Lejay et al., 1999, 2003; Matt et al., 2001), several lines of evidence indicate that the diurnal changes in NH$_4^+$ uptake by Citrus plants are not due to light per se, but are illustrative of a regulation exerted by photosynthates on root transport systems. First, the activity of the NH$_4^+$-dependent to photosynthesis. Finally, the HATS activity was found to be markedly reduced by shading, showing that the effect of light is quantitative, as expected for a control by photosynthesis.

Taken together, the present results reveal that regulation of root NH$_4^+$ uptake by light in a woody species like Citrus bears strong similarity with what has been described for
root NH$_4^+$ and NO$_3^-$ uptake in herbaceous plants (Delhon et al., 1996; Gazzarrini et al., 1999; Lejay et al., 1999, 2003). However, an original observation made from the present experiments is the strong parallelism found between diurnal changes in NH$_4^+$ HATS activity, photosynthetic activity, and sugar accumulation in root tissues. For all these processes, a peak was noticed at the same time in the middle of the photoperiod, suggesting an almost immediate dependency of NH$_4^+$ uptake on downward transport of sucrose. Although both root NH$_4^+$ and NO$_3^-$ uptake rates are known to be affected within hours by a change in either light intensity or CO$_2$ concentration in the atmosphere (Gastal and Saugier, 1989; Delhon et al., 1996; Matt et al., 2001), the response is often more pronounced for NH$_4^+$ uptake (Matt et al., 2001), which also shows steeper diurnal variations than NO$_3^-$ uptake (Ourry et al., 1996; Gazzarrini et al., 1999; Lejay et al., 1999; Kumar et al., 2003). These observations highlight a particularly important role of photosynthesis in regulating root NH$_4^+$ acquisition, a conclusion that may be related...
to the fact that, contrarily to NO₃⁻, exogenous NH₄⁺ must be assimilated in the roots. Given the low levels of NH₄⁺ accumulated in tissues, this means that one carbon skeleton must be available in roots for incorporating each NH₄⁺ ion taken up by the transport systems. This strong requirement, which is not valid for any other mineral nutrient acquired by the roots, is a convincing reason for assuming a fast and marked response of root NH₄⁺ uptake to any environmental change affecting photosynthesis. The present data provide new insight on this point by showing that NH₄⁺ HATS also responds to the endogenous regulation of photosynthesis that occurs in the absence of environmental changes. Indeed, a decline in CO₂ fixation at the end of the day in plants under artificial light of constant intensity is often reported to be due to circadian rhythm or feedback inhibition of photosynthesis (Rolland et al., 2002). That NH₄⁺ HATS activity follows photosynthetic activity under these circumstances confirms that the diurnal changes in root NH₄⁺ uptake are ultimately determined by the variations of carbohydrate synthesis and export to the roots (Riens et al., 1994; Delhon et al., 1996).

Another interesting observation was that continuous light suppressed the diurnal changes in NH₄⁺ HATS activity, but did not seem to result in an increased NH₄⁺ uptake by the plant over a 24 h period (compare Figs 1 and 6). Indeed, the stimulation of NH₄⁺ HATS activity during what was the usual night period was compensated for by a reduction in NH₄⁺ uptake rate during the rest of the day. This indicates that, despite its stringent control on root NH₄⁺ uptake systems, photosynthesis during the normal day/night cycle was not limiting the overall NH₄⁺ acquisition by the plant. This may suggest that extended duration of photosynthesis was also compensated for by a lower photosynthetic activity, to yield the same daily amount of C fixed by the shoot. Alternatively, other regulatory mechanisms such as feedback repression by N metabolites (Rawat et al., 1999; von Wiren et al., 2000a; Cerezo et al., 2001b; Loqué and von Wirén, 2004) may have also prevented any increase in total NH₄⁺ uptake by enhanced C acquisition in response to continuous light. Regardless of the mechanism involved, the present data confirm that modifying diurnal regulation of uptake

---

**Fig. 10.** Correlation between ¹⁵NH₄⁺ influx, *CitAMT1* gene expression, and accumulation of sucrose in the roots when exogenous organic acids were supplied in the dark period. (A) Time-course of ¹⁵NH₄⁺ influx during the dark period of the light/dark cycle in plants supplied with a complete nutrient solution containing 1 mM NH₄NO₃ with or without carboxylic acids. 2-Oxoglutarate and malate were supplied at a concentration of 10 mM at the beginning of the dark period and the plants were harvested 5 h later. The HATS-mediated ¹⁵NH₄⁺ influx was assayed at 200 μM external ¹⁵NH₄⁺ concentration. The LATS-mediated ¹⁵NH₄⁺ influx was calculated by subtracting the influx measured at 0.2 mM ¹⁵NH₄⁺ from that measured at 5 mM, and the values shown are means of 12 replicates ±SE. (B) Real-time PCR analysis of the expression of *CitAMT1*. The plants were 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 measured in the same samples. Each bar represents average data with standard error bars from two independent experiments (n=4). (C) Sucrose concentrations in the roots after 5 h of exogenous supply of 2-oxoglutarate and malate at the beginning of the dark period. The results shown are means of three replicates ±SE.
CitAMT1 expression is sucrose-stimulated and may account for the regulation of NH$_4^+$ HATS by photosynthesis

Interestingly, it was found that light/dark conditions or sucrose supply affected the activity of the HATS, but not that of the LATS (Figs 1, 2, 4, 7, 8). Thus, absence of light or low C status of the roots does not lead to a general decline in root NH$_4^+$ acquisition that may be due to energy limitation or shortage of transpiration, but results in a down-regulation of specific uptake systems. Furthermore, shading of the plants modified the kinetic properties of the HATS (both $K_m$ and $V_{max}$; Table 1), suggesting that even within this transport system, specific transporters may be more particularly regulated by photosynthesis than others.

Although other putative members of the CitAMT gene family have not been investigated in this study, the present data show that CitAMT1 could be one of the transporters responsible for the regulation of NH$_4^+$ HATS activity by photosynthesis. Indeed, CitAMT1 mRNA accumulation in the roots is markedly stimulated by both light and sucrose (Figs 1, 2, 4, 7, 8). This finding suggests that the control exerted by light and sugars acts at the molecular level, and is a key regulatory process for expression of NH$_4^+$ transporter genes in the roots. Moreover, a strong correlation was found between NH$_4^+$ HATS activity and CitAMT1 transcript level in response to both light treatments and sugar supply to the roots. Therefore, we hypothesize that regulation of root NH$_4^+$ influx by photosynthesis is achieved, at least partly, by changes in CitAMT1 expression. Since a very similar pattern of contents in endogenous sugars in the root and photosynthesis rate is also observed, this further suggests that sugars are the signal molecules modulating CitAMT1 expression as a function of shoot photosynthesis, thereby providing a mechanistic basis for the physiological link between nitrogen and carbon metabolism in plants. Similar results were reported in Arabidopsis (Gazzarini et al., 1999; Lejay et al., 2003), tomato (von Wirén et al., 2000b), and rice (Kumar et al., 2003), where AMT1 genes were also shown to be induced by light and/or sugar, and to display diurnal changes in expression which parallel those of root NH$_4^+$ uptake. Although transcriptional regulation of AMT transporters seems to be a general feature (Loqué and von Wirén, 2004), induction by light and sugars occurs for only one or two specific members within each AMT multigene family in all species investigated to date. In most cases, the other AMT genes respond to other stimuli. In A. thaliana for instance, light and sugars stimulate root expression of AtAMT1.2 and AtAMT1.3, but not that of AtAMT1.1, which is markedly induced by N-starvation (Gazzarini et al., 1999; Rawat et al., 1999). In rice, only OsAMT1.3 was shown to display marked diurnal changes in root expression, while OsAMT1.1 and OsAMT1.2 (but not OsAMT1.3) were up-regulated by N limitation (Kumar et al., 2003). Differential regulation of the various AMTs was also found in other species such as tomato (von Wirén et al., 2000b) and Lotus japonicus (D’Apuzzo et al., 2004), suggesting that each AMT gene may be responsible for modulating root NH$_4^+$ uptake in response to one specific endogenous or exogenous signal (light, C status of the plant, NH$_4^+$ availability, N status of the plant). Furthermore, data available in A. thaliana confirm that the root NH$_4^+$ or NO$_3^-$ transporters regulated at the mRNA level by a given stimulus are those responsible for the response of NH$_4^+$ or NO$_3^-$ HATS to this stimulus. For instance, knock-out mutants deficient for the N starvation-induced AtAMT1.1 gene have lost part of the up-regulation of the NH$_4^+$ HATS by N starvation (Kaiser et al., 2002; Loqué et al., 2006). The same holds true for a mutant lacking the sugar-induced AtNRT2.1 NO$_3^-$ transporter gene, where the response of the NO$_3^-$ HATS to light treatments and sucrose supply are markedly attenuated as compared with the wild type (Lejay et al., 2003). Although the above considerations highlight the transcriptional control of CitAMT1 expression as an important regulatory level for root NH$_4^+$ uptake in Citrus plants, the present results do not rule out that post-transcriptional mechanisms may also be involved (Rawat et al., 1999; Loqué and von Wirén, 2004).

Sucrose-specific regulation of CitAMT1 expression

Altogether, the present data indicate that the regulation of root NH$_4^+$ uptake is strongly similar between herbaceous plants and a woody species like Citrus, at both physiological and molecular levels. CitAMT1 is thus expected to play the same role as AtAMT1.2 and AtAMT1.3 in A. thaliana, or as OsAMT1.3 in rice, in ensuring the control of the NH$_4^+$ HATS activity by photosynthesis. However, an original observation arising from the present work is that CitAMT1 expression and NH$_4^+$ HATS activity seem to be specifically stimulated by sucrose, and neither by other soluble sugars like glucose and fructose, nor by carboxylic acids like 2-oxoglutarate and malate (Figs 8, 9). Accordingly, the best correlation between CitAMT1 expression and root concentrations of sugars was found with sucrose, and not with glucose or fructose (Figs 5, 8).

The lack of effect of 2-oxoglutarate and malate on CitAMT1 expression may be surprising, because carboxylic acids are the carbon skeletons used for amino acid synthesis. However, the present data agree with those previously reported in A. thaliana by Lejay et al. (2003), which showed that expression of both sucrose-inducible AtAMT1.2 and AtAMT1.3 genes is not increased in response to an exogenous supply of 2-oxoglutarate or malate.
The effect of sugars other than sucrose on AtAMT1.2 and AtAMT1.3 expression was not investigated by these authors, and remains unknown. Nevertheless, the present finding of a specific regulation of the NH$_4^+$ HATS by sucrose in Citrus markedly differentiates this transport system from the NO$_3^-$ HATS, which is stimulated indifferently by sucrose, glucose, or fructose in A. thaliana (Lejay et al., 2003). Glucose stimulation of root NO$_3^-$ uptake was also reported in other species (Hänisch Ten Cate and Breteler, 1981; Delhon et al., 1996). Furthermore, sugar induction of Arabidopsis, encoding a major component of the NO$_3^-$ HATS (Cerezo et al., 2001a; Filleur et al., 2001), is not specific to sucrose and has been related to C metabolism downstream of the hexokinase step in glycolysis (Lejay et al., 2003).

Why should NH$_4^+$ uptake be specifically regulated by sucrose, while root NO$_3^-$ uptake is controlled by downstream compounds of sucrose metabolism is unknown. However, sucrose is by far the main form of C transported in the phloem. It thus appears to be a very good candidate as a signalling molecule for co-ordinating the activity of N uptake systems with the C feeding of the roots. Furthermore, several signalling pathways have been identified that are responsible for the sugar regulation of gene expression in plants (Rolland et al., 2002). Among these pathways, sucrose-specific signalling is well documented for the control of various physiological processes such as starch synthesis and degradation (Loreti et al., 2000; Fernie et al., 2001), fructan synthesis (Müller et al., 2000), and anthocyanin synthesis (Solfanelli et al., 2006). Sucrose-specific sensing acts at the molecular level and modulates the expression of several genes of C metabolism, including those encoding α-amylase (Loreti et al., 2000), sucrose:fructan-6-fructosyltransferase (Müller et al., 2000), enzymes of the anthocyanin biosynthetic pathway (Solfanelli et al., 2006), or the light-regulated ATB2 transcription factor (Rook et al., 1998). Most interestingly, sucrose acts as a signal molecule regulating the expression of one of its own transport system, the phloem proton-sucrose symporter that plays a major role in sucrose transport from source to sink organs (Chiou and Bush, 1998). Thus, the present finding that sucrose may also specifically regulate NH$_4^+$ transporter genes in the roots suggests that it is a pivotal signal governing both assimilate partitioning from source organs, and assimilate utilization in sink organs.

Acknowledgements

This work was supported by the Ministerio de Ciencia y Tecnología (BF12003–06948) and the Ministerio de Educación, Cultura y Deporte (Grant AP2002–3620). This work was supported in part by the Pla de Promoció de la Investigació de la Universitat Jaume I 2004 (P1 1A2004–22). We thank the Servicio Central de Instrumentación Científica (SCIC) of the Universitat Jaume I where $^{15}$N analysis was performed.

References


Downloaded from https://academic.oup.com/jxb/article/58/11/2811/609867 by guest on 06 March 2022


