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Research Paper

Regulation of Nitrate Transport in Citrus Rootstocks Depending on Nitrogen Availability

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KEY WORDS

Citrus, inducible high affinity transport system (iHATS), constitutive high affinity transport system (cHATS), nitrate uptake, regulation

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ABSTRACT

Previously, we reported that in Citrus plants, nitrate influx through the plasmalemma of roots cells follows a biphasic pattern, suggesting the existence of at least two different uptake systems, a high and low affinity transport system (HATS and LATS, respectively). Here, we describe a novel inducible high affinity transport system (iHATS). This new nitrate transport system has a high capacity to uptake nitrate in two different *Citrus* rootstocks (Cleopatra mandarin and Troyer citrange). The iHATS was saturable, showing higher affinity than constitutive high affinity transport system (cHATS) to the substrate NO_3^-. The V_{max} for this saturable component iHATS was higher than cHATS, reaching similar values in both rootstocks.

Additionally, we studied the regulation of root NO_3^- uptake mediated by both HATS (iHATS and cHATS) and LATS. In both rootstocks, cHATS is constitutive and independent of N-status. Concerning the regulation of iHATS, this system is upregulated by NO_3^- and down-regulated by the N status and by NO_3^- itself when plants are exposed to it for a longer period of time. LATS in Cleopatra mandarin and Troyer citrange rootstocks is repressed by the N-status.

The use of various metabolic uncouplers or inhibitors indicated that NO_3^- net uptake mediated by iHATS and LATS was an active transport system in both rootstocks.

ABBREVIATIONS

DCCD, (N-N'-Dicyclohexyl-carbodiimide); DES, (Diethylstilbestrol); 2,4-DNP, (2,4-dinitrophenol); K_m, the external ion concentration giving half of the maximum rate (μM); MES, (2-[N-Morpholino]ethane-sulfonic acid); $[NO_3^-]_0$, external nitrate concentration; TRIS (Tris(hydroxymethyl)-aminomethane); V_{max}, the calculated maximum rate of ion influx (μmol NO_3^- g^{-1} root fresh weight h^{-1}).

INTRODUCTION

Plants can extract and use a wide range of inorganic and organic forms of nitrogen (N) from soils. However, except in agricultural systems fertilized with urea, nitrate (NO_3^-) and ammonium (NH_4^+) are believed to provide the bulk of the N resource available to the plants. Productivity in agricultural systems is highly dependent on the availability of N for uptake by roots. Roots of higher plants can absorb organic sources of nitrogen like amino acids and low molecular weight compounds,[1-2] however mineral N is mainly acquired from the soil like ammonium and nitrate salts.

Physiological studies have demonstrated that powerful regulatory mechanism operate at the whole plant level, so that in the long term, nitrate uptake depends on internal factors related to N demand of the plant, rather than on nitrate availability in the soil volume. It has been shown for a number of plant species that influx of NO_3^- involves al least three different transport systems.[3-4] When NO_3^- is available at low concentrations (<1 mM) two different system transports are operating, one is constitutive (cHATS) and the other is inducible (iHATS) and operates only after prior exposure to nitrate. Both systems follow saturable kinetic patterns and display low K_m values. cHATS has a higher affinity for NO_3^-, but iHATS as an enhanced uptake capacity.[5-7] An additional transporter, a LATS, exhibits linear non-saturable kinetics depending on increasing external nitrate concentration.[5] In *Arabidopsis* and *Brassica napus* an inducible component for a nitrate LATS has been reported.[8-9] The LATS for nitrate has been traditionally considered constitutive, this hypothesis is supported by membrane depolarization studies.[10]

Table 1 **Effect of inhibitors on NO_3^- net uptake into Cleopatre mandarine roots**

	cHATS		iHATS		LATS	
Treatment	**NO_3^- net uptake**	**% inhibition**	**NO_3^- net uptake**	**% inhibition**	**NO_3^- net uptake**	**% inhibition**
Control	0.103±0.010		0.197±0.012		0.417±0.030	
2,4-DNP	0.051±0.001*	50	0.003±0.001*	98	0.109±0.011*	74
DCCD	0.036±0.002*	65	0.056±0.003*	72	0.148±0.012*	64
DES	0.031±0.012*	70	0.043±0.002*	78	0.217±0.020*	48

The cHATS, iHATS and cHATS+LATS activities were measured in 0.2 mM NO_3^-, uninduced seedlings, 0.2 mM NO_3^-, induced seedlings by 3 d, and 3 mM NO_3^-, uninduced seedlings, respectively. iHATS and LATS were calculated by subtracting the NO_3^- net uptake measured at 0.2 mM NO_3^- in uninduced seedlings from these measured at 0.2 mM NO_3^- in induced seedlings and, 3 mM NO_3^- in uninduced seedlings, respectively. Each data point is the average of 24 replicates with ± SE. *Significant at 5% level respect to the control. NS not significant. NO_3^- net uptake is expressed as μmol g^{-1} root FW h^{-1}.

Table 2 **Effect of inhibitors on NO_3^- net uptake into Troyer citrange roots**

	cHATS		iHATS		LATS	
Treatment	**NO_3^- net uptake**	**%inhibition**	**NO_3^- net uptake**	**% inhibition**	**NO_3^- net uptake**	**% inhibition**
Control	0.098 ± 0.010		0.127 ± 0.012		0.386 ± 0.030	
2,4-DNP	0.046 ± 0.001*	53	0.002 ± 0.001*	98	0.116 ± 0.011*	70
DCCD	0.031 ± 0.002*	68	0.038 ± 0.003*	70	0.154 ± 0.012*	60
DES	0.027 ± 0.012*	72	0.038 ± 0.002*	70	0.193 ± 0.020*	50

The cHATS, iHATS and cHATS+LATS activities were measured in 0.2 mM NO_3^-, uninduced seedlings, 0.2 mM NO_3^-, induced seedlings by 3 d, and 3 mM NO_3^-, uninduced seedlings, respectively. iHATS and LATS were calculated by subtracting the NO_3^- net uptake measured at 0.2 mM NO_3^- in uninduced seedlings from these measured at 0.2 mM NO_3^- in induced seedlings and, 3 mM NO_3^- in uninduced seedlings, respectively. Each data point is the average of 24 replicates with ± SE. *Significant at 5% level respect to the control. NS not significant. NO_3^- net uptake is expressed as μmol g^{-1} root FW h^{-1}.

Concerning to the regulation of root N uptake, there is a general agreement on the hypothesis that feedback repression exerted by the nitrogen nutritional status of the plant[11-24] is involved in the control of NH_4^+ and NO_3^- uptake systems.

In contrast to the large number of communications on N uptake kinetics and its regulation in herbaceous species,[4,25-34] it only has been reported in spruce,[7] *Quercus suber*[35] and Citrus concerning woody species.[23,36-39] Complex interactions govern nitrate availability and N demand during root development and concerning root architecture. The discrepancy between field observations and conclusions drawn from physiological approaches probably results from our lack of knowledge on the processes involved and in the regulation of nitrate uptake.

The HATS for NO_3^- uptake is sensitive to metabolic inhibitors and appears to be an active transport system.[5,40-42] The mechanism of energy coupling for active NO_3^- transport by HATS has been investigated in a limited number of species by means of electrophysiological studies.[10,43-44] Nitrate absorption was associated with depolarization of cell membrane electrical potential difference ($\Delta\Psi$), which was inducible by NO_3^- and saturable with respect to exogenous NO_3^- concentration.[10] These observations are consistent with a mechanism for NO_3^- uptake by the HATS involving a $2H^+$:$1NO_3^-$ symport moved by the energy derived from the proton gradient generated by the plasma membrane H^+-ATPase.[43-45] However, very little information has been reported about the energy dependence for the NO_3^- transport, especially in fruit trees in which NO_3^- uptake plays a preponderant role after mineral fertilization, when NO_3^- levels in soil are raised during a limited period of time.[36]

The aim of the present report was to improve the general knowledge about NO_3^- uptake and their regulation in two different Citrus rootstocks widely used in agriculture, We have characterized the nitrate transport system and its regulation in induced and uninduced Citrus rootstocks, namely Troyer citrange (salt sensitive) and Cleopatra mandarin (salt tolerant).[46]

MATERIAL AND METHODS

Plant material. Cleopatra mandarin (*Citrus reshni*) and Troyer citrange (*Citrus sinensis* L. Osbeck x *Poncirus trifoliata* Blanco) seeds were germinated under greenhouse conditions in 0.5 L pots filled with fine sand, and irrigated twice a week with distilled water. One-month-old seedlings were watered with N-free Hoagland's solution[47] supplemented with 1 mM NH_4NO_3. Temperatures ranged between 16–18°C (night) and 25–27°C (day). Relative humidity was maintained at approximately 80%. Prior to the experiments, three-month-old Cleopatra mandarin or Troyer citrange seedlings with a single shoot were selected for uniformity of size, de-potted, and transferred for seven days to aerated Hoagland solution 1 mM NH_4NO_3 on hydroponic culture to level up their nutritional status. To study the NO_3^- uptake mediated by HATS, seedlings were separated into two different groups, one group was grown in nitrogen-free solution for seven days to make sure only NO_3^- constitutively expressed transport systems continued working (uninduced seedlings), and the other group was grown in nitrogen-free solution for seven days and induced with 0.2 mM KNO_3 for three days (induced seedlings) (based on the results obtained in the regulation section). To study the NO_3^- mediated by LATS, seedlings were separated into two different groups, one group was grown in nitrogen-free solution for seven days (uninduced seedlings) and the other group was grown in a complete Hoagland's solution for seven days and pre-treated with 3 mM KNO_3 for one day (pre-treated seedlings). To maintain similar K^+ and Ca^{2+} concentration in the nutrient solutions, K_2SO_4 and $CaSO_4$ were added to compensate the absence of 1.5 mM KNO_3 and 3 mM $Ca(NO_3)_2$ of Hoagland solution. The pH of the nutrient solutions was adjusted to 6.0 with 1 M KOH.

Measurement of NO_3^- uptake. Nitrate uptake was determined by placing six seedlings in a pyrex 200 mL glass containing uptake solution (Hoagland solution N-free added with K_2SO_4 and $CaSO_4$, 1.0 mM MES (pH 6.0) and NaNO3 at concentrations ranging

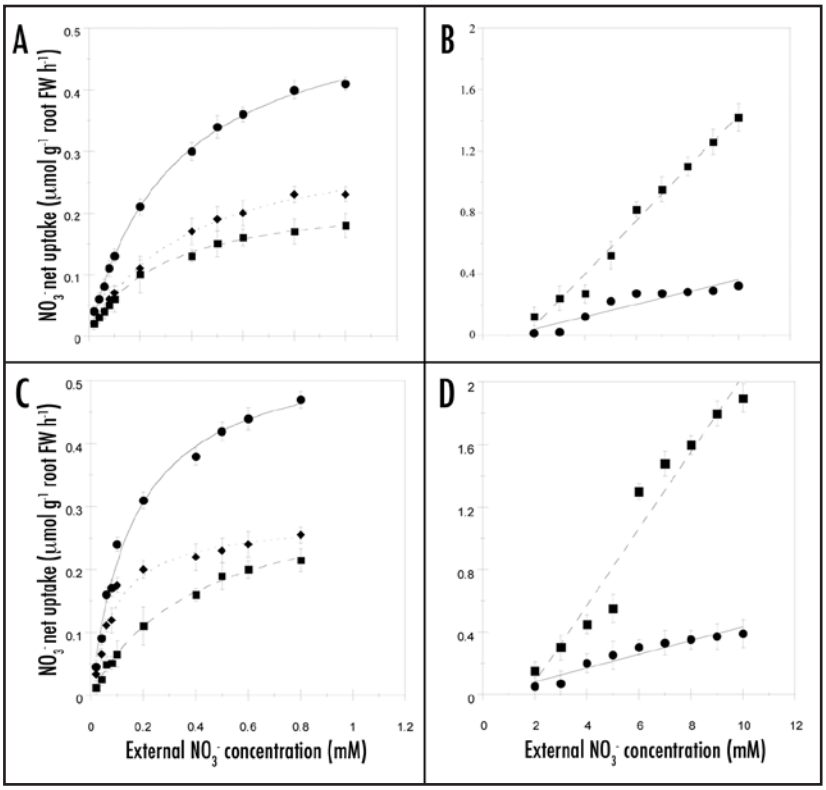
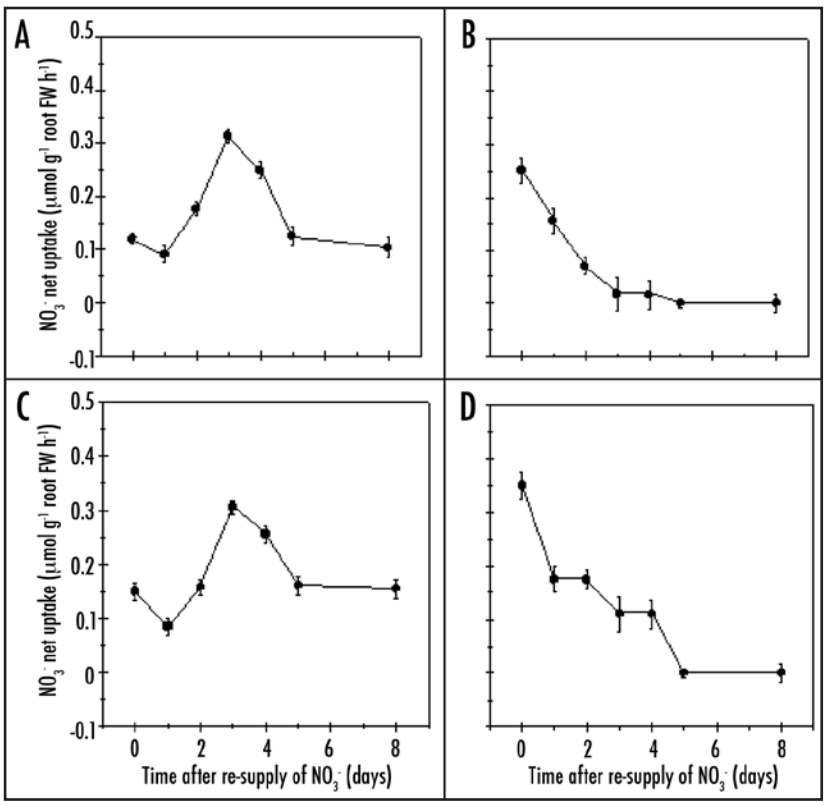


Figure 1. Kinetics of NO_3^- net uptake into Cleopatra mandarine (A and B) and Troyer citrange (C and D) roots in the low [(A–C) • cHATS+iHATS; + iHATS; ◇ cHATS] and high NO_3^- concentration range (B–D) • LATS of pre-treated seedlings; ◇ LATS of uninduced seedlings). iHATS is estimated by subtracting cHATS from cHATS+iHATS. NO_3^- net uptake measured above 1 mM external NO_3^- concentration is considered to be the combined contributions of HATS+LATS. LATS is estimated by subtracting cHATS V_{max} from HATS+LATS. Each data point is the mean of 24 replicates with SE values shown as vertical bars.



between 0.01 and 10 mM). Solutions were aerated vigorously. Water losses by transpiration and evaporation were compensated by frequent additions of water, maintaining the solution volume approximately constant. Aliquots (0.25–1 mL) were taken at 30 min intervals up to 3–6 h depending on the external NO_3^- concentration. Uptake rates were determined by measuring the disappearance of NO_3^- from the uptake solutions as a function of time, calculated by linear regression. Nitrate was determined spectrophotometrically by measuring absorbance at 212 nm.[48] The nitrate uptake rate was expressed as µmol NO_3^- g^{-1} root fresh weight h^{-1}. Based on our previous results[36] NO_3^- uptake above 1 mM $[NO_3^-]_0$, measured NO_3^- uptake appeared to result from the participation of two transportsystems (HATS + LATS). Thus, The NO_3^- mediated by LATS was calculated by subtracting the measured NO_3^- uptake at concentrations >1 mM $[NO_3^-]_0$ and the calculated V_{max} for the HATS. External NO_3^- concentrations of 0.2 mM and 3 mM were selected to assay the activities of both HATS and HATS+LATS, respectively, in the following studies.[36]

Kinetics of NO_3^- uptake. The kinetics of NO_3^- uptake as a function of $[NO_3^-]_0$ were measured in uninduced seedlings with $[NO_3^-]_0$ ranging from 10 µM to 10 mM, in induced seedlings with $[NO_3^-]_0$ ranging from 10–800 µM and in pre-treated seedlings with $[NO_3^-]_0$ ranging from 1–10 mM. The double reciprocal plots of the uptake rates versus substrate concentrations were subjected to linear regression analysis. The Michaelis-Menten kinetic constants (K_m and V_{max}), in both uninduced and induced seedlings, were calculated from these regression equtions in the concentration range of 0.01–1 mM $NO3^-$.[49]

Regulation of NO_3^- uptake. Re-supply experiments were performed to investigate the regulation of NO_3^- uptake after NO_3^- starvation. Uninduced seedlings were transferred for eight days into N-free Hoagland solutions supplemented with 0.2 or 3 mM KNO_3. These solutions were renewed daily to prevent depletion. Nitrate uptake was measured daily after re-supply at both 0.2 and 3 mM $[NO_3^-]_0$ in seedlings transferred into solutions 0.2 or 3 mM KNO_3, to estimate the activities of both HATS and LATS, respectively.

Metabolic inhibitor studies. The effect of 2,4-DNP (metabolic inhibitor) and DCCD and DES (plasmalemma H^+-ATPase inhibitors) was studied in both uninduced and induced seedlings. The compound 2,4-DNP was added to the uptake solutions at a concentration of 20 µM. For the plasmlemma H^+-ATPase assays, the seedlings were preincubated with 0.1 mM of either DCCD or DES for 1 h and were added in the same

Figure 2. Time course of NO_3^- net uptake in roots of uninduced seedlings of Cleopatra mandarine and Troyer citrange after re-supply of NO_3^- at 0.2 mM (A and C, respectively) or 3 mM (B and D, respectively). NO_3^- net uptake was measured at both 0.2 (iHATS) and 3 mM NO_3^- (cHATS+LATS). The value calculated of LATS only in Cleopatra mandarine and Troyer citrange (B and D, respectively) was obtained by subtracting the values of V_{max} of each rootstock from HATS+LATS (data not shown). Each data point is the average of 18 replicates with SE values shown as vertical bars.

concentration to the uptake solutions. Nitrate concentration for the uptake solutions was 0.2 mM in both uninduced and induced seedlings or 3 mM in uninduced seedlings. For experiments with DES or DCCD, all solutions (including control solutions) contained 0.25% (v/v) ethanol.

External pH effect. The effect of pH on NO_3^- uptake was studied in uninduced seedlings grown hydroponically as described above. Uptake solutions contained 1 mM MES-TRIS (pH 4, 5, 6, 7, 8 or 9) and 0.2 or 3 mM $NaNO_3$. The uptake measurements were carried out as mentioned above.

RESULTS

Kinetics of NO_3^- uptake. When external NO_3^- concentrations are below 1 mM, nitrate uptake fits a typical Michaelis-Menten curve, reaching saturation at 0.5 mM external NO_3^- concentration, typical of the activity of a saturable HATS (Fig. 1A and C), similar nitrate uptake patterns were described previously in uninduced Cleopatra mandarin and Troyer citrange seedlings.[36]

When seedlings were exposed to a temporary deprivation of NO_3^-, they exhibited lower NO_3^- uptake rates than seedlings induced by exposure to NO_3^- after the starvation period (Fig. 1A and C). NO_3^- uptake was mediated by cHATS when seedlings were exposed to a temporary lack of NO_3^- (uninduced seedlings) and NO_3^- uptake was mediated by iHATS + cHATS when seedlings were induced by NO_3^- (induced seedlings). iHATS is estimated by subtracting cHATS from cHATS+iHATS.

The apparent K_m values of the low concentration saturable systems for NO_3^- uptake in uninduced (cHATS) and induced (iHATS) in Cleopatra mandarin seedlings were 281 ± 8 and 225 ± 10 μM and the corresponding V_{max} values were 0.25 ± 0.01 and 0.32 ± 0.02 μmol g^{-1} root FW h^{-1}, respectively (Fig. 1A). Figure 1C shows similar kinetics constant to Troyer citrange in both cHATS and iHATS transport systems when compared with Cleopatra mandarin. These values were 0.26 ± 0.01 μmol g^{-1} root FW h^{-1} for V_{max} and 315 ± 12 μM for K_m in the cHATS and 0.33 ± 0.01 μmol g^{-1} root FW h^{-1} for V_{max} and 204 ± 15 μM for K_m in the iHATS. Kinetic constant values for HATS to uninduced seedlings for both rootstocks were similar to those shown previously by Cerezo et al.[36]

In the high concentration range (1–10 mM), NO_3^- net uptake increased almost linearly with external NO_3^- concentration in both Cleopatra mandarin and Troyer citrange seedlings (Fig. 1B and D), indicating the action of a non-saturable LATS as it was described by Cerezo et al.[36]

In this study, after 24 h of exposure with a 3 mM NO_3^- solution, the uptake rate constants in pre-treated seedlings were 0.040 and 0.044 μmolg^{-1} root FW h^{-1} mM^{-1} for Cleopatra mandarin and Troyer citrange, respectively, which represented a reduction of 77% and 82% with respect to uninduced seedlings. Average value of NO_3^- uptake (0.25 ± 0.03 μmolg^{-1} root FW h^{-1}) was near to V_{max} in cHATS (Fig. 1B and D), which represents 20% of NO_3^- net uptake measured at 3 mM external NO_3^- concentration.

Regulation of NO_3^- uptake by NO_3^- resupplies. To study the regulation of NO_3^- uptake by NO_3^- resupplies, Cleopatra mandarin or Troyer citrange uninduced seedlings were transferred to 0.2 mM NO_3^- solution for 8 days resulted an increased activity of the HATS, where maximum stimulation was reached after 3 days (0.312±0.012 and 0.305 ± 0.010 μmolg^{-1} root FW h^{-1} for Cleopatra mandarin and Troyer citrange, respectively). Stimulation then decreased to a minimum of

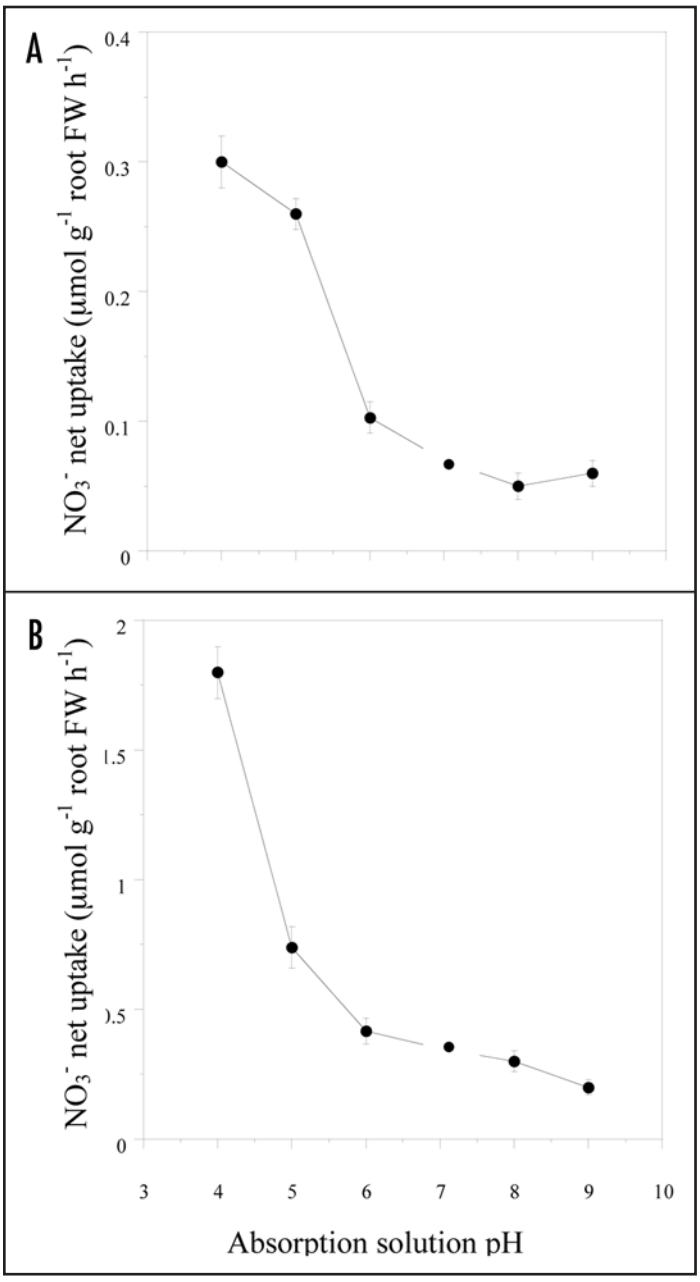


Figure 3. Effect of the pH of the absorption solution on NO_3^- net uptake into roots of Cleopatra mandarine seedlings. For the cHATS (A), NO_3^- net uptake was measured at 0.2 mM NO_3^- in uninduced seedlings. For the HATS+LATS, NO_3^- net uptake was measured at 3 mM NO_3^- in uninduced seedlings. The value of LATS only (B) was obtained by subtracting the values of HATS from HATS+LATS (data not shown) for each treatment. Each data point is the average of 18 replicates with SE values shown as vertical bars.

NO_3^- uptake equal at NO_3^- uptake in uninduced seedlings (0.103 ± 0.002 and 0.155 ± 0.022 μmol g^{-1} root FW h^{-1}) for Cleopatra mandarin and Troyer citrange, respectively (Fig. 2A and C).

The opposite response was observed for the calculated LATS-mediated NO_3^- uptake in both Cleopatra mandarin and Troyer citrange seedlings, which decreased upon transfer of the uninduced seedlings to N-free Hoagland solutions supplemented with 3 mM KNO_3 (Fig. 2B and D). The inhibition of the LATS activity by NO_3^- re-supply was 30% in both rootstocks after one day.

After three days, NO_3^- uptake was similar to V_{max} in both rootstocks, 0.25 ± 0.01 to Cleopatra mandarin (Fig. 1A) and 0.26 ± 0.01 μmol g^{-1} root FW h^{-1} to Troyer citrange (Fig. 1C).

LATS values (Fig. 2B and D) were calculated by subtracting the values of cHATS V_{max} of each rootstock from HATS + LATS. The NO_3^- uptake observed to $[NO_3^-]_0$ 3 mM after three days of resupply of 3 mM KNO_3 were reduced in both rootstocks. The values were lower than 0.12 and 0.30 μmol g^{-1} root FW h^{-1} to Cleopatra mandarin and Troyer citrange, observed to 0.2 mM $[NO_3]_0$ after three days of resupply of 0.2 mM KNO_3 (Fig. 2A and C), which indicates that iHATS is actively inhibited in the presence of NO_3^- to 3 mM.

Effect of metabolic inhibitors on NO_3^- uptake. To study the effect of metabolic inhibitors on NO_3^- uptake, an ATP-synthesis inhibitor (2,4-DNP) and two plasmalemma ATPase inhibitors (DCCD and DES) were used. The effect of these compounds was tested in uninduced and induced Cleopatra mandarin and citrange Troyer seedlings. The presence of 2,4-DNP prevented the uptake of NO_3^- by cHATS, iHATS and LATS from 50–98%. In the same conditions, treatment of roots with either DCCD or DES, which have been shown to inhibit the plasmalemma H^+-ATPase, also inhibited root nitrate absorption on all transport systems from 48%–85% (Tables 1 and 2).

Effect of external pH on NO_3^- uptake. Both the HATS-mediated and the LATS-mediated NO_3^- uptake in uninduced Cleopatra mandarin seedlings displayed a strong dependence upon external pH, with a marked optimum in the acidic range. The NO_3^- uptake at both 0.2 mM and 3 mM $[NO_3^-]_0$ were markedly inhibited (by more than 75%) when the solution pH was raised from pH 4 to 7 (Fig. 3A and B). Similar results were found in Troyer citrange (24). These results showed that the uptake rate was reduced approximately by 70% in both HATS and LATS transport systems, when the solution pH was raised from pH 4 to 7.

DISCUSSION

Nitrate uptake in Troyer citrange and Cleopatra mandarin is controlled by three transport systems. Two of them are HATS, cHATS and iHATS, that show Michaelis-Menten kinetics, the third is a LATS that is a linear non-saturable system. cHATS is constitutive and independent of N-status. In Citrus, the role of cHATS is probably to enable the cytoplasmic concentration of nitrate to rise to a level sufficient for the induction of the higher capacity iHATS as described before in barley by Behl et al.[50]

For the regulation of iHATS, this system is under feedback regulation by the N-status of the plant, being upregulated after 1–3 d of NO_3^- exposure, and downregulated between 3–8 d of NO_3^- exposure. Moreover, our data provide evidence that in both rootstocks, the LATS is repressed by NO_3^-.

Our results show that the activities of the three transport systems (cHATS, iHATS and LATS) are differently affected either by the N-status of the seedlings or by NO_3^- itself (Fig. 2). In other plants,[5,51-53] a period of induction of several hours was usually required before the effect of resupplying NO_3^- became apparent on cHATS. However, in both Cleopatra mandarin and Troyer citrange, the time of exposure to NO_3^- required for induction was unusually long, since up to three days were necessary for maximal response. This is coincidental with the slow induction observed in other woody species such as spruce.[7] Moreover, the inductive enhancement factor for V_{max} was lower than that observed in other plants in which flux increases at least 5 to 30 times.[5-7] These results suggest that in Citrus, the iHATS activity is NO_3^- upregulated and N-downregulated, whereas cHATS is not affected by NO_3^- exposure.

Very little information exists about the LATS. Several reports indicate that different LATS are regulated differently by N present in plants.[54-55] Different authors have shown that the LATS is constitutive and does not require induction by nitrate, as it is shown by both kinetic[5] and electrophysiological[10] studies, but this does not mean that the LATS is not under-regulation, because it is influenced by N demand.[25] We found that after resupply, NO_3^- uptake mediated by LATS decreased in Cleopatra mandarin and Troyer citrange seedlings, indicating that LATS is repressed in plants under high N status. These results suggest that LATS is N-repressive and does not work in plants pre-treated with 3 mM NO_3^- according to results shown previously by Cerezo et al.[22] This effect could be explained by a N-downregulation of the LATS by NO_3^- itself or by amino acids.[14,56-58]

In some species HATS for NO_3^- uptake is sensitive to metabolic inhibitors and appears to be an active transport system[5,4] based on electrophysiological studies.[10,43-44] These studies are consistent with a mechanism for NO_3^- uptake by the HATS involving a $2H^+$:$1NO_3^-$ symport moved by the energy derived from the proton gradient generated by the plasma membrane H^+-ATPase.[10,43-45]

Results in both Cleopatra mandarin and Troyer citrange seedlings support this proposal since both cHATS and iHATS were inhibited by a metabolic uncoupler (2,4-DNP) and by inhibitors of H^+ translocation ATPases (DCCD and DES) (Tables 1 and 2). This is further supported by the pH response of the NO_3^- transport system, which was inhibited at alkaline pH values (Fig. 3A). The observation of an acidic pH optimum coincides with previous studies of the pH dependence of nitrate uptake.[44]

The LATS was characterized as constitutive[5,10] and relatively insensitive to metabolic inhibition. However, considering the determinations of root cytoplasmic NO_3^- concentrations[56] and the transmembrane electrical potential differences measured by Glass et al.[10] in barley root cells, it is very unlikely that passive influx of NO_3^- occurs via the LATS. Glass et al.[10] suggested that this system is thermodynamically active and capable of transporting NO_3^- against its electrochemical potential gradient. These authors also presented evidence indicating that the LATS for NO_3^- uptake is probably mediated by an electrogenic proton cotransport system. Our results are consistent with this hypothesis, we found that LATS for NO_3^- uptake in Cleopatra mandarin and Troyer citrange seedlings roots was also inhibited by alkaline external pH (Fig. 3B) and appeared to be very sensitive to metabolic uncouplers (2,4-DNP) and inhibitors of H^+ translocation ATPases (DCCD and DES). These results support the possibility that LATS is an active transport system, linked to the H^+-ATPase. All systems are carrier-mediated, probably by an electrogenic proton cotransport system.

In unfertilised soil, cultivated areas usually maintain a concentration of NO_3^- in soil below 1 mM depending on soil moisture and other factors. Taking all together we propose that under these field conditions, the HATS would play a major role in NO_3^- nutrition and the induction of a nitrate uptake system. Under low rising nitrate concentrations, nitrate uptake takes place through a cHATS, which seems to function as a sensing mechanism for nitrate in the environment of the roots that activates a system with increased inducible capacity (iHATS). After fertilization, when the NO_3^- level in soil is considerably raised, the LATS enables the plant to absorb large amounts of NO_3^- in a limited period of time, which are probably

accumulated through different reserve N chemical forms in leaves or other N-storage organs.

A major challenge for the future will be to discover the function of the regulatory mechanism by which the plant monitors its internal status and transduces the signals to modulate the expression and/or activity of the net nitrate transports.

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