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Regulation of Apoplastic NH_4^+ Concentration in Leaves of Oilseed Rape[1]

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Regulation of apoplastic NH_4^+ concentration in leaves of oilseed rape (*Brassica napus* L.) was studied using a vacuum-infiltration technique that allowed controlled manipulations of the apoplastic solution. In leaves infiltrated with NH_4^+-free solution, the apoplastic NH_4^+ concentration returned in less than 1.5 min to the preinfiltration level of 0.8 mM. Infiltrated $^{15}NH_4^+$ was rapidly diluted by $^{14}NH_4^+/^{14}NH_3$ effluxed from the cell. The exchange rate of $^{15}N/^{14}N$ over the apoplast due to combined ^{14}N efflux from the symplast and ^{15}N influx from the apoplastic solution was 29.4 μmol g^{-1} fresh weight h^{-1} between 0 and 5 min after infiltration. The net uptake of NH_4^+ into the leaf cells increased linearly with apoplastic NH_4^+ concentrations between 2 and 10 mM and could be partially inhibited by the channel inhibitors La^{3+} and tetraethylammonium and by Na^+ and K^+. When apoplastic pH increased from 5.0 to 8.0, the steady-state apoplastic NH_4^+ concentration decreased from 1.0 to 0.3 mM. Increasing temperature increased the rate of NH_4^+ net uptake and reduced the apoplastic steady-state NH_4^+ concentration. We conclude that the apoplastic solution in leaves of oilseed rape constitutes a highly dynamic NH_4^+ pool.

NH_4^+ is constantly generated in large quantities in plant leaves by processes such as photorespiration, nitrate reduction, protein turnover, and lignin biosynthesis (Joy, 1988; Leegood et al., 1995). Refixation of NH_4^+ takes place mainly in the chloroplasts and is catalyzed by the chloroplastic isoform of Gln synthetase, GS_2 (Leegood et al., 1995). In addition to being a central metabolic intermediate, NH_4^+ may be translocated to the leaves from the roots (Cramer and Lewis, 1993; Mattsson and Schjoerring, 1996).

The rapid turnover of NH_4^+ in plant leaves leads to the establishment of a finite NH_4^+ concentration in the leaf apoplastic solution (Husted and Schjoerring, 1995). This concentration and the concentration of H^+ determines the size of the NH_3 compensation point (i.e. the NH_3 mole fraction in the air within the substomatal cavities; Farquhar et al., 1980; Husted and Schjoerring, 1996). The NH_3 compensation point ranges between 0.1 and 20 nmol mol^{-1} air and is thus of the same order of magnitude as the naturally occurring atmospheric NH_3 concentration (Sutton et al., 1994). At an NH_3 compensation point of 5 nmol mol^{-1}, for example, this would under conditions of equilibrium correspond to an apoplastic NH_4^+ concentration of 1 mM at 20°C and pH 5.8 (Husted and Schjoerring, 1996). The existence of an NH_3 compensation point implies that vegetation has a major influence on the transport and budgets of atmospheric NH_3, a pollutant with damaging environmental impacts (Langford and Fehsenfeld, 1992; Dentener and Crutzen, 1994; Sutton et al., 1995).

The concentration of NH_4^+ in the leaf apoplastic solution is very sensitive to leaf N status and external N supply. Therefore, the apoplastic NH_4^+ concentration may be about 10 times higher in oilseed rape (*Brassica napus* L.) plant leaves treated with high N than in leaves treated with low N (Husted and Schjoerring, 1996). Barley plants having access to NH_4^+ in the root medium have higher apoplastic NH_4^+ concentrations than plants absorbing NO_3^-, and the leaf apoplastic NH_4^+ concentration increases with the NH_4^+ concentration in the root medium (Mattsson and Schjoerring, 1996). Inhibition of Gln synthetase leads to a rapid and very substantial increase in apoplastic NH_4^+ (Husted and Schjoerring, 1995), and barley mutants with reduced Gln synthetase activity have increased apoplastic NH_4^+ relative to wild-type plants (Mattsson et al., 1997).

Despite the importance of leaf apoplastic NH_4^+ concentration in NH_4^+ recovery and plant-atmosphere NH_3 exchange, very little information is available concerning the transport of NH_4^+ between the leaf apoplast and symplast. In leaf discs of bean, Raven and Farquhar (1981) observed that uptake of methylammonium (an NH_4^+ analog) could not be accounted for by passive diffusion but seemed to be mediated by some kind of energy-requiring transport system. In roots of various plant species as well as in *Chara corallina*, a high-affinity transport system showing Michaelis-Menten kinetics with a K_m of approximately 15 to 40 μM and a low-affinity transport system showing a linear response to external NH_4^+ have been demonstrated (Ritchie, 1987; Glass et al., 1997). Considering the relatively high concentrations of NH_4^+ (0.5–1.5 mM) frequently encountered in the leaf apoplastic solution of oilseed rape plants, the low-affinity system appears to be central in NH_4^+ transport. In roots the low-affinity transport system has been proposed to be a uniport, with fluxes driven by the electrochemical gradient across the plasma membrane (Wang et al., 1994). This uniport may be a specific NH_4^+ channel, a K^+ channel, or a shared cation channel (e.g. a

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Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DES, diethylstilbestrol; TEA-Cl, tetraethylammonium chloride.

K^+/NH_4^+ channel, as indicated by Avery et al. [1992] and Schachtman et al. [1992]). Ninnemann et al. (1994) isolated and characterized a gene for a high-affinity NH_4^+ transporter that was highly expressed in both roots and leaves of Arabidopsis.

A further complicating factor concerning NH_4^+ transport in leaves relative to that in roots is the possible existence of a large efflux component due to diffusion of dissolved NH_3. Even under conditions in which the intracellular NH_4^+ concentration is 10 to 100 times lower than the extracellular concentration, a high pH in the cytoplasm (7.0–7.5; Martin et al., 1982) and in the chloroplasts (approximately 8.0 in light) relative to that in the apoplastic solution (approximately 6.0) may maintain a gradient of dissolved NH_3 directed toward the apoplast.

The objective of the present work was to investigate the response of NH_4^+ transport between the apoplast and symplast of leaf cells in oilseed rape to variations in apoplastic NH_4^+ concentration. The hypothesis tested was that the apoplastic NH_4^+ concentration is highly regulated and rapidly attains a steady-state level under changing conditions. A vacuum-infiltration method was used to manipulate the apoplastic NH_4^+ concentration and to introduce various transport inhibitors into the apoplast. Effects of controlled changes in different parameters such as temperature and pH on the steady-state NH_4^+ concentration and NH_4^+ net transport were elucidated. Finally, the stable isotope ^{15}N was used to assess the contribution of bidirectional NH_3/NH_4^+ transport over the plasma membrane to the maintenance of apoplastic NH_4^+ homeostasis.

MATERIALS AND METHODS

Seeds of oilseed rape (*Brassica napus* L. cv Global) were germinated in the dark on wet filter paper for 4 d prior to planting in 0.0025-m^3 self-watering pots (four plants per pot). The pots were filled with a growth medium consisting of a 1:1 mixture of soil to sand and containing 0.15 mol NH_4NO_3 per pot, supplemented with additional nutrients as described by Husted and Schjoerring (1995). Plants were grown in a greenhouse at a day/night temperature cycle of approximately 18°C/14°C (70% ± 5% RH) under a 16-h photoperiod with a PPFD > 400 μmol m^{-2} s^{-1}.

Before experiments, fully developed green leaves were cut off at the stem and their petioles were cut with a sharp blade under deionized water. Leaves were thereafter transferred to a growth chamber 1 h before experiments to allow adjustment to the environmental conditions under which the experiments were later carried out. Unless otherwise specified, the growth chamber had 70% ± 5% RH, a temperature of 20°C ± 1°C, and a PPFD of 475 ± 5 μmol m^{-2} s^{-1}.

Extraction and Analysis of Apoplastic Solution

A leaf disc of approximately 1.0 g was washed in deionized water and infiltrated with different solutions adjusted to 350 mosmol with sorbitol. Infiltration was performed in a 50-mL syringe mounted in a hydraulic infiltrator designed in our laboratory. The infiltrator was programmed to expose the leaf disc to 5 atm of pressure for 8 s, followed by vacuum, and this procedure was repeated three times. The leaf disc was then blotted dry with thin paper tissues, and the apoplastic solution was collected in microcentrifuge vials by centrifuging the leaf disc at 2000*g* for 10 min at 5°C. Cytoplasmic contamination of the apoplast during the extraction procedure was between 0.1% and 0.7%, as assessed on the basis of measurements of the activity of the marker enzyme malate dehydrogenase (EC 1.1.1.37; Husted and Schjoerring, 1995).

Apoplastic air volume was determined by infiltrating the leaf disc with a high-viscosity silicone fluid (polydimethylsiloxane: viscosity, 5 centistoke, density, 0.904 g cm^{-3}; Dow Corning, Poole, UK). The air volume was calculated as the increase in weight of the leaf disc after infiltration, corrected for the density of the silicone oil. The fraction of leaf apoplastic solution in the extracellular space of the leaf disc was determined by infiltrating the apoplast with indigo carmine (50 μM indigo-5,5′disulfonic acid, Sigma) dissolved in 50 mM phosphate buffer at pH 6.2 and adjusted to 350 mosmol with sorbitol. After the dye had infiltrated, the apoplastic solution was isolated by centrifugation, and the dilution of the indigo carmine solution was determined spectrophotometrically at 610 nm.

Cation concentrations in the apoplastic extracts were measured by isocratic HPLC using an IC-Pak C column (Waters-Millipore) at 30°C with a flow rate of 1.0 mL min^{-1} and an eluent containing 0.1 mM EDTA, 2.5 mM 18-crown-6-ether (Sigma), and 4.0 mM HNO_3.

Measurements of pH in apoplastic extracts were conducted in a microcentrifuge tube using a microelectrode (ΦSmart ISFET Micro Probe, Beckman).

The osmolality of solutions used for infiltration was measured on a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany). All solutions were prepared from ultrapure water (Milli-Q, Millipore) with an 18.2 mΩ resistance.

Apoplastic NH_4^+ Homeostasis

The steady-state apoplastic NH_4^+ concentration was determined after infiltration with either 0 or 0.8 mM NH_4Cl in 150 mM Mes buffer, pH 6.20. The apoplastic solution was extracted 1.5, 2.5, and 4.0 min after infiltration.

To assess the bidirectional transport of NH_3/NH_4^+ over the plasma membrane, leaf discs were infiltrated with 1 mM $^{15}NH_4Cl$ (98% ^{15}N) in an unbuffered solution containing 280 mM sorbitol (350 mosmol). The leaf discs were then incubated for 5, 10, 15, or 25 min before extraction of the apoplastic solution. During the incubation period the leaf discs were placed on a soaked piece of filter paper in a zippered plastic bag to avoid evaporation of leaf water. The plastic bags were placed in a growth chamber (same conditions as described above).

The ^{15}N abundance in the extracted apoplastic solution was determined by the Dumas combustion method in a system consisting of an elemental analyzer (ANCA-SL, Europa Scientific, Crewe, UK) coupled to a mass spectrometer (20–20 tracer, Europa Scientific). Because of the low quantity of apoplastic solution obtained from the leaf disc,

it was necessary to spike the samples with 10 μg of N in the form of $(NH_4)_2SO_4$ to facilitate analysis within the detection limit of the mass spectrometer. Samples and spiking solution were mixed in tin capsules and freeze-dried prior to analysis.

NH_4^+ Net Transport at Different NH_4^+ Concentrations in the Apoplastic Solution

Leaves were infiltrated with 150 mM Mes buffer, pH 6.20, containing NH_4Cl in concentrations between 1 and 80 mM. The duration of the incubation was 4 min.

Influence of Inhibitors on NH_4^+ Net Uptake

NH_4^+ net uptake was investigated in the presence of 100 μM of the ATPase inhibitor DES and 20 μM of the protonophore CCCP (both from Sigma). The inhibitors were dissolved in ethanol in a 100× stock solution and added to a buffer solution to give a final ethanol concentration of 1%. The buffer contained 150 mM Mes, pH 6.2, and 10 mM NH_4Cl. Controls received 1% ethanol without inhibitors. Leaves were pretreated for 1 h by petiole feeding in the presence of 100 μM DES, 20 μM CCCP, or control solution prior to the experiments.

The influence of the two channel blockers, La^{3+} and TEA-Cl, on the net uptake of NH_4^+ was tested by infiltration with 150 mM Mes buffer, pH 6.2, containing either 10 mM NH_4Cl and 10 mM $LaCl_3$ or 10 mM NH_4Cl and 10 mM TEA-Cl. Control leaves were infiltrated with 10 mM NH_4Cl only. Leaves were not pretreated with the inhibitors.

Effects of K^+ and Na^+ on NH_4^+ Net Uptake

Leaf discs were infiltrated and incubated for 4 min with a solution containing 150 mM Mes buffer, pH 6.2, 10 mM NH_4Cl, and 0, 50, or 100 mM of KCl or NaCl.

Influence of pH and Net H^+ Transport between Apoplast and Symplast on Steady-State NH_4^+ Concentration in the Apoplastic Solution

The effect of pH in the apoplastic solution on the steady-state concentration of NH_4^+ was investigated by infiltrating leaf discs with 150 mM Mes buffer adjusted to pH 5.0 and 6.0, or 150 mM Tes buffer adjusted to pH 7.0 and 8.0, followed by incubation of the leaf discs for 4 min.

The net H^+ transport during the period of infiltration was determined by measuring the pH in the infiltration solution before and after infiltration (maximum difference 0.4 pH unit; Fig. 5b). The amount of protons required to cause this pH change was subsequently quantified by titration of 100 mL of the buffered infiltration solution, corrected for the dilution with solution already present in the apoplast before the infiltration. The buffer capacity of the latter solution was negligible relative to that of the infiltration buffer solution (data not shown).

Effect of Temperature on Steady-State Apoplastic and Symplastic NH_4^+ Concentrations and Net NH_4^+ Transport between Apoplast and Symplast

Leaves were preincubated at temperatures ranging from 5°C to 35°C for 1 to 2 h in a growth chamber with a RH of 70% ± 5% and a PPFD of 475 ± 5 μmol m^{-2} s^{-1}. The leaves were then infiltrated with a solution containing 150 mM Mes buffer, pH 6.2, and either 0 or 10 mM NH_4Cl and incubated for 4 min at the same temperature used during the preincubation. Infiltration with 0 mM NH_4^+ was used to determine the steady-state apoplastic NH_4^+ concentration, and infiltrations with 10 mM NH_4^+ were used to determine the net uptake of NH_4^+. The solutions were adjusted to the respective temperature prior to infiltration, and all experimental work was carried out at the specified temperature (except centrifugation, which took place at 5°C). Tissue NH_4^+ was measured after 1.0 g of leaf material was ground in an ice-cooled mortar in the presence of 8 mL of 0.1 M H_2SO_4 and acid-washed sand. The extract was shaken for 30 min on ice and then centrifuged at 30,000*g* for 10 min at 5°C. The supernatant was collected, pH adjusted to 6.0 with 0.2 M $KHCO_3$, and filtered on a 0.45-μm polysulfone filter. The filtered extract was analyzed for NH_4^+ concentration by HPLC.

Calculations

The NH_4^+ concentration in the apoplast immediately after infiltration was calculated by the following equation:

$$C_{start} = C_{ini} \times V_{sol} + C_{in} \times (1 - V_{sol}) \quad (1)$$

where D_{ini} is the molar NH_4^+ concentration in the apoplastic solution prior to infiltration, V_{sol} is the volume fraction (L L^{-1}) of apoplastic solution in the extracellular space, and C_{in} is the molar NH_4^+ concentration in the infiltration solution.

To obtain the net uptake of NH_4^+ over the plasma membrane per unit leaf fresh weight, the volume of extracellular space (V_{apo}, L g^{-1}), including both apoplastic water and air, was calculated as:

$$V_{apo} = V_{air} + V_{air} \times V_{sol} \quad (2)$$

where V_{air} is the volume fraction (L g^{-1}) of the extracellular air space. Finally, the net uptake ($F_{NH_4^+}$) was calculated following the equation:

$$F_{NH_4^+} = \frac{C_{start} - C_{ex} \times V_{apo}}{\Delta t} \quad (3)$$

where C_{ex} is the molar NH_4^+ concentration in the apoplastic solution at the conclusion of the experiment and Δt is the duration in hours of the experimental period. The experimental period was defined as starting at infiltration and ending at the start of centrifugation. The shortest possible duration of the experimental period was 1.5 min, which corresponds to the minimum time required to infiltrate and transfer the leaf disc to the centrifuge.

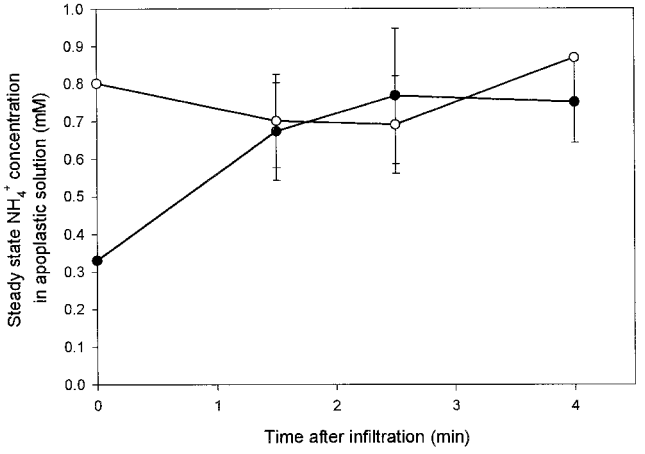

Figure 1. Time course of apoplastic NH_4^+ concentration in leaf discs of oilseed rape infiltrated at 20°C with either 0 mM (●) or 0.8 mM (○) NH_4Cl in a 150 mM Mes buffer solution, pH 6.2, adjusted to 350 mosmol with sorbitol. The concentration at time 0 after infiltration with 0 mM NH_4Cl was corrected for the NH_4^+ already present in the apoplast. Values are the means ± SE of four replicates.

RESULTS

The volume of apoplastic air in the leaves ranged from 0.20 to 0.25 mL g^{-1} fresh weight. The corresponding range for apoplastic water was 0.06 to 0.10 mL g^{-1} fresh weight. The apoplastic NH_4^+ concentration in newly sampled leaves was approximately 0.8 mM.

NH_4^+ Homeostasis in Leaf Apoplastic Solution

The apoplastic solution in leaves infiltrated with an NH_4^+-free solution attained in less than 1.5 min an NH_4^+ concentration of 0.8 mM (Fig. 1). The apoplastic NH_4^+ concentration remained at this level throughout the rest of the 4-min experimental period. No changes in apoplastic NH_4^+ concentration were observed upon infiltration with a solution containing 0.8 mM NH_4^+ (Fig. 1).

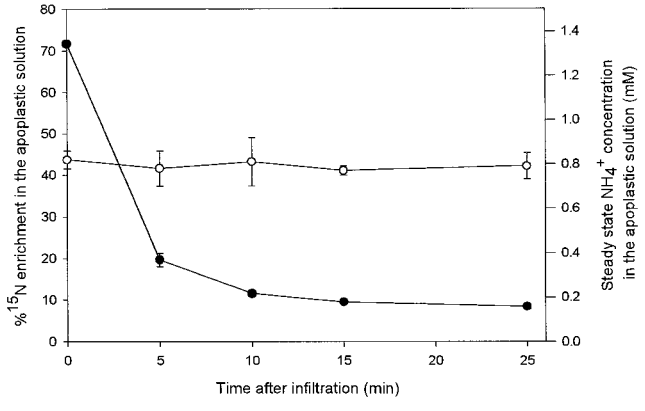

Figure 2. Time course of ^{15}N excess (●) and NH_4^+ concentration (○) in the apoplastic solution of leaf discs of oilseed rape infiltrated with 1.0 mM $^{15}NH_4Cl$ solution adjusted to an osmotic potential of 350 mosmol with sorbitol. Values are the means ± SE of six replicates.

Table I. *Exchange rate of $^{15}N/^{14}N$ over the plasma membrane determined by measuring the dilution of ^{15}N with ^{14}N (n = 6)*

Exchange rates were calculated on the basis of the ^{15}N-enrichment measurements illustrated in Figure 2.

Interval	Exchange Rate of $^{15}N/^{14}N$
min	*μmol g^{-1} fresh wt h^{-1}*
0–5	29.42 ± 3.95
5–10	19.87 ± 4.35
10–15	6.22 ± 1.79
15–20	3.86 ± 0.68

Introduction of a solution containing 1.0 mM ^{15}N-enriched NH_4^+ into the apoplast, resulting in an initial ^{15}N excess of 72 atom % in the apoplastic NH_4^+ pool, was followed by a very rapid dilution of the ^{15}N with ^{14}N (Fig. 2). After 5 min the ^{15}N excess was reduced to 20.0 atom %, and after 25 min it was reduced to 8.4 atom %. During the same period the apoplastic NH_4^+ concentration remained constant at 0.8 mM (Fig. 2), and total apoplastic N, including all organic and inorganic N compounds, remained at 7.1 mM (data not shown). In the time intervals 0 to 5, 5 to 10, 10 to 15, and 15 to 25 min after infiltration, the decline in ^{15}N atom % excess amounted to 75%, 41%, 18%, and 12%, respectively, when expressed relative to the ^{15}N excess at the start of each period. The corresponding exchange rate of $^{15}N/^{14}N$ over the apoplast due to the combined ^{14}N efflux from the symplast and ^{15}N uptake from the apoplastic solution was 29.4 μmol g^{-1} fresh weight h^{-1} between 0 and 5 min after infiltration and 3.9 ± 0.7 μmol g^{-1} fresh weight h^{-1} between 15 and 25 min after infiltration (Table I).

Response of NH_4^+ Net Uptake to Increasing Apoplastic NH_4^+ Concentration

The net uptake of NH_4^+ over a 4-min experimental period responded linearly to increasing NH_4^+ concentrations

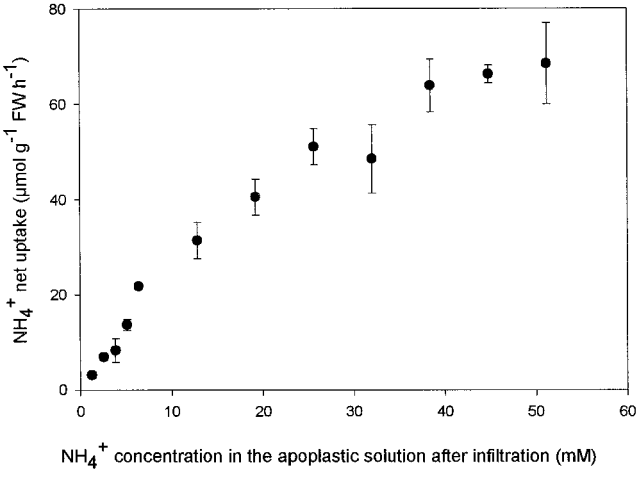

Figure 3. Net uptake of NH_4^+ from the apoplastic solution in leaf discs of oilseed rape infiltrated for 4 min at 20°C with 150 mM Mes buffer solutions, pH 6.2, adjusted to an osmotic potential of 350 mosmol with sorbitol and different concentrations of NH_4Cl. Values are the means ± SE of four replicates. FW, Fresh weight.

up to about 10 mM (Fig. 3). At higher concentrations the NH_4^+ net uptake started to saturate, becoming close to saturation at concentrations greater than approximately 40 mM NH_4^+ (Fig. 3).

Effect of Inhibitors and Competing Cations on NH_4^+ Net Uptake

Neither the ATPase inhibitor DES nor the protonophore CCCP had any effect ($P > 0.05$) on the net NH_4^+ uptake (Fig. 4a). Conversely, the unspecific channel-blocker La^{3+} and the specific K^+-channel-blocker TEA-Cl reduced ($P < 0.05$) the NH_4^+ net uptake (Fig. 4, c and d). The reduction caused by La^{3+} was 30%, and that of TEA-Cl was 47%.

Increasing the concentrations of K^+ or Na^+ resulted in a decrease in NH_4^+ net uptake (Fig. 4b). The inhibition caused by K^+ was 50% at 100 mM KCl in the infiltration solution. A similar concentration of NaCl resulted in only a 20% decline of NH_4^+ net uptake.

Effect of Apoplastic pH on Steady-State Apoplastic NH_4^+ Concentration and Proton Flux

The steady-state concentration of NH_4^+ in the apoplastic solution decreased with increasing pH between 5.0 and 8.0 (Fig. 5a). For each pH increment the NH_4^+ concentration declined by approximately 30% ($P < 0.01$).

Apoplastic pH also affected the net transport of H^+ between the apoplast and the symplast (Fig. 5b). At approximately pH 6.5 the net transport of H^+ was zero. Below this pH value, the leaf cells had a net uptake of H^+, whereas at higher pH values the net H^+ transport was in the opposite direction. The magnitude of the net H^+ transport was 20.4 nmol g^{-1} fresh weight h^{-1} at pH 5.0 and -120 nmol g^{-1} fresh weight h^{-1} at pH 8.0 (Fig. 5b).

Effect of Temperature on NH_4^+ Net Uptake and Apoplastic NH_4^+ Concentration

The net uptake of NH_4^+ from the apoplastic solution and into the symplast increased almost 3-fold with temperature in the interval from 5°C to 35°C (Fig. 6a). Over the same range of temperatures the steady-state concentration of NH_4^+ in the apoplastic solution decreased from approximately 1.0 to 0.2 mM (Fig. 6b), while the tissue NH_4^+ concentration remained almost constant at about 5.3 mM (Fig. 6c). The Q_{10} value (the ratio of rates at temperatures differing by 10°C) for net NH_4^+ uptake was 1.69 between 5°C and 15°C and decreased to about 1.2 between 25°C and 35°C (Table II). The greatest sensitivity of NH_4^+ net uptake and steady-state NH_4^+ concentration to changing temperature was observed between 10°C and 15°C (Fig. 6, a and b).

DISCUSSION

NH_4^+ Homeostasis in Leaf Apoplastic Solution

Both the rapid dilution of infiltrated $^{15}NH_4^+$ with $^{14}NH_4^+$ (Fig. 2) and the rapid adjustment to steady-state NH_4^+ concentration after infiltration with NH_4^+-free solution (Fig. 1) suggest a substantial efflux of NH_3/NH_4^+ from the leaf cells into the apoplastic solution. However, since no increase in steady-state apoplastic NH_4^+ concentration occurred over time, the NH_3/NH_4^+ effluxed from the cell was recirculated back into the cell. The apparent decrease in NH_4^+ recirculation rate over time (Table I) was due to the dilution of $^{15}NH_4^+$ with existing $^{14}NH_4^+$ in the leaf plus incorporation of ^{15}N into the organic pool. Substantial amounts of NH_3/NH_4^+ are generated in photorespiration and during lignin biosynthesis. In the latter process NH_3/NH_4^+ is released directly in the apoplast (Nakashima et al., 1997), and photorespiratory NH_3/NH_4^+ is released in the mitochondria (Leegood et al., 1995). Since

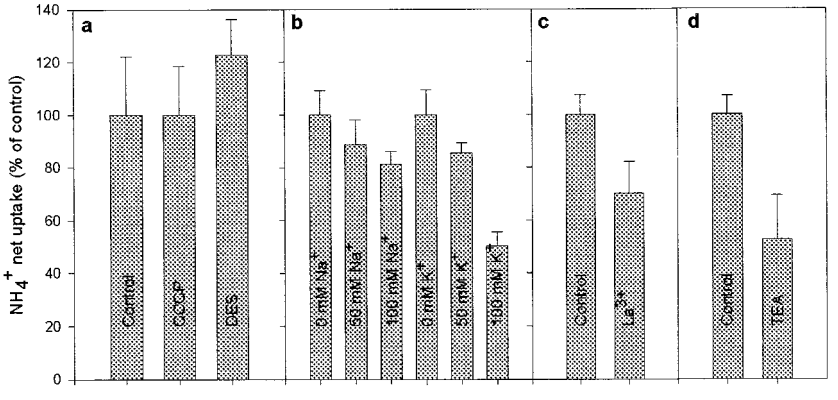

Figure 4. Effect of different inhibitors on NH_4^+ uptake from the apoplastic solution in leaf discs of oilseed rape. a, 20 μM CCCP (a protonophore) and 100 μM DES (an ATPase inhibitor) supplied by petiole feeding during a 1-h pretreatment period and subsequently added to the infiltration solution. b, KCl and NaCl added in increasing concentrations to the infiltration solution. c, 10 mM of the nonspecific channel blocker La^{3+} ($LaCl_3$). d, 10 mM specific K^+-channel blocker TEA-Cl. In addition to the specified inhibitor the infiltration solution contained 150 mM Mes, pH 6.2, and 10 mM NH_4Cl and was adjusted to an osmotic potential of 350 mosmol with sorbitol. The experiments were carried out at 20°C with a incubation period of 4 min. Values are the means ± SE of four replicates.

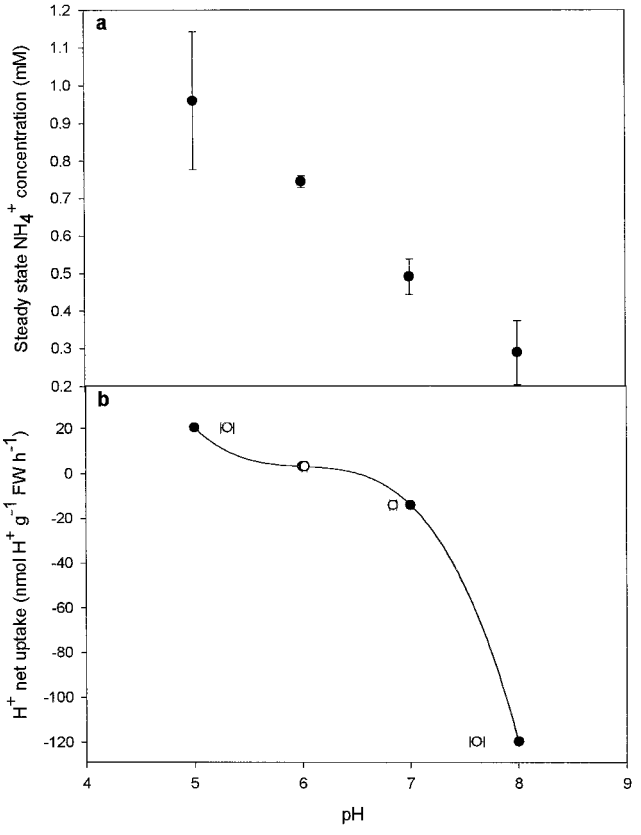

Figure 5. Effect of apoplastic pH on apoplastic NH_4^+ concentration and H^+ net uptake from the apoplastic solution in leaf discs of oilseed rape. a, Steady-state apoplastic NH_4^+ concentration at different pH values in the apoplastic solution. Leaf discs were infiltrated for 4 min with 150 mM Mes buffer, pH 5.0 and 6.0, or 150 mM Tes buffer, pH 7.0 and 8.0, adjusted to an osmotic potential of 350 mosmol with sorbitol. b, H^+ net uptake from the apoplastic solution during the infiltrations described in a. ●, pH in the infiltration solution prior to infiltration; ○, pH in the solution extracted after 4 min. Values are the means ± SE of four replicates. FW, Fresh weight.

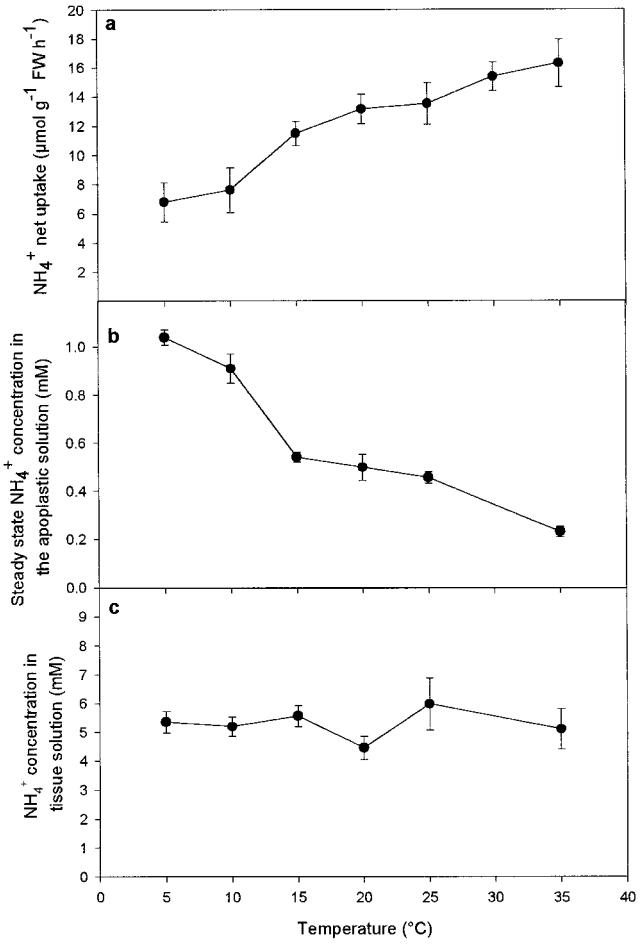

Figure 6. The temperature response of NH_4^+ net uptake from the apoplastic solution (a), steady-state apoplastic NH_4^+ concentration (b), and tissue-water NH_4^+ concentration in leaf discs of oilseed rape (c). Leaf discs were infiltrated for 4 min at the specified temperature with 150 mM Mes buffer, pH 6.2, containing 10 mM NH_4Cl and adjusted to an osmotic potential of 350 mosmol. Prior to the experiments both leaves and infiltration solutions were placed for 1 h at the same temperature as that used during the infiltration. Values are the means ± SE of four replicates. FW, Fresh weight.

biomembranes are highly permeable to NH_3 (Kleiner, 1981), the dissolved NH_3 may escape to the apoplast on its way back to the chloroplasts for reassimilation. A higher pH in cytoplasm, mitochondria, and chloroplasts than in the apoplast (Kurkdjian and Guern, 1989) would sustain an outwardly directed gradient of dissolved NH_3 even in cases in which the NH_4^+ concentration in the extracellular solution was higher than that in the cytoplasm or organelles. In root cells cytoplasmic NH_4^+ concentration can be up to 40 mM (Wang et al., 1993a), and leaf cells of oilseed rape can achieve high NH_4^+ concentrations (Finnemann and Schjoerring, 1998).

NH_4^+ Uptake from Leaf Apoplastic Solution

The net uptake of NH_4^+ from the leaf apoplastic solution into the mesophyll cells of oilseed rape increased linearly with apoplastic NH_4^+ concentration up to approximately 10 mM (Fig. 3). Because of the substantial efflux component (Fig. 2), the actual NH_4^+ influx was considerably higher

Table II. *Q_{10} values for NH_4^+ net flux at 10°C intervals between 5°C and 35°C (n = 4)*

Q_{10} values were calculated on the basis of the results shown in Figure 6a.

Temperature Range	Q_{10}
°C	
5–15	1.69
10–20	1.49
15–25	1.20
20–30	1.38
25–35	1.22

than the recorded NH_4^+ net uptake. Influx rates of NH_4^+ in both *Lemna gibba* and rice roots were smaller than those observed in the present study for leaf cells and did not show any sign of saturation at external NH_4^+ concentrations even up to 40 mM (Ullrich et al., 1984; Wang et al., 1993b). The much higher NH_4^+ uptake in leaf cells may be related to the requirement for a rapid retrieval of effluxed NH_3 originating from photorespiration and from NH_4^+ liberated in the apoplast during lignin biosynthesis.

NH_4^+ uptake in roots takes place via both a high- and a low-affinity transport system, with the former saturating at less than 1 mM (Glass et al., 1997). In the present study it was not possible to investigate NH_4^+ uptake below 0.8 mM, because apoplastic NH_4^+ rapidly attained a steady-state concentration of 0.8 mM, even after infiltration with an NH_4^+-free solution (Fig. 1). Although high-affinity NH_4^+ transport was not investigated, high levels of mRNA from the *AMT1* gene, which codes for a high-affinity NH_4^+ transporter, were found in leaves of Arabidopsis (Ninnemann et al., 1994), suggesting that a high-affinity NH_4^+-transport system may also be present in the closely related oilseed rape species.

Neither the ATPase inhibitor DES nor the protonophore CCCP affected the net uptake of NH_4^+ (Fig. 4a), suggesting that NH_4^+ uptake via the low-affinity system in leaf cells of oilseed rape is independent of both plasma membrane ATPase activity and the establishment of a proton gradient. Tyerman et al. (1995) and Mouritzen and Rosendahl (1997) found that a channel-like transporter on the symbiotic interface of N_2-fixing pea transported NH_4^+ independently of a proton gradient. In contrast, CCCP inhibited low-affinity NH_4^+ influx in rice roots by approximately 30% (Wang et al., 1993b). The inhibition of the low-affinity NH_4^+ uptake by K^+ and the inhibitors La^{3+} and TEA-Cl (Fig. 3) indicates that the NH_4^+ transport took place via a K^+ channel or a specific NH_4^+ channel closely related to a K^+ channel, as was previously proposed by Ketchum and Poole (1990), Schatchtman et al. (1992), Terry et al. (1992), and Wegner et al. (1994). The fact that a 10-fold excess in apoplastic K^+ concentration over that of NH_4^+ inhibited NH_4^+ net uptake by only approximately 50% (Fig. 3b) suggests a higher affinity for NH_4^+ relative to K^+. Since the K^+ concentration in the leaf apoplastic solution was typically more than 10 times higher than the concentration of NH_4^+ (data not shown), a relatively high affinity for NH_4^+ would be required for efficient NH_4^+ retrieval. A close relationship between an NH_4^+ channel and a K^+ channel would also be expected. Uozumi et al. (1995) showed that only minor site mutations in the P-domain of the inward-rectifying K^+ channel (KAT1) from Arabidopsis expressed in yeast increased the NH_4^+ conductance of the channel to 1 order of magnitude higher than that of K^+.

Effect of Apoplastic pH and Temperature

The increase in steady-state apoplastic NH_4^+ concentration (Fig. 5a) and the decrease in net NH_4^+ uptake at decreasing pH in the apoplastic solution (Munn and Jackson, 1978; Vessey et al., 1990; Dyhr-Jensen and Brix, 1996) most likely reflects a depolarization of the membrane potential and closing of channels following increased net uptake of H^+ (Fig. 4b; Poole, 1978; Kurkdjian and Guern, 1989; Seto-Young and Perlin, 1991; Yan et al., 1992). Enhanced release of cell wall-bound NH_4^+ following increases in H^+ concentration did not contribute significantly to the higher steady-state NH_4^+ concentration at decreasing pH, because only a very small amount of NH_4^+ was bound to the cell walls (data not shown; Husted and Schjoerring, 1995).

The highest sensitivity of net NH_4^+ uptake to temperature change was observed at temperatures from 10°C to 15°C (Fig. 6a), which is in agreement with results previously reported for roots of oilseed rape and rice (Macduff et al., 1987; Wang et al., 1993b). The observed increase in net NH_4^+ uptake at increasing temperature was far too high to be caused solely by an effect on NH_4^+ diffusion, indicating that temperature affected the transport mechanism by opening and/or closing channels (Colombo and Cerana, 1993). In accordance with the stimulating effect of temperature on NH_4^+ net uptake, the steady-state apoplastic NH_4^+ concentration declined (Fig. 6).

In conclusion, our data show that the apoplastic solution in leaves of oilseed rape constitutes a highly dynamic NH_4^+ pool. NH_4^+ is constantly added to this pool via NH_3 efflux from the mesophyll cells. The efflux of NH_3 imposes requirements for an efficient NH_4^+-retrieval system in the leaf cell plasma membrane. This retrieval system includes a transporter with channel-like properties and is able to respond very rapidly to perturbations in apoplastic NH_4^+ concentration, thereby maintaining apoplastic NH_4^+ homeostasis. Documentation of the rapid NH_4^+/NH_3 recirculation over the plasmalemma of mesophyll cells is a new contribution to understanding the dynamics and physiological implications of NH_3/NH_4^+ transport and turnover in plants. The discovery of channel-mediated NH_4^+ transport in leaves calls for further investigation of the genetic and molecular basis for this transport.

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