Plant Physiol. (1997) 113: 451–461

Differential Ammonia-Elicited Changes of Cytosolic pH in Root Hair Cells of Rice and Maize as Monitored by 2′,7′-bis-(2-Carboxyethyl)-5 (and -6)-Carboxyfluorescein-Fluorescence Ratio[1]

Harald Kosegarten*, Franz Grolig, Josef Wieneke, Glen Wilson, and Bernd Hoffmann

Institut für Pflanzenernährung, Südanlage 6, Justus-Liebig-Universität, D-35390 Giessen, Germany (H.K., G.W.); Institut für Allgemeine Botanik und Pflanzenphysiologie, Senckenbergstrasse 17, Justus-Liebig-Universität, D-35390 Giessen, Germany (F.G.); Institut für Radioagronomie, Forschungszentrum Jülich GmbH, D-52425 Jülich (J.W.); and Carl Zeiss, Tazendpromenade 1, D-07740 Jena, Germany (B.H.)

Intact hair cells of young rice (*Oryza sativa* L.) and maize roots (*Zea mays* L.), grown without external nitrogen, were specifically loaded with 2′,7′-bis-(2-carboxyethyl)-5 (and -6)-carboxyfluorescein acetoxymethyl ester to monitor fluorescence ratio cytosolic pH changes in response to external ammonia (NH_4^+/NH_3) application. In neutral media, cytosolic pH of root hairs was 7.15 ± 0.13 (*O. sativa*) and 7.08 ± 0.11 (*Z. mays*). Application of 2 mM ammonia at external pH 7.0 caused a transient cytosolic alkalization (7.5 ± 0.15 in rice; 7.23 ± 0.13 in maize). Alkalization increased with an increase of external pH; no pH changes occurred at external pH 5.0. The influx of ^{13}N-labeled ammonia in both plant species did not differ between external pH 5.0 and 7.0 but increased significantly with higher pH. Pretreatment with 1 mM 1-methionine sulfoximine significantly reduced the ammonia-elicited pH increase in rice but not in maize. Application of 2 mM methylammonia only caused a cytosolic pH increase at high external pH; the increase in both species compared with the ammonia-elicited alkalization in 1-methionine sulfoximine-treated roots. The differential effects indicate that cytosolic alkalization derived from (a) NH_3 protonation after passive permeation of the plasma membrane and, particularly in rice, (b) additional proton consumption via the glutamine synthetase/glutamate synthase cycle.

Many higher plants suffer from nutrition with ammonia[2] as the exclusive N source (Moritsugu and Kawasaki, 1982). The cellular mechanism of ammonia toxicity is still not completely understood. Poor growth at low pH, as observed with various plant species fed exclusively with NH_4^+, most likely is caused by effects of low pH rather than by the ammonium ion per se (Findenegg, 1987). At high external pH, plant tissues often accumulate ammonia and the toxic effects emerge as soon as the ammonia level increases (Mehrer and Mohr, 1989). Also at high external pH, the NH_4^+/NH_3 equilibrium shifts in favor of NH_3, a molecule that readily permeates biomembranes (Kleiner, 1981; Macfarlane and Smith, 1982). ^{31}P-NMR revealed that at higher concentrations (5–10 mM) and at high pH (pH 8.0) NH_3 diffuses into maize root tip cells and rapidly increases the cytosolic pH (Roberts et al., 1982). Uncoupling of the proton pumps inside the cell due to entry of NH_3 has been suggested as a toxic mechanism (Lee and Ratcliffe, 1991; Roberts and Pang, 1992).

Not all plants show ammonia toxicity symptoms under exclusive ammonia nutrition. Comparative physiological studies of plants with differential sensitivity to ammonia may help to identify the regulatory mechanism(s) affected and to unravel how plant cells cope with this adverse condition. In the present study we used rice (*Oryza sativa* L.) and maize (*Zea mays* L.), which react differentially to exclusive ammonia nutrition. Rice plants adapt well to exclusive ammonia nutrition in flooded rice fields with reductive conditions (Sasakawa and Yamamoto, 1978; Patnaik and Rao, 1979). Many investigations have shown that rice assimilates ammonia very efficiently (Dijkshoorn and Ismunadji, 1972; Magalhães et al., 1995). As the pH of the flood water increases to about 10.0 at noon (Mikkelsen et al., 1978), the concentration of NH_3 increases. Under these conditions, assimilation of ammonia in the rice root might contribute to the avoidance of toxic effects. In contrast to rice, the growth of maize considerably decreases with ammonia as the sole N source (Findenegg, 1987).

Cytosolic pH seems to be a cellular parameter crucially affected by the entry of ammonia. Therefore, continuous monitoring of cytosolic pH appeared to be a promising approach to study the cellular regulations involved. Various techniques (NMR, microelectrode, 5,5-dimethyloxazolidine dione) have been applied to measure cytosolic pH in plant cells, each with specific advantages. Fluorescent indicators frequently have been used in various types of animal cells (Musgrove et al., 1986; Wolosin et al., 1988; Mozingo and Chandler, 1990) and in unicellular organisms

[1] This work was supported by the Deutsche Forschungsgemeinschaft.

* Corresponding author; fax 49–641–99–39199.

[2] In this paper the terms ammonia and methylammonia are used when the protonation state is not important. Otherwise, the chemical formulae (NH_3 or NH_4^+; CH_3NH_2 or $CH_3NH_3^+$) are given.

Abbreviations: BCECF, 2′,7′-bis-(2-carboxyethyl)-5 (and -6)-carboxyfluorescein; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5 (and -6)-carboxyfluorescein acetoxymethyl ester; GOGAT, glutamate synthase; GS, Gln synthetase; MSO, 1-Met sulfoximine.

(Dixon et al., 1989; Efiok and Webster, 1990), but only rarely in suspension-cultured cells (Horn et al., 1992; Sakano et al., 1992) and in cells from whole organs of higher plants (Pheasant and Hepler, 1987; Irving et al., 1992).

We have established a technique to monitor cytosolic pH changes in intact roots and single root hairs by the fluorescent indicator BCECF, and we describe the effects of extracellular ammonia (2 mM) at low and high pH levels on cytosolic pH in rice and maize, respectively. To differentiate between NH_4^+ uptake and NH_3 diffusion being responsible for cytosolic pH changes, the effects of methylammonia (2 mM) at low and high external pH levels on cytosolic pH were monitored. In addition, uptake of ^{13}N-labeled ammonia at low and high external pH was measured. In the presence of the Gln synthetase inhibitor MSO (Wedler and Horn, 1976; Leason et al., 1982), cytosolic pH changes were studied when ammonia assimilation was blocked.

MATERIALS AND METHODS

Chemicals

BCECF and BCECF-AM were obtained from Molecular Probes (Eugene, OR). All other chemicals were from Sigma.

Plant Material and Culture

For pH measurements, rice (*Oryza sativa* L., IR 36) and maize seeds (*Zea mays* L., cv Blizzard) were soaked with 0.5 mM $CaSO_4$ (24 h) and then germinated in the dark in a humid chamber at 22°C for 4 and 2 d, respectively. The seedlings were transferred to a nutrient solution (1 mM K_2SO_4, 0.1 mM NaCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$) lacking any N for 20 h. For ^{13}N influx measurements, the seeds were germinated in vermiculite for 6 d and then the seedlings were cultured for 2 d in N-free nutrient solution (pH 6.0, pH stat control with 10 mM $Ca(OH)_2$). The basic nutrient solution was as described by Wieneke (1992).

Loading of BCECF into Root Cells and Fluorimetric pH Measurement in Roots

To monitor pH changes in the cytosol by means of fluorescence ratio (Rink et al., 1982), the pH-dependent fluorescent dye BCECF was chosen because of a pK_a (7.0) close to the cytosolic pH. The fluorescence ratio of BCECF is insensitive to pH changes in the range 2.0 to 6.5, i.e. in the vacuolar pH range. The dye is typically used as a dual excitation indicator, with excitation at 500 nm near the absorption maximum and a second excitation near the isosbestic point (439 nm) at 450 nm. The emission was recorded at 530 nm.

BCECF-AM, the lipophilic acetoxymethyl ester, was used for loading the root cells. The dye ester crosses the plasma membrane and is cleaved by cytosolic esterases (Paradiso et al., 1987). BCECF is released as the free acid of which the deprotonated form prevails in the protoplasm. Prior to dye loading, the roots of the seedlings were mounted into a capillary tube with the root tip at the bottom. Then, buffer solution with 5 mM Mes/KOH, pH 5.0, was pumped continuously through the capillary tube. The tube was fixed at an angle of 45° to the beam of the excitation light. The emitted light was observed at a 45° angle. A long-pass filter (OG 515, Schott, Mainz, Germany) was used to eliminate reflected light. The autofluorescence of the root tissue was determined in continuous flow before the root cells were loaded with the fluorescent dye. The roots were incubated (1–5 min) in 10 μM BCECF-AM in buffer solution until the emission intensity obtained from excitation at 500 nm had reached about 350 (arbitrary units).

After dye loading the root was washed with 5 mM Mes/KOH, pH 5.0, for about 60 min. A decrease of the fluorescence ratio during washing from about 2.5 to about 0.9 ratio units (corresponding to a pH ≤ 6.0) was due to diffusion of some fluorescent dye into the acidic vacuole (Brauer et al., 1995).

To localize the dye within the root and the root cells, dye-loaded roots were washed, plasmolyzed by 1 M sorbitol, and inspected by fluorescence microscopy (Leica Diaplan, Leitz, Wetzlar, Germany) with epifluorescence facility, using filter Cübeleitz Ploemopak L3 (excitation BP 450–490 nm; dichronic FT 510 nm; emission BP 525/20), XBO 75-W/2 Xenon lamp, NPL Fluotar 10/0.45 oil, and NPL Fluotar 40/1.32 oil. In the epifluorescence configuration the excitation light is applied to the probe through the microscope objective (which in this case also serves as a condensor).

Cellular pH Measurements by Microfluorimetry in Vivo

Because of dye interference between the vacuole and cytosol, with roots only relative changes of cytosolic pH (i.e. not absolute values) could be monitored by the method described above. Root hairs have the major cytosolic portion at their tips and the vacuole compartment at their base. Therefore, the pH of the cytosolic cap could be measured under an inverted fluorescence microscope (Axiovert 135, Zeiss). The root was mounted in a homemade chamber. Dye loading and cytosolic pH measurements were carried out in continuous flow. The autofluorescence of the cytosolic caps of rice and maize was measured before loading and amounted to about 1 to 2% of the fluorescence after dye loading.

A XBO 75-W/2 Xenon lamp was used for epifluorescence. The caps were illuminated with the following filter combination: excitation filters (BP 436/10 and BP 485/20) were changed with a fast filter wheel at a frequency of 50 Hz. The light was beam-split to the probe via the dichroic mirror FT 510. Emitted fluorescence was filtered by a bandpass filter (BP 515–565) for cutting off excitation light and nonspecific red fluorescence.

A measuring diaphragm of 5 × 5 μm was positioned on the region of the cytosolic caps. The illumination field diaphragm was about 2 μm larger than the measuring diaphragm. The cytosolic pH of up to three root hairs was measured simultaneously. The series of records were taken from various root hair caps by use of a motorized microscope stage (DC 100 × 90; Zeiss) over a period of about 60 min, with a temporal resolution of about 4 s.

Calibration of pH-Dependent BCECF-Fluorescence Ratio

The pH dependence of the fluorescence ratio of BCECF in buffer solutions (50 mM Mes/KOH, pH 5.0–6.7; 50 mM Hepes/KOH, pH 6.9–9.0) measured with the fluorescence photometer is shown in Figure 1A. The ratio of fluorescence intensity resulting from excitation at 500 and 450 nm shows a clear pH dependency in the pH range from 6.5 to 8.0.

In vivo calibration was carried out according to the method of Thomas et al. (1979) using nigericin in the presence of high K^+ concentrations to equilibrate the pH gradient across the plasmalemma. The whole root was suspended for 10 min in a medium containing 125 mM KCl and 50 mM buffer. The pH of the cytosolic cap was equilibrated to the buffer pH range of 4.0 to 8.0 (50 mM Mes/Tris, pH 4.0–6.3; 50 mM Hepes/Tris, pH 6.7–8.0) by the addition of 1 μM nigericin. Each buffered external pH equilibration was realized with a separate root. The measurements were carried out in continuous flow by positioning the cytosolic caps of about 15 different root hairs. Each pH equilibration comprised 150 single signals for each of the excitation wavelengths (485 and 436 nm) from which the fluorescence ratio was determined. The fluorescence emission was measured at 515 to 565 nm. In a further experiment the pH dependency of BCECF (1 μM) was analyzed for the pH range 6.5 to 8.0 in buffer solutions (50 mM Hepes/Tris, pH 6.5–8.0) that contained 125 mM KCl.

The pH dependence of buffered dye solutions and for intracellularly trapped BCECF as obtained with the fluorescence microscope are shown in Figure 1B. The curves resemble that obtained with the fluorescence photometer in Figure 1A. However, the ratio values in Figure 1A are lower than in Figure 1B. This was caused by using other excitation wavelengths and by working with different types of excitation lamps. The ratio of energy output of longer to shorter wavelengths was lower using the xenon discharge lamp (fluorescence photometer, Perkin-Elmer) compared with the DC-operating xenon high-pressure lamp (fluorescence microscope, Zeiss). Since intracellular and extracellular BCECF revealed the same pH dependency between pH 6.75 and 8.0, unknown pH values within that range were calculated by interpolation of the calibration curve.

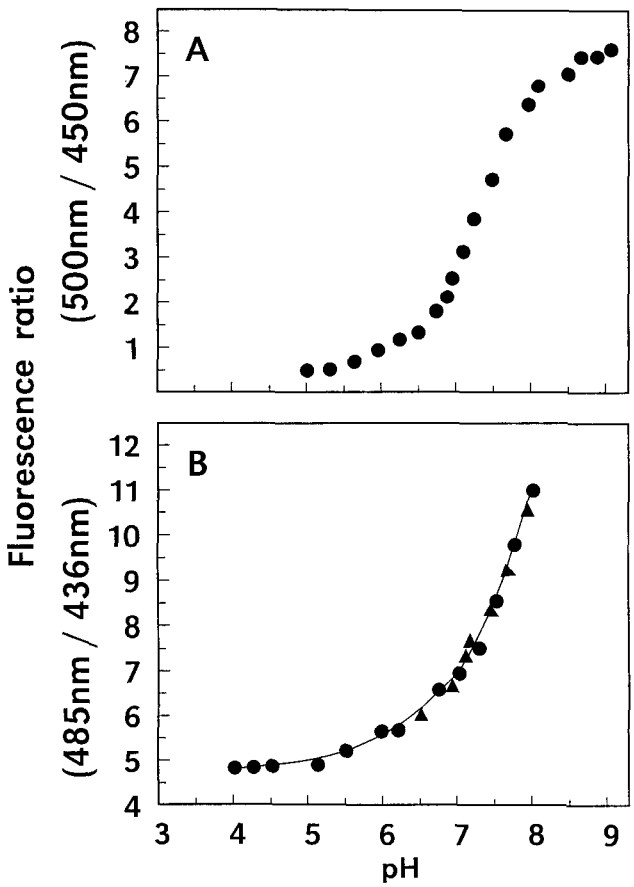

Figure 1. *Calibration curves. A, Relating fluorescence ratio obtained with the fluorescence photometer (excitation at 500 and 450 nm) to the pH of buffered dye solutions (50 mM Mes/KOH, pH 5.0–6.7; 50 mM Hepes/KOH, pH 6.9–9.0). Measurements were carried out in capillary tubes of 50 μm i.d. B, Relating fluorescence ratio obtained with the fluorescence microscope (excitation at 485 and 436 nm) to pH. ▲, BCECF in buffer solutions (50 mM Hepes/Tris, pH 6.5–8.0) containing 125 mM KCl. Measurements were carried out in capillary tubes of 50 μm i.d. ●, Intracellularly located BCECF; BCECF in root hair cells of rice equilibrated at 22°C in 50 mM buffers (Mes/Tris, pH 4.0–6.3; Hepes/Tris, pH 6.7–8.0) containing 125 mM KCl and 1 μM nigericin.*

Effect of BCECF Concentration on the Fluorescence Ratio

High concentrations of fluorescent dyes may cause an "inner filter effect," particularly in the case of a relatively long path length (Tanke et al., 1982). The root hair diameter usually varies between 5 and 17 μm (Dittmer, 1949). To simulate this short optical path length, we examined the effect of increasing BCECF concentrations on the fluorescence ratio by using capillary tubes of 50 μm i.d. Dye solutions of various concentrations were buffered with 100 mM Mes/KOH at pH 6.0. Because there was no change of the fluorescence ratio with increasing concentrations of BCECF from 2 to 100 μM (data not shown), and since our loading conditions were controlled, we could exclude a concentration effect on the fluorescence ratio caused by high dye concentrations inside the root hair cell.

Application of Ammonia and Methylammonia to Root Cells at Different pH Levels with and without MSO Pretreatment

Cytosolic pH changes were studied on a root segment of about 1.5 cm in length, starting from the area where root hair development begins. Measurements were made on root hairs about 5 mm away from the calyptra. After the dye was loaded and the roots were washed for about 60 min with 5 mM Mes/KOH at pH 5.0, the effects of 1 mM $(NH_4)_2SO_4$ and of 2 mM $(CH_3)NH_3Cl$ on cytosolic pH were investigated with 5 mM Mes/Tris or 5 mM Hepes/Tris at various pH levels. The effects of the various buffer solutions on cytosolic pH were recorded as a control.

The same experiments were done in the presence of MSO. For this purpose the roots were pretreated with 1 mM MSO for 5 h (Fentem et al., 1983) in 5 mM Mes/KOH (pH 5.0), and measurements were then carried out in the presence of MSO as described above.

Influx Measurements with ^{13}N-Labeled Ammonia

$^{13}NH_4^+$ was produced from cyclotron-produced $^{13}NO_3^-$ (Wieneke, 1992) by reduction with Devarda's alloy in a small apparatus under strongly alkaline conditions. $^{13}NH_3$ was flushed by N_2 gas flow into a trap with 5 mM H_2SO_4 and the solution was finally adjusted to about pH 5.0. No ^{18}F contamination could be detected.

Experimental sets with four plants each were preincubated for 10 min in 5 mM Mes/KOH buffered nutrient solution (pH 5.0) with 2 mM $(NH_4)Cl$ and then for 10 min in N-free solution with various pHs, buffered at pH 5.0 with 5 mM Mes/KOH, and at pH 7.0, 8.4, and 9.4 with 5 mM Hepes/KOH. ^{13}N-labeling was carried out on 80 mL (rice) or 130 mL (maize) of aerated [^{13}N]ammonia-labeled nutrient solution at the various pH levels for 10 min followed by a 3-min exchange in unlabeled nutrient solution with identical pH. In one experiment uptake rates were examined in buffer solutions with 5 mM Mes/Tris at pH 5.0 (experimental set with six plants). Plant roots were then excised and measured for ^{13}N by ordinary γ-counting (Wallac 1480, EG & G, Munich, Germany). Calculations of uptake are based on specific activity and on corrections to an arbitrary starting point for isotopic decay. The length and the diameter of the root hairs were determined to be 474.73 ± 146.36 and 6.66 ± 0.3 μm, respectively, for rice and 141.83 ± 55.34 and 11.22 ± 1.52 μm, respectively, for maize (±SD, n = 100).

Statistics and Presentation of Data

Figures 4, 5, and 7 show original curves of whole roots; at least five equivalent experiments were conducted. Statistical information is given for the ratio level before $(NH_4)_2SO_4$ was added, for the cytosolic pH change after addition of $(NH_4)_2SO_4$ at pH 7.0, and for the pH response after 10 min. The curves in Figure 6 represent average curves obtained from about 15 root hairs of one root; this experiment was repeated three times. The mean cytosolic pH levels were calculated from 45 root hairs (n = 45). Mean values of three repetitions (Table I) and four repetitions (Table II) are given. All other experiments were carried out twice and gave the same results.

RESULTS

Monitoring of BCECF-Fluorescence Ratio in Root Hair Cells

Fluorescence microscopy of the young roots revealed that BCECF specifically sequestered into the root hairs (Fig. 2). Dye fluorescence in the root hairs of both species originated from the protoplasm and not from the apoplast, which is obvious after plasmolysis (Fig. 2B).

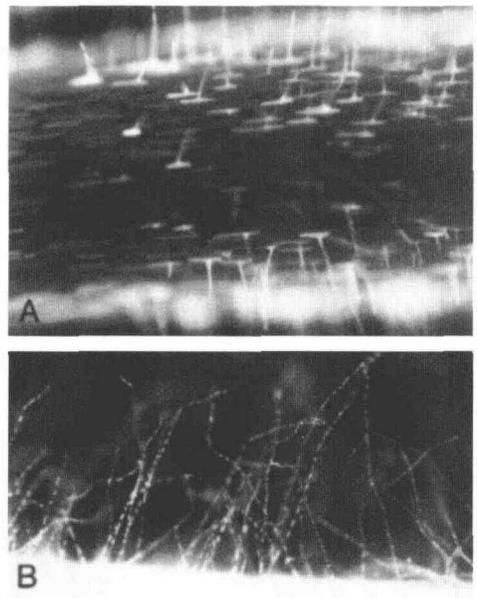

Figure 2. Fluorescence micrographs of young roots of *Z. mays* (A) and *O. sativa* (B), with root hairs loaded with BCECF. The maize root (A, tip toward left) is shown at the transition between the elongation and maturation zones where the root hairs emerge. Only the root hairs are loaded with BCECF. The rice root hairs (B) were plasmolyzed with 1 M sorbitol, showing that the fluorescent dye is confined to the protoplast.

Figure 3A shows the fluorescence emission at 530 nm as related to excitation wavelengths ranging from 400 to 510 nm. Fluorescence of a BCECF-loaded rice root (trace 1) and fluorescence of the root without BCECF (root autofluorescence, trace 2) are shown. The difference spectrum between both (Fig. 3B, trace 2) agrees well with spectra of BCECF dissolved in buffers at pH 6.0 and 5.75, respectively (Fig. 3B, traces 1 and 3). After dye loading and washing, the BCECF-fluorescence ratio (500/450 nm) of roots attained a constant level of about 0.9 ± 0.17 (±SD, n = 5) for rice and 1.0 ± 0.23 (±SD, n = 5) for maize. This ratio corresponded to a pH ≤6.0 (Fig. 1A) and implied (a) that fluorescence from BCECF derived from both the cytosol and the acidic vacuole, (b) that the vacuolar pH of root hairs was lower than the mixed pH of 6.0, and (c) that because pH sensitivity of BCECF fluorescence is restricted to pH ≥6.5 (Fig. 1A), changes in BCECF fluorescence could only derive from the cytosol. Because of the contribution of BCECF fluorescence from the vacuole, it was possible to detect only relative changes of cytosolic pH, not to measure absolute values in the roots.

Differential Effects of Ammonia at Different External pH on Cytosolic pH in Root Hairs of Rice and Maize

The effects of 2 mM ammonia on cytosolic pH of root hairs were tested at external pH 5.0 and 7.0 (Figs. 4 and 5). Cytosolic pH was not affected by NH_4^+ at pH 5.0. A change of external pH from 5.0 to 7.0 resulted in only a minor change of cytosolic pH, but pH increased immediately after application of NH_4^+/NH_3 at external pH 7.0 in

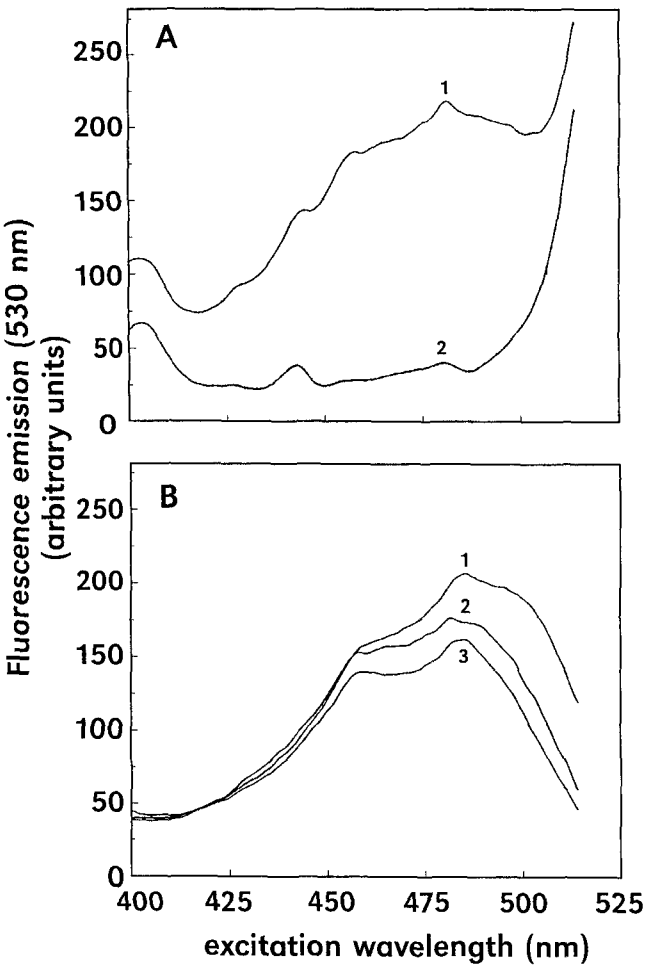

Figure 3. Fluorescence emission of BCECF (530 nm) depends on excitation. A, Trace 1, Fluorescence of BCECF-loaded root; trace 2, Root autofluorescence. B, Traces 1 and 3, Fluorescence of BCECF in 50 mM Mes/Tris and 125 mM KCl at pH 6.0 and pH 5.75, respectively; trace 2, difference spectrum (total fluorescence minus autofluorescence) of a young rice root. The increase of signal intensity at excitation wavelengths ≥500 nm is caused by light scattering of the excitation beam into the analyzing monochromator. The root was loaded and washed as described in "Materials and Methods."

both species. The pH peak for rice was attained within 80 s (Fig. 4, trace 1), and that of maize was reached within 110 s (Fig. 5, trace 1). The increase of the fluorescence ratio in rice was 1.87 ± 0.41 units (±SD; n = 5) (Table I), which then declined quickly, and 60 ± 2.31% (±SD; n = 5) of this cytosolic pH increase was reduced within 10 min (Fig. 4, trace 1). In maize roots without MSO treatment, the increase of the cytosolic pH within 1 to 2 min (0.94 ± 0.25 ratio units, ±SD, n = 5; Table I), as well as the pH restoration after 10 min (Fig. 5, trace 1), was less pronounced than in rice roots (without MSO treatment). After about 50 min the same level of fluorescence ratio as observed before application of ammonia at pH 7.0 was attained in both species.

Pretreatment of rice roots with MSO resulted in a much lower increase of the cytosolic pH of 0.91 ± 0.37 ratio units

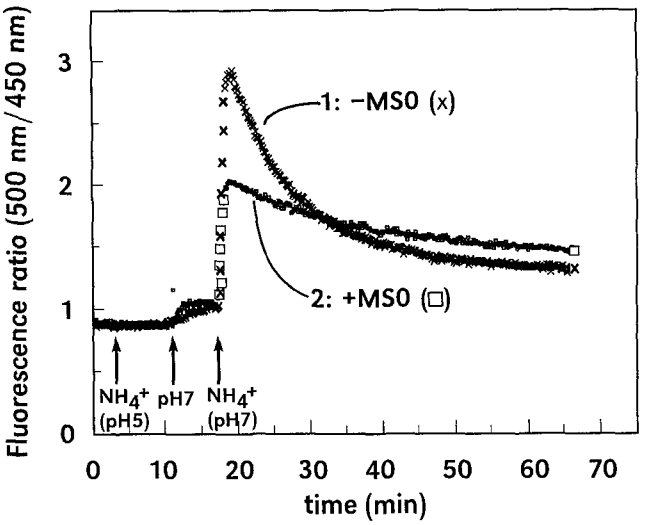

Figure 4. Time course of ammonia-elicited changes in BCECF-fluorescence ratio of young rice roots in the presence or absence of 1 mM MSO. The roots were washed after dye loading for 60 min with 5 mM Mes/KOH, pH 5. Trace 1, Root without MSO treatment. Trace 2, Root pretreated with MSO (1 mM, 5 h). First arrow indicates addition of 1 mM $(NH_4)_2SO_4$ at pH 5.0. Second arrow indicates medium change to 5 mM Hepes/Tris at pH 7.0. Third arrow indicates same medium including 1 mM $(NH_4)_2SO_4$.

(±SD; n = 5) than in the roots without MSO and in a less pronounced pH regulation (Fig. 4, trace 2). It is interesting that MSO pretreatment of maize roots (Fig. 5, trace 2) gave almost the same alkalization as roots without MSO (0.70 ± 0.31 ratio units; ±SD, n = 5), and the pH decline curves in maize roots (with and without MSO) were similar to the

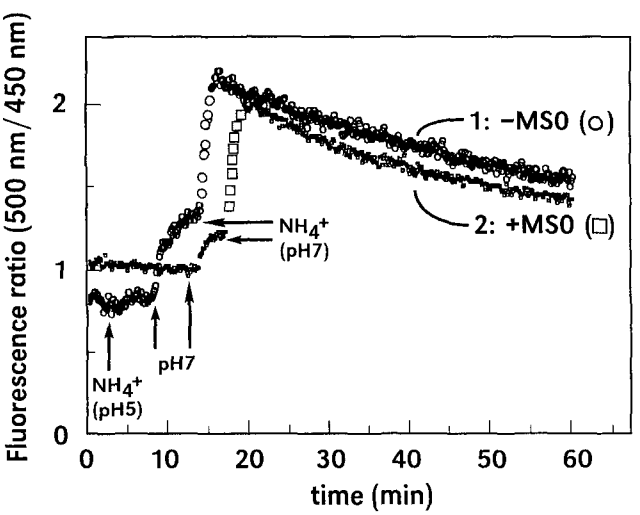

Figure 5. Time course of ammonia-elicited changes in BCECF-fluorescence ratio of young maize roots in the presence or absence of 1 mM MSO. The roots were washed after dye loading for 60 min with 5 mM Mes/KOH, pH 5.0. Trace 1, Root without MSO treatment. Trace 2, Root pretreated with MSO (1 mM, 5 h). First arrow indicates addition of 1 mM $(NH_4)_2SO_4$ at pH 5.0. Second and third arrows indicate medium change to 5 mM Hepes/Tris at pH 7.0. Fourth and fifth arrows indicate same medium including 1 mM $(NH_4)_2SO_4$.

Table I. *Increase of BCECF-fluorescence ratio (500/450 nm) in roots of rice and maize (n = 3), respectively, after exposure to 2 mM ammonia at various external pH levels*

The Δ fluorescence ratio represents the difference between the ratio level directly before and the maximal fluorescence ratio after ammonia application. The NH_3 concentrations as corresponding to the various external pH levels were calculated according to the Henderson-Hasselbalch equation.

External pH	NH_3 Concentration	Δ Fluorescence Ratio ± SD	
		O. sativa	*Z. mays*
	μM		
5.0	—[a]	—	—
6.0	1.1	0.24 ± 0.03	—
6.5	3.6	0.44 ± 0.05	0.08 ± 0.01
7.0	11.2	1.87 ± 0.41[b]	0.94 ± 0.25[b]
7.7	54.8	3.47 ± 0.66	1.37 ± 0.10
8.2	163.7	3.62 ± 0.47	2.32 ± 0.37
8.5	302.0	5.31 ± 0.37	3.24 ± 0.34
9.0	719.9	5.62 ± 0.21	4.20 ± 0.03

[a] —, No alkalization effect. [b] $n = 5$.

decline in MSO-treated rice roots (Fig. 4, trace 2). The pH peak for rice pretreated with MSO was attained within 105 s (Fig. 4, trace 2), and that of maize was attained within 165 s (Fig. 5, trace 2).

In Table I the cytosolic pH increase after application of 2 mM ammonia is shown for various pH levels in the uptake solution. The cytosolic pH increased with an increase of the pH in the outer solution and increased further the more NH_3 was produced at high pH. At all pH levels ≥6.0 the cytosolic pH increase in rice was significantly higher than in maize.

The cytosolic pH of the caps of the root hair cells was 7.15 ± 0.13 (±SD; $n = 45$) for rice and 7.08 ± 0.11 (±SD; $n = 45$) for maize prior to the NH_4^+/NH_3 treatment (Fig. 6). As observed for the root, the cytosolic pH of the individual root hair cells was not altered by NH_4^+ at pH 5.0 (not shown in Fig. 6) but increased immediately after application of NH_4^+/NH_3 at pH 7.0 in both rice and maize (Fig. 6). The cytosolic alkalization of the root hair cells yielded a pH of 7.5 ± 0.15 (±SD; $n = 45$) in rice and 7.23 ± 0.13 (±SD; $n = 45$) in maize root hair cells after addition of NH_4^+/NH_3 at pH 7.0. The steep increase in cytosolic pH in rice declined within about 20 min to the normal level, whereas there was no comparable decline in maize within the same period (Fig. 6). The same differential MSO effects as observed with roots were found on single root hairs. For the cytosolic pH level and the pattern of pH restoration individual differences were obtained between various root hairs (data not shown).

Effects of Methylammonia at Different External pH on Cytosolic pH in Root Hairs of Rice and Maize

This experiment was conducted as a control for the ammonia-elicited pH changes observed at low and high external pH (Figs. 4 and 5; Table I). In Figure 7 the effects of ammonia and of methylammonia (2 mM) at pH 5.0 and at high pH in the uptake solution (pH 7.0 in the case of ammonia and pH 8.45 in the case of methylammonia) on the cytosolic pH of rice and maize root hairs are shown. As with ammonia, addition of methylammonia at pH 5.0 did not affect cytosolic pH. Both ammonia (pK_a 9.25) and methylammonia (pK_a 10.7) alkalized the cytosol at high external pH. The level of cytosolic pH increase was the same for ammonia application in MSO-treated roots (rice, 0.91 ± 0.37; maize, 0.70 ± 0.31 ratio units; ±SD, $n = 5$) and for methylammonia application in the roots without MSO pretreatment (rice, 0.97 ± 0.22; maize, 0.93 ± 0.22 ratio units; ±SD, $n = 5$).

Uptake of ^{13}N-Labeled Ammonia into Roots of Rice and Maize at Different External pH

Table II shows the effect of external pH on the influx rate of ^{13}N-labeled ammonia in both plant species. In rice the ammonia uptake rates were higher than in maize. The uptake rates did not differ between pH 5.0 and 7.0 in either plant species but increased significantly with higher pH in the outer solution.

DISCUSSION

Methodological Aspects of Cytosolic pH Measurement with BCECF in Root Hairs

Various methods have been developed to measure cytosolic pH. Reviews from Kurkdjian and Guern (1989), Guern et al. (1991), and Felle (1993) describe and compare these methods in detail. In the present work we have elaborated a fluorescence technique to monitor continuously cytosolic pH changes of massive roots and of root hair cells by the fluorescence ratio of the pH-sensitive dye BCECF (pK_a 7.0).

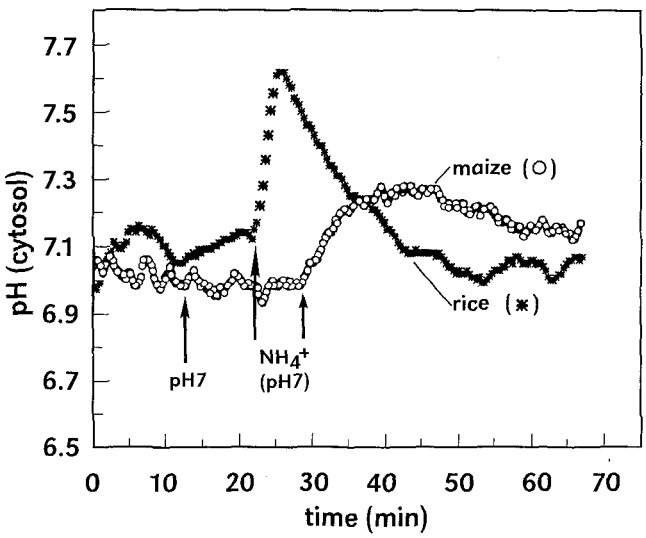

Figure 6. Ammonia-elicited pH changes in the cytosol of single root hair cells of rice (*) and maize (○) at pH 7.0 in the uptake solution. The pH was measured in a fluorescence microscope by positioning the measuring diaphragm in the region of the cytosolic cap. First arrow indicates change of medium from 5 mM Mes/Tris, pH 5.0, to 5 mM Hepes/Tris, pH 7.0; at the second and third arrow 1 mM $(NH_4)_2SO_4$ was added.

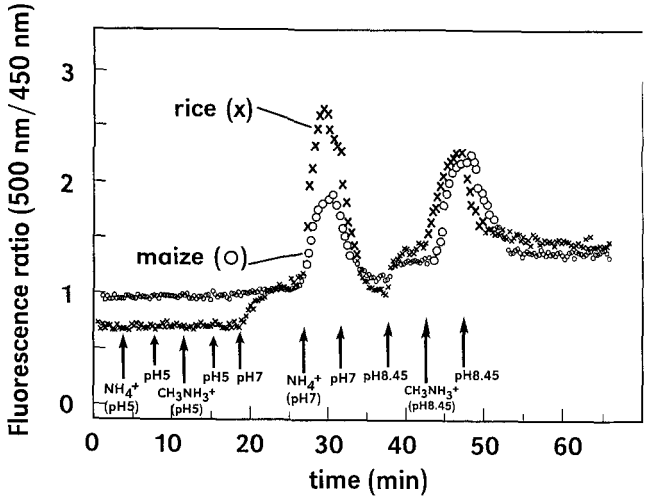

Figure 7. Ammonia- and methylammonia-elicited cytosolic pH changes (ΔBCECF-fluorescence ratio) of young rice and maize roots. The roots were washed for 60 min with 5 mM Mes/KOH, pH 5.0. Arrows (from left to right) indicate starting points of the following solution changes: 1 mM $(NH_4)_2SO_4$ in 5 mM Mes/Tris, pH 5.0; 5 mM Mes/Tris, pH 5.0; 2 mM CH_3NH_3Cl in 5 mM Mes/Tris, pH 5.0; 5 mM Mes/Tris, pH 5.0; 5 mM Hepes/Tris, pH 7.0; 1 mM $(NH_4)_2SO_4$ in 5 mM Hepes/Tris, pH 7.0; 5 mM Hepes/Tris, pH 7.0; 5 mM Hepes/Tris, pH 8.45; 2 mM CH_3NH_3Cl in 5 mM Hepes/Tris, pH 8.45; and 5 mM Hepes/Tris, pH 8.45.

The excitation spectrum of BCECF in loaded hair cells of young roots (Fig. 3B, trace 2) was identical to that of the fluorochrome in buffer solutions with a pH range of 5.75 to 6.0 (Fig. 3B, traces 1 and 3). After loading, the fluorescence ratio (500/450 nm) decreased slowly but steadily (data not shown), indicating progressive protonation of the dye. This shift most likely derived from diffusion of cytosolic dye into the vacuole. As reported for sea urchin eggs (Mozingo and Chandler, 1990), heterogeneous subcellular distribution of BCECF impedes exact measurement of cytosolic pH in compartmented cells. Similar difficulties have been encountered during measurements of plant cytosolic $-\log[Ca^{2+}]$ with Indo-1 (Russ et al., 1991).

The pH of higher plant vacuoles ranges from pH 2.0 to 6.0 (Kurkdjian and Guern, 1989; Guern et al., 1991; Echeverria et al., 1992). We have measured a mixed pH value of ≤6.0 of roots (Figs. 4, 5, and 7), which results from BCECF in the cytosol near its pK_a value of 7.0 and from dye in the acidic vacuole. BCECF is in a pH-insensitive form at the acidic pH levels of the vacuole (Fig. 1A). Therefore, changes of the fluorescence ratio measured on whole roots cannot derive from the vacuole.

Upon application of ammonia at high external pH, in both species, the internal pH immediately increased (Figs. 4, 5, and 7). As described above, alkalization in the vacuole due to protonation of NH_3 cannot be monitored with BCECF if the vacuolar pH is lower than 6.0. Because of restoration of vacuolar pH via tonoplast proton pumps, vacuolar pH was partially recovered (Bertl et al., 1984) and did not reach cytosolic pH levels (Gerendas et al., 1990) when a weak base accumulated in the vacuole. If the observed transient alkalization were caused by protonation of NH_3 in the vacuole, then the pH increase in MSO-treated roots (Figs. 4 and 5, trace 2) should be higher than that observed in roots without MSO treatment because of a higher accumulation rate of ammonia in the vacuole (Lee and Ratcliffe, 1991). Actually, however, the internal pH increase was reduced upon MSO treatment in rice and did not change in maize as compared with roots without MSO (Figs. 4 and 5, trace 2). Therefore, uptake of the weak base NH_3 into the vacuole certainly had not increased the vacuolar pH to values ≥6.5, at which BCECF fluorescence is pH-sensitive (Fig. 1A). On the contrary, BCECF located in the cytosol is in the pH-sensitive form and, therefore, the major fluorescence ratio changes observed in roots derived from the cytosol. However, the invariant vacuolar fluorescence output of the pH-insensitive form of BCECF contributed to the overall fluorescence ratio, leading to an underestimation of the cytosolic pH. Hence, exact determination of the cytosolic pH was only possible with cytosolic caps of single root hairs (Fig. 6).

The micrographs of Figure 2 demonstrate that specifically the root hairs of young *Z. mays* and *O. sativa* roots were loaded with BCECF. The esterases needed for cytosolic cleavage of the membrane-permeable acetoxymethyl ester of BCECF may be particularly active and thus promote preferential accumulation in these rapidly growing cells. Because of specific sequestration of BCECF into root hairs only (Fig. 2), the fluorescence ratio changes of roots carrying the root hairs (Figs. 4 and 5, trace 1) are in accordance with those obtained from cytosolic caps of single root hair cells (Fig. 6) and thus represent the mean response of several hundred root hairs.

We could equilibrate external and internal pH of root hair cells by addition of nigericin in the presence of high potassium concentrations. As described for Ehrlich ascites tumor cells (Thomas et al., 1979), the calibration curve for BCECF in the root hair cap was similar to that obtained in dye solution (Fig. 1B). The cytosolic pH of root hair cells measured in neutral external solution was 7.15 ± 0.13 and 7.08 ± 0.11 for rice and maize, respectively. This agrees well with reported cytosolic pH values of various plant tissues and cells obtained with different techniques, all being close to neutral between pH 7.0 and 7.5 (reviewed by Felle, 1988). Depending on intracellular zonation of the distal part of the root hair cells, we cannot completely exclude the fact that we have also monitored minor contributions of BCECF fluorescence that originated from the vacuole. In this case we would have underestimated the cytosolic pH of the cytosolic portion of the root hair cap. A promising approach to avoid dye diffusion into the vacuole seems to be coupling BCECF to dextran (Miller et al., 1992).

Without knowing about the buffering capacity of the root hair cells, the pH changes monitored with our method indicated only whether protons are consumed or produced but was not indicative of the rate of H^+ release or consumption. The higher cell surface of rice root hairs (rice, 9.99 ± 3.10 mm^2; maize, 5.19 ± 1.67 mm^2; ±SD, n = 100) may lead to higher uptake rates of ammonia, as compared with maize (Table II). Since rice and maize root hair cells are highly vacuolated, the cell surface-to-cytosolic volume

Table II. *Effect of external pH on uptake of ^{13}N-labeled ammonia into 8-d-old rice and maize roots grown at pH 6.0 in N-free nutrient solution (n = 4)*

The experimental solution was buffered with Mes/KOH and in one case with Mes/Tris[a] at pH 5.0.

Plant Species	External pH	Uptake Rate ± SD
		μmol g^{-1} fresh wt h^{-1}
O. sativa	5.0	13.49 ± 3.24
	5.0[a]	15.58 ± 4.30[a]
	7.0	11.34 ± 2.17
	8.4	21.38 ± 1.84
	9.4	26.11 ± 3.19
Z. mays	5.0	5.10 ± 0.84
	7.0	6.12 ± 1.02
	8.4	14.41 ± 0.82
	9.4	16.81 ± 0.97

[a] $n = 6$.

ratio is probably similar. Since alkalization of the cytosol was the same in the MSO-treated roots of both species after ammonia application (Figs. 4 and 5, trace 2) and also after application of methylammonia (Fig. 7), the differential cell surface presumably had no effect on the cytosolic pH increase. The faster pH increase in rice (±MSO) may indicate a lower buffering capacity than in maize (±MSO).

Mechanism(s) of Cytosolic Alkalization upon Ammonia Application at Different External pH in Rice and Maize

Differential effects on cytosolic pH may be expected, depending on whether NH_4^+ or NH_3 are applied to the tissue. In addition, depending on the physiological state of the cell and on the cell type, differential cellular pH responses have been reported when NH_4^+ was added at low external pH to the tissue (Gerendas et al., 1990; Carroll et al., 1994). Contrary to the ^{31}P-NMR technique used by these authors, our method can monitor fast reactions without time delay upon ammonia application; moreover, since the root hair cells specifically sequestered the dye, the ammonia effects could be studied in a single cell type.

Whereas cytosolic pH did not change with NH_4^+ application at external pH 5.0 (Figs. 4 and 5, trace 1), it increased immediately upon addition of NH_4^+/NH_3 at pH 7.0 in both plant species (Figs. 4 and 5, trace 1, and 6). Inhibition of GS by pretreating the roots with MSO significantly reduced the ammonia-elicited pH increase in rice but not in maize (Figs. 4 and 5, trace 2). Three mechanisms, which could be involved in the transient alkalization, are discussed below.

At high pH the NH_4^+/NH_3 equilibrium shifts in favor of NH_3, which readily permeates the plasma membrane (Kleiner, 1981; Macfarlane and Smith, 1982). After permeation NH_3 causes cytosolic alkalization by being protonated. $^{13}NH_4^+$-influx studies of Wang et al. (1993) indicate that NH_4^+ is the major N form crossing the plasmalemma. NH_4^+ uptake decreases the plasma membrane potential (Herrmann and Felle, 1995), and it may be argued that depolarization stimulated the plasmalemma proton pump.

NH_4^+ is readily assimilated in the cytosol of roots via the GS/GOGAT cycle. The overall reaction for the GS/GOGAT steps consumes protons (Mengel, 1991; Singh, 1995) during transfer of the NH_2 group from Gln onto 2′-oxoglutarate, producing two molecules of glutamate (Fig. 8). Hence, the net effect of the GS/GOGAT cycle would be to increase the cytosolic pH.

Until now conclusive data on uptake rates of NH_4^+ and NH_3 at an elevated pH have been lacking. There are data that are consistent with NH_4^+ uptake (Schlee and Komor, 1986; Wang et al., 1993), but others indicate NH_3 permeation through the plasmalemma (Lycklama, 1963; Ullrich et al., 1984). In our experiments similar uptake rates of ^{13}N-labeled ammonia were observed at pH 5.0 and 7.0 in rice and maize, respectively (Table II). In contrast to pH 5.0, however, a significant cytosolic alkalization was found in both species at pH 7.0. At this pH the ammonia equilibrium concentration of NH_3 increases to approximately 1% of the NH_4^+ concentration. Therefore, we assume that the cytosolic alkalization observed at external pH 7.0 (and not found at external pH 5.0) results from internal protonation of NH_3 rather than from NH_4^+ uptake. Our interpretation

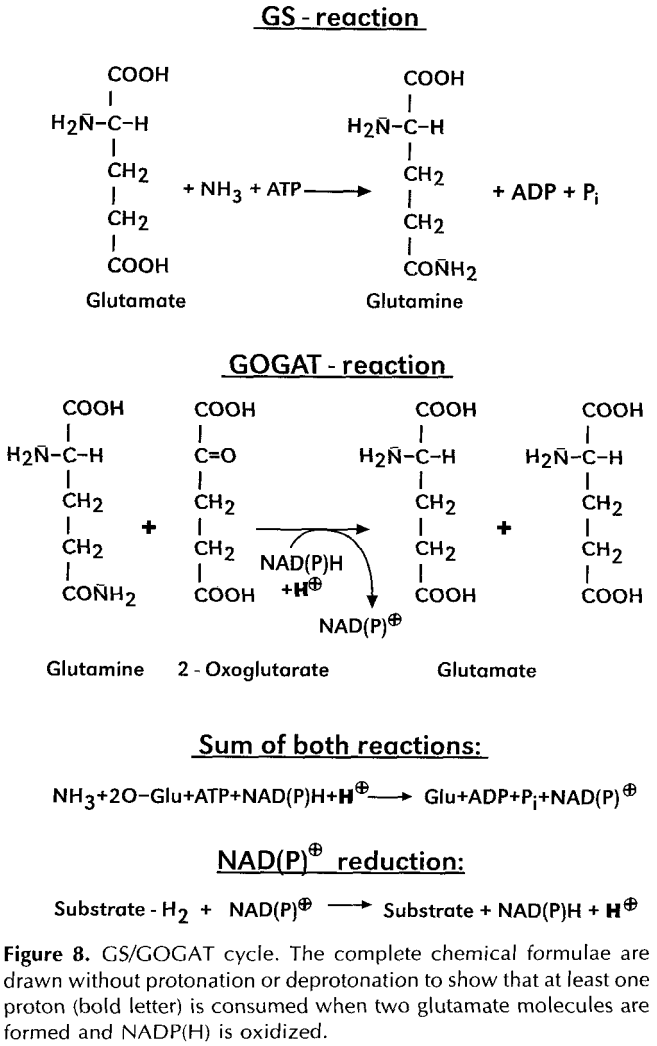

Figure 8. GS/GOGAT cycle. The complete chemical formulae are drawn without protonation or deprotonation to show that at least one proton (bold letter) is consumed when two glutamate molecules are formed and NADP(H) is oxidized.

is supported by the finding that the increase of cytosolic pH increased with the amount of NH_3 in the uptake solution (Table I) and that ammonia toxicity to crops increases with pH and total ammonia concentration (Bennett, 1974). Also, application of methylammonia at external pH 8.45 (1% of the total methylammonia concentration is CH_3NH_2) yielded a sharp cytosolic alkalization, whereas at external pH 5.0 no pH change was monitored (Fig. 7). Because of the low affinity of methylammonia to the ammonia transporter (Kosola and Bloom, 1994), the fast cytosolic alkalization effect (Fig. 7) probably was caused by CH_3NH_2 being protonated after diffusion rather than by $CH_3NH_3^+$ uptake. Since methylammonia is not metabolized, the increase of cytosolic pH after application of methylammonia in roots without MSO (Fig. 7) was the same as after addition of ammonia under conditions in which GS/GOGAT was completely inhibited by MSO (Fig. 4, trace 2). According to the method of Fentem et al. (1983), MSO incubation periods of 5 h totally inhibit the GS activity, and in our experiments a longer MSO incubation did not result in other pH effects, as was found for rice and maize, respectively (Figs. 4 and 5, trace 2). In contrast to NH_3 permeation, the uptake of NH_4^+ at first should decrease cytosolic pH because of deprotonation of NH_4^+ at the cytosolic pH level. Uptake of NH_4^+ decreases the membrane potential (Herrmann and Felle, 1995), but stimulation of the proton pump at elevated cytosolic pH is impeded because of lack of protons (substrate) for the pump (Felle and Bertl, 1986).

From our results we cannot exclude NH_4^+ uptake at high pH. However, the increase of [^{13}N]ammonia uptake rates at high external pH (Table II) is consistent with NH_3 entry and differs from uptake rates reported by Wang et al. (1993). In 3-week-old ammonia-grown rice roots they found with increasing pH a decrease of ^{13}N uptake via the low-affinity transport system. In contrast to our plant material, their seedlings were well supplied with ammonia. We therefore assume that our seedlings had a high demand for N and hence at high external pH any NH_3 diffusing into the cytosol was quickly assimilated, thus maintaining a steep diffusion gradient for NH_3. Since the $^{13}NH_4^+$ uptake rate at pH 5.0 in the outer solution did not differ between the experimental sets in which Mes was buffered with KOH and Tris, respectively (Table II), a K^+ effect on the NH_4^+ uptake rate in our experiments can be excluded.

Crucial for the actual external NH_3 concentration at the plasma membrane is the pH in the root apoplast. The apoplast pH is usually different from that in the bulk solution (reviewed by Guern et al., 1991), and plasmalemma proton pumps contribute much to depress the pH in the root apoplast (Sentenac and Grignon, 1987; Toulon et al., 1992). Such pumps, however, apparently can be concentrated at particular sites (Bouche-Pillon et al., 1994) where selective transporters for nutrient uptake prevail, e.g. a high-affinity NH_4^+ transporter (Ninnemann et al., 1994). Uptake of NH_3 presumably does not occur via transport systems but via diffusion of NH_3 across the membrane (Kleiner, 1981). Such diffusion may well occur at microsites devoid of H^+-ATPase and with a relatively high pH (reviewed by Guern et al., 1991).

It is apparent from pretreatment of rice roots with the GS inhibitor MSO that the assimilation of ammonia by the GS/GOGAT system enhanced the NH_3-elicited, transient cytosolic alkalization. Increase of cytosolic pH upon application of 2 mM NH_4^+/NH_3 at pH 7.0 was significantly higher in rice roots (Fig. 4, trace 1) than in maize roots (Fig. 5, trace 1). Cytosolic alkalization reached a pH level of 7.5 ± 0.15 in *O. sativa* (about 0.35 pH unit increase) in comparison with 7.23 ± 0.13 in *Z. mays* root hairs (about 0.15 pH unit increase; Fig. 6). Herrmann and Felle (1995) found an alkalization of about 0.1 pH unit in *Sinapis alba* root hairs upon addition of 1 mM NH_4^+/NH_3 at pH 7.0. In view of the differential effect of MSO in rice versus maize roots, we suggest that the larger alkalization in rice roots derived from higher efficacy of the GS/GOGAT pathway as compared with maize roots. In vitro investigations have shown that the activity of GS in rice roots is much higher than in maize (Magalhães and Huber, 1989, 1991). Accordingly, pretreatment of intact rice roots with MSO drastically reduced the cytosolic pH increase (Fig. 4, trace 2), whereas MSO applied to the maize root had no significant effect on cytosolic pH increase (Fig. 5, trace 2). The faster increase of pH in rice cells may indicate that NH_3 diffusion into the cytosol is promoted by a highly active GS/GOGAT pathway maintaining a steep gradient for NH_3 entry into the cells. The higher [^{13}N]ammonia uptake rates of rice versus maize at high external pH (Table II) corroborate this interpretation.

Cytosolic pH did not change upon addition of NH_4^+ at external pH 5.0 in either plant species (Figs. 4 and 5, trace 1). In contrast to the supposed passive NH_3 permeation at pH 7.0 leading to a sudden pH shift, uptake of NH_4^+ at pH 5.0 certainly is controlled by selective transporters (Ninnemann et al., 1994). One may speculate that under these controlled conditions the cytosolic buffer capacity could balance the NH_4^+-elicited pH changes that occurred. $NAD(P)^+$ produced by the GS/GOGAT cycle will be quickly reduced, yielding one H^+, which balances the proton consumed by GS/GOGAT. If, however, the rate of ammonia uptake is high and less controlled (pH ≥ 7.0 in the outer solution), the reduction of $NAD(P)^+$ initially cannot cope with the consumption of H^+ via GS/GOGAT, leading to the observed transient pH increase in the cytosol of rice root hairs. Moreover, preliminary investigations in our laboratory have shown that an increase of the cytosolic pH from 7.0 to 7.5 stimulated the GS activity in rice but not in maize. This activation may also contribute to the pronounced alkalization in the cytosol of rice roots after application of ammonia at pH 7.0 but not at pH 5.0 in the uptake solution.

Possible Mechanism for pH Restoration

The decline of cytosolic alkalization in rice and maize root hair cells was much faster in rice (Figs. 4, trace 1, and 6) than in maize (Figs. 5, trace 1, and 6); it was also found at higher cytosolic pH levels than those shown in Figure 6. In both species (Figs. 4 and 5) the cytosolic pH was restored after about 50 min. This reaction was observed in roots and, with individual differences, in single root hairs (Fig. 6). A

similar pH restoration was found in *S. alba* root hairs by Herrmann and Felle (1995). Various mechanisms may be involved in this pH regulation. The "biochemical pH-stat" (Davies, 1973, 1986) plays a major role in cytosolic pH regulation (Kurkdjian and Guern, 1989; Guern et al., 1991), and we presume that in case of NH_3-elicited alkalization, these reactions contribute to cytosolic pH restoration. Clearly, further work is needed to unravel this complex backregulation.

CONCLUSIONS

We have applied the BCECF-fluorescence ratio technique to measure cytosolic pH changes in single root hairs and to monitor such changes in roots with populations of hundreds of root hairs. Although contribution of dye fluorescence from the vacuole impeded exact determination of cytosolic pH, relative changes of cytosolic pH could be obtained readily and, due to high cell number, with high statistical significance from the respective root segment. The cytosolic pH changes were largely congruent for single root hairs and whole roots. Precise measurement of cytosolic pH was possible only in the cytosolic cap of the single root hair but required the aid of sophisticated microscopy equipment.

A higher, transient cytosolic alkalization upon application of ammonia at high external pH was found in rice as compared with maize. In rice, the cytosolic pH increase was significantly reduced by MSO. This hints at a more efficient GS/GOGAT pathway in rice, possibly reflecting constitutive adaptation to natural NH_3 overflow.

ACKNOWLEDGMENTS

We wish to thank Prof. Dr. Dr. h.c. K. Mengel (Institute of Plant Nutrition, Giessen, Germany) and Prof. Dr. H.H. Felle (Institute of Botany, Giessen, Germany) for critical reading of the manuscript, and Dr. J. Bohring (Institute of Plant Nutrition, Giessen, Germany) for assistance with data presentation. The rice seeds were kindly donated by Dr. G.S. Khush, International Rice Research Institute, Manila, Philippines.

Received August 26, 1996; accepted November 1, 1996.

LITERATURE CITED

Bennett AC (1974) Toxic effects of aqueous ammonia, copper, zinc, lead, boron, and manganese on root growth. *In* EW Carson, ed, The Plant Root and Its Environment. University of Virginia, Charlottesville, pp 197–217

Bertl A, Felle H, Bentrup FW (1984) Amine transport in *Riccia fluitans*. Cytoplasmic and vacuolar pH recorded by a pH-sensitive microelectrode. Plant Physiol **76:** 75–78

Bouche-Pillon S, Fleurat-Lessard P, Fromont J-C, Serrano R, Bonnemain J-L (1994) Immunolocalization of the plasma membrane H^+-ATPase in minor veins of *Vicia faba* in relation to phloem loading. Plant Physiol **105:** 691–697

Brauer D, Otto J, Tu S-I (1995) Selective accumulation of the fluorescent pH indicator, BCECF, in vacuoles of maize root-hair cells. J Plant Physiol **145:** 57–61

Carroll AD, Fox GG, Laurie S, Philipps R, Ratcliffe RG, Stewart GR (1994) Ammonium assimilation and the role of γ-aminobutyric acid in pH homeostasis in carrot cell suspensions. Plant Physiol **106:** 513–520

Davies DD (1973) Control of and by pH. Symp Soc Exp Biol **27:** 513–529

Davies DD (1986) The fine control of cytosolic pH. Physiol Plant **67:** 702–706

Dijkshoorn W, Ismunadji M (1972) Nitrogen nutrition of rice plants measured by growth and nutrient content in pot experiments. 3. Changes during growth. Neth J Agric Sci **20:** 133–144

Dittmer HJ (1949) Root hair variations in plant species. Am J Bot **36:** 152–155

Dixon GK, Brownlee C, Merrett MJ (1989) Measurement of internal pH in the Coccolithophore *Emiliania huxleyi* using 2′,7′-bis-(2-carboxyethyl)-5 (and -6-)-carboxyfluorescein acetoxymethyl ester and digital imaging microscopy. Planta **178:** 443–449

Eccheverria E, Burns J, Felle HH (1992) Compartmentation and cellular conditions controlling sucrose breakdown in mature acid lime fruits. Phytochemistry **31:** 4091–4095

Efiok BJS, Webster DA (1990) Respiratory driven Na^+ electrical potential in the bacterium *Vitreoscilla*. Biochemistry **29:** 4734–4739

Felle HH (1988) Short-term pH regulation in plants. Minireview. Physiol Plant **74:** 583–591

Felle HH (1993) Ion-selective microelectrodes: their use and importance in modern plant cell biology. Bot Acta **106:** 5–12

Felle HH, Bertl A (1986) Light-induced cytoplasmic pH changes and their interrelation to the activity of the electrogenic proton pump in *Riccia fluitans*. Biochim Biophys Acta **848:** 176–182

Fentem PA, Lea PJ, Stewart GR (1983) Action of inhibitors of ammonia assimilation on amino acid metabolism in *Hordeum vulgare L.* (cv Golden Promise). Plant Physiol **71:** 502–506

Findenegg GR (1987) A comparative study of ammonium toxicity at different constant pH of the nutrient solution. Plant Soil **103:** 239–243

Gerendas J, Ratcliffe RG, Sattelmacher B (1990) ^{31}P Nuclear magnetic resonance evidence for differences in intracellular pH in the roots of maize seedlings grown with nitrate or ammonium. J Plant Physiol **137:** 125–128

Guern J, Felle HH, Matthieu Y, Kurkdjian A (1991) Regulation of intracellular pH in plant cells. Int Rev Plant Cyt **127:** 111–173

Herrmann A, Felle HH (1995) Tip growth in root hair cells of *Sinapis alba L.*: significance of internal and external Ca^{2+} and pH. New Phytol **129:** 523–533

Horn MA, Meadows RP, Apostol I, Jones CR, Garenstein DG, Heinstein PF, Low PS (1992) Effect of elicitation and changes in extracellular pH on the cytoplasmic and vacuolar pH of suspension-cultured soybean cells. Plant Physiol **98:** 680–686

Irving HR, Gehring CA, Parish RW (1992) Changes in cytosolic pH and calcium of guard cells precede stomatal movement. Proc Natl Acad Sci USA **89:** 1790–1794

Kleiner D (1981) The transport of NH_3 and NH_4^+ across biological membranes. Biochim Biophys Acta **639:** 41–52

Kosola KR, Bloom AJ (1994) Methylammonium as a transport analog for ammonium in tomato (*Lycopersicon esculentum L.*). Plant Physiol **105:** 435–442

Kurkdjian A, Guern J (1989) Intracellular pH: measurement and importance in cell activity. Annu Rev Plant Physiol Plant Mol Biol **40:** 271–303

Leason M, Cunliffe D, Parkin D, Lea PJ, Miflin BJ (1982) Inhibition of pea leaf glutamine synthetase by methionine sulphoximine, phosphinotricin and other glutamate analogues. Phytochemistry **21:** 855–857

Lee RB, Ratcliffe RG (1991) Observations on the subcellular distribution of the ammonium ion in maize root tissue using in vivo ^{14}N-nuclear magnetic resonance spectroscopy. Planta **183:** 359–367

Lycklama JC (1963) The absorption of ammonium and nitrate by perennial rye-grass. Acta Bot Neerl **12:** 361–423

Macfarlane JJ, Smith FA (1982) Uptake of methylamine by *Ulva rigida*: transport of cations and diffusion of free base. J Exp Bot **33:** 195–207

Magalhães JR, Huber DM (1989) Ammonium assimilation in different plant species as affected by nitrogen form and pH control in solution culture. Fertil Res **21:** 1–6

Magalhães JR, Huber DM (1991) Response of ammonium assimilation enzymes to nitrogen form treatments in different plant species. J Plant Nutr **14:** 175–185

Magalhães JR, Huber DM, Lee TC, Tsai CY (1995) Ammonium assimilation in rice based on the occurrence of ^{15}N and inhibition of glutamine synthetase activity. J Plant Nutr **18:** 1861–1873

Mehrer I, Mohr H (1989) Ammonium toxicity: description of the syndrome in *Sinapis alba* and the search for its causation. Physiol Plant **77:** 545–554

Mengel K (1991) Ernährung und Stoffwechsel der Pflanze. Gustav Fischer Verlag, Jena, Germany, p 142

Mikkelsen DS, de Datta SK, Obcemea WN (1978) Ammonia volatilization losses from flooded rice soils. Soil Sci Soc Am J **42:** 725–730

Miller DD, Callaham DA, Gross DJ, Hepler PK (1992) Free Ca^{2+} gradient in growing pollen tubes of *Lilium*. J Cell Sci **101:** 7–12

Moritsugu M, Kawasaki T (1982) Effect of solution pH on growth and mineral uptake in plants under constant pH condition. Ber Ohara Inst Landw Biol Okayama Univ **18:** 77–92

Mozingo NM, Chandler DE (1990) The fluorescent probe BCECF has a heterogenous distribution in sea urchin eggs. Cell Biol Int Rep **14:** 688–699

Musgrove E, Rugg C, Hedley D (1986) Flow cytometric measurement of cytoplasmic pH: a critical evaluation of available fluorochromes. Cytometry **7:** 347–355

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

Paradiso AM, Tsien RY, Machen TE (1987) Digital image processing of intracellular pH in gastric oxyntic and chief cells. Nature **325:** 447–450

Patnaik S, Rao MV (1979) Sources of nitrogen for rice production. *In* International Rice Research Institute, Nitrogen and Rice. Los Banos, Laguna, Philippines, pp 25–43

Pheasant DJ, Hepler PK (1987) Intracellular pH change is not detectable during the metaphase/anaphase transition in *Tradescantia stamen* hair cells. Eur J Cell Biol **43:** 10–13

Rink TJ, Tsien RY, Pozzan T (1982) Cytoplasmic pH and free Mg^{2+} in lymphocytes. J Cell Biol **95:** 189–196

Roberts JKM, Pang MKL (1992) Estimation of ammonium ion distribution between cytoplasm and vacuole using nuclear magnetic resonance spectroscopy. Plant Physiol **100:** 1571–1574

Roberts JKM, Wemmer D, Ray PM, Jardetzky O (1982) Regulation of cytoplasmic and vacuolar pH in maize root tips under different experimental conditions. Plant Physiol **69:** 1344–1347

Russ U, Grolig F, Wagner G (1991) Changes of cytoplasmic free Ca^{2+} in the green alga *Mougeotia scalaris* as monitored with indo-1, and their effect on the velocity of chloroplast movements. Planta **184:** 105–112

Sakano K, Yazaki Y, Mimura T (1992) Cytoplasmic acidification induced by inorganic phosphate uptake in suspension-cultured *Catha ranthus* roseus cells. Measurement with fluorescent pH indicator and ^{31}P-nuclear magnetic resonance. Plant Physiol **99:** 672–680

Sasakawa H, Yamamoto Y (1978) Comparison of the uptake of nitrate and ammonium by rice seedlings—influence of light, temperature oxygen concentration, exogenous sucrose, and metabolic inhibitors. Plant Physiol **62:** 665–669

Schlee J, Komor E (1986) Ammonium uptake by *Chlorella*. Planta **168:** 232–238

Sentenac H, Grignon C (1987) Effect of H^+ excretion on the surface pH of corn root cells evaluated by using weak acid influx as a pH probe. Plant Physiol **84:** 1367–1372

Singh RP (1995) Ammonia assimilation. *In* HS Srivastava, RP Singh, eds, Nitrogen Nutrition in Higher Plants. Associated Publishing, New Delhi, pp 189–203

Tanke HJ, van Oostveldt P, van Duijn P (1982) A parameter for the distribution of fluorophores in cells derived from measurement of inner filter effect and reabsorption phenomena. Cytometry **82:** 359–369

Thomas JA, Buchsbaum RN, Zimniak A, Racker E (1979) Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. Biochemistry **18:** 2210–2218

Toulon V, Sentenac H, Thibaud J-B, Davidian J-C, Moulineau C, Grignon C (1992) Role of apoplast acidification by the H^+ pump. Effect on the sensitivity to pH and CO_2 of iron reduction by roots of *Brassica napus L.* Planta **186:** 212–218

Ullrich WR, Larsson M, Larsson C-M, Lesch S, Novacky A (1984) Ammonium uptake in *Lemna gibba G1*, related membrane potential changes, and inhibition of anion uptake. Physiol Plant **61:** 369–376

Wang M, Siddiqi MY, Ruth TJ, Glass ADM (1993) Ammonium uptake by rice roots. II. Kinetics of $^{13}NH_4^+$ influx across the plasmalemma. Plant Physiol **103:** 1259–1267

Wedler FC, Horn BR (1976) Catalytic mechanisms of glutamine synthetase enzymes. J Biol Chem **251:** 7530–7538

Wieneke J (1992) Nitrate fluxes in squash seedlings measured with ^{13}N. J Plant Nutr **15:** 99–124

Wolosin JM, Alvarez LJ, Candia LA (1988) Cellular pH and Na^+-H^+ exchange activity in lens epithelium of *Bufo marinus* toad. Am J Physiol **255:** C595–C602