

Localization and Capacity of Proton Pumps in Roots of Intact Sunflower Plants¹

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ABSTRACT

Proton extrusion by roots of intact sunflower plants (*Helianthus annuus* L.) was studied in nutrient solutions or in agar media with a pH indicator. Proton extrusion was enhanced by either iron deficiency, addition of fusicoccin, or single salt solutions of ammonium or potassium salts. The three types of proton extrusion differ in both localization along the roots and capacity. From their sensitivity to ATPase inhibitors it seems justified to characterize them as proton pumps driven by plasma membrane ATPases.

Enhanced proton extrusion induced by preferential cation uptake from $(\text{NH}_4)_2\text{SO}_4$ or K_2SO_4 was uniformly distributed over the whole root system. In contrast, the enhancement effect of fusicoccin was confined to the basal root zones and that of iron deficiency to the apical root zones. Also the rates of proton extrusion per unit of root fresh weight differed remarkably and increased in the order: Fusicoccin \ll K_2SO_4 $<$ $(\text{NH}_4)_2\text{SO}_4$ $<$ iron deficiency.

Under iron deficiency the average values of proton extrusion for the whole root system are 5.6 micromoles H^+ per gram fresh weight per hour; however, for the apical root zones values of about 28 micromoles H^+ can be calculated. This high capacity is most probably related to the iron deficiency-induced formation of rhizodermal transfer cells in the apical root zones. It can be assumed that the various types of root-induced acidification of the rhizosphere are of considerable ecological importance for the plant-soil relationships in general and for mobilization of mineral nutrients from sparingly soluble sources in particular.

In higher plants, electrogenic proton pumps are localized in various membranes, such as the plasma membranes, tonoplast or mitochondrial membranes (1–4, 7). It has been recently established that Mg-ATPases can function as electrogenic proton pumps (26). These ATPases can be distinguished by different sensitivities to anions (Cl^- , NO_3^-), monovalent cations, and inhibitors (1, 3, 4, 10). Plasma membrane and tonoplast ATPase catalyze proton extrusion from the cytosol into the apoplast (free space) and the vacuole, respectively. Both electrogenic pumps may generate electrical and chemical gradients across membranes (25). Therefore, electrogenic proton pumps can serve as the driving force for energy-dependent transmembrane movement of solutes, such as ions, sugars or amino acids. Furthermore, proton pumps are involved in the hormone-induced plant cell enlargement (17, 18) as well as in regulation of cytoplasmic pH (24).

Active proton extrusion by the plasma membrane ATPase into the free space of root cells (cortex) is also of particular ecological

importance. Acidification of the soil/root interface (rhizosphere) not only mobilizes mineral nutrients (e.g. P, Fe, Zn; [5, 20]) but also affects the activity of microorganisms such as *Rhizobium* (16) or pathogenic fungi (23, 27). This acidification of the rhizosphere can be easily demonstrated by agar techniques and depends on plant species, plant age, form and level of nutrient supply, and buffer capacity of the soil (13). Furthermore, deficiency of phosphorous (8) or iron (12, 22) may lead to an acidification of the rhizosphere by proton extrusion.

For the ecological role of proton extrusion two main factors are important, the localization along roots and the rate of proton extrusion. For roots of intact plants there are only a few reports on localization of proton efflux pumps using an indicator dye technique, e.g. in relation to extension growth (18, 28), geotropism (15), and to iron deficiency (12).

In this paper we report on results with intact sunflower plants on both the rate of proton extrusion and localization of proton pumps along roots as induced by iron deficiency, FC² and different nutrient supply. In a following paper the properties of the iron deficiency-induced proton pumps will be described in more detail.

MATERIALS AND METHODS

Growth of plants. Seeds of sunflower (*Helianthus annuus* L. cv Sobrid) were germinated for 3 d in quartz sand moistened with saturated CaSO_4 solution and subsequently transferred to a continuously aerated nutrient solution (25 plants per 1.5-L plastic vessel) of the following composition (m):

K_2SO_4 , 0.15×10^{-3} ; $\text{Ca}(\text{NO}_3)_2$, 0.5×10^{-3} ; MgSO_4 , 0.2×10^{-3} ; KH_2PO_4 , 0.1×10^{-3} ; H_3BO_3 , 1.0×10^{-5} ; MnSO_4 , 1.0×10^{-7} ; ZnSO_4 , 5.0×10^{-8} ; CuSO_4 , 5.0×10^{-8} ; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 5.0×10^{-9} .

Two days later the plants were transferred to light-tight plastic vessels (2 plants/vessel) containing the above mentioned nutrient solution with 0.1 mM FeEDTA (control plants) or without Fe (–Fe). The nutrient solution was adjusted to pH 6.0 by a few drops of NaOH (0.1 M), continuously aerated, and changed every day to ensure a nearly constant supply of nutrients. After 7 d growth without iron, swelling of the root tips could be observed as the first symptom of iron deficiency. One or 2 d later, the typical iron deficiency-induced pH decline took place. Therefore, 9- to 11-d-old plants were used for the studies on proton extrusion induced either by iron deficiency or FC or forms of mineral nutrients, especially of nitrogen.

Both preculture and the experiments were performed in a growth chamber with a day/night regime of 16/8 h, and a light intensity of 8 klux (30 w/m²; fluorescent tubes OSRAM L 40 w/

² Abbreviations: FC, fusicoccin; DCCD, dicyclohexylcarbodiimide; DES, diethylstilbestrol.

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25 white and 40 w/77 Fluora, ratio 3:1). The temperature was 26/22°C; the RH approximately 70 to 75%.

Measurement of the pH Changes with pH Electrodes. For measuring the root-induced pH changes, individual plants were transferred to black plastic boxes into which a glass beaker was inserted filled with 150 ml of either single salt solutions or of complete nutrient solution. Both solutions were continuously aerated. The pH changes of the solutions over the time period were plotted by an automatic pH printer with 6 pH electrodes (METROHM). In addition, the pH was checked manually with a portable pH meter.

FC (10 μM) or ATPase inhibitors (DCCD, DES) were added to these solutions. For this purpose, stock solutions were prepared of FC and the inhibitors, dissolved in ethanol. The final ethanol concentration in the experimental solutions never exceeded 0.025% (v/v). To exclude an unspecific effect of ethanol, the same ethanol concentration was also used in the controls.

The amount of protons released by the roots was calculated by titration of the solutions at the end of the experiments with 10 mM NaOH to the initial pH value. Prior to the determination of the fresh weight of roots or of excised root tips, the excess water was removed by blotting with filter paper.

Measurement of Oxygen Consumption. In some experiments, proton extrusion and oxygen consumption in the rooting medium were simultaneously measured by a pH electrode and an oxygen electrode (Clark type; E090, WTW, Weilheim, West-Germany). For this purpose a 150-ml glass vessel with both electrodes installed was filled with complete nutrient solution or single salt solution. The inlets for the plant stems and the electrodes in the lid of the glass container were air sealed with plastic rubber material. The solution (kept at 25°C) was continuously mixed by a magnetic stirrer and root damage was prevented by shielding with a polyester tissue placed on the bottom of the glass vessel. Oxygen uptake was expressed in $\mu\text{mol O}_2/\text{g}$ fresh weight root \cdot h.

Localization of the Proton Extrusion along Roots. To localize the proton extrusion along single roots of intact sunflower plants, an agar technique described elsewhere (9) was applied, using a pH indicator (bromocresol purple, 0.006%) dissolved in an agar medium (0.75% agar). The agar medium contained either the complete nutrient solution or the single salts as indicated.

In measuring pH changes in intact plant systems, the agar technique is the more sensitive method compared to the commonly used pH electrodes. This is also the case even when small solution volumes (150 ml/plant) were used. Proton extrusion along the roots was seen (purple \rightarrow yellow color of the pH indicator) either after a few minutes (Fe deficiency-induced) or after 2 to 4 h (FC, K_2SO_4 , or $[\text{NH}_4]_2\text{SO}_4$).

Each experiment was carried out two or three times with three to four replicates. Thus, the results presented are representative of at least six individual plants.

RESULTS

Different Localization of Proton Extrusion along Roots. As shown in Figure 1, depending upon the type of medium or pretreatment of plants, proton extrusion is localized in different zones along the roots of intact sunflower plants. In iron-adequate plants, $(\text{NH}_4)_2\text{SO}_4$ (Fig. 1A) and to a lesser extent also K_2SO_4 (Fig. 1B) and KCl (not shown here) leads to an acidification along the whole root system. No acidification takes place in iron-adequate plants in case of solutions of nitrate salts such as KNO_3 or $\text{Ca}(\text{NO}_3)_2$ (not shown here) or the complete nutrient solution with N source as nitrate only (Fig. 1C). Addition of 10 μM FC to complete nutrient media of iron-adequate plants induced acidification only in the basal zones of the root system (Fig. 1D) and not in the elongation zones behind the root tips as had been expected. In contrast, the acidification of the medium of iron-

deficient sunflower plants is confined to the apical zones of the roots (Fig. 1E). More or less identical patterns were obtained when single salts of nitrate were used instead of complete nutrient solution (data not shown).

Besides the differences in localization of the proton extrusion along the roots, there are also distinct differences in the rate of extrusion. Under iron deficiency, acidification (yellow color of the pH indicator) was clearly visible after 10 to 20 min, whereas it took about 1 h and 2 h before acidification could be observed with ammonium nitrogen and FC, respectively.

Rate of Proton Extrusion by Various Types of Proton Pumps. The different rates of proton extrusion as indicated by the agar technique (Fig. 1) were measured directly in solution culture (Table I). In iron-adequate plants, some proton consumption took place in complete nutrient solution. Addition of 10 μM FC decreased proton consumption, but no net proton extrusion could be measured. The FC-induced acidification—as shown with the highly sensitive agar technique—is obviously insufficient to be measured in a larger volume of nutrient solution. In single salt solutions of KCl or sulfate the net proton extrusion was between 1.8 and 2.0 $\mu\text{mol H}^+/\text{g}$ fresh weight \cdot h and thus still lower than in ammonium salt solutions (3.4–3.6 $\mu\text{mol H}^+$). In contrast to K_2SO_4 , in Na_2SO_4 solutions the net proton extrusion was negligible.

The highest rate of proton extrusion (5.6 $\mu\text{mol H}^+/\text{g}$ fresh weight \cdot h) occurred in roots of iron-deficient plants (Table I). Taking into account that the iron deficiency-induced proton extrusion is restricted to the apical root zones (~20% of the whole root system of 10-d-old sunflower seedlings), actual rates per unit root weight in these zones can be calculated to be as high as 28 $\mu\text{mol H}^+/\text{g}$ fresh weight root tips \cdot h, i.e. about eight times higher than the rate obtained using ammonium salts with a uniform acidification along the whole root system (Fig. 1A).

Effect of Various ATPase Inhibitors. As shown in Table I, proton extrusion, either induced by iron deficiency or potassium and ammonium salts, is completely inhibited by the ATPase inhibitors 5 μM DCCD and 50 μM DES. This inhibition occurs rapidly and is complete within 10 to 20 min (Fig. 2). This indicates that both types of proton extrusion, driven either by iron deficiency or imbalance in cation/anion uptake in iron-adequate plants, are probably ATP-dependent proton pumps.

In contrast to the inhibition of proton extrusion, the ATPase inhibitor DCCD has no effect on root respiration as measured by the O_2 consumption of the roots (Fig. 2). Even after 3 h and with the highest chosen DCCD concentration (50 μM), no inhibition of oxygen consumption was detectable (results not shown).

DISCUSSION

Proton extrusion along individual roots of intact plants is well documented in relation to root extension growth (18, 28) and geotropism (15). In both instances the proton extrusion is confined to the subapical root zones where extension growth takes place. In this paper, in roots of intact plants, three other types of proton extrusion are described which differ in both localization and capacity. From the sensitivity to inhibitors (Table I; Fig. 2) it seems justified to characterize them as proton pumps driven by plasma membrane-bound ATPases.

Enhancement of proton extrusion induced by preferential cation uptake is a well known phenomenon, especially when ammonium nitrogen is supplied (20) or in legumes relying on nitrogen fixation (13). This cation-induced proton extrusion is more or less uniformly distributed along the whole root system (Fig. 1) as is to be expected in roots of rapidly growing young plants with unlimited supply of nutrients at the root surface. In solid substrate such as soils, however, exhaustion zones of nutrients along growing roots can lead to different rates of proton extrusion (acidification) along the roots (13).

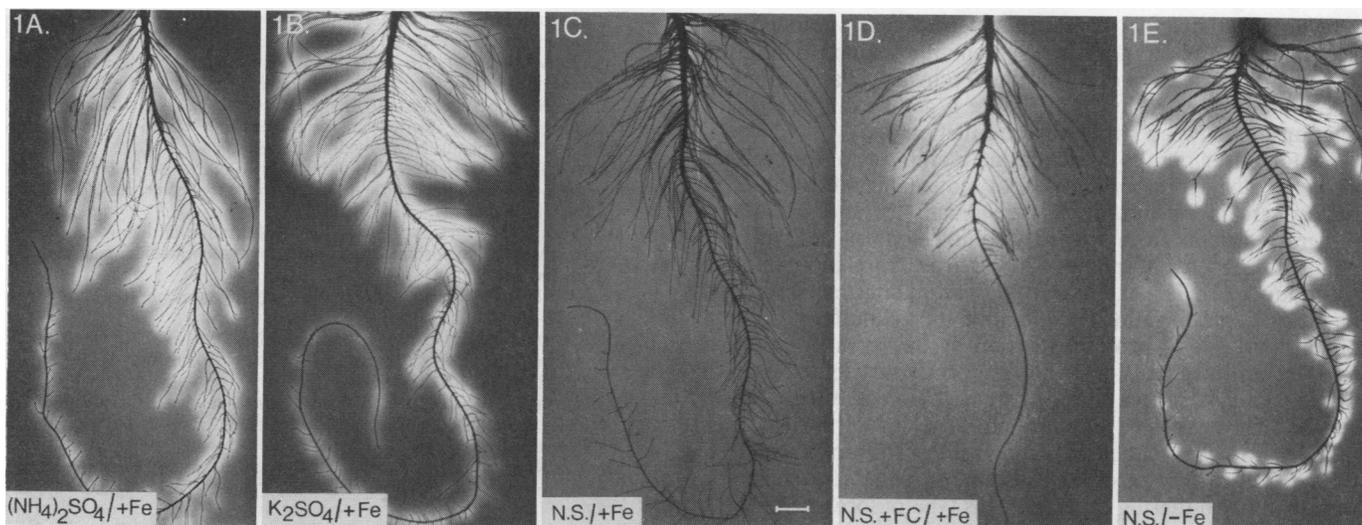


FIG. 1. Localization of proton extrusion along roots of 10-d-old sunflower plants into single salt solutions or nutrient solution (N. S.). A to D Iron-adequate plants (+Fe); E, iron-deficient plants (-Fe). Proton extrusion caused by preferential cation uptake (A, B), fusicoccin (D), and iron deficiency (E). Roots of intact plants (precultured ± 0.1 mM FeEDTA) were embedded in agar medium (pH 6.0) with the pH indicator bromocresol purple. The yellow color (white in the photographs) indicates acidification of the agar medium to pH 5.0 and lower. The agar medium contained nutrient solution (N as nitrate) or single salts (0.5 M) as indicated on the photographs. Photographs were taken after 2 h (A-D) and after 20 min (E), respectively. Bar = 1 cm.

Table I. Rates of Proton Extrusion by the Roots of 10-d-old Sunflower Seedlings of Different Iron Nutritional Status (Preculture ± 0.1 mM FeEDTA) as Affected by Various Salt Solutions, ATPase Inhibitors, and FC

Each value represents the mean of six replicates \pm SD.

Treatment	Control	+ DCCD (5 μ M)	+ DES (50 μ M)
$\mu\text{mol H}^+/\text{g fresh wt} \cdot \text{h}$			
Fe-adequate plants			
Complete nutrient solution	-0.8 ± 0.3	ND ^a	ND
Complete nutrient solution + 10 μ M FC	≤ 0.1	ND	ND
KNO ₃ 1.0 mM	-0.3 ± 0.2	ND	ND
KCl 1.0 mM	$+1.8 \pm 0.5$	-0.9 ± 0.2	-0.8 ± 0.3
K ₂ SO ₄ 0.5 mM	$+2.0 \pm 0.5$	-0.8 ± 0.1	-0.8 ± 0.2
Na ₂ SO ₄ 0.5 mM	$+0.1 \pm 0.2$	ND	ND
NH ₄ Cl 1.0 mM	$+3.4 \pm 1.2$	-0.6 ± 0.2	-0.6 ± 0.2
(NH ₄) ₂ SO ₄ 0.5 mM	$+3.6 \pm 1.2$	-0.5 ± 0.1	-0.6 ± 0.2
Fe-deficient plants			
Complete nutrient solution (except Fe)	$+5.6 \pm 1.3$	-0.3 ± 0.1	-0.3 ± 0.1

^a Not determined.

Enhanced proton extrusion in iron-deficient roots is, on the other hand, confined to the apical root zones. This has certain similarities with the enhanced proton extrusion in the zones of proteoid root formation in *Lupinus albus* (5). Enhanced proton extrusion has also been described in rape (*Brassica napus*) in response to phosphorus deficiency (8). However, the exact localization of the proton extrusion in rape roots is not yet known.

With one exception (19), all studies on the effect of FC have been performed using isolated plant tissue, e.g. hypocotyl or root segments (11). From these results, FC-induced proton extrusion was expected to occur in the apical root zones. But, as has been shown (Fig. 1D), using intact plants, the FC-induced proton extrusion of roots is restricted to the basal zone. To our knowledge this is the first report on the localization of the FC effect in roots of intact plants.

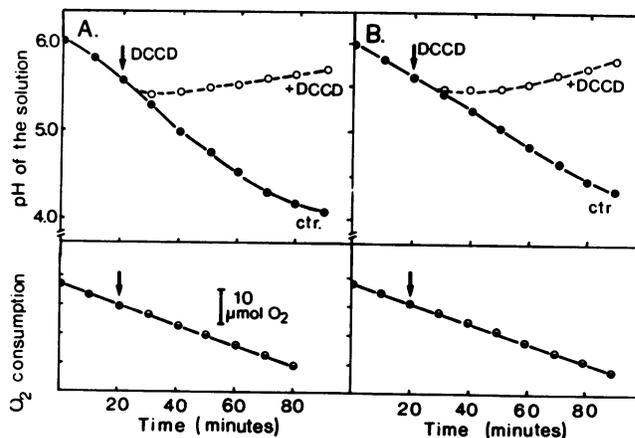


FIG. 2. Effect of the ATPase inhibitor 5 μ M DCCD on proton extrusion and O₂ consumption ($\mu\text{mol O}_2/\text{g fresh wt} \cdot \text{h}$) of roots of 10-d-old sunflower seedlings. A, Iron-deficient plants in complete nutrient solution; B, iron-adequate plants in (NH₄)₂SO₄ solution (0.5 mM). Values represent the mean of six experiments.

Besides localization of the proton efflux pumps, their capacity for proton extrusion is of particular interest. Reports for intact plants are rare in this respect. Rates of proton extrusion in relation to preferential cation uptake between 1.8 and 3.6 $\mu\text{mol H}^+/\text{g fresh weight} \cdot \text{h}$ (Table I) are in good agreement with rates reported in literature varying between less than 1.0 $\mu\text{mol H}^+$ (14) and 4.0 $\mu\text{mol H}^+$ (6, 19). In iron-deficiency roots the rates of proton extrusion are even higher (Table I). Considering the fact that the iron deficiency-induced enhanced proton efflux is confined to the apical root zones (Fig. 1), rates of about 28 $\mu\text{mol H}^+/\text{g fresh weight} \cdot \text{h}$ can be calculated for these root zones. These are the highest values reported so far in roots of intact plants. The exceptionally high rate of proton extrusion in these root zones is correlated with the formation of rhizodermal transfer cells (9, 21). The enormous enlargement of the surface area of the plasma membrane at the external surface of rhizodermal transfer cells is most probably the prerequisite for the high rates of proton extrusion in these root zones (21). If proton efflux

rates are calculated for only the rhizodermal cells, values between 80 and 150 $\mu\text{mol H}^+/\text{g fresh weight} \cdot \text{h}$ are obtained. This correlation of the formation of rhizodermal transfer cells with the high rate of proton extrusion is therefore a good example of close relationship between structure and function.

Proton efflux pumps in the plasma membrane of root cells are also of ecological importance. Root-induced acidification of the rhizosphere is an important mechanism for mobilization of mineral nutrients from sparingly soluble sources such as phosphate from rock phosphates (20) or iron from inorganic Fe^{III} compounds (5). The extent of acidification and the corresponding nutrient mobilization depend upon the pH buffering capacity of soils and rate of proton extrusion by the roots. If at a given rate this proton extrusion is not uniformly distributed over the whole root system but confined to certain root zones, the efficiency can be expected to be much higher in terms of nutrient mobilization. Consequently, strong acidification of a small volume of soil in the rhizosphere should be possible even in soils with high pH buffering capacity. This type of local acidification is a typical property of plant species with proteoid roots such as *Lupinus albus* (5) and an inducible mechanism in roots of dicots in response to iron deficiency (12).

The mechanism of the induction of the proton efflux in response to iron deficiency is still not understood (22). The role of the shoot in the activity of the proton efflux pump induced by iron deficiency will be the subject matter of a following paper.

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