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Fe Uptake Mechanism in Fe-Efficient Cucumber Roots[1]

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ABSTRACT

Fe-efficient plants respond to iron stress both by morphological and physiological modifications. In roots of a Fe-efficient plant (*Cucumis sativus* L.) grown in the presence or in the absence of iron, the capacity to acidify the external medium, change in the transmembrane electrical potential, and the ATPase activity have been determined. Roots from plants grown in the absence of iron showed a great capacity to acidify the external medium, a higher transmembrane electrical potential difference (−145 millivolts, *versus* −105 millivolts), and a higher ATPase activity (+30%). The administration of Fe^{2+}, but not Fe^{3+}, caused a block of the acidification capacity, a great decrease in the transmembrane electrical potential difference in root cells, and a large inhibition of the ATPase activity of isolated microsomal membrane vesicles.

Plants respond to iron stress in different ways. Morphological and physiological modifications can be seen in roots of the so-called Fe-efficient species (15). Morphological modifications include increase in root hair formation and swelling of the root tips (10, 18). Physiological responses include enhanced acidification of the external medium (8, 11, 19), increased amount of reduction of Fe^{3+} to Fe^{2+} at the root surface (3, 17), and increased organic acid synthesis (11). All of these phenomena may be useful for absorbing inorganic iron compounds and making iron available for plant metabolism (2). The mechanism by which plants generally promote cation uptake is the negativization of the PD[2] through the activation of H^+ extrusion mechanism(s) at the plasmalemma. H^+ extrusion might be linked to the activity of an H^+-ATPase and/or to an electron transport system, even though a direct involvement in PD genesis has not yet been demonstrated for the latter mechanism (4, 7, 12). In Fe-efficient species an electron transport system for the reduction of Fe^{3+} was demonstrated (1), but it is not clear if this mechanism is also involved in the acidification of the medium and in the genesis of the PD. Sijmons *et al.* (21) demonstrated that incipient ferric reduction in Fe-efficient roots of *Phaseolus vulgaris* corresponded to a depolarization of the PD and hypothesized that a transport of electrons and protons across the plasma membrane occurred at a ratio of $2e^-/H^+$. In this paper we evaluated the role of the H^+-ATPase and of the Fe-reductase in the proton extrusion activity and the PD genesis.

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[2] Abbreviation: PD, transmembrane electrical potential.

MATERIALS AND METHODS

Growth of Plants

Cucumber seeds (*Cucumis sativus* L. cv Marketer from F.1li Ingegnoli, Milan) were sown in agriperlite, watered with $CaSO_4$ (0.1 mM), allowed to germinate in the dark at 26°C for 4 d, and then transferred to a nutrient solution that had the following composition (mM): 2 $Ca(NO_3)_2$, 0.75 K_2SO_4, 0.65 $MgSO_4$, 0.5 KH_2PO_4, 1×10^{-2} H_3BO_3, 1×10^{-3} $MnSO_4$, 5×10^{-4} $CuSO_4$, 5×10^{-4} $ZnSO_4$, 5×10^{-5} $(NH_4)_6Mo_7O_{24}$ and 0.1 Fe-EDTA (if added); pH was adjusted to 6.0 to 6.2 with NaOH. Hydroponic cultures were maintained in a greenhouse and the growing solution was changed weekly. Plants became chlorotic after approximately 1 week of culture in −Fe, and in the second week no further deficiency symptoms were observed.

Determination of Acidification Capacity

Roots from an 8 to 15 d hydroponic culture were used. After excision, roots were washed in distilled water, transferred to 50 mL Erlenmeyer flasks that were submerged in a temperature regulated shaking-bath which was maintained at 26°C and had a shaking rate of 100 oscillations/min. After preincubation in 10 mL of 5 mM sucrose and 0.5 mM $CaSO_4$ (pH 6.2) for 60 min, the solution was changed with 10 mL of identical fresh solution containing different forms of Fe; 0.1 mM $FeSO_4$(Fe^{2+}) and 0.1 mM FeEDTA or 0.05 mM $Fe_2(SO_4)_3$ (Fe^{3+}) were used. During our incubation times (120 min) no precipitation of Fe hydroxide was observed when $Fe_2(SO_4)_3$ was used. The pH of the medium was recorded with a Radiometer pHM 64 pH-meter.

For roots grown in the presence of Fe, the preincubation was performed in the presence of 0.1 mM FeEDTA.

Determination of PD

PD measurements were performed in accordance with standard procedures, as described by Cocucci *et al.* (6) in a 1.5 mL lucite cuvette, under continuous flow (about 10 mL/min) of the aerated, regulated (26°C) medium (5 mM sucrose, 0.5 mM $CaSO_4$ [pH 6.2]). Four 4 cm long root segments were preincubated for 60 min in the above aerated medium. After preincubation, the PD was measured for 1 h (time required for a constant PD value); then Fe salts were added and their effects on PD recorded (0.1 mM $FeSO_4$ [Fe^{2+}] and 0.1 mM FeEDTA or 0.05 mM $Fe_2[SO_4]_3$ [Fe^{3+}] were utilized). During our experimental time, no precipitation of Fe hydroxide was observed when $Fe_2(SO_4)_3$ was used. Membrane potentials were measured by inserting the microelectrodes into the cortical

cells at about 1 cm from the root tip. Glass electrodes were filled with 3 M KCl. Only electrodes with electrical resistance in the range of 9 to 14 MΩ were used for the measurements.

Isolation of Microsomal Fraction

Roots from 8 to 15 d hydroponic culture were harvested, washed in distilled water, and homogenized in an ice-cold mortar in 250 mM sorbitol, 25 mM Tris-Mes (pH 8.0), 3 mM EGTA, 5 mM 2-mercaptoethanol, and 1 mg/mL BSA (4 mL/g). Microsomal membrane vesicles were essentially prepared as described by Zocchi and Hanson (22). The 80,000g microsomal fraction was resuspended in 250 mM sorbitol, 2.5 mM Tris-Mes (pH 7.2), layered over 10% (w/w) Dextran T-70 prepared in 250 mM sorbitol, 2.5 mM Tris-Mes (pH 7.2), and centrifuged for 2 h at 70,000*g* in a Beckman SW 41 rotor. The membranes at the interface were collected, diluted 10 times in 250 mM sorbitol, 2.5 mM Tris-Mes (pH 7.2), and centrifuged at 80,000*g* for 30 min. The pelleted membranes were resuspended in 250 mM sorbitol, 2.5 mM Tris-Mes (pH 7.2) and used for the ATPase assay. The cytologic origin of the microsomal membranes was not determined.

ATPase Activity

ATPase activity of microsomal membranes was measured as Pi released from ATP, according to Hodges and Leonard (9), at pH 6.5 in a 0.5 mL final volume containing 30 mM Tris-Mes, 3 mM Tris-ATP, 3 mM $MgSO_4$, and 25 to 50 μg protein vesicles at 27°C for 30 min.

RESULTS

Plants were grown in hydroponic culture in the presence (+Fe roots) or absence (−Fe roots) of iron. Roots were used after the onset of medium acidification (about 5–6 d after transfer to the culture solution). Figure 1 compares the capacity of −Fe and +Fe roots to acidify the external medium. Only −Fe roots were able to acidify the medium (Fig. 1A), while +Fe roots even after 2 h of iron deficiency had not shown this capacity (Fig. 1B). Administration of different ionic forms of iron gave different responses. Fe^{2+} strongly inhibited acidification of the medium in −Fe roots, while Fe^{3+} showed only a slight effect; +Fe roots did not show any appreciable response to either Fe^{2+} or Fe^{3+}. Similar results were obtained by using either FeEDTA or $Fe_2(SO_4)_3$ as a source of Fe^{3+} ions (data not shown).

The electrical potential difference is a good parameter for evaluating ion fluxes across membranes. The stronger acidification capacity showed by −Fe roots corresponded to a more negative PD value (−145 mV) than that of the +Fe roots (−105 mV) (Fig. 2, A and B). Different ionic forms of iron influenced the PD value in different ways. Figure 2A shows that the presence of Fe^{2+}, in −Fe roots, strongly depolarized the cells (down to −85 mV), whereas the presence of Fe^{3+} had a smaller effect; in +Fe roots the depolarizing effect of Fe^{2+} was very small, whereas Fe^{3+} had a small hyperpolarizing effect. Similar results were obtained by using either FeEDTA or $Fe_2(SO_4)_3$ as a source of Fe^{3+} (data not shown). Acidification of the medium and PD might have different origins: that is through the activity of a plasma membrane H^+-ATPase and/or an electron transport across the plasma membrane, perhaps in this case linked to the reduction of the ferric form. The above data suggested the possibility of an increased activity of the ATPase during Fe-deficiency. This was tested for directly. Table I shows that the ATPase activity extracted from −Fe roots was about 30% higher than that

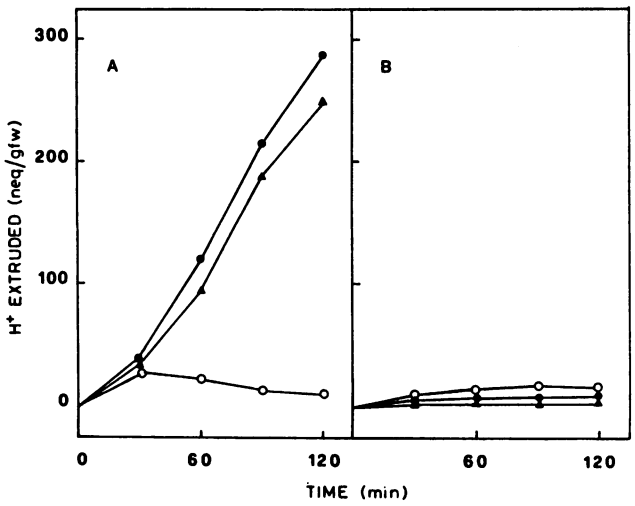

Figure 1. Acidification of the medium in −Fe (A) and +Fe (B) roots. Effect of Fe^{2+} (○) and Fe^{3+} (▲) form. Iron was added as sulfate salts at 0.1 mM concentration. (●) control. Each point is the mean of five independent experiments. SE did not exceed ±6%.

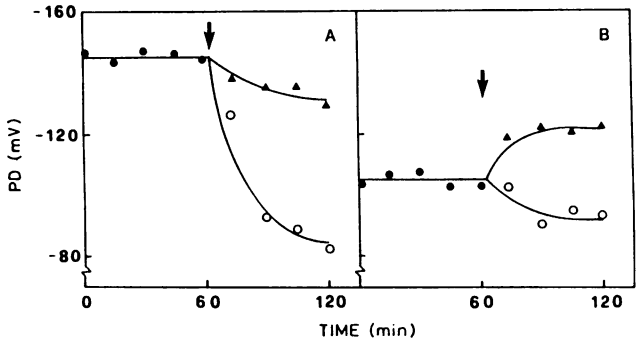

Figure 2. Transmembrane electrical potential difference (●) in −Fe (A) and +Fe (B) roots. Segments were incubated in 5 mM sucrose, 0.5 mM $CaSO_4$ (pH 6.2). At the arrows Fe^{2+} (○) or Fe^{3+} (▲) was added, as sulfate salts, at 0.1 mM final concentration. Each point is the mean of five independent experiments. SE did not exceed ±6%.

Table I. *ATPase Activity in Microsomal Fractions*

Microsomal fractions were extracted from roots grown in the absence or in the presence of iron. ATPase activity was measured at 27°C in 30 mM Tris-Mes, 3 mM $MgSO_4$, and 3 mM ATP-Tris (pH 6.5). Values are the means of four experiments run in triplicate ± SE.

Treatment	ATPase Activity	
	−Fe	+Fe
	μmol Pi/mg protein 30 min	
Control	13.8 ± 0.8	10.9 ± 1.3
+ KCl (50 mM)	17.3 ± 1.0	15.3 ± 1.0

Table II. *Effect of Fe^{2+} on the ATPase Activity*

ATPase activity was measured as in Table I on microsomal fractions extracted from roots grown in the absence or in the presence of iron. Fe^{2+} was added as sulfate salt. Values are the means of four independent experiments. SE did not exceed ±5%.

Treatment	ATPase Activity	
	−Fe	+Fe
	μmol Pi/mg protein 30 min	
Control	14.3	9.6
$+Fe^{2+}$ 5×10^{-5}	14.3 (0)[a]	9.8 (0)
10^{-4}	7.9 (45)	6.6 (31)
2×10^{-4}	4.3 (70)	3.4 (64)
4×10^{-4}	4.2 (71)	3.5 (63)

[a] Numbers in parentheses represent % inhibition.

Table III. *Effect of Fe^{3+} on the ATPase Activity*

ATPase activity was measured as in Table I on microsomal fractions extracted from roots grown in the absence or in the presence of iron. Fe^{3+} was added as sulfate salt. Values are the means of four independent experiments. SE did not exceed ±5%.

Treatment	ATPase Activity	
	−Fe	+Fe
	μmol Pi/mg protein 30 min	
Control	11.5	7.4
$+Fe^{3+}$ 5×10^{-5}	10.8 (6)[a]	7.0 (5)
10^{-4}	11.2 (2)	7.1 (4)
2×10^{-4}	10.6 (8)	7.1 (4)
4×10^{-4}	ND	ND

[a] Numbers in parentheses represent % inhibition.

extracted from +Fe roots. This activity was stimulated by KCl to the same extent in both of the extracts. Tables II and III show the effect of different ionic forms of iron on the ATPase activity: Fe^{2+} at very low concentration strongly inhibited the ATPase activity (about 50% at 10^{-4} M), while Fe^{3+} had little effect. Other divalent cations (Ni^{2+} and Co^{2+}) tested did not show any inhibition even at 1 mM concentrations.

DISCUSSION

The increased acidification capacity induced by iron deficiency in Fe-efficient plants has long been considered a way by which plants decrease the pH of the rhizosphere and make iron more available for absorption (2). The data presented in this paper extend this view by demonstrating the close correlation between acidification of the external medium, hyperpolarization of the transmembrane electrical potential, and increased H^+-ATPase activity. In fact, plants grown in the absence of iron showed all these parameters to a much greater extent than the corresponding plants grown in the presence of iron. The increased acidification of the external medium in −Fe roots (Fig. 1, A and B) was well correlated with the larger PD value (Fig. 2, A and B) and with the higher ATPase activity (Table I). Moreover, Fe^{2+}, the ionic form readily taken up (5), which induced in −Fe roots a decrease in the medium acidification capacity and in the PD value, also inhibited the ATPase activity. It is very interesting to point out that, in −Fe roots, an appreciable effect on both parameters was also demonstrated for the Fe^{3+} form, probably as a consequence of the reduction to Fe^{2+}, its uptake, and its effect on PD and ATPase activity. It cannot be ruled out that the depolarizing effect in the presence of Fe^{3+} may be due to an imbalance in the transport of electrons and protons across the plasma membrane by the Fe-reductase, in agreement with Sijmons *et al.* (21). In any case, the absence of a Fe^{3+} effect on medium acidification suggests that the described electron transport across the plasma membrane is not linked to the bulk of proton extrusion. The small hyperpolarizing effect of the Fe^{3+} form in +Fe roots (Fig. 2B) may be explained, in agreement with Sijmons *et al.* (21), by hypothesizing that the imbalance in the ratio of electrons and protons ($2e^-/H^+$) transported across the plasma membrane by the Fe-reductase activity increases proton availability in the cytoplasm and activates the H^+-ATPase mechanism generating a hyperpolarization. This is in agreement with the hypothesis of other authors (4, 13, 20) of an indirect control of redox mechanisms on the ATPase activity. This interpretation might be accepted if one hypothesized that in this material two H^+/ATPases are operating that are differently affected by the cytoplasmic Fe^{2+} concentration.

In conclusion, our data suggest that Fe-efficient plants increase Fe uptake capacity through the activation of an ATPase involved in medium acidification and PD genesis and of an electron transport system useful to the iron reducing activity. The *in vitro* inhibition of the ATPase and the *in vivo* inhibition of proton extrusion by Fe^{2+} suggest that this mechanism is controlled by the concentration of Fe^{2+} in the cytoplasm. Moreover, since in −Fe roots proton extrusion operates in the presence of a highly negative value of PD, while in +Fe roots and in other materials (14, 16) proton extrusion appears to be inhibited by PD hyperpolarization, it is suggested that in −Fe roots this mechanism is released from a direct or indirect control by PD.

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