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Proteins under the Control of the Gene for Fe Efficiency in Tomato

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ABSTRACT

Fe-deficient dicotyledons develop Fe-efficiency reactions, such as proton extrusion and ferric chelate reduction activity, which are located in the plasma membranes of the root epidermal cells. The fer mutant of tomato (*Lycopersicon esculentum* Mill.) cannot develop these reactions. Membranes were isolated from roots of wild-type (FER) and mutant (fer) tomato plants grown on nutrient solution with high and low Fe concentrations. Two proteins were identified which are synthesized under the control of the FER gene.

Dicotyledonous plants grown with limited iron increase their capacity to mobilize and absorb iron from the soil. This "Fe-efficiency" response may include formation of extra root hairs, development of rhizodermal transfer cells, excretion of protons, and the capacity to reduce extracellular ferric chelates (Turbo reductase) and to take up ferrous ions at high rates (17). These developments are regulated in the roots (5), although the shoot can exert influence (15). The regulation mechanism is unknown.

A mutant tomato has been isolated which is unable to develop these Fe-efficiency reactions (6, 7, 11); it develops severe chlorosis on normal soils (20). The mutation is recessive and affects a gene (FER; 20) that segregates in a Mendelian fashion. Apparently, FER codes for an essential factor in the regulation of Fe-efficiency reactions.

Iron efficiency in dicotyledons may be likened to the regulation of iron uptake by *Escherichia coli* in which a single protein controls synthesis of a siderophore and the receptor for the ferric-siderophore complex (1). This protein is activated when it binds ferrous ions and then blocks transcription of the regulated genes by binding to *cis*-regulatory elements.

I propose that the FER gene codes for a protein, comparable to the fur protein in *E. coli*, which controls the transcription of genes that mediate the Fe-efficiency response. In contrast to the *E. coli* fur protein, however, the FER protein is an activator, because the recessive mutation lacks the response.

To understand how plants control their iron uptake, it is essential to characterize the FER protein. Identification through its Fe-binding properties is difficult, since many proteins bind ferrous and ferric ions and the FER protein is probably rare. An alternative is to isolate proteins which are controlled by FER and then search for the regulatory components through DNA-binding assays. The best characterized Fe-efficiency reactions, proton extrusion by a proton-pumping ATPase (10, 18) and the ferric chelate reduction system (Turbo reductase; 3, 4), are located in the epidermal plasma membranes of young roots. The proteins that make part of these systems should be absent or present at low levels in roots grown with sufficient iron and in the fer mutant. The present work was aimed at the identification of one or more or these proteins.

MATERIALS AND METHODS

Growth of Plants. Seeds of tomato (*Lycopersicon esculentum* Mill.) cv "Floradel" (genotype FER) and T3820fer (genotype fer) were a gift from Dr. V. Römheld, University of Hohenheim, Stuttgart, FDR. They were sown on vermiculite moistened with nutrient solution containing 5 mM $Ca(NO_3)_2$, 2 mM KH_2PO_4, 1 mM KCl, 0.5 mM $MgSO_4$, 0.5 mM $(NH_4)_2SO_4$, 60 μM Fe-HEDTA[2], 50 μM H_3BO_3, 6 μM $MnCl_2$, 1.5 μM $ZnSO_4$, 0.6 μM $CuCl_2$, and 0.3 μM $(NH_4)_6Mo_7O_{24}$, at 25°C and a 16 h light/8 h dark light regime. After 2 weeks the plants were transferred to water culture, seven plants per container with 4 L nutrient solution, which was regularly refreshed. The light regime was 16 h light/8 h dark (cool-white fluorescent at 30 W/m^2); the temperature was 22 to 27°C. The concentrations of Fe-HEDTA in "high Fe" nutrient solution was 60 μM; this yielded dark green shoots for both varieties. "Low Fe" nutrient solution contained 0.5 μM Fe-HEDTA for FER plants, and 5 to 15 μM for fer plants, depending on the conditions in the growth room; both varieties had yellow-green shoots with this treatment.

Preparation of Root Membranes. Young roots (apical 5–10 cm) were harvested from 5- to 7-week-old plants, 1 d after the nutrient solution was renewed. They were rinsed with ice-cold water, dipped dry with Kleenex, and weighed. All further treatments were performed at 4°C. Thirty g roots were cut with a razor blade into 1-cm pieces and mixed with 100 mL 0.5 M sucrose, 50 mM Hepes-KOH, 5 mM ascorbate, 3 g polyvinylpolypyrrolidone, 2 mM DTT (pH 7.5). After vacuum infiltration the mixture was homogenized for 2 × 10 s with a Moulinex vegetable mixer equipped with razor blades. The homogenate was filtered through two layers of Miracloth and centrifuged for 10 min at 10,000*g*. The supernatant was then subjected to 30 min centrifugation at 50,000*g*. The precipitate was suspended in 9 mL 0.33 M sucrose, 5 mM K-phosphate, 2 mM KCl (pH 7.8). Of this homogenate, 9 mL were layered on 27 g "phase mixture" (6.2% in PEG and dextran, containing 2 mM KCl [14]). After shaking for 15 s, the mixture was centrifuged for 5 min at 3,000*g*. The upper layer was washed twice with 15 mL lower layer of "phase system" (14). The final upper layer was diluted with 100 mL of 0.5 M sucrose, 50 mM Hepes-KOH (pH 7.5), and after 1 h centrifugation at 100,000*g*, the resulting pellet was resuspended in the buffer ("membranes").

ATPase Activity was determined in 160 μL 250 mM sucrose, 25 mM Tris-Mes, 50 mM KCl, 3 mM $MgSO_4$, 3 mM Na-ATP,

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[2] Abbreviation: HEDTA, hydroxyethylenediaminetriacetic acid.

0.025% Triton X-100 (pH 6.0) at 25°C. After 1 h, the mixture was cooled to 0°C, and 60 μL ice-cold 33% (w/v) TCA were added, followed by 5 min centrifugation at 0°C. Of the supernatant, 180 μL were added to 180 μL 1 M KOH at room temperature, followed by 120 μL 1% SDS, 300 μL 3.3 mM $(NH_4)_6Mo_7O_{24}$ in 1.6 N H_2SO_4, and 200 μL 170 mM ascorbic acid, 2.5 mM $SnCl_2$ in 0.6 N H_2SO_4. After 1 h the A_{680}–A_{500} was determined.

Table I. *ATPase and Cyt Oxidase Activities of Membrane Preparations from Roots of FER and fer Genotype Tomatoes, Used for Two-Dimensional Gel Electrophoresis Shown in Figure 1*

All activities were measured in the presence of Triton X-100.

Enzyme Activity	Source of Membrane Preparation			
	Floradel (FER) grown on		T3820fer grown on	
	High Fe	Low Fe	High Fe	Low Fe
	nmol·min⁻¹·mg protein⁻¹			
ATPase				
Control	66	118	61	95
+ 30 μg/mL oligomycin	56	112	51	74
+ 0.1 mM vanadate	21	22	26	30
Cyt oxidase	40	36	43	25

Cyt Oxidase was determined in 50 mM K-phosphate (pH 7.5), 0.02% Triton X-100, 23 μM reduced Cyt *c* from horse heart, at 25°C. The rates were determined at 22 μM Cyt c_{red}. Protein was determined with 0.1 strength Bradford reagent (Bio-Rad) + 0.003% Triton X-100, with BSA as reference.

Protein was extracted from membranes with phenol (8), omitting phenylmethylsulfonyl fluoride, with 100 mM DTT instead of mercaptoethanol. The final pellet was left to dry on air until the smell of acetone had disappeared; it was then immediately solubilized as described (8).

Two-Dimensional Electrophoresis was performed according to Hurkman and Tanaka (8) and Bauw *et al.* (2) with modifications. Samples containing 10 to 30 μg protein were loaded at the acid end of the electrofocusing gel and overlaid with 10 μl 8 M ureum, 1% ampholytes (pH 3–10), 5% Nonidet P-40, 100 mM DTT. The anodic solution was 10 mM H_3PO_4; the cathodic solution was 20 mM NaOH. The voltage used for electrofocusing was brought to 400 V in 15-min steps of 100 V; total duration was 5.5 h. SDS electrophoresis was performed in 12% acrylamide, overlaid with 4% stacking gel. After placing the electrofocusing gel on the stacking gel, it was covered with 60 mM Tris-HCl (pH

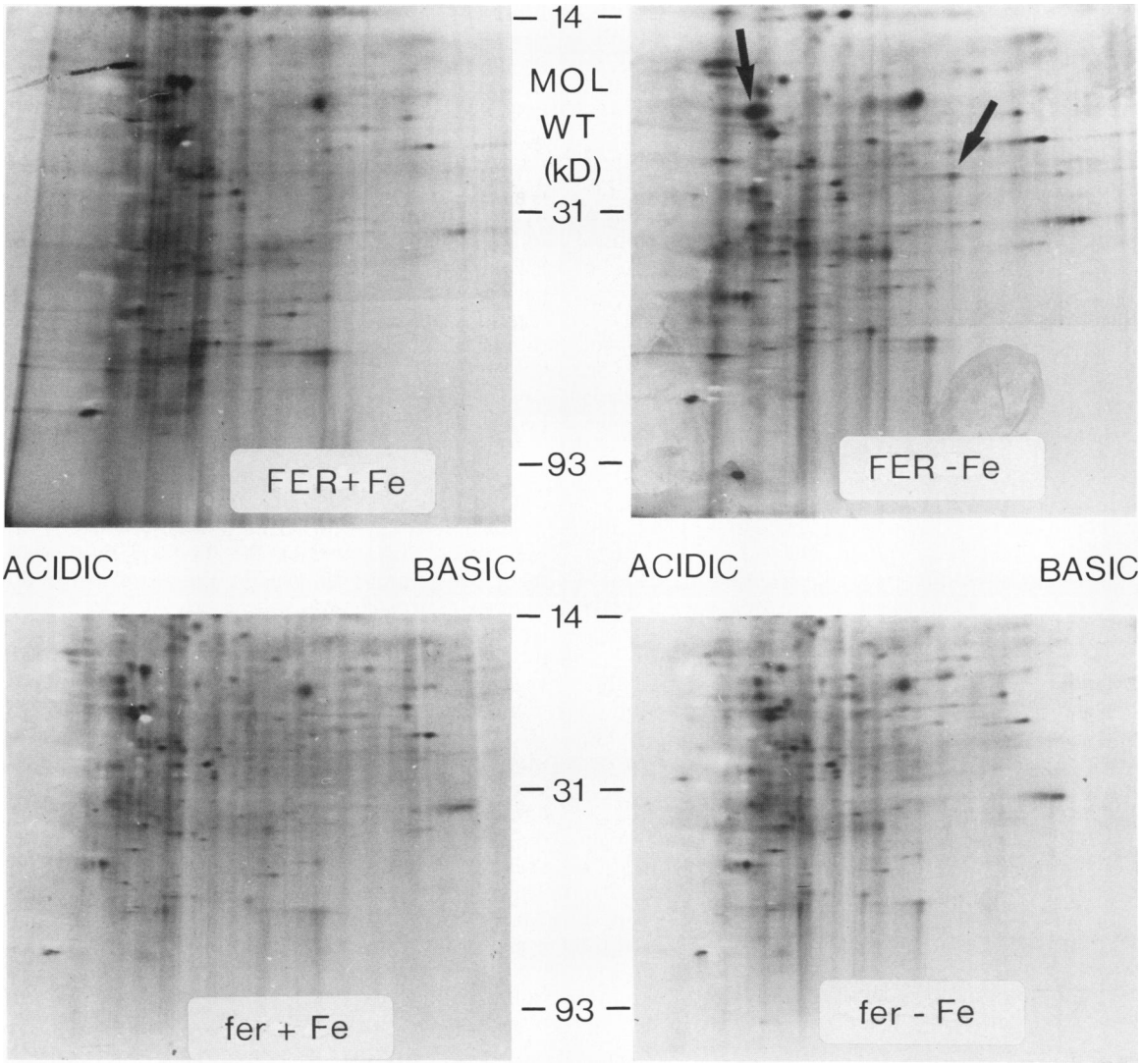

FIG. 1. Two-dimensional electrophoresis gels of membrane proteins from roots of FER (top) and fer (bottom) genotype tomatoes grown on high Fe (left) and low Fe (right) levels. The arrows indicate proteins synthesized under the control of the FER gene.

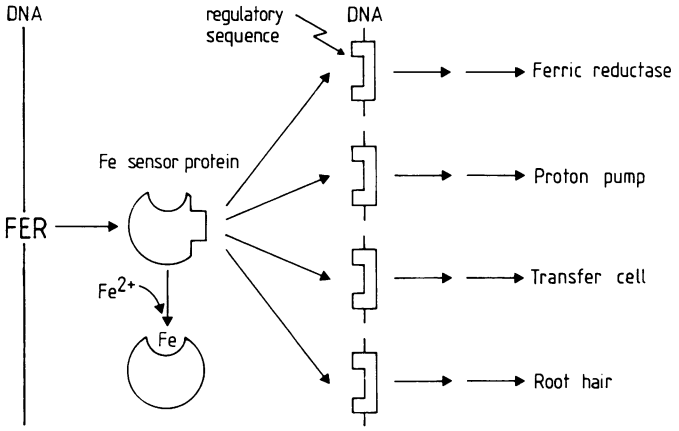

FIG. 2. Hypothesis for the regulation of Fe-efficiency reactions in tomato. The FER gene encodes a regulatory protein that can bind to common sequence elements which activate genes involved in Fe-efficiency reactions, inducing transcription. The regulatory protein can bind ferrous ions and, in doing so, changes its conformation so that it can no more bind to the genes' regulatory sequences.

6.8), 2% SDS, 100 mM DTT, 10% glycerol, 0.002% bromophenol blue. The anodic and cathodic buffers were 25 mM Tris, 195 mM glycine, 0.1% SDS. All acrylamide solutions used were filtered over charcoal and 0.22-μm filters. Gels were fixed overnight in 25% (v/v) isopropanol, 10% (v/v) acetic acid, washed four to five times with water for 2 d, and stained with silver (19).

RESULTS AND DISCUSSION

Shoots of the tomato variety T3820 genotype fer and of the wild type variety Floradel (genotype FER) could not be distinguished when the plants were grown at high Fe concentrations (60 μM). At 0.5 μM Fe, the FER type showed slight symptoms of chlorosis; the roots developed the classical Fe-efficiency reactions. At this low Fe concentration, the mutant type fer developed severe chlorosis and stopped growth. With 5 to 15 μM Fe, it developed a yellow-green habitus comparable to FER grown with 0.5 μM. The mutant did not develop any of the known Fe-efficiency reactions.

Membranes were isolated from young roots, in order to identify proteins synthesized under control of the FER gene. Under low Fe conditions, FER plants develop rhizodermal transfer cells, where proton extrusion and ferric reduction take place at the plasma membranes (13). Fe-deficiency-induced rhizodermal transfer cells (12), like transfer cells in general (16), contain many mitochondria. Thus, under conditions where Fe-deficiency-induced proteins are expected, mitochondrial contamination should be greatest. Therefore, the two-phase method of plasma membrane isolation was chosen, which yields membrane preparations with minimal mitochondrial contamination (14). In addition, in this procedure a step was omitted that increases yield but also the risk of contamination, *i.e.* extraction of the first lower phase with fresh upper phase. The ATPase activity of all preparations was 10 to 25% inhibited by oligomycin. This suggests mitochondrial contamination, but Cyt oxidase activities did not correspond to oligomycin sensitivity of each preparation. Kasamo (9) reported 19% inhibition by oligomycin of purified root plasma membrane ATPase from *Phaseolus mungo*; possibly the same holds for tomato. I therefore used Cyt oxidase as an indicator of mitochondrial contamination and for further analysis used only preparations with an activity lower than 50 nmol·min^{-1}·mg $protein^{-1}$ (Table I). The ATPase of the membranes was not inhibited by nitrate (data not shown).

The two-dimensional electrophoresis protein patterns of membranes from the same type of tissue varied somewhat. However, protein patterns from FER grown on high and on low Fe showed a number of consistent differences. Some proteins present in the high Fe preparations were absent in those from low Fe. This is most likely due to inhibition of the synthesis, or increased lability, of (Fe-) proteins. On the other hand, two proteins were always present in the low Fe preparations and absent in high Fe preparations (Fig. 1, top). The same two proteins were also absent in the preparations from the fer mutant, whether grown on high or on low Fe (Fig. 1, bottom). Therefore, these proteins are not part of a stress response to growth inhibition by a general nutrient deficiency. Their expression depends on FER, which may be a transcription activating factor as depicted in Figure 2. Alternatively, the Fe-binding protein, which detects Fe deficiency, is a cytoplasmic protein that activates the transcription factor through modification. In this case, the FER-encoded product can be either protein.

The two induced proteins can now be used as a starting point to unravel the regulation of the Fe-efficiency response in dicotyledons.

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