

Iron and Copper Nutrition-Dependent Changes in Protein Expression in a Tomato Wild Type and the Nicotianamine-Free Mutant *chloronerva*¹

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The nicotianamine-deficient mutant *chloronerva* resembles phenotypically an Fe-deficient plant despite the high accumulation of Fe in the leaves, whereas it suffers from Cu deficiency in the shoot. Two-dimensional electrophoretic separation of proteins from root tips and leaves of wild-type *Lycopersicon esculentum* Mill. cv Bonner Beste and the mutant grown with and without Fe showed a number of consistent differences. In root tips of the Fe-deficient wild type and the Fe-sufficient as well as the Fe-deficient mutant, the expression of glyceraldehyde-3-phosphate dehydrogenase, formate dehydrogenase, and ascorbate peroxidase was increased. In leaves of the Fe-sufficient and -deficient mutant, Cu-containing chloroplastic and cytosolic superoxide dismutase (Cu-Zn) and plastocyanin (Cu) were nearly absent. This low plastocyanin content could be restored by supplying Cu via the xylem, but the superoxide dismutase levels could not be increased by this treatment. The differences in the protein patterns between wild type and mutant indicate that the apparent Fe deficiency of mutant plants led to an increase in enzymes involved in anaerobic metabolism as well as enzymes involved in stress defense. The biosynthesis of plastocyanin was diminished in mutant leaves, but it was differentially induced by increased Cu content.

Strategy I plants (dicots and nongraminaceous monocots) respond to Fe deprivation by thickening of root tips, differentiation of transfer cells, enhanced proton extrusion, and increased activity of the rhizodermal Fe(III) reductase (Bienfait et al., 1989). Fe-deficient plants of BB also exhibit these responses to Fe deficiency. However, independent of the Fe supply, chl_n behaves like an Fe-deficient plant, although it accumulates Fe, Mn, Zn, and Cu in different organs. Fe and Mn are accumulated in shoots and roots, but Cu accumulation is restricted to the roots (Pich et al., 1994). Additionally, young leaves of chl_n develop intercostal chlorosis. The biochemical basis of this apparent Fe deficiency is the lack of NA, a non-protein amino acid found in all multicellular plants investigated up to this time, the exception of the tomato mutant chl_n (Rudolph et al., 1985). NA is also a key substance in the biosynthesis of MAs (strategy II). NA

forms stable complexes with divalent ions such as Fe, Cu, Zn, and Mn (Beneš et al., 1983; Anderegg and Ripberger, 1989). Because of its high affinity for Cu, it has been suggested that NA may transport Cu in or into the xylem or possibly mobilize Cu in the roots (Pich et al., 1994; Pich and Scholz, 1996). In addition, NA may function as a chelator of Fe in phloem transport (Scholz, 1989; Schmidke and Stephan, 1995).

The physiology of Fe uptake has been extensively studied (Marschner et al., 1986), but little is known about Fe-dependent gene expression and regulation in strategy I plants. In strategy II plants (grasses), some Fe-deficiency-specific (Ids) clones have been found using differential hybridization. The putative open reading frame of Ids 1 encodes a protein that contains two Cys-rich regions and may have a function in the regulation of MAs synthetic genes or the gene encoding the Fe(III)-MAs transporter (Okumura et al., 1991). Ids 2 and 3 were identified as 2-oxoglutarate-dependent dioxygenases. Nakanishi et al. (1993) suggested that the hydroxylation process that leads to synthesis of MAs may be catalyzed by the dioxygenase (Nakanishi et al., 1993; Okumura et al., 1994). In addition, the activities of NA synthase (Higuchi et al., 1994) and NA aminotransferase (Kanazawa et al., 1994) are strongly induced in Fe-deficient barley roots, and in this way these enzymes take part in regulation of Fe uptake. For plants surviving under conditions of heat stress, mineral stress, or anaerobiosis, it is important to adapt to environmental variations through regulation of enzyme activities and gene expression.

In this paper we report the differential expression of proteins isolated from the Fe-sufficient and Fe-deficient wild-type tomato plant BB and its mutant chl_n. The goal of our research is the identification of proteins involved in Fe acquisition.

Abbreviations: AP, ascorbate peroxidase; BB, *Lycopersicon esculentum* Mill. cv Bonner Beste; chl_n, *Lycopersicon esculentum* Mill. mutant *chloronerva*; FDH, formate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAs, mugineic acid-family phytosiderophores; NA, nicotianamine (2S:3'S:3''S)-N-[N-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]-azetidine-2-carboxylic acid); PC, plastocyanin; SOD, superoxide dismutase.

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MATERIALS AND METHODS

Plant Growth

Tomato seedlings of BB and chl_n were grown under controlled climatic conditions as described by Stephan and Procházka (1989). At d 12, plants were transferred into boxes with aerated Hoagland nutrient solution including different Fe-EDTA concentrations: BB(+Fe), 10 μM ; BB(-Fe), 0 μM ; chl_n(+Fe), 10 μM ; and chl_n(-Fe), 2 μM . Twenty-one days after sowing, 20-mm root tips and the youngest and oldest leaves were harvested, crushed in liquid nitrogen, and stored at -80°C until use. In the case of Cu-feeding experiments, roots of 17-d-old BB(+Fe) and chl_n(+Fe) plants were cut, and the remaining shoots were incubated for 4 d in Hoagland nutrient solution with 10 μM Fe-EDTA and 5 μM Cu before harvest. Daily, a small disc from the stem was removed with a razor blade to avoid occlusion of xylem vessels by callus.

Protein Extraction

Protein extractions were performed according to Meyer et al. (1988). The precipitates were dissolved in a buffer containing 100 mM Tris/HCl, pH 7.8, 2.5% SDS, 20% glycerol, and 5% mercaptoethanol. Protein extracts were heated for 3 min at 100°C and sonicated for 20 s with Vibra cell (Bioblock Scientific, Vannes, France). Protein concentrations were determined using the Lowry assay after TCA precipitation (Lowry et al., 1951).

Protein Electrophoresis

One-dimensional electrophoresis was performed according to Laemmli (1970). Two-dimensional electrophoresis, performed according to O'Farrell (1974), was modified using the SE600 2-D Electrophoresis System (Hoefer, San Francisco, CA). IEF was accomplished in glass tubes (150 \times 3 mm), closed with Parafilm (American National Can, Neenah, WI) at the bottom, and inserted into a small chamber with 8 M urea. To prepare the gel mixture for 10 IEF gels, 8.25 g of urea, 3 mL of H₂O, 2.0 mL of acrylamide stock (30:5.7% [w/v] acrylamide:bisacrylamide), 3.0 mL of 10% Triton X-100 stock, 0.6 mL of ampholines, pH range 5 to 8, and 0.15 mL of ampholines, pH range 3.5 to 10 (Pharmacia), were added and swirled until the urea was completely dissolved. Twenty-five microliters of 10% ammonium persulfate and 20 μL of *N,N,N',N'*-tetramethylethylenediamine were added, and the solution was loaded into the gel tubes. Overlay solutions and prefocusing were carried out according to O'Farrell (1974). Fifty micrograms of total protein were loaded and gels were run at 300 V for 1 h, at 500 V for 18 h, and at 600 V for 1 h. After IEF the disc gels were extruded into 4 mL of equilibration buffer containing 62.5 mM Tris/HCl, pH 6.8, 1.2% SDS, 10% glycerol, and 5% mercaptoethanol and shaken at room temperature for 20 min. Subsequently, the equilibration buffer was renewed and the IEF gel was stored at -80°C until use. For the second dimension, a 10 to 20% exponential gradient gel (160 \times 180 mm plates and 1.5 mm thick) was prepared and allowed to stand overnight. Ten milliliters of stacking gel

mixture contained 2.4 mL of Rotiphorese Gel 30 (Roth, Karlsruhe, Germany), 2.4 mL of 0.5 M Tris/HCl, pH 6.8, 100 μL of 10% SDS, 50 μL of 10% ammonium persulfate, and 10 μL of *N,N,N',N'*-tetramethylethylenediamine. Gels were run for about 6 h at 60 mA constant current until the dye front reached the bottom. One-dimensional gels were stained with Coomassie blue, and two-dimensional gels were stained with silver according to Jungblut and Seifert (1990). Premixed protein molecular weight standards, low range (Boehringer, Mannheim), were used for one- and two-dimensional electrophoresis. The chosen figures were selected from several representative two-dimensional electrophoresis gels of three independent protein extractions from plants grown in series.

Electroblotting and Automated N-Terminal Sequencing

Electroblotting onto a PVDF membrane (Immobilon-P^{SO}, Millipore) was performed according to Towbin et al. (1979) using a Transphor Electrophoresis Unit TE 42 (Hoefer). Protein spots were detected after staining, according to Eckerskorn and Lottspeich (1993), and were cut out of the membrane exactly along the borders of the protein spots. Proteins were sequenced with a gas-phase sequencer LF 3400 (Beckman) using a standard sequencing procedure. The resulting phenylthiohydantoin amino acids (Pth-Xaa) were analyzed on-line using the HPLC system "Gold" (Beckman). Homology searches against GenBank and SWISS-PROT databases, release April 1995, were performed using the software packages PC/Gene (IntelliGenetics, Zurich, Switzerland), HIBIO DNASIS, and HIBIO PROSIS (Hitachi Software, Tokyo, Japan).

Micronutrient Analyses

Atomic absorption spectrometric analyses were conducted according to Stephan et al. (1995).

RESULTS

BB and chl_n developed different phenotypes when grown with various Fe supplies in the Hoagland nutrient solution. The Fe-deficient wild type showed chlorotic leaves and thickened root tips in contrast to the Fe-sufficient wild type. On the other hand, Fe-sufficient and Fe-deficient mutant chl_n exhibited retarded growth of the whole plant, stunted roots, thickened root tips, and intercostal chlorosis of young leaves. These mutant symptoms were enhanced under Fe deficiency.

One-Dimensional Electrophoresis of Root and Leaf Proteins

In comparisons of one-dimensional electrophoresis gels prepared from 20 mm-root tips of Fe-sufficient and -deficient BB and chl_n (Fig. 1), a 98-kD protein was identified that is nearly absent in the root tips of Fe-sufficient BB, compared to Fe-deficient BB or Fe-sufficient and Fe-deficient chl_n. Attempts to obtain amino acid sequence information for this polypeptide failed, presumably because the N terminus was blocked. One-dimensional electrophoresis

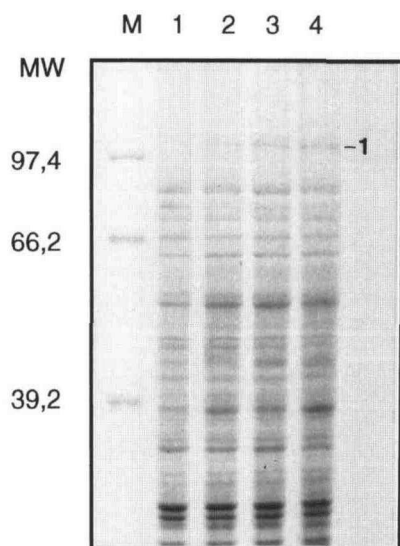


Figure 1. One-dimensional electrophoresis gel from 20-mm root tips of BB and chlIn grown under varying Fe supplies. Lane 1, BB(+Fe); lane 2, BB(-Fe); lane 3, chlIn(+Fe); lane 4, chlIn(-Fe). For details of Fe supply, see "Materials and Methods." Protein 1 is nearly absent in the root tips of Fe-sufficient BB compared to Fe-deficient BB or Fe-sufficient and Fe-deficient chlIn. Lane M, Molecular weight standards. MW, Molecular weight.

of leaf proteins from the Fe-sufficient or -deficient wild type and mutant showed no significant differences (data not shown).

Two-Dimensional Electrophoresis of Root Proteins

Two-dimensional electrophoresis gels prepared from 20-mm root tips of Fe-sufficient or -deficient BB and chlIn showed different protein patterns. In root tips of Fe-sufficient BB, the proteins 2, 3, 4, and 5 were present to a much lesser extent, compared to the root tips of Fe-deficient BB and Fe-sufficient and Fe-deficient chlIn (Figs. 2 and 3). After electroblotting and amino acid sequence analysis, proteins 3, 4, and 5 could be identified on the basis of N-terminal homology to sequences in the SwissProt and GenBank databases. The N-terminal fragment of protein 3 has 91.7% identical amino acids to glycolytic GAPDH from *Zea mays*. Fifteen amino acids of protein 4 are completely identical to those of FDH from *Solanum tuberosum*, and protein 5 has 76.9% identical N-terminal amino acids to AP from *Arabidopsis thaliana* (Tables I and II). A sequence in protein 2 included the amino acids NGKIKI, which have been found to be highly conserved for GAPDH. However, due to many variables in the overall amino acid content, the sequence information for protein 2 could not be used for a reliable database search.

Two-Dimensional Electrophoresis of Leaf Proteins

In leaves of Fe-sufficient or Fe-deficient BB and chlIn, the pattern of various proteins differed (Figs. 4 and 5). The amounts of proteins 6, 7, and 9 were low in the mutant

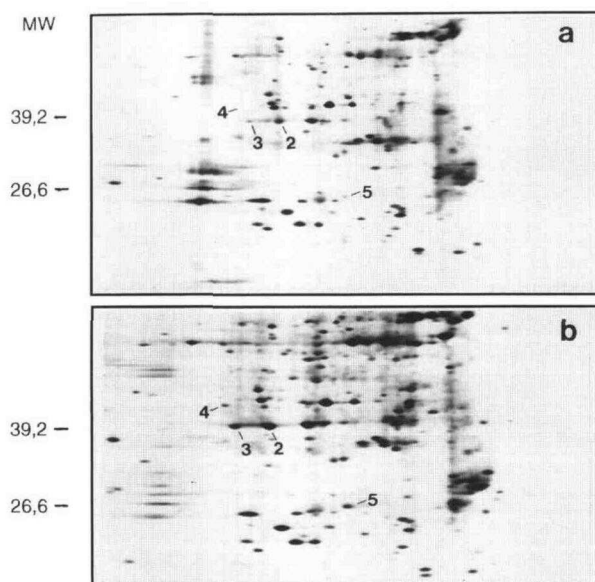


Figure 2. Two-dimensional electrophoresis gel from 20-mm root tips of BB grown with 10 μM Fe-EDTA (a) and without Fe-EDTA in the Hoagland nutrient solution (b). In root tips of Fe-sufficient BB, proteins 2, 3, 4, and 5 were greatly decreased compared to the root tips of Fe-deficient BB. MW, Molecular weight.

leaves compared to Fe-sufficient and Fe-deficient wild-type leaves. Protein 6 was identified by N-terminal sequencing as a chloroplast SOD with 93.3% identity to a Cu-Zn-dependent SOD precursor from *L. esculentum*. Protein 7 showed 92.3% identity to Cu-Zn-dependent cytosolic SOD from *L. esculentum*, and protein 9 showed 86.7% identity to

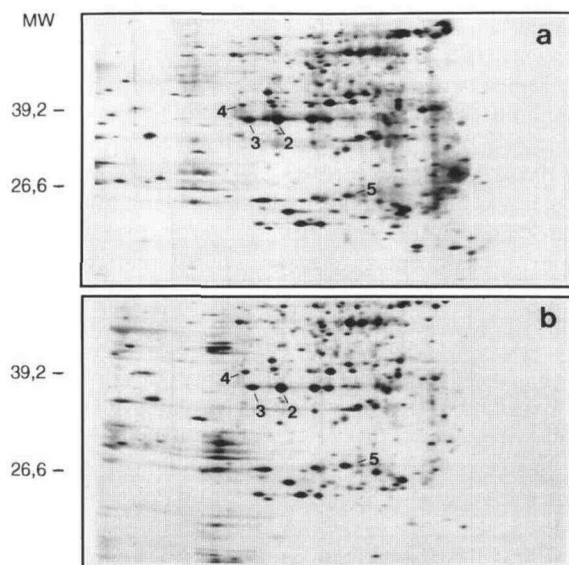


Figure 3. Two-dimensional electrophoresis gel from 20-mm root tips of chlIn grown with 10 μM Fe-EDTA (a) and with 2 μM Fe-EDTA in the Hoagland nutrient solution (b). There are no differences in the protein contents between the root tips of Fe-sufficient and Fe-deficient chlIn. MW, Molecular weight.

Table I. Protein homology data for N-terminal sequenced proteins 2, 3, 4, 5, 6, 7, and 9, differentially expressed in root tips and leaves of BB and chl_n grown under varying iron nutrition

	Homology Data	Identity	aa Overlap	Enzyme
Protein 2	(G) N (G) K I K (I) X (I) N (S) X X K X 10	91.7%	12 aa	GAPDH
Protein 3	N G K I K I G I N G F (C) R X X : : : : : : : : X :			
G3PC_Maize	M G K I K I G I N G F G R I 10			
Protein 4	L Q A S P G P K K I V G V F Y X : : : : : : : : : : X	100%	15 aa	FDA
MISTFDHD	L Q A S P G P K K I V G V F Y 24 38			
Protein 5	G K Y P T V S E E Y L K A V . X : : : : . : : : X	76.9%	11 aa	AP
ATASCOR	K N Y P T V S E D Y K K A V 20			
Protein 6	A T K K A V A V L K G N X N V X : : : : : : : : . : X	93.3%	15 aa	SOD Cu-Zn chloroplast precursor
SODP_LYCES	A T K K A V A V L K G N S N V 64 75			
Protein 7	V K A V A V L N S R E G V X : : : : : : . : : X	92.3%	13 aa	SOD cytosolic
SODC_LYCES	V K A V A V L N S S E G V 10			
Protein 9	L E V L L Q G D D G X L A F I X : : : : : : : : : : X	86.7%	15 aa	PC
PLAS-LYCES	L E V L L G G D D G S L A F I 80			

PC from *L. esculentum* (Tables I and III). Because of the high homology between the Fe- and Cu-Zn-dependent SODs, a multiple sequence alignment was done (Table IV). The result clearly shows that at least in the N terminus no homologies between the Fe-dependent SODs and the Cu-Zn-dependent SODs could be found. On the other hand, there are distinct differences in the amino acid sequences between cytosolic and chloroplast SODs. Therefore, the two SODs identified in this report both belong to the family of Cu-Zn-dependent enzymes, with protein 6 being localized in the chloroplast and protein 7 in the cytosol. Furthermore, the expression of another protein, designated

protein 8, has been found to be increased in the mutant leaves with and without Fe supply, but it was nearly absent in the wild-type leaves with and without Fe supply. This protein, however, was blocked N terminally and no sequence information was obtained.

Cu Concentration in Root Tips and Leaves of BB and chl_n Grown under Various Fe and Cu Supplies

Root tips of Fe-sufficient wild-type plants contained 889 compared to 422 nmol Cu (g dry weight)⁻¹ in leaves. In BB grown under Fe deficiency, Cu accumulated to 6420 nmol

Table II. Proteins of 20-mm root tips, differentially expressed in BB and chl_n grown under varying Fe supply, identified on the basis of homology in the SWISS-PROT and GenBank databases

BB(+Fe) was supplied with 10 μ M Fe-EDTA, BB(-Fe) was supplied with no Fe-EDTA, chl_n(+Fe) was supplied with 10 μ M Fe-EDTA, and chl_n(-Fe) was supplied with 2 μ M Fe-EDTA.

Protein	BB (+Fe)	BB (-Fe)	chl _n (+Fe)	chl _n (-Fe)	Identified Enzyme	Subcellular Localization	Ref.
1	-	+	+	+	N-terminal blocked, 98 kD		
2	-	+	+	+	37 kD		
3	-	+	+	+	GAPDH of <i>Z. mays</i>	Cytoplasmic	Brinkmann et al. (1987)
4	-	+	+	+	FDH of <i>S. tuberosum</i>	Mitochondrion	Colas des Francs-Small et al. (1993)
5	-	+	+	+	AP of <i>A. thaliana</i>	Cytoplasm	Kubo et al. (1992)

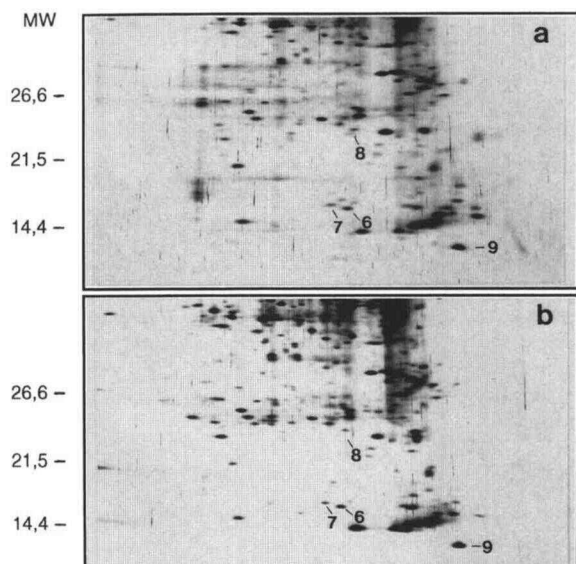


Figure 4. Two-dimensional electrophoresis gel from the youngest leaves of BB grown with 10 μM Fe-EDTA (a) and without Fe-EDTA in the Hoagland nutrient solution (b). There are no differences in the protein contents of proteins 6, 7, 8, and 9 between Fe-sufficient and Fe-deficient BB. MW, Molecular weight.

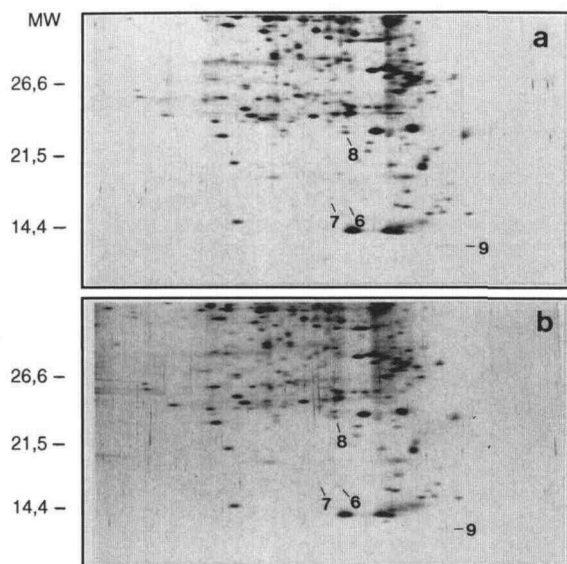


Figure 5. Two-dimensional electrophoresis gel from the youngest leaves of chlN grown with 10 μM Fe-EDTA (a) and with 2 μM Fe-EDTA in the Hoagland nutrient solution (b). There are no differences in the protein contents of proteins 6, 7, 8, and 9 between Fe-sufficient and Fe-deficient chlN. MW, Molecular weight.

Cu (g dry weight) $^{-1}$ in the roots but no increase was seen in leaves. After feeding the wild type via the xylem with 5 instead of 0.3 μM Cu, no significant increase in the Cu content was detected in the leaves compared to plants grown under normal Cu supply. In root tips of Fe-sufficient and Fe-deficient chlN, Cu accumulated but the leaves contained only about 30 nmol Cu (g dry weight) $^{-1}$, which indicates the leaves were severely Cu deficient. However, after feeding chlN with 5 μM Cu via xylem, the Cu content increased about 8-fold up to 245 nmol Cu (g dry weight) $^{-1}$ in the leaves (Table V).

Two-Dimensional Electrophoresis of Mutant Leaf Proteins after Cu Feeding

The two-dimensional protein electrophoresis gel of leaf material harvested from chlN after Cu feeding showed an increase in the amount of the Cu-containing enzyme PC (protein 9) but no changes of the Cu-Zn-dependent SODs compared to chlN grown under normal Cu supply (Fig. 6).

DISCUSSION

In the present study, we have investigated changes in protein patterns induced by Fe deficiency in the tomato cultivar BB and the mutant chlN. In Fe-deficient root tips of BB, the concentration of a number of polypeptides was increased compared to Fe-sufficient controls. In general, the pattern of polypeptides in root tips of chlN regardless of Fe status corresponded to that of Fe-deficient BB. Three polypeptides showing increased concentrations under Fe-deficiency stress were identified by N-terminal sequencing to be GAPDH, FDH, and AP. Based on the known functions of these enzymes, we speculate here on the function they may have in the response of roots to Fe deficiency.

GAPDH

The glycolytic enzyme GAPDH catalyzes the first step in the second phase of glycolysis. It is known that heat stress (reviewed by Lindquist and Craig, 1988) and anaerobiosis (Russell and Sachs, 1982) lead to an increase in the gene

Table III. Leaf proteins, differentially expressed in BB and chlN grown under varying Fe supply, identified on the basis of homology in the SWISS-PROT and GenBank databases

BB(+Fe) was supplied with 10 μM Fe-EDTA, BB(-Fe) was supplied with no Fe-EDTA, chlN(+Fe) was supplied with 10 μM Fe-EDTA, and chlN(-Fe) was supplied with 2 μM Fe-EDTA.

Protein	BB (+Fe)	BB (-Fe)	chlN (+Fe)	chlN (-Fe)	Identified Enzyme	Subcellular Localization	Ref.
6	+	+	-	-	SOD Cu-Zn precursor of <i>L. esculentum</i>	Chloroplast	Perl-Trevers et al. (1988)
7	+	+	-	-	SOD Cu-Zn of <i>L. esculentum</i>	Cytoplasmic	Perl-Trevers et al. (1988)
8	-	-	+	+	N-terminal blocked		
9	+	+	-	-	PC precursor of <i>L. esculentum</i>	Chloroplast	Detlefsen et al. (1989)

Table IV. Multiple sequence alignment of different Fe- and Cu-Zn-containing chloroplast and cytosolic SODs and the differentially expressed proteins 6 and 7

The alignment clearly shows that at least in the N terminus no homologies could be found between the Fe-dependent SODs and the Cu-Zn-dependent SODs. Distinct differences exist in the amino acid sequences between cytosolic and chloroplast SODs.

		Homology Data	SODs
1.	SODF_ARATH	--GKPLEHI IHSTYNGDLLPAFNNAQAawnH	Chloroplast (Fe), <i>A. thaliana</i>
2.	SODF_NICPL	--GKTLEDI ILVTYNKGAPLPAFNNAQAawnH	Chloroplast (Fe), <i>N. plumbagenifolia</i>
3.	SODF_SOYBN	--GKSLEEE IIVTSYNKGDILPAFNNAQVvWH	Chloroplast (Fe), <i>Glycine max</i>
4.	SODC_LYCES	-----KA--VAVLNSSEGVSGT	Cytosolic (Cu-Zn), <i>L. esculentum</i>
5.	SODC_MAIZE	-----KA--VAVLNAGTD-VKGT	Cytosolic (Cu-Zn), <i>Z. mays</i>
6.	SODC_ARATH	-----KG--VAVLNSSEGVVVG	Cytosolic (Cu-Zn), <i>A. thaliana</i>
7.	PROT_7	-----VKA--VAVLNSREGV	
8.	SODP_LYCES	---KPL--TVFAATKKA--VAVLKGNNSVEGV	Chloroplast (Cu-Zn), <i>L. esculentum</i>
9.	SODP_PETHY	---KPF--IVFAATKKA--VAVLKGTNSVEGV	Chloroplast (Cu-Zn), <i>Petunia hybrida</i>
10.	PROT_6	-----ATKKA--VAVLKGNXNV	

expression of GAPDH and the expression of other glycolytic genes. This "coincides with morphological changes in the mitochondria. . . of barley aleurone layers (Belanger et al., 1986) and soybean root tips (Chen et al., 1988). It has been surmised that glycolytic gene induction is necessary for the survival of these organisms when mitochondrial functions are compromised either by heat stress or reduced oxygen levels" (Sachs and Ho, 1986; Webster and Murphy, 1988) or increased ATP turnover (Russell and Sachs, 1989). An impairment of Fe-containing enzymes of oxidative metabolism by the lack of Fe or at least biologically active Fe in the case of *chl*n possibly causes an increase in anaerobic metabolism, resulting in a greater ability to synthesize ATP.

FDH

Similar to GAPDH, the concentration of the dimeric enzyme FDH is also minimal in Fe-deficient root tips and in *chl*n tips regardless of Fe status. FDH catalyzes the oxidation of formate to CO₂ in the presence of NAD⁺. It is known that formate is produced by potato tubers and roots under anaerobic stress, which led Colas des Francs-Small et al. (1993) to suggest a correlation between FDH activity and anaerobic metabolism. In addition, higher-plant mitochondria from nonphotosynthetic plant tissue might contain a formate-producing fermentation pathway similar to those described in bacteria and algae (Colas des Francs-Small et al., 1993).

Because of the observed changes in the protein concentrations of GAPDH and FDH in relation to Fe deficiency, heat stress, and anaerobiosis, the increased synthesis of

these enzymes might be more generally regarded in a framework of stress physiology.

AP

AP is involved in cellular defense mechanisms against oxidative stress by its ability to catalyze H₂O₂ reduction. It is known that the AP activity is induced by environmental stresses such as ozone exposure and drought (Kubo et al., 1992). The cytosolic AP is highly expressed in Fe-deficient wild type as well as in the Fe-deficient and Fe-sufficient mutant. In the case of *chl*n, the increased AP activity correlated with high Fe and Cu concentrations in the root tips and enhanced free oxygen radicals (Haber-Weiss reaction; Pich and Scholz, 1993). Although in Fe-deficient BB the protein concentration of AP is higher compared to Fe-sufficient wild type, the measured enzyme activity was lower in Fe-deficient BB (Pich and Scholz, 1993), suggesting an as-yet unknown posttranslational regulation or inactivation. As with GAPDH and FDH, the increase in AP is likely to be an example of a general response to stress.

In leaves of Fe-sufficient and Fe-deficient *chl*n, the Cu-containing cytoplasmic and chloroplast SODs (Cu-Zn) as well as PC were nearly absent or present at very low levels compared to the Fe-sufficient and Fe-deficient wild type. Normally, it is very difficult to induce Cu deficiency in higher plants because Cu is a ubiquitous metal ion. In root tips of *chl*n, Cu accumulates, but the leaves continuously suffer from Cu deficiency (Table V). These results are in agreement with the results from a previous investigation (Pich and Scholz, 1995), which leads to the conclusion that Cu deficiency in leaves seems to be an additional symptom

Table V. Cu concentrations nmol Cu (g dry weight)⁻¹ in root tips and leaves of BB and *chl*n grown under varying Fe supply (+Fe/-Fe) and after supply with 5 μM Cu via the xylem

For details of Fe supply, see "Materials and Methods." Values are means of two or three individual analyses; SD at 5% level. For Fe-sufficient root tips, only one measurement each was carried out.

Plant	+Fe		-Fe		+5 μM Cu
	Root tips	Leaves	Root tips	Leaves	Leaves
BB	889.2	422.0 ± 6	6420 ± 313	432.2 ± 74.8	468.5 ± 31.5
<i>chl</i> n	1804.5	32.82 ± 0.41	5754 ± 441	35.07 ± 4.85	244.5 ± 0.3

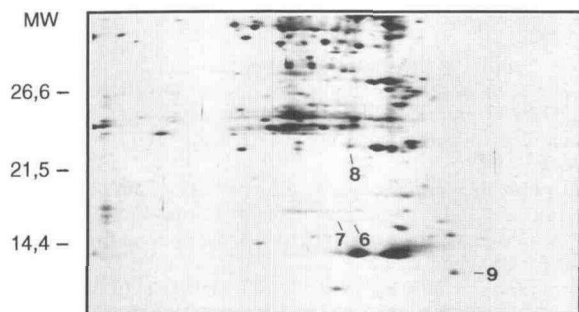


Figure 6. Two-dimensional electrophoresis gel from the youngest leaves of *chl n* after feeding with 5 μM Cu and with 10 μM Fe-EDTA in the Hoagland nutrient solution via the xylem. The protein content of protein 9 is increased as compared to Figure 5. MW, Molecular weight.

exhibited by *chl n*, presumably because a lack of NA results in an inability to mobilize Cu in the roots for xylem transport to the leaves. After feeding *chl n* with 5 μM Cu via the xylem, the leaf Cu concentration increased about 8-fold compared to that of leaves from *chl n* grown under normal Cu supply via the roots, supporting the Cu mobilization function for NA. Under the same experimental conditions, the Cu concentration of BB(+Fe) leaves did not increase, indicating the maintenance of a Cu homeostasis in which NA plays an important role (Stephan et al., 1996).

SOD

The Cu/Zn-containing SOD catalyzes the dismutation of superoxide radicals to oxygen and H_2O_2 . Previous work demonstrated that the mRNA level of Cu/Zn-dependent SOD in *Saccharomyces cerevisiae* as well as its activity were reduced in cells grown in Cu-deficient medium (Thiele, 1992). Therefore, our results are in agreement with the postulation that Cu plays an important role as a cofactor in SOD protein synthesis and/or protein stability.

PC

The Cu-dependent protein PC acts as an electron carrier between PSI and PSII. In *Chlamydomonas reinhardtii* its accumulation is regulated by environmental Cu levels. PC accumulates under conditions of Cu sufficiency due to an increase in the stability of the Cu metalloprotein. However, under environmental conditions of low Cu availability, PC levels decreased (Merchant and Bogorad, 1986). Changes in the amounts of SODs and PC could be a consequence of either posttranslational incorporation of the essential Cu into the metalloproteins, the induction of the biosynthesis by Cu, or both mechanisms (Thiele, 1992). After feeding *chl n* with 5 μM Cu, it accumulated in the leaves, resulting in an increase in the Cu-containing protein PC but not in chloroplastic and cytoplasmic SODs. In this way, it seems possible that the Cu concentration might not be sufficient for the induction of SOD biosynthesis, whereas for post-transcriptional incorporation and stabilization of PC mRNA, the amount of Cu seems sufficient. Therefore, the expression of Cu-responsive genes may be a quantitative

response to cellular metal concentration, not only in microorganisms (Thiele, 1992) but also in higher plants.

In summary, GAPDH, FDH, and AP have been suggested to play a role in the Fe-deficiency stress response in root tips of the mutant *chl n* but not to be specifically associated directly with the *chloronerva* mutation, since the protein content of these three enzymes is as high as in the Fe-deficient wild type. In *chl n* leaves the Cu deficiency is an additional effect caused by the lack of NA and resulting in an inability to transport Cu sufficiently from the roots via the xylem to the leaves and leading to a change in the protein content of cytosolic and chloroplast SODs and PC. The amount of these proteins seems to depend on the cellular Cu concentration and is not directly involved in the *chl n* mutation. Our data have shown that the NA-less mutant *chl n* not only behaves like an Fe-deficient plant but, in some cases, also behaves like a Cu-deficient plant.

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