Plant Cell Physiol. 40(3): 273–280 (1999)

Iron Deficiency Induced Changes in Ascorbate Content and Enzyme Activities Related to Ascorbate Metabolism in Cucumber Roots

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Ascorbate content and the activities of ascorbate free-radical reductase (AFR-R) and ascorbate peroxidase (APX) were investigated in order to determine whether they are affected under Fe deficiency. Plasma membrane vesicles, cell wall and cytosolic fractions were isolated from the roots of cucumber (*Cucumis sativus* L.) plants grown in the absence or in the presence of Fe. Plasma membrane vesicles showed NADH-dependent reducing activities with Fe^{3+}-citrate, ferricyanide and AFR as electron acceptors. Only AFR-R activity was stimulated ca. 3-fold in Fe deficient plasma membranes. No significant change in cytosolic AFR-R activity was induced by Fe depletion, while the activity of cytosolic APX was more than twice that of the non-deficient control. Furthermore, the content of ascorbate (AA) was enhanced ca. 1.7-fold in Fe deficient roots. These results indicate that metabolic changes resulting in enhanced AA levels and activities of AFR-R and APX in the roots could be related to plant responses to Fe deficiency stress.

Key words: Ascorbate — Ascorbate free-radical reductase (EC 1.6.5.4) — Ascorbate peroxidase (EC 1.11.1.11) — *Cucumis sativus* — Fe deficiency — Plasma membrane.

Enhanced ferric reduction and H^+-extrusion are the most typical root responses to Fe deficiency in dicotyledonous and non-graminaceous monocotyledonous plant species (Marschner et al. 1987). Ferric reduction is mediated by reductase bound to the plasma membrane (PM) of root epidermal cells (Chaney et al. 1972, Buckhout et al. 1989, Brüggemann et al. 1990, Holden et al. 1992, Rabotti and Zocchi 1994). Bienfait (1985) discerned two types of PM-bound redox-systems: one (termed the standard reductase) that can reduce only external electron acceptors with high redox potential like ferricyanide, and another (termed the turbo reductase) that can reduce both ferricyanide and ferric chelates. The standard reductase is thought to be constitutive in all plants, while the turbo reductase is induced by Fe deficiency in the epidermis of young lateral roots. However, recent studies (Buckhout et al. 1989, Brüggemann et al. 1990, Holden et al. 1991) could not confirm that biochemically distinct reductase is synthesized under Fe deficiency stress. According to Moog and Brüggemann (1994) NADH-dependent constitutive iron reductase activity is increased either by enzyme activation or by induced protein synthesis.

The specific activities of PM-bound NADH-FeCh-R and NADH-FeCN-R are enhanced 1.3 to 3.1-fold in Fe deficient roots, depending on the plant species and nature of ferric chelator. In intact roots, however, ferric reduction is stimulated more than 20 times in response to Fe starvation (reviewed by Moog and Brüggemann 1994). The apparent quantitative difference between in vitro and in vivo results is thought to be a dilution effect related to the spatial localization of Fe stress response in the root rather than an alternative mechanism for supplying Fe reduction potential: whereas PM are isolated from whole roots, enhanced Fe reduction is only confined to the root tips (Buckhout et al. 1989).

Ascorbate (AA) is a common constituent of the plant cell, which is synthesized in the cytosol and then translocated to the apoplast (Rautenkranz et al. 1994, Takahama 1996, Takahama and Oniki 1992). When AA serves as electron donor, its oxidation is prevalently restricted to ascorbate free-radical (AFR) production (Arrigoni et al. 1981). NADH:ascorbate free-radical reductase (AFR-R) (NADH:AFR:oxidoreductase) is considered essential in maintaining the ascorbate system in reduced state (Borraccino et al. 1986, Hossain and Asada 1985, Navas et al. 1994). AFR-R has been found in chloroplasts (Hossain et al. 1984), but also in nonphotosynthetic tissue. Soluble AFR-R has been purified from cucumber fruit (Hossain and Asada 1985), soybean root nodules (Dalton et al. 1992), potato tubers (Borraccino et al. 1986) and pea (Murthy and Zilinskas 1994). cDNA clones encoding AFR-R have been isolated from cucumber, pea and tomato (Grantz et al. 1995, Murthy and Zilinskas 1994, Sano and Asada 1994). The molecular size of cytosolic AFR-R from cucumber and pea is around 47 kDa and is composed of 433 amino acid residues as deduced from cDNA. The

Abbreviations: AA, ascorbic acid; AAO, ascorbic acid oxidase; AFR-R, ascorbate free-radical reductase; APX, ascorbate peroxidase; BPDS, bathophenanthrolinedisulfonate; BTP, bis-Tris propane; CW, cell wall; DHA, dehydroascorbic acid; FeCh-R, ferric chelate reductase; FeCN-R, ferricyanide reductase; IDP, inosine 5'-diphosphate; PM, plasma membrane; PMSF, phenylmethylsulfonyl fluoride; PPDF, photosynthetic photon density flux; SOD, superoxide dismutase.

purified enzyme has been characterized to be a FAD-enzyme (Hossain and Asada 1985, Asada 1997). Evidence for the existence of distinct AFR-R in association with the PM of glyoxysomes, spinach leaves and maize roots has been presented (Bérczi and Møller 1998, Bowditch and Donaldson 1990, Luster and Buckhout 1988). PM-bound AFR-R was suggested to participate in AA reduction in both the cytosol and apoplast (Bérczi and Møller 1998). AFR-induced activation of transplasma membrane redox system was found to be related to cell elongation, nutrient uptake and proton excretion from onion roots (González-Reyes et al. 1992, 1994, Hidalgo et al. 1989).

Major function of AA is to protect plant cells against oxidative damage. Oxygen free radicals may be scavenged directly by AA. Furthermore, ascorbate peroxidase (APX) utilizes AA as electron donor for reduction of H_2O_2 under normal and stress conditions (Asada 1992, Klapheck et al. 1990, Mittler and Zilinskas 1991). APX is a hemoprotein, which is localized in chloroplasts in thylakoid-bound and soluble forms, but also in other compartments of leaf cell and in nonphotosynthetic tissues (Asada 1997). Cytosolic APX has been purified from pea shoots (Mittler and Zilinskas 1991). Glyoxysomes and peroxisomes contain APX in a membrane-bound form (Bunkelmann and Trelease 1996). The participation of active oxygen species in changing the Fe^{3+}/Fe^{2+} ratio in the roots has been studied, but it is not well understood (Brüggemann and Moog 1989, Brüggemann et al. 1990, Cakmak et al. 1987, Rabotti and Zocchi 1994). It is also not known if APX may play a role in Fe stress responses of plants. Tomato plants grown under Fe deficient conditions have shown increased amount of APX-protein in the roots (Herbik et al. 1996), but APX activity was found to be lower than in the Fe sufficient plants (Pich and Scholz 1993).

In this work we have studied the effects of Fe nutritional status on AA content and enzyme activities related to the AA metabolism in cucumber roots. Our results revealed increased AA content and activities of PM-bound AFR-R and cytosolic APX in the Fe deficient roots, indicating that the ascorbate system is activated under Fe deficiency.

Materials and Methods

Plant culture—Cucumber (*Cucumis sativus* L. cv. Seriki No. 2) seeds were germinated and grown in quartz sand moistened with saturated $CaSO_4$ solution for 7 d. Seedlings were then transplanted and grown for 7 d in plastic vessels (four plants per pot), containing 3 liters of continuously aerated nutrient solution (pH 6.0) with the following composition (mM): 2 $Ca(NO_3)_2$, 0.7 K_2SO_4, 0.5 $MgSO_4$, 0.1 KCl, 0.1 KH_2PO_4, and (μM): 10 H_3BO_3, 1 $MnSO_4$, 0.5 $CuSO_4$, 0.5 $ZnSO_4$, 0.05 $(NH_4)_6Mo_7O_{24}$, plus or minus 0.1 mM Fe^{3+}EDTA. Plants were maintained in a growth chamber at 23°C, with a photoperiod of 16 h light/8 h dark, and with a PPDF of 450 μmol m^{-2} s^{-1} (metalhalide lamps, Iwasaki MT 400 DL/BUD). Four days after growing without Fe, an acidification of the nutrient solution was detected, and during the remaining three days of the growing period the pH had decreased about 0.6 pH unit per day. Plants were harvested 13–14 d after planting when the leaves showed symptoms of Fe deficiency.

Plasma membrane isolation—The preparation of microsomes was modified from the method developed by Garbarino and DuPont (1988). Roots were excised, rinsed in distilled water and homogenized in a mortar at 4°C with a buffer (300 mM sucrose, 50 mM Tris-Cl, 8 mM EDTA, 0.2% (w/v) BSA, pH 8.0) supplemented with 4 mM DTT, 2 mM PMSF, and 1.5% (w/v) insoluble PVP. A medium to tissue ratio of 2 ml (g FW)$^{-1}$ was used. After filtration through four layers of cheesecloth, the homogenate was centrifuged at 10,000 × *g* for 20 min and the supernatant was again centrifuged at 50,000 × *g* for 35 min. The resulting microsomal fraction was suspended in 300 mM sucrose, 5 mM K-phosphate (pH 7.8), 5 mM KCl, 0.1 mM EDTA, and 1 mM DTT. The PM vesicles were purified from microsomes by partitioning in an aqueous polymer two phase system with a final composition of 6.2% (v/w) Dextran T 500, 6.2% (v/w) PEG 3350, 300 mM sucrose, 5 mM K-phosphate (pH 7.8), 5 mM KCl, and 0.1 mM EDTA (Yamashita et al. 1994). Partitioning was obtained by repeated inversion and the phases were separated by centrifugation at 700 × *g* for 5 min. To increase the purity of the fraction, the upper phase was partitioned twice with fresh lower phase. DTT was added to the first phase only, to reduce oxidative browning. The second upper phase was diluted 5-fold with a dilution buffer of 300 mM sucrose, 5 mM HEPES-BTP (pH 7.0), and membranes were pelleted at 80,000 × *g* for 40 min. The resulting pellet was resuspended in dilution buffer and stored at −80°C.

Cell wall and cytosol isolation—Excised roots were homogenized in a mortar at 4°C with a grinding buffer (300 mM sucrose, 50 mM Tris-Cl, 8 mM EDTA, pH 8.0) supplemented with 4 mM DTT and 2 mM PMSF. A medium to tissue ratio of 4 ml (g FW)$^{-1}$ was used. The homogenate was filtered through four layers of cheesecloth. Crude cell walls were sedimented at 1,000 × *g* for 10 min (4°C) in a swinging bucket rotor. The pellet was suspended in grinding buffer (without PMSF) and sonicated with ASTRASON W-380 for 1 min with a 50% pulse cycle at 0°C. The sonicated suspension was centrifuged at 1,000 × *g* for 10 min. The pellet was suspended in 10 mM Na-phosphate (pH 6.0). The washing step was repeated twice. The resulting pellet was suspended in the washing buffer and stored at −80°C. The supernatant after crude cell wall sedimentation was centrifuged at 80,000 × *g* for 60 min and the resulting upper cytosolic fraction was stored at −80°C.

Measurement of Fe^{3+}-reducing capacity of intact plants—In vivo Fe^{3+}-reduction by the roots of intact plants was determined, using BPDS as a reagent to detect Fe^{2+}. The assay solution contained 0.5 mM $CaSO_4$, 5 mM MES (pH 5.8), 0.1 mM Fe^{3+}EDTA, and 0.3 mM BPDS. The reaction was performed in aluminum foil-wrapped 100 ml glass vials at 23°C for 60 min. During the experiment plant shoots were illuminated and the medium was aerated continuously. The absorption of the assayed solution was measured at 535 nm. The molar extinction coefficient of 22.1 mM^{-1} cm^{-1} was used to calculate $Fe^{2+}(BPDS)_3$ concentration (Chaney et al. 1972). The absorption of blank without plants was subtracted from the measured value to correct for occasional photoreduction.

Enzyme assays—In vitro Fe^{3+}-chelate reduction by freeze-stored PM was measured by a modified method of Holden et al.

(1991). Enzyme activity was determined at 26°C in 1 ml of 250 mM sucrose, 15 mM MES-BTP (pH 6.0), 250 μM Fe^{3+}-citrate (20 : 1 citrate-KOH : $FeCl_3$, pH 6.0), 0.03% Triton X-100, 125 μM BPDS, and 10 μg PM-protein. The reaction was started with 160 μM NADH (prepared as a 16 mM stock in 15 mM HEPES-KOH, pH 7.5). The formation of $Fe^{2+}(BPDS)_3$ was followed spectrophotometrically at 535 nm for 3 min. In some experiments, when indicated, BPDS was omitted from the assay medium and NADH oxidation was monitored at 340 nm.

Ferricyanide reductase was determined by a modified method of Buckhout and Hrubec (1986), following NADH oxidation. The assay was conducted at 26°C with 10 μg PM-protein in a reaction mixture (1 ml) containing 250 mM sucrose, 15 mM MES-BTP (pH 6.0), 0.3 mM ferricyanide, 0.03% Triton X-100, and 160 μM NADH.

NADH-ascorbate free radical reductase (AFR-R) was assayed as described by Luster and Buckhout (1988) with some modifications. The oxidation of NADH was measured at 340 nm in a reaction mixture (1 ml) consisting of 50 mM BTP-HEPES (pH 8.0), 1 mM AA, 16 mUnits of ascorbate oxidase (Sigma Chemical Co.), 0.01% Triton X-100, and 18 μg enzyme protein. The reaction was started by the addition of 0.16 mM NADH following a 3 min incubation period at 30°C. Ascorbate oxidase activity was confirmed daily by monitoring the absorbance of 0.1 mM ascorbate with 16 mUnits of ascorbate oxidase, buffered with 50 mM BTP-HEPES (pH 8.0), at 267 nm. An extinction coefficient of 6.22 $mM^{-1}\ cm^{-1}$ was used in calculation of the enzyme activity.

Ascorbate oxidase (AAO) activity was determined by the method of Takahama and Oniki (1994) with an oxygen Clark-type electrode at 25°C. The reaction mixture (1 ml) contained 10 mM Na-phosphate (pH 6.0), 0.5 mM EDTA, and 20 μg protein. The reaction was started by the addition of 1 mM AA.

Ascorbate peroxidase (APX) was determined according to Asada (1984). Excised roots were homogenized in a mortar with a grinding medium (4 ml $(g\ FW)^{-1}$) consisting of 100 mM K-phosphate (pH 7.8), 2 mM AA, and 5 mM EDTA. The cytosolic fraction was isolated as described above. APX was measured by following the decrease in absorbance at 290 nm as AA was oxidized ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$). The reaction mixture (1 ml) contained 50 mM K-phosphate (pH 7.0), 0.25 mM AA, and 10–20 μg protein at 25°C. The reaction was started by the addition of 0.1 mM H_2O_2.

Guaiacol peroxidase (POX) was measured spectrophotometrically at 26°C by following the formation of tetraguaiacol at 470 nm ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$). The reaction mixture (1 ml) consisted of 30 mM K-phosphate (pH 7.0), 2 mM H_2O_2, and 2.7 mM guaiacol. The reaction was started by the addition of 5 μg cytosolic protein.

Marker enzymes measured were respectively, vanadate-sensitive ATPase (PM), latent IDPase (Golgi apparatus), nitrate-sensitive ATPase (tonoplast), azide-sensitive ATPase and Cyt *c* oxidase (mitochondria), and antimycin A-insensitive NADPH-Cyt *c* reductase (ER). ATPase activity was determined as described by Yamashita et al. (1994), in the presence and absence of 0.1 mM Na_3VO_4, 1 mM NaN_3, and 50 mM KNO_3, respectively. Latent IDPase activity was measured in the same manner as the ATPase activity, except for the addition of 3 mM IDP as a substrate. The activities of NADPH-Cyt *c* reductase and Cyt *c* oxidase were determined by following the reduction or oxidation of Cyt *c*, respectively at 550 nm ($\varepsilon = 18.8\ mM^{-1}\ cm^{-1}$) (Yapa et al. 1986).

Solubilization of membrane-bound AFR-R—Membrane proteins were solubilized according to the method of Brüggemann and Moog (1989) with some modifications. Frozen (−80°C) PM in a dilution buffer were thawed and equal volume of the buffer, containing 1 M KCl was added. Membranes were pelleted at 100,000 × *g* for 45 min. The pellet was resuspended in a medium (300 mM sucrose, 5 mM HEPES-BTP, pH 7.0), and 1% Triton X-114 was added at a protein : detergent ratio of 1 : 10. After 30 min on ice, membranes were centrifuged at 100,000 × *g* for 60 min and the supernatant and pellet were analyzed for AFR-R activity.

Determination of ascorbate (AA) and dehydroascorbate (DHA)—AA and DHA were determined by the color development with 2,4-dinitrophenylhydrazine. Excised roots (1 g FW) were homogenized in a mortar with 1 ml of 6% metaphosphoric acid and 2.8 M acetic acid. The homogenate was diluted to 50 ml with a solution consisting of 3% metaphosphoric acid and 1.4 M acetic acid, and centrifuged at 2,000 × *g* for 10 min. AA and DHA in the supernatant, as well as in the standard solution were determined immediately. The reaction mixture, containing 1 ml of sample solution, 1 ml of 0.26 M thiourea in 5% metaphosphoric acid, and 0.5 ml of 0.1 M dinitrophenylhydrazine in 4.5 M H_2SO_4, was incubated for 3 h at 37°C. After cooling on ice, 2.5 ml of 16 M H_2SO_4 were added to the assay media and incubated at room temperature for 30 to 40 min. The color development was monitored spectrophotometrically at 540 nm. Total AA was determined as DHA, after oxidation with one drop of 7 mM Na-2,6 dichlorophenolindophenol. AA was calculated as a difference between total AA and DHA.

Protein determination—Proteins in the PM and cytosol were determined by the method of Bradford (1976) using BSA as a standard. Protein in the CW was estimated by a modification of the Lowry procedure (Markwell et al. 1978). The samples were solubilized in 0.1 M NaOH for 20 h at 4°C prior to protein analysis. Protein in Triton X-114-containing samples was measured according to Schaffner and Weissmann (1973).

Data are given as means ± SD, and the number of experiments and replications is specified in the tables.

Results

The capacity of intact roots to reduce exogenous Fe^{3+}-chelate was tested. In response to Fe deficiency, the rate of Fe^{3+}-reduction increased 26-fold (i.e., from 2.64 ± 0.36 to 68.9 ± 1.6 nmol $(g\ FW)^{-1}\ min^{-1}$ for control and Fe depleted plants, respectively), which is within the ranges reported for other dicotyledonous plants (Moog and Brüggemann 1994).

The aqueous polymer two-phase partitioning technique is known to provide highly purified plasma membrane vesicles. To evaluate the purity of the PM fraction used in this study we analyzed the activities of membrane marker enzymes. Table 1 shows the enzyme activities in the PM and starting microsomal fractions. Vanadate-sensitive ATPase, a PM marker enzyme, was enriched ca. 3-fold in this fraction compared to the microsomes. Membrane contamination by ER (NADPH-Cyt *c* reductase) and Golgi apparatus (latent IDPase) was minimal, with 2% or less recovery in the PM fraction of the respective marker enzyme. Mitochondrial membranes were virtually absent: only 0.13% of the total Cyt *c* oxidase activity was reco-

Table 1 Specific activities of marker enzymes in microsomal and plasma membrane (PM) fractions from Fe deficient roots

Marker enzyme	Specific activity [nmol (mg protein)$^{-1}$ min^{-1}]		Recovery in PM, %
	Microsomes	PM	
Vanadate-sensitive ATPase	595 ±65	1,760 ±5	17
Latent IDPase	590 ± 7	74 ±9	0.7
NADPH-cytochrome *c* reductase	55.0± 0.7	22.2±1.5	2.3
Cytochrome *c* oxidase	39 ± 3	0.9±0.3	0.13

Data are the means±SD of one out of two preparations (n=2) with similar results.

vered in the PM fraction and no marked azide-sensitive ATPase activity was detected (Table 1, 2). The absence of contamination by tonoplast membranes was demonstrated by the lack of nitrate-inhibited ATPase in the PM. There was no significant difference in the PM isolated from Fe sufficient and Fe deficient roots regarding the Mg^{2+}-ATPase activity and the effect of the inhibitors (vanadate, azide, nitrate and molybdate) (Table 2). Qualitative and quantitative evidence for a similar purity in PM fractions from Fe sufficient and Fe deficient roots was also reported by Buckhout et al. (1989) and Susín et al. (1996).

The capacity of isolated PM to reduce FeCN, Fe^{3+}-citrate, and AFR was compared by measuring the rate of NADH oxidation (Table 3). AFR was the least active of the acceptors tested, but the rate of NADH oxidation was 3-times higher in PM isolated from Fe deficient roots than in the control roots. When the electron acceptor was a Fe^{3+}-chelate, the rate of NADH oxidation was increased by only 10% (FeCN) and 8% (Fe^{3+}-citrate) in PM isolated from Fe deficient roots compared to Fe sufficient roots (Table 3). The PM reducing activity with respect to FeCN and Fe^{3+}-citrate was also evaluated by monitoring $Fe^{2+}(BPDS)_3$ formation. In these experiments, Fe deficiency induced activation of the PM-bound ferric reductase was ca. 20% or less compared to the control (i.e., 80.7±1.8 to 94.3±2.9 nmol (mg protein)$^{-1}$ min^{-1} with respect to Fe^{3+}-citrate).

To characterize AFR-R binding to PM, isolated membrane vesicles were washed with 500 mM KCl and subsequently solubilized with 1% Triton X-114, at a protein to detergent ratio of 1 : 10. While the salt washing removed 29% of the protein, the detergent solubilized 48% of the total protein. After solubilization of PM proteins, the specific activity of AFR-R increased 2.3-fold, from 26.8 to 61.5 nmol (mg protein)$^{-1}$ min^{-1}. Total activity of the enzyme before and after solubilization was 8.04 and 8.82 nmol min^{-1}, respectively. No AFR-R activity was detected in the pellet.

Further biochemical characterization of PM-bound and solubilized AFR-R was performed (Table 4). The PM-bound enzyme was latent in the absence of 0.01% Triton X-100. The enzyme activity was 4-times higher in the presence of 160 μM NADH as an electron donor compared to 160 μM NADPH. This property was similarly displayed by soluble potato AFR-R (Borraccino et al. 1986), while in maize root PM, AFR-R showed about the

Table 2 Effect of inhibitors on ATPase activities in PM fractions from Fe sufficient and Fe deficient cucumber roots

Inhibitor	ATPase activity [nmol (mg protein)$^{-1}$ min^{-1}]			
	Fe sufficient		Fe deficient	
	Activity	%	Activity	%
Control	649±28	100	711±19	100
+Na_3VO_4 (0.1 mM)	73± 1	11	53± 3	7
+NaN_3 (1.0 mM)	623±12	96	638± 9	90
+KNO_3 (50 mM)	713±18	110	726±25	102
+Na_2MoO_4 (1.0 mM)	611±10	94	620[a]	87

Data are the means±SD of one out of two preparations (n=2) with similar results.
[a] SD less than 1.

Table 3 Effect of electron acceptors on the reductase activity in PM fractions from Fe sufficient and Fe deficient cucumber roots

Electron acceptor	Reductase activity [nmol NADH (mg protein)$^{-1}$ min^{-1}]		Increase[a], %
	Fe sufficient	Fe deficient	
Ferricyanide	770 ±7	844 ±33	10
Fe-citrate	53.6±3.7	58.1± 0.1	8
AFR	7.4±1.5	22.6± 5.5	205

Data for AFR are the means±SD of 3 independent experiments (n=5). Data for FeCN and Fe-citrate are the means±SD of one out of three preparations (n=3) with similar results.
[a] Increase is determined as [(Fe deficient)−(Fe sufficient)/(Fe sufficient)]×100.

same specific activities with either donor (Luster and Buckhout 1988). Neither FAD nor FMN at 100 nM had any marked influence on the rate of NADH oxidation. The PM-bound AFR-R activity was not affected by 10 μM 2,4-D (data not shown), although Morré et al. (1995) reported that auxins (IAA and 2,4-D) stimulated NADH oxidase activity in soybean PM. Unlike the PM-bound reductase, solubilized AFR-R showed no activity when NADPH was substituted for NADH as an electron donor.

Iron deficiency affected enzyme activities of AA metabolism (Table 5). The cytosolic APX activity was increased over 2 times compared to Fe sufficient roots, whereas guaiacol peroxidase activity was not affected. The cytosolic AFR-R and AA oxidase activities were enhanced by 22 and 27%, respectively, while AA oxidase in the cell wall fraction was virtually unchanged. AFR-R activity was 17 times higher in the cytosol than in the PM, but the difference between Fe deficient and Fe sufficient roots was strongly pronounced in PM fraction.

The changes in AA and DHA levels in the roots as affected by Fe deficiency were examined (Table 6). Fe deficient roots were enriched both, in AA (by factor of 1.7) and DHA (by factor of 3.2), while the ratio of AA to (AA+DHA) was decreased by 23% compared to Fe sufficient roots.

Table 4 Effect of detergent, pyridine dinucleotides, FMN and FAD on ascorbate free-radical reductase activity in isolated PM from Fe deficient cucumber roots and after solubilization of PM with 1% Triton X-114

Assay condition	Activity, % of control	
	PM-bound	Solubilized
Control	100	100
−Triton X-100	0	n.d.
−NADH+NADPH (160 μM)	22	0
+FAD (100 nM)	111	100
+FMN (100 nM)	111	79

Control rates are 26.8 (PM-bound) and 61.5 nmol (mg protein)$^{-1}$ min^{-1} (solubilized).
n.d.=not determined.

Discussion

Our investigations revealed typical response reactions in Fe deficient cucumber plants: enhanced ability of the roots to reduce extracellular ferric chelates and acidification of the growth medium. However, in contrast to many reports (Brüggemann et al. 1990, Buckhout et al. 1989, Holden et al. 1991, Moog and Brüggemann 1994, Rabotti and Zocchi 1994) we were not able to detect pronounced increase of the PM-bound NADH-FeCh-R activity in Fe deficient roots. Susín et al. (1996) have suggested that the failure to observe a significant enhancement of PM-bound NADH-FeCh-R activity in the Fe deficient sugar beet roots may reside in the loss, during PM preparation, of a cytoplasmic cofactor needed for the enzyme to be active.

The orientation of the PM vesicles prepared by phase-partitioning is predominantly right-side-out (cytoplasmic face inside) (Rubinstein and Luster 1993) and NADH-FeCh-R activity is 70–80% latent in isolated PM vesicles in the absence of Triton X-100 (Askerlund and Larsson 1991, Brüggemann et al. 1990, Buckhout et al. 1989, Holden et al. 1991). Recently, it has been suggested that in vitro both, the donor and acceptor sites for NADH-FeCh-R activity are located on the internal surface of the PM, and unlike the native membranes, no trans-oriented flow of electrons is detectable in isolated PM vesicles (Schmidt and Bartels 1997). It is possible that the loss of integrity of the redox system or the exposure of some nonphysiological active sites in isolated PM vesicles may result in reduced enzyme activity.

The components of the transmembrane electron transport chain in root PM remain unknown. We have observed that the NADH-FeCh-R activity was reduced by 63% during 6 min preincubation of PM-vesicles with 0.03% Triton X-100 in the absence of substrates and was

Table 5 Specific activities of ascorbate oxidase (AAO), ascorbate peroxidase (APX), peroxidase (PX) and ascorbate free-radical reductase (AFR-R) in Fe sufficient and Fe deficient cucumber roots

Enzyme	Fraction	Specific activity [nmol (mg protein)$^{-1}$ min^{-1}]		Increase[a], %
		Fe sufficient	Fe deficient	
Ascorbate oxidase	Cell wall	1,870± 50	1,680±270	−10
Ascorbate oxidase	Cytosol	1,940±110	2,470± 90	27
Ascorbate peroxidase	Cytosol	389± 97	882± 71	127
Guaiacol Peroxidase	Cytosol	1,020± 55	882± 48	−14
AFR reductase	Cytosol	324± 12	395± 19	22

Data are the means±SD (n=3 for AAO and APX and n=4 for PX and AFR-R).
[a] Increase is determined as [(Fe deficient)−(Fe sufficient)/(Fe sufficient)] × 100.

completely recovered in the presence of FAD, but not of FMN (Zaharieva et al. 1997). These findings suggest that native enzyme may require FAD for the electron transport.

An important question that has not been resolved yet is whether several different reductase activities in the root PM may be responsible for the reduction of Fe in vivo (Lesuisse and Labbe 1992). We found elevated AA levels in Fe deficient roots. In nutrient solution, AA reduces Fe^{3+}-chelates of varying stability at high rates (Römheld and Marschner 1983). The role of AA in regulating the redox processes in root is not well documented. Ascorbate-loaded PM-vesicles have been used to study their ability to reduce extracellular, nonpermeating electron acceptors. The investigations provided evidence for a transplasma membrane electron transport from AA to AFR, FeCN and Fe^{3+}-citrate (Askerlund and Larsson 1991, Hassadim et al. 1987, Horemans et al. 1994). These results point to a possible role of AA in regulating the redox state of cell wall, PM surface and cytoplasm (Navas et al. 1994). The purified AFR-R from cucumber fruits catalyzes the reduction of FeCN in addition to AFR (Sano et al. 1995). Thus, NADH:FeCN-R activity is at least partly accounted for by AFR-R. PM isolated from maize roots contains AFR-R activity, which is involved in the regeneration of AA (Luster and Buckhout 1988). Bérczi and Møller (1998) have found AFR-R that is strongly associated with the inner (cytoplasmic) surface of the spinach leaf PM. They suggested that the role of the enzyme would be to facilitate the recovery of AA pool at both sides of the membrane surface. Our studies also confirmed that in addition to the root cytosolic AFR-R, there is AFR-R, part of which remained bound to the PM after a wash with 500 mM KCl and was only released from the PM after treatment with 1% Triton X-114. Furthermore, no AFR-R activity was determined in the PM in the absence of Triton X-100. These results suggest that both donor and acceptor sites of the enzyme are present at the inner (cytoplasmic) surface of the isolated PM vesicles. Plant AFR-R has been characterized to be a FAD enzyme (Hossain and Asada 1985). We found that the activities of both PM-bound and solubilized AFR-R were not markedly affected by the addition of FAD or FMN into the assay medium, indicating that flavin is tightly bound to the apoprotein and is not released dur-

Table 6 Ascorbate (AA) and dehydroascorbate (DHA) content and redox state of ascorbate (AA (AA+DHA)$^{-1}$) in Fe sufficient and Fe deficient cucumber roots

Ascorbates	Content [nmol AA (g FW)$^{-1}$]		Increase[a], %
	Fe sufficient	Fe deficient	
AA+DHA	422 ±6	939 ±36	123
AA	272 ±5	465 ±25	71
DHA	150 ±2	474 ±11	216
AA (AA+DHA)$^{-1}$	0.64±0.01	0.49± 0.03	−23

Data are the means±SD (n=3).
[a] Increase is determined as [(Fe deficient)−(Fe sufficient)/(Fe sufficient)] × 100.

ing the isolation of PM and solubilization of PM-bound AFR-R by the detergent. Observed 3-fold stimulation of the PM-bound AFR-R activity in Fe deficient roots suggests an activation of the processes related to keeping AA in reduced state in the vicinity of the membrane surface. It would be interesting to further evaluate whether enhanced AFR-R activity under Fe deficiency could result in cells having an increased capacity for reductive Fe uptake.

Observed higher levels of AA, DHA and APX activity may indicate an increased production of active oxygen species in cucumber roots deprived of Fe. The mechanisms involved in the formation of active oxygen species under Fe deficiency and their effect on Fe reduction are not well understood. When Fe is completely excluded from the growth medium O_2 could be one of the electron acceptors, resulting in O_2^- as well as H_2O_2 formation (Rabotti and Zocchi 1994). SOD was reported to inhibit PM-bound FeCh-R activity and Fe^{3+}-reduction by intact roots, suggesting that O_2^- radicals may contribute to the reduction of Fe^{3+} (Brüggemann et al. 1990, Cakmak et al. 1987, Rabotti and Zocchi 1994). Catalase has been found to eliminate the inhibitory effect of SOD, indicating that H_2O_2 produced in SOD-catalyzed disproportionation of O_2^- may oxidize part of Fe^{2+} in a Fenton reaction and thus counteract Fe^{3+}-reduction (Brüggemann et al. 1990). Rabotti and Zocchi (1994) have reported much higher guaiacol peroxidase activity in the PM and CW from Fe deficient cucumber roots compared to control roots and proposed that the possible role of increased peroxidase activity is to eliminate Fe^{2+} reoxidation by H_2O_2. According to Asada (1992) plants mainly use APX as the enzyme scavenging H_2O_2. It is likely that APX may have important function in controlling H_2O_2 levels in the roots subjected to Fe stress. The enhanced APX activity in Fe deficient roots would defend against excessive H_2O_2 production and hence prevent Fe^{2+} reoxidation. To our knowledge this paper provides the first direct evidence for changes of ascorbate metabolism in the roots induced by Fe deficiency stress. This information could be a useful starting point for further investigations on the role of ascorbate system in the roots and its possible link to Fe reduction and acquisition.

This work was carried out while T. Zaharieva stayed at the Research Institute for Bioresources, Okayama University, Japan, which is gratefully acknowledged. H. Matsumoto and T. Zaharieva express their gratitude to Japan Society for the Promotion of Science for the award of the fellowship. The authors are also grateful to Dr. Isao Hasegawa (Dept. Agric. Chem., Cell Agric. Vet. Med., Nihon University) for his helpful advice in the assay of ascorbic acid

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(Received November 5, 1998; Accepted December 12, 1998)