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Does a voltage-sensitive outer envelope transport mechanism contribute to the chloroplast iron uptake?

Ádám Solti[1]*, Krisztina Kovács[2], Brigitta Müller[1], Saúl Vázquez[3+], Éva Hamar[1], Hong Diep Pham[1], Brigitta Tóth[4], Javier Abadía[3], Ferenc Fodor[1]

[1]Department of Plant Physiology and Molecular Plant Biology, Institute of Biology, Faculty of Sciences, Eötvös Loránd University, Pázmány P. sétány 1/C, Budapest 1117, Hungary

[2]Laboratory of Nuclear Chemistry, Department of Analytical Chemistry, Institute of Chemistry, Faculty of Sciences, Eötvös Loránd University, Pázmány P. sétány 1/A, Budapest 1117, Hungary

[3]Department of Plant Nutrition, Aula Dei Experimental Station, Spanish Council for Scientific Research (CSIC), P.O. Box 13034, Zaragoza 50080, Spain

[4]Department of Agricultural Botany, Crop Physiology and Biotechnology, Institute of Crop Sciences, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen, Böszörményi út 138, Debrecen 4032, Hungary

[+]Present institution: Faculty of Science, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Sutton Bonington, Leicestershire, LE12 5RD, UK

*corresponding author: adam.solti@ttk.elte.hu; Tel. +36-1-3722500 /8614; Fax. +36-1-381-21-64

Main Conclusion

Based on the effects of inorganic salts on chloroplast Fe uptake, the presence of a voltage-dependent step is proposed to play a role in Fe uptake through the outer envelope.

Abstract

Although iron (Fe) plays a crucial role in chloroplast physiology, only few pieces of information are available on the mechanisms of chloroplast Fe acquisition.

Here, the effect of inorganic salts on the Fe uptake of intact chloroplasts was tested, assessing Fe and transition metal uptake using bathophenantroline-based spectrophotometric detection and plasma emission coupled mass spectrometry, respectively. The microenvironment of Fe was studied by Mössbauer spectroscopy.

Transition metal cations (Cd^{2+}, Zn^{2+}, Mn^{2+}) enhanced, whereas oxoanions (NO_3^-, SO_4^{2-} and BO_3^{3-}) reduced the chloroplast Fe uptake. The effect was insensitive to diuron (DCMU), an inhibitor of chloroplast inner envelope associated Fe uptake. The inorganic salts affected neither Fe forms in the uptake assay buffer nor those incorporated into the chloroplasts. The significantly lower Zn and Mn uptake compared to that of Fe indicates that different mechanisms/transporters are involved in their acquisition.

The enhancing effect of transition metals on chloroplast Fe uptake is likely related to outer envelope-associated processes since divalent metal cations are known to inhibit Fe^{2+} transport across the inner envelope. Thus, a voltage-dependent step is proposed to play a role in Fe uptake through the chloroplast outer envelope on the basis of the contrasting effects of transition metal cations and oxoaninons.

Abbreviations

apoLhcII, Light Harvesting Complex II apoprotein; BPDS, bathophenantroline disulphonate; Chl, chlorophyll; CCCP, Carbonyl cyanide m-chlorophenyl-hydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; $\Delta\Psi$, transmembrane electrochemical potential; EDTA, Ethylenediaminetetraacetic acid; FRO, Ferric chelate Reductase Oxidase protein; HEPES, 4-

(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ICP-MS, Inductively Coupled Plasma Mass Spectrometry; IE, inner envelope; OE, outer envelope; OEP, Outer Envelope Protein; PM, plasma membrane; PPFD, photosynthetic photon flux density; RbcL, Rubisco large subunit; VDAC1, Voltage-Dependent Anion Chanel 1; VHA, V-type H^+ ATPase.

Key words: chloroplast; envelope membrane; iron metabolism; Mössbauer spectrosopy; voltage-dependent transport

Introduction

Metal ions are essential micronutrients for all living organisms including plants. Among the transition metals, iron (Fe) is the most abundant in plant tissues. In shoot tissues of plants with normal Fe supply, up to 80–90% of the total Fe is found in the chloroplasts (Terry and Abadía 1986; Morrissey and Guerinot 2009), and thylakoid membranes themselves contain approximately 60% of total leaf Fe (Castagna et al. 2009). The major Fe-sinks in the chloroplasts are proteins binding non-heme Fe, Fe-S clusters and heme cofactors. The absence of Fe induces strong deficiency symptoms, the so-called Fe chlorosis. The biosynthesis of chlorophyll (Chl) and Fe-S clusters as well as the assembly of pigment–protein complexes is strongly hampered by Fe deficiency, leading to a decrease in photosynthetic capacity and productivity of plants (Andaluz et al. 2006; Timperio et al. 2007; Abadía et al. 2011; Basa et al. 2014). Fe is also required for the activity of Fe-containing enzymes involved in protection against oxidative stress, and therefore anti-oxidative defence mechanisms can be affected when Fe is in short supply (Latifi et al. 2005; Tewari et al. 2005).

In contrast with the wealth of data on Fe root acquisition (Abadía et al. 2011) and long distance transport (Rellán-Álvarez et al. 2010), our knowledge on transition metal uptake by leaf cells and organelles are still scarce (for review see: Krämer et al. 2007; Palmer and Guerinot 2009; Abadía et al. 2011). In the mesophyll apoplast and symplast, Fe can occur in the form of citrate or nicotianamine (NA) complexes (Weber et al. 2007; Álvarez-Fernández et al. 2014), but their participation and importance in the leaf cell Fe uptake and transport processes is still not known. Leaf mesophyll cells are known to take up both Fe^{2+} and Fe^{3+} (Nikolić and Römheld 2007), and although the whole process is not well understood yet, at least part of the apoplasmic Fe^{3+} can undergo reduction mediated by FRO reductases for an effective Fe uptake (Jeong et al. 2008) similarly to root Fe aquisition.

Concerning the chloroplasts, photosynthetic organelles of endosymbiotic origin, Fe uptake may differ from that of eukaryotic cells, since Fe should cross two different membranes, the chloroplast outer (OE) and inner envelopes (IE). The first protein found to be involved in chloroplast Fe acquisition was PIC1/TIC21 (Duy et al. 2007a). PIC1 is localised in the IE of chloroplasts in *Arabidopsis*, was shown to be a component of the IE protein translocon machinery (Teng et al. 2006), and is a member of a larger ‘Fe-import’ complex together with the NiCo protein (Duy et al. 2011). Based on results obtained with PIC1 overexpressing lines,

it also seems to regulate chloroplast Fe metabolism (Duy et al. 2011). Another important member of the Fe uptake machinary is the chloroplast ferric chelate oxidoreductase (FRO7), in the absence of which chloroplasts were not able to take up Fe from the cytoplasm (Jeong et al. 2008). The chloroplast FRO has been recently localised in the IE (Solti et al. 2014). In fact, the chloroplast FRO and IE uptake machinery must work in a close cooperation, because no free Fe^{2+} accumulates during the uptake process (Solti et al. 2012). Bughio et al. (1997) and Solti et al. (2012) showed that Fe uptake of barley (*Hordeum vulgare*) and sugar beet (*Beta vulgaris*) chloroplasts, respectively, were light/photosynthesis dependent, since both was blocked by a PSII inhibitor. Shingles et al. (2002) found the importance of inwardly directed proton gradient in Fe^{2+} movement across the chloroplast IE membrane.

Despite some Fe metabolism related transporters have been discovered in the past few years, no transport protein participating in the movement of Fe^{3+} across the chloroplast OE has been described by now (Inoue 2011; Breuers et al. 2011, López-Millán et al. 2016). OE membrane, the first barrier that regulates solute transport in and out of the chloroplasts, includes various transport proteins and protein complexes (Gutierrez-Carbonell et al. 2014). Most of them are descendant of prokaryotic ancestors (Reumann and Keegstra 1999), and similar to those occuring in Gram-negative bacteria. Gram-negative bacteria take up Fe in Fe^{3+}-siderophore chelated forms across the plasma membrane (PM). This uptake of Fe^{3+}-siderophores is voltage sensitive (Braun 2003). In *Escherichia coli*, the main component in the Fe uptake across the outer membrane is the FecA, a TonB-dependent Fe^{3+}-citrate receptor/gated channel, which is energised via a proton gradient across the cytoplasmatic membrane through cytoplasmatic membrane integrated proteins (Duy et al. 2007b; Braun and Herrmann 2007; Marshall et al. 2009). β-barrel, pore-forming transport proteins, such as TOC75, are abundant in the OE (Inoue 2007; Duy et al. 2007b; Breuers et al. 2011; Gutierrez-Carbonell et al. 2014). Among OE proteins, the presence of a specific channel for amino acids (OEP16), an ATP- and substrate-regulated channel (OEP21), a cation-selective channel (OEP37) and an unspecific channel (OEP24) have been already approved (Duy et al. 2007b; Breuers et al. 2011; Gutierrez-Carbonell et al. 2014). The outer envelope proteins and the function of OEPs have been discussed several times (Soll et al. 2000; Bölter and Soll 2001; Duy et al. 2007b; Breuers et al. 2011), but the significance of voltage-regulation in the Fe uptake of intact chloroplasts is not clear yet.

The aim of this study was to test whether voltage-sensitive transport through OE may have a function in the Fe acquisition of intact chloroplasts. In particular, we studied how transition metal cations and oxoanions influence the Fe uptake of intact chloroplasts.

Materials and Methods

Plant material

Sugar beet (*Beta vulgaris* L. cv. Orbis) plants were grown in hydroponics in a climate chamber with 14/10 h light (160-200 μmol m^{-2} s^{-1} photosynthetic photon flux density, PPFD)/dark periods, 24/22 °C and 70/75% relative humidity in modified ¼ strength Hoagland solution with 10 μM Fe^{3+}-citrate (Fe:citrate = 1:1, Reanal Kft., Hungary) as Fe source (Solti et al. 2012). Mature leaves of plants having 7-8 leaves were used for isolation of chloroplasts.

Chloroplast isolation, determination of purity and intactness

Sugar beet chloroplasts were isolated and purified on a stepwise sucrose gradient as described in Solti et al. (2012), and see also the Supplementary Material 1. Chloroplast density was determined by counting in a Nikon Optiphot-2 microscope. Chloroplasts were solubilised in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 2% DTT, 10% glycerol, and 0.001% bromophenol blue at room temperature for 30 min. Proteins were separated in 10–18% gradient polyacrylamide gels in a MiniProtean apparatus (BioRad) using a constant current of 20 mA per gel at 6 °C. Protein concentration of samples was determined by comparing the area density with that of a standard mixture using Phoretix 4.01 software (Phoretix International, Newcastle upon Tyne, UK).

To detect mitochondrial contamination and estimate the integrity of the chloroplasts, protein blots were carried out. Membrane proteins separated by SDS-PAGE were transferred to Amersham™ Protran™ Premium 0.2 um NC blotting membranes (Amesham-Pharmacia,

Germany) in a 25 mM Tris, pH 8.3, 192 mM glicine, 20% (v/v) methanol and 0.02% (m/v) SDS at 4 °C using 90 V constant voltage (<0.4 A) for 3 h.

The purity of chloroplast preparations was checked with rabbit polyclonal antibody against mitochondrial alternative oxidase (AOX 1/2, a mitochondrial inner envelope marker, Lang et al. 2011) (Agrisera AG, Vännäs, Sweden). Mitochondrial sign at ~ 34 kDa was identificated according the manufacturer's informations (for more information, please visit: http://www.agrisera.com/en/artiklar/aox1_2-plant-alternative-oxidase-1-and-2.html). In order to estimate the integrity of the chloroplasts, membranes were decorated with rabbit polyclonal antibodies against apoLHCII (a gift from Dr. Udo Johanningmeier, Bohum Universität, Germany) and RbcL (Rubisco large subunit, form I and form II; Agrisera AG, Vännäs, Sweden). Antibodies were dissolved in 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% gelatine following the manufacturer's instructions. Horseradish peroxidase- (HRP-) conjugated goat-anti-rabbit IgG (BioRad, Inc.) was used to detect bands following the manufacturer's instructions. Chloroplast integrity was estimated by comparing the RbcL/apoLhcII ratio in solubilized leaf tissues and chloroplast samples as in Solti et al. (2012).

Measurements of Fe uptake

Iron uptake was assessed from the total chloroplast Fe content before and after a 30 min Fe uptake assay. Total chloroplast Fe was measured with BPDS according to Solti et al. (2012). The assay was carried out with 0.5 ml chloroplast suspension (100 µg chlorophyll (Chl) ml^{-1}; approximately 76000 ± 9500 chloroplasts μl^{-1}) in uptake buffer (50 mM HEPES-KOH, pH 7.0, 330 mM sorbitol, 2 mM $MgCl_2$), and 100 µM Fe^{3+}-citrate (Fe:citrate 1:1; Reanal Kft., Hungary) was used as Fe source. In order to test the effects of inorganic salts on the Fe uptake process, one of the following inorganic salts: KCl, K_3BO_3, KNO_3, K_2SO_4, $CdSO_4$, $CdCl_2$, $ZnSO_4$, $ZnCl_2$ and $MnCl_2$ was also added to the Fe uptake medium at a concentration of 500 µM. Transition metal cations were also tested as chloride salts at a concentration of 200 µM. K^+ and Cl^- were present both in the isolation buffer and the uptake assay medium in mM concentrations. To uncouple chloroplast envelope membrane $\Delta\Psi$, 5 µM of the ionophore CCCP (carbonyl cyanide m-chlorophenyl-hydrazone) was added to the Fe uptake assay medium. The reduction-based Fe uptake across the chloroplast IE membrane was disrupted by

using 10 µM of the photosynthetic electron transport inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), which blocks NADPH production by the photosynthetic electron transport chain. Values obtained with the Fe uptake medium free of any added inorganic salts and inhibitors are referred to as 'control' values throughout the paper. Iron uptake was initiated by illuminating the samples with 160 µmol m^{-2} s^{-1} PPFD white light, and terminated by placing the samples in ice to the dark. Fe uptake values are expressed in attomol (amol; 10^{-18} mol) Fe taken up $chloroplast^{-1}$.

Determination of element concentrations in chloroplasts

Chloroplast samples, taken before and after the Fe uptake assay, were washed in washing buffer containing 50 mM HEPES-KOH (pH 7.0), 330 mM sorbitol, 2 mM EDTA, 2 mM $MgCl_2$. The total Fe content of chloroplasts was determined after reduction by 100 µM ascorbic acid and Fe^{2+} complex formation with 300 µM BPDS as in Solti et al. (2012), using an absorption coefficient of 22.14 mM^{-1} cm^{-1} for the Fe(II)-BPDS complex (Smith et al. 1952). For determination of the concentrations of other elements, chloroplast samples taken before and after the uptake assays were washed in washing buffer, resuspended in uptake buffer and dried for one week at 60 °C. Samples were digested by HNO_3 for 30 min at 60 °C and then in H_2O_2 for 90 min at 120 °C. After filtration by MN 640W paper, ion contents were measured using ICP-MS (Inductively Coupled Plasma Mass Spectrometer, Thermo-Fisher, USA).

Mössbauer spectroscopy

Changes in the chemical microenvironment of ^{57}Fe were assessed using Mössbauer spectroscopy (Solti et al. 2012). After 30 min incubation in uptake buffer supplemented with $^{57}Fe^{3+}$-citrate, chloroplasts were washed in washing buffer to remove any excess Fe adsorbed on the organelle surface. Concentrated chloroplast suspensions were placed in a conventional constant acceleration type Mössbauer spectrometer (Wissel) in a liquid nitrogene bath cryostat at 80 K. A $^{57}Co(Rh)$ source of ~10^9 Bq activity was used, and the spectrometer was calibrated with α-Fe at room temperature. Evaluation of spectra was carried out using the MOSSWIN code (Klencsár et al. 1996). The Mössbauer parameters calculated for the spectral components

were: isomer shift (δ, mm s^{-1}), quadrupole splitting (Δ, mm s^{-1}) and partial resonant absorption areas (S_r, %). These parameters provide information on the electron densities at the Mössbauer nuclei (including also the valence state) and on the magnitude of any electric field gradients (indicating the coordination number of the resonant atom). Quantitative analytical information for the different species found can be obtained from the relative spectral areas (Greenwood and Gibb 1971).

Statistical analysis

Fe uptake measurements were carried out with three technical repetitions in each of three to four biological repetitions. To analyse statistical differences between means a Student's t-test was applied. To compare multiple treatments, one-way ANOVA was performed with a Tukey-Kramer multiple comparison *post hoc* test, using InStat v. 3.00 (GraphPad Software, Inc.).

Results

Intactness of chloroplasts

To determine the intactness of chloroplasts, the ratio of RbcL to apoLhcII was followed and compared during the whole isolation process, i.e. in leaves, leaf homogenates, first chloroplast pellets and in class I and class II chloroplast fractions (Fig. 1). Purified class I chloroplast fractions were free of mitochondrial contamination (no sign of AOX 1/2 was detected according to westen blots). Although the first chloroplast pellet contained larger amount of damaged chloroplast, intact class I chloroplasts could be purified by sucrose gradient centrifugation. Comparing the RbcL/apoLhcII ratio of chloroplast samples to that of leaves, the intactness of class I chloroplast was 96.8±8.3% (for calculations, see Supplementary Material 1), while the soluble Rubisco escaped from the chloroplast stroma of damaged class II chloroplast. When chloroplasts were subjected to Fe uptake assay, inorganic salts did not influenced significantly the intactness of the chloroplasts (see Supplementary Material 2).

Influence of inorganic salts on the chloroplast iron uptake

Chloroplasts were able to take up Fe from a medium containing Fe^{3+}-citrate independently of the inorganic ions present. Though a 30-min incubation in the Fe uptake medium in the presence of Cd^{2+}, Mn^{2+}, Zn^{3+}, NO_3^-, SO_4^{2-} or BO_3^{2-} did not cause significant changes in the chloroplast intactness (Fig. S2, Table S2), the Fe uptake varied in the presence of different ions. When salts were applied at 500 µM concentrations, transition metal cations (Cd^{2+}, Zn^{2+}, Mn^{2+}) generally enhanced the Fe uptake whereas anions added as K^+ salts (NO_3^-, SO_4^{2-} and BO_3^{3-}) decreased it (Fig. 2). Among the metal cation chloride salts, $ZnCl_2$ was the most effective in stimulating Fe uptake. It was followed by $CdCl_2$, whereas $MnCl_2$ had a weakly significant effect ($P<0.05$). Using K^+ salts, effects of anions were independent of their valence: Cl^- did not influence the chloroplast Fe uptake and the inhibition by NO_3^- and BO_3^{3-} was similar, whereas the presence of SO_4^{2-} decreased strongly the uptake of Fe. When the transition metal cations and SO_4^{2-} were present together in the uptake medium (transition metals were added in forms of sulphate salts) they apparently had antagonistic effects, leading to an intermediate Fe uptake, i.e. to values between those measured with K_2SO_4 and the metal chloride salts.

The chloroplast Fe uptake was also affected by the concentration of transition metal cations in the uptake medium, Fe uptake being more enhanced at a concentration of 200 µM than at 500 µM (Fig. 3). The differences in Fe uptake using 200 and 500 µM metal concentrations was 22±4% in average.

To uncouple chloroplast envelope membrane $\Delta\Psi$, CCCP was added to the Fe uptake assay medium. The ionophore CCCP eliminated the Fe uptake capacity of chloroplasts both in darkness (not shown) and in light (Fig. 4), since hardly any measurable changes were found in the Fe content of chloroplasts irrespectively of the inorganic metal salt used. The reduction-based Fe uptake was disrupted by the photosynthetic electron transport inhibitor DCMU, which blocks NADPH production. In the absence of any additional inorganic salts, DCMU significantly decreased the Fe uptake of control chloroplasts under light conditions during a 30-min incubation, which dropped from 570±78 to 120±15 amol Fe $chloroplast^{-1}$ (a 79% decrease, Fig. 4). Using inorganic metal salts, a DCMU-induced decrease in Fe uptake was also found (Fig. 4) but the tendency of changes was the same as without DCMU (Fig. 2): KCl and KNO_3 induced no singificant change, transition metal cations (except Mn^{2+}) and

oxoanions significantly increased and decreased the Fe uptake, respectively. The percentage of changes were also similar to the case when inorganic salts were applied in the absence of DCMU.

ICP studies on the element content of chloroplasts and uptake of transition metals

Numerous elements were detected in isolated chloroplasts, with Ca, Mg and S being present in high amounts (Table 1). Potassium content was somewhat higher but comparable to that of Na. Among the essential transition metals, two groups could be distinguished, with Fe and Zn being approximately five- to ten-fold more abundant when compared to Cu, Mn and Mo. Among non-essential metals, the chloroplast contents of Al and Ba were high and comparable to those of Fe and Zn. Other non-essential transition metals were present in lower amounts. Incubation of chloroplasts in the control Fe uptake medium for 30 min in the light did not change the element contents considerably (the Cu and Cr content showed a small but significant increase only), except that a 3.4 fold-increase in their Fe content.

The uptake of transition metals into the chloroplasts was also monitored by ICP-MS. The incubation of chloroplasts in Fe uptake medium supplemented with transition metals in the form of chloride at 500 μM concentrations for 30 min in the light led to a significant uptake of each metal (Fig. 5). The uptake of Zn and Mn were significantly lower than that of Fe, whereas the Cd uptake was comparable to that of Fe uptake in the control assay. After the 30 min incubation period in the presence of 500 μM $ZnCl_2$, $MnCl_2$ and $CdCl_2$, the final metal contents taken up were 112±4, 146±5 and 515±17 amol chloroplast^{-1} for Zn, Mn and Cd respectively, whereas that of Fe ranged from 883±3 to 1168±4 amol Fe chloroplast^{-1}.

Mössbauer analysis of Fe forms

The Fe chemical forms in the assay medium were not altered in the presence of the applied inorganic salts. In the uptake medium, the $^{57}Fe^{3+}$-citrate solution showed only one component, with hyperfine parameters δ=0.47(1) mm s^{-1} and Δ=0.64(1) mm s^{-1}, typical of high spin Fe^{3+}-carboxylate complexes (Solti et al. 2012) (Fig. 6A). The presence of anions did not have any effect on the Mössbauer spectrum, with no additional quadrupole doublets appearing on the spectra; as an example, when adding 500 μM SO_4^{2-} the only component had hyperfine

parameters δ=0.48(1) mm s^{-1}, Δ=0.65(1) mm s^{-1} (Fig. 6B). Similar results were found with other anions (not shown).

The chemical forms of the ^{57}Fe taken up by chloroplasts from the 100 µM $^{57}Fe^{3+}$-citrate assay medium during 30-min incubation in the light were also studied by Mössbauer spectroscopy (Fig. 7). The spectra consisted of a broadened quadrupole doublet which was fitted to the superposition of two doublets with the following hyperfine parameters: δ=0.46(1) mm s^{-1}, Δ=1.06(4) mm s^{-1} (Fe_A component) and δ=0.48(1) mm s^{-1}, Δ=0.61(3) mm s^{-1} (Fe_B component) (Fig. 6A). These components have been assigned to heme groups or Fe_4S_4 (Fe_A) and high spin Fe^{3+}-carboxylate complexes, respectively, with the latter being assigned to Fe^{3+}-citrate (Fe_B) having passed the OE membrane but not yet metabolized (Solti et al. 2012). The relative abundance of Fe_A component accounted for approximately one third of the total Fe present in the sample (35±10%). The addition of anions or cations to the uptake medium did not result in any change in the Mössbauer spectra when compared to control samples containing only $^{57}Fe^{3+}$-citrate: as an example, the Fe chemical forms in the chloroplasts were not altered by the addition of 500 µM Zn^{2+} (Fig. 7B). Similar results were found with other metals (not shown). Signals that could be assigned to high spin Fe^{2+} compounds (e.g., $[Fe(H_2O)_6]^{2+}$ or Fe^{2+}-carboxylates) or ferritins were not found in any of the chloroplast samples analyzed.

Discussion

Though chloroplasts contain a large part of the total shoot Fe, and Fe plays a crucial role in chloroplast structure and function, only few pieces of information are available about the *in vivo* mechanisms and regulation of Fe acquisition by chloroplasts (Nouet et al. 2011). In particular, no information is available on the role of the chloroplast OE in the chloroplast Fe uptake process. Since chloroplasts are organelles of endosymbiotic origin, and Fe transport across the outer membrane of the evolutionally related Gram-negative bacteria is a membrane potential driven step, we tested whether voltage-sensitive transport through OE may also have a function in the Fe acquisition of intact chloroplasts.

Transition metals and oxoanions affect Fe uptake in chloroplasts

The presence of inorganic salts in the assay medium influenced the chloroplast Fe uptake but did not affect chloroplast integrity. The Fe forms in the uptake buffer were unchanged as judged by Mössbauer spectroscopy. This latter is in line with the fact that Fe^{3+}-citrate has a much higher stability constant (K_i=11.5) than Zn^{2+}-citrate (K_i=5.0), Mn^{2+}-citrate (K_i=4.2) or Cd^{2+}-citrate (K_i=3.8) under the conditions used (Fodor 2002), which makes the occurrence of metal-citrate complexes other than Fe^{3+}-citrate unlikely. Furthermore, the presence of divalent metal cations, as well as that of NO_3^-, SO_4^{2-} and BO_3^{3-}, had no effect on the Fe forms incorporated into chloroplasts as judged by Mössbauer spectroscopy, since i) only two signals were found, corresponding to Fe^{3+}-carboxylates (Fe_B) and Fe-S centers or heme (Fe_A), the same signals as reported previously in untreated chloroplasts, and ii) the ratio of Fe_A/Fe_B component was similarly unchanged (Solti et al. 2012). These results, obtained in the presence of relatively high concentrations of transition metal ions that are known to interact with Fe homeostasis *in vivo* (Pilon et al. 2009; Kobayashi and Nishizawa 2012), suggest that the effects of these metals on Fe uptake may reside mainly on transport processes through the chloroplast envelope, but not on a different allocation of Fe forms inside the chloroplast.

Iron uptake in intact chloroplasts was enhanced by transition metal cations, whereas it was hampered by oxoanions. These results were the opposite to those of Shingles et al. (2002) who reported that divalent transition metal cations such as Zn^{2+} inhibited the movement of Fe^{2+} across the chloroplast IE membrane. In addition, the effect of cations and anions were similar in the absence and presence of DCMU. Iron uptake into the chloroplasts is light/photosynthesis dependent, and photosynthesis inhibitors such as DCMU, are known to eliminate the majority of chloroplast Fe uptake capacity (Bughio et al. 1997; Solti et al. 2012). The reason for the inhibitory effect of DCMU is that photosynthetically produced NADPH is necessary to fuel the chloroplast IE Fe^{3+}-chelate reductase enzyme (Solti et al. 2014), which is an essential component in the chloroplast Fe uptake process (Jeong et al. 2008). Based on Mössbauer spectroscopy studies, the DCMU-insensitive Fe uptake has been postulated as a Fe-pool that has moved across the OE membrane and accumulated between the OE and IE membranes (Solti et al. 2012). Taken together the opposite effect of divalent cations on Fe uptake of intact chloroplasts compared to IE (Fig. 2 *versus* Shingles et al. 2002), the inhibitory effects of DCMU on the Fe movement across IE membrane (Solti et al. 2012) and the similar influence of transition metal cations and oxoanions on the DCMU-sensitive and -insensitive Fe uptake (Fig. 4), necessarily, an additional regulatory role of OE in Fe uptake is strongly supported.

May a bacterial type, voltage-dependent Fe uptake mechanism exist in the chloroplast OE?

To the best of our knowledge, no results have been published so far on the mechanism of Fe movement across the chloroplast OE. Data obtained by Mössbauer spectroscopy indicated that Fe crossed the OE membrane in a chelated, Fe^{3+}-citrate form, which accumulated in the inter-envelope space before reduction (Solti et al. 2012). The similarities that Gram-negative bacteria, including cyanobacteria, share with chloroplasts may aid to understand the Fe uptake mechanism of chloroplasts. In Gram-negative bacteria, voltage sensitivity of the uptake of Fe^{3+}-siderophores was found (Braun 2003), where changes in the PM $\Delta\Psi$ regulates the pore opening of the Fe^{3+}-siderophore transporter in the outer membrane (Braun and Hantke 2011). In Gram-negative bacteria, the Ton system contributes to the transfer of the energy, originates from the polarization of the cytoplasmic membrane, to the OM transporters (Braun, 2014). A voltage-sensitive mechanism may be also expected to facilitate the Fe uptake in chloroplasts, since chloroplasts polarise their membrane systems similarly to free living Gram-negative bacteria (Shingles et al. 2002). Chloroplasts are known to maintain $\Delta\Psi$ and ΔpH across the IE membrane (Shingles and McCarty 1994; Pottosin and Dobrovinskaya 2015). $\Delta\Psi$ (positive intermembrane space) was shown to be an inwardly rectifying driving force for the Fe^{2+} movement across the IE membrane (Shingles et al. 2002). Nevertheless, the polarization of the IE membrane (accumulation of positive charges in the intermembrane space) necessarily polarizes the OE membrane as well. Here, using the uncoupling ionophore CCCP, the Fe uptake of chloroplasts was fully abolished, which supports the previous observations on the voltage-dependency of Fe uptake mechanism. Nevertheless, CCCP, being a hydrophobic compound, not only uncouples the $\Delta\Psi$ of the chloroplast IE membrane but can also incorporate into chloroplast OE, thus eliminating any possible additional effects of the inorganic salts. In the chloroplast OE, the OEP24 voltage-sensitive β-barrel protein, similar to the mitochondrial voltage-dependent anion channel (VDAC) proteins (Röhl et al. 1999; Clausen et al. 2004), have been reported This may have a number of distinct functions (Homblé et al. 2012). The presence of these proteins in the OE is also supported by the recent chloroplast envelope proteome analysis work of Gutierrez-Carbonell et al. (2014). Therefore, a voltage-dependent Fe-complex transport mechanism can also be postulated for the chloroplast OE. We hypothesize that the presence of transition metal cations and oxoanions can change the polarisation of the OE membrane (depolarize and hyperpolarize it,

respectively), possibly due to the lower permeability of transition metal cations or oxoanions compared to the ions normally present, such as K^+ and Cl^-, which have relatively low surface charge and thus a smaller hydrate coat. A similar effect of inorganic ions was also observed on the PM solute transport activity of root cells (Wang et al., 2011).

The effect of transition metals on chloroplast Fe uptake is quality- and concentration-dependent

Chloroplasts require transition metals such as Zn, Mn and Cu to be functional (Shcolnick and Keren 2006). In fact, our results indicate that chloroplasts take up available transition metals from the assay medium, and their uptake increasing at higher concentrations. Whereas Cu and Zn are known to be taken up by P-type ATPases (Abdel-Ghany et al. 2005; Finazzi et al. 2014) and by HMA1 (Kim et al. 2009), respectively, no data have been published yet on the mechanism of chloroplast uptake of Mn, as well as for non-essential metals such as Cd (Nouet et al. 2011). The significantly higher uptake of Cd^{2+} compared to Zn^{2+} and Mn^{2+} supports that different chloroplast uptake mechanisms should be involved in both cases. Cadmium was shown to be taken up by plant cells in competition to Ca^{2+} (Perfus-Barbeoch et al. 2002; Rodríguez-Serrano et al. 2009), and thus chloroplasts may also take up Cd^{2+} mediated by Ca^{2+} transporters. In spite of the fact that Zn^{2+} and Mn^{2+} inhibit Fe^{2+} uptake by the IE competitively (Shingles et al. 2002), the relatively low uptake of Zn^{2+} and Mn^{2+} even when they are present in high concentrations in the uptake medium makes unlikely that chloroplast Zn^{2+} and Mn^{2+} uptake may occur through the Fe transport system. Among the transition metal cations tested, Zn^{2+} has the highest surface charge (i.e. the smallest ionic radius, the ionic radius for Cd^{2+}: 0.97 Å, for Mn^{2+}: 0.80 Å, and for Zn^{2+}: 0.74 Å), so that the size of its hydrate coat is the largest. A larger hydrate coat in the case of free Zn^{2+} may cause a (higher) size-exclusion in the movement across the OE membrane leading to a longer lasting depolarisation, which in turn results in a higher enhancement of the chloroplast Fe uptake.

Higher transition metal concentrations in the uptake medium resulted in a relatively lower enhancement of Fe uptake (Fig. 3). While transition metal cations stimulated chloroplast Fe uptake, they are known to inhibit Fe^{2+} movement across the chloroplast IE membrane competitively (Shingles et al. 2002). A similar inhibitory effect was also found on the process of Fe uptake through bacterial PM (Moreau et al. 1998). The reason for the lower stimulating

effect at higher metal concentrations may be the sum of the stimulation of Fe uptake through the OE and the competitive inhibition of the Fe uptake through the IE.

Conclusion

We propose that a voltage-dependent Fe^{3+}-complex transport system is involved in the Fe^{3+}-citrate transport across the chloroplast OE. Our proposal is based on the DCMU-insensitive and uncoupling ionophore (CCCP)-sensitive stimulating effects of transition metal cations (Cd^{2+}, Zn^{2+}, Mn^{2+}) and inhibitory effects of oxoanions (NO_3^-, SO_4^{2-}, BO_3^{3-}) on the Fe uptake of intact chloroplasts. The enhancement effect of transition metals on the Fe uptake is quality- and concentration dependent. The lower stimulation at higher concentrations may be connected with the transition metal uptake into the inter-envelope space, and thus their inhibitory effect on the Fe^{2+} uptake system of the IE.

Author contribution statement

Á.S. conceptualized the study, designed the experiments, participated in most of the experimental work and wrote the manuscript. KK. performed the Mössbauer spectroscopy studies. B.M. grew the experimental plants, contributed to the chloroplast number calculations, protein polyacrylamide gel electrophoresis and western blots. S.V. contributed to the experiments focusing on the effects of inorganic ions. É.H. and H.D.P. contributed to the experiments focusing on the effect of inhibitors on the iron uptake of chloroplasts. B.T. carried out the determination of element concentrations in chloroplasts by ICP-MS. J.A. and F.F. contributed in the conceptualization of the study, design of the experiments and helped to edit the manuscript. All authors have read and approved the article.

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Table 1 Element contents (in amol chloroplast^{-1}) in freshly isolated chloroplasts (A) and in chloroplasts after incubation in the Fe uptake assay medium in the light for 30 min (B). Statistical differences between means (Student's t-test, $P<0.05$) are indicated (*).

	A	**B**
Al	317.3±72.3	317.3±43.3
B	359.4±20.9	323.2±2.7*****
Ba	244.7±40.7	241.2±3.2
Ca	134873.3±19728.3	127210.6±27189.4
Cd	2.4±0.6	2.1±0.7
Cr	6.1±0.8	7.8±0.5*****
Cu	20.9±8.8	26.9±1.0*
Fe	233.7±12.2	803.0±7.8*****
K	7652.2±442.5	8584.8±3079.3
Li	17.2±1.9	17.3±2.2
Mg	20027.2±2348.7	20859.1±2674.8
Mn	59.5±7.7	61.8±10.7
Mo	53.0±5.2	48.3±3.8*****
Na	4484.0±631.6	4026.6±721.0
P	2239.8±148.5	2326.1±174.0
S	27101.1±1063.7	26928.0±2292.0
Sr	87.3±16.0	84.2±3.1
Zn	342.7±25.6	320.7±51.1

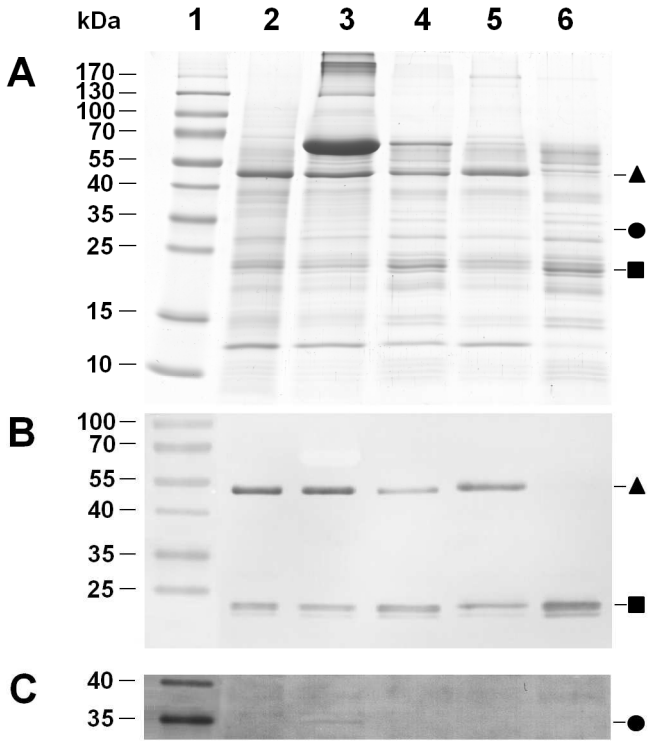

Figure 1 Coomassie stained solubilized proteins on polyacrylamide gel (A), combined immunoblot against RbcL and apoLhcII (B), and immunoblot against AOX 1/2 (C). Samples were 1 – molecular weight standard; 2 – leaf; 3 – leaf homogenate; 4 – first chloroplast pellet; 5 – class I chloroplasts; 6 – class II chloroplasts. As for molecular weight standards, Fermentas Page Ruler Prestained Protein SM0671 was used. Marks are: triangle – RbcL; circle – AOX 1/2; square – apoLhcII. Lanes on protein gels and immunoblots were loaded with 20 µg solubilised protein except sample (3), where the lane was loaded with 20 µg solubilised proteins over the bovine serum albumin (at ~66 kDa) originating from the isolating buffer.

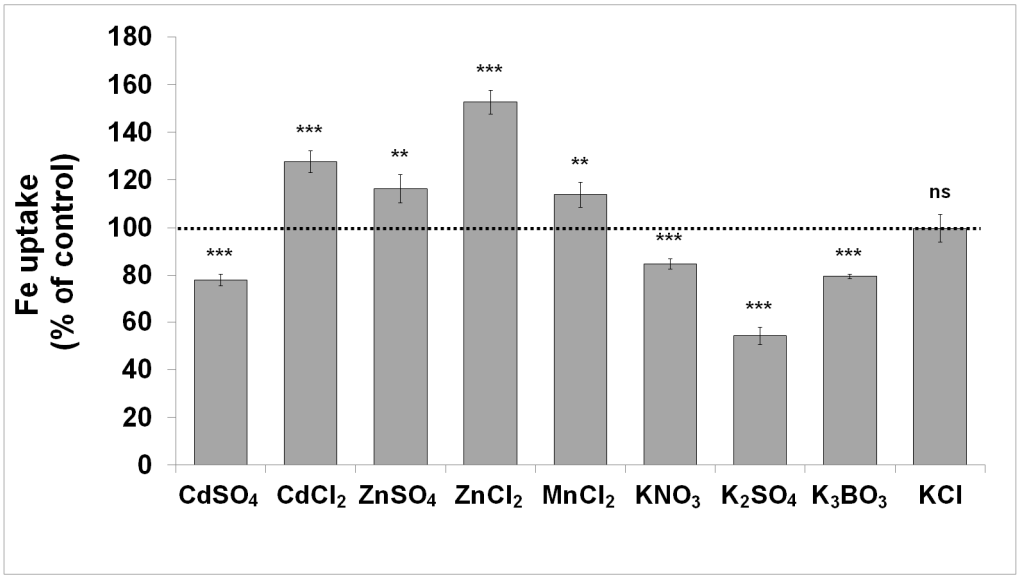

Figure 2 Chloroplast Fe uptake in the presence of inorganic salts at 500 µM concentrations during a 30-min incubation period in the light. The chloroplast Fe uptake in the control, free of any added inorganic salts, was 570±78 amol Fe chloroplast^{-1}. Statistical differences between each treatment and the control, free of any added inorganic salts, are marked above columns (Student's t-test); ***: P<0.001, **: P<0.05, ns: not significant. To compare the effects of the different salts, one-way ANOVA was performed with Tukey-Kramer *post-hoc* test, and changes among treatments were found to differ significantly (P<0.05).

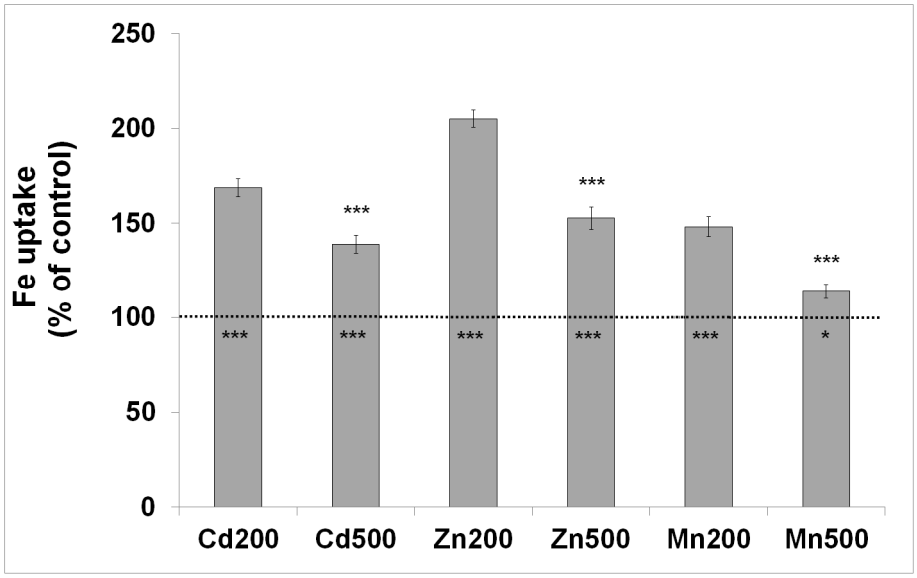

Figure 3 Effect of the transition metals Cd, Zn and Mn, used as chloride salts and at two different concentrations (200 and 500 µM, indicated as Me200 and Me500) on the uptake of Fe by chloroplasts during a 30-min incubation period in the light. The chloroplast Fe uptake in the control, free of any added inorganic salts, was 570±78 amol Fe chloroplast^{-1}. Statistical differences between each treatment and the control, free of any added inorganic salts, are marked within each column (Student's t-test); *: P<0.10, ***: P<0.01. Also, significant differences in the 500 µM treatments *vs.* the 200 µM ones (Student's t-test) are marked by arterisks above the columns; ***: P<0.01. To compare the effects of the different salts, one-way ANOVA was performed with Tukey-Kramer *post-hoc* test, and changes among treatments were found to differ significantly (P<0.05).

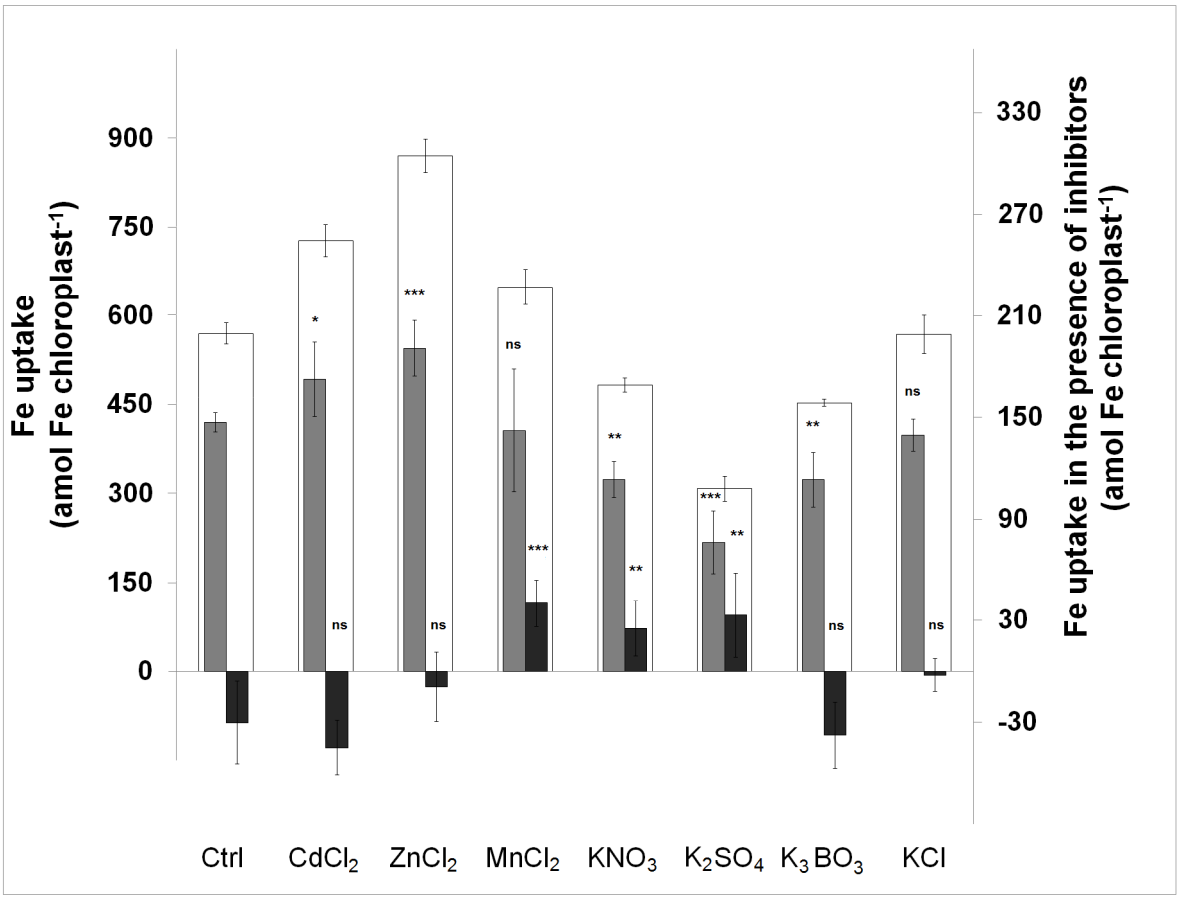

Figure 4 Effect of DCMU (grey columns, right y axis) and CCCP (black columns, right y axis) on the Fe uptake of chloroplasts in the presence of inorganic salts at 500 µM concentrations during 30-min incubation in the light compared to the samples containing no inhibitors (white columns, left y axis). Statistical differences between each treatment and the control, free of any added inorganic salts but containing the given inhibitor (grey and black columns) are marked above the column (Student's t-test); ***: P<0.001, **: P<0.05, ns: non significant. To compare the effects of the different salts in the presence of inhibitors, one-way ANOVA was performed with Tukey-Kramer *post-hoc* test, and Fe uptake in the corresponding DCMU and CCCP treatments were found to differ significantly (P<0.05).

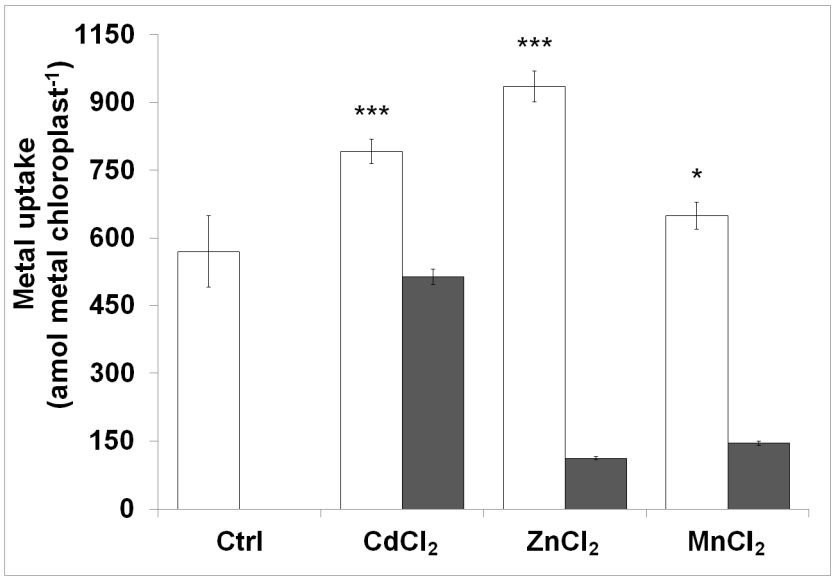

Figure 5 Chloroplast Fe uptake (white columns) and transition metal uptake (black columns) from a Fe uptake medium without and with 500 µM transition metal cations during a 30-min incubation period in the light. Significant differences from the control (free of any added inorganic salts) values (Student's t-test) are marked by asterisks above columns; *: $P<0.1$, ***: $P<0.01$. To compare the uptake of different metals, one-way ANOVA was performed using a Tukey-Kramer *post-hoc* test, and they were found to differ significantly ($P<0.05$).

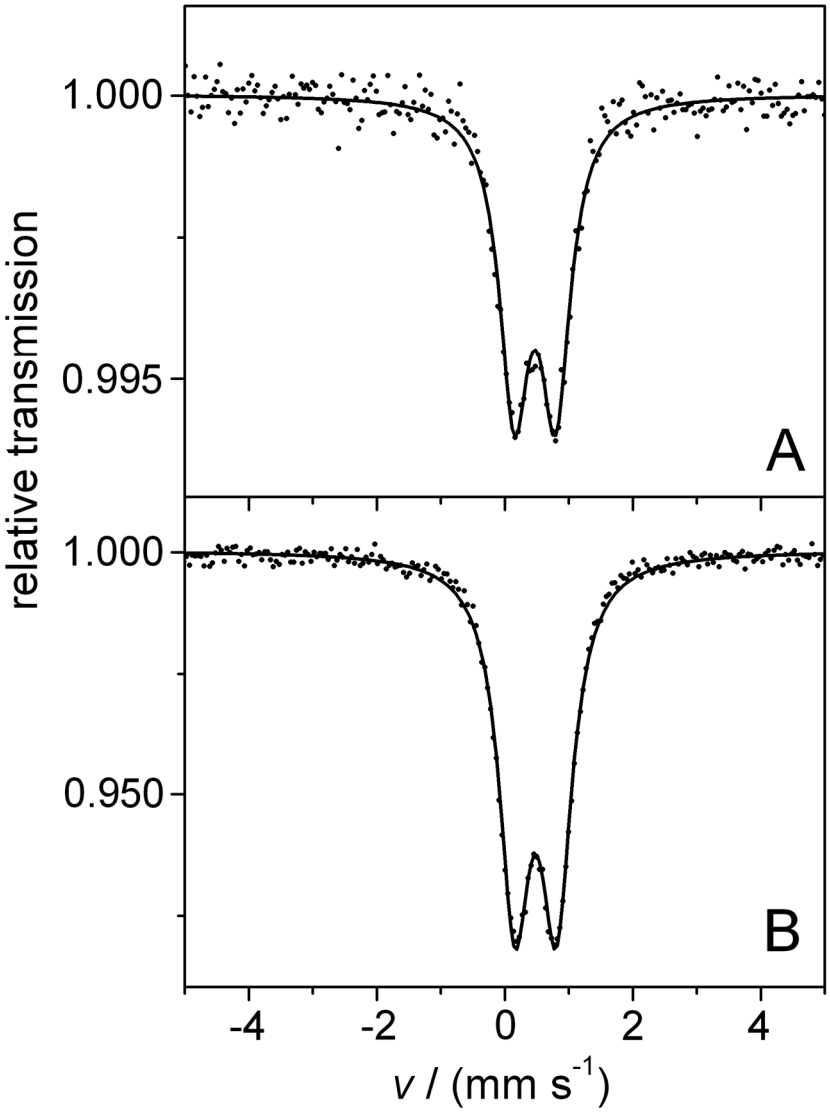

Figure 6 Mössbauer spectra of $^{57}Fe^{3+}$-citrate 1:1.1 complexes in uptake medium without (A) and with a five times higher amount of $K_2SO_4^{2-}$ over Fe (B). Evaluation and calculations of parameters for the spectral components, including isomer shift, quadrupole splitting and partial resonant absorption areas were calculated and fitted the MOSSWIN code.

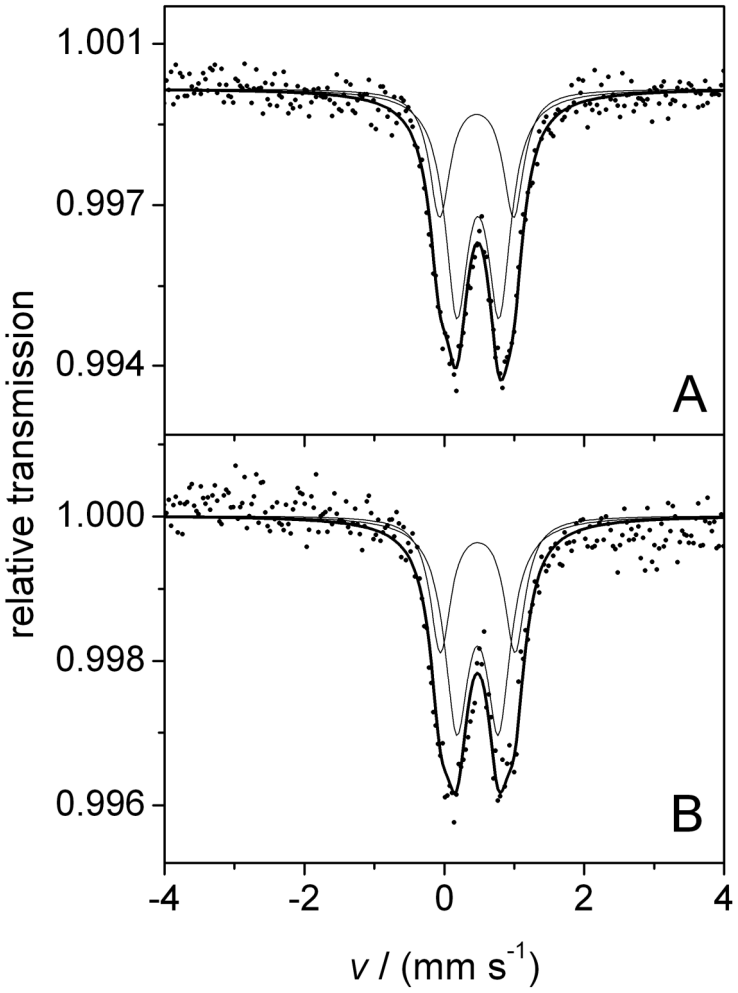

Figure 7 Mössbauer spectra of chloroplasts after 30-min incubation in the light in Fe uptake assay medium , in the absence (A) and in the presence (B) of 500 μM $ZnCl_2$. Evaluation and calculations of parameters for the spectral components, including isomer shift, quadrupole splitting and partial resonant absorption areas were calculated and fitted the MOSSWIN code.