A Metal-binding Member of the Late Embryogenesis Abundant Protein Family Transports Iron in the Phloem of *Ricinus communis* L.*

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The transport of metal micronutrients to developing organs in a plant is mediated primarily by the sieve elements. Ligands are thought to form complexes with the free ions in order to prevent cellular damage, but no binding partners have been unequivocally identified from plants so far. This study has used the phloem-mediated transport of micronutrients during the germination of the castor bean seedling to identify an iron transport protein (ITP). It is demonstrated that essentially all $^{55}$Fe fed to seedlings is associated with the protein fraction of phloem exudate. It is shown that ITP carries iron *in vivo* and binds additional iron *in vitro*. ITP was purified to homogeneity from minute amounts of phloem exudate using immobilized metal ion affinity chromatography. It preferentially binds to Fe$^{3+}$ but not to Fe$^{2+}$ and also complexes Cu$^{2+}$, Zn$^{2+}$, and Mn$^{2+}$ *in vitro*. The corresponding cDNA of ITP was cloned using internal peptide fragments. The deduced protein of 96 amino acids shows high similarity to the stress-related family of late embryogenesis abundant proteins. Its predicted characteristics and its RNA expression pattern are consistent with a function in metal ion binding. The ITP from *Ricinus* provides the first identified micronutrient binding partner for phloem-mediated long distance transport in plants and is the first member of the late embryogenesis abundant protein family shown to have such a function.

The metal micronutrients iron, zinc, manganese, and copper are imported into plants by specific uptake systems in the plasmalemma of root cells (1). The xylem then transports the ions from roots to mature organs of the shoot with the transpiration stream. It is thought that organic acids such as citric acid chelate iron in order to increase its solubility in the xylem (2). However, the development of young and growing organs depends essentially on micronutrient supply mediated by the sieve elements (3). Reasons for this include the low transpiration rate of developing organs such as shoot apices and the inability of the still incompletely developed xylem vessels to provide the micronutrients required for proper growth (4).

The phloem-mediated transport of iron and other micronutrients is well established, especially during periods of mineral deficiency and enhanced demand for micronutrients during flowering or seed filling (5). The seedlings of castor bean are completely supplied with all micronutrients via the phloem during the first week of germination (6). However, the chemical association of these metals during phloem transport is still controversial. The physicochemical properties of transition metal micronutrients cause severe problems for the transport of their free ion forms in sieve tubes. The ions of iron, copper, manganese, and zinc are highly reactive species that require masking of their electrical charge in the cytoplasm and phloem. Iron availability in particular undergoes easily changes of valence, and its redox changes favor the generation of highly reactive oxygen species via the Fenton reaction (7). Moreover, Fe$^{2+}$ and particularly Fe$^{3+}$ are extremely insoluble at alkaline pH values. Since the prevalent pH of phloem sap is between pH 7 and 8, free iron would immediately precipitate with amionic phloem constituents (2).

It has been suggested that iron, manganese, copper, and zinc move principally in an ionic form (8), whereas other authors have found manganese bound in an organic complex with a molecular mass of 1–1.5 kDa and zinc in a negatively charged complex (9). A detailed characterization of these transport species was not reported. Iron has been suggested to travel in the phloem of castor bean (*Ricinus communis* L.) in a ferric complex with a molecular mass of 2.4 kDa (10). Specific chemical properties and observations of some transport characteristics favored the metal complexes of nicotianamine, a plant-endogenous chelator, as the transport species in sieve tubes (11, 12). However, in the last few years an increasing number of findings indicated the involvement of peptides and/or proteins in the transport of iron and other micronutrients in the phloem (13–15). The seedling of castor bean (*R. communis* L.) is an established model system for phloem analysis (2, 16, 17); we report the purification and cloning of the corresponding cDNA of a protein from phloem exudate that transports iron *in vivo* in the phloem and is able to bind iron, copper, manganese, and zinc *in vitro*. To the best of our knowledge, this finding represents the first molecular identification of a micronutrient transport protein from plant phloem.

**EXPERIMENTAL PROCEDURES**

**Collection of Sieve Tube Exudate and $^{55}$Fe Labeling in Vivo—Seedlings of *R. communis* L. cv. “Carmenica” (Jelitto Staedensamen, Hamburg, Germany) were grown in the dark under sterile conditions as previously described (6). After removal of the endosperm, the hypocotyls of the seedlings were cut at the apical hook marking the beginning of the exudation period. The cotyledons were immediately submerged upside down in a 10-ml glass beaker containing 5 mM MES$^+$ buffer, pH 4.5.**

\[ \text{MES} = 4\text{-morpholineethanesulfonic acid; Tricine, N-}2\text{-hydroxy-1,1-bis(hydroxymethyl)ethyglycine; ITP, iron transport protein; LEA, late embryogenesis abundant; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline.} \]

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1 The abbreviations used are: MES, 4-morpholineethanesulfonic acid; Tricine, N-2-hydroxy-1,1-bis(hydroxymethyl)ethylglycine; ITP, iron transport protein; LEA, late embryogenesis abundant; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline.
5.5, and $^{55}$FeCl$_3$ or $^{65}$ZnCl$_2$ (Amersham Biosciences). The remaining part of the hypocotyl between the cotyledons and hook extended 2–3 cm above the surface of the beaker and allowed sampling of phloem exudate without contamination by the feeding solution (6, 16, 17). Volumes and specific activities are given in the respective figure legends. Phloem exudate was obtained from the distal hypocotyl ends. As demonstrated in Fig. 1A, radioactive signals on blots were identified with the Bio Image Analyzer. Radioactive signals on protein blots were localized and quantified with the Bio Image Analyzer BAS 2000 (Fuji).

**Molecular Weight Fractionation and Sucrose and Protein Determination—**Size fractionation of phloem sap components (500 µl) was carried out with NAP-5 (Amersham Biosciences) columns containing Sephadex G-25 DNA grade matrix equilibrated with 10 mM ammonium bicarbonate buffer. Eluate fractions of 200 µl were analyzed for sucrose, protein, $^{55}$Fe, or $^{65}$Zn. Sucrose was determined photometrically with the anthrone method after dilution of phloem exudate as described (16). Protein concentrations were determined in parallel using two described methods (18, 19).

**Gel Electrophoresis, Protein Staining, and Blotting—**All electrophoretic separations were carried out on Mini-Protein II electrophoresis cells (Bio-Rad) and run at 80 V. For the native PAGE, the Laemmli system (20) was used, but omitting SDS in buffers and gels. Proteins were separated on 12.5 and 17.5% gels, with a stacking gel of 4% polyacrylamide. Proteins were electroblotted onto nitrocellulose membranes washed three times for 10 min in TBS buffer (0.02 M Tris, 0.15 M NaCl) of pH 7.0. Membranes were then incubated in TBS buffer for 30 min, followed by incubation for 45 min in 15 ml of TBS buffer containing either 33.3 kBq ml$^{-1}$ $^{55}$FeCl$_3$ or $^{65}$ZnCl$_2$. Unbound metal ions were removed by washing with TBS. After drying, the membranes were evaluated with the Bio Image Analyzer.

**Protein Cleavage and Sequencing—**High molecular weight fractions containing pure ITP were concentrated with a Vivastep concentrator (VivaScience), run in a native one-dimensional PAGE, and subsequently electroblotted on a PVDF membrane and stained with Coomassie Blue. From the protein proved to be N-terminally blocked, it was cleaved on the PVDF membrane by the protease LysC. The eluted peptides were separated by reversed phase high pressure liquid chromatography. The amino acid sequence of the cleavage peptides was determined with the protein sequencer. For Northern analysis total RNA was isolated according to Chomczynski and Sacchi (30). 5 µg of RNA per lane were separated on 1.3% agarose gels containing 10% formaldehyde and blotted onto nylon membrane (Hybond-N$^+$, Amersham Biosciences). Hybridization was carried out using the full-length ITP cDNA as a $[^32]$PdATP-labeled probe, and specific activities are given in the respective figure legends. Phloem exudate was collected at the distal hypocotyl ends. As demonstrated in Fig. 1A, radiolabeled iron was present in the sieve tube exudate within 30 min after the start of feeding. Its concentration reached a plateau after 2 h, resembling saturation kinetics.

**RESULTS**

**Phloem-mediated Iron and Zinc Transport in Vivo—**Iron transport in the phloem was analyzed using 7-day-old seedlings of *R. communis*. At this developmental stage, all micro-nutrients are exported from the endosperm across the apoplastic space into the cotyledons and from there to the growing seedling parts via the phloem. *Ricinus* cotyledons were inoculated in $^{55}$FeCl$_3$ or $^{65}$ZnCl$_2$ solution, and phloem exudate was collected at the distal hypocotyl ends. As demonstrated in Fig. 1A, radiolabeled iron was present in the sieve tube exudate within 30 min after the start of feeding. Its concentration reached a plateau after 2 h, resembling saturation kinetics. The combination of whether or not zinc was in the phloem is in part associated with the high or low molecular weight fraction was answered by molecular size exclusion chromatography, with a cut-off of 5 kDa. The concentrations of protein and sucrose were used as markers for high and low molecular weight compounds in the eluted fractions. Radiolabeled iron exactly and exclusively co-eluted with the protein fraction under these conditions.
tions (Fig. 1B). In contrast, radiolabeled zinc only eluted with the low molecular weight fraction, as marked by the presence of sucrose (Fig. 1C). The slightly earlier elution of zinc might indicate an association with a ligand somewhat larger than sucrose.

To verify whether iron was bound to proteins in this fraction, the cotyledons were incubated in $^{55}$FeCl$_3$ as before, and the phloem exudate was separated by SDS-PAGE and in parallel by native PAGE. With both methods, signals of radioactive iron were found in three positions. These signals were localized on the SDS gel near the starting point, near the 17-kDa marker (ITP), and below the 11-kDa marker protein (Fig. 2A). Signals on the native gel were more diffuse but appeared in a pattern similar to the SDS gel (Fig. 2B). When the autoradiography patterns were compared with Coomassie Blue stainings of the same gels, only the central 17-kDa signal consistently co-localized with a protein band under denatured and native conditions. After gel excision, the 17-kDa band, but not the high and low molecular bands, yielded detectable protein for peptide sequencing (see below). This suggests that the other two bands might have contained no proteins or were artifacts resulting from the high viscosity of phloem exudate. We conclude from these experiments that iron and zinc are both transported via the phloem in Ricinus seedlings but are associated with different ligands found in the high and low molecular weight fractions, respectively. Iron in the phloem is associated at least in part with a 17-kDa protein that was consequently termed ITP (for iron transport protein) and further analyzed.

Evidence for Iron Binding of ITP—The presence of iron in the putative ITP was directly identified using two specific staining methods and in vitro labeling with $^{55}$FeCl$_3$. Phloem exudate was collected from Ricinus seedlings without feeding of radiolabeled iron. First, proteins were separated on native PAGE, blotted to PVDF membranes, and stained with Coomassie Blue (Fig. 3A) or different iron-specific reagents. The chromogenic ligand Ferene S forms blue complexes with iron and has a strong enough affinity to remove iron from proteins. When Ferene S was applied to the blots, the Ferene-iron complex was
Ferene S, diaminobenzoic acid, and 55FeCl₃ detection at the it migrated with a molecular mass of 17 kDa (Fig. 4) demonstrated by excision of the corresponding Coomassie Blue labeling. The identity of these signals with the 17-kDa ITP was in vivo very same gel position where ITP was found after autoradiography (Fig. 4, A and B). Binding assay for ITP after incubation of the membrane in TBS buffer with 33 kBq of 55FeCl₃ ml⁻¹, corresponding to an iron concentration of 0.4 μM; ITP-bound radioactivity was visualized by a Bio Image Analyzer. C, ITP bands were excised from a native gel as shown in A and loaded singly (1) or doubly (2) on an SDS-PAGE gel that was Coomassie Blue-stained for size determination of the protein. D, one and two excised bands were separated by SDS-PAGE, blotted to PVDF membrane, and stained by iron-specific Ferene S together with 2.5 μg (1c) and 5 μg (2c) of myoglobin as positive control. Note that myoglobin runs as a single band and below ITP as compared with native PAGE in Fig. 3.

confirming the iron-binding property of ITP (Fig. 4D). Final confirmation was derived from internal amino acid sequences obtained after tryptic digestion of protein bands that were cut from native gels as well as from sequences of purified ITP (see below; Fig. 8). It is concluded that ITP was the only protein in phloem exudate analyzed under these conditions that carried detectable iron in vivo and was able to bind additional iron in vitro. These results also suggest that ITP has the capacity to bind several iron atoms, unless the ITP fraction consisted of iron-loaded and unloaded protein molecules.

Protein Purification and Functional Characterization—ITP was purified for further characterization of its metal-binding properties and the molecular identification of the polypeptide. 1 ml of freshly collected phloem exudates (containing 0.2–0.25 mg of protein) was desalted and passed through a HiTrap metal affinity column containing a ferric iron-loaded matrix. Bound proteins were eluted by a step gradient of increasing concentrations of imidazole, and the fractions were analyzed by SDS-PAGE. ITP was first eluted with fraction 2, reaching its maximum concentration and purity in fraction 3, which corresponds to 150 mM imidazole (Fig. 5, Fe⁴⁺). During stripping of the column with EDTA for regeneration, more ITP eluted, indicating a very high affinity for Fe⁴⁺. In total, not more than 5 μg of ITP were purified from 1 ml of phloem exudate per experiment. The purification was also carried out with an affinity column that had been loaded with ferrous ions. The comparison of both protein patterns after PAGE indicated that a smaller number of proteins had bound to Fe²⁺ than to Fe³⁺. The imidazole elution did not yield ITP in any fraction (Fig. 5, Fe²⁺).

The affinity of ITP for other transition metals that function as plant micronutrients was investigated using correspond-
FIG. 5. Purification of ITP from phloem exudates using immobilized metal affinity chromatography. A HiTrap metal affinity column was loaded with different metals or without metal as indicated. Phloem sap proteins in HEPES buffer were applied to the column and eluted with an imidazole gradient. Aliquots (10 μl) of eluted fractions were separated by Tricine SDS-PAGE (16.5%) and silver-stained. The entire gel is shown for eluted fractions from the Fe3⁺ column, whereas only the size range between 10 and 20 kDa is given for the other columns. Left and right lanes contained molecular weight markers; lane U shows untreated phloem sap; E represents fractions from regeneration of the columns with EDTA.

FIG. 6. Binding of affinity-purified ITP to various metals. A, ITP was affinity-purified using either a Fe³⁺ (top panel) or a Zn²⁺ HiTrap column and was washed from imidazole by ultrafiltration; increasing volumes (μl) were subjected to dot-blotting to PVDF membranes. Iron-purified ITP-bound [⁵⁵]Fe and zinc-purified ITP were able to bind [⁶⁵]Zn after incubation with 2.22 kBq ml⁻¹ [⁵⁵]Fe and [⁶⁵]Zn, respectively. B, ITP was affinity-purified by copper, ferric iron, manganese, or zinc columns and shown to bind [⁵⁵]Fe. BSA (10 μg/ml) served as a negative control for metal binding.

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untranslated regions of 80 and 115 bp, respectively. A stop codon at positions 56–58 upstream of the start codon indicated that the sequence was full-length with respect to the translation product. The derived 96 amino acids gave rise to a calculated molecular mass of 10.9 kDa and a predicted pI of 6.7. All three peptides obtained by amino acid sequencing were found in the translated sequence, proving unequivocally the identity of ITP from in vivo labeling experiments, affinity purification, and the protein encoded by the cDNA.

Amino acid sequence comparison of ITP with protein data bases revealed high homology to the group of late embryogenesis abundant (LEA) proteins. Highest homology was found with the SRC1 protein from soybean (31) that contains 60% identical amino acids and matches length and pI of ITP very closely (Fig. 8A). The ITP protein is very hydrophilic (86% polar or charged amino acids) and is dominated by α-helical structures. Exactly two-thirds of the amino acids are the four amino acids His, Lys, Gly, and Glu, whereas six amino acids (Val, Trp, Cys, Phe, Gln, and Arg) are completely missing. The C termi-
mRNA with an estimated size of 580 bp was found in all seedling parts and in leaves of 4-week-old Ricinus plants, being most abundant in roots (Fig. 8B). The purification of ITP and cloning of its cDNA represent the first molecular and functional identification of an iron transporting and micronutrient-binding protein in the phloem and the first member of the LEA protein family with this function.

**DISCUSSION**

The distribution of micronutrients to developing organs of plants depends to a great extent on phloem transport. The chemical properties of the free metal ions, in particular of iron, demand ligands that provide solubility and shielding during phloem-mediated long distance transport to the nutrient sinks. Until now, no such binding partner had been clearly identified. In this study, an iron transport protein has been purified from phloem exudates, and its cDNA has been cloned using Ricinus as a model for phloem transport analysis. The following evidence supports the conclusion that ITP is a transport species for iron in the phloem. 1) Essentially all iron in the phloem is associated with the high molecular weight fraction; 2) ITP-iron complexes rapidly appear in pure phloem exudate after feeding of cotyledons with $^{56}$Fe; 3) ITP carries iron in *vivo* and is able to bind additional iron in *vitro*; 4) purified ITP binds preferentially Fe$^{3+}$, the dominant iron species in the phloem; and 5) the amino acid sequence of ITP suggests it is capable of binding several metal ions per molecule.

When Ricinus cotyledons were fed with $^{56}$Fe or $^{65}$Zn, the radioactivity obtained after transport through the sieve tubes was associated with different molecular size fractions, indicating separate ligands and transport mechanisms for the two micronutrients. This agrees with findings that describe a high affinity nickel- and zinc-binding peptide from pumpkin phloem sap with a molecular mass of 4 kDa (14). The reports of iron transport species of 2.4 and 2.8 kDa in phloem sap from pea and adult Ricinus plants (10, 13) were not confirmed by our experiments. However, none of these peptides were further characterized until now.

After electrophoretic separation of $^{55}$Fe-labeled sieve tube exudate, three signals were detected. Only one of them, which we called ITP, was consistently labeled in *vivo* and was able to bind additional iron in *vitro*. Although no protein was detected in either the high or the low molecular weight eluant in feeding experiments and no protein other than ITP was purified by immobilized metal affinity chromatography, the possibility cannot be excluded that other factors with different biochemical nature and properties contribute to iron transport in the phloem. The quick appearance of the ITP-Fe$^{3+}$ complex at the distant exudation site after the beginning of feeding of cotyledons indicates that ITP was indeed responsible for iron transport and was already present in the seedling, as would be expected for a physiological function during germination. This also rules out any induction by the experimental set-up, such as wounding.

Purified ITP showed a preference for Fe$^{3+}$ but also bound zinc, copper, and manganese, whereas in *vivo* it only complexed with iron. The fact that $^{65}$Zn was exclusively associated with the low molecular weight fraction in feeding experiments suggests that the binding of ITP to zinc in *vitro* might be a consequence of the different chemical environment, such as pH and concentrations, or the absence of additional phloem ligands. Thus, the name iron-binding protein is justified, although the *in vivo* binding properties to copper and manganese are not known and could only be determined by feeding experiments with radioactive isotopes of these metals. Interestingly, ITP only weakly bound to columns loaded with Fe$^{2+}$. This preference of ITP for ferric iron fits with the finding that only 4% of the total iron in the phloem exudate of castor bean seedling is ferrous iron (33). The apparent lack of ferrous iron binding points to a possible cooperation with the chelator nicotianamine in phloem loading and unloading of iron. The formation constant of the Fe(III)-nicotianamine complex of 20.6 (12) is higher than that of the Fe(II)-nicotianamine complex (12.8) (34), but the Fe$^{2+}$-nicotianamine complex possesses an unusual kinetic stability (12). It is tempting to speculate that nicotianamine could play the role of a shuttle by chelation of Fe$^{3+}$ from ITP-bound Fe$^{3+}$ during loading and unloading, thereby maintaining a low but significant steady-state ferrous concentration in the phloem as suggested in Ref. 10.

Because of the function of ITP in micronutrient binding and iron transport, we purified the protein and cloned the corresponding full-length cDNA. The derived amino acid sequence included all three internal peptide sequences and thus proved that the experimentally demonstrated metal binding ability and the encoded protein are the same. The unusual amino acid composition of ITP suggests that the differences between apparent and predicted molecular weight and pI are due to altered gel mobility. ITP consists of only 14% nonpolar amino acids and might therefore exhibit aberrant electrophoretic behavior. Data base searches place ITP into a group that is collectively referred to as LEA/RAB/dehydrin proteins (35), a family of highly hydrophilic proteins generally involved in dehydration processes (36, 37). Like many LEA proteins, ITP also lacks Cys and Trp residues. The highest homology of ITP was found with SRC1 from soybean, which is induced by high and low temperatures, drought, wounding, and viral infection in leaf and stem (30). In ITP, only four amino acids (Glu, His, Lys, and Gly) compose 75% of the SRC1 protein, which consists primarily of repeats of a degenerate tetrapeptide motif. This motif is also present in ITP, although not as often as in SRC1. The ITP gene is expressed in all seedling parts investigated. Interestingly, the expression in fully developed leaves suggests a transport function of ITP in adult plants as well.

Metal binding has never been observed for members of the LEA protein family to date (32); thus, our results describe a new function for this protein class. It is assumed that their polar composition and random structure enable them to bind water and act as compatible solutes (36, 37). The metal binding ability of ITP may be a consequence of the high abundance of the four amino acids mentioned. In particular, His constitutes 19% of the protein and may act like a polyhistidine tag used for protein fusion and subsequent nickel affinity purification. Indeed, ITP was able to bind to nickel affinity columns (data not shown). Amino acid comparison assigns ITP to group 2 of the LEA proteins, called dehydrins. However, homologous features are only partially conserved in ITP and are difficult to assess because of the strong variability of lengths and composition of the known dehydrins (32). Further investigations will focus on the expression patterns of the ITP gene during ontogenesis, characterization of the metal binding affinity of the ITP protein, and the possible interaction with nicotianamine.

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