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The nicotianamine molecule is made-to-measure for complexation of metal micronutrients in plants

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The non-proteinogenic amino acid nicotianamine (NA) is ubiquitous among plants. In meristematic tissues it reaches concentrations of about 400 μmol (g fresh weight)$^{-1}$. NA forms complexes, among others, with the metal micronutrients (MN) copper, zinc, iron and manganese (log K_{MeNA} 18.6–8.8). Calculations of the dissociation curves of the metal–NA complexes based on the complex formation constants and on the acid dissociation constants of NA revealed their stability at the neutral or weak alkaline pH of cytoplasm and sieve tube sap. For the Mn–NA complex, dissociation begins at about pH 6.5, for all others dissociation occurs at more acid pHs. Thus, metal–NA complexes could theoretically persist also in the apoplasm and in xylem sap. The octanol water partition coefficient of NA is about 1 and those of its metal complexes are in the range of 0.3–0.4. The reason for this shift is perhaps the negative charge of the complexes. The higher lipophilicity of the free NA indicates that the NA supply to sites of requirement is faster than the removal of the complexes as long as membranes are an integral part of the transport paths. Changing phloem transport rates of MN–NA complexes by manipulation of the cotyledon apoplasm of *Ricinus communis* L. suggest a competition of MN for NA at the site(s) of phloem loading. Thus, NA could control MN transport via phloem including recirculation.

Keywords: chelation, metal complex stability, metal micronutrients, nicotianamine, phloem transport

Introduction

The non-proteinogenic amino acid nicotianamine (NA) was first detected by Noma *et al.* (1971) in tobacco leaves, *Nicotiana tabacum* L. cv. Hicks. Later its chemical structure was determined as (2*S*:3′*S*:3″*S*)-*N*-[*N*-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]-azetidine-2-carboxylic acid (Figure 1) by Kristensen and Larsen (1974) who isolated this compound from beech nuts, *Fagus silvatica* L. These discoveries did not lead immediately to investigation of the function(s) of NA in plant metabolism. However, when Buděšínský *et al.* (1980) identified NA with the so-called 'normalizing factor' for the mutant *chloronerva* of *Lycopersicon esculentum* Mill. cv. Bonner Beste, its physiological significance became obvious, at least considering the abnormalities of the *chloronerva* phenotype. NA is probably ubiquitous among plants (Procházka & Scholz 1984, Rudolph *et al.* 1985). In young growing tissues concentrations of several hundred μM NA were reported (Stephan *et al.* 1990, 1994, Pich *et al.* 1994).

The NA-auxotroph mutant *chloronerva* is semilethal. Its growth is strongly retarded as compared with wild-type plants. The leaves are abnormally shaped, the roots are stunted and tips thickened. The most conspicuous feature is an intercostal chlorosis of young leaves. The pattern of chlorophyll-containing areas of these leaves coincides with the pattern of iron distribution (Scholz 1965). The mutant develops some symptoms typical of a plant suffering iron deficiency, although the shoot organs, especially the older leaves, contain large amounts of iron ('apparent iron deficiency'; Scholz 1967, Stephan & Grün 1989, Pich *et al.* 1991). Normal growth can be completely restored by application of NA to roots or leaves. This characteristic is termed 'phenotypical normalization' (Scholz *et al.* 1988a, 1992).

The mutant *chloronerva* is the only known NA-free plant. Thus, it became an excellent genotype to use in investigations of NA function(s). NA forms stable complexes with some divalent transition metals (Beneš *et al.* 1983, Anderegg & Ripperger 1989; Table 1). Therefore, a relation was

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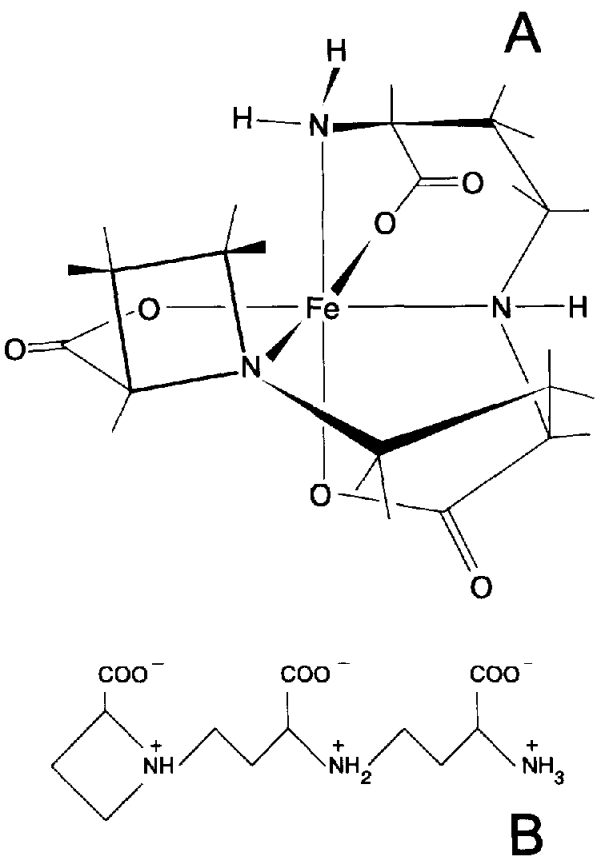

Figure 1. NA. (**A**) Spatial configuration of the complex with a central ferrous ion and three carboxylic and three amino groups as ligands giving the necessary octahedral coordination. (**B**) Plain formula of the molecule.

postulated between the metal complex formation capacity of NA and its biological function(s), especially with regard to the plant's iron metabolism (Schreiber 1986, Stephan 1995). This hypothesis was supported by the results of a test of 29 NA analogues to bring about the 'phenotypical normalization'. Only derivatives capable of forming hexadentate complexes, a prerequisite of high complex stability, showed biological activity (Scholz *et al.* 1988b). Furthermore, the chemically-synthesized (+)-NA enantiomer possessed a bioactivity comparable to that of the naturally occurring (−)-NA. This implies, that NA does not exert its function via a stereospecific binding to a macromolecular surface such as a membrane or a receptor protein, but performs its role in plant metabolism as a metal chelating agent transporting iron (Ripperger *et al.* 1982).

The physico-chemical characteristics of NA known to date, and the manifold physiological and biochemical observations on the mutant *chloronerva* were used by Stephan & Scholz (1993) to suggest the hypothesis that NA fulfills its function(s) by complexing iron and other transition metals, making these metals available for symplasmic transport especially in phloem sap. First evidence supporting the phloem transport hypothesis of NA was provided by Scholz (1989). The NA-free *chloronerva* was enabled to re-mobilize iron and to translocate it to sites of requirement, i.e. to the growing shoot apex and to the root tips, only by external application of NA. Recently evidence has shown that NA and the metal micronutrients (MN) copper, zinc, iron and manganese are co-transported in the sieve tube sap of castor bean seedlings (*Ricinus communis* L.) in a constant stoichiometric ratio of about 1.25 (Schmidke & Stephan 1995) and that NA is involved in the xylem translocation of copper (Pich *et al.* 1994).

It is still unknown whether the metal–NA complexes suggested for MN transport in phloem and apoplasm are really stable enough at the pH's existing in plant tissues and cells and to which extent they are able to penetrate membranes. Thus, we have therefore calculated their dissociation equilibria and estimated the octanol water partition coefficients (K_{ow}) of NA and its metal complexes. Furthermore, we investigated the influence of NA scarcity in the cotyledon apoplasm on the MN transport rates in the sieve tubes to test the necessity of NA in phloem loading/transport of MN.

Materials and methods

Calculation of the pH dependence of metal–NA complex stability

For estimation of the real concentrations of the assumed metal–NA complexes in biological systems one must know the concentrations of the different NA ligand species present in a biological solution, especially that of the fully deprotonated form (NA^{3-}). Since ligand species cannot easily be predicted, it is necessary to introduce the term α_H. Its product with $[NA^{3-}]$ gives the sum of the different ligand species not bound to the metal ion (Anderegg & Ripperger 1989) (equation 1).

$$[NA^{3-}]\alpha_H = [NA^{3-}] + [HNA^{2-}] + [H_2NA^-] + [H_3NA] + [H_4NA^+] + [H_5NA^{2+}] + [H_6NA^{3+}] \quad (1)$$

By substitution one obtains an expression for α_H in terms

Table 1. Stability constants ($\log K_{MeNA}$) of NA with various divalent transition metal ions

Mn(II)	Fe(II)	Co(II)	Zn(II)	Ni(II)	Cu(II)	References
8.8	12.1	14.8	14.7	16.1	18.6	Beneš *et al.* (1983)
	12.8		15.4			Anderegg & Ripperger (1989)

Table 2. Acid dissociation constants of nicotianamine (H_6L^{3+}) at 25°C and $I = 0.1$ (KNO_3) mol dm^{-3}

pK_1	pK_2	pK_3	pK_4	pK_5	pK_6	References
—	—	—	6.97	9.13	9.75	Beneš *et al.* (1983)
-	-	2.2	7.01	9.14	10.17	Anderegg & Ripperger (1989)

The values of pK_1 and pK_2 could not be obtained as the acids involved are practically completely dissociated.

of the acid dissociation constants K_i of NA (Table 2), as shown in equation (2),

$$\alpha_H = 1 + K_6^{-1}[H^+] + K_6^{-1}K_5^{-1}[H^+]^2 + K_6^{-1}K_5^{-1}K_4^{-1}[H^+]^3 + K_6^{-1}K_5^{-1}K_4^{-1}K_3^{-1}[H^+]^4 + \cdots \quad (2)$$

and the H^+ concentration. The terms containing K_2 and K_1 have been omitted because their relative contribution is insignificant. From the total metal concentration $[Me_{tot}]$ (equation 3),

$$[Me_{tot}] = [Me^{2+}] + [MeNA^-] \quad (3)$$

the total NA concentration $[NA_{tot}]$ (equation 4),

$$[NA_{tot}] = [MeNA^-] + \alpha_H[NA^{3-}] \quad (4)$$

and from the complex formation constants K_{MeNA} (equation 5),

$$K_{MeNA} = \frac{[MeNA^-]}{[Me^{2+}][NA^{3-}]} \quad (5)$$

it follows that the concentrations of metal NA complexes are related by the following expression (equation 6):

$$[MeNA^-] = K_{MeNA}([Me_{tot}] - [MeNA^-]) \frac{[NA_{tot}] - [MeNA^-]}{\alpha_H} \quad (6)$$

After transformation this expression can be solved as a quadratic equation. We performed these calculations using the PC program MENACONC.

Estimation of the octanol water partition coefficients

The partition of NA and its complexes with copper, zinc, iron and manganese between 1-octanol and water was carried out according to Fujita *et al.* (1964). Portions of 25 ml of 1-octanol saturated with ultra-pure water and ultra-pure water saturated with 1-octanol were vigorously shaken for 5 min at room temperature. The concentrations of the compounds used (2 μM in the aqueous phase) were chosen so that NA could be determined with sufficient accuracy. Only the NA concentrations in the water phase were determined and those in the octanol phase were obtained by difference. The metal–NA complexes were formed by mixing equal volumes of equimolar solutions of NA and $CuSO_4$, $ZnCl_2$, $(NH_4)_2Fe(SO_4)_2$ and $MnCl_2$, respectively. Thin layer chromatography was used to test the formation of the complexes. The Fe(II) salt solution was always freshly prepared under nitrogen to avoid oxidation because NA forms a complex under the given conditions only with divalent iron. The partition coefficients were calculated as

$$K_{OW} = \frac{c_{octanol}}{c_{water}}$$

Source and estimation of NA

NA was synthesized according to Procházka & Rudolph (1988) from azetidine-2-carboxylic acid. This amino acid was analysed by a modified procedure of automated amino acid analysis as described earlier (Pich *et al.* 1994).

Determination of the metal contents

The metal contents of experimental material were analysed by atomic absorption spectrometry as described elsewhere (Schmidke & Stephan 1995).

Culture of castor bean seedlings and sieve tube sap collection

Seven-day-old seedlings of castor bean (*Ricinus communis* L.) were cultivated at 27°C in the dark under sterile conditions. Sieve tube sap was obtained by a cut at the hypocotyl hook. Two series of experiments were performed. During the first experiments, the cotyledons remained embedded in the endosperm which was placed in moist vermiculite during the 2 h of the sap collection procedure. In the second experiment, the endosperm was removed 2 h before the beginning of sap collection and the cotyledons were incubated in 5 mM MES buffer at pH 6.0. This pH value coincides closely with the apoplasm pH of the cotyledons *in vivo* as estimated in incubation experiments with buffer solutions of different pHs. The incubation was continued also during the entire sap collection period. The sap was collected in micropipettes and frozen until determination of NA and metal contents. For details, see Schmidke & Stephan (1995).

Results

pH dependence of the metal–NA complex stability

Only copper, zinc, iron and manganese were studied here for the calculation of the pH dependent dissociation behaviour. The other transition metals whose complex stability constants had been already estimated (Table 1), were omitted. The present calculations are based on the complex formation constants given by Beneš *et al.* (1983) because only these authors estimated the values of all metals mentioned here. The acid dissociation constants were taken from Anderegg & Ripperger (1989); these authors reported more comprehensive measurements of these parameters than others.

The complexes exist undissociated in the alkaline pH range (> pH 7); they begin to dissociate with increasing

proton concentration. The MN complex stability towards increasing acidity increases with increasing stability constant, in the order $Mn < Fe < Zn < Cu$. An example is given for assumed concentrations of 100 μM for NA as well as for the metals (Figure 2). A dissociation of 1% is observed with Mn^{2+} at pH 6.55, with Fe^{2+} at pH 5.41, with Zn^{2+} at pH 4.54 and with Cu^{2+} at pH 3.26. On the other hand 99% of the complexes are dissociated with Mn^{2+} at pH 4.51, with Fe^{2+} at pH 3.41, with Zn^{2+} at pH 2.59 and with Cu^{2+} at pH 1.50.

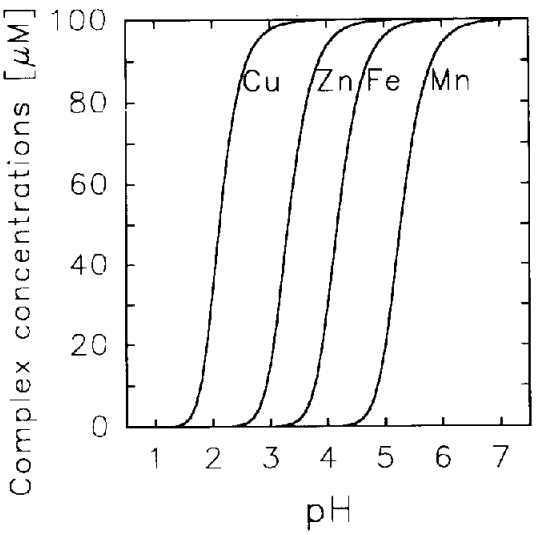

Figure 2. Complex concentrations (μM) of different heavy metals with NA as depending on the pH. The concentrations were calculated based on the complex formation constants K_{MeNA} (Benes *et al.* 1983) and the acid dissociation constants $pK_{3,\ldots,6}$ (Anderegg & Ripperger 1989) of NA by means of the PC program MENACONC. For the example given here metal and NA concentrations of 100 μM were assumed.

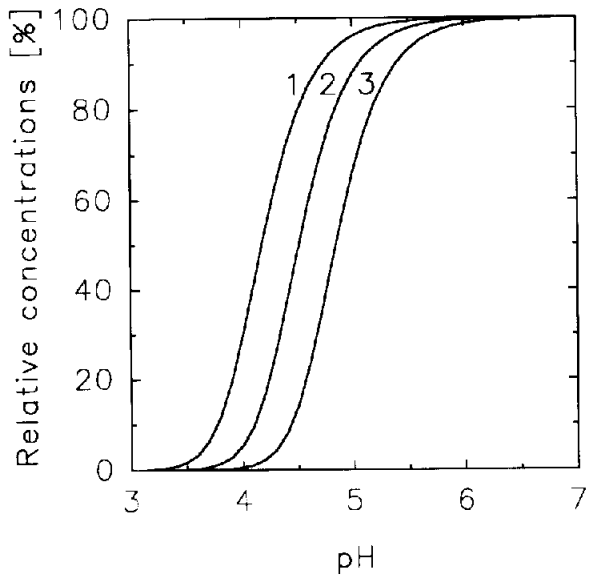

Figure 3. Relative concentrations of the iron–NA complex (%) depending on the pH and the concentrations of both complex partners at a constant ratio of 1:1. A value of 100% corresponds to the maximum concentration related to the absolute concentrations of the complex partners. **1**, Fe and NA each 100 μM; **2**, Fe and NA each 10 μM; **3**, Fe and NA each 1 μM. Further details in the legend of Figure 2.

The course of dissociation with increasing concentrations of metal ion and ligand is demonstrated in Figure 3 for the element iron. An increase of the 1:1 molar ratio from the 1 μM level to the 100 μM level leads to less competition between metal and protons. The level of 50% dissociation is shifted by about one half pH unit. If one complex partner is maintained at the 1 μM level and the concentration of the other one is increased up to 100 μM, a similar shift of complex stability can be established in the direction of the more acid pH range (Figure 4). This shift progressively increases if the concentration of one of the complex partners drops below 5 μM (Figure 5).

Octanol water partition coefficients

The K_{OW} of NA was determined to be a little less than 1. The coefficients decreased to about one-third by complex formation with metals, with the exception of iron. The Fe–NA complex proved to have similar lipophilic characteristics to the free NA under our experimental conditions (Figure 6).

NA:metal ratios in the sieve tube sap

The transport rates of the individual MN in the phloem sap were differently affected by removal of the endosperm. The higher the complex stability constant of a metal ion with NA, the lesser was the decrease of its phloem transport rate (relative transport rate). Only manganese, the element with the lowest complex constant studied, did not follow

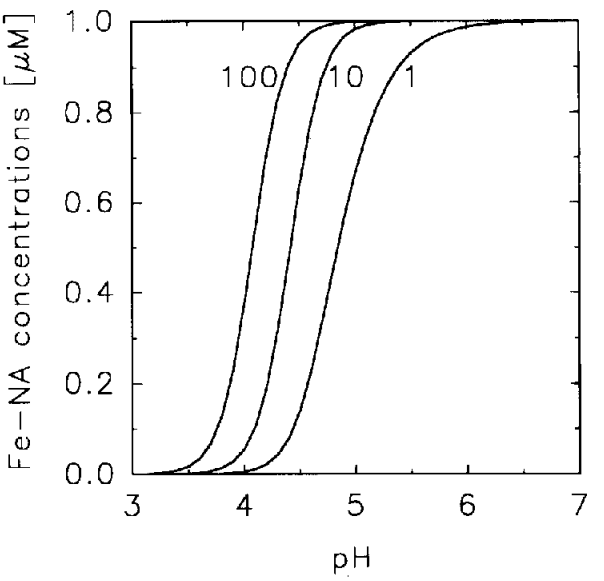

Figure 4. Concentrations of the iron–NA complex depending on the pH and the molar ratios of both complex partners over the concentration range 1–100 μM. The ratios are given in μM Fe to 1 μM NA or vice versa.

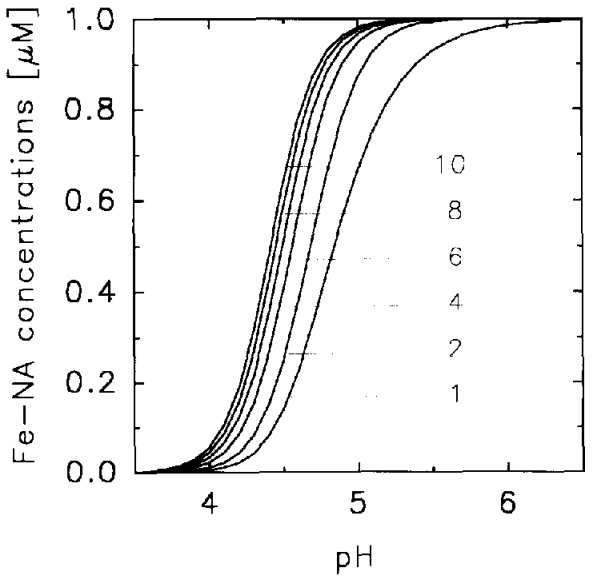

Figure 5. Concentrations of the iron–NA complex depending on the pH and the molar ratios of both complex partners over the concentration range 1–10 μM. The numbers indicate the ratios in μM Fe to 1 μM NA or vice versa.

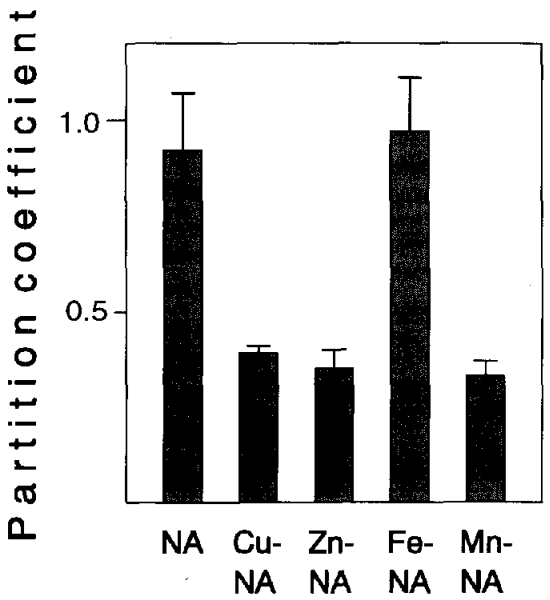

Figure 6. 1-Octanol water partition coefficients of NA and the metal–NA complexes. For details see the experimental section. Each column represents the mean of three experiments. The bars indicate the $SD_{5\%}$.

this pattern (Figure 7). The different changes of the phloem transport rates of the individual metals observed are not correlated with a general decrease of MN concentrations in the sieve tube sap. These were even increased in the cases of copper and zinc (Schmidke & Stephan 1995).

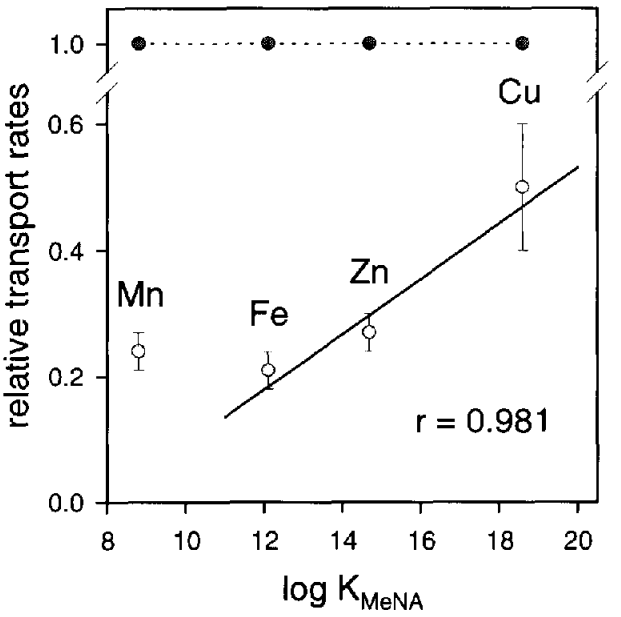

Figure 7. Correlation between the decrease of the phloem transport rates of copper, zinc, iron and manganese after the removal of the endosperm from 7-day-old castor bean seedlings, expressed as relative transport rates, and their complex formation constants (log K_{MeNA}) with the endogenous ligand NA (see Table 1). The transport rates in seedlings with persisting endosperm correspond to 1.0. The bars indicate the $SD_{5\%}$.

Discussion

Our experiments have clearly shown that the NA complexes of all four metals exist in aqueous solutions practically undissociated at pH $\geqslant$ 6 (Figures 2–5). Thus, these complexes could exist in the sieve tube sap as well as in the cytoplasm at the neutral to weakly alkaline pH prevailing there (Hoffmann *et al.* 1992). This is also supported by the occurrence of NA and MN in phloem exudates at almost stoichiometric concentrations (Schmidke & Stephan 1995). These complexes have also a high probability to exist in the apoplasm and in the xylem transpiration stream (Pich *et al.* 1994). However, the complexes of manganese and iron could be partially dissociated at a pH below 6 especially at low concentrations (Figures 4 and 5). This fact could become important in the course of remobilization and retranslocation of MN from shoot organs via the phloem sap if it includes passage through the leaf apoplasm.

The decrease of the K_{OW} of NA by complex formation with metals (Figure 6) is probably the result of a change of the charge of the molecule and not of a change in molecular conformation. Although its hydrophilic ligand groups are directed into the centre of the molecule to bind the metal atom, as shown in the model (Figure 1A), the degree of lipophilicity decreased. The NA solution used for the partition experiments had a pH of 7.1. This value corresponds almost exactly to the isoelectric point of the amino acid NA. It follows that the NA molecule is virtually

neutral (i.e. without charge), and thus, as lipophilic as possible. In contrast, the metal complexes bear one negative charge. This could be the reason for their increased hydrophilicity. A consequence for the transport through membranes is that free NA could permeate membranes faster than its metal complexes if membrane transport proteins are not involved in their movement.

According to the K_{ow} of NA and its metal complexes, as well as its characteristics as non-ionized compound or as weak acids, respectively, NA should be about equally transported in phloem and xylem, whereas the complexes tend to be optimally mobile in the phloem, following the simulation model to predict phloem transport based on the physicochemical properties of the solutes (Kleier 1988, Bromilow 1994) presuming there are no specific carriers for NA and its complexes. The fact, that synthetic (+)-NA revealed the same bioactivity in a test with the NA-free tomato mutant *chloronerva* (Ripperger *et al.* 1982) as the naturally occurring (−)-NA suggests that there is no participation of a (stereospecific) membrane transport protein in NA transport. For the MN-NA complexes, regarded as weak acids, the sieve tubes could act as an ion trap ensuring an effective retention and so guaranteeing effective long distance transport to the sink region(s).

The K_{ow} of the Fe–NA complex differed from those of the other metal complexes obtained in the partition experiments. It canot be excluded that iron was oxidized during the shaking procedure in spite of the careful preparation of the Fe(II) salt solution to avoid oxidation. As a consequence, if ferrous ions were replaced by ferric ones, no complex could be formed. The K_{ow} measured under this condition would be that of metal-free NA. This dilemma is difficult to avoid in such an *in vitro* system including an ion able to change its valence state so easily.

We showed that the decrease of MN transport rates, in the sieve tubes, varied from one metal to the other. Furthermore, a close correlation is seen between the decrease of the metal translocation rate and the complex formation constant of the metal involved (Figure 7). This would suggest a competition of the metal ions for NA at or near to the site of their phloem loading, if NA becomes scarce. A decrease in NA concentration in the apoplasm is perhaps caused in such exudation experiments by access to the cotyledon apoplasm as a result of the removal of the endosperm which is inevitably coupled with a dilution effect of apoplasm solutes. For manganese this correlation seems to be of only little significance. This can be caused by the relatively low affinity of this metal for NA (Table 1), but also by its low concentrations in the sieve tube exudate (Schmidke & Stephan 1995). On the other hand, these results offer strong support for the observation that NA is the preferred ligand for copper even at the acid pH of the xylem exudate of tomato, and thus a copper translocator for the far distance transport from the roots to the shoot (Pich *et al.* 1994).

The arguments mentioned above suggest the suitability of the NA molecule as the MN transporter in symplasm and phloem. Moreover, they explain previous observations, such as the inability to retranslocate iron via the phloem of the NA-less tomato mutant *chloronerva* (Scholz 1989), and the stoichiometry between MN and NA in the phloem exudate of *R. communis* L. seedlings (Schmidke & Stephan 1995). The latter finding can be seen as one of the best prerequisites for the existence of metal–NA complexes in the phloem sap. The observation of iron-phosphorus deposits in phloem cells and chloroplasts of mesophyll cells of the mutant *chloronerva*, but not in wild-type plants, by EDAX indicates that iron is unprotected in the absence of the chelator NA and precipitates (Becker *et al.* 1995).

The formation of metal complexes with other amino acids which also occur in the sieve tube sap, and partially in much higher concentrations than NA, can be neglected because of their low complex formation constants (Irving & Williams 1953, Sillén & Martell 1964, O'Sullivan 1972) as well as the drastic decrease of their concentrations after removal of the storage endosperm (Schobert & Komor 1989) which only insignificantly influenced the MN NA concentration ratios in phloem exudate as outlined in our previous paper (Schmidke & Stephan 1995).

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