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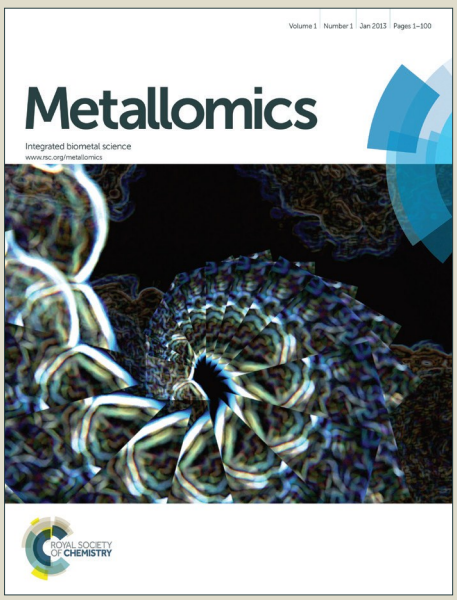

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Involvement of nitrogen functional groups in high-affinity copper binding in tomato and wheat root apoplasts: spectroscopic and thermodynamic evidence

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Carboxylic groups located in plant cell walls (CW) are generally considered to be the main copper binding sites in plant roots, despite the presence of other functional groups. The aim of this study was to investigate sites responsible for copper binding in root apoplasts, i.e. CW and outer surface of the plasma membrane (PM) continuum. Binding sites in root apoplasts were investigated by comparing isolated CW of a monocotyledon (*Triticum aestivum L.*) and dicotyledon (*Solanum lycopersicum L.*) crop with their respective whole roots. Copper speciation was examined by X-ray absorption (XAS) and 13C-nuclear magnetic resonance spectroscopies while the affinity of ligands involved in copper binding was investigated by modeling copper sorption isotherms. Homogeneous speciation and binding of copper was found in wheat and tomato root apoplasts. Only Cu-N and Cu-O bonds were detected in wheat and tomato root apoplasts. Nitrogen/oxygen ligands were identified in slightly higher proportions (40-70%) than single oxygen ligands. Furthermore, low- and high-affinity binding sites contributed in an almost equivalent proportion to copper binding in root apoplasts. The high-affinity N functional groups embedded in root apoplasts participated in copper binding in the same magnitude than the low-affinity carboxylic groups.

Introduction

Copper (Cu)–like seven other micronutrients–is essential for plant development [1]. Copper is a redox-active transition metal that occurs as Cu^{2+} and Cu^{+} depending on the physiological conditions. It participates in many biological processes such as photosynthesis, mitochondrial respiration, perception of ethylene and cell wall (CW) remodeling. The optimum Cu concentration range in plants is narrow, as Cu deficiency and toxicity are generally considered to occur below 5 and above 20 $\mu g.g^{-1}$ dry weight in shoots, respectively. Consequently, plants must finely regulate Cu uptake and homeostasis [2].

Substantial repetitive Cu inputs in agricultural areas (fungicides, organic waste spreading, etc.), associated with the low mobility of Cu in soil, lead to large-scale Cu contamination of soils with the potential occurrence of Cu phytotoxicity [3, 4].

Plant roots are primarily exposed to Cu contamination in soil. Cu accumulates almost entirely within the rhizodermis and outer cortex of roots [5, 6] and is mainly located in the apoplastic compartment [7, 8]. Measurements obtained via X-ray absorption spectroscopy (XAS) and pectinase treatment independently suggested that Cu was mainly bound to polygalacturonate-like groups located in CW [5, 9]. These experimental findings support the hypothesis of an increased rigidity of outer root CW tissues due to the strong binding of Cu to pectins, resulting in a slower elongation rate of outer cells compared to stele and inner cortex cells, as well as ruptures in the rhizodermis and outer cortex of roots. These processes are currently considered to be the underlying mechanistic of Cu toxicity to roots (i.e. Cu rhizotoxicity).

Plant roots are known to be particularly sensitive to Cu, so as to Cu(II) (hereafter referred to as Cu) was found to be the third most rhizotoxic metal cation out of 26 [10]. Kopittke et al., [11] showed that the rhizotoxicity of metal cations was linearly related to the binding strength of metals to hard ligands (i.e. ligands containing oxygen or nitrogen such as carboxyl, phosphate, carbonyl, phenolic and amine groups) in the root apoplasm. Surprisingly, Cu is one of the four metal cations exhibiting “unexpectedly high rhizotoxicity”. Indeed, Cu is substantially more toxic than predicted from the strength with which it binds to hard ligands [11]. The strength of metals binding to hard ligands was mainly calculated in the hard ligand scale (i.e. HLScale) from the strength of cation binding

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Electronic Supplementary Information (ESI) available: details on the X-ray absorption spectroscopy data acquisition and treatment are available in ESI, as well as potentiometric titrations, modelling procedure and additional data.

to 12 hard ligands, including almost exclusively carboxyl and hydroxyl groups [12]. We took this hard ligand theory into consideration when investigating Cu speciation and sorption in roots and we put forward two hypotheses. First, Cu ligands such as phosphate and amine groups could significantly contribute to Cu binding in addition to pectin-borne carboxyl groups. Then, the Cu binding strength of root apoplasts could be underestimated by the current HLScale owing to the under-representation of amine groups as high-affinity Cu binding sites.

To assess the relevance of these two hypotheses, we jointly determined the speciation and sorption of Cu in root apoplasts. Tomato (*Solanum lycopersicum* L.) and wheat (*Triticum aestivum* L.) were chosen as model species of dicots and monocots, respectively. Indeed, it is well known that dicots have a higher pectin concentration and overall binding site density than monocots. To date, there are no reliable *in situ* and highly-resolved analytical techniques which enable to decipher trace element speciation in plants exposed to environmentally realistic concentrations and to investigate the relative contribution of CW and plasma membranes in metal binding. Therefore, experiments were carried out on CW isolated from roots and on roots still containing CW and PM in order to evaluate their respective contributions to the whole root Cu binding capacity. The main functional groups involved in Cu sorption within roots were further identified by XAS and nuclear magnetic resonance (NMR). The affinity of root materials for Cu was estimated by fitting sorption data with an advanced geochemical model initially devoted to the binding of metal cations on humic substances.

Experimental

Plant growth and isolation of root cell walls

The procedures used to get healthy plants and isolated root CW were already outlined by Guigues et al. [13]. Briefly, seeds of bread wheat (*Triticum aestivum* cv. Premio) and tomato (*Solanum lycopersicum* cv. Moneymaker) were grown in hydroponic conditions for 21 days. At harvest, roots were subdivided into homogenous subsamples and then stored frozen. After thawing, a subsample of roots were rinsed with 1 mM $Ca(NO_3)_2$ to eliminate cytosolic compounds related to membrane leakage during thawing and oven-dried at 50°C (to constant mass). Dried roots (hereafter referred to as roots) contained both CW and PM. The remaining rinsed root subsamples were kept moist for CW isolation.

Cell walls were isolated from wheat and tomato roots using Triton X 100 (1% v/v) for 30 d. Isolated CW were then washed for 10 d in 1 mM $Ca(NO_3)_2$ and stored at 4°C. The efficiency of the isolation procedure was checked by measuring the loss of calcium, Cu, iron, phosphorus and potassium and by performing NMR measurements on roots and CW [13].

Batch experiments regarding copper sorption on roots and cell walls

Before the copper sorption experiments, roots and CW were stirred in HNO3 solution at pH 3 for 1 h to remove highly bound or precipitated cations (e.g. iron and aluminium), and then rinsed twice with ultrapure water (18.2 MΩ) for 30 min and finally oven-dried at 50°C to constant mass. Copper was added as $Cu(NO_3)_2$ in batch experiments. Under our experimental conditions with pH < 5, preliminary speciation calculations showed that Cu^{2+} accounted for > 99 % of total Cu in solution. Root and CW exposure to Cu was thus representative of root exposure to Cu in soil solution with pH < 6.5 for which Cu^{2+} accounts for > 95% of total inorganic Cu[14].

Experiment 1

50 mg (dry mass basis) of wheat and tomato roots or CW was shaken end-over-end for 24 h at 25°C in 250 ml of 0.05 M NaNO3 at pH 4.7 (± 0.3), with the initial Cu concentration ranging from pCuT 6.2 to 4.8 (pCuT = -log10 [CuT]). The suspension was filtered (Whatman grade 4 filter) and roots and CW were rinsed thrice with 100 ml of 0.05 NaNO3 through a Büchner funnel. Sorption experiments were performed in triplicate. Replicates reserved for XAS analysis were snap-frozen in liquid N2 and stored at -18°C. The two remaining replicates were oven-dried at 50°C until constant mass was achieved, ground using a porcelain mortar and were digested for Cu analysis through hot mixing of 69% HNO3 and 30% H2O2 (TraceSELECT ultra, Sigma-Aldrich). The Cu concentration in the digest was determined by inductively coupled plasma mass spectrometry (ICP-MS, NexION 300X Perkin Elmer). Blanks and certified reference materials (rye-grass roots ERM-CD 281, EnviroMAT Driking water EP-L-3 and groundwater ES-H-2) were included in the digestion and analysis procedures. The measurement uncertainty was under 15%.

Experiment 2

50 mg (dry mass basis) of wheat and tomato roots was shaken end-over-end for 24 h at 25°C in 250 ml of 0.05 M NaNO3 at pH 4.5 (± 0.2), with the initial Cu concentration ranging from pCuT 5.1 to 3. The suspension was filtered (Whatman grade 4 filter) and roots were rinsed thrice with 100 ml of 0.05 NaNO3 through a Büchner funnel. The Cu concentration in the initial and equilibrium solution was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Jobin Hyvon Horiba J 38). Blanks and in-house reference samples were included in the analyses. The measurement uncertainty was under 15%. The amount of Cu bound to wheat and tomato roots was calculated according to the difference in the quantity of Cu recovered in the initial solution and in the solution in chemical equilibrium with roots.

Experiment 3

A dry mass of 10 (± 0.5) mg of wheat and tomato roots or CW was shaken end-over-end for 24 h at 25°C in 25 ml of 0.03 M NaNO3 at pH 4.5 (± 0.3), with the initial Cu concentration ranging from pCuT 7.3 to 3 M. Sorption experiments were performed in duplicate. After a short plant material sedimentation time (a few minutes), the supernatant was

removed for analysis. The Cu concentration in the initial and equilibrium solution was determined by ICP-MS. For each data point, a summary of the initial and equilibrium Cu concentrations and measured pH are presented in Table S1. Blanks and certified reference material (EnviroMAT Drinking Water EP-L-3 and Groundwater ES-H-2) were included in the analyses. The measurement uncertainty was under 10%. The amount of Cu bound to wheat and tomato roots or CW was calculated according to the difference in the quantity of Cu recovered in the initial solution and in the solution in chemical equilibrium with roots or CW.

Copper speciation in roots and cell walls determined by X-ray absorption spectroscopy

Copper K-edge X-ray absorption spectra for reference compounds and plant samples from experiment 1 were obtained on the French Absorption spectroscopy beamline in Material and Environmental sciences (FAME Beamline) at European Synchrotron Research Facility (ESRF) [15]. Frozen roots and CW were ground and pressed into pellets in liquid N_2 (77K), with special care taken to keep the pellets frozen in liquid N_2 until the XAS analysis to avoid artificial speciation changes. Pellets from frozen-hydrated samples and reference compounds were transferred to an He cryostat and cooled to 10K to prevent radiation. Spectra for the root and CW samples are the sum of 1 to 12 scans of 45 min each, depending on the Cu concentration recorded in fluorescence mode using a 30-element solid-state Ge detector (Canberra). Each scan was focused on a different specimen position to reduce the risk of beam damage and obtain representative spectra. The data normalization and analysis procedures are reported in the supporting information (Methods S1).

NMR identification of Cu binding functional groups

Dried roots from experiment 2 were analyzed by solid state ^{13}C CP-MAS NMR spectroscopy. Since Cu(II) is paramagnetic, the resonance of C atoms in its vicinity will be broadened beyond detection. This property can be used to identify Cu binding sites on root material by monitoring functional groups experiencing signal loss upon the addition of Cu(II). Note that, due to the lack of sensitivity of conventional pulse NMR, Cu quantities necessary to observe a significant effect are well above the concentrations that would be expected in a natural environment. However, these experiments can help identify trends and differences in behavior between the root material samples. Spectra were obtained with a Bruker Avance WB 400-MHz spectrometer at 101.6 MHz. Typical acquisition parameters included a spin rate of 10 kHz, 2 ms contact time and 2 s recycling delays. Depending on the sample, 10-18×10^3 scans were recorded. Free Induction Decays, i.e. the time domain NMR signal, were processed with MestReNova software; data treatment consisted in 50 Hz line broadening and standard baseline and phase correction. Semi-quantitative analysis was performed by spectra decomposition into Gaussian peaks using IGOR PRO 5.0 software. In parallel, attempts to obtain usable ^{15}N NMR data failed because N accounted for only 1 to 5 % of the tomato and wheat CW and root material weights. Considering the low sensitivity of the ^{15}N nucleus, this led to unreasonably long acquisition times.

Modeling copper sorption on roots, cell walls and plasma membranes

Copper sorption on wheat and tomato roots and CW was characterized by simulating data from potentiometric titrations (Figure S1) [13] and from experiment 3 with the Humic Ion-Binding Model VII, hereafter referred to as Model VII [16]. Model VII was originally designed to simulate the sorption properties of two humic substances, namely a fulvic and a humic acid, depicted as a regular array of type 1 and 2 binding sites. The density (L_{Hi}, $cmol_c.kg^{-1}$) of type-1 sites was arbitrarily set as twofold above the density of the type-2 sites (i.e. $L_{H1} = 2 \times L_{H2}$). Protons and metal ions compete for sorption on these sites with metal ions being able to form mono-, bi- and tri-dendate complexes. Electrostatic effects are accounted for in Model VII by approximating the diffuse layer/bulk solution system with a Donnan model.

As roots and CW are solid organic matters, we parameterized their sorption properties on the basis of the default conformation parameters defined for humic acid (HA) in Model VII (see Methods S2 for details on the modelling procedure). CO_2 partial pressure was assumed to be that of the ambient atmosphere ($10^{-3.5}$ atm) and the temperature was set at 25°C for the calculations. The theoretical potentiometric titrations and Cu sorption isotherms of the outer surface of wheat and tomato root cell PM were calculated according to the difference between the root and CW data (Figs. S2 and S3). The modelling procedure (Methods S2) was also implemented on the theoretical potentiometric titrations and Cu sorption isotherms of the outer surface of root cell PM.

Results

Copper speciation in roots and cell walls

Cu sorption in wheat and tomato roots and CW measured in batch experiments 1 and 3 were similar (Figure S4), thus demonstrating that Cu speciation studied by XAS could be related to the Cu sorption characterized with Model VII.

Analyses of samples from experiment 1 indicated that Cu sorbed (Cu_{ads}) in wheat and tomato roots or CW ranged from 61 to 891 $mg.kg^{-1}$ (Table S2) as a function of the initial Cu concentration (pCu_T 6.2 to 4.8). For the same pCu_T, Cu_{ads} for tomato was equal to or slightly higher than Cu_{ads} for wheat.

The shape of XANES and EXAFS spectra and Fourier transforms (FT) for wheat and tomato roots were very similar to those of CW (Figure 1 and 2), regardless of the adsorbed Cu concentration (Table S2). All XANES spectra gave an intense absorption maximum at 8997 eV (feature A) and a weak feature at 8978 eV (feature B) like the XANES spectum of Cu(II)-malate (Figure 1), indicating that Cu was predominantly bound as Cu(II) in wheat and tomato roots and CW [6, 17].

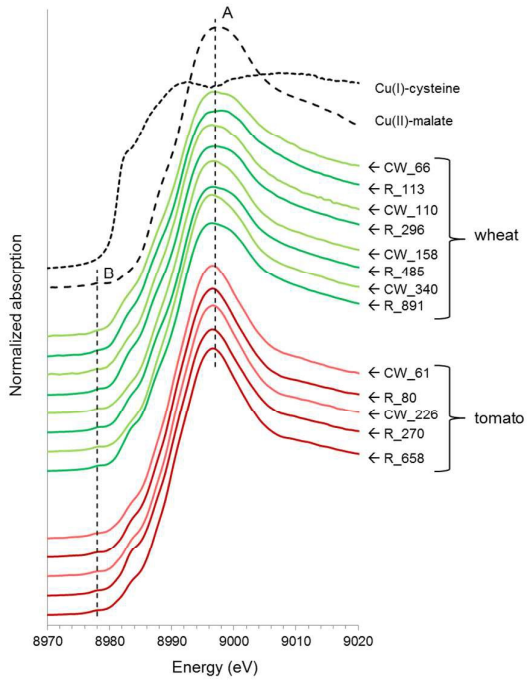

Figure 1 Normalized Cu K-edge X-ray absorption near-edge spectroscopy (XANES) spectra for wheat and tomato roots (R) and cell walls (CW) and for two reference compounds, i.e. Cu(I)-cysteine and Cu(II)-malate. The number in the sample name refers to the concentration of Cu bound to the plant material (mg.kg^{-1} initial dry roots).

All EXAFS spectra exhibited a shoulder at 5.6 Å^{-1}, which was more or less pronounced depending on the sample (Figure 2 a, feature C). This shoulder was also observed on the Cu(II)-histidine spectrum but not on that of Cu(II)-formate. The wheat spectra could be differentiated from those of tomato by a shift in the first oscillation (between 2.9 and 3.7 Å^{-1}) of 0.12 Å^{-1} (Figure S5). This shift was also observed between Cu(II)-histidine and Cu(II)-formate.

All FT presented a first peak at around 1.5 Å (feature D, distance uncorrected for phase shift) representing the first coordination shell Cu-O which could be easily distinguished from Cu-S (feature E, at 1.8 Å as observed for the FT of Cu(I)-cysteine, Figure 2b). Furthermore, the FT of wheat and tomato roots and CW exhibited a signal close to that of Cu(II)-histidine (features F and G). A characteristic peak (feature H), visible on the Cu(II)-formate spectrum was also visible on the FT of tomato roots and CW (Figure 2b).

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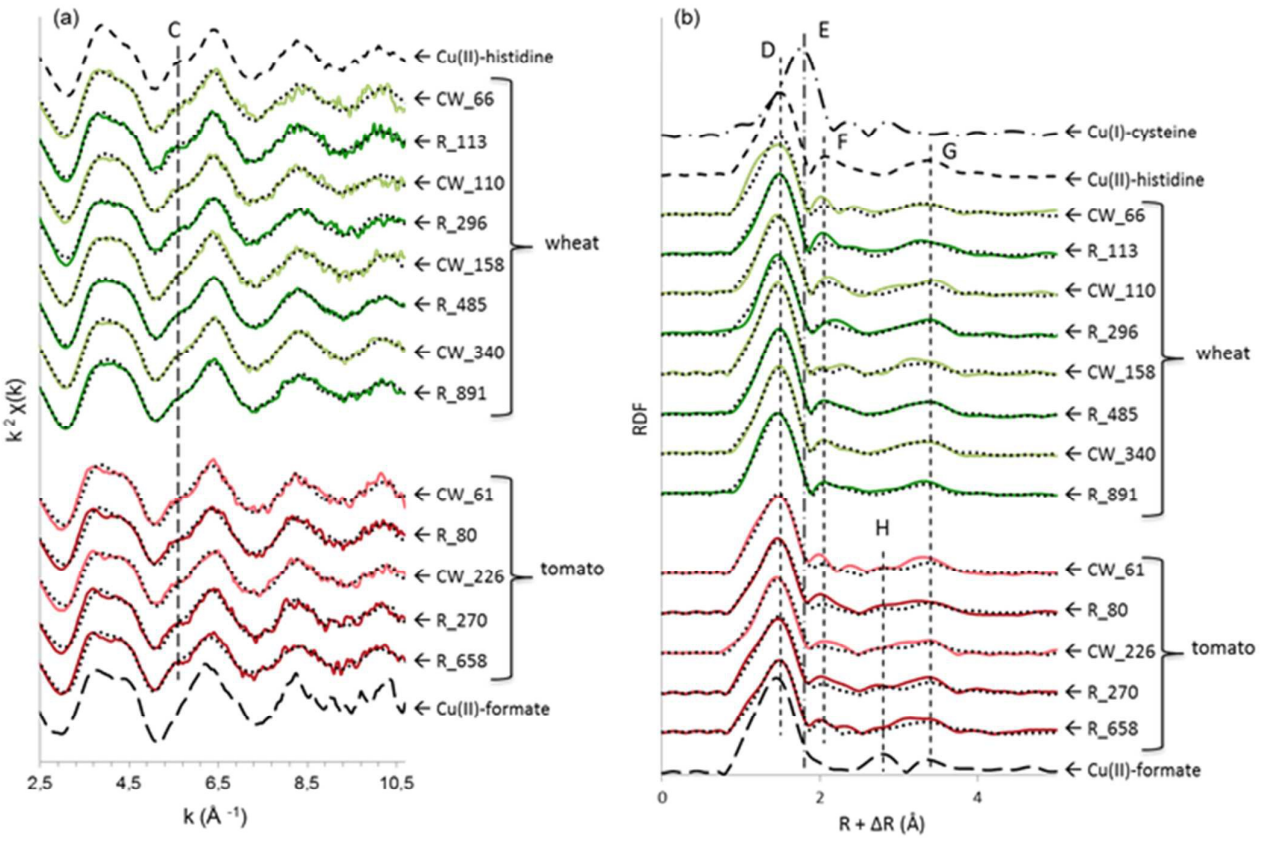

Figure 2 Cu K-edge k2-weighted extended X-ray absorption fine structure (EXAFS) spectra (a) and their radial distribution function (RDF) (b) for wheat and tomato roots (R) and cell walls (CW) and for three reference compounds, i.e. Cu(II)-histidine, Cu(II)-formate and Cu(I)-cysteine. Solid lines represent experimental spectra and dotted lines are the best linear combination fits. The distances are uncorrected for phase shifts. The number in the sample name refers to the Cu adsorbed in the plant material (in mg.kg-1 initial dry roots).

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No significant differences in Cu speciation in wheat or tomato roots and CW were found by linear combination fitting (LCF) analysis (Figs. 2 and 3 and Table S2). All of the best fits were obtained by combining Cu(II)-histidine with one or more reference compounds. Cu(II)-histidine represented 37 to 73% and 42 to 57% of the EXAFS spectra for wheat samples and tomato samples respectively (Figure 3, Table S2). The attempts to model CW and root samples with Cu(II)-histidine reference compound always significantly decreased NSS. For example, in wheat roots containing 485 mg Cu kg^{-1} (R_485), adding Cu(II)-histidine reference improved the spectral match and decreased the NSS by 43%, with the best statistical agreement obtained with a mixture of 65% Cu(II)-histidine, 18% Cu(II)-malate and 17% Cu(II)-malonate.

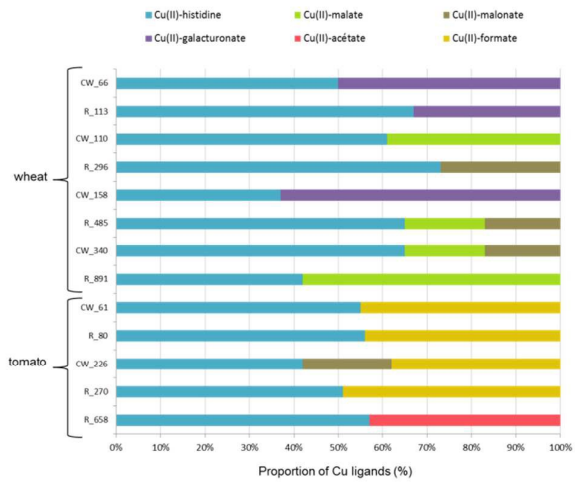

Figure 3 Proportion of each Cu ligand derived from the best fit of the Cu K-edge k2-weighted extended X-ray absorption fine structure (EXAFS) spectra for wheat and tomato roots (R) and cell walls (CW).

In addition to Cu(II)-histidine, Cu(II)-formate was identified from the spectra as the second compound in tomato roots and CW, i.e. 35 to 49%, except for tomato R_658, where the best fit was obtained with Cu(II)-acetate. In addition to Cu(II)-histidine, the reference compounds used in the LCF analysis of wheat roots and CW spectra included Cu(II)-galacturonate, Cu(II)-malate or Cu(II)-malonate regardless of the pCu_T.

^{13}C-NMR identification of functional groups involved in Cu binding

Cu sorption in wheat and tomato roots measured in batch experiments 2 and 3 looked similar (Figure S4), demonstrating that the functional groups involved in Cu binding as identified by ^{13}C-NMR could be related to the Cu binding site affinity characterized with Model VII.

As expected, the ^{13}C-NMR signal for all samples was dominated by root carbohydrates, i.e. resonances of 60-110 ppm (cellulose, hemicellulose, pectin, etc.) which accounted for 60% of the C detected in tomato and 80% in wheat and the line around 172 ppm (uronate and protein carboxyls/amides) (Figure 4a and b). Total phenols accounted for around 1-2% of the detected C, and aromatic compounds including lignins and aromatic amino acids jointly represented less than 5% in all cases, which was negligible.

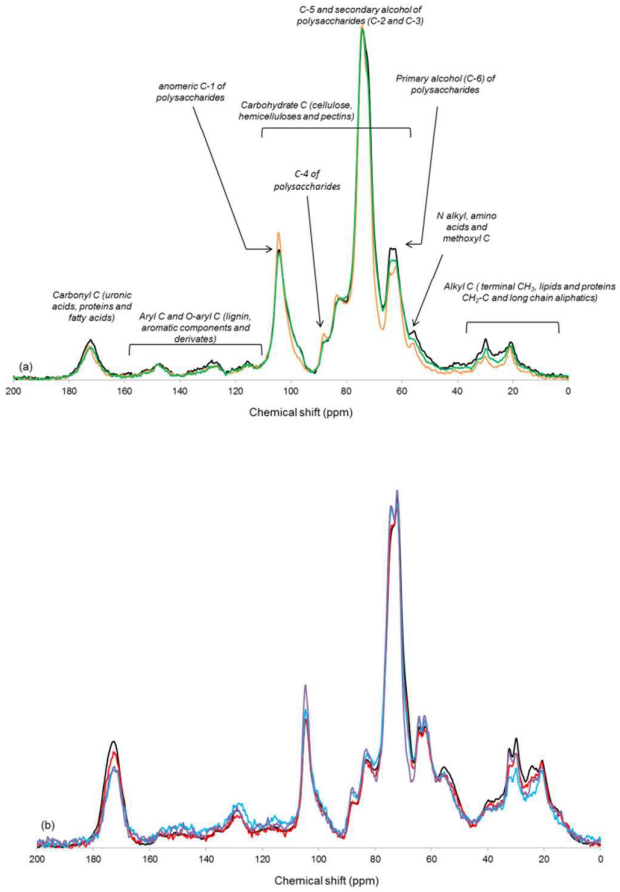

Figure 4 13C-NMR spectra for (a) wheat roots containing 0 (black line), 4855 (orange line) and 8023 (green line) mg.kg-1 dry mass and (b) tomato roots containing copper bound at 0 (black line), 832 (red line), 2785 (blue line) and 9864 (purple line) mg.kg-1 dry mass.

The major difference in root composition when comparing tomato and wheat samples was the proportion of alkyl C, presumably proteins. This fraction of aliphatic C accounted for approximately 30% in tomato roots (Figure 4b) whereas this proportion was only around 15% in wheat roots (Figure 4a). It should be kept in mind, however, that these were rough estimates since aliphatic amino acids such as valine (Val), threonine (Thr), proline (Pro) and isoleucine (Ile) have resonances of 60 to 70 ppm, i.e. the carbohydrate region, and could not be distinguished from cellulose type material under our experimental conditions.

Regarding tomato roots, the addition of increasing amounts of Cu led to a decrease in peak intensities in alkyl and carbonyl/carboxyl regions (Figure 4b). The intensity variations observed between 0 and approximately 60 ppm could not be converted with sufficient confidence into variations of the proportions since, assuming that they were due to Cu binding by proteins, only a limited fraction of C could potentially be impacted. Moreover, there was an overlap between contributions at around 55 and 60 ppm which complicated the determination of alkyl vs. carbohydrate compounds. Semi-quantitative analysis was possible, however, for carboxyl resonance variations around 172 ppm: when the Cu concentration increased from 0 to 9864 mg.kg^{-1} (dry mass), the surface area of the peak had decreased by approximately 30%. In wheat, equivalent amounts of added Cu(II) caused reduction of only around 10% of the contribution around 172 ppm, showing that Cu binding to carboxyl was less prevalent for this material (Figure 4a). Like tomato, the intensity of variations in the alkyl region was hard to transform into meaningful variations of the proportions. An interesting feature was the decrease in intensity of the line at around 62 ppm. As it seems unlikely that the primary alcohol of a hexose moiety specifically binds Cu, this decrease could be attributed to the loss of signal of Val, The, Pro and Ile amino acids, thereby suggesting that root proteins have a specific composition.

Modeling the acidic properties of roots, cell walls and plasma membranes

Parameterization of the two humic-acid (HA-like) model enabled us to almost neatly fit (*RMSR* < 1.9 cmol$_c$.kg^{-1}) the potentiometric titrations obtained experimentally for roots and CW, and theoretically for wheat and tomato PM (Table 1, Figure 5 and Figure S2). PROSECE [13] and two HA model had a similar goodness of fit, thus strengthening the relevance of the parameterization used herein.

Wheat and tomato roots and PM as well as wheat CW were each described by four p*Ka* (Table 1). For HA_I- and HA_{II}-borne sites, p*Ka* ranged from 3.4 to 7.5 and from 7.2 to 10.0, respectively. Tomato CW were characterized by only three p*Ka*, i.e. 3.6 and 6.0 for HA_I-borne sites and 9.75 for HA_{II}-borne sites. Only one out of the four distribution terms, i.e. *Δ*p*Ka*, was set at a value different from zero to adequately fit the data for each root material. As this was extensively introduced and discussed by Guigues et al. [13], these acidic properties along with additional NMR data suggested that carboxyl, amine, and phosphate groups could be involved as CW and PM binding sites.

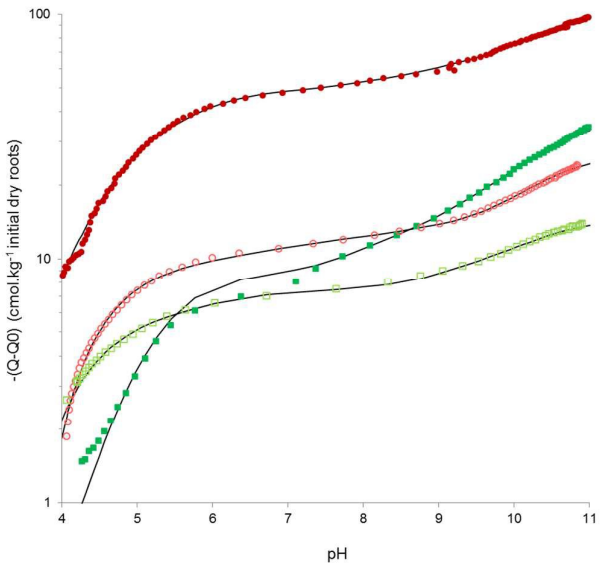

Figure 5 Potentiometric titrations for roots (filled symbols) and cell walls (empty symbols) of wheat (squares) and tomato (circles) expressed in charge (Q) corrected according to the initial charge (Q0). Solid lines refer to the fitting curves obtained with the two humic-acid (HA) model, as described in the Experimental section.

3.4 Copper sorption in roots, cell walls and plasma membranes

Irrespective of the root material considered in batch experiment 3, the adsorbed Cu concentration increased with the increasing total Cu concentration in solution (Figure 6). When expressed in root and CW dry mass, respectively, the quantity of Cu bound to roots and CW was the same, indicating that the sorbed amounts of Cu were nearly equivalent (results not shown). In accordance with the binding site density (Table 1), wheat roots and CW bound less Cu at a given Cu concentration in equilibrated solution and they were more rapidly Cu saturated than tomato roots and CW (Figure 6). Very similar findings were obtained for the theoretical sorption of Cu on PM (Figure S3). At a given solution Cu concentration, 28 to 49% of root-bound Cu was bound to PM while the remainder was bound to CW (not showed).

Modeling copper sorption in roots, cell walls and plasma membranes

The two HA model was able to quite neatly fit Cu sorption in wheat roots, CW and PM (*RMSR* = 76 mg.kg^{-1}) over the entire Cu concentration range in solution and in tomato (*RMSR* = 4.5 mg.kg^{-1}) for $pCu_T > 4.5$ (Figure 6, Figure S3). For $pCu_T < 4.5$, the two HA model deviated from the experimental data points for tomato roots, CW and PM. While the two HA model could be specifically parameterized to fit the tomato data for $pCu_T < 4.5$ in solution (data not shown), the modeling results are discussed below on the basis of the parameterization used to fit the data for $pCu_T > 4.5$, which corresponds to the environmentally-relevant range of root-exposed Cu concentrations in soil solution (Table 2).

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Table 1 Acidic properties modelled from experimental data for wheat and tomato roots, cell walls (CW) and derived for plasma membranes (PM) from theoretical potentiometric titrations. The total root and CW binding site densities (cmolc.kg-1 initial dry roots) were obtained by fitting the experimental data with PROSECE software [13], those of PM were derived from the previous results. The acidic site density of type 1(LH1) was twofold that of type 2 (LH2). The stability constants (pKai) and the distribution terms (ΔpKai) were obtained by fitting the experimental data with the two humic-acid model, as described in the Material and Methods.

		HA_I						HA_{II}						Total site density
		Type 1			Type 2			Type 1			Type 2			
		L_{H1}	pKa_1	ΔpKa_1	L_{H2}	pKa_2	ΔpKa_2	L_{H1}	pKa_1	ΔpKa_1	L_{H2}	pKa_2	ΔpKa_2	
Wheat	Roots	8.2	4.6	1.5	4.1	7.4	0	16.1	10.0	0	8.1	9.1	0	36.5
	CW	5.5	3.4	0	2.8	4.9	2.0	2.3	9.8	0	4.6	8.6	0	15.2
	PM	6.4	5.2	2.5	3.2	7.5	0	3.9	10.0	0	7.8	9.5	0	21.3
Tomato	Roots	30.3	4.0	0	15.1	5.1	0	34.5	9.8	0	17.3	8.3	1.8	97.2
	CW	10.6	3.6	0	5.3	6.0	3.5	8.5	9.75	0	4.3	9.75	0	28.7
	PM	20.4	4.1	0	10.1	4.6	0	25.3	9.6	0	12.7	7.2	1.8	68.5

Table 2 Copper sorption properties modelled for wheat and tomato roots, cell walls (CW) on the basis of experimental data and derived from theoretical sorption data for plasma membranes (PM). The intrinsic equilibrium constant (logKCu) and heterogeneity parameter (ΔLK2Cu) were obtained by fitting the experimental data with the two humic-acid (HA) model, as described in the Material and Methods. The intrinsic equilibrium constants (logKCu,2) were derived from logKCu,1, as given in equation (1) .

		HA_I			HA_{II}		
		Type 1	Type 2		Type 1	Type 2	
		$LogK_{Cu,1}$	$LogK_{Cu,2}$	$\Delta LK2_{Cu,I}$	$LogK_{Cu,1}$	$LogK_{Cu,2}$	$\Delta LK2_{Cu,II}$
Wheat	Roots	2.45	3.9	0.8	7.0	6.4	≤ 0.2
	CW	2.1	3.0	≤ 0.1	7.0	6.1	≤ 0.5
	PM	2.0	2.9	≤ 0.4	7.0	6.7	≤ 0.1
Tomato	Roots	2.1	2.7	≤ 0.2	7.0	5.9	≤ 0.1
	CW	2.3	3.8	≤ 0.2	7.5	7.5	≤ 0.2
	PM	1.9	2.1	≤ 0.1	6.0	4.5	0

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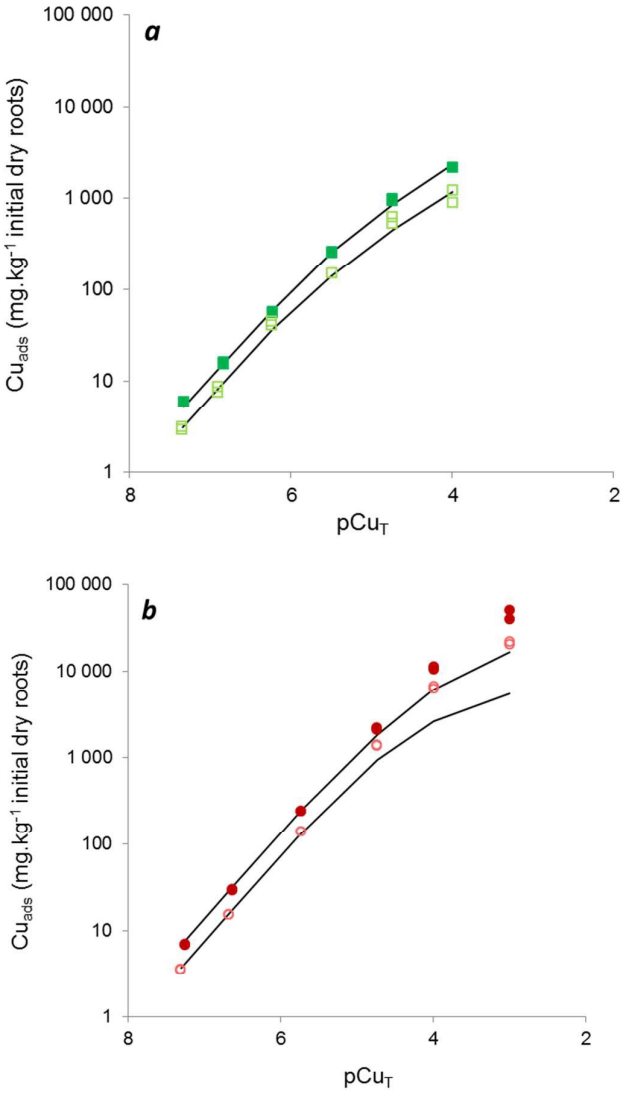

Figure 6 Copper binding by wheat (a) and tomato (b) roots (filled symbols) and cell walls (empty symbols). Solid lines refer to the fitting curves obtained with the two humic-acid (HA) model, as described in the Experimental section.

For HA_I sites, $\log K_{Cu,1}$ and $\log K_{Cu,2}$ ranged from 1.9 to 2.45 and from 2.1 to 3.9, respectively (Table 2). The intraspecific comparison revealed that HA_I sites of PM had lower intrinsic equilibrium constants than those of both wheat and tomato roots and CW. For HA_{II} sites, $\log K_{Cu,1}$ and $\log K_{Cu,2}$ ranged from 6.0 to 7.5 and from 4.5 to 6.7, respectively. For the HA_{II} sites of tomato, intrinsic equilibrium constants obtained for PM were lower than those of roots and CW. For HA_{II} sites of wheat, the $\log K_{Cu,1}$ of PM was equal to those of roots and CW and the $\log K_{Cu,2}$ of PM was slightly higher than those of roots and CW. An interspecific comparison revealed that equilibrium constants of roots and PM were higher for wheat than for tomato. In contrast, equilibrium constants of CW were higher for tomato than for wheat. The heterogeneity parameters ($\Delta LK2_{Cu}$) for both HA_I and HA_{II} sites were fairly low, i.e. 0.8 for wheat roots or not different from zero for all other wheat and tomato root materials.

Copper distributions between HA_I and HA_{II} sites obtained with the two HA model revealed a slightly higher contribution of HA_{II} sites to Cu binding in wheat roots and a similar contribution between HA_I and HA_{II} sites for tomato roots (Figure S6). While HA_I sites were overriding in wheat and tomato CW and tomato PM, HA_{II} sites prevailed in wheat PM.

Discussion

Dual copper coordination with carboxyl and nitrogen functional groups

There is increasing evidence that hard ligands other than carboxylic and hydroxyl groups could be highly involved in metal sorption within root apoplasts. Potentiometric titration of root CW revealed cation binding sites with p*Ka* higher than around 7.5, i.e. presumably attributed to phenolic and N functional groups (such as imidazole and amine groups) [18]. In addition to cation binding sites present in root CW, cation binding sites on the outer surface of cell PM could also have a role. Based on an investigation of the paramagnetic effect of cobalt (Co) on ^{31}P- and ^{13}C-NMR signals of phosphatidylserine, McLaughlin et al. [19] estimated that Co(II) was bound to the surface of the model membrane as a phosphate complex (13%), amine-carboxyl chelate complex (32%) and carboxyl complex (54%). A substantial number of Cu binding sites on root-cell PM may hence involve not only carboxyl groups but also phosphate, phenolic and N functional groups.

It is generally assumed that it is hard to distinguish between Cu-N and Cu-O coordination for biomolecules with XAS due to their similar atomic number. However, the Cu-N distance (≈2.00 Å) is on average slightly longer than the Cu-O distance (≈1.95 Å) [20]. The two types of binding environment can thus be differentiated on the basis of these different distances (Fig. S8 in Collin et al. [6]). Cu-N and Cu-O bonds have been recently distinguished in biota. Indeed, Manceau et al. [21] and Collin et al. [6] succeed in showing that Cu was bound to histidine residues in *T. arvense* root CW and in *Phyllostachys fastuosa* roots, respectively.

In line with these results, we found that Cu(II)-histidine-like species were ubiquitous in all samples but one and (co-)dominated Cu speciation in 10 out of the 13 root and CW samples (Figure3, Table S2). In the Cu(II)-histidine reference compound, Cu was simultaneously bound to O and N atoms borne by carboxylic and amine groups [22]. This reflects the involvement of amino acid residues present in CW [23] and on the outer surface of PM [24]. In agreement, Rubino and Franz [25] recently reviewed the contribution of histidine as one of the three main ligands (with cysteine and methionine) involved in Cu(II) coordination in a wide range of proteins such as those involved in Cu reductase activity and Cu transport at the surface of cell plasma membrane of root cells.

Copper was also associated with O atoms in wheat and tomato roots and CW (Figure3, Table S2). While the Cu-O local environment was ubiquitous in all samples, it (co-)dominated Cu speciation in only 6 out of the 13 samples. Copper was likely bound to carboxyl and alcohol groups, as in Cu(II)-galacturonate and Cu(II)-malate. This environment likely reflects the participation of CW polysaccharides such as pectins and of periplasmic arabinogalactan in Cu binding [26]. Such CW sites have already been identified as Cu binding sites in roots of cowpea exposed to Cu for 24 h [5].

Beyond the contribution of Cu-N and Cu-O first coordination shells, no other Cu ligand was detected by XAS analysis in wheat and tomato roots and CW. The absence of a Cu-phosphate bond suggests that PM phospholipids were not involved in Cu binding or that the proportion was under the detection threshold (i.e. below 10%). Indeed, Cu has a low affinity for phosphate groups relatively to carboxyl and N functional groups, in contrast to zinc [5, 27]. Moreover, we did not detect a Cu-sulfur bond either. This bond has been detected for plants that cope with Cu by internal sequestration via several S-rich ligands (e.g. metallothioneins) or by biomineralization of copper sulfide [6]. As the dry roots and CW we studied were metabolically-inactive, these physiologically-driven processes were likely not active.

The homogeneity of Cu speciation, whatever the plant species and root apoplastic compartment, was further supported by the values fitted for $\Delta LK2_{Cu}$, in the two HA model. This parameter was designed to account for the presence at low concentration of binding sites exhibiting a very high affinity for metals in humic substances, thus enabling simulation of strong binding behavior at low metal concentration [16]. The $\Delta LK2_{Cu}$ values fitted in the two HA model (≤ 0.8; Table 2) were much lower than those reported for humic substances (2.34 for Cu), suggesting that such sites are either absent from root apoplasts or can be neglected.

High-affinity copper binding

XAS analysis showed that Cu is coordinated in root apoplasts either with a single O ligand mainly involving carboxyl groups or with both O and N ligands involving carboxyl and N functional groups presumably of amino acids. Copper has a much higher affinity for synthetic and natural ligands with both carboxylic and N functional groups (e.g. ethylene diamine tetraacetic acid, histidine, nicotianamine) than for ligands with carboxyl groups only [28, 29].

In line with these considerations, the two HA model was parameterized for wheat and tomato roots by considering that 34 to 47% of binding sites were low-p*Ka* sites (i.e. type 1 and 2 in HA_I), presumably corresponding to carboxyl-like groups and having low-affinity Cu binding constants ranging from $\log K_{Cu}$ 2.1 to 3.9. In addition, the two HA model was further parameterized by considering that 53 to 66% of binding sites were high-p*Ka* sites (i.e. type 1 and 2 in HA_{II}), presumably corresponding to N functional groups and having high-affinity Cu binding constants ranging from $\log K_{Cu}$ 5.9 to 7 (Tables 1 and 2). Note that these high-affinity Cu binding sites presumably corresponding to N functional groups have even higher Cu binding constants than those of similar high-p*Ka* sites commonly described in humic substances ($\log K_{Cu}$ 4.8-5.6) but which likely correspond to phenolic groups [16].

The findings of the two HA model further suggested that high-affinity Cu binding sites markedly contributed to Cu complexation especially when considering the root surfaces where these high-affinity sites bound 40-60% of the total complexed Cu (Figure S6a and b).

Compared to carbohydrates that usually represent 55-90% of the rhizodermal and hypodermal root CW, structural proteins represent less than 35% in dry mass [30]. This questions whether these structural proteins were sufficient to provide the quantity of N functional groups necessary to bind 40-60% of total complexed Cu. The maximal amount of Cu bound to high-affinity sites ranged from 1 cmol kg^{-1} in wheat CW to 40 cmol kg^{-1} in tomato roots (Figures 6 and S6). In comparison, the N concentration ranged from 70 cmol kg^{-1} in wheat CW to 360 cmol kg^{-1} in tomato roots. As one atom of Cu is usually coordinated with approximately 3 N atoms in 3D protein structures [31], this means that approximately 3-4% of total N in wheat CW and roots and 30-40% of total N in tomato CW and roots have to be involved to bind 40-60 % of total complexed Cu.

Relative contribution of cell walls and plasma membranes

The comparison of Cu sorption in roots and CW suggested that PM bound only 28 to 49% of Cu in wheat and tomato roots. This lower quantitative contribution of PM over CW to Cu complexation is surprising at first sight as PM was previously showed to bear 60-70% of the total binding site density in wheat and tomato roots [13]. This first suggests that cation binding sites in CW exhibit a much higher affinity for Cu than those located at the outer surface of PM. This hypothesis is relevant for tomato as the $\log K_{Cu}$ of CW sites were 0.4 to 3 units higher than those of PM sites (Table 2). However, for wheat the $\log K_{Cu}$ of CW sites were either only slightly higher by 0.1 units (for HA_I sites and type-1 sites of HA_{II}) or lower by 0.6 units than the $\log K_{Cu}$ of PM sites.

The weak Cu binding strength of PM in wheat roots could otherwise be related to electrostatic effects that were shown to highly influence the ion concentration on PM surfaces [32, 33]. Indeed, the net negative charge of isolated CW was higher

than that of roots for wheat when the solution pH was lower than 5.5 (Figure 5), thus indicating that PM were positively charged in sorption experiments performed at pH 4.5-5. Wheat root PM may hence have exerted a repulsive effect on Cu^{2+} thus lowering their Cu binding strength.

An additional hypothesis that may explain the greater contribution of CW over PM to Cu complexation is the spatial arrangement of these two compartments. By design, any metal cations interacting with roots first interact and bind within CW and after passing through the CW the remaining non-bound metal cations then interact and bind on the outer surface of PM. Consequently, we hypothesize that Cu, which exhibits a high affinity for binding sites in root apoplasts, first and thus mainly accumulates in CW even though CW have a lower binding site density and Cu-affinity than PM as observed for wheat.

It should be noted that the comparison we made between roots and CW material to reveal the relative contribution of CW and plasma membranes in Cu binding on root apoplasts was, however, limited by potential experimental artefacts. While the ^{13}C-NMR data suggested that the carbohydrate structure was preserved during the CW isolation procedure [13], we likely concomitantly extracted some soluble and labile proteins from CW by using $Ca(NO_3)_2$ [34]. In addition, plasma membrane leakage during freezing and thawing of roots made the functional groups on both the outer and inner surface of plasma membranes potentially available for Cu binding. Due to these potential experimental artefacts, further studies should be conducted to investigate the relative contribution of CW and plasma membranes in metal binding on root apoplasts with *in situ* and highly-resolved analytical techniques such as Nano-SIMS [35].

Interspecific comparison

Tomato roots exhibited a 2.7-fold higher binding site density than wheat roots [13]. Calculations performed with the two HA model suggest that the binding sites had a higher affinity for Cu (i.e. higher $\log K_{Cu}$) in wheat roots than in tomato roots (Table 2). The higher mean contribution of the Cu-histidine reference in the LCF of EXAFS spectra (Figure 3) for wheat (62 ± 14 %) than for tomato (55 ± 3 %) could suggest a higher contribution of amino acids to root Cu binding in wheat as compared to tomato. This preferential binding to N functional groups in wheat was further supported by the NMR data. Indeed, the addition of Cu led to modifications primarily in the spectral region below 60 ppm, i.e. where signals from amino acids were expected, although the modifications were hard to quantify, whereas the resonance of carboxyl C around 172 ppm was generally unaffected (Figure 4a). In addition, the high affinity of N functional groups for Cu on wheat roots is further supported by a trend in the LCF results, showing a decrease of the histidine contribution with increasing Cu concentration, i.e. when N functional groups became progressively saturated. No such trend is apparent with the tomato root materials for which the ratio of the relative contributions of N functional vs. carboxyl groups did not evolve with the Cu concentration. The fact that the NMR signal of both N functional and carboxyl groups are simultaneously affected with increasing Cu concentration is consistent with the LCF results for tomato. Although the amount of N in tomato roots was roughly twofold higher than in wheat, our spectroscopic data did not support preferential binding of Cu to N-groups for tomato.

This suggests that the nature and thus affinity for Cu, and/or (steric) accessibility of N binding sites differed in tomato and wheat roots.

The HLScale underestimated the nitrogen functional groups contribution

How could the HLScale, which is defined with a single value, account for both the low- and high-affinity sites we identified in root apoplasts? The HLScale may correlate with both low- and high-affinity sites detected for a range of metal cations. However, this assertion could not be tested herein as we focused our investigation solely on Cu. Alternatively, the Cu binding strength classification on the HLScale shows that Cu^{2+} was one of the divalent cations exhibiting the highest binding strength toward hard ligands, but it remained (much) lower than the binding strength of trivalent cations [12]. This behavior is typical of the binding strength of Cu to most hard ligands, with the notable exception of some amino acids that are known to play an important role in metal transport to and within plants. For instance, Cu exhibits an exceptionally high affinity for phytosiderophores exuded by monocot roots and some histidine-rich sequences of metal-transport proteins located on the outer surface of PM [36, 37]. In comparison to synthetic N ligands (e.g. EDTA, NTA or ATP), Cu exhibited with these biologically-relevant amino acids and proteins a binding strength close to or even exceeding that of trivalent cations such as Fe^{3+}.

Owing to the substantial contribution of amino acid residues in Cu binding in root apoplasts, we suggest that the HLScale likely underestimates the high affinity of these binding sites for Cu. This may partly explain the underprediction of the unexpectedly high Cu rhizotoxicity underlined by Kopittke et al.[11].

Conclusions

Our combined modeling and spectroscopic investigation findings revealed a dual local environment of Cu in wheat and tomato root apoplasts. As expected, CW pectins were identified as one of the major Cu binding sites. We found no evidence of PM phospholipid involvement. More surprisingly, Cu speciation was (co)dominated by N functional groups within CW and PM. These N groups exhibited a high affinity for Cu and hence markedly contributed to Cu complexation in root apoplasts. Furthermore, we suggest that the HLScale should be adjusted to better account for the contribution of biologically relevant N functional groups, which could help to explain the unexpectedly high rhizotoxicity exhibited by Cu.

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