Influence of CdCl_2 and CdSO_4 supplementation on Cd distribution and ligand environment in leaves of the Cd hyperaccumulator *Noccaea (Thlaspi) praecox*

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Abstract

Background and aims The aim was to investigate whether different Cd salts in the nutrient solution of the Cd/Zn hyperaccumulator *Noccaea (Thlaspi) praecox* alter leaf Cd distribution and Cd ligand environment, and plant fitness.

Methods Plants were grown for 8 weeks with 100/300 μM CdCl_2 or CdSO_4 . Leaf biomass, and total chlorophyll, anthocyanin, Cd, Cl, S and P concentrations were monitored. Cd localisation and ligand environment in leaves were analysed using quantitative synchrotron-based micro-X-ray fluorescence imaging,

and Cd K-edge X-ray absorption fine structure and Cd $\text{L}_{3\text{-edge}}$ micro-X-ray absorption near-edge structure measurements.

Results Cd uptake and plant fitness were comparable for CdCl_2 and CdSO_4 treatments, and depended on applied Cd concentration. In all treatments, Cd preferentially accumulated with high concentrations of Cl in vacuoles of large vacuolarised epidermal cells, bound mainly to oxygen-based (O)-ligands. In the mesophyll of CdCl_2 -treated plants, Cd was preferentially sequestered in vacuoles, while for CdSO_4 , Cd accumulated preferentially in the apoplast. In the symplast, O-ligands increased with increasing Cd concentrations; in the apoplast, sulphur-based (S)-ligands prevailed.

Conclusions Cd partitioning between leaf mesophyll apoplast and symplast and the Cd ligand environment in *N. praecox* depend on the Cd salt type and concentration added to the nutrient solution.

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Introduction

Cadmium (Cd) can have detrimental effects on plant growth and development even at very low concentrations. Nevertheless, a few plant species have evolved the ability to accumulate and tolerate Cd concentrations that

exceed 0.1 % dry shoot mass without showing stunted growth and/or other toxicity symptoms. *Noccaea* (*Thlaspi*) *caerulescens* from Ganges, France, and *Noccaea* (*Thlaspi*) *praecox* from Žerjav, Slovenia, are considered to be the most Cd-tolerant plant species, and they show the highest Cd hyperaccumulation capacities (Cosio et al. 2004; Vogel-Mikuš et al. 2005; Xing et al. 2008). In addition, *Arabidopsis halleri* (Cosio et al. 2004; Zhao et al. 2006) and *Sedum alfredii* (Tian-Hua and You-Zhang 2003; Tian et al. 2011) can accumulate significant amounts of Cd without detrimental effects on plant growth and development.

Various localisation techniques have been used to investigate the mechanisms of Cd accumulation and tolerance in model Cd-hyperaccumulating plants (Wójcik et al. 2005; Van Belleghem et al. 2007; Vollenweider et al. 2006; Isaure et al. 2006; Vogel-Mikuš et al. 2008; Pongrac et al. 2009; Tian et al. 2011). These have put special emphasis on relating the Cd distribution patterns at the tissue and cell levels to the Cd-tolerance mechanisms. Cd-localisation studies with different Cd-hyperaccumulating and Cd-non-accumulating species have shown variable distribution patterns among the different leaf-cell types. In the non-accumulating *A. thaliana*, Cd was detected in tracheids, but not in mesophyll tissue (Van Belleghem et al. 2007). Similarly, in *Salix viminalis*, the pectin-rich layers of the collenchyma cell walls of the leaf veins represent the main Cd sink (Vollenweider et al. 2006). Leaf veins and trichomes have also been shown to be the main storage sites of Cd in *A. halleri* (Isaure et al. 2006). In the hyperaccumulating ecotype of *Sedum alfredii*, Cd was mainly localised in the stem cortex and pith, while in the non-accumulating ecotype, Cd was restricted to vascular bundles (Tian et al. 2011). In *N. caerulescens*, Cd was localised in vacuoles of spongy and palisade mesophyll cells, and of some epidermal cells (Wójcik et al. 2005), while Zn was distinctly localised in vacuoles of large storage epidermal cells (Frey et al. 2000).

A recent study showed that Cd distribution patterns within *N. caerulescens* leaf tissues are governed by differential expression of the metal transporters in particular cell types (Leitenmaier and Küpper 2011). Large leaf epidermal storage cells show almost 14-fold greater Cd uptake rates when compared to small epidermal cells and mesophyll cells. This has been attributed to the differences in transport rates over the cell plasma membranes and the expression patterns of their

metal transporters. This mainly involves the TcZNT5 metal transporter, which is more abundant in the plasma membrane of epidermal storage cells than in other cell types (Leitenmaier and Küpper 2011). Although epidermal storage cells can have higher metal concentrations than mesophyll cells, it is the mesophyll tissue layer that harbours the greater total mass of metal (Ma et al. 2005; Vogel-Mikuš et al. 2008; Ebbs et al. 2009). The involvement of the leaf mesophyll in metal hyperaccumulation and tolerance is reasonable, as mesophyll cells are much more abundant and are positioned between the vascular bundles (representing a source of metals in the leaves after xylem loading) and the epidermal cells (Ebbs et al. 2009). In a study that compared Cd sorption and transport characteristics in the leaf mesophyll layer of the *N. caerulescens* ecotypes of Prayon (Zn/Cd hyperaccumulator) and Ganges (Cd/Zn hyperaccumulator) (Ebbs et al. 2009), a clear difference in Cd efflux between these two ecotypes was shown, with Prayon showing higher efflux rates. This suggests that the Cd taken up by Ganges mesophyll cells might be more effectively sequestered into the vacuoles, which decreases the need for efflux to achieve homeostasis in mesophyll cells (Ebbs et al. 2009). The Prayon and Ganges ecotypes differ also according to the Cd ligand environment in mesophyll cells, where Ganges showed a significantly higher portion of sulphur-based Cd ligands (S-ligands), while Prayon showed a larger portion of oxygen-based Cd ligands (O-ligands), which was attributed to the cell-wall components (Ebbs et al. 2009). This supports differential Cd localisation in these two *Noccaea* genotypes. Ebbs et al. (2009) concluded that the innate differences in the sorption and transport characteristics of the mesophyll layer in the leaves of the Ganges and Prayon ecotypes appear to be controlled by numerous factors, such as the developmental age of the plants, the concentration of Cd to which these cells are exposed, the duration of the Cd exposure, and the Cd status of the leaf cells before and during the exposure (Ebbs et al. 2009).

Accordingly, there are also implications that as well as different cell types having particular physiological properties, the metal uptake and distribution patterns in leaves might be related to the plant growth conditions, and especially to the presence of different cations and anions in the nutrient solution. In field-collected Cd/Zn hyperaccumulator *N. praecox*, for example, the highest concentrations of Cd were in

the vacuoles of the mesophyll cells (Vogel-Mikuš et al. 2008), while in a hydroponic experiment where *N. praecox* plants were grown in the presence of high Cd and low Zn concentrations, Cd was mainly localised in epidermal tissues (Pongrac et al. 2010). The methodology of the latter study, however, did not define Cd localisation at the cellular and subcellular levels.

When metals are added to the nutrient solution, they are always added in the form of soluble metal salts. In the studies of Cd uptake, transport and compartmentalisation, three Cd salts have usually been used: CdSO_4 (Küpper et al. 2004; Ebbs et al. 2009; Pongrac et al. 2009), CdCl_2 (Tolrà et al. 2006) and $\text{Cd}_2(\text{NO}_3)_3$ (Isaure et al. 2006). This addition of metal salts into nutrient solutions not only alters the cation concentrations, but also the anion concentrations; however, not much attention has been paid to this issue in studies of the mechanisms of metal uptake and tolerance.

Chlorine (in the form of chloride, Cl^-), for example, is an essential element for plants, as it acts as a major osmotically active solute in the vacuole and it is involved in both turgor and osmoregulation. In the plant-cell cytoplasm, Cl^- regulates the activities of enzymes and acts as a counter anion, with Cl^- fluxes implicated in stabilisation of the membrane potential, and regulation of pH gradients and electrical excitability (White and Broadley 2001). Chloride applied to leaves in the form of NaCl was shown to promote Cd accumulation in durum wheat grain, which was attributed to the Cl^- complexing of Cd and thus to a reduction in the positive charge on the metal ion (Ozkutlu et al. 2007). Charge reduction or removal (e.g., CdCl_2^0 species) increases the diffusivity/lipophilicity of Cd and enhances its penetration through the membranes of leaf epidermal cells (Ozkutlu et al. 2007). In metal-hyperaccumulating plants, however, the possible role of Cl^- in metal detoxification has been overlooked, as low-molecular-weight ligands have been shown to bind metals in leaf tissues. These have included organic acids (e.g., malic, citric and oxalic acids) stored in vacuoles, and histidine, nicotianamine, phytic acid, metallothioneins, phytochelatins and low-molecular-weight thiols, such as glutathione, cysteine and others (Salt and Krämer 2000; Callahan et al. 2006; Hernandez-Allica et al. 2006).

Sulphur, on the other hand, has been shown to be actively involved in Cd detoxification in metal-

hyperaccumulating plants. Although phytochelatins do not have any crucial role in Cd detoxification, a significant portion of Cd has been seen to be bound to thiol groups, especially in young leaves (Küpper et al. 2004), roots (Hernandez-Allica et al. 2006; Vogel-Mikuš et al. 2010) and seeds (Vogel-Mikuš et al. 2010). Enhanced uptake of S, was reported in the *N. caerulescens* Ganges ecotype, as a defence mechanism against Cd toxicity (Küpper and Kochian 2010).

Studies of the distribution of metals within plant tissues are crucial for understanding the physiology of metal hyperaccumulation and tolerance. Numerous methods are available, each of which offers different degrees of complexity, sensitivity, and spatial resolution. The spatial distribution of metals within biological tissues is usually investigated using micro-analytical techniques that are based on the emission of characteristic X-rays, as excited either by charged particles (electrons, protons or heavier particles) or photons (synchrotron radiation) (Khan et al. 1984; Vázquez et al. 1992; Frey et al. 2000; Küpper et al. 2000; Cosio et al. 2005; Wójcik et al. 2005; Isaure et al. 2006; Vogel-Mikuš et al. 2008; Pongrac et al. 2010). Synchrotron-based micro-X-ray fluorescence (SR μ -XRF) is a unique technique, in that it allows the mapping of element distributions at the tissue, cellular and sub-cellular levels. In-situ detection of the chemical forms of elements can also be achieved by micro-X-ray absorption near-edge structure spectroscopy (μ -XANES).

The only drawback of element distribution imaging using SR μ -XRF is the absence of suitable software for fully quantitative analyses. Therefore, studies that have used this technique have only provided element distribution maps that were constructed by displaying the number of counts integrated over the energy window corresponding to the strongest $K\alpha$ or $L\alpha$ lines of the mapped elements (Isaure et al. 2006; Zhang et al. 2011; Tian et al. 2011), or by integration after X-ray spectra deconvolution, using software such as PyMCA (Solé et al. 2007), and normalisation according to dwell time and beam current (Tolrà et al. 2011; Regvar et al. 2011). By using a fully quantitative analysis, it would be possible to calculate the element concentrations (in $\mu\text{g g}^{-1}$ dry weight) and to provide quantitative element distribution maps of tissues and cells. In this way, element distribution maps of tissues and cells of differentially treated plants and those recorded under different experimental conditions

would become comparable. In particular, only fully quantitative analyses would allow the correction of the features that result from absorption and enhancement of the X-ray fluorescence lines in the sample matrix and from the uneven thickness of the sample. For the purpose of fully quantitative SR μ -XRF element distribution analysis, a software based on the fundamental parameters of XRF spectroscopy has been developed in our laboratory. Therefore, the resulting maps of the element distributions can now be presented in a fully quantitative manner.

The aims of this study were to: (i) compare Cd, Cl, S and P uptake and plant fitness, and Cd ligand environment (using Cd K-edge X-ray absorption fine structure [EXAFS]) in the leaves of the Cd/Zn hyper-accumulator *N. praecox* when grown in hydroponic solution supplemented with 100 μ M and 300 μ M CdCl₂ or CdSO₄; (ii) investigate the Cd distribution patterns and Cd ligand environment in the epidermis and mesophyll of mature leaves of the plants supplemented with the different Cd salts (CdCl₂ or CdSO₄), using fully quantitative SR μ -XRF imaging and Cd L₃-edge μ -XANES, respectively; and (iii) relate Cd distribution patterns and Cd ligand environment to the Cd-tolerance mechanisms. We hypothesised that addition of different Cd concentrations as these different Cd salts (CdCl₂ and CdSO₄) will influence the physiological responses of the plants (biomass production, and total chlorophyll, anthocyanin, Cd, Cl, S and P concentrations) as well as the Cd distribution and Cd ligand environment in the leaves.

Materials and methods

Plant growth conditions

Mature seeds of *N. praecox* were collected at a Pb-, Cd- and Zn-polluted site in Slovenia (Vogel-Mikuš et al. 2005) and sown in vermiculite. The seeds were germinated in growth chambers with a 16-h day period, a light intensity of 160 μ mol m⁻² s⁻¹, day/night temperatures of 21 °C/19 °C, and relative humidity of 50 to 60 %. After 30 days, the seedlings were transferred to the modified nutrient solution (Tolrà et al. 1996) (four seedlings per one-L pot) containing 3 mM KNO₃, 1 mM NH₄H₂PO₄, 2 mM Ca(NO₃)₂, 0.5 mM MgSO₄, 66 μ M FeEDTA, 50 μ M KCl, 46 μ M H₃BO₃, 9 μ M MnSO₄, 1.5 μ M CuSO₄,

0.14 μ M (NH₄)Mo₇O₂₄, and 15 μ M ZnSO₄. pH of nutrient solution was adjusted to 6.5 and monitored twice per week. After 14 days of adaptation, the nutrient solution was supplemented with 100 μ M or 300 μ M CdCl₂ or CdSO₄, and pH was again adjusted to 6.5 and the plants were grown for a further 8 weeks. According to Geochem-EZ (<http://www.plantmineralnutrition.net/Geochem>) calculations 79 % and 78 % of Cd was in ionic form in CdSO₄ and CdCl₂ supplemented plants, respectively. The additions of CdCl₂ or CdSO₄ resulted in modified S and Cl concentrations in the nutrient solution. The nutrient solution supplemented with 100 μ M CdCl₂ or CdSO₄ contained 525 μ M and 625 μ M SO₄²⁻, respectively, and 250 μ M and 50 μ M Cl⁻, respectively. The nutrient solution supplemented with 300 μ M CdCl₂ or CdSO₄ contained 525 μ M and 825 μ M SO₄²⁻, respectively, and 650 μ M and 50 μ M Cl⁻, respectively. One set of plants was retained in the control nutrient solution, which cumulatively consisted of 525 μ M SO₄²⁻ and 50 μ M Cl⁻. The nutrient solution was replaced every 7 days, and was constantly aerated using an air pump. The experiment was repeated twice, with 5–10 plants per treatment.

Leaf biomass, chlorophylls and anthocyanins

Upon harvesting, the leaves were rapidly frozen in liquid nitrogen, freeze-dried and weighted. In freeze-dried leaves, the total chlorophyll concentrations were determined photometrically in 80 % acetone extracts, according to Monni et al. (2001), with calculations according to Lichtenthaler and Buschmann (2001). The concentrations of anthocyanins were determined as described by Lindoo and Caldwell (1978). The total contents of the chlorophylls and anthocyanins were calculated by multiplying the leaf biomass of a particular plant by its total chlorophyll or anthocyanin concentration, expressed as μ g plant⁻¹.

Leaf bulk element analysis

The concentrations of Cd, Cl, S and P in the leaves were determined by XRF spectroscopy. The freeze-dried plant material was pulverised and homogenised in a mortar. Depending on the available material, 100 mg to 500 mg of the powdered leaves were pressed into pellets using a pellet die and hydraulic press. ²⁴¹Am (25 mCi) and ⁵⁵Fe (25 mCi) (Isotope

Products Laboratories, Valencia, USA) were used as the primary excitation sources for the analysis of Cd and of Cl, S and P, respectively. The fluorescence radiation emitted was collected using an energy dispersive X-ray spectrometer, equipped with a Si(Li) detector (Canberra, 157 Meriden, USA), with a 25- μm -thick Be window. The energy resolution of the spectrometer at count rates below $1,000 \text{ c s}^{-1}$ was 175 eV at 5.9 keV. The XRF analysis of Cd was performed in air, and of P, Cl and S under vacuum, and the samples were irradiated for 1,000 s to 5,000 s to obtain spectra with sufficient statistics (Nečemer et al. 2008). The analysis of the X-ray spectra was performed using the analysis of X-ray spectra by an iterative least squares programme (van Espen and Janssens 1993), as included in the quantitative X-ray analysis system software package (Vekemans et al. 1994). Element quantification from the measured spectra was performed using the quantitative analysis of environmental samples based on fundamental parameters (Kump et al. 2007). Quality assurance for the element analyses was performed using standard reference materials: NIST SRM 1573a (tomato leaves as a homogenised powder); CRM 129 (hay powder); and OU-10 (geological sample of longmyndian greywacke, GeoPT24, certified through an international proficiency test of the International Association of Geoanalysts), all analysed in the form of pressed pellets.

Statistical analysis

Statistical analysis of the plant biomass, chlorophyll, anthocyanin and mineral concentrations and their corresponding contents was carried out with the Statistica Statsoft, version 7.0, software. The significances of the differences among the means of the different treatments were evaluated by one-way ANOVA, and comparisons among the means were carried out using Duncan's *post-hoc* test, at a significance level of $p \leq 0.05$.

Cd K-edge X-ray absorption fine structure spectrometry

The freeze-dried *N. praecox* leaves included young, mature and old leaves, which were homogenised in a mortar with liquid nitrogen and packed into 5-cm plastic holders for the Cd K-edge EXAFS measurements

(Vogel-Mikuš et al. 2010). These measurements were carried out in transmission detection mode at beamline C of the Hamburg Synchrotron Radiation Laboratory (HASYLAB) of the German Electronic Synchrotron (DESY). The beamline was equipped with a Si 311 two-crystal monochromator with about 2 eV resolution at the Cd K edge (26,711 eV). Higher harmonics were eliminated by detuning the monochromator crystals to 60 % of the rocking curve maximum, using the beam-stabilisation feedback control. The intensity of the monochromatised beam was monitored with three 10-cm-long ionisation detectors. These were filled with krypton, the first to a pressure of 190 mbar, and the second and third to a pressure of 1,000 mbar. The samples were placed between the first pair of detectors, with the reference Cd metal foil between the last pair, to check the stability of the energy scale. The absorption spectra were measured within the interval $[-250 \text{ eV to } 1,000 \text{ eV}]$ relative to the Cd K edge. In the XANES region, equidistant energy steps of 0.5 eV were used, while for the EXAFS region, equidistant k-steps ($\Delta k \approx 0.03 \text{ \AA}^{-1}$) were adopted, with an integration time of 1 s step^{-1} . The Cd K-edge EXAFS spectra recorded on the samples of *N. praecox* leaves were analysed with the IFEFFIT package (Ravel and Newville 2005), as described in detail in Vogel-Mikuš et al. (2010).

Sample preparation for mapping element distributions

Optimally developed leaves were detached from the plants. Leaf fragments of $5 \text{ mm} \times 2 \text{ mm}$ were quickly excised with a razor blade from the upper part of each leaf, across the main vein of the leaf, and embedded on stainless steel needles (diameter, 2 mm), dipped into tissue-freezing medium (Jung, Leica), and rapidly frozen in propane cooled with liquid nitrogen (Vogel-Mikuš et al. 2008). The frozen samples were transferred to a Leica CM3050 cryotome (Leica, Bensheim, Germany) in liquid nitrogen, and were sectioned into 20- μm -thick sections. The cryotome chamber and head temperature were maintained between $-30 \text{ }^{\circ}\text{C}$ and $-25 \text{ }^{\circ}\text{C}$ during the sectioning. Individual sections were placed in aluminium containers between two layers of pre-cooled fine structured filter paper and fixed with a pre-cooled stainless steel cover, to ensure the flatness of the sections. Aluminium containers with leaf cross-sections were transferred in liquid nitrogen to an Alpha 2–4 Christ freeze dryer and freeze-dried at a temperature that gradually increased from

–180 °C to –25 °C, and a pressure of 0.12 mbar, for 2 days. After freeze-drying, the leaf cross-sections were adjusted to room temperature and placed in a desiccator until further handling. Prior to the measurements, freeze-dried leaf cross-sections were mounted on stainless steel sample holders between two layers of 4 µm ultralene foil (SPEX SamplePrep).

Mapping of element distributions using synchrotron micro-X-ray fluorescence spectrometry

The mapping of the element distributions in the leaves of the differently treated *N. praecox* plants was performed using a scanning X-ray microscope at the ID21 beamline of the European Synchrotron Radiation Facility, Grenoble, France. For the mapping, two to four regions per treatment were selected in the leaf cross-sections, although the element distribution maps of only the representative specimens are presented.

The X-ray beam delivered by the undulator was monochromatised using a Si 111 double crystal monochromator, and focused to a submicron probe ($0.3 \times 0.7 \mu\text{m}^2$) using zoneplate micro-focusing. The X-ray fluorescence emission of the sample was collected using an 80 mm² XFlash 5100 silicon drift diode (Bruker, Germany). The excitation energy for the scan was set to 3.55 keV (i.e., above the Cd L₃ edge and below the potassium K edge, to avoid the strong potassium signal), and the distribution maps of Cd, S, Cl and P were simultaneously recorded.

In selected leaf tissue cross-sections regions of 100 µm × 100 µm, 100×100 pixels were selected using a video-microscope installed in an experimental chamber, and scanned with a micro-beam under vacuum, with a dwell time of 300 ms pixel^{–1}. The dead time of the measurements was below 5 %. XRF spectra were recorded using an X-Ray Instrumentation Associates acquisition system. In the quantification procedure, the recorded X-ray spectra were first deconvoluted using the PyMca software (Solé et al. 2007), then corrected for dead time, and normalised per beam current in the ring. Element intensities with their uncertainties were obtained in a matrix form, with data for each measured pixel in a single row. The quantification procedure applied was an extension of the quantification used in the conventional XRF analysis (Quantitative analysis of environmental samples; Kump et al. 2007) and was based on fundamental parameters. The fundamental parameters used were

taken from the database of Elam et al. (2002), and the system of equations for measured elements was based on the relationship initially developed by Sherman (1955). The sample thickness was calculated from the scattering peak. The calibration of the XRF system was performed with a set of thin (a few 10's of µgcm^{–2} thick) standard samples (Al, CuSx, CdSe) obtained from Micromatter, and the respective geometry and/or instrumental constants were calculated (He and Van Espen 1991). Additionally, the geometric constant of the same value was also obtained from the measured incident photon beam by the photodiode placed behind the sample, and considering the solid angles extended from the sample to the detector. Using the fundamental parameters, the respective element sensitivities were then calculated. The uncertainties introduced by the fundamental parameters and the calibration procedure were partially compensated for if the experimental sensitivities were used in further quantifications. The measurements were validated by scanning multi-element standard reference materials: NIST SRM 1573a, CRM 129 and OU-10. The certified and measured values are presented in Supplementary Information Table S1.

Development of the software for quantitative analysis of the SR µ-XRF data (QA-SR-µ-XRF; © P. Kump) that is based on the fundamental parameters of XRF spectroscopy has enabled us to study the concentration relationships of Cd, S, Cl and P at the tissue and cellular levels, as well as to compare the element concentrations in tissues and cells under different treatments, and the element distribution maps recorded under different conditions. It should be however emphasised that the absolute element concentrations obtained by QA-SR-µ-XRF reflect the local concentrations of the excised parts of the leaves (the region at the midrib vein, as shown in Vogel-Mikuš et al. 2008), and these are therefore sometimes not very consistent with the bulk element concentrations measured for whole leaves. Nevertheless basic trends in element (e.g. Cd) partitioning between apoplast and symplast can be seen from these maps that can be related to the cellular Cd tolerance mechanism.

Co-localisation analysis

Co-localisation of Cd with Cl, S and P was analysed using the ImageJ programme with the 'intensity correlation analysis' plug-in, which generated the

Pearson's correlation coefficients (r) (http://www.macbiophotonics.ca/imagej/colour_analysis.htm), and the 'intensity highlighter' plug-in, which generated co-localisation images. Quantitative 100×100 pixels, 8-bit element maps were generated using ImageJ, from the matrices obtained after quantitative SR μ XRF imaging, which were used for the co-localisation analysis. The Pearson's correlation coefficients ranged from 1 to -1 , where 1 represents perfect correlation/co-localisation, and -1 represents perfect exclusion; 0 represents random localisation.

Cd L₃-edge micro-X-ray absorption near-edge structure spectrometry

Cd L₃-edge μ -XANES spectra were recorded in the energy region from 3.50 keV to 3.66 keV, in selected parts of the apoplast and symplast of epidermal and mesophyll cells, and in the phloem region of the mature leaves, as also for the Cd model compounds, prepared as described below. The monochromatic beam of the ID21 beamline was focused with the zoneplate ($0.3 \times 0.7 \mu\text{m}^2$) for the Cd L₃-edge μ -XANES measurements in the apoplast and symplast, while in the phloem region and for the Cd model compounds, the beam was focused with a pinhole of 50 and 100- μm diameter, respectively. The exact energy calibration was established with absorption measurements on a 2- μm -thick Cd metal foil, measured in transmission mode, by assigning the first inflection point of the Cd L₃ edge of the Cd foil to 3,538 eV (Isaure et al. 2006, 2010).

The Cd model compounds were Cd-pectin, Cd-glutathione, Cd-malate, Cd-oxalate, Cd-phytate and Cd-citrate. These were prepared as liquid-complexed compounds, with the solutions for the CdCl₂ salts prepared at a metal:ligand (M:L) molar ratio of 1:130 for pectin and 1:10 for all of the other ligands. The reference materials for CdSO₄ were prepared at a M:L molar ratio of 1:300 for pectin and 1:10 for all of the other ligands. The pH of the prepared Cd solutions with pectin was adjusted to 5.0, with glutathione to 2.7, and with malate and phytate to 5.1 and 3.6, respectively, as reported in Isaure et al. (2006). The solutions of Cd references were then frozen in liquid nitrogen and freeze-dried at -40°C and 0.12 mbar. The freeze-dried material was pulverised in a mortar and pressed into pellets using a hydraulic press. The non-complexed standards of CdS, CdSO₄, and CdCl₂

(Sigma-Aldrich, Merck) were spiked with microcrystalline cellulose, homogenised, and pressed into pellets.

The Cd L₃-edge XANES of the reference Cd compounds were prepared in the form of homogeneous pellets with the total absorption thickness (μd) of about 2.5 above the investigated Cd L₃ edge. These were measured at room temperature in fluorescence and transmission detection mode at the EXAFS beamline, Synchrotron Elettra, Trieste, Italy. A Si 111 double crystal monochromator was used with about 0.4 eV resolution at 3.5 keV. Higher-order harmonics were effectively eliminated by detuning the monochromator crystals to 40 % of the rocking curve maximum. The intensity of the monochromatic X-ray beam was measured by three 30-cm-long consecutive ionisation detectors, filled with the following gas mixtures: N₂ at 85 mbar and He at 1915 mbar; N₂ at 460 mbar, and He at 1340 mbar; N₂ at 880 mbar and He at 1120 mbar, respectively. The samples were mounted on the sample holder placed between the first and second ionisation detectors, rotated by 45° to allow simultaneous fluorescence and transmission detection. The fluorescence signal was detected with a silicon drift diode detector (80 mm²) with a Be window (Ketek, Germany). The absorption spectra were measured within the interval $[-150 \text{ eV to } 100 \text{ eV}]$ relative to the Cd L₃ edge (3,358 eV). Equidistant energy steps of 0.25 eV were used with an integration time of 10 s step⁻¹. In all of the experiments, the exact energy calibration was established with simultaneous absorption measurements on 0.1- μm -thick Cd metal deposited on 3- μm -thick Al foil placed between the second and third ionisation chambers. The absolute energy reproducibility of the measured spectra was $\pm 0.02 \text{ eV}$.

Results

Growth responses and chlorophyll and anthocyanin concentration and content

The biomass of the leaves increased significantly (by around 120 %) in plants treated with 100 μM CdCl₂/CdSO₄ (100Cd plants), when compared to the controls, while in plants treated with 300 μM CdCl₂/CdSO₄ (300Cd plants) the biomass was not statically different from the controls (Fig. 1a). There was no difference in the leaf biomass of plants treated

Fig. 1 **a** Leaf biomass, **b** total chlorophylls concentrations and contents, and **c** total anthocyanins concentrations and contents of *N. praecox* plants treated with 100 μM and 300 μM CdCl_2 and CdSO_4 (means $\pm$ se; $n=10$). Different letters above columns represent statistically significant differences; Duncan's test ($p < 0.05$). DW, dry weight

with equal concentrations of CdCl_2 or CdSO_4 (Fig. 1a).

Due to the significant differences in leaf biomass between the control and 100Cd-treated plants, and hence possible dilution/concentration effects, we use

here the measure of the content of the total chlorophylls, anthocyanins, Cd, Cl, S and P in the leaves, expressed as mg plant^{-1} (chlorophylls, anthocyanins) or $\mu\text{g plant}^{-1}$ (Cd, Cl, S, P).

The chlorophyll and anthocyanin concentrations (mg g^{-1} dry weight [DW]) were measured in the leaves to estimate the levels of stress in the Cd-treated plants. The concentrations of the total chlorophylls decreased significantly in the Cd-treated plants (Fig. 1b). However the content of total chlorophylls in the 100Cd plants (regardless of the Cd salt used) remained comparable to that of the controls, while in the 300Cd plants, the total chlorophylls content decreased significantly (Fig. 1b). Despite this decrease in the total chlorophylls, the mean ratios between the chlorophyll a and chlorophyll b concentrations remained comparable between the control (2.3 ± 0.05) and the Cd treated plants (up to 2.5 ± 0.09 in 300CdSO₄ plants).

The plants treated with 100Cd showed an intensive purple coloration of the leaves, while this was not seen in the 300Cd plants (irrespective of the Cd salt added). An increased anthocyanin concentration was seen especially in the 100CdCl₂ plants, and the increase was even more pronounced when calculated as anthocyanin content (Fig. 1c). For the other treatments, the concentration and content of the anthocyanins did not differ significantly from those of the controls. In the 300CdSO₄ plants, there was a slight increase in the anthocyanin content, when compared to the 300CdCl₂ plants; however, this increase was not statistically significant, due to the high variability of the anthocyanin concentrations between individual plants.

Element concentrations and contents in leaves

The Cd concentrations in the leaves correlated positively with those supplied into the nutrient solution, and they were independent of CdCl_2 or CdSO_4 in the nutrient solution (Fig. 2a). Control plants contained traces of Cd, probably because of the Cd burden from the seeds that were originally collected at the metal-polluted site (Vogel-Mikuš et al. 2007). The Cd content (i.e., as $\mu\text{g Cd plant}^{-1}$) did not differ between the 100CdCl₂ and 100CdSO₄ plants, while in the 300Cd plants, there was slightly higher (but not statistically significant) Cd content seen for the 300CdSO₄ plants than for the 300CdCl₂ plants.

Significantly higher S concentrations were seen in the 300Cd plants than in the 100Cd plants, again

Fig. 2 Concentrations and contents of **a** cadmium (Cd), **b** sulphur (S), **c** chlorine (Cl), and **d** phosphorus (P) in leaves of *N. praecox* plants treated with 100 μ M and 300 μ M CdCl₂ and CdSO₄ (means $\pm$ se; control, $n=10$). Different letters above columns represent statistically significant differences; Duncan's test ($p < 0.05$). DW, dry weight

irrespective of the Cd salt applied (Fig. 2b). The S content, on the other hand, was slightly higher in plants grown in CdSO₄-enriched solutions, especially in the 300CdSO₄ plants.

The Cl concentrations strongly correlated with Cl⁻ concentrations in the nutrient solution, with higher concentrations in the CdCl₂-treated plants (Fig. 2c). The highest Cl content was however found in the 100CdCl₂ plants.

The P concentrations were the highest in the 300Cd plants, irrespective of the Cd salt added (Fig. 2d). The highest P content was, however, in the 100Cd plants.

Cadmium ligand environment in the leaves of *N. praecox*: Cd-K EXAFS

The Cd K-edge EXAFS spectra recorded on the freeze-dried and homogenised leaf material following the different treatments were analysed with the IFEFFIT package (Ravel and Newville 2005). The Fourier transform magnitudes of the EXAFS spectra showed two compound peaks in the R range between 1 Å and 3 Å (Fig. 3a), which indicated that the average local structures around the Cd atoms in the leaves following all four of the treatments were very similar. The quantitative structural information of the local Cd neighbourhood (type and average number of neighbour atoms and their distances from the Cd atom) and the Debye–Waller factor of neighbouring shells (as a measure of the thermal and structural disorder in the shells), were obtained by quantitative EXAFS analysis. For this, the model EXAFS function was fitted to the measured EXAFS spectra. To achieve this, we constructed model EXAFS functions *ab initio*, from a set of scattering paths of the photoelectron obtained in a tentative spatial distribution of the neighbour atoms with the FEFF6 programme code (Rehr et al. 1992). The model comprised oxygen and sulphur atoms in the first coordination shell, and carbon atoms in the second. The atomic species of the neighbours were identified in the fit by their specific scattering factors and phase shifts.

We introduced three variable parameters for each shell of neighbours into the model: the shell

Fig. 3 **a** Fourier transform magnitudes of the k^3 weighted EXAFS spectra (solid line) measured on leaves of *N. praecox* plants treated with 100 μM and 300 μM CdCl_2 and CdSO_4 , together with the best-fit EXAFS model (dashed line) calculated in the k range from 3 $\text{\AA}^{-1}$ to 11 $\text{\AA}^{-1}$, and the R range from 1.1 $\text{\AA}$ to 3.1 $\text{\AA}$. The spectra are displaced vertically for clarity. **b** Percentages of Cd O-ligands and S-ligands (as indicated), based on quantitative EXAFS analysis

coordination number (N), the distance (R), and the Debye–Waller factor (σ^2). In addition, the common shift of energy origin, ΔE_0 , was allowed to vary. The amplitude reduction factor ($S_0^2=0.9$; Vogel-Mikuš et al. 2010) was fixed during the fitting. For all of the samples, there was very good agreement between the model and the experimental spectrum using the k range from 3 $\text{\AA}^{-1}$ to 11 $\text{\AA}^{-1}$ and the R range from 1.1 $\text{\AA}$ to 3.1 $\text{\AA}$. To minimise the relatively large uncertainties of the coordination numbers and the Debye–Waller factors due to the high correlations between these parameters in the fit of each individual spectrum, a simultaneous fit of all of the measured spectra was performed. In the simultaneous relaxation, some of the parameters within the groups of similar spectra were constrained to common values; in particular, the common energy shift ΔE_0 , the Cd–O, Cd–S and Cd–C interatomic distances, and the corresponding Debye–Waller factors. The best-fit structural parameters are listed in Supplementary Information Table S2. The quality of the fit is shown in Fig. 3a.

For all of the treatments, the majority of the Cd was found to be bound to O-ligands, with only a minor part bound to S-ligands. Carbon atoms were detected in the second coordination sphere. The Cd–O, Cd–S and Cd–C distances were slightly longer than those reported in our previous study of Cd complexation in vegetative tissues of *N. praecox* (Vogel-Mikuš et al. 2010). The presence of carbon atoms in the second coordination shell can be ascribed to complexing of Cd with thiol groups (Cd–S–C–R), which forms thiolate complexes

(Strasdeit et al. 1991; Pickering et al. 1999), or to carboxyl or hydroxyl organic groups (Cd–O–C–R) (Vogel-Mikuš et al. 2010).

Slightly more Cd atoms were bound to S-ligands in the 300Cd plants than in the 100Cd plants (Fig. 3b). In the 300CdSO₄ plants, there was 5 % more Cd S-ligands than in the 300CdCl₂ plants, while in the 100Cd plants there was no difference in the ligand environment between the CdSO₄ and CdCl₂ plants (Fig. 3b).

Cadmium, S, Cl and P localisation in the leaves of *N. praecox*: SR μ -XRF

Element localization studies were performed on mature *N. praecox* leaves. Higher concentrations of Cd were observed in the leaf tissue of the 300Cd plants than for the 100Cd plants (Table 1, Figs. 4, 5, 6 and 7), which is in line with the bulk Cd analyses (Fig. 2a). In all of the treatments, Cd was preferentially localised in large vacuolarised epidermal cells, although in the CdSO₄ plants, relatively high concentrations of Cd were seen also in the epidermal apoplast. The symplast/apoplast ratios in the epidermis of the 100CdCl₂ and 300CdCl₂ plants were 2.8 and 3.4, respectively, while in the 100CdSO₄ and 300CdSO₄ plants, these ratios were 1.2 and 2.0, respectively (Table 1, Figs. 4, 5, 6 and 7). This indicated that the symplast Cd concentrations increased with the increased Cd supply, and they were higher in the CdCl₂ plants than in the CdSO₄ plants.

Table 1 Concentrations of cadmium (Cd), sulphur (S), chlorine (Cl) and phosphorus (P) calculated from quantitative SR μ -XRF element distribution maps in the apoplast and symplast of theepidermis and mesophyll, as shown in green in the images of representative samples of *N. preacox* leaf tissues. Means and standard deviations (in parentheses), $n=2-4$

Element conc.	Treatment	Epidermis		Mesophyll	
		Apoplast	Symplast	Apoplast	Symplast

Cd ($\mu\text{g g}^{-1}$)	100 CdCl ₂	1730 (250)	4900 (1700)	430 (151)	600 (200)
	300 CdCl ₂	2080 (437)	7220 (1516)	1570 (518)	1960 (647)
	100 CdSO ₄	1060 (350)	1250 (400)	1440 (650)	1070 (300)
	300 CdSO ₄	6840 (970)	13900 (2502)	2100 (220)	835 (300)
S ($\mu\text{g g}^{-1}$)	100 CdCl ₂	410 (60)	320 (45)	810 (267)	340 (112)
	300 CdCl ₂	1160 (290)	970 (243)	2550 (145)	2510 (90)
	100 CdSO ₄	1060 (159)	1430 (215)	2480 (347)	2570 (360)
	300 CdSO ₄	8080 (485)	6590 (395)	7550 (440)	8820 (640)
Cl ($\mu\text{g g}^{-1}$)	100 CdCl ₂	4800 (670)	5100 (710)	2090 (21)	1920 (19)
	300 CdCl ₂	6780 (48)	7800 (65)	3908 (39)	4240 (42)
	100 CdSO ₄	1140 (22)	870 (25)	1540 (15)	1180 (12)
	300 CdSO ₄	4930 (730)	7900 (1180)	1390 (380)	830 (150)
P ($\mu\text{g g}^{-1}$)	100 CdCl ₂	820 (115)	600 (85)	1310 (432)	530 (175)
	300 CdCl ₂	2170 (586)	1754 (474)	1300 (328)	620 (81)
	100 CdSO ₄	1260 (378)	840 (252)	1210 (436)	578 (208)
	300 CdSO ₄	2120 (430)	930 (251)	2610 (530)	1440 (430)

For the mesophyll, the $100 \mu\text{m} \times 100 \mu\text{m}$ scans of the element distributions were taken in the palisade layer (Figs. 5, 6 and 7). The Cd distribution differed significantly between the CdCl₂ and CdSO₄ plants (Figs. 5, 6 and 7). In the CdCl₂ plants, the Cd was mainly localised in the symplast. The symplast/apoplast ratios were 1.4 and 1.2 in the 100CdCl₂ and 300CdCl₂ plants, respectively (Table 1, Figs. 5, 6 and 7). In the CdSO₄ plants, however, there were significantly higher Cd concentrations in the apoplast (Table 1, Figs. 5, 6 and 7), with symplast/apoplast Cd ratios of 0.7 and 0.4, as calculated for the 100CdSO₄ and 300CdSO₄ plants, respectively. In both of the CdSO₄ treatments, Cd accumulated in the apoplastic space in the form of local deposits that were rich in P and S (maps shown only for 300CdSO₄ plants; Fig. 6).

The lowest concentrations of S were seen in the 100CdCl₂ plants, and the highest in the 300CdSO₄ plants, which is in line with the bulk analyses (Table 1, Fig. 2b). In the epidermis, there were higher concentrations of S in the apoplast of the CdCl₂ plants (Table 1), with the symplast/apoplast ratio of 0.8. In the 100CdSO₄ plants, there were higher proportions of S stored in the symplast (symplast/apoplast ratio, 1.3), while in the 300CdSO₄ plants, there was a higher proportion stored in the apoplast (symplast/apoplast ratio, 0.8). However, the absolute symplastic S concentration was much higher in the 300CdSO₄ plants than in the 100CdSO₄ plants (Table 1). Similar to the epidermis, the S concentrations in the mesophyll depended strongly on the amount of SO₄²⁻ added to the nutrient solution, with lower concentrations in the

Fig. 4 **a** Video image of epidermal/palisade mesophyll tissues of mature leaves of *N. praecox* plants treated with 300 μM CdSO_4 . UE-upper epidermis, LVC-large vacuolarized epidermal cells, SE-sub-epidermis, PM-palisade mesophyll. The green square denotes the region where SR $\mu\text{-XRF}$ element distribution maps (scan size $100\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$) were recorded at excitation

energy of 3.55 keV. **b** Areal density of the sample calculated from the scattering peak. **c-f** Quantitative SR $\mu\text{-XRF}$ distribution maps of cadmium (Cd), sulphur (S), chlorine (Cl), and phosphorus (P). Quantitative analysis was performed using the QA- $\mu\text{-XRF}$ programme. **g-i** Co-localisation maps for Cd-S, Cd-Cl and Cd-P. White areas denote co-localisation

CdCl_2 plants than in the CdSO_4 plants. The proportion of S in the symplast increased with the Cd and S supply (Table 1), with symplast/apoplast ratios of 0.4 for 100CdCl_2 , 1.0 for 300CdCl_2 and 100CdSO_4 , and 1.2 for 300CdSO_4 plants. Especially high S concentrations were seen in the mesophyll symplast of the 300CdSO_4 plants; however, S-rich deposits were also seen in the apoplast (Table 1, Figs. 6 and 7).

The highest concentrations of Cl were seen in the symplast of large vacuolarised epidermal cells,

together with Cd (Table 1, Figs. 4 and 7); the symplast/apoplast ratios for Cl were 1.1 and 1.2 for 100CdCl_2 and 300CdCl_2 plants, respectively. In the epidermis of the 100CdSO_4 plants, the apoplastic localisation prevailed, while in 300CdSO_4 plants, Cl was again found at very high concentrations in the epidermal symplast. In the mesophyll, there was more Cl in the apoplast, with the exception of the 300CdCl_2 plants, where higher Cl concentrations were seen for the symplast.

Fig. 5 **a** Video image of palisade mesophyll tissues of mature leaves of *N. praecox* plants treated with 300 μM CdCl_2 . UE-upper epidermis, SE-sub-epidermis, PM-palisade mesophyll. The green square denotes the region where SR $\mu\text{-XRF}$ element distribution maps (scan size $100\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$) were recorded at excitation energy of 3.55 keV. **b** Areal density of the sample

calculated from the scattering peak. **c-f** Quantitative SR $\mu\text{-XRF}$ distribution maps of cadmium (Cd), sulphur (S), chlorine (Cl), and phosphorus (P). Quantitative analysis was performed using the QA- $\mu\text{-XRF}$ programme. **g-i** Co-localisation maps for Cd-S, Cd-Cl and Cd-P. White areas denote co-localisation

Phosphorus mainly accumulated in the apoplast of the epidermis and mesophyll, for all of the treatments (Table 1, Figs. 5, 6 and 7). In line with the bulk analyses (Fig. 2d), there were higher P concentrations in the 300Cd plants, with preferential accumulation in the apoplast, and especially in the 300CdSO₄ plants.

The element concentrations were also measured in the phloem tissues, as they are good indicators of the mobile element fraction. The highest Cd concentrations

were in the 300Cd plants, and there were no differences between the CdCl₂ and CdSO₄ plants. Higher Cl concentrations were seen in the CdCl₂ plants, while no such trend was seen for S. Interestingly, higher S concentrations were seen in the 300CdCl₂ than the 300CdSO₄ plants. In the phloem of the CdSO₄ plants, however, there was an increase in P concentration when compared to the CdCl₂ plants, for both the 100Cd and 300Cd plants (Table 2).

Fig. 6 **a** Video image of palisade mesophyll tissues of mature leaves of *N. praecox* plants treated with 300 μM CdSO_4 . SE-sub-epidermis, PM-palisade mesophyll. The green square denotes the region where SR $\mu\text{-XRF}$ element distribution maps (scan size $100\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$) were recorded at excitation energy of 3.55 keV. **b** Areal density of the sample calculated from the

scattering peak. **c-f** Quantitative SR $\mu\text{-XRF}$ distribution maps of cadmium (Cd), sulphur (S), chlorine (Cl), and phosphorus (P) Quantitative analysis was performed using the QA- $\mu\text{-XRF}$ programme. **g-i** Co-localization maps for Cd-S, Cd-Cl and Cd-P. White areas denote co-localisation

Co-localisation analysis

The co-localisation analysis was performed to resolve the relationships/correlations in the distributions between the Cd and S, and Cl and P elements. Pearson's co-localisation coefficients (r) were calculated in the regions that were shown to be encircled in the element distribution maps, separately for the apoplast and symplast, and separately for the epidermis and mesophyll, as shown in Table 1.

In the epidermis, the Cd co-localised with Cl in the symplast and apoplast under all of the treatments (Figs. 4 and 7; in Fig. 7, yellow). The Pearson's correlation coefficients (r) calculated for Cd and Cl for the epidermal symplast of the 100CdCl₂, 300CdCl₂, 100CdSO₄ and 300CdSO₄ plants were 0.92, 0.82, 0.50 and 0.96, respectively, and for the epidermal apoplast, these were 0.74, 0.61, 0.67 and 0.85, respectively. Co-localisation between Cd and S was not apparent from the r calculated in the epidermis

Fig. 7 **Top left panel:** Cd-L₃ edge μ -XANES spectra recorded in *N. praecox* leaf epidermis, for the mesophyll of plants treated with 100 μ M CdCl₂ and 300 μ M CdSO₄, and the phloem of plants treated with 100 μ M and 300 μ M CdCl₂ and CdSO₄, and Cd-L₃ edge μ -XANES spectra of model compounds (CdCl₂, Cd-malate, Cd-oxalate, Cd-citrate, Cd-pectin, and Cd-glutathione, as indicated). **Top right panel:** Quantitative SR μ -XRF distribution maps for Cd (red), S (blue) and Cl (green), recorded at excitation energy of 3.55 keV. Crosses indicate the points where the Cd-L₃ edge μ -XANES spectra were recorded.

Yellow indicates overlap of Cd and Cl, and purple indicates overlap of Cd and S. **a** Epidermis of plants treated with 100 μ M CdCl₂; **b** epidermis of plants treated with 300 μ M CdSO₄; **c** mesophyll of plants treated with 100 μ M CdCl₂; and **d** mesophyll of plants treated with 300 μ M CdSO₄. **Bottom panel:** e Percentage of O-ligands and S-ligands in the apoplast and symplast (as indicated) of plants treated with 100 μ M CdCl₂ and 300 μ M CdSO₄, as obtained from the Cd-L₃ edge μ -XANES spectra after linear combination fit analysis

(Figs. 4 and 7b). However, as can be seen in Fig. 4, where Cd is represented by red and S by green, and

where white represents co-localisation pixels, it can be seen that Cd was strongly co-localised with S in the

Table 2 Concentrations of cadmium (Cd), sulphur (S), chlorine (Cl) and phosphorus (P) calculated from quantitative SR μ -XRF element distribution maps in the leaf phloem region, and proportions of Cd-sulphur and Cd-oxygen ligands calculated from the Cd-L₃ μ -XANES spectra after linear combination fit

El. conc./treatment	100 CdCl ₂	300 CdCl ₂	100 CdSO ₄	300 CdSO ₄
Cd ($\mu\text{g g}^{-1}$)	530 (100)	1310 (200)	540 (60)	1320 (200)
S ($\mu\text{g g}^{-1}$)	2000 (230)	3900 (720)	2300 (350)	2020 (290)
Cl ($\mu\text{g g}^{-1}$)	3480 (460)	3100 (440)	1000 (140)	1500 (200)
P ($\mu\text{g g}^{-1}$)	3980 (460)	5340 (660)	5740 (800)	10800 (1410)
Cd S-ligands (%)	37	23	8	13
Cd O- ligands (%)	63	77	92	87
Cd S/O-ligands	0.59	0.30	0.09	0.15

cytoplasm of the large vacuolarised cells, but not in the vacuole. In the CdCl₂ plants there was positive co-localisation between P and Cd in the epidermal apoplast (100CdCl₂, $r=0.40$; 300 CdCl₂, $r=0.42$), while in the CdSO₄ plants no such co-localisation was seen.

In the mesophyll of all of the treatments, Cd co-localised with Cl in the symplast, with $r=0.34$, 0.41 , 0.82 and 0.62 for the 100CdCl₂, 300CdCl₂, 100CdSO₄ and 300CdSO₄ plants, respectively. In the mesophyll apoplast, Cd co-localised with Cl only in the 300CdCl₂ plants ($r=0.84$), and with S only in the 300Cd plants. The Cd-S r for the apoplast was 0.78 and 0.42 for the 300CdCl₂ and 300CdSO₄ plants, respectively, while for the symplast, r was 0.73 and 0.38 in the 300CdCl₂ and 300CdSO₄ plants, respectively. Interestingly, in the CdSO₄ plants, there was strong co-localisation between Cd and P in the apoplast (intracellular spaces), which was especially pronounced in the 300 CdSO₄ plants ($r=0.79$; Figs. 6 and 7d; $r=0.44$ for the 100CdSO₄ plants). There was no co-localisation of Cd and P in mesophyll of the 100CdCl₂ plants, while in the 300CdCl₂ plants, co-localisation of Cd and P was seen in some parts of the apoplast (Fig. 5).

Cadmium ligand environment in leaves of *N. praecox* at the sub-cellular level: Cd L₃-edge μ -XANES

The Cd L₃-edge μ -XANES spectra were recorded in selected points of epidermal and mesophyll cells of the

analysis. Maps represent quantitative distributions of Cd in phloem region mapped in the *N. praecox* leaf cross-section, as indicated in video-image (top left). Means and standard deviations (in parentheses), $n=2-4$

100CdCl₂ and 300CdSO₄ plants, as indicated in Fig. 7, and in the phloem region for all four of these treatments (Table 2). The points for Cd L₃-edge μ -XANES measurements were selected locally in the Cd-rich parts of the apoplast and symplast. All of the Cd L₃-edge μ -XANES spectra obtained from the plant cells, as well as a selected set of Cd L₃-edge μ -XANES spectra of standard reference Cd compounds, are shown in Fig. 7. The types of Cd bonding can be identified from the characteristic shapes of the edge features and the pre-edge resonances. The Cd in the plant cells was expected to be bound to different organic ligands, so the most probable candidates were selected in a set of reference compounds on the basis of the existing literature (Isaure et al. 2006, 2010). The relative amounts of the Cd ligands at the selected point in the cell was determined with the linear combination fit method (Ravel and Newville 2005), where the Cd L₃-edge μ -XANES spectra measured in the plant cells were modelled with a linear combination with the Cd L₃-edge μ -XANES spectra of the chosen reference Cd compounds (Isaure et al. 2006, 2010). In all of these cases, the best fit was obtained with the spectrum of Cd-glutathione (Cd-S-C-R coordination) and the spectrum of Cd-pectin (Cd-O-C-R coordination). The quality of the fit is illustrated in Supplementary Information Figure S1. All of the other combinations with the rest of the reference spectra were rejected in the fitting procedure, or they led to fits with significantly

higher R-factors, which measure the quality of the fitting (Ravel and Newville 2005).

In all of the treatments, Cd was bound to O-ligands and S-ligands (Fig. 7), as already shown by the bulk Cd K-edge EXAFS analysis. It has to be emphasised that these data obtained from the EXAFS analysis represent the Cd ligand environment at the level of the whole leaf, which included young, mature and old leaves, while the Cd L₃-edge μ -XANES data represent the Cd ligand environment of the locally Cd-enriched sites chosen in mature leaf cross-sections.

In the epidermal cells, the O-ligands prevailed over the S-ligands in both vacuoles and apoplast, although a slightly higher proportion of S-ligands was seen in the 100CdCl₂ than in the 300CdSO₄ plants (Fig. 7e). In the mesophyll symplast of the 100CdCl₂ plants, the S-ligands prevailed over the O-ligands (Fig. 7e), while in the apoplast it was not possible to record the Cd L₃-edge μ -XANES spectra with high enough signal-to-noise ratio due to Cd concentrations that were too low. In the mesophyll symplast of the 300CdSO₄ plants, there were more or less equal portions of S-ligands and O-ligands (Fig. 7e), while in the apoplast of the 300CdSO₄ plants, where the Cd was accumulated at the highest concentrations, the S-ligands strongly prevailed. Despite strong co-localisation of Cd and P in the mesophyll apoplast of the 300CdSO₄ plants, coordination of Cd to P compounds was not confirmed.

The Cd ligand environment was examined also in the phloem tissues (Table 2, Fig. 7). Higher proportion of S-ligands was seen in the CdCl₂ than in the CdSO₄ treated plants irrespective of the Cd tissue concentrations (Table 2). This was in line also with the higher S concentrations seen in the 300CdCl₂ than in the 300CdSO₄ plants (Table 2).

Discussion

Growth responses and concentrations of chlorophylls and anthocyanins

The treatments of *N. praecox* plants with 100 μ M CdCl₂/CdSO₄ resulted in significantly increased leaf biomass, as already observed by Pongrac et al. (2010). Then, in plants treated with 300 μ M CdCl₂/CdSO₄ the leaf biomass was comparable to that of the control treatment, which indicates the high tolerance of this plant species to Cd. The increase in leaf biomass of the

100Cd plants was much more pronounced, as is typical in hormesis, where low levels of intoxicants can stimulate plant growth, most probably in order to dilute the intoxicants in tissues, and high levels cause intoxication, and so have negative effects on biomass production. Similarly, in Cd hyperaccumulating *N. caerulescens*, Cd supplementation enhanced leaf biomass (Pongrac et al. 2009) and stimulated the activities of some enzymes, such as carbonic anhydrase (Liu et al. 2008). This suggests that Cd stimulates the growth of the Cd-hyperaccumulating *Noccaea* ecotypes, however, the exact mechanism remains unknown.

The total chlorophyll concentrations in the leaves of *N. praecox* decreased with the addition of CdCl₂/CdSO₄ to the nutrient solution, as compared to the controls, similar to what was seen in pot experiments performed by Pongrac et al. (2009), while supplementation of the plants with the different Cd salts in our experiment did not influence the total chlorophyll concentrations. In the 100Cd plants, however, the total chlorophyll content (as μ g plant⁻¹) was comparable to the control, which indicates that the decrease in the chlorophyll concentration was probably related to the increase in the plant biomass. In the 300Cd plants, on the other hand, the total chlorophyll content was significantly lower than in the control, which indicates the development of Cd-toxicity symptoms, although the visible chlorosis was not very pronounced. Cd has been reported to degrade chlorophyll in plants. Barylá et al. (2001) suggested that Cd can directly affect enzymes of the chlorophyll synthesis pathway, or can interfere with the correct assembly of the pigment-protein complexes of the photosystems. In addition, the interference by Cd of the Fe supply might additionally inhibit chlorophyll synthesis (Bao et al. 2012). In *N. praecox*, however, the chlorophyll degradation appears only at very high Cd tissue concentrations, pointing to very efficient detoxification mechanisms.

The only difference seen between the CdCl₂ and CdSO₄ treatments was an increase in the total anthocyanins concentrations for the 100CdCl₂ plants. Accumulation of anthocyanins has been shown to be induced by Cd in many plant species (Dai et al. 2012; Mobin and Khan 2007), in order to increase the antioxidant capacity against Cd-induced oxidative stress (Dai et al. 2012). Although anthocyanins are predominantly stored in vacuoles, in *Lactuca sativa* and *Pseudowintera colorata*, they have been shown to be in cytosolic spaces prior to their transport into the

vacuole. Their presence in the cytosol might therefore be related to the scavenging of superoxide radicals and H_2O_2 . Metal complexation with anthocyanins has been suggested for Mg (Kondo et al. 1992), Fe (Everest and Hall 1921), Al (Takeda et al. 1985), Mo and W (Hale et al. 2002). In each case, the binding of these metals resulted in a colour change from pink to blue. Cadmium, on the other hand, did not induce colour changes in anthocyanins extracted from *Brassica oleracea* (Hale et al. 2002); however, the anthocyaninless variety C1-108 contained significantly lower levels of Cd than the high anthocyanin variety C1-67 (Hale et al. 2002). Hale et al. (2002) therefore proposed that anthocyanins have a role in the sequestration of metal ions in peripheral tissues. However, further studies are needed to determine their role in metal-hyperaccumulating plants. In the 300Cd *N. praecox* plants, which on average contained up to 1 % Cd, the content of anthocyanins decreased significantly comparing to the 100Cd plants. This might be connected to the higher level of intoxication of the leaf tissues in the 300Cd plants, and consequentially a decreased ability to synthesise secondary metabolites, as observed by Krupa et al. (1996).

Cd, S, Cl and P accumulation and the Cd ligand environment

Hydroponically grown *N. praecox* plants can accumulate up to 1.2 % Cd in their leaves, which is the highest reported Cd concentration in this plant species (Vogel-Mikuš et al. 2005; Tolrà et al. 2006; Pongrac et al. 2009). This is related to the high Cd concentrations supplied to the nutrient solution (300 μM) and to the duration of the experiment (8 weeks). Cadmium concentrations in leaves can be increased by increasing the Cd concentrations supplemented to the nutrient solution, regardless of the Cd salt used. When calculating the Cd contents (as $\mu\text{g plant}^{-1}$), however, the highest Cd values (of up to 25,000 $\mu\text{g plant}^{-1}$) were found in the 300CdSO₄ plants.

Sulphur and Cl concentrations in the leaves reflected the concentrations added to the nutrient solution. Interestingly, in the 300Cd plants, increased P concentrations were seen. However, the higher P contents seen in the 100Cd plants indicates that the increased P concentrations in the 300Cd plants probably results from a concentration effect, due to the lower leaf biomass production, rather than to changed uptake rates, as has been

reported for Mg, Ca and S in *N. caerulea* (Küpper and Kochian 2010).

According to the Cd K-edge EXAFS analysis that reflects the average Cd ligand environment in bulk *N. praecox* leaf tissues, including young, mature and old leaves, the O-ligands prevailed over the S-ligands under all treatments, as already observed in field-collected plants (Vogel-Mikuš et al. 2010). In those field-collected plants, the Cd was approximately 80 % bound to O-ligands, namely the carboxyl and hydroxyl groups of organic acids and cell wall components, and only 20 % to S-ligands. In the 100Cd plants, an even greater proportion of oxygen ligands was observed (89 %), while in the 300Cd plants, the proportion of O-ligands slightly decreased, on account of the S-ligands, which, however, still did not exceed 18 %. When comparing the 300CdSO₄ plants and the 300CdCl₂ plants, there was greater S-ligands involvement in the 300CdSO₄ plants. We can thus conclude that as well as the leaf developmental stage, with higher proportion of S-ligands found in young leaves and higher proportion of O-ligands found in old leaves (Küpper et al. 2004), the Cd ligand environment can also depend on the Cd tissue concentration/content and the S supply. An increase in the Cd tissue concentration might induce longer retention of Cd in the cytosol, where it binds to strong S-ligands (especially in young leaves; Küpper et al. 2004) so as not to interfere with vital processes, such as photosynthesis and cellular respiration. On the other hand, the S supply represents the basis for synthesis of S compounds/ligands, which can be altered if the plants are exposed to S starvation.

Cadmium localisation and ligand environment at the tissue and cellular levels

Cadmium localisation at the tissue and cellular levels

Cadmium was in all treatments preferentially localised in vacuoles of large vacuolarised epidermal cells of the mature leaves, reaching up to 5 % DW in some parts of the measured cells, depending on the Cd concentrations in the nutrient solution, as already shown by others (Vázquez et al. 1992; Wójcik et al. 2005). One of the main mechanisms of detoxification of toxic metals in leaves is accumulation of the metals in metabolically less active tissues, such as the epidermis. However, when taking into account the relative

weight portions of the particular leaf tissues of *N. praecox* (epidermis 18.3 %, mesophyll 74.8 %, veins 6.9 %), according to Vogel-Mikuš et al. (2008), a significant portion of Cd is also stored in the mesophyll in Cd hyperaccumulating *Noccaea* ecotypes. A recent study has shown that the Cd distribution patterns within leaf tissues of the Zn/Cd hyperaccumulator *N. caerulescens* are governed by differential expression of metal transporters, mainly TcZNT5, that are much more abundant in the plasma membrane of epidermal storage cells (large vacuolarised cells) than in the other cell types (Leitenmaier and Küpper 2011). In our study high Cd concentrations were also seen in the epidermal cell walls (up to 0.7 % DW), making the apoplast an additional sink for Cd, as already shown (Vogel-Mikuš et al. 2008). High levels of Cd accumulation in the apoplast indicate that besides vacuolar sequestration, efflux (Ebbs et al. 2009) might have an important role in the detoxification of the cytosol of epidermal cells in *N. praecox*.

In large vacuolarised epidermal cells, Cd was strongly co-localised with Cl, regardless of the treatment applied. In the 100CdCl₂ plants, more Cd and Cl was accumulated in the epidermal symplast than in the 100CdSO₄ plants, while in the 300Cd plants, the situation was just the opposite (Table 1). This might be partly explained by the high size variability of the epidermal cells, and the small number of scan repetitions, due to the limited beamtime assigned. The diameter of the epidermal cells has been shown to be linearly related to Zn concentrations in particular cells (Küpper et al. 1999). This indicates that larger epidermal cells may accumulate higher concentrations of elements such as Cd and Cl, as seen also in the present study. The presence of high Cl concentrations in the epidermal symplast, namely in the vacuoles, was already noted in field grown *N. praecox* plants (Vogel-Mikuš et al. 2008), but at that time, not much attention was paid to this.

Building up of Cl⁻ in vacuoles influences the uptake of cations, in order to maintain electroneutrality over the tonoplast. In addition, Cl was shown to stimulate proton pumping vacuolar type ATP-ase (V-type ATP-ase), a multimeric enzyme that is localised in endomembranes of eukaryotic cells and that establishes an electrochemical H⁺ gradient. In plants, V-type ATP-ase has been identified at the vacuolar membrane (Luttge and Ratajczak 1997), where it can be coupled with cation/H⁺-antiporters, energising cation (and also

Cd²⁺) uptake into the vacuoles (Golldack and Dietz 2001). Among the other cation/H⁺-antiporters, CAX1 and CAX2 are possible candidates for Cd vacuolar sequestration in *N. praecox* (Hirschi et al. 1996).

Intimate connections between Cd and Cl accumulation in the symplast and the influences of CdCl₂ and CdSO₄ supplementation on Cd distribution, were even more pronounced in the palisade mesophyll. In the CdCl₂ plants, Cd was preferentially localised in the vacuoles of the palisade mesophyll, while in the CdSO₄ plants containing lower amounts of Cl⁻ and higher amounts of SO₄²⁻ (which does not stimulate V-type ATP-ase activity to that extent as Cl⁻; Broadley et al. 2012), more Cd was accumulated in the apoplast. Similar differences in the Cd distributions in the mesophyll were also seen in two *N. caerulescens* ecotypes, Prayon and Ganges. In the Prayon ecotype, there were higher efflux rates of Cd from the mesophyll cells, so consequentially more Cd was accumulated in the mesophyll apoplast, while the Ganges ecotype had a greater ability to sequester Cd in the mesophyll vacuoles (Leitenmaier and Küpper 2011). The difference between these two ecotypes was attributed to the differential expression and distribution of metal transporters (Leitenmaier and Küpper 2011). In addition to this explanation, our data indicate that the rate of influx or efflux, and thus the final Cd distribution pattern between apoplast and symplast in the *N. praecox* leaf mesophyll, might also be influenced by the amount of Cl⁻ in the plant tissues.

As mentioned previously, in the CdSO₄ plants, Cd accumulates in the mesophyll apoplast in a form of S-rich and P-rich deposits. This was especially pronounced in the 300CdSO₄ plants, where higher Cd concentrations were seen than in the 100CdSO₄ plants. In non-accumulating plants species, additional supplementation of nutrient solution with P increased the proportion of Cd compartmentalised in the cell-wall fraction, and especially in the roots (Jiang et al. 2007; Qiu et al. 2011). It has been suggested that P addition increases the contents of electronegative ions, such as HPO₄²⁻ and H₂PO₄⁻, which can lead to binding of Cd²⁺ onto cell-wall components through a variety of mechanisms, including adsorption, complexation, precipitation and crystallisation, thus reducing the migratory ability of Cd within plants (Jiang et al. 2007; Qiu et al. 2011). As well as P, high levels of co-localisation were also seen between Cd and S in the 300Cd plants. To resolve the Cd ligand environment of local Cd deposits,

Cd L₃-edge μ -XANES spectra were recorded in particular parts of the leaf cells.

*Cadmium ligand environment at the cellular level:
Cd L₃-edge μ -XANES*

Cd-L₃ μ -XANES spectra were recorded in Cd-enriched parts of the apoplast and symplast of the mature leaves, so the data here reflect only the local, rather than the average, Cd ligand environment in the leaves, as obtained by Cd K-edge EXAFS. The best linear combination fits of the measured Cd L₃-edge μ -XANES spectra were obtained by introducing Cd-glutathione (Vogel-Mikuš et al. 2010; Küpper et al. 2004) and Cd-pectin, rather than Cd-malate or Cd-citrate (Ueno et al. 2005; Küpper et al. 2004). In line with the data obtained, high amounts of pectin components were found in the epidermal leaf tissues of the field grown *N. praecox* plants, using Fourier transformed infrared micro-spectroscopy (Regvar et al. unpublished). This indicates that in *N. praecox*, pectin compounds can provide a significant portion of R-COOH and R-OH sites for the binding of Cd, especially in the cell walls. In the vacuoles, on the other hand, other organic acids which are more abundant than polysaccharide compounds are more likely candidates for Cd binding. It has to be emphasised, however, that the Cd L₃-edge μ -XANES spectra were not used here to obtain definitive information on the in-vivo Cd coordination, but simply as a tool to compare the Cd ligand environment across possible classes of ligands that have been identified previously in the literature (Isaure et al. 2006, 2010). When the fingerprint approach is used to find combinations of different ligand molecules to fit μ -XANES spectra, the results should be interpreted with care. Different compounds can have relatively similar spectra. Cd L₃-edge μ -XANES spectra of Cd-O-R and Cd-S-R compounds differ only in the Cd-L₃ pre-edge structure, which is present in Cd-O-R and absent in Cd-S-R compounds, while CdCl₂ has an additional pre-edge peak after the first pre-edge structure (as we showed in Fig. 7). Here, we therefore avoided assigning Cd ligands to particular chemical molecules, and rather assigned them only according to the first neighbouring Cd atoms, as S-ligands and O-ligands.

Despite the presence of high Cl concentrations and significant Cd-Cl co-localisation, in the large vacuolarised epidermal and mesophyll cells of the 100CdCl₂

and 300CdSO₄ plants, Cd was bound to O-ligands and S-ligands, with no Cl-ligands seen. The CdCl₂ was probably dissociated already in the nutrient solution under the given conditions, and in the plant tissues the Cl did not co-precipitate back with Cd. CdCl₂ therefore does not have a role in facilitating Cd uptake and transport in *N. praecox* through the mechanisms of charge reduction or removal (i.e., the CdCl₂⁰ species), which increase the diffusivity/lipophilicity of Cd and enhances its ability to penetrate through membranes, as proposed by Ozkutlu et al. (2007), but more likely has a role in Cd vacuolar sequestration through activation of V-ATP-ases by chloride (Broadley et al. 2012).

In the vacuoles of epidermal cells, when comparing the 100CdCl₂ and 300CdSO₄ plants, the percentages of O-ligands increased with increasing Cd concentrations. Cadmium has much higher affinity for binding to thiol (R-SH) than hydroxyl (R-OH) and carboxyl (R-COOH) groups, so Cd²⁺ ions bind primarily to the R-SH groups. When the R-SH pool is saturated, the Cd²⁺ ions bind further to O-ligands. The size of the R-SH pool primarily depends on the S nutrition, the rate of sulphate to thiol conversion, the plant species, and environmental factors, such as temperature, light and water availability, and they can also vary in the presence of different stress factors (Hawkesford et al. 2012). The occurrence of Cd toxicity symptoms and decreases in plant biomass in the 300Cd plants when compared to the 100Cd plants might be related to saturation of the R-SH pool, as well as to the limited capacity of Cd storage in metabolically less-active compartments.

In the mesophyll symplast, namely the vacuole, there was a relatively high portion of S-ligands (58 % in the 100CdCl₂ plants; 49 % in the 300CdSO₄ plants), possibly in order to protect the highly sensitive metabolic processes from damage caused by free Cd²⁺. These observations are in contrast to the results obtained in our previous study, in which a high prevalence of O-ligands in isolated mesophyll tissues was seen using Cd-K EXAFS analysis (Vogel-Mikuš et al. 2010). In this previous study, however, the average Cd ligand environment was determined in isolated mesophyll tissues that contained 1700 $\mu\text{g g}^{-1}$ Cd (4900 $\mu\text{g g}^{-1}$ in vacuoles) (Vogel-Mikuš et al. 2008, 2010), while in the present study, the average Cd concentrations in the mesophyll vacuoles varied between 600 $\mu\text{g g}^{-1}$ and 850 $\mu\text{g g}^{-1}$. This indicates again that the proportion of

S-ligands in the symplast lowers with increasing Cd tissue concentrations, as observed in epidermal cells.

In the mesophyll apoplast of the 300CdSO₄ plants, where the Cd concentrations in particular deposits can reach more than 1 %, there was 63 % Cd S-ligands. This indicates that strong Cd-S complexes are not present only in the symplast (cytosol), but also in the cell walls and intracellular spaces. The proposed mechanism of accumulation of Cd-S rich deposits in cell walls and intracellular spaces might be through the efflux (Ebbs et al. 2009) of Cd-S-R complexes from the cytoplasm, or Cd might bind to the R-SH compounds stored in the cell walls already before entering the symplast, when it is transported from the xylem via apoplastic pathway to the water evaporation sites. The Cd-S complexes stored in the mesophyll apoplast of *N. praecox* were also enriched in P, but coordination of Cd with phosphate groups was not confirmed by the Cd L₃-edge μ -XANES here. Phosphorus compounds might help to fix Cd-S-R complexes to insoluble concretes, and in this way prevent their dissolving and reuptake into the mesophyll cells. Further studies of these complexes are needed, however, to resolve their final compositions.

The Cd ligand environment was studied also in the phloem, and was related to the treatment applied. Cadmium O-ligands strongly prevailed, although the relative proportions of the S-ligands and O-ligands decreased with increasing Cd concentrations, as was already observed in the leaf symplast. Interestingly, in the CdCl₂ plants, more Cd was bound to S-ligands than in the CdSO₄ plants, irrespectively of the Cd concentrations applied. This could be connected to the higher mobility of the Cd-S complexes, or to the higher concentrations of R-SH compounds in the phloem of CdCl₂ plants, when compared to the CdSO₄ plants. Sulphate reduction predominately takes place in the leaves, precisely in the chloroplast, so R-SH compounds are loaded into the phloem in the leaf mesophyll to be transported to the roots (Hawkesford et al. 2012). As low molecular weight thiols have a crucial role in Cd detoxification in the roots of Cd hyperaccumulator *N. caerulescens* (Hernandez-Allica et al. 2006), exposure to Cd increases the need for organic S compounds in the roots, which probably leads to increased R-SH phloem loading. Our results indicate that addition of CdCl₂ versus CdSO₄ can cause perturbations in sulphur metabolism that result in an increased proportion of Cd-S ligands in phloem; however, further studies are needed to resolve this.

Conclusions

- In hydroponically grown *N. praecox*, leaf biomass, total chlorophylls and anthocyanins vary according to the Cd concentrations supplemented in the nutrient solution, although they did not depend on the type of Cd salt applied.
- Cadmium, Cl and S concentrations in the leaves correlate well with the amounts of these elements supplied in the nutrient solution, while increased P concentrations were seen in the 300Cd plants.
- Cadmium is mainly bound to O-ligands and to a lesser extent to S-ligands in the leaves, as determined by Cd K-edge EXAFS. The proportion between O-ligands and S-ligands, however, depends on the Cd concentrations and the Cd salt applied, with 300CdSO₄ plants showing the highest proportion of S-ligands, probably on the account of Cd-S binding in young leaves.
- Large epidermal vacuolarised cells represent the main sink for Cd accumulation in *N. praecox* mature leaves, irrespectively of the Cd salt applied. The proportion of O-ligands versus S-ligands (as determined by Cd L₃-edge XANES) increases with increasing Cd concentrations.
- The Cd distribution patterns seen in the leaf mesophyll are strongly related to the CdCl₂/CdSO₄ treatments. CdCl₂ stimulated the uptake of Cd into the vacuoles of mesophyll cells, where a high proportion of the Cd was coordinated to S-ligands. In the presence of CdSO₄, efflux of Cd from the mesophyll symplast was more pronounced, resulting in accumulation of Cd-S rich deposits in the cell walls and intercellular spaces.
- Addition of CdCl₂ to the nutrient solution altered the Cd ligand environment in the phloem, when compared to the CdSO₄ plants, with higher proportions of S-ligands seen in the CdCl₂ plants.

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Supplementary Information

Table S1. Element concentrations in the standard reference materials NIST SRM 1573a, OU-10 and CRM 129, as analysed by quantitative SR μ -XRF at the ID21, of the European Synchrotron Radiation Facility, Grenoble, France. The uncertainties of the measurement are given in parentheses.

Element	NIST 1573a		OU-10		CRM 129	
	Measured (ppm)	Certified (ppm)	Measured (ppm)	Certified (ppm)	Measured (ppm)	Certified (ppm)
Mg	11200 (3180)	12000	10800 (3180)	10700	1710 (2150)	1450 (40)
Al	1660 (750)*	598 (12)	78500 (13700)*	58100	270 (550)	nd
Si	2800 (560)	nd	317000 (54200)	341000	1480 (350)	nd
P	nd	nd	nd	nd	2160 (410)	2360 (70)
S	9400 (1630)	9600	135 (110)	nd	3540 (620)	3160 (40)
Cl	6430 (1110)	6600	35 (55)	nd	9230 (1590)	nd

NIST 1573a, tomato leaves, as a homogenized powder, analysed in the form of a pressed pellet

OU-10, Longmyndian greywacke (GeoPT24), analysed in the form of a pressed pellet; certified through an international proficiency test of the International Association of Geoanalysts

CRM 129, hay, as a homogenized powder, analysed in the form of a pressed pellet

* Increased values are the consequence of K-K α escape. Even if the excitation energy was set below K-K α , the tail of the potassium peak can still be detected in the spectra when the K concentrations are high.

nd, not determined

Table S2. Parameters of the nearest coordination shells around the Cd atoms in the *N. praecox* leaves, indicating the number of nearest neighbor atoms (N), the distances (R), and the Debye-Waller factors (σ^2). The best fits in the k range from 3 Å⁻¹ to 11 Å⁻¹ and the R range from 1.0 Å to 3.1 Å are obtained with an amplitude reduction factor S_0^2 of 0.9. The uncertainties of the last digits are given in parentheses.

Cd neighbour	N	R (Å)	σ^2 (Å²)
100 µM CdCl₂			
O	4.0(5)	2.30(1)	0.009(3)
S	0.5(4)	2.53(1)	0.008(4)
C	1.1(5)	3.10(2)	0.006(3)
C	0.5(4)	3.18(2)	0.006(3)
300 µM CdCl₂			
O	4.7(5)	2.30(1)	0.009(3)
S	0.7(4)	2.53(1)	0.008(4)
C	0.9(5)	3.10(2)	0.006(3)
C	0.7(4)	3.18(2)	0.006(3)
100 µM CdSO₄			
O	5.0(5)	2.30(1)	0.009(3)
S	0.6(4)	2.53(1)	0.008(4)
C	0.3(5)	3.10(2)	0.006(3)
C	0.6(4)	3.18(2)	0.006(3)
300 µM CdSO₄			
O	4.6(5)	2.30(1)	0.009(3)
S	1.0(6)	2.53(1)	0.008(4)
C	1.0(6)	3.18(2)	0.006(3)

Supplementary Information Figure legend

Figure S1. Linear combination fitting for the Cd-L₃ edge μ -XANES spectra recorded in **a** the epidermis cell wall of plants treated with 300 μ M CdSO₄, and **b** the mesophyll cell wall of plants treated with 300 μ M CdSO₄. The best fits were obtained through introduction of the spectra of Cd-pectin (Cd-O-C-R coordination) and Cd-glutathione (Cd-S-C-R coordination) into the linear combination fit model.

Figure S1.