MicroXANES tomography reveals cell-specific changes of Zn ligands in leaves of Turnip Yellow Mosaic Virus infected plants

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HK had the idea of doing μ XANES on plant samples in tomographic mode and supervised the study. Ana Mijovilovich made the Geopixe fits, the XANES analysis, and wrote the first draft of the manuscript, which was revised by all other authors. Ana Mijovilovich, DB, GF, KS, JG and HK did the measurements at the synchrotron. Archana Mishra did the metal treatments of the plants. Ana Mijovilovich, Archana Mishra and HK prepared the plant samples for the μ XANES measurements. EA prepared most of the model compounds, the others were done by Ana Mijovilovich and HK. DB did the tomographic reconstructions.

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ABSTRACT

As many metals are essential for plants, excess or deficiency of them or alteration of their uptake, translocation, sequestration or physiological use can have severe consequences for plant growth and fitness. Therefore, investigating the distribution and speciation of metals in tissues is essential to understand plant physiology. We present a method based on non-destructive Synchrotron X-ray microtomography combined with microspectroscopy for studying metal distribution and speciation in plant tissues. By using the Maia detector system and the high flux of the undulator beam at the P06 beamline of the PETRA III synchrotron (at DESY), it was possible to record μ XANES for every voxel of a tomogram. The metal coordination in regions of interest within the tissue samples could be determined by comparing the XANES with spectra of relevant reference compounds. Metal distribution and coordination were measured in shock frozen hydrated plant leaves in a cryostream, avoiding sample preparation artefacts like liquid cell content redistribution that occurs with other preparation methods, unequal distribution of stains in staining assays, sample degradation by beam damage and thawing, etc. A spatial resolution of 5 μ m was selected, which is sufficient to resolve all leaf tissues (epidermis, palisade mesophyll, spongy mesophyll, veins), larger cells and biomineralization hotspots.

As an application example, we studied the effect of infection with Turnip Yellow Mosaic Virus (TYMV) on the Zn distribution and the Zn speciation in duplicates of *Noccaea ochroleucum*. This non-accumulator plant grown with 100 μ M Zn had enough metal to allow collecting significant spectroscopic data. We found that the TYMV infected samples formed biomineralization crystallites, showing strong spectroscopic similarity to Zn silicate.

Keywords: microspectroscopy; microtomography; XANES; Zinc; *Noccaea ochroleucum*

1. INTRODUCTION

Zinc is an essential metal for plants. It is present in more than 1200 proteins (reviewed by Andresen et al., 2018) [1]. As a few examples, Zn works as a Lewis base in carbonic anhydrase, a protein involved in the conversion of CO₂ into bicarbonate, as well as in purple acid phosphatases (PAPs). It also has structural functions, but no redox function.

Hyperaccumulator plants use a different strategy to cope with high metal content than non-accumulators [2]. Non-accumulators use strong ligands for detoxification. Phytochelatins, to some extent glutathione, and oxalate are used as storage ligands. Hyperaccumulators, in contrast, usually use weak O/N ligands like small organic acids (malate, citrate) and histidine, for storage (Zn: Salt et al., 1999 [3]; Cd+Zn [4]; Küpper et al., 2004 [5]; Cu: Küpper et al., 2009 [6]). In most hyperaccumulators, the main accumulation occurs in the vacuoles of large epidermal cells [7-10]. For transport, hyperaccumulators and non-accumulators use strong ligands like nicotianamine [2]. Hyperaccumulators seem to react as non-accumulators when facing a high concentration of a non-accumulated metal. This was observed in a study of copper accumulation in *N. caerulea*, which showed mostly sulfur ligands, but also nicotianamine and biomineralization as copper oxalate [11].

It has been established by various studies that hyperaccumulators store metal(loid)s in their above-ground parts as a mechanism of defense against herbivores and pathogens (review e.g. by Leitenmaier and Küpper, 2013) [2]. Nevertheless, the response of the metal metabolism to pathogen infection in hyperaccumulator plants, as well as non-accumulator plants, is not known. A poised question is whether plants that normally do not hyperaccumulate Zn would show a redistribution, or maybe enhanced accumulation, in response to the viral infection. Modification of a non-hyperaccumulator's ionome (mineral nutrient and trace element composition) [4] after viral infection has been observed in Tobacco plants infected with *Xylella fastidiosa* [12].

During synchrotron measurements, preserving the integrity of the plant specimen is essential for the study of metal distribution within the plants. This typically requires shock-freezing and measuring in frozen-hydrated state, as discussed earlier

[7,5,13]. The synchrotron X-ray measurement is non-destructive, in contrast to most other analytical techniques that destroy the sample like LA-ICP-MS.

Synchrotron X-ray analysis is advantageous over other widely-used methods to obtain an image of the metal distribution in plant tissue, such as staining with a dye or construction of metal-responsive fluorescent proteins. Fluorescent proteins and dyes bind easily to weakly complexed metal atoms but have little access to metals bound to proteins and enzyme active sites. The active metal site of an enzyme has high affinity and specificity for a metal, which hampers the binding of the fluorescent protein or dye.

X-Ray Absorption Near Edge Structure (XANES) spectroscopy probes the transition from a core level to the bound state and valence orbitals, providing a tool to get insight into chemical information [14-18]. The energy position of the absorption edge changes with the oxidation state. The intensity and shape of the absorption edge results from the convolution of the transitions from the 1s to the Lowest Unoccupied Molecular Orbitals (LUMOs). The coordination of the metal center can be determined from the comparison with the spectra of reference compounds. Components of the absorption edge region, such as the edge inflection point (which defines the edge position), the maximum intensity of the absorption edge region (often called the white line) and the structure after the edge are distinctive features used for the compound speciation using XANES. Several mathematical approaches exist for analyzing compound speciation. Linear combination fitting (LCF) and principle component analysis (PCA) have been incorporated in many XAS fitting packages [19-21]. In LCF, the ability to determine and quantify the species is largely limited by the data quality and the availability of all significant reference materials with sufficiently different XANES spectra. This approach has been successful in plants to determine the species even from the very lifetime broadened edges of Cd and Zn [22,3,5], as well as Cu [23,11] and As [13]. In a standard XANES spectrum collected from bulk, species down to 10% can be quantified [24]. Species in amounts less than 5% or with strong similarity with other species can still be discerned with PCA [25].

Fluorescence images of metals can be obtained by just scanning along a line followed by an incremental step in orthogonal direction (2D raster scan) or followed by an incremental orthogonal rotation (tomography mode). While a 2D raster scan

generates a 2D projection along the depth of the sample and is a direct measurement, tomography allows to measure a virtual cross-section into the sample depth, but requires a mathematical reconstruction of the object image. Here, the composition of both μ XRF tomogram slices with the chemical information at every pixel elucidated from the XANES spectra allows determination of the distribution of both the metal concentration and its chemical species. By correlating the spatial distribution of metal speciation with the tissue and its function, it is possible to draw conclusions about the transport and storage mechanisms of metal(loid)s in the plants [13, 26]. In our first study [13], the As speciation was determined in the non-accumulator *Ceratophyllum demersum* using confocal μ XANES. As appeared as arsenite, arsenate and As bound to glutathione and phytochelatins. The space resolution and statistics obtained allowed distinguishing between epidermis, mesophyll and veins. When the metal concentration is low, however, the setup with confocal optics and only one single detector does not provide enough sensitivity. The Maia detector with 384 elements in backscattering geometry can overcome the limitation of low statistics by providing a large solid angle of fluorescence capture. Improving detection sensitivity also minimizes the beam damage per detected photon [27]; however the Maia setup does not allow confocal measurement.

A proof of concept study on tomography coupled to μ XANES was performed in larvae of the fruit fly, *Drosophila melanogaster*, for Cu^{2+} and Cu^{3+} species [28]. That study was done on freeze-dried samples measured at room temperature. The room temperature measurement raises concerns about beam damage and sample preparation artefacts like metal redistribution during drying in plant tissues. Compared to 2D mapping, one important advantage of single-slice tomography on frozen-hydrated samples (which also yields a 2D image as the final result after reconstruction) is, that the layer that is irradiated by the beam is cooled with optimal heat transfer by the non-irradiated tissue layers above and below the measured layer.

The current study shows the advantages and conundrums of measuring XANES in tomography mode for intact frozen plant tissues with cellular resolution. We show that measuring XANES in fluorescence tomography mode with a large solid-angle detector solves the problems of low sensitivity of confocal measurements and the missing depth resolution of non-confocal lateral raster scan measurements. As an

1 application example, in this work we investigated how an infection with the Turnip
2 Yellow Mosaic Virus (TYMV) affects the Zn speciation in *N. ochroleucum*, which
3 normally accumulates Ni but not Zn. We show that spectromicroscopy is feasible even
4 on this non-accumulator plant when grown with 100 μ M Zn. However, spectroscopic
5 imaging of micrometer sized objects like the biomineralization dots is challenging due
6 to the low signal to noise ratio, problems with accurate sample positioning (drift), etc.
7 For single slice tomography it is crucial to reduce temperature gradients which allow
8 small objects to drift out of the scanned area during the measurement.

2. MATERIALS & METHODS

2.1. Plant material and growth conditions

Noccaea ochroleucum (formerly called *Thlaspi ochroleucum*, common name of the genus: “pennycress”), plants were grown in a phytochamber with a sinusoidal light and temperature cycle with a maximum photon flux density of $125 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ and 25°C at noon and 18°C at night. For germination, the seeds were spread on moistened perlite : vermiculite (3:1), stratified at 4°C in the dark for two weeks, then germinated at $20-25^{\circ}\text{C}$. The 3 weeks old seedlings were transferred to plastic pots with full-strength hyperaccumulator hydroponic nutrient solution (HHNS), as described in detail by Küpper et al. (2007) [29]. Plants were treated with $100 \mu\text{M}$ Zn. The nutrient solution was continuously renewed with a flow rate of $250 \text{ ml.d}^{-1}.\text{plant}^{-1}$, and the leaves were sampled after 3 months.

Virus inoculum was prepared for manual inoculations by homogenizing a small amount of infected leaf tissue (Chinese cabbage young mature leaves) in phosphate buffer. When *Noccaea ochroleucum* plants were transferred on hydroponics under metal treatments up to 8 weeks, a pair of healthy leaves was then chosen for inoculation. Typically, the 2nd or 3rd pair of newest leaf was dusted with carborundum powder and the virus homogenate was applied by gently stroking it onto the leaf surface with a sterile cotton bud.

2.2. Plant sample mounting

Plants infected with the Turnip Yellow Mosaic Virus (TYMV) and untreated control samples were measured at PETRA III beamline P06. Samples from two independent experiments were measured to account for biological variability, and duplicate samples of the same experiment were measured as well (i.e. four samples in total). Selected leaf was cut from the petiole and transferred/dipped to water until sampling. For sampling first midrib was cut with the sharp blade (surgical) and the whole length of leaf beside the mid rib was sectioned as a sample. Leaf cuts made in water were mounted into polyimide capillaries for the synchrotron tomography. The intact tissue was flash-frozen in isopentane previously cooled to -140°C in a liquid N_2 bath, and subsequently stored in liquid N_2 . Samples were transported in a dry shipper and kept frozen at all times.

2.3. Preparation of model compounds

The following solution samples were prepared to be used as XANES reference compounds: 5 mM Zn chloride (pH not adjusted) as aquo complex, 5 mM ZnCl_2 + 50 mM reduced glutathione (adjusted to pH7) as Zn-GSH complex, 5mM ZnCl_2 + 50 mM histidine (pH 7) as Zn-histidine complex, 5 mM ZnCl_2 + 50 mM citric acid (pH 4) as Zn-citrate complex, 0.8 mM ZnCl_2 + 1.2 mM nicotianamine (pH 7) as Zn-nicotianamine complex, 5 mM ZnCl_2 + 50 mM Na-metasilicate (pH 8) to produce Zn-silicate suspension, 5 mM ZnCl_2 + ortho-phosphoric (pH 8) to produce Zn-phosphate suspension, and 5mM ZnCl_2 + 50 mM phytate (pH 5.5 and pH 7) for Zn-phytate complex. The liquid references were mixed with 20% glycerol to minimize ice formation and were frozen in the same polyimide capillaries and with the same method as the leaf samples.

2.4. μ XANES tomography

The measurements were performed in the microprobe endstation at beamline P06 at the PETRA III synchrotron radiation source at DESY (Hamburg, Germany) [30]. The undulator beam was focused using Kirkpatrick-Baez (KB) mirrors to approx. 500×500 nm spot size. The incoming photon flux was monitored with an ionization chamber before the KB mirrors. The intensity of the transmitted radiation was monitored with a Passivated Implanted Planar Silicon (PIPS) diode behind the sample. An in-line long-distance digital microscope with 2 μm resolution was used for sample alignment. Due to the long counting times needed for biological samples, only single slice tomograms resulting in a 2D image of the leaf were collected for 122 energy values in the energy range 9.660- 9.950 keV with a collection interval of 5 μm and thickness of the size of the beam (2 μm). We used 90 rotation angles of 4 degrees (full 360 degrees rotation of the sample). Energies were selected using a cryogenically cooled Si(111) double crystal monochromator with 1.4×10^{-4} dE/E band pass. Data was collected in backward scattering geometry using a Maia 384C detector system [27]. Collection time per pixel was 1.5-2 ms, while one tomogram for a single energy step was about 3 minutes. Samples were kept frozen during several hours of measurements by a cryostream (Oxford Cryosystem 700) with flow of N_2 gas. The cryostream imposed a distance from

sample to the detector face of 17.5-19.5 mm, longer than the optimal 10 mm. After a couple of hours of operation, the temperature gradient in the metal sample support became stable, giving tomograms with no significant height drift for big plant cells. The effect on small (biomineral) particles is discussed in the Results and Discussion sections.

A high-resolution tomogram of the non-infected sample (control) was acquired, with a step size of 0.25 μm and a beam size of approx. 500 x 500 nm, a counting time of 1 ms per pixel, at an excitation energy of 16.5 keV, to validate the tissue identification in the μXANES tomograms. In this high spatial resolution image cell types are easily identified by their shape and distribution (shown in Fig. 1). The total collection time of this high resolution 2D image was approximately 5 hours.

The long times required for the collection of a single μXANES tomogram limited the number of samples that could be measured. In order to get good statistics for the μXANES tomograms, the number of rotation angles was reduced and the sample was moved out of focus to achieve a beam size approximately equal to the collection interval of 5 μm in order to avoid under-sampling artifacts. With this measurement strategy plant species grown with 100 μM Zn and accumulating approx. 5000 ppm Zn, gave enough fluorescence to be able to collect μXANES tomograms within the available beamtime.

2.5. Data processing

The data analysis workflow is shown in Flowchart S1 (Appendix). Sinogram images were processed with GeoPIXE [31,32]. The tomogram images were reconstructed from the sinograms using the maximum likelihood expectation maximization (MLEM) algorithm [33,34]. From the Zn fluorescence images stacks, the XANES spectra at different locations on the samples were extracted using Fiji [35]. We show the regions selected to build the XANES spectra from the average of all pixels selected in Fig. 3, Fig. S6 (Appendix) and Fig. S7 (Appendix). The selection was done one region at a time for each sample, but for displaying purposes we have superimposed all regions on one image using PhotoImpact X3 (Corel Corporation, Ottawa, Canada). We also show the XANES spectrum extracted from the average of the spectra from all the pixels of the whole leaf. The possibility of radiation damage was studied by running up to 4

consecutive XANES tomography scans. No radiation damage was found. XANES spectra were processed with ATHENA [21] by fitting two splines, one at the low energy side of the edge and another for the high energy side, and normalizing the absorption edge to 1. The edge region was binned to 0.25 eV, and the entire XANES was smoothed by 3 iterations of interpolation. This was done in order to diminish the influence of the statistical noise on the component analysis. PCA analysis was performed with ATHENA [21]. PCA was run for an ensemble of 20 plant spectra corresponding to the different tissues and plants (control and virus-treated) and 9 reference compounds, which resulted in a set of the 5 most important components. The target transform revealed which reference compounds were most likely to be represented in the whole ensemble of samples. Unimportant components show a poor match between the experimental spectrum of the reference compound and the target transform, and were discarded. The linear combination fitting was performed in SIGMAPLOT (Systat Software, San Jose, CA, USA) by including the 5 components pointed as most likely by PCA, plus Zn silicate, which scored low in PCA, but was added to account for the mineralization. The fitting was performed on a 110 eV range around the edge. The Levenberg-Marquardt algorithm was employed for the optimization and the goodness of the fit was assessed by the coefficient of determination R_{sq} given by $1 - (SSE/SST)$ where SSE is the sum of squared error and SST is the sum of squared total. The best fit gave the R_{sq} closest to 1.

3. RESULTS

For each energy point of the XANES spectrum a tomogram of the Zn fluorescence was collected. The tomogram corresponding to the energy of the maximum intensity of the Zn K_{α} edge in the XANES spectrum, was used to visualize the tissues and to choose the regions of interest for the extraction of XANES spectra from the energy stack of images.

3.1. XANES of reference compounds

In order to be able to explain the Zn speciation in the plant samples, their XANES spectra were compared with model compounds. Zn complexes were prepared as reference compounds for those ligands that are known to be present in plant tissues and that may bind, according to the knowledge of their chemical properties, to Zn. They were recorded in the same tomographic scanning mode as the plant samples. Normalized XANES spectra for the reference compounds are shown in Fig. 2. The spectra of Zn histidine and Zn citrate are shown together with those measured in a standard XAS beamline equipped with a Si(111) monochromator and a 13-element HP Ge fluorescence detector [5] in Fig. S2 (Appendix). The structure of the XANES profiles were preserved after tomographic reconstruction of the virtual cross sections. The reference compounds for Zn histidine, Zn chloride, Zn phytate (pH 5.5 and 7) are like those shown by Monsanto et al. [24] measured in a XAS beamline with a Si(111) monochromator and a Ge fluorescence detector. For example, the XANES for Zn coordinated to low Z ligands (O or N) shows a high intensity edge. When sulfur is bound, as in the Zn-GSH reference, a lower edge intensity is found as well as a shift of the edge to lower energies. The Zn silicate showed features after the edge characteristic of some scattering of the ejected photoelectron in a crystalline structure and an edge position characteristic of tetrahedral Zn [36].

3.2. Plant samples

The TYMV treated sample showed a different accumulation pattern compared to the control sample, with biomineralization dots, possibly composed of small particles of Zn silicate. Those dots appear mostly in the palisade mesophyll cells. Table 1 and 2 show

the composition for each tissue type. Epidermal tissues and palisade mesophyll showed an increase in the Zn phosphate due to the virus infection, while the spongy mesophyll cells showed no significant change. Zn bound histidine was more abundant for the virus treated sample in all tissues compared to the control sample. The XANES spectrum of Zn citrate is very similar in shape and intensity to that of Zn chloride. While the individual presence of each is difficult to assess, the presence of these octahedral low Z ligand species is reduced in the virus infected samples for all tissues. Though also listed in table 1 and 2, minor components (less than 10%) are below the limit of detection of the method [24].

In spite of the lower spatial resolution of 5 μm (with respect to former measurements on leaves at 0.5 μm), the resolution was sufficient to distinguish the palisade mesophyll cells from spongy mesophyll and visualizing the upper and lower epidermis, which allowed to select specific areas for XANES spectra extraction as seen in Fig. 3, Fig. S6 (Appendix) and Fig. S7 (Appendix).

3.3. Specific tissue information from μXANES tomography

Fig. 4 shows the XANES spectrum summed over the area of the whole leaf compared to specific tissues. By selecting the whole leaf, the spectrum obtained resembled the bulk X-ray Absorption Spectroscopy (XAS) measurement done with spot sizes of hundreds of micrometers in the vertical direction times several millimeters in the horizontal. The whole leaf spectrum resembled the spectrum of the palisade cells since they constitute most of the leaf, and in *N. ochroleucum*, contrary to *N. caerulea*, they show the highest concentration of metal. Therefore, interesting details of other tissues, like the biomineralization dots inside the spongy mesophyll, or the epidermis, were not visible in the whole leaf spectrum.

3.4. Plant μXANES

The spectra extracted from selected zones in the samples are shown in Fig. 5. For both the controls and infected plants, the spectra of upper and lower epidermis, palisade mesophyll and spongy mesophyll tissue were obtained. Due to the lower metal content found in the vein, the XANES of veins showed much more noise than from the

other tissues, so that ligand proportions were less accurate and due to the high noise in the data only for one sample of each kind (virus and control) was fitted.

In the palisade mesophyll of one control sample, the spectra were extracted for different area sizes and are shown in Fig. S4 (Appendix) and Fig. S5 (Appendix). There was no noticeable difference in the spectra down to a few pixels (see Table S1 - Appendix), even when the data becomes noisy (leading to oscillations in the smoothed data for an energy above 9.70 keV, shown in Fig. S4 (Appendix) for region 2 -2.5 pixels).

PCA analysis revealed the most likely reference compounds to be present in the complete ensemble of samples. The PCA target transform indicated that Zn chloride, Zn histidine, Zn nicotianamine, Zn phosphate, and Zn citrate are all represented in the composition of the virus treated samples (see Fig. S8 - Appendix).

The linear combination fitting results show the main components for each tissue. Minor components (around and less than 10%) or similar components are difficult to discern due to the low statistics of the data, as already stated by Monsanto et al. [24].

3.4.1. COMPOSITION OF THE VIRUS TREATED PLANT

Fig. 6A shows proportions obtained from the fit for all tissues. The corresponding table with the mean and Rsqr-factors is shown in Table S1 (Appendix). For the biomineralization dots, the quality of the data does not allow a proper fit. This can be seen in Fig. S9 (Appendix) for the spectra. An attempt of a fit including the 6 references yielded mainly Zn silicate (36%), followed by Zn histidine (29%) and Zn-nicotianamine (27%), but the overall quality of the fit was poor with a Rsqr-factor of 95%. Fig. 7 shows the spectra together with its best fit for the other tissues of the TYMV infected plant. Epidermis tissues (upper and lower) were averaged due to its similarity. The most abundant were Zn-histidine and Zn-phosphate with about 45% and 42% respectively. Similar results were obtained for the palisade and spongy mesophylls. For the vein, the main components were Zn histidine with 66% and Zn phosphate with 29%. XANES of two replicates of the TYMV plant are shown together in Fig. S1 (Appendix).

3.4.2. COMPOSITION OF THE CONTROL PLANT

Fig 6B, corresponding to Table S2 (Appendix), shows the weights for each Zn component of the control plants, and Fig. 8 shows the spectrum together with its best fit for all the tissues of one of the control plant replicates. In the average of the upper and lower epidermis, the main components were Zn histidine (26%) and Zn phosphate (26%), Zn citrate with 22% and Zn chloride with 11%. In the palisade mesophyll and in the spongy mesophyll cells, the ligand proportions were somewhat similar to the epidermis, with an increase of around 8% of the histidine for both tissues, and also an increase of 10% of the citrate in the palisade mesophyll. Zn silicate was either not present, or present with negligible values for all tissues. For the vein, only one sample gave enough statistics to attempt a fit. The results showed mainly Zn-chloride (11%), Zn-phosphate (52%) and Zn-histidine (33%).

4. DISCUSSION

4.1. μ XANES Tomography to study metals in intact tissues - general aspects

Since biological tissues consist mostly of light elements (C, O, N), hard X-rays will penetrate a long distance, and the resulting images consist of the fluorescence of a stack of cells. For water, which constitutes 90% of most hydrated plant tissues, 41% of the intensity is absorbed at 10 keV after 1 mm, as calculated with HEPHAESTUS [21] from the McMaster tables [39] and its update [40]. Collecting XANES for each pixel in tomography mode down to a resolution of a few microns, either full 3D or single slice 2D, drastically improves the analytical power of the spectroscopy. It is possible to measure μ XANES on 2D scans (raster scans) of thin sections as well, but this bears the high risk of sample preparation artefacts.

Tomography allows measuring the interior of specimens without the need of cutting to unveil a surface. However, the size of the specimen is a limiting factor. The higher the resolution of the tomography, the smaller the specimen should be, otherwise resulting in unfeasible measuring times at current data acquisition rates. Some specimens like *Ceratophyllum demersum* [13] have small leaves that can be introduced in the capillary, but most specimens require cutting of tissue blocks. These blocks must not be too small in order to prevent cutting damage on the measured cells. In those cases, tomography also brings an advantage. In tomography, the outer layer of the sample functions as a protecting layer of the internal leaf, whose cells are intact. Thus, tomography provides a unique way to avoid collecting data that suffer from cutting artefacts.

The reference compounds measured in tomography mode using the Maia detector showed equivalent XANES spectra to bulk spectra previously collected on a conventional (non-imaging) XAS beamline using the same monochromator type and a 13-element Ge solid-state fluorescence detector. This indicates that the Maia detector and GeoPIXE fitting of the fluorescence spectra produce XANES spectra free of artefacts in biological samples. This is important to note since the Maia detector operates in backscattering geometry which results in a higher scattering signal in comparison to conventional fluorescence detectors in 90° geometry. Also the pile up generated at high count rates has to be considered, which result from the high intensity beam that is required for measuring diluted samples (see Fig. S2 - Appendix).

The reference compounds were chosen to model ligands that are known to occur in the cytoplasm (with pH around 7.4) as well as ligands present in the vacuole (with pH around 4 to 6). Histidine as an amino acid residue in proteins is well known to be a ligand to metals in all kinds of metalloproteins (plant-specific review: Andresen et al., 2018 [1]), and free histidine has been found to play a role in metal hyperaccumulation by plants [41,3,2]. Zn phytate at two pH values (5.5 and 7) was included since its presence has been determined in *Noccaea caerulea* roots and shoots [24].

The edge position and shape of the absorption edge are the main features for the compound speciation. Diluted zinc chloride solution, which is mostly aquo complex as Cl^- is only a weak ligand, is rather indistinguishable from Zn-citrate. Zn-phosphate has a similar edge energy position and similar shape at the absorption edge with slight differences in the second resonance. Zn-phytate at low pH is different from high pH. Both Zn-histidine and Zn-nicotianamine show distinct XANES spectra, suitable for compound speciation. Zn is known to coprecipitate with silicon as Zn silicate in *Arabidopsis* (= *Cardaminopsis*) *halleri* grown in Zn polluted soils [42]. However, the exact nature of the biomineral can be more complex. Unfortunately the quality of the spectra did not allow a good fit. All biomineralization dots comprised a few pixels. The XANES of each individual dot was obtained, and some representatives of each spectrum type found are shown in Fig. S3 (Appendix). The spectra of such small objects appear with a huge variation that hinders identification. PCA of the biomineralization dots and the highest Zn concentration areas of the replicate of the virus treated samples gave Zn silicate, Zn nicotianamine and Zn histidine as most likely present in the sample ensemble. PCA analysis indicates that the reference liquid solution of Zn silicate is not well represented by the biomineral. Therefore, the exact nature of the biomineral dots requires further studies.

Measuring small features like the biomineral particles is a challenge regarding stability of the optical and mechanical components of the beamline. In the measurement on frozen samples in open air using a vertical cryojet, temperature gradients are generated in the metal supports. Before these have reached equilibrium, they cause a slow drift in the vertical axis, which moves the object in/out of the beam generating higher/lower counts with time. As the tomograms at different energies are

collected sequentially, for different regions of the energy scan region such displacements are different, which results in a distorted spectrum. Such a distortion was observed in some of the biomineralization dots. Such distortions of spectra were not observed in the palisade mesophyll, because the palisade mesophyll occupies a large volume of the leaf tissue. This problem in measuring spectroscopy on very small objects at non-ambient temperatures could be minimized by measuring in a cryochamber at temperature equilibrium. A suitable vacuum sample chamber with cryogenic cooling and highly efficient X-ray detector is currently developed at the P06 beamline, but not operational yet. Besides, the Maia detector imposes geometric restrictions that hamper its use in a cryochamber. The drift problem could be easily corrected for full 3D tomography, where 2D projections can be (also vertically) aligned before tomographic reconstruction. However, the long scanning times required for 4D data collection make 3D μ XANES unfeasible with current beamlines.

Anyhow, at the tissue level and even for zones comprising a few pixels, the single slice tomography allows collecting μ XANES data of good enough quality for composition analysis of the leaf tissues. Knowing the binding of the metal from the composition analysis gives insight in the physiology of the plant after the virus infection.

4.2. The effect of TYMV in metal distribution of *N. ochroleucum*

The virus did not affect the metal distribution and speciation for all leaf tissues in the same way. In the epidermis, Zn appears mainly bound as Zn histidine and Zn phosphate, for both the virus treated and the control plants. For the palisade and spongy mesophyll tissues, the virus treated plant had more Zn bound to histidine than the control plant. Due to the large variation between replicates it was not possible to conclude about differences between palisade and spongy mesophyll tissue in each plant. The relative standard deviation among samples was high (up to 141% for the control).

The most significant difference in the virus treated *N. ochroleucum* was the presence of biomineralization Zn dots, absent in the control plant. Biomineralization in normal plants is one of the defense mechanism against toxic metal concentration [43].

Noccaea caerulescens, which is related to *N. ochroleucum*, hyperaccumulates Zn and Cd but not Cu. When exposed to high Cu concentration, biomineralization as Cu-oxalate occurred [11]. Hyperaccumulators may also form biominerals. Zn silicate in the cytoplasm and nuclei of leaf cells has been observed in the Zn and Cd hyperaccumulator *Arabidopsis* (= *Cardaminopsis*) *halleri* [42]. *N. ochroleucum* does not hyperaccumulate, but only moderately accumulates Zn (hyperaccumulation defined as >1% in dry mass). We speculate that the virus infection induces a higher re-distribution of Zn that ends as biomineral. The exact interaction of the virus infection with the biomineralization process is a topic for future research. Knowledge about biomineralization in plants is interesting because of its ecological significance in biogeochemical process in soils, detoxification of heavy metals, and as defense from herbivores [43].

In summary, the work shows that after the infection with TYMV, the non-accumulator *N. ochroleucum* biomineralizes Zn mainly in the spongy mesophyll cells. The Zn species identified in tissues other than the biomineral dots are mainly Zn histidine and Zn phosphate. This is in strong contrast to the dominance of organic acids (plus aquo complex) repeatedly found in hyperaccumulator plants [2,3,5]. Zn phosphate has been recently found in the non-accumulator *Arabidopsis lyrata* [44]. Biologically, the presence of (hardly bioavailable) phosphates and of biominerals makes Zn in *N. ochroleucum* less toxic to herbivores. Thus, in this species, contrary to hyperaccumulators like *N. caerulescens*, Zn is not very suitable as an elemental defense. Like in the earlier studies using bulk XAS [2,3,5], in this study no sulfur ligands could be identified in any of the leaf tissues.

This work proves the feasibility of cell specific metal speciation using μ XANES tomography with a spatial resolution down to 5 μ m in intact frozen plant tissues. Measuring with higher spatial resolution, but constant radiation dose would be made possible by new developments in detectors with larger solid angle of detection combined with faster data acquisition. This would also enable to perform spectroscopy in non hyperaccumulator plants grown with less than 100 μ M Zn of this work.

A study with high resolution tomography, as shown here for the control sample (Fig. 1), with a pixel size of 0.25 μ m, is planned. This will give further insight to the sub-cellular distribution of Zn.

5. Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:...'.

References

- [1] E. Andresen, E. Peiter, H. Kupper, Trace metal metabolism in plants, *J. Exp. Bot.* 69 (2018) 909–954. doi:10.1093/jxb/erx465.
- [2] B. Leitenmaier, H. Küpper, Compartmentation and complexation of metals in hyperaccumulator plants, *Front. Plant Sci.* 4 (2013). doi:10.3389/fpls.2013.00374.
- [3] D.E. Salt, R.C. Prince, A.J.M. Baker, I. Raskin, I.J. Pickering, Zinc Ligands in the Metal Hyperaccumulator *Thlaspi caerulescens* As Determined Using X-ray Absorption Spectroscopy, *Environ. Sci. Technol.* 33 (1999) 713–717. doi:10.1021/es980825x.
- [4] D.E. Salt, I. Baxter, B. Lahner, Ionomics and the study of the plant ionome., *Annu. Rev. Plant Biol.* 59 (2008) 709–733. doi:10.1146/annurev.arplant.59.032607.092942.
- [5] H. Küpper, A. Mijovilovich, W. Meyer-Klaucke, P.M.H. Kroneck, Tissue- and age-dependent differences in the complexation of cadmium and zinc in the cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype) revealed by X-ray absorption spectroscopy, *PLANT Physiol.* 134 (2004) 748–757. doi:10.1104/pp.103.032953.
- [6] H. Küpper, B. Goetz, A. Mijovilovich, F.C. Küpper, W. Meyer-Klaucke, Complexation and Toxicity of Copper in Higher Plants. I. Characterization of Copper Accumulation, Speciation, and Toxicity in *Crassula helmsii* as a New Copper Accumulator, *PLANT Physiol.* 151 (2009) 702–714. doi:10.1104/pp.109.139717.
- [7] H. Küpper, F.J. Zhao, S.P. McGrath, Cellular compartmentation of zinc in leaves of the hyperaccumulator *Thlaspi caerulescens*, *PLANT Physiol.* 119 (1999) 305–311. doi:10.1104/pp.119.1.305.
- [8] H. Kupper, E. Lombi, F.J. Zhao, G. Wieshammer, S.P. McGrath, Cellular compartmentation of nickel in the hyperaccumulators *Alyssum lesbiacum*, *Alyssum bertolonii* and *Thlaspi goesingense*, *J. Exp. Bot.* 52 (2001) 2291–2300. doi:10.1093/jexbot/52.365.2291.
- [9] B. Frey, C. Keller, K. Zierold, Distribution of Zn in functionally different leaf epidermal cells of the hyperaccumulator *Thlaspi caerulescens*, *Plant. Cell Environ.* 23 (2000) 675–687. doi:10.1046/j.1365-3040.2000.00590.x.
- [10] B. Leitenmaier, H. Küpper, Cadmium uptake and sequestration kinetics in individual leaf cell protoplasts of the Cd/Zn hyperaccumulator *Thlaspi caerulescens*, *PLANT CELL Environ.* 34 (2011) 208–219. doi:10.1111/j.1365-3040.2010.02236.x.

- 1 [11] A. Mijovilovich, B. Leitenmaier, W. Meyer-Klaucke, P.M.H. Kroneck, B. Goetz, H.
2 Küpper, Complexation and Toxicity of Copper in Higher Plants. II. Different
3 Mechanisms for Copper versus Cadmium Detoxification in the Copper-Sensitive
4 Cadmium/Zinc Hyperaccumulator *Thlaspi caerulescens* (Ganges Ecotype), *PLANT*
5 *Physiol.* 151 (2009) 715–731. doi:10.1104/pp.109.144675.
- 6 [12] F. Navarrete, L. De La Fuente, Zinc Detoxification Is Required for Full Virulence
7 and Modification of the Host Leaf Ionome by *Xylella fastidiosa*., *Mol. Plant. Microbe.*
8 *Interact.* 28 (2015) 497–507. doi:10.1094/MPMI-07-14-0221-R.
- 9 [13] S. Mishra, G. Wellenreuther, J. Mattusch, H.-J. Staerk, H. Küpper, Speciation and
10 Distribution of Arsenic in the Nonhyperaccumulator Macrophyte *Ceratophyllum*
11 *demersum*, *PLANT Physiol.* 163 (2013) 1396–1408. doi:10.1104/pp.113.224303.
- 12 [14] G.N. George, B. Hedman, K.O. Hodgson, An edge with XAS, *Nat. Struct. Biol.*
13 (1998). doi:10.1038/1336.
- 14 [15] R.W. Strange, M.C. Feiters, Biological X-ray absorption spectroscopy (BioXAS): a
15 valuable tool for the study of trace elements in the life sciences, *Curr. Opin. Struct.*
16 *Biol.* (2008). doi:10.1016/j.sbi.2008.06.002.
- 17 [16] F.M.F. De Groot, AWARD PAPER: XANES spectra of transition metal compounds,
18 in: *J. Phys. Conf. Ser.*, 2009. doi:10.1088/1742-6596/190/1/012004.
- 19 [17] H.A. Castillo-Michel, C. Larue, A.E. Pradas del Real, M. Cotte, G. Sarret, Practical
20 review on the use of synchrotron based micro- and nano- X-ray fluorescence mapping
21 and X-ray absorption spectroscopy to investigate the interactions between plants and
22 engineered nanomaterials, *Plant Physiol. Biochem.* (2017).
23 doi:10.1016/j.plaphy.2016.07.018.
- 24 [18] F. Porcaro, S. Roudeau, A. Carmona, R. Ortega, Advances in element speciation
25 analysis of biomedical samples using synchrotron-based techniques, *TrAC - Trends*
26 *Anal. Chem.* (2018). doi:10.1016/j.trac.2017.09.016.
- 27 [19] T. Ressler, WinXAS: A program for X-ray absorption spectroscopy data analysis
28 under MS-Windows, *J. Synchrotron Radiat.* 5 (1998) 118–122.
29 doi:10.1107/S0909049597019298.
- 30 [20] S.R. Wasserman, P.G. Allen, D.K. Shuh, J.J. Bucher, N.M. Edelstein, EXAFS and
31 principal component analysis: a new shell game., *J. Synchrotron Radiat.* 6 (1999) 284–
32 286. doi:10.1107/S0909049599000965.

- [21] B. Ravel, M. Newville, ATHENA, ARTEMIS, HEPHAESTUS: data analysis for X-ray absorption spectroscopy using IFEFFIT, *J. Synchrotron Radiat.* 12 (2005) 537–541. doi:10.1107/S0909049505012719.
- [22] D.E. Salt, R.C. Prince, I.J. Pickering, I. Raskin, Mechanisms of Cadmium Mobility and Accumulation in Indian Mustard., *Plant Physiol.* 109 (1995) 1427–1433.
- [23] H. Kuepper, B. Goetz, A. Mijovilovich, F.C. Kuepper, W. Meyer-Klaucke, Complexation and Toxicity of Copper in Higher Plants. I. Characterization of Copper Accumulation, Speciation, and Toxicity in *Crassula helmsii* as a New Copper Accumulator, *PLANT Physiol.* 151 (2009) 702–714. doi:10.1104/pp.109.139717.
- [24] A.C. Monsanto, P. Kappen, Y. Wang, P.J. Pigram, A.J.M. Baker, C. Tang, In vivo speciation of zinc in *Noccaea caerulescens* in response to nitrogen form and zinc exposure, *Plant Soil.* 348 (2011) 167–183. doi:10.1007/s11104-011-0887-7.
- [25] M. Gräfe, E. Donner, R.N. Collins, E. Lombi, Speciation of metal(loid)s in environmental samples by X-ray absorption spectroscopy: A critical review, *Anal. Chim. Acta.* 822 (2014) 1–22. doi:https://doi.org/10.1016/j.aca.2014.02.044.
- [26] G. Sarret, P. Saumitou-Laprade, V. Bert, O. Proux, J.L. Hazemann, A. Traverse, M.A. Marcus, A. Manceau, Forms of Zinc Accumulated in the Hyperaccumulator *Arabidopsis halleri*, *PLANT Physiol.* 130 (2002) 1815–1826. doi:https://doi.org/ 10.1104/pp.007799.
- [27] C.G. Ryan, D.P. Siddons, G. Moorhead, R. Kirkham, P.A. Dunn, A. Dragone, G. De Geronimo, Large detector array and real-time processing and elemental image projection of X-ray and proton microprobe fluorescence data, *Nucl. Instruments Methods Phys. Res. Sect. B Beam Interact. with Mater. Atoms.* 260 (2007) 1–7. doi:https://doi.org/10.1016/j.nimb.2007.01.271.
- [28] S.A. James, R. Burke, D.L. Howard, K.M. Spiers, D.J. Paterson, S. Murphy, G. Ramm, R. Kirkham, C.G. Ryan, M.D. de Jonge, Visualising coordination chemistry: fluorescence X-ray absorption near edge structure tomography., *Chem. Commun. (Camb).* 52 (2016) 11834–11837. doi:10.1039/c6cc06747f.
- [29] H. Kuepper, A. Parameswaran, B. Leitenmaier, M. Trtilek, I. Setlik, Cadmium-induced inhibition of photosynthesis and long-term acclimation to cadmium stress in the hyperaccumulator *Thlaspi caerulescens*, *NEW Phytol.* 175 (2007) 655–674. doi:10.1111/j.1469-8137.2007.02139.x.

- [30] U. Boesenberg, C.G. Ryan, R. Kirkham, D.P. Siddons, M. Alfeld, J. Garrevoet, T. Núñez, T. Claussen, T. Kracht, G. Falkenberg, Fast X-ray microfluorescence imaging with submicrometer-resolution integrating a Maia detector at beamline P06 at PETRAIII, J. Synchrotron Radiat. 23 (2016) 1550–1560. doi:10.1107/S1600577516015289.
- [31] C.G. Ryan, D.R. Cousens, S.H. Sie, W.L. Griffin, G.F. Suter, E. Clayton, Quantitative pixe microanalysis of geological material using the CSIRO proton microprobe, Nucl. Instruments Methods Phys. Res. Sect. B Beam Interact. with Mater. Atoms. 47 (1990) 55–71. doi:https://doi.org/10.1016/0168-583X(90)90047-X.
- [32] C.G. Ryan, R. Kirkham, R.M. Hough, G. Moorhead, D.P. Siddons, M.D. de Jonge, D.J. Paterson, G. De Geronimo, D.L. Howard, J.S. Cleverley, Elemental X-ray imaging using the Maia detector array: The benefits and challenges of large solid-angle, Nucl. Instruments Methods Phys. Res. Sect. A Accel. Spectrometers, Detect. Assoc. Equip. 619 (2010) 37–43. doi:https://doi.org/10.1016/j.nima.2009.11.035.
- [33] P.P. Bruyant, Analytic and Iterative Reconstruction Algorithms in SPECT, J. Nucl. Med. 43 (2002) 1343–1358. http://jnm.snmjournals.org/content/43/10/1343.abstract.
- [34] W. De Nolf, F. Vanmeert, K. Janssens, XRDUA: Crystalline phase distribution maps by two-dimensional scanning and tomographic (micro) X-ray powder diffraction, J. Appl. Crystallogr. (2014). doi:10.1107/S1600576714008218.
- [35] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.Y. Tinevez, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, Fiji: An open-source platform for biological-image analysis, Nat. Methods. (2012). doi:10.1038/nmeth.2019.
- [36] G.A. Waychunas, C.C. Fuller, J.A. Davis, J.J. Rehr, Surface complexation and precipitate geometry for aqueous Zn(II) sorption on ferrihydrite: II. XANES analysis and simulation, Geochim. Cosmochim. Acta. 67 (2003) 1031–1043. doi:https://doi.org/10.1016/S0016-7037(02)01280-2.
- [37] J. Rose, I. Moulin, A. Masion, P.M. Bertsch, M.R. Wiesner, J.Y. Bottero, F. Mosnier, C. Haehnel, X-ray absorption spectroscopy study of immobilization processes for heavy metals in calcium silicate hydrates. 2. Zinc, Langmuir. (2002). doi:10.1021/la001302h.

- [38] J.P. Veiga, M.O. Figueiredo, A XANES study on the structural role of zinc in ancient tile glazes of Portuguese origin, *X-Ray Spectrom.* 37 (2008) 458–461. doi:10.1002/xrs.1089.
- [39] J.H. McMaster, W. H. Kerr, Del Grande, N., Mallett, J.H. and Hubbell, Compilation of X-Ray Cross Sections, Lawrence Livermore National Laboratory Report UCRL-50174 Section II Revision I (1969) available from National Technical Information Services L-3, U.S. Dept. of Commerce, 1969.
- [40] A. Shaltout, H. Ebel, R. Svagera, Update of photoelectric absorption coefficients in the tables of McMaster, *X-Ray Spectrom.* 35 (2006) 52–56. doi:10.1002/xrs.815.
- [41] U. Krämer, J.D. Cotter-Howells, J.M. Charnock, A.J.M. Baker, J.A.C. Smith, Free histidine as a metal chelator in plants that accumulate nickel, *Nature.* 379 (1996) 635. <https://doi.org/10.1038/379635a0>.
- [42] D. Neumann, U. zur Nieden, Silicon and heavy metal tolerance of higher plants., *Phytochemistry.* 56 (2001) 685–692.
- [43] H. He, E.J. Veneklaas, J. Kuo, H. Lambers, Physiological and ecological significance of biomineralization in plants, *Trends Plant Sci.* 19 (2014) 166–174. doi:10.1016/j.tplants.2013.11.002.
- [44] G. Sarret, G. Willems, M.-P. Isaure, M. A. Marcus, S. C. Fakra, H. Fre'rot, S. Pairis, Nicolas Geoffroy, A. Manceau, P. Saumitou-Laprade. Zinc distribution and speciation in *Arabidopsis halleri* x *Arabidopsis lyrata* progenies presenting various zinc accumulation capacities. *New Phytol.* 184 (2009): 581–595. doi: 10.1111/j.1469-8137.2009.02996.x

Figure captions

Figure 1. Tomogram of the control plant in high resolution. Contrast was adjusted to reduce noise with Fiji [35]. Tomogram acquired with a step size of 0.25 μm and beam size of 0.5 μm .

Figure 2. XANES of reference compounds: Zn chloride, Zn citrate (pH 4), Zn phosphate, Zn histidine (pH 7), Zn nicotianamine (pH 7), Zn phytate (pH 5.5), Zn phytate (pH 7), Zn GSH (pH7.1), and Zn silicate (pH 8).

Figure 3. Tomograms of: a) plant infected with TYMV and b) control plant. The areas selected for spectroscopy are shown. Tomograms were acquired with a step size of 5 μm and thickness of the size of the beam (2 μm). The tomogram corresponding to the energy of the maximum of the Zn $K\alpha$ edge is shown.

Figure 4. XANES of the whole leaf for the plant infected with TYMV, showing the similarity to the palisade mesophyll and differences compared to the upper epidermis.

Figure 5. XANES spectra of: a) plant infected with TYMV and b) control plant. For each plant XANES of upper and lower epidermis, palisade mesophyll and spongy mesophyll cells are shown. Only the vein for the control plant is shown (see text).

Figure 6. Bar graphs of the Linear combination fitting results for: a) the TYMV treated plants and b) control (non-infected) plant. Numerical values, mean and R_{sqr} -factors are shown in Table S1 and S2 (Appendix).

Figure 7. Linear combination fitting for the XANES of the plant infected with TYMV. The best fit is shown for: a) epidermis, b) the palisade mesophyll, c) vein and d) spongy mesophyll.

- 1 Figure 8. Linear combination fitting for the XANES of the control plant. The best fit of
- 2 the combinatorial analysis is shown for: a) epidermis, b) the palisade mesophyll, and c)
- 3 spongy mesophyll.
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- 5 Flowchart 1. Flow diagram for the experiment and data analysis.
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- 8 Figure S1. XANES of two replicates of the TYMV plant and control plant, for the
- 9 palisade mesophyll.
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- 11 Figure S2. XANES of a) Zn citrate and b) Zn histidine. The measurement 2002 in former
- 12 D2 beamline at DORIS (Hamburg, Germany) in XAS set-up is compared to data from
- 13 2017-2018 at P06 beamline at PETRA (Hamburg, Germany) in tomography mode.
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- 15 Figure S3. XANES spectra for several representative biomineralization dots.
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- 17 Figure S4. XANES spectra (top left) and Linear combination fitting for the XANES of the
- 18 control plant palisade mesophyll for regions 1 (top right), region 2 (bottom left) and a
- 19 subset of 5 pixels of region 2 (bottom right). The best fit is shown.
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- 21 Figure S5. Tomogram of the control plant palisade mesophyll. The areas selected for
- 22 spectroscopy are shown: A) region 1, B) region 2, c) a subset of 5 pixels of region 2.
- 23 Tomograms were acquired with a step size of 5 μm and thickness of the size of the
- 24 beam (2 μm). The tomogram corresponding to the energy of the maximum of the Zn
- 25 $\text{K}\alpha$ edge is shown.
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- 27 Figure S6. Tomograms of replicate samples for the control plant. Tomograms were
- 28 acquired with a step size of 5 μm and thickness of the size of the beam (2 μm). The
- 29 tomogram corresponding to the energy of the maximum of the Zn $\text{K}\alpha$ edge is shown.
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- 31 Figure S7. Tomograms of replicate samples for the TYMV treated plant. Tomograms
- 32 were acquired with a step size of 5 μm and thickness of the size of the beam (2 μm).

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1596 1 The tomogram corresponding to the energy of the maximum of the Zn $K\alpha$ edge is
1597 2 shown.
1598 3
1600 4 Figure S8. Target transforms for all reference compounds in the PCA analysis of the
1601 5 complete ensemble of samples.
1602 6
1603 7 Figure S9. Linear combination fitting “attempt” for the XANES of the biomineralization
1604 8 dots in the plant infected with TYMV.
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MicroXANES tomography reveals cell-specific changes of Zn ligands in leaves of Turnip Yellow Mosaic Virus infected plants

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Flowchart 1. Flow diagram for the experiment and data analysis.

Table S1. Linear combination fitting results for the palisade mesophyll of one control plant for three selected regions with different sizes: region 1, region 2 and a 5 pixels subset of region 2.

CONTROL Sample 288	Region 1 (%)	Region 2 (%)	Region 2 (%) 5 pixels
Zn chloride	26	33	23
Zn citrate	26	28	39
Zn phosphate	0	0	0
Zn nicotianamine	21	33	38
Zn histidine	27	6	0
Zn silicate	0	0	0
Rsqr	0.9971	0.9969	0.9900

Table S2. Linear combination fitting results for the TYMV treated plants. Numbers have been rounded for both replicates: Rep1 and Rep2 and their mean value.

	Epidermis (%)	Palisade mesophyll (%)	Spongy mesophyll (%)	Vein (%)
Zn chloride				
Rep1	1	0	3	0
Rep2	8	8	9	-
Mean	5	4	6	0
Zn citrate				
Rep1	10	2	1	2
Rep2	5	9	9	-
Mean	8	6	5	2
Zn phosphate				
Rep1	44	39	21	29
Rep2	40	25	27	-
Mean	42	32	24	29
Zn nicotianamine				
Rep1	0	0	0	3
Rep2	0	9	10	-
Mean	0	5	5	3
Zn histidine				
Rep1	45	55	63	66
Rep2	47	49	45	-
Mean	46	52	54	66
Zn silicate				
Rep1	0	4	12	0
Rep2	0	0	0	-
Mean	0	2	6	0
Rsqr				
Rep1	0.9975	0.9970	0.9971	0.9944
Rep2	0.9968	0.9944	0.9968	-

Figure S1. XANES of two replicates of the TYMV plant and control plant, for the palisade mesophyll.

Figure S2. XANES of a) Zn citrate and b) Zn histidine. The measurement 2002 in former D2 beamline at DORIS (Hamburg, Germany) in XAS set-up is compared to data from 2017-2018 at P06 beamline at PETRA (Hamburg, Germany) in tomography mode.

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Figure S4. XANES spectra (top left) and Linear combination fitting for the XANES of the control plant palisade mesophyll for regions 1 (top right), region 2 (bottom left) and a subset of 5 pixels of region 2 (bottom right). The best fit is shown.

Figure S5. Tomogram of the control plant palisade mesophyll. The areas selected for spectroscopy are shown: A) region 1, B) region 2, c) a subset of 5 pixels of region 2. Tomograms were acquired with a step size of 5 μm and thickness of the size of the beam (2 μm). The tomogram corresponding to the energy of the maximum of the Zn $K\alpha$ edge is shown.

Figure S6. Tomograms of replicate samples for the control plant. Tomograms were acquired with a step size of 5 μm and thickness of the size of the beam (2 μm). The tomogram corresponding to the energy of the maximum of the Zn $K\alpha$ edge is shown.

Figure S7. Tomograms of replicate samples for the TYMV treated plant. Tomograms were acquired with a step size of 5 μm and thickness of the size of the beam (2 μm). The tomogram corresponding to the energy of the maximum of the Zn K α edge is shown.

Figure S8. Target transforms for all reference compounds in the PCA analysis of the complete ensemble of samples.

Figure S9. Linear combination fitting “attempt” for the XANES of the biomineralization dots in the plant infected with TYMV.