Imaging element distribution and speciation in plant cells

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To maintain cellular homeostasis, concentrations, chemical speciation, and localization of mineral nutrients and toxic trace elements need to be regulated. Imaging the cellular and subcellular localization of elements and measuring their *in situ* chemical speciation are challenging tasks that can be undertaken using synchrotron-based techniques, such as X-ray fluorescence and X-ray absorption spectrometry, and mass spectrometry-based techniques, such as secondary ion mass spectrometry and laser-ablation inductively coupled plasma mass spectrometry. We review the advantages and limitations of these techniques, and discuss examples of their applications, which have revealed highly heterogeneous distribution patterns of elements in different cell types, often varying in chemical speciation. Combining these techniques with molecular genetic approaches can unravel functions of genes involved in element homeostasis.

Spatial and chemical information of mineral element homeostasis

Plants take up a range of mineral elements from the soil, some of which are essential for growth, whereas others are non-essential [1]. Deficiencies of essential elements are a major limiting factor for crop production in many areas worldwide, whereas excessive accumulation of both essential and non-essential elements can lead to phytotoxicity [1]. Accumulation of some elements, such as cadmium (Cd) [2] and arsenic (As) [3], in the edible parts of crops may pose a significant risk to human health well before phytotoxicity occurs. By contrast, there is a need to increase essential micronutrients, such as iron (Fe) and zinc (Zn), in plant-

based foods to alleviate their deficiencies in humans [4]. Plant nutrition research aims to understand how minerals are acquired, transported, distributed, stored, and used in plants. This knowledge is important not only for sustainable agricultural production but also for ensuring the nutritional quality and safety of agricultural products [5].

Analyses of total elemental concentrations can now be performed using high-throughput platforms to reveal the ionic profile (see [Glossary](#)) of plant tissues [6]. Although the total concentrations of minerals can provide information about the capacity for uptake and translocation, it is well recognized that minerals are distributed heterogeneously across different cell types [7]. Not only may the total concentrations vary at the tissue, cellular, and subcellular scales but also the chemical speciation of minerals may vary. This spatial information is crucial for understanding the homeostasis of minerals, particularly how different cell types and, fundamentally, different genes function in controlling the distribution, complexation, and storage of minerals, and how these processes vary among diverse plant species in the ecophysiological context.

A range of techniques are available for mapping element distribution at various spatial scales. Traditional methods, such as energy-dispersive X-ray microanalysis (EDX) and proton (particle)-induced X-ray emission (PIXE), have been useful in mapping the cellular distributions of macronutrients or metals and metalloids that accumulate to high concentrations in hyperaccumulating plants (e.g., [8,9]). Visualizing the spatial distribution of micronutrients or toxic trace elements in non-hyperaccumulating plant species is much more challenging because of their low concentrations. Obtaining reliable *in situ* information about the chemical speciation of mineral elements presents an even greater challenge. These tasks have been greatly facilitated by the novel uses of imaging or analytical techniques that offer greater sensitivity, spatial resolution, or capability for chemical speciation, such as synchrotron-based X-ray absorption or fluorescence and mass spectrometry-based techniques. Here, we review the advantages and limitations of these techniques and discuss examples

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Glossary

Absorption edge: a sharp discontinuity in the graph of the absorption coefficient of a substance plotted against the wavelength of X-rays being absorbed. It represents the minimum energy necessary to free electrons from particular shells of the atoms of interest.

Beamline: the instrumentation that generates and transports synchrotron radiation to an experimental end station where the appropriate radiation is selected, focused, and directed on a sample mounted on a stage. It also includes appropriate detection systems for the signals generated from the sample.

Chemical speciation: the distribution of an element among defined chemical species in a system. Chemical species refer to a specific form of an element defined as electronic or oxidation state, and/or complex or molecular structure.

Complexation: formation of a coordination entity consisting of a metal or metalloid center and its ligands.

Ecophysiological: the interrelationship between the physiology of an organism and its environment.

Edge positions: the energy of the incident X-ray where the absorption edge is observed.

Electronegativity: the tendency of an atom or a functional group to attract electrons towards itself.

Energy-dispersive X-ray microanalysis (EDX): a microanalytical technique that uses the characteristic spectrum of X-rays emitted by the specimen after excitation by high-energy electrons to obtain information about its elemental composition.

Fast fluorescence detector technologies: devices that are able to detect the fluorescence signal originating from a sample efficiently.

Fourier transformation: a mathematical transformation employed to transform signals between time or spatial domain and frequency domain.

Freeze substitution: the process of replacing ice in a frozen sample with alcohol or another solvent at sub-zero temperatures.

High-pressure freezing: the rapid freezing of a sample under the application of pressure, which allows samples 200–300 μm in size to be frozen with minimal formation of damaging ice crystals.

Hyperaccumulating plants (or hyperaccumulators): refer to plant species that are able to accumulate and tolerate large concentrations of metals or metalloids in their aboveground parts. The concentration thresholds used to define hyperaccumulation vary among metals or metalloids, but usually are more than two orders of magnitude higher than those attained by normal non-hyperaccumulating plant species growing on uncontaminated soils.

Image stacks: a dataset where maps of the same areas are collected at different incident energies (e.g., across the absorption edge of an element).

Incident beam: a wave or particle beam which intercepts a sample.

Ionic profile: the feature of the mineral nutrient and trace element composition of an organism.

K_{α} line: refers to the emission line when an electron transitions from a 2p orbital of the second or 'L' shell to the innermost 'K' shell.

K-edge: the absorption edge of the K shell electrons.

Laser ablation system: instrumentation for removing material from a solid surface by irradiating it with a laser beam.

Laser microdissection (LMD) instrument: instrumentation for isolating specific cells of interest from microscopic regions of tissue or organisms using laser beam.

Lateral resolution: the ability of a system to distinguish two points in the direction perpendicular to the direction of an incident beam.

Metalloids: a chemical element that has properties bordering those of metals and non-metals (e.g., arsenic and selenium).

Non-hyperaccumulating plants: plant species that are not hyperaccumulating plants (see above).

Proton (particle)-induced X-ray emission (PIXE): emission of X-rays specific to an element in a specimen when it is irradiated with an ion (proton or α particle) beam, used as a technique to obtain information about the elemental composition of a sample.

Rastering: scanning of a sample in a defined pattern.

Secondary ion mass spectrometry (SIMS): emission of ions from the surface of a sample after bombardment with a high-energy primary ion beam. Secondary ions from the sample are analyzed in a mass spectrometer to build up a chemical map of the sample surface. Isotopes as well as molecular species can be detected.

Spatial resolution: the ability of a system to distinguish the position of two points in a 3D space.

Synchrotron: a particular type of particle accelerator where electrons are accelerated to almost the speed of light to produce electromagnetic radiation (i.e., synchrotron radiation).

Synchrotron-based X-ray absorption: a technique based on the absorption profile of a sample as function of the energy of an incident X-ray beam generated at a synchrotron.

Synchrotron X-ray fluorescence (S-XRF): a mapping technique based on the detection of the fluorescence emitted by a sample while it is rastered through an X-ray beam generated at a synchrotron.

Tomographic techniques: techniques that allow the reconstruction of virtual sections within a specimen.

of their uses in imaging the distributions of minerals, particularly micronutrients or trace elements, and for probing their chemical speciation in plant cells.

Synchrotron-based techniques

Synchrotron facilities provide high-intensity photon sources that are >10 orders of magnitude brighter than those generated by conventional X-ray tubes [10,11]. Owing to this characteristic, synchrotron-based techniques are highly sensitive and can be used to detect a wide range of elements with a high spatial or lateral resolution. The sensitivity increases with the atomic number, meaning that the techniques are ideally suited to investigations on trace elements and heavy metals or metalloids. Furthermore, these techniques require minimal sample preparation and can be used to probe hydrated plant samples and to investigate element speciation *in situ*.

There are two main types of synchrotron-based techniques that can be used to analyze plant samples in the context of this review: X-ray fluorescence (XRF, or S-XRF for synchrotron XRF) for imaging elemental distribution and X-ray absorption spectrometry (XAS) for analyzing chemical speciation of elements (Figure 1). Both techniques involve irradiating the sample with X-rays of a definite energy, which excite and eject the core electrons of atoms with a binding energy lower than the incident X-ray. As a result, a vacancy is created in a core orbital, which is then filled by an electron from a higher energy orbital, releasing the excess energy in the form of fluorescence. The energy of this fluorescence signal is characteristic for each element. By rastering a sample through an X-ray beam, fluorescence spectra can be recorded at each position and used to generate XRF elemental maps. A step-by-step guide on the use of S-XRF for elemental mapping in plants is provided in [12]. In an XAS experiment, the energy of the incident X-ray beam is progressively increased while keeping the sample in the same position and the fluorescence signal is collected incrementally. As the energy approaches the binding energy of the core electrons of the element of interest, absorption of the incident beam progressively increases and so does the emitted fluorescence. XAS spectra are therefore generated by detecting and recording the absorption or fluorescence at each energy point. An XAS spectrum is conventionally divided into two parts, the X-ray absorption near-edge structure (XANES) and the extended X-ray absorption fine structure (EXAFS) (Figure 1), covering the energy range from approximately –50 to +200 eV of the absorption edge and from the absorption edge to approximately +800 eV, respectively [10]. XANES is particularly sensitive to the oxidation states of elements and the electronegativity of the ligands, whereas EXAFS can provide information about the coordination chemistry such as the identity and number of the coordinating atoms and the interatomic distance.

In general, there are two types of beamlines used for analyzing elements in plant samples: one uses a millimeter X-ray beam to assess the average speciation of the element of interest in the sample (usually homogenized) and the other uses a micro-focused X-ray beam (tens of nm to a few μm) to collect spatial information of intact specimens at the cellular or subcellular scales (Figure 1).

Figure 1. Synchrotron-based techniques for analyzing elemental distribution and speciation in plant tissues. The images of cowpea root, wheat grain, and rice grain are reproduced, with permission, from [37,75,84]. The rice node image is based on unpublished data from the authors.

The penetrating nature of X-rays means that 2D elemental maps are a planar compression of the volume probed by the beam. Therefore, elemental maps should be interpreted keeping in mind the morphology of the sample in relation to the variation in thickness and the overlaying tissues. One way to overcome this effect is to use thin sections of uniform thickness (e.g., [13,14]). Conversely, the penetrating nature of the X-rays enables the use of tomographic techniques to map elements in virtual 2D sections or in 3D through mathematical reconstructions [15].

The intensity of the X-ray beam means that long exposure may cause damage to the specimen. This has the potential to cause significant artifacts in both element distribution and, perhaps more problematically, chemical speciation. For example, photo-reduction of redox-sensitive elements (e.g., As and Se) may occur during synchrotron beam exposure, causing speciation artifacts [16–18]. It is therefore essential to preserve the sample using cryogenic conditions or preferably by reducing the radiation dose. This can be achieved by using fast detector technologies that drastically reduce the exposure duration and radiation doses [19].

Mass spectrometry-based techniques

Secondary ion mass spectrometry

Secondary ion mass spectrometry (SIMS) is a surface-sensitive technique that uses an energetic primary ion beam to remove particles from the top few atomic layers of a sample surface. The ions emitted during this bombard-

ment, known as secondary ions, are analyzed in a mass spectrometer to give information about the elemental or molecular distribution within the sample. The NanoSIMS is a specific type of SIMS instrument that has been designed for high lateral resolution (down to 50 nm) imaging while still maintaining high mass resolution (the ability to distinguish between species with nominally the same mass) and high sensitivity (mg/kg range). It is possible to detect most elements in the periodic table, from hydrogen to uranium, as well as their different isotopes. These characteristics offer many advantages for the analysis of elemental distributions in plant cells at the cellular and, crucially, subcellular scale, and enables the use of stable isotope labeling to track element uptake and compartmentalization [20]. The main limitations of this instrument are that it is difficult to analyze some elements, such as Zn, Cd, and Mn, because of poor secondary ion yield, the difficulty of quantifying actual concentrations, and the inability to obtain chemical state information [21]. The field of view is limited and mapping large areas is relatively slow, although it has become more rapid with the advances in automation and beam control available on newer instruments. Although SIMS is a destructive technique, which can be a disadvantage for some samples, it also enables depth profiling through a sample to build up a 3D volume.

SIMS must be conducted under an ultra-high vacuum, which imposes limitations on the form of the sample. Fresh tissue cannot be analyzed because of the high water content, instead it must be rapidly cryo-fixed and analyzed

either frozen-hydrated by cryo-SIMS [20] or, if the instrument does not have a cryo-stage, as is the case for the NanoSIMS, subsequently fixed at low temperature, dehydrated with acetone, and embedded in resin [22]. Sample preparation is the most crucial step in successfully analyzing elemental distributions in plant cells with SIMS analysis [23]. High-pressure freezing followed by freeze substitution has been found to be the best method to preserve cellular and subcellular structures as well as elemental distributions of plant cells for NanoSIMS analysis [14,22,24].

Laser-ablation inductively coupled plasma mass spectrometry

Laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) can be used to investigate element distribution in solid sample materials by using a focused laser beam in an argon atmosphere under normal pressure. Sample material is ablated by the laser beam and the laser-induced aerosol is transported with a carrier gas (He or Ar) into the inductively coupled plasma ion source where ionization takes place. The charged ions are detected by their mass-to-charge ratio, m/z , in the mass spectrometer. The spatial resolution of the common laser ablation systems generally ranges from 50 to 300 μm [25]. A high resolution can be achieved using a laser beam with a smaller spot size, although this may lead to insufficient energy focused on the sample and, hence, limited ablation of the material. A laser microdissection (LMD) instrument can substitute a laser ablation system to substantially enhance the spatial resolution [26].

Imaging the cellular and subcellular distribution of elements

The techniques described above have been used to image element distribution at the tissue and cellular scales of plant samples. Many of the earlier studies focused on hyperaccumulator plants that are able to accumulate large concentrations of metals or metalloids. These studies revealed, for example, Cd and Zn accumulation in the trichomes of *Arabidopsis halleri* [27,28], Zn in the epidermal cells of the leaves of pennycress (*Thlaspi praecox*) [9], Ni in the trichomes [29] and the epidermal vacuoles [22] of *Alyssum* species, Se in the trichomes of milkvetch (*Astragalus bisulcatus*) [30], As in the vacuoles of Chinese brake fern (*Pteris vittata*) fronds [31], and Tl in the veins of candytuft (*Iberis intermedia*) leaves [32].

More recently, there has been a shift in attention to studying essential or toxic trace elements in non-hyperaccumulator plants: some examples are described below. Fluorescence tomography images obtained at different energies corresponding to different oxidation states of As show a strong accumulation of arsenate [As(V)] in the iron plaque formed on the root surfaces of aquatic plants such as cattail (*Typha latifolia*) [33] and rice (*Oryza sativa*) [34] (Figure 2A), suggesting that iron plaque provides a barrier to the entry of As(V) into these plants but is less effective in blocking the uptake of arsenite [As(III)], probably owing to a weaker adsorption of As(III) by the plaque [35]. Inside rice roots, As(III) is preferentially stored in the vacuoles of the pericycle and endodermis cells and is strongly colocalized with S

as revealed by NanoSIMS analysis [24] (Figure 2B). This colocalization is consistent with a vacuolar sequestration of As(III)–thiol (e.g., phytochelatins) complexes. Using S-XRF with a fast detector, the As distribution has been imaged in fresh hydrated roots of cowpea (*Vigna unguiculata*) following short-term exposure to either As(III) or As(V), showing a strong accumulation of As in the region that is likely to be endodermis and/or pericycle cells [36] (Figure 2C). The same approach has been applied to mapping the *in situ* distribution of Zn, Cu, Ni, Mn, and Se in hydrated tissues [17,37–39]. A recent study combining S-XRF and NanoSIMS has revealed that As is localized in the vacuoles of the companion cells within the phloem of various types of vascular bundles of the rice node, internode, and leaf sheath [14] (Figure 2D,E). A strong colocalization of As with S is again evident. Thus, the companion cells possibly provide the most important storage capacity of As in rice aboveground tissues, implying that the biosynthesis of phytochelatins and the vacuolar transport of As(III)–phytochelatin complexes are likely to be much enhanced in this cell type. This distribution pattern also supports the notion that inorganic As is transported to the grain mainly via the phloem [40,41], although with a restricted mobility, presumably because of the vacuolar sequestration. In rice grain, information obtained largely through fluorescence tomography indicates that inorganic As [mainly As(III)] accumulates preferentially in the ovular vascular traces (OVTs) (Figure 2F), which are the conducting tissues transporting nutrients and water to the grain [42–44]. By contrast, methylated As species such as dimethylarsinic acid (DMA) are much more mobile during phloem transport and can permeate into the endosperm of rice more easily than inorganic As [40,44,45].

Mapping the cellular distribution of Cd in non-hyperaccumulator plants is difficult because of its low tissue concentrations and the high energy of its K_{α} line (which is not always accessible at microspectroscopy beamlines). In attempts to overcome these difficulties, researchers have often used artificially high concentrations of Cd that cause severe phytotoxicity and therefore diminish the environmental and physiological relevance of the results obtained (see [46]). Cd has been shown to accumulate in the vascular bundles of roots and leaf trichomes of *Arabidopsis thaliana* after plants were exposed to a high concentration of Cd [47]. Using S-XRF, Cd has also been shown to accumulate in the xylem of the enlarged vascular bundles (EVBs) and in the parenchyma cell bridge surrounding the EVBs in the nodes of rice grown in contaminated soil [48]. By contrast, Zn accumulates preferentially in the parenchyma cell bridge bordering the EVBs and the diffuse vascular bundles (DVBs) [48]; NanoSIMS analysis revealed that Zn was localized to the vacuoles of these cells [14]. Fe and Mn are localized to the fundamental parenchyma cells in the nodes of rice, where there is a strong colocalization of Fe and P inside the vacuoles, possibly in insoluble forms [14] (Figure 2D,E), which may explain the antagonistic effect of P on Fe nutrition [49]. The differences among Cd, Zn, Fe, and Mn distribution in the rice node are striking, even though Cd is known to share some common transporters with the other metals [2]. A possible explanation lies in the different chemical speciation of these metals.

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Figure 2. Mapping elemental distribution at the cellular and subcellular scales. **(A)** Synchrotron X-ray fluorescence (S-XRF) 2D tomography of a rice root growing in an As-contaminated soil, showing the accumulation of Fe and As(V) on the surface iron plaque [34]. **(B)** NanoSIMS imaging of Si, S, and As in a rice root thin section showing colocalization of As and S in the vacuoles of pericycle and endodermal cells, and Si in the apoplast of endodermal cells [24]. Abbreviations: En, endodermis; Pc, pericycle; SE, secondary electron image; Xy, xylem vessel. **(C)** S-XRF imaging with a fast detector of fresh hydrated roots of cowpea exposed As(V) or As(III) for 24 h [36]. **(D)** S-XRF imaging of a rice node thin section showing distinct distribution patterns of As (red), Zn (green), and Fe (blue). Abbreviations: EVB, enlarged vascular bundles; DVB, diffuse vascular bundles; FP, fundamental parenchyma; LVB, large vascular bundles [14]. **(E)** NanoSIMS imaging of the DVB and FP regions marked as areas 1 and 2 in (D), showing strong colocalization of As and S in the vacuoles of the companion cells of the phloem in the DVBs and strong colocalization of Fe and P in the vacuoles of FP cells [14]. Abbreviations: CC, companion cells; ST, sieve tubes. **(F)** S-XRF 3D tomography of a rice grain showing the accumulation of As in the ovular vascular trace (OVT) [42]. **(G)** S-XRF 3D tomography of Fe, Mn, and Zn in the seeds of wild type *Arabidopsis* (Col-0) and the *vit1-1* mutant [50]. Reproduced, with permission, from [14,24,34,36,42,50].

Mature seeds are ideal for S-XRF or NanoSIMS analysis because they are largely and naturally dehydrated and therefore require minimal sample preparation. Fluorescence tomography has been used to characterize elemental distribution in the seeds of wild type *A. thaliana* and a mutant defective in the vacuolar iron transporter 1 (VIT1) [50]. Whereas bulk analyses revealed no significant differences in the total Fe concentrations, the mutant seed exhibited an Fe distribution pattern different to that in the wild type. Iron in wild type seeds is strongly localized to the provascular strands of the hypocotyl, radicle, and cotyledons, probably inside the vacuoles (Figure 2G). By contrast, Fe is not detected in these cells in the *vit1* mutant seeds and is instead located more diffusely in the hypocotyl and radicle, and in the epidermal cells of the cotyledons. This investigation has unraveled the function of VIT1 in vacuolar Fe storage, which is crucial for seedling development after germination. Fluorescence tomography and high-resolution S-XRF has been used to investigate the elemental distribution of wild type *Arabidopsis* seeds and embryos, and of mutants of endomembrane cation exchangers (CAXs) and overexpression lines [13]. Higher concentrations of Ca were found in the seed coat and in the embryo of the *cax* mutants compared with that found in the wild type; the mutants also had reduced partitioning of Ca into organelles and higher levels of Ca in the cytosol. High resolution (0.15 μm) S-XRF analysis of thin sections of embedded embryos showed strikingly different patterns of elemental distribution. In wild type seeds, Mn is strongly localized to a sub-epidermal layer of cells of the cotyledons, Fe to the organelles (probably the vacuoles) of the endodermal cells of the radicles and the vasculature of the cotyledons, and Zn and Ca are uniformly distributed to all of the cells of the embryo [13]. CAX mutation also alters the distribution of Mn. These examples demonstrate the power of combining state-of-the-art imaging techniques with molecular genetic methods to understand the gene functions and cellular homeostasis of elements.

SIMS or NanoSIMS has been used to image a wide range of elements in cereal grains at the cellular and subcellular scales, showing strong localization of minerals with phytate granules in the aleurone cells [51,52], of Se in the protein matrix surrounding the starch granules, of As in the sub-aleurone cells, and in hotspots in the OVT region of the rice grain [45]. Stable isotope ^{70}Zn labeling and LA-ICP-MS has shown that ^{70}Zn strongly accumulates in the crease vascular tissue of wheat (*Triticum aestivum*) grain, suggesting that the translocation from the maternal to filial tissues may be a bottleneck for Zn accumulation in the endosperm [53]. For Fe and Zn biofortification in cereals, it is important to increase the concentrations of Fe and Zn in the endosperm because this is the part that is mostly consumed by humans. Overexpression of nicotianamine synthase and ferritin genes can increase Fe concentration in the rice endosperm [54]. In LA-ICP-MS analysis, Fe in the endosperm of the transgenic rice appears to accumulate in spots, probably as a consequence of spatially restricted ferritin accumulation [54].

Tracing the movement of elements

One of the advantages of mass spectrometry-based techniques such as SIMS and LA-ICP-MS is the ability to distinguish and quantify different isotopes, thus enabling stable isotopes to be used as tracers. NanoSIMS has been used to reveal the uptake and competition between rhizosphere microorganisms and plant roots for ^{15}N labeled ammonium [55,56]. Pulsing cut stems of bean (*Phaseolus vulgaris*) plants with ^{26}Mg , ^{41}K , ^{44}Ca , and H_2^{18}O and using cryo-SIMS to image their distribution at different times revealed a rapid exchange of cations between xylem vessels and the adjacent xylem parenchyma cells, and a slow exchange with cambium and phloem; the three cations also exhibited different exchange kinetics [57,58]. In these studies, samples were shock-frozen in melting propane, fractured, and analyzed under cryo-conditions, thus preserving the cellular structure and *in situ* element distribution.

Probing chemical speciation of elements

XAS analyses can be performed with bulk samples (usually freeze-dried or, preferably, powdered frozen-hydrated samples) to gain overall information about speciation, or about specific spots of a sample at the micrometer or sub-micrometer scale using micro-focused XANES and EXAFS. However, owing to the complexity of biological samples, the speciation information obtained is often more accurate with regard to the oxidation state and the type of binding ligands than the exact identities of the ligand molecules. For example, XANES can distinguish As(V), As(III), As(III)–glutathione, and As(III)–phytochelatin, but not the numerous variants of As(III)–phytochelatin or other As(III)–thiol complexes that have been detected in plant tissues based on *ex situ* extraction and ICP-MS and electrospray mass spectrometry analysis [59]. There are numerous examples of using XAS to investigate the chemical speciation of elements in plant tissues; some examples are described below.

Most plant species appear to be able to reduce As(V) to As(III) rapidly, resulting in the dominance of trivalent As inside plant cells [60]. Arsenic is found to be present mainly as As(III) coordinated with thiol compounds in non-hyper-accumulators (e.g., [61–63]), but predominantly as free arsenite or arsenate in As hyperaccumulating ferns (e.g., [31,64,65]). A recent study employing the fluorescence-XANES imaging technique showed dramatic changes in the As speciation from the outer to the inner layers of cells of rice and wheat roots that were exposed to As(V) in the growth medium [66]. As(V) was found only in the rhizodermis, whereas the As(III)–thiol complexes dominated in the cortex and the stele [66]. Confocal XANES is another technique that can be used to target chemical species analysis to specific spatial regions of cells in intact plant tissues [59]. Using this technique, it has been shown that As preferentially accumulated in the epidermal cells of the aquatic plant hornwort (*Ceratophyllum demersum*) with As(III)–phytochelatin, As(III)–glutathione, and uncomplexed As(III) and As(V) as the main chemical species of As [59]. A study has been performed that compared As speciation in rice grain by *ex situ* extraction followed by high-performance liquid chromatography–ICP-MS

Figure 3. Probing chemical speciation of elements in plant tissues. **(A)** Synchrotron X-ray absorption near edge structure (XANES) arsenic *K*-edge spectra of arsenic standards and five rice grain samples [67]. Abbreviations: As(III), arsenite; As(III)-GSH, arsenite-glutathione complex; As(V), arsenate; DMA, dimethylarsinic acid. **(B)** Zinc *K*-edge extended X-ray absorption fine structure (EXAFS) spectra of three Zn standards, *Arabidopsis halleri*, *Arabidopsis lyrata*, and their F1 or F2 progeny leaves after Fourier transformation [74]. **(C)** Average first-shell zinc (Zn)-O distance determined by shell simulations based on the EXAFS spectra in (B) as a function of total Zn concentration [74]. Abbreviation: DW, dry weight. Reproduced, with permission, from [67,74].

analysis and *in situ* bulk XANES [67] (Figure 3A). The samples had low As concentrations ranging from 0.20 to 0.34 $\mu\text{g/g}$. This study provided evidence of good agreement between the two techniques and demonstrated the high sensitivity of XANES offered by modern synchrotron beam-lines. In rice grains, As is present as As(III), As(III)-thiol complexes, As(V), and DMA [43,67].

Cadmium is coordinated mainly with S (probably phytochelatins or glutathione) in the roots of *A. thaliana* and mustard (*Brassica juncea*) [47,68], but with O or N ligands in the trichomes [47]. In the rice node, Cd is coordinated mainly with S in the xylem of the enlarged vascular bundles, but with both S and O ligands in the phloem [48]. Similar to As hyperaccumulators, Cd hyperaccumulators do not appear to use thiol complexation as the main mechanism to detoxify Cd given that Cd is found to be coordinated mainly to O or N ligands [69–72].

Depending on plant species, type of tissues and growth conditions, common ligands for binding Zn in plant cells include organic acids (O ligands), histidine, phosphate, or phytate [28,38,69,73]. For instance, EXAFS has been used

to investigate the speciation of Zn in parent plants and in the F₁ and F₂ progenies from crosses between the Zn hyperaccumulator *Arabidopsis halleri* and its non-hyperaccumulator relative *Arabidopsis lyrata* [74]. Figure 3B shows the Zn EXAFS spectra after Fourier transformation of Zn standards and plant samples. Shell simulations were then used to determine the structural parameters for the first and second Zn coordination shells, which were found to be well fitted with oxygen and carbon as nearest and next nearest atomic neighbors, respectively, as would be expected in Zn-organic acid complexes. The results indicated a correlation between total Zn in plants and the proportion of Zn in octahedral coordination (Figure 3C). This is indicative of a dominant complexation by organic acids, where Zn is sixfold coordinated, in Zn-accumulating plants. In plants that accumulated less Zn, EXAFS analysis revealed a larger proportion of Zn being fourfold coordinated, probably with the cell wall and phosphate (Figure 3C). In aleurone cells of wheat grain, EXAFS showed that Fe and Zn were complexed by phytates; Fe was coordinated octahedrally by six O atoms and fewer

than two P atoms, whereas Zn was coordinated tetrahedrally by four O atoms and approximately 1.5 P atoms [75].

In the case of Cu, Cu(II) is reduced to Cu(I) and coordinated to S, possibly as Cu(I)–metallothionein complexes in tomato (*Solanum lycopersicum*) and oat (*Avena sativa*) plants [76] and in alpine pennycress (*Nocca caerulescens*; formerly *Thlaspi caerulescens*), which hyperaccumulates Zn and Cd but not Cu [77]. The coordination chemistry and accessible geometries of Cu(I)–thiolate complexes are highly plastic, a feature that allows Cu(I) to be bound by various chaperones and delivered to target proteins requiring Cu [78]. There is also evidence that some Cu in *N. caerulescens* is bound by nicotianamine or is biomineralized [77]. In contrast to Cu non-accumulators that rely on S ligands for complexation, swamp stonecrop (*Crassula helmsii*), a Cu-accumulating aquatic plant, binds Cu almost exclusively through O-rich ligands, probably organic acids [79]. Apoplastic binding of Cu with pectin is also important, particularly in the cell walls of the rhizodermis and the outer cortex of roots [38]. A general pattern that has emerged from comparing metal and metalloid non-accumulators with hyperaccumulators is that strong ligands such as thiol compounds are often used in plant species that are metal or metalloid non-accumulators but are rarely observed in the hyperaccumulators, perhaps because of the cost constraint for the production of such ligands in large quantities.

Concluding remarks and future perspectives

The cellular status of mineral elements can be defined by three main characteristics: concentration, chemical speciation, and localization; regulation of all three attributes is essential to element homeostasis. The examples described above demonstrate the capabilities of synchrotron and mass spectrometry-based techniques to acquire spatial and chemical information about elements at the cellular or subcellular scales. They reveal the highly heterogeneous distribution patterns of elements in different cell types or subcellular organelles, often varying in chemical speciation. Such information can be used to guide future studies to identify the genes that control element distribution and chemical speciation. Synchrotron-based imaging techniques have already been used to reveal the functions of genes involved in the homeostasis of some nutrients or trace elements [13,50]. Owing to the time required for spatial elemental mapping and the availability of beam or machine time, these techniques are suited to studies comparing a limited number of samples (e.g., mutants, overexpressing lines, and their wild type). Screening large arrays of small seeds (e.g., of *A. thaliana*) for element concentrations is possible using S-XRF [80]; however, it would be difficult to adopt a similar high-throughput approach for other types of plant samples.

As the technologies continue to advance, more novel applications to plant science can be foreseen. For example, chemical speciation at the subcellular scale with synchrotron nanoprobe focusing the beam to <100 nm is possible. This would need to be performed on thin cryo-sections of plant samples to avoid changes in speciation due to dehydration and embedding, and to overcome the penetrating nature of the X-rays that can distort the spatial

information for geometrically undefined samples. With the advent of fast fluorescence detector technologies, novel approaches that unify S-XRF mapping and XAS speciation are also becoming available. These are based on S-XRF mapping at different energies to obtain speciation rather than elemental maps. This approach in itself is not new given that the distribution of different species of metalloids such as As and Se have been investigated previously by taking advantage of the significant differences in edge positions for different redox states of these elements [31,34,81]. However, with fast detector systems it is now possible to collect image stacks of the same area at many different energies, which has opened the door to speciation mapping for elements having more subtle differences in spectral features between chemical species. From these energy stacks, XANES spectra can then be extracted at each pixel or groups of pixels. This approach, named fluorescence-XANES imaging, has been successfully tested with mineral samples [82] and has also recently been used to analyze plant samples [66].

In the future, although challenging, it may be possible to fit a cryo-stage to the NanoSIMS to avoid complicated sample preparation protocols and ensure minimum elemental redistribution. Further improvements in spatial resolution below 50 nm will be limited by the ionization process and the collection efficiency of the instrument. For LA-ICP-MS, spatial resolution can be substantially increased, to the nanoscale, with the development of near field-ICP-MS that introduces a tapered probe into the laser radiation field to induce local laser ablation on the sample surface [83]. This technique offers the potential of high resolution and low cost, although the technique has not yet been applied to mapping plant tissues.

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References

- 1 Marschner, P. (2012) *Marschner's Mineral Nutrition of Higher Plants*, Academic Press
- 2 Clemens, S. *et al.* (2013) Plant science: the key to preventing slow cadmium poisoning. *Trends Plant Sci.* 18, 92–99
- 3 Zhao, F.J. *et al.* (2010) Arsenic as a food-chain contaminant: mechanisms of plant uptake and metabolism and mitigation strategies. *Annu. Rev. Plant Biol.* 61, 535–559
- 4 White, P.J. and Broadley, M.R. (2009) Biofortification of crops with seven mineral elements often lacking in human diets – iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytol.* 182, 49–84
- 5 von Wirén, N. (2011) Grand challenges in plant nutrition. *Front. Plant Sci.* 2, 4
- 6 Salt, D.E. *et al.* (2008) Ionomics and the study of the plant ionome. *Annu. Rev. Plant Biol.* 59, 709–733
- 7 Conn, S. and Gilliam, M. (2010) Comparative physiology of elemental distributions in plants. *Ann. Bot.* 105, 1081–1102
- 8 Küpper, H. *et al.* (1999) Cellular compartmentation of zinc in leaves of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiol.* 119, 305–311

- 9 Vogel-Mikus, K. *et al.* (2008) Comparison of essential and non-essential element distribution in leaves of the Cd/Zn hyperaccumulator *Thlaspi praecox* as revealed by micro-PIXE. *Plant Cell Environ.* 31, 1484–1496
- 10 Sarret, G. *et al.* (2013) Use of synchrotron-based techniques to elucidate metal uptake and metabolism in plants. *Adv. Agron.* 119, 1–82
- 11 Lombi, E. and Susini, J. (2009) Synchrotron-based techniques for plant and soil science: opportunities, challenges and future perspectives. *Plant Soil* 320, 1–35
- 12 Donner, E. *et al.* (2013) Mapping element distributions in plant tissues using synchrotron X-ray fluorescence techniques. In *Plant Mineral Nutrients: Methods and Protocols. Methods in Molecular Biology (Vol. 953)* (Maathuis, F.J.M., ed.), pp. 143–159, Springer
- 13 Punshon, T. *et al.* (2012) The role of CAX1 and CAX3 in elemental distribution and abundance in *Arabidopsis* seed. *Plant Physiol.* 158, 352–362
- 14 Moore, K.L. *et al.* (2014) Combined NanoSIMS and synchrotron X-ray fluorescence reveals distinct cellular and subcellular distribution patterns of trace elements in rice tissues. *New Phytol.* 201, 104–115
- 15 de Jonge, M.D. and Vogt, S. (2010) Hard X-ray fluorescence tomography – an emerging tool for structural visualization. *Curr. Opin. Struct. Biol.* 20, 606–614
- 16 Lombi, E. *et al.* (2011) In situ analysis of metal(loid)s in plants: state of the art and artefacts. *Environ. Exp. Bot.* 72, 3–17
- 17 Wang, P. *et al.* (2013) In situ speciation and distribution of toxic selenium in hydrated roots of cowpea. *Plant Physiol.* 163, 407–418
- 18 George, G.N. *et al.* (2012) X-ray-induced photo-chemistry and X-ray absorption spectroscopy of biological samples. *J. Synchr. Radiat.* 19, 875–886
- 19 Lombi, E. *et al.* (2011) Trends in hard X-ray fluorescence mapping: environmental applications in the age of fast detectors. *Anal. Bioanal. Chem.* 400, 1637–1644
- 20 Metzner, R. *et al.* (2008) Imaging nutrient distributions in plant tissue using time-of-flight secondary ion mass spectrometry and scanning electron microscopy. *Plant Physiol.* 147, 1774–1787
- 21 Moore, K.L. *et al.* (2012) Elemental imaging at the nanoscale: NanoSIMS and complementary techniques for element localisation in plants. *Anal. Bioanal. Chem.* 402, 3263–3273
- 22 Smart, K.E. *et al.* (2010) High-resolution elemental localization in vacuolate plant cells by nanoscale secondary ion mass spectrometry. *Plant J.* 63, 870–879
- 23 Chandra, S. *et al.* (2000) Subcellular imaging by dynamic SIMS ion microscopy. *Anal. Chem.* 72, 104a–114a
- 24 Moore, K.L. *et al.* (2011) NanoSIMS analysis reveals contrasting patterns of arsenic and silicon localization in rice roots. *Plant Physiol.* 156, 913–924
- 25 Wu, B. and Becker, J.S. (2012) Imaging techniques for elements and element species in plant science. *Metallomics* 4, 403–416
- 26 Becker, J.S. *et al.* (2010) Scaling down the bioimaging of metals by laser microdissection inductively coupled plasma mass spectrometry (LMD-ICP-MS). *Int. J. Mass Spectrom.* 294, 1–6
- 27 Fukuda, N. *et al.* (2008) Micro X-ray fluorescence imaging and micro X-ray absorption spectroscopy of cadmium hyper-accumulating plant, *Arabidopsis halleri* ssp *gemma*, using high-energy synchrotron radiation. *J. Anal. Atom. Spectrom.* 23, 1068–1075
- 28 Sarret, G. *et al.* (2002) Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. *Plant Physiol.* 130, 1815–1826
- 29 McNear, D.H. *et al.* (2005) Application of quantitative fluorescence and absorption-edge computed microtomography to image metal compartmentalization in *Alyssum murale*. *Environ. Sci. Technol.* 39, 2210–2218
- 30 Freeman, J.L. *et al.* (2006) Spatial imaging, speciation, and quantification of selenium in the hyperaccumulator plants *Astragalus bisulcatus* and *Stanleya pinnata*. *Plant Physiol.* 142, 124–134
- 31 Pickering, I.J. *et al.* (2006) Localizing the biochemical transformations of arsenate in a hyperaccumulating fern. *Environ. Sci. Technol.* 40, 5010–5014
- 32 Scheckel, K.G. *et al.* (2007) Synchrotron X-ray absorption-edge computed microtomography imaging of thallium compartmentalization in *Iberis intermedia*. *Plant Soil* 290, 51–60
- 33 Blute, N.K. *et al.* (2004) Arsenic sequestration by ferric iron plaque on cattail roots. *Environ. Sci. Technol.* 38, 6074–6077
- 34 Seyfferth, A.L. *et al.* (2010) Arsenic localization, speciation, and co-occurrence with iron on rice (*Oryza sativa* L.) roots having variable Fe coatings. *Environ. Sci. Technol.* 44, 8108–8113
- 35 Chen, Z. *et al.* (2005) Direct evidence showing the effect of root surface iron plaque on arsenite and arsenate uptake into rice (*Oryza sativa*) roots. *New Phytol.* 165, 91–97
- 36 Kopittke, P.M. *et al.* (2012) Examination of the distribution of arsenic in hydrated and fresh cowpea roots using two- and three-dimensional techniques. *Plant Physiol.* 159, 1149–1158
- 37 Lombi, E. *et al.* (2011) Fast X-ray fluorescence microtomography of hydrated biological samples. *PLoS ONE* 6, e20626
- 38 Kopittke, P.M. *et al.* (2011) In situ distribution and speciation of toxic copper, nickel, and zinc in hydrated roots of cowpea. *Plant Physiol.* 156, 663–673
- 39 Kopittke, P.M. *et al.* (2013) Distribution and speciation of Mn in hydrated roots of cowpea at levels inhibiting root growth. *Physiol. Plant.* 147, 453–464
- 40 Carey, A.M. *et al.* (2010) Grain unloading of arsenic species in rice. *Plant Physiol.* 152, 309–319
- 41 Zhao, F.J. *et al.* (2012) Arsenic translocation in rice investigated using radioactive ⁷³As tracer. *Plant Soil* 350, 413–420
- 42 Carey, A.M. *et al.* (2011) Phloem transport of arsenic species from flag leaf to grain during grain filling. *New Phytol.* 192, 87–98
- 43 Lombi, E. *et al.* (2009) Speciation and distribution of arsenic and localization of nutrients in rice grains. *New Phytol.* 184, 193–201
- 44 Zheng, M.Z. *et al.* (2013) Differential toxicity and accumulation of inorganic and methylated arsenic in rice. *Plant Soil* 365, 227–238
- 45 Moore, K.L. *et al.* (2010) NanoSIMS analysis of arsenic and selenium in cereal grain. *New Phytol.* 185, 434–445
- 46 Van Belleghem, F. *et al.* (2007) Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. *New Phytol.* 173, 495–508
- 47 Isaure, M.P. *et al.* (2006) Localization and chemical forms of cadmium in plant samples by combining analytical electron microscopy and X-ray spectromicroscopy. *Spectrochim. Acta B: Atom. Spectrom.* 61, 1242–1252
- 48 Yamaguchi, N. *et al.* (2012) Role of the node in controlling traffic of cadmium, zinc, and manganese in rice. *J. Exp. Bot.* 63, 2729–2737
- 49 Zheng, L.Q. *et al.* (2009) Physiological and transcriptome analysis of iron and phosphorus interaction in rice seedlings. *Plant Physiol.* 151, 262–274
- 50 Kim, S.A. *et al.* (2006) Localization of iron in *Arabidopsis* seed requires the vacuolar membrane transporter VIT1. *Science* 314, 1295–1298
- 51 Heard, P.J. *et al.* (2002) Determination of the elemental composition of mature wheat grain using a modified secondary ion mass spectrometer (SIMS). *Plant J.* 30, 237–245
- 52 Moore, K.L. *et al.* (2012) Localisation of iron in wheat grain using high resolution secondary ion mass spectrometry. *J. Cereal Sci.* 55, 183–187
- 53 Wang, Y.X. *et al.* (2011) Stable isotope labelling and zinc distribution in grains studied by laser ablation ICP-MS in an ear culture system reveals zinc transport barriers during grain filling in wheat. *New Phytol.* 189, 428–437
- 54 Wirth, J. *et al.* (2009) Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. *Plant Biotechnol. J.* 7, 631–644
- 55 Clode, P.L. *et al.* (2009) In situ mapping of nutrient uptake in the rhizosphere using nanoscale secondary ion mass spectrometry. *Plant Physiol.* 151, 1751–1757
- 56 Jones, D.L. *et al.* (2013) Competition between plant and bacterial cells at the microscale regulates the dynamics of nitrogen acquisition in wheat (*Triticum aestivum*). *New Phytol.* 200, 796–807
- 57 Metzner, R. *et al.* (2010) Tracing cationic nutrients from xylem into stem tissue of French bean by stable isotope tracers and cryo-secondary ion mass spectrometry. *Plant Physiol.* 152, 1030–1043
- 58 Metzner, R. *et al.* (2010) Contrasting dynamics of water and mineral nutrients in stems shown by stable isotope tracers and cryo-SIMS. *Plant Cell Environ.* 33, 1393–1407
- 59 Mishra, S. *et al.* (2013) Speciation and distribution of arsenic in the nonhyperaccumulator macrophyte *Ceratophyllum demersum*. *Plant Physiol.* 163, 1396–1408
- 60 Zhao, F.J. *et al.* (2009) Arsenic uptake and metabolism in plants. *New Phytol.* 181, 777–794
- 61 Pickering, I.J. *et al.* (2000) Reduction and coordination of arsenic in Indian mustard. *Plant Physiol.* 122, 1171–1177
- 62 Bluemlein, K. *et al.* (2008) Can we trust mass spectrometry for determination of arsenic peptides in plants: comparison of LC-ICP-

- MS and LC-ES-MS/ICP-MS with XANES/EXAFS in analysis of *Thunbergia alata*. *Anal. Bioanal. Chem.* 390, 1739–1751
- 63 Castillo-Michel, H. *et al.* (2011) Localization and speciation of arsenic in soil and desert plant *Parkinsonia florida* using μ XRF and μ XANES. *Environ. Sci. Technol.* 45, 7848–7854
 - 64 Webb, S.M. *et al.* (2003) XAS speciation of arsenic in a hyper-accumulating fern. *Environ. Sci. Technol.* 37, 754–760
 - 65 Lombi, E. *et al.* (2002) Arsenic distribution and speciation in the fronds of the hyperaccumulator *Pteris vittata*. *New Phytol.* 156, 195–203
 - 66 Kopittke, P.M. *et al.* (2013) Laterally resolved speciation of arsenic in roots of wheat and rice using fluorescence-XANES imaging. *New Phytol.* <http://dx.doi.org/10.1111/nph.12595>
 - 67 Maher, W. *et al.* (2013) Measurement of inorganic arsenic species in rice after nitric acid extraction by HPLC-ICPMS: verification using XANES. *Environ. Sci. Technol.* 47, 5821–5827
 - 68 Salt, D.E. *et al.* (1995) Mechanisms of cadmium mobility and accumulation in Indian mustard. *Plant Physiol.* 109, 1427–1433
 - 69 Küpper, H. *et al.* (2004) 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, 748–757
 - 70 Huguet, S. *et al.* (2012) Cd speciation and localization in the hyperaccumulator *Arabidopsis halleri*. *Environ. Exp. Bot.* 82, 54–65
 - 71 Tian, S.K. *et al.* (2011) Cellular sequestration of cadmium in the hyperaccumulator plant species *Sedum alfredii*. *Plant Physiol.* 157, 1914–1925
 - 72 Vogel-Mikus, K. *et al.* (2010) Complexation of cadmium in seeds and vegetative tissues of the cadmium hyperaccumulator *Thlaspi praecox* as studied by X-ray absorption spectroscopy. *Plant Soil* 331, 439–451
 - 73 Salt, D.E. *et al.* (1999) Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. *Environ. Sci. Technol.* 33, 713–717
 - 74 Sarret, G. *et al.* (2009) Zinc distribution and speciation in *Arabidopsis halleri* $\times$ *Arabidopsis lyrata* progenies presenting various zinc accumulation capacities. *New Phytol.* 184, 581–595
 - 75 Neal, A.L. *et al.* (2013) Iron and zinc complexation in wild-type and ferritin-expressing wheat grain: implications for mineral transport into developing grain. *J. Biol. Inorg. Chem.* 18, 557–570
 - 76 Ryan, B.M. *et al.* (2013) Copper speciation and isotopic fractionation in plants: uptake and translocation mechanisms. *New Phytol.* 199, 367–378
 - 77 Mijovilovich, A. *et al.* (2009) Complexation and toxicity of copper in higher plants. II. Different mechanisms for copper versus cadmium detoxification in the copper-sensitive cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype). *Plant Physiol.* 151, 715–731
 - 78 Pushie, M.J. *et al.* (2012) The fictile coordination chemistry of cuprous-thiolate sites in copper chaperones. *Biochim. Biophys. Acta Bioenerg.* 1817, 938–947
 - 79 Küpper, H. *et al.* (2009) 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, 702–714
 - 80 Young, L.W. *et al.* (2006) A high-throughput determination of metal concentrations in whole intact *Arabidopsis thaliana* seeds using synchrotron-based X-ray fluorescence spectroscopy. *J. Synchr. Radiat.* 13, 304–313
 - 81 Pickering, I.J. *et al.* (2000) Quantitative, chemically specific imaging of selenium transformation in plants. *Proc. Natl. Acad. Sci. U.S.A.* 97, 10717–10722
 - 82 Etschmann, B.E. *et al.* (2010) Reduced As components in highly oxidized environments: evidence from full spectral XANES imaging using the Maia massively parallel detector. *Am. Mineral.* 95, 884–887
 - 83 Wu, B. and Becker, J.S. (2011) Imaging of elements and molecules in biological tissues and cells in the low-micrometer and nanometer range. *Int. J. Mass Spectrom.* 307, 112–122
 - 84 Johnson, A.A.T. *et al.* (2011) Constitutive overexpression of the *OsNAS* gene family reveals single-gene strategies for effective iron- and zinc-biofortification of rice endosperm. *PLoS ONE* 6, e24476

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