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Functional characterisation of metal(loid) processes *in planta* through the integration of synchrotron techniques and plant molecular biology

Erica Donner^{1,2,*}, Tracy Punshon^{3,*}, Mary Lou Guerinot³, and Enzo Lombi¹

¹Centre for Environmental Risk Assessment and Remediation, University of South Australia, Building X, Mawson Lakes Campus Mawson Lakes, South Australia, SA-5095 Australia

²CRC CARE, P.O. Box 486, Salisbury, South Australia 5106, Australia

³Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA

Abstract

Functional characterisation of the genes regulating metal(loid) homeostasis in plants is a major focus of crop biofortification, phytoremediation, and food security research. This paper focuses on the potential for advancing plant metal(loid) research by combining molecular biology and synchrotron-based techniques. Recent advances in x-ray focussing optics and fluorescence detection have greatly improved the potential of synchrotron techniques for plant science research, allowing metal(loids) to be imaged *in vivo* in hydrated plant tissues at sub-micron resolution. Laterally resolved metal(loid) speciation can also be determined. By using molecular techniques to probe the location of gene expression and protein localisation and combining it with this synchrotron-derived data, functional information can be effectively and efficiently assigned to specific genes. This paper provides a review of the state of the art in this field, and provides examples as to how synchrotron-based methods can be combined with molecular techniques to facilitate functional characterisation of genes *in planta*.

Keywords

X-ray fluorescence; tomography; speciation; functional genomics; plants; metals

Metal(loid)s in plants

All organisms, including plants, use homeostatic mechanisms to control metal(loid) uptake, transport, accumulation, and detoxification at the cellular and organismal level [1]. Current efforts to understand the molecular mechanisms controlling these functions in plants are driven by issues of both deficiency and toxicity. This is because the elements falling within this category include a range of essential nutrients required for healthy plant growth and crop nutritional value (e.g. iron (Fe), manganese (Mn)), as well as some non-essential elements that act detrimentally as food chain contaminants (e.g. arsenic (As), cadmium (Cd)). Furthermore, some metal(loid)s function both as micronutrients and contaminants depending on their concentration (e.g. copper (Cu), selenium (Se), zinc (Zn)). By understanding the mechanisms controlling metal uptake, distribution and storage in plants, goals to genetically engineer biofortified crop species for improved nutrition [e.g. 2,3] and hyperaccumulator species for phytoremediation [e.g. 4–6] can be significantly advanced.

Corresponding author: Erica Donner, erica.donner@unisa.edu.au.

*Erica Donner and Tracy Punshon contributed equally to this manuscript.

Transgenic plants designed to accumulate lower concentrations of key food chain contaminants (e.g. As, Cd) in edible organs could also be produced to improve food quality [7].

Previous research is testament to the complexity of plant metal(loid) homeostasis. Different plant species and even different populations of the same species show huge variability in their capacity to tolerate and accumulate metals [e.g. 8,9], and research suggests that the genes regulating these processes may not necessarily be species specific or novel, but rather differently expressed and regulated in different species and populations [7]. Using molecular tools, researchers have identified numerous metal(loid) transporters, with a broad range of substrate specificities. As transport underpins both uptake and storage, functional characterisation of the genes regulating metal(loid) transport is a major focus of current research and, as documented in the following sections of this paper, a broad suite of techniques can be used in this endeavour.

This paper focuses on the potential for advancing plant metal(loid) research by combining molecular biology and synchrotron-based techniques. Recent advances in x-ray focussing optics and fluorescence detection have greatly improved the potential of synchrotron techniques for plant science research, allowing metal(l)oids to be imaged *in vivo* in hydrated plant tissues at sub-micron resolution [10]. Laterally resolved metal(loid) speciation can also be determined. By using molecular techniques to probe the location of gene expression and protein localisation and combining it with this synchrotron-derived data, functional information can be effectively and efficiently assigned to specific genes. This paper provides a review of the state of the art in this field, and provides examples as to how synchrotron-based methods can be combined with molecular techniques to facilitate functional characterisation of genes *in planta*.

Current methods used to assess physiological processes and gene functions in plants

Environmental Conditions

Numerous approaches are used to determine the physiological processes resulting from the disruption of a gene involved in plant metal(loid) homeostasis. The most basic and reliable method involves making measured changes to the growth conditions of mutant and wild type plants, and comparing their responses. This is done either by depriving or inundating the plant with the metal(loid) of interest. The manipulation of growth conditions can either be direct; by altering the formulation of the growth media, or indirect by altering pH or the concentration of other competing elements thought to use the same transport proteins. This approach has been effective in characterizing genes responsible for root-to-shoot transport of arsenic (As) by looking at phosphorus (P) uptake characteristics. Due to chemical similarities in ionic radii and charge [11], pentavalent As is transported via high affinity phosphate transporters [12–15]. Tolerance and sensitivity to metal(loid)s are diagnostic of phenotypes; when a gene encoding a metal(loid) transport protein involved in uptake is disrupted or silenced, transport ceases and tolerance to high external concentrations can result. Likewise, when a gene encoding a protein involved in storage is disrupted, metal(loid)s accumulate to toxic levels in the cytosol causing hypersensitivity. Subtle phenotypes are more challenging to find however, particularly in situations when gene expression is low, or occurs at certain developmental stages or times of the day, or when absence of the gene product is compensated for by upregulation of other genes or by the presence of functionally redundant genes.

In metal(loid) studies, growth phenotypes can range from visual symptoms of nutrient deficiency such as stunting, chlorosis and necrosis, to the death of the plant. Many of these phenotypes can be measured; such as measuring chlorophyll a and b concentration to quantify chlorosis for example; or by measuring fresh and dry biomass, leaf area index and root elongation production to quantify growth inhibition. Some symptoms are particularly revealing of specific processes; for instance, phenotypes such as interveinal chlorosis can indicate that iron (Fe) is not being transported out of the vascular system, pointing to very specific transporters and biosynthesis pathways; in this case nicotianamine [16–18].

Cellular metal concentrations above homeostatic optimums prompt the production of reactive oxygen species (ROS), causing oxidative damage and cell death. Iron is a particularly potent generator of ROS; the intimate connection between Fe metabolism and oxidative stress drives the tight regulation of Fe concentration and distribution within the cell [19]. Direct measurement of ROS and cellular responses to ROS (superoxide, lipid peroxidation and the accumulation of hydrogen peroxide) are part of the suite of tests used in metal(loid) gene characterization. Direct measurement of ROS and superoxide in vivo is technically challenging, involving the use of electron paramagnetic resonance (EPR) to measure changes in the chemical forms of the oxidizable metals [20]. Significantly easier assays are available for the measurement of hydrogen peroxide and lipid peroxidation [21]; in particular histochemical staining for hydrogen peroxide of intact tissues can show the cellular location of oxidative stress. Infiltration of plant tissues with 3,3-diaminobenzidine (DAB) is a simple assay that results in a brown polymerization product from reaction with hydrogen peroxide in the presence of peroxidase [20].

Gene Expression

The site of expression and the site of action of a particular gene usually closely coincide; therefore expression information is part of the fundamental battery of tests in gene characterization. Standard methods involve the use of reporter-gene constructs; for example tagging the gene with the jellyfish green fluorescent protein (GFP), causing cells expressing the gene to glow green under blue light [22, 23]. Additionally the GUS reporter system (β -glucuronidase staining) operates on the principle of producing an easily identifiable colour upon expression [24]. To help determine where in the cell proteins are being expressed, various marker constructs have been developed that can be used in conjunction with a tagged protein of interest [25–27]. Additional techniques include immunolocalization using a specific antibody raised against the protein of interest. The magnitude of gene expression increases when the gene product is needed in response to environmental or physiological cues, determined via measurement of RNA via Northern Blot or reverse transcription polymerase chain reaction (RT-PCR), or measurement of proteins via Western Blot.

Transient Cell-Based Expression Systems

Studying a gene in a single cell can provide fundamental information on gene function removed from the complications of a multicellular organism. The bacteria *Escherichia coli* and the yeast *Saccharomyces cerevisiae* are commonly used for investigating cell-autonomous gene function. Many plant metal transporters such as the essential iron transporter IRT1 (Iron Regulated Transporter 1) were first identified by their ability to complement a yeast metal transport mutant [28]. Although IRT1 was originally identified as an Fe transporter, we now know from complementation and uptake studies in both yeast and *Arabidopsis* that IRT1 is also able to transport Cd, Mn and Zn in addition to Fe [29, 30]. Similarly the vacuolar iron transporter VIT1 from *Arabidopsis* was expressed in yeast cells in which the orthologous gene CCC1 (Calcium Cross Complementor 1) had been silenced, rescuing the hypersensitivity phenotype displayed when mutant yeast cells were grown in

Fe-enriched media [31]. This demonstrated that the function of VIT1 - a tonoplast Fe transporter - and CCC1 were the same.

In addition to yeast, plant protoplasts (cells lacking a cell wall) are a robust transient expression system commonly used in functional genomics [32]. Protoplasts have been used to localize fluorescently tagged proteins, to demonstrate protein-protein interactions using BiFC (bimolecular fluorescence complementation) and to demonstrate regulatory gene circuits. Macromolecules such as DNA, RNA and proteins can be delivered to the protoplast via PEG-calcium fusion, electroporation or microinjection, allowing the manipulation of metabolic pathways, signal transduction, transcription and translation. Mesophyll protoplasts isolated from *Arabidopsis* retain many of the cellular activities and physiological responses of the intact host plants, unlike undifferentiated suspension culture cells.

Measurement of metal content

While growth tests can be used to indicate the function of metal(loid) responsive genes, ultimately direct measurement of metal(loid) concentration or abundance plays a critical part in gene characterization. There are numerous techniques available for quantifying elemental content. They can be divided into volume-averaged and spatially resolved techniques. In the former, the elemental content is determined in a complex mixture of tissues; usually entire organisms or organs are dissolved, and the concentration is diluted by the sample volume. The latter involves in situ measurement involving imaging of specific tissues or cells. Additionally, there are several techniques which blur the distinction between volume-averaged and spatially-resolved techniques; cell fractionation and purification can systematically isolate subcellular organelles for subsequent bulk metal analysis, and laser ablation systems can be used to introduce samples for bulk analysis in a raster-scanned manner very similar to elemental mapping.

In general, the volume-averaged metal analysis technique of choice is inductively coupled plasma mass spectroscopy (ICP-MS). Here, a liquid sample is sprayed into a plasma of argon gas at 7000°C, completely atomizing the sample, which is then quantified using a secondary electron multiplier detector. Solid samples must be dissolved completely before analysis, which can introduce a false positive result if the extracting solution contains metal(loid)s of interest, or a false negative result if the dissolution is incomplete. The benefits of ICP-MS are the incredibly low detection limits; in the region of parts per trillion (ppt) or and parts per quadrillion (ppq) for many elements, combined with very high accuracy and precision of measurement. Spatially resolved methods include synchrotron x-ray fluorescence mapping and tomography, both of which are described later in this article.

Metal-responsive fluorophores

In conjunction with confocal microscopy, ion sensitive probes or fluorophores can be used to image the cellular or subcellular location of metals. These reagents signal the presence of metals with the production of fluorescence, which can be imaged in three dimensions within the cell. Fluorophores are available for a limited number of metals, including Zn, Ca, Cu, Fe, Cd, Pb and Hg [33]. Fluorophores are particularly well established for imaging cellular Zn; Sinclair et al [34] used Zinpyr-1 to help characterize two Zn-transporting P-type ATP-ases, HMA2 and HMA4, in the root vasculature of *Arabidopsis*. This study tested the reliability of the fluorophore by measuring the fluorescence of plants grown under Zn excess and Zn-deprivation conditions, and found that patterns of fluorescence were consistent with the expression of HMA2 and HMA4. Sarret et al [35] used Zinquin to show the accumulation of Zn at the base of tobacco (*Nicotiana tabacum* L. cv Xanthi) trichomes. Kawachi et al [36] used FuraZin-1 to show active Zn transport into vacuoles in AtMTP-1-expressing yeast cells. Other Zn fluorophores include TSQ (6-methoxy-(2-p-toluenesulfonamido) quinoline)

and the ZnAF family fluorophores, which differ on the basis of their membrane permeability. Phen Green SK has been used to demonstrate the transport of Cu^{2+} through thylakoid membranes of pea (*Pisum sativum*) chloroplasts [37]. In this case it was the quenching of the PGSK fluorescence in the lumen of the chloroplasts by Cu^{2+} that signified transport. Mito-CS1 is a new targetable fluorescent probe that can selectively detect labile Cu^{2+} in mitochondria of living cells [38]. It is selective for Cu^{2+} over other abundant mitochondrial metal ions, including Fe^{2+} and Zn^{2+} .

There are fewer non-fluorescent chemosensors currently in use, although the Prussian blue histopathological stain, also known as Perl stain is a particularly convenient and simple assay to look for the distribution pattern of Fe in tissue, and has been applied successfully in numerous studies [39–41].

Conventional applications of synchrotron techniques in plant science research

Two synchrotron-based techniques have dominated the investigation of metal(loid) distribution and speciation in plants: x-ray fluorescence (XRF) and x-ray absorption spectroscopy (XAS). These two approaches provide complimentary information and their integration is set to increase significantly in the nearfuture with the development of techniques such as XANES imaging [10].

Mapping plant metal(loid) distributions using synchrotron x-ray fluorescence

Synchrotron-XRF (SXRF) has mainly been employed for mapping elemental distributions in two dimensions (2D). This is done by scanning a specimen through a laterally-resolved x-ray beam, and collecting and analysing the x-ray fluorescence signal emitted from the sample. As reported in recent reviews [e.g. 11,42] the main advantages of this technique are due to its sensitivity (in the sub mg/kg range), the multielemental nature of the information collected, and the possibility to conduct the measurements at ambient temperature and pressure. The excellent detection limits achievable for plant materials using this technique are due to the intrinsic characteristics of the synchrotron radiation (high brilliance and coherence of the beam), the nature of the samples (investigation of high Z elements in a matrix dominated by light elements), and the existing availability of sensitive fluorescence detectors.

Lateral resolution of SXRF is largely controlled by the optics used to focus the x-ray beam. Typically, spot sizes between one and a few μm are achievable using Kirkpatrick-Baez (KB) focussing mirrors, and sub- μm spot sizes have also been achieved at some beamlines [43]. Lateral resolutions in the range of a few tens of nm can be achieved through the use of Fresnel zone-plates (ZP), however, some trade-offs are incurred. In comparison with the KB mirrors, ZP have lower focusing efficiencies (i.e. lower photon fluxes are achievable), and the shorter focal length of the ZP arrangement makes XAS investigations challenging as the focal point shifts with changing incident energy. It should be noted here that while the choice of optics largely determines the lateral resolution of the analyses, it is usually the thickness of the sample that dictates the spatial resolution. This is because, for transition metals and metalloids, the x-ray utilised for the analysis can penetrate deeply into the sample and the resulting fluorescence signal is generated by the interaction of the x-ray beam with the entire volume in the path of the beam. This fact significantly limits the spatial resolution of the analysis. For instance, if SXRF analysis of a leaf is conducted by rastering the specimen through an x-ray beam, it is impossible to ascertain whether the fluorescence signal obtained originates from the lower or upper epidermis, or from other structures within the leaf [e.g. 44,45]. For this reason, when plant tissues overlap, as is almost always the

case, preparation of thin sections is generally required to allow more specific information to be discerned. In the case of inherently dry specimens, such as mature seeds or wood, thin sections can be obtained without embedding [e.g. 46], but this is not the case for highly hydrated samples. Therefore, one of the main advantages of synchrotron techniques over methods such as PIXE or SIMS which must be done under vacuum, i.e. the possibility to perform the analysis under physiologically relevant hydration conditions, is in practice negated for highly hydrated samples by the penetrating nature of the x-ray.

On the other hand, the penetrating nature of the x-ray through biological samples can be harnessed to allow the investigation of internal metal distribution through 2D and 3D fluorescence μ -computed tomography (μ -CT). In this case, a sample is scanned repeatedly across a transect at numerous different angles (typically being rotated in a step-wise manner through 0–180°) and elemental sinograms are obtained [45]. After computer assisted reconstruction, images of element distributions within a virtual 2D cross section can be generated. The process can be repeated in adjacent areas to generate 3D element distributions. An alternative approach to μ -tomography is based on a confocal geometry that makes use of glass polycapillary half-lenses [e.g. 47,48]. In both cases however, the specimen is analysed for a considerable time at the same position. For instance, in the case of 2D fluorescence μ -tomography the sample is scanned tens of times across the same transect, albeit at different angles. As a consequence, the scanning time and the direct beam damage have, until very recently, precluded the possibility of performing this analysis on highly hydrated biological samples. This approach has therefore largely been limited to seeds [31,49] or dehydrated samples of leaves [e.g. 50] or roots [e.g. 51]. As will be described in a later section of this paper, elemental imaging of highly hydrated plant tissues is now possible, but the information obtained, albeit collected under physiologically relevant conditions, is nevertheless still limited by the penetrating nature of the x-rays and the complex 3D structure of plant organs.

Determining plant metal(loid) speciation using synchrotron techniques

In addition to element distribution, the speciation of metals and metalloids is of vital importance in understanding their metabolism in plants as well as the toxicity of contaminants and the availability of micronutrients. However, this information is not generally attainable through XRF. Notable exceptions relate to the possibility to distinguish between some species when metalloids with very distinct oxidation states are investigated. For instance, in the case of Se the incident energy of the beam can be tuned sequentially to the absorption edge of organic Se species (e.g. selenomethionine or selenocysteine), selenite and selenate with SXRF maps obtained at each energy. Using this approach [52] could successfully investigate the distribution of organic Se and selenate in the Se hyperaccumulator *Astragalus bisulcatus*. A similar approach was used to investigate the distribution of As species in *Pteris vittata* and *Raphanus sativus* [53,54]. However, in most cases the position of the absorption edges or the presence of a significant number of different species would require scanning the samples at many different energies; an approach that until recently has been considered too time consuming and too likely to cause beam damage to be pursued. Consequently, metal(loid) speciation in plants has generally been investigated using XAS techniques such as XANES (x-ray absorption near edge spectroscopy) and EXAFS (extended x-ray absorption fine structure spectroscopy). These two techniques are based on the photo-electric effect and provide information regarding the oxidation state and binding environment of the element of interest.

A number of reviews are available on the fundamentals of XAS [e.g. 55,56]; therefore the theoretical background of these techniques will not be discussed in detail here. Experimentally, an unfocussed (for bulk analysis) or focussed (for μ -XAS) x-ray beam is

tuned to a range of energies covering the absorption edge of the element of interest. The spectrum obtained is the average sum of the fluorescence signal originating from the species present in the illuminated volume. It should be clear that in the case of μ -XAS the caveats previously discussed for SXRF relative to the volume explored by the beam also apply in this case. As for SXRF, XAS can be conducted on fresh, hydrated samples.

However, in such cases the issue of beam damage must be carefully considered as XANES spectra collected over 10–20 minutes are not uncommon, particularly in cases where element concentrations are low. For instance, Scheckel et al. [44] observed beam-induced sample damage during replicated *in vivo* XANES analysis over the same region of tissue, and Lombi et al. [11] recently reported an example of *in beam* photo-reduction of As in rice grains.

Another common approach to XAS analysis relies on the use of cryostats to allow the analysis of frozen hydrated tissues. The advantages of this approach are improved signal:noise ratios, a possible reduction in beam damage, and the possibility to homogenise the samples to collect more representative bulk spectra (it should be clear that in this case samples should be homogenised frozen and transferred in this state to the cryostat).

Under physiologically relevant conditions, the most important limitations to be considered with regards to element speciation in plants by XAS are related to the possibility of beam damage and to the representativeness of the μ XAS data collected. While the former limitation has been discussed above, the latter needs further discussion, because in addition to the issues of lateral vs. spatial resolution discussed earlier, the heterogeneous distribution of metal(loid) species in a sample is also problematic. For instance, Lombi et al. [46] reported widely different As K-edge XANES in rice grains when spot analyses were conducted only a few μ m apart. Bulk analysis can be more representative but does not provide spatial information and is not sensitive enough to ascertain the presence of species representing less than 5–10% of the total element investigated.

This brief discussion about the application of conventional synchrotron techniques in plant metal(loid) research should have been sufficient to highlight the potential, but also the limitations, of synchrotron methods when applied to highly hydrated plant materials under physiologically relevant conditions. In the next section we will summarise the state-of-the-art and discuss how very recent developments in synchrotron techniques may provide solutions to some of the problems described above.

Synchrotron radiation studies under physiologically relevant conditions

A number of studies have investigated metal(loid) speciation in highly hydrated plant organs under physiologically relevant conditions, and a summary of the relevant literature is reported in Table 1. This listing clearly shows that ‘bulk’ speciation studies have generally been conducted using frozen hydrated tissues. In such cases, the metal(loid) speciation is preserved as it occurs under real physiological conditions even though the analysis is clearly not conducted *in vivo*. Isaure et al. [57] studied the speciation of Cd in roots and trichomes of *Arabidopsis thaliana*. Interestingly, both frozen-hydrated samples and freeze-dried samples were studied, and no significant differences in speciation were found between the two sets of samples. However, this kind of information is still very limited across various elements, plant species and tissues. Therefore, until more information becomes available, the use of frozen specimens should still be the preferred option if feasible.

In contrast, studies investigating the distribution and/or local speciation of metal(loid)s through laterally resolved techniques have mainly been conducted using freshly harvested plant parts. Possibly the earliest investigation of metal distribution and speciation conducted

in hydrated plant samples at room temperature was published by Yun et al. [58]. In that study, elemental distribution and Mn speciation in roots infected by a mycorrhizal fungus were investigated using a microfocussed beam at the Advanced Photon Source (beamline 2-ID-D). A few studies, have been conducted truly *in vivo* by inserting a part of the plant (generally a leaf) in the path of the beam while the plant was still growing in a pot. The first of these experiments was conducted by Scheckel et al. [44] who analysed the speciation and distribution of Tl in leaves of *Iberis intermedia*. This approach was subsequently used by Hokura et al. [59] who investigated the speciation and distribution of As in the hyperaccumulator fern *Pteris vittata*. As noted earlier, a few studies [53,54,60] have also used the shift in absorption edges of As and Se compounds to obtain species distribution maps by analysing the samples at 2–3 different incident energies.

Generally, most SXRF experiments reported in the literature have been conducted using intact plant parts (i.e. not using thin sections) and therefore the results obtained suffer somewhat from the ‘volume distortions’ explained earlier in this paper. Only a very few studies used approaches that were able to investigate metal distribution in virtual sections. Bulska et al. [61] used confocal μ -XRF to examine the 2D distribution of Se in fresh samples of *Allium cepa*, whilst Scheckel et al. [62] used differential absorption μ -CT to determine the 3D distribution of Tl in fresh and freeze-dried leaves of *Iberis intermedia*. The latter approach was only possible because of the Tl hyperaccumulating abilities of this species (with total Tl concentrations up to 1% DW) and the results clearly showed that the reconstructions were much clearer for the freeze-dried leaves than for the fresh specimens.

Finally, two very recent experiments have reported results obtained by 2D fluorescence μ -CT of highly hydrated plant samples. Both these experiments were conducted at the x-ray fluorescence microspectroscopy (XFM) beamline at the Australian Synchrotron and took advantage of the recently commissioned 384-element Maia detector [63]. The annular geometry of this detector coupled with ‘on-the-fly’ (continuous) scanning allows drastically reduced data acquisition times with transit times per pixel as short as ~ 50 μ s. Using this technology Lombi et al. [64] and Kopittke et al. [65] recently reported the 2D distribution of Cu, Zn and Ni in virtual cross sections of *Vigna unguiculata* roots. In order to beat the onset of beam-damage, transit times of 3.9 ms/pixel were required, and transit times double that rate (7.8 ms/pixel) were observed to result in significant beam-damage (Figure 1) [64]. It should be considered that, since the acquisition of 2D tomographic information requires a transect across the samples to be scanned at many different angles, the overall transit time per pixel is considerable. In the case of the studies mentioned above, 200 rotations (i.e. 200 line scans) were collected, which translates to an overall transit time per pixel of just below 0.8–1.6 s with the latter value already causing beam damage. This result is in line with the finding reported by Scheckel et al. [44] who reported visible beam damage on fresh roots mapped with a dwell time of 1 s/pixel. Information regarding beam-induced damage is extremely scant but the results discussed here indicate that this could be a substantial problem where highly hydrated samples are concerned, and it is certainly an issue which should be more thoroughly considered. Having said this, it is likely that recent developments in detector technology may, in the near future, begin to overcome these issues [10].

Use of synchrotron-based methods for functional characterisation *in planta*

Synchrotron radiation-based imaging techniques have enormous potential as tools in functional gene characterization, and much of this potential remains as yet unrealized. By analyzing differences in sub micron-scale metal(loid) distribution, abundance and/or speciation in wild-type and mutant organisms, gene function can potentially be revealed. The first example of the use of SXRF imaging in gene characterization was published by Kim et al [31]. These authors used microtomography to show disruption in wild-type Fe

distribution in mature Arabidopsis seeds in which the gene for a vacuolar Fe transporter (*AtVIT1*) had been silenced. Mutant seeds were unable to load Fe around the vasculature in preparation for germination, whereas wild-type seeds stored Fe almost exclusively around the vasculature. This resolved the primary function of the Fe transporter, and the distribution of Fe correlated strongly with *VIT1* expression in seed.

Microtomography has also been used to check for disrupted Fe distribution in seeds from a double loss-of-function mutant *ysl1ysl3* (Yellow Stripe-Like) [66]. This family of proteins transports metals that bind to the metal chelator nicotianamine. YSL1 and YSL3 in particular are involved in seed development and metal loading into the seed; therefore it was intuitive in this case to look for disruption in seed Fe localization. Seeds collected from *ysl1ysl3* contained reduced levels of Fe, Zn and Cu, and were unable to mobilize Zn and Cu from the veins of young and senescing leaves. The lower Fe abundance in the double mutant and normal vasculature-associated localization of Fe was confirmed using SXRF microtomography of mature dry seed. Recently, Johnson et al [67] used SXRF mapping to show that constitutively overexpressing nicotianamine synthase genes in rice (in particular *OsNAS2*) enhanced Fe and Zn abundance in the edible portion of polished white rice grains (endosperm). Overexpression of *NAS2* in rice increased endosperm Fe concentrations to the much sought after required level for Fe-sufficiency from a rice-based diet of between 14–19 $\mu\text{g g}^{-1}$, representing a four-fold increase in Fe over wild-type rice.

Using two different synchrotron beam lines, Punshon et al. [68] used both SXRF microtomography and mapping to show the subtle mislocalization of calcium (Ca) in *cax1cax3* double loss-of-function mutants in Arabidopsis. *CAX1* and *CAX3* encode for endomembrane cation exchangers, primarily transporting Ca^{2+} , but which may also be involved in Mn transport. For this study, *in vivo* data from microtomography of seed was compared to mapping data collected from resin-embedded sectioned embryos, showing good agreement in elemental distribution between the two approaches (Figure 2a,2b). The sub-epidermal distribution of Mn seen here, first observed by Kim et al [31], has also been observed in the seeds of other species (*Thlaspi caerulescans* R.Tappero, personal communication) analyzed by SXRF mapping and microtomography, and using histochemical staining methods [40]. Closer observation of sub-epidermal cells at the highest possible resolution (Figure 2c) clearly shows numerous spherical, Mn-replete subcellular bodies, which also contain Ca and Zn. Ordinarily these bodies are free of Fe (Figure 2c), although these cells are the primary location of Fe storage in *vit1-1* loss-of-function mutants [31]. The function of this distinct Mn localization to the abaxial side (underside) of the embryonic leaf is unknown; genes encoding Mn transporters for this tissue layer have not been found among numerous mutants for disrupted Fe transport. The ionic radius of Mn^{2+} is between Ca^{2+} and Mg^{2+} and close to that of Fe^{2+} , and Mn can therefore share transporters with these elements [69]. Pittman [69] pointed out that genes from the natural resistance-associated macrophage protein (Nramp) family, specifically *AtNramp1*, *AtNramp3* and *AtNramp4* are able to transport Mn. However, Arabidopsis seeds from *nramp1-1* and *nramp3nramp4* loss-of-function mutants have a wild-type Mn distribution (Figure 2d), indicating that these genes are not responsible for Mn storage in the embryonic leaf. There is a requirement for Mn in the oxygen evolving complex of photosystem II [70], although it seems intuitive that Mn stored for that purpose would localize to palisade cells.

The studies done by Kim et al [31], Punshon et al [68] and Johnson et al [67] using SXRF all have implications in the field of biofortification, where genetic manipulation of Fe, Zn and Ca responsive genes is used to increased the concentration of bioavailable forms of these widely limiting nutrients in plant-based foods. However, the application of SXRF as a tool in plant molecular genetics goes beyond biofortification to both pure plant science and

applied areas where metal(loid)s limit or enhance plant growth, or contaminate plant-based foods.

Although the use of SXRF imaging as a phenotyping tool in molecular genetic studies is still relatively new, it is intuitive that knowledge of gene expression is more important in guiding the analysis than differences in bulk metal(loid) concentrations. Mislocalization of elements is not always associated with changes in bulk concentration. In the case of *VIT1* for example [31], the high level of gene expression in mature seed suggested that perturbations would most likely impact seed metal abundance or distribution, despite bulk metal analysis showing no differences. Online microarray data are available for the Arabidopsis genome [71] and other model organisms [72] that allow researchers to analyze the location, growth stage and level of expression of their gene of interest, or conversely, to find the identity of genes expressed in specific organs and at specific development stages. Punshon et al [68] used online microarray data to show the expression levels of Ca transporters CAX1 and CAX3 throughout seed development, and were able to break down expression into the seed coat and embryo. Although the capability exists, patterns of elemental accumulation uncovered by SXRF imaging have yet to be used to guide searches for expressed genes.

The majority of molecular genetics studies using SXRF have focused on seed, which are ideal samples for SXRF microtomography because of their stability over extended periods of time. As mentioned earlier, it has been technically challenging to collect *in vivo* data from fresh stem, leaf and particularly the roots of plants, where metal(loid) homeostasis genes are also expressed. The low stability of hydrated tissue has been the primary issue, and even once stabilized, inherently low abundances of micronutrient elements and beam damage from the extended dwell time needed for biological samples have been further stumbling blocks. However, recent concurrent developments in fast detectors and low-overhead data acquisition systems have significantly reduced sample analysis times. This has major implications for the utility of SXRF imaging in the plant sciences. The use of array detectors, multiple silicon drift detectors and the Maia-384 massively parallel detector [73, 74] have dramatically increased count rates and presented the need to handle much larger amounts of data. Implementation of fly scanning is now rapidly replacing traditional data collection methods, where processing time and motorized stage movement overheads significantly added to the required analysis times. With fly scanning, data is handled ‘on-the-fly’, and processing is event-based. Each event is tagged by detector identity and scan position and the event stream is processed in real time, with no time overhead for processing [10]. The shorter dwell time per point (reduced from several seconds down to fractions of a second) means that the sample stage can move continuously rather than stopping and starting, reducing stage movement overhead from motorized stage movement. The recent work by Punshon et al [68] also used a unique 180° detector geometry in combination with fly scanning to eliminate self-absorption for low atomic number elements, such as Ca (Figure 2a). These improvements have made fresh tissue analysis possible, allowing access to all of the tissues of the plant, and will allow the greater level of replication needed when analyzing biological samples.

Future challenges and perspectives

As documented above, recent advances in synchrotron-based x-ray fluorescence technology have greatly increased the potential for using hard x-ray synchrotron techniques to conduct plant science research under physiologically relevant conditions. Nevertheless, significant challenges still exist, particularly in relation to sample preparation. The advent of fast detectors has solved some of the problems related to beam damage of hydrated tissues, but in order to collect information on sub-cellular structures without the complications of concurrently imaging overlapping features, ultra-thin sectioning is required. This is not

possible for hydrated tissues unless they are frozen, in which case the sections need to be transferred frozen to a cryostage for measurement. Alternatively, cryo-fixation and resin embedding followed by sectioning is possible, but must be done without altering the speciation and distribution of the metals during the sample preparation. In this case, sample fixation continues to be as much of a challenge for synchrotron techniques as it is for other methods requiring extensive sample preparation. An alternative approach for obtaining sub-cellular resolution information is the use of very high resolution tomography using a sub-micron beam, however this has not yet been achieved with hydrated plant tissues. Other challenges to be addressed include issues relating to self-absorption in tomography, especially where lower *z* elements are the focus, or where plant organs with a substantial bias in one dimension (e.g. leaves) are to be examined. Future efforts should also be directed towards integrating sub-cellular research using x-ray techniques with infra-red based research. This could provide greater insight into metabolic processes involving organic molecules to control metal(loid) homeostasis. For example, the production of chelator molecules in response to the spatially resolved presence of metals could be examined. Finally, time requirements for synchrotron based research are also still an issue. Beamlines suitable for plant science research are generally oversubscribed and access is granted on a competitive basis. This precludes the use of these techniques for high-throughput phenotypic screening, and highlights the need for careful design of synchrotron-based experiments to maximise the use of beamtime. Integrating the results from multiple techniques together to maximise the insights gained poses a rewarding challenge for future research.

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Figure 1.

2D map for Zn (in magenta) and Compton (green) collected after two tomography scans were performed with transit times per pixel of 3.9 and 7.8 ms (a). Beam induced damage is visible on the root where the tomography data was collected at the lower speed (upper arrow in a). The sinogram of 200 rotations acquired over 360° is reported in (b) and the tomographic reconstruction in (c). The cowpea root was exposed to 40 μM Zn for 24 h, excised and immediately inserted in a polyimide capillary partially filled with water. The capillary is visible in figure (a and b) as an envelope around the root and as the green circle in (c). White scale bars are 100 μm . Modified from Lombi et al. [64]

Figure 2.

Elemental distribution in Arabidopsis seeds collected by SXR imaging at two different resolutions (beam height $\times$ width shown in μm). The distribution of the elements of interest were conserved between *in vivo* microtomography (a) and raster-scanned resin-embedded, 1 μm -thick sectioned embryos (seed coat removed prior to embedding) (b). Subcellular organelles enriched with Mn (c) were imaged in greater detail by reducing the step size. Although likely candidates, *nramp1-1* and *nramp3nramp4* double mutants do not have disrupted Mn distribution (d). Images are individually scaled normalized fluorescence.

Table 1

Studies investigating metal(loid) speciation and/or distribution in highly hydrated plant organs under physiologically relevant conditions (presented in chronological order).

Target element	Plant species	Analysis	Frozen/fresh	Reference
Cd	<i>Brassica juncea</i>	EXAFS	frozen	Salt et al. [75]
Cu	<i>Larrea tridentate</i>	EXAFS	frozen	Polette et al. [76]
Mn	<i>Plantago Lanceolata</i>	μ -XRF, μ -XANES	fresh	Yun et al. [58]
Zn	<i>Thlaspi caerulescens</i>	EXAFS	frozen	Salt et al. [77]
Ni	<i>Thlaspi goesingense</i>	XANES	frozen	Kraemer et al. [78]
As	<i>Brassica juncea</i>	EXAFS	frozen	Pickering et al. [53]
Se	<i>Astragalus bisulcatus</i>	μ -XRF (2 energies) μ -XANES	fresh	Pickering et al. [52]
Mo	<i>Brassica juncea</i>	EXAFS	frozen	Hale et al. [79]
Cr	<i>Trifolium brachycalycinum</i>	μ -XRF, μ -XANES	fresh	Howe et al. [80]
As	<i>Pteris vittata</i>	EXAFS	frozen	Webb et al. [81]
Tl	<i>Iberis intermedia</i>	μ -XRF, μ -XANES	fresh	Scheckel et al. [44]
Pb	<i>Nicotiana tabacum</i>	μ -XRF	fresh	Fuhrman and Lanzirotti [82]
Se	<i>Alium cepa</i>	Confocal μ -XRF, μ -XANES	fresh	Bulska et al. [61]
Se	<i>Astragalus bisulcatus</i>	μ -XRF (2 energies) μ -XANES	fresh	Freeman et al. [83]
As	<i>Pteris vittata</i>	μ -XRF, μ -XANES	fresh	Hokura et al. [84]
Cd	<i>Arabidopsis thaliana</i>	XANES	frozen	Isaure et al. [57]
As	<i>Pteris vittata</i>	μ -XRF (3 energies) XANES	fresh	Pickering et al. [60]
Tl	<i>Iberis intermedia</i>	Absorption μ -CT	fresh	Scheckel et al. [62]
Ni, Co	<i>Alyssum murale</i>	μ -XRF	fresh	Tappero et al. [50]
Ni	<i>Stackhousia trynoidii</i>	μ -XRF, μ -EXAFS	frozen	Ionescu et al. [85]
As	<i>Raphanus sativus</i>	μ -XRF (2 energies) XANES	frozen	Smith et al. [54]
Ni	<i>Leptoplax emarginata</i> , <i>Thlaspi caerulescens</i>	EXAFS	frozen	Montarges-Pelletier et al. [86]
Cd	<i>Arabidopsis halleri</i>	μ -XRF	fresh	Hokura et al. [59]
Cs	<i>Arabidopsis thaliana</i>	μ -XRF, μ -XANES	fresh	Marmioli et al. [87]
Fe, Zn, Mn, Cu	<i>Oryza sativa</i>	μ -XRF	fresh	Takahashi et al. [88]
Hg	<i>Medicago sativa</i>	EXAFS	frozen	Carrasco-Gil et al. [89]
Cu, Zn	<i>Vigna unguiculata</i>	EXAFS, μ -CT	frozen/fresh	Kopittke et al. [65]
Zn, Ni	<i>Vigna unguiculata</i>	μ -CT	fresh	Lombi et al. [64]
Pb	<i>Sedum alfredii</i>	EXAFS	frozen	Tian et al. [90]