Multi-element bioimaging of *Arabidopsis thaliana* roots

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One sentence summary

A new technique for imaging of ion transport and distribution in *Arabidopsis thaliana* roots is presented.

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Author contributions

D.P.P., S.H., J.K.S and D.E.S. conceived the research plans; M.G.M.A. prepared and supplied plant mutants. D.P.P., A.C. and S.H. designed the experiments; D.P.P. and A.C. performed all experiments; D.P.P., A.C., S.H. and J.K.S. analyzed the data; D.P.P, A.C., S.H. and J.K.S. drafted the article, M.G.M.A. and D.E.S. complemented the writing with contributions from all authors. The final manuscript was approved by all authors.

Abstract

Better understanding of root function is central for development of plants with more efficient nutrient uptake and translocation. We here present a method for multi-element bioimaging at the cellular level in roots of the genetic model system *Arabidopsis thaliana*.

Using conventional protocols for microscopy we observed that diffusible ions such as potassium (K^+) and sodium (Na^+) were lost during sample dehydration. Thus, we developed a protocol which preserves ions in their native, cellular environment. Briefly, fresh roots are encapsulated in paraffin, then cryo-sectioned and freeze dried. Samples are finally analyzed by Laser Ablation-Inductively Coupled Plasma-Mass Spectrometry (LA-ICP-MS), utilizing a specially designed internal standard procedure. The method can be further developed to maintain the native composition of proteins, enzymes, RNA and DNA, making it attractive in combination with other omics techniques.

To demonstrate the potential of the method we analyzed a mutant of *A. thaliana* unable to synthesize the metal chelator nicotianamine (NA). The mutant accumulated substantially more zinc (Zn) and manganese (Mn) than the wild type in the tissues surrounding the vascular cylinder. For iron (Fe) the images looked completely different, with Fe bound mainly in the epidermis of the WT plants, but confined to the cortical cell walls of the mutant.

The method offers the power of ICP-MS to be fully employed, thereby providing a basis for detailed studies of ion transport in roots. Being applicable to *A. thaliana*, the molecular and genetic approaches available in this system can now be fully exploited in order to gain a better mechanistic understanding of these processes.

Introduction

Investigations of the localization of inorganic elements in young plant roots may answer a range of important and unresolved questions with respect to root functionality and plant nutrient transport. To date, our understanding of how plants control radial root transport of essential plant nutrients and toxic elements is mainly circumstantial, relying on changes in shoot or shoot-to-root concentration ratios or analyses of xylem sap composition. Roots of *A. thaliana* have a simple cellular organization and are unrivalled in their ability to be imaged by confocal microscopy as they are very thin (diameter ≈ 120 μm) and have a low background fluorescence. This has led to an amazingly detailed understanding of the growth and development of roots. Unfortunately, the fragile nature of these roots constitutes a major challenge when trying to understand the processes that drive nutrient uptake at the same level of detail. The method we present here for element bioimaging of *A. thaliana* roots is a critical step in utilizing the potential of combining targeted genetic modifications and bioimaging at the cellular level, in order to unravel the complexities of how roots selectively acquire and translocate mineral nutrients from the soil.

The uptake and radial transport of inorganic ions is tightly controlled by transport proteins varying in selectivity, affinity and capacity. However, it has recently been demonstrated that also physical barriers in the root systems play a pivotal role in regulating ion uptake, for example by the epidermis at the root surface as well as by the lignin and suberin barriers adjacent to the endodermis (Hosmani et al., 2013; Pfister et al., 2014; Kamiya et al., 2015; Barberon et al., 2016). The cells of the endodermis are sealed by the Casparian strip, a lignin-based barrier which restricts apoplastic movement of ions and water into the vascular bundles. Further control of ion movement into the stele (and ultimately to the leaves via the xylem), is achieved by suberin deposition along the cell walls of the endodermis (Baxter et al., 2009; Geldner, 2013). Radial transport of inorganic ions may also be impeded by lack of proper ligands, for example by nicotianamine (NA) or other organic acids. The functional effects that root barriers and ligands have on nutrient acquisition are still elusive, and probably constitute a combined response to various genetic determinants that are yet to be understood at the mechanistic level. A better understanding of radial root transport of essential nutrients is a prerequisite to improve nutrient uptake efficiency in crops and optimize the use of natural resources in agriculture. Likewise, better knowledge about transport of potentially toxic trace elements such as cadmium and arsenic will help improve food safety.

Different techniques are available for elemental imaging, i.e. the *in situ* visualization of elements, typically divided into mass spectrometry (MS)-based techniques (e.g. nano-Secondary Ion Mass Spectrometry (nano-SIMS) and LA-ICP-MS), or synchrotron X-ray based techniques (e.g. Energy dispersive X-ray microanalysis (EDX), Proton-induced X-ray emission (PIXE), X-ray fluorescence (XRF) or X-ray absorption spectrometry (XAS)) (Zhao et al., 2014). Relative to the SIMS and the synchrotron X-ray based techniques, LA-ICP-MS offers a range of advantages in terms of low detection limits and high sensitivity for many elements (Becker et al., 2010) (see Discussion for more details). LA-ICP-MS is also much more accessible and has substantially lower running costs than any of the competitive techniques.

Because of the requirement of dry samples, the usefulness of LA-ICP-MS for root analysis has long been hampered. For this reason, LA-ICP-MS analysis of plant materials has to date mostly been applied to naturally dry samples, like seeds and cereal grains (Lombi et al., 2011; Olsen et al., 2016). Analysis of hydrated samples, like roots, pose major sample preparation challenges, with respect to maintaining biological structure and the native composition of the elements therein, during drying. Uncontrolled drying of e.g. cross sections or longitudinal sections of young roots causes disruption of most cells, mainly due to the sudden loss of turgor and the resulting ion leakage. In order to preserve sample integrity, specimens for microscopy are typically dehydrated with a slow, gradual exchange of water with ethanol, acetone or tetrabutylalcohol (Feder and Obrien, 1968; Beeckman and Viane, 2000). Following dehydration, it is a common practice to embed the specimens in a block, typically a resin or paraffin block, prior to sectioning. However, young plant roots, like the ones of the genetic model plant *A. thaliana*, are extremely fragile and they easily disintegrate during such sample preparation. In addition, for elemental bio imaging these procedures are problematic since some ions will leak out of the tissue during prolonged soaking in organic solvents (Fourie and Peisach, 1977; Davies et al., 1991). It is unclear, however, how dehydration affect leakage and displacement of different elements and to what degree. In theory, ions with a low valence and little or no interaction with other compounds (e.g. K^+ and Na^+), should be highly diffusible and easily lost from the tissue, whereas di- and trivalent cations such as Mn^{2+}, Zn^{2+} , Fe^{2+} and Fe^{3+} should be less prone to leakage due to covalent bonding or coordination with various ligands.

In order to maintain not only the elements present in the root tissue, but also the integrity of various chemical components (*i.e.* ligands, proteins, nucleic acids and metabolites), rapid freezing of the

root is an attractive alternative to dehydration. After freezing, samples can be sectioned on a cryotome and then freeze-dried prior to analysis, alternatively freeze-dried first and then sectioned (Bhatia et al., 2004). In order to be able to prepare very thin sections (<5 μm), while still maintaining cell structures and element composition, freeze-substitution (FS) has also been employed (Siegele et al., 2008; Smart et al., 2010). This technique is based on ultra-rapid freezing followed by slow substitution of the ice with acetone, then chemical fixatives, like osmium tetroxide (Smart et al., 2010) or tetrahydrofuran (Palsgard et al., 1994). It has been shown, however, that the localization of highly diffusible ions, like K^+, Na^+, Ca^{2+} and magnesium (Mg^{2+}) may be significantly altered during freeze substitution (Smart et al., 2010).

For ordinary cryo-sectioning, the initial freezing and subsequent sectioning is typically done in OCT media (Optimal Cutting Temperature; Tissue-Tek, Sakura Finetek, Tokyo, Japan), which is a glycol-based freezing media (containing polyvinyl alcohol and polyethylene glycol, PEG) that facilitates fast freezing and mechanical support of the specimen during the following sectioning. Upon transfer of the sections to glass-slides, the section melts briefly, hereby adhering to the surface of the glass-slide. In this critical process, melted OCT may cover small specimens, partly or fully. OCT is highly water soluble and can easily be washed off, however with the risk of also washing off leachable ions. Also, the hygroscopic OCT media has a high osmotic potential, which means that it may cause water and/or ion diffusion upon direct contact with the root during the time that passes from excision to freezing in liquid nitrogen, with the risk of ion displacement inside and/or outside of the specimen.

In order to meet these challenges, we have developed a novel sample preparation method. We show that encapsulation of the fresh tissue with paraffin prior to freezing and cryo-sectioning is an essential step in order to avoid displacement of elements. Further, we also show that the method is applicable to the very small and fragile roots of *A. thaliana*. The method was tested using *nas1nas2nas3nas4* quadruple mutants (*NAS*: *N*icotian*A*mine *S*ynthase), which are unable to synthesize the metal chelator nicotianamine (NA). Upon cultivation in hydroponics, the leaves of these mutants display symptoms typical for iron (Fe) deficiency (Schuler et al., 2012). Total element concentration and xylem sap analyses, in combination with elemental bioimaging of the roots, showed that NA deficiency has different effects on radial and long distance transport of Fe *viz*. Zn and Mn, which in turn appears to be related to xylem loading processes as well as the affinity of NA and other ligands to these different metal ions.

By enabling the examination of *A. thaliana* roots, the genetic model plant of choice in plant science, the methodological developments described here pave the way for a range of new possibilities for investigating ion uptake, transport and compartmentation in root tissues. Moreover, the method works for any elemental ion present in the tissue, including any added isotope, be it essential plant nutrients or any other element taken up by plant roots.

Results

The effect of dehydration on the loss of nutrient ions

Initially, we attempted to perform elemental bioimaging analyses on cross sections prepared from *A. thaliana* roots which had been dehydrated with ethanol, using a standard protocol for microscopy sample preparation (Feder and Obrien, 1968; Beeckman and Viane, 2000). We observed that the resulting ion intensities, especially for K and Mg, were surprisingly weak and reasoned that substantial losses of these ions probably had occurred during the prolonged dehydration procedure in ethanol. To confirm this, roots of 6-week-old wild-type *A. thaliana* were subjected to dehydration with ethanol (see Materials and Methods). After dehydration, the roots were freeze-dried, digested and analyzed by ICP-OES. Control samples were freeze-dried immediately after harvest, digested and analyzed by ICP-OES. As can be seen in Table 1, the dehydration treatment induced a substantial loss of particularly K, but also more than 70% of divalent ions like Ca and Zn were lost. This clearly shows that conventional dehydration protocols are not suitable for elemental bioimaging analyses.

Cryo-sectioning to maintain tissue structure during sample preparation

We reasoned that instant freezing of the *A. thaliana* roots would maintain the native ionic composition and if kept frozen throughout sectioning and the subsequent drying, no ions would change place within the tissues, nor be lost from the sample (*i.e.* leak out of the root). After sectioning, during the transfer to glass slides, we observed that the specimens were covered, partly or fully, by the OCT embedding media (Fig. 1C). When analyzing these OCT-covered samples with LA-ICP-MS, very low sensitivity was obtained and it was very difficult to assign the ion signals to specific tissue structures within the specimens (data not shown).

In order to solve this, paraffin was used to encapsulate the whole roots, preventing the ions from leaking out of the tissues and at the same time keeping the hygroscopic OCT media physically separated from the sample, which appeared to be highly critical in order to avoid osmotic movement of water and ions from the tissue. The paraffin coating was also a successful way to keep the surface of the specimen free from OCT, while maintaining the turgor and integrity of the root cells. The microscopy images clearly showed that the structure of the root were nicely preserved after drying, with the major tissues types including the epidermis, cortex cells, endodermis and vascular

tissues still being clearly visible (compare Fig. 1A; cross section in wet condition, to Fig. 1B; cross section in dry condition). The paraffin ring coating the root could also be seen, as well as the surrounding OCT media, efficiently kept away from the surface of the specimen (Fig. 1B). Note that the cross section in Fig. 1C was also first frozen, then sectioned and dried, however without the paraffin encapsulation. The UV image (Fig. 1D) shows lignified and suberized tissues; thickenings characteristic of the Casparian strip and suberization observed in the endodermis.

Preparation and application of internal standard

An internal standard based on an element which is not normally found in root tissues is important for comparison of ion intensities between samples and analytical runs, and to validate that the tissue is quantitatively ablated. Ideally, the applied internal standard should have a similar ionization potential as the target analyte. For this reason Rubidium (Rb; ionization potential 4.18 eV) was chosen since it has a similar ionization potential as K (4.34 eV), which is the element we focused on during method development, as K is an ideal proxy for highly diffusible ions since it does not bind strongly to any ligands and adsorbs poorly to the negative charges in the cell walls. We tested many different strategies for the application of an internal Rb standard. These included a couple of approaches for applying an Rb-containing solution to a glass slide, for example by drying a drop onto the glass slide, applying it with a brush and then dry it, or by spraying it onto the surface. In order to reduce surface tension of the water droplets, we also tried different mixtures of water and ethanol (20-60%) in the solution. All of these attempts were more or less unsuccessful since the standard solution applied in these ways was not evenly distributed on the surface (data not shown). Next, we tried injecting the Rb-solution into a marker pen, allowing it to equilibrate overnight, and then using it for drawing a line onto the surface of the polyethylene naphthalate (PEN) membrane covered glass slide. When ablating this colored line with LA-ICP-MS, we clearly detected Rb as both its ^{85}Rb and ^{87}Rb isotopes. The distribution was much better than in our previous attempts (i.e. the drying, brushing or spraying). However, there was still a 15-20% relative standard deviation (RSD) of the Rb-signal across the ablated lines containing the internal standard (data not shown). In order to apply a more uniform amount of Rb-standard below the sample, we finally developed a method based on injection of the Rb solution directly into an inkjet cartridge (Fig. 2, see also in Materials and methods). The standard was then printed five times on a transparent plastic sheet, ensuring a thick, homogenous layer. When ablating this surface, the Rb-signal was very stable, and

we consistently recorded RSD-values between 4 and 6 % across the whole rectangle (data not shown).

Optimization of LA settings

The next step was to optimize the LA settings for the analysis of the paraffin-coated, cryo-sectioned and dried specimens, which now had an internal Rb-standard placed underneath them. In LA, there is a trade-off between resolution and sensitivity, both with respect to the energy level of the laser beam, and also the thickness of the root slice. Using a high energy setting for the laser beam (50% or more of the maximum energy; corresponding to >3 J cm^{-2}) might result in inferior resolution, since a high-energy pulse will spread out more than a corresponding low-energy pulse. Conversely, a lower energy would minimize this spread, helping to maintain a high spatial resolution. However, using too low energy would result in less material being ablated, which of course reduces the intensity of the signal since less material would reach the plasma of the ICP-MS. Roots are composed of tissues with very different hardness. Typically cell walls and cells in the stele are much more dense and rigid than cells in the cortex. If tissue removal during laser ablation was not complete, variations in hardness might bias the results, as it would be impossible to tell whether differences in the obtained signal intensities are due to differences in tissue hardness or in absolute ion concentrations. For this reason, we optimized the LA settings for a complete removal of the tissue in the cross sections, but using the lowest possible laser energy, in order to maximize spatial resolution. We tried different thicknesses of the sections, in combination with different laser energies. As can be seen in Fig. 3, 16-µm thick cross sections of the roots were quantitatively removed using laser energy levels of 2.36 or 1.40 J cm^{-2} (corresponding to 40 and 30% of the maximum energy), whereas 0.59 J cm^{-2} (corresponding to 20% of the maximum energy) was not sufficient to obtain a quantitative ablation of the tissue.

Next, we investigated the influence of sample thickness on the sensitivity (Fig. 4). From the same root, 20, 16 or 12 µm thick sections were ablated using approximately 1.40 J cm^{-2} energy in the laser beam. All the sections were quantitatively ablated using these settings (see insert of the 20 µm thick section before and after ablation; Fig. 4). However, as expected, the signal intensity of ^{39}K was much stronger in the 16 and 20 µm thick sections, compared to the 12 µm thickness. Hence, in

246 order to get the best possible resolution (from a quantitative ablation) and the maximum signal
247 intensity from the same ablation, sections should be 16-20 µm thick.

248 Since we applied the internal standard underneath the cross sections, another criterion for a
249 successful ablation was that the laser beam should also penetrate into the internal standard below
250 the root section, without hitting the glass slide.

251

252 A non-quantitative ablation is seen in Fig. 5B, where the low energy (0.6 J cm^{-2}) ablated some root
253 material, but nothing of the underlying Rb-standard, analyzed as the ^{85}Rb isotope. When using 1.40
254 J cm^{-2} energy in the laser beam, all the soft tissues of the root were ablated, together with the Rb-
255 standard below these areas (Fig. 5A). However, in the areas with hard tissues (cell walls and stele)
256 only a part of the root tissue was ablated, hence the laser beam did not penetrate into the Rb-
257 standard below these tissue structures, leaving them without any Rb-signal (Fig. 5A). The higher
258 energy used in Fig. 5C (2.4 J cm^{-2}) ablated all the root tissues quantitatively and penetrated down to
259 the Rb-standard below, now generating an even Rb-signal throughout the area of the cross section.
260 Therefore, in order to obtain a quantitative ablation and at the same time reach down to the Rb-
261 standard, we had to use this higher energy level. The ^{85}Rb-signal could now be used to correct the
262 obtained values, where the lower left image (Fig. 5D) is the raw ^{66}Zn signal from the same section
263 as in Fig. 5C, and the lower, right image is the ^{66}Zn signal, again from the same section as in Fig
264 5C, now corrected on the basis of the ^{85}Rb-signal (Fig 5E). It should be noted that Zn has an
265 ionization potential of 9.39 eV, which differs considerably from that of Rb (4.18 eV). However, the
266 internal standard was not used for absolute quantification in this case, but only to account for
267 possible signal drift and to document a quantitative ablation *viz*. where all tissue material are
268 transported as an aerosol to the ICP-MS for detection

269

270 **Multi elemental bio imaging with LA-ICP-MS**

271 Following the method optimization described above, a cross section of a wild-type *A. thaliana* root
272 was analyzed by LA-ICP-MS, using a 5 µm resolution (spot size). In Fig. 6, a microscopy image
273 and the corresponding distribution of $^{39}K^{+}$ are shown, revealing its relative concentrations in the
274 different tissues. The ^{39}K signal in this image was not corrected relative to the Rb signal since it was
275 not used for any intercomparisons. Zn and Mn were also analyzed in the same root section,

underlining the multi-elemental capacity possible with LA-ICP-MS (data not shown). Any other elements present in the tissue can in principle also be analyzed, given that it has a concentration above the detection limit. Depending on ionization potentials and isotopic abundances of the elements, 5 to 10 elements may in total be monitored in the same analytical run. All essential plant nutrients, except for nitrogen (N) and chloride (Cl) can be analyzed. In addition, other relevant elements like Na, aluminum (Al), cadmium (Cd) and arsenic (As) can all be studied with the method.

Bio-imaging of NAS quadruple mutants

The applicability of the method was tested with a case study, using 6-week-old quadruple *A. thaliana nas1nas2nas3nas4* mutants (*nas4x*) and wild type (WT) plants of the same age. The *nas4x* mutants displayed severe deficiency symptoms of interveinal chlorosis, especially in the youngest leaves, even though both mutant and WT were grown with the full spectrum of mineral nutrients. (Fig. 7).

The total concentrations of Zn and Mn in the leaves clearly showed that the *nas4x* mutant was very inefficient in long distance, root-to-shoot transport of these two elements (Table 2). In *nas4x* the shoot concentration of Zn was approximately half that observed in WT, and for Mn the decrease was 28% ($P<0.05$ both elements). Interestingly, no significant difference in shoot Fe concentration was found between the WT and the *nas4x* mutant ($P>0.05$), even though *nas4x* displayed what could be interpreted as Fe deficiency symptoms. Hence with respect to root-to-shoot transport of Fe the *nas4x* mutant was similar to WT.

The elemental composition of the xylem sap confirmed the shoot data in Table 2, with Zn and Mn concentrations being significantly lower in the xylem sap of *nas4x* compared to WT ($P<0.05$). Again, Fe was different from the other three elements, showing no significant difference in xylem sap concentration between the WT and the mutant ($P>0.05$). Furthermore, there were no significant differences in the potassium (K) or magnesium (Mg) concentrations in the xylem sap of the *nas4x* and WT plants, supporting that the xylem sap flow rates were similar (data not shown).

Using LA-ICP-MS we observed that the *nas4x* mutant accumulated substantially more Zn than WT in all root tissues, including in the stele, endodermis, cortex and epidermis (Fig. 9, middle). This

evidences a severely reduced capacity for radial ion transport, and depressed xylem loading of Zn in the *nas4x* mutant, which was also corroborated by the lower Zn concentrations in the xylem sap and in the shoots (Table 2 and Fig. 8). The total root concentration of Zn was 63% higher in the roots of *nas4x* compared to WT ($P<0.05$) (Table 2), which is consistent with the LA-ICP-MS imaging. The Mn image (Fig. 9, right) also indicated an impaired radial transport in the *nas4x* mutant, with more Mn accumulating in the cortex and around the vascular tissues than in the WT.

Similar to Zn, the total root Fe was significantly increased in *nas4x* compared to WT ($P>0.05$) (Table 2). However, unlike Zn, this increase in root Fe was mainly due to increased accumulation of Fe in the cortex and cortical cell walls. This contrasts with Fe accumulation in the WT which primarily occurs in the epidermis. The images in Fig. 9 may seem to indicate a higher total Fe concentration in the WT, which would be in contrast to the results in table 2, showing 56% higher Fe concentration in the *nas4x* roots. However, the accumulated signal counts (the sum of all counts throughout the cross section) was in fact slightly higher in the mutant, and in addition these counts appeared in a smaller tissue area than in the WT. Interestingly, neither *nas4x* nor WT accumulated Fe in and around the stele, which could explain the similar whole-shoot and xylem sap concentrations.

Discussion

Elemental imaging can be performed with many different analytical techniques. All of these techniques have their benefits and shortcomings with respect to sample preparation, element coverage, sensitivity and spatial resolution.

The X-ray based techniques act very differently on a sample in comparison with the MS-based techniques, since the X-rays penetrate into the sample and any surrounding material, whereas the MS-based techniques ablate only the top layer of the specimen. In X-ray based techniques it is thus important not to smear the signals from the top cell layers with signals from the deeper layers of the sample. In order to avoid this, very thin sections have to be prepared (<5 μm thick) which typically compromise sensitivity. However, new approaches have enabled direct analysis on hydrated roots, with a minimum of sample preparation, using S-XRF(Lombi et al., 2011). However, prolonged exposure to X-rays causes damage to the tissue sample, which might alter the distribution of elements (Zhao et al., 2014).

With respect to spatial resolution, nano-SIMS outcompete all other imaging techniques, reaching as low as a 50 nm spatial resolution (Moore et al., 2012), whereas X-ray based techniques reach between 1 and 2 μm and LA-ICP-MS systems typically ranging from 5 to 200 μm (Becker et al., 2010). New LA designs, however, like the near-field LA-type, can go below 5 μm, and substitution of the LA-unit with a laser micro dissection unit has been used in order to improve resolution down to 1 μm (Becker et al., 2010).

Since MS-based techniques mainly rely on the first ionization potential for each element, these techniques offer a wide range of elements to be tested, including both light and heavy atoms. Additionally, MS-based techniques offer the analysis of stable isotopes. Due to technical improvements, including the development of the triple quadrupole ICP-MS, traditionally problematic atoms with high ionization energies or atoms affected by strong interferences, such as sulphur (S) and phosphorus (P), can now be analyzed with low background noise, offering greatly improved sensitivities (Balcaen et al., 2015). With X-ray based techniques sensitivity generally decreases with atomic number, since the lighter elements (e.g. K, Na, Mg, Al) have low emission energies, and thus will be difficult to detect even at relatively small depths within the sample. Heavier elements (e.g. Cu, silver (Ag) and gold (Au)) have much higher emission energies which will be able to pass through larger distances within a sample, resulting in a higher sensitivity (Zhao

et al., 2014). One of the main limitations with SIMS is the poor sensitivity for detection of certain elements such as Zn, Mn and Cd. This is due to a low secondary ion yield of these particular elements (Moore et al., 2012). With detection limits observed in the sub μg g^{-1} level for many elements, LA-ICP-MS is more sensitive than either SIMS or X-ray based techniques (Becker et al., 2010). Relative to SIMS and synchrotron X-ray based techniques, LA-ICP-MS is also much more accessible and has substantially lower running costs.

The combined use of synchrotron X-ray based techniques and tomography analyses have enabled direct analysis on hydrated roots (Lombi et al., 2011). The concentrations of seven different elements were analyzed in roots of cow pea, and using a mathematical model, images and estimated concentrations in virtual cross sections could be displayed (Wang et al., 2013). As mentioned, one of the main challenges with this analytical approach has been the tissue damage from X-ray treatment during prolonged analyses. However, with new, fast detectors much progress has been made in recent years, reducing the exposure times from hours to minutes (Lombi et al., 2011). This approach reduces the radiative damage of highly sensitive hydrated tissue samples and allows construction of ultra-thin 2D images and in perspective the technique might enable 3D imaging using confocal detection (Lombi et al. (2011). The experiments by Lombi et al. (2011) and Wang et al. (2013) were conducted in concentration ranges relevant for environmental toxicology, but it should be noted that these conditions was far higher than the physiological concentration ranges normally used for cultivating plants. The Zn supply in Wang et al. (2013), for example, was 40 times higher than that considered to be a normal Zn supply in hydroponics. Thus, for understanding ion transport processes under physiological nutrient concentrations, the high sensitive of LA-ICP-MS offers a number of major advantages relative to state-of-the-art synchrotron techniques.

Sample preparation and LA-ICP-MS analysis

Analyzing hydrated samples imposes major challenges with respect to sample preparation, since the sample needs to be dried prior to LA-ICP-MS analysis. After drying, both the biological structures and the elemental ion composition of the specimen need to be maintained in order for the analysis to be meaningful (Moore et al., 2012). Also, absolutely planar sections need to be obtained, since LA-ICP-MS is sensitive to sample topography and matrix composition. Hence, with respect to sample preparation, LA-ICP-MS analyses face similar challenges as nano-SIMS (Moore et al.,

2012; Moore et al., 2014). As was shown here and elsewhere, existing protocols for microscopy are not designed for maintaining the ionic composition of plant samples. In roots of the grass *Festuca rubra*, an average of 78% of the Zn was lost during dehydration and fixation processes (Davies et al., 1991), which is consistent with the findings presented here (Table 1). Hence, we conclude that the use of standard protocols for microscopy drains element ions out of the root tissues, leading to erroneous conclusions about ion distribution and concentration.

Freezing is an attractive alternative to dehydration, fixing both cellular structures and ions in their native states. Some SIMS and LA-ICP-MS instruments have the capability to analyze samples in their frozen, hydrated state, thus avoiding any sample preparation artefacts (Metzner et al., 2010). However, apart from the fact that only very few instruments have this highly specialized facility, it has been reported that such analyses typically are cumbersome and technically challenging to perform since they suffer from serious analytical artefacts related to hardness, background noise and interference (Metzner et al., 2010; Moore et al., 2012). Hence, for most element bioimaging techniques, freezing followed by water removal is still the preferred method of sample preparation (Smart et al., 2010; Moore et al., 2014).

During freezing there is a risk of ice crystal formation which may rupture the tissues, causing ion leakage and displacement, resulting in a poor quality imaging. Fast, high pressure freezing (HPF), typically at ~200 MPa/-196^{o}C, is frequently used to overcome such problems, as it decreases the ice crystal formation, also in the deeper layers of the sample (Moore et al., 2012). Here, we did not use HPF, yet we observed little or no ice formation in the specimens after plunge freezing in liquid nitrogen. Most probably, this was due to the small size of the *A. thaliana* roots which enabled rapid freezing. We also pre-cooled the OCT media in order to facilitate the fastest possible freezing, minimizing ice crystal formation.

In order to perform cryo sectioning, the use of a cutting medium is needed for thin, fragile roots. For larger roots, direct freezing without support can be done in liquid propane, cooled by liquid nitrogen, and subsequently cut in cross sections. (Schneider et al., 2002).

An alternative to traditional cryo sectioning with OCT is freeze substitution. Freeze substitution replaces the water with an organic solvent, typically acetone, after which the acetone in turn is substituted with a resin or another fixative. Once embedded, the sample can be sliced in thin sections, down to a thickness of 1 μm. Even though this technique is regarded as the state-of-the-art

sample preparation technique (Moore et al., 2012; Zhao et al., 2014) it has some disadvantages, and the localization of highly diffusible ions like K^+, Na^+, Ca^{2+} and Mg^{2+} may still be significantly altered during the acetone substitution and the following chemical fixation (Smart et al., 2010). In Smart et al. (2010), the Ca signal was stronger than the K signal in the cytosol, suggesting misplacement of these elements during sample preparation (Smart et al., 2010). In another study, about 90 % of the nickel (Ni) ions in leaf tissues were lost during freeze-substitution with tetrahydrofuran (Budka et al., 2005). Here, we have shown using the highly mobile K^+ ion as a proxy for ions that are easily lost from the tissue, that the elemental ion composition can be maintained with a simple encapsulation of the root tissue with paraffin, prior to freezing and sectioning on a cryotome. After cryo-sectioning, the cross sections were put in an ordinary freezer overnight. During storage for 16 hours, we found that ice sublimation occurred, which dried the samples completely. Hence, dry cross sections were obtained without having to move them to a freeze-drier facility, without adding solvents and without pressure or temperature changes imposed on the samples, which all could alter the distribution of elements.

The application of an internal standard proved useful in order to record reproducible images and enabling comparisons between the results recorded in separate analytical runs. For normalization of topographic differences or differences in tissue hardness, an element already present in the tissue can be used. Carbon (^{13}C) has frequently been used in other LA-ICP-MS studies (Becker et al., 2010; Becker et al., 2010). In our root sections, the signal from ^{13}C was very weak, which made this approach unreliable. Also, roots may have large differences in suberin content, depending on their nutrient status (Barberon et al., 2016), and since suberin is a carbon-rich compound, the use of this element for normalization could be a significant source of error. Here, we decided to apply Rubidium (Rb) as an internal standard, since we knew that it was not present in our nutrient solutions. Rb was chosen due to its similarity to K in terms of its ionization potential (First ionization potentials; K=4.34 eV, Rb=4.18 eV). In practice, and in future analyses, any element or isotope can be used in the way described here, which enables comprehensive multi element fingerprinting analyses. The normalization shown in fig. 5D and E indicates that only minor differences in the images occur upon normalization with the Rb-signal, yet for day-to-day comparisons it is a very valuable tool, since there are typically quite large variations in ICP-MS sensitivity on a day-to-day basis. As stated above, Zn has a different ionization potential (9.39 eV) than Rb (4.18 eV), hence can only be used for monitoring instrumental changes in signal strength

444 and in this case also to document a quantitative ablation process. For semi or absolute
445 quantification, an element with an ionization potential closer to Zn would have to be used.

446

447 **Method validation using NAS mutants**

448 We tested the developed method on the *nas4x* mutant which is unable to synthesize nicotianamine
449 (NA); an essential ligand for metal ion homeostasis in plants (von Wiren et al., 1999; Takahashi et
450 al., 2003). Changes in *NAS* gene expression have been shown to strongly affect the transport and
451 distribution of Fe, Zn, Cu and Mn (Klatte et al., 2009; Deinlein et al., 2012). Consistent with these
452 previous observations, the *nas4x* quadruple mutants showed interveinal chlorosis, mainly present in
453 the youngest leaves, which is a well-known characteristic of mineral nutrient deficiencies (Fig. 7;
454 see also (Takahashi et al., 2003; Schuler et al., 2012). However, our analysis of the total element
455 concentrations in the shoots revealed deficiencies of Zn and Mn, but not of Fe (Table 2; see also
456 (Klatte et al., 2009; Schuler et al., 2012)). It should be noted that we did not separate the shoot into
457 young and old leaves, so these observations are based on the average concentration of the whole
458 shoot. Schuler et al. (2012) observed that the nas4x mutant seemed to have a defect with respect to
459 Fe delivery to sink organs which may explain the marked interveinal chlorosis observed in the
460 young leaves of our plants (Fig. 7).

461 Xylem sap data confirmed that the root-to-shoot transport of Fe was less affected by NA deficiency
462 than that of Zn and Mn (Fig. 8). Substantial increases in citrate levels have previously been reported
463 for *nas* mutants and might be the reason for the absence of reduction in shoot Fe concentration,
464 since citrate is the preferred Fe ligand at the low pH of the xylem sap (von Wiren et al., 1999;
465 Rellan-Alvarez et al., 2008; Schuler et al., 2012). The low xylem concentrations of Zn and Mn point
466 towards a pivotal role of NA in xylem loading of these elements, but not for Fe. A significant
467 decrease in xylem Zn concentration was observed in *A. halleri* RNAi lines with depressed NA
468 content [27]. Speciation analyses of the xylem sap revealed that the main Zn ligands, both in WT
469 and RNAi lines, were malate and/or citrate, not NA (Cornu et al., 2015). Using LA-ICP-MS
470 bioimaging, we show that Zn accumulated particularly in and around the stele in the *nas4x* mutant
471 (Fig. 9, middle), which is consistent with the obtained xylem and shoot data (Table 2 and Fig. 8)
472 and the proposed primary role for NA in loading Zn into the xylem. Fe in the same root section
473 from *nas4x* showed no such accumulation in and around the stele (Fig. 9, left), suggesting that NA

is not required for xylem loading of Fe. The *A. thaliana* mutant *frd3*, which lacks a functional citrate transporter, was reported to have approximately 40% lower citrate concentrations in the xylem sap and showed Fe accumulation in the root central cylinder (Schuler et al., 2012), which underlines the importance of citrate for xylem-mediated root-to-shoot Fe transport [28]. The fact that *nas4x* mutant plants developed symptoms of interveinal chlorosis indicative of Fe deficiency, despite having similar whole-shoot Fe concentrations as the WT, suggests that NA is involved in phloem-based partitioning of Fe after unloading from the xylem. Consequently, insufficient amounts of Fe reach the chloroplasts in the interveinal cells of the young, newly developed leaves, leading to chlorosis. Thus, using LA-ICP-MS to image the steady state distribution of Fe and Zn in the *nas4x* mutant has provided *in planta* evidence of a role for NA in loading Zn but not Fe into the xylem. Our data clearly show that LA-ICP-MS is a powerful technique to study the consequences of changes in available ligands for ion transport and xylem loading at the cellular level. As such, the method can reveal important processes that would have been otherwise overlooked.

Conclusions

We conclude that LA-ICP-MS constitutes a powerful technique for biological investigations of intra- and intercellular ion transport. Moreover, ICP-MS offers a wide range of elements to be analyzed, including light elements and biologically important elements such as Zn and Mn, which have poor sensitivity in synchrotron X-ray fluorescence based techniques and in Secondary Ion Mass Spectrometry (SIMS), respectively. Also, since the presented method is applicable to the small and fragile roots of *A. thaliana*, the full range of molecular, genetic and cell biological approaches available in this genetic system can be utilized. For example, a broad selection of mutants with alterations in ion transporter function or root cellular morphology can be studied, allowing a deeper understanding of the functional role of different cell-types, extracellular barriers such as Casparian strips and suberin lamellae, and a cellular-level resolution of the major ion transport pathways. As such, research on agricultural nutrient use efficiency and plant adaptability to adverse environmental conditions will benefit from the possibilities that ICP-MS based bioimaging method offers. Since the method can be further developed to maintain also the native composition of proteins, enzymes, RNA and DNA, the protocol may be useful in combination with other omics techniques.

Materials and methods

Plant cultivation

Arabidopsis thaliana accession Columbia-0 (Col-0) and the *A. thaliana nas1nas2nas3nas4 (nas4x)* quadruple mutant were used for all experiments. The *nas4x* mutant was obtained by crossing homozygous T-DNA insertion mutants for *nas1* (SALK_082174), *nas2* (SALK_066962), *nas3* (SALK_106467) and *nas4* (SALK_135507) (see TAIR at www.arabidopsis.org) in different combinations to obtain initially double and subsequently triple mutants, two of which were crossed to obtain the quadruple mutant. Seeds were sterilized in 50% ethanol for 1 min and in 2.5% NaClO (Klorin original, Colgate-Palmolive A/S, Denmark) with 0.05% (v/v) Triton X-100 for 10 min, then rinsed three times with Milli-Q water. Hereafter they were stratified for 48 h at 4 °C in the dark. Seeds were germinated in distilled water and one-week-old plants were transferred to a hydroponic system containing 0.25 mM $CaCl_2$, 1mM KH_2PO_4, 0.05 mM KCl, 0.25 mM K_2SO_4, 1mM $MgSO_4$, 0.1 mM NaFe-EDTA, 2 mM NH_4NO_3, 30 µM H_3BO_3, 5 µM $MnSO_4$, 1 µM $ZnSO_4$, 1 µM $CuSO_4$, 0.7 µM $NaMoO_4$, 1 µM $NiSO_4$. The pH of the solution was adjusted to 5.8 by KOH (2M). Nutrient solutions were replaced every second day. All plants were grown in a controlled environment with 8h light (90 µmol m^{-2} s^{-1}), 22℃ :19℃ (day: night) and 75% humidity.

Total element concentration

Whole roots were gently dried on a napkin, and then freeze-dried at -45℃, 1 mbar for 36 hours together with the shoots. Hereafter, each sample was weighed. All leaves of the shoot were treated as the same sample, *i.e.* we did not separate between young and old leaves. All samples were digested with 500 µL HNO_3 (70 %) and 250 µL H_2O_2 (15 %), and subsequently diluted to 10 mL. Elemental analysis was performed with an ICP-OES (Agilent 5100, Manchester, U.K.), using external calibration, drift check samples and certified reference material for optimal data quality (Olsen et al., 2016).

Xylem sap analysis

Half an hour before the onset of light in the climatic chamber, the plants were cut just below the leaf rosette with a ceramic knife. The first µL of xylem sap was discarded, hereafter 3-6 µL were collected from each replicate. Each sample was diluted one time with 7% HNO_3 (2 µL xylem sap + 2 µL HNO_3), giving a final volume of 4 µL with an acid concentration of 3.5%. Two µL of this sample was injected directly into the ICP-MS in flow injection analysis mode using an inert HPLC (Ultimate 3000, Thermo Scientific) as auto sampler and injector, 3.5% HNO_3 as mobile phase and a flow rate of 0.3 mL min^{-1} (Olsen et al., 2016). The concentration was measured using external calibration, injected using the same conditions as described for the samples.

Conventional root tissue dehydration

The entire root was cut off from the shoot and put into a 50-mL Eppendorf tube filled with FAA fixative, where it was left in the fridge overnight (+6^0C). The FAA fixative contained (for 100 mL): 52 mL of Ethanol (96 %), 5 mL of acetic acid, 5 mL of formaldehyde (37 %) and 28 mL of MilliQ-water. The next day all the FAA fixative was discarded and the sample was washed twice in Milli-Q water, and then soaked in 50% ethanol for 30 min. Hereafter the sample was transferred to 70% ethanol, 96 % ethanol and 100% ethanol, respectively, each time for 30 min. The last step was a transfer to a new solution of 100 % ethanol, where the sample was kept for 3 hours. All the steps were performed at room temperature.

Sample preparation for LA-ICP-MS analysis

The root samples for LA-ICP-MS analyses were all cut approximately 2 cm from the tip of the primary root. The length of each piece of primary root was between 6 and 8 mm, and any lateral roots were cut off from this root piece. After gentle drying on a napkin, the root piece was dipped in melted paraffin (70ºC), which was then allowed to harden for 5 seconds. The paraffin-coated root was then put in a handmade aluminum-foil mold containing non-frozen, but pre-cooled OCT medium (4ºC) (Optimal Cutting Temperature; Tissue-Tek, Sakura Finetek, Tokyo, Japan), in which the root segment was aligned horizontally and submerged in OCT. The mold with its contents was then put in liquid nitrogen in order to freeze it instantaneously. After freezing, the solid OCT block was transferred to a cryotome (Leica CM050S, St. Gallen, Switzerland), precooled to -25 ºC, where it was mounted for sectioning. Cross-sections 12-20 µm thick were cut and transferred with a thin paint brush onto membrane-covered glass slides (MembraneSlide 1.0 PEN, Carl Zeiss Microscopy,

Göttingen, Germany). For transfer of the sections, the downside of the glass was slightly warmed up with a finger, allowing the section to briefly melt onto the glass slide, ensuring uniform adhesion to the membrane in a horizontal position. Immediately after this critical step, the section was deep frozen again inside the cryotome. In order to evaluate the quality of the root sections, some samples were visualized with a light microscope. These samples were always discarded, since they were completely thawed, hence considered unfit for further analysis. If the sample check in the microscope was satisfactory, new sections were cut and immediately left to dry in the freezer overnight.

Preparation and application of internal standard

In order to apply an internal standard, we used an inkjet printer (Brother, DCP-J412 model, Stockholm, Sweden). Briefly, a small hole was drilled in the magenta ink cartridge and 0.5 mL of ink was removed using a syringe with a needle. Hereafter, 0.5 mL of an Rb-solution (10 000 mg L^{-1}, Peak Performance certified reference material, CPI International, U.S.A.) was injected, and the hole was sealed with glue. The ink cartridge was left to equilibrate overnight, and then it was installed into the inkjet printer. A magenta-colored rectangle was printed on regular transparent plastic paper. The same rectangle was printed on the same area of the paper five times, ensuring a thick, very homogenous application of Rb-containing ink. An approximately 1 x 2.5 cm rectangle was cut from the plastic paper and carefully inserted under the PEN membrane of the membrane-covered PEN-microscopy slide, which had carefully been opened on one side with a scalpel (Fig. 2).

LA-ICP-MS analysis

The LA-ICP-MS analyses were performed with a nanosecond LA unit (NWR193, New Wave Research, Fremont, CA, USA) equipped with an ArF excimer laser source operating at 193 nm using the following key settings: Energy: 1.4-2.3 J cm^{-2} (30-40 % of maximum energy), Scan speed: 10 µm s^{-1}, Repetition rate: 40-60 Hz and spot size 5 µm. All elemental signals were obtained with an Agilent 7900 ICP-MS (Agilent technologies, Manchester, U.K.), operated in He-mode. The isotopes analyzed were ^{85}Rb, ^{39}K, ^{24}Mg, ^{55}Mn, ^{56}Fe and ^{66}Zn, using an integration time of 0.1 s. The

key settings on the ICP-MS were: Sample cone depth: 5 mm, Carrier gases 1 mL/min, Octopole collision gas (He): 1 mL/min

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Table 1. Total element concentration in the roots of 6 weeks old *A. thaliana* wild-type plants, sequentially dehydrated with ethanol, then dried, digested and analyzed by ICP-OES. The right hand column shows the concentration changes compared to roots from control plants, *i.e.* plants that were not dehydrated with ethanol (n=4).

Elements	Fresh root (µg g^{-1} dry weight)	Dehydrated root (µg g^{-1} dry weight)	% difference
Mg	5382 ± 760	602 ± 217	-88.8
K	74513 ± 4222	2212 ± 409	-97.0
Ca	10998 ± 2944	2873 ± 856	-73.9
Mn	166 ± 34	32 ± 9	-80.9
Zn	1144 ± 36	87 ± 13	-92.4

Table 2. Total element concentration in shoots and roots of 6 weeks old wild-type (WT) and *nas4x* mutant plants of *A. thaliana*, cultivated under normal nutrient conditions in a hydroponic system. All samples were dried, di gested and then analyzed by ICP-OES (n=3).

	WT shoot (µg g^{-1} DW)	*nas4x* shoot (µg g^{-1} DW)	*nas4x* shoot (% diff. from WT)	P	WT root (µg g^{-1} DW)	*nas4x* root (µg g^{-1}DW)	*nas4x* root (% diff. from WT)	P
Mn	412 ± 14	322 ± 5	-21.9	<0.001	182 ± 28	272 ± 87	49.2	0.167
Fe	198 ± 86	168 ± 46	-15.4	0.618	9890 ± 528	15400 ± 2720	55.7	0.026
Zn	156 ± 12	73 ± 5	-53.0	<0.001	1000 ± 262	1630 ± 182	63.1	0.027

Fig 1. Cross sections from *A. thaliana* roots. (A) A fresh cross section that was first encapsulated in paraffin, frozen and cryo-sectioned, then stained with toluidine blue (TBO) and viewed under a microscope in wet condition. (B and C) Cross sections that were either encapsulated in paraffin (B), or not (C), prior to freezing, cryo-sectioning and freeze-drying. (D) UV image of a fresh section showing suberized and lignified tissues. The images to the left were captured with bright field microscopy (BF) and the image to the right was captured with UV fluorescence microscopy (450-490 nm). Abbreviations: Ep=epidermis, Co=Cortex, Va=vascular tissues and En=Endodermis. The scale bars represent 50 µm.

Fig 2. Schematic illustration of the procedure for application of a rubidium (Rb) standard underneath a cross section from *A. thaliana* roots. The cross section was first encapsulated in paraffin, cryo-sectioned and then freeze-dried. The Rb-standard was applied on a transparent polymer with an ink-jet printer and then manually inserted in the PEN membrane envelope, under the cross section.

Fig 3. Optimization of LA-ICP-MS settings in order to obtain a quantitative ablation and to achieve the best possible spatial resolution when analyzing cross sections from *A. thaliana* roots. All three cross sections were 16 µm thick and they were analyzed with decreasing energy levels (right to left) and otherwise identical settings. The energy levels in the laser beam were 2.36, 1.40 and 0.59 J cm^{-2}, respectively. The upper images (A) are bright field microscopy images, the middle images (B) are microscopy images taken after the ablation and the lower images (C) represent the distribution and ion intensity of potassium (K), analyzed as ^{39}K with LA-ICP-MS. The red and blue color spectrum in (C) represent high or low intensities, respectively (range: 2000-80000 counts). Scale bars represent 50 µm.

Fig 4. The impact of thickness of cross sections from *A. thaliana* roots on the ion intensity. Sections with 20, 16 and 12 µm thickness were analyzed with 1.4 J cm^{-2} energy in the laser beam. All samples, including the 20 µm thick section, were quantitatively ablated (upper far left: before ablation, lower far left: after ablation). The color spectrum represent an ion intensity map of potassium (K), analyzed as $^{39}K^{+}$ (range: 2000-80000 counts). Scale bars represent 50 µm.

Fig 5. (A) An ion intensity image of $^{85}Rb^{+}$ in the upper half of a 20 µm thick cross section from *A. thaliana* roots, analyzed by LA-ICP-MS with 1.4 J cm^{-2} in the laser beam. (B) An ion intensity map of $^{85}Rb^{+}$ in the lower half of the same section as (A), analyzed with 0.6 J cm^{-2} in the laser beam. (C) A $^{85}Rb^{+}$ ion intensity map analyzed with 2.4 J cm^{-2} in the laser beam. (D) An ion intensity map of $^{66}Zn^{+}$ from the same cross section as (C). (E) The distribution and ion intensity of $^{66}Zn^{+}$ in the same cross section as (C), where the values have been corrected, using the $^{85}Rb^{+}$ signals as internal standard. The color spectrum represent high to low ion intensities ($^{85}Rb^{+}$, range: 10-5000 counts (A, B and C), $^{66}Zn^{+}$ range: 0-150, (D and E)). Scale bars represent 50 µm.

Fig 6. A 20 µm thick cross section from *A. thaliana* roots encapsulated in paraffin, cryo-sectioned and freeze-dried prior to analysis. The image to the left is a UV fluorescence image (450-490 nm) taken before ablation, where the red background color originates from a rubidium (Rb)-standard placed below the cross section. The image to the right shows the distribution and ion intensity of potassium ($^{39}K^{+}$) ions in the same sample, analyzed by LA-ICP-MS, where the color spectrum represent high to low ion intensities, respectively (range: 2000 – 80000 counts). The scale bar represents 50 µm.

Fig 7. (Large image) Wild-type (WT) and a *nas4x* quadruple mutant *A. thaliana* plants, 6 weeks old, cultivated in a hydroponic system with standard nutrient conditions. The mutant (left) display interveinal chlorosis which is a known symptom of iron (Fe) deficiency. (Small image) Full size image of the WT and the *nas4x-2* mutant plants.

Fig 8. Xylem sap concentration of micro nutrients in 6 weeks old wild type (WT) and *nas4x* mutant plants of *A. thaliana* (n=3) The plants were cultivated under standard nutrient conditions in a hydroponic system.

Fig 9. Twenty µm thick cross sections from roots of *A. thaliana*; wild-type (WT)(upper images) and *nas4x* (lower images) plants, analyzed by LA-ICP-MS. The plants were 6 weeks of age and were cultivated under normal nutrient conditions in a hydroponic system. The color spectrum represents high to low ion intensities of ^{56}Fe (left) and ^{66}Zn (middle) and ^{55}Mn (right), in the ranges 4000-20000, 200-500and 40-400, respectively. The bar represents 50 µm.

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