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One "OMICS" to integrate them all: ionomics as a result of plant genetics, physiology and evolution

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Abstract The ionome concept, which stands as the inorganic composition of an organism, was introduced 15 years ago. Since then, the ionomics approaches have identified several genes involved in key processes for regulating plants ionome, using different methods and experimental designs. Mutant collections and natural variation in the model plant species *Arabidopsis thaliana* have been central to the recent discoveries, which are now being the basis to move at a fast pace onto other models such as rice and non-model species, aided by easier, lower-cost of genomics. Ionomics and the study of the ionome also needs integrations of different fields in plant sciences such as plant physiology, genetics, nutrition and evolution, especially plant local adaptation, while relying on methods derived from chemistry to physics, and thus requiring interdisciplinary, versatile teams. Here we review the conceptualization of the ionome as an integrated way of viewing elemental accumulation, and provide examples that highlight the potential of these approaches to shed light onto how plants regulate the ionome. We also review the main methods used in multi-element quantification and visualization in plants. Finally, we indicate what are the likely next steps to move the ionomics field forward.

Keywords Ionome · Elemental profiling · ICP · Natural variation · X-ray fluorescence

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1 Introduction

The *ionome* is defined as the mineral nutrient and trace element composition of an organism, including both essential and non-essential elements, metals and non-metals, extending on the term *metallome* (Salt et al. 2008). The study of the ionome is called *ionomics*, which aims at quantifying as many elements as possible in individual samples, and using that information to understand how physiology, development, genetics and the environment change the inorganic composition of organs, tissues, cell types and single cells of target organisms. Since the concept was proposed (Lahner et al. 2003), ionomics has been applied to many different plant species, including *Arabidopsis thaliana* (L.) Heynh. (Arabidopsis) (Chao et al. 2014a; Huang and Salt 2016), *Oryza sativa* L.

(rice) (Pinson et al. 2015), *Zea mays* L. (maize) (Baxter et al. 2014), *Glycine max* (L.) Merr. (soybean) (Ziegler et al. 2013), *Brassica napus* L. (rapeseed) (Thomas et al. 2016), as well as the yeast *Saccharomyces cerevisiae* (Yu et al. 2012) and human cells (Malinouski et al. 2014). Combined with the power of genetics and genomic approaches, high-throughput ionomics profiling has been used to understand the *genetic mechanisms that regulate the plant ionome* (for a comprehensive review on the advances of ionomics in the last years, see Huang and Salt 2016). The commonly used approaches are mutant screening to identify genes that are key to elemental accumulation (Kamiya et al. 2015; Hindt et al. 2017) and natural variation screening to find alleles that explain differences in the ionome of diverse genotypes (Huang and Salt 2016; Yan et al. 2016; Chen et al. 2018). In particular, *natural variation* (i.e., distinct ecotypes of a species collected that can show different genetic and physiological mechanisms adapted to their particular, diverse environments) has been shown to be very useful to identify genes that are linked to high/low concentrations of elements (Huang and Salt 2016). Therefore, the ionomic profile of plants grown in a common environment may reflect adaptations to their native local environment (Baxter et al. 2010; Huang and Salt 2016).

The ionome concept can be construed as individual elements that act independently or as a combination of multiple elements that vary as an interdependent network (Baxter 2015). Of course, often the concentration of elements does vary independently and many studies found that single genes can control accumulation of only one element in plant tissues (Chao et al. 2012; Huang and Salt 2016). On the other hand, there are situations in which one unique gene can regulate the concentrations of multiple elements. The IRON REGULATED TRANSPORTER 1 (AtIRT1), for example, is a high affinity iron (Fe^{2+}) transporter induced under Fe deficiency conditions (Eide et al. 2005; Barberon et al. 2011). Although crucial to Fe acquisition, AtIRT1 has broad substrate specificity, being able to transport other divalent cations such as Zn^{2+}, Mn^{2+}, Cd^{2+} and Co^{2+} (Korshunova et al. 1999). As a result, plants under physiologically relevant low Fe conditions show increases in leaf Zn, Mn, Cd and Co concentrations, due to up-regulation of *AtIRT1* (Baxter et al. 2008). Strikingly, Fe concentration itself is similar in leaves of plants grown under Fe deficient and Fe sufficient conditions, demonstrating that the ionomics profile can show signatures that are more informative to access the plant Fe status than measuring Fe concentration alone (Baxter et al. 2008).

Many single gene mutations cause multi-element changes in Arabidopsis. AtESB1 (ENHANCED SUBERIN 1) is part of the machinery necessary to form Casparian Strip domain in the endodermis, and *esb1* mutants show disordered strips (Hosmani et al. 2013). This mutant also shows altered leaf concentrations for many elements, presumably due to increased apoplastic diffusion through the Casparian strip, which results in altered communication between the root apoplast and the xylem, and therefore changes in root-to-shoot translocation (Baxter et al. 2009; Hosmani et al. 2013; Ricachenevsky et al. 2018a). Mutant plants lacking AtMYB36, a transcription factor that orchestrates the Casparian strip formation, also have multiple elemental changes in leaves associated with loss of strips (Kamiya et al. 2015). Another example is the significant ionomics changes in the *sic1* mutant, which shows differences in concentration of several elements in leaves. The *sic1* mutant causative gene is a choline transporter named CTL1, which is necessary for plasmodesmata formation, vesicle sorting and correct localization of metal transporters in roots (Guo et al. 2017). Thus, it is clear that the elements in the ionome can act interdependently.

The ionome can therefore be viewed as a snapshot of the plant underlying genetics and physiology. Here we discuss the contributions of ionomics to plant physiology, with a special focus on (1) how natural variation in genetics can affect the ionome; (2) how these changes can be linked to variation in physiological processes and local adaptation; (3) what are the analytical tools used to probe the plant ionome; and (4) the future prospects for the field of ionomics.

2 Ionomics as a tool for understanding the genetic basis underlying elemental accumulation in plant tissues

Scientific interest in understanding the plant ionome comes from different fields, including: (i) *evolutionary biology* and the knowledge regarding local adaptation of natural populations; (ii) *nutrient use efficiency* of crops, which if increased will allow lowering reliance on heavily used fertilizers that are inefficiently

absorbed by plants; (iii) *environmental monitoring*, through phytoremediation and phytoindication (technologies that use plants to clean up soil, air and water, and to predict the environmental status, respectively); (iv) *biofortification*, which aims the improvement of the nutritional quality of food crops; (v) and *food safety*, by the avoidance of heavy metals accumulation in plants edible parts. On overview of ionomics workflow is shown in Fig. 1.

In particular, Arabidopsis and rice are the two most explored plant model species, and ionomics findings have relied on both (Huang and Salt 2016). Arabidopsis is the plant model per excellence, while rice is a major staple food, and a target for biofortification (Sperotto et al. 2012; Ricachenevsky et al. 2015). Rice grains are also a dietary source of the toxic metalloid arsenic (As) and of cadmium (Cd), which can accumulate depending on how plants are cultivated (Pinson et al. 2015). Thus, understanding the rice grain ionome can positively affect human nutrition and food quality/safety. Here we focus on the most recent examples of ionomics studies that uncovered genes linked to natural variation in these species, to show the potential of natural variation as a source of new alleles of known genes, which can aid in the study of gene function and metabolic pathway regulation.

2.1 Importance of ionomics for plant nutrition

The ionomics approach has already significantly contributed to our understanding of plant physiology, specifically in the plant nutrition field. The multi-element nature of the used techniques is excellent for finding how perturbations in abundance of one element affect others. Examples include interplay between molybdenum (Mo) and iron (Fe) (Vigani et al. 2017); how rice grain nutrients are affected by toxic arsenic (As) species (Punshon et al. 2018); and to the identification of casual genes involved in natural variation of nutrient concentration in rice seeds, including Mo, sodium (Na) and nitrogen (N) (Yang

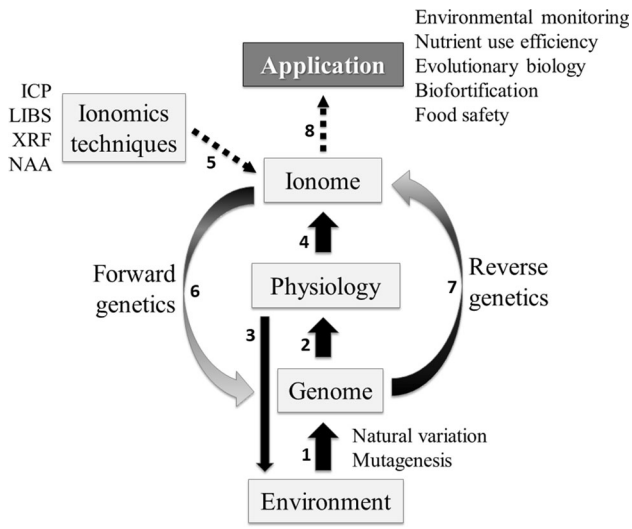

Fig. 1 Biological processes affecting the *plant ionome* and main steps for *ionomics studies*. *(1)* The environment (e.g.: water and nutrient supply, water and soil pollution, soil composition, stress) provide selective pressure, resulting in genomic variations in plant populations. Mutations in the genome can arise spontaneously during replication, by environmental mutagenic agents; or can be generated artificially for identifying interesting genes related to the ionome. *(2)* Mutations can affect elements absorption and distribution. *(3)* In turn, plants can differentially affect the bioavailability of elements in the surrounding environment (e.g.: by secreting chelators in the rhizosphere) affecting the elemental profile in plant tissues, which can affect selection. *(4)* The ionome is determined, thus, by its underlying physiology, which is determined by the genome and environment interaction. *(5)* The ionome can be analyzed by several methods. *(6)* The genetic basis of elemental accumulation can be studied by forward genetics, when regulatory genes are identified from a set of ionomics mutants or natural variation (i.e., from phenotype to genotype); or *(7)* by reverse genetics, when a gene is mutated (or has its expression modified) and the ionomics phenotype is characterized (i.e., from genotype to phenotype). *(8)* The obtained knowledge can be applied for understanding plant evolution, improve plant nutrition, environmental monitoring (phytoremediation and bioindication), and to increase food safety

et al. 2018, Huang et al. 2018 *in press*). Below we discuss some examples of such studies.

2.1.1 *APR2 controls natural variation in sulfur and selenium content*

As one of the essential macronutrients, sulfur is vital for plant growth and development. It is taken up as sulfate and is assimilated into cysteine, an amino acid at the crossroads of primary metabolism, protein synthesis and the formation of low molecular weight sulfur-containing defense compounds, such as glutathione and phytoalexins (Rausch and Wachter 2005).

The existence of natural variation in sulfur (S) content was first shown by Loudet et al. (2007), who has linked the variation in sulfate content to AtAPR2 (ADENOSINE 5′-PHOSPHOSULFATE REDUCTASE), one of the three isoforms of a key enzyme of assimilatory sulfate reduction pathway (Setya et al. 1996) in Arabidopsis. Loudet et al. (2007) noticed that Bay-0 accession accumulates more sulphate than Shahdara and attributed this difference to a single-SNP difference in the coding sequence that results in a single amino acid change (alanine by glutamate) interfering with binding of the reductant to the thioredoxin-like active site. By sequencing the *AtAPR2* allele of more than 60 Arabidopsis natural accessions these authors identified nine distinct protein haplotypes, in which the polymorphism in $AtAPR2^{Shahdara}$ remained unique. Then, some genotypes were transformed with the Shahdara and Bay-0 alleles, showing that the AtAPR2 enzyme is three to four times less active in genotypes containing the Shahdara allele than in genotypes containing the Bay-0 allele. The authors also identified an interesting example of physiological interaction between quantitative trace loci (QTL) and environment: the allelic effect of Shahdara tends to be stronger with nitrogen (N) limitation in the soil, probably due the tight interconnection of sulfate assimilation with N metabolism. Under this condition, AtAPR activity and mRNA accumulation of *AtAPR* isoforms are reduced compared with accumulation under high-N conditions (Koprivova et al. 2000), leading to higher sulphate content in Shahdara, compared to Bay-0. Only under N-limiting conditions the parental accession Shahdara accumulates more sulfate than Bay-0 and other accessions. Furthermore, only the $AtAPR2^{Shahdara}$ haplotype substantially reduced the total APR activity, suggesting that under high-N conditions, the AtAPR2 reduction capacity in Shahdara is adequate to prevent sulfate accumulation, but when the expression of *AtAPR2* is reduced owing to the effect of low-N conditions, the activity is no longer sufficient, and sulfate accumulates (Loudet et al. 2007). The accumulation of the major low-molecular weight thiol glutathione, which is important for plant stress defense (Noctor et al. 1998), was lower in Shahdara than in Bay-0 leaves. Glutathione synthesis is dependent on availability of cysteine, so this finding could be seen as an indirect effect of reduction of AtAPR activity in Shahdara. These insights on sulphate assimilation may have an impact on sulfur fertilizer use and stress defense improvement (Loudet et al. 2007).

Selenium (Se), an S analogue, is incorporated into biomolecules in plants in place of S via its assimilatory pathway. Although plants do not require Se, plant-based Se is an important source of this essential element for animals (Roman et al. 2014). Because of that, biofortification of edible plant parts with Se may be a good strategy to minimize Se deficiency in animals, highlighting the importance of studies on natural variation to identify genotypes that accumulate Se in tissues. Chao et al. (2014b) evaluated the genetic architecture of variation in total leaf S and Se concentrations from a set of 349 Arabidopsis accessions (Baxter et al. 2010) and identified other variant of the enzyme AtAPR2 with strongly diminished catalytic capacity in the accession Hodonín. This accession presents S and Se concentrations 2-fold higher compared to Col-0 genotype, and a decrease of S flux from sulfate into the reduced sulfur compounds, cysteine and glutathione, and into proteins. The authors confirmed that the catalytic capacity of AtAPR2 varies by four orders of magnitude across the *A. thaliana* species range, and that variation is driven by the shoot, since S accumulates only in plants with a loss of AtAPR2 activity in the leaves. Chao et al. (2014b) propose that the reduced-function alleles of APR2 are adaptive to soils with elevated sulfate concentrations, where the capacity to limit the energy-demanding reduction of sulfate may have a fitness advantage. For example, the european accessions possessing the weak alleles of *AtAPR2* were collected from central Europe and Scandinavia, regions known to have experienced high levels of sulfate deposition due to acid rain from the 1950s to the 1970s (Menz and

Seip 2004). Although these observations are intriguing, more work is needed to establish if these *AtAPR2* weak alleles are really adaptive, and if so, to what conditions. Uncovering this new role for natural genetic variation in APR2 opens up a new avenue for exploring natural variation in S metabolism for the manipulation of dietary selenium concentrations in crop plants (Chao et al. 2014b).

2.1.2 *Natural variation in root length under low Fe conditions is linked to FRO2*

Fe is essential for key processes in plants, including electron transfer reaction in mitochondria and chloroplast, and for chlorophyll synthesis. Fe is highly abundant in the soil, but frequently is not available for uptake, and thus plants have evolved specific mechanisms for Fe absorption. Conversely, Fe can also become toxic if accumulated, and thus plants have to fine-tune Fe concentration in tissues (Sperotto et al. 2012; Ricachenevsky and Sperotto 2014). These mechanisms are being studied in detail for many years, with mutants that show different responses to low Fe and/or change Fe concentration in tissues well characterized in model species (for reviews see Hindt and Guerinot 2012; Brumbarova et al. 2015). However, much less is known on Fe homeostasis natural variation.

Recently, a panel of 134 Swedish Arabidopsis accessions was analysed for root growth under Fe deficiency (Satbhai et al. 2017). Genome-wide association for root length under low Fe linked the observed variation to FERRIC REDUCTASE/OXIDASE 2 (*FRO2*) gene, a membrane-bound enzyme that reduces Fe^{3+} to Fe^{2+} in the rhizosphere, a key step in Fe uptake that increases Fe solubility (Robinson et al. 1999). Longer roots under low Fe, but not under control conditions, were correlated with increased *AtFRO2* expression and ferric chelate activity (Satbhai et al. 2017). Complementation of a *fro2* loss-of-function mutant using natural alleles showed that *AtFRO2* is the causal gene of the longer roots phenotype under low Fe. Interestingly, the panel was analysed for FRO haplotypes, establishing that that there is allelic heterogeneity for *AtFRO2* (i.e., different *AtFRO2* alleles that result in the same phenotype). Polymorphisms in these haplotypes are regulating *AtFRO2* expression but not its coding sequence. Moreover, it was shown that *AtFRO2* alleles that cause increased expression under Fe deficiency confer increased seedling vigour in alkaline soils (Satbhai et al. 2017). Thus, these data indicate that natural allelic variation in FRO2 in Arabidopsis and other plants might be an interesting avenue for generating Fe deficiency-tolerant genotypes.

2.1.3 *NRX1 is the causal gene for high Mg sensitivity*

Magnesium (Mg) is the 8th most abundant mineral element on earth and the 4th abundant mineral element in plants following nitrogen, potassium and calcium (Maguire and Cowan 2002). Mg is particularly important to plants since it is involved in macromolecules stabilization and chlorophyll synthesis, also acting as a cofactor of a series of enzymes involved in photosynthetic carbon fixation and metabolism (for reviews see Maathuis 2009). Even being an essential macronutrient, high Mg in soils can affect crop growth and development, and one of the reasons is that Ca^{2+} and Mg^{2+} compete for the same transporters in the plasma membrane, leading to calcium deficiency in plant tissues, as shown by Tang et al. (2015) in Arabidopsis. Calcium is an essential plant macronutrient with key structural and signalling roles. Its anions (Ca^{2+}) act as a stabilizing element in the membranes, a strengthening agent in cell walls, and a secondary messenger for a multitude of signals (for reviews see White and Broadley 2003).

The gene *AtNRX1* (ARABIDOPSIS NUCLEOREDOXIN) was recently identified as the major source of sensitivity to high levels of Mg^{+2} by negatively regulating Ca uptake under this condition (Niu et al. 2018). Compared with wild type, mutants *nrx1* supplied with high Mg (10 mM) showed higher Ca concentrations in the plant; higher cytosolic Ca^{2+} concentrations during root elongation; and higher fresh weight and lateral-root number. They also showed increased Mg concentration, since *AtNRX1* is co-expressed with *AtMRS2*–3 (MAGNESIUM TRANSPORTER 4), a transport gene related to Mg^{2+} nutrition (Gebert et al. 2009), indicating that Mg^{2+} transporter genes have interactions with *AtNRX1*, as proposed by Niu et al. (2018). The authors highlight that the discovery could help to breed/select crops that can adapt to high Mg^{2+} soils such as serpentine ones (high ratio of Mg^{2+}: Ca^{2+}), in semi-arid regions (where water deficiency can lead to Mg accumulation) or Mars regolith (which are known to

have high levels of magnesium sulfate and is a potential medium for plant growth in bioregenerative life support systems) (Niu et al. 2018).

2.2 Food safety, environmental monitoring and evolution studies under the light of ionomics

The plant ionome includes not only essential elements, but also toxic and trace elements that can accumulate in plant tissues. Some of these can be found in edible tissues such as leaves and seeds, and thus can be ingested by animals and humans, resulting in toxicity (da Rosa Couto et al. 2018; Nachman et al. 2018). Ionomics approaches can quantify accumulation of these elements, and studies have linked causative genes to variation in their accumulation in Arabidopsis, which are discussed below.

2.2.1 HMA3 and HMA4 drive natural variation in shoot cadmium and zinc accumulation

Cadmium (Cd) is a trace element and one of the most toxic heavy metals to living beings, and can enter humans diet leading to serious diseases such as cancer (Huff et al. 2007), low bone density, and renal dysfunctions (Nordberg et al. 2002). Plants can accumulate high Cd concentrations in aerial tissues, including vegetative (such as leaves) and reproductive (such as seeds) what may represent something good or bad, depending on the point of view: plants that accumulate high Cd in the above-ground, harvestable tissues, may be successfully used in the phytoremediation of environments contaminated with this element; by the other hand, if this high accumulation occurs in edible parts of staple foods, such as rice grains, Cd may be transferred through food chain causing health disorders in humans and other living beings (Shimbo et al. 2001). Zinc (Zn) holds similar chemical properties with Cd and, because of that, they are often substrate for the same transporters (Chao et al. 2012), and thus their homeostasis control can share some common mechanisms. For this reason, understanding the mechanism of Cd and Zn accumulation in plants and finding genes involved in natural variation is important to reduce its potential toxicity (Chao et al. 2012).

The ionomics profile of the aforementioned 349 wild Arabidopsis accessions (Baxter et al. 2010) revealed a fourfold variation in leaf Cd accumulation when these accessions were grown in a controlled experiment. By combining forward genetics techniques, Chao et al. (2012) identified a P-type ATPase transporter expressed mainly in the root tonoplast, the HEAVY METAL ATPASE 3 (*HMA3*), as the major locus responsible for the variation in leaf Cd accumulation. This transporter is localized to the tonoplast and mediates the efflux of Cd and other divalent cations from the cytosol into the vacuoles mainly in root cells, decreasing translocation to shoots. This study showed the existence of 10 major natural HMA3 protein haplotypes, which results in changes in protein coding sequences, and presumably in transporter activity (Chao et al. 2012). Presumably, lower AtHMA3 function leads to lower Cd compartmentalization in root vacuoles, and higher Cd translocation to shoots and probably seeds (Ricachenevsky et al. 2018a). It was shown that natural variation of *AtHMA3* rice ortholog *OsHMA3* directly affects Cd concentration in grains (Miyadate et al. 2011; Ueno et al. 2010; Yan et al. 2016).

Zn is an essential micronutrient for plants, but may be toxic in high concentrations, so Zn concentration in plant cells is finely regulated (Lin and Aarts 2012; Ricachenevsky et al. 2015). HMA3 also acts on Zn efflux from citosol into root vacuoles, but the ectopic expression of functional *HMA3* alleles reduces Cd concentration in the shoot with no effects in Zn concentration in aboveground tissues of Arabidopsis and rice (Chao et al. 2012; Sasaki et al. 2014). Chao et al. (2012) hypothesized that plants sense changes in cytosolic Zn due to variation in AtHMA3 function, altering expression of genes related to Zn uptake, transport and compartmentalization to maintain Zn homeostasis in the shoot. In fact, the ectopic overexpression of functional *OsHMA3* in rice promoted the up-regulation of genes involved in the Zn uptake/translocation belonging to the ZIP family (ZINC-REGULATED TRANSPORTER/IRON-REGULATED TRANSPORTER PROTEINS) such as *OsZIP4*, *OsZIP5*, *OsZIP8*, *OsZIP9*, and *OsZIP10* (Sasaki et al. 2014) keeping Zn levels in shoots in wild type and *OsHMA3*-overexpressors similar. The *A. thaliana* Col-0 accession has a natural loss-of-function allele of *AtHMA3*. However, other accessions contain naturally functional allele of *AtHMA3* (Chao et al. 2012). Expressing a functional allele of *AtHMA3* from its native promoter in the Col-0 accession revealed

genes that are up-regulated in response to native AtHMA3 function, such as *AtZIP3* (ZINC TRANSPORTER 3), *AtMTP1* (METAL TOLERANCE PROTEIN 1), and the transcription factor *AtbZIP19* (BASIC-REGION LEUCINE-ZIPPER 19) (Pita-Barbosa and Salt D.E. unpublished data).

Based on the same Arabidopsis natural variation screen (Baxter et al. 2010), Chen et al. (2018) identified two accessions that accumulate significantly lower Zn in leaves compared to other genotypes. Sequence analysis revealed that a deletion in the third exon of *AtHMA4* (HEAVY METAL ATPASE 4) and polymorphisms in the promoter region of the same gene are responsible for the loss of function/low expression of this transporter, driving the low Zn shoots concentration observed. *AtHMA4* and *AtHMA2* are a duplicated gene pair that code for plasma membrane transporters shown to be required for Zn and Cd xylem loading and root-to-shoot translocation (Hussain et al. 2004). The two natural AtHMA4 alleles and the *hma4-2* null mutant allele when expressed in the Col-0 background show enhanced resistance to a combination of high Zn and high Cd in the growth medium, raising the possibility that variation at *AtHMA4* may play a role in environmental adaptation (Chen et al. 2018). Interestingly, the rice ortholog *OsHMA2* was shown to have a similar function to AtHMA2/AtHMA4 (Takahashi et al. 2012), and thus it may also be a target to find natural variation for Zn translocation to shoots.

2.2.2 *HAC1 drives natural variation in shoot arsenic*

Arsenic (As) is a toxic metalloid and is listed as a class-one carcinogen (NRC 2001). Humans are exposed to As mainly through drinking water and food, and the development of low arsenic-containing food is of great importance to guarantee human food safety. Considering that arsenic is also very toxic to plants (Pita-Barbosa et al. 2015, 2019), it needs to be detoxified after entering the root cells. This process depends on the previous reduction of arsenate to arsenite by arsenate reductase enzyme. Once reduced, arsenite is chelated by phytochelatins (PC) and the complexes PC-arsenite are immobilized into vacuoles by ABCC-type transporters (Song et al. 2010).

The previously cited 349 natural variation accessions screening (Baxter et al. 2010) showed that the leaf As concentrations range from 0.15 to 3.49 μg g^{-1} dry weight (Chao et al. 2014a). The genetic locus controlling natural variation in leaf accumulation of arsenic was identified as HAC1 (HIGH ARSENIC CONTENT 1) (Chao et al. 2014a), which was also independently identified by Sánchez-Bermejo et al. (2014) and named as ATQ1 (ARSENATE TOLERANCE QTL 1). This locus encodes an arsenate reductase, which is present in the root epidermis and pericycle. In the epidermis, HAC1 functions to reduce arsenate to arsenite, facilitating efflux of arsenic as arsenite back into the soil and limiting both its accumulation in the root symplast and transport to the shoot. In the pericycle, arsenate reduction by HAC1 may play a role in limiting arsenic loading into the xylem (Chao et al. 2014a).

Natural variation at the *AtHAC1* locus accounts for a significant proportion of the species-wide diversity in leaf arsenic accumulation in Arabidopsis when plants are grown in soil containing environmentally relevant trace concentrations of arsenic. Chao et al. (2014a) also identified a rare allele in Kr-0 accession, which accounts to elevated leaf As due to a nucleotide polymorphism that generates a non-functional AtHAC1 protein that is not present in any other accession screened to date. In roots of Col-0 wild-type plants 98% of the total accumulated arsenic is present as arsenite, whereas in roots of Kr-0 plants arsenite accounts for between 79 and 83% of total root arsenic. It is important to highlight that earlier studies proposed that plant ACR2 proteins, which are homologs of the yeast (*S. cerevisiae*) arsenate reductase, may be responsible for arsenate reduction in plant cells (Bleeker et al. 2006; Dhankher et al. 2006; Ellis et al. 2006; Duan et al. 2007). So another important finding provided by Chao et al. (2014a) is that ACR2 arsenate reductase in *A. thaliana* plays no detectable role in arsenic metabolism and does not interact epistatically with AtHAC1, since arsenic metabolism in the *acr2 hac1* double mutant is disrupted in an identical manner to that described for the *hac1* single mutant.

Rice is the most important dietary source of As (Mondal and Polya 2008; Meharg et al. 2009), and there is evidence linking high As exposure in rice with genotoxic effects in humans (Banerjee et al. 2013). The findings about the source of natural variation in Arabidopsis allowed the identification of rice orthologs in the last two years (Shi et al. 2016). The arsenate reductases *OsHAC1;1* and *OsHAC1;2* are

predominantly expressed in roots, with OsHAC1;1 being abundant in the epidermis, root hairs, and pericycle cells while OsHAC1;2 is abundant in the epidermis, outer layers of cortex, and endodermis cells of rice. Knocking out *OsHAC1;1* or *OsHAC1;2* decreased the reduction of arsenate to arsenite in roots, reducing arsenite efflux to the external medium, while double mutants showed greater effects. In contrast, overexpression of either *OsHAC1;1* or *OsHAC1;2* increased arsenite efflux, reduced As accumulation, and enhanced arsenate tolerance. When grown under aerobic soil conditions, overexpression of either *OsHAC1;1* or *OsHAC1;2* also decreased As accumulation in rice grain (Shi et al. 2016).

2.2.3 *Ionomics importance for understanding tolerance to salinity and evolution*

Soil salinity is an important source of stress that impacts plant growth and productivity, causing yield losses in crops around the world. It promotes dehydration in non-tolerant plants, due decreased water uptake by roots under this condition. Moreover, high concentrations of NaCl in tissues are toxic in most plant species. Considering that salinity is widespread in the environment, plants have evolved diverse mechanisms to regulate NaCl accumulation and keep the absorption of important nutrients that are present in lower concentrations in the soil (Munns and Tester 2008).

In a screening of 6000 fast-neutron-mutagenized Arabidopsis plants grown under unstressed conditions, Lahner et al. (2003) identified 51 mutants with altered shoot elemental profiles. One of these ionomic mutants was shown to harbor a deletion in *AtHKT1* (HIGH-AFFINITY POTASSIUM TRANSPORTER 1), responsible for the elevated shoot Na^+ phenotype of this mutant (Gong et al. 2004). *AtHKT1* is known to encode a Na^+ transporter localized in the vascular tissues throughout the plant, with expression being highest in the roots. This transporter regulates Na^+ accumulation in shoots by unloading Na^+ from the xylem in both roots and shoots and recycling Na^+ to the roots via the phloem (Uozumi et al. 2000; Rus et al. 2006; Berthomieu et al. 2003).The first genetic mapping of natural variation in Na concentrations in Arabidopsis was carried by Rus et al. (2006), who identified a novel *AtHKT1* allele from two Arabidopsis accessions, Ts-1 and Tsu-1, collected from coastal regions of Spain and Japan, respectively. The $AtHKT1^{Ts\text{-}1}$ and $AtHKT1^{Tsu\text{-}1}$ allele is responsible for the higher shoot Na^+ accumulation in these accessions compared with the reference accession Col-0 via the downregulation of *AtHKT1* expression in root, the primary site for HKT1 function in regulating shoot Na^+. A deletion in a tandem repeat sequence approximately 5 kb upstream of AtHKT1 (the promoter region) is the factor driving the differential expression of AtHKT1 in both accessions, resulting in enhanced NaCl tolerance. After that, several studies were conducted to better explain the HKT1 control of Na homeostasis in different species, such as *Solanum lycopersicum* L. (tomato) (Jaime-Pérez et al. 2016), *Vitis vinifera* L. (grapevine) (Henderson et al. 2018), *Triticum* spp. L. (wheat) (Xu et al. 2018), maize (Zhang et al. 2018), and even the carnivorous plant *Dionaea muscipula* Ellis—the "venus flytrap" (Böhm et al. 2016), in which *HKT1* expression and sodium uptake activity are induced upon prey contact.

Ionomics has also been applied to evolution studies regarding salt tolerance. Busoms et al. (2018) recently showed the role of naturally evolved *HKT1;1* allelic variant in the adaptation of Arabidopsis populations to fluctuating salinity dynamics in nature by resequencing 77 individuals from several salinity gradients along the Catalonian coast. The study integrated the obtained data with 1135 worldwide Arabidopsis genomes for a detailed understanding of the demographic and evolutionary dynamics of naturally evolved salinity tolerance. The authors found that genotypes holding the alleles responsible for low expression of *HKT1;1* in roots (leading to high leaf sodium phenotype—$HKT1;1^{HLS}$) were recent migrants that have moved specifically into Iberian areas where soil sodium levels fluctuate widely due to geography and rainfall variation. The proportion of plants expressing these alleles positively correlates with soil sodium level over time, so the plants are better adapted to salinity. Also, $HKT1;1^{HLS}$ plants clusters with high-sodium accumulator accessions worldwide, suggesting that *HKT1;1* is under fluctuating selection in response to climate variations and is a worldwide determinant in adaptation to saline conditions.

Genome doubling has been occurring during plant evolution and has driven the adaptive radiation of these organisms (Parisod et al. 2010). In this context, ionomics analysis was a key strategy used to explain

how plant polyploidization confers tolerance to salinity in Arabidopsis as a result of lower sodium/potassium concentration in tissues. During the analysis of the elemental composition of leaves from the aforementioned set of 349 Arabidopsis accessions (Baxter et al. 2010), the autotetraploid accession Wa-1 (from Warsaw, Poland) had the highest concentration of leaf potassium (K) and the K analogue rubidium (Rb) (Chao et al. 2013). The Wa-1 diploid obtained by haploid induction (Ravi and Chan 2010) showed reduced K and Rb concentrations, in relation to the tetraploid progenitor (Chao et al. 2013). The same phenotypic response was observed in haploids Col-0 and Ler—also prepared by haploid induction (Ravi and Chan 2010), compared to diploid progenitors (Chao et al. 2013). By using colchicine, Chao et al. (2013) also induced tetraploidy in eight natural diploids and the same phenotype was observed: the higher the ploidy, the higher K and Rb content in leaves. The reciprocal grafting technique (Rus et al. 2006) revealed that leaf K is controlled by root ploidy, independently of the ploidy of the shoot. The work conducted by Chao et al. (2013) also reinforced that plants harbouring higher potassium/sodium ratios are more tolerant to salinity (Munns and Tester 2008): tetraploid (Wa-1) grown in nutrient media supplemented with 200 mM NaCl showed increased rate of survival compared to diploids (Col-0 and Ler). The genotype Wa-1 also produced significantly more seeds under elevated salinity, proving that polyploidy can represent a reproductive advantage in saline environments (Chao et al. 2013).

3 Analytical technologies required for ionomics studies

Ionomics requires the application of high-throughput elemental analysis technologies that allow the measurement of elemental content and/or distribution in the organism of interest. The choice of the suitable technique is determined by several factors including the expected sample throughput, the elements to be measured, dynamic quantification range, sensitivity of the instrument, the available amount of sample, complexity of sample preparation, reliability, cost per sample as well as required resolution of elemental distribution (Salt et al. 2008). Various techniques can be used to measure the elemental composition, however, the most common methods used to perform the simultaneous quantification of multiple elements are Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), X-Ray Fluorescence (XRF), Synchrotron based micro X-Ray Fluorescence (micro-SXRF), Laser-Induced Breakdown Spectroscopy (LIBS), and Neutron Activation Analysis (NAA).

3.1 Inductively coupled plasma techniques

In Inductively Coupled Plasma (ICP) techniques, the high-temperature plasma is used to ionise the analyte atoms for their detection by either Optical Emission Spectroscopy (ICP-OES)—also called Atomic Emission Spectroscopy (ICP-AES)—or by Mass Spectrometry (ICP-MS). Both ICP-OES and ICP-MS allow simultaneous quantification of up to 60 elements in the sample. Generally, the samples analysed by these ICP techniques must be liquid which require transforming solid samples such as leaf tissue or grains into the liquid. It is achieved by performing an acid digestion in a heat block that allows digesting hundreds of samples in relatively very short time. Both ICP-OES and ICP-MS instruments offer a high sample throughput as they can be used with autosamplers, so the multielement analysis of one sample can be performed in less than 5 min. Therefore, these systems enable the efficient analysis of large sample batches, which is ideal to perform an ionomic profiling of thousands of plants (Danku et al. 2013). For example, the ICP-OES was used to determine the elemental content of approximately 4385 mutant yeast strains over 2 years (Eide et al. 2005), to study the changes in elemental profiles in maize plants exposed to neutral and alkaline salt stress (Guo et al. 2017), and ionomic responses under salt stress in wild and cultivated barley plants (Wu et al. 2013). Some researchers hired ICP-OES and ICP-MS together to determine the concentration of 29 major and trace elements in roots, stems and leaves of *Nicotiana langsdorffii* Weinm. wild-type and mutant genotypes exposed to abiotic stresses (Ardini et al. 2013).

Although ICP-OES is an excellent tool for high throughput elemental profiling, it is ICP-MS that has become the most largely used tool to study the plant ionome over last 15 years, since the concept of ionome was introduced (Lahner et al. 2003). The main

advantages of ICP-MS over ICP-OES is its greater sensitivity, that allows element detection at ng/g (parts-per-trillion) level, where the lower detection limit for ICP-OES is µg/g (parts-per-billion, ppb). Also, a wide dynamic range which for cutting edge ICP-MS instruments is up to 12 orders of magnitude, where ICP-OES instruments offer only up to 6 order of magnitude. These differences allow the use of smaller sample sizes for ICP-MS analysis and eliminate the need of sample dilution even if a sample contains analytes of great differences in concentration as wider dynamic linear range allows determination of low and high concentrated elements at the same time. Other advantages of ICP-MS over ICP-OES are that individual isotopes can be measured, and the use of collision cell technology allows the efficient removal of polyatomic spectral interferences that used to be one of the main drawbacks of ICP-MS technique. Therefore, ICP-MS was hired multiple times to perform high throughput elemental profiling of many plants including Arabidopsis (Lahner et al. 2003; Campos et al. 2017), rice (Norton et al. 2010; Zhang et al. 2014; Pinson et al. 2015), maize (Baxter et al. 2013, 2014; Mascher et al. 2014), soybean (Ziegler et al. 2013), rapeseed (Thomas et al. 2016), *Lotus japonicus* (Regel) K. Larsen (Chen et al. 2009), and vegetable crops including carrot, radish, turnip, komatsuna, bok-choy, nabana, napa cabbage, garland chrysanthemum, podded pea, green pea and kidney bean (Watanabe et al. 2016).

The ICP-MS can be also coupled to laser ablation introduction system (LA-ICP-MS) that allows acquiring information about the ionome of solid sample by using a focused laser beam in an argon (Ar) atmosphere. It ablates the sample material, which is then transported with the argon carrier gas to the plasma of ICP-MS. The lateral resolution of LA-ICP-MS imaging is depended on the spot size of the focused laser beam and it ranges from 50 to 300 µm for the common laser ablation system (Wu and Becker 2012). Although, LA-ICP-MS imaging does not have the spatial resolution of other imaging techniques such as scanning electron microscopy or X-ray microanalysis, but it presents enough spatial distribution for qualitative and quantitative imaging of metals and metalloids and quite often it does not require any sample preparation. It presents also high sensitivity, large linear responses as well as the ability for isotopic analysis (Pozebon et al. 2017). Therefore, LA-ICP-MS has been successfully applied for qualitative and quantitative elemental mapping of biological tissues such us rodent brain sections (Hare et al. 2017). However, there are not that many applications of LA-ICP-MS in plant sciences, probably due to the fact that the laser ablation process takes place in helium (He) or Ar atmosphere with a certain flow rate which may cause dehydration of the sample during the measurement, thus affecting the ion intensity signals of measured by ICP-MS analytes (Wu and Becker 2012). Therefore, imaging of elements in mainly low water plant tissues by LA-ICP-MS has been reported by different authors in recent years, such as for quantitative imaging of nutrients in the leaves of *Elsholtzia splendens* Nakai ex F.Maek. (Wu et al. 2009), multi-elemental bioimaging of durum wheat grains (Persson et al. 2016a), evaluation of the translocation and accumulation of metals including Cd, Cu, Fe and Mn in sunflower seeds (Pessoa et al. 2017), and determination of changes in the mineral composition and distribution within Se biofortified olives (D'Amato et al. 2018). However, new techniques are being developed to allow the imaging of elements in the plants tissues with high water content (Persson et al. 2016b). Although, LA-ICP-MS is gaining more interest for multielement imaging of different plant tissues over last years, it has not been used yet in the high-throughput manner for ionomics screening of arrayed samples.

3.2 Laser-induced breakdown spectroscopy

Another type of atomic emission spectroscopy, Laser-Induced Breakdown Spectroscopy (LIBS), allows the determination of elemental content of samples in the form of solids, liquids, gels, gases, plasmas and biological material such as blood, teeth or *leaf*. In this technique, the highly focused laser beam is used to excite the atoms in the sample. When atoms are coming back to the lower energy state they emit the light of characteristic for each element frequencies that is detected and related by the use of certified reference material to the concentration of an element (Singh and Thakur 2007). Although LIBS is still a new and little explored for measuring nutrients in plants, it has started gaining more attention over recent years (Van Maarschalkerweerd and Husted 2015). LIBS was successfully used to measure the concentration of Ca, Mg, P, K, Na, B, Fe, Cu, Mn, Zn and other elements in

leaves of different plants such as sugarcane, soy, citrus, coffee, maize, wheat, barley, rape, poppy, eucalyptus, mango, bean, banana, lettuce, brachiaria, pearl millet, grape, rubber tree, tomato, cucurbit seeds and many others (Pouzar et al. 2009; Nunes et al. 2010; de Carvalho et al. 2015; Singh et al. 2017). LIBS was also used as a diagnostic tool to monitor the changes in the content of Ca, K, Na and Fe for rapid detection of drought stress in wheat and gardenia (Kunz et al. 2017) and to identify the nutritional changes provoked by bacterial disease in the citrus leaves and soybean (Ranulfi et al. 2017, 2018).

3.3 X-ray fluorescence

X-ray fluorescence (XRF) is a spectroscopic technique that allows the measurement of concentration of multiple elements in biological samples. In this technique the sample is exposed to high energy X-rays or gamma rays that excite the atoms in the sample, and during the relaxation they emit the X-rays of lower energy. The energy and intensity of emitted light is characteristic for each element, therefore, it can be used to detect and quantify elements in a complex mixture. The sensitivity of XRF increases with the atomic number, thus trace elements and heavy metals or metalloids are easily detectable even in very low concentrations, with the limits of quantifications down to a few parts-per-million (ppm) for the heaviest elements. In order to quantify elements such as S, P, K, Mg, Ca, Na and Cl, higher concentrations of these elements are required in the sample. Generally, XRF is a non-destructive method that requires minimal sample preparation, therefore, the analysis can be performed on hydrated living plants in situ. However, because the measurement can be affected by the particle size and sample density and homogenous sample produce more precise analytical data, the samples e.g. leaf material are often ground and prepared as flat disc before the analysis (Van Maarschalkerweerd and Husted 2015). Ten years before the concept of the ionome was defined and introduced, XRF was successfully used for the first multi-elemental screening of over 100,000 mutagenized Arabidopsis seedlings, identifying three mutants with altered ionomes (Delhaize et al. 1993). A synchrotron-based XRF was also used for a high-throughput determination of Mn, Fe, Ni, Cu and Zn concentrations in whole intact Arabidopsis seeds. A rapid screening proof-of-concept experiment demonstrated that XRF could be used for identification of seeds with mutant ionomic phenotypes (Young et al. 2006). Recent applications of XRF techniques are focused on the optimisation of methodology that will allow quantification of elemental content in low amount of biological material or in high-throughput manner. For example, the concentrations of P, Si and Cl were determined by XRF in the random subset of 84 rice mutagenized plants (Sevanthi et al. 2018), while the total reflexion X-ray fluorescence (TXRF) was used for rapid and simultaneous quantification of K, Ca, S, Mn, and Sr in Arabidopsis leaf in the amount of tissues as low as 0.3 mg of dry weight (Hohner et al. 2016). The energy dispersive X-ray fluorescence (EDXRF) was optimised to allow a high-throughput multi-elemental profiling of grains at low cost. This methodology was used to determine the concentration of K, Mg, P and Fe in grains of 266 Afghan wheat landraces that allowed the identification of the best wheat genotypes in terms of elemental content that can be used in the crop improvement (Kondou et al. 2016). It was also demonstrated that the EDXRF has an ability to accurately measure the concentration of Na, Mg, P, S, Cl, K, Ca, Fe, Mn, Cu, Zn in forages such as alfalfa hays, grass hays and corn silages, therefore it can be used as cost-effective and simple method in small forage laboratories (Berzaghi et al. 2018). The XRF techniques also allow the imaging of element distribution in biological tissues. Synchrotron XRF was used to obtain the elemental maps of As, Fe, Zn, Mn, Cu, K, Ca and Ni in rice grains (Punshon et al. 2018), seeds of Arabidopsis (Ricachenevsky et al. 2018b) and *Nicotiana tabacum* L. (tobacco) (Hermand et al. 2014) as well as leaves of *Medicago trunculata* (Punschon et al. 2013). Whereas, μ-XRF allowed the localisation and a semi-quantitative evaluation of content of As, Zn, Fe, Mn, Ca, K, S, P, Mg and Si in tobacco seedlings (Capobianco et al. 2018). Similarly to ICP techniques, XRF techniques present the throughput required for ionomics screen and additionally allow the non-destructive elemental profiling that with portable device can be performed in the greenhouse or in the field.

3.4 Neutron activation analysis

The neutron activation analysis (NAA) is a nuclear process used for qualitative and quantitative elemental

analysis of unknown, bulk samples. In NAA, the sample is irritated with neutrons which causes formation of radionuclides that decay with time, emitting characteristic gamma radiation that can be detected, and gamma rays at a particular energy are indicative of the presence of a specific radionuclide. Therefore, the analysis of gamma-ray spectra yields the concentration of various elements in the samples. NAA is a non-destructive method allowing simultaneous determination of about 25-30 major and trace elements in geological, environmental and biological samples in ppb-ppm range. The analysis by NAA can be performed on the sample without its prior preparation, however, the main drawback of this technique is that it requires access to a nuclear reactor as a source of neutrons. Nevertheless, NAA has been used for elemental analysis of plants for more than 45 years (Salt et al. 2008; Singh et al. 2013). NAA was used to determine the concentration of multiple elements in the leaves and roots of many different medicinal plants (Yamashita et al. 2005; Lokhande et al. 2010; Fei et al. 2010) and to determine the concentration of Mn, K, As and Br in Thai jasmine rice grown in different cultivation areas in the northeast Thailand (Kongsri et al. 2016). NAA combined with Laser-Induced Breakdown Spectroscopy (LIBS) was also used to determine the ions present in the leaves of *Populus trichocarpa* Torr. and A.Gray ex. Hook. (Martin et al. 2017). However, in comparison to other techniques such as ICP-MS, ICP-OES and XRF, NAA has not yet been used for a high-throughput elemental profiling of different mutants, genotypes, and accessions.

4 Trends in ionomics

The concept of the ionome has been initially applied to study the biology and homoeostasis of nutrients in different plant species and to *S. cerevisiae* with an analysis of knockout and overexpression alleles of all identified genes in the yeast genome (Eide et al. 2005; Yu et al. 2012). However, recently, the study of the ionome has been also embraced by researchers from many different fields. The ionomics studies have been reported in bacteria (Muller et al. 2016), fish (Yoshida et al. 2014; Wei et al. 2018), different mammalian species including horse, pig, cattle, goat, cat, dog, rat, mouse, hamster (Ma et al. 2015; Hadsell et al. 2018) as well as humans (Gonzalez-Dominguez et al. 2014; Konz et al. 2017).

Today the developments in cell biology move our mechanistic understanding of biological processes to the cellular level, and single cell analysis is becoming a new frontier in 'omics' (Wang and Bodovitz 2010). Substantial progress has been made in the areas of single-cell genomics (Kashtan et al. 2014; Hemberg 2018), transcriptomics (Tang et al. 2011) and metabolomics (Misra et al. 2014; Fessenden 2016). For example, single cell genotyping can identify abnormal, mutated cells in a tissue or organism, which has a great potential for some diagnostic applications, especially to identify cancer cells or genetic defects in oocytes and sperms used for in vitro fertilisation (Galler et al. 2014). The determination of elemental content of the single cells is becoming of the high importance in the light of recent findings showing that the contents of the essential elements e.g. Fe or Cu in biological tissues or cell are associated with various patho-physiological conditions (Wang et al. 2015). Concerning the plant science, information about the elemental content of single cells could substantially increase our knowledge of mineral nutrient and trace element homeostasis in plants by delivering a comprehensive insight into the processes occurring at the cellular level and giving a more accurate representation of cell-to-cell variation. Recently, single-cell transcriptome profiling of $\sim$ 4000 cells of Arabidopsis roots has been reported (Shulse et al. 2018). Similarly, single-cell ionomics should be possible given the current developments in the field.

Although the ionome concept was proposed 15 years ago, the field has been developing mostly for model species, all which have sequenced genomes and allow mapping of causative genes of interesting traits easily. With the increased capacity to access genomic information for non-models, ionomics should be performed for many more plants species, helping to understand conserved and unique adaptations and mechanisms for elemental distribution and accumulation in comparison to the standard, well-explored models Arabidopsis and rice. It is important to highlight that ionomics approaches can be applied even to non-model species that lack genetic resource such as sequenced genomes, transcriptomes, mutant databases, and established protocols. In particular, ionomics approaches aiming the genus *Oryza*, which has now 11 species with available genomes (Ohyanagi

et al. 2016), plus more than 3,000 rice cultivars sequences (Wang et al. 2018), could be successful in finding new alleles useful for biofortification, food safety and stress tolerance (Ricachenevsky and Sperotto 2016; Menguer et al. 2017). Another focus will be plant groups with unique adaptations such as macrophytes, which can have small, tractable genomes, are easily maintained and are ubiquitous in the environmental, and thus should have great diversity for natural variation studies (Wang et al. 2014; Wiegleb et al. 2015). Since the data discussed here suggests selection is acting at the same loci and resulting in similar ionomic phenotypes across species, the information described here comprises a powerful pathway to translate discoveries in Arabidopsis into crops and other plants (Huang and Salt 2016).

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