Plant Ionomics: From Elemental Profiling to Environmental Adaptation

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ABSTRACT

Ionomics is a high-throughput elemental profiling approach to study the molecular mechanistic basis underlying mineral nutrient and trace element composition (also known as the ionome) of living organisms. Since the concept of ionomics was first introduced more than 10 years ago, significant progress has been made in the identification of genes and gene networks that control the ionome. In this update, we summarize the progress made in using the ionomics approach over the last decade, including the identification of genes by forward genetics and the study of natural ionomic variation. We further discuss the potential application of ionomics to the investigation of the ecological functions of ionomic alleles in adaptation to the environment.

Key words: nutrient homeostasis, natural variation, ionomics, Arabidopsis thaliana, Casparian strip, adaptation


INTRODUCTION

The ionome is defined as “the mineral nutrient and trace element composition of an organism, and represents the inorganic component of cellular and organismal systems” (Salt et al., 2008). Since the concept of the ionome was first proposed more than 10 years ago (Lahner et al., 2003), significant progress has been made in the field of ionomics, in which high-throughput elemental profiling is combined with genetics to identify the genes that control the ionome (Danku et al., 2013). A milestone study in the development of ionomics was carried out in the genetic model plant Arabidopsis thaliana (Lahner et al., 2003; with commentaries by Hirschi, 2003 and Rea, 2003). In this study inductively coupled plasma mass spectrometry (ICP-MS) was used to analyze the leaf ionome of many thousands of plants, leading to the identification of multiple ionomic mutants. Interestingly, only 11% of the mutants identified showed alterations in a single element, supporting the concept that ionomic networks in plants are coordinately regulated and need to be viewed as a whole. This was further reinforced by the discovery of ionomic regulatory networks involved in both Fe and P homeostasis (Baxter et al., 2008b), and this concept was recently reviewed by Baxter (2015). Ionomic studies have now been performed in many plant species, including rice (Norton et al., 2010; Zhang et al., 2014; Pinson et al., 2015), maize (Baxter et al., 2013, 2014; Mascher et al., 2014; Gu et al., 2015), barley (Wu et al., 2013), soybean (Ziegler et al., 2013), Lotus japonica (Chen et al., 2009), tomato (Sanchez-Rodriguez et al., 2010), and others (Watanabe et al., 2007; White et al., 2012; Parent et al., 2013). The concept of the ionome has also been applied to Saccharomyces cerevisiae with an ionomic analysis of knockout and overexpression alleles of all identified genes in the yeast genome (Eide et al., 2005; Yu et al., 2012). Recently, ionomic studies in humans (Sun et al., 2012; Malinouski et al., 2014) and other animals (Yoshida et al., 2014; Ma et al., 2015) have also been reported. By coupling genetics with high-throughput elemental profiling, ionomics has led to the identification of numerous genes controlling the ionome. Furthermore, ionomics has allowed the discovery of genes controlling natural variation in the plant ionome. The application of technical advances in genetic mapping approaches such as deletion mapping (e.g., Baxter et al., 2009) and DNA microarray-based bulk segregant analysis (BSA) (e.g., Gong et al., 2004; Chao et al., 2011) has greatly aided in the success of ionomics, and the application of mapping by sequencing (e.g., Kamiya et al., 2015) is set to further accelerate these successes. Meanwhile, technical advances in imaging the cellular and subcellular localization of elements has started to allow a cell-type and tissue-specific understanding of the ionome (reviewed by Zhao et al., 2014).

In this update, we highlight progress over the past decade in the use of ionomics to identify genes controlling the ionome (Table 1). We also discuss the potential application of this approach to characterize the functions of natural ionomic loci in adapting to the edaphic (soil) environment.
IDENTIFICATION OF IONOMIC GENES BY FORWARD GENETICS

A systematic screen for mutants with altered leaf ionomic profiles was carried out by Lahner et al. (2003). In this proof-of-concept screen, 338 putative mutants with alterations in the leaf accumulation of single or multiple elements were identified from a screen of 4747 M2 fast neutron (FN) mutant plants. Of these 338 putative mutants, 51 were confirmed after rescreening of all M3 families. Chen et al. (2009) also performed a similar large ionomic screen of 2000 mutagenized M2 *Lotus japonica* plants. In such large experiments, plants have to be grown in different experimental blocks (e.g., plant cultivation trays) repeatedly over many months to allow the harvesting and analysis of thousands of plant samples. It is notable that the ionome may also vary at different growth stages. To be successful it is thus essential to minimize the variation introduced from the growth media (such as different batches of soil), the growth stage, and the growth environment. In our screen of FN mutagenized *A. thaliana*, the wild-type and the known ionomic mutant *frd3* were included in each plant growth tray as controls and plants were harvested at 5 weeks old.

### Table 1. Summary of Ionomic Genes.

<table>
<thead>
<tr>
<th>Mutant/accession name</th>
<th>Ionomic phenotype</th>
<th>Causal gene</th>
<th>TAIR ID</th>
<th>Gene description</th>
<th>Identification approach</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>frd3</td>
<td>High Mn and Co</td>
<td>FRD3</td>
<td>AT3G08040</td>
<td>Citrate transporter</td>
<td>Forward genetics</td>
<td>Delhaize, 1996; Rogers and Guerinot, 2002; Durrett et al., 2007</td>
</tr>
<tr>
<td>esb1-1</td>
<td>Low Ca and Mn; high Na, S, K, As, Se, and Mo</td>
<td>ESB1</td>
<td>AT2G28670</td>
<td>Dirigent domain-containing protein</td>
<td>Forward genetics</td>
<td>Baxter et al., 2009; Hosmani et al., 2013</td>
</tr>
<tr>
<td>myb36-1</td>
<td>Low Ca, Mn, and Fe; high Na, Mg, and Zn</td>
<td>MYB36</td>
<td>AT5G57620</td>
<td>MYB domain transcription factor</td>
<td>Forward genetics</td>
<td>Kamiya et al., 2015</td>
</tr>
<tr>
<td>tsc10a-1</td>
<td>Low Mg, Ca, Fe, and Mo; high Na, K, and Rb</td>
<td>TSC10A</td>
<td>AT3G06060</td>
<td>3-Ketodihydrospargagine reductase</td>
<td>Forward genetics</td>
<td>Chao et al., 2011</td>
</tr>
<tr>
<td>sgn3</td>
<td>Low K; high Mg</td>
<td>SGN3</td>
<td>AT4G20140</td>
<td>Receptor-like kinase</td>
<td>Forward genetics</td>
<td>Pfister et al., 2014</td>
</tr>
<tr>
<td>sgn1</td>
<td>High Mg</td>
<td>SGN1</td>
<td>AT1G61590</td>
<td>Kinase</td>
<td>Forward genetics</td>
<td>Alissimone, J., Fujita, S., et al., unpublished results</td>
</tr>
<tr>
<td>nakr1-1/npcc6</td>
<td>High Na, K, and Rb</td>
<td>NaKR1</td>
<td>AT5G02600</td>
<td>Metal binding protein</td>
<td>Forward genetics</td>
<td>Tian et al., 2010</td>
</tr>
<tr>
<td>cpr5</td>
<td>Low K</td>
<td>CPR5</td>
<td>AT5G64930</td>
<td>Constitutive expression of pathogen resistance</td>
<td>Forward genetics</td>
<td>Bortolino et al., 2011</td>
</tr>
<tr>
<td>Ts-1, Tsu-1</td>
<td>High Na</td>
<td>HKT1;1</td>
<td>AT4G10310</td>
<td>Sodium transporter</td>
<td>Natural variation</td>
<td>Rus et al., 2006; Baxter et al., 2010</td>
</tr>
<tr>
<td>Ts-1, Se-0</td>
<td>High Co</td>
<td>FPN2</td>
<td>AT5G03570</td>
<td>Ferroportin metal efflux protein</td>
<td>Natural variation</td>
<td>Morrissey et al., 2009</td>
</tr>
<tr>
<td>Shahdara, Hod</td>
<td>High sulfate, S, and Se</td>
<td>APR2</td>
<td>AT1G62180</td>
<td>5’-Phosphosulfate reductase</td>
<td>Natural variation</td>
<td>Loudet et al., 2007; Chao et al., 2014b</td>
</tr>
<tr>
<td>Bay-0</td>
<td>High sulfate</td>
<td>ATPS1</td>
<td>AT3G22890</td>
<td>ATP sulfurylase</td>
<td>Natural variation</td>
<td>Koprivova et al., 2013</td>
</tr>
<tr>
<td>Ler-0, Shahdara</td>
<td>Low Mo</td>
<td>MOT1</td>
<td>AT2G25680</td>
<td>Molybdenum transporter</td>
<td>Natural variation</td>
<td>Tomatsu et al., 2007; Baxter et al., 2008a; Poormohammad Kiani et al., 2012</td>
</tr>
<tr>
<td>CS28181</td>
<td>Low Cd</td>
<td>HMA3</td>
<td>AT4G30120</td>
<td>Heavy metal ATPase</td>
<td>Natural variation</td>
<td>Chao et al., 2012</td>
</tr>
<tr>
<td>Kas-1; Kr-0</td>
<td>High As</td>
<td>ATQ1/HAC1</td>
<td>AT2G21045</td>
<td>Arsenate reductase</td>
<td>Natural variation</td>
<td>Sanchez-Bermejo et al., 2014; Chao et al., 2014a</td>
</tr>
</tbody>
</table>

### Molecular Plant

Plant Ionomics
Figure 1. Hierarchical Clustering of *A. thaliana* FN Mutants and Natural Accessions Based on the Leaf Ionome.
The clustering was performed using the percentage difference from Col-0 grown in the same cultivation tray.

(A) Hierarchical clustering of *A. thaliana* FN mutants. The causal genes for cloned mutants are listed on the top, with the same color highlighting the mutants in the first row. Different soil batches or plant cultivation trays in which the mutants were grown are shown in different colors in the second and third rows, respectively. The remaining rows represent the elements quantified in each mutant. The wild-type Col-0 and the frd3 mutant were used as controls and grown in all plant cultivation trays.

(B) Hierarchical clustering of 349 *A. thaliana* natural accessions. Three accessions, Fab-2, Cvi-2, and Ts-1, were grown in each plant cultivation tray as controls and are highlighted by various colors in the first row. The second row highlights the accessions with causal genes that have been cloned, and accessions are shown in the same colors as the elements and causal gene names in the far right column. Different colors in the third row indicate different plant cultivation trays. The remaining rows represent the elements quantified in each accession.
reinforcing the conclusion that mutants with a similar ionomic phenotype are reliably clustered together based on this phenotype.

The enhanced suberin1-1 (esb1-1) mutant (Baxter et al., 2009) is the first ionomic mutant to be cloned from the forward genetic screen reported by Lahner et al. (2003), who originally named it 145:01. ESBI encodes a dirigent-domain-containing protein. Mutation of ESBI causes the disruption of the normal deposition of lignin required for Casparian strip formation, leading to ectopic deposition of lignin and suberin at the endodermis (Hosmani et al., 2013; with commentary by Halpin, 2013). The esb1 mutant displays a multielement ionomic phenotype, with accumulation of lower concentrations of Ca, Mn, and Zn and higher levels of Na, S, K, As, Se, and Mo in leaves. A second mutant isolated by Lahner et al. (2003) named 112:50, which also shows a complex multielement ionomic phenotype, was recently identified as myb36-1 (Kamiya et al., 2015; with commentary by Franke, 2015). Detailed analysis revealed that the transcriptional regulator MYB36 directly regulates the expression of the genes involved in the formation of Casparian strips, including ESBI, Casparian strip domain proteins (CASPs), and Peroxidase 64 (PER64) (Kamiya et al., 2015). Loss of function of MYB36 leads to the absence of Casparian strips and deposition of ectopic lignin and suberin at the endodermis. These changes lead to an elevated concentration of Na, Mg, and Zn, and a decreased concentration of Ca, Mn, and Fe in the leaves (Kamiya et al., 2015). Ectopic expression of MYB36 is sufficient to build Casparian strip-like structures in the cortex and the epidermis. Even though the Casparian strip-like structures are not perfectly formed, it would be interesting to determine whether and how such additional structures affect the leaf ionome. The tsc10a-1 mutant (Chao et al., 2011), which alters sphingolipid biosynthesis, is another mutant showing enhanced suberin deposition in the root from the screen of Lahner et al. (2003), in which it was originally named 71:13. Even though the detailed mechanism of enhanced suberin deposition in this mutant is not clear, the tsc10a mutant shows a clearly altered leaf ionome, including increases in Na, K, and Rb and decreases in Mg, Ca, Fe, and Mo (Chao et al., 2011). Given the significant role the Casparian strip is now known to play in regulating the leaf ionome (Baxter et al., 2009; Hosmani et al., 2013; Pfister et al., 2014; Kamiya et al., 2015), it would be of great interest to determine whether and how such additional structures affect the leaf ionome. The tsc10a mutant and investigate whether TSC10A is required for the formation of Casparian strips. In a forward genetic screen for endodermal barrier mutants, the schengen3 (sgn3) mutant was isolated, which displayed a defect in Casparian strip formation (Pfister et al., 2014). Unlike the Casparian strip mutants esb1, myb36, and the double mutant casp1 casp3 (Hosmani et al., 2013), sgn3 only has a Casparian strip defect and no ectopic endodermal suberin or lignin. The sgn3 mutant shows several ionomic alterations, including low K and high Mg in leaves (Pfister et al., 2014). Interestingly, the Lahner et al. (2003) screen also identified the 117:20 mutant with reduced K and increased Mg. This mutant has recently been identified as a new allele of sgn3 (Hermans, C., and Salt, D.E., unpublished results) (Figure 1A), further highlighting the importance of the Casparian strip in controlling the leaf ionome. The fact that alteration of a common biological system, such as endodermal diffusion barriers, leads to similar ionomic phenotypes raises the intriguing possibility that hierarchical clustering of ionomic mutants could help to identify novel mutants in the same biological pathway.

This is supported by the fact that different alleles of esb1, myb36, and tsc10a cluster together independent of the plant cultivation tray or soil batch in which they were cultivated (Figure 1A). Uncharacterized mutants that cluster with mutants disrupted in known processes of interest, such as Casparian strip development, could therefore be prioritized for cloning.

As discussed above, many ionomic mutants have turned out to be Casparian strip mutants, indicating the critical role of Casparian strips in maintenance of the ionome in plants. It is now becoming clear that the Casparian strip-beating endodermis acts as a barrier in plant roots to control the selective entry of water and mineral nutrients into the vascular system and translocation to the shoot (Geldner, 2013). The endodermis not only prevents mineral nutrients entering the stele from the cortex, but also stops the leak of mineral nutrients from the stele back to the cortex. When Casparian strips are not formed correctly, ions at high concentration in the cortex and low concentration in the stele will leak into the stele, whereas ions that are high in the stele and low in the cortex will leak out of the stele. Therefore, the complex changes observed in the leaf ionome in Casparian strip mutants are likely to be dependent on the gradient of ions across the endodermis (Kamiya et al., 2015). Loss of SGN3 function causes Casparian strips with gaps to form, leading to reduced leaf K and increased Mg, suggesting that the concentration of K is normally higher in the stele compared with the cortex, while the concentration of Mg is lower in the stele relative to the cortex. The ionomic changes of esb1 and myb36 are complex, which reflects the fact that these two mutants not only have defects in Casparian strip formation but also compensatory ectopic deposition of lignin and suberin at the endodermis. Such ectopic deposition of lignin and suberin would be expected to affect both apoplastic and transmembrane ion transport across the endodermis. Indeed, recent studies have shown that endodermal suberization is dynamic and responsive to mineral nutrient status (Barberon et al., 2016).

The forward genetic screen for ionomic mutants reported by Lahner et al. (2003) also identified several non-Casparian strip mutants. The nakr1-1 (for sodium potassium root defective 1-1, also called nppc6), reported by Lahner et al. (2003) as 136:31, is one of these mutants. NAKR1 encodes a soluble metal binding protein and is required for the loading of Na and K into the phloem (Tian et al., 2010). The mutant nakr1-1 accumulates higher level of Na, K, and Rb, although the molecular function of NakR1 remains unknown. Another mutant from this screen named 126:45 was identified as a new loss-of-function allele of CONSTITUTIVE EXPRESSER OF PATHOGENESIS RELATED GENES 5 (CPR5), which accumulate low K in leaves. The low-K phenotype is independent of both the elevated salicylic acid and jasmonic acid that is required for the local resistance to pathogens in the cpr5 mutants (Borgli et al., 2011). The molecular mechanisms underlying the link between CPR5 and the low-K phenotype are currently unclear. However, increased expression of various CYCLIC NUCLEOTIDE GATED CHANNELS (CNGCs) in the shoot of cpr5 and decreased expression of the high-affinity potassium transporter HAK5 in the roots may constitute part of the mechanism (Borgli et al., 2011).

An additional forward genetic screen of 3162 EMS mutagenized plants (D.E. Salt, unpublished results), following the protocol of
Lahner et al. (2003), but with plants grown under either Fe or phosphate deficiency, identified a further 36 leaf ionomic mutants. Genetic analyses of these mutants has identified new alleles of BRUTUS (Hindt, M.N., et al., unpublished results), SOS1 (Brazelton, J., and Salt, D.E., unpublished results), and MOT1 (Andreotta, M.A., and Salt, D.E., unpublished results). Recent work from the laboratory of Niko Geldner (Alassimone, J., Fujita, S., et al., unpublished results) has identified Schengen-1 as a kinase that plays a critical role in Casparian strip formation. Interestingly, analysis of two high Mg mutants identified in our recent screen revealed them both to be loss-of-function allele of sgn1 (Kamiya, T., and Salt, D.E., unpublished results). Work on novel mutants with high leaf S and high leaf Na from this same screen is also ongoing to identify and characterize the genes involved.

Importantly, the bulk segregant approach in F2 mapping populations has also failed to work. Generally this has been due to a lack of penetrance of the ionomic mutant phenotype in F2 progeny of an outcross mapping population. This suggests that natural variation in the outcross parent can suppress the mutant phenotype. The recent use of background mutations in the mutagenized line as markers for mapping (Abe et al., 2012) means that a backcrossed F2 mapping population can now be used, overcoming issues of low mutant phenotype penetrance in an outcrossed F2 population.

**IDENTIFICATION OF IONOMIC ALLELES FROM NATURAL ACCESSIONS**

Genetic variation in natural accessions provides abundant resources for identification of quantitative trait locus (QTL) and causal genes. In our study of 349 A. thaliana accessions from the HapMap population (Baxter et al., 2010), we observed large variation in most of the 19 elements we quantified (Figure 1B). Unsupervised hierarchical clustering of these accessions based on their leaf ionomic phenotypes revealed that the clustering of accessions is largely independent of the experimental blocks (plant cultivation trays) in which they were grown. Rather, the accessions tend to be clustered together based on their ionomic profiles. This is supported by the clustering of the three accessions Fab-2 (low Zn), Cvi-2 (high Mo), and Ts-1 (high Na) with known leaf ionomic profiles that were included in each plant cultivation tray as controls (Figure 1B). The robustness of this clustering is further supported by the fact that chemical analogs, such as S and Se, and K and Rb, which are known to co-vari in A. thaliana (Broadley et al., 2010), are grouped together, as would be expected for elements that are likely regulated by the same processes in plants. The macronutrients Ca and Mg are also known to be co-regulated in A. thaliana (Broadley et al., 2010), and these elements are also grouped together in our clustering (Figure 1B). Taken together, these results affirm further validation of the high-throughput ionomic approach, providing justification for the use of this set of natural accessions for identification of ionomic QTLs and causal genes.

Traditional linkage mapping or QTL mapping using biparental crosses made between accessions from the phenotypic extremes have been successful in identifying genes responsible for variation in several ionomic traits. The HKT1;1 gene encoding an Na transporter was identified to be the causal gene controlling high leaf Na in the A. thaliana accessions Ts-1 and Tsu-1 (Rus et al., 2006). Reduced expression of HKT1;1 is responsible for the overaccumulation of Na in the leaves of these accessions, possibly driven by a deletion in the promoter (Rus et al., 2006; Baxter et al., 2010). Whether variation in the coding regions of HKT1;1 also plays a role is unclear. Using a similar approach, a polymorphism in the coding region of FPN2 (also known as IREG2), a ferroportin upregulated by Fe deficiency (Schaaf et al., 2006), was identified to be causal for variation in leaf Co in accessions Ts-1 and Se-0 (Morrissey et al., 2009). Further work suggested that this variation in FPN2 is involved in alterations in Fe homeostasis (Morrissey et al., 2009). Mapping using DNA microarray-based BSA in phenotyped F2 populations has also been successful at identifying Adenosine 5’-Phosphosulfate Reductase2 (APR2) as the causal gene for variation in total leaf S and Se in the Hod A. thaliana accession (Chao et al., 2014b). The variation in amino acid sequence of APR2 changes the enzymatic rate, resulting in variation in sulfate and selenate reduction and higher accumulation of S and Se. Furthermore, an analysis of APR2 alleles across 855 A. thaliana accessions revealed 11 haplotypes with amino acid changes in conserved regions of the protein. Enzymatic analysis revealed that activity of APR2 varies by four orders of magnitude across the A. thaliana species. However, the reason for such variation remains unknown (Chao et al., 2014b). Interestingly, this F2 mapping approach sometimes fails to identify clear QTLs. The most striking example of this was the failure to identify the genetic basis of elevated K in the Wa-1 accession. Further analysis revealed that this was because it was the cytotype and not the genotype of Wa-1 that was driving elevated leaf K. To our surprise, the ploidy level of the root was controlling variation in leaf K (Chao et al., 2013).

A second successful approach used to identify ionomic QTLs is to use recombinant inbred lines (RILs) developed by crossing two parental lines and then selfing for multiple generations to generate essentially immortal homzygous lines. QTL analyses in the Bay-0 × Shahdara RIL population also identified APR2 as being causal for a QTL controlling variation in leaf sulfate concentrations (Loudet et al., 2007). This RIL population was further used to identify ATP sulfurylase 1 (ATPS1) as a second gene controlling variation in leaf sulfate accumulation (Koprivova et al., 2013). Unlike the variation in the coding region of APR2, which drives the S phenotypes (Loudet et al., 2007; Chao et al., 2014b), a deletion in the first intron of ATPS1, resulting in lower ATPS1 transcript accumulation, is responsible for the ATPS1 controlled sulfate phenotype (Koprivova et al., 2013). Thus, so far, natural variation in the total S or sulfate accumulation in A. thaliana is controlled by APR2 and ATPS1, two key enzymes in the sulfate reduction and assimilation pathway.

Combining bulk segregant-based mapping in an F2 population with analysis of RILs has also proved to be a fruitful approach (Baxter et al., 2008a). To identify the causal locus driving low Mo in the Ler-0 accession, we performed DNA microarray-based BSA on a phenotyped Col-0 × Ler-0 F2 mapping population. This was followed by using selected RILs from an existing Col-0 × Ler-0 RIL population with informative recombination break points to fine-map the causal locus. This revealed that variation at Molybdenum Transporter 1 (MOT1) is responsible for natural variation of leaf Mo in Ler-0 (Tomatsu et al., 2007; Baxter et al., 2008a). The functional
polymorphism in MOT1 in Ler-0 is a 53-bp deletion in the promoter (Tomatsu et al., 2007; Baxter et al., 2008a). However, further analysis of the MOT1 promoter in 283 accessions revealed a total of six non-coding structural polymorphisms (Forsberg et al., 2015). Of these six polymorphisms, two were significantly associated with variation in leaf Mo across a species-wide collection of 340 accessions. The 53-bp deletion originally identified in Ler-0 is associated with decreased leaf Mo, and a duplicated 330-bp insertion in the promoter that has undergone a 4-bp deletion was associated with elevated leaf Mo. These polymorphisms appear to act to control Mo in leaves by either decreasing or increasing MOT1 expression, respectively. However, an amino acid polymorphism in MOT1 from the A. thaliana Sha accession is fully associated with the phenotypic effect of this weak allele across ~300 genotyped accessions, suggesting that polymorphisms in the coding region of MOT1 are also important (Poormohammad Kiani et al., 2012).

The major limitation of linkage mapping or QTL mapping is that it only exploits the variation between two genotypes (biparental cross) or among a few genotypes (multiple accession intercross). However, allelic diversity is much higher in natural populations. Genome-wide association (GWA) analysis is an ideal approach for exploiting this extensive allelic variation in natural populations to uncover important genotype-to-phenotype links. GWA has become a powerful approach to isolate the QTLs or genes controlling phenotypic variation in plants, such as flowering time and disease resistance (Weigel and Nordborg, 2015). Several natural alleles controlling ionomic traits have also been identified using GWA mapping. In the first comprehensive GWA study in A. thaliana, 107 traits including the leaf concentration of 18 elements were analyzed in 96–192 accessions (Atwell et al., 2010). Significant single nucleotide polymorphisms (SNPs) associated with leaf Na were identified in the genomic region surrounding HKT1;1, a gene encoding a known Na transporter. However, significant SNPs for other elements were not identified, perhaps because of the small numbers of accessions (n ~ 93) used in the ionomic portion of the study. Using a larger set of 349 accessions genotyped at ~250,000 SNPs, which represents a large proportion of the species-wide diversity in A. thaliana (Baxter et al., 2010), we have identified four ionomic loci using GWA mapping. HKT1;1 was identified as controlling 32% of the variation in leaf Na accumulation across this species-wide collection (Baxter et al., 2010). Using GWA mapping coupled with linkage mapping and transgenic complementation, natural variation in leaf Cd accumulation in A. thaliana was revealed to be primarily governed by allelic diversity in the coding region of HMA3 (Chao et al., 2012). Variation at the HMA3 locus accounts for 30% of the total variation of leaf Cd in the 349 accessions. HMA4 is also a key protein required for Cd accumulation and tolerance in A. thaliana (Hussain et al., 2004; Mills et al., 2010) and Arabidopsis halleri (Hanikenne et al., 2008). However, genetic variation at HMA4 appears to not be a major contributor to natural variation in leaf Cd accumulation in A. thaliana (Chao et al., 2014a, 2014b).

GWA analysis was also successful at identifying the HAC1 gene in controlling variation in As concentration in leaves (Chao et al., 2014a). HAC1 encodes an arsenate reductase, which has also been shown to control natural variation of arsenate tolerance in A. thaliana (Sanchez-Bermejo et al., 2014). Variation at HAC1 accounts for 18% of the total variation in leaf As accumulation in this species-wide collection of A. thaliana. GWA mapping is powerful at detecting alleles that have large trait effects and occur at relatively high frequency in the population. However, this approach is not always successful if the causal allele is rare in the population. For example, APR2 was not identified in a GWA scan for genetic associations with variation in total leaf S accumulation, most likely because the weak APR2 allele is rare in this collection of 349 accessions (Chao et al., 2014b). Furthermore, traditional GWA mapping is based on the mean difference in phenotype between alternative genotypes at each marker and is unable to detect causal associations if the genes they tag have multiple alleles with opposite effects on traits. Such alleles can, however, be detected if GWA mapping is based on the phenotypic variance between the alternative genotypes, an analysis known as variance heterogeneity GWA, or vGWA. The identification of MOT1 as the causal gene for variation in leaf Mo is one of the successful examples of vGWA (Shen et al., 2012). The strong vGWA signal observed at MOT1 was further fine-mapped and determined to be driven by the presence of three independent genetic polymorphisms all in strong linkage disequilibrium (LD) with the markers displaying the genetic variance heterogeneity. Two of the three genetic polymorphisms are promoter variants of MOT1, and the third a variant located ~25 kb downstream of MOT1. The fact that one variant tags three different minor alleles with different effects on the mean Mo concentration explains the increased phenotypic variance of the group of accessions with these genotypes (Forsberg et al., 2015). It is this allelic heterogeneity around MOT1 that makes it difficult to detect significant SNPs associated with leaf Mo in 96 accessions (Atwell et al., 2010), and to barely detect significant SNPs in the 340 accessions (Forsberg et al., 2015), when using mean-based GWA analysis. Using a multi-locus mixed modeling approach to reanalyze the leaf Na data of Baxter et al. (2010) determined that the optimal model contained not one but six SNPs that together explained 52.6% of the total phenotypic variation. This was taken as evidence for allelic heterogeneity also at HKT1;1 (Segura et al., 2012). To be comprehensive, GWA approaches will therefore require consideration of the allelic heterogeneity that may underlie ionomic traits. Given the relatively small LD of 10 kb (Kim et al., 2007) in A. thaliana, GWA mapping offers a method to rapidly map to only a handful of candidate genes. In our four successful examples of using GWA mapping to identify loci controlling variation in Na, Cd, Mo, and As, we found the peak associated SNPs to be within the coding region of HKT1;1, 3.1 kb from HMA3, 15.4 kb from MOT1, and 19.8 kb from HAC1. Therefore, based on our experience a combination of traditional linkage mapping in biparental crosses with parents chosen from the phenotypic extremes for rare alleles, coupled with GWA mapping (with models to detect single and multiple loci), appears to be a good approach for identification of the loci controlling natural ionomic variation.

IONOMICS AND ENVIRONMENTAL ADAPTATION

As sessile organisms, plants have evolved sophisticated mechanisms to adapt and survive in a diverse range of environments,
including highly heterogeneous soils. Plants take up most of the mineral nutrients and trace elements they require from the soil. Therefore, the ionomic profile of plants grown in a common environment may reflect adaptations to their native local environment (Baxter et al., 2010; and commentary by Anderson and Mitchell-Oluds, 2010). As discussed above, several natural alleles controlling the variation of ionomic traits have been identified. The identification of such natural allelic variants may allow a new approach to investigate the molecular basis of adaptation to native soils. Recent work by Busoms et al. (2015) has identified clear signals of local adaptation in A. thaliana to elevated soil salinity at the coast in Catalonia, with salt-tolerant coastal populations and salt-sensitive inland populations being separated by less than 30 km. Clear selective edaphic agents that act over short distances to maintain differentiated subpopulations of A. thaliana therefore do exist, and it is not unreasonable that at least in some cases this local adaptation could be driven by ionomic loci.

HKT1;1 is one of the candidate genes potentially involved in adaptation to saline soil. The Na transporter HKT1;1 functions in Na homeostasis by removing Na$^+$ from the xylem sap to regulate Na accumulation in the leaves (Munns and Tester, 2008). Natural accessions with a weak allele of HKT1;1 accumulate higher concentrations of Na, and this allelic variation in the strength of HKT1;1 appears to be modulated at the level of gene expression (Rus et al., 2006; Baxter et al., 2010). Furthermore, the ability to accumulate Na in leaves was found to be strongly associated with coastal habitats and other potential saline soils (Baxter et al., 2010). Accessions with elevated leaf Na containing the weak allele of HKT1;1 are therefore more likely to grow in potentially saline-affected soils, suggesting a role for HKT1;1 in adaptation to salt salinity. Multiple haplotypes containing weak alleles of HKT1;1 exist (Baxter et al., 2010), suggesting repeated evolution to saline-affected environments. However, since the salinity level in the native habitat where each accession was collected is not available, further fieldwork is required to confirm that allelic variation at HKT1;1 is in fact linked to soil salinity. Using a multiple-year field-based reciprocal transplant experiment, we have recently shown that A. thaliana accessions collected near the coast generally outperform those from adjacent inland habitats when grown under high salinity, indicating a local adaptation to soil salinity (Busoms et al., 2015). The next step is to perform a high-density collection of the adjacent coastal and inland populations and the soils from the plants’ native habitats. Genotyping the HKT1;1 locus in the population and determining the salinity level of the local soil could reveal whether HKT1;1 functions in adaptation to soil salinity. Such studies are currently ongoing.

Mo is an essential element for plants and is required for enzymes involved in nitrate assimilation, sulfate detoxification, purine catabolism, and abscisic acid biosynthesis (Mendel, 2011). The bioavailability of Mo for plant growth is largely dependent on soil pH, and Mo deficiency is often observed in acidic soils. On the other hand, excess Mo could also have an impact on plant development (Kaiser et al., 2005). Across the A. thaliana species there is significant functional variation of MOT1 that is involved in controlling variation in Mo accumulation (Baxter et al., 2008a; Forsberg et al., 2015). Furthermore, this variation also controls variation in tolerance to acid soils in A. thaliana (Poormohammad Kiani et al., 2012). Such allelic diversity suggests that MOT1 may play an important role in adaptation. Indeed, West Asian A. thaliana accessions with the weak allele of MOT1 occur in their native habitat on soils with high water-extractable Mo content (Poormohammad Kiani et al., 2012). It is notable that Copper Transporter 6 (COPT6), nearby the MOT1 loci, is also associated with natural variation in leaf Mo accumulation (Forsberg et al., 2015). COPT6 is thought to play a role in Cu redistribution when Cu is limited in the environment (Garcia-Molina et al., 2013), and MOT1 has been shown to be upregulated under Cu deficiency (Billard et al., 2014). Therefore, it is possible that MOT1 and COPT6 may play integrated roles in environmental adaptations that involve both Cu and Mo.

It is largely unknown whether other natural ionomic alleles also play a role in adaptation to the corresponding elements in soils, such as APR2 and ATPS1 for S, HMA3 for Cd, or HAC1 for As, and further studies are required to unravel this. However, it is notable that natural ionomic variation in different species tends to be controlled by genes from the same gene family, in some cases sharing the same type of causal polymorphisms. For example, variation in shoot Na concentration is controlled by HKT1;1 in A. thaliana (Rus et al., 2006; Baxter et al., 2010), HKT1;5/SKC1 in rice (Ren et al., 2005), and HKT1;5-A in wheat (James et al., 2006; Munns et al., 2012), all belonging to the class 1 subfamily of HKT transporters (Platten et al., 2006). Variation in the coding region of HKT1;5/SKC1 is responsible for variation in salt tolerance and shoot Na and K concentrations in rice (Ren et al., 2005), while variation in the promoter region of HKT1;1 appears to drive the high leaf Na in A. thaliana (Rus et al., 2006). The variation of leaf Mo in Mimulus guttatus is controlled by the homolog of A. thaliana MOT1 (Lowry et al., 2012). HMA3 is responsible for controlling variation in Cd accumulation in both A. thaliana (Chao et al., 2012) and rice (Jeno et al., 2010). Interestingly, the functional polymorphisms in HMA3 in both A. thaliana and rice are in the coding regions. Such conservation suggests that selection is acting at the same loci across species, with similar ionomic consequences. Such conservation should provide a powerful pathway to translate discoveries of ionomic loci in A. thaliana into crops and other plants.

**IONOMIC BIG DATA AND THE IHUB**

Taking advantage of high-throughput elemental profiling, we are able to analyze the leaf ionome of more than 1000 samples per week sustainably over multiple years. In the past decade, we have analyzed 205 169 unique A. thaliana samples for 18 to 20 elements. This represents approximately 4 million publicly accessible data points from 15 369 unique lines/accessions, and includes 11 478 FN mutagenized plants, 28 357 T-DNA mutagenized plants (representing 3452 genes), 21 960 EMS mutagenized plants, and 40 388 wild-type plants including 753 different accessions and 1474 inbred lines. All data can be downloaded from the IHUB at www.ionomicshub.org. To effectively utilize this scale of data it is essential to develop data management tools to appropriately store, retrieve, and analyze such data. We have developed the IHUB cyberinfrastructure to facilitate the management of these large ionomic datasets (Baxter et al., 2010).
The iHUB provides integrated workflow control for data acquisition and validation, data storage, data search and retrieval, and data visualization. Given that the plant ionome is highly dependent on the growth stage, tissue, and environment in which plants are cultivated, the iHUB captures all information relating to cultivation, harvesting, sample preparation, elemental analysis, and data processing. Such information includes the samples’ genotype, planting and harvesting date, tissue type, growth media and watering conditions, growth environment, and ICP-MS analytical conditions. Common genotypes are also included in each plant cultivation tray. This provides the ability to compare samples from plants cultivated and analyzed years apart. The iHUB also contains tools for data discovery, data annotation, data sharing, and data publication. Public users are able to search the data based on either line/accession name or ionomic phenotype. Multiple sets of selected data can be combined into unique datasets, which can be associated with a stable digital object identifier for citation in publications. Workgroups can also be set up to share private datasets between collaborators. An Ionomics Atlas (Dragut et al., 2012) accessed via the iHUB has also been developed to connect the leaf ionome of *A. thaliana* populations with their landscape distribution, which includes ionomic data and associated genetic loci for 348 accessions of the HapMap collection, and the geographic location, climate data (temperature, humidity, pressure, and precipitation) and soil properties of the habitat where the accessions were collected. The Ionomics Atlas allows users to access and explore the interconnection of ionomic, genomic, and environmental data through a Google Maps interface. Such a tool allows an investigation of the potential role of natural ionomic variation in adaptation to the landscape. Currently the iHUB not only includes ionomic data of *A. thaliana* but also data of other species, including rice (Zhang et al., 2014; Pinson et al., 2015), soybean, maize, and yeast (Yu et al., 2012). The iHUB is useful to directly test gene function and identify potentially interesting mutants for further study. Since it first went live in August 2007, the iHUB has had 15 687 unique users from 2217 cities in 124 countries.

**CONCLUSIONS AND PERSPECTIVES**

Ionomics has emerged over the last decade as a powerful tool for successfully identifying genes and gene networks that control mineral nutrient and trace element homeostasis. Such success has required the timely integration of the analytical methods of high-throughput elemental profiling with modern molecular genetics. The molecular, cellular, and physiological functions of genes discovered using this approach are starting to be unravelled, although the road from gene to function (and publication) can still be a long one. Ionomics has also yielded advances in our understanding of the loci that control natural ionomic variation. However, the ecological functions of allelic variants of ionomic loci, if any, are still largely unknown. With the release of the RegMap panel of 1307 *A. thaliana* accessions (Horton et al., 2012), which combines both regional and species-wide collections with accurate information on the collection site of each accession, and the increasing availability of whole-genome sequences of more than 1000 accessions (http://1001genomes.org/), it is now becoming possible to probe the potential ecological functions of ionomic loci across the genome. Such opportunities are leading to what might be termed “landscape ionomics,” which will require the coupling of ionomics, population genomics, and field-based ecological studies.

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