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Ionomics: The functional genomics of elements

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Abstract

Ionomics is the study of elemental accumulation in living systems using high-throughput elemental profiling. This approach has been applied extensively in plants for forward and reverse genetics, screening diversity panels, and modeling of physiological states. In this review, I will discuss some of the advantages and limitations of the ionomics approach as well as the important parameters to consider when designing ionomics experiments, and how to evaluate ionomics data.

Keywords: ionomics; maize; *Arabidopsis;* mineral nutrition

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INTRODUCTION TO IONOMICS

Elements, along with nucleic acids, proteins, and metabolites, are an essential building block of the living cell and are involved in almost every process in an organism. Understanding the functions and dynamics of elements is therefore critical for understanding how life works. For plants, which must take up all elements except carbon and oxygen from the soil environment, control of the uptake and distribution of elements from the local environment is crucial for survival. *Ionome* is defined as 'the mineral nutrient and trace element composition of an organism, representing the inorganic component of cellular and organismal systems' [1]. It is a dynamic network of elements that are controlled by the physiology and biochemistry of the plant, which are ultimately controlled by the genome, in response to the environment. Improvements in inductively coupled plasma (ICP) spectroscopy techniques have enabled the measurement of the ionome in a high-throughput fashion in a process called ionomics. The initial ionomics studies focused on identifying or characterizing mutants in the model organisms *Arabidopsis* [2] and yeast [3]. In recent years, the ionomics approach has been extended to *Lotus japonica* [4, 5], as well as to broad surveys of many plant species [6]. Currently, there are at least six labs [4, 7–9] taking an ionomics approach to elemental accumulation in the species mentioned as well as rice (*Oryza sativa*), maize (*Zea mays*), soybean (*Glycine max*), mouse (*Mus musculus*), worm (*Caenorhabditis elegans*), and human cell lines.

To date, the largest ionomics project is the *Arabidopsis* ionomics project at the Purdue Ionomics facility, which has analyzed the leaf ionome of >125 000 plants (http://www.ionomicshub.org). This project has several different components: (i) forward genetics mutant screens of fast neutron [2] and EMS mutagenized plants; (ii) reverse genetic screens of T-DNA lines selected from collections because they contain disruptions in transporters, kinases, or unknown genes or because they were submitted by members of the community; (iii) screens of natural accessions; and (iv) screens of F2 or RIL populations derived from ionomic mutants from (i) or diverse accessions identified in (iii). These efforts have led to the identification of several ionome-altering genes [10–14]. The need to keep track of all of the data generated from this project led to the development of the Purdue Ionomics Information Management System (PiiMS) (http://www.ionomicshub.org [7]). This web-based data management system allows for the collection of all of the relevant

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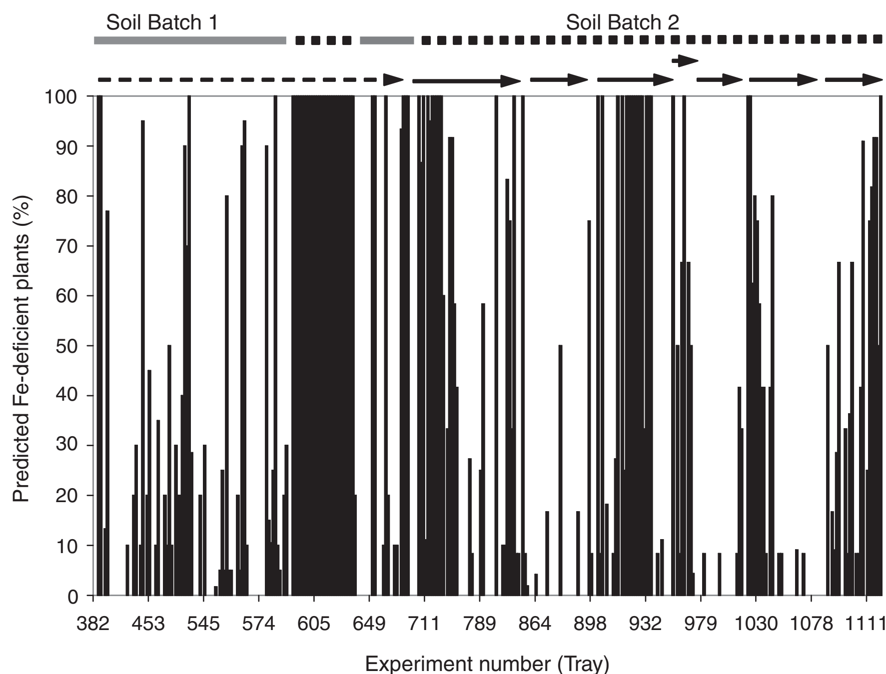

Figure 1: Logistic regression model (from [15]) predictions of Fe deficiency of *Arabidopsis* plants across multiple experiments. Data for the shoot concentrations of Mn, Co, Zn, Mo, and Cd from *Arabidopsis* Col-0 grown in 357 different experiments (median $n = 12$ per experiment) from 04/21/2003 through 04/12/2007 were analyzed using the logistic regression model (simple model) and the data presented as the percentage of *Arabidopsis* plants predicted as Fe-deficient in each experiment. Over time different soil batches were used to grow the plants, and these are represented on the graph as a thick gray (batch 1) or dashed (batch 2) line. Also, during this extended period of experiments Fe was included in the fertilization solution as either Fe-tartrate (dashed arrow) or Fe-HBED (solid arrows). For Fe-HBED each new stock solution of Fe-HBED is represented by a different arrow.

metadata (e.g., line name, catalogue name, soil batch, and watering solution) associated with the data produced by the spectrometers. The metadata collected was essential for Baxter *et al.*'s [15] use of the ionome to characterize the elemental environment that plants were grown in. Logistical regression models based on the leaf accumulation of five and six elements were better predictors of the Fe and P status of the soil than the accumulation of Fe and P themselves. When applied to over 3 years of data from PiiMS, the Fe model was able to detect changes in the soil batch and watering solution (Figure 1, Baxter and Salt, unpublished results). This demonstrates that the ionome can be used as a probe of the soil environment that plants are grown in as well as a phenotype for the identification of gene functions. The ionome is strongly influenced gene × environment interactions, as QTL analysis of RILs grown in different environments reveals large numbers of different loci ([8], Baxter *et al.*, unpublished). Therefore, tracking environmental change is essential for making sense of genetics experiments.

Why ionomics?

There are three properties of ionomic analysis that make it attractive as a profiling technology for knowledge generation. (i) *Cost-effective:* The per-sample cost can be as low as \$10, and will likely continue to fall as high-throughput methodology improves. (ii) *High throughput:* With an autosampler, hundreds of samples can be run on an ICP per day. This throughput enables genetics and modeling studies, which frequently require thousands of samples per project. (iii) *Comprehensive:* Like transcript profiling, ionomic profiling can simultaneously measure most of the relevant components within the class of molecules under study. Proteomics and metabolomics are currently limited to subsets of the total complement in a sample. As these components are all essential parts of the cell and organism, measuring a comprehensive set ensures that alterations in the physiology of the plant are detected, assuming the relevant tissue is being analyzed with enough replicates and highly sensitive detection.

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The advantages of measuring multiple elements

When performing genetics and modeling experiments, there are clear benefits to measuring multiple components like proteins, transcripts, and metabolites. Elements are similar, with the advantages that they are relatively cheap to measure and the whole class of components can be measured in a single run. Whether sets of elements are treated as a multivariate trait or as multiple independent components, having more elements increases the power and efficiency of the experiment. The multiple-element Fe and P models from Baxter [15] are better at predicting the physiological state of the growth media than individual elements, as random or other environmental variation in a single element cannot fool the model. Chemical analogs, such as S/Se, Ca/Sr, and K/Rb, form one class of element relationships, although some plants can discriminate between some analogs [16]. Other element relationships arise from shared responses to biochemical or physiological alterations in the plant. When elements are treated as independent traits, profiling a single population can be treated as multiple independent experiments and QTLs can be identified for each element [8, 17]. A combination of these two approaches was used to analyze F2 plants from a cross of the 14501 ionomics mutant in the Col-0 background to the Ler-0 accession. Plants were divided into pools based on their Ca and B phenotypes, while separate pools were constructed for high and low Mo phenotypes. Bulk segregant mapping (BSA) was used to map the *enhanced suberin biosynthesis 1* (*esb1*) in the Ca/B pools [13] and the *molybdenum transporter* 1^{Ler} (*mot1*^{Ler}) allele in the Mo pools [12]. The *esb1* mutant has increased suberin in the root, which appears to reduce radial solute transfer from the apoplast to the xylem and reduce transpiration. These two phenomena are likely to be the cause of the nine-element ionomic phenotype observed in this mutant.

HOW IONOMICS WORKS

In this review, I will focus on the most common method of ionomic profiling: ICP-MS or OES analysis of digested plant samples. For a more comprehensive review of other approaches to ionomic profiling, see Salt *et al.* [1]. I will start with a short overview of the process and continue with a detailed discussion of several aspects important to the success of an ionomic study.

The process of sample preparation for ionomic analysis is fairly straightforward. Samples must be digested down to their elemental components in trace metal grade acid. The digestion volume must be considerably larger than the sample size, and the digestion should be carried out in a plastic fume hood to prevent contamination. These factors dictate that samples must be small (or the available vented clean space massive) if a high-throughput analysis is going to be carried out. ICP-OES is more tolerant of dissolved solids and therefore is more flexible with digestion procedures. However, ICP-OES is less sensitive than the ICP-MS, which is a significant consideration with small samples. The standard *Arabidopsis* sample size at the Purdue Ionomics facility is 5–10 mg dry weight, or a couple of small leaves. We have developed procedures to sample single dry rice and maize grains and soybeans as well as batches of 5–10 rice grains in the same 16 mm diameter × 100 mm height borosilicate tubes. Hansen *et al.* have recently published a method for high-throughput analysis of smaller samples using a microwave digestion procedure [9]; however, this method has yet to be applied to a large profiling experiment to asses the tradeoffs with the methods described in this section. For downstream analysis, the amount of tissue actually digested needs to be obtained, either by direct weighing or through a weight calculation (see below). After digestion, the samples can be run on the ICP, which has the capacity to measure dozens of elements, although the elements measured in a given run depend on several factors (see below). With modern autosamplers, hundreds of samples can be analyzed in a single run. Post-run data processing to incorporate standards and controls is a critical part of large experiments, as daily calibrations of the instruments and variations in the growth environment can have significant effects on the data produced.

Obtaining a weight

For small (e.g., 5–10 mg) samples, the process of obtaining a weight can be challenging. Dry samples of that size are too small to be easily handled, so the samples need to be fresh when put in the tube. Drying the samples in the tubes can alter the weight of the tube, necessitating control tubes to be used to correct the tared weight of the sample tubes. Even on a five-place balance that measures down to tenths of a milligram, there can be significant error in the milligram range. To obtain even

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that level of accuracy requires ~30 s of stabilization time per sample, which is prohibitive when working with hundreds of samples. To circumvent this problem, Lahner *et al.* [2] devised a method of calculating the weight of a sample based on the elemental profile (described at http://www.ionomicshub.org). This method discards highly variable elements and averages the weights calculated from the remaining elements. The calculation usually averages more than six independently measured elements, and is therefore quite robust to alterations in the ionome. Since the total amount of each element is measured, the calculation is not confounded by alterations in the form of the element, an issue that might prevent this method from working with metabolite data. The method works quite well when calibrated by 6–10 actual weights in each run and produces more accurate weights than a five-place balance. The development of this method is one of the efficiencies that allowed the Purdue Ionomics facility to analyze over 125 000 *Arabidopsis* leaf samples in the past 6 years.

It is important to emphasize that this method only works if the samples are the same tissue and are approximately the same size. The calculation assumes that the elements are distributed homogenously throughout the sample, which is a reasonable assumption for a full leaf or an *Arabidopsis* seed. However, for crop grains, which have been under strong selection to increase carbohydrate, protein, and/or oil, this assumption is unlikely to hold true. Changes in the endosperm starch concentration of cereal grains may not alter the total amount of elements accumulating in the seed, thereby changing the calculated concentration in the seed. It is unlikely that alterations in starch, protein, and oil content will affect each element equally. For example, in the analysis of seven RIL lines from the B73xIl14h NAM population that were still segregating at the Sugary locus, the locus responsible for common sweet corn, several elements were significantly different between the su+ and su− kernels within each line, while others showed little difference (Baxter and T. Rocheford, unpublished results, Figure 2). This property of crop seeds therefore adds an additional level of complexity to ionomic analysis and may necessitate the weighing of each sample. Fortunately, there is considerably more technology available for handling crop seeds, which might allow for automating these tasks.

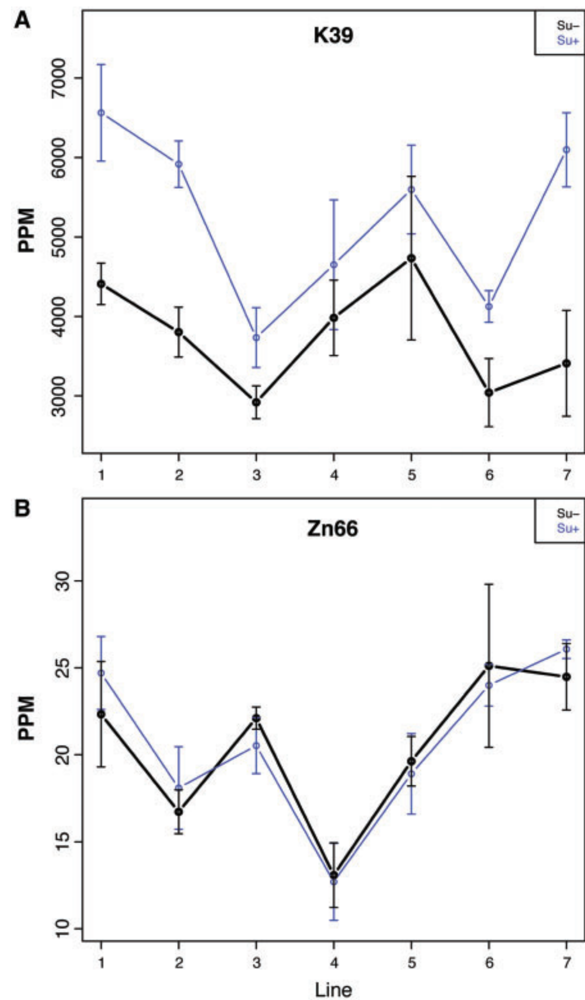

Figure 2: The K (**A**) and Zn (**B**) concentrations of maize kernels from seven RILs of the Il14hxB73 nested association mapping population where the Sugary (Su) locus is segregating ($n = 10–12$ per line, $n = 4–8$ for su+/su− within each line). Error bars indicate standard deviation. In a linear model with *line* and *Su* as factors, *line* was significant ($P < 1 \times 10^{-5}$) for both elements and 9 of the 14 other elements measured, while *Su* was significant for K and five other elements.

Measured elements

The elements that are measured in a given experiment are a function of the following variables: the concentration and species of the elements in the growth medium, the type of growth media, the species and tissue under study, the amount of sample, and the instrument and analysis method. If elements are not present in the media or are unavailable to the plant, they will not be detected in the analysis. In order to consistently measure some elements in *Arabidopsis* leaves, the Purdue Ionomics facility spikes the soil with a solution containing sub-toxic

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concentrations of As, Cd, Co, Li, Ni, and Se (described in [2]). If elements are present at low levels or are not well taken up by the plants, their detection will depend on the sensitivity of the instrument and the size of the sample analyzed. Several factors can contribute to the sensitivity of detection for a given element in ICP spectroscopy. The background signal can vary depending on the sample preparation; for example, samples digested in sodium borosilicate tubes have a much higher background for Na and B than samples digested in the more expensive Teflon tubes. In ICP-MS, polyatomics arising from other elements can create interferences (e.g., $Ar^{40}O^{16}$or $Ca^{40}O^{16}$ can interfere with Fe^{56}). These issues are not present in the less sensitive ICP-OES, although there can be interferences from elements whose spectral emissions are near each other.

The elemental composition of different tissues can vary widely; for example, Na is routinely detectable in leaves but present in very low concentrations in corn seeds. Which tissue is analyzed will have a large effect on the size of the sample and the ease of preprocessing. If a large sample like a full leaf from a crop plant is analyzed, the sample must be homogenized, likely by grinding in liquid N_2, and subsampled, which will greatly increase the per-sample cost and time. Roots have similar issues, with the added difficulty that soil-grown roots need to be extensively cleaned to prevent soil contamination of the sample. Subsampling during tissue collection by techniques such as leaf punching can be used for high-throughput analysis, with two potential complications: potential contamination from the hole punch due to metal-on-metal friction (metal scissors should not be used to harvest ionomics samples for the same reason) and variability in the tissue sampled due to difficulty in identifying the same section of tissue on different plants.

Normalizing large experiments

Genetics and modeling studies such as those discussed in the introduction comprise thousands of samples, which necessitate multiple growth batches and runs on the ICP. Variation in the growth media and the calibrations of the instrument as well as drift of the instrument within the experiment are inevitable and must be accounted for if the complete dataset is to yield useful data. The instrument variation can be mitigated by the use of matrix matched liquid standards that are repeatedly run throughout each run. Since digested samples are quite stable, any variation observed in these control samples can be attributed to alterations in the machine. As the types of molecular interferences that complicate a particular analysis are highly dependent on the composition of the sample (e.g., $Ca^{40}O^{16}$ is much more of a problem when Ca concentrations are orders of magnitude higher than Fe concentrations), it is important that the standard is composed of very similar samples. With the machine variance largely accounted for using this approach, control lines grown in every batch can be used to assay the variance in growing conditions. For induced mutant screens, where each line is almost genetically identical to the parent, the parent can be used as a single control line. However, if the control line for a given batch is skewed, it could be because the whole batch is skewed or because just the control line is skewed (see Figure 4). For this reason, it is preferable to have multiple control lines in each batch, allowing for full batch corrections. Diverse genotypes respond differently to alterations in the environment, which makes multiple control lines critical for experiments analyzing diverse germplasm. For example, in a screen of 360 *Arabidopsis* accessions, the Purdue Ionomics facility used four control lines ($n = 6$ plants) in every 108-plant tray, leaving room for 14 test lines ($n = 6$). Figure 3 shows the tray-to-tray variation we observed in the four control accessions for Cu and Rb across the 25 trays. To normalize for tray-to-tray variation that affects all or most lines in a similar fashion, we created a normalization factor for all four control lines individually and averaged them. This accounts for large variations like those found in trays 1483–86 for Cu and 1479 for Rb (Figure 3A and C), but does not account for what appear to be line-specific effects, such as those seen for Fab-2 in trays 1481–2 (Figure 3D). How to incorporate these multiple controls into the analysis is an active area of research. Nevertheless, it will always be preferable to have more than one or two control lines, even if it reduces the number of test lines that can be run. For field-grown plants, for which the time and effort costs are higher than for lab-grown plants, the benefits of multiple control lines must be balanced against the work required to produce the extra lines.

Picking a tissue

Given all of the above factors, the ideal tissue for most ionomic analysis is either leaves or seeds. Even though roots are an incredibly important tissue for elemental uptake, they have several

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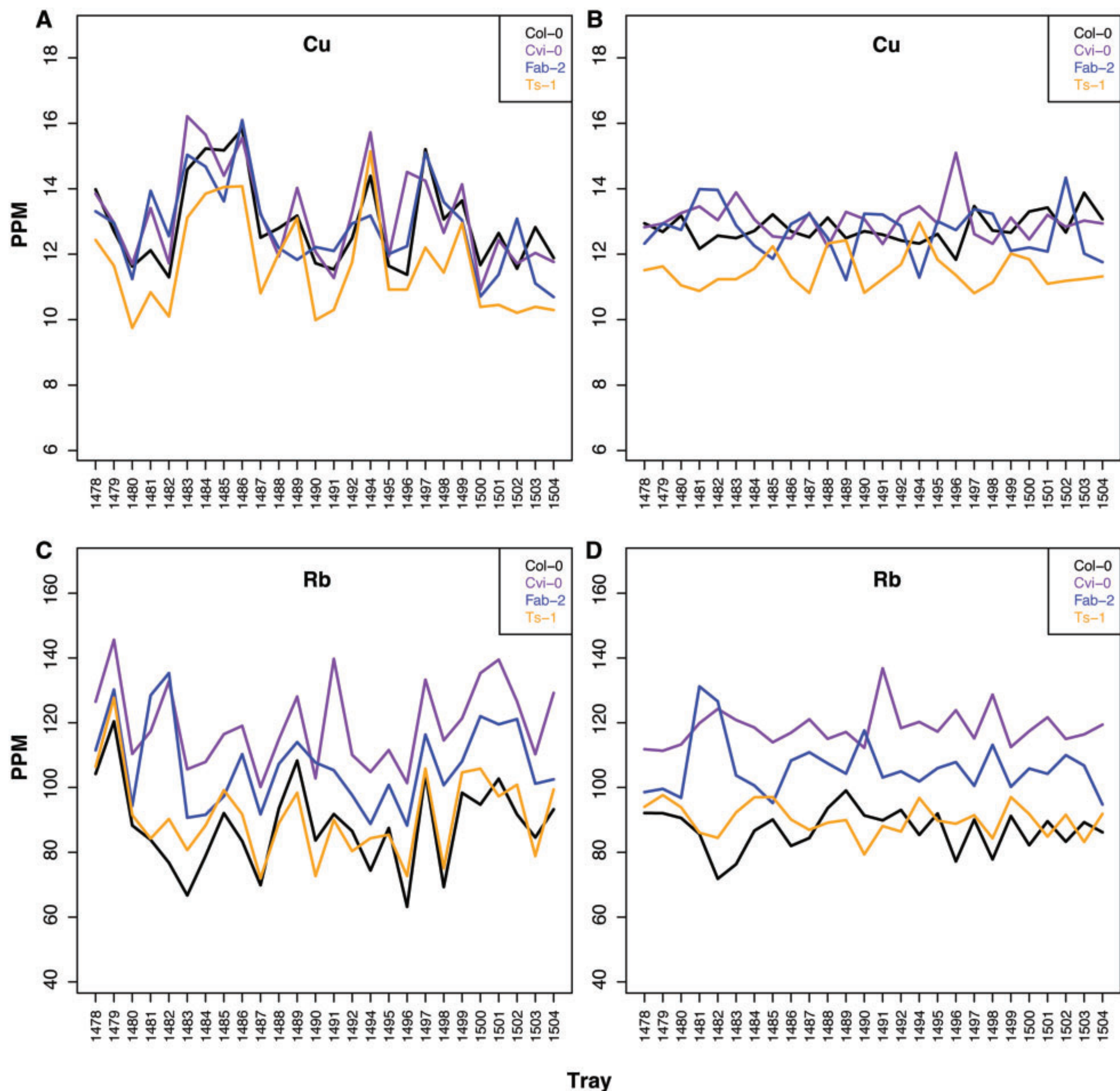

Figure 3: The concentration of Cu and Rb in the four control *Arabidopsis* accessions (Col-0, Cvi-0, Fab-2, and Ts-1) planted ($n = 6$) in each tray of a 360 accession screen. (**A** and **C**) show the mean concentration before the normalization procedure. (**B** and **D**) show the mean concentration after each line was used to create a tray-specific normalization factor, and the four factors for each tray were averaged. Data available at http://www.ionomicshub.org, trays 1478–1504.

drawbacks for ionomic analysis. As pointed out above, soil contamination greatly affects ionomic measurements. Unlike transcriptional profiling, where the plant transcripts can be distinguished from soil organism transcripts by their sequence, there is no way to distinguish soil elements from plant elements. One way to avoid contamination is to grow plants in hydroponics, which would also have the advantage of being a precisely definable growth medium, thereby reducing the variability in the experiment. However, hydroponic growth is more labor intensive, and hydroponics are an extremely artificial environment for land plants, and Fe-P plaques can accumulate on the root surface, limiting the relevance of any discoveries.

Despite the difficulties in analyzing roots directly using the ionomic approach, ionomics is an excellent way to analyze root phenotypes. Unlike proteins, transcripts, and metabolites, ions in leaves or seeds

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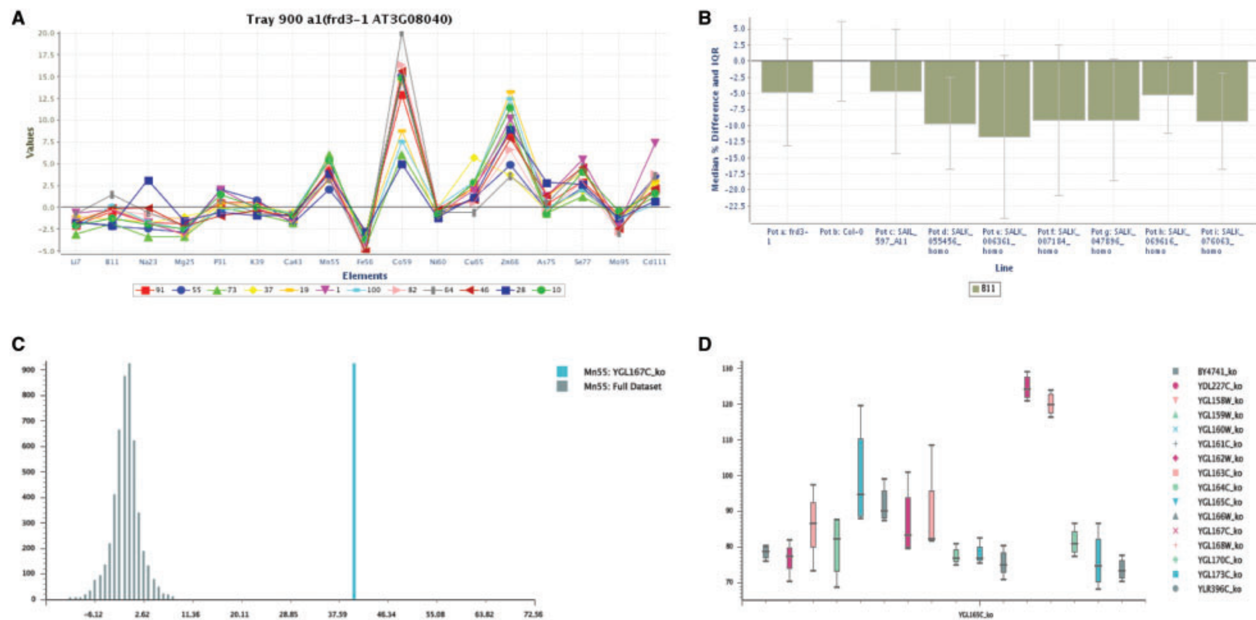

Figure 4: Different methods of displaying ionomics data from http://www.ionomicshub.org. (**A**) *Z*-score plot of *frd3-1*, the positive control used in many *Arabidopsis* trays. The number of standard deviations away from the average of a control line. (**B**) A percent change plot of B from a tray of T-DNA lines. Note that all lines are slightly low, indicating that the Col-0 reference is slightly high in B in this tray and the low B of these lines should be disregarded. (**C**) A histogram of Mn values from the yeast knockout collection with the line YGL167C indicated. (**D**) Boxplots of data from the plate where YGL167C was run. The colored boxes denote the interquartile range containing 50% of the values (25–75%) while the whiskers denote the extremes of the values.

must have traveled there through the plant. By the time an element reaches a leaf or seed, it has been liberated from its soil environment, been taken up by the root, crossed multiple membranes, and moved through the plant's vasculature, and it may have been bound to the cell wall or sequestered to a subcellular component like the mitochondria, ER, or vacuole. Alterations in these processes in the root can be detected as changes in the accumulation of elements in the leaves. The leaves and seeds can therefore be viewed as a summary tissue for many different plant processes. Indeed, many of the ionomic genes and physiological responses identified by profiling leaves are actually acting in the roots. The Fe and P models from Baxter *et al.* [15] detect alterations in the soil environment and the plant's response to them. The ionomic changes are driven by biochemical responses that occur in the roots, although there is thought to be shoot-to-root signaling contributing to the response. The ionome-altering effect of disrupting the HKT1, MOT1, FPT2, and ESB1 genes occurs in the roots, as shown by expression patterns and grafting experiments [11–14].

Whether to analyze leaves or seeds depends on the species being studied and the particular needs of the experiment. For *Arabidopsis*, young leaves of 5-week-old vegetative stage plants can be reproducibly harvested with ease, while the seeds, which can come from different shoots of different lengths that dried on different days, can be quite variable. Maize, on the other hand, grows rapidly, and small differences in germination and growth can have large effects in young plants, so picking a reproducible leaf for large plants can be difficult. However, due to thousands of years of breeding, maize kernels are a very reproducible development stage with relatively uniform properties.

DATA ANALYSIS

As with any high-information content phenotyping platform, there are multiple ways to approach the ionomics data. All of the aspects of the ionomics process mentioned in the previous section should be considered by researchers when trying to interpret ionomics data. One of the ways to do this is to ensure that the researcher looks at more than just

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the particular line that they are interested in, as other lines will help provide context. However, with 17 + datapoints for each line, it is difficult to compare all the elements for multiple lines. Instead, looking at the subsets of the data in different ways can give a fuller picture. There are four data display methods available at http://www.ionomicshub.org: *Z*-score plots, percent change plots, boxplots, and histograms (Figure 4).

For looking at data from induced mutants (T-DNAs, EMS, RNAi, etc.), where there is a known reference to be used for a control, *Z*-score plots can be used to look at the effect on all elements on a common scale (number of standard deviations away from the average of the control) (Figure 4A). When all the individual samples are plotted, the user can get a good feel for which elements are reproducibly altered. The drawback of this approach is that a limited number of lines can be compared, making it difficult to view all of the data for a single experiment. The *Z*-score plots will also display false positives due to variation among the controls. When 17 + elements are measured in each experiment over hundreds of experiments, instances where the control line in a single element is altered due to random variations will be fairly common. Looking at the percent change (Figure 4B) or boxplots (Figure 4D) for the tray in question will reveal whether the difference in question is significant or artifactual. Percent change plots and boxplots are similar: percent change plots are best used for comparing to a known reference, while boxplots simply show the distributions for a group of lines and are more suited for natural accessions where there is no true reference. When testing the hypothesis that two natural accessions are different, growing and analyzing them in the same tray is preferable, but frequently researchers want to know how a given line compares to the distribution of the population it comes from, a question histograms (Figure 4C) are ideally suited to answer. Currently, the boxplots and histograms are only implemented in the yeast database at ionomicshub.org and will be moved to the other databases as part of a system upgrade.

Key Points

- Ionomics is a relatively inexpensive yet comprehensive physiological profiling technique, especially for research questions involving mineral nutrition, making it well suited for plant studies.
- Using the appropriate combinations of instrument, tissue, and controls will enable researchers to harness the power of ionomics for genetics, gene discovery, and modeling.

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