**QTL x environment interactions underlie ionome divergence in switchgrass**

Li Zhang1\*, Alice MacQueen1\*, Jason Bonnette1, Felix B. Fritschi2, David B. Lowry3, Thomas E. Juenger1

1Department of Integrative Biology, University of Texas at Austin, Austin TX 78712; 2Division of Plant Sciences, University of Missouri, Columbia MO 65211; 3Department of Plant Biology and DOE Great Lakes Bioenergy Research Center, Michigan State University, East Lansing, MI 48824

\*corresponding authors. Email: lz5943@utexas.edu or alice.macqueen@utexas.edu

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**Summary**

* Ionomics provides a snapshot of the functional status of a biological organism and captures information about its physiological status under different conditions. We evaluate genetic variation in the ionome in outbred, perennial switchgrass (*Panicum virgatum*) in three environments across the species’ native range, and explore patterns of genotype-by-environment interactions (GxE).
* 725 clonally replicated genotypes of an outbred F2 mapping population, created from deeply diverged upland and lowland switchgrass ecotypes, were grown at three common gardens. Abundances of 18 mineral elements were determined for whole tillers using ICP-MS. These abundances were used to identify quantitative trait loci (QTL) with and without QTL-by-environment interactions (QTLxE) using a multi-environment QTL mapping approach.
* Element content varied significantly both within and between switchgrass ecotypes, and GxE was present at both the trait and QTL level. 14 of 18 element contents were under some genetic control, and 77 QTL were detected for these elements. 74% of QTL colocalized multiple elements, half of QTL exhibited significant QTLxE, and roughly equal numbers of QTL had significant differences in magnitude and sign of their effects across environments.
* The switchgrass ionome is under moderate genetic control and is controlled by loci with highly variable effects across environments.

Key words: GxE, QTLxE, conditional neutrality, antagonistic pleiotropy, bioenergy, reaction norm

**Introduction**

Plants take up most of the elements of the ionome from soil, which is highly heterogeneous across multiple spatial scales (Huang & Salt, 2016). Studies in many plant species have examined the genetic architecture of the ionome and discovered strong genetic effects underlying divergence in elemental composition, and many quantitative trait loci (QTL) in genetic mapping experiments (Buescher *et al.*, 2010; Lowry *et al.*, 2012; Zhang *et al.*, 2014; Shakoor *et al.*, 2016). Studies in *A. thaliana*, where transgenic manipulation is possible, have identified several causal genes controlling elemental variations (Rus *et al.*, 2006; Morrissey *et al.*, 2009; Chao *et al.*, 2014). Recent work in *A. thaliana* has also shown signals of local adaptation to soil salinity, which could be driven by genetic loci that affect the ionome (Busoms *et al.*, 2015). Regardless of plant species, studying genetic variation in the ionome can provide insights into how plants adapt to the highly variable soils that comprise the natural landscape, and can lead to the discovery of genes involved in elemental accumulation, including transporters, transcription factors, and metal binding proteins (Rus *et al.*, 2006; Baxter *et al.*, 2008; Baxter *et al.*, 2010; Baxter & Dilkes, 2012). However, the ionome of an individual depends not only on its genetic makeup, but also on the environment it experiences. Genetic variation in the makeup of the ionome between environments is a type of GxE.

The pattern of phenotypic expression of a single genotype across a range of environments is known as a *reaction norm*. Reaction norms make two important points about GxE explicit: first, that the phenotype expressed by a given genotype depends on the environmental context, and second, that the phenotypic effect in a given environment depends on the genotype in question (Gomulkiewicz & Kirkpatrick, 1992). The reaction norm of a particular genotype and its underlying genetic architecture are heritable properties of the genome and can evolve. Alleles of a gene that affect a reaction norm can do so, and thus exhibit GxE, in multiple ways (Des Marais *et al.*, 2013). For continuous phenotypes like elemental abundances, which have a given mean and standard deviation in two environments for a reference allele, the alternate allele of that gene can affect the magnitude or the sign of the phenotypic effect in one environment relative to the second. *Differential sensitivity* occurs when the magnitude of the phenotypic effect of an allele depends on the environment. Conditional neutrality is the most extreme case of differential sensitivity, which occurs when an allele affects the magnitude of the phenotype in one environment and not in another. *Antagonistic pleiotropy* occurs when the sign of the phenotypic effect of an allele depends on the environment. Studies of several biological systems in their natural environments have found that local adaptation is more often caused by conditional neutrality than antagonistic pleiotropy at the level of the QTL (Des Marais *et al.*, 2013; Wadgymar *et al.*, 2017). When alleles have been identified for ionomic traits, transcription factors and transporters are often involved (Mickelbart *et al.*, 2015).

Identifying molecular mechanisms causing GxE in the plant ionome has been difficult. GxE could not be examined in the many previous studies that identified ionomic QTL in a single environment (Loudet *et al.*, 2007; Norton *et al.*, 2010; Baxter *et al.*, 2014; Zhang *et al.*, 2014; Gu *et al.*, 2015). These studies have largely focused on charactering the elemental accumulation of various plant tissues or species, and have led to valuable knowledge on the genetic control of element accumulation in plants. However, they offered limited insights into how the ionome interacts with environment. More recently, studies have begun to identify GxE and QTL-by-environment interactions (QTLxE) for the plant ionome (Phuke *et al.*, 2017; Veley *et al.*, 2017; Ziegler *et al.*, 2017; Fikas *et al.*, 2019). Thus far, these studies have been limited to biparental crosses or diversity panels with limited numbers of genotypes, particularly in short-lived, inbred crop species such as rice and maize. Studies of GxE in the ionome in outbred, perennial systems may reflect different patterns of GxE, as these plants must cope with heterogenous environments, including non-optimal abundances of essential and non-essential elements, over their longer lifespans.

Switchgrass (*Panicum virgatum*) is a outbred, perennial species with wide environmental adaptation across the eastern half of North America and high biomass productivity across a large geographic range (Casler *et al.*, 2007). Switchgrass was selected as a model bioenergy species by the U.S. Department of Energy (DOE) in 1991 (Wright & Turhollow, 2010), not only because of its high productivity across environments, but also its ecosystem services associated with carbon sequestration, soil erosion and wildlife biodiversity (McBride *et al.*, 2011). Switchgrass has substantial morphological diversity over its native range, including highly divergent southern lowland and northern upland ecotypes. The southern lowland ecotype of switchgrass is typically adapted to wet and riparian areas of southern United States, tends to be more biomass-productive, nutrient-use-efficient, heat-tolerant, and pathogen-resistant than the northern upland ecotype (Porter Jr, 1966; Aspinwall *et al.*, 2013; Uppalapati *et al.*, 2013; Lowry *et al.*, 2014), while the northern upland ecotype is often adapted to dry areas of mid and northern latitudes, and tends to be more freezing-tolerant (Hultquist *et al.*, 1997; Casler, 2012; Peixoto & Sage, 2016).

In this study, we expand the scope of GxE research in ionomics by evaluating the genetic architecture and reaction norms of the ionome in switchgrass. We use an outbred, F2 mapping population derived from a four-parent cross of lowland and upland ecotypes (Milano *et al.*, 2016). We clonally propagated and planted the four parents, the two F1 genotypes, and approximately 750 F2 individuals at three common gardens, then quantified the accumulation of 18 elements. The 18 elements included macronutrients (Mg, P, K, Ca), analogues of macronutrients (Rb, Sr), micronutrients (B, Mn, Fe, Co, Cu, Zn, Se, Mo), and elements that can be harmful to plant growth (Na, Al, As, Cd). With these data, we evaluated the reaction norms of particular QTL for elements in the ionome. Our results allow us to address the following questions: 1) What is the genomic basis for variation in elemental abundances in the switchgrass ionome? 2) What fraction of QTL for distinct elements co-localize, suggesting possible common genetic architectures underlying their abundances? 3) How frequently do ionomic QTL show GxE? 4) Which QTL colocalize with candidate genes, suggesting avenues for future molecular characterization of the switchgrass ionome?

**Materials and Methods**

**Experimental Design and Phenotyping**

The details of the creation of the mapping population can be found in Milano *et al*. (2016). In brief, the genetic mapping population was produced from two initial crosses of two pairs of highly divergent southern lowland and northern upland ecotypes: lowland AP13 (A) x upland DAC6 (B), and lowland WBC3 (C) x upland VS16 (D). The F1 hybrids (A x B, C x D) were then intercrossed reciprocally to create the outbred four-way mapping population (F2).

The details of experimental design are described in Lowry *et al*. (2019). Briefly, the grandparents, F1 hybrids, and the F2 progeny were propagated clonally in 3.8-L pots at the Brackenridge Field Laboratory, Austin, TX in 2013-2015, and then transported to and planted at three field sites in May-July of 2015. Weed cloth was used to suppress weeds, and holes were cut in a honeycomb fashion for planting of the experimental plants. Edge effects were prevented with a row of border plants. Plants were hand-watered as needed through the summer of 2015 to facilitate establishment. The three sites (Austin, Texas, hereafter TX; Columbia, Missouri, hereafter MO; and Hickory Corners, Michigan, hereafter MI) had distinct soil and climatic conditions. TX site (30.384°N, -97.73°W) has clay soil, MO (38.897°N, -92.22°W) and MI (42.420°N, -85.37°W) sites have loam soil. The contents of mineral P, K, Ca, Mg and Na were measured from a soil sample consisting of equally mixed proportions of soil from three locations spanning the entire garden on the diagonal, sampled at six inches in depth. These soil profiles were conducted by the Soil, Water, and Forage Testing Laboratory at Texas A&M University. The soil profiles for P, K, Ca, Mg, and Na were 8, 285, 16865, 222 and 11 ppm at TX site; 19, 106, 2351, 332 and 12 ppm at MO site; and 32, 41, 2154, 108 and 10 ppm at MI site (see also Table 2). The average temperatures in 2016 for TX, MO, and MI sites were 21.9, 13.6, 10.4 °C, respectively. The annual precipitations in 2016 for TX, MO and MI sites were 829, 928, and 975 mm, respectively.

Samples of whole tillers of approximately 700 plants were collected at each of the three sites at the end of growing season in 2016, and sent to Danforth plant science center for elemental contents assay of 18 elements (P, K, Ca, Mg, Rb, Sr, Mn, Zn, Cu, Co, Fe, Mo, B, Se, Al, Na, Cd, and As). Details of the process can be found in Ziegler *et al*. (2013). Briefly, tissue samples were weighed and digested in nitric acid at room temperature overnight, and then heated at 100 °C for 3 hours. Total analyte contents were measured by ICP-MS (Perkin Elmer NexION 350D). Measurements were corrected for potential variation in sample preparation and instrument drift using both internal standards and matrix matched controls as described in Ziegler *et al*. (2013). Outliers and negative values yielded due to machine error were further excluded from analysis.

**Genotyping and Map Construction**

Details on the genetic map construction can be accessed on https://datadryad.org/stash/dataset/doi:10.5061/dryad.ghx3ffbjv (Lovell *et al.*, 2020) and in Bragg *et al.* (2020). In brief, Illumina fragment paired end libraries from each of the four grandparents were aligned to the *P. virgatum* reference genome v5 via bwa *mem* (Li & Durbin, 2009) and used for single-nucleotide polymorphism (SNP) calling. Then a kmer-based approach was used to capture multiple variant and distinguish each grandparent when genotyping the progeny. The resulting genotype matrix was polished via sliding windows across the physical V5 switchgrass genome position and markers were re-ordered within linkage groups (Lowry *et al.*, 2019; Lovell *et al.*, 2020).

**Heritability Estimates and Genetic Correlation**

Narrow-sense heritability (*h2*) was estimated as *Va/Vp*, where *Va* is the additive variance attributable to genetic relatedness, and *Vp*is the total phenotypic variance. *h2* was estimated for each ionomic element at each site using the additive kinship matrix, which was obtained based on marker genotypic information. Genetic correlations between sites for each element were also estimated using the kinship matrix in a similar way. These two processes were implemented via the Sommer package (Covarrubias-Pazaran, 2016) in R (2020). Details on the implementation of the Sommer, particularly the multivariate mixed model (i.e., mmer) can be found in Lowry *et al*. (2019). Briefly, for *h2* estimation, ionomic phenotypes at each site were used as response variables in a linear mixed model with the kinship matrix modeled as a random effect to estimate the additive genetic variance for each genotype. For genetic correlation estimation, multivariate combinations of ionomic phenotypes from the three sites were used as response variables, and similarly the kinship matrix was modeled as a random effect and used to estimate the additive genetic covariance among phenotypes. We further tested for GxE on the trait level using the same multivariate mixed model. In other words, we tested whether *Va* differed by site for each element. Specifically, we used a likelihood-ratio test to compete two models. The first model (i.e., main effect model) assumed that there is no GxE and that a single additive genetic variance plus the fixed effect for environment is sufficient for modeling the data. The alternative model (i.e., unstructured model) assumed that GxE exists and freely estimates a unique additive genetic variance and covariance (an unstructured variance-covariance matrix) within and across environments. Significance of the likelihood-ratio test for GxE was assessed at the level of *α* = 0.05.

**Multi-environment QTL Mapping**

Details of the mapping procedures and implementation for the four-way population are described in Malosetti *et al*. (2013), Lowry *et al*. (2019), and Bragg *et al*. (2020). In brief, a multienvironment mixed model implemented in Genstat v.19 (2020) was fit for each ionomic element to identify QTL and potential QTL x E interactions:

where *μ* represents the population mean; *E* represents the environment effect; , represents the total effect from the additive effect from the first grandparent (i.e., the difference between *A* (AP13) and *B* (DAC) alleles, , the second grandparent (i.e., the difference between *C* (WBC) and *D* (VS16) alleles, , and the dominance effect (i.e., the intralocus interaction, ; represents the QTL × environment interactions; and *e* represents the error term. Genome-wide QTL and QTL x E significance was assessed at *α* = 0.05 with a Bonferroni correction (Li & Ji, 2005).

**Candidate Gene Search and GO Enrichment Analyses**

We consider the genes located in the 1.5-LOD confidence intervals around the detected significant QTL as candidate genes. We then determined if homologs from rice (v7), *A. thaliana* (TAIR 10), and a curated list of genes that affect the plant ionome (Whitt *et al.*, 2020) were overrepresented in our QTL regions. The annotation file for switchgrass was accessed on JGI (Joint Genome Institute) Phytozome 13 website: https://njp-spin.jgi.doe.gov/. The Gene Ontology (GO) enrichment analysis was conducted using Fisher’s exact test for each GO term via R package ‘topGO’ (Alexa. & Rahnenfuhrer., 2020). GOs with adjusted *p* < 0.05 were considered significant.

**Results**

**The genetic basis of elemental content variation and covariation at three common gardens**

To explore the genetic component of ionomic variation in switchgrass, we determined 18 elemental compositions for both the F0 ‘grandparent’ genotypes and for the clonally replicated, outbred F2 genotypes at three common gardens. Average element content varied over six orders of magnitude: Co, Se, Mo, and Cd had the lowest accumulation (~1x10-2 µg g-1 dry weight) and K had the highest accumulation (~1x104 µg g-1 dry weight). After correction for multiple testing, eleven of the 18 element abundances differed significantly between the four grandparents (AP13, DAC6, WBC, and VS16) at one or more garden (Table 1). Three element abundances (Ca, P, Na) differed significantly between the four grandparents at every garden after correction for multiple testing, and Sr and Mg abundances also differed at every garden before this correction (Table 1). Interestingly, there were just as many significant differences in element\*garden content (16) between the two lowland genotypes, AP13 and WBC, as there were between the upland and lowland parents. In contrast, there were only two significant differences in element\*garden content between the two upland parents.

In the F2 genotypes, variation in the content of each element followed a continuous, unimodal distribution within each garden (Figure 1a). Within gardens, the majority of the element contents were not strongly phenotypically correlated (r < 0.5); fewer than 3% of element pairs had positive correlations greater than 0.5 (Supplemental Table S1). Among these, Ca content was positively correlated with Sr at each site (0.8-0.9), and Al content was positively correlated with Fe content at MI (0.8) and TX (0.5).

All element abundances had low to moderate heritabilities (0 < *h2* < 0.6, Figure 1b). The majority of the elements (Na, Mg, Al, P, K, Ca, Mn, Fe, Cu, Zn, Se, Rb, Sr, Mo, and Cd) had moderate heritabilities (0.2 < *h2* < 0.6) for at least one garden, while B, Co, and As had low heritabilities (*h2* < 0.2) everywhere. There were moderate heritabilities for 8 elements in the TX garden (none unique to TX), 12 elements at the MO garden (Na and Al content were moderately heritable only at MO), and 15 elements at the MI garden (K, Zn, Se and Cd content were moderately heritable only at MI). The low heritabilities of some elements at certain sites (B, K, Co, As, and Se) were due to both the large error variance (*Ve*) and the near zero additive genetic variance (*Va*) for these elemental contents (Supplemental Table S2). Likelihood-ratio tests between models with genetic effects only and models with genetic and GxE effects indicated that GxE existed for 16 of the 18 elements (all but B and Se) at the trait level (*p* < 0.05). Thus, switchgrass exerted genetic control of elemental accumulation in an environmentally-sensitive fashion for the majority of the elements of the ionome.

The distributions of all 18 element abundances also differed significantly among gardens (all *p* < 0.002, Welch one-way tests, Table 2). These distinct phenotypic distributions were undoubtedly affected by soil element abundances, which varied in ways that affected plant element content in both intuitive (Ca, K) and non-intuitive (Mg, P, Na) fashions (Table 2). They were also underlain by moderate to strong positive genetic correlations for the majority of the elements among sites (Supplemental Table S3). Positive genetic correlations less than one indicate the presence of GxE at the trait level, and likely magnitude-changing instead of sign-changing patterns of GxE at the level of QTL across the common gardens for the elemental accumulations. Only one negative genetic correlation was observed, for B content in the TX and MO gardens (-0.46). Negative correlations indicate a possible trade-off in loci controlling B content; however, B content heritability was low at both of these gardens, reducing our power to identify QTL. The genetic correlations for two elements (As and Se) could not be determined because the content of these elements had close to zero genetic variance.

We next identified QTL and QTLxE interactions using independent multi-environment mixed models for each of the 18 elements. We detected 77 significant QTL with LOD thresholds above 3.5 for 14 elemental compositions (Figure 2a, and Supplemental Table S4). 38 (49%) of these QTL exhibited QTLxE (Supplemental Table S4). No significant QTL were detected for B, As, Co and Se, almost certainly because of the low heritabilities of these four elemental contents (Figure 1b). The remaining elements had between two (Na, Fe, Mo, Cd) and 14 (P) QTL regions. We divided the 18 elements into four types: macronutrients, micronutrients, non-essential analogues to nutrients, and potentially harmful elements. If QTL had been equally distributed across the elements, we would have expected 17, 34, 8, and 17 QTL in these classes, respectively. However, there was an over-enrichment for QTL for macronutrients (2.05x, binomial test *p* < 0.001) and non-essential analogues (1.99x, binomial test *p* = 0.002) relative to this expectation, and an under enrichment for micronutrients (0.50x, binomial test *p* < 0.001) and potentially harmful elements (0.47x, binomial test *p* = 0.013).

**QTL colocalization across elements of the ionome**

Using our 77 QTL, we nextidentified QTL where distinct elements co-localized. Co-localization suggests either linked genes affecting element accumulation, or co-transport of elements using the same genetic architecture. The latter is more plausible for elements that are most commonly bioavailable in the soil as similar ions. We considered QTL colocalizing if there was any overlap in the genomic region with LODs within 1.5-LOD of the maximum LOD score. Twenty-one sets of QTL colocalized, and 20 QTL (26.0%) did not overlap another ionomic QTL, and hence were singletons (Figure 2b). Mg was the only element with a majority of singleton QTL, with both more non-colocalizing and fewer colocalizing QTL than expected (chi-square test, *p* = 0.005). P had the most colocalizing QTL. Colocalizing P QTL always colocalized with elements which are most abundant in soil as cations with 1+ or 2+ charge. Ca QTL always colocalized, either with P (2 QTL) or with elements most abundant in soil as 2+ or 3+ cations (3 QTL). Al QTL also always colocalized, with Sr in 3 of 4 QTL, and with Fe for both Fe QTL. The partial co-localization of QTL between Ca and Sr, and between Al and Fe, may underlie some of the high phenotypic correlation in these traits in the F2 genotypes (Supplemental Table S1). Three QTL sets colocalized four or more elements. One of these sets was located at 6.63Mb – 33.56Mb on Chr02N with Ca, Zn, Rb and Sr QTL, one at 0.97Mb – 41.75Mb on Chr04N that included Mg, K, Fe, and Al QTL, and the third at 33.91Mb – 51.66Mb on Chr07K that included Al, Ca, Mn, Fe, Zn, and Sr QTL (Figure 2a).

**Ionomic QTLxE frequencies and QTL reaction norms**

We next explored patterns of effect sizes, and types of QTLxE, in our 77 QTL, particularly in our 38 QTL exhibiting QTLxE (Figure 3, and Supplemental Figure S1). The design of the crosses that generated the four-way population also allowed us to quantify differences in allelic effects for two distinct lowland vs. upland crosses, AP13 vs. DAC (A x B) and WBC vs. VS16 (C x D). In addition to looking at patterns of GxE within these crosses, we could also determine if we had captured variation in effects between these crosses, for both QTL with and without QTLxE effects. For the 39 QTL without QTLxE, most effects (75%) had the same direction in both lowland vs. upland contrasts (Supplemental Figure S1). Thus, most QTL without QTLxE exhibited differences in QTL effects between the upland and lowland sets of parents, and few exhibited differences in QTL effects between the two upland or the two lowland parents. Of the ten QTL without QTLxE but with within-ecotype variation, two QTL were singletons, and four colocalized with elements which had no significant QTLxE. The remaining four QTL colocalized with elements which did have QTLxE. If these four colocalizing QTL are due to loci that affect the content of multiple elements, then these QTL represent an interesting case of GxE caused by changes in pleiotropy at that locus.

For the 38 QTL (i.e., 76 allelic contrasts) with QTLxE, 35 contrasts (46%) had differential sensitivity in effects (i.e., a magnitude change) across gardens, and 15 of these contrasts were statistically significant after a multiple testing correction (*t*-test, *p* < 0.000198, Supplemental Figure S1). These differentially sensitive effects were present both in one or two lowland vs. upland allelic contrasts. For instance, the effect of QTL 5K@51.99 for Na content, a potentially harmful element, was differentially sensitive in both allelic contrasts (Figure 3a), while the effect of QTL 2N@10.06 for Mn content, a micronutrient, was differentially sensitive only in the A x B contrast (Figure 3b). The other 41 allelic contrasts (54%) exhibited antagonistic pleiotropic effects (i.e., a sign change) across gardens, and 13 of them were statistically significant after a multiple testing correction (*t*-test, *p* < 0.000198, Supplemental Figure S1). The majority of the antagonistic effects were present in only one contrast. For example, the effects of QTL 2N@72.03, and 9N@24.08 for Rb, a macronutrient analog, were antagonistic for the A x B contrast, but not the C x D contrast (Figure. 3c). Overall, element QTL with QTLxE did not have consistent patterns across environments. For example, the QTL 2N@78.05 and 3K@26.18 for P, an important macronutrient, had the largest effects in TX, while the other two QTL 3N@56.03 and 4K@6.08 for P had the largest effect in MO (Figure. 3d).

**Ionomic QTL colocalization with candidate genes**

To explore avenues for future molecular characterization of the switchgrass ionome, we determined the genetic content of the 77 QTL intervals for genes and gene ontology (GO) terms. We first examined QTL colocalization with candidate genes from ionomic mapping studies in other plant species, and found six important candidate genes (Supplemental Table S5) in the QTL intervals affecting element accumulation in switchgrass. For example, *Pavir.9NG231800*, a homolog of *MOT1*, is located within the 1.5-LOD interval of the largest Mo content QTL (Chr09N@43.81). *MOT1*, which encodes a molybdate transporter, is responsible for the natural variation in Mo accumulation in *A. thaliana* and in rice (Baxter *et al.*, 2008; Huang *et al.*, 2019), and may play an important role in adaptation to acidic soils (Poormohammad Kiani *et al.*, 2012). *Pavir.7kg416470*, a homolog of *HKT1*, was a candidate gene in the QTL interval on Chr07K which colocalized for six elements. *HKT1* encodes a Na transporter, and is responsible for the variation of Na content in *A. thaliana* (Rus *et al.*, 2006; Baxter *et al.*, 2010), rice (Ren *et al.*, 2005), and wheat (Munns *et al.*, 2012). Interestingly, this candidate gene was in the QTL interval for Al, Ca, Fe, Mn, Sr, and Zn, and did not contain a QTL for Na content in our mapping population. Candidate genes for heavy metal-associated ATPases, which are homologs of *HMA* in *A. thaliana* and rice, were found in Cu (Chr01K@14.42 and Chr07K@26.27), Cd (Chr02N@85.72), and Zn (Chr02N@71.96) content QTL intervals. These genes are responsible for copper, cadmium and zinc, and zinc and cadmium transport, respectively. A sixth candidate gene, *Pavir.9KG014451*, was associated with the homolog of the *A. thaliana* *MYB36*. *MYB36* is aMYB domain transcription factor that regulates the expression of the genes involved in the formation of Casparian strips. The absence of Casparian results in the changes in leaf content of Na, Mg, Zn, Ca, Mn, and Fe in *A. thaliana* (Kamiya *et al.*, 2015). This candidate gene was in the QTL colocalizing Ca (Chr09K@20.05), Mg (Chr09K@18.15), and Mn (Chr09K@20.05) content.

To elucidate the cellular pathways associated with ion content in switchgrass, we also looked at GO term enrichment based on the gene content in our 77 QTL. We identified 405 unique enriched GO terms across the ionomic traits (*p* < 0.05). Overall, these QTL regions were enriched for GO terms of DNA-binding transcription factor activity, heme binding, and oxidoreductase activity (Supplemental Table S6). Among the macronutrients and analogs of macronutrients, the QTL regions of Mg were significantly enriched for GO terms of carbohydrate binding, protein transport, cell wall biogenesis, and signal peptide processing, among the 34 ontologies. Mg is involved in protein synthesis (approximately 75% of leaf Mg) and associated with chlorophyll pigments (15-20% of total Mg), mainly functioning as a cofactor for a series of enzymes involved in photosynthetic carbon fixation and metabolism (Cakmak & Kirkby, 2008; White & Broadley, 2009). K QTL regions were significantly enriched for GO ontologies of oxidoreductase activity, calcium and iron ions binding, and in particular, antioxidant activity. K, as a constituent of the plant structure, has a regulatory function in several biochemical processes related to protein synthesis, carbohydrate metabolism, and enzyme activation. K can enhance antioxidant defense in plants, which protects plants from oxidative stress in adverse environments (Hasanuzzaman *et al.*, 2018).

Among the micronutrients, Mn content QTL intervals were significantly enriched for GO ontologies of photosynthesis, mitochondria, carbohydrate binding, the photosystem I reaction center, and electron transfer activity. Mn functions as a major contributor to various biological systems including photosynthesis, respiration, and nitrogen assimilation in plants among other functions (Andresen *et al.*, 2018; Alejandro *et al.*, 2020). Cu content QTL regions were significantly enriched for GO ontologies of cell wall macromolecular catabolic process, oxidoreductase activity, calcium ion binding, and regulation of transcription among the 36 ontologies. Cu is an essential cofactor for numerous proteins, an essential player in electron transport, and involved in chloroplastic and mitochondrial Cu transport and homeostasis. Cu is also involved in the control of cellular redox state (a major Cu-binding protein is the Cu/Zn superoxide dismutase) and the remodeling of the cell wall (Cohu & Pilon, 2010; Andresen *et al.*, 2018). Among the elements potentially harmful to plant growth, Cd QTL regions were significantly enriched for GO ontologies of metal ion binding, photosynthesis (light harvesting), and cell growth among others. Cd, as one of the most toxic and non-essential heavy metals for plants, can displace essential metals (such as Zn, Fe and Ca) from a wealth of metalloproteins and disturb normal physiological processes. It can also cause severe developmental aberrance such as chloroplast structure change, reactive oxygen species (ROS) production and cell death (Wan & Zhang, 2012).

**Discussion**

Ionomics has been a powerful tool for determining the elemental status of plants, assessing homeostasis, and evaluating the genetic architecture responsible for ionomic variation. With its unprecedented scale, our study not only examined the genetic basis of the ionome but also how individual ionomic loci responded to different environments (i.e., expressed GxE) in perennial switchgrass. We detected 77 significant QTL across the 18 elements, half of which had significant QTLxE effects. This indicated the importance of the environmental context in elemental content variation at the QTL level. We observed common QTL colocalization between elements, which supported a partially shared regulatory network for element uptake, transportation, or accumulation. Understanding the genetic architecture of elemental accumulation in our outbred population is the first step in uncovering the potential for ionomic adaptation in switchgrass in response to divergent environmental conditions.

Genotype by environment interactions are common across many different phenotypes, species, and environments. Previous work has found that GxE is often caused by differential sensitivity in response to the environment, and that antagonistic pleiotropy (or trade-offs) at the whole-genome level are relatively rare or weak (Des Marais *et al.*, 2013; Wadgymar *et al.*, 2017; Lowry *et al.*, 2019). Our study found not only conditional neutral effects, but substantial antagonistic pleiotropy (54%) across the ionomic QTL with QTLxE, indicating that alleles had opposing effects on element content in different environments. This result suggests that the plant ionome may play an important role in local adaptation, as both model and empirical work have suggested that there should be strong trade-offs involved in local adaptation at the level of QTL (Felsenstein, 1976; Bradshaw & Schemske, 2003; Kawecki & Ebert, 2004). Our cross design also allowed us to compare allelic effects for two distinct lowland vs. upland crosses and determine if there was variation in effects between these crosses. Interestingly, some ionomic QTL showed differential sensitivity in one cross but antagonistic pleiotropy in the other. This suggests that the same set of loci may not be consistently responsible for divergence between lowland and upland switchgrass ecotypes, and implies that substantial ionomic variation also exists within upland and lowland ecotypes. In essence, these results suggest that different loci contribute to ionomic variation across the range of the species, and that ionomic divergence among ecotypes was not based on fixed differences between the ecotypes.

QTL for multiple elements typically colocalized in our study. This may not be surprising, as maintaining ion homeostasis requires a network of ion uptake, transportation, trafficking, and sequestration mechanisms, and not all genes in this regulatory network will be ion-specific (Clemens, 2001).We saw substantial colocalization of P QTL with cation QTL, always with elements most abundant in soil as cations with 1+ or 2+ charge. Phosphorus is a component of key molecules of plants such as ATP, nucleic acids, and the form of phosphorous most readily accessed by plants, inorganic P, is likely co-transported with positively charged ions (Schachtman *et al.*, 1998). Colocalization of P QTL with cation QTL in our study might thus reflect co-transportation of P and cations at the gene level. Indeed, we found a few cation transporters annotated for *A. thaliana* in the P QTL intervals, including high-affinity K+ transporter, ZIP metal ion transporter family, and Ctr copper transporter family. P QTL colocalized with K and/or Ca QTL at three positions (8K@10.7, 9K@60.9, and 9N@2.4). P, K, and Ca are all macronutrients, which plants need in large quantities. Although different populations may have adapted to soil types with different quantities of these elements, the need for these macronutrients in large quantities could have facilitated the evolution of similar or shared mechanisms or network to take up these elements from soils, thus yielding colocalizing QTL. Alternatively, colocalization could be coincidental and/or simply due to multiple linked genes. In support of this view, P also had many QTL that were singletons (5 non-colocalizing QTL out of 14), as did the important macronutrient Mg (6 non-colocalizing QTL out of 9). P and Mg deficiencies in soils are often widespread (Maathuis, 2009); thus, an potential adaptive scenario is that switchgrass plants were under stronger selection to increase uptake or tolerate lower levels of accumulation of these two macronutrients, the segregation of which drove the increase in variation for content of these elements and led to ion-specific QTL. Indeed, our study identified significantly more QTL for macronutrients than expected (2.05x enrichment, binomial test *p* < 0.001). Identification of these QTL and their reaction norms is the first step in testing hypotheses of local adaptation in natural environments.

We detected fewer QTL than expected for micronutrients (0.5x, binomial test *p* < 0.001), and most micronutrient QTL colocalized with QTL of other elements. Taken together, these results suggest that there may have been only weak selection on accumulation of micronutrients in the grandparents of this population. It is possible that switchgrass obtains sufficient quantities of these micronutrients from any soil. We also found little variation in content of harmful elements, and fewer QTL than expected for harmful elements (0.47x, binomial test *p* = 0.013). It may be that harmful elements impose such strong selection that beneficial alleles have been fixed, and deleterious alleles purged, at least in the populations from which our four grandparents were sampled. Alternatively, harmful elements may not be present in sufficient quantities in the commonly encountered soils for the four grandparents, and thus there may have been only weak selection against specific or non-specific accumulation of these elements. We also found more QTL than expected for non-essential analogues (1.99x, binomial test *p* = 0.002). The non-essential analogue Sr was phenotypically correlated with its chemical analogs Ca at every garden, and they shared colocalized QTL at the two large clusters on Chr02N and Chr07K in our cross. Strong correlations between Sr and Ca have been reported in other species (Broadley & White, 2012; Shakoor *et al.*, 2016). The colocalization of QTL of Sr with other elements also likely reflects its non-essential nature, in that it is seldom the target of uptake by plants, and instead only accumulates via non-ion-specific mechanisms.

We found multiple candidate genes which may affect ionome content in our QTL regions and provide targets for future fine-mapping research in switchgrass. Among these, we found a homolog of *HKT1*, *Pavir.7kg416470*, in the QTL on Chr07K. This candidate gene was in the QTL interval for the six elements, Al, Ca, Fe, Mn, Sr, and Zn, but not in either of the two Na accumulation QTL intervals. *HKT1*, which encodes Na transporter, was responsible for the variation in Na accumulation in *A. thaliana* (Rus *et al.*, 2006; Baxter *et al.*, 2010), rice (Ren *et al.*, 2005; Kobayashi *et al.*, 2017), wheat (Munns *et al.*, 2012), and maize (Zhang *et al.*, 2018). However, Na accumulation in these studies were assayed in plant leaves, while Na accumulation in our study was assayed from whole tillers, which included both leaves and shoots. It seems likely that different tissues could accumulate elements at different levels, but our data represents a composite picture of several tissues. In addition, soil Na was not particularly variable in our gardens (i.e., 11, 12, and 10 ppm for TX, MO and MI, respectively), and some of these elements do compete with Na uptake from soil (Mass *et al.*, 1972; Cramer *et al.*, 1989; Tuna *et al.*, 2007). It is also possible that the lack of variability of soil Na relative to these other elements masked a QTL effect for Na but allowed detection of this QTL for other elements.

Overall, our results suggest that ionomic variation, and ionomic variation across environments, is common in switchgrass. This variation, controlled by a combination of genes and the environment, offers critical material for adaptation of switchgrass metabolism and development across different environments. Future work should explore if ionomic variation is locally adaptive in switchgrass, which will help realize the potential of ionomics in studying adaptation to varying environments.

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**Author contributions**

F.B.F., T.E.J., and D.B.L. designed research; J.B., F.B.F, D.B.L, and T.E.J. performed research; L.Z. and A.M analyzed data; L.Z. and A.M. wrote the paper with comments and editing by all co-authors.

**Data Availability**

The data, R scripts, Genstat outputs, and other outputs can be found on Github: https://github.com/Alice-MacQueen/fourway-ionomics. The phenotypic correlation between elements at each garden is presented in Supplemental Table S1. The variance partitioning between additive genetic variance and environmental variance in heritability estimation for each element at each garden is presented in Supplemental Table S2. The genetic correlation among sites for each element is presented in Supplemental Table S3. The identified QTL with confidence intervals are presented in Supplemental Table S4. The candidate genes are listed in Supplemental Table S5 (in a separate Excel), and the significant GO terms are included in Supplemental Table S6 (in a separate Excel). The effects of QTL identified for each element across gardens is presented in Supplemental Figure S1.

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Table 1. Element accumulation (µg g-1) means, standard errors, and comparisons by Welch one-way test of the four F0 ‘grandparent’ individuals at the TX, MO, and MI gardens.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Element | Site | AP13 | DAC | VS16 | WBC | P-Valuea |
| macronutrient |  | MI | 1614±48 | 2046±102 | 1163±48 | 1454±123 | <0.0001\* |
| Ca | MO | 1445±47 | 1395±80 | 1101±24 | 1736±155 | 0.0002\* |
|  | TX | 2947±149 | 5293±362 | 3953±156 | 2168±82 | <0.0001\* |
|  | MI | 72581±3741 | 46184±1711 | 31615±3024 | 66643±12666 | <0.0001\* |
| K | MO | 54865±5417 | 44609±11478 | 24143±8032 | 83190±10820 | 0.0419 |
|  | TX | 54414±5221 | 59728±13856 | 39167±5242 | 67527±7067 | 0.0525 |
|  | MI | 1367±50 | 1011±73 | 1059±50 | 1686±112 | <0.0001\* |
| Mg | MO | 857±25 | 767±47 | 784±50 | 1497±117 | 0.0175 |
|  | TX | 949±55 | 1333±101 | 1154±42 | 1027±52 | 0.0182 |
|  | MI | 296±10 | 391±21 | 386±18 | 441±24 | <0.0001\* |
| P | MO | 615±41 | 378±43 | 346±5 | 851±39 | <0.0001\* |
|  | TX | 316±12 | 758±53 | 650±41 | 300±16 | <0.0001\* |
| analogue |  | MI | 1.509±0.084 | 0.966±0.112 | 0.728±0.07 | 3.026±0.284 | <0.0001\* |
| Rb | MO | 2.923±0.162 | 1.245±0.129 | 0.94±0.036 | 3.719±0.222 | <0.0001\* |
|  | TX | 1.565±0.123 | 1.5±0.305 | 1.451±0.21 | 2.079±0.203 | 0.1951 |
|  | MI | 3.831±0.14 | 5.834±0.977 | 3.258±0.201 | 3.709±0.333 | 0.0418 |
| Sr | MO | 9.093±0.575 | 8.81±0.768 | 6.27±0.221 | 9.684±0.899 | 0.0011 |
|  | TX | 6.362±0.263 | 8.866±0.287 | 9.502±0.482 | 5.601±0.231 | <0.0001\* |
| micronutrient |  | MI | 3.417±0.247 | 4.12±1.188 | 3.294±0.431 | 3.32±0.502 | 0.9330 |
| B | MO | 3.402±0.704 | 3.196±0.673 | 3.319±2.247 | 2.476±0.273 | 0.6658 |
|  | TX | 4.925±0.421 | 7.211±0.432 | 6.852±0.537 | 4.402±0.319 | 0.0005\* |
|  | MI | 0.029±0.002 | 0.066±0.016 | 0.046±0.007 | 0.026±0.004 | 0.0356 |
| Co | MO | 0.219±0.057 | 0.321±0.186 | 0.145±0.025 | 0.168±0.036 | 0.6059 |
|  | TX | 0.082±0.008 | 0.149±0.047 | 0.189±0.122 | 0.11±0.033 | 0.4476 |
|  | MI | 3.223±0.144 | 5.333±0.261 | 4.919±0.125 | 3.332±0.164 | <0.0001\* |
| Cu | MO | 8.715±0.538 | 12.848±4.019 | 8.03±0.291 | 9.919±0.836 | 0.1985 |
|  | TX | 4.205±0.229 | 6.152±0.727 | 4.141±0.403 | 5.094±0.378 | 0.0729 |
|  | MI | 32.33±1.21 | 41.7±3.58 | 34.27±1.84 | 30.199±1.448 | 0.0458 |
| Fe | MO | 39.64±2.4 | 83.06±52.69 | 32.4±1.78 | 45.761±6.237 | 0.1069 |
|  | TX | 51.5±2.75 | 78.42±12.89 | 50.78±7 | 44.089±4.489 | 0.1662 |
|  | MI | 47.3±2.14 | 52.22±3.88 | 53.39±3.76 | 33.605±2.882 | 0.0009 |
| Mn | MO | 67.04±3.74 | 70.9±7.88 | 101.45±24.06 | 76.523±7.952 | 0.5783 |
|  | TX | 25.56±1.49 | 39.85±3.61 | 38.86±3.17 | 14.212±1.221 | <0.0001\* |
|  | MI | 0.046±0.002 | 0.039±0.003 | 0.051±0.003 | 0.041±0.003 | 0.0603 |
| Mo | MO | 0.087±0.004 | 0.056±0.005 | 0.053±0.015 | 0.122±0.009 | 0.0143 |
|  | TX | 0.092±0.011 | 0.044±0.005 | 0.053±0.007 | 0.117±0.018 | 0.0004\* |
|  | MI | 0.01±0.004 | 0.012±0.004 | 0.007±0.002 | 0.041±0.003 | 0.1384 |
| Se | MO | 0.042±0.003 | 0.05±0.017 | NA | 0.122±0.009 | 0.1384 |
|  | TX | 0.044±0.004 | 0.048±0.01 | 0.038±0.006 | 0.117±0.018 | 0.1384 |
|  | MI | 7.51±0.934 | 7.54±0.406 | 11.39±2.796 | 8.136±1.636 | 0.6080 |
| Zn | MO | 22.43±3.802 | 11.36±0.912 | 11.58±0.898 | 28.504±10.996 | 0.0754 |
|  | TX | 49.34±13.966 | 110.91±86.947 | 15.75±2.458 | 18.849±1.185 | 0.1489 |
| harmful |  | MI | 48.79±2.46 | 69.19±14.38 | 59.73±5.04 | 49.204±3.266 | 0.1845 |
| Al | MO | 102.17±10.24 | 95.78±30.36 | 77.56±10.51 | 84.231±5.996 | 0.5187 |
|  | TX | 68.36±5.2 | 100.48±16.74 | 77.55±7.45 | 56.923±4.699 | 0.0656 |
|  | MI | 0.01±0.001 | 0.019±0.004 | 0.012±0.001 | 0.011±0.001 | 0.1384 |
| As | MO | 0.016±0.003 | 0.022±0.017 | NA | 0.022±0.003 | 0.1384 |
|  | TX | 0.011±0.001 | 0.017±0.005 | 0.012±0.001 | 0.01±0.001 | 0.1384 |
|  | MI | 0.016±0.001 | 0.022±0.002 | 0.012±0.001 | 0.013±0.002 | 0.0027 |
| Cd | MO | 0.03±0.011 | 0.028±0.01 | 0.015±0.006 | 0.017±0.002 | 0.6142 |
|  | TX | 0.002±0 | 0.003±0 | 0.002±0 | 0.002±0 | 0.0216 |
|  | MI | 50.5±3.48 | 8.67±1.64 | 12.71±4.98 | 47.892±6.147 | <0.0001\* |
| Na | MO | 160.83±7.53 | 11.87±1.43 | 10.08±1.31 | 59.685±7.239 | <0.0001\* |
|  | TX | 122.87±12.37 | 35.46±5.04 | 65.56±14.28 | 124.885±15.271 | <0.0001\* |

aStars in this column indicate p-values that are significant after a Bonferroni correction for 54 independent Welch one-way tests.

Table 2. Element accumulation (µg g-1) means ± standard errors of the outbred F2 mapping population, and comparisons by Welch one-way test at the three common gardens.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Elementa | TX garden | MO garden | MI garden | P-valueb |
|  | Ca | 3768±35 | 1420±12 | 1408±15 | <0.001\* |
|  | Soil Ca | 16865 | 2351 | 2154 | CL: 180c |
|  | K | 60162±882 | 60032±1010 | 55912±958 | 0.002\* |
|  | Soil K | 285 | 106 | 41 | CL: 125c |
| macronutrient | Mg  Soil Mg | 1530±14  222 | 1144±8  332 | 1309±11  108 | <0.001\*  CL: 50c |
| P  Soil P | 421±4  8 | 485±7  19 | 294±3  32 | <0.001\*  CL: 50c |
| macronutrient  analogue | Rb | 1.788±0.027 | 2.436±0.026 | 1.087±0.019 | <0.001\* |
| Sr | 8.459±0.073 | 8.534±0.078 | 3.846±0.04 | <0.001\* |
| micronutrient | B | 5.565±0.059 | 2.645±0.046 | 3.233±0.06 | <0.001\* |
| Co | 0.065±0.001 | 0.14±0.004 | 0.028±0 | <0.001\* |
| Cu | 4.926±0.058 | 8.325±0.117 | 3.801±0.036 | <0.001\* |
| Fe | 43.48±0.4 | 32.88±0.41 | 27.69±0.25 | <0.001\* |
| Mn | 27.46±0.31 | 80.63±0.97 | 48.27±0.58 | <0.001\* |
| Mo | 0.053±0.001 | 0.059±0.001 | 0.032±0 | <0.001\* |
| Se | 0.047±0.001 | 0.039±0.001 | 0.009±0.001 | <0.001\* |
| Zn | 18.819±0.349 | 10.995±0.147 | 6.509±0.096 | <0.001\* |
| Al | 58.96±0.73 | 76.17±0.71 | 41.06±0.5 | <0.001\* |
| potentially  harmful | As | 0.01±0 | 0.013±0 | 0.01±0 | <0.001\* |
| Cd | 0.003±0 | 0.024±0.001 | 0.03±0.001 | <0.001\* |
| Na  Soil Na | 70.46±1.47  11 | 25.56±0.53  12 | 9.72±0.17  10 | <0.001\* |

aWhen the element indicated is prefaced by the word ‘Soil’ the row contains average soil elemental content at this garden.

bStars in this column indicate p-values that are significant after a Bonferroni correction for 18 independent Welch one-way tests.

cCL: Critical level. The point at which the Soil, Water, and Forage Testing Laboratory of Texas A&M University recommends no additional nutrient input.

**List of figures**

Figure 1. (a) Phenotypic variation (half violin plots) of ionomic content traits for the mapping population (F2) at the three field sites (TX, MO, and MI). (b) Heritability of each ionomic content trait at each of the three field sites (TX, MO, and MI).

Figure 2. (a) QTL with 1.5-LOD supportive intervals for each ionomic trait using the multi-environment QTL model from Genstat. (b) UpSet plot showing patterns in elemental content QTL colocalization between elements.

Figure 3. QTL effects (reaction norms) across the three field sites (TX, MO, and MI) for a sample of representative elements: (a) Na (potentially harmful element), (b) Mn (micronutrient), (c) Rb (macronutrient analog), and (d) P (macronutrient). A x B represents the lowland AP13 x upland DAC cross, C x D represents the lowland WBC x upland VS16 cross.