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

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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. Concentrations of 18 mineral elements were determined in whole post-anthesis tillers using ICP-MS. These measurements were used to identify quantitative trait loci (QTL) with and without QTL-by-environment interactions (QTLxE) using a multi-environment QTL mapping approach.
* Element concentrations varied significantly both within and between switchgrass ecotypes, and GxE was present at both the trait and QTL level. Concentrations of 14 of the 18 elements 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, previous work has provided limited insights into how the ionome varies in natural environments. 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).

To date, there has been limited progress in identifying the molecular mechanisms causing GxE in the plant ionome. 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 characterizing the elemental accumulation of various plant tissues or species, and though they have led to valuable knowledge on the genetic control of element accumulation in plants, they offer 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). 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 (*Oryza sativa*) and maize (*Zea mays*). 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 an 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 because it can be grown on marginal soils, which would minimize biofuel competition with food crops for arable land [6,25]. 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 the southern United States and tends to be more biomass-productive and nutrient-use-efficient than the northern upland ecotype (Porter Jr, 1966; Aspinwall *et al.*, 2013; Uppalapati *et al.*, 2013; Lowry *et al.*, 2014). In contrast, 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). Ionomics research in switchgrass has identified significant differences in elemental uptake between lowland and upland ecotypes for many elements (Yang *et al.*, 2009), including lower nutrient concentrations in lowland ecotypes; however, the genetic basis of this divergence has yet to be mapped. Nutrient elements are always removed along with harvested biomass; reduced nutrient removal necessitates lower fertilizer inputs to maintain plant productivity and thus promotes sustainable biofuel agriculture. High levels of some elements, particularly alkali metals, can negatively affect the downstream conversion to bioenergy and increase the cost of bioenergy production (Gouzaye *et al.*, 2014; de Koff & Allison, 2015; Serapiglia *et al.*, 2016). However, marginal soils are likely to vary more in their elemental compositions than traditional arable land, making understanding GxE in the switchgrass ionome all the more essential to identify genes that can promote nutrient-efficient growth in these environments. Understanding the genetics of ionomic concentration divergence between switchgrass ecotypes across their native range will help breeders develop switchgrass as a sustainable biofuel species.

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 plant macronutrients (Mg, P, K, Ca), plant micronutrients (B, Mn, Fe, Co, Cu, Zn, Se, Mo), analogues of macronutrients (Rb, Sr), and non-essential elements that can harm (Al, As, Cd, Na) or benefit plant growth (Na) (Marschner, 2012). 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 colocalize, 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. Woven ground cover (Sunbelt 3.2 OZ, Dewitt Company) 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, with no further supplemental irrigation after this point. The three common garden locations (Austin, Texas, hereafter TX; Columbia, Missouri, hereafter MO; and Hickory Corners, Michigan, hereafter MI) have distinct soil and climatic conditions. The TX garden (30.384°N, -97.73°W) has clay soil, the MO (38.897°N, -92.22°W) garden has a silt loam soil, and the MI (42.420°N, -85.37°W) garden has a loam soil. The concentrations of mineral P, K, Ca, Mg and Na at each of the three sites were measured on a soil sample consisting of equally mixed proportions of soil samples (0-15 cm depth) from three locations spanning the entire garden on the diagonal. Soil samples were analyzed by the Soil, Water, and Forage Testing Laboratory at Texas A&M University (http://soiltesting.tamu.edu). The concentrations of P, K, Ca, Mg, and Na were 8, 285, 16865, 222 and 11 ppm at the TX garden; 19, 106, 2351, 332 and 12 ppm at MO garden; and 32, 41, 2154, 108 and 10 ppm at the MI garden (see also Table 2). The average temperatures in 2016 at the TX, MO, and MI gardens were 21.9, 13.6, 10.4 °C, respectively. The annual precipitation in 2016 at the TX, MO and MI gardens were 829, 928, and 975 mm, respectively.

Samples of whole post-anthesis tillers of approximately 700 plants were collected at each of the three sites at the end of the 2016 growing season, after approximately 1.5 years of growth in each common garden. Tiller samples were first ground with a knife mill (Wiley Model 4, Thomas Scientific) to pass through a screen size of 2 mm and subsequently ground with an inducted air abrasion mill (Cyclone Mill, UDY corporation) to pass through a 1 mm screen. The milled samples were homogenized and aliquots were sent to the Donald Danforth Plant Science Center to determine tissue concentrations 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. Elemental concentrations 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 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 concentration variation and covariation at three common gardens**

To understand the genetic component of ionomic variation in switchgrass, we determined concentrations of 18 elements for both the F0 ‘grandparent’ genotypes and for the clonally replicated, outbred F2 genotypes at three common gardens. Average element concentration varied over six orders of magnitude: Co, Se, Mo, and Cd had the lowest concentrations (~1x10-2 µg g-1 dry weight) and K had the highest concentration (~1x104 µg g-1 dry weight). After correction for multiple testing, concentrations of 11 of the 18 elements differed significantly between the four grandparents (AP13, DAC6, WBC, and VS16) at one or more garden (Table 1). Concentrations of three elements (Ca, P, Na) differed significantly between the four grandparents at every garden after correction for multiple testing, and Sr and Mg concentrations also differed at every garden before this correction (Table 1). Interestingly, there were just as many significant differences in element\*garden concentrations (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 concentrations between the two upland parents (data not shown).

In the F2 genotypes, variation in the concentration of each element followed a continuous, unimodal distribution within each garden (Figure 1a). Within gardens, the majority of the element concentrations 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 concentration was positively correlated with Sr concentration at each site (0.8-0.9), and Al concentration was positively correlated with Fe concentration at MI (0.8) and TX (0.5).

All element concentrations had low to moderate heritabilities (0 < *h2* < 0.6, Figure 1b). The majority of the elements (K, Ca, Mg, P, Mn, Fe, Zn, Cu, Mo, Se, Sr, Rb, Na, Al, 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 concentration were moderately heritable only at MO), and 15 elements at the MI garden (K, Zn, Se and Cd concentration 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 the concentrations of these elements (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 concentrations 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 concentrations and availability, which varied in ways that affected plant element concentrations 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 gardens (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 concentrations. Only one negative genetic correlation was observed, for B concentration in the TX and MO gardens (-0.46). Negative correlations indicate a possible trade-off in loci controlling B concentration. It should be noted, however, that B concentration heritabilities were 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 concentrations 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 concentrations of 14 elements (Figure 2a, and Supplemental Table S4). Thirty-eight (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 the tissue concentrations of these four elements (Figure 1b). The remaining elements had between two (Na, Fe, Mo, Cd) and 14 (P) significant QTL. We determined if the number of QTL we identified varied by element type by dividing the 18 elements into four types: macronutrients, micronutrients, non-essential analogues to nutrients, and other non-essential elements. The presence of more elemental QTL than expected indicates ecotype-specific genetic divergence, while the presence of fewer than expected might indicate that purifying selection has removed genetic variation for these 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 were more QTL than expected for both macronutrients (2.05x, binomial test *p* < 0.001) and non-essential analogues (1.99x, binomial test *p* = 0.002), and fewer QTL than expected for micronutrients (0.50x, binomial test *p* < 0.001) and other non-essential 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 colocalized. Co-localization suggests either linked genes affecting element accumulation, or co-transport of elements using the same ion channel. The latter is more plausible for elements that are most commonly bioavailable in the soil as similar ions. We considered QTL to colocalize 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 colocalization 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 the 77 QTL, particularly the 38 QTL exhibiting QTLxE (Figure 3, and Supplemental Figure S1). The design of the crosses that generated the four-way population allowed quantification of differences in allelic effects for two distinct lowland vs. upland crosses, AP13 vs. DAC (A x B) and WBC vs. VS16 (C x D). Thus, 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 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. These four QTL may well be caused by multiple linked loci; however, if these four colocalizing QTL are due to single loci that affect the concentration of multiple elements, then these QTL represent an interesting case of GxE caused by changes in pleiotropy at a single locus.

For the 38 QTL, and 76 allelic contrasts with QTLxE, 35 contrasts (46%) had differential sensitivity in their reaction norm 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 observed in either one or both lowland vs. upland allelic contrasts for the same QTL. For instance, the effect of QTL 2N@24.04 for the macronutrient Ca was differentially sensitive in both allelic contrasts (Figure 3a), while the effect of QTL 2N@10.06 for the micronutrient Mn 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 these contrasts 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 allelic contrast. For example, the effects of QTL 3K@36.09 for the micronutrient Zn were antagonistic for the C x D contrast, but not the A x B contrast (Figure 3c). Overall, QTL for the same element with QTLxE did not have similar patterns across environments. For example, the QTL 2N@78.05 and 3K@26.18 for the macronutrient P 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 concentration in switchgrass. For example, *Pavir.9NG231800*, a homolog of *MOT1*, is located within the 1.5-LOD interval of the largest Mo concentration 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 concentration 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) QTL intervals. These genes are responsible for Cu, Cd and Zn transport. A sixth candidate gene, *Pavir.9KG014451*, was associated with the homolog of *A. thaliana* *MYB36*. *MYB36* is aMYB domain transcription factor that regulates the expression of genes involved in the formation of the Casparian strip. The absence of the Casparian strip results in changes in leaf concentrations 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) concentrations.

To elucidate the cellular pathways associated with ion concentrations in switchgrass, we also looked at GO term enrichment based on the gene content in the 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), is associated with chlorophyll (15-20% of total Mg), and functions 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 ion binding, and in particular, antioxidant activity. K 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 concentration 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 concentration 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. Cu is also involved in the control of cellular redox state (a major Cu-binding protein is the Cu/Zn superoxide dismutase) and remodeling of the cell wall (Cohu & Pilon, 2010; Andresen *et al.*, 2018). Among non-essential elements, Cd QTL regions were significantly enriched for GO ontologies of metal ion binding, photosynthesis (light harvesting), and cell growth among others. Cd is one of the most toxic heavy metals for plants and 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 is a powerful tool for determining the elemental status of plants, and can be combined with mapping populations to determine the genetic architecture responsible for variation in elemental composition. Our study not only examined the genetic basis of the switchgrass ionome, but also how individual ionomic loci responded to three environments (i.e., expressed GxE) across the native range of this perennial species. 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 concentration variation at the QTL level. We observed common QTL colocalization between elements, which supports a partially shared regulatory network for element uptake, transportation, or accumulation, as previously suggested (Baxter *et al.*, 2014; Dhanapal *et al.*, 2018). Understanding the genetic architecture of elemental accumulation in our outbred population of divergent switchgrass ecotypes is the first step in uncovering the potential for ionomic adaptation in switchgrass across variable 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 differentially sensitive effects, but also substantial antagonistic pleiotropy (54%) across the ionomic QTL with QTLxE, indicating that alleles commonly had opposing effects on element concentrations 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 found substantial colocalization of P QTL with cation QTL, always with elements most abundant in soil as cations with 1+ or 2+ charge. P is a component of key molecules of plants such as ATP, nucleic acids, and the form of P 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-transport 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 networks 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 macronutrient Mg (6 non-colocalizing QTL out of 9). P and Mg deficiencies in soils are often widespread (Maathuis, 2009); thus, a 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 concentrations 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 concentration of potentially harmful elements (Al, As, and Cd), and fewer QTL than expected for these 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 the four grandparents were sampled. Alternatively, harmful elements may not be present in sufficient quantities in the commonly encountered soils and in the three common garden 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 analog Ca at every garden, and they shared colocalized QTL at the two large clusters on Chr02N (at the top) 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 within our QTL regions which may affect element concentrations. These candidate genes 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. The identification of loci that affect nutrient concentration in these environments will facilitate the development of switchgrass varieties with high nutrient-use efficiency for sustainable biofuel production. When combined with harvested biomass, plant elemental concentrations can be linked to nutrient removal from the soil and impact biofuel conversion efficiency and future soil fertility.

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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.H.M. analyzed data; L.Z. and A.H.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 concentration (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 | 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 | 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 | 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\* |
| micronutrient |  | 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 | 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 | 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 |
|  | 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 | 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 | 0.046±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 | 0.029±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 | 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 | 3.831±0.14 | 5.834±0.977 | 3.258±0.201 | 3.709±0.333 | 0.0418 |
| analogue | 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\* |
|  | 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 | 50.5±3.48 | 8.67±1.64 | 12.71±4.98 | 47.892±6.147 | <0.0001\* |
| other | 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\* |
|  | 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 |

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

Table 2. Element concentration (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 |
| macronutrient | K | 60162±882 | 60032±1010 | 55912±958 | 0.002\* |
| Soil K | 285 | 106 | 41 | CL: 125c |
| Ca | 3768±35 | 1420±12 | 1408±15 | <0.001\* |
| Soil Ca | 16865 | 2351 | 2154 | CL: 180c |
| 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 |
| micronutrient | Mn | 27.46±0.31 | 80.63±0.97 | 48.27±0.58 | <0.001\* |
| Fe | 43.48±0.4 | 32.88±0.41 | 27.69±0.25 | <0.001\* |
| Zn | 18.819±0.349 | 10.995±0.147 | 6.509±0.096 | <0.001\* |
| Cu | 4.926±0.058 | 8.325±0.117 | 3.801±0.036 | <0.001\* |
| B | 5.565±0.059 | 2.645±0.046 | 3.233±0.06 | <0.001\* |
| Mo | 0.053±0.001 | 0.059±0.001 | 0.032±0 | <0.001\* |
| Co | 0.065±0.001 | 0.14±0.004 | 0.028±0 | <0.001\* |
| Se | 0.047±0.001 | 0.039±0.001 | 0.009±0.001 | <0.001\* |
| analogue | Sr | 8.459±0.073 | 8.534±0.078 | 3.846±0.04 | <0.001\* |
| Rb | 1.788±0.027 | 2.436±0.026 | 1.087±0.019 | <0.001\* |
| other | Na  Soil Na | 70.46±1.47  11 | 25.56±0.53  12 | 9.72±0.17  10 | <0.001\* |
| Al | 58.96±0.73 | 76.17±0.71 | 41.06±0.5 | <0.001\* |
| 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\* |

aWhen the element indicated is prefaced by the word ‘Soil’ the row contains average soil elemental concentration 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. The genetic component of phenotypic variation in element concentrations (µg g-1) across three common gardens (TX: orange; MO: green; MI: blue) (a) Phenotypic variation in element concentrations for the mapping population (F2). (b) Heritability of each element concentration.

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Figure 2. The overlapping genomic distributions of QTL for element concentrations (µg g-1). (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 concentration QTL colocalization between elements. Vertical barplot shows the number of QTL that colocalize for each combination of elements, represented by the filled circles connected by lines when more than one element colocalizes. Horizontal barplot shows the number of QTL for each element.



Figure 3. Representative differentially sensitive and antagonistically pleiotropic reaction norms for element concentrations (µg g-1) QTL effects across three common gardens (TX, MO, and MI). Two allelic contrasts are shown: A x B panels show QTL effects for the lowland AP13 x upland DAC cross, and C x D panels show QTL effects for the lowland WBC x upland VS16 cross. (a) Ca (macronutrient): 2N@24.04 shows differential sensitivity in both allelic contrasts. (b) Mn (micronutrient): 2N@10.06 shows differential sensitivity in one allelic contrast, (c) Zn (micronutrient): 3K@36.09 shows antagonistic pleiotropy in one allelic contrast, and (d) P (macronutrient): has five QTL with QTLxE with distinct reaction norms at each QTL.