Integrating co-expression networks with GWAS detects genes driving elemental accumulation in maize seeds

Robert J. Schaefer1, Jean-Michel Michno1,2, Joseph Jeffers3, Owen Hoekenga4, Brian Dilkes5, Ivan Baxter6,7\*, Chad L. Myers1,3\*

# 

1. Biomedical Informatics and Computational Biology Graduate Program, University of Minnesota, Minneapolis, MN, USA
2. Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN, USA
3. Department of Computer Science, University of Minnesota, Minneapolis, MN, USA
4. Cayuga Genetics Consulting Group LLC, Ithaca, NY, USA
5. Department of Biochemistry, Purdue University, West Lafayette, IN, USA
6. Donald Danforth Plant Science Center, St. Louis, MO, USA
7. USDA-ARS Plant Genetics Research Unit, St. Louis, MO, USA

\* Corresponding Authors: Ivan Baxter, [ivan.baxter@ars.usda.gov](mailto:ivan.baxter@ars.usda.gov);

Chad L. Myers, [cmyers@cs.umn.edu](mailto:cmyers@cs.umn.edu)

# Abstract

Genome wide association studies (GWAS) have identified thousands of loci linked to hundreds of traits in many different species. However, for most loci, the causal genes and the cellular processes they contribute to remain unknown. This problem is especially pronounced in species where functional annotations are sparse. Given little information about a gene, patterns of expression are a powerful tool for inferring biological function. Here, we developed a computational framework called Camoco that integrates loci identified by GWAS with functional information derived from gene co-expression networks. We built co-expression networks from three distinct biological contexts and establish the precision of our method with simulated GWAS data. We applied Camoco to prioritize candidate genes from a large scale GWAS examining the accumulation of 17 different elements in maize seeds demonstrating the need to match GWAS datasets with co-expression networks derived from the appropriate biological context. Furthermore, our results show that simply taking the closest genes to significant GWAS loci will often lead to spurious results, indicating the need for proper functional modeling and a reliable null-distribution when integrating these high throughput data types. We performed functional validation on two genes identified by our approach using mutants and annotate other high priority candidates with ontological enrichment and curated literature support, resulting in a targeted set of candidate genes that drive elemental accumulation in maize grain.

# Introduction

Genome wide association (GWA) studies are a powerful tool for understanding the genetic basis of trait variation. This approach has successfully helped identify the genetic bases of hundreds of important traits in different species, including important yield-relevant traits in crops. Sufficiently powered GWA studies often identify tens to hundreds of loci, containing hundreds of single nucleotide polymorphisms (SNPs) associated with a trait of interest (McMullen et al. 2009), although many SNPs reside outside annotated gene boundaries (Wallace et al. 2014). In *Zea mays* (maize) alone, GWA studies have identified nearly 40 genetic loci for flowering time (Buckler et al. 2009), 89 loci for plant height (Peiffer et al. 2014), 36 loci for leaf length (Tian et al. 2011), 32 loci for resistance to southern leaf blight (Kump et al. 2011), and 26 loci for kernel protein (Cook et al. 2012). These studies describe the genetic architecture of and statistical associations between many loci and a trait of interest, but the identification of causal genes and functional alleles remains a challenge. The molecular identities of genes responsible for trait variation also ascribes function to biological pathways and processes that are affected by the identified variants. Linkage disequilibrium (LD), which powers GWAS, also acts as a major hurdle that limits the identification of causal genes. Genetic markers are identified by a GWA study, but can be physically displaced from the causal mutation. Thus, a GWA “hit” can implicate many causal genes at each associated locus. In maize, LD varies between 1kb to over 1 million bases (Gore et al. 2009). In other crop species (Morrell et al. 2005; Caldwell et al. 2006) and humans (Reich et al. 2001; Koch, Ristroph, and Kirkpatrick 2013) it can extend even farther. Furthermore, gene regulatory regions play a significant role in functional variation (Wray 2007), which means that causal variants can be physically distant from the gene whose expression is affected. Several QTL comprised of non-coding sequences have been previously reported in maize (Clark et al. 2006; Castelletti et al. 2014; Louwers et al. 2009). These challenging factors mean that even when a variant is strongly associated with a trait, many plausible candidate genes are implicated because of their location. These issues are multiplied when studying complex traits involving the coordinated effects of many loci throughout the genome. Narrowing candidates to likely causal genes through prior knowledge is exacerbated in crop species, where gene annotation is largely incomplete. For example, mutant analysis of maize reveals that only ~1% of genes have functional annotations (Andorf et al. 2015).

Even when a short list of candidate genes is identified for a locus, it is difficult to integrate disparate genomic data types that help identify strong candidate genes influencing a trait. The interpretation and narrowing of large lists of candidates linked to SNPs with strong association to complex traits bottlenecks our mechanistic understanding of how natural variation in genes influences traits. Advanced mapping populations, developed in crops and model species, enabled the rapid identification of hundreds of loci that affect traits critical to important issues such as worldwide food supply and crop nutritional quality. Yet, we lack the tools to understand the wealth of information linking genotypic variation to phenotype. Although we expect that genes interact to affect phenotypical changes, GWA studies do not explicitly model interactions between genes.

RNA expression data provides an informative and easily measurable source of information about gene function. Simultaneous assessment of all genes’ expression profiles in different contexts, such as during development or within different genetic backgrounds, helps establish how a gene’s expression is linked to its biological function. In general, we expect that the cellular machinery underlying specific processes or traits require many genes with highly similar expression profiles. Additionally, variation in the pattern or intensity of gene expression can underlie variation in observed phenotypes, even where protein sequences maybe identical. Analysis of co-expression in the form of gene networks has identified functionally related genes, including in several crop species (R.J. Schaefer et al. 2014; Mochida et al. 2011; Obayashi et al. 2014; Sarkar, Kim, and Grover 2014; Zheng and Zhao 2013; Ozaki et al. 2010; Ruth Swanson-Wagner et al. 2012).

Because co-expression provides a measure of functional relationships, it has the potential to serve as a powerful means of interpreting GWAS candidate loci. We expect that a set of genes contributing to the same biological process will affect variation in a given phenotype through guilt-by-association (Wolfe, Kohane, and Butte 2005). Thus, genetic variation discovered by GWAS is likely encoded by co-regulated genes, and these data sets should non-randomly overlap. By directly integrating GWAS data with co-expression networks, candidate genes linked to GWAS SNPs can be prioritized based on putative functional information captured by the gene co-expression network and simultaneously annotated to biological processes. Though not all functional relationships are captured using co-expression (Ritchie et al. 2015), the comparison of these data have the potential to provide highly informative, and sometimes exclusive, clues about genes that have been otherwise not been studied. This principle has been used successfully with other types of networks, such as protein-protein interactions (M. Li et al. 2008), and co-expression has been used as a basis for understanding GWA studies in mouse and human (Calabrese et al. 2017; Bunyavanich et al. 2014; Taşan et al. 2014). It is, however, often unclear what the null expectations are for overlapping two sets of genes that were selected based on their performance in functional assays. A simple ranking or overlap is likely to identify genes present in co-expression networks and GWAS candidate gene lists, but a thoughtful consideration of overlap expectations is required to provide confidence that such an overlap could not be found by picking two lists at random.

We developed a freely available, open source computational framework called Camoco (**C**o-**A**nalysis of **mo**lecular **co**mponents), designed for integrating GWAS candidate lists with gene co-expression networks to prioritize individual candidate genes. Camoco evaluates candidate SNPs and corresponding association statistics derived from a typical GWAS study, then identifies a ranked list of high confidence candidate genes embedded in strongly connected functional modules where multiple members of those modules are associated with the phenotype of interest.

We applied this approach in maize, which serves as a genetic model system and is one of the most important agricultural crops in the world. We focused on quantitative phenotypes measuring the accumulation of 17 different elements in the maize grain ionome (Al, As, B, Ca, Cd, Fe, K, Mg, Mn, Mo, Na, Ni, Rb, S, Se, Sr and Zn). Plants must take up all elements except carbon and oxygen from the soil, making the plant ionome a critical component in understanding plant environmental response (I. Baxter 2010), grain nutritional quality (Guerinot and Salt 2017) and plant physiology (I. R. Baxter et al. 2008). We evaluated multiple different types of co-expression networks for supporting the application of Camoco. To demonstrate the efficacy of our approach, we simulated GWA studies using Gene Ontology (GO) terms to establish maize specific SNP-to-gene mapping parameters as well as a robust null model for GWAS-expression network overlap. This approach confirms the overlap between functional modules captured by co-expression networks and GWAS candidate SNPs for the maize grain ionome. We present high-confidence candidate genes identified for a variety of different ionomic traits, test single gene knockouts demonstrating the utility of this approach, and highlight several best practices on integrating co-expression networks and GWAS data.

# Results

## A framework for integrating GWAS results and co-expression networks

We developed a computational framework called Camoco that integrates GWAS outputs with co-expression networks to prioritize high-confidence causal genes associated with a phenotype of interest. Camoco’s rationale is that genes which function together in a biological process, as identified by a GWA study, should also have non-random structure in co-expression networks which capture the same biological function. Our approach takes a list of SNPs associated with a trait of interest and a table of gene expression values as input, and produces a list of high priority candidate genes near GWAS peaks that have evidence of strong co-expression as output.

The three major components of the Camoco system are a module for SNP-to-gene mapping (Fig. 1A), tools for construction and analysis of co-expression networks (Fig. 1B), and an "overlap" algorithm that integrates GWAS-derived candidate genes with co-expression networks and identifies high priority candidate genes with strong co-expression support across multiple GWAS loci (Fig. 1C) (see Materials and Methods for details on each component). The overlap algorithm identifies high-priority genes by identifying the subset of genes that are highly connected to other genes also implicated by GWAS SNPs.

To accomplish this, we implemented and evaluated two different network scoring metrics: subnetwork density and subnetwork locality (Eq.1 and Eq.2). Subnetwork density measures the average interaction strength between all pairwise combinations of genes near GWAS peaks. Subnetwork locality measures the specificity of co-expression-derived interactions for a given candidate gene to all genes in the GWAS-derived subnetwork as compared to all other genes in the genome (See Materials and Methods for score details). For an input GWAS trait and gene expression data set, statistical significance for both density as well as locality is determined by generating a null-distribution based on generating random GWAS traits (n=1000) with the same number of implicated loci and corresponding candidate genes. This null-distribution is used to derive a p-value for the observed subnetwork density and locality for all putative causal genes (Fig. 1D). The end result of this process is, for a given input GWAS trait, Camoco produces a ranked list of candidate causal genes for both network metrics, and a false discovery rate that indicates the significance of the observed overlap between each candidate causal gene and the co-expression network. Using this integrated approach, the number of candidate genes prioritized for follow-up validation is reduced to those with two levels of functional evidence. Our method can be applied to any trait and species where GWAS and gene expression experiments are applicable.

### Fig. 1

### Schematic of the Camoco framework

The Camoco framework integrates genes identified by SNPs associated with complex traits with functional information inferred from co-expression networks. **(A)** A typical GWAS result for a complex trait identifies several SNPs (circled) passing the threshold for genome-wide significance indicating a multi-genic trait. SNP-to-gene mapping windows identify a varying number of candidate genes for each SNP. Candidate genes are identified based on user-specified window size and a maximum number of flanking genes surrounding a SNP (e.g. 50kb and 2 flanking genes designated in red). **(B)** Independently, gene co-expression networks identify interactions between genes uncovering an unbiased survey of putative biological co-function. Network interactions are identified by comparing gene expression profiles across a diverse set of accessions (e.g. experimental conditions, tissue, samples). Dense subnetworks indicate sets of genes with strongly correlated gene expression profiles. **(C)** Co-analysis of co-expression interactions among GWAS trait candidate genes identifies a small subset of genes with strong network connections. Blue lines indicate genes that have similar co-expression patterns, indicating co-regulation or shared function. Starred genes are potential candidate genes associated with GWAS trait based on SNP-to-gene mapping and co-expression evidence. Red stars indicate genes that are not the closest to the GWAS SNP (non-adjacent) that may have been missed without co-expression evidence. **(D)** Statistical significance of subnetwork interactions is assessed by comparing co-expression strength among genes identified from empirical GWAS datasets to random networks containing the same number of genes. In the illustrated case, the more interesting subnetwork has both high density as well as locality.

## Generating co-expression networks from diverse transcriptional data

A co-expression network derived from the biological context that generates the phenotypic variation subjected to GWAS is a key component of our approach. A well-matched co-expression network will describe functional relationships identify coherent subsets of GWAS-implicated genes. We and others have shown that co-expression networks generated from expression data derived from different contexts capture different functional information (Robert J. Schaefer, Briskine, Springer, and Myers 2014; R. Swanson-Wagner et al. 2012). For example, experiments measuring changes in gene expression can explore environmental adaptation, developmental and organ based variation, or variation in expression that arises from population and ecological dynamics (see (R.J. Schaefer, Michno, and Myers 2016) for review). For some species, published data contains enough experimental accessions (i.e. samples, tissues, conditions, etc.) to build networks from these different types of expression experiments. We reasoned that these different sources of expression profiles likely have a strong impact on the utility of the co-expression network for interpreting genetic variation captured by GWAS. Using this rationale, we constructed several different co-expression networks independently, and assessed the ability of each to produce high-confidence discoveries using our Camoco framework.

All datasets used here were generated from whole-genome RNA-Seq analysis. The first dataset (ZmPAN) targeted expression variation between diverse maize accessions, and was built from whole-seedling transcriptomes on a panel of 503 diverse maize inbred lines from a previously published dataset (Hirsch et al. 2014). Briefly, Hirsch et al. chose these lines to represent major heterotic groups within the US, sweet corns, pop corns, and exotic maize lines while seedling tissue was chosen due to the number of represented tissues. The second dataset (ZmSAM) examined genotypic variation of different tissues and across developmental time points. Whole genome RNA-Seq transcriptome profiles from 76 different diverse tissues and developmental time points in the maize reference accession, B73, were used to build a second gene co-expression network using a publically available dataset (Stelpflug et al. 2015). Finally, we created a third dataset (ZmRoot hereafter) as part of the ionomics GWAS research program. These data measure genotypic variation existing within the root, which serves as the primary uptake and delivery system for all the measured elements. Gene expression was measured from mature roots in a collection of 46 genotypically diverse maize inbreds.

### Table 1

#### Significantly Co-expressed GO Terms

Co-expression was measured among genes within each GO term that had co-expression data in each network, using both density (Eq.1) and locality (Eq.2). Significance of co-expression metrics was assessed by comparing values to 1000 random gene sets of the same size.

Co-expression networks for each dataset were constructed from gene expression matrices using Camoco (see Supp. File 1 and Materials and Methods for specific details on building these networks). Once built, several summary statistics were evaluated from interactions that arise from genes in the network (Supp. Fig. 1-3). Co-expression was measured among genes within the same Gene Ontology (GO) term to establish how well density and locality captured terms with annotated biological functions.

Density and locality were measured for subnetworks consisting of the set of genes co-annotated to each GO term and compared to scores from 1000 random sets of genes of the same size (See Table 1; Supp. Table 1 for full data). In total, 818 GO Terms of the 1078 tested (76%) were comprised of gene sets that were significantly co-expressed (p ≤ 0.01) in at least one network using density or locality relative to the randomized gene lists of the same size. Broken down by network as well by co-expression score, there was substantial co-expression among GO Terms for both density as well as locality in each network. Density was significant for the most GO terms in the ZmRoot network while locality performed best in ZmPAN (See Table 1). Taking into account terms captured by both scores or by either score, overlap between the two co-expression metrics was comparable. As previously reported (Robert J. Schaefer, Briskine, Springer, Myers, et al. 2014), GO terms that exhibit strong co-expression between members often do so in only a subset of the networks (Supp. Table 1). Thus, the expression data that provides information about biological process and nature of the co-expression score used determine the network’s experimental context and influenced the subset of GO terms with significantly co-expressed members. Overall, while density and locality recover different GO terms as significantly co-expressed sets of genes, there are substantially more co-expressed GO terms, for either score, than was found by size-matched randomly generated sets of genes (Supp. Table 1).

### Table 2

#### Gene co-expression network cluster assignments

Gene clusters were calculated by running Markov-Clustering (MCL) on the co-expression matrix. Cluster values designate network specific gene clusters and are not compared across networks.

In addition to detecting strong co-expression among genes previously annotated by functional processes, unsupervised network clustering using the Markov-Clustering algorithm (Dongen 2000) showed distinct modules within each network. A large number of clusters were significantly enriched (hypergeometric p-value < 0.01; Supp. Table 3) for genes that are co-annotated for the same GO term. Not all clusters identified previously annotated gene sets. Many strongly co-expressed clusters lacked any previously annotated function (Table 2; Supp. Table 3), potentially identifying novel co-regulated biological processes. Additionally, all networks exhibited a truncated power law distribution in the number of significant interactions (degree) each gene possessed (Supp. Fig. 1-3), which is typical of biological networks (Ghazalpour et al. 2006).

### Accounting for *cis-* gene interactions

Camoco models the integration of GWAS candidates with co-expression interactions by directly assessing the density or locality of interaction among candidate genes near GWAS SNPs. However, the process of mapping SNPs to surrounding candidate genes has inherent complications that can strongly influence subnetwork co-expression calculations. While we assume that most informative interactions among candidate genes are between GWAS loci, cis-regulatory elements and other factors can lead to co-expression between linked genes and produce skewed distributions in density and locality calculations, which can in turn bias co-expression statistics. Identifying significant overlap between GWAS loci and co-expression networks requires a distinction between co-expression among genes that are close to each other on a chromosome (*cis*-) compared to those genes which are not (*trans*-).

To assess the impact of *cis-* co*-*expression, network interactions for genes located on different chromosome (*trans-*interactions) were compared to cis­-interactions for pairs of genes less than 50 kb apart. The groups’ distributions indicate that *cis-* genes are much more likely to have strong co-expression interaction scores than *trans-* genes (Fig 2). This bias toward linked genes is especially pronounced for strong positive co-expression, where we observed substantially stronger enrichment for linked gene pairs compared to *trans-* genes (e.g. Z-score ≥ 3, see Fig 2 inset).

### Fig 2

#### Cis vs Trans Co-Expression Network Interactions

Comparing distributions of co-expression network interaction scores between *cis-* and *trans-* sets of genes. Distribution densities of *trans-* gene pairs (green) show interactions between genes on separate chromosomes. Distribution densities of *cis-* gene pairs (blue) show interactions between genes with less than 50kb intergenic distance. Inset figures show Z-score values greater than 3.

The enrichment of significant co-expression among *cis*- genes, likely due to shared *cis*-regulatory sequences, prompted us to remove *cis-* interactions when examining co-expression relationships among candidate genes identified by GWAS SNPs in Camoco. To account for possible cis-regulation within network metrics described here, only interactions that span different GWAS loci (*trans-*) are included in density and locality calculations for GWAS-network overlap calculation (See Materials and Methods).

## Evaluation of the Camoco framework

To explore the limits of our co-expression based approach, we examined factors that influence overlap detection between co-expression networks and genes linked to GWAS loci. In an idealized scenario, SNPs identified by GWAS map directly to true causal genes, all of which exhibit strong co-expression network interactions (Fig 3). But in practice, SNPs can affect regulatory sequences or be in linkage disequilibrium (LD) with the functionally important allele, leading to a large proportion of SNPs occurring outside of genic regions (Wallace et al. 2014).

We evaluated two major factors that influence SNP-to-gene mapping. The first of these is the total number of functionally related genes in a subnetwork, representing the fraction of genes involved in a causal biological process, that are simultaneously identified by GWAS. In cases where too few genes represent any one of the underlying causal processes, our proposed approach does not perform well. For example, only a single locus in a 10 gene biological process reaches genome-wide significance due to penetrance, limited allelic variation in the mapping population, or extensive gene by environment interactions. We refer to this as the *missing candidate gene rate (MCR)*, or, in other words, the fraction of genes involved in the causal process not identified by the GWAS in question (Fig 3**B**; Eq. 6).

A second key challenge in using Camoco to identify causal genes from GWAS loci is the fact that each associated SNP can be potentially linked with a large number of candidate genes. Thus, in cases where the linked regions are large (i.e. imperfect SNP-to-gene mapping), the framework’s ability to confidently identify subnetworks of highly co-expressed causal genes may be compromised. One would expect to find scenarios where the proposed approach does not work simply because there are too many non-causal genes implicated by linkage within each GWAS locus, such that the co-expression signal among the true causal genes is diminished by the false candidates linked to those regions. We refer to this as the *false candidate gene rate (FCR)*, the fraction of all genes linked to GWAS loci that are not causal genes (Fig 3**C**; Eq. 7).

To explore the limits of our co-expression-based approach with respect to these factors, we simulated scenarios where we could precisely control both MCR and FCR. In practice, neither of these quantities can be controlled — MCR is a function of the genetic architecture of the phenotype as well as the degree of power within the study population of interest, and FCR is a function of recombination frequency in the GWA study population.

We evaluated the expected performance of the Camoco framework for a range of both parameters by simulating ideal GWAS scenarios using co-expressed GO terms (p < 0.05; Table 1). These ideal cases were then either subjected to a subset of genes being replaced by random genes (i.e. to simulate MCR but conserve term size) or adding functionally unrelated genes using SNP-to-gene mapping (i.e. to simulate FCR introduced by linkage). In both cases, simulated GWAS candidates (GO term set members) were subjected to varying levels of either FCR or MCR while tracking the number of GO terms that remained significantly co-expressed at each level. These simulations enabled us to explore a broad range of settings for these key parameters and establish whether our proposed approach had the potential to work in the maize GWAS setting.

### Fig 3

#### Simulating GWAS-network overlap using Gene Ontology Terms

Several GWAS scenarios were simulated to assess the effect of noise on co-expression network overlap. Panel (**A**) shows an ideal GWA study where SNPs (blue points) map directly to candidate genes within the same biological process (i.e. a GO Term) and have strong co-expression (green lines). Signal is defined as the co-expression among the genes exclusive to the GO term. Noise in the overlap between GWAS and co-expression networks was introduced by varying two parameters: missing candidate gene rate (MCR) and false candidate gene rate (FCR). Panel **B** demonstrates the effect of a large proportion of missing candidate genes (e.g. MCR=2/5) on network signal. Likewise, panel (**C**) shows the effect of false candidate genes (FCR) on network overlap, either through false positive GWAS SNPs (orange points) or through imperfect SNP-to-Gene mapping (e.g. FCR=3/8). Here the additional candidate genes introduce co-expression noise (orange lines) that impedes the identification of network structure.

### Simulated GWAS datasets show robust co-expression signal to MCR and FCR

Subnetwork density and locality were measured for significantly co-expressed GO terms containing between 50 and 150 genes in each network at varying levels of MCR (see Supp. Table 4). At each MCR level, density and locality among the remaining genes were compared to 1000 random sets of genes of the same size. The proportion of initial GO terms that remained significantly co-expressed was recorded for each network (see Fig. 4, red-curve; See Supp. Fig. 4**A** for absolute term numbers). GO terms were also split into two starting groups based on strength of initial co-expression: moderate (0.001 < p-val ≤ 0.05; blue curve) and strong (p-val ≤ 0.001; violet curve).

### Fig. 4

#### Strength of co-expression among GO Terms at varying levels of MCR

Subnetwork density and locality were measured for all GO terms with strong initial co-expression (p≤ 0.05) comparing co-expression in GO Terms to 1,000 random networks of the same size. Co-expression density and locality were compared again (n=1000) with varying missing candidate rate (MCR), with a percentage of genes removed from the term and replaced with random genes. Curves decline with increased MCR, as the proportion of strongly co-expressed GO terms (p-val ≤ 0.05, n=1000) decreases compared to the initial number of strongly co-expressed terms in each network (Red curve). GO terms in each network were also split into two subsets based on initial co-expression strength: ‘Strong’, initial co-expression p ≤ 0.001 (Blue curve), and ‘Moderate’, initial 0.001 < p ≤ 0.05 (Purple curve). In all three networks, GO terms with stronger initial co-expression were more robust to missing candidate genes.

As expected, strength of co-expression among GO terms decreased as MCR increased. Fig 3 shows the decay in the proportion of GO terms that exhibit significant co-expression at increasing levels of MCR (red curve). In general, the decay of signal is similar between density and locality, where signal initially decays slowly until approximately 60% MCR, when signal quickly diminishes.

We observed that the initial strength of co-expression impacted the rate at which co-expression signal decayed. Signal among strongly co-expressed GO terms (p-val ≤ 0.001; violet curve) decayed at a substantially lower rate than moderately co-expressed GO terms, indicating that this approach is robust for GWAS datasets with moderate levels of missing genes when co-expression among true candidate genes is strong. Co-expression signal in relation to MCR was also compared between GO terms split by the number of genes within the term (See Supp. Fig. 4**B-C**), which did not influence the rate at which co-expression signal decayed.

Likewise, the effect of FCR was simulated. Significantly co-expressed GO terms encompassing between 50 and 150 genes (MCR=0) with significant co-expression (p<0.05; see Supp. Table 4) were selected. The nucleotide position of the starting base pair of each true GO term gene were used as input for our SNP-to-gene mapping protocol for identifying GWAS candidates (See Materials and Methods). Subnetwork density and locality were calculated for the expanded gene sets that corresponded to the simulated candidate genes at each SNP-to-gene mapping combination in each network to evaluate the decay of co-expression signal as FCR increases (Fig. 5).

### Fig. 5

#### Simulated GWAS: SNP-to-gene Mapping Density Signal Robustness

Strongly co-expressed GO terms (density or locality p-value ≤ 0.05) were used to simulate the effect of FCR on GWAS results. False candidates were added to GO terms by including flanking genes near true GO term genes according to SNP-to-gene mapping (windowing) parameters. Boxplots show effective FCR of GO terms at each SNP-to-gene mapping parameter. Signal plots show the proportional number GO terms that remain significant at FCR ≥ x (red curve). GO terms in each network were also split into two subsets based on initial co-expression strength: ‘Strong’, initial co-expression p ≤ 0.001 (Blue curve), and ‘Moderate’, initial 0.001 < p ≤ 0.05 (Purple curve). In all three networks, GO terms with stronger initial co-expression were more robust to missing candidate genes.

Candidate genes were added by varying the window size for each SNP up to 50kb, 100kb and 500kb upstream and downstream, and varying the maximum number of flanking genes on each side to 1, 2, and 5. Given the number of additional candidate genes introduced at each SNP-to-gene mapping combination, FCR was calculated for each GO term at each window size (See Fig. 5 box-plots). The least permissive SNP-to-gene mapping combination, 50kb and 1 flanking gene, incurred an average of over 50% false candidate genes. As SNP-to-gene mapping parameters become more permissive, FCR increases quickly (Fig. 5 bottom).

Co-expression signal in relation to FCR was assessed by comparing subnetwork density and locality in each GO term at different SNP-to-gene mapping parameters for each of the three co-expression networks to random subnetworks with the same number of genes (n=1000) (Fig. 5 top). The proportion of significantly co-expressed GO terms decayed at higher levels of FCR (See Supp. Fig. 5**A** for absolute term numbers). The minimum FCR level for most GO terms was ~50% as the most stringent SNP-to-gene mapping (50kb/1 flank) approximately doubled the number of candidate genes. Two additional scenarios were considered where signal was further split based on the initial co-expression strength: moderate (0.001 < p-val < 0.05; blue curve) and strong (p-val < 0.001; violet curve).

Despite high initial false candidate rates, co-expression signal among GO terms remained significant even at 60-70% FCR. Similar to results with MCR, GO terms with stronger initial co-expression were more likely to remain significantly co-expressed at higher FCR levels. Co-expression signal in relation to FCR was also compared between GO terms split by the number of genes in the term (See Supp. Fig. 5**B-C**), which did not differentiate the rate at which co-expression signal decayed.

When true candidate genes identified by GWAS were strongly co-expressed, as simulated here, a substantial number of false positive SNPs or an introduction of false candidate genes through uncertainty in SNP-to-gene mapping can be tolerated and network metrics still detected the underlying co-expressed genes sets using our method. These results indicate that in GWAS scenarios where the majority of SNPs do not perfectly resolve to candidate genes, systematic integration with co-expression networks can efficiently filter out false candidates introduced by SNP-to-gene mapping if the underlying causative loci are strongly co-expressed. Moreover, in instances where several interleaving genes exist between strongly associated SNPs in LD with each other and the true causative allele, true causal candidates can be detected using co-expression networks as a functional filter for candidate gene identification.

The potential for using this approach, however, is highly dependent on the linkage disequilibrium of the organism in question, the genetic architecture of the trait being studied, and the degree of co-expression between causative loci. Simulations provide both insight on the feasibility of using Camoco to evaluate overlap between co-expression networks and GWA studies as well as a survey of the SNP-to-gene mapping parameters that should be used when using this approach (See Discussion for more detail). In the context of maize, simulations performed here suggest that systematic integration of co-expression networks to interpret GWAS results will increase the precision with which causal genes associated with quantitative traits in true GWAS scenarios can be identified.

## Prioritizing causal genes driving elemental accumulation in maize grain

Identifying the biological processes underlying the elemental composition, also known as the ionome, of plant tissues can lead to a better understanding of plant adaptation as well as improved crops (I. Baxter and Dilkes 2012). High throughput analytic approaches such as inductively coupled plasma-mass spectrometry (ICP-MS) can measure elemental concentrations for multiple elements and are scalable to thousands of samples a week. Using ICP-MS, we measured the accumulation of 17 elements in maize kernels from the nested association mapping (NAM) population (Buckler et al. 2009). We processed kernels from grow-outs from 4 different geographic locations as described in depth by Zielger et al. (Ziegler et al. 2017). To reduce environmental specific factors, the SNPs used in this study were from GWAS was performed on the all locations models for elements reported by Ziegler et al.

### 

Significant SNPS associated with the maize grain Ionome were mapped to candidate genes by collapsing SNPs with overlapping windows down to effective SNPs, then taking genes upstream and downstream of the effective SNP up to the flank limit.

Briefly, the maize nested association mapping (NAM) panel was used to map associated loci. Approximately 30 million SNPs and small copy-number variants were projected onto the association panel and used to perform a GWAS for each of the 17 elements. Over four thousand SNPs were significantly associated (through a resampling procedure (Valdar et al. 2009), with a resampling model inclusion probability > 0.05) with elemental accumulation (Table 3; see Materials and Methods). These SNPs were used as input to Camoco to generate candidate genes from the maize filtered gene set (FGS; n=39,656) for each element using a range of SNP-to-gene mapping parameters: 50kb, 100kb, and 500kb windows (up/downstream) limited to 1, 2 or 5 flanking genes (upstream and downstream of SNP; See Fig. 1A). In total, 4,243 statistically significant SNPs were associated with maize grain ionome traits. Summing the potential candidate genes across all 17 traits implicates between 5,272 and 22,927 unique genes depending on the SNP-to-gene mapping parameters used (between 13% and 57% of the maize FGS respectively). On average, each trait’s significantly associated SNPs identified 118 non-overlapping windows across the ten chromosomes of maize (i.e. effective loci, see Materials and Methods) and these implicate an average of 612 candidate genes per element (Table 3).

### Camoco identifies high priority candidate causal genes under ionomic GWAS loci

Given the large number of candidate genes associated with elemental accumulation, we used Camoco to integrate network co-expression with effective loci identified by GWAS for each of the 17 elemental traits separately. By combing candidate gene lists with the three gene expression data sets (ZmPAN, ZmRoot and ZmSAM) and two co-expression network approaches (locality and density), we discovered high priority candidate genes driving elemental accumulation in maize (See Fig. 1C). For each network-trait combination, Camoco identified a ranked list of prioritized candidate causal genes, each associated with an FDR that reflects the significance of co-expression connecting that candidate gene to genes near other loci associated with a single trait (Supp. Table 5). We defined a set of high-confidence discoveries by reporting candidates that were discovered at a false discovery rate (FDR) < 30% in at least two SNP-to-gene mapping parameter settings (e.g.: 50kb/1Flank and 100kb/1Flank), denoted as the high priority overlap (HPO) set (see Supp. Table 6 and Materials and Methods).

### Fig. 6

#### Number of intervening genes between HPO gene and GWAS locus

The distribution of positional candidates and HPO genes. Panel A shows the distribution in the number of positional candidates between each of the 610 HPO genes and an effective locus (note: intervening gene could also be an HPO gene). Panel B candidate genes near GWAS SNPs were ranked by their absolute distance to effective loci. The distribution shows rank of the absolute distance (either upstream or downstream) of HPO gene. Both inset plots show the lower end of the distributions.

By these criteria we found strong evidence of co-expression for 610 HPO genes that were positional candidates among the 17 ionomic traits measured (1.5% maize FGS). The number of HPO genes discovered varied significantly across the traits we examined, with between 2 and 209 HPO genes for a given element considering either density of locality in any network (Table 4; Either:Any column). HPO genes discovered by Camoco were often non-adjacent to GWAS effective loci, either having genes intervening the HPO candidate and the effective locus or positional candidates that were closer either upstream or downstream of the GWAS locus (See schematic in Fig. 1**C**). Of the 610 HPO genes, 297 had zero intervening genes (Fig. 6**A**). The remaining 313 HPO genes had between 1 and 54 intervening genes, though the majority (292 HPO genes) had 10 or fewer intervening genes. We observed similar results when considering candidate genes’ absolute distances to the effective locus (Fig. 6**B**), demonstrating that Camoco can identify candidates with strong co-expression evidence that would not have been selecting by choosing the closest positional candidate. Candidate genes can be prioritized based on patterns of co-expression rather than biasing towards any previous annotation (See Discussion).

### Table 4

#### Maize Grain Ionome High Priority Candidate Genes

Gene specific density and locality metrics were compared to (n=1000) random sets of genes of the same size to establish a 30% False Discovery rate. Genes were considered candidates if they were observed at 2 or more SNP-to-gene mappings (i.e. HPO). Candidates in the "Either" column are HPO genes discovered by either density or locality in any network. The number of genes discovered for each element is further broken down by co-expression method (density, locality, both) and by network (ZmPAN, ZmSAM, ZmRoot). Candidates in the "Both" column were either discovered by density and locality in the same network or in different networks (Any). Note: zero elements had HPO genes using “Both” methods in the ZmPAN or ZmSAM networks.

### Co-expression networks derived from variation across genotypically diverse accessions support stronger candidate gene discoveries

The variation in the number of genes discovered by Camoco depended on which co-expression network was used as the basis for discovery. The ZmRoot co-expression network proved to be the strongest input, discovering genes for 15 of the 17 elements (absent in Ni and Rb) for a total of 335 HPO genes, ranging from 1 to 126 per trait (Supp. Table 6). In contrast, the ZmSAM network, which was constructed based on a tissue and developmental expression atlas collected exclusively from the B73 accession, supported the discovery of candidate genes for only 8 elements (B, Ca, K, Mg, Ni, P, Rb and Se) for a total of 74 HPO genes, ranging from 1 to 52 per trait (Supp. Table 6). The ZmPAN network, constructed from whole-seedlings (pooled tissue) across 503 different accessions, provided intermediate results, supporting high-confidence candidate discoveries for 10 elements (Al, As, Cd, Mg, Mn, Mo, Se, Sr and Zn) for a total of 228 HPO genes, ranging from 1 to 97 per trait (Supp. Table 6). The relative strength of the different networks for discovering candidate causal genes was consistent even at stricter FDR thresholds (e.g. FDR ≤ 0.10; Supp. Table 6).

### Fig. 7

#### HPO Genes for Cd and Se in the ZmRoot Network

The strongest 100,000 interactions were used to visualize global clustering of genes (n=7,844) in the ZmRoot network. A force directed algorithm positioned genes (**A**; green nodes) showing approximate boundaries (dotted black circles) of the top 10 MCL clusters (Supp. Table 2). The ZmRoot network view was filtered to possible candidate genes (blue nodes) identified from SNP-to-gene mapping (Table 3) for Cd and Se (**B** and **C** respectively). Network edges were removed from the visualization in panels B and C, though MCL cluster boundaries were preserved. HPO genes for each element (highlighted in red) co-localize to specific clusters.

Fig. 7 visualizes the discovery process for HPO genes in the ZmRoot network. Genes were organized in a global view using the strongest 100,000 interactions using a force-directed layout algorithm to show high level clustering (Fig. 7A). For two elements, Cd and Se, possible candidate genes from SNP-to-gene mapping for each element (Fig. 7B-C, blue nodes) span many of the MCL clusters identified in the network (dotted ellipses). The HPO genes, in contrast, discovered by density and locality networks co-localize to a small number of MCL clusters (red nodes).

### Density and locality network metrics provide complementary information

As part of the Camoco pipeline, both density and locality were assessed on a gene specific level to measure the strength of a given candidate causal gene’s co-expression relationships with genes in other GWAS-identified loci (See Eq.3 and Eq.4). Gene specific density simply measures the fraction of observed co-expression interactions to total possible co-expression relationships between the candidate gene and genes linked to other GWAS-identified loci, while gene specific locality normalized genes interactions to account for the proportion of interactions between the candidate gene and the rest of the genome (i.e. genes not near a GWAS locus). Overall, density identified more HPO genes than locality. For example, across all traits and networks, 391 HPO candidate genes were discovered using density while 247 HPO candidate genes were discovered using locality (See Table 4, Density:Any and Locality:Any). Interestingly, identified high-confidence genes were largely complementary, both in terms of which traits they produced results on and for which network. Among the two sets of genes (391 and 247 genes, respectively), 26 HPO genes were discovered in common (Table 4: Both:Any). While this overlap is statistically significant (p ≤ 1.5e-13; hypergeometric), the large number of uniquely discovered genes suggests the two measures are capturing largely complementary biological information from co-expression subnetworks. Indeed, when we measured the direct correlation of gene-specific density and locality measures across several GWAS traits and GO terms, we observed very weak positive but significant correlations (Supp. Figure 6). Density was most effective at identifying HPO genes within the GWAS-linked loci when using the ZmRoot network (326 HPO genes using density vs. 11 HPO gene using locality). Locality provided stronger results on the ZmPAN network (228 HPO genes using locality and 0 HPO genes using density). We observed that the utility of the locality metric appeared to be linked to the number of accessions used to construct the network (Supp. Table 7), suggesting that the differences between networks in locality may simply reflect the number of samples used to generate them (See Discussion).

### Most candidate causal genes are trait-specific

One important question is the extent to which putative causal genes overlap across different ionomic traits. It is plausible that some mechanisms affecting elemental accumulation are shared by multiple elements. We compiled the complete set of HPO genes discovered for each element and assessed overlap across the complete set of 17 elements (Table 5). Most of the discovered HPO genes are element specific, with relatively little overlap between elements (Table 5). However, a limited number of element pairs did exhibit statistically significant overlap including Cd, sharing significant overlap with 7 other elements (Al, Cu, K, Mg, Mo, Se and Sr), and Se, sharing significant overlap with 3 other elements (As, Cd and Mg), and Mo, sharing significant overlap with 2 other elements (Al, Cd). These candidate genes represent important potential modulators of elemental composition and are worthy of further study (Supp. Table 8).

### Table 5

#### Element HPO candidate gene overlap

Overlap between the 610 HPO genes discovered between different elements by either density or locality and in any network. The diagonal shows the number of HPO genes discovered for each element. Values in the upper triangular (green) show the number of genes that overlap between elements. The values in the lower triangular designate the p-values (hypergeometric) for overlap between the two sets of HPO genes. Red cells indicate significance with Bonferroni correction.

### Enrichment analysis of putative causal genes

To explore the broader biological processes represented among HPO genes, we performed Gene Ontology enrichment analysis on the candidate lists revealing enrichments for 5 elements (Supp. Table 9). For example, Sr was enriched for “anion transport” (GO: 0006820; p≤0.008) and “metal ion transmembrane transporter activity” (GO:0046873; p≤0.015). Possibly driven by insufficient functional annotation of the maize genome, these enrichment results were limited and zero elements passed a strict multiple-test correction (Bonferroni). To compensate for the sparsity of annotations, we used the HPO gene set discovered for each trait to identify the set of highly-connected co-expression network neighbors, designated the HPO+ sets. Inclusion in HPO+ was determined by a gene’s aggregate connectedness to the HPO set (see Materials and Methods). The HPO+ sets for several of the ionomic traits showed strong GO enrichment, many of which had terms that passed strict multiple-test correction, including Al, As, Cd, Cu, Fe, K, P, Se, Sr, and Zn (Supp. Table 10). Several of the enriched GO terms were common across HPO+ sets for different elements (Fig. 8). For example, we found enrichment for a collection of GO terms related to ion transport (GO:0006811) including anion transport (GO: 0006820) and potassium ion transport (GO:0006813), and others (GO:0015849, GO:0015711, GO:0046942, GO:0006835), which were supported by enrichments from multiple elements (Al, Cd, Fe, Sr) (See Fig. 8; “Transport” cluster). We also observed a set of 6 elements whose HPO+ sets (Al, Cd, Cu, K, Se, Sr) were enriched for GO terms related to chromatin organization (e.g. GO:0006325, GO:0071824, GO:0034728, GO:0006334, See Fig. 8; “Chromatin Organization” cluster). This may result from changes in cell cycle or endoreduplication control in roots, which is expected to alter the accumulation of multiple elements (Chao et al. 2011).

### Fig. 8

#### Gene Ontology Biological Process Enrichment for the Ionome

The HPO+ gene sets were analyzed for GO enrichment in the “Biological Process” namespace. Each node represents a GO term organized hierarchically in a tree with directed edges designating parent terms. Shaded terms were enriched for HPO+ genes (p < 0.05; hypergeometric). Dotted circles represent curated functional terms describing the enriched nodes in clade of the tree. Each clade is annotated with the ionomic terms represented in the GO enrichment.

Several of the observed GO enrichments were trait-specific, including collections of GO terms reflecting “Chemical Response” (Se), “Microtubule Movement” (As), “Adhesion” (Cu), and “Saccharide Metabolism” (P). For example, the “Saccharide Metabolism” collection of GO term enrichments was driven by 5 HPO+ genes for P, one of which was *tgd1* (GRMZM2G044027; See Supp. Table 10). Mutations in the Arabidopsis ortholog for *tgd1* caused the accumulation of triacylglycerols and oligogalactolipids and showed a decreased ability to incorporate phosphatidic acid into galactolipids (Fan et al. 2015) which may alter P accumulation directly or via phosphatidic acid signaling (Katagiri et al. 2005). TGD1 is an ATP-binding cassette (ABC) transporter known to transport other substrates, including inorganic and organic cations and anions (Roston et al. 2012). The *tgd1* gene was present in the HPO set, and the other four other genes were identified as strongly connected neighbors (HPO+) in the co-expression network. Two are of unknown function, GRMZM2G018241 and GRMZM2G030673, and the other two are involved in cellulose synthesis, GRMZM2G122277 and GRMZM2G177631. We should note that these enriched GO terms demonstrated the deficiencies of automated annotation approaches. Terms related to “blood coagulation” and “regulation of body fluid levels”, which were likely due to annotations translated to maize genes based on protein sequence homology in humans, were recovered. While, at face value, these term descriptions are inapplicable in plant species, the fact that these terms contained HPO genes as well as strong network co-expression suggests that these annotations assigned through orthology might be capturing misnamed biological signal and further refined via co-expression evidence (see Discussion).

In general, using co-expression networks to expand the neighborhood of the high-confidence candidate causal genes and then assessing the entire set for functional coherence through GO enrichment is a productive strategy for gaining insight into what processes are represented. Yet, this approach is particularly challenging in the annotation-sparse maize genome, where only ~1% of genes have mutant phenotypes (Lawrence et al. 2004). GO terms were too high-level or insufficiently described to help distinguish causal genes. However, terms discovered here contain genes that act in previously described pathways known to impact elemental traits. With greater confidence that subnetworks containing HPO genes contained coherent biological information, we refined our analysis by curating HPO genes for their involvement in specific biological processes, namely those that are known or suspected to affect the transport, storage, and utilization of elements.

## GA-signaling DELLA domain transcription factors influence the ionome of maize

One of the high-confidence candidate genes, which appeared in the HPO sets comparing Cd and the ZmRoot network, is the Gibberellin (GA) signaling component and DELLA- and GRAS-domain transcription factor *dwarf9* (GRMZM2G024973, *d9*; (Winkler and Freeling 1994)). *d9* is one of two DELLA paralogs in the maize genome, the other is *dwarf8* (GRMZM2G144744; *d8*), both of which can be mutated to dominant negative forms that display dwarf phenotypes and dramatic suppression of GA responses (Lawit et al. 2010). Camoco ranked *d9* among the high-confidence candidates for Cd, but not *d8*, though bothgenes are present in the root-based co-expression network (ZmRoot). *Dwarf8* and *d9* shared moderate but positive co-expression (ZmRoot: Z=1.03; ZmPAN: Z=1.04). Given the indistinguishable phenotypes of the known dominant mutants of *d8* and *d9*, the most likely explanation for this result is that there was allelic variation for *d9* in the GWAS panel, but not *d8*. The GA biosynthetic enzyme Ent Kaurene synthase (GRMZM2G093603) encoding the *dwarf5* locus (Fu et al. 2016) also affected the concentration of seed Cd and appeared among the HPO genes for Sr in the ZmRoot network. This gene is required for the biosynthesis of bioactive GA via ent-Kaurene, strongly suggesting that GA signaling in roots shapes the ionome and alters the accumulation of Cd in seeds, with potential impacts on human health.

### Fig. 9

#### Ionomic profiles of D8 and D9 mutants

Boxplots displaying ICP-MS values for D8 and D9 along with null segregating siblings (Sib8 and Sib9). P-values indicated statistical differences between mutants and wild-type siblings while asterisks (\*\*) indicate significant differences in a joint analysis between dwarf and wild-type.

To test for the impact of GA signaling on the ionome, and provide single-locus tests, we grew the dominant GA-insensitive mutants *D9-1* and *D8-mpl* and their null segregating siblings (Sib9 and Sib8). The dominant *D8-mpl* and *D9-1* alleles have nearly equivalent effects on above-ground plant growth and similar GA-insensitivity phenotypes in shoots (Winkler and Freeling 1994). Both mutants were obtained from the maize genetics co-op and crossed three times to inbred B73 to generate BC2F1 families, segregating 1:1 for the dwarf phenotype. Ears from phenotypically dwarf and phenotypically wild-type siblings were collected and processed for single-seed ionomic profiling using ICP-MS (Fig. 9). Both dwarf lines had significantly different elemental compositions from their wild type siblings. A joint analysis by t-tests between least-squared means comparing dwarfs and wild-types revealed that Cu, Fe, P, and Sr were higher in the dwarf than wild-type seeds (Designated with two asterisks in Fig. 9). In addition to the elements that were different in the joint model, *D9-1* was also significantly different from its wild-type siblings for Na content and seed weight (p < 0.05; t test). Dominant mutants of *d8* are expressed at lower levels than *d9* in the root but many fold higher levels in the shoot (qteller.com; (Wang et al. 2009)). D8 was also was significantly different from its sibling in Cd and Mo accumulation. It is possible that *D8-mpl* has a shoot-driven effect on Mo accumulation in the seed, but previous work (Asaro et al. 2016) identified a large-effect QTL affecting Mo and containing the Mot1 gene a mere 22 Mb away from D8. As the allele at Mot1 is unknown in the original *D8-mpl* genetic background, we cannot rule out linkage drag carrying a Mot1 allele cannot be ruled out. This dominant-negative allele of D9 did not recapitulate the Cd accumulation effect of the linked GWAS QTL that formed the basis for its discovery as a high-confidence candidate gene by Camoco, but the D8-mpl allele did, and our data demonstrate that both D8 and D9 have broad effects on ionomic phenotypes.

### Fig. 10

#### Co-expression network for D9 and cadmium HPO genes

Co-expression interactions among high priority candidate (HPO) genes were identified in the ZmRoot network for Cd and visualized at several levels. Panel **A** shows local interactions among the 126 cadmium HPO genes (red nodes). Genes are positioned based on chromosomal position and are arranged in a circle to visualize inter-locus interactions. Interactions among HPO genes and Della9 (D9; GRMZM2G024973) are highlighted in yellow. Panel **B** shows a force directed layout of d9 with HPO neighbors. Circled genes show sets of genes with previously known roles in elemental accumulation.

Genes co-expressed with D9 were investigated to determine which among these were associated with ionomic traits and seed Cd levels. In the ZmRoot network, D9 had strong co-expression interactions with 38 other HPO genes (Fig. 10A). Among these were the maize Shortroot paralogs (GRMZM2G132794) and a second GRAS domain transcription factor (GRMZM2G079470). Both of these, as well as the presence of many cell cycle genes among the co-expressed genes and ionomics traits affecting genes, raised the possibility that, like in Arabidopsis (Wild et al. 2016), DELLA-dependent processes, responsive to GA, both shape the architecture of the root and the ionome. In Arabidopsis, DELLA expression disrupts Fe uptake and loss of DELLA prevents some Fe-deficiency mediated root growth suppression. Our finding that constitutive DELLA activity in the roots, affected by the D9-1 and D8-mpl mutants, resulting in excess Fe points to a conserved role for the DELLA domain transcription factors and GA signaling in Fe homeostasis in maize, a plant with an entirely different Fe uptake system than *A. thaliana*. However, the direction of the effect was opposite to that observed in *A. thaliana*. Future research into the targets of the DELLA proteins in maize will be required to further address these differences.

Remarkably, the HPO co-expression network associated with D9 in the roots contained three genes with expected roles in the biosynthesis and polymerization of phenylpropanoids (Monaco et al. 2013). The genes encoded by enzymes that participate in phenylpropanoid biosynthesis CCR1 (GRMZM2G131205) and the maize LigB paralog (GRMZM2G078500) as well as a laccase paralog were co-expressed with D9 (GRMZM2G336337). LigB, which in Angiosperms is only known to be required for the formation of a pioneer specialized metabolite of no known function in *A. thaliana*, was linked to QTL for multiple ions including Cd, Mn, Zn, and Ni. CCR1, however, was only found for Cd. The Laccase 12 gene (GRMZM2G336337) was also a multi-ionomic hit with linked SNPs affecting Cd, Fe, and P. Genes co-expressed with D9 also were identified in the ZmPAN network. Consistent with the hypothesis that maize DELLA regulated the type II iron uptake mechanism used by grasses, the nicotianamine syntase3 gene (GRMZM2G439195, ZmPAN-Cd), which is required for making the type II iron chelators, was both a Cd GWAS hit and substantially co-expressed with D9 in the ZmPAN network such that it contributed to the identification of D9 as an HPO gene for Cd.

## Camoco identifies GWAS candidates for ion accumulation in maize seeds

In addition to the mutant analysis of HPO genes identified by our approach, we manually examined the literature evidence supporting the association of candidate genes with ionomic traits. In addition to genes with known roles in elemental homeostasis, HPO genes for some ionomic traits included multiple genes encoding known members of the same pathway or protein complex. This suggests that biological signal was enriched by our novel combination of expression level polymorphisms and GWAS, and provided evidence of novel associations between multiple pathways and elemental homeostasis.

For example, one gene with highly pleiotropic effects on the maize kernel ionome is *sugary1* (*su1*; GRMZM2G138060) (I. R. Baxter et al. 2014). Genetic polymorphisms that affect seed compartment proportions or the major storage constituents are expected to contribute disproportionately to variation in seed ionomic contents. Within the NAM population, functional variation for *su1* can be found in the B73 x IL14H subpopulation. For this reason, six IL14H RIL’s that were still segregating for the recessive *su1* allele were previously tested for ionomic effects (I. R. Baxter et al. 2014). This demonstrated that segregation for a loss of function allele at *su1*, on the cob, affected the levels of P, S, K, Ca, Mn, Fe, As, Se, and Rb in the seed (I. R. Baxter et al. 2014). The *su1* gene was present among the HPO genes for Se accumulation (Supp. Table 6) based on the root co-expression network (ZmRoot-Se). The *su1* locus was only identified in the HPO set for the element Se, but was linked to significant NAM GWAS SNPs for the elements P, K, and As. Thus, of the eight elements that were identified as co segregating with the *su1* allele in the IL14H RIL population and measured in the NAM panel, four were associated with *su1* variation in association panel. It is possible that *su1*, expressed in multiple plant compartments including the roots (qteller.com;(Wang et al. 2009)), might also affect the seed ionome through effects beyond a dramatic loss of seed starch. This may result from coordinate regulation of the encoded isoamylase and other root-expressed determinants of S and Se metabolism, or from unexpected coordination between root and seed expression networks. The finding that HPO network neighbors for P were enriched among carbohydrate biosynthetic enzymes favors the former of these two hypotheses (see Fig. 8).

Our combined analysis of loci linked GWAS SNPs and gene co-expression networks identified many HPO genes for Se accumulation. Several genes with known effects on the ionome, or known to be impacted by the ionome, were identified within this HPO set. For example, one candidate gene, GRMZM2G327406, encodes an Adenylyl-sulfate kinase (adenosine-5'-phosphosulfate (APS) kinase 3), which is a key component of the sulfur and selenium assimilation pathway and plays a role in the formation of the substrate for protein and metabolite sulfation (ZmRoot-Se). At another locus, Camoco identified a cysteine desulfurulyase (GRMZM2G581155), critical for the metabolism of sulfur amino acids and the biosynthesis of the 21st amino acid selenocysteine, as an HPO gene (ZmRoot-Se).

Based on the work of Chao et al. in Arabidopsis, alterations in cell size and cell division in the root are expected to have effects on K accumulation in leaves (Chao et al. 2011). Two of the four subunits of the polycomb repressive complex 2 (PRC2), known to act on the cell cycle via the retinoblastoma-related proteins (RBRs), were identified as HPO genes for the K analog Rb. Both *msi1* (GRMZM2G090217; ZmSAM-Rb) and *fie2* (GRMZM2G148924; ZmSAM-Rb), members of the PRC2, are co-expressed in the ZmSAM network. The RBR-binding E2F-like transcription factor encoded by GRMZM2G361659 (ZmSAM-Rb) was also found, further indicating that cell cycle regulation via these proteins’ interactions could provide a common mechanism for these associations. Histone deacetylases from the RPD3 family are also known to interact with RBR protein. The RPD3-like *histone deacetylase 2* from maize was identified in the same HPO set (GRMZM2G136067; ZmSAM-Rb). The Arabidopsis homologs of both MSI and this histone deacetylase are known to act as histone chaperones, and the latter directly binds Histone H2B. Remarkably, histone H2B (GRMZM2G401147; ZmSAM-Rb) was also an HPO hit. Lastly, an Actin utilizing SNF2-like *chromatin regulator18* (GRMZM2G126774 ZmSAM-Rb) was identified as another SAM-Rb hit. This mirrors the similar finding of GO enrichment for chromatin regulatory categories in the HPO+ enrichment analysis presented above. Taken together, these demonstrate a strong enrichment for known protein-protein interactors important for chromatin regulation and cell cycle control among the HPO set for the K analog Rb.

Many transporters with known roles in ionome homeostasis were also identified among the HPO genes. Among these were a P type ATPase transporter of the ACA P2B subfamily 4 (GRMZM2G140328; ZmRoot-Sr) encoding a homolog of known plasma membrane localized Ca transporters in multiple species (I. Baxter et al. 2003), an ABC transporter homolog of the family involved in organic acid secretion in the roots from the As HPO set (GRMZM2G415529; ZmRoot-As) (Badri et al. 2007), and a pyrophosphate energized pump (GRMZM2G090718; ZmPAN-Cd). Several annotated transporters were identified in the HPO sets for multiple elements, including a sulfate transporter (GRMZM2G444801, ZmRoot-K), a cationic amino acid transporter (AC207755.3\_FG005; ZmPAN-Cd, ZmPAN-Mo), and an inositol transporter (GRMZM2G142063; ZmRoot-Fe, ZmRoot-Cd, ZmRoot-Sr).

Cadmium was well measured and affected by substantial genetic variance (Ziegler et al. 2017). We detected the largest number of HPO candidate genes for Cd (209 genes; See Table 4). Among these were the maize *glossy2* gene (GRMZM2G098239, ZmPAN-Cd), responsible for a step in the biosynthesis of hydrophobic barriers (Tacke et al. 1995). This implicates the biosynthesis and deposition of hydrophobic molecules in the root in accumulation of ions and may point to root processes, rather than epicuticular waxes deposition, as the primary mode by which these genes affect water dynamics. An ARR1-like HPO gene, GRMZM2G067702, was also associated with Cd (ZmRoot). Previous work has shown that ARR genes from Arabidopsis are expressed in the stele where they regulate the activity of HKT1 (Mason et al. 2010). This gene was expressed at the highest level in the stele at 3 days after sowing (DAS).

Integrating GWAS data with co-expression networks resulted a set of 610 HPO genes primed for functional validation (1.5% of the maize FGS). The further curated subset of genes described above all have previous demonstrated roles in elemental accumulation, yet only represent a small proportion of the HPO genes discovered by Camoco. Functional validation is expensive and time consuming. Combining data driven approaches such as network integration with expert biological curation is an extremely efficient means for the prioritization of genes driving complex traits like elemental accumulation.

# Discussion

### The effects of linkage disequilibrium

We demonstrated that gene expression data, through the construction of co-expression networks, can be used as an effective basis for prioritizing causal genes from GWAS. Our approach addresses a challenging bottleneck in the process of translating large sets of statistically associated loci into shorter lists based on mechanistic understandings of these traits. Marker SNPs identified by a GWA study provide an initial lead on a region of interest, but due to linkage disequilibrium, the candidate region can be quite broad and implicate many potentially causal genes. In addition to LD, many SNPs identified by GWAS studies lie in regulatory regions far from their target genes (Clark et al. 2006; Castelletti et al. 2014; Louwers et al. 2009). Additionally, factors such as bias due to cis- co-expression, as well as different co-expression metrics and networks, need to be considered to identify co-expression signal.

These factors can result in a very large (upwards of 57% of all genes here) and ambiguous set of candidate genes, even where a locus is identified solely by GWAS. A common approach to interpreting such a locus is through manual inspection of the genome region of interest with a goal of identifying candidate genes whose function is consistent with the phenotype of interest, which can introduce bias in the discovery process and completely ignores uncharacterized genes. For non-human and non-model species like maize, this situation is especially challenging because most of these organisms’ genomes remain functionally uncharacterized. Our approach leverages the orthogonal use of gene expression data, which can be readily collected for most species of interest, to add an important interpretation and prioritization filter to the output of a GWA study with additional experimental evidence used to rank plausible candidate genes based on this second logical filter.

We demonstrate that Camoco successfully identified subsets of genes linked to candidate SNPs that also exhibit strong co-expression with genes near other candidate SNPs. The resulting gene sets (HPO genes) reflect groups of co-regulated genes that can be used to infer broader biological processes where genetic variation affects the phenotype of interest. Indeed, using Camoco, we found strong evidence for HPO gene sets in 13 of 17 of our examined elemental accumulation phenotypes (with 5 or more HPO genes). These high priority sets of genes represent a small fraction of the candidates implicated by the GWAS for each phenotype (see Table 3 and Table 4). Importantly, the inclusion of co-expression data efficiently filtered out many of the candidates implicated only due to SNP-to-gene mapping. Previous studies in maize found that while LD decays rapidly in maize (~1kb), variance can be large, as the functional allele segregates in a small number of lines (Wallace et al. 2014). Additionally, Wallace et al. showed that causal polymorphisms are likely to reside in regulatory regions, outside of exonic regions. Until we precisely understand the regulatory landscape in species and lines of interest, even the most powerful GWA studies will identify polymorphisms that implicate genes many base pairs away. Here, we find that the large majority of HPO genes, implicated with both GWAS and gene expression, were rarely the closest genes to identified SNPs, and thus would not have been identified by simply prioritizing genes closest to each marker SNP (Fig. 6).

### Establishing performance expectations of Camoco

It is important to note caveats to our approach. For example, phenotypes caused by genetic variation in a single or small number of genes, or conversely, that are caused by a diverse set of otherwise functionally unrelated genes are not good candidates for our approach. The core assumption underpinning Camoco is that multiple genetic variants in different genes are involved in a common biological process which individually cause phenotypic variation. We expect this assumption holds for many phenotypes (supported by the fact that we have discovered strong candidates for the most traits examined), but we expect that certain traits, and individual causal genes, will violate this assumption. For these traits and genes, Camoco cannot be applied. Additionally, expression data used to build networks does not fully overlap with genomic data included in GWAS. For example, of the 39,656 genes in the maize filtered gene set ([ftp.maizesequence.org](ftp://ftp.maizesequence.org)), 11,718 genes did not pass quality control qualifications and were absent from the three co-expression networks analyzed here, and thus would never be included in the HPO set even if significant SNPs were tagging them.

### Camoco discovered gene sets are as coherent as GO terms

To evaluate the expected performance of our approach using Gene Ontology, we observed an interesting trend. We used sets of genes annotated to the same GO term as a gold standard for groups of functionally coherent genes. We simulated the effect of imperfect SNP-to-gene mapping by assuming that subsets of these GO terms were identified by a simulated GWAS trait in which the neighboring genes (encoded nearby on the genome) were added to simulate the scenario where we could not resolve the causal gene from linked neighboring genes. This analysis was useful as it established the boundaries of possibility for our approach, i.e. how much noise in terms of false candidate genes can be tolerated before the entire premise of our approach fails. As described in Fig. 5, this analysis suggests a sensitivity of ~40% using a SNP-to-gene mapping rule of a +/-500 kb window and up to 2 flanking genes (nearly 75% false candidates due to SNP-to-gene mapping). This result suggests that we would not be likely to discover processes as coherent as GO terms if linkage extended beyond this point.

At the same window and flanking parameters noted above, we made significant discoveries (genes with FDR < 0.30) for 7 of 17 elements (41%) using the density metric in the ZmRoot network. This success rate is remarkably consistent with predictions made from GO simulations at the same window and flanking parameters. Intriguingly, HPO gene sets alone were not significantly enriched for GO term genes, indicating that while the HPO gene sets and GO terms exhibited similar patterns of gene expression, their described gene sets are mutually exclusive. Gene sets did not exhibit GO term enrichment until the HPO gene sets were supplemented with co-expression neighbors (HPO+), though the descriptions were high level. We speculate that this is due to discovery bias in GO annotations used for our evaluation – which were largely curated from model species and assigned to maize through orthology. There are likely many maize-specific processes and phenotypes not yet annotated in ontologies, yet that have strong co-expression evidence and can be assigned functional annotations through GWAS.

Our analysis shows that the loci linked to ionomic GWAS loci are as coherent as many maize genes co-annotated GO terms, many of which have strong literature support for being involved in elemental accumulation. Indeed, one of the key motivations of our approach are the limited species-specific gene ontologies for crop genomes like maize, and this result emphasizes the extent of this limitation. Where current functional annotations, such as GO, rely highly on orthology, future curation pipeline could rely on species-specific curations that utilize both GWAS and co-expression data.

Beyond highlighting the challenges of a genome lacking precise functional annotation, these results also suggest an interesting direction for future work. Despite maize genes’ limited ontological annotations, many genome-wide association studies have been enabled by powerful mapping populations (e.g. NAM (McMullen et al. 2009)). Our results suggest that these sets of loci, combined with a proper mapping to the genes they represent, could serve as a powerful resource for gene function characterization. Systematic efforts to curate the results from such genome-wide association studies, filter gene sets with Camoco and similar tools, and provide public access in convenient forms would be worthwhile. Maize is exceptional in this regard, due to its excellent genomic tools and powerful mapping populations. Accordingly, there are several other crop species with rich population genetic resources but limited genome functional annotation that could also benefit from this approach.

### Co-expression context matters

Using our approach, we evaluated 17 ionomic traits for overlap with three different co-expression networks. Two of the co-expression networks were generated from gene expression profiles collected across a diverse set of individuals (ZmRoot, ZmPAN) and performed substantially better than the ZmSAM network, which was based on a large collection of expression profiles across different tissues and developmental stages derived from a single reference line (B73). We emphasize that this result is not a reflection of the data quality or even the general utility of the co-expression network derived the tissue/developmental atlas. Evaluations of this network showed a similar level of enrichment for co-expression relationships among genes involved in the same biological processes (Table 1) and had very similar network structure (Table 2). Instead, our results indicate that the underlying processes driving genotypic variation associated with traits captured by GWAS are better captured by transcriptional variation observed across genetically diverse individuals. Indeed, despite networks having similar levels of GO term enrichment (Table 1), the actual GO terms that drive enrichment are quite different (Supp. Table 1), which is consistent with our previous analysis demonstrating that the experimental context of co-expression networks strongly influences which biological processes it captures (Robert J. Schaefer, Briskine, Springer, and Myers 2014).

Between the two co-expression networks based on expression variation across genotypically diverse individuals, we observed differences depending on which tissues were profiled. Our co-expression network derived from sampling of root tissue across a diverse set of individuals (ZmRoot) provided the best performance at our chosen FDR (Table 4), producing a total of 335 (326 from density and 11 from locality, 2 in both) HPO candidate genes as compared to 228 (all from locality) HPO candidate genes produced by the ZmPAN network, which was derived from expression profiles of whole seedlings. This result affirms our motivation for collecting tissue-specific gene expression profiles— we expected that processes occurring in the roots would be central to element accumulation phenotype (which were measured in kernels). The difference between the performance of these two networks was modest, however, and much less significant than the difference between the developmental/tissue atlas-derived network and the diverse genotype-derived network. Furthermore, we do not expect that the ZmRoot nor the ZmPAN networks fully describe elemental accumulation processes. While ions are initially acquired from soil via the root system, we do not directly observe its accumulation in the seed. The datasets presented here could further be complemented by additional tissue specific data such as genotypically diverse seed or leaf networks.

The ZmRoot and ZmPan’s networks’ performances were strongly influenced by their respective network metrics. Specifically, HPO gene discovery in the ZmRoot network was driven by the density metric while performance of the ZmPAN network relied on the locality metric (Table 4). However, in both networks, locality and density’s positive correlation (Supp. Figure 6) implies that these two metrics are complementary. This relationship was also observed for the density and locality of GO terms. Table 1 shows that both metrics had similar overall performance, each capturing ~40% of GO terms in each network. However only ~25% were captured by both metrics, indicating that certain biological processes are reflective of each metric. In addition to tissue source differences between the ZmRoot and ZmPAN networks, the number of experimental accessions drastically differed between the networks (503 accessions in ZmPAN and 48 in ZmRoot) which influenced the performance of network metrics. We showed that locality was sensitive to the number of accessions used to calculated co-expression (Supp. Table 7) and thus could explain the bias between network metric and the number of input accessions. This result also suggests that that the 46 accessions in ZmRoot did not saturate this approach for co-expression signal, and that expanding the ZmRoot dataset to include 503 accessions would result in greater power to detect overlap and the identification of more true positives using locality.

In general, our results strongly suggest that co-expression networks derived from expression profiling of genetically diverse individuals, as opposed to deep expression atlases derived from focus on a single reference genotype, will be more powerful for interpreting candidate genetic loci identified in a GWA studies. Furthermore, our findings suggest that where it is possible to identify relevant tissues for a phenotype of interest, tissue-specific expression profiling across genetically diverse individuals is an effective strategy. Identifying the best co-expression context for a given GWAS has important implications for data generation efforts in future studies.

# Conclusion

Here, we integrate co-expression network with loci associated with elemental accumulation in maize grain. We built three different co-expression networks and simulated their ability to detect co-expression using GO terms, and used these networks to identify patterns of co-expression in a set of measuring association for 17 different elemental traits. This resulted in the discovery of 610 high-confidence candidate causal genes. These candidate gene sets were enriched for bioprocesses related to the ionome. Although most high-confidence candidate genes are uncharacterized and worthy of further study, we did find linkage between ionomic traits and alleles at genes that have previously been demonstrated to affect the plant ionome. We validated our approach using genes and pathways not previously demonstrated to affect the ionome in maize, and demonstrated that GA signaling through the DELLA-domain transcription factors broadly impacted the plants’ elemental profiles. Our approach successfully prioritizes causal genes underlying GWAS-identified loci based solely on gene expression data, and enables the functional interpretation of otherwise uncharacterized genes associated with complex traits.

# Materials and Methods

## Software implementation of Camoco

Camoco (Co-analysis of Molecular Components) is a python library that includes a suite of command line tools to inter-relate and co-analyze different layers of genomic data. Specifically, it integrates genes present near and around GWAS loci with functional information derived from gene co-expression networks. Camoco builds and analyzes co-expression networks from gene transcript expression data (i.e. RNA-Seq), but can also be utilized on other expression data such as metabolite, protein abundance, or micro-array data.

This software implements three main routines: 1) construction and validation of co-expression networks from a counts or abundance matrix, 2) SNPs or other loci mapping onto genes, and 3) a method to assess the *overlap* of co-expression among candidate genes near significant GWAS peaks.

Camoco is open source and freely available under the terms of the 'MIT license'. Full source code, software examples as well as instructions on how to install and run Camoco are available at <http://github.com/schae234/Camoco>.

Camoco version 0.5.0 was used for this article.

## Construction quality control of co-expression networks

### ZmPAN: A genotypically diverse, PAN genome co-expression network

Camoco was used to process the FPKM table reported by Hirsh et al. and to build a co-expression network. The raw gene expression data were passed through Camoco’s quality control pipeline using parameters specified in Supp. File 1. After QC, the network was built using 24,756 genes. For each pairwise combination of genes, a Pearson Correlation Coefficient (PCC) was calculated across FPKM profiles to produce ~306 million possible network edges (Supp. Fig. 1A), then mean centered and standard normalized (Z-score hereafter) to allow cross network comparison (Supp. Fig. 1B). A global significance threshold of Z ≥ 3 was set on co-expression interactions to calculate gene degree and other conventional network measures.

To assess overall network health, several approaches were taken. First, a Z-score of edges between genes co-annotated in the maize Gene Ontology (GO) terms was compared to edges in 1000 random terms containing the same number genes. Supp. Fig. 1C shows the distribution of p-values compared to empirical Z-score of edges within a GO term. With a nominal p-value cutoff of 0.05, the PAN co-expression network had 11.9-fold more GO terms than expected with a p-value ≤ 0.05, suggesting that edges within this co-expression network capture meaningful biological variation. Degree distribution, or the number of interactions per gene, is also as expected within the network. Supp. Fig. 1D shows empirical degree distributions compared to the power law, exponential, and truncated power law distributions. Typically, the degree distributions of biological networks are best fit by a truncated power law distribution, which is consistent with the ZmPAN genome co-expression network (Ghazalpour et al. 2006).

### ZmSAM: A Maize RNA-Seq Tissue Atlas co-expression network

Publicly available gene expression data was generated from downloaded from Stelpflug et. al (Stelpflug et al. 2015). In total, 22,691 genes passed quality control metrics specified in Supp. File 1. Like the *ZmPAN* network described above, gene interactions were calculated between each pairwise combination of genes to produce ~257 million network edges. A global significance threshold of Z ≥ 3 was set on co-expression interactions to differentiate significantly co-expressed gene pairs.

Supp. Fig. 2A shows before network edge score normalization. Network interaction scores were mean centered and standard normalized (Supp. Fig. 2B). The ZmSAM network shows a 10.8-fold enrichment for strong edge scores (p ≤ 0.05) between genes annotated in Gene Ontology terms (Supp. Fig. 2C). A final network health check shows that the empirical degree distribution of the ZmSAM network is consistent with previously characterized biological networks (Supp. Fig. 2D).

### ZmRoot: A Genotypically Diverse Maize Root Co-Expression Network

Root RNA was extracted and sequenced from 48 diverse maize lines using TruSeq stranded RNA library prep and Illumina HiSeq 100bp paired end RNA Sequencing (RNASeq) reads. Raw reads were deposited into the short read archive (SRA) under Project number PRJNA304663. Reads were pre-processed using a standard mapping pipeline. Raw reads were passed through quality control using the program AdapterRemoval (Lindgreen 2012), which collapses overlapping reads into high quality, single reads while also trimming residual PCR adapters. Reads were mapped to the Maize 5b reference genome using BWA (H. Li and Durbin 2009; Schubert et al. 2014) PCR duplicates were detected and removed, and then realignment was performed across detected insertions and deletions resulting in between 14 and 30 million high-quality, unique nuclear reads per sample. Two samples were dropped due to low coverage, bringing the total number of samples to 46.

Quantification of gene expression levels into fragments per kilobase per million reads (FPKM) was done using a modified version of HTSeq that quantifies both paired- and unpaired-end reads (Anders, Pyl, and Huber 2014), available at http://github.com/schae234/MixedHTSeq. Raw FPKM tables were imported into Camoco and passed through the quality control pipeline. After QC steps (Supp. File 1), 25,260 genes were included in co-expression network construction containing ~319 million interactions. Supp. Fig. 3A shows raw PCC scores while Supp. Fig. 3B shows Z-scores after standard normal transformation. Similar to ZmPAN and ZmSAM co-expression among GO terms was compared to random gene sets of the same size as GO terms (1000 instances) showing a 13.5-fold enrichment for significantly co-expressed GO Terms (Supp. Fig. 3C). The degree distribution of the ZmRoot network closely follows a truncated power law, similar to the aforementioned networks (Supp. Fig. 3D).

## SNP-to-Gene mapping and effective loci

Two parameters are used during SNP-to-gene mapping: candidate window size and maximum number of flanking genes. First, windows were calculated both upstream and downstream of input SNPs. SNPs with overlapping windows were collapsed down into *effective loci* containing the contiguous genomic intervals of all overlapping SNPs, including windows both upstream and downstream of the effective locus’ flanking SNPs (e.g. locus 2 in Fig. 1A). Effective loci were cross referenced with the maize 5b functional gene set (FGS) genome feature format (GFF) file (http://ftp.maizesequence.org/release-5b/filtered-set/ZmB73\_5b\_FGS.gff.gz) to convert effective loci to candidate gene sets containing all candidate genes within the interval of the effective SNP and also including up to a certain number of flanking genes both upstream and downstream from the effective SNP. For each candidate gene identified by an effective locus, the number of intervening genes was calculated from the middle of the candidate gene to the middle of the effective locus. Candidate genes were ranked by the absolute value of their distance to the center of their parental effective locus. Algorithms implementing SNP-to-gene mapping used here are accessible through the Camoco command line interface.

## Calculating subnetwork density and locality

Co-expression was measured among candidate genes using two metrics: density and locality. Subnetwork *density* is formulated as the average interaction strength between *all* (un-thresholded) pairwise combinations of gene-gene interactions that occur between input genes normalized for the number of total pairs among input genes:

### Eq.1

Where X-bar is the mean sub-network interaction score, E(X) is the expected network interaction score, σ(X) is the standard deviation of network interactions, and N is the number of interactions in the sub-network.

Network *locality* assesses the proportion of significant co-expression interactions (Z ≥ 3) that are locally connected to other subnetwork genes, compared to the number of global network interactions. To quantify network locality, both local and global degree is calculated for each gene within a sub-network. To account for degree bias, where genes with a high global degree are more likely to have more local interactions, a linear regression is calculated on local degree using global degree (local ~ global) and regression residuals for each gene are analyzed:

### Eq.2

Gene specific density is calculated by considering subnetwork interactions on a per-gene basis:

### Eq.3

Gene locality residuals can be interpreted independently to identify gene specific locality:

### Eq.4

We note that, here, interactions among genes originating from the same effective GWAS locus (i.e. cis-interactions) were removed from density and locality calculations due to a bias in cis co-expression. During SNP-to-gene mapping, candidate genes retained information containing a reference back to the parental GWAS SNP. A software flag within Camoco removes interactions derived from the same parental SNP from co-expression score calculations.

Statistical significance of subnetwork density and locality was assessed by comparing subnetwork scores to 1000 random sets of candidate genes, conserving the number of input genes. Using these randomizations, p-values were calculated for co-expression scores.

## Simulating GWA studies using Gene Ontology (GO) terms

GO (Harris et al. 2004) annotations were downloaded for maize genes from http://ftp.maizesequence.org/release-4a.53/functional\_annotations/. Co-annotated genes within a GO term were treated as true causal genes identified by a hypothetical GWA study. Terms between 50 and 100 genes were included to simulate the genetic architecture of a multi-genic trait. In each co-expression network, terms having genes with significant co-expression (p-value ≤ 0.05; density or locality) were retained for further analysis. Noise introduced by imperfect GWAS was simulated using two different methods to decompose how noise affects significantly co-expressed networks.

### Missing Candidate Rate

### Eq. 6

### False Candidate Rate

### Eq. 7

### Simulating Missing candidate gene rate (MCR):

The effects of MCR were evaluated by subjecting GO terms with significant co-expression (p<0.05; described above) to varying levels of missing candidate rates. True GO term genes were replaced with random genes at varying rates (MCR: 0%, 10%, 20%, 50%, 80%, 90%, 100%). The effect of MCR was evaluated by assessing the number of GO terms that retained significant co-expression (compared to n=1000 randomizations) at each level of MCR.

### Adding false candidate genes by expanding SNP-to-gene mapping parameters

To determine how false candidates due to imperfect SNP-to-gene mapping affected the ability to detect co-expressed candidate genes linked to a GWAS trait, significantly co-expressed GO terms were assessed at the different SNP-to-gene mapping parameters (50kb 100kb, 500kb and 1, 2, or 5 flanking genes). Effective FCR at each SNP-to-gene mapping parameter setting was calculated by dividing the number of true GO genes with candidates identified after SNP-to-gene mapping. Since varying SNP-to-gene mapping parameters changes the number of candidate genes considered within a term, each term was considered independently for each parameter combination.

## Maize Ionome GWAS

Elemental concentrations were measured for 17 different elements in the maize kernel using inductively coupled plasma mass spectrometry (ICP-MS) as described in Ziegler et al. (Ziegler et al. 2017). Outliers were removed from single seed measurements using median absolute deviation (Davies and Gather 2012). Basic linear unbiased predictors (BLUPs) for each elemental concentration were calculated across different environments and estimate variance components (Hung et al. 2012). Joint linkage analysis was run using TASSEL version 3.0 (Bradbury et al. 2007) with over 7,000 SNPs obtained by genotype-by-sequencing (GBS) (Elshire et al. 2011). An empirical p-value cutoff was determined by performing 1,000 permutations where the BLUP phenotype data was shuffled within each NAM family before joint-linkage analysis was performed. The p-value corresponding to a 5% false discovery rate was used for inclusion of a QTL in the joint linkage model.

Genome wide association was performed using stepwise forward regression implemented in TASSEL version 4.0, similar to other studies (Wallace et al. 2014; Cook et al. 2012; Tian et al. 2011). Briefly, genome wide association was performed on a chromosomal-by-chromosome basis. To account for variance explained by QTL on other chromosomes, the phenotypes used were the residuals from each chromosome calculated from the joint-linkage model fit with all significant joint-linkage QTL except those on the given chromosome. Association analysis for each trait was performed 100 times by randomly sampling, without replacement, 80% of the lines from each population.

The final input SNP dataset contained 28.9 million SNPs obtained from the maize HapMap1 (Gore et al. 2009), the maize HapMap2 (Chia et al. 2012), as well as an additional ~800,000 putative copy-number variants from analysis of read depth counts in HapMap2 (Wallace et al. 2014; Chia et al. 2012). These ~30 million markers were projected onto all 5,000 lines in the NAM population using low density markers obtained through GBS. A cutoff p-value value (p ≤ 1e-6) was used from inclusion in the final model. SNPs associated with elemental concentrations were considered significant in they were selected in more than 5 of the 100 models (Resample model inclusion probability (RMIP)) (Valdar et al. 2009)

## Identifying ionome high priority overlap (HPO) genes and HPO+ genes

Gene specific density and locality were calculated for candidate genes identified from the 17 ionome GWAS traits as well as for 1000 random sets of genes of the same size. Gene specific metrics were converted to the standard normal scale (Z-score) by subtracting the average gene specific score from the randomized set and dividing by the average randomized standard deviation. A false discovery rate was established by incrementally evaluating the number of GWAS candidates discovered at a Z-score threshold compared to the average number discovered in the random sets. For example, if 10 GWAS genes had a gene specific z-score of 3 and an average of 2.5 randomized genes (in the 1000 random sets) had a score of 3 or above, the FDR would be 25%.

High priority overlap (HPO) candidate genes for each element were identified by requiring candidate genes to have a co-expression FDR ≤ 30% in two or more SNP-to-gene mapping scenarios in the same co-expression network using the same co-expression metric (i.e. density or locality).

HPO+ candidate gene sets were identified by taking the number of HPO genes discovered in each element (n genes) and querying each co-expression network for the set of (n) genes that had the strongest aggregate co-expression. For example, for the 18 HPO genes for P, an additional 18 genes (36 total) were added to the HPO+ set based on co-expression in each of the networks. Genes were added based on the sum of their co-expression to the original HPO set.

## Reduced accession ZmPAN networks

Both the ZmPAN and ZmRoot networks were rebuilt using only the 20 accessions shared between the 503 ZmPAN and 46 ZmRoot experimental data sets. The ZmPAN network was also built using the common set of 20 accessions as well as 26 accessions selected from the broader set of 503 to simulate the number of accessions used in the ZmRoot network. Density and locality were assessed in these reduced accession networks using the same approach as the full datasets.

# References

Anders, Simon, Paul Theodor Pyl, and Wolfgang Huber. 2014. “HTSeq - A Python Framework to Work with High-Throughput Sequencing Data.” *Bioinformatics (Oxford, England)* 31 (2): 166–69. doi:10.1093/bioinformatics/btu638.

Andorf, Carson M, Ethalinda K Cannon, John L Portwood, Jack M Gardiner, Lisa C Harper, Mary L Schaeffer, Bremen L Braun, et al. 2015. “MaizeGDB Update: New Tools, Data and Interface for the Maize Model Organism Database.” *Nucleic Acids Research*, gkv1007. doi:10.1093/nar/gkv1007.

Asaro, A., G. Ziegler, C. Ziyomo, O. Hoekenga, B. Dilkes, and I. Baxter. 2016. “The Interaction of Genotype and Environment Determines Variation in the Maize Kernel Ionome.” *G3&amp;#58; Genes|Genomes|Genetics* 6 (December): 4175–83. doi:10.1534/g3.116.034827.

Badri, D. V., V. M. Loyola-Vargas, C. D. Broeckling, C. De-la-Pena, M. Jasinski, D. Santelia, E. Martinoia, et al. 2007. “Altered Profile of Secondary Metabolites in the Root Exudates of Arabidopsis ATP-Binding Cassette Transporter Mutants.” *Plant Physiology* 146 (2): 762–71. doi:10.1104/pp.107.109587.

Baxter, Ivan. 2010. “Ionomics: The Functional Genomics of Elements.” *Briefings in Functional Genomics* 9 (2): 149–56. doi:10.1093/bfgp/elp055.

Baxter, Ivan, and Brian P Dilkes. 2012. “Elemental Profiles Reflect Plant Adaptations to the Environment.” *Science (New York, N.Y.)* 336 (6089): 1661–63. doi:10.1126/science.1219992.

Baxter, Ivan R., Gregory Ziegler, Brett Lahner, Michael V. Mickelbart, Rachel Foley, John Danku, Paul Armstrong, David E. Salt, and Owen A. Hoekenga. 2014. “Single-Kernel Ionomic Profiles Are Highly Heritable Indicators of Genetic and Environmental Influences on Elemental Accumulation in Maize Grain (Zea Mays).” *PLoS ONE* 9 (1). doi:10.1371/journal.pone.0087628.

Baxter, Ivan R, Olga Vitek, Brett Lahner, Balasubramaniam Muthukumar, Monica Borghi, Joe Morrissey, Mary Lou Guerinot, and David E Salt. 2008. “The Leaf Ionome as a Multivariable System to Detect a Plant’s Physiological Status.” *Proceedings of the National Academy of Sciences of the United States of America* 105 (33): 12081–86. doi:10.1073/pnas.0804175105.

Baxter, Ivan, Jason Tchieu, Michael R Sussman, Marc Boutry, Michael G Palmgren, Michael Gribskov, Jeffrey F Harper, and Kristian B Axelsen. 2003. “Genomic Comparison of P-Type ATPase Ion Pumps in Arabidopsis and Rice 1” 132 (June): 618–28. doi:10.1104/pp.103.021923.dresa.

Bradbury, Peter J, Zhiwu Zhang, Dallas E Kroon, Terry M Casstevens, Yogesh Ramdoss, and Edward S Buckler. 2007. “TASSEL: Software for Association Mapping of Complex Traits in Diverse Samples.” *Bioinformatics (Oxford, England)* 23 (19): 2633–35. doi:10.1093/bioinformatics/btm308.

Buckler, Edward S, James B Holland, Peter J Bradbury, Charlotte B Acharya, Patrick J Brown, Chris Browne, Elhan Ersoz, et al. 2009. “The Genetic Architecture of Maize Flowering Time.” *Science (New York, N.Y.)* 325 (5941): 714–18. doi:10.1126/science.1174276.

Bunyavanich, Supinda, Eric E Schadt, Blanca E Himes, Jessica Lasky-Su, Weiliang Qiu, Ross Lazarus, John P Ziniti, et al. 2014. “Integrated Genome-Wide Association, Coexpression Network, and Expression Single Nucleotide Polymorphism Analysis Identifies Novel Pathway in Allergic Rhinitis.” *BMC Medical Genomics* 7 (1): 48. doi:10.1186/1755-8794-7-48.

Calabrese, Gina M., Larry D. Mesner, Joseph P. Stains, Steven M. Tommasini, Mark C. Horowitz, Clifford J. Rosen, and Charles R. Farber. 2017. “Integrating GWAS and Co-Expression Network Data Identifies Bone Mineral Density Genes SPTBN1 and MARK3 and an Osteoblast Functional Module.” *Cell Systems* 4 (1). Elsevier Inc.: 46–59.e4. doi:10.1016/j.cels.2016.10.014.

Caldwell, Katherine S., Joanne Russell, Peter Langridge, and Wayne Powell. 2006. “Extreme Population-Dependent Linkage Disequilibrium Detected in an Inbreeding Plant Species, Hordeum Vulgare.” *Genetics* 172 (1): 557–67. doi:10.1534/genetics.104.038489.

Castelletti, Sara, Roberto Tuberosa, Massimo Pindo, and Silvio Salvi. 2014. “A MITE Transposon Insertion Is Associated with Differential Methylation at the Maize Flowering Time QTL Vgt1.” *G3 (Bethesda, Md.)* 4 (5): 805–12. doi:10.1534/g3.114.010686.

Chao, D.-Y., K. Gable, M. Chen, I. Baxter, C. R. Dietrich, E. B. Cahoon, M. L. Guerinot, et al. 2011. “Sphingolipids in the Root Play an Important Role in Regulating the Leaf Ionome in Arabidopsis Thaliana.” *The Plant Cell* 23 (3): 1061–81. doi:10.1105/tpc.110.079095.

Chia, Jer-Ming, Chi Song, Peter J Bradbury, Denise Costich, Natalia de Leon, John Doebley, Robert J Elshire, et al. 2012. “Maize HapMap2 Identifies Extant Variation from a Genome in Flux.” *Nature Genetics* 44 (7). Nature Publishing Group: 803–7. doi:10.1038/ng.2313.

Clark, Richard M, Tina Nussbaum Wagler, Pablo Quijada, and John Doebley. 2006. “A Distant Upstream Enhancer at the Maize Domestication Gene tb1 Has Pleiotropic Effects on Plant and Inflorescent Architecture.” *Nature Genetics* 38 (5): 594–97. doi:10.1038/ng1784.

Cook, Jason P, Michael D McMullen, James B Holland, Feng Tian, Peter Bradbury, Jeffrey Ross-Ibarra, Edward S Buckler, and Sherry a Flint-Garcia. 2012. “Genetic Architecture of Maize Kernel Composition in the Nested Association Mapping and Inbred Association Panels.” *Plant Physiology* 158 (2): 824–34. doi:10.1104/pp.111.185033.

Davies, Laurie, and Ursula Gather. 2012. “The Identification of Multiple Outliers.” *Journal of the American Statistical Association*, February. Taylor & Francis Group. http://www.tandfonline.com/doi/abs/10.1080/01621459.1993.10476339.

Dongen, Stijn van. 2000. “MCL: A Cluster Algoithm for Graphs.” Center for Information Workshop.

Elshire, Robert J, Jeffrey C Glaubitz, Qi Sun, Jesse A Poland, Ken Kawamoto, Edward S Buckler, and Sharon E Mitchell. 2011. “A Robust, Simple Genotyping-by-Sequencing (GBS) Approach for High Diversity Species.” *PloS One* 6 (5): e19379. doi:10.1371/journal.pone.0019379.

Fan, Jilian, Zhiyang Zhai, Chengshi Yan, and Changcheng Xu. 2015. “Arabidopsis TRIGALACTOSYLDIACYLGLYCEROL5 Interacts with TGD1, TGD2, and TGD4 to Facilitate Lipid Transfer from the Endoplasmic Reticulum to Plastids.” *The Plant Cell* 27 (October): tpc.15.00394. doi:10.1105/tpc.15.00394.

Fu, Jingye, Fei Ren, Xuan Lu, Hongjie Mao, Meimei Xu, Jörg Degenhardt, Reuben J. Peters, and Qiang Wang. 2016. “A Tandem Array of *Ent* -Kaurene Synthases in Maize with Roles in Gibberellin and More Specialized Metabolism.” *Plant Physiology* 170 (2): 742–51. doi:10.1104/pp.15.01727.

Ghazalpour, Anatole, Sudheer Doss, Bin Zhang, Susanna Wang, Christopher Plaisier, Ruth Castellanos, Alec Brozell, et al. 2006. “Integrating Genetic and Network Analysis to Characterize Genes Related to Mouse Weight.” Edited by Greg Gibson. *PLoS Genetics* 2 (8). Public Library of Science: e130. doi:10.1371/journal.pgen.0020130.

Gore, Michael a, Jer-Ming Chia, Robert J Elshire, Qi Sun, Elhan S Ersoz, Bonnie L Hurwitz, Jason a Peiffer, et al. 2009. “A First-Generation Haplotype Map of Maize.” *Science (New York, N.Y.)* 326 (5956): 1115–17. doi:10.1126/science.1177837.

Guerinot, Mary Lou, and David E Salt. 2017. “Fortified Foods and Phytoremediation . Two Sides of the Same Coin 1” 3755.

Harris, M a, J Clark, a Ireland, J Lomax, M Ashburner, R Foulger, K Eilbeck, et al. 2004. “The Gene Ontology (GO) Database and Informatics Resource.” *Nucleic Acids Research* 32 (Database issue): D258-61. doi:10.1093/nar/gkh036.

Hirsch, Candice N, Jillian M Foerster, James M Johnson, Rajandeep S Sekhon, German Muttoni, Brieanne Vaillancourt, Francisco Peñagaricano, et al. 2014. “Insights into the Maize Pan-Genome and Pan-Transcriptome.” *The Plant Cell* 26 (1): 121–35. doi:10.1105/tpc.113.119982.

Hung, H-Y, C Browne, K Guill, N Coles, M Eller, A Garcia, N Lepak, et al. 2012. “The Relationship between Parental Genetic or Phenotypic Divergence and Progeny Variation in the Maize Nested Association Mapping Population.” *Heredity* 108 (5): 490–99. doi:10.1038/hdy.2011.103.

Katagiri, Takeshi, Kanako Ishiyama, Tomohiko Kato, Satoshi Tabata, Masatomo Kobayashi, and Kazuo Shinozaki. 2005. “An Important Role of Phosphatidic Acid in ABA Signaling during Germination in Arabidopsis Thaliana.” *Plant Journal* 43 (1): 107–17. doi:10.1111/j.1365-313X.2005.02431.x.

Koch, Evan, Mickey Ristroph, and Mark Kirkpatrick. 2013. “Long Range Linkage Disequilibrium across the Human Genome” 8 (12). doi:10.1371/journal.pone.0080754.

Kump, Kristen L, Peter J Bradbury, Randall J Wisser, Edward S Buckler, Araby R Belcher, Marco a Oropeza-Rosas, John C Zwonitzer, et al. 2011. “Genome-Wide Association Study of Quantitative Resistance to Southern Leaf Blight in the Maize Nested Association Mapping Population.” *Nature Genetics* 43 (2): 163–68. doi:10.1038/ng.747.

Lawit, Shai J., Heidi M. Wych, Deping Xu, Suman Kundu, and Dwight T. Tomes. 2010. “Maize Della Proteins Dwarf plant8 and Dwarf plant9 as Modulators of Plant Development.” *Plant and Cell Physiology* 51 (11): 1854–68. doi:10.1093/pcp/pcq153.

Li, Heng, and Richard Durbin. 2009. “Fast and Accurate Short Read Alignment with Burrows-Wheeler Transform.” *Bioinformatics (Oxford, England)* 25 (14): 1754–60. doi:10.1093/bioinformatics/btp324.

Li, Min, Jian-er Chen, Jian-xin Wang, Bin Hu, and Gang Chen. 2008. “Modifying the DPClus Algorithm for Identifying Protein Complexes Based on New Topological Structures.” *BMC Bioinformatics* 9 (1). BioMed Central: 398. doi:10.1186/1471-2105-9-398.

Lindgreen, Stinus. 2012. “AdapterRemoval: Easy Cleaning of next-Generation Sequencing Reads.” *BMC Research Notes* 5 (1): 337. doi:10.1186/1756-0500-5-337.

Louwers, Marieke, Rechien Bader, Max Haring, Roel van Driel, Wouter de Laat, and Maike Stam. 2009. “Tissue- and Expression Level-Specific Chromatin Looping at Maize b1 Epialleles.” *The Plant Cell* 21 (3): 832–42. doi:10.1105/tpc.108.064329.

Mason, Michael G., Deepa Jha, David E. Salt, Mark Tester, Kristine Hill, Joseph J. Kieber, and G. Eric Schaller. 2010. “Type-B Response Regulators ARR1 and ARR12 Regulate Expression of AtHKT1;1 and Accumulation of Sodium in Arabidopsis Shoots.” *Plant Journal* 64 (5): 753–63. doi:10.1111/j.1365-313X.2010.04366.x.

McMullen, Michael D, Stephen Kresovich, Hector Sanchez Villeda, Peter Bradbury, Huihui Li, Qi Sun, Sherry Flint-Garcia, et al. 2009. “Genetic Properties of the Maize Nested Association Mapping Population.” *Science (New York, N.Y.)* 325 (5941). AAAS: 737–40. doi:10.1126/science.1174320.

Mochida, Keiichi, Yukiko Uehara-Yamaguchi, Takuhiro Yoshida, Tetsuya Sakurai, and Kazuo Shinozaki. 2011. “Global Landscape of a Co-Expressed Gene Network in Barley and Its Application to Gene Discovery in Triticeae Crops.” *Plant & Cell Physiology* 52 (5): 785–803. doi:10.1093/pcp/pcr035.

Monaco, Marcela K., Taner Z. Sen, Palitha D. Dharmawardhana, Liya Ren, Mary Schaeffer, Sushma Naithani, Vindhya Amarasinghe, et al. 2013. “Maize Metabolic Network Construction and Transcriptome Analysis.” *The Plant Genome* 6 (1): 0. doi:10.3835/plantgenome2012.09.0025.

Morrell, Peter L, Donna M Toleno, Karen E Lundy, and Michael T Clegg. 2005. “Low Levels of Linkage Disequilibrium in Wild Barley (Hordeum Vulgare Ssp. Spontaneum) despite High Rates of Self-Fertilization.” *Proceedings of the National Academy of Sciences of the United States of America* 102 (7): 2442–47. doi:10.1073/pnas.0409804102.

Obayashi, T., Y. Okamura, S. Ito, S. Tadaka, Y. Aoki, M. Shirota, and K. Kinoshita. 2014. “ATTED-II in 2014: Evaluation of Gene Coexpression in Agriculturally Important Plants.” *Plant and Cell Physiology* 55 (1): e6–e6. doi:10.1093/pcp/pct178.

Ozaki, Soichi, Yoshiyuki Ogata, Kunihiro Suda, Atsushi Kurabayashi, Tatsuya Suzuki, Naoki Yamamoto, Yoko Iijima, et al. 2010. “Coexpression Analysis of Tomato Genes and Experimental Verification of Coordinated Expression of Genes Found in a Functionally Enriched Coexpression Module.” *DNA Research : An International Journal for Rapid Publication of Reports on Genes and Genomes* 17 (2): 105–16. doi:10.1093/dnares/dsq002.

Peiffer, Jason A, Maria C Romay, Michael A Gore, Sherry A Flint-Garcia, Zhiwu Zhang, Mark J Millard, Candice A C Gardner, et al. 2014. “The Genetic Architecture of Maize Height.” *Genetics*, February. doi:10.1534/genetics.113.159152.

Reich, David E, Michele Cargill, Stacey Bolk, James Ireland, Pardis C Sabeti, Daniel J Richter, Thomas Lavery, et al. 2001. “Linkage Disequilibrium in the Human Genome” 9 (Table 1): 199–204.

Ritchie, Marylyn D., Emily R. Holzinger, Ruowang Li, Sarah A. Pendergrass, and Dokyoon Kim. 2015. “Methods of Integrating Data to Uncover Genotype–phenotype Interactions.” *Nature Reviews Genetics* 16 (2). Nature Publishing Group: 85–97. doi:10.1038/nrg3868.

Roston, Rebecca L., Jinpeng Gao, Monika W. Murcha, James Whelan, and Christoph Benning. 2012. “TGD1, -2, and -3 Proteins Involved in Lipid Trafficking Form ATP-Binding Cassette (ABC) Transporter with Multiple Substrate-Binding Proteins.” *Journal of Biological Chemistry* 287 (25): 21406–15. doi:10.1074/jbc.M112.370213.

Sarkar, Neelam K., Yeon-Ki Kim, and Anil Grover. 2014. “Coexpression Network Analysis Associated with Call of Rice Seedlings for Encountering Heat Stress.” *Plant Molecular Biology* 84 (1–2): 125–43. doi:10.1007/s11103-013-0123-3.

Schaefer, R.J., R. Briskine, N.M. Springer, and C.L. Myers. 2014. “Discovering Functional Modules across Diverse Maize Transcriptomes Using COB, the Co-Expression Browser.” *PLoS ONE* 9 (6). doi:10.1371/journal.pone.0099193.

Schaefer, R.J., J.-M. Michno, and C.L. Myers. 2016. “Unraveling Gene Function in Agricultural Species Using Gene Co-Expression Networks.” *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*. doi:10.1016/j.bbagrm.2016.07.016.

Schaefer, Robert J., Roman Briskine, Nathan M. Springer, and Chad L. Myers. 2014. “Discovering Functional Modules across Diverse Maize Transcriptomes Using COB, the Co-Expression Browser.” *PLoS ONE* 9 (6): 99193. doi:10.1371/journal.pone.0099193.

Schaefer, Robert J., Roman Briskine, Nathan M. Springer, CL Chad L. Myers, H Wei, S Persson, T Mehta, et al. 2014. “Discovering Functional Modules across Diverse Maize Transcriptomes Using COB, the Co-Expression Browser.” Edited by Frederik Börnke. *PLoS ONE* 9 (6). Public Library of Science: 99193. doi:10.1371/journal.pone.0099193.

Schubert, Mikkel, Luca Ermini, Clio Der Sarkissian, Hákon Jónsson, Aurélien Ginolhac, Robert Schaefer, Michael D Martin, et al. 2014. “Characterization of Ancient and Modern Genomes by SNP Detection and Phylogenomic and Metagenomic Analysis Using PALEOMIX.” *Nature Protocols* 9 (5): 1056–82. doi:10.1038/nprot.2014.063.

Stelpflug, Scott C., Sekhon Rajandeep, Brieanne Vaillancourt, Candice N. Hirsch, C. Robin Buell, Natalia De Leon, and Shawn M. Kaeppler. 2015. “An Expanded Maize Gene Expression Atlas Based on RNA-Sequencing and Its Use to Explore Root Development.” *The Plant Genome*, no. 608: 314–62. doi:10.3835/plantgenome2015.04.0025.

Swanson-Wagner, R., R. Briskine, R. Schaefer, M.B. Hufford, J. Ross-Ibarra, C.L. Myers, P. Tiffin, and N.M. Springer. 2012. “Reshaping of the Maize Transcriptome by Domestication.” *Proceedings of the National Academy of Sciences of the United States of America* 109 (29). doi:10.1073/pnas.1201961109.

Swanson-Wagner, Ruth, Roman Briskine, Robert Schaefer, Matthew B. Hufford, Jeffrey Ross-Ibarra, C. L. Myers, P. Tiffin, and N. M. Springer. 2012. “Reshaping of the Maize Transcriptome by Domestication.” *PNAS* 109 (29). National Acad Sciences: 11878–83. doi:10.1073/pnas.1201961109/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1201961109.

Tacke, Eckhard, Christian Korfhage, Detlef Michel, Massimo Maddaloni, Mario Motto, Simona Lanzini, Francesco Salamini, and Hans???Peter ???P D??ring. 1995. “Transposon Tagging of the Maize Glossy2 Locus with the Transposable Element En/Spm.” *The Plant Journal*. doi:10.1046/j.1365-313X.1995.8060907.x.

Taşan, Murat, Gabriel Musso, Tong Hao, Marc Vidal, Calum a Macrae, and Frederick P Roth. 2014. “Selecting Causal Genes from Genome-Wide Association Studies via Functionally Coherent Subnetworks” 12 (2). doi:10.1038/nmeth.3215.

Tian, Feng, Peter J Bradbury, Patrick J Brown, Hsiaoyi Hung, Qi Sun, Sherry Flint-Garcia, Torbert R Rocheford, Michael D McMullen, James B Holland, and Edward S Buckler. 2011. “Genome-Wide Association Study of Leaf Architecture in the Maize Nested Association Mapping Population.” *Nature Genetics* 43 (2): 159–62. doi:10.1038/ng.746.

USDA. 2016. “Crop Production 2015 Summary.”

Valdar, William, Christopher C Holmes, Richard Mott, and Jonathan Flint. 2009. “Mapping in Structured Populations by Resample Model Averaging.” *Genetics* 182 (4): 1263–77. doi:10.1534/genetics.109.100727.

Wallace, Jason G, Peter J Bradbury, Nengyi Zhang, Yves Gibon, Mark Stitt, and Edward S Buckler. 2014. “Association Mapping across Numerous Traits Reveals Patterns of Functional Variation in Maize.” *PLoS Genetics* 10 (12). Public Library of Science: e1004845. doi:10.1371/journal.pgen.1004845.

Wang, X., A. A. Elling, X. Li, N. Li, Z. Peng, G. He, H. Sun, Y. Qi, X. S. Liu, and X. W. Deng. 2009. “Genome-Wide and Organ-Specific Landscapes of Epigenetic Modifications and Their Relationships to mRNA and Small RNA Transcriptomes in Maize.” *The Plant Cell Online* 21 (4): 1053–69. doi:10.1105/tpc.109.065714.

Wild, Michael, Jean Michel Davi??re, Thomas Regnault, Lali Sakvarelidze-Achard, Esther Carrera, Isabel Lopez Diaz, Anne Cayrel, Guillaume Dubeaux, Gr??gory Vert, and Patrick Achard. 2016. “Tissue-Specific Regulation of Gibberellin Signaling Fine-Tunes Arabidopsis Iron-Deficiency Responses.” *Developmental Cell* 37 (2): 190–200. doi:10.1016/j.devcel.2016.03.022.

Winkler, Rodney G, and Michael Freeling. 1994. “Physiological Genetics of the Dominant Gibberellin-Nonresponsive Maize Dwarfs, Dwart8 and Dwart9.” *Planta* 193: 341–48. doi:10.1007/BF00201811.

Wolfe, Cecily J, Isaac S Kohane, and Atul J Butte. 2005. “Systematic Survey Reveals General Applicability Of ‘guilt-by-Association’ within Gene Coexpression Networks.” *BMC Bioinformatics* 6 (January): 227. doi:10.1186/1471-2105-6-227.

Wray, Gregory A. 2007. “The Evolutionary Significance of Cis-Regulatory Mutations.” *Nature Reviews. Genetics* 8 (3). Nature Publishing Group: 206–16. doi:10.1038/nrg2063.

Zheng, Zhi-Liang, and Yihong Zhao. 2013. “Transcriptome Comparison and Gene Coexpression Network Analysis Provide a Systems View of Citrus Response to ‘Candidatus Liberibacter Asiaticus’ Infection.” *BMC Genomics* 14 (1): 27. doi:10.1186/1471-2164-14-27.

Ziegler, Greg, Philip J. Kear, Di Wu, Catherine Ziyomo, Alexander E. Lipka, Michael Gore, Owen Hoekenga, and Ivan Baxter. 2017. “Elemental Accumulation in Kernels of the Maize Nested Association Mapping Panel Reveals Signals of Gene by Environment Interactions.” *bioRxiv*, no. May.

# Supplementary Figures

## Supp. Fig. 1

### ZmPAN Network Health

Global network health of the maize PAN genome (ZmPAN) co-expression network. **(A)** Raw Pearson correlation coefficient distribution of all co-expression interactions. **(B)** Fisher transformed, variance stabilized and mean centered network interactions. **(C)** A Volcano plot showing empirical density for genes in each GO term compared to the corresponding p-value derived from measuring density in 1000 random gene sets of the same size. **(D)** Degree distribution of ZmPAN genome co-expression network compared to power law, exponential, and truncated power law distributions.

## Supp. Fig. 2

### ZmSAM Network Health

Global network health of the maize ZmSAM co-expression network. **(A)** Raw Pearson correlation coefficient distribution of all co-expression interactions. **(B)** Variance stabilized and mean centered network interactions. **(C)** A Volcano plot showing empirical density for genes in each GO term compared to the corresponding p-value derived from measuring density in 1000 random gene sets of the same size. **(D)** Degree distribution of Tissue/Developmental co-expression network compared to power law, exponential, and truncated power law distributions.

## Supp. Fig. 3

### ZmRoot Network Health

Global network health of the maize ZmRoot co-expression network. **(A)** Raw Pearson correlation coefficient distribution of all co-expression interactions. **(B)** Variance stabilized and mean centered network interactions. **(C)** A Volcano plot showing empirical density for genes in each GO term compared to the corresponding p-value derived from measuring density in 1000 random gene sets of the same size. **(D)** Degree distribution of ZmRoot co-expression network compared to power law, exponential, and truncated power law distributions.

## Supp. Fig. 4

### MCR supplemental figure

Panel (A) shows the absolute number of GO terms that remain significantly co-expressed at varying levels of MCR in each network. Red curves show all GO terms with an initial co-expression p-value ≤ 0.05. Blue and purple curves show GO terms with either moderate or strong initial co-expression (at MCR=0). Panels (B-C) shows the percent and absolute number of GO terms that remain significantly co-expressed at varying levels of MCR. The red curves show small GO terms (50 ≤ n < 65), the blue curve shows medium sized GO terms (65 ≤ n < 80) and the purple curve shows large terms (80 ≤ n < 100).

## Supp. Fig. 5

### FCR supplemental figure

Panel (A) shows the absolute number of GO terms that remain significantly co-expressed at varying levels of FCR in each network. Red curves show all GO terms with an initial co-expression p-value ≤ 0.05. Blue and purple curves show GO terms with either moderate or strong initial co-expression. Panels (B-C) shows the percent and absolute number of GO terms that remain significantly co-expressed at varying levels of FCR. The red curves show small GO terms (50 ≤ n < 65), the blue curve shows medium sized GO terms (65 ≤ n < 80) and the purple curve shows large terms (80 ≤ n < 100).

## Supp. Figure 6

### Distribution of Pearson correlation coefficients between gene specific density and locality

Pearson correlation was measured between gene specific density and locality in each network for both ionome elements as well as GO terms. PCCs between metrics were calculated by grouping sets of genes in either Ionome elements (e.g. Al, Fe, etc.) or GO Terms at the same SNP-to-gene mapping parameters (50, 100 and 500 kb Window Size and 1,2, and 5 gene flank limits). The distribution shows the PCCs between the metrics aggregated across all SNP-to-gene mapping parameters.

# Supplementary Files

## Supp. File 1

### Quality control and Co-expression networks overview

This file contains log information, quality control parameters, and network build parameters for gene co-expression networks.

# Supplementary Tables

## Supp. Table 1

### Full gene ontology term density and locality p-values

Density and locality scores were measured between genes within each GO Term. Subnetwork p-values were generated for both density and locality by comparing each term’s metric to n=1000 randomized gene sets of the same size.

## Supp. Table 2

### Network MCL cluster gene assignments

Clusters in all three networks were identified using the MCL algorithm. Genes in each network were assigned to cluster IDs. Lower cluster IDs have a larger number of genes.

## Supp. Table 3

### Network MCL cluster GO enrichment

Enrichment of genes co-annotated for GO terms in each MCL cluster.

## Supp. Table 4

### Network signal of GO terms with various levels of MCR/FCR.

Co-expression among co-annotated GO terms genes was compared to random gene sets of the same size to generate a p-values. Noise was introduced by varying the missing candidate rate (MCR) or false candidate rate (FCR). Missing candidates were removed in proportion to the values in the table, while false candidates were introduced using SNP-to-gene mapping values (see WindowSize and FlankLimit). FCR values are reported as averages across 10 percent quantiles (see Fig. 5).

## Supp. Table 5

### Maize Grain Ionome GWAS Network Overlap Candidate Genes

Candidate genes were identified by extracting candidate genes in each co-expression network (ZmSAM, ZmPAN or ZmRoot) using SNP-to-gene mapping for each Element (using WindowSize and FlankLimit). Co-expression (density or locality) among all genes within a subnetwork was compared to randomized gene sets of the same size to establish p-values. Gene specific z-scores were computed by comparing the empirical gene-specific density (Eq.3) or locality (Eq.4) to the average density or locality observed in randomized gene sets, then correcting for standard deviation. False discovery rates (FDR) were calculated for candidate genes with positive gene-specific co-expression values by comparing the number of genes discovered at a z-score cutoff to the average number of genes discovered in randomized sets.

## Supp. Table 6

### Maize Grain Ionome GWAS High Priority Overlap (HPO) Candidate Genes

High priority overlap (HPO) genes were identified by calculating gene-specific density or locality (Method column) for each element at different SNP-to-gene mapping parameters (see WindowSize and FlankLimit columns). At a FDR cutoff of 30%, genes were defined as HPO if they were observed at two or more SNP-to-gene mapping parameters.

## Supp. Table 7

### HPO genes discovered with networks built from accessions subsets

The number of HPO genes discovered in full ZmPAN (503 accessions) and ZmRoot (46 accessions) networks were compared to networks built with a subset of accessions. Both ZmPAN and ZmRoot networks were re-built using a common set of 20 accessions. The ZmPAN network was re-built using 46 accessions consisting of the 20 common accessions and either 26 random or 26 CML biases accessions to simulate the number used in the full 46 accession ZmRoot network. Each network analyzed for HPO genes in the 17 GWAS elements.

## Supp. Table 8

### Multiple Element HPO gene list

The number commonly discovered HPO genes, hypergeometric p-values of set overlap, and GRMZM IDs across multiple elements.

## Supp. Table 9

### Element Gene Ontology Enrichment

HPO genes for each element were tested for enrichment among genes co-annotated for Gene Ontology (GO) terms (hypergeometric test). Bonferroni correction is included as a column, treating each GO term as an independent test.

## Supp. Table 10

### HPO plus neighbors Gene Ontology Enrichment

Elemental HPO gene sets were supplemented with an additional set of highly connected neighbors equal to the number of genes in the HPO set. These HPO+ gene sets were tested for enrichment among genes annotated for Gene Ontology terms (hypergeometric test).