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Linking Metabolic QTLs with Network and cis-eQTLs Controlling Biosynthetic Pathways

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Phenotypic variation between individuals of a species is often under quantitative genetic control. Genomic analysis of gene expression polymorphisms between individuals is rapidly gaining popularity as a way to query the underlying mechanistic causes of variation between individuals. However, there is little direct evidence of a linkage between global gene expression polymorphisms and phenotypic consequences. In this report, we have mapped quantitative trait loci (QTLs) controlling glucosinolate content in a population of 403 Arabidopsis Bay × Sha recombinant inbred lines, 211 of which were previously used to identify expression QTLs controlling the transcript levels of biosynthetic genes. In a comparative study, we have directly tested two plant biosynthetic pathways for association between polymorphisms controlling biosynthetic gene transcripts and the resulting metabolites within the Arabidopsis Bay × Sha recombinant inbred line population. In this analysis, all loci controlling expression variation also affected the accumulation of the resulting metabolites. In addition, epistasis was detected more frequently for metabolic traits compared to transcript traits, even when both traits showed similar distributions. An analysis of candidate genes for QTL-controlling networks of transcripts and metabolites suggested that the controlling factors are a mix of enzymes and regulatory factors. This analysis showed that regulatory connections can feedback from metabolism to transcripts. Surprisingly, the most likely major regulator of both transcript level for nearly the entire pathway and aliphatic glucosinolate accumulation is variation in the last enzyme in the biosynthetic pathway, AOP2. This suggests that natural variation in transcripts may significantly impact phenotypic variation, but that natural variation in metabolites or their enzymatic loci can feed back to affect the transcripts.

Introduction

A longstanding goal in genetics is to unravel the molecular and genetic bases of complex traits such as disease resistance, growth, and development. While phenotypic variation in natural populations is largely quantitative and polygenic, understanding this variation is complicated by the interaction of environmental and genetic factors [1,2]. Quantitative trait mapping, the most common approach to analyze complex traits, measures the association of genetic markers with phenotypic variation, delineating quantitative trait loci (QTLs) [1,3]. Advances in statistical models, improvements in marker technology, and expanding genomic resources have lead to increasingly refined QTL maps for a wide array of traits, ranging from development and morphology to metabolism and disease resistance [4–10]. In spite of these considerable efforts, the molecular basis of many quantitative traits remains unknown.

Recently, our understanding of quantitative traits has been enhanced by genomic approaches that use microarray technology to measure global transcript levels in mapping populations and map expression QTL (eQTL) [11–13]. Whole-genome eQTL analysis in yeast, mice, and humans has revealed that gene expression traits are highly heritable, and can have surprisingly complex underlying genetic architecture [13–15]. Recently, similar global analysis of gene expression was conducted in two independent A. thaliana recombinant inbred line (RIL) mapping populations [16,17]. These studies revealed large numbers of both cis- and trans-acting eQTL, with evidence of nonadditive genetic variation and transgressive segregation, consistent with results from animal systems. In addition, network eQTL analysis in the Bay × Sha RIL population showed that transcript variation was controlled by variation in specific biological networks including both biosynthetic and signal transduction pathways.
Author Summary

Natural genetic variation and the resulting phenotypic variation between individuals within a species have been of longstanding interest in wide-ranging fields. However, the molecular underpinnings of this phenotypic variation are relatively uncharted. Recently, genomics methodologies have been applied to understanding natural genetic variation in global gene expression. This, however, did not resolve the connection between variation in gene expression and the resulting physiological phenotype. We used two metabolic pathways within the model plant Arabidopsis to show that it is possible to connect genomic analysis of genetic variation to the resulting phenotype. This analysis showed that the connection between gene expression and metabolite variation were complex. Finally, the major regulators of gene expression variation for these pathways are two biosynthetic enzymes: GSL.Elong, GSL.ALK, and GSL.OX.

[18]. These studies present a detailed picture of variation in gene expression and its underlying genetic architecture, but the relationship between transcript levels and the resultant phenotypic variation remains poorly understood.

Testing the connection between eQTLs and downstream phenotypic variation requires a phenotype with detailed molecular and quantitative genetic information. Metabolic phenotypes are ideal for these studies, because these traits are highly variable and can be accurately measured using high-throughput techniques [5,19,20]. Knowledge of biochemical pathways enables comparison between the transcript level of a biosynthetic gene and downstream metabolic phenotypes. This engenders detailed hypotheses about the basis of metabolic variation that incorporate biochemical relationships, flux concepts, and transcriptional regulation [21].

Derived traits generated from the raw metabolite accumulation data can provide unique insights into the metabolic network [22,23]. These derived traits can include the sum of related metabolites, providing information about the whole pathway, or the ratio of two metabolites related as precursor and product can be used to query variation in a specific enzymatic process.

We test our ability to link eQTLs with phenotypic variation using two secondary metabolite pathways responsible for the synthesis of aliphatic and indolic glucosinolates within A. thaliana. These metabolites play an important role in plant defense against herbivory, and have chemopreventive activity in the human diet. An improved understanding of the genetic basis of glucosinolate variation thus affects evolution and in the human diet. An improved understanding of the genetic basis of glucosinolate variation thus affects evolution and in the human diet. An improved understanding of the genetic basis of glucosinolate variation thus affects evolution and in the human diet.

For compounds that are undetected intermediates, chemical names only are provided. For detected compounds, both the structure and chemical name are provided. The position of known genetic loci controlling biosynthetic variation is shown in italics. (A) The pathway and genes responsible for the production of the core glucosinolate structure from tryptophan (indolic glucosinolates) and methionine (aliphatic glucosinolates). (B) The chain elongation cycle for aliphatic glucosinolate production. Each cycle of these reactions adds a single carbon to a 2-oxo-acid, which is then transaminated to generate homo-methionine for aliphatic glucosinolate biosynthesis. The GSL.Elong QTL alters this cycle through variation at the MAM1, MAM2, and MAM3 genes that leads to differential glucosinolate structure and content [32]. (C) The enzymes and genetic loci controlling aliphatic glucosinolate side chain modification within the Bay-0 × Sha RIL population. Side-chain modification is controlled by variation at the GSL.ALK QTL via cis-eQTLs at the AOP2 and AOP3 genes. The Coi-0 and Sha accessions express AOP2 to produce alkenyl glucosinolates. In contrast, the Ler and Bay-0 accessions express AOP3 to produce hydroxyl glucosinolates. Coi-0 is null for both AOP2 and AOP3, producing only the precursor methylsulfinyl glucosinolates [31]. The GSL.OX QTL appears to be controlled by cis-eQTLs regulating flavin-monoxygenase enzymes that oxygenate a methythio to methylsulfinyl glucosinolate [33].

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Figure 1B and 1C) [31–33]. Additional QTLs have been identified which, according to current knowledge, are not associated with known biosynthetic genes [30,34]. As such, the aliphatic and indolic glucosinolate metabolic pathways provide a useful model system to link phenotypic QTLs with eQTLs.

To compare phenotypic QTLs and eQTLs, we measured the accumulation of aliphatic and indolic glucosinolates in the Bay × Sha RIL population. In addition to 14 and 11 metabolic QTL for the indolic and aliphatic metabolites, respectively, several epistatic interactions were detected. Using the same seeds and developmental stages as the
previous eQTL mapping in the Bay × Sha RIL population allowed us to compare metabolite QTL and eQTL locations [17]. Comparing metabolite QTLs with network eQTLs controlling the expression of aliphatic and indolic glucosinolate biosynthetic pathways indicates that all network eQTLs collocate with metabolic QTLs, but the inverse statement is not true, as some QTLs are detected only for metabolite traits. We obtained evidence that variation in biosynthetic enzymes and possibly transcription factors can control natural variation for transcript levels of glucosinolate biosynthetic genes. The heritability of metabolic traits was on average lower than that for transcript levels, suggesting that metabolite accumulation may be more susceptible to environmental factors. This detailed picture of glucosinolate accumulation and modification shows that eQTLs can be associated with changes in the resulting phenotype, allowing us to generate testable mechanistic hypotheses regarding the interplay between expression variation and downstream phenotypic variation.

Results

Metabolite Trait Distributions

We measured glucosinolate production by the A. thaliana accessions, Bayreuth (Bay-0) and Shahdara (Sha), the parents of the Bay × Sha RIL population. The Bay-0 and Sha accessions differed in both the quantity of glucosinolates and the specific structures synthesized, verifying that the Bay-0 × Sha RIL population is potentially informative for analyzing the relationship of eQTLs to metabolic variation. The glucosinolate profile of Sha is similar to that previously published for the Cape Verdi Islands (Cvi-1) accession, which forms predominantly three carbon (C3) and four carbon (C4) alkenyl glucosinolates, with high total aliphatic glucosinolate content (Tables 1, S1, and S2) [19,30].

In contrast, Bay-0 resembles Landsberg erecta (Ler), which contains mostly C3 hydroxy glucosinolates, with lower total aliphatic glucosinolate content (Table 1) [19,30]. The parental accessions also differed in partitioning of indolic glucosinolates into different structures, with Bay-0 producing significantly more 4-methoxy-indol-3-ylmethyl glucosinolate (Table 1).

We measured the average glucosinolate content within the Bay-0 × Sha RILs and compared the trait distribution among 403 RILs to the Bay-0 and Sha parental means (Table 1). Some RILs accumulated two aliphatic glucosinolates (4-methylsulfinylbutyl [4-MSO] and 4-methylthiobutyl [4-MT]) that are not found in the parental accessions. Transgressive segregation for this biosynthetic capability was previously observed in the Ler x Cvi RIL population and shown to result from epistasis between the GSLAOPlp and GSL.Elong loci [6,30–32,35–37]. In the Sha parent, the AOP2 enzyme fully converts all 4-MSO into but-3-enyl glucosinolate, preventing the detectable accumulation of 4-MSO within Sha. In Bay-0, 4-MSO does not accumulate due to the Bay-0 allele at the AOP2 locus, where the enzyme cannot convert 4-MSO (Figure 1) [31]. In addition to transgressive segregation for biosynthetic potential, there is transgressive segregation for glucosinolate levels. For this population, the transgressive segregation for the quantity of glucosinolates produced is almost entirely negative, as the RIL population includes numerous lines producing less total aliphatic glucosinolate than either parental accession, but no lines that accumulate average levels higher than the Sha parent (Table 1). This is especially striking for total indolic glucosinolate content, where all of the RILs were significantly lower than both parents. Because the Bay-0 and Sha parents were grown concurrently with the RILs, this is not due to environmental effects. This observation of negative transgressive segregation contrasts with the Ler × Cvi RIL population, where both positive and negative transgressive segregation was observed, with RILs producing both greater and lesser quantities of glucosinolates than either parental accession [30]. Given that the GSLAOPlp and

Table 1. Variation in Aliphatic Glucosinolates within Bay-0, Sha, and the Bay-0 × Sha RILs

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Glucosinolate</th>
<th>Abbreviation</th>
<th>Bay-0 Mean</th>
<th>SE</th>
<th>Sha Mean</th>
<th>SE</th>
<th>RIL Percentile</th>
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<tr>
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<td>-</td>
<td>0.00</td>
<td>-</td>
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<td>0.69</td>
<td>0.10</td>
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Mean glucosinolate content in μmol cm⁻² is shown for Bay-0 and Sha grown concurrently with RILs, as well as the 95th and 5th percentile values for the RILs. SE shows the standard error of the measurement. Pathway indicates whether the glucosinolate is derived from methionine (aliphatic) or tryptophan (indolic). Total aliphatic and total indolic are the sum of all aliphatic and indolic glucosinolates, respectively.

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Aliphatic Glucosinolate QTLs

Analysis of aliphatic glucosinolate variation among the RILs identified 11 QTLs that control either aliphatic glucosinolate content or the partitioning of aliphatic glucosinolates into particular structures (Figure 3). The QTLs affecting the largest number of aliphatic glucosinolate and causing the largest phenotypic differences were the previously identified GSL.AOP and GSL.Elong loci (Tables S4 and S5). However, these loci appear to modify in trans the transcript levels for a broad set of aliphatic glucosinolate biosynthetic genes identified eight cis-eQTL clusters that all coincided with aliphatic glucosinolate metabolite QTLs (GSLALPH.I.0, GSLALPH.I.73, GSLALPH.II.15, GSLALPH.III.42, GSLALPH.III.10, GSL.AOP, GSL.Elong, and GSL.Elong; Figures 3 and 4 and Table S6). Two of these eQTLs, GSL.AOP and GSL.Elong, are known to be cis-eQTLs controlling the expression of four biosynthetic genes [31]. However, these loci did not significantly change the values (unpublished data).

Indolic Glucosinolate QTLs

For the indolic glucosinolate metabolism, we identified eight QTLs involved in the accumulation of indolic glucosinolates, three QTLs controlling the ratio of glucosinolates to each other (GSL.Elong and GSL.AOP), and the QTL influences only 3C aliphatic glucosinolate accumulation, and the GSLALPH.I.0 QTL controls ratio traits involving 4-MT and 8C glucosinolate (Figure 4 and Tables S4 and S5). These QTLs highlight the ability of derived traits to provide unique insights into metabolic variation.

Mapping eQTLs controlling transcript levels for the known aliphatic glucosinolate biosynthetic genes identified eight eQTL clusters that all coincided with aliphatic glucosinolate metabolite QTLs (GSLALPH.I.0, GSLALPH.I.73, GSLALPH.II.15, GSLALPH.III.42, GSLALPH.III.10, GSL.AOP, GSL.Elong, and GSL.Elong; Figures 3 and 4 and Table S6). Two of these eQTLs, GSL.AOP and GSL.Elong, were also detected using the network mean z-score for the aliphatic glucosinolate biosynthetic gene network. The network mean z-score is a derived trait obtained by averaging across the aliphatic glucosinolate transcripts within a RIL (Table S7) [18]. As the aliphatic glucosinolate transcripts are believed to participate in a highly coregulated network, this derived trait is potentially informative regarding network control [18,38,39]. These six network eQTLs appear to control transcript levels of the pathway in trans, but whether trans functionality occurs via metabolite feedback or transcriptional mechanisms remains to be elucidated (Figure 4). The eQTLs detected in the network analysis predominantly controlled biosynthetic enzymes acting early in the aliphatic glucosinolate pathway—in the elongation and core biosynthetic stages—and not the secondary modification stages (Figures 4 and S1). Of the other three metabolite QTLs that did not show a network eQTL, the GSLALPH.I.20 QTL overlapped with a cis-eQTL.
for UGT74B1, and the other two did not coincide with loci affecting the expression of any known biosynthetic genes.

**Enzyme-Encoding Genes and Network eQTLs**

Variation at the side-chain–modifying GSL.AOP and GSL.Elong loci controlled the accumulation of most metabolites and transcripts (Figures 3 and 4). Neither locus had been previously identified as impacting transcript accumulation for the whole glucosinolate biosynthetic network. Both GSL.AOP and GSL.Elong are controlled by cis-eQTLs leading to differential enzyme expression. As in Cvi, the Sha GSL.AOP locus expresses the AOP2 enzyme, leading to alkenyl glucosinolate production, higher glucosinolate content, and elevated transcript levels for most aliphatic glucosinolate genes (Figure 4 and 5). We used Col-0 (which is null for AOP2 and AOP3) transformed with a functional AOP2 gene from *Brassica oleracea* to test if the presence of functional AOP2 transcript can affect both metabolite and transcript levels [40]. AOP2 from *B. oleracea* conducts the same reaction as AOP2 from Sha and is also associated with elevated aliphatic glucosinolate content in *Brassica*, allowing us to test the conservation of this locus across the two species [40,41].

Introduction of a transcript encoding functional AOP2 results in the production of alkenyl glucosinolates and a statistically significant doubling of total aliphatic glucosinolate content, as is the case with the presence of a functional AOP2 transcript contributed by the Sha allele at the GSL.AOP QTL (Figure 5). This supports the hypothesis that the Sha allele at the GSL.AOP QTL controls metabolite and transcript levels for aliphatic glucosinolates due to increased expression of the AOP2 gene. This suggests the presence of a previously unrecognized regulatory effect of AOP2, whereby it controls transcript accumulation for most biosynthetic genes potentially through transcription factors. While we could not detect any micro-RNA signatures within the AOP2 transcript or gene, it remains to be shown whether the metabolite and transcript effect is due to the enzymatic activity of AOP2 or

![Figure 3. QTL Summary for Aliphatic Glucosinolates](image)

QTL position for aliphatic glucosinolate biosynthetic gene expression estimates and aliphatic glucosinolate metabolite accumulation is shown on the x-axis with the Roman numeral representing the chromosome number (I–V), while the arabic numeral shows the CM position on that chromosome. All five *Arabidopsis* chromosomes are represented contiguously. Names of the QTL positions that were shown by ANOVA to be statistically significant are included within the figure for reference.

(A) The left-hand y-axis and the dotted line show the number of genes in the aliphatic glucosinolate biosynthetic pathway (22 total) that have an eQTL at a given position as determined within QTL Cartographer. The right-hand y-axis and solid line shows the likelihood ratio (LR) trace for direct QTL analysis of the average transcript level across the genes for the aliphatic glucosinolate biosynthetic pathway as estimated using the mean z-score approach. The dashed and solid horizontal lines show significance thresholds (α = 0.05) as estimated by 1,000 permutations. Breaks in lines show the end of chromosomes.

(B) The QTLs for the nine aliphatic glucosinolate metabolites were mapped independently using the average across all experiments as well as the average within each experiment. QTL positions for all indolic glucosinolate traits were then summed across experiments to identify metabolite QTL “hotspots.”

(C) The QTLs for the 11 derived summation aliphatic glucosinolate metabolite traits were mapped independently using the average across all experiments as well as the average within each experiment. QTL positions for all indolic glucosinolate traits were then summed across experiments to identify metabolite QTL “hotspots.”

(D) The QTLs for the 39 derived ratio aliphatic glucosinolate metabolite traits were mapped independently using the average across all experiments as well as the average within each experiment. QTL positions for all indolic glucosinolate traits were then summed across experiments to identify metabolite QTL “hotspots.”

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some other regulatory signature within the AOP2 transcript. The association of both GSLAOP and GSL.Elong with eQTLs for the majority of aliphatic glucosinolate biosynthetic genes and metabolites (Figure 4) suggests a regulatory interplay between the metabolites directly synthesized by these enzymes and transcript levels for the aliphatic glucosinolate biosynthetic genes.

**Epistasis for Transcripts versus Metabolites**

We tested the identified QTLs for pairwise epistatic interactions controlling metabolite accumulation, partitioning, or transcript levels. This analysis identified at least one pairwise epistatic QTL interaction for all metabolites, with variation in at least half of the metabolites controlled by interactions between GSLAOP, GSL.Elong, and GSL.V.66 (Figure 4). The most common pairwise interaction was detected between GSLAOP and GSL.Elong, controlling 7 of 9 aliphatic glucosinolate metabolites (Figure 4). In contrast to the metabolites, most expression traits did not identify epistatic eQTL interactions. The few transcripts traits that identified epistatic eQTL interactions encode biosynthetic enzymes functioning in the early steps of the elongation cycle: MAM1, BCAT4, and an Aconitase. This suggests that genes in the elongation cycle may be regulated differently from the rest of the aliphatic glucosinolate pathway genes (Figures 1, 4, and S1).
To investigate the nature of the epistatic interaction between GSL.AOP and GSL.Elong, we calculated mean pheno-
typic values for the RILs containing each of the allelic
combinations at these two loci (Figure 6). The
interaction had a negative epistatic effect on the
total content of both aliphatic and indolic glucosinolates,
with lines possessing the nonparental allelic combination of
GSL.AOP from Bay-0 and GSL.Elong from Sha exhibiting
significantly lower glucosinolate content than either parent
(Figure 6). Lines possessing Sha alleles at both loci had the
highest glucosinolate accumulation.

The GSL.AOP and GSL.Elong loci also control the network
expression mean z-score for the aliphatic biosynthetic gene
network, but did not exhibit a pairwise epistasis for this trait
(Figure 4). In contrast, the genes in the methionine
elongation cycle did identify an epistatic interaction between
GSL.AOP and GSL.Elong (Figure 6; BCAT4 is shown as an
example). This lack of an epistatic effect on aliphatic glucosinolate network expression is not likely a statistical
artifact, as the pattern of the group means shows a striking
difference between aliphatic glucosinolate accumulation and
network expression of the aliphatic biosynthetic genes
(Figure 6). Substitution of the Bay-0 GSL.ElongC3 allele for
the Sha allele in a background containing the Sha
GSL.AOPAlk allele leads to increased accumulation of aliphatic glucosino-
late biosynthetic transcripts but lower aliphatic glucosinolate
content (Figure 6). This suggests that these two loci regulate
both transcript and metabolite accumulation via distinct
mechanisms.

Indolic Glucosinolate QTLs

We analyzed the indolic glucosinolate pathway as a second
test of our ability to link eQTLs altering transcript levels for
biosynthetic genes with metabolite accumulation QTLs. A total of 13 QTLs were identified that control the accumulation of indolic glucosinolates, their partitioning into particular structures, or both (Figure 7 and Table S8). These loci affect one or more of the indolic glucosinolate traits in this population, with the directionality of allelic effects mixed, so that Bay-0 alleles at some loci increase trait values while the Bay-0 alleles at other loci decrease the metabolite trait values (Table S8). Interestingly, the GSLINDOLIC.IV.8 and GSLINDOLIC.V.20 QTLs map to the same genomic locations as the previously known GSLAOP and GSL.ELONG loci, which control aliphatic glucosinolate variation. Transgenic analysis confirmed that simulating the Sha GSLAOP allele by introducing the AOP2 gene into a null background increases total indolic glucosinolate content by about 30% ($p = 0.035$). This shows that variation at GSLAOP affects indolic glucosinolate metabolism and that there is cross-talk between the pathways for indolic and aliphatic glucosinolate production.

Testing the identified indolic metabolite QTLs for pairwise epistatic interactions identified numerous epistatic interactions affecting indolic glucosinolate accumulation. Notably, the GSLINDOLIC.II.15 QTL was detected as epistatically interacting with five other QTLs (GSLINDOLIC.III.7, GSLINDOLIC.III.60, GSLINDOLIC.IV.36, GSLINDOLIC.V.45, and GSLINDOLIC.V.59; Table S8). These epistatic interactions affect the partitioning of the indolic glucosinolates into two distinct methoxylated derivatives without significantly altering the total indolic glucosinolate content. GSLINDOLIC.II.15 might encode or regulate enzymes responsible for this methoxylation (Table S8). A regulation hypothesis is supported by the fact that the GSLINDOLIC.II.15 region contains a massive trans-acting eQTL that influences transcript levels for more than 5,000 genes [17].

Analysis of eQTLs controlling transcript levels of individual indolic glucosinolate biosynthetic genes identified four eQTL clusters (GSLINDOLIC.III.60, GSLINDOLIC.IV.8, GSLINDOLIC.IV.45, and GSLINDOLIC.V.59; Figure 7 and Tables S6 and S7). Because the indolic glucosinolate biosynthetic genes are also believed to be coregulated, we estimated pathway expression mean $z$-value to map network QTLs. This identified the same four loci, plus three additional QTLs affecting expression of the indolic gene network (GSLINDOLIC.II.15, GSLINDOLIC.III.7, and GSLINDOLIC.V.5). All three of these network-specific eQTLs colocalized with metabolite QTLs, supporting the ability of the $z$-scale network approach to derive biological information. All seven eQTLs colocalize with loci that control either the accumulation or partitioning of the indolic glucosinolates produced by these genes. This suggests that the eQTLs controlling transcript levels for the indolic glucosinolate biosynthetic genes also affect variation in their metabolite products. Again, it remains to be tested whether the QTLs primarily affect transcript levels, causing downstream metabolite effects, or if the polymorphisms first affect the metabolites, influencing transcript levels through some form of feedback. There are also indolic metabolite QTLs, such as GSLINDOLIC.IV.36, with no detectable impact on gene expression traits.

**Discussion**

We used the *Arabidopsis* aliphatic and indolic glucosinolate biosynthetic pathways to test our ability to link QTLs controlling metabolic and expression polymorphisms. These metabolic pathways are well characterized with near complete identification of most biosynthetic enzymes and genes, as well as detailed quantitative genetic analysis [25,27]. A direct comparison of metabolite QTL and eQTL maps reveals...
that for both pathways, all eQTLs collocate with metabolite QTLs (Table 2, Figures 2 and 7). This suggests that expression differences for genes comprising these two pathways are predictive of phenotypic differences. However, the predictive capacity of eQTLs is not perfect, as indicated by the presence of metabolite-specific QTL clusters that do not associate with eQTL clusters.

Metabolite-specific QTL clusters cannot be explained by the superior power of the metabolite QTL analysis, which used 403 RILs versus the 211 phenotyped for transcript level, as restriction of the metabolite analysis to the set of 211 RILs included in the expression QTL analysis did not alter this result (unpublished data). In addition, higher heritability of transcript levels versus metabolite traits creates a higher statistical likelihood of failure to detect metabolite QTLs than eQTLs (Figure 2). Although the eQTL and metabolite QTL analysis were done at different times, all experiments used seed from the same mothers, and were grown in the same growth chamber under similar growth conditions. As such, environmental variance was minimized and is unlikely to explain this result.

We therefore feel that biological processes, rather than statistical or experimental considerations, explain these metabolite-specific QTLs. Metabolite-specific QTLs could be caused by metabolic feedback mechanisms, whereby a product is responsible for fine-tuning the activity of a rate-limiting enzyme in the biosynthetic pathway. Alternatively, metabolite-specific QTLs may identify loci controlling flux of substrates into the pathway. In addition, polymorphisms that affect post-translational regulation or structural variation in enzymes could lead to metabolite QTLs independent of loci controlling transcript levels. A similar result would be observed if the metabolite QTLs were caused by variation in other biosynthetic pathways that affect substrate availability, or by eQTLs affecting an unknown biosynthetic gene. Segregating variation in pathways responsible for the catabolic turnover of glucosinolates would also affect metabolite accumulation without affecting biosynthetic gene expression. The presence of QTLs that exclusively affect metabolites and not expression traits illustrates the complexity involved in extrapolating variation in gene expression to changes in metabolite content.

Network eQTL Identity

The three most consistently detected QTLs for aliphatic glucosinolate content and transcript variation were GSL.AOP, GSL.Elong, and GSL.V.66. Two of these network QTLs, AOP and Elong, contain cis-eQTLs controlling variation in transcript levels for enzymes located at the beginning and end of the aliphatic glucosinolate pathway. Neither AOP nor Elong has previously been associated with regulation of other glucosinolate biosynthetic or regulatory genes. This suggests that the accumulation of aliphatic glucosinolates is regulated by multiple mechanisms functioning such that enzyme variation can feed back to alter transcript accumulation (Figure 1). The GSL.AOP QTL is likely controlling glucosinolate content and expression through natural variation in the AOP2 gene (Figure 5). Altering the expression of the AOP2 transcript, the enzyme at the end of the aliphatic glucosinolate pathway might pull carbon into aliphatic glucosinolate production (Figures 4–6). In addition, introducing the tran-
script for AOP2 increases transcript level for the aliphatic biosynthetic pathway, suggesting that regulation also occurs by control of gene expression and potentially metabolic fluxes through the beginning of the aliphatic glucosinolate pathway (Figures 5 and 6). It is possible that a metabolite produced by or used by the AOP2 enzyme may have the capacity to regulate transcript accumulation for the rest of the biosynthetic network through feedback. Direct regulation of transcript accumulation by metabolites has been noted for a variety of riboswitches [42-45].

For the GSL.Elong network QTLs, the comparison of eQTLs to metabolite QTLs suggests that variation in transcript levels for at least two biosynthetic genes (MAM1 and MAM3) at GSL.Elong directly causes metabolic variation, and that this biosynthetic variation may alter transcript regulation for nearly the entire biosynthetic pathway. Specifically, lines that contain 4C glucosinolates have lower transcript levels for almost all biosynthetic genes. One possibility is that a metabolic intermediate produced in the GSL.Elong\textsuperscript{bc} background negatively regulates gene expression. Alternatively, both the GSL.AOP and GSL.Elong loci could possess tightly linked second loci that cause the observed transcriptional polymorphisms and also interact epistatically. Testing the interaction between biosynthetic loci and transcript levels will require careful genetic manipulation with full transcriptome analysis.

Comparing the remaining network eQTLs to candidate glucosinolate transcriptional regulators shows that the GSL.ALIPH.I.66 QTL overlaps the physical position of the MYB28 transcription factor, which has a large-effect cis-eQTL [17,39]. Likewise, the GSL.INDOLIC.I.45 and GSL.INDOLIC.I.59 QTLs overlap the physical position of the indolic transcription factors, ATR1 and ATR2, and both genes have cis-eQTLs [17,29,46]. This suggests that these three QTLs may be explained by variation in the expression of these known regulatory genes. In addition, the GSL.ALIPH.I.10 and GSL.ALIPH.III.10 QTLs overlap with the physical position for the aliphatic glucosinolate transcriptional regulators AtDOF1.1 and IQD1, respectively [28,47]. Neither gene exhibits a cis-eQTL, suggesting that if these genes are responsible for GSL.ALIPH.I.10 and GSL.ALIPH.III.10 QTLs, it is potentially due to an activity polymorphism. Alternatively, small changes in transcript levels for these transcription factors might lead to large changes in network regulation (Figure S1).

The GSL.ALIPH.II.15 QTL, a major network eQTL for transcript levels for both aliphatic and indolic glucosinolates pathways, does not overlap any known biosynthetic or regulatory genes. This locus does colocalize with a massive eQTL cluster controlling the expression of several thousand genes [17]. This suggests that this region may be highly pleiotropic and its effects on glucosinolate content may be indirect. These results suggest that eQTLs can control metabolite production through a variety of direct and indirect regulatory mechanisms.

cis-eQTLs and QTLs for Specific Metabolites

While most eQTLs co-located with QTL clusters controlling several metabolites, there were also instances in which a statistically significant association between expression and metabolic phenotypes was limited to one or few metabolic traits. For example, SGT74B1 catalyzes glycosylation of the characteristic glucosinolate backbone structure, and its expression is controlled by a single eQTL at GSL.ALIPH.I.20 [48]. This large-effect cis-eQTL maps to a 100-kb interval that includes the physical position of SGT74B1. While we might predict that variation in the expression of SGT74B1 would influence production of multiple aliphatic glucosinolates, this locus was only identified as controlling one metabolic trait, the accumulation of but-3-ethyl glucosinolate. This gene is not specific to the synthesis of but-3-ethyl glucosinolate, as previous work has demonstrated that SGT74B1 has a broad biochemical capacity to glucosylate glucosinolates [48]. Instead, the lack of detected effects in this study for this eQTL on the accumulation of other glucosinolates is likely because genotypes accumulating but-3-ethyl glucosinolate also exhibit the highest level of total aliphatic glucosinolates within this population (Table 1). This suggests that the SGT74B1 expression polymorphism is only limiting when flux across the biosynthetic pathway is maximized. Consequently, unexpected factors directly related to biochemical pathway connectivity and flux can interfere with our ability to directly associate eQTLs for biosynthetic genes with specific metabolite QTLs. As such, eQTLs are not predictive in all contexts.

QTL Causality

This comparative analysis of eQTLs to metabolic QTLs has provided novel insights, including the identification of the enzymes AOP2 and MAM1 as well as the transcription factor MYB28 as potential regulators of transcript accumulation for the complete aliphatic glucosinolate biosynthetic pathway. In addition, differential expression of the ATR1 and ATR2 transcription factors have been implicated as the underlying cause of QTLs controlling the indolic glucosinolate pathway. Data presented in this study validate the potential of the enzyme AOP2 to control aliphatic glucosinolate gene expression. The other regulatory roles hypothesized above remain to be verified. Identification and validation of the molecular causes of the 17 different QTLs identified in this study will require a complex mixture of experiments ranging from transgenic complementation to validate the gene, promoter swaps to validate the difference in gene expression, and recombination mapping to more precisely identify the causal polymorphism [49].

Trait Heritability and Epistasis

For glucosinolates and their biosynthetic genes, we observed significant differences between the estimated heritability of metabolite accumulation and transcript levels, respectively (Figure 2). Expression traits had consistently higher broad sense heritability than the accumulation of individual metabolites or metabolite summation traits. Because genetically identical individuals were used for both experiments, there is no difference in the amount of genotype variation available to transcript and metabolite traits. Thus, environmental inputs may affect metabolic traits more strongly than transcripts. The mechanic link between sequence polymorphism and variation in transcript levels may have fewer intervening processes than a causal sequence polymorphism and metabolic variation. The presence of additional regulatory processes, both metabolic and post-transcriptional, may allow greater environmental heterogeneity effects on metabolite accumulation. It is also possible that the results obtained for the glucosinolate pathway are not indicative of typical transcript or metabolite heritability.
There is evidence that glucosinolate production is under diverse selection pressures, favoring high levels of plasticity in glucosinolate accumulation mediated by environmental stimuli such as nutrient and water availability and wounding [50–52]. This would require that metabolic traits be influenced by subtle environmental heterogeneity, leading to reduced estimates of their heritability. It is therefore important to expand this analysis to other metabolic pathways to determine the extent to which these conclusions are generalizable.

We also observed that metabolic traits identified significantly more epistatic interactions than the corresponding transcripts. Regulatory processes that occur between transcript accumulation and metabolite accumulation, e.g., metabolic feedback, post-transcriptional regulation, and enzyme activity regulation, may increase regulatory interactions between loci, leading to metabolic traits showing higher levels of epistasis. The constant adjustment of metabolite flux through complex networks may also enhance the potential for epistasis. This finding may be specific to the glucosinolate system. A broader metabolomics analysis in comparison to eQTLs for all known enzymatic loci will be required to test whether these differences in heritability and epistasis are a general feature of transcriptomic and metabolomic networks. If this is the case, a detailed modeling approach may contribute to understanding differences in genetic architecture between metabolic and transcript networks.

Conclusions

In this report, we show that it is possible to relate natural variation at the transcript and metabolite levels for two glucosinolate biosynthetic networks. Furthermore, this analysis shows that the comparison of eQTLs to metabolite QTLs within an a priori–defined framework can identify complex regulatory mechanisms whereby variation in enzymes or metabolites may feed back to alter transcript accumulation. For aliphatic glucosinolates, the beginning and end of the biosynthetic pathway interact to control the whole pathway. These feedback associations can lead to the rapid generation of new hypotheses about the regulation of biosynthetic networks, but also show that the de novo reconstruction of biosynthetic relationships from metabolite data will require great care. In all cases, variation in gene expression also affected the resultant metabolites, although extrapolating the effects of gene expression on metabolism requires caution due to interplay of biochemical mechanisms. Combining different genomics datasets will greatly expand our ability to understand both the regulation of metabolism and the relationship between transcription and metabolism.

Materials and Methods

Mapping populations. The Bay × Sha population of 403 A. italiana RILs [53] was used to map QTLs controlling individual and total glucosinolate content for both the aliphatic and indolic glucosinolates. The QTL on Chromosome V for total content of aliphatic glucosinolates in the August 2005 experiment is also presented elsewhere for clarity (Sonderby, Hansen, Halkier, and Kliebenstein, unpublished data). Further, 211 of these lines have been analyzed for variation in gene expression and used to map QTLs controlling transcript levels [17,18], allowing comparison of QTLs controlling metabolite accumulation with transcript levels for the underlying biosynthetic genes.

Plant growth conditions. Seeds were imbied and cold-stratified at 4 °C for 3 d to break dormancy. Two complete plantings were grown simultaneously in neighboring growth chambers to provide independent biological replicates for each experiment. The full experiment was replicated three times between March of 2004 and August of 2005, providing six glucosinolate measurements for most lines, totaling nearly 2,600 measurements. The replicates were labeled May 2004, May 2005, and August 2005. For the May 2004 and August 2005 experiments, plants were grown in flats with 36 cells per flat, and maintained under short-day conditions in controlled environment growth chambers. For the May 2004 experiment, plants were grown in flats with 96 cells per flat, and maintained under short-day conditions in controlled environment growth chambers. For the same growth chambers, similar growth conditions, and assaying glucosinolate content at the same developmental stage analyzed in the eQTL mapping experiment maximizes our ability to compare the metabolic QTL results with eQTLs for the biosynthetic genes [17,18]. At 35 days after germination, a fully expanded mature leaf was harvested, ground, digitally photographed, and analyzed for glucosinolate content as described below at the same age as the plants used for eQTL analysis [17,18].

Analysis of glucosinolate content. The glucosinolate content of excised leaves was measured using a previously described high-throughput analytical system [30,34]. Briefly, one leaf was removed from each plant, digitally photographed, and placed in a 96-well microtiter plate with 500 µl of 90% methanol and one 3.8-mm stainless steel ball-bearing. Tissue was homogenized for 5 min in a microtissue homogenizer, centrifuged, and the supernatant was then transferred to a 96-well filter plate with 50 µl of DEAE sephadex. The sephadex-bound glucosinolates were eluted by incubation with sulfatase. Individual desulfoglucosinolates within each sample were separated and detected by high-performance liquid chromatography (HPLC)–diode-array detection, and identified and quantified by comparison to purified standards. Tissue area for each leaf was measured using Image J with scale objects included in each digital image [55]. The glucosinolate traits are reported per square centimeter of leaf area. There was no significant variation detected for leaf density within these lines (unpublished data).

In addition to the content of individual glucosinolates, we developed a series of sumation and ratio traits based on prior knowledge of the glucosinolate pathways (Table S1) [56]. For instance, the ratio of 3-MT, 3-MSO, and 3-OHP and allyl glucosinolates were summed (sum3C) to provide an estimate of the content of 3C aliphatic glucosinolates within these lines (Figure 1C and Table S1). This enables the detection of QTLs that specifically alter 3C glucosinolate accumulation irrespective of specific side-chain modification. The ratio traits were created to measure the efficiency of partitioning a class of glucosinolates into particular structures. For example, the ratio aliphatic glucosinolate to total 3C glucosinolates (aliphatic/3C) allows discrimination of the efficiency of production of 3C alkenyl glucosinolates independent of the accumulation of 3C glucosinolates (Figure 1C and Table S1). These ratios and summation traits allow us to isolate the effects of variation at individual steps of glucosinolate biosynthesis from variation affecting the rest of the biosynthetic pathway [56]. For each glucosinolate trait, we determined the average value per RIL per experiment for QTL mapping. Because there was no significant difference in the variance of the traits between the experiments, we also calculated the average value per RIL across all three experiments for all traits (Table S2). Heritability of each glucosinolate trait was estimated using the general linear model procedure within SAS (http://www.sas.com) where broad sense heritability was defined as $\sigma^2_{e} + \sigma^2_{g}$ (Table S1), where $\sigma^2_{e}$ is the estimated genetic variance for the metabolite among different genotypes in this sample of RILs, and $\sigma^2_{g}$ is the estimated phenotypic variance for the metabolite [2].

Analysis of gene expression QTL. We used previously published biochemical and coexpression data to identify all known or predicted genes encoding glucosinolate biosynthetic enzymes [27,31,33,35,58]. For these purposes, the indolic and aliphatic glucosinolate pathways are considered to be independent biosynthetic processes. This appears to reflect the biological reality, as the two pathways use different genes and amino acid precursors [25,27]. Gene families were separated into genes involved in aliphatic or indolic glucosinolate pathways based on biochemical or phenotypic data where possible [59–61]. Where this was not possible, coexpression with the known indolic or aliphatic glucosinolate genes was combined with published biochemistry to separate gene family members into their respective pathways [38]. This gene list involved in aliphatic and indolic glucosinolate biosynthesis (Table S3).

Heritability, eQTL position, eQTL effect, and transcript levels for individual transcripts were obtained using the RMA estimated
expression values from the previously published analysis of gene expression in the Bay × Sha population [17]. To conduct network expression analysis, transcript levels for each biosynthetic gene within each RIL were standardized as z-scores. This is done by taking the transcript level for a gene within a RIL, subtracting the mean transcript level for that gene among the RILs, then dividing the result by the standard deviation of transcript levels across the RILs [18]. The mean z-score for the aliphatic and indolic glucosinolate biosynthetic genes was calculated within each RIL for each replicate [18]. This pathway mean z-score per RIL per replicate was then used to estimate heritability of the aliphatic and indolic glucosinolate pathway gene expression as described for the metabolites. The mean z-score per RIL across replicates was calculated and used to map QTLs controlling transcript levels of the aliphatic and indolic glucosinolate biosynthetic pathways (Table S4). Because these global transcription studies were conducted in the same mapping population, we grew the plants under the same conditions and in the same growth chambers, it was possible to compare the expression and metabolite data.

QTL analysis. The Bay × Sha RIL population has been previously genotyped [33], and additional markers were obtained from the expression QTL analysis [62] as well as markers specific for the GSL.AOP and GSL.Elong loci [9,30]. To maximize our ability to detect all possible QTLs, we used the averages from each experiment, May 2004, May 2005, and August 2005, as well as the average across all experiments to conduct four QTL mapping tests. This was done for all individual glucosinolate traits, summation traits, and ratio traits (Table S1). The four averages for each trait were independently used for QTL mapping within Windows QTL Cartographer v2.5 (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm) [63–65]. Composite interval mapping was used to identify QTLs within Window QTL Cartographer v2.5 with a 10-cM window and an interval mapping increment of 2 cM. Forward regression was used to identify five cofactors per quantitative trait. The declaration of statistical significance for QTLs is based on permutation-derived empirical thresholds using 1,000 permutations for each trait mapped [66,67]. The Eqtl module of QTL Cartographer was used to automatically identify the location of each significant QTL for each trait from each experiment and the whole experiment average (Tables S5–S7) [65]. Composite interval mapping with permutations to assign significance using the underlying trait distribution is fairly robust at handling normal or near-normal traits as found for most of our traits [68]. In addition, all data from the three different experiments were used in the multitrait composite interval algorithm within QTL Cartographer v2.5.

QTL clusters were identified by using the QTL summation approach, where the position of each QTL for each trait for each experiment is indicated by a 1, and the number of traits controlled by a QTL at a given position is totaled [18]. This summation was conducted using four groups: Group I, all aliphatic glucosinolate metabolite traits; Group II, all eQTLs for aliphatic glucosinolate biosynthetic gene expression; Group III, all indolic glucosinolate metabolite traits; and Group IV, all eQTLs for indolic glucosinolate biosynthetic genes. These QTL clusters identified a set of defined genetic positions that were then named with respect to their position and whether they affected aliphatic or indolic glucosinolate content (Tables S5–S7). The QTLs at the previously characterized and cloned AOP and Elong loci were named as such [30–32,69].

To further validate each QTL identified and query for potential epistasis, we conducted an analysis of variance (ANOVA) using all experiments. For each trait, the markers most closely associated with each significant QTL for that trait were used as main effect cofactors. In addition, experiment (May 2004, May 2005, and August 2005) was used as a main effect cofactor. An automated SAS script was then developed to directly test all main effects as well as all possible pairwise interactions, including experiment × marker (QTL) and marker (QTL) × QTL interactions. Significance values were corrected for multiple testing within a model using false discovery rate (q < 0.05) in the automated script. The script returned all significance values as well as QTL main-effect estimates in terms of allelic substitution values (Tables S5–S7). No significant three-way interactions were identified.

AOP2 analysis. Two independent homoygous lines containing functionally expressed AOP2 transcript from B. oleracea expressed from a 35S promoter were obtained in the Col-0 background that is null for AOP2 and AOP3 [31,40]. These two lines were grown in a random block design with wild-type Col-0 as the control for glucosinolate content and by ATH1 Affymetrix microarrays (http://www.affymetrix.com) for altered transcript levels [17,18]. For total aliphatic glucosinolate content, six individual plants per line were measured and ANOVA used to test for altered glucosinolate accumulation. The complete experiment was conducted twice. As there was no difference between the independent transgenic lines, this factor was removed from the model. From each experiment, two independent RNA samples from Col-0 and two independent RNA samples from Col-0::AOP2 were obtained and hybridized with ATH1 Affymetrix microarrays as described [17,18]. Transcript levels for the genes involved in aliphatic glucosinolate biosynthesis (Table S3) were obtained and used in a targeted ANOVA testing the effect of the AOP2 transcription. p-values were tested for significance against a false discovery rate of 0.05 using this subset of genes [70].

Supporting Information

Figure S1. Gene Family and Transcription Factor eQTLs for the Aliphatic Glucosinolates

QTL position information for the FMOs, aconitases, and putative glucosinolate regulatory factors are presented as described in Figure 3. Found at doi:10.1371/journal.pgen.0030162.sg001 (1.2 M TIF).

Table S1. Heritability of Glucosinolate Variation within the Bay × Sha RILs

For all traits, ANOVA was conducted to test for the effect of variation between the RILs (Line), Experiment, Flat within an Experiment (Rep(Exp)) and an interaction between Line and Experiment. The p-values, sums of squares, and percent of total variance are presented. Found at doi:10.1371/journal.pgen.0030162.st001 (38 KB XLS).

Table S2. Mean Values for Glucosinolate Traits in Each RIL

The mean value and standard deviation for each trait as measured within the described experiments is provided. Trait abbreviations are as listed in Table S1. All glucosinolate values are in μmol g⁻¹. Line represents the specific Bay × Sha RIL and N is the number of measurements obtained for this RIL. Found at doi:10.1371/journal.pgen.0030162.st002 (841 KB XLS).

Table S3. Gene lists for Glucosinolate Pathway Genes

The genes defined as being involved in glucosinolate biosynthesis are presented along with their pathway. Functional proof indicates whether the gene has been experimentally validated as playing a role in glucosinolate production or if the function is predicted. Found at doi:10.1371/journal.pgen.0030162.st003 (29 KB XLS).

Table S4. Metabolic QTLs for Aliphatic Glucosinolate Traits

The average trait value per RIL across experiments along with the trait value per RIL for each individual experiment was used for QTL mapping. The presence of each locus in a specific experiment can be shown under “QTL Detection in Given Experiment.” The MT QTL column shows the complete results from the multitrait composite interval mapping within QTL Cartographer. M means that the QTL was identified as a main effect, while I means that it had an experiment specific interaction, and MI shows that it was found with both main and interaction effects using multitrait composite interval mapping. The data from each experiment were used to conduct ANOVA to test each QTL using the nearest marker as the main effect, with significance presented under “Main Effect” column. For each marker/QTL, the model tested for a QTL × Experiment interaction, “Esp Interaction.” The model tested all pairwise marker × marker interactions for evidence of epistasis. The significance of each pairwise test is presented under the columns marked “Pairwise Assessment of Epistasis.” Finally, the model was used to estimate the allele substitution effect of each locus as shown. NS represents nonsignificant results within this model. Found at doi:10.1371/journal.pgen.0030162.st004 (53 KB XLS).

Table S5. QTLs for Aliphatic Glucosinolate Summation Traits

QTLs detected for each aliphatic summation glucosinolate trait are presented as a separate table. The average trait value per RIL across experiments along with the trait value per RIL for each individual experiment was used for QTL mapping; the presence of a QTL in a specific experiment is shown under “QTL Detection in Given Experiment.” The MT QTL column shows the complete results from the multitrait composite interval mapping within QTL Cartographer. M means that the QTL was identified as a main effect, while I means that it had an experiment specific interaction, and MI shows that it was found with both main and interaction effects using multitrait composite interval mapping. The data from each experiment were
used to test each QTL using the nearest marker as main effect, with significance determined by this ANOVA presented under the “Main Effect” column. For each marker/QTL, the same model tested for a QTL × Experiment interaction, “Exp Interaction.” This model also tested all pairwise marker × marker interactions for evidence of epistasis. The significance of each pairwise test is presented under the columns marked “Pairwise assessment of Epistasis.” Finally, the model estimated the allele substitution effect of each locus. For all cells, NS represents nonsignificance within the model. Found at doi:10.1371/journal.pgen.0030162.st005 (50 KB XLS).

Table S6. eQTL for Glucosinolate Biosynthetic Genes

Significant eQTLs previously detected for each glucosinolate biosynthetic gene. The position in chromosome and cM is presented along with the genes’ identities. In addition, the additive effect of the Bay-0 allele at each eQTL is provided. Found at doi:10.1371/journal.pgen.0030162.st007 (27 KB XLS).

Table S7. Average Gene Network Expression (Z-Score)

The mean z-score for each RIL for the aliphatic glucosinolate and indolic glucosinolate biosynthetic pathways are presented for the 211 RILs where there are available data. Found at doi:10.1371/journal.pgen.0030162.st007 (27 KB XLS).

Table S8. Metabolic QTLs for Indolic Glucosinolate Traits

QTLs detected for each indolic glucosinolate trait are presented as a separate table. The average trait value per RIL across experiments along with the trait value per RIL for each individual experiment was used for QTL mapping, and the presence of a QTL in a specific experiment is shown under “QTL Detection in Given Experiment.” The 3D eQTL Cartograph shows the complete results from the multivariate composite interval mapping within QTICartographer. M means that the QTL was identified as a main effect, while I means that it had an experiment specific interaction, and MI shows that it was found with both main and interaction effects using multivariate composite interval mapping. The data from each experiment were used within an ANOVA to test each QTL using the nearest marker as main effect; significance is presented under the “Main Effect” column. For each marker/QTL, the same model tested for a QTL × Experiment interaction, “Exp Interaction.” Finally, the same model tested all pairwise marker × marker interactions for evidence of epistasis. The significance of each pairwise test is presented under the columns marked “Pairwise assessment of Epistasis.” Finally, the model was used to estimate the allele substitution effect of each locus as shown. For all cells, NS represents nonsignificance within the model. Found at doi:10.1371/journal.pgen.0030162.st008 (32 KB XLS).

Accession Numbers

The microarray dataset used in this study has been deposited at European Bioinformatics Institute ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under numbers E-TABM-125 and E-TABM-224. All gene identifiers are listed in Table S3.

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