The Molecular Basis of Quantitative Genetic Variation in Central and Secondary Metabolism in Arabidopsis

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ABSTRACT

To find the genes controlling quantitative variation, we need model systems where functional information on physiology, development, and gene regulation can guide evolutionary inferences. We mapped quantitative trait loci (QTLs) influencing quantitative levels of enzyme activity in primary and secondary metabolism in Arabidopsis. All 10 enzymes showed highly significant quantitative genetic variation. Strong positive genetic correlations were found among activity levels of 5 glycolytic enzymes, PGI, PGM, GPD, FBP, and G6P, suggesting that enzymes with closely related metabolic functions are coregulated. Significant QTLs were found influencing activity of most enzymes. Some enzyme activity QTLs mapped very close to known enzyme-encoding loci (*e.g.*, hexokinase, PGI, and PGM). A hexokinase QTL is attributable to *cis*-acting regulatory variation at the *AtHXK1* locus or a closely linked regulatory locus, rather than polypeptide sequence differences. We also found a QTL on chromosome *IV* that may be a joint regulator of GPD, PGI, and G6P activity. In addition, a QTL affecting PGM activity maps within 700 kb of the PGM-encoding locus. This QTL is predicted to alter starch biosynthesis by 3.4%, corresponding with theoretical models, suggesting that QTLs reflect pleiotropic effects of mutant alleles.

ONE of the long-standing controversies in population genetics is the nature of the evolutionary forces influencing genetic variation for quantitative traits and enzyme polymorphisms. Such variation could be neutral (Ayala and Hartl 1993; Schaeffer and Miller 1992) or experiencing selection because of deleterious (Nachman *et al.* 1994; Templeton 1996), advantageous (Clark 1994; McDonald and Kreitman 1991; Mitchell-Olds 1996a,b), or stabilizing (Houle *et al.* 1996) effects on fitness. To understand the evolutionary forces influencing quantitative trait variation, we must first identify the actual genes that are responsible for quantitative variation within or among populations.

To find the genes controlling quantitative variation, we need model systems where functional information on physiology, development, and gene regulation can guide evolutionary inferences (Mackay and Fry 1996). Genetic variation in enzyme activity provides such an experimental system. Interpretation of physiological genetic data may be simpler than for other quantitative traits. The genes controlling physiological variation belong to two conceptual categories: "structural genes," such as enzyme-encoding loci and associated proteins, and "regulatory genes," a functionally diverse category of loci that influence expression of structural genes (Holton and Cornish 1995) by a variety of mechanisms, including transcription factors, promoter and enhancer elements, hormone synthesis, protein kinases and phosphatases, and allosteric alteration of enzyme kinetics. Regulatory factors may exert simultaneous coordinated control on several traits; such pleiotropic effects cause a "genetic correlation" between activity levels for several enzymes. Alternatively, when physiological variation is attributable to allelic differences at enzyme-encoding loci, it may be because of coding changes that alter the polypeptide sequence of enzymes or because of *cis*-acting regulatory variation at the enzyme locus.

Several methods can provide information on the genes that influence quantitative trait variation. First, quantitative genetics can estimate patterns of genetic variation and covariation among traits. Second, lines carrying single transposon or T-DNA inserts permit quantification of pleiotropic effects of mutations influencing several traits (Clark *et al.* 1995a; Lyman *et al.* 1996). Third, candidate gene studies focus on loci that are thought to have functionally important effects that may influence quantitative traits (Eanes *et al.* 1996; Lagziel *et al.* 1996; Lai *et al.* 1994; Templeton *et al.* 1987). In many cases, the chief disadvantage of candidate gene analysis is our limited understanding of physiology and development of most quantitative traits. However, other traits, such as growth in mice, are burdened by an intractably large number of candidate genes (Keightley *et al.* 1996). Finally, this article focuses on quantitative trait locus (QTL) mapping, which can provide informa-

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tion on the chromosomal locations of unknown genes that influence quantitative variation.

QTL mapping can characterize effects of completely unknown loci and may identify candidate genes that are worthy of further study (*e.g.*, Long *et al.* 1995). In physiological genetics, a QTL that affects activity of only a single enzyme and maps near the enzyme-encoding locus may be caused by allelic variation at the enzyme locus itself. Alternatively, a QTL that affects several related enzymes in a pathway suggests a regulatory factor that jointly regulates expression of these enzymes. Unlike conventional quantitative genetics, QTL mapping can identify the separate effects of multiple regulatory loci, except in cases of tight linkage. Despite these advantages, QTL mapping rarely provides information on the actual genes that are responsible for quantitative trait variation (Mackay and Fry 1996). This limitation occurs because many unknown genes reside in the intervals between molecular markers (Bevan *et al.* 1998), and because we do not understand physiology and development of most quantitative traits.

In addition, QTL mapping provides an incomplete and possibly biased view of the genes that are responsible for quantitative variation. Most QTL mapping experiments have little power to detect factors with small phenotypic effects (Beavis 1994). Chromosome regions are more likely to be statistically significant when they contain several tightly linked QTLs in coupling phase. Epistatic QTLs are difficult to detect because they require large numbers of statistical tests and stringent significance thresholds. Also, depending on statistical methods, pleiotropic QTLs may be more easy to detect because of their multivariate phenotype. Despite these limitations, QTL mapping is the best method available to begin a search for the genetic basis of quantitative trait variation.

In this article, we use QTL mapping of physiological variation in Arabidopsis to study the molecular basis of quantitative trait variation. *Arabidopsis thaliana* provides a genetically tractable experimental system for studying physiology as a model quantitative trait. Quantitative levels of enzyme activity are genetically variable among ecotypes, and this genetic segregation can be studied in recombinant inbred (RI) lines. Many enzyme-encoding loci are cloned, and their chromosomal locations are identified on linkage and physical maps. We ask (1) Is there genetic variation and covariation for enzyme activity levels in several pathways? (2) Can we identify QTLs that influence enzyme activity levels? (3) Do enzyme activity QTLs correspond to enzyme-encoding or regulatory genes? (4) Are activity differences at enzyme-encoding loci caused by changes in polypeptide sequence?

MATERIALS AND METHODS

We used 94 RI lines from the Columbia × Landsberg *erecta* (Col × Ler) cross (Lister and Dean 1993). F_8 RI seeds were obtained from the Nottingham Arabidopsis Stock Centre. Data on RFLP marker genotypes for 64 molecular markers with approximately even spacing, averaging 9 cM between adjacent markers, were a gift from C. Lister and C. Dean. Plants were grown in potting soil mix with timed-release fertilizer (Osmocote) in 96-celled flats at a density of 507 plants m^{-2} under 14-hr day length from cool white and GrowLux fluorescent bulbs in a controlled environment growth room. Each flat contained one seed from each of 94 RI lines and the Col and Ler parents. We used a randomized complete blocks design with 20 flats (N = 1920 seeds). We planted only a single seed in each cell, so that possible planting errors or contamination could be detected by the presence of more than one seedling per cell. After planting, flats were cold stratified at 4° for 5 days, moved to the growth room, and assayed for glycolytic enzymes 18 days later and for defensive enzymes the next day. One leaf was removed each day for enzyme assays and determination of total protein.

Enzyme activity assays: We measured quantitative levels of enzyme activity for six glycolytic enzymes: glucose-6-phosphate dehydrogenase (GPD), fructose bisphosphatase (FBP), phosphoglucose isomerase (PGI), phosphoglucomutase (PGM), glucose-6-phosphatase (G6P), and hexokinase (HXK). In addition, we assayed activity levels of four enzymes that may be involved in plant defense against insects or pathogens: peroxidase (PER), shikimic dehydrogenase (SDH), myrosinase (MYR), and chitinase (CHI; Broglie *et al.* 1991; Graham and Graham 1991; Keith *et al.* 1991; Mitchell-Olds *et al.* 1996; Mithen *et al.* 1995; White and Antoniw 1991; Ye *et al.* 1990).

Enzymes were assayed using previously published methods modified for use with 96-well microplates and a UV micro plate reader (model 3550-UV; Bio-Rad, Richmond, CA). Leaves were removed and placed in 1.2-ml strip tubes containing cold extraction buffer (50 mm Tris-HCl, pH 7.5) and four steel ball bearings. The tubes were placed in an 8 × 12 microtiter format rack and kept on ice until all tubes were filled. The tubes were securely capped, and the rack of tubes was secured into a paint shaker and shaken for 45 sec. The tubes in racks were centrifuged 10 min at 3300 rpm. Using an octapipet, the supernant was removed to a fresh tube on ice.

All enzymes were assayed using modifications of previously published methods (deVeau *et al.* 1992; Gierow and Jergil 1982; Lee 1982; Rao *et al.* 1990; Wang *et al.* 1991). Interpretation of *in vitro* assays of enzyme activity, such as the V_{max} values reported here, requires several caveats (see discussion). It is possible that assays of enzyme activity in crude homogenates might reflect the presence of interfering molecules (Gillespie 1991). Because of these concerns, we first tested enzyme assays with or without a Sephadex G50 desalting step to remove low–molecular-weight contaminants and endogenous substrates. We found very similar results using both methods, so we do not use desalting for most enzymes in the current experiments. The one exception is MYR, where we do use spin column desalting before the MYR assay to remove endogenous substrate (glucosinolates), ascorbate, and other low–molecular-weight molecules.

The change in absorption caused by the reduction of NADP or the oxidation of NADH was measured spectrophotometrically at 340 nm and 25° unless otherwise noted. After centrifugation, plant extract (200 μl for CHI; 100 μl for GPD, FBP, HXK, SDH, and MYR; 50 μl for G6P, PGI, and PGM; 20 μl for PER; and 10 μl for quantification of total protein) and freshly prepared reaction mix were pipetted into the individual wells of microtiter plates, followed by the addition of substrate in a total volume of 250 μl. The reaction mixtures for the individual assay methods were as follows:

GPD: 50 mm Tris-HCl, pH 7.7, 5 mm $MgCl_2$, and 1 mm

NADP. The reaction was started with the addition of 3 mm G6P.

Fructose 1,6-biphosphatase (FBP): The reaction mixture was as for GPD, except that 3 U/ml GPD and 4 U/ml PGI were also included and the reaction was started with the addition of 1 mm FBP.

PGM: 50 mm Tris-HCl, pH 8.0, 5 mm $MgCl_2$, 1 mm NADP, 3 U/ml GPD, and 0.1 mm glucose 1,6-bisphosphate. The reaction was started with the addition of 1 mm glucose 1-phosphate.

PGI: 100 mm Tris-HCl, pH 8.0, 2 mm $MgCl_2$, 1 mm NADP, 1 U/ml GPD, and 5 mm fructose 6-phosphate was the substrate.

HXK: 100 mm Tris-HCl, pH 8.0, 2 mm $MgCl_2$, 1 mm NADP, 15 U/ml GPD, 20 mm KCl, and 0.5 mm ATP. The reaction was started with the addition of 0.5 mm glucose.

G6P: 12.5 mm sodium cacodylate, 8 U/ml peroxidase, 15 U/ml glucose oxidase, 10 mm phenol, and 0.4 mm 4-aminoantipyrine. The change in absorption was measured at 490 nm after the addition of 5 mm G6P.

Peroxidase activity was measured using a modification of the standard procedure using guaiacol as substrate (Pütter 1974). The reaction mixture contained 80 mm sodium phosphate, pH 6.0, 10 mm hydrogen peroxide, 10 mm guaiacol, and crude extract (usually 20–40 μl) in a total volume of 250 μl. The reaction was started by the addition of the substrate, guaiacol, and the change in OD at 450 nm was measured for 5 min at room temperature.

Balinsky and Davies' (1961) method was modified and used to measure SDH activity. The reaction mixture contained 100 mm Tris HCl, pH 9.0, 1 mm NADP, 2 mm shikimic acid, and crude extract (usually 100–150 μl) in 250 μl total volume. The reaction was started by the addition of the shikimic acid, and the change in OD at 340 nm was measured for 10 min at room temperature.

Myrosinase activity (Siemens and Mitchell-Olds 1997) was determined using crude plant extracts that were first desalted on Sephadex G50. A Sephadex G50 slurry (300 μl) was placed in the wells of 96-well micro plates with membrane bottoms (Loprodyne 3.0 mm Silent Monitor plate; Pall Corp., East Hills, NY) and centrifuged to almost dryness. A total of 100 ml of crude extract was added, eluted by centrifugation (Berry and Kauvar 1993), then again eluted with an additional 25 μl H_2O, giving a final eluted volume of 100 μl. To assay myrosinase activity, 50 ml color reagent (containing 6.5 U/ml glucose oxidase, 0.4 mm 4-aminoantipyrine, 1.25 U/ml peroxidase, and 4.4 mm phenol in 136 mm Imidazole buffer, pH 7.0) and 25 μl 5 mm sinigrin were added to the eluate. The reaction mixture was incubated at room temperature for 40 min. The change in OD at 490 nm was then measured for 20 min.

CHI activity was measured using a modification of Boller and Mauch's (1988) method. Crude plant extract was incubated 24 hr at 37° with 2 mm regenerated chitin (Molano *et al.* 1977) in 20 mm sodium acetate, pH 4.5. Incubations consisted of 200 ml extract + 50 ml chitin suspension in 1.2-ml strip tubes. After incubation, the tubes were centrifuged 15 min at 3300 rpm, and 150 ml of supernant was removed to a fresh 1.2-ml tube containing 15 μl 1.0 m potassium phosphate, pH 7.1, and 10 μl 30 mg/ml cytohelicase (suspended in 10 mm KCl + 1 mm EDTA, pH 6.8). Samples were gently mixed and incubated 2 hr at room temperature. The *N*-acetylglucosamine produced was then determined using the method of Reissig *et al.* (1955). Borate buffer, pH 9.8, was added to a final concentration of 133 mm. After mixing, samples were heated to 105° for 3 min, cooled in ice water, and then brought to room temperature. Freshly diluted *p*-dimethylamino-benzaldehyde (0.5 ml) was then added to each sample. The samples were mixed and 200 ml transferred to the wells of a 96-well plate. Plates were covered and incubated at 37° for a 30 min. OD was measured at 595 nm, and equivalents of *N*-acetylglucosamine were determined using a standard curve after correcting for reagent blanks.

Protein concentration was determined using the method of Bradford (1976). Finally, to control for differences in sample preparation, we transformed activity levels for each enzyme to "specific activity" per unit of total protein in each sample. In the remainder of this article, all references to enzyme activity are for specific activity, which are normalized with respect to the quantity of total protein in each sample.

All reagents and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO), except for the protein assay reagent, which was from Bio-Rad.

Statistical methods: When quantitative traits are measured on RI lines, analysis of variance (ANOVA) can quantify the proportion of total variation that is attributable to genetic differences. This proportion is the broad-sense *heritability*, which ranges from 0 to 1. In addition, allelic differences might have simultaneous effects on several traits because of pleiotropy. For example, some families might have high genetic values for two traits because they carry an allele that upregulates both traits, while other genotypes might have low values of both traits because of pleiotropic effects of this locus. Such genetic effects are quantified by the *genetic correlation*, which ranges from -1 to $+1$. In RI lines, nonzero genetic correlations may reflect pleiotropy, but they could also be caused by linkage disequilibrium (statistical nonindependence) of tightly linked QTLs. Thus, standard quantitative genetics provides information on the amount of genetic variation (heritability) and on shared gene action affecting several traits (genetic correlations).

We tested for genetic differences among RI lines by randomized complete blocks ANOVA using FLAT and LINE as random effects. Because lines were not replicated within flats, it was necessary to assume that the LINE × FLAT interaction was absent. This assumption is reasonable because flats were grown in a small area within a controlled environment growth chamber and rotated during the experiment. Genetic correlations were estimated from the correlation of family means after eliminating a few families that had low sample sizes because of poor germination. For ANOVA and genetic correlations, we used Bonferroni significance thresholds to provide a conservative correction for multiple statistical tests (Rice 1989). With 45 pairwise correlations, a tablewise 5% significance threshold uses $\alpha = 0.05 / 45 = 0.0011$.

QTL effects were estimated and tested by least squares interval mapping (Haley and Knott 1992; Jansen 1993; Zeng 1994) using the family mean for each RI line. Conditional probabilities of QTL genotypes given molecular marker data were calculated using SYSTAT BASIC and analyzed with SYSTAT, assuming doubled recombination fractions and no interference in F8 lines (Mitchell-Olds 1996a). Because maximum likelihood (Paterson *et al.* 1991; Stuber *et al.* 1992) and least-squares (Mitchell-Olds 1996a; Visscher *et al.* 1996) interval mapping may produce artifactual statistical "humps" within the intervals between markers (Mitchell-Olds 1995a), QTLs were judged to be significant only if they exceeded the significance threshold at a molecular marker.

When information was available on map location of enzyme-encoding loci, we first tested for QTLs at the coding loci using *a priori* planned tests, with a sequential Bonferroni correction for *N*, the number of known loci encoding a particular enzyme. Otherwise, we considered two approaches for determining a genomewide 5% significance threshold. We report QTLs exceeding Lander and Botstein's (1989) LOD = 2.3 threshold for five chromosomes and a marker spacing of 9 cM. An LOD of 2.3 is asymptotically equivalent to an *F*-ratio of 10.6 (Haley and Knott 1992). In addition, we conducted Monte Carlo simulations using the RI lines and marker genotypes used in this study, performing 500 simulations with a normally

distributed pseudorandom variable as the quantitative trait. In each simulation, we identified the marker with the largest *F*-ratio, and we established a 5% significance threshold as the *F*-ratio that was exceeded in only 5% of these simulations (Routman and Cheverud 1997). This 5% critical *F*-ratio equals 11.78. Simulations indicate that the suggested critical value of Lander and Botstein corresponds to a *P* value of 8.8%. Thus, although the Lander and Botstein significance threshold is slightly liberal, it provides a fair approximation in this instance. We used a sequential model-building approach: after initial identification of significant QTLs, remaining markers were checked again for additional significant QTLs when added to the existing statistical model. We also tested for epistasis, but pairwise interactions among significant QTLs never approached significance.

DNA sequence comparisons: *AtHXK1* cDNAs have been sequenced in both the Landsberg *erecta* and the Columbia ecotypes that gave rise to this RI mapping population. Landsberg DNA sequence was obtained from GenBank (accession number ATU28214). Columbia cDNA sequence was obtained from a contig of ESTs assembled by The Institute of Genomic Research (web address http://www.tigr.org/tdb/at/atest.html). These two sequences were identical.

RESULTS

Several enzyme-encoding loci were mapped by BLAST homology searches (Recipon *et al.* 1995) to genomic sequences with known map positions. Cytosolic-*PGI* is 23,732 bp from *DFR* on chromosome *5* (P1 clone MJB21, accession number AB007647), and *PGI-b* is on chromosome *4* (accession number Z99708). *G6P* homologues exist on P1 clone MIK22 (accession number AB005236) on chromosome *5* and BAC F3I6 (accession number AC002396) on chromosome *I*. *PGM* is found on chromosome *5* (P1 clone MIO24, accession number AB010074) ~700 kb from marker m435, with another homologue on chromosome *I* (BAC T26J12, accession number AC002311). *GPD* homologues exist on chromosome *1* (BAC F3I6, accession number AC002396, and BAC F14J9, accession number AC003970) and on chromosome *5* (P1 clone MIK22, accession number AB005236). Finally, an *FBP* homologue exists on chromosome *5* (P1 clone MSJ1, accession number AB008268).

Because of incomplete germination, we obtained enzyme activity data from 1097 plants, totaling 13,127 assays for enzyme activity or protein concentration. All enzymes studied showed highly significant quantitative genetic variation in this segregating population (Table 1). Next, we examined patterns of genetic correlations among activity levels of pairs of enzymes (Table 2). Even after a conservative Bonferroni correction for multiple statistical tests, 40% (18 out of 45) of pairwise genetic correlations were significantly different from zero. All significant genetic correlations were positive. This positive genetic correlation cannot result from variation in sample preparation because it reflects characteristics of particular RI genotypes, and because we analyzed specific activity of each enzyme, normalized with respect to the quantity of total protein in each sample. Positive genetic correlations were especially noticeable among activity levels of five glycolytic enzymes, PGI, PGM, GPD, FBP, and G6P. This suggests that enzymes that have closely related metabolic functions are influenced by genetic segregation of loci that jointly regulate this area of metabolism. On the other hand, genetic correlations among defensive enzymes or between glycolytic and defensive enzymes showed fewer significant correlations. This may indicate greater genetic independence among branches of metabolism that have less functional similarity.

TABLE 1

ANOVA for each trait

Enzyme	*F*-ratio	R^2
PGI	2.10	0.55
PGM	5.14	0.46
GPD	4.86	0.53
FBP	3.66	0.43
G6P	2.64	0.65
HXK	1.59	0.34
PER	2.13	0.27
SDH	7.38	0.47
MYR	3.52	0.34
CHI	2.30	0.25

Table shows *F*-ratios resulting from genetic variation among RI lines and total R^2 for each trait from ANOVAS containing main effects resulting from flat and RI lines. All $N \geq 1076$; d.f. $\geq 95, 961$. All $P < 0.00001$, except HXK, $P = 0.00053$.

Of 10 enzymes studied, we mapped significant QTLs affecting the activity of 7 enzymes (Table 3). Most of the QTLs had significant effects on only a single enzyme. There was no hint of epistasis among these QTLs. Individual QTLs explained up to 26% of the genetic variation. Taken together, QTLs explain up to 48% of the genetic variation for activity of a given enzyme. In some cases, enzyme activity QTLs mapped very close to known enzyme-encoding loci (*e.g.*, *AtHXK1*, *PGI-b*, and *PGM*). Alternatively, some known enzyme-encoding loci did not have significant effects on enzyme activity (*e.g.*, *G6P*, *PGI-c*, and *CHI*). This is not surprising, given the low rate of nucleotide polymorphism in Arabidopsis. In some instances, information on map location of coding loci was insufficient to determine whether some QTLs correspond to enzyme-encoding or regulatory loci. Fortunately, this is changing rapidly. Finally, there was no hint of statistically significant epistasis among QTLs (not shown).

We also asked whether the magnitude of estimated QTL effects differed between enzymes of primary and secondary metabolism. Seven glycolytic QTL had an average activity difference of 9.3%, while four QTLs influencing enzymes of secondary metabolism caused average effects of 25.5% (standard errors 2.6 and 3.5%, respectively; Table 3). The magnitude of QTLs affecting secondary metabolism is significantly larger ($F = 13.70$;

TABLE 2

Genetic correlations among enzyme activity levels

	PGI	PGM	GPD	FBP	G6P	HXK	PER	SDH	MYR
PGM	**0.39**								
GPD	**0.57**	**0.63**							
FBP	**0.36**	**0.53**	**0.52**						
G6P	**0.42**	0.20	**0.50**	**0.38**					
HXK	0.08	−0.21	−0.12	0.08	0.24				
PER	0.32	0.07	0.18	0.13	0.32	**0.39**			
SDH	0.34	**0.39**	**0.49**	**0.69**	0.18	0.04	0.23		
MYR	**0.45**	0.24	**0.43**	0.28	0.20	0.01	0.34	**0.35**	
CHI	0.23	0.07	0.11	−0.11	0.21	**0.36**	**0.59**	−0.10	0.20

Genetic correlations among enzyme activity levels, estimated from correlations of family means of RI lines. $N = 83$. Statistically significant correlations are shown in boldface, using Bonferroni correction for multiple tests.

d.f. = 1, 9; $P = 0.005$ by ANOVA). The generality of this result should be interpreted with caution, however, because we found only significant QTL for two enzymes of secondary metabolism, MYR and SDH.

TABLE 3

Enzyme activity QTL

Enzyme	Marker	Location	*F*-ratio	Difference (%)
PGM	M435	V:112	**22.66**	16.2
GPD	G3845	IV:56	**22.07**	15.0
PGI	M488	I:6	**12.15**	3.6
	G3845	IV:56	10.77	3.3
G6P	G3845	IV:56	**26.19**	8.4
	G4715B	V:60	11.40	−5.9
	MI291B	V:74	**42.33**	12.5
HXK	G3088	IV:80	4.93	−5.9
MYR	G3786	I:19	**29.79**	21.1
	MI358	III:50	**21.52**	21.8
SDH	G4026	I:85	**18.52**	38.3
	G4532	II:20	10.97	−13.7

For each trait, this table shows the most significant molecular markers, their locations, the *F*-ratios attributable to each marker, and the percentage difference in enzyme activity between QTL homozygotes carrying Columbia or Landsberg alleles. Positive difference values indicate higher activity for the Columbia allele. QTL for HXK and PGI activity are significant based on *a priori* planned tests at the known enzyme-encoding loci using sequential Bonferroni significance thresholds (*AtHXK1* near marker G3088, $P = 0.029$; *PGI-b* near marker G3845, $P = 0.0015$). *F*-ratios shown in boldface exceed the genome-wide 5% significance threshold determined by Monte Carlo simulation. Remaining markers exceed the approximate threshold of Lander and Botstein (1989) and should be regarded as marginally significant. Two potential G6P QTLs at V:60 and V:74 are reported because, even though they are in close proximity, they have effects of opposite sign. No statistically significant QTL were found for FBP, PER, or CHI. All d.f. $\geq 1, 73$. Total R^2 attributable to molecular markers for enzymes with significant QTL:PGM, 0.23; GPD, 0.23; PGI, 0.24; G6P, 0.48; HXK, 0.06; MYR, 0.39; SDH, 0.32. Individual QTL explained up to 26% of the genetic variation.

DISCUSSION

Despite the importance of quantitative genetic variation in many areas of biology (Mitchell-Olds 1995b), we have little understanding of the molecular basis of genetic polymorphisms that control this variation. To find the genes that actually control variation in quantitative traits, we must begin with well-known physiological and developmental pathways in genetically tractable model organisms (*e.g.*, Cheverud *et al.* 1996; Long *et al.* 1995; Shook *et al.* 1996). Although mutant screens and transgenic studies of Arabidopsis (Meyerowitz and Somerville 1994) provide extensive information on the genes that *can* influence phenotype when manipulated experimentally, QTL-mapping studies address a different question: Which polymorphic loci *actually do* influence naturally occurring phenotypic variation among ecotypes? Although these natural polymorphisms provide the foundation for evolutionary changes in plant breeding and wild species, we have little understanding of the molecular basis of quantitative trait variation. To address this question, we examined quantitative genetic variation in levels of enzyme activity for six glycolytic and four defensive enzymes in a recombinant inbred population from a cross between Arabidopsis ecotypes.

Some QTLs provide suggestive evidence regarding the causes of quantitative genetic variation. For example, HXK is encoded by two loci in Arabidopsis (Jang *et al.* 1997). A QTL influencing levels of HXK activity maps very near to one of these loci, *AtHXK1*, at 80 cM on chromosome *IV*. This suggests that activity variation may be attributable to allelic differences at the *AtHXK1* locus. If so, activity variation could result from coding changes that alter the polypeptide sequence of this hexokinase locus or from *cis*-acting regulatory variation at the *AtHXK1* locus. We can distinguish between these alternatives because *AtHXK1* cDNA sequences are available from both Landsberg *erecta* and Columbia parents (see materials and methods). Both ecotypes have identical cDNA sequences, so polypeptide sequence dif-

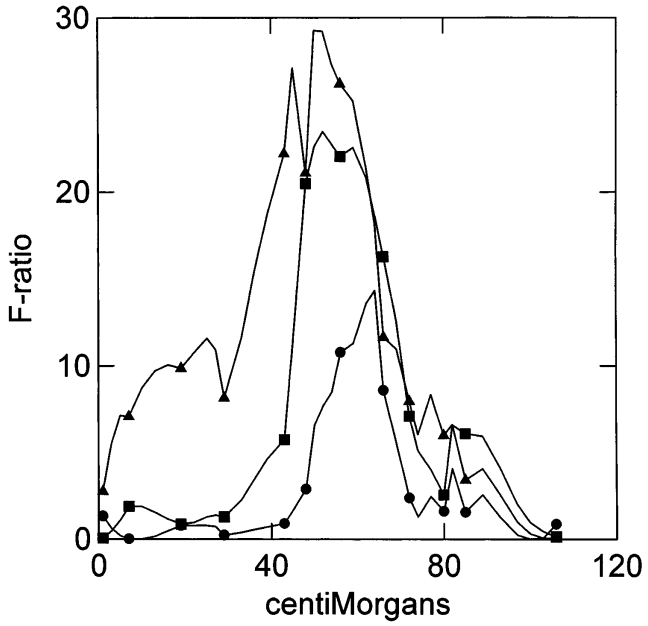

Figure 1.—Evidence for a QTL on chromosome *IV* that jointly regulates activity of PGI, GPD, and G6P. *F*-ratios are from least-squares interval mapping. Test statistics assume that PGI has an additional QTL at m488 on chromosome *I*, and G6P has two additional QTL at G4715B and MI291B on chromosome *V*. Symbols indicate marker locations. PGI, GPD, and G6P are shown with circles, squares, and triangles, respectively.

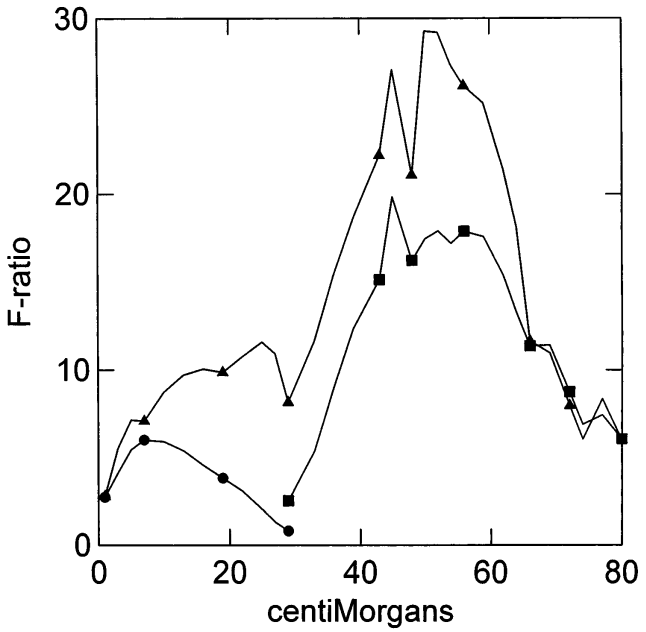

Figure 2.—G6P activity may be influenced by two QTL on chromosome *IV*. The top line shows test statistics for a single QTL on chromosome *IV* (triangles). Alternatively, the lower lines assume that there is a known QTL on chromosome *IV* and test for the existence of a second QTL nearby. Evidence for a QTL on the left end of chromosome *IV* is indicated with circles, assuming that another QTL is located at 56 cM. Alternatively, evidence for a QTL near the middle of chromosome *IV* is indicated with squares, assuming that another QTL is located at 19 cM. All test statistics assume that G6P is influenced by additional QTL at G4715B and MI291B on chromosome *V*.

ferences at this HXK-encoding locus cannot be responsible for this enzyme activity QTL. Consequently, this QTL must be caused by *cis*-acting regulatory variation at the *AtHXK1* locus or by a closely linked regulatory locus.

Functional studies of HXK-encoding loci provide an additional perspective on the molecular basis of quantitative trait variation. Jang *et al.* studied transgenic plants over- and underexpressing *AtHXK* and found alterations in flowering time, an important life history trait in Arabidopsis (Mitchell-Olds 1996a). Similarly, *CAM1* and *PGM* mutants alter both starch accumulation and age at first flowering (Caspar 1994; Eimert *et al.* 1995). This suggests that pleiotropy may have an important influence on the maintenance of quantitative variation in natural populations (Caballero and Keightley 1994) and that the number of QTLs affecting some traits may be very large. Furthermore, if central metabolism often influences expression of quantitative trait variation, then candidate gene approaches must be closely integrated with QTL mapping to narrow the list of candidate genes to a tractable number of loci that reside within chromosomal regions that have been identified by QTL analyses.

We also found one QTL at 56 cM on chromosome *IV* that may be a joint regulator of GPD, PGI, and G6P activity (Figure 1). This chromosome region has positively correlated effects on activity of each of these enzymes. In QTL mapping, it is extremely difficult to exclude the possibility that a chromosome region with effects on multiple traits might be caused by several tightly linked loci (Mackay and Fry 1994; Zeng 1994), each affecting only a single trait. These positively correlated effects fit the pattern expected from a regulatory locus exerting coordinated control on several enzymes. However, it is also plausible that this region contains several tightly linked loci with physiologically independent effects on glycolytic metabolism because the *PGI-b* enzyme–encoding locus has been identified in this chromosomal region (GenBank accession number Z97337). Physiological studies in Drosophila using genetic correlations, mutation accumulation lines, and insertional mutagenesis (Clark 1990; Clark *et al.* 1995a,b) also suggest an important role for regulatory variation in the evolution of metabolism. A positive genetic correlation between GPD and G6P activity is consistently observed in *Drosophila melanogaster* (Clark and Keith 1988; Clark *et al.* 1995b; Miyashita and Laurie-Ahlberg 1984). Clearly, however, there is also substantial genetic variation that could support independent evolution of individual enzyme activities (Table 1).

G6P activity may be influenced by two QTLs on chromosome IV, separated by ~35 cM (Figure 2). This possi-

bility was analyzed by least squares interval mapping, controlling for additional QTLs on chromosome *V*. Even controlling for a possible factor near 19 cM, there is strong evidence for a QTL influencing G6P near 56 cM on chromosome *IV*. Alternatively, controlling for a factor near 56 cM, there is suggestive evidence for a second factor near 19 cM. (However, this possible QTL does not exceed the significance thresholds used in Table 3). Further experimental work would be necessary to validate that QTL. Fortunately, the current experimental results provide an *a priori* prediction that could be tested on new segregating lines scored for only a few markers.

We identified a QTL affecting PGM activity near marker m435 on chromosome V, which is ~700 kb from the PGM-encoding locus (GenBank accession number AB010074). Previous work by Neuhaus and Stitt (1990) allow us to estimate the effects of this QTL on flux through the starch biosynthetic pathway. The flux control coefficient *Z* (Keightley and Kacser 1987) quantifies the relationship between activity of a particular enzyme and flux through a pathway. With simple models of metabolism, the flux-control coefficients for a pathway sum to 1.0. Under light-saturating conditions, the control coefficient for PGM on starch biosynthesis is 0.21 (Neuhaus and Stitt 1990) in wild-type plants. However, PGM activity strongly limits starch biosynthesis ($Z \sim 1.0$) in heterozygous mutant plants with only a single functional *PGM* allele. In the Col × Ler cross, *PGM* homozygotes for the Col and Ler alleles differ by ~16% in PGM activity, so this QTL is predicted to alter starch biosynthesis by 3.4%. Therefore, because the effects of this QTL are large for one trait (PGM activity) and small for another (flux), the QTL corresponds with theoretical models suggesting that many QTLs reflect pleiotropic effects of mutant alleles (Kondrashov and Turelli 1992).

On average, QTLs influencing enzymes of secondary metabolism had effects more than twice as large as QTLs affecting primary metabolism. Only a small number of enzymes and QTLs were available for this comparison, so the generality of this result should be interpreted with caution. Nevertheless, this trend conforms with physiological and evolutionary intuition. In many cases, enzymes of secondary metabolism may be under weaker selection or experience geographically heterogeneous selection pressures (Thompson 1994) in comparison to enzymes of primary metabolism, which may be essential for growth and survival (*e.g.*, Neuhaus and Stitt 1990).

Interpretation of *in vitro* assays of enzyme activity requires several caveats (Gillespie 1991). V_{max} may provide an imperfect approximation of actual enzyme activity under physiological conditions. Large differences in enzyme activity may have small effects on fitness or metabolic flux (Dykhuizen *et al.* 1987). Metabolic control theory (Kacser and Burns 1981; Keightley and Kacser 1987) proposes that flux through a pathway may be poorly correlated with activity of particular enzymes. Furthermore, estimates of control coefficients are specific to particular physiological and genetic environments, and they may be altered in different environmental conditions or in some mutant backgrounds (Kruckeberg *et al.* 1989; Neuhaus and Stitt 1990).

Despite these caveats, physiological population genetics can make important contributions to understanding the molecular basis of quantitative variation (Clark *et al.* 1995a,b; Stam and Laurie 1996). Transient activity of individual enzymes may be very important when physiological fluctuations result from environmental variation (Gillespie 1991). For example, control coefficients of glycolytic enzymes change depending on light intensity (Kruckeberg *et al.* 1989), which is highly variable in the field. In addition, genetic experiments have demonstrated that *in vitro* assays can bring functional interpretation and genetic prediction to quantitative genetics. Using the methods used here, we have shown that selection on physiology can cause genetically correlated changes in ecologically important traits and vice versa (Mitchell-Olds *et al.* 1996; Siemens and Mitchell-Olds 1998). For these reasons, population studies of physiological variation can provide useful evolutionary information.

How well have we succeeded in understanding the molecular basis of quantitative variation in a model system with known physiological pathways in a genetically tractable organism? We found extensive genetic variation for each physiological trait, as well as positive genetic correlations, suggesting that enzymes with closely related metabolic functions are influenced by loci that jointly regulate a pathway (questions 1 and 2 posed in the Introduction). We mapped one QTL with positively correlated effects on activity of GPD, PGI, and G6P, which may represent a joint regulator of these enzymes. Another QTL influencing HXK enzyme activity mapped near the *AtHXK1* enzyme-encoding locus. Because *AtHXK1* cDNA sequences are identical in Ler and Col, this HXK activity QTL is attributable to linked regulatory variants rather than to coding changes (partial information regarding questions 3 and 4 posed in the Introduction).

However, even in this experimentally tractable model system, these conclusions are limited by our ignorance regarding the actual genes that reside on chromosome intervals and by our incomplete understanding of gene regulation. Mackay and Fry (1996) discuss experimental approaches required to identify the actual loci responsible for quantitative trait variation. Improved functional information on gene regulation and availability of genomic sequence will facilitate these efforts. For example, an Arabidopsis population of 1000 F_2 plants (representing 2000 independent gametes) would include 20 recombination events per centimorgan. In such a population, the average interval between adjacent recombination points would be <10 kb and would

typically contain only two loci (Bevan *et al.* 1998). As genomic sequence becomes available in Arabidopsis, it will become feasible to identify the actual loci responsible for quantitative trait variation.

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