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A Combined-Cross Analysis Reveals Genes With Drug-Specific and Background-Dependent Effects on Drug Sensitivity in *Saccharomyces cerevisiae*

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ABSTRACT

Effective pharmacological therapy is often inhibited by variable drug responses and adverse drug reactions. Dissecting the molecular basis of different drug responses is difficult due to complex interactions involving multiple genes, pathways, and cellular processes. We previously found a single nucleotide polymorphism within cystathionine β-synthase (*CYS4*) that causes multi-drug sensitivity in a vineyard strain of *Saccharomyces cerevisiae*. However, not all variation was accounted for by *CYS4*. To identify additional genes influencing drug sensitivity, we used *CYS4* as a covariate and conducted both single- and combined-cross linkage mapping. After eliminating numerous false-positive associations, we identified 16 drug-sensitivity loci, only 3 of which had been previously identified. Of 4 drug-sensitivity loci selected for validation, 2 showed replicated associations in independent crosses, and two quantitative trait genes within these regions, *AQY1* and *MKT1*, were found to have drug-specific and background-dependent effects. Our results suggest that drug response may often depend on interactions between genes with multi-drug and drug-specific effects.

RESPONSE to pharmacological therapy varies and is often highly heritable (Evans and Johnson 2001; Evans and McLeod 2003; Ingelman-Sundberg *et al.* 2007). Variable drug responses make it difficult to achieve optimal dosing and frequently result in adverse drug reaction, a major cause of death in hospitalized patients (Lazarou *et al.* 1998). In addition to impacting drug therapy, adverse drug reactions can limit or even eliminate the use of a drug (Shah 2006). Consequently, understanding the genetic basis of variable drug responses is important to both mitigating adverse drug reactions and developing new or improved pharmacological therapies. Although many pharmacogenetic variants have been identified from surveys of candidate genes and pathways (Katz and Bhathena 2009), there have been only a few studies that have conducted genomewide mapping (Dolan *et al.* 2004; Watters *et al.* 2004; Perlstein *et al.* 2006; Duan *et al.* 2007; Huang *et al.* 2007; Kim and Fay 2007; Perlstein *et al.* 2007; Bleibel *et al.* 2009; Shukla *et al.* 2009), and many of these have focused on chemotherapy-induced cytotoxicity in human lymphoblastoid cell lines, which in some instances may be susceptible to false-positive associations due to low repeatability (Choy *et al.* 2008). Furthermore, identification of individual genes and their causal variants in human cell lines is a significant challenge. Thus, there is still an incomplete picture of the genes, pathways, and processes responsible for both pharmacokinetic (absorption, distribution, metabolism, and excretion of a drug) and pharmacodynamic (physiological or biochemical effect of a drug) variation.

Saccharomyces cerevisiae has proved to be a useful system for pharmacological research. The yeast deletion collection has been used to identify a compound's mechanism of action as well as its indirect effects on basic biological processes (Baetz *et al.* 2004; Giaever *et al.* 2004; Lum *et al.* 2004). Many yeast genes that function in detoxification of xenobiotic compounds through drug transport and metabolism have been identified (Balzi and Goffeau 1995; Decottignies and Goffeau 1997; Wolfger *et al.* 2004; Moye-Rowley 2005; Barreto *et al.* 2006). In addition, many yeast genes that function in pleiotropic drug resistance are homologous to human genes involved in multi-drug resistance to chemotherapy (Kuchler and Thorner 1992; Wolfger *et al.* 2001; Gottesman *et al.* 2002). However, genes responsible for population genetic variation may be different from those identified through mutant screens since naturally occurring alleles may be neomorphic or have effects that are small or dependent on genetic background. Furthermore,

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many drug-sensitive phenotypes may result from the combined effects of multiple genes that show very small or no effects by themselves.

Linkage mapping has generated significant insight into the genetic architecture and molecular basis of variable drug responses between different yeast strains. Two recent studies examined growth differences in the presence of 31 and 104 different compounds and found that drug sensitivity was often due to the combined effects of drug-specific as well as multi-drug-sensitive quantitative trait loci (QTL; Kim and Fay 2007; Perlstein *et al.* 2007). In addition to known mutations segregating at *HO*, *URA3*, *HAP1*, and *LEU2*, the two studies each identified a major-effect gene causing multi-drug sensitivity. Perlstein *et al.* (2007) found a nonsynonymous polymorphism within *PHO84*, an inorganic phosphate transporter, that caused sensitivity to 25/104 compounds. *PHO84* is a member of the major facilitator superfamily of transporters, which includes human genes in the solute carrier family 22 (*SLC22*) that are important for hepatic and renal excretion of cationic drugs (Koepsell 2004). Kim and Fay (2007) found a nonsynonymous polymorphism within *CYS4*, an enzyme in the cysteine biosynthesis pathway that is required for glutathione biosynthesis. Attachment of glutathione to a drug is one of the major mechanisms by which cells detoxify xenobiotic compounds (Hayes *et al.* 2005). Thus, both genes affect the pharmacokinetic response to multiple drugs.

QTL with small and/or drug-specific effects also contribute to variable drug responses (Kim and Fay 2007; Perlstein *et al.* 2007). However, identification of small-effect genes can be complicated by the simultaneous segregation of other QTL, particularly those of large effect. Studies of other quantitative traits have shown that the effects of a QTL can be small in isolation but much larger in combination with other segregating QTL (*e.g.*, Steinmetz *et al.* 2002; Deutschbauer and Davis 2005; Gerke *et al.* 2009). Thus, identification of small-effect QTL may depend on accounting for interactions with those of large effect.

One approach to identifying small or background-dependent QTL is to generate recombinants that are fixed for the major QTL through backcrosses or introgression (*e.g.*, Sinha *et al.* 2008). An alternative approach, and the one implemented here, is to identify associations after statistically removing the effects of the major QTL (*e.g.*, Brem *et al.* 2005). To map genes affecting drug sensitivity while controlling for the large effects of a multi-drug-sensitive allele of *CYS4*, we conducted both single- and combined-cross linkage scans using *CYS4* as a covariate. After eliminating many false-positive associations, we identified two genes, *AQY1* and *MKT1*, that show drug-specific and background-dependent effects. Our results show how drug sensitivity can be mediated by a combination of genes with multi-drug and drug-specific effects.

MATERIALS AND METHODS

Strains, media, drugs, genotyping, and phenotyping: Previously collected genotype and phenotype data are described in Kim and Fay (2007). M22 and YPS163 are homothallic diploids derived from monosporic clones isolated from a vineyard in Italy and an oak tree in Pennsylvania, respectively, and S288c is a diploid laboratory strain (*ho/ho, ura3*Δ *EcoRV-Stu1/ura3-52*).

In this study, an independent set of new recombinant strains was generated using three diploid hybrid strains—MY, MS, and YS—generated by mating M22 (M), YPS163 (Y), and S288c (S) to one another by tetrad dissection, pairing spores, and selecting diploids by observation of shmooing. Hybrid strains were sporulated, tetrads were dissected, and a total of 80, 76, and 87 recombinant strains, each from a single spore of a different tetrad, were obtained from the MY, MS, and YS hybrids, respectively.

Strains were grown on rich medium (2% yeast extract, 1% peptone, 2% dextrose) and drug sensitivity was measured in rich medium by comparison of growth in the presence and absence of each drug. Strains were grown overnight, diluted in rich medium, grown for 2 hr, treated with either water or drug, and then grown for 20 hr in an iEMS incubator (30°), shaker (1200 rpm), and plate reader (Labsystems, Helsinki, Finland). OD_{600} was used to quantify cell density every 2 min, and drug resistance was measured by the delay in growth caused by the drug relative to water treatment. The growth delay was measured by the difference in the time point at which maximum growth rate was observed while controlling for initial cell density (Kim and Fay 2007). Final drug concentrations were 2 mm for idazoxan hydrochloride (Drug 9), dipropyldopamine (Drug 12), phenylephrine (Drug 22), and aminoguanidine (Drug 27) and 12.5 μm for palmitoyl-dl-carnitine (Drug 31).

QTL mapping: Genome linkage scans were conducted using the Haley–Knott regression algorithm implemented in the statistical software package, R/QTL (Broman *et al.* 2003). Both single-cross and combined-cross scans were conducted. The single-cross scans were based on recombinants from the MY, MS, and YS crosses, and the combined-cross scans were based on combinations of the single crosses MY–MS, MS–YS, and MY–YS, by assuming Y = S, M = Y, and M = S for the three combined crosses, respectively. The phenotypic variance was normalized from each cross separately before merging. The regression model for a single cross was $y_i = \beta_0 + \beta_1 Q_i + \varepsilon_i$, where i is the strain, β_0 and β_1 are regression coefficients, and Q_i is the QTL genotype. The regression model for a combined-cross analysis was $y_i = \beta_0 + \beta_1 Q_i + \beta_2 C_i + \beta_3 Q_i C_i + \varepsilon_i$, where C_i is an indicator for the cross origin. In each case in which *CYS4* showed significant linkage, a second linkage scan was conducted using the genotype of the *CYS4* causal polymorphism as a covariate. The regression model for a single-cross analysis was $y_i = \beta_0 + \beta_1 Q_i + \beta_2 CYS4_i + \beta_3 Q_i CYS4_i + \varepsilon_i$, where $CYS4_i$ is the genotype of the *CYS4* causal polymorphism. Significant log odds ratio (LOD) scores were determined using the false discovery rate (FDR) from permutations of 1000 shuffled phenotypes. For models with *CYS4* as a covariate, we conducted conditional permutations by including intact *CYS4* genotypes during permutation. The FDR for a given LOD cutoff was estimated by the number of QTL from the shuffled data divided by the number from the real data.

QTL interactions and cross-specific QTL: Significant interactions between a QTL and *CYS4* were obtained using a two-QTL regression model based on *CYS4* and imputed QTL genotypes as implemented in the fitqtl module of R/QTL (Broman *et al.* 2003). For the 10 QTL identified in a single cross (Table 2), we analyzed pairs of crosses together to test

whether the effects of a QTL were cross-specific. For a QTL identified in a single cross, we tested two sets of combined crosses that included the cross in which the QTL was originally identified. Cross-specific QTL were identified by a significant interaction (likelihood-ratio test, $P < 0.05$) between the effects of a QTL and the cross in which it was segregating for both combined crosses. QTL that showed cross-specific interactions within one or neither of the combined crosses were not considered cross-specific.

QTL validation: QTL were validated in an independent set of 80, 76, and 87 segregants from the MY, MS, and YS crosses, respectively. Segregants were genotyped at the four candidate QTL [chromosome 4 (893,678 bp), chromosome 12 (766,637 bp), chromosome 14 (531,242 bp), and chromosome 16 (894,088 bp)] by sequencing. Sensitivity to Drug 9 and Drug 27 was measured in the MY segregants for validation of the chromosome 4 and chromosome 16 QTL. Sensitivity to Drug 12 and Drug 31 was measured in the MY–SY segregants for validation of the chromosome 12 and chromosome 14 QTL. Significant associations between genotypes and phenotypes were tested by analysis of variance (ANOVA; $P < 0.05$) using the *CYS4* genotype as a covariate.

Reciprocal hemizygosity test: Two or more independent deletions of *MKT1* and *AQY1* were generated within YPS163 (*MAT*α, *trp1*Δ*::hghMX*) and BY4741 (an S288c derivative with genotype *MAT***a**, *ura3*Δ, *met15*Δ, *his3*Δ, *leu2*Δ) using a *kanMX* deletion cassette (Wach *et al.* 1994). Hemizygotes (163/Δ, Δ/4741, and 163/288, where 163 refers to the YPS163 background and 288 refers to the S288c background) for *MKT1* and *AQY1* were generated by mating on rich media followed by selection of diploids on complete dropout media without trytophan, uracil, histidine, and leucine. Drug sensitivity was measured as described above except that Drugs 12, 22, and 27 were used at a final concentration of 4 mM, and for *AQY1*, 2 or 6 mM propargylglycine (PPG) was supplemented to rich medium to phenocopy the effects of *CYS4* (Kim *et al.* 2009). Significant differences between reciprocal hemizygotes were tested by a *t*-test for *MKT1* and by ANOVA for *AQY1* to account for the different concentrations of PPG.

RESULTS

QTL mapping: In a previous study (Kim and Fay 2007), we examined the genetic basis of 31 variable drug responses using three sets of 45 recombinant strains (Figure 1A) generated from each pairwise cross of three strains of *S. cerevisiae*: M22 (vineyard isolate), YPS163 (oak tree isolate), and S288c (laboratory strain). Linkage analysis of 198 markers was used to map QTL for sensitivity to each drug, measured by the drug-dependent delay in growth from nearly continuous measurements of cell density in the presence and absence of each drug. A total of 56 QTL were identified at an FDR of 1% from linkage analysis of each drug in each cross separately. The 56 QTL represent 8 unique loci after QTL for different drugs or crosses were combined if they were within the same marker interval or within 15 cM of one another. We previously showed that *CYS4*-I123N underlies one of these QTL and causes a large-effect, multi-drug-sensitive phenotype for 25 of the 31 drugs (Kim and Fay 2007). With the exception of the QTL corresponding to *CYS4*, the 7 remaining QTL affect sensitivity to only one or a small number of drugs.

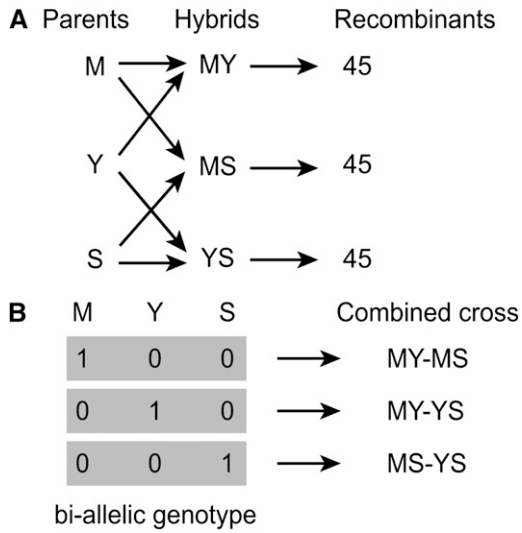

Figure 1.—Schematic of the three-way cross and combined-cross analysis. (A) Three parental strains, M22 (M), YPS163 (Y), and S288c (S), were used to generate three hybrids and 45 homozygous recombinant strains for each cross. (B) Combined-cross analysis was conducted assuming biallelic genotypes for each pair of crosses.

To identify QTL underlying drug sensitivity while controlling for the effects of *CYS4*, we conducted linkage analysis using the genotype of the *CYS4* causal polymorphism as a covariate. The inclusion of *CYS4* as a control variable removes any phenotypic differences that can be attributed to *CYS4* such that any additional factors that are found have at least some effect that is independent of *CYS4*. After combining QTL from different drugs or crosses into unique loci, 15 QTL were identified at a 5% FDR (Table 1). Of these 15 QTL, 11 were new QTL, and 2 of the 8 original QTL, chromosome 4 (893,678 bp) and chromosome 11 (634,178 bp), were no longer significant (supporting information, Table S1). This result raises the possibility that some of the previously identified QTL were false positives. However, the lack of overlap between the two sets of QTL could also be the result of lower power due to the small number of recombinant strains.

In a three-way cross design, each QTL is expected to segregate in two of the three crosses. However, many of the QTL were identified in only a single cross. Only 4 of the 19 QTL identified with or without *CYS4* as a covariate were identified independently in two crosses. Interestingly, 3 of these correspond to loci with known mutations: *CYS4*, *HO*, *URA3*. *HO*, and *URA3* are deleted in S288c and were identified as QTL in both crosses involving S288c. QTL that were identified in only one of the three crosses could be the result of false negatives due to low power or could be the result of epistasis. A QTL may show cross-specific effects if there are pairwise epistatic interactions with another QTL that is segregating in only one of the two relevant crosses or, more generally, if the effects of a QTL are dependent on

TABLE 1

QTL identified by four linkage models

Model	Covariate		
	None	*CYS4*	Combined[a]
Single cross[b]	0	6	6
Combined cross[b]	2	17	17
Both models[c]	8	9	12
Total	10	32	35

[a] Total number of unique QTL identified with or without *CYS4* as a covariate.

[b] QTL identified only in the single-cross or only in the combined-cross model; see MATERIALS AND METHODS for model descriptions.

[c] QTL identified by both the single- and the combined-cross models.

multiple loci in the genetic background. For example, a QTL caused by an allele of M22 that shows an interaction with a QTL caused by an allele of S288c may be detected only in the M22–S288c or the M22–YPS163 cross, depending on whether the interaction makes the QTL more or less easy to identify.

A combined-cross analysis has increased power over a single-cross analysis due to its larger sample size and makes it possible to explicitly test for QTL with cross-specific effects (LI *et al.* 2005). A combined-cross analysis was conducted on all three pairs of crosses: MY–MS, MY–YS, and MS–YS, where M, Y, and S represent the M22, YPS163, and S288c parental strains. To conduct a linkage scan using data from two crosses, we normalized each set of phenotypes and recoded the genotypes to be biallelic. For example, the combined data from the M22–YPS163 (MY) and M22–S288c (MS) crosses were recoded such that $Y = S$ to identify QTL caused by M22-specific alleles (Figure 1B). Using *CYS4* as a covariate, the combined-cross analysis identified 26 unique QTL. The 26 QTL include 9 of the 15 QTL identified in the single-cross analysis as well as another 17 new QTL (Table 1). Together, the four QTL mapping models identified 35 unique QTL on the basis of 31 drug traits (Table 1 and Table S1).

QTL validation: Four QTL were selected for validation using a set of 80, 76, and 87 new recombinant strains derived from the MY, MS, and YS crosses, respectively. *CYS4* was genotyped as a positive control and for the covariate analysis. Of the four QTL selected for validation, chromosome 4 (893,678 bp) was identified in both the single- and combined-cross analysis but was not found to be significant in any of the models that used *CYS4* as a covariate; chromosome 12 (766,637 bp) was identified in only the combined cross using *CYS4* as a covariate; and chromosome 14 (531,242 bp) and chromosome 16 (894,088 bp) was identified by all four linkage models.

Two of the four QTL, chromosome 14 (531,242 bp) and chromosome 16 (894,088 bp), were validated in the independent set of recombinant strains (Figure 2 and Table S1). Drug 27 (aminoguanidine) showed a significant association with the chromosome 16 QTL in the MY cross (ANOVA, $P = 0.0007$). For the chromosome 14 QTL, Drugs 12 and 22 did not show a significant association in the YS cross, but Drug 31 (palmitoyl-DL-carnitine) showed a significant association in the MY–YS combined-cross analysis with *CYS4* as a covariate (ANOVA, $P = 0.015$; Figure 2). Although the chromosome 14 QTL affected sensitivity to Drug 31 but not to Drugs 12 and 22, subsequent experiments (see below) showed that *MKT1*, a gene within the QTL interval, affects sensitivity to both Drugs 12 and 22. The difference between the two validated and two false-positive QTL is unlikely to be caused by the type of linkage analysis; the two validated QTL were identified in both the single- and the combined-cross analysis with and without *CYS4* as a covariate. However, the two false-positive QTL showed smaller (secondary) effects relative to the largest-effect (primary) QTL. The chromosome 4 QTL was identified in the MY cross and was secondary to the effects of *CYS4*. The chromosome 12 QTL was identified in the MY–YS combined-cross analysis and was secondary to the validated chromosome 14 QTL.

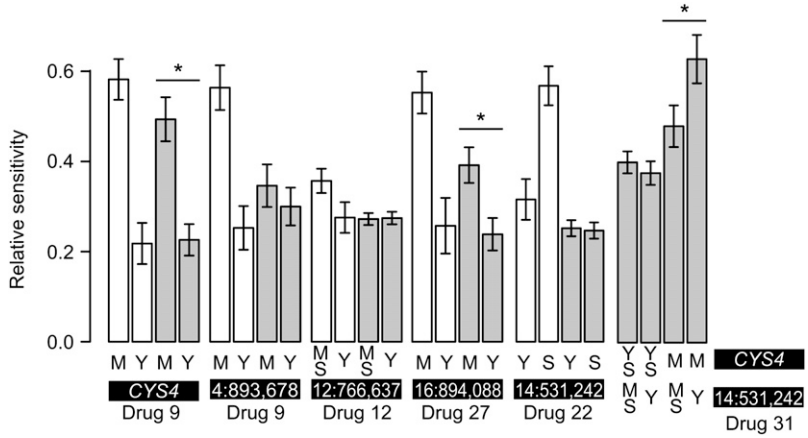

FIGURE 2.—Validation of selected QTL in an independent set of recombinant strains. Estimated effects of *CYS4* and four other QTL on drug sensitivity in the original (open bars) and newly derived (shaded bars) recombinant strains. Each bar shows the phenotype mean and the standard error defined by the genotypes (M, Y, and S) at each QTL. Asterisks indicate QTL with significant effects in the independent set of recombinant strains.

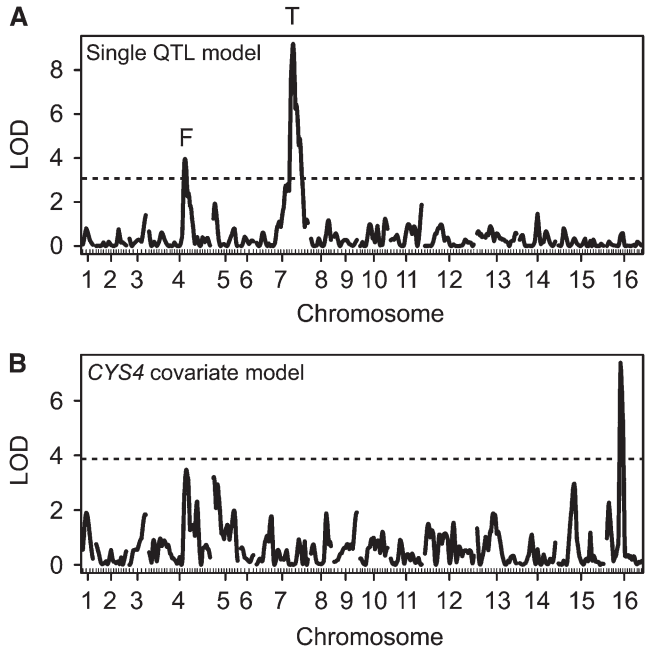

FIGURE 3.—Linkage mapping with *CYS4* as a covariate controls for false-positive associations. Genomewide linkage analysis using a single QTL model (A) and a model with *CYS4* as a covariate (B) for sensitivity to Drug 15 (hydrocortisone 21-hemisuccinate sodium) in the MY cross. Validated and false-positive QTL are labeled by T and F, respectively. Dashed lines show LOD cutoffs required for a false discovery rate of 1% (A) and 5% (B).

The false-positive QTL can be attributed to inappropriate linkage models. Most linkage models do not control for the effects of multiple QTL segregating in a single cross. When sample sizes are small and the effects of a true QTL are large, false-positive associations can attain genomewide significance if genotypes at one locus are correlated with or shadow those at a true QTL. The false-positive chromosome 4 QTL is likely a shadow QTL caused by a chance correlation with *CYS4* since the chromosome 4 QTL was identified only in models that lacked *CYS4* as a covariate. As expected for a shadow QTL, genotypes at the chromosome 4 QTL were significantly correlated with those at *CYS4* (Pearson correlation, $P = 0.0014$) in the original but not in the replicated recombinant strains. Comparison of linkage with and without *CYS4* as a covariate shows that the chromosome 4 QTL completely disappears when *CYS4* is used as a covariate and that another QTL appears on chromosome 16 (Figure 3).

To help eliminate potentially false-positive QTL, we removed all QTL with secondary effects to another primary QTL identified in the same linkage scan. After this filter, a total of 19 unique QTL remained. Three QTL occur at positions with known mutations: chromosome 7 (789,201 bp) corresponds to *CYS4*, chromosome 4 (112,957 bp) corresponds to *HO*, and chromosome 5 (104,539 bp) corresponds to *URA3*. Of the remaining 16 QTL (Table 2), 12 were identified exclusively by linkage analysis that used *CYS4* as a covariate, 1 was exclusively identified without *CYS4*, and 3 were identified by both methods. Six QTL were exclusively identified by the combined-cross analysis, 6 others were exclusively identified by the single-cross analysis, and 4 others were identified by both.

Pleiotropic and drug-specific QTL: QTL that cause resistance to different drugs and map to the same location can be explained by a single pleiotropic QTL or by two linked QTL. Because our marker interval is not dense enough to distinguish between these two possibilities (KNOTT and HALEY 2000), we defined QTL as being potentially pleiotropic if they were within the same marker interval or within 15 cM of one another. Three of the 16 QTL are potentially pleiotropic, involving sensitivity to between two and five drugs (Table 2). Interestingly, the QTL that correspond to known mutations in *CYS4*, *URA3*, and *HO* show extensive pleiotrophy as they were identified on the basis of linkage to 25, 7, and 5 drug-sensitivity phenotypes, respectively.

Cross-specific QTL and epistatic interactions with *CYS4*: Epistasis may be responsible for cross-specific QTL and QTL identified using *CYS4* as a covariate. Since 15/16 QTL were all identified using *CYS4* as a covariate, we tested each for interactions with *CYS4*.

Four QTL showed significant interactions with *CYS4* (ANOVA Bonferroni corrected $P < 0.05$, Table 2). Three of the four were exclusively identified in the single-cross analysis. These pairwise epistatic interactions can explain cross-specific QTL. For example, in the MY cross, the effect of the chromosome 14 (451,294 bp) QTL is observed only in combination with the M22 drug-sensitive allele of *CYS4* (Figure 4A), providing an explanation for why it was detected in the MY but not in the YS cross.

Cross-specific QTL may also result from more complex, multilocus interactions present in one cross but not in another. Another possibility is that cross-specific QTL could be due to a QTL that has effects in two crosses, but due to power is significant in only one. To distinguish between these possibilities, we tested for cross-specific effects of a QTL in a combined-cross analysis (see MATERIALS AND METHODS). Cross-specific effects were identified if both sets of combined crosses showed an interaction between the effect of the QTL and the cross in which it was segregating. For example, if a QTL was detected in the MY cross, combined linkage analysis was performed on the MY + MS crosses and the MY + YS crosses. If the M-allele is sensitive and Y and S are resistant alleles, the MY + MS crosses should show no significant cross-by-QTL interaction term, whereas the MY + YS cross should show a significant interaction term because M and S are encoded (incorrectly) as being the same allele. Thus, the expected pattern in the absence of any background effects is a significant cross-specific term in one but not in both combined crosses. If both

TABLE 2

QTL with the highest LOD score in any single scan

Chromosome (bp)	LOD[a]	Interval[b] (kb)	Drug	Cross	*P*(QTL × *CYS4*)	Cross-specific[d]
2 (413,404)	5.1	367–460	1	MY-SY	0.46	
4 (512,797)	6.9	482–520	3	All except MY[c]	0.074	Yes (MY, MS, YS)
4 (1,170,947)	5.5	1140–1224	29	MY	3.8×10^{-6}*	NA
5 (265,654)	4.2	219–310	4	MS	0.23	NA
7 (886,487)	4.8	838–929	2	MY	0.021	Yes (MY)
8 (33,410)	4.8	1–81	21	MY	0.0014*	NA
8 (479,987)	4.9	448–514	1	MY–MS	0.52	
12 (461,183)	5.6	406–500	19	MY–MS	0.0059	
13 (317,754)	5.3	287–546	4	MY–MS	0.055	
14 (451,294)	5.1	407–476	10, 16	MY, MY–MS, MY–YS	4.1×10^{-7}*	No (MY, YS)
14 (531,242)	3.9	429–569	12, 22	YS[c], MY–YS, MS–YS	0.11	Yes (YS)
15 (181,894)	6.5	121–194	31	MY–MS	0.013	
15 (381,869)	4.9	322–431	12	MY	0.022	NA
15 (642,515)	4.7	588–679	23	MY–YS	0.60	
16 (382,638)	4.5	363–418	5, 15, 22, 24, 26	MY	0.00011*	NA
16 (894,088)	6.3	860–922	27	MY, MY–YS	0.32	No (MY, YS)

*The significance of including an interaction term between the QTL and *CYS4* after Bonferroni correction is <0.05.
[a] The maximum LOD for each unique QTL.
[b] The intersection of pleiotropic QTL intervals defined by a 1.5-drop in LOD score, preferably from an individual cross.
[c] QTL identified without covariate; all others were identified using *CYS4* as a covariate.
[d] The cross in parentheses indicates in which cross a QTL showed cross-specific effects. NA indicates that the test was not applicable since cross-specific effects were not detected in either of the two combined cross analyses.

combined crosses showed a significant cross-by-QTL interaction term, we inferred that the difference between the M and Y alleles is different from that between the M and S alleles. The simplest explanation is that the effect of the M allele depends on genetic background. However, it is also possible that there are three QTL alleles such that the difference between the M and Y alleles is not the same as the difference between the M and S alleles.

Three of five QTL showed cross-specific effects in both combined-cross analyses (Table 2). Five of the 10 QTL that were identified in single crosses were not discernible because they were not significant in either of the combined-cross analyses with a cross-specific term. Two QTL showed results consistent with low power. One of the QTL showing cross-specific effects was the validated chromosome 14 QTL where the Y allele causes sensitivity in the YS cross but not in the MY cross (Figure 4B). The cross-specific effects of the Y allele may be due to complex epistasis since there is no simple epistatic interaction with *CYS4*. Another cross-specific QTL, chromosome 7 (886,487 bp), showed a marginally significant interaction with *CYS4* ($P = 0.02$, uncorrected for multiple tests) suggesting that this interaction may be responsible for the observed cross-specific effects (Table 2).

Three functionally distinct QTL alleles: QTL with cross-specific effects may in some cases be due to three different alleles in the three parental strains. Distinguishing between epistasis and three alleles will ultimately require cloning the gene underlying a cross-specific QTL. However, if the three alleles are sufficiently different from one another, then they should produce significant effects in all three crosses. One of the cross-specific QTL [chromosome 4 (512,797 bp), sensitivity to lithium chloride] showed evidence for three alleles on the basis of significantly different effects in all three crosses (Figure 5). While both the M and Y

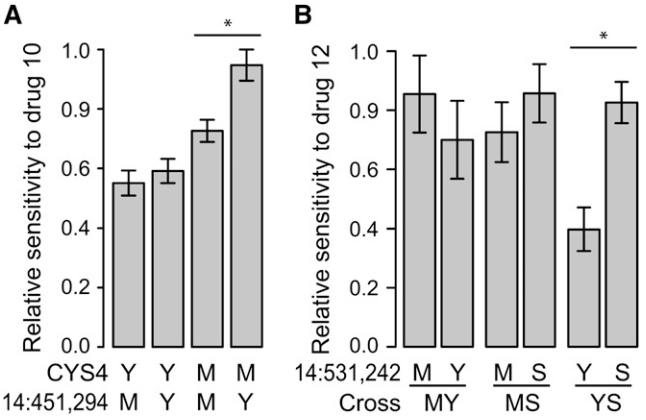

FIGURE 4.—Examples of epistatic and cross-specific QTL. (A) A significant interaction between chromosome 14 (451,294 bp) QTL and *CYS4* is shown by sensitivity to Drug 10 (fluphenazine) for four different groups of segregants on the basis of genotypes at *CYS4* and at the chromosome 14 QTL (Y or M alleles in the MY cross). (B) Cross-specific effects of the QTL at chromosome 14 (531,242 bp) is shown by relative sensitivity to Drug 12 (dipropyldopamine) for two groups of segregants on the basis of the QTL genotypes for the MY, MS, and YS crosses. Asterisks indicate a significant difference.

alleles are sensitive relative to the S allele, the chromosome 4 QTL also shows a significant effect in the MY cross alone. The 95% confidence interval from the posterior probability distribution of this QTL covers a 61-kb region on chromosome 4 that includes the *ENA* genes, which are P-type ATPases involved in the efflux of sodium and lithium ions and which vary in copy number among strains (Wieland *et al.* 1995). Since the lab strain has three tandemly repeated *ENA* genes, it is possible that the three alleles correspond to different alleles of *ENA* and/or to differences in *ENA* copy number.

***MKT1* and *AQY1* underlie cross-specific and drug-specific QTL:** The validated chromosome 14 (531,242 bp) QTL covers an interval of 141 kb and includes *MKT1*. An S288c-specific amino acid change within *MKT1* has been shown to affect high-temperature growth (Sinha *et al.* 2006), sporulation efficiency (Deutschbauer and Davis 2005), sensitivity to DNA damage (Demogines *et al.* 2008), and genomewide changes in gene expression (Smith and Kruglyak 2008). Using a reciprocal hemizygosity test (Steinmetz *et al.* 2002), we found that the S288c allele of *MKT1* also causes sensitivity to Drug 12, dipropyldopamine (*t*-test, $P = 0.035$), and Drug 22, phenylephrine (*t*-test, $P = 0.047$), and so is at least partially responsible for the chromosome 14 QTL (Figure 6A). This effect is absent in the presence of PPG, an inhibitor of *CYS3* (Washtien and Abeles 1977) that phenocopies the M22 allele of *CYS4* (Kim *et al.* 2009), which is consistent with the absence of an effect in the MS cross where both the S288c allele of *MKT1* and the M22 allele of *CYS4* segregate (Figure 4B).

The validated chromosome 16 (894,088 bp) QTL covers a 62-kb interval and includes *AQY1*. *AQY1* is an aquaporin that mediates water transport across cell membranes and S288c carries an allele of *AQY1* that fails to influence water transport and confers resistance to osmotic stress due to the combined effects of two amino acid mutations (Bonhivers *et al.* 1998). A reciprocal hemizygosity test (Figure 6B) showed that YPS163 carries an allele of *AQY1* that causes sensitivity to Drug 27, aminoguanidine (ANOVA, $P = 0.00014$), but not to Drugs 12 or 22. Interestingly, Figure 2 shows that the YPS163 allele is more resistant than that of M22 whereas Figure 6B shows that the YPS163 allele is more sensitive than that of S288c. These differences suggest the presence of three different alleles or two alleles with opposite effects that are dependent on the genetic background. Consistent with three functionally distinct alleles, there are three amino acid differences between the YPS163 and S288c alleles of *AQY1*, two of which (M121V and T255P) are functional in combination (Bonhivers *et al.* 1998), and there are three other amino acid differences between the M22 and YPS163 alleles of *AQY1* (R42K, V53A, and G226S). Alternatively, another gene in the QTL interval may also affect drug sensitivity.

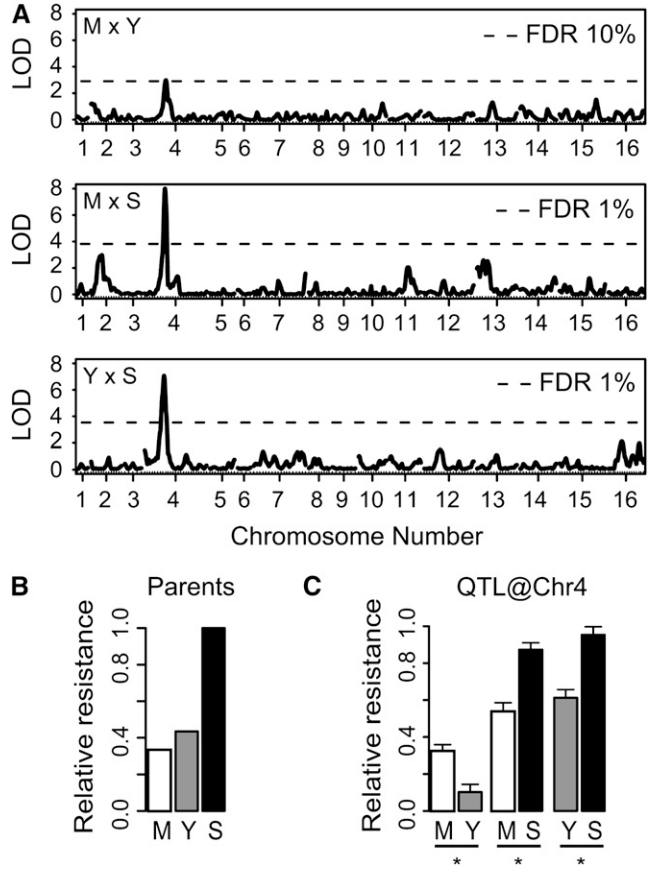

Figure 5.—A QTL on chromosome 4 shows evidence for three alleles underlying lithium chloride resistance. (A) The chromosome 4 (512,797 bp) QTL was identified in the MS and YS crosses at an FDR of 1% and in the MY cross at an FDR of 10%. (B) Parental phenotypes. (C) The effect size of the chromosome 4 QTL estimated from each cross shows that no two parental alleles are equivalent to one another. Asterisks indicate a significant difference.

DISCUSSION

Most quantitative traits are thought to be influenced by numerous genes of small effect. However, genes with small and/or background-dependent effects are much more difficult to identify than those of large effect and constitute a significant challenge to understanding the molecular basis of a trait. Using a combination of different mapping methods, we identified 16 QTL underlying sensitivity to 18 different pharmacological compounds. These QTL differ from a previously identified large-effect, multi-drug-sensitive allele of *CYS4* (Kim and Fay 2007) in that their effects are smaller, drug-specific, and often background-dependent as a consequence of their interaction with *CYS4* or the genetic background of the cross in which they segregate. Moreover, we identified two genes underlying drug-specific effects: *MKT1*, involved in translational regulation (Lee *et al.* 2009), and *AQY1*, a water channel protein (Laizé *et al.* 1999). Our results highlight the diversity of molecular mechanisms underlying variable drug responses.

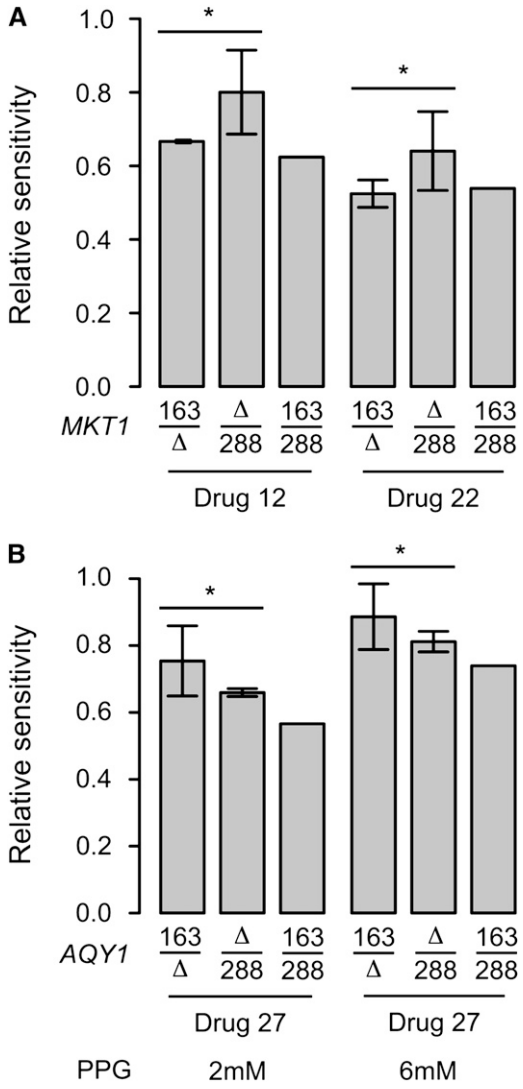

Figure 6.—*MKT1* and *AQY1* underlie two validated QTL. Relative drug resistance of reciprocal hemizygotes with the S288c allele deleted (163/Δ), the YPS163 allele deleted (Δ/288), or neither allele deleted (163/288). Asterisks indicate significant differences between 163/Δ and Δ/288. Relative sensitivity of 163/288 is the mean of two technical replicates.

Using four mapping methods, we identified 35 unique QTL. However, a significant number are likely false positives due to shadow or ghost QTL that can arise due to chance correlations between a true QTL and an unlinked locus (Doerge and Churchill 1996; Cheverud *et al.* 2004). When the true QTL has a large effect, even a slight correlation with another locus can result in a significant association and a false-positive QTL unless the effects of the first QTL are included in the test for association. A number of statistical methods have been designed to eliminate these false positives either by conditioning on other markers in the genome (Jansen 1993; Zeng 1993) or by conditional permutation tests (Doerge and Churchill 1996). In our study, the small sample size and large marker interval result in considerable uncertainty in the location and the effect size of a QTL. To account for these uncertainties, we used the causal polymorphism within *CYS4* as a covariate in the linkage analysis and we considered only primary QTL as candidates, *i.e.*, the QTL with the largest effect in a single linkage scan. This approach reduced the number of unique QTL from 35 to 19. Although some true positives may have been eliminated, the validated QTL (Figure 2) and shadow QTL (Figure 3) suggest that many of the QTL that were eliminated were false positives. In addition to removing false positives, the *CYS4* covariate model increased the number of QTL identified. Fourteen of the 16 QTL shown in Table 2 were only identified by using *CYS4* as a covariate, suggesting that *CYS4* also obscured real associations at other loci.

Combined-cross analysis provides increased power and resolution due to larger sample sizes but also provides a means of identifying QTL with effects that depend on their genetic background (Li *et al.* 2005; Blanc *et al.* 2006; Guo *et al.* 2006; Jagodic and Olsson 2006; Malmanger *et al.* 2006). A number of lines of evidence suggest that many of the drug-sensitivity QTL that we identified have effects that depend on their genetic background. First, 6 of the 16 QTL shown in Table 2 were identified only in the single-cross analysis despite the larger sample size and the lower FDR cutoff (5% *vs.* 1%) used in the combined-cross analysis. Three of these QTL showed significant interactions with *CYS4*. This provides one explanation as to why they were identified in only a single cross; if the QTL is dependent on *CYS4*, its effects should be absent in a cross where the M22 allele of *CYS4* is not segregating. Second, in the combined-cross analysis, three of five cases that could be tested showed a significant interaction between the effect of the QTL and the cross in which it segregated. Although this type of epistasis is difficult to experimentally confirm, our results support the value of combined-cross analysis in identifying context-dependent QTL.

Most of the QTL showed drug-specific effects. Of the 16 QTL in Table 2, 13 showed significant associations with sensitivity to one drug. Although low statistical power may also contribute to this pattern, it is unlikely to account for all of the drug specificity. The two QTL that correspond to known mutations in *URA3* and *HO* showed effects similar to other drug-specific QTL but were found to be associated with seven and five drugs, respectively. The identification of QTL that correspond to mutations thought to be irrelevant to growth in rich medium is consistent with previous studies (Brem *et al.* 2002; Perlstein *et al.* 2007) and provides further evidence that deficiencies may often have pervasive yet subtle effects (Hillenmeyer *et al.* 2008).

The identification of *MKT1* and *AQY1* highlights the diversity of molecular mechanisms by which cells are made drug sensitive. *MKT1* was first identified as a gene required for maintenance of the K2 killer toxin through

propagation of satellite double-stranded RNA of an L-A virus (Wickner 1980, 1987). Mktp has been shown to involved in post-transcriptional regulation of *HO* (Tadauchi *et al.* 2004) and in Puf3p-dependent regulation of cytoplasmic processing bodies (P-bodies) (Lee *et al.* 2009). An amino acid polymorphism (G30D) within *MKT1* has been shown to influence high-temperature growth (Steinmetz *et al.* 2002), sporulation efficiency (Deutschbauer and Davis 2005), cell morphology (Nogami *et al.* 2007), sensitivity to DNA damage (Demogines *et al.* 2008), the frequency of petite mutants (Dimitrov *et al.* 2009), and the expression of numerous genes (Smith and Kruglyak 2008). We found *MKT1* affects sensitivity to the dipropyldopamine (Drug 12), a dopamine agonist. In humans, catechol-*O*-methyltransferase transfers a methyl group from *S*-adenosylmethionine to the catechol group of a variety of compounds (Männistö and Kaakkola 1999). The dependence of catecholamine metabolism on *S*-adenosylmethionine may explain why the effects of *MKT1* are dependent on the M22 allele of *CYS4*, which inhibits the cysteine/methionine biosynthesis pathway and upregulates *S*-adenosylmethionine (Kim *et al.* 2009).

AQY1 encodes an aquaporin that transports water across cell membranes (Bonhivers *et al.* 1998) and improves freeze tolerance in vegetative cells (Tanghe *et al.* 2002, 2004) but inhibits freeze tolerance in spores (Sidoux-Walter *et al.* 2004). Recently, aquaporins have been shown to also conduct other small molecules, such as carbon dioxide, nitric oxide, and ammonia (Wu and Beitz 2007). Mutations in *AQY1* increase permeability to ammonia and methylamine by enlarging the central aromatic/arginine restriction within the water channel (Beitz *et al.* 2006). This raises the possibility that the amino acid differences between the YPS163 and S288c alleles of *AQY1* affect the permeability of the cell wall to aminoguanidine (CH_6N_4), which is only slightly larger than methylamine (CH_3NH_2). However, it is also possible that a reduction in water permeability indirectly affects sensitivity to aminoguanidine, an inhibitor of advanced glycosylation end products (Nilsson 1999).

In addition to *MKT1* and *AQY1*, other genes may contribute to the chromosome 14 (531,242 bp) and chromosome 16 (894,088 bp) QTL. *MKT1* occurs in a QTL hotspot; nine quantitative trait genes have been identified within a ~60-kb region on chromosome 14 (Steinmetz *et al.* 2002; Ben-Ari *et al.* 2006; Heck *et al.* 2006; Dimitrov *et al.* 2009). Five of the genes, *MKT1*, *END3*, *SAL1*, *PMS1*, and *SWS2*, span a 10-kb region and are adjacent to one another. The other three genes, *RAS2*, *RHO2*, and *FKH2*, lie 10–25 kb upstream or downstream of the five adjacent genes. *END3* and *RHO2* affect high-temperature growth (Steinmetz *et al.* 2002); *RAS2*, *PMS1*, *SWS2*, and *FKH2* affect sporulation efficiency (Ben-Ari *et al.* 2006); *SAL1* affects the frequency of petite mutants (Dimitrov *et al.* 2009); and *PMS1* affects the sensitivity to DNA damage (Heck *et al.* 2006). Thus, it is quite plausible that one or more of these genes also influences drug sensitivity. Interestingly, the chromosome 14 (531,242 bp) QTL interval includes all of the genes in the QTL hotspot. Although no other quantitative trait genes have been found near *AQY1*, our results do not exclude this possibility.

In summary, our results support a model whereby drug sensitivity is often mediated by an interaction among multiple genes, including those with both multidrug-sensitive and drug-specific effects. This model is supported by a substantial number of drug-sensitivity QTL with *CYS4*-dependent and cross-specific effects. The prevalence of these interactions within the context of an entire population is relevant to identifying the molecular basis of pharmacogenetic variation in humans and has significant implications for the complexity of treatment through personalized medicine.

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A Combined-Cross Analysis Reveals Genes With Drug-Specific and Background-Dependent Effects on Drug Sensitivity in *Saccharomyces cerevisiae*

Hyun Soek Kim and Justin C. Fay

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TABLE S1

Complete list of 35 unique QTL identified using any of the four linkage models

Chromosome	Coordinate	Drug	Cross	Method (S,C,W,O)[a]	Highest LOD score[b]	Validation[c]	Known mutations	Identified in Kim & Fay (2007)
2	253,368	26	MY-YS	CW	No			
2	413,404	1, 15, 19	MY-YS	CW	Yes			
4	112,957	5, 12, 19, 27, 28	MY, YS, MY-YS, MS-YS	SO, SW, CO, CW	Yes		*HO*	yes
4	512,797	3	MS, YS, MY-MS, MS-YS, MY-YS	SO, CO	Yes			yes
4	893,678	9 drugs	MY, MY-MS, MY-YS	SO, CO	No	No (P = 0.46)		yes
4	1,170,947	4, 29	MY	SW	Yes			
5	104,539	7 drugs	MS, YS, MS-YS, MY-YS, MY-MS	SO, SW, CO, CW	Yes		*URA3*	yes
5	265,654	4	MS, MY-MS	SW, CW	Yes			
6	97,971	10	MY-YS	CW	No			
7	578,340	16	MY-MS	CW	No			
7	789,201	25 drugs	MY, MS, MY-MS, MS-YS, MY-YS	SO, SW, CO, CW	Yes	Yes (P = 5.1e-6)	*CYS4*	yes
7	886,487	2	MY	SW	Yes			
8	33,410	10, 16, 21	MY, MY-MS	SW, CW	Yes			
8	479,987	1, 12, 13	MY, MY-MS, MY-YS	SW, CW	Yes			
10	134,562	23	MY-YS	CW	No			
10	507,960	6	MY-YS	CW	No			
10	639,988	10	MY-YS	CW	No			
11	354,172	10, 26	MY-MS	CO, CW	No			
11	634,178	6, 21	MY, MY-MS	SO, CO	No			yes
12	386,115	10	MY-YS	CW	No			
12	461,183	1, 5, 19, 31	MY-MS, MY-YS	CW	Yes			

12	505,525	15	MY-YS	CW	No		
12	766,637	12, 27	MY-YS, MS-YS	CW	No	No (P = 0.93)	
12	861,030	24	MS, MY-MS	SW, CW	No		
13	91,118	10	MY-YS	CW	No		
13	317,754	4	MY-MS	CW	Yes		
13	370,679	2	MY	SW	No		
14	451,294	10, 16	MY, MY-MS, MY-YS	SW, CW	Yes		
14	531,242	12, 22, 31	YS, MY-YS, MS-YS	SO, CO, CW	Yes	Yes (P* = 0.015)	yes
15	181,894	11, 31	MY-MS	CO, CW	Yes		
15	381,869	12	MY	SW	Yes		
15	642,515	23	MY-YS	CW	Yes		
16	382,638	5, 15, 22, 24, 26	MY	SW	Yes		
16	486,321	10, 12	MY	SW	No		
16	894,088	27	MY, MY-YS	SO, SW, CO, CW	Yes	Yes (P = 7.00e-4)	yes

[a] S: Individual cross, C: combined cross, W: with *CYS4* as a covariate, O: without *CYS4* as a covariate.

[b] QTL has the highest LOD score in the linkage scan.

[c] P-values from ANOVA of an independent set of recombinant strains.