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Dissecting the Pleiotropic Consequences of a Quantitative Trait Nucleotide

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Abstract

The downstream consequences of a single quantitative trait polymorphism can provide important insight into the molecular basis of a trait. However, the molecular consequences of a polymorphism may be complex and only a subset of these may influence the trait of interest. In natural isolates of *Saccharomyces cerevisiae*, a nonsynonymous polymorphism in cystathione beta-synthase (*CYS4*) causes a deficiency in both cysteine and glutathione that results in rust-colored colonies and drug-dependent growth defects. Using a single nucleotide allele-replacement, we characterized the effects of this polymorphism on gene expression levels across the genome. To determine whether any of the differentially expressed genes are necessary for the production of rust-colored colonies, we screened the yeast deletion collection for genes that enhance or suppress rust coloration. We found that genes in the sulfur assimilation pathway are required for the production of rust color but not the drug-sensitivity phenotype. Our results show that a single quantitative trait polymorphism can generate a complex set of downstream changes, providing a molecular basis for pleiotropy.

Keywords

QTN; *CYS4*; pleiotropy; gene expression; hydrogen sulfide

Introduction

The molecular basis of phenotypic variation in natural populations is important to understanding both molecular and phenotypic evolution. Yet, dissecting the molecular basis of a trait is difficult as it involves not only mapping quantitative trait polymorphisms but also understanding how each polymorphism affects a trait by influencing the activity of other genes, pathways and cellular processes involved in the production of a trait. For example, a quantitative trait polymorphism in *MKT1* affects high temperature growth (Sinha *et al.*, 2006), sporulation efficiency (Deutschbauer & Davis, 2005), DNA damage sensitivity (Demogines *et al.*, 2008), as well as numerous gene expression levels (Smith & Kruglyak, 2008), but the mechanisms by which the *MKT1* polymorphism affects these traits have only begun to be characterized (Lee *et al.*, 2009).

The relationship among molecular, cellular and organismal changes caused by quantitative trait polymorphisms is key to obtaining a general understanding of the molecular basis of a trait. Changes in gene expression provide a rich measurement of molecular variation and have provided valuable insights into the genes and molecular processes related to a trait of interest, e.g. (Cavalieri *et al.*, 2000; de Haan *et al.*, 2002; Fay *et al.*, 2004; Schadt *et al.*,

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2003). However, the relationship between gene expression and other phenotypes may often be complex; changes in gene expression that are tightly correlated with a trait may not be involved in the production of the trait. In yeast, changes in the environment cause many genes to change expression even though most are not required to survive or grow in the new environment (Birrell *et al.*, 2002; Giaever *et al.*, 2002). However, environmentally induced changes in gene expression may be different from genetic variation in gene expression segregating in natural populations since the latter is heritable and shaped by natural selection. Thus, while there are numerous examples of changes in gene expression with phenotypic consequences (Carroll *et al.*, 2001; Wray *et al.*, 2003), relatively little is known about the extent to which the effects of a quantitative trait polymorphism are mediated by downstream changes in gene expression.

Identifying the molecular consequences of a single quantitative trait polymorphism is difficult when there are other polymorphisms segregating within the genetic background. In yeast, allele-replacement strains have been used to measure the precise effects of a number of quantitative trait polymorphisms on gene expression levels (Brown *et al.*, 2008; Smith & Kruglyak, 2008; Yvert *et al.*, 2003). Each polymorphism resulted in numerous gene expression changes, but the role that these changes play in generating organismal phenotypes is difficult to know. Indeed, only a subset of the downstream molecular consequences of a quantitative trait polymorphism may mediate its effects. Thus, even when changes in gene expression can be attributed to a single quantitative trait polymorphism, correlative and causative changes must be distinguished to fully understand the molecular basis of a trait.

In yeast, hydrogen sulfide production varies among strains and has a negative impact on wine quality (Jiranek *et al.*, 1995; Spiropoulos & Bisson, 2000). We previously found that a strain isolated from a vineyard in Italy, M22, expresses genes in the sulfur assimilation pathway at high levels and produces high levels of hydrogen sulfide, H_2S, a metabolite produced by the sulfur assimilation pathway (Fay *et al.*, 2004). H_2S producing strains have been shown to produce dark-brown colonies when grown in the presence of lead acetate (Ono *et al.*, 1991) or on BiGGY medium which uses bismuth as an indicator for sulfide production (Jiranek *et al.*, 1995). Similarly, M22 produces reddish-brown or rust-colored colonies in the presence of copper sulfate. Previous work has shown that a wild-type laboratory strain produces brown-colored colonies when grown on copper containing synthetic complete medium due to copper-sulfur mineral complexes on the cell surface (Yu *et al.*, 1996). These studies support the hypothesis that rust coloration is due to overproduction of hydrogen sulfide leading to the precipitation of hydrogen sulfide and copper ions (Fay *et al.*, 2004). However, the relationship between expression of the sulfur assimilation genes, hydrogen sulfide production and colony color depends on the environment and strain background (Linderholm *et al.*, 2008; Spiropoulos *et al.*, 2000).

To dissect the relationship between changes in gene expression and the production of rust-colored colonies, we conducted a genetic analysis using M22. Based on a cross between M22 and two strains that are white-colored in the presence of copper sulfate, we found rust coloration co-segregates with another M22 phenotype, a drug-dependent delay in growth that occurs across a variety of pharmacological compounds (Kim & Fay, 2007). Quantitative trait mapping, complementation, and a single nucleotide allele-replacement showed that an amino acid polymorphism in cystathione beta-synthase (*CYS4*), I123N, causes both drug-sensitivity (Kim & Fay, 2007) and rust-colored colonies (Fig. S1). *CYS4* encodes the first step in the cysteine biosynthesis pathway, which generates cysteine from homocysteine. (See Fig. 3 for an overview of the pathway.)

Cysteine regulates degradation of Met4 (Menant *et al.*, 2006), a transcriptional activator of genes in the sulfur assimilation pathway. The sulfur assimilation pathway incorporates sulfur from sulfate into homocysteine via hydrogen sulfide (Thomas & Surdin-Kerjan, 1997). Thus, a deficiency in cysteine provides a mechanism by which the *CYS4* polymorphism could cause feedback up-regulation of the sulfur assimilation pathway and high levels of hydrogen sulfide production. If rust coloration is caused by a reaction between copper and hydrogen sulfide ions, rust coloration may be mediated by up-regulation of the sulfur assimilation pathway. However, the mechanism by which rust color is produced could be more complex; both cysteine and glutathione play critical roles in monitoring and regulating the oxidative/reductive state of a cell (Toledano *et al.*, 2007), so deficiencies in cysteine and glutathione could result in alteration of other cellular processes that are required for the production of rust colored colonies.

Here, we describe changes in gene expression caused by a nonsynonymous polymorphism in *CYS4* and show that the majority of differentially expressed genes are not required for the production of rust color. We also show that while genes in the sulfur assimilation pathway are required for the rust coloration phenotype, they are not required for the drug-sensitivity phenotype, indicating that the sulfur assimilation pathway plays a role in mediating the pleiotropic effects of the *CYS4* polymorphism.

Materials and Methods

Strains and Media

Rich medium [2% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) dextrose, with or without 2% (w/v) agar] was supplemented with copper sulfate (1mM) and propargylglycine (320μM PPG) to generate PPG medium for the colony color assays. For chemical complementation, L-cysteine and L-glutathione (Sigma, USA) were dissolved in distilled water, filter-sterilized, and added to YPD medium. M22 is a homothallic diploid isolated from a vineyard in Italy (Fay & Benavides, 2005). An allele replacement strain (M22-*CYS4::N123I*) was generated by directly transforming M22-*CYS4*Δ by fusion PCR fragments containing the desired *CYS4* allele and by selecting on complete media followed by color selection (Kim & Fay, 2007). Deletion of *CYS4*, *MET10*, *MET2*, and *MET17* in M22 was generated using the *kanMX* deletion cassette and selected on the rich medium supplemented with G418 (Wach *et al.*, 1994).

Expression analysis

Overnight cultures of M22, M22-*CYS4*Δ, and M22-*CYS4::N123I* were diluted in fresh YPD to 10% (v/v) and grown for 3 hours at 30°C. Total RNA was isolated using an RNeasy mini kit (QIAGEN, USA), reverse transcribed, labeled with Cy3 or Cy5 fluorescent dyes (Genisphere, USA), and hybridized to epoxy-coated slides (MWG Biotech, USA) spotted with 6388, 70mer oligos (Qiagen-Operon, USA). Arrays were scanned using a ScanArray Express laser scanner (Perkin Elmer, USA). Microarrays were manufactured and hybridizations were completed in the microarray core facility in Washington University's Genome Center. A dye-swap was performed for one of the three replicates of each strain, where Cy3 instead of Cy5 was used to label the reference RNA, a pool of RNA from all samples. Individual array was normalized using the LOWESS algorithm to control for intensity dependent dye effect (Dudoit *et al.*, 2002). The log transformed median ratio of normalized two channel intensities were used for further analysis. Significant differences in gene expression among strains (M22, M22-*CYS4*Δ, and M22-*CYS4::N123I*) were estimated using analysis of variance with the model: $y_i = \mu + V_i + \varepsilon_i$, where y_i is the ratio of transcripts in strain i compared to the reference pool, μ is the average ratio across all strains, V_i is the effect of strain i on the transcript ratio, and ε_i is the error. Significant variation in gene

expression among the strains was identified using the false discovery rate (FDR) method of Benjamini-Hochberg (Benjamini & Hochberg, 1995). Tukey's posthoc honest significant difference method implemented in R (http://cran.r-project.org/) was used to identify which of the three pairs of strains were different from one another. Gene expression data have been deposited in Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) and are accessible through the GEO Series accession number GSE14102.

Enhancer/suppressor screen

The 4664 homozygous *kanMX*-deletion strains (BY4743, *MAT*a/α, *his3*Δ1/*his3*Δ1, *leu2*Δ0/*leu2*Δ0, *lys2*Δ0/*LYS2*, *MET15*/*met15*Δ0, *ura3*Δ0/*ura3*Δ0) were grown on YPD agar plates (384/plate) for 24 hours at 30°C. Colonies were replica-plated to PPG medium using a RoToR robot (Singer Instruments, UK). Photographs were taken after 24 hours growth, images were cropped and red and green channels were saved separately using Photoshop CS2 (Adobe Systems Inc. USA). Spotfinder (Saeed *et al.*, 2003) was used to grid the colonies and measure color intensities. Rust coloration of each strain was measured using the green channel intensities divided by size of the colony. To control for edge effects from the plate, each colony was normalized by either the median of the row or column of the 384 strains in each plate depending on which deviated more from the overall background. Enhancers and suppressors were identified by those strains showing a green intensity of less than 0.80 and greater than 1.20, respectively, from photographs of the strains grown on PPG medium. At these cutoffs, the deletion strains showed slight but noticeably darker or lighter levels of rust color. At a cutoff of 0.60 and 1.30, the strains showed large, easily recognized effects on rust coloration. To assess the significance of deletions with slight effects on rust coloration, a subset of 1,494 strains were phenotyped in replicate. The replicates showed an average standard error of 0.012 and there were no cases where a single green intensity measurement showed a deviation greater than 0.20 from the mean of the five replicates. The probability of observing a single value below 0.80 or above 1.20 from permutations of the error, as measured by each strains deviation from the genotype mean, was 2.7×10^{-4} and 2.6×10^{-4}, respectively.

Gene ontology analysis

Significantly enriched classes of genes were identified using gene ontology terms and P-values from the hypergeometric distribution followed by Bonferroni correction as implemented in the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). For the analysis of enhancers and suppressors, named genes in the homozygous deletion collection were used as the background set by which significantly enriched gene ontology terms were identified.

Results

A deficiency in cysteine biosynthesis causes rust coloration in M22

To characterize the molecular mechanisms by which a single amino acid polymorphism within cysteine beta-synthase (Cys4) causes rust coloration. We compared two strains that are identical except for the causative nonsynonymous site within *CYS4*. We previously showed that the *CYS4* amino acid polymorphism causes a deficiency in glutathione, which can be synthesized from cysteine, and that supplementation with either cysteine or glutathione eliminates drug-sensitivity (Kim & Fay, 2007). Similarly, both cysteine and glutathione suppress rust-coloration in M22 and strains lacking *CYS4* (Fig. 1). Since glutathione can be converted back into cysteine (Ganguli *et al.*, 2007), these results imply that rust coloration is mediated by a deficiency in intracellular levels of either glutathione or cysteine.

Differential gene expression caused by a quantitative trait polymorphism

To characterize the molecular consequences of the *CYS4* amino acid polymorphism, we measured genome-wide changes in gene expression levels. We compared expression profiles of M22 (rust-colored) to an allele replacement strain (M22-*CYS4::N123I*, white-colored). For reference, we also compared the expression profile of the M22 allele-replacement strain to M22 with *CYS4* deleted (M22-*CYS4*Δ, rust-colored). From three replicate comparisons of M22 to M22-*CYS4::N123I* we identified 24 genes differentially expressed at a false discovery rate (FDR) of 5% and 141 genes at a FDR of 20% (Table S1). Compared to the white-colored allele-replacement, deletion of *CYS4* caused a larger set of 45 and 189 changes in gene expression at a 5% and 20% FDR, respectively (Table S1). The average fold change for the 24 and 45 differentially expressed genes was 2.1 and 2.3, respectively. The intersection between the two sets of genes is large; 21 genes intersect at a 5% FDR and 75 genes intersect at a 20% FDR. The larger number of expression differences between M22-*CYS4*Δ and the allele replacement as well as the large and directionally consistent overlap (71/75) with those expression differences between M22 and the allele replacement is consistent with the M22 allele of *CYS4* being a hypomorph, as suggested by previous observations based on glutathione levels (Kim & Fay, 2007).

The 75 genes that differ between the two rust-colored strains and the white allele-replacement strain are significantly enriched for genes involved in sulfur metabolic processes ($P = 1.3\times10^{-4}$) and siderophore-iron transport ($P = 0.0058$, see Methods). Genes that function in sulfur-assimilation and metabolism of sulfur-containing amino acids, methionine and cysteine are up-regulated in M22 and M22-*CYS4*Δ (Table 1), consistent with cysteine-dependent feedback regulation by Met4 (Menant *et al.*, 2006). Genes that function in siderophore-iron transport are likely up-regulated in response to glutathione deficiency since glutathione is required for maturation of cytoplasmic iron-sulfur containing proteins and glutathione deficiency causes activation of iron transporters by Aft1 and Aft2 (Rutherford *et al.*, 2005;Sipos *et al.*, 2002).

Identification of enhancers and suppressors of rust coloration

To identify genes that affect rust coloration, we screened the yeast deletion collection (Winzeler *et al.*, 1999) for enhancers and suppressors of the rust coloration phenotype. Since the deletion collection is white in the presence of copper sulfate, we used propargylglycine (PPG), which inhibits cystathionine gamma-lyase (*CYS3*) and cysteine biosynthesis, to phenocopy the M22 allele of *CYS4*. Figure S2 shows that similar to a deficiency in *CYS4*, PPG causes decreased levels of glutathione and makes it possible to identify deletions that enhance or suppress rust coloration.

Out of 4664 homozygous diploid deletion strains, we identified 120 enhancers and 304 suppressors of rust coloration (Fig. 2, Table S2). Of the 98 enhancers that are named genes, 23 are involved in protein catabolism through endosomal or vacuolar functions, and the most significant gene ontology enrichment is ubiquitin-dependent protein catabolic processes via the multivesicular body pathway ($P = 5.8\times10^{-7}$, Table 2). However, the four strongest enhancers are all genes involved in the production of cysteine. *CYS3* and *CYS4* generate cysteine from homocysteine, *DUG2* generates cysteine by degradation of glutathione (Ganguli *et al.*, 2007), and *PDX3* produces pyridoxal 5′-phosphate (Vitamin B6), a prosthetic group required for Cys4 function (McClean *et al.*, 2000). The list of enhancers also includes a number of genes that function in methionine or cysteine biosynthesis (Table 2), and is enriched for genes involved in sulfur amino acid metabolic processes ($P = 0.003$). The enhancement of rust coloration by deletion of genes involved in cysteine biosynthesis as well as two genes involved in glutathione degradation, *DUG2* and *DUG3*, suggest that rust coloration results from low levels of cysteine rather than glutathione (Fig. 3).

Suppressors of rust coloration include 267 named genes, the majority of which are related to mitochondrial functions (Table 2). Many of the mitochondrial related genes function in the ribosome (29 genes), cytochrome complexes (12 genes), translation (6 genes) and ATP synthase (10 genes). Although there is enrichment for genes involved in cellular respiration ($P = 2.0\times10^{-15}$) and genes that function in the mitochondria ($P = 1.1\times10^{-35}$), there are many genes required for respiration that did not suppress rust coloration. Thus, the suppression of rust color is not simply due to the reduced rate of growth of petite mutants. In addition to mitochondrial related genes, there is also enrichment for genes involved in gene expression ($P = 7.2\times10^{-8}$) and sulfate assimilation ($P = 3.0\times10^{-5}$). All six genes that function in the assimilation of sulfur from sulfate into homocysteine are suppressors of rust coloration (Table 2, Fig. 3). The last step in the sulfur assimilation pathway is the formation of homocysteine from hydrogen sulfide and O-acetyl-L-homoserine by Met17, which is an enhancer of rust coloration. The suppression of rust coloration by deletion of genes that lead to the production of hydrogen sulfide combined with the enhancement of rust coloration by deletion of *MET17* indicates that hydrogen sulfide is a key metabolite involved in the production of rust coloration (Fig. 3).

Differentially expressed genes necessary for the production of rust coloration

If genes that are differentially expressed as a result of the *CYS4* polymorphism play a role in generating the rust coloration phenotype, then deletion of these genes should either enhance or suppress rust coloration. Seven of the 120 enhancers and eight of the 304 suppressors were differentially expressed between both the rust (M22, M22-*CYS*Δ) and white-colored (M22-*CYS4::N123I*) strains. Genes that are up-regulated in M22 should suppress rust coloration when deleted if their expression levels are involved in the production of rust color. Of the eight suppressors, seven are expressed at higher levels in the rust-colored strains and five of these function in the conversion of sulfate into hydrogen sulfide before assimilation into homocysteine (Fig. 3). The other two suppressors are *TUF1* and *YTA12*, required for translation of mitochondrial proteins and assembly of mitochondrial enzyme complexes, respectively. Only one of the enhancers, *SWI3*, was expressed at lower levels in the two rust-colored strains.

The identification of differentially expressed genes necessary for the production of rust coloration provides insight into the mechanism by which the *CYS4* polymorphism results in rust coloration. The up-regulation of both enhancers and suppressors in the same biochemical pathway suggests that hydrogen sulfide is involved in the production of rust coloration. To confirm that hydrogen sulfide production is required for rust coloration in M22, we deleted *MET2*, *MET17* and *MET10* in M22. The suppression of rust coloration by *MET10* and enhancement of rust coloration by *MET2* or *MET17* shows that rust coloration in M22 depends on hydrogen sulfide production (Fig. 4). To confirm the requirement for mitochondrial function in M22 and test whether mitochondrial function is required for hydrogen sulfide production, we deleted both *TUF1* and *YTA12* in M22. Surprisingly, neither deletion suppressed rust coloration. Rust coloration of M22 was also unaffected by deletion of two other genes, *MRPL38* and *MRPS17*, that are required for rust coloration in the deletion collection background.

Pleiotropy at the molecular level

In addition to rust coloration, the M22 allele of *CYS4* also causes a delay in growth upon exposure to a variety of different pharmacological compounds (Kim & Fay, 2007). While both the rust coloration and drug sensitivity phenotypes are caused by a deficiency in cysteine/glutathione, the molecular basis of these two traits may not be identical. To determine whether any of the differentially expressed genes that function in sulfur assimilation or iron transport affect drug-sensitivity, we measured growth in the presence

and absence of atenolol, a beta-adrenergic receptor antagonist that causes a delay in growth dependent on the *CYS4* amino acid polymorphism (Kim & Fay, 2007). Four of the genes involved in the production of sulfur-containing amino acids and two of the genes involved in iron transport increase drug resistance when deleted. However, most of the genes in the sulfur assimilation pathway that affect rust coloration do not affect drug-sensitivity (Table 1). In contrast, deletions of genes required for glutathione biosynthesis, *CYS3, CYS4* and *GSH1*, increase drug-sensitivity. Thus, while both rust coloration and drug-sensitivity result from a deficiency in cysteine, they each have a distinct molecular basis since they are enhanced and suppressed by different sets of genes.

Discussion

Dissecting the molecular mechanisms by which a single quantitative trait polymorphism influences a trait of interest provides insight into those biochemical or signaling pathways that underlie a trait. We identified a complex set of downstream changes in gene expression caused by a pleiotropic quantitative trait polymorphism. We then showed that a subset of the differentially expressed genes in the same biochemical pathway is required for the production of rust coloration but not drug-sensitivity. These results provide insight into the molecular basis of pleiotropy.

The molecular basis of rust coloration

The suppression of rust coloration by deletion of genes required for hydrogen sulfide production and enhancement of rust coloration by deletion of genes involved in utilization of hydrogen sulfide implies that rust coloration is mediated by hydrogen sulfide production. Copper-dependent changes in yeast colony color are thought to be due to copper-sulfur mineralization at the cell surface (Ashida *et al.*, 1963; Yu *et al.*, 1996). Consistent with this possibility, electron micrographs show dark granules at the cell surface in M22 but not the white-colored M22 allele replacement strain (Fig. S3). However, hydrogen sulfide production may not be sufficient for the production of rust color. The rust-colored compound may also depend on other cellular perturbations such as a deficiency in glutathione, a potent antioxidant, or altered level of metabolites in the S-adenosylmethionine pathway (Christopher *et al.*, 2002). In the yeast deletion collection background but not M22, rust coloration is dependent on some aspect of mitochondrial function. The large number of suppressors with mitochondrial functions makes it difficult to know what mitochondrial function is required for rust coloration. One possibility is that CuS mineralization occurs in the mitochondrion or requires iron-sulfur containing proteins made in mitochondria (Kispal *et al.*, 1999). However, it is also possible that mitochondrial mutants suppress rust coloration by affecting H_2S production, either by reducing the need for glutathione or by affecting the sulfur assimilation genes through mitochondrial retrograde regulation (Liu & Butow, 2006).

In M22, hydrogen sulfide production may be elevated due to differential regulation of the sulfur assimilation pathway. Alternatively, the M22 allele of *CYS4* may cause homocysteine utilization to be the rate limiting step in sulfur assimilation. In this scenario, up-regulation of the sulfur assimilation genes would have little or no effect on hydrogen sulfide levels. Consistent with this latter possibility, up-regulation of the sulfur assimilation pathway in another Italian wine strain due to a frameshift in the extracellular amino acid sensor, *SSY1* (Brown *et al.*, 2008), does not lead to rust coloration or noticeable levels of hydrogen sulfide production (Kyle Brown, personal communication).

Our enhancer and suppressor screen implies that hundreds of genes with a wide range of biological functions affect rust coloration. In many cases, enhancement and suppression of rust coloration may be mediated by changes in cysteine/glutathione homeostasis and hydrogen sulfide production. Evidence for this possibility can be found in the overlap

between enhancers and suppressors of rust coloration and two other screens of the yeast deletion collection. A screen for genes involved in glutathione homeostasis identified 276 genes that excrete high levels of glutathione and one of the largest functionally related groups contains genes involved in the secretory pathway or vacuolar protein sorting (Perrone *et al.*, 2005), similar to enhancers of rust coloration. Of the 276 genes, 96 overlap with enhancers or suppressors of rust coloration. Another screen for genes involved in hydrogen sulfide production identified 89 genes, many of which function in the biosynthesis of sulfur containing amino acids (Linderholm *et al.*, 2008). Of the 89 deletions that cause high levels of sulfite reductase activity, 15 overlap with enhancers of rust coloration. These comparisons imply that some but not all of the enhancers and suppressors of rust coloration affect cysteine/glutathione levels and hydrogen sulfide production in the absence of PPG induced inhibition of cysteine biosynthesis.

The role of gene expression in pleiotropy

We found a large number of genes that are differentially expressed as a consequence of the *CYS4* polymorphism. The up-regulation of genes that suppress rust coloration when deleted implies that the differential expression of genes in the sulfur assimilation pathway plays an important role in the production of rust color. However, other *CYS4* induced changes in gene expression may have no downstream phenotypic consequences. For example, the up-regulation of genes that enhance drug-sensitivity when deleted, e.g. *CYS3* or *GSH1*, suggests that some expression changes are a response to alterations in cysteine homeostasis but are not responsible for drug-sensitivity. Finally, some *CYS4* induced changes in gene expression may only have phenotypic consequences under certain environmental conditions; e.g. rust coloration is only observed in the presence of copper sulfate but up-regulation of the sulfur assimilation genes occurs in both the presence and absence of copper sulfate (Fay *et al.*, 2004). Thus, the differential expression of siderophore iron transporters may also produce a downstream phenotype when measured under the right condition. The up-regulation of *SIT1* and *FIT2* combined with the higher level of drug-resistance when deleted implies that the differential expression of these two genes may be involved in drug sensitivity.

Implications for phenotypic variation in other yeast isolates

What is the relevance of our results to phenotypic variation in other strains of yeast? While the rust coloration phenotype is rare, progeny of M22 crossed with two white-colored strains show a continuous distribution of rust coloration phenotypes in those strains that inherit the M22 allele of *CYS4* (Fig. S4). Furthermore, natural yeast isolates show significant variation in levels of intracellular glutathione (Fig. S5), which affects metabolism of a wide range of pharmacological compounds (Kim & Fay, 2007), and hydrogen sulfide, which affects wine production (Mendes-Ferreira *et al.*, 2002). Thus, our results provide insight into the genes, pathways and processes that affect population variation in cysteine/glutathione metabolism and hydrogen sulfide production. However, we also found evidence for significant effects of genetic background. Deletions of four mitochondrial genes suppress rust coloration in BY4743, the strain used to make the deletion collection, but do not suppress rust coloration in M22. Thus, differences in genetic background may have resulted in missed M22-specific enhancers or suppressors, and presents a significant challenge to obtaining a comprehensive understanding of the molecular basis of cysteine-related phenotypes.

How common are pleiotropic, background dependent quantitative trait nucleotides? A nonsynonymous polymorphism in *MKT1* shares many of the characteristics of the *CYS4* polymorphism: extensive pleiotropy, background dependent effects and numerous changes in gene expression levels (Demogines *et al.*, 2008; Deutschbauer *et al.*, 2005; Sinha *et al.*, 2006; Smith & Kruglyak, 2008). However, small-effect polymorphisms may have quite

different characteristics. For example, quantitative trait polymorphisms with small effects show less pleiotropy than those of large effect (Wagner *et al.*, 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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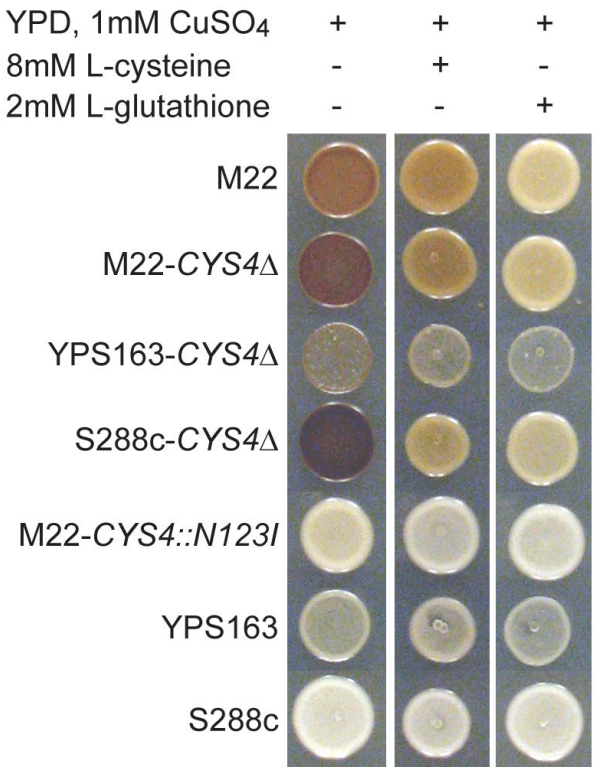

Fig. 1.

Cysteine and glutathione suppress rust coloration. Deletion of *CYS4* in M22, S288c and YPS163 results in rust coloration, similar to M22, on rich medium supplemented with 1mM copper sulfate. The level of rust coloration is reduced in medium supplemented with 8mM L-cysteine or 2mM L-glutathione. Rust coloration is difficult to see in YPS163 which shows reduced growth in the presence of copper sulfate.

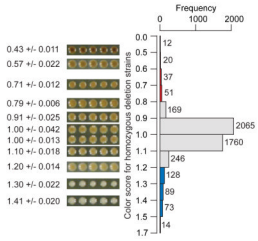

Fig. 2.

Phenotypic distribution of enhancers and suppressors of rust coloration in the homozygous yeast deletion collection. Rust coloration was measured by the average intensity of the green channel from RGB photographs of a strain divided by the median of all strains in the same row or column (See methods for details). Enhancers are shown in red and suppressors in blue. Photographs of selected strains show five replicates per strain illustrating the mean and standard deviation of the rust coloration phenotype.

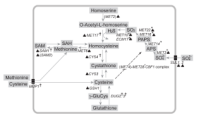

Fig. 3.

Genes involved in sulfur assimilation and biosynthesis of cysteine and glutathione. Asterisks and crosses indicate suppressors and enhancers of rust color, respectively. Genes not included in the screen are shown in parenthesis. Genes up-regulated in M22-*CYS4::N123I* or in M22-*CYS4*Δ are indicated by a triangle. Dotted lines show ubiquitin mediated negative regulation of Met4 by cysteine and positive regulation of the sulfur assimilation pathway by the Met4 complex.

Abbreviations: APS (adenosine 5′-phosphosulfate), PAPS (3′-phosphoadenosine-5′-phosphosulfate), SAM (S-adenosylmethionine), γ-GluCys (γ-glutamylcysteine), SAH (S-adenosylhomocysteine)

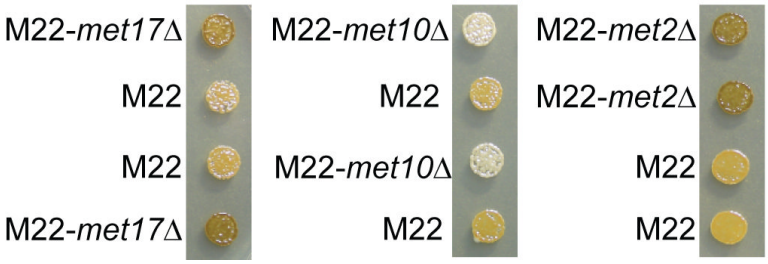

Fig. 4.

Deletion of *MET2* or *MET17* enhances rust coloration and deletion of *MET10* suppresses rust coloration in M22. The four meiotic progeny of each heterozygous M22 deletion strain were plated on rich medium with 1mM $CuSO_4$ and labels show strain genotypes.

Table 1

Rust coloration and drug sensitivity are affected by different set of *CYS4* induced genes

		Gene expression relative to M22-*CYS4::N123I*		Gene deletion phenotype	
Gene class	**Gene name**	**M22**	**M22-*CYS4Δ***	**Rust color**	**Drug sensitivity**
Sulfate transport	*SUL1*	4.1	16.7*		resistant
	SUL2	1.9	3.1*		
	OAC1	1.4*	1.1		
Sulfate assimilation	*MET3*	7.1*	3.3	WW	
	ECM17	6.0**	5.1**	WW	
	MET10	4.5**	4.5**	WW	resistant
	MET22	3.1*	2.0	W	
	MET16	2.4*	1.9*	W	
Homocysteine biosynthesis	*MET17*	4.6**	2.5**	R	
	IRC7	1.8**	1.4**		resistant
	MET2	1.6	2.1**		
Methionine biosynthesis	*MET6*	5.1*	2.0		
S-AdoMet biosynthesis	*SAM1*	1.7*	1.2	RR	
	SAM2	1.8*	2.0*		resistant
Methionine salvage	*ADI1*	1.7*	1.0		
Cysteine biosynthesis	*CYS4*	2.5**	0.05**	RR	sensitive
	CYS3	1.5*	1.6*	RR	sensitive
Glutathione biosynthesis	*GSH1*	1.6	2.1*		sensitive
Siderophore-iron transport	*ARN1*	1.5**	3.1**		
	SIT1	2.9**	2.7**		resistant
	ENB1	2.5*	2.4*		
	FIT2	1.6	8.5**		resistant

		Gene expression relative to M22-*CYS4::N123I*		Gene deletion phenotype	
Gene class	**Gene name**	**M22**	**M22-*CYS4Δ***	**Rust color**	**Drug sensitivity**
	FIT3	1.4	7.8**		

Gene expression ratios are the average of three replicates and the mean squared error is 0.13, averaged across genes. Rust coloration and drug sensitivity phenotypes were measured using strains from the homozygous diploid deletion collection. RR indicates a strong enhancer (< 0.6) and WW a strong suppressor (> 1.3), as measured by the intensity of the green channel from photographs. R indicates a moderate enhancer (< 0.8) and W a moderate suppressor (> 1.2). Drug sensitivity was measured by the growth-delay induced by 2mM atenolol supplemented to rich medium. Strains with less than 4 hours of grown-delay are classified as resistant and those with more than 9 hours of growth-delay are classified as sensitive.

** Genes significant at 5% FDR

* genes significant at 20% FDR

Table 2

Functional classification of enhancers and suppressors

Pathway/function	Subclass	Enhancers	Suppressors
Sulfate-assimilation & sulfur metabolism	Enzyme	*SAM1, CYS3/4, DUG2/3, MET17, GLO2*	*MET3/10/14/16/22, ECM17*
	Regulation	*BUL1, STP1*	*MET28/32*
	Siroheme biosynthesis		*MET1/8*
	Purine and adenosine biosynthesis	*ADE1*	*ADE3/5/6*
	Transport	*MUP1*	
	SAM dependent methyl transfer	*CHO2*	
Vacuolar, golgi, protein sorting		23 genes	
Mitochondrial			221 genes
Total		**120 genes**	**304 genes**