

# Manifold anomalies in gene expression in a vineyard isolate of *Saccharomyces cerevisiae* revealed by DNA microarray analysis

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**Genome-wide transcriptional profiling has important applications in evolutionary biology for assaying the extent of heterozygosity for alleles showing quantitative variation in gene expression in natural populations. We have used DNA microarray analysis to study the global pattern of transcription in a homothallic strain of *Saccharomyces cerevisiae* isolated from wine grapes in a Tuscan vineyard, along with the diploid progeny obtained after sporulation. The parental strain shows 2:2 segregation (heterozygosity) for three unlinked loci. One determines resistance to trifluoro-leucine; another, resistance to copper sulfate; and the third is associated with a morphological phenotype observed as colonies with a ridged surface resembling a filigree. Global expression analysis of the progeny with the filigreed and smooth colony phenotypes revealed a greater than 2-fold difference in transcription for 378 genes (6% of the genome). A large number of the overexpressed genes function in pathways of amino acid biosynthesis (particularly methionine) and sulfur or nitrogen assimilation, whereas many of the underexpressed genes are amino acid permeases. These wholesale changes in amino acid metabolism segregate as a suite of traits resulting from a single gene or a small number of genes. We conclude that natural vineyard populations of *S. cerevisiae* can harbor alleles that cause massive alterations in the global patterns of gene expression. Hence, studies of expression variation in natural populations, without accompanying segregation analysis, may give a false picture of the number of segregating genes underlying the variation.**

The advent of DNA microarray technology (“DNA chips”) has made possible the analysis of global patterns of gene expression and revealed unexpected networks of coordinated regulation (1). These studies have, in turn, stimulated renewed interest in the interactions among metabolic pathways and the control of metabolic flux (2, 3). Most experiments thus far have dealt with comparisons of patterns of gene expression of organisms with the same genotype grown under different conditions or at different stages of the cell cycle (4–8). Virtually nothing is known about the extent of variation in either the levels or patterns of global gene expression among organisms isolated from natural environments. Is there significant variation in gene expression? How many genes and how much variation? If there is variation, what are the molecular mechanisms? Finding answers to these questions would open new vistas for molecular evolutionary biology, because it may reveal how much adaptive evolution can be attributed to changes in gene regulation.

In this paper we compare gene expression in parent and progeny of a natural isolate of *Saccharomyces cerevisiae* cultured under standard conditions. The parental isolate was obtained from a damaged grape berry in a Montalcino vineyard in Tuscany (9). Although wine yeast is rarely found on grapes with an unbroken skin, viable cells are found in about one-third of damaged berries, inside of which they establish a little fermentation chamber (10). There is known to be a great deal of functional heterozygosity among vineyard isolates. For example, when vineyard isolates are sporulated and their progeny tested

for growth on the sugars sucrose, maltose, and galactose, approximately 67% of the isolates segregate for the inability to use at least one of these sugars (9). Although about 70% of vineyard isolates are homothallic (11) and thus begin their diploid phase as complete homozygotes after sporulation, change of mating type, and mother-daughter cell fusion, they persist asexually for long enough periods to accumulate a significant load of mutations; these are resolved only at the next sporulation, constituting the “genome renewal” process (12).

The natural isolate that we have examined is known to be heterozygous for two resistance genes and a gene affecting colony morphology. Comparison of the global patterns of gene expression between the parental strain and its progeny, and among the progeny themselves, revealed an unexpectedly large number of genes whose level of expression showed major differences. Differences in level of expression of at least a factor of 2 were observed for 378 genes, or 6% of the genome. Many of the overexpressed genes are associated with amino acid biosynthesis or transport of sulfur or ammonia. Most of these differences are part of a suite of correlated traits, indicated by the fact that comparisons among the progeny revealed only eight segregating loci that differed in expression by 2-fold or more. We conclude that natural vineyard populations of *S. cerevisiae* can harbor alleles that cause massive alterations in the global patterns of gene expression.

## Materials and Methods

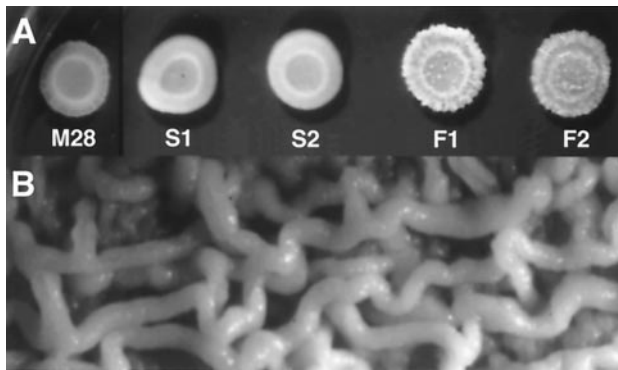
**DNA Microarray Construction.** A set of clones containing 6218 verified ORFs of the yeast genome were obtained from Research Genetics (Huntsville, AL) and amplified to levels required for preparation of DNA microarrays by PCR (6). Some of the longer ORFs were amplified with the GIBCO/BRL Elongase Amplification Kit (Life Technologies, Rockville, MD), using 40 cycles of 1 min at 95°C denaturation, 1 min at 55°C annealing, and 10 min at 68°C elongation. We obtained an amplified product confirmed by agarose gel electrophoresis for 98% of the ORFs. The amplified DNA was precipitated with isopropanol, washed with 70% EtOH, and resuspended in 25  $\mu$ l Micro Spotting Solution (Telechem, Sunnyvale, CA). The DNA was spotted on CMT-GAPS amino-silane-coated glass slides (Corning), using a microarraying robot with a 16-pin head constructed from a design by Patrick O. Brown (<http://cmgm.stanford.edu/pbrown/>).

**Extraction of mRNA.** RNA was extracted from flash-frozen pellets of yeast cultures grown aerobically at 30°C in a shaker at 225 rpm

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**Fig. 1.** (A) Colony morphologies of the parental strain M28 and the smooth (S1, S2) and filigreed (F1, F2) segregants. The colonies range in size from 1.0 to 1.2 cm. (B) Convoluted surface of a filigreed colony. The diameter of the filament is approximately 0.2 mm.

to an optical density of 0.8 in 1% yeast extract, 2% peptone, and 2% dextrose, using the hot-acid-phenol method. The mRNA was purified using the Qiagen (Chatsworth, CA) Extraction Kit.

**Preparation of cDNA and Hybridizations.** For each sample, the cDNA was prepared with GIBCO/BRL reverse transcriptase (Life Technologies) and 1.5  $\mu\text{g}$  of mRNA, labeled separately with the fluorochromes cyanine-3 and cyanine-5, essentially as described (13), and purified. Each competitive hybridization was performed twice, with the fluorochromes switched in the two replicates.

**Data Acquisition and Analysis.** Fluorescent DNA bound to the microarray was detected with a GenePix 4000 microarray scanner (Axon Instruments, Foster City, CA), using the GENEPIX 4000 software package to locate spots in the microarray. To avoid artificially inflated values of overexpression due to near-zero values in the denominator, fluorescence intensity values were adjusted by subtracting background from foreground after correcting the background for the lesser fluorescence of negative control spots on the same slide. To eliminate signals that are most prone to estimation error, any spot was excluded from analysis if both the cyanine 3 fluorochrome and cyanine 5 fluorochrome fluorescence signals were within two standard deviations of the distribution of intensities of the background pixels for that spot. Exclusion of these spots is a conservative measure, but including them has no effect on the conclusions presented here. Expression values were normalized by scaling the cyanine 5 fluorochrome values so that the mean cyanine 5 fluorochrome and cyanine 3 fluorochrome values of control spots were equal, which reduces the error variance. Differences in expression of 2-fold or more can be reproduced reliably. The data were analyzed using the program GENESPRING (Silicon Genetics, Redwood City, CA).

## Results

**Phenotypic and Genetic Characterization.** When grown on rich agar medium, the parental strain, designated M28, shows a colony morphology with a slightly ridged surface, delicately filigreed, as compared with the smooth colony surface observed in most laboratory strains and natural isolates (Fig. 1A). When M28 is induced to sporulate, the resulting diploid progeny show 2:2 segregation of a more extreme filigreed phenotype. Although homothallism is an obstacle to conventional genetic analysis, the 2:2 segregation indicates simple Mendelian inheritance, and it implies that the parental genotype is heterozygous for a single recessive allele associated with the extreme filigreed phenotype. We also found that M28 is heterozygous for unlinked alleles

conferring resistance to 200  $\mu\text{g}/\text{ml}$  of 5,5,5-trifluoro-D,L-leucine and 500  $\mu\text{g}/\text{ml}$  copper sulfate (data not shown). Fig. 1A shows the smooth (S1 and S2) versus filigreed (F1 and F2) colony morphology in one tetrad from the parental strain M28. It differs from a filamentous phenotype previously described (14, 15) in that the morphology is expressed under certain conditions in the heterozygous parent as well as in the homozygous diploid, and it is observed in both rich agar medium (1% yeast extract/2% peptone/2% dextrose) and upon growth in liquid (1% yeast extract, 2% peptone, and 2% dextrose). Microscopic observation of the surface of the filigreed colony reveals that the cells are associated in a tubular, convoluted manner resembling that of multicellular tissue (Fig. 1B).

Although differing dramatically in colony morphology, the filigreed and smooth segregants have indistinguishable growth rates in liquid culture. Cytological analysis of unsynchronized growing cultures of the filigreed genotypes late in logarithmic phase showed dense clumps and long chains of unseparated parental and progeny cells. All of the buds in a given aggregate are of approximately the same size. As the culture becomes older the cells elongate and the phenotype becomes more evident. This cellular phenotype can account for the filigreed surface of the colonies, as well as for the tendency of the filigreed strain to form thin, tissue-like wisps of gossamer material in liquid cultures.

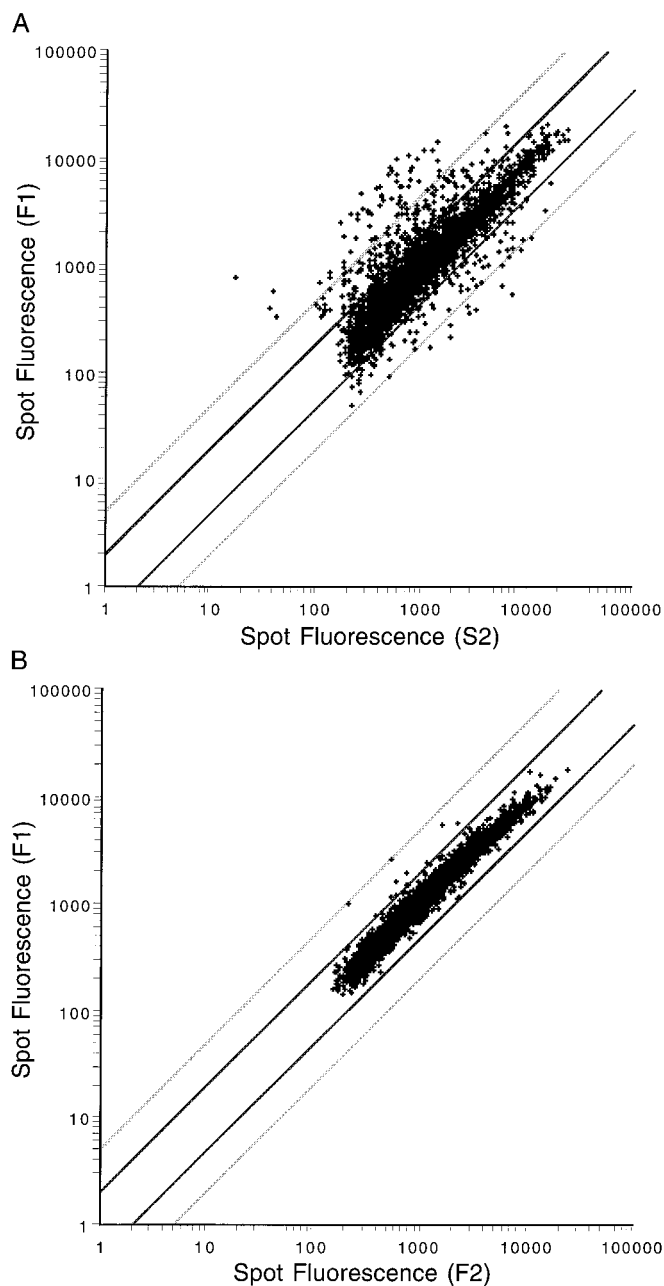
The expression of the filigreed phenotype on agar depends on the composition of the medium, temperature, and age of the colony. The phenotype is less pronounced in colonies grown on minimal medium. In homozygous genotypes, the phenotype is more extreme at 4°C or 18°C than at 28°C, and it is not expressed in cells grown at 37°C. Even the heterozygous M28 parent shows the filigreed phenotype at 4°C and 18°C (data not shown), and there is some expression after about 7 days, even at 28°C (Fig. 1A).

Genetic modifiers also affect the expression of the filigreed phenotype. This effect is evident in the tetrad in Fig. 1A, in which the four spores show a somewhat different phenotype. The differences become more pronounced with the passage of time. Colonies derived from S2 remain smooth, whereas those from S1 begin to show papillae. Colonies derived from F2 develop a more extreme filigreed phenotype over time, whereas those derived from F1 remain more constant.

**Genome Expression Profiles.** To assess the level of heterozygosity for alleles having marked effects on the level of gene transcription, we carried out competitive hybridizations. M28 was compared with each of its four progeny; each of F1 and F2 was compared with each of S1 and S2; and F1 was compared with F2.

Much to our surprise, we found a massive amount of expression variation among the progeny. Fig. 2A shows the expression profile observed in the comparison of F1 (the least extreme filigreed phenotype) against S2 (the most smooth phenotype). In this comparison, 153 genes (2.5% of the genome) were overexpressed in F1 by a factor of 2 or more, and 225 (3.6% of the genome) were underexpressed by a factor of 2 or more. The comparison of F2 against S2 gave virtually identical results, in both the number and the identity of the genes affected (data not shown).

To assess how many of these differences are due to segregating heterozygosity, we compared F1 with F2 (Fig. 2B). In this comparison only eight genes (0.1% of the genome) differed in expression by 2-fold or more. This number of genes is an overestimate of half the number of segregating genes (since some of the differences could be due to pleiotropy), from which it can be deduced that, with 95% confidence, no more than 13 genes would be expected to cosegregate by chance. We conclude that the vast majority (at least 97%) of the expression differences between F1 and S2 segregate as a suite of traits resulting from



**Fig. 2.** Log-log scatterplot of fluorescence measured for mRNA labeled with either cyanine-3 or cyanine-5 fluorochrome in a competitive hybridization on a microarray containing 6218 ORFs. Spots with at least one fluorescence signal significantly above background are plotted for (A) F1 against S2 and (B) F1 against F2. Dark lines represent a 2-fold difference in expression; light lines represent a 5-fold difference in expression.

a single gene, a small number of genes, or a group of linked genes.

Many of the highly overexpressed genes in Fig. 2A are associated with amino acid biosynthesis. However, transcripts from genes such as *LEU4*, *HIS5*, and *ARG4* and others involved in amino acid biosynthesis tend to be more variable than other types of transcripts in repeated experiments with the laboratory strain BY4743 (8). This strain has two mutations (*his3* and *leu2*) that may affect the expression of other genes for amino acid biosynthesis, whereas M28 is prototrophic. Nevertheless, to be conservative, we focused attention on

ORFs whose expression differed by 3-fold or more in a manner that could be reproduced in independent experiments. The relevant comparisons are F1 with S2, F2 with S2, and F1 with M28. Collation of the gene expression profiles revealed 26 ORFs that are overexpressed at least 3-fold in all three comparisons. Fig. 3 shows the expression of these 26 ORFs in the comparisons F1 versus S2 (front), F2 versus S2 (rear), and F1 versus M28 (middle), ranked in order of their mean level of expression across all three experiments. Note that the expression levels in F1 versus M28 are highly correlated with the other comparisons, which means that M28 has approximately the same expression levels as S2 under these growth conditions; this inference is confirmed by the direct comparison of S2 with M28.

We focus the subsequent discussion primarily on the genes in Fig. 3 and others as relevant. Unless otherwise stated, the specific levels of expression cited in the following sections are for the comparison of F1 with S2, but the results and interpretation are consistent across all of the experimental hybridizations. A complete listing of overexpressed and underexpressed genes is available as additional information from the editorial office and on our web site (<http://www.oeb.harvard.edu/hartl/lab>).

**Amino Acid Biosynthesis.** A total of 19 of the 26 most highly overexpressed genes in Fig. 3 are involved in amino acid biosynthesis, particularly methionine (12 *MET* genes), leucine (*LEU1*), histidine (*HIS4*, *HIS5*), arginine (*ARG4*, *CPA2*), and serine (*SER3* and *SER33*, which encode isozymes of phosphoglycerate dehydrogenase). Another 3 of the 26 are involved in sulfate (*SUL1*, *SUL2*) or ammonia (*MEP2*) transport. The remaining four are *ADE17*, which encodes an isozyme of 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; *DLD3*, encoding lactate dehydrogenase; *OAC1*, encoding a mitochondrial oxaloacetate transport protein; and *YNL276C*, an ORF with an unidentified function.

Gene expression in the methionine pathway is shown in Fig. 4. Both sulfur transporters, encoded in the genes *SUL1* and *SUL2*, are up-regulated in F1 relative to S2 by factors of 7.1 and 5.0, respectively. *MET3* is overexpressed by 12.7-fold; it is the most highly overexpressed gene in F1. Overexpression of other genes in the pathway is consistent with the positive regulator *MET28* (16) being overexpressed 4.0-fold, whereas the negative regulator *MET30* (17) is not overexpressed (1.3-fold). Functionally related to the methionine pathway and sulfite assimilation is siroheme biosynthesis, in which *MET1*, the gene for the first step, is overexpressed by a factor of 4.1.

On the other hand, *SAM1* and *SAM2*, which are involved in the synthesis of *S*-adenosylmethionine (AdoMet) downstream of methionine, are not overexpressed (1.2 and 1.3, respectively). Because the negative regulator *MET30* is induced by AdoMet, the 1.3-fold expression of *MET30* implies that AdoMet does not accumulate. Although *CYS3* and *CYS4*, which are involved in cysteine synthesis from the methionine pathway, are not overexpressed (1.3 and 1.0, respectively), the ORFs *YHR112C* and *YGL184C*, thought to be involved in the reverse reaction, are up-regulated by 2.1-fold and 2.7-fold, respectively, which implies that cysteine is shunted into the methionine pathway. The genes for histidine biosynthesis are also markedly up-regulated, with factors of overexpression of 2.3 for *HIS1*, 2.1 for *HIS2*, 1.9 for *HIS3*, 5.8 for *HIS4*, 2.8 for *HIS5*, 1.5 for *HIS6*, and 2.2 for *HIS7*. The substrate of Ade17p, 5-aminoimidazole-4-carboxamide ribonucleotide, is produced in the pathway of histidine biosynthesis by His7p, and *ADE17* is overexpressed 7.7-fold.

Steps in the leucine biosynthetic pathway are also up-regulated. *LEU1* is among the 26 leading overexpressed genes in Fig. 2, and *LEU2* is overexpressed by 3.5-fold. On the other hand, the key regulatory gene *LEU4* is not overexpressed. Many genes

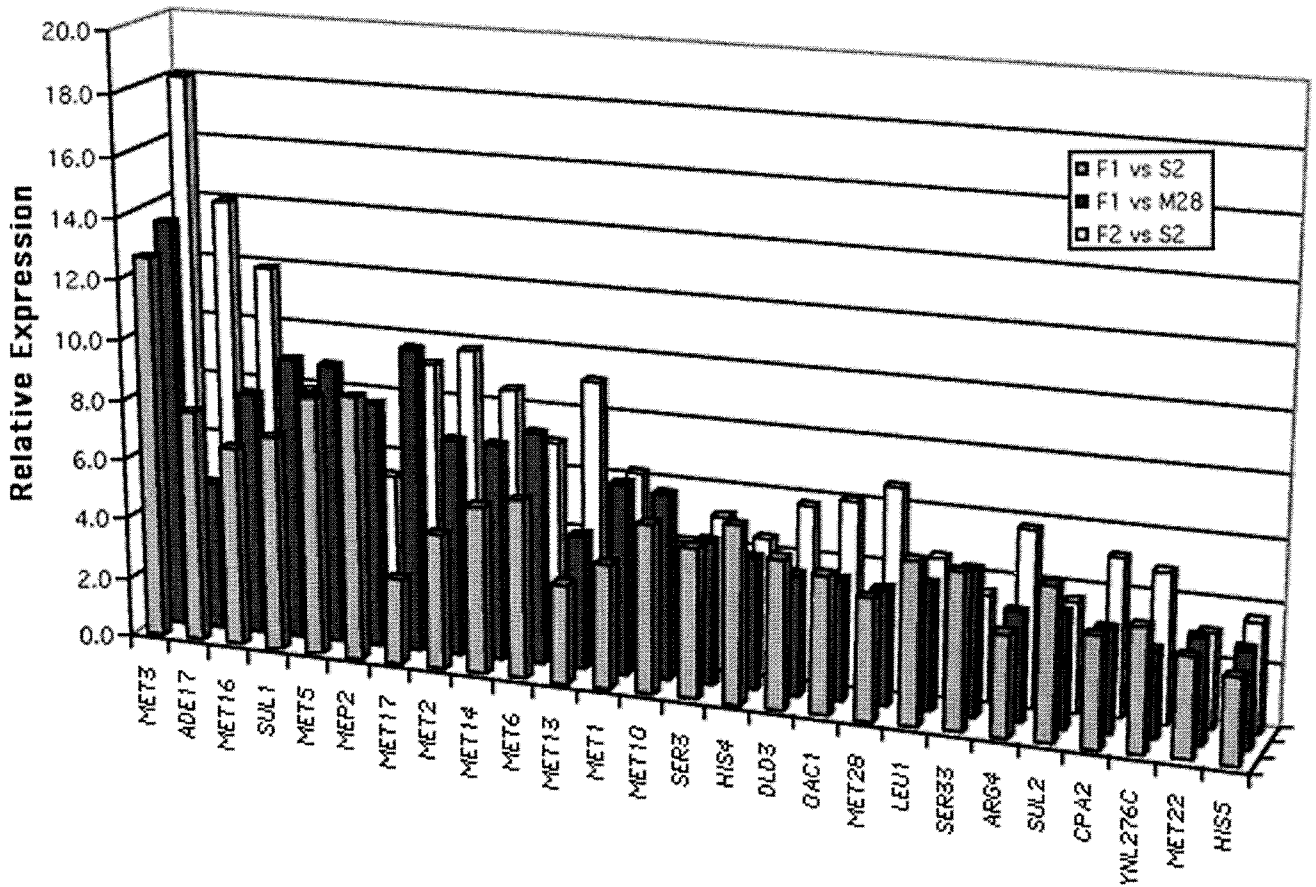


Fig. 3. Relative expression levels of all genes overexpressed 3-fold or more in comparisons of F1 with S2 (front), F2 with S2 (rear), and F1 with the parental strain M28 (middle). The overexpressed genes are arranged in order of their mean expression level across all three comparisons.

in the branched-chain amino acid biosynthetic pathway are also overexpressed: *BATI* is overexpressed 3.9-fold, and other genes in the isoleucine-valine pathway are overexpressed by factors of about 2. Excess leucine is metabolized into products including

isoamyl alcohol (18), a substance previously shown to induce filamentous growth in liquid culture (19).

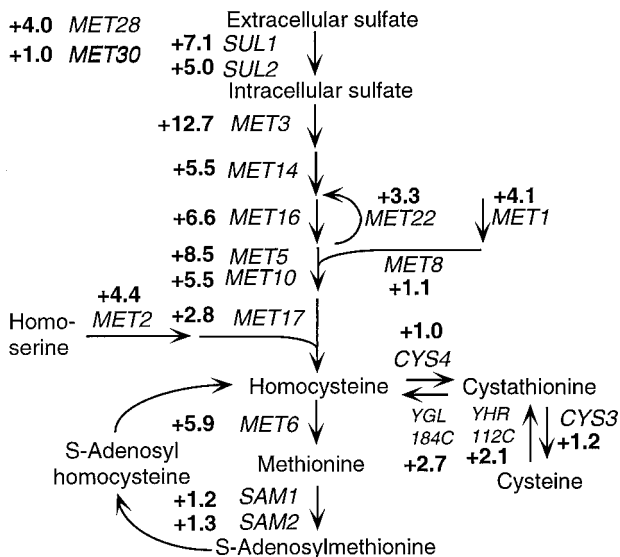


Fig. 4. Key steps related to the biosynthesis of methionine. The numbers are the relative levels of expression of the indicated genes.

**Bud and Filament Formation.** The filigreed growth habit encouraged us to examine ORFs implicated in morphogenesis. *RIM9* is overexpressed 3.5-fold. Its product regulates Rim101p and, through this mechanism, the meiotic activator *IME3*. Null mutants have a smooth colony morphology and are defective in invasive growth (20, 21). Also overexpressed 5.6-fold is the ORF *YOR225W*, which has been shown to be induced in haploid filamentous growth, where its mRNA level is significantly increased in cells overproducing Tec1p compared with *tec1* null mutants (22). Because *TEC1* is not overexpressed in the filigreed strains (0.82-fold), overexpression of *YOR225W* results from a different mechanism in this case. We note that *PHD1*, which is the main transcriptional regulator of filamentous growth in the morphogenetic pathway induced by ammonia starvation (23), is not markedly up-regulated (1.6-fold), nor is *MYO4* (1.5-fold), which in laboratory strains is reported to cause morphological abnormalities, including failure of cells to separate (24).

**Ammonia and Energy Metabolism.** Depletion of ammonia is among the metabolic costs of a high rate of amino acid biosynthesis, and although the ammonia transporter gene *MEP3* is not overexpressed (1.2-fold), *MEP1* and *MEP2* are overexpressed by factors of 2.5 and 8.7, respectively. Ammonia starvation through deletion of these genes has been reported to induce *PHD1* and filamentous growth (25). Among the 26 most highly overexpressed sequences is *OAC1* (4.5-fold), which links mitochondrial

metabolism with sulfur metabolism; the product of this gene is involved in small-molecule mitochondrial transport and is responsible for moving oxalacetate, sulfate, thiosulfate, and malonate through the mitochondrial membrane (26).

Several key enzymes in glycolysis and the pentose shunt are also overexpressed in F1 and F2 relative to S2. These include the gene *PFK27* (2.4-fold), which encodes isozyme 2 of 6-phosphofructo-2-kinase, and the genes for both isozymes of 6-phosphogluconate dehydrogenase, *GND1* (2.5-fold) and *GND2* (2.9-fold). *GPD2*, encoding glycerol-3-phosphate dehydrogenase (NAD<sup>+</sup>), is also up-regulated (2.1-fold).

**Down-Regulated Genes.** By the same stringent criterion used for the overexpressed ORFs, we identified the greatly underexpressed ORFs as those that are at least 3-fold underexpressed in all three comparisons of F1 with S2, F2 with S2, and F1 with M28. This criterion yields five genes. Four of these encode amino acid permeases (*BAP2* and *BAP3* for branched-chain amino acids, *GNP1* for glutamine, and *DIP5* for dicarboxylic amino acids). The remaining entry is *CHAI1*, which encodes a catabolic serine (threonine) dehydratase.

**Overall Expression Patterns.** Restricting our attention only to the comparison of F1 with S2 (Fig. 2A), 153 genes were overexpressed in F1 by a factor of 2 or more. The top 50 of these include all 26 genes in Fig. 3, along with *RIM9*, *GND2*, and still more genes involved in amino acid biosynthesis (*ARG1*, *BAT1*, *LEU2*, *GDH1*, and *GDH3*). Similarly, in the comparison of F1 with S2, 225 genes were underexpressed by a factor of 2 or more; the 20% of these with the lowest expression levels include all five of the greatly underexpressed genes identified in the previous comparisons, along with still more genes involved in amino acid transport (*ALP1*, *BAT2*, *BAP3*, *JEN1*, and *PUT4*) and, curiously, *GDH2*. Interestingly, among the eight genes with outlier expressions in the comparison F1 against F2 (Fig. 2B) is *GAP1*, a general amino acid permease, which is expressed 2.0-fold higher in F2 than in F1. This higher rate of expression could account for the somewhat more extreme filigreed phenotype of F2.

## Discussion

In this paper we demonstrate the feasibility and power of an approach combining classical Mendelian segregation analysis with microarray-based genomics to study variation in gene expression in a natural isolate of yeast from wine grapes. The approach holds promise for studies of quantitative variation in natural populations as well as applications in biotechnology. We find a large number of genes that are up-regulated or down-regulated by a factor of 2 or more, but the number of segregating loci is very small. Hence most of the metabolic differences segregate as a suite of traits. This conclusion is consistent with the overexpression of entire pathways (amino acid biosynthesis) or the underexpression of genes with similar functions (amino acid transport).

Up-regulation of the methionine biosynthetic pathway and alterations in sulfur and nitrogen assimilation are cardinal transcriptional indicators of the metabolic phenotype. This result might be significant in light of recent findings that demonstrate a connection between the methionine pathway regulators Met30p and Met4p and progression through the cell cycle (27–29), with consequent effects on morphogenesis (30). In any case, the interrelated suite of metabolic traits highlights the systemic interconnection between fundamental regulatory pathways acting at the level of transcription. Further analysis of this metabolic phenotype promises to be of great interest in shedding light on the still-obscure connections

between amino acid biosynthetic pathways and nutrient-mediated control of fundamental cellular events.

The scatterplot in Fig. 2B indicates that the parental strain M28 is not heterozygous for many genes that markedly affect the level of transcription. Only eight genes in the entire genome show overexpression or underexpression by a factor of 2 or more. However, we have identified two drug-resistance alleles that segregate 2:2 in M28. One confers resistance to trifluoroleucine. This trait segregates independently of the filigreed phenotype, but F1 and F2 happen to be resistant. Overexpression of *LEU4* has been reported to be associated with resistance in laboratory mutants (31), but *LEU4* is not markedly overexpressed in F1 and F2. The other heterozygous resistance allele confers resistance to copper sulfate. Adaptation of metabolism to sulfur in natural isolates is not surprising. For as long as 200 years, Tuscan vintners have been treating vineyards with copper sulfate to inhibit the growth of molds on the grapes, and sodium sulfite, potassium metabisulfite, and sulfur dioxide are widely used during and after fermentation to stabilize the wine and kill bacteria. Hence, it is likely that human intervention to control molds and bacteria has resulted in selection of vineyard yeasts for resistance to these agents. Resistance to copper sulfate in M28 also segregates independently of the filigreed phenotype, and S1 and F1 happen to be resistant. Although the copper transporters *CTR1* and *CTR3* are both underexpressed 1.8-fold in F1 relative to S2, which suggests a possible role in copper sulfate resistance, the genes are both underexpressed to the same level in F2, which is sensitive. Therefore, if *CTR1* and *CTR3* are involved in the resistance at all, their underexpression alone is not sufficient. The somewhat variable nature of the filigreed phenotype in different tetrads implies that modifiers of the phenotype are also heterozygous in M28. It is possible that some heterozygous genes that exist in M28 have less than a 2-fold effect on the transcriptional profile, which is why so little evidence of segregation is apparent in Fig. 2B.

The key result is that the wholesale differences in amino acid metabolism segregate 2:2 as a suite of traits. This suite of traits may be due to a single gene, a very small number of independent genes, or a group of linked genes. Whether the suite of metabolic traits is related to the filigreed phenotype we do not know. It is possible that, by chance, the metabolic and morphological phenotypes are completely independent and that they happened to cosegregate in this particular tetrad. Overall, differences in transcription of 2-fold or greater were observed for 378 genes, approximately 6% of the genome, representing a massive readjustment of metabolism reflected principally in increased expression of genes for amino acid biosynthesis and the transport of sulfur and ammonia, and decreased expression of amino acid permeases. The principal implication of this finding is that studies of expression variation in natural populations, without accompanying segregation analysis, may give a false picture of the true level of heterozygosity for genes affecting levels of transcription.

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