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Letter

Expression evolution in yeast genes of single-input modules is mainly due to changes in *trans*-acting factors

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Both *cis*- and *trans*-regulatory mutations contribute to gene expression divergence within and between species. To estimate their relative contributions, we examined two yeast strains, BY (a laboratory strain) and RM (a wild strain), for their gene-expression divergence by microarray. Using these data and published ChIP-chip data, we obtained a set of single-regulator-regulated genes that showed expression divergence between BY and RM. We randomly selected 50 of these genes for further study. We developed a step-by-step approach to assess the relative contributions of *cis*- and *trans*-variations to expression divergence by using pyrosequencing to quantify the mRNA levels of the BY and RM alleles in the same culture (co-culture) and in hybrid diploids. Forty genes showed expression divergence between the two strains in co-culture, and pyrosequencing of the BY/RM hybrid diploids showed that 45% (18/40) can be attributed to differences in *trans*-acting factors alone, 17.5% (7/40) mainly to *trans*-variations, 20% (8/40) to both *cis*- and *trans*-acting factors, 7.5% (3/40) mainly to *cis*-variations, and 10% (4/40) to *cis*-acting factors alone. In addition, we replaced the BY promoter by the RM promoter in each of 10 BY genes that were found from our microarray data to have expression divergence between BY and RM, and in each case our quantitative PCR analysis revealed a *cis* effect of the promoter replacement on gene expression. In summary, our study suggests that *trans*-acting factors play the major role in expression evolution between yeast strains, but the role of *cis* variation is also important.

[Supplemental material is available online at www.genome.org.]

Functional variation of a gene among individuals in a species can be due to nucleotide differences in coding regions or in regulatory elements. To date, most studies of molecular evolution have examined changes in protein-coding sequences (see Li 1997). Recently, several reviews argued that mutations in transcriptional regulation may have been a major cause for phenotypic evolution (Carroll 2000; Purugganan 2000; Stern 2000; Tautz 2000; Wray et al. 2003). Some studies have shown that natural genetic variations are heritable and can cause significant differences in gene expression (Brem et al. 2002; Cowles et al. 2002; Rockman and Wray 2002; Schadt et al. 2003). However, evolutionary changes in transcriptional regulation are still poorly understood.

Gene expression changes can arise from *cis*- or *trans*-changes, or both. Variation(s) in *cis*-regulatory elements can be defined as polymorphism(s) in functional motifs of the promoter region or the DNA element(s) located within the transcribed region of the gene and/or a short distance outside of the transcribed region that influences the gene-expression level or timing or the mRNA stability. On the other hand, *trans*-regulatory variations are changes that affect the timing, level, or activity of the transcription factors (TFs) or other regulators that control the expression of the target genes (Yvert et al. 2003; Wittkopp et al. 2004). Thus, the effect of *cis*-variation should be allele specific, whereas *trans*-variation can affect both alleles in a diploid.

The relative contributions of *cis*- and *trans*-regulatory variations to expression evolution remain controversial. Several studies have found cases where intraspecies divergence in gene expression arose from mutations in *cis*-regulatory elements, such as in the cases of *Gpdh* and *Cyp6g1* (the cytochrome P450 family) in *Drosophila melanogaster* (Laurie-Ahlberg and Bewley 1983; Daborn et al. 2002), beta-glucuronidase in *Mus domesticus* (Bush and Paigen 1992), and prolactin in the tilapia, *Oreochromis niloticus* (Streelman and Kocher 2002). Furthermore, recent studies reported that both intra- and interspecies divergences in gene expression during development among *D. melanogaster*, *Drosophila yakuba*, and *Drosophila simulans* are mainly due to *cis*-variations (Rifkin et al. 2003; Osada et al. 2006) and that the variations in expression levels of 29 genes between *D. melanogaster* and *D. simulans* are mainly due to *cis*-regulatory changes (Wittkopp et al. 2004).

However, using linkage analysis to map the genetic changes responsible for the expression differences of 2294 genes between the yeast strains BY4716 (a laboratory strain) and RM11-1a (a wild strain), Brem et al. (2002) and Yvert et al. (2003) found that the expression divergence of 1716 genes (~75%) between these

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two strains seemed to be mainly due to changes in *trans*-acting factors. More recently, Ronald et al. (2005) found that ~25% of the 5727 genes that they have tested contain local regulatory variation between these two strains. By measuring allele-specific expression for 77 of these local regulatory variation-containing genes in a hybrid diploid with quantitative real time PCR, they estimated that in 52%–78% of these genes, local regulatory variation acts in *cis*. However, in some special circumstances, local regulatory variation identified through linkage analysis actually conferred *trans*-regulatory effects (Ronald et al. 2005; Rockman and Kruglyak 2006). Combined results from these studies suggest that *trans*-variations play a major role in expression divergence in yeast, with contributions from *cis*-variations. This is a very interesting finding because it is in contrast to the conclusion of the studies in *Drosophila* (Rifkin et al. 2003; Wittkopp et al. 2004; Osada et al. 2006). However, the studies in both *Drosophila* and yeast have not attempted to identify the direct target genes of a regulator, except for the case of Amn1P (Brem et al. 2002; Ronald et al. 2005), and the regulatory pathways of these regulators have not been investigated in detail. Here, we used a different approach to study the relative contributions of *cis*- and *trans*-regulatory mutations to expression evolution in yeast.

First, we chose yeast single-input module (SIM) genes, each of which is putatively regulated by a single transcription factor (TF). Since a SIM gene is regulated by a single immediate-upstream TF, the chance for a change to occur in its *trans*-acting factors would, on average, be smaller than that for a multiple input module (MIM) gene, because a MIM gene is regulated by more TFs, and thus, likely by more *trans* factors than a SIM gene. For this reason, we hypothesize that the role of *trans* factors is less important for SIM genes than for MIM genes. Moreover, it would be simpler to figure out the molecular changes that cause the observed expression change in a SIM gene than in a MIM gene. To identify SIM genes we used data obtained by the ChIP-chip technique that combines chromatin-immunoprecipitation (ChIP) with DNA microarrays (chip) to map the promoter and its corresponding TFs (Lee et al. 2002; Harbison et al. 2004). Note that the current ChIP-chip data cannot guarantee that each of these genes is a true SIM gene, so they should be taken as "putative" SIM genes. However, such a gene is unlikely to be regulated by many TFs.

Second, we used two different budding yeast strains BY4741 (denoted as BY) and RM11-1a (RM). These two strains proliferate rapidly and have propagated under different environmental conditions for decades. As a result, these two strains display substantial divergence in gene expression and are ideal for studying expression divergence within species.

Third, we took advantage of the fact that yeast can propagate as haploids or diploids and that the pyrosequencing technique can be used to estimate the relative expression levels of two alleles in an mRNA pool if there is nucleotide polymorphism in the coding region. We first used microarrays to identify and select SIM genes that showed expression differences between the BY and RM strains. Next, we used pyrosequencing to check the expression differences of each selected gene when the two strains were grown in separate cultures, and selected those genes that showed significantly different expression levels for further analysis. The expression differences could be due to a combination of differences in environmental factors such as glucose concentration and genetic (*trans* or *cis*) factors. Then, we grew the two strains in the same culture (co-culture) and used pyrosequencing to estimate the relative expression levels of the two alleles of a gene (one from each of the two strains). This co-culturing should remove the environmental effects.

Fourth, we formed a BY/RM hybrid diploid and used pyrosequencing to study the relative expression levels of the two alleles of a gene in the diploid (where the two alleles are from the two different strains). This procedure should remove the effects of *trans* factors, so the remaining differences, if any, should be due to the *cis* effects.

Finally, for some of the genes selected we have also swapped the promoters of the two strains to verify the *cis* effects.

In summary, the experimental procedure we used provided a rigorous means to separate the *cis* and *trans* effects. As will be seen, our conclusion is that *trans* factors play a major role for the expression divergence between the two yeast strains, even for SIM genes. However, it will become clear that *cis* effects are also common. Since we are still in the process of obtaining data for MIM genes, we cannot yet test our hypothesis that the role of *trans* factors is less important for SIM genes than for MIM genes.

Results

SIM genes that show different expression levels between strains

We used microarrays to compare gene expression profiles of BY and RM cells from exponentially growing cells. There were 1360 genes that showed significant ($P < 0.5 \times 10^{-4}$) expression differences between the two strains (Supplemental Table 1). We used a collection of *Saccharomyces cerevisiae* TFs and their target genes predicted from ChIP-chip data (Harbison et al. 2004). This database includes 203 known TFs and their downstream target genes, from which we collected 1049 genes, each of which is uniquely bound by only one of 72 TFs at a significance level of $P < 10^{-3}$ (Harbison et al. 2004). By incorporating the single-input modules inferred from the ChIP–chip data (Lee et al. 2002; Harbison et al. 2004) with our expression data, we identified 219 putative single-regulator regulated (SIM) genes that showed different expression levels between the BY and RM strains (Supplemental Table 2).

Allele quantification of SIM genes

For pyrosequencing analyses, we randomly selected 50 SIM genes from a pool of 80 genes in Supplemental Table 2, for which the regulatory domain and DNA-binding domain of the upstream TFs are known (see Supplemental Table 3 for the gene list and DNA sequence comparison). An additional selection criterion used was that the gene should have polymorphism(s) in the transcribed region between BY and RM, which can be used as the target for pyrosequencing analysis.

We used pyrosequencing to check the expression differences in each selected gene between the BY and RM strains grown in separate cultures. We found that eight of the 50 selected genes showed similar expression levels, while the remaining 42 genes showed significantly different expression levels between the BY and RM strains (Supplemental Table 4). The observed expression differences in separate cultures could be due to a combination of environmental and genetic factors such as glucose concentration, *trans* factors, or *cis* factors. Next, we used pyrosequencing to quantify the relative expression levels of the RM/BY alleles of these 50 SIM genes when the strains are grown together in co-culture. We found four of these 50 genes did not show significant expression difference in both separate cultures and co-culture;

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these four genes were removed from further analysis. In addition, we found that six of the remaining 46 genes have similar allele expression levels under co-culture (RM/BY ≈ 1; Supplemental Table 4), but show different expression levels when grown separately. Since this co-culturing system should remove the environmental effects, our results indicated that 13% (6/46) of the expression divergence observed under separate cultures was mainly due to interactions between genetic factors and environmental factors. Interestingly, there were five genes that showed a similar expression level in separate cultures of the BY and RM strains, but showed significant expression difference in the BY and RM co-culture system. The nonsignificant difference in expression level under separate cultures could be in part due to large standard errors (two of the five genes had particularly large standard errors) or due to stronger effects of environmental factors than genetic factors (Supplemental Table 4). When grown in separate cultures, the RM strain grows faster and consumes glucose at a faster rate, and this difference in glucose concentration may affect the expression of some genes.

To further characterize the effects of *cis*- and *trans*-variations on the 40 genes that showed significant expression differences between the BY and RM alleles in the co-culture system, and are therefore likely to be affected by the genetic variations between BY and RM, we generated a BYa/RMα and a RMa/BYα hybrid diploid strain and examined the relative expression levels of the BY and RM alleles of the 40 genes in each strain by pyrosequencing (see Methods). Because the *trans*-acting effects should be the same in the same genetic background, an equal level of expression of the BY and RM alleles in a hybrid diploid indicates differences in *trans*-regulation. Therefore, if *trans*-regulatory divergence completely explains the expression difference of a given gene between the two strains, the allele-specific expression will be approximately the same in the hybrids. On the other hand, if the allele-specific expression of a given gene is different in the hybrid, the difference can be attributed to *cis*-regulatory divergence. If *cis*-regulatory divergence completely explains the expression difference of a given gene between the two strains, the allele-specific expression ratios in the hybrid and parental strains should be approximately equal ($RM_{parent}/BY_{parent} = RM_{hybrid}/BY_{hybrid}$). If the expression levels of the BY and RM alleles in a hybrid are different and the ratio RM_{parent}/BY_{parent} is different from RM_{hybrid}/BY_{hybrid}, the expression difference between the BY and RM alleles should be due to a combination of *cis*- and *trans*-regulatory effects. Our results, which are summarized in Table 1, showed that 45% (18/40) of these 40 cases can be attributed to the differences in *trans*-acting factors alone, 17.5% (7/40) mainly to *trans*-variations, 20% (8/40) to both *cis*- and *trans*-acting factors, 7.5% (3/40) mainly to *cis*-variations, and 10% (4/40) to *cis*-acting factors alone (Table 1).

In summary, variations in expression or sequence of *trans*-acting factors were mainly responsible for 62.5% of the expression difference observed between the BY and RM strains. In total, *trans*-acting factors affected 90% of the genes that showed expression differences between the BY and RM strains if the minor *trans*-effects were also included, while the corresponding value for *cis*-factors is only 55%.

Confirmation of the *cis*-regulatory effect by promoter swapping

We performed promoter-swapping experiments to confirm the *cis*-effects inferred by the SIM gene DNA sequence comparisons and microarray data. The candidate genes for promoter swapping were selected by three criteria: (1) the expression is significantly different between the BY and RM strains, (2) the expression of the corresponding upstream TF is similar for the BY and RM strains, and (3) the binding domain and activation domain of the TF have no nonsynonymous mutation between the BY and RM strains. Thus, these genes were selected to minimize *trans* effects and enhance *cis* effects. For each gene selected, there were SNPs identified in the intergenic region (which usually includes the promoter region) between the BY and RM strains, though the SNPs did not fall into the predicted TF-binding site (Fig. 1). The expression regulation of SIM genes appears to be more complicated than we expected, so we examined whether the *cis*-variation affected the gene expression after the intergenic region of the BY allele was replaced with the intergenic region of the RM allele. Seventeen genes with a significant difference between the BY and RM strains in expression level in the array data ($P < 1.0 \times 10^{-4}$) were selected for promoter-region swapping, and we succeeded in 10 cases; we did not have successful transformants for the remaining seven genes. The sequence comparisons of the promoter regions of these 10 genes are shown in Figure 1. There are 33, 24, 6, 6, 6, 5, 5, 5, 9, and 6 nucleotide differences in the promoter regions of YCR075C, YDL137W, YEL052W, YEL053C, YFL004W, YGR157W, YLR438W, YML075C, YOR306C, and YPL157W between BY and RM. After the replacement of the BY promoter sequence (300–800 bp) with the RM sequence, the expression of the gene was determined by quantitative real time PCR (qRT-PCR).

The relative expression levels of RM and BY (RM/BY, Column 4) and of the Swap strain and BY (Swap/BY, column 6) determined by qRT–PCR are summarized in Table 2. (Some of the specific promoter swapping had several different transformants that were from different transformation events, and they showed the same phenotype and similar qRT-PCR results. The qRT-PCR results for four of the 10 genes were actually obtained from two transformants.) For comparison, the RM/BY expression ratios estimated from our microarray data and pyrosequencing data are also listed in Table 2 (column 2). Although the RM/BY expression ratios estimated from microarray, real-time PCR, and pyrosequencing data are not identical, they do show the same trend; e.g., if the RM/BY ratio from the microarray data was <1, it was also <1 from the real time PCR and pyrosequencing data (Table 2). Under the assumption of no effect of *cis*-variation on the expression level of a gene, the Swap/BY ratio is expected to be 1. Therefore, our data indicate the presence of *cis*-effect in each of the 10 genes studied, because the Swap/BY ratio is different from 1 for all of the 10 genes (Table 2). For gene YDL137W, the estimated Swap/BY ratio is 1.45, which is similar to the estimate for the RM/BY ratio from qRT-PCR data (1.44, Table 2). This observation suggests that the BY allele with the promoter of the RM allele showed an expression level similar to that of the RM allele. In other words, the lower expression level of the RM allele was mainly due to its promoter region, so that the expression difference between the RM and BY alleles was mainly due to *cis*-effects. A similar comment applies to the gene YEL053C. However, for the remaining eight genes, the Swap/BY ratio was different from the RM/BY ratio in each case, so there was also a *trans*-effect in each case (Table 2). Therefore, for these eight genes, the expression divergence between BY and RM is due to variations in both promoter sequences and upstream *trans*-regulatory factors. This conclusion is largely consistent with the pyrosequencing data summarized in Table 1, though the data in Table 2 indicates a

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Table 1. Pyrosequencing results of the 40 genes that showed significant expression divergence between BY and RM

ORF[a]	Separate culture	Co-culture	BY(a) × RM(α) hybrid	RM(a) × BY(α) hybrid	Effect
YBL050W	0.92 ± 0.025	0.66 ± 0.057	1.03 ± 0.082	0.99 ± 0.050	*trans* alone
YDL084W	0.83 ± 0.019	0.86 ± 0.033	1.03 ± 0.034	1.04 ± 0.038	*trans* alone
YER103W	0.59 ± 0.063	0.53 ± 0.031	1.05 ± 0.048	1.03 ± 0.053	*trans* alone
YGL198W	0.95 ± 0.019	0.92 ± 0.032	1.02 ± 0.033	1.02 ± 0.032	*trans* alone
YGL248W	0.84 ± 0.066	0.80 ± 0.064	1.06 ± 0.031	1.05 ± 0.041	*trans* alone
YGR241C	1.07 ± 0.062	1.21 ± 0.090	1.00 ± 0.014	0.97 ± 0.044	*trans* alone
YHR146W	0.78 ± 0.030	0.61 ± 0.051	0.95 ± 0.026	0.98 ± 0.057	*trans* alone
YJL130C	1.01 ± 0.045	1.18 ± 0.037	1.00 ± 0.051	1.00 ± 0.042	*trans* alone
YJR077C	2.14 ± 0.151	1.95 ± 0.162	1.00 ± 0.034	1.00 ± 0.038	*trans* alone
YKL072W	0.49 ± 0.092	0.47 ± 0.080	1.04 ± 0.051	0.96 ± 0.057	*trans* alone
YKR054C	0.66 ± 0.098	0.67 ± 0.107	0.99 ± 0.076	1.02 ± 0.082	*trans* alone
YLR223C	0.85 ± 0.031	0.68 ± 0.024	0.99 ± 0.039	0.97 ± 0.052	*trans* alone
YNL094W	0.69 ± 0.044	0.70 ± 0.038	1.00 ± 0.045	1.03 ± 0.026	*trans* alone
YNL113W	1.12 ± 0.039	0.91 ± 0.029	1.00 ± 0.040	0.99 ± 0.047	*trans* alone
YNR050C	0.88 ± 0.029	0.81 ± 0.044	1.00 ± 0.044	1.00 ± 0.073	*trans* alone
YOL021C	1.02 ± 0.020	0.91 ± 0.040	1.00 ± 0.092	1.03 ± 0.093	*trans* alone
YOR326W	0.83 ± 0.064	0.90 ± 0.038	0.97 ± 0.026	0.97 ± 0.042	*trans* alone
YPR154W	0.80 ± 0.046	0.63 ± 0.046	0.98 ± 0.037	1.04 ± 0.050	*trans* alone
YAL037W	0.54 ± 0.032	0.78 ± 0.045	1.09 ± 0.036	1.10 ± 0.035	major *trans* effect
YCR075C	0.52 ± 0.028	0.62 ± 0.041	1.09 ± 0.036	1.06 ± 0.025	major *trans* effect
YDR232W	1.64 ± 0.120	1.58 ± 0.084	1.17 ± 0.050	1.19 ± 0.053	major *trans* effect
YLR438W	1.96 ± 0.104	2.70 ± 0.192	1.54 ± 0.049	1.57 ± 0.054	major *trans* effect
YML075C	4.11 ± 0.294	3.77 ± 0.449	1.21 ± 0.028	1.25 ± 0.050	major *trans* effect
YOL144W	0.69 ± 0.057	0.67 ± 0.078	1.19 ± 0.031	1.12 ± 0.045	major *trans* effect
YPL264C	0.37 ± 0.059	0.56 ± 0.080	1.09 ± 0.034	1.05 ± 0.021	major *trans* effect
YFL016C	0.83 ± 0.077	0.85 ± 0.025	0.83 ± 0.035	0.85 ± 0.029	*cis* alone
YFL037W	0.97 ± 0.044	0.89 ± 0.032	0.87 ± 0.033	0.88 ± 0.039	*cis* alone
YGL200C	1.05 ± 0.015	1.10 ± 0.042	1.10 ± 0.025	1.10 ± 0.039	*cis* alone
YIL117C	0.61 ± 0.056	0.83 ± 0.041	0.87 ± 0.046	0.86 ± 0.042	*cis* alone
YDL124W	0.35 ± 0.083	0.25 ± 0.026	0.40 ± 0.037	0.42 ± 0.072	major *cis* effect
YMR318C	0.67 ± 0.055	0.71 ± 0.021	0.77 ± 0.048	0.84 ± 0.040	major *cis* effect
YOR306C	0.46 ± 0.043	0.48 ± 0.025	0.55 ± 0.031	0.60 ± 0.060	major *cis* effect
YEL052W	0.62 ± 0.038	0.70 ± 0.065	0.83 ± 0.063	0.86 ± 0.038	both *cis* and *trans*
YGR157W	0.52 ± 0.030	0.57 ± 0.040	0.77 ± 0.021	0.80 ± 0.042	both *cis* and *trans*
YJR078W	0.38 ± 0.041	0.28 ± 0.066	1.54 ± 0.086	1.60 ± 0.065	both *cis* and *trans*
YKL152C	0.63 ± 0.091	0.64 ± 0.095	0.77 ± 0.041	0.78 ± 0.035	both *cis* and *trans*
YOL140W	1.18 ± 0.070	1.28 ± 0.033	1.11 ± 0.042	1.12 ± 0.041	both *cis* and *trans*
YOR049C	0.28 ± 0.054	0.28 ± 0.066	0.57 ± 0.064	0.64 ± 0.080	both *cis* and *trans*
YOR150W	1.72 ± 0.102	1.43 ± 0.155	1.16 ± 0.059	1.24 ± 0.057	both *cis* and *trans*
YPL157W	0.67 ± 0.055	0.63 ± 0.041	0.88 ± 0.035	0.83 ± 0.049	both *cis* and *trans*

Data presented are the expression ratios of the RM/BY alleles in separate cultures, in co-culture, and in two BY × RM hybrids. Each reaction was repeated at least three times from different RNA and cDNA samples.
[a]Systematic name.

somewhat more important role of *cis* variation than inferred from the result in Table 1. Note, however, that the 10 genes were selected for promoter replacement using the three criteria mentioned above. These criteria can lead to an overestimate of the contribution of *cis* elements to expression divergence.

Relationship between sequence polymorphism and *cis* versus *trans* effects

In comparing the DNA sequences of the two strains, we found that the polymorphism rate in the upstream intergenic regions of the genes whose expression divergence is affected mainly by *cis*-variation is higher than that in the upstream regions of the genes whose expression divergence was affected mainly by *trans*-variation (0.0102 vs. 0.0051 nucleotide differences per site, $P < 0.05$). All of the genes whose expression level is affected mainly by *cis*-regulatory variations show polymorphisms in their promoter (upstream) regions and in their coding regions (Table 3); however, in the coding regions, the polymorphism rate in genes with *cis*-regulatory variation is not significantly different from that in the genes with *trans*-regulatory variation (0.0047 vs. 0.0028). Similarly, Ronald et al. (2005) observed a greater polymorphism rate (0.0071) in the upstream regions of the 1233 genes showing self-linkage, compared with upstream regions of 3949 genes without self-linkage (0.0040); the increase was not restricted to the region most likely to contain regulatory elements, but extended for at least 1 kb upstream of the genes. They also observed that the polymorphism rate was also higher in the coding regions of genes showing self-linkage (0.0044 vs. 0.0029).

Discussion

Our allele quantification analysis with pyrosequencing showed that *trans*-acting factors affect most of the expression differences observed between the BY and RM strains, in accordance with the observation of Brem et al. (2002), Yvert et al. (2003), and Ronald et al. (2005). Indeed, our data suggested that 90% of genes with expression difference between BY and RM are affected by *trans*-acting regulatory factors, though half of them are also influenced by differences in *cis*-acting regulatory elements. Taken together, our results show that even for SIM genes, which likely have only one or few immediate upstream TFs, *trans*-regulatory variation plays a major role in expression divergence between the two yeast strains. Therefore, it is reasonable to speculate that *trans*-variation is a major determinant in the genome-wide expression

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divergence in the yeast *S. cerevisiae*. Yvert et al. (2003) reported that 1716 of the 2294 genes that showed expression differences between BY and RM are not self-linked in the genome. The data suggested that 75% (1716/2294) of the genes are affected by *trans*-variation between BY and RM. Their results also suggested that most regulatory genetic variation does not reside in the TFs themselves, because most of the *trans*-variation effect observed between BY and RM does not map to the immediate upstream TFs. In some special circumstances, local regulatory variation identified through linkage analysis actually confers *trans*-regulatory effects (Ronald et al. 2005; Rockman and Kruglyak 2006). Ronald et al. (2005) examined 77 genes that showed self-linkage for the presence of allele-specific expression (ASE) in a diploid hybrid of the two parent strains, BY and RM (*cis*-variance will cause allele-specific expression in a diploid hybrid). Their results showed that 57% (44) of the 77 assayed genes have ASE, suggesting that *trans*-acting local variation is likely to be responsible for a minority of the self-linkages tested. Overall, these data and our data suggest that *trans*-variation plays a major role in expression divergence in yeast.

It has been suggested that promoters may be more evolvable than coding regions (Gerhart and Kirschner 1997; Carroll et al. 2001). Even though our allele quantification with pyrosequencing showed that only 10% of SIM genes were affected by the variations in *cis*-regulatory elements alone, the data also showed that 45% of SIM genes were affected by variations in both *cis*- and *trans*-regulatory elements. Indeed, the expression in promoter-swapped strains showed that the polymorphisms in the promoter region contributed to the expression divergence of the two yeast strains in each of the 10 genes studied (Table 2). On the other hand, a change in a regulator may affect the expression of multiple downstream genes. One example in our study is the *HAP4* gene. The *HAP4* mRNA level in the wild strain is 18% higher than the level in the laboratory strain, and it may be responsible or partially responsible for the expression difference of 42% (8/19) of its target genes between the BY and RM strains (our microarray data). *HAP4* was suggested to act as a target of carbon-source regulation, because its mRNA level increased about 10-fold during nonfermentative conditions (Forsburg and Guarente 1989; DeRisi et al. 1997). Evolution of *trans*-acting factors may be related to the relaxation of selection pressure or adaptation to the rich medium growth condition in the laboratory strain (Gu et al. 2005).

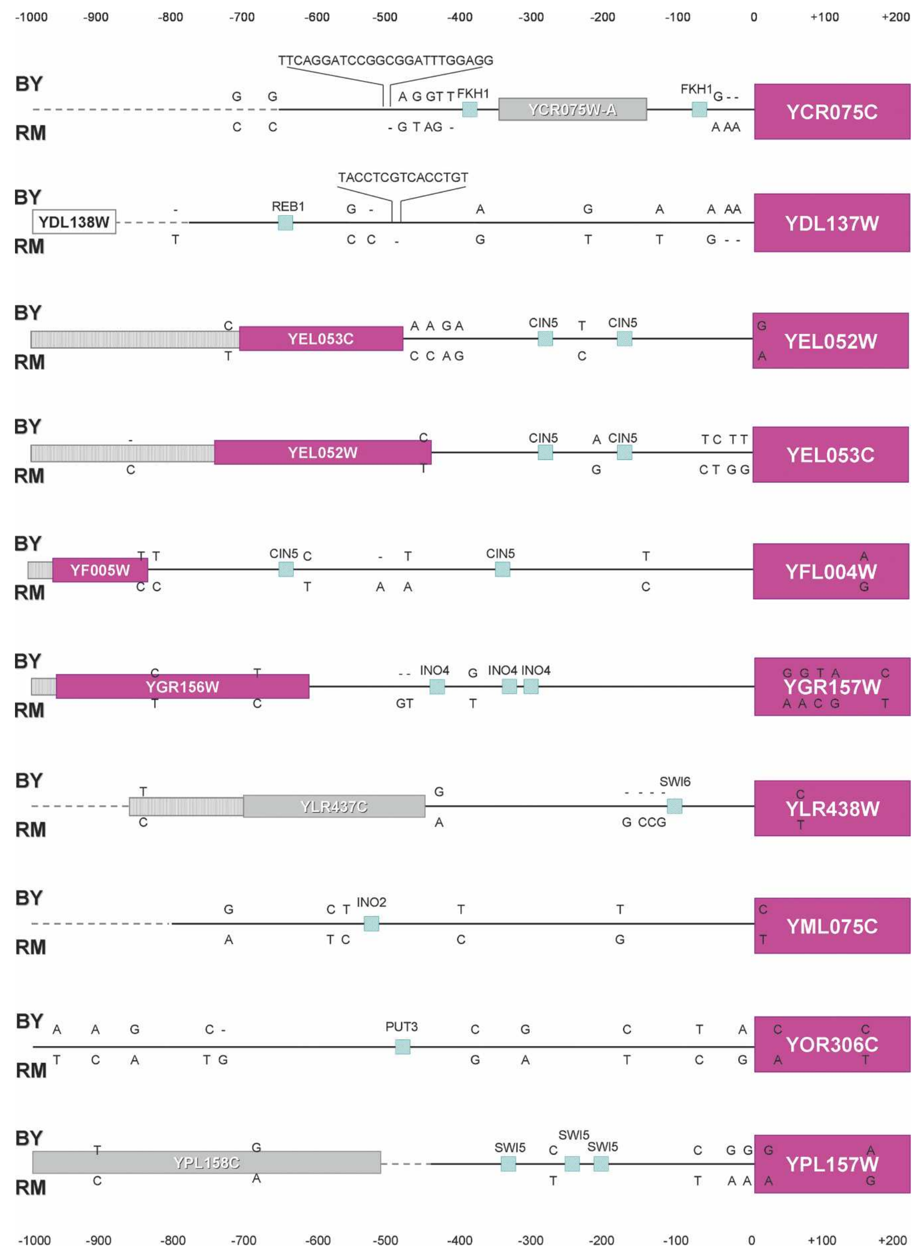

Figure 1. Physical map of 10 promoter regions of the BY and RM strains. The SNP positions of BY and RM were labeled; a dash denotes the absence of nucleotide. Black lines and pink boxes denote the swapped promoter region and the coding region, respectively. Gray boxes denote putative (uncharacterized) genes. Dotted line and checker denote the unswapped intergenic region and the coding region, respectively. The blue cubes indicate the TFs binding site quoted from the SGD database with the data of Harbison et al. (2004).

Cis-regulatory variations are more readily studied at the molecular level compared with *trans*-regulatory variations. The effect of *trans*-regulatory variations is difficult to pursue at the molecular level because *trans*-regulatory variations could be due to differences in any combination of expression level, DNA-binding affinity, cofactor interaction, and post-translation modification of upstream factors.

The influence of changes in *cis*- and/or *trans*-regulatory elements during evolution is still a controversial issue. The conclusion can vary from study to study, depending on the organism studied (Brem et al. 2002; Schadt et al. 2003; Yvert et al. 2003;

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Table 2. Relative gene expression levels of the SIM genes between promoter-swapped strains and the BY4741 strain

	Ratio RM/BY						
SIM gene[a]	Arrays	Pyroseq.	ΔΔCt RM/BY	Ratio RM/BY (95% CI)	ΔΔCt Swap/BY	Ratio Swap/BY (95% CI)	Effect
YCR075C	0.74	0.52	0.59 ± 0.16	0.67 (0.53–0.81)[b]	−0.11 ± 0.09	1.08 (0.96–1.2)	major *trans* effect
YDL137W	1.27	NA	−0.52 ± 0.10	1.44 (1.24–1.64)[b]	−0.53 ± 0.03	1.45 (1.39–1.51)[c]	*cis* alone
YEL052W	0.83	0.62	1.04 ± 0.18	0.49 (0.35–0.63)[c]	0.57 ± 0.13	0.67 (0.55–0.79)[c]	both *cis* and *trans*
YEL053C	0.84	NA	0.60 ± 0.23	0.67 (0.45–0.89)[b]	0.64 ± 0.12	0.64 (0.54–0.74)[c]	*cis* alone
YFL004W	0.68	NA	1.00 ± 0.23	0.50 (0.32–0.68)[c]	−0.40 ± 0.17	1.33 (1.01–1.65)	both *cis* and *trans*
YGR157W	0.66	0.52	1.71 ± 0.27	0.31 (0.19–0.43)[c]	0.92 ± 0.32	0.54 (0.28–0.80)[b]	both *cis* and *trans*
YLR438W	1.31	2.70	−1.57 ± 0.31	3.02 (1.74–4.3)[b]	0.12 ± 0.03	0.92 (0.88–0.96)[c]	major *trans* effect
YML075C	1.97	3.77	−1.18 ± 0.07	2.27 (2.05–2.49)[c]	−0.38 ± 0.08	1.30 (1.14–1.46)[b]	major *trans* effect
YOR306C	0.58	0.46	1.42 ± 0.21	0.38 (0.28–0.48)[c]	0.25 ± 0.08	0.84 (0.74–0.94)[b]	major *trans* effect
YPL157W	0.93	0.67	0.31 ± 0.13	0.81 (0.67–0.95)[b]	0.08 ± 0.08	0.95 (0.91–0.99)[b]	major *trans* effect

Note: For real time PCR data, ratio (RM/BY) or (Swap/BY) = $2^{[-\Delta\Delta Ct]}$, ΔΔCt = [ΔCt(RM or Swap) − ΔCt(BY)]. If ratio (Swap/BY) = 1, *trans*-effect alone; if ratio (Swap/BY) ≠ 1, *cis*-effect was involved. If ratio (RM/BY) = ratio (Swap/BY), *cis*-effect alone; if ratio (RM/BY) ≠ ratio (Swap/BY), both *cis* and *trans*. If the 95% confidence intervals for RM/BY and Swap/BY have only a minor overlap, we conclude that both *cis*- and *trans*-effects were involved. The null hypothesis of Swap/BY = 1 was tested by the two-tail Student's *t*-test and so was the null hypothesis of RM/BY = Swap/BY. Each reaction was repeated at least three times from different RNA and cDNA samples.
[a]Systematic name.
[b]Significantly different from 1 ($P < 0.05$) by *t*-test.
[c]Significantly different from 1 ($P < 0.01$) by *t*-test.

Morley et al. 2004; Wittkopp et al. 2004; Hughes et al. 2006; Osada et al. 2006). Results from studies of *Drosophila* showed that the majority of expression divergence observed both between and within species is due to the variations in the *cis*-regulatory elements (Wittkopp et al. 2004; Hughes et al. 2006; Osada et al. 2006). The contribution of *trans*-differences observed between species of fruit flies might not be the original source of expression divergence, but may have accumulated after speciation (Wittkopp et al. 2004). On the other hand, since the yeast data are from different strains in *S. cerevisiae*, the *trans* variation considered here represents within-species polymorphism.

Methods

Yeast strains and maintenance

The laboratory strains BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) are descendants of S288C. The wild strains RM11-1a (*MATa leu2Δ0 ura3Δ0 ho::KAN*) and RM11-1α (*MATα lys2Δ0 ura3Δ0 ho::KAN*) are gifts from Dr. Lee Hartwell (Fred Hutchinson Cancer Research Center, Seattle, WA). They were haploid strains derived from Bb32(3), a natural isolate collected by Robert Mortimer (Mortimer et al. 1994). WL201 is a hybrid strain of BY4741 × RM11-1α, and WL202 is a hybrid strain of BY4742 × RM11-1a; both were constructed in our lab. Yeast strains were maintained on YPAD.

Growth conditions and extraction of nucleic acids

Yeast strains were grown in YPAD and harvested at the mid-log phase. Overnight yeast cultures were used to prepare the starting cultures with $OD_{600} = 0.1$ and were grown in YPAD medium at 30°C with 250 rpm shaking. The yeast cells were harvested at the $OD_{600} = 1.0$, and the total RNAs were extracted by the hot acid phenol-chloroform method. The mRNA was isolated by using the QIAGEN Oligotex mRNA purification kit (QIAGEN) following the manufacturer's instructions.

Microarray analysis

Each microarray experiment was conducted with 0.5 µg of purified mRNA from each strain. The purified mRNA and oligo dT primers were used for cDNA synthesis with aminoallyl-modified dUTP (Sigma). The reverse transcription was performed following the manufacturer's instructions of the Super-script II kit (Invitrogen). The cDNA samples were cleaned up by Microcon-30 microconcentrators (Millipore) and were labeled with either Cy3 or Cy5 by coupling reactions for microarray experiments. The labeled cDNA samples were purified with the Qiaquick PCR purification kit (QIAGEN) and combined as a cy3/cy5 paired sample. The probes, 40-mer yeast oligos from MWG, were spotted on UltraGAPS coated slides (Corning), and were cross-linked to the slide by UV before use. The hybridization of cDNA samples to the probes was done with the MAUI hybridization System (Biomicro) at 42°C for 16 h. The microarray was scanned with GenePix 4000B microarray scanner (Axon Instruments) with the GenePix 5 software package. Each experiment was repeated eight times. Dye-swapping was also performed in each set of experiments to eliminate dye bias (a total of 16 slides). The microarray data obtained from the GenePix 5 were further normalized by the local regression intensity-dependent normalization method (the LOWESS fit), and the scale normalization method.

In order to extract the effect of interest and filter noise resulting from differences in dye-labeling efficiency and variation between/within each array, we apply the two-stage strategy: (1)we use the standard normalization procedure to normalize log ratios of the raw data, and (2) we use an ANOVA model for the normalized log ratios to estimate the significant levels of the effect of interest.

For the first stage, the composite normalization strategies between/within each array in Yang et al. (2002) can be summarized by the following statistics:

$$\log_2 Y_{gr} = \left[\left[\frac{\log_2 Ratio_{igr} - \log_2 \alpha_i(A)}{\beta_i}\right]\right]$$

The term $\log_2 Y_{gr}$ is the normalized log ratio, which is normalized by the location normalization within array and scale normalization between arrays, $Ratio_{igr} = R_{igr}/G_{igr}$, where R_{igr} and G_{igr} represent the raw data of red and green fluorescent intensity in array i for gene g and replicate r, $A = \log_2\sqrt{R_{igr}G_{igr}}$, the $\log_2(\alpha_i(A))$ term means intensity-dependent normalization, the normalization within array, which can be estimated by the LOWESS fit method, and β_i is the scale normalization, the normalization between ar-

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Table 3. Expression level of TFs (microarray data) and polymorphism rate of the downstream SIM genes

TF	Expression ratio RM/BY	No. of aa changes in TF CD	Target gene[a]	No of nt changes in promoter of target gene	No. of nt changes in Target gene CDs	Effect
CIN5	1.020	2	YEL052W	5	12	both *cis* and *trans*
CIN5	1.020	2	YOR049C	6	7	both *cis* and *trans*
GCN4	1.011	2	YOL140W	5	8	both *cis* and *trans*
HSF1	1.093	6	YKL152C	1	1	both *cis* and *trans*
INO4	1.030	0	YGR157W	2	25	both *cis* and *trans*
REB1	1.082	0	YOR150W	12	1	both *cis* and *trans*
SWI5	1.008	3	YPL157W	4	4	both *cis* and *trans*
SUM1	0.940	0	YJR078W	4	7	both *cis* and *trans*
FKH1	0.948	4	YFL037W	2	3	*cis* alone
GCN4	1.011	2	YIL117C	5	3	*cis* alone
HSF1	1.093	6	YFL016C	9	8	*cis* alone
REB1	1.082	0	YGL200C	3	1	*cis* alone
PUT3	1.104	8	YOR306C	11	13	major *cis* effect
YAP7	1.031	0	YDL124W	13	9	major *cis* effect
YAP7	1.031	0	YMR318C	8	3	major *cis* effect
CIN5	1.020	2	YAL037W	0	2	major *trans* effect
FKH1	0.948	4	YCR075C	3	3	major *trans* effect
GCN4	1.011	2	YPL264C	0	3	major *trans* effect
INO2	1.021	0	YML075C	2	11	major *trans* effect
REB1	1.082	0	YOL144W	2	14	major *trans* effect
STB5	1.139	5	YDR232W	6	6	major *trans* effect
SWI6	0.973	4	YLR438W	3	4	major *trans* effect
CBF1	0.604	2	YNL094W	2	3	*trans* alone
DAL82	0.928	2	YOL021C	2	2	*trans* alone
FKH1	0.948	4	YDL084W	0	3	*trans* alone
FKH1	0.948	4	YKR054C	3	45	*trans* alone
GCN4	1.011	2	YJL130C	2	17	*trans* alone
GCN4	1.011	2	YNR050C	4	2	*trans* alone
HSF1	1.093	6	YER103W	6	1	*trans* alone
HSF1	1.093	6	YPR154W	0	2	*trans* alone
REB1	1.082	0	YBL050W	4	1	*trans* alone
REB1	1.082	0	YGL198W	4	3	*trans* alone
REB1	1.082	0	YGL248W	1	1	*trans* alone
REB1	1.082	0	YGR241C	6	5	*trans* alone
REB1	1.082	0	YHR146W	8	10	*trans* alone
REB1	1.082	0	YKL072W	0	1	*trans* alone
REB1	1.082	0	YLR223C	3	4	*trans* alone
REB1	1.082	0	YNL113W	17	1	*trans* alone
REB1	1.082	0	YOR326W	1	6	*trans* alone
SUM1	0.940	0	YJR077C	4	3	*trans* alone

[a]Systematic name.

rays, which can be estimated by the median absolute deviation *MAD* (see Yang and Speed 2002; Yang et al. 2002).

For the second stage, we first write down the model and then explain the implication. The ANOVA model for the normalized log ratios is, according to Equation 1 of Landgrebe et al. (2006):

$$\log_2 Y_{gr} = \gamma_{Rg} - \gamma_{Gg} + \tau_{kg} - \tau_{k'g} + \varepsilon_{gr},$$

where γ_{Rg} and γ_{Gg} represent the dye-gene interaction term for the dye effect on Cy5 and Cy3 dyes, respectively, $\tau_{kg} - \tau_{k'g}$ indicates the term of interest that reflects gene-specific differences in expression for two varieties (k,k') (like two varieties of BY and RM strains in this paper), and ε_{gr} denotes the noise. For modeling aspects and applications, see Landgrebe et al. (2006).

The above model can be represented in the matrix form and the corresponding test statistics can be derived for detecting the significant difference in gene expression between BY and RM:

$$\underset{\sim}{Z}_g = X\underset{\sim}{\beta}_g + \underset{\sim}{\varepsilon}_g,$$

$$t_g = (\tau_{1g} - \tau_{2g}) / \sqrt{s_g^2 l^T (X'X)^{-1} l},$$

where $Z_{gr} = \log_2 Y_{gr}$, $\underset{\sim}{\beta}_g^T = [\gamma_{Rg}\ \gamma_{Gg}\ \tau_{1g}\ \tau_{2g}]$, X is the corresponding design matrix of the model; l^T = (0 0 1 -1), and s_g^2 is the sample variance of g. Similar to Landgrebe et al. (2006), we assume that the error is normally distributed with mean 0 and a common variance, so that the test statistics follows a t-distribution with the degree of freedom of $v_g = n_g$-rank(X), where n_g is the number of observations on gene g and rank(X) is the rank of the design matrix. The corresponding significant level, the P-value, can be calculated from the above test statistic for each of the genes under study.

Collection of single TF-regulated genes

We used a collection of *Saccharomyces cerevisiae* transcriptional regulators and their target genes predicted from ChIP-chip data (http://jura.wi.mit.edu/young_public/regulatory_code/GWLD.html) (Harbison et al. 2004). This database includes 203 known TFs and their downstream target genes, from which we collected 1049 genes, each of which is uniquely regulated by only one of 72 TFs at a significance level of $P < 10^{-3}$.

Polymorphisms in TFs and promoter regions

The DNA sequences of the BY4741 strain were downloaded from the *Saccharomyces* Genome Database (http://www.yeastgenome.

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org/). Sequences for TFs and promoters of their target genes from RM11-1a were determined using an ABI 377A automated DNA sequencer and BigDye sequencing kits (Applied Biosystems) in our lab or obtained from Broad Institute (http://www.broad.mit.edu/annotation/). The DNA sequences of TFs and the promoter regions (intergenic regions) and coding sequences of downstream target genes in both strains were aligned.

Allele quantification with pyrosequencing

An aliquot of 5 µg of total RNA from each strain was used for cDNA synthesis. The reverse transcription was carried out with oligo-dT primers and the Super-script II kit (Invitrogen) following the manufacturer's instructions. After identification of strain-specific nucleotide differences, a 150–200-bp fragment from each gene in two hybrid strains, WL201 and WL202, and in the co-culture of parental strains, BY4741 and RM 11-1a, were amplified and sequenced using primer sequences listed in Supplemental Table 5. Pyrosequencing reactions were used to measure the relative abundances of the two alleles in genomic DNA and in cDNA samples from both parental and hybrid pools and were performed according to the manufacturer's instructions (http://www.pyrosequencing.com/). Pyrosequencing software reports a peak height directly proportional to the number of molecules incorporated into the growing DNA chain. The ratio of allele-specific frequencies (RM_{parent}/BY_{parent}, RM_{hybrid}/BY_{hybrid}), which corresponds to the relative abundances of the BY and RM alleles in the starting sample, was also reported by the pyrosequencing software, PSQ 96MA 2.1.1. The cDNA ratios were normalized with genomic DNA measurements as described in Wittkopp et al. (2004). Because both alleles are extracted and measured in a single sample, this method is insensitive to differences in extraction efficiency and eliminates the need for control genes or quantification of total RNA recovery.

Let BY_{hybrid} and RM_{hybrid} be the expression levels of the BY and RM alleles in the hybrid diploid and let BY_{parent} and RM_{parent} be the expression levels of the BY and RM alleles when the two strains are grown in the same culture. Then, the *trans*- and *cis*-effects on the expression differences between BY_{parent} and RM_{parent} can be judged by the following guidelines:

1. If $RM_{hybrid} = BY_{hybrid} \rightarrow$ *trans*-effect alone
2. If $RM_{hybrid} \neq BY_{hybrid}$ and
   (a) $RM_{parent}/BY_{parent} = RM_{hybrid}/BY_{hybrid} \rightarrow$ *cis*-effect alone
   (b) $RM_{parent}/BY_{parent} \neq RM_{hybrid}/BY_{hybrid} \rightarrow$ both *cis*- and *trans*-effects

The null hypothesis of $RM_{hybrid}/BY_{hybrid} = 1$ was tested by the two-tail Student's *t*-test, and so was the null hypothesis of $RM_{parent}/BY_{parent} = RM_{hybrid}/BY_{hybrid}$.

Because the *trans*-acting effects should be the same in the same genetic background, an equal expression level of the BY and RM alleles in a hybrid diploid indicates differences in *trans*-regulation. Therefore, if *trans*-regulatory divergence completely explains the expression difference of a given gene between the two strains, the allele-specific expression will be approximately the same in the hybrids. However, if the allele-specific expression of a given gene is different in the hybrid, the difference can be attributed to *cis*-regulatory divergence. If *cis*-regulatory divergence completely explains the expression difference of a given gene between the two strains, the allele-specific expression ratios of hybrid and parental strains should be approximately equal ($RM_{parent}/BY_{parent} = RM_{hybrid}/BY_{hybrid}$). If the expression levels of the BY and RM alleles in a hybrid are different and the ratio RM_{parent}/BY_{parent} is different from RM_{hybrid}/BY_{hybrid}, the expression difference between the BY and RM alleles should be due to a combination of *cis*- and *trans*-regulatory effects

To further characterize the major effecter in the group of genes affected by both *trans*- and *cis*-variations between BY and RM, we divide the distance from the ratio RM_{parent}/BY_{parent} to 1 into three sections: (1) major *trans*-effect, if RM_{hybrid}/BY_{hybrid} is close to 1; (2) both *trans*- and *cis*-effect, if RM_{hybrid}/BY_{hybrid} is close to the middle between 1 and RM_{parent}/BY_{parent}; (3) major *cis*-effect, if RM_{hybrid}/BY_{hybrid} is close to RM_{parent}/BY_{parent}. In some cases, RM_{hybrid}/BY_{hybrid} is considerably higher than 1, while RM_{parent}/BY_{parent} is lower than 1. In such a case, if the distance from RM_{hybrid}/BY_{hybrid} to 1 is similar to that from RM_{parent}/BY_{parent} to 1, the expression difference is due to both *cis*- and *trans*-effect, and if the distance from RM_{hybrid}/BY_{hybrid} to 1 is substantially larger than from RM_{parent}/BY_{parent} to 1, it is major *cis*-effect.

If the *trans*-acting factors are the major effecter for the expression divergence between the BY and RM alleles observed in the co-culture conditions, the expression levels of the BY and RM alleles will be approximately equal in a hybrid diploid genetic background, because the *trans*-acting effects should be the same in the same genetic background. Therefore, if *trans*-regulatory divergence explains the majority of the expression difference of a given gene between the two strains, the allele-specific expression will be close to that in the hybrids. However, if *cis*-regulatory divergence explains the majority of the expression difference of a given gene between two strains, the allele-specific expression ratios of hybrid could be close to or higher than the parental strains ($RM_{parent}/BY_{parent} \approx RM_{hybrid}/BY_{hybrid}$).

Swapping of promoter elements

The construction was done by PCR-based mutagenesis involving two sequential steps (Gray et al. 2004). The gene of interest was first replaced by an *URA3* cassette with ~45 bp flanking homologous regions to the gene of interest at both ends. The transformation is performed by the LiOAc/SS Carrier DNA/PEG method (Gietz and Woods 2002) and transformants were selected as Ura^+ colonies. The insertion of *URA3* at the targeted site was confirmed by diagnostic PCR and sequencing. The inserted *URA3* was further replaced by a second transformation with the appropriate fragment into the *URA3*-inserted strain. The transformants were selected by 5-FOA counter selection. Only the strains that carried the desired sequence will survive and form colonies on the medium with 5-FOA (1 µg/mL). The constructions at the targeted site were confirmed by diagnostic PCR and sequencing.

Expression shift examined by real-time PCR

An aliquot of 0.5 µg of purified mRNA from each strain was used for cDNA synthesis and the reverse transcription was carried out with oligo-dT or random hexamer primers following the manufacture's instructions of the Super-script II kit (Invitrogen). Real-time PCR analyses were carried out in a final volume of 25 µL that contained 2 µL of diluted cDNA (1 ng/µL), 1 µL each of gene-specific forward and reverse primers (5 µM), and 12.5 µL of SYBR Green Supermix from Bio-Rad with 40 cycles of 95°C for 15 sec and 60°C for 1 min. The primers were designed by using the Primer Express software from Applied Biosystems. The relative expression of each gene was normalized to that of the *Act1* gene (ΔCt) and quantified with the $\Delta\Delta Ct$ relative quantification method and the relative expression ratio was calculated following ABI's suggestion, ratio (RM/BY) = $2^{[-\Delta\Delta Ct]}$. The amplification efficiency of each primer pair was tested by using twofold serial dilutions of the templates as suggested by Applied Biosystems and the amplification efficiency of the target gene and the refer-

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ence gene were approximately equal. The influence of *cis*- or *trans*-effect was determined by the same guideline as used for analyzing the pyrosequencing data.

1. if ratio Swap/BY = 1 → *trans*-effect
2. if ratio Swap/BY ≠ 1 and
   (a) if ratio RM/BY = ratio Swap/BY → *cis*-effect alone
   (b) if ratio RM/BY ≠ ratio (Swap/BY) → both *cis*- and *trans*-effect

The null hypothesis of Swap/BY = 1 was tested by the two-tail Student's *t*-test, and so was the null hypothesis of RM/BY = Swap/BY.

Primers

Primers used for gene disruption, promoter swapping, and allele quantification are listed in Supplemental Table 5.

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