Transcriptional cosuppression of yeast *Ty1* retrotransposons

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Cosuppression, the silencing of dispersed homologous genes triggered by high copy number, may have evolved in eukaryotic organisms to control molecular parasites such as viruses and transposons. *Ty1* retrotransposons are dispersed gene repeats in *Saccharomyces cerevisiae*, where no cosuppression has been previously observed. *Ty1* elements are seemingly expressed undeterred to a level as high as 10% of total mRNA. Using *Ty1*-*URA3* reporters and negative selection with 5-fluoroorotic acid, it is shown that *Ty1* genes can undergo transcriptional cosuppression that is independent of DNA methylation and polycomb-mediated repression. Expression of *Ty1*-related genes was shown to be in one of two states, the coexpressed state with all *Ty1*-related genes transcribed or the cosuppressed state with all *Ty1*-related genes shut off, without uncoordinated or mosaic expression in any individual cell. Rapid switches between the two states were observed. A high copy number of *Ty1* elements was shown to be required for the initiation of *Ty1* homology-dependent gene silencing, implying that *Ty1* gene expression is under negative feedback control. *Ty1* transcriptional repressors facilitated the onset of *Ty1* cosuppression, and the native *Ty1* promoters were required for *Ty1* cosuppression, indicating that *Ty1* cosuppression occurs at the transcriptional level.

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Cosuppression, which has been defined as high gene copy number-triggered homology-dependent gene silencing (Jorgensen 1995), may have evolved in eukaryotes to control invasive molecular parasites such as viruses and transposons (Wolfe and Matzke 1999). Cosuppression first came to attention when attempts to boost gene expression in transgenic plants resulted in cosilencing of both the transgenes and the homologous endogenous genes. Cosuppression can be distinguished from other forms of gene silencing by the high target gene copy number-dependent initiation and homology-dependent spreading. Cosuppression has been observed in many organisms, and it appears in two forms, posttranscriptional gene silencing (PTGS, also known as RNA interference or RNAi) and transcriptional gene silencing (TGS). In PTGS, homologous genes are silenced by homologous RNA duplex-mediated mRNA degradation (Grant 1999; Hamilton and Baulcombe 1999; Bass 2000; Hammond et al. 2000; Zamore et al. 2000). The mechanisms of TGS are poorly understood. For example, a *white* promoter-*Adh* gene fusion construct was integrated into the *Drosophila* genome, and it was found that an increased copy number of the fusion gene resulted not only in reduced expression of the fusion genes, but also in reduced ex-

pression of the endogenous *Adh* gene (Pal-Bhadra et al. 1997). This cosuppression is sensitive to the dosage of *Polycomb*-group genes, and the transcriptionally repressive Polycomb proteins are found associated with the cosuppressed fusion genes. In plants and *Neurospora*, DNA methylation is frequently detected at repetitive genes undergoing TGS (Assaad et al. 1993; Rossignol and Faugeron 1994). Although it is not clear whether the association of Polycomb proteins or DNA methylation is the cause or the consequence of TGS, the role of chromatin structure has been the focus of investigation (Vaucheret and Fagard 2001). However, neither DNA methylation nor *Polycomb* homologs are present in *Saccharomyces cerevisiae*, and thus different mechanisms would be required for any observed cosuppression in budding yeast. It has been suggested that PTGS and TGS may function in synergy (Ingelbrecht et al. 1994; Wasenegger et al. 1994; Al-Kaff et al. 1998; Jones et al. 1999). In plants, an RNA duplex containing promoter sequences can trigger TGS accompanied by de novo methylation of the promoter (Mette et al. 2000), implying a mechanistic connection between TGS and PTGS.

Ty1 elements in *S. cerevisiae* are retrotransposons. The coding region of *Ty1* is flanked by two 340-bp-long terminal repeats (LTRs, or δ elements) that function as the transcriptional promoter and terminator, respectively. The coding region consists of two overlapping open reading frames: *TYA1* (*GAG*), encoding a structural

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capsid protein (CA); and *TYB1* (*POL*), encoding protease (PR), integrase (IN), and reverse transcriptase (RT). Replication or transposition of *Ty1* requires the following steps. RNA polymerase II transcribes *Ty1* elements from LTR to LTR, and the RNA is polyadenylated and transported into the cytoplasm. *TyA* and *TyA-TyB* are translated, and the production of *TyA-TyB* involves a programmed translational frame shift at a frequency of 3% (Belcourt and Farabaugh 1990). *TyA* and *TyA-TyB* associate with *Ty1* mRNA to form virus-like particles (VLPs), wherein *TyA* is processed into CA and *TyA-TyB* is processed into CA, PR, IN, and RT. *Ty1* protein processing is catalyzed by PR and is essential for transposition (Youngren et al. 1988; Merkulov et al. 1996, 2001). Reverse transcription occurs in the VLP, using tRNA_i^{Met} as a primer (Eichinger and Boeke 1988; Chapman et al. 1992), and the resultant double-stranded *Ty1* cDNA is then transported into the nucleus (of the same cell), where either nonhomologous integration catalyzed by IN or homologous recombination with a chromosomal *Ty1* occurs.

The transcription of *Ty1* elements is under the control of both mating type and the invasive/filamentous growth MAP-kinase pathway. Mating-type control refers to the higher level of *Ty1* expression seen in haploids compared to diploids, and the *a1/α2* heterodimeric repressor, which is only present in diploid cells, is responsible for this regulation. Binding sites for the *a1/α2* repressor have been identified in the region of *Ty1* nucleotides 815–927 adjacent to the *Mcm1* binding sites (Errede et al. 1987). The invasive/filamentous growth MAP-kinase pathway is activated by nitrogen starvation in diploid cells and by an unknown environmental element in haploid cells, and activity of this signal transduction cascade requires the *Ste11*, *Ste7*, and *Kss1* kinases. Activation of this pathway results in activation of the *Ste12* and *Tec1* transcription factors. The *Ty1* regulatory region of nucleotides 384–433 contains binding sites for the *Ste12* and *Tec1* DNA-binding factors (Company et al. 1988; Baur et al. 1997; Madhani and Fink 1997). The level of *Ty1* mRNA is about 10-fold lower in wild-type diploid cells compared with haploids (Elder et al. 1981), and similar decreases are seen in *ste7*, *ste11*, *ste12*, and *tec1* haploid mutants compared with wild-type haploids (Dubois et al. 1982; Laloux et al. 1990). Additionally, *TYA1-lacZ* reporters are activated by a constitutive MAPKK kinase *Ste11-4* or by nitrogen starvation (Morillon et al. 2000). *Ty1* transcription is also activated by DNA-damaging agents in an *RAD9*-dependent but *DUN1*-independent fashion (Rolfe et al. 1986; Bradshaw and McEntee 1989; Staleva and Venkov 2001).

Most *S. cerevisiae* strains have about 30 copies of the *Ty1* element dispersed throughout the genome, and *Ty1* mRNA expression can be as high as 10% of total mRNA. Cosuppression has never been observed previously for *Ty1* elements, although the repeated, dispersed nature of *Ty1* elements makes them prime candidates for cosuppression. We report here the detection of abolished expression of *Ty1*-related genes using *Ty1-URA3* reporters plus 5-fluoroortic acid (5-FOA) counterselection. Expres-

sion of the entire family of *Ty1*-related genes was shown to be in one of two states, either all genes expressed or all genes not expressed, without uncoordinated or mosaic expression in any individual cell. Rapid switches between the two states were observed. Investigations in a strain lacking any endogenous *Ty1* elements showed that abolished expression of a *Ty1-URA3* reporter could only be detected in the presence of a high-copy plasmid carrying the *Ty1* element, and this requirement for high *Ty1* copy number established this phenomenon as cosuppression. *Ty1* transcriptional repressors facilitated the onset of *Ty1* cosuppression, and the native *Ty1* promoter was required for *Ty1* cosuppression, indicating that *Ty1* cosuppression occurs at the transcriptional level. The transcription of *Ty1* elements in a cell appeared under striking digital control with *Ty1* transcriptional repressors enhancing the frequency of *Ty1* cosuppression rather than reducing the amplitude of *Ty1* transcription, which contradicts the conventional wisdom of *Ty1* transcriptional factors regulating the transcriptional amplitude of *Ty1* elements.

Results

Off-regulation of *Ty1-URA3* reporters

In-frame *Ty1-URA3* fusions (driven by the *Ty1* promoter) were constructed as reporters for the detection of potential *Ty1* cosuppression. *URA3* was chosen as the key component for the reporters to take advantage of the fact that both positive and negative selection schemes are available for selecting cells that either do or do not express *Ura3* (Boeke et al. 1987). Cells expressing *Ura3* can grow without uracil. Because cells expressing *Ura3* convert 5-fluoroortic acid (5-FOA) to a toxic compound, only cells lacking the *Ura3* enzyme can grow on media containing 5-FOA. A bacterial *Tn3* minitransposon, *Tn3URA3OT*, carrying the *URA3* ORF plus a transcriptional terminator, was randomly inserted into a cloned *Ty1* element (*pHSS6:Ty1*) via in vitro transposition in *Escherichia coli* (Fig. 1; Seifert et al. 1986; Burns et al. 1994). The resultant *Ty1-URA3* fusions were excised by *NotI* restriction digestion and introduced into a *ura3-1* yeast strain (W303A) via homologous recombination with chromosomal *Ty1* elements, and strains carrying in-frame *Ty1-URA3* fusions were selected on SC-Ura plates. The *Ura*⁺ transformants were first purified on nonselective YEPD plates, and then tested for growth on SC + 5-FOA. 5-FOA survivors were retested for growth on SC-Ura to rule out the possibility that the 5-FOA survivors had suffered a mutation in the *Ty1-URA3* gene.

If there is cosuppression of the *Ty1* genes, the partially homologous *Ty1-URA3* fusion gene may be turned off or off-regulated in cells undergoing *Ty1* cosuppression. Cells with the *Ty1-URA3* reporter could be 5-FOA-resistant because expression of the *Ty1-URA3* reporter was abolished by cosuppression. Many such *Ura*⁺ and 5-FOA^r (Ufo) strains with different *TYA1-URA3* in-frame fusions at various (if not all) *Ty1* loci were, indeed,

Figure 1. The Ufo scheme to detect Ty1 cosuppression.

obtained. The phenotype of these Ufo strains was reversible, as they were able to survive repeated sequential opposing Ura⁺ and 5-FOA⁺ selections. The Ufo phenotypes of two Ty1-URA3 strains (Y32 and Y33, carrying reporters Ty1-URA3-32 and Ty1-URA3-33, respectively) are illustrated in Figure 2A. The strains formed colonies on SC-Ura and SC + 5-FOA after 3 d of incubation at 30°C. Quantitative titration/plating experiments revealed that only fractions of Y33 cells from a colony grown on nonselective SC medium formed colonies on SC-Ura (64% ± 13%) and SC + 5-FOA (16% ± 3%) plates. Microscopic examinations confirmed that a significant fraction of the cells plated on selective media failed to divide (Table 1, lines 2 and 3). The failure of ~10% of the plated cells to divide on SC-Ura unequivocally pointed to the existence of an intrinsic population of cells that do not express Ty1-URA3-33. This result ruled out that potential leakiness of 5-FOA selection allowed Ura⁺ cells to form colonies on 5-FOA or that 5-FOA induced the off-regulation of Ty1-URA3-33. Experiments examining plating efficiencies of strain Y32, with a different Ty1-URA3 reporter integrated at a different chromosomal location gave similar results (data not shown). It should be noted that the Y33 5-FOA⁺ colony formation efficiency of 16% underreports the fraction (64%) of Y33 cells undergoing transient Ty1 cosuppression (see below).

Southern hybridization analysis showed that Y32 and Y33 each contained a single URA3 gene fusion (Fig. 3A, bottom). Ty1-URA3-32 was cloned and sequenced, and found to be an insertion of URA3OT at nt +635 of yGRW Ty1-1. Ty1-URA3-33 was cloned and sequenced, and

found to be an insertion of URA3OT at nt +902 of a Ty1 element unique to W303. When Y33 (MAT α Ty1-URA3-33 ura3-1) was backcrossed to W303B (MAT a ura3-1), two Ura⁺ and two Ura⁻ segregants were obtained for each tetrad, confirming a single in-frame Ty1-URA3 in Y33. All Ura⁺ segregants were MAT α , suggesting Ty1-URA3-33 is closely linked to the MAT locus. All Ura⁺ segregants were also capable of growth on 5-FOA (conferring the Ufo phenotype), showing that the Ufo phenotype segregated as a single Mendelian trait and that Ty1-URA3-33 was sufficient for the Ufo phenotype. Y33, as either a Ura⁺ or 5-FOA⁺ population, was backcrossed to W303B, and in both cases the Ufo phenotype appeared in half of the segregants. This indicates that the phenotype is not stable through meiosis and that there is no detectable genetic difference between the two phenotypic populations.

To address whether a Ty1-URA3 reporter must be chromosomally located to be off-regulated, plasmid D806 with the Ty1-URA3-33 on a YCp[LEU2] vector was constructed. W303A carrying D806 displayed the Ufo phenotype on SC-Leu-Ura and SC-Leu + 5-FOA media (Fig. 2B), indicating that cosuppression could block expression of the reporter present on a plasmid. However, the expression of an independently expressed URA3 embedded in the Ty1 element at the same position in either orientation (on D1261 and D1262) could not be blocked by cosuppression, indicating that an in-frame Ty1-URA3 fusion is required for the Ufo phenotype. Similar results were obtained in an S288C strain, eliminating the possibility that the cosuppression is specific to the W303 strain background.

Although a remote possibility, it is conceivable that the URA3 sequence itself plays a role in the decreased expression of the Ty1-URA3 fusion. To test this possibility, a strain with a Ty1-TRP1 in-frame fusion gene was constructed and tested for growth on a medium with 5-fluoroanthranilic acid (FAA), which kills TRP1⁺ cells (Toyn et al. 2000). The strain was able to grow on the FAA medium (Fig. 2C), indicating that cosuppression can block the expression of the Ty1-TRP1 reporter. This shows that off-regulation of a Ty1-related gene does not depend on the URA3 sequence.

Concerted off-regulation of Ty1-related genes

Although the growth of the Ty1-URA3 strains on media containing 5-FOA indicates the absence of Ura3 activity, these experiments do not address whether the growth resulted from reduced levels of Ty1-URA3 mRNA or not. Northern assays were performed to determine the mRNA levels of Ty1-URA3-33 and ACT1 (as a control; Fig. 3A, right). The results show that Ty1-URA3-33 mRNA is expressed when Y33 cells are grown in the absence of uracil (SC-Ura), but is absent from Y33 cells grown on medium containing 5-FOA (SC + 5-FOA).

Because one hallmark of cosuppression is homology-dependent gene silencing, it is critical to determine whether genes sharing homology with Ty1-URA3-33 are off-regulated in concert with Ty1-URA3-33. Northern

Figure 2. Off-regulation of *Ty1* reporters. (A) The Ufo phenotype of *Ty1-URA3* strains (Y32 and Y33) was determined by a titration/plating assay. Cells from colonies formed on YEPD were titrated and then plated onto SC, SC-Ura, and SC + 5-FOA plates. A *ura3-1* strain, W303A, grew in the presence of 5-FOA but not in the absence of uracil. A *URA⁺* strain, DY4073, grew in the absence of uracil but not in the presence of 5-FOA. Fractions of Y32 and Y33 cells readily formed colonies on both SC-Ura and SC + 5-FOA plates in 3 d at 30°C. 5-FOA⁺ Y32 and Y33 colonies were all tested Ura⁺. A microscopic view (100×) with undivided cells, two microcolonies, and a partial macrocolony of Y33 on SC + 5-FOA is shown on the right. (B) *YCplac111 [LEU2]*, D806 (*YCp[LEU2]:Ty1-URA3-33*), D1261 (*YCp[LEU2]:ty1::URA3(A)*), and D1262 (*YCp[LEU2]:ty1::URA3(B)*) were transformed into W303B and an S288C strain, YPH499. Transformants from colonies formed on SC-Leu were titrated and then plated onto SC-Leu, SC-Leu-Ura, and SC-Leu + 5-FOA plates. 5-FOA⁺ W303B D806 and YPH499 D806 colonies were all tested Ura⁺. (C) Growth patterns of a *Ty1-TRP1KL* strain (Y1203, carrying a *Ty1* element with the ORF of *TRP1* from *Kluyveromyces lactis* inserted at nt +902 of *Ty1*) and two isogenic control strains were determined by a titration/plating assay. Cells from colonies formed on YEPD were titrated and then plated onto SC, SC-Trp, and SC + FAA plates. Cells of W303A (*trp-1*) grew in the presence of FAA but not in the absence of tryptophan. Cells of a *TRP⁺* strain, Y1149, grew in the absence of tryptophan but were killed by FAA. Fractions of Y1203 cells readily formed colonies on both SC-Trp and SC + FAA plates in 3 d at 30°C. FAA⁺ Y1203 colonies were all tested Trp⁺.

assays were performed to determine the mRNA levels of genes sharing homology with *Ty1-URA3-33*, the endogenous *Ty1* genes, and the native *ura3-1* in the same cells (Fig. 3A, right). The results show that *Ty1-URA3-33* and *Ty1* mRNAs are coregulated. They were expressed in Y33 cells grown in the absence of uracil (SC-Ura), but were absent in Y33 cells grown in the presence of 5-FOA (SC + 5-FOA). Genes unrelated to *Ty1*, such as *ura3-1* and *ACT1*, were expressed normally in Y33 cells grown in SC + 5-FOA. A modest threefold reduction of *ura3-1* mRNA was observed in Y33 cells grown in SC + 5-FOA, and it was likely to be the result of Ppr1-mediated metabolic repression by uracil in the medium (Denis-Duphil 1989). The abolished expression of *Ty1*-related genes was also detected in *ppr1* cells in which *ura3-1* mRNA did not depend on the concentration of uracil (data not shown). A Southern assay of genomic DNA showed that the *Ty1-URA3-33* reporter remains intact in the Y33 cells grown in the SC + 5-FOA medium, eliminating the possibility that loss of the *Ty1-URA3-33* reporter causes the absence of *Ty1-URA3-33* mRNA. Except for a yet to be understood fuzziness of *Ty1-URA3-32* Northern signals, the same results were obtained with Y32 (Fig. 3A,

left). These results show that *Ty1* homology is required for the cosuppression.

The *Ty1* homology-dependence was confirmed in two control experiments that showed the specificity of the *Ty1-URA3* plus 5-FOA reporting method. Cells with the *ura3-1* allele can grow on 5-FOA, and cells with *URA3* at a telomeric location can grow on 5-FOA because of telomeric gene silencing (Gottschling et al. 1990). Growth of these cells on 5-FOA did not result in loss of *Ty1* expression (Fig. 3B), showing that growth on 5-FOA per se does not cause loss of *Ty1* mRNA.

If the expression of *Ty1*-related genes in a cell is not coordinated, loss of one *Ty1*-related gene's expression will always be associated with expression of some other *Ty1*-related genes in the cell to result in a mosaic pattern of *Ty1* expression. In other words, mosaic expression of *Ty1* elements in a cell is not compatible with the coordinated off-regulation of *Ty1*-related genes observed here, and the above results show that *Ty1* elements are either all expressed or all turned off with no mosaic expression of *Ty1* elements in the same cell. Growth of *Ty1-URA3* cells in SC + 5-FOA selects those cells not expressing *Ty1* elements, growth of *Ty1-URA3* cells in

Table 1. Microscopic plating efficiencies

Culture history		Y33 (<i>Ty1-URA3</i>)	W303A (<i>ura3-1</i>)	DY4073 (<i>URA</i> +)
1	From SC to SC	(98 ± 1)%	(98 ± 1)%	(99 ± 1)%
2	From SC to SC-Ura	(90 ± 1)%	(0 ± 0)%	(98 ± 1)%
3	From SC to SC + 5-FOA	(22 ± 2)%	(98 ± 1)%	(0 ± 0)%
4	From SC-Ura to SC	(98 ± 1)%	n.d.	(98 ± 1)%
5	From SC-Ura to SC-Ura	(93 ± 1)%	n.d.	(98 ± 1)%
6	From SC-Ura to SC + 5-FOA	(11 ± 1)%	n.d.	(0 ± 0)%
7	From SC + 5-FOA to SC	(23 ± 1)%	(99 ± 1)%	n.d.
8	From SC + 5-FOA to SC-Ura	(17 ± 2)%	(0 ± 0)%	n.d.
9	From SC + 5-FOA to SC + 5-FOA	(22 ± 1)%	(98 ± 2)%	n.d.

Cells from a colony formed on a medium were plated onto the same medium or a different one. The plated cells were incubated at 30°C overnight and then observed under a light microscope. Microscopic plating efficiency was calculated as the fraction of cells divided at least once on a new medium. The presented efficiencies were averaged from four experiments.

SC-Ura enriches those cells expressing *Ty1* elements and growth of *Ty1-URA3* cells in SC or YEPD results in a mixture of the two populations.

Another hallmark of cosuppression is that a high copy number of the target gene is required to reduce gene expression (Jorgensen 1995). To examine the role of *Ty1* copy number, a *Ty0* strain that completely lacks *Ty1* and *Ty2* sequences was used (Wilke and Adams 1992). A *Ty0* strain was transformed with the *Ty1-URA3-33* reporter and either a single-copy centromere plasmid with a *Ty1* element (*YCp[LYS2]:Ty1*) or a multicopy plasmid with a *Ty1* element (*YEp[LYS2]:Ty1*), and the strains were assayed for the ability to grow on 5-FOA (Fig. 4). The results clearly show that expression of the *Ty1-URA3-33* reporter can be turned off by the *YEp:Ty1* high-copy plasmids but not by the *YCp:Ty1* single-copy plasmid. This result shows that high copy number is required for the abolished expression of the *Ty1-URA3-33* reporter and qualifies the coordinated off-regulation of *Ty1*-related genes as bona fide cosuppression. If an epigenetic factor unrelated to *Ty1* blocks the expression of *Ty1* elements to cause *Ty1* cosuppression, it is difficult to imagine that high *Ty1* copy number is required for *Ty1* cosuppression. Therefore, the requirement of high *Ty1* copy number rules out the involvement of such a *Ty1*-independent epigenetic factor and points to interactions among *Ty1* elements as the key in *Ty1* cosuppression. If a *Ty1* gene product mediates the interactions among *Ty1* elements to initiate *Ty1* cosuppression, it would imply negative feedback control of *Ty1* gene expression.

A key role of the native *Ty1* promoters in *Ty1* cosuppression

Given that the cosuppression-sensitive *Ty1-URA3* reporters are driven by *Ty1* promoters, it is important to

Figure 3. Northern assays. (A) Coordinated off-regulation of *Ty1*-related genes. Y32 and Y33 were grown in SC-Ura and SC + 5-FOA to the mid-log phase (all in triplicates). Total yeast RNA and genomic DNA were extracted from the cells of each culture, and subjected to Northern and Southern analyses, respectively. The Northern blot was probed with a ³²P-labeled 1.1-kb *HindIII* DNA fragment of *URA3*, a ³²P-labeled 1.3-kb *SalI-KpnI* DNA fragment of *Ty1*, and a ³²P-labeled 1.5-kb *BamH-HindIII* DNA fragment of *ACT1*. No low-molecular-weight *Ty1* signals were detected after an extended exposure (data not shown). The genomic DNA samples were digested with *EcoRI*, resolved on a 0.8% TAE agarose gel, and blotted onto a nitrocellulose membrane. The Southern blot was probed with the same *URA3* probe. The signal-containing region of an autoradiograph is shown here with the signals of *ura3-1* (~12 kb), *Ty1-URA3-33* (~11 kb), and *Ty1-URA3-32* (~6 kb) indicated. (B) 5-FOA treatment and the off-regulation of *Ty1* elements. W303A (*ura3-1*), DY4073 (*URA* +), Y33 (*Ty1-URA3*), and UCC16 (*Tel:URA3*) were grown in liquid media as indicated. Total RNA was extracted from the cells and subjected to Northern analysis as described above. (C) *Ty1* mRNA levels in mutant cells not undergoing *Ty1* cosuppression. Isogenic *MATα* *Ty1-URA3-33* strains Y33 (wild-type; lanes 8, 12), Y443 (*gcn5Δ*; lanes 5, 9), Y442 (*ste12Δ*; lanes 6, 10), Y461 (*ste11Δ*; lanes 7, 11), Y33 with *YCp50-LEU2* (lanes 1, 3), and Y33 with *YCp50-LEU2:MATA* (lanes 2, 4) were grown in liquid media as indicated; total RNA was extracted and subjected to Northern analysis as described above.

Figure 4. *Ty1* copy number-dependent off-regulation of *Ty1-URA3-33*. Y1144, a *Ty0* strain with *YCp[LEU2]Ty1-URA3-33*, was transformed with D1264 (*YCp[LYS2]:Ty1*), D1242 (*YEp[LYS2]*), D1251 (*YEp[LYS2]:Ty1*), D1256 (*YEp[LYS2]:pPGK-Ty1* over-expressing *Ty1* from the strong *PGK1* promoter), and D1257 (*YEp[LYS2]:pGAL-Ty1*). Transformants from SC-Leu-Lys plates were serial-diluted and spotted onto the indicated solid media and incubated at 30°C for 3 d. 5-FOA^r Y1144 D1251 colonies were all tested Ura^r.

determine the role of the native *Ty1* promoters in *Ty1* cosuppression. *Ty1*-related genes driven by heterologous promoters were constructed and tested for cosuppression. *YEp:pPGK-Ty1* failed to cosuppress *Ty1-URA3-33* (Fig. 4), indicating that the native *Ty1* promoters are required for *Ty1* elements to initiate *Ty1* cosuppression. Neither the ORF of *Ty1-URA3-33* driven by the *MET3* promoter nor *URA3-Ty1* gene fusions driven by the *URA3* promoter were capable of generating a Ufo phenotype (data not shown), indicating that the native *Ty1* promoter is also required for a *Ty1*-related gene to become a target of *Ty1* cosuppression. Although the *Ty0* strain lacks *Ty1* and homologous *Ty2* elements, it still has many δ elements (Wilke and Adams 1992), which remain following homologous recombination between the two *Ty1* δ elements (Knight et al. 1996). The fact that cosuppression was not seen in the *Ty0* strain shows that multiple copies of δ elements are not sufficient to initiate *Ty1* cosuppression, and that multiple copies of full-length *Ty1* elements are needed for *Ty1* cosuppression. Moreover, *YEp:pGAL-Ty1* with no *Ty1* transcription (due to the repression of *pGAL* by glucose in the medium) could not cosuppress *Ty1-URA3-33* (Fig. 4), implying that expression of *Ty1* elements is required for the initiation of *Ty1* cosuppression. These experiments point to a key role of the native *Ty1* promoters in *Ty1* cosuppression.

Digital control of *Ty1* transcription and *Ty1* TGS

Because the *Ty1* promoters contain *cis*-acting elements required for *Ty1* transcriptional regulation, the requirement of the native *Ty1* promoters for *Ty1* cosuppression implies that *Ty1* cosuppression occurs at the level of

transcription. If *Ty1* cosuppression is transcriptional, changes in *Ty1* transcriptional factors should affect *Ty1* cosuppression. Experiments were performed to determine how changes in *trans*-acting *Ty1* transcriptional regulators affect *Ty1* cosuppression. The diploid-specific transcriptional repressor *al*/ α 2 lowered the average *Ty1* mRNA level by a factor of 10 in unsorted cells (a mixture of *Ty1*-expressing and non-expressing cells; Fig. 3C, cf. lane 2 with lane 1; Elder et al. 1981). The effect of the *Ty1* transcriptional repressor *al*/ α 2 on *Ty1* cosuppression was investigated. Y33 with *YCp50-LEU2:MATa* displayed a colony formation efficiency on SC-Leu + 5-FOA 16-fold higher than that of Y33 with *YCp50-LEU2* (Table 2, cf. line 2 with line 1). These results show that *Ty1* cosuppression occurs much more easily in diploid cells where *Ty1* elements are transcriptionally repressed. To see if there is a positive correlation between *Ty1* transcriptional repression and the 5-FOA^r colony formation efficiency of *Ty1-URA3-33* cells, *Ty1* cosuppression was examined in *gcn5*, *ste12*, and *ste11* mutants that lack *Ty1* transcriptional activators (Fig. 3C, lanes 5–12). *STE12* and *STE11* encode components of the invasive/filamentous growth MAP-kinase pathway that stimulates *Ty1* transcription; and *GCN5* encodes the catalytic subunit of the SAGA histone acetyltransferase protein complex, a general transcriptional activator. The level of *Ty1* mRNA in unsorted isogenic *gcn5* Δ cells was 5% of that in unsorted Y33 cells (Fig. 3C, cf. lane 5 with lane 8) and *gcn5* Δ increased the 5-FOA^r colony formation efficiency of *Ty1-URA3-33* cells by a factor of 4.9 (Table 2, cf. line 3 with line 6). *Ty1* mRNA levels in unsorted isogenic *ste12* Δ and *ste11* Δ cells were at 9% and 5% of that in unsorted Y33 cells, respectively (Fig. 3C, cf. lanes 6 and 7 with lane 8; Dubois et al. 1982). *ste12* Δ and

Table 2. 5-FOA^r colony formation efficiencies of *Ty1-URA3-33* strains

	Strain	Genotype	5-FOA medium	5FOA ^r colony formation efficiency
1	Y33 <i>YCp50-LEU2</i>	<i>MATa</i>	SC-Leu + 5-FOA	(2.7 $\pm$ 0.1)%
2	Y33 <i>YCp50-LEU2:MATa</i>	<i>MATa/a</i>	SC-Leu + 5-FOA	(46 $\pm$ 5)%
3	Y443	<i>MATa gcn5</i> Δ	SC + 5-FOA	(93 $\pm$ 2)%
4	Y442	<i>MATa ste12</i> Δ	SC + 5-FOA	(82 $\pm$ 5)%
5	Y461	<i>MATa ste11</i> Δ	SC + 5-FOA	(92 $\pm$ 1)%
6	Y33	<i>MATa</i>	SC + 5-FOA	(16 $\pm$ 3)%

5-FOA^r colony formation efficiency was calculated as the number of colonies formed on SC-Leu + 5-FOA (or SC + 5-FOA) divided by the number of colonies formed on SC-Leu (or SC). The presented efficiencies were averaged from three experiments.

ste11Δ mutations increased the 5-FOA⁺ colony formation efficiency of *Ty1-URA3-33* cells 4.1- and 4.8-fold, respectively (Table 2, cf. lines 4 and 5 with line 6).

The above results indicate that the lowered average *Ty1* mRNA levels in unsorted cells of the aforementioned pseudodiploid and haploid mutants are at least partially caused by increased fractions of cells undergoing *Ty1* cosuppression. To investigate the possibility that the elevated *Ty1* cosuppression frequencies are solely responsible for the lowered *Ty1* mRNA levels in the strains, the effects of *Ty1* transcriptional regulators on the *Ty1* mRNA levels at the cosuppressed state and the coexpressed state were investigated. *Ty1* elements in the pseudodiploid cells and the *gcn5*, *ste12*, and *ste11* mutants were found completely cosuppressed with *Ty1-URA3-33* (data not shown), indicating that *Ty1* transcriptional regulators do not set the *Ty1* mRNA level at the cosuppressed state. This result also rules out the possibility that the increased fractions of 5-FOA⁺ cells are the result of diminished toxicity of 5-FOA to cells with lower levels of *Ty1-URA3-33*. The levels of *Ty1* mRNA in the Y33 pseudodiploid, *Ty1-URA3-33 gcn5Δ*, *Ty1-URA3-33 ste12Δ*, and *Ty1-URA3-33 ste11Δ* haploid cells grown without uracil were found to be 82%, 67%, 90%, and 86% of that in Y33 cells grown without uracil, respectively (Fig. 3C, lanes 3,4,9–12). It is conceivable that actual *Ty1* mRNA levels in the pseudodiploid and mutant haploid cells with *Ty1* expression are even higher, as it is more difficult to enrich Ura⁺ cells from higher backgrounds of Ura⁺ cells. It was concluded that *Ty1* mRNA levels in the pseudodiploid and mutant haploid cells with *Ty1* expression were detected at levels experimentally indistinguishable from that in the wild-type haploid. Growth of *URA⁺ gcn5Δ*, *URA⁺ ste11Δ*, *URA⁺ ste12Δ* haploids and *URA⁺ MATa/α* pseudodiploids in media with or without uracil did not result in different *Ty1* mRNA levels for each strain (data not shown), ruling out the possibility that the uracil prototrophy selection per se regulates the level of *Ty1* mRNA. The same result was obtained with a true *Ty1-URA3-33* diploid isogenic strain, Y250 (data not shown). These results indicate that *Ty1* transcriptional regulators play no role in setting the *Ty1* mRNA level at either the cosuppressed state or the coexpressed state. Rather, the elevated *Ty1* cosuppression frequencies are solely responsible for the lowered *Ty1* mRNA levels in unsorted diploid, *ste12Δ*, *ste11Δ*, and *gcn5Δ* haploid cells.

In other words, the transcription of *Ty1* elements is under striking digital control with *Ty1* transcriptional repressors enhancing *Ty1* cosuppression frequency rather than reducing *Ty1* transcriptional amplitude in cells expressing *Ty1* elements. Digital control of *Ty1* transcription contradicts the conventional wisdom that *Ty1* transcriptional factors regulate *Ty1* transcriptional amplitude and extends the probabilistic enhancer action (enhancers increasing the number of expressing cells rather than the level of gene expression per cell; Walters et al. 1995, 1996; Magis et al. 1996) to dispersed gene repeats.

The observations of a *Ty1* transcriptional repressor fa-

cilitating the onset of the cosuppressed state and *Ty1* transcriptional activators obstructing the onset of the cosuppressed state indicate that turning off *Ty1* transcription is a key step in *Ty1* cosuppression. Digital control of *Ty1* transcription along with the aforementioned requirement of the native *Ty1* promoters for *Ty1* cosuppression establishes *Ty1* cosuppression as TGS.

Calculating the frequency of transient *Ty1* cosuppression

Plating efficiency experiments (Table 1) were performed to determine what fraction of Y33 cells with the *Ty1-URA3-33* reporter is undergoing cosuppression. These experiments also allowed the calculation of the rate at which a cosuppressed *Ty1-URA3-33* reporter comes on (the on-switch rate), and the rate at which an expressed *Ty1-URA3-33* reporter is extinguished (the off-switch rate). In these experiments cells from a colony grown on a specific medium (SC, SC-Ura, or SC + 5-FOA) were resuspended in sterile water and plated onto the same medium or a different one. After overnight incubation cells were examined microscopically and plating efficiency was calculated as the fraction of cells that divided at least once on the new plate.

The following two numbers were used to calculate the fraction of Y33 cells with the *Ty1-URA3-33* reporter undergoing cosuppression. First, the *Ty1* mRNA level of Y33 cells grown in SC-Ura is 2.5-fold higher than when grown in SC (Fig. 3B,C). Second, 11% of Y33 cells from a colony grown under selection on SC-Ura were able to divide on SC + 5-FOA (Table 1, line 6), indicating that uracil prototrophy selection was only able to enrich Y33 cells with *Ty1* expression to a purity of 89%. Together, these results translate to saying that 36% (89%/2.5) of the Y33 cells grown in SC, without selection, express *Ty1*-related genes. Thus, the remaining 64% of Y33 cells grown in SC medium are undergoing *Ty1* cosuppression. The fraction of Y33 cells not expressing *Ty1* is surprisingly high.

Data from the plating experiments allowed three independent calculations of the on-switch rate of *Ty1-URA3-33* in Y33 cells, and similar results were obtained from these calculations. First, the on-switch rate can be calculated by monitoring the fate of Y33 cells grown on SC + 5-FOA. Only 23% of Y33 cells from colonies grown under selection on SC + 5-FOA divided on nonselective SC (Table 1, line 7), and only 22% of the Y33 cells grown on SC + 5-FOA divided when plated on the same medium, SC + 5-FOA (Table 1, line 9). These results indicate that cosuppressed Y33 cells, which do not express the *Ty1-URA3-33* reporter, frequently lose the cosuppressed state and express *Ty1-URA3-33* to cause cell death on 5-FOA. The fact that the live and 5-FOA-resistant cells in Y33 colonies grown on SC + 5-FOA continued to switch allowed a direct calculation of the on-switch rate. Of the plated Y33 cells from colonies grown on SC + 5-FOA, 17% divided on SC-Ura (Table 1, line 8), and this translates into an on-switch rate of 0.74 (17%/23%) per cell generation.

Second, the on-switch rate can also be calculated by monitoring the actions taken by unsorted Y33 cells after they are plated on SC-Ura. Of the 90% of Y33 cells grown on SC that divided on SC-Ura (Table 1, line 2), 36% were cells already expressing *Ty1-URA3-33* at the time of plating according to the calculation above. It can be calculated that 54% (90% – 36%) of the Y33 cells grown nonselectively on SC were cosuppressed at the time of plating but underwent on switches on SC-Ura to express *Ty1-URA3-33*. This translates into an on-switch rate of 0.84 (54%/64%) per cell generation.

Third, the on-switch rate can also be calculated by monitoring the actions taken by the unsorted Y33 cells after they are plated on SC + 5-FOA. Only 22% of Y33 cells grown nonselectively on SC divided when plated on SC + 5-FOA (Table 1, line 3), indicating that only 34% (22%/64%) of the originally cosuppressed cells remained cosuppressed for one cell generation. This calculates to an on-switch rate of 0.66 (1 – 0.34) per cell generation.

Microscopic examination of Y33 cells plated on SC + 5-FOA showed both colonies and microcolonies (Fig. 2A, right). The existence of these microcolonies indicates that *Ty1-URA3-33* was not expressed in the founding cell of a microcolony, allowing division on 5-FOA, but that rapid on switches sometimes caused all progeny of a cosuppressed cell to be killed by 5-FOA. This also provides an explanation for the Y33 5FOA⁺ colony formation efficiency (16%) being lower than the microscopic 5-FOA-plating efficiency (22%).

It was difficult if not impossible to determine the rate of off switches. Although the plating efficiency of Y33 cells grown on SC-Ura and then replated on the same medium was determined (Table 1, line 5), this value could not be used to directly measure the off-switch rate. Among these cells, those that underwent off switches after being replated onto SC-Ura were tolerated because they could undergo the aforementioned rapid on switches and eventually divide on SC-Ura. Because co-expressed Y33 cells that express *Ty1-URA3-33* can not survive long enough to undergo off switches on SC + 5-FOA, the microscopic 5-FOA-plating efficiency of Y33 cells from SC-Ura (Table 1, line 6) does not reflect the fraction of coexpressed cells undergoing off switches on SC + 5-FOA. Nonetheless, an estimate for the off-switch rate can be made as follows. Assuming that Y33 cells grown in SC are at equilibrium with 64% cosuppressed cells and 36% coexpressed cells, the number of cosuppressed cells undergoing on switches (64% × 0.74 per cell generation) should equal the number of *Ty1*-expressing cells undergoing off switches (36% × the off-switch rate). This allows an estimation of the off-switch rate to be 1.3 per cell generation. In summary, both the on-switch and off-switch rates are extremely high, consistent with the idea that *Ty1* cosuppression lacks mitotic stability.

Discussion

Ty1 transcriptional cosuppression is documented in this report as a novel cosuppression. On one hand, it has the key characteristics of classical TGS in plants: induction

by high gene copy number, homology-dependence, and a possible function of molecular parasite control (see below). On the other hand, *Ty1* cosuppression displays unique features. TGS in plants is usually mitotically stable, whereas *Ty1* cosuppression is transient. TGS in plants is usually associated with DNA methylation. Because there is no DNA methylation in *S. cerevisiae*, *Ty1* cosuppression is the first clear example of DNA-methylation-independent TGS. Multicopy promoter sequences are sufficient to induce TGS in plants (Vaucheret and Fagard 2001), but multicopy δ elements fail to initiate *Ty1* cosuppression (Fig. 4).

Cosuppression-mediated negative feedback control of Ty1 transcription

The initiation of *Ty1* cosuppression by high *Ty1* copy number suggests the existence of a negative feedback control mechanism regulating *Ty1* transcription. The high switch rates of *Ty1* cosuppression point to a dynamic pattern of *Ty1* transcription in vivo. *Ty1* transcription in a wild-type haploid is blocked by cosuppression at such a high frequency (1.3 per cell generation) that *Ty1* elements are mostly cosuppressed (for 64% of a cell cycle). This pattern of *Ty1* transcription is consistent with the idea of cosuppression mediating negative feedback control of *Ty1* expression. It is possible that a yet to be identified *Ty1* gene product inhibits *Ty1* transcription and acts to initiate *Ty1* cosuppression. The production of this putative *Ty1* gene product is expected to depend on the structure or function of the native *Ty1* promoter, as *pPGK-Ty1* and *PGAL-Ty1* failed to initiate *Ty1* cosuppression (Fig. 4). *Ty1* reverse transcriptase is inhibited by 0.2 mg/mL phosphonoformic acid (PFA; Lee et al. 2000). However, this concentration of PFA does not affect *Ty1* cosuppression in Y33 cells (data not shown), showing that *Ty1* cDNA does not function as the feedback signaling molecule. Experiments are in progress to determine what minimal *Ty1* sequences are required to initiate cosuppression in a *Ty0* strain, and this may shed light on the *Ty1* gene product that initiates *Ty1* cosuppression.

Cosuppression and Ty1 transposition

Because *Ty1* transposition can be mutagenic, there must be selective pressure to evolve mechanisms regulating *Ty1* transposition. *Ty1* transposition is largely limited to noncoding regions of the genome (Devine and Boeke 1996). However, such target restriction is not 100% effective, and *Ty1* transpositions into coding regions do occur (Natsoulis et al. 1989). Therefore, there is selective pressure for mechanisms to reduce the activities leading to *Ty1* transposition. Indeed, *Ty1* elements in wild-type cells are dormant under normal growth conditions. The dormancy involves specific host genes, and mutations in *FUS3*, *RAD52*, *CDC9*, *SSL2*, and *RAD3* cause *Ty1* hypertransposition (Conte et al. 1998; Lee et al. 1998, 2000; Conte and Curcio 2000; Rattray et al. 2000). *Ty1* trans-

position is induced by stressful conditions such as nitrogen starvation through the activation of the invasive/filamentous growth pathway (Morillon et al. 2000) or DNA damage through the activation of the Rad9-mediated DNA-damage-response pathway (Bradshaw and McEntee 1989; Staleva and Venkov 2001). *Ty1* elements may bring their host the benefit of adaptive mutagenesis (Morillon et al. 2000) to improve the fitness of the host (Wilke and Adams 1992; Knight et al. 1996). Moreover, *Ty1* elements can play a role in DNA repair (an extreme form of adaptive mutagenesis), as *Ty1* cDNA fragments have been found in repair patches following double-stranded DNA breaks (Moore and Haber 1996; Teng et al. 1996; Yu and Gabriel 1999). *Ty1* cosuppression seems to play an important role in suppressing *Ty1* hypertransposition. Preliminary experiments show abolished *Ty1* transcriptional cosuppression associated with all the known mutations and environmental conditions that cause *Ty1* hypertransposition (Y.W. Jiang, unpubl.).

Chromatin-remodeling and DNA-methylation-independent TGS

It is commonly assumed that transcription of repetitive genes in euchromatic regions is automatically coregulated. However, our previous studies have shown that transcriptional coregulation of two solo δ elements in a cell is not a given, and that it involves chromatin remodeling. *ACT3/ARP4* encodes a subunit of two chromatin-remodeling protein complexes, the NuA4 histone acetyltransferase complex (Galarneau et al. 2000) and the Ino80 ATP-dependent chromatin-remodeling complex (Shen et al. 2000). We reported that a recessive allele of *ACT3/ARP4* (*act3-3*) caused mosaic expression of two δ elements in the same cell (Jiang and Stillman 1996). It will be interesting to see whether chromatin remodeling plays a role in the transcriptional coregulation of the δ elements in *Ty1* cosuppression.

DNA methylation had been regarded as the most important aspect of chromatin remodeling in TGS of plants; however, it was recently reported that mutations of an *Arabidopsis* gene, *MOM*, reactivated transcription from heavily methylated and previously silenced loci, separating transcriptional activity from methylation pattern (Amedeo et al. 2000). The predicted *MOM* gene product is a nuclear protein of 2001 amino acids containing a region similar to the ATPase region of the chromatin-remodeling Swi2/Snf2 family, and it is suggested that *MOM* acts downstream of DNA methylation. Further work is needed to determine whether any chromatin-remodeling activity is required for *Ty1* cosuppression, which must be independent of DNA methylation because there is no DNA methylation in *S. cerevisiae*.

Materials and methods

YEPD, SC, SC-Ura, and SC + 5-FOA media preparation, cloning, yeast transformation, and Northern procedures were performed as previously described (Jiang and Stillman 1996). The preparation of SC + FAA (Toyn et al. 2000) and PCR-mediated site-

directed mutagenesis (Howorka and Bayley 1998) were performed according to published protocols.

Plasmids

A *Bam*HI-*Kpn*I linker (5' > ggatccgtcgaggggggcccgtacc > 3') and *URA3OT* as a 900-bp *Kpn*I-*Hind*III fragment from pPSG-B (Silar and Thiele 1991) were ligated into the *Bam*HI/*Hind*III sites of an *Eco*RI-less m-*Tn3* (Seifert et al. 1986) to create D467. The *Hind*III site in D467 was destroyed to create D468 *pTn3URA3OT*. The 11-kb *Eco*RI fragment of D547 containing *Ty1-URA3-33* was cloned into the *Eco*RI site of a *YCplac111[LEU2]* vector to create D804. The 6-kb *Xba*I-*Eco*RI fragment of D547 containing *Ty1-URA3-33* was cloned into a *YCplac111[LEU2]* vector to create D806. The 600-bp *Bam*HI-*Stu*I fragment of pJJ242 was ligated with the 11-kb *Bam*HI-*Stu*I fragment of D806 to create D1261 [*YCp[LEU2]:ty1::URA3(A)*] with wild-type *URA3* (driven by its own promoter) inserted at nucleotide 902 of *Ty1* in the same orientation. The 1.2-kb *Bam*HI-*Aat*II fragment of *pTn3:URA3* was ligated with the 11-kb *Bam*HI-*Aat*II fragment of D806 to create D1262 [*YCp[LEU2]:ty1::URA3(B)*] with wild-type *URA3* (driven by its own promoter) inserted at nucleotide 902 of *Ty1* in the opposite orientation. The 3.8-kb *Bam*HI-*Bgl*II fragment of pNKY51 (Alani et al. 1987) containing a *URA3* popout was cloned into the *Bam*HI site of *pTn3* to create D611 *pTn3:URA3* (popout). The genomic DNA of yeast strain 337 was digested with *Hind*III to completion and ligated into the *Hind*III site of pHSS6 to create a genomic library of 337. The genomic library was transformed into the *leu6B* *E. coli* strain KC8 for *Leu*⁺ transformants from which D623 contains *LEU2* from strain 337 was isolated. D623 was mutagenized with *Tn3:URA3* popout carried by D611 to create an *leu2::URA3* (popout) library D672. The 8-kb *Not*I fragment of D572 containing *Ty1* was cloned into the *Not*I site of pRS317 to create D1264 [*YCp[LYS2]:Ty1*]. The 2- μ plasmid as an *Hpa*I fragment was cloned into the *Sma*I site of YDp-K to create D766. The *Sal*I site in D766 was converted into an *Not*I site to create D772. The super polylinker of pSL1180 as an *Hind*III-*Eco*RI fragment was cloned into the *Hind*III/*Eco*RI sites of pHSS6 to create D825 (pHSS6 with Superlinker). The *Sma*I fragment of D825 was deleted to create D1241. The 2.3-kb *Not*I-*Aat*II fragment of D772 containing the pUC backbone was replaced with D1241 as a 2.2-kb *Not*I-*Aat*II fragment to create D1242 [*YEp[LYS2]*]. The 8-kb *Not*I fragment of D572 containing *Ty1* was cloned into the *Not*I site of D1242 to create D1251 [*YEp[LYS2]:Ty1*]. The 1.5-kb *Hind*III-*Xho*I fragment of pL2L2 [*YEp:pPGK-L-A[TRP1]*; Wickner et al. 1991] containing *pPGK1* was cloned into the *Hind*III/*Xho*I sites of M1498 [*YEplac181[LEU2]* with BSKSII] to create D745 [*YEp[LEU2]*]. The *Ty1* coding region plus 3'- δ as an *Xho*I-*Apa*I fragment from D570 [*YEp[LEU2]:Ty1*] was cloned into the *Xho*I/*Apa*I sites of D745 to create D848 [*YEp[LEU2]:pPGK-Ty1*]. D848 was digested with *Apa*I and ligated to an *Apa*I to *Not*I converter (5' > gcggccgcggcc > 3') to create D1253. The 8-kb *Not*I fragment of D1253 containing *Ty1* was cloned into the *Not*I site of D1242 to create D1256 [*YEp[LYS2]:pPGK-Ty1*]. D571 [*YEp[LEU2]:pGAL-Ty1*] was digested with *Apa*I and ligated to an *Apa*I to *Not*I converter (5' > gcggccgcggcc > 3') to create D1255. The 8-kb *Not*I fragment of D1255 containing *Ty1* was cloned into the *Not*I site of D1242 to create D1257 [*YEp[LYS2]:pGAL-Ty1*]. The 4.5-kb *Kpn*I-*Sac*I fragment of D871 containing *STE11* was cloned into the *Kpn*I/*Sac*I sites of BS KS⁺ to create D872. The 2-kb *Eco*RI-*Pst*I fragment of D872 containing the *STE11* ORF was replaced by the 700-bp *Eco*RI-*Pst*I fragment of pJJ248 containing *TRP1* to create D873 [*ste11::TRP1* in BS].

Jiang

Table 3. Yeast strains

Strain	Genotype	Background	Source
W303A	<i>MATα ade2 can1 his3 leu2 trp1 ura3-1</i>	W303	R. Rothstein
W303B	<i>MATα ade2 can1 his3 leu2 trp1 ura3-1</i>	W303	R. Rothstein
DY4073	<i>MATα ade2 can1 his3 leu2 lys2 trp1</i>	W303	D.J. Stillman
Y32	<i>MATα Ty1-URA3-32 ade2 can1 his3 leu2 trp1 ura3-1</i>	W303	This study
Y33	<i>MATα Ty1-URA3-33 ade2 can1 his3 leu2 trp1 ura3-1</i>	W303	This study
Y1149	<i>MATα ade2 can1 his3 leu2 ura3</i>	W303	This study
Y1203	<i>MATα Ty1-TRP1KL ade2 can1 his3 leu2 ura3</i>	W303	This study
UCC16	<i>MATα adh4::URA3 ade2 can1 his3 leu2 trp1 ura3</i>	W303	D. Gottschling
Y442	<i>MATα Ty1-URA3-33 ste12::Leu2 ade2 can1 his3 leu2 trp1 ura3</i>	W303	This study
Y461	<i>MATα Ty1-URA3-33 ste11::TRP1 ade2 can1 his3 leu2 trp1 ura3</i>	W303	This study
Y443	<i>MATα Ty1-URA3-33 gcn5::HIS3 ade2 can1 his3 leu2 trp1 ura3</i>	W303	This study
YPH499	<i>MATα ade2 gal3 his3 leu2 lys2 trp1Δ1 ura3-52</i>	S288C	P. Heiter
337	<i>MATα Ty0 gal3 ura3 ρ^-</i>	337	C.M. Wilke
Y200	<i>MATα Ty0 gal3 lys2 ura3 ρ^-</i>	337	This study
Y211	<i>MATα Ty0 gal3 leu2 lys2 ura3 ρ^-</i>	337	This study
Y1140	<i>MATα Ty0 gal3 leu2 lys2 ura3</i>	337	This study
Y1144	<i>MATα Ty0 gal3 leu2 lys2 ura3 YCp [LEU2]Ty1-URA3-33</i>	337	This study

Strain constructions

The strains used in this study are listed in Table 3. The coding region of the *Kluyveromyces lactis* *TRP1* gene was PCR-amplified from pBS1479 with primers D1238 (5' > ACAT CAAATTTTACAAAACCTCGAATCTCGGTGGTATTATTC tgctcgtaaagtgtgtgttg > 3') and D1239 (5' > TGAGTTCAT CATCAGTGTATCTGACGTACGGGTTTCCGTTTACTG TCGtgaattcctgcagcc > 3') to create a *Ty1-TRP1KL* in-frame gene fusion knock-in construct (with the junction at nucleotide 902 of *Ty1*). Y1150 was a *Trp*⁺ transformant of a W303 strain with the *Ty1-TRP1KL* knock-in construct DNA, and recombination between the knock-in construct and a single *Ty1* element was verified by Southern (with a *Ty1-TRP1KL* probe) and successful cloning of a full-length *Ty1-TRP1KL*. Y1150 was backcrossed once to obtain Y1203. Y442 was a *Leu*⁺ sterile transformant of Y33 transformed with pSUL16 *ste12::LEU2* gene disrupter (Fields and Herskowitz 1987) digested with *SacI* and *SphI*. A W303 *gcn5::HIS3* strain DY5925 (a gift from D.J. Stillman, University of Utah, Salt Lake City) was crossed with Y33 to create Y443. Y461 was a *Trp*⁺ sterile transformant of Y33 transformed with a D873 *ste11::TRP1* gene disrupter digested with *SacI* and *KpnI*. P. Philippsen and M. Ciriacy created *Ty0* strain 337 by deleting the only *Ty1* element from a wild isolate (Wilke and Adams 1992). Y200 was an α -amino adipate-resistant *lys2* mutant of *Ty0* strain 337. Y200 was transformed with the *leu2::URA3* (popout) library D672 linearized with *NotI* for *Ura*⁺*Leu*⁻ transformants from which a 5-FOA-resistant strain (Y211) was then obtained. The ρ defect of Y211 was corrected by cytoduction (Lancashire and Mattoon 1979) with a *kar1-1* mutant MY686 (a gift from C.M. Rose, Princeton University, New Jersey) to obtain ρ + α -amino adipate-resistant Y1140, whose *Ty0* status was verified by a Southern with a 2.3-kb *Sall-EcoRI* *Ty1* probe that recognizes both the *Ty1* and *Ty2* ORFs. Y1140 was transformed with D804 to generate Y1144.

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