Genome-wide Expression Analysis of Sulfite Tolerance Genes in *Saccharomyces Cerevisiae*

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Abstract. Sulfite is an important food preservative that is widely used in wine brewing, and the mechanism of sulfite metabolism in some strains of *Saccharomyces cerevisiae* has been reported. However, there is still something unclear in the sulfite metabolism, because there are different mechanisms in the different strains. To study the sulfite metabolism in the transcriptional network and gene expression of *S. cerevisiae* strains, *SSU1* and *FZF1* genes were identified from genome sequences of yeast strains, and the transcriptional profile was mined from cDNA microarray. The fifth zinc finger of *FZF1* protein was found to be another vital region for regulation, just as the first and fourth fingers previously reported. The functional difference of *SSU1* protein in all surveyed strains might be related to the variation sites at 19, 52, 164, 291 and 344, and that of *FZF1* protein might be related to the variation sites at 100, 107, 115, 120 and 258. The sulfur-tolerant capability of EC1118, UWOPS03-461-4, UWOPS05-217-3, UWOPS05-227-2, YPS128 and YPS606 strains was higher than the other strains surveyed, according to the CAI value of *SSU1* and *FZF1* genes. The evolutions of *SSU1* and *FZF1* genes were pushed by the mutation pressures. The expression levels of *SSU1* and *FZF1* genes might be enhanced by *ROX1*, *ADR1*, *YPR015C*, *HAP4* and *GIS1* genes. In addition, the function of *YPR015C* was validated, and the *YPR015C* gene was firstly found to play an important role in regulating sulfite metabolism.

1. Introduction
Sulfite was a potentially toxic but normal metabolite produced during reductive sulfite assimilation in yeast, which was used as a food preservative [1-3]. Sulfite was also used to mediate the stabilities of wine flavor and quality [4,5]. Therefore, it is important to study the metabolism mechanism of sulfite in yeast.

The sulfite metabolism in yeast has been reported to be regulated by the genes, *SSU1*, *FZF1* and *GRR1*. The previous studies showed that *SSU1* encoded a plasma membrane protein previously implicated in sulfite metabolism [1,2,5]. The *FZF1* gene of *Saccharomyces cerevisiae* encoded a five-zinc-finger transcription factor involved in sulfite tolerance, and
was found to be a positive regulator of SSU1 transcription [6]. SSU1p mediated efflux of the free form of sulfite, and multi-copy SSU1 or FZF1 could result in producing more efflux of free sulfite than SSU1 null mutant [2]. Aa et al. (2006) pointed out that the SSU1 and FZF1 genes were highly variable [7]. The SSU1 expression pattern was induced by the lack of differences among promoter regions [5]. There was a common translocation in wine yeast that formed a locus called SSU1-R, which was highly tolerant to sulfite [8]. More recently, another translocation ADH1-SSU1 was found to increase SSU1 gene expression and confer sulfite tolerance [9].

Transcription factors play an important role in gene expression. Chua et al. (2006) analyzed the effects of 55 yeast transcription factors on the growth phenotype [10]. According to the transcription factor analysis, non-coding changes affected the expression of FZF1, whereas coding changes affected the expression of SSU1, a sulfite efflux pump activated by FZF1 [11]. Although the mechanism of interaction between the SSU1 and FZF1 has been reported, little information about the function network of transcription factors affected by the expression of SSU1 and FZF1 genes were known. The study of the transcription factor network associated with sulfite tolerance was useful and vital for further interpreting the metabolism mechanism of sulfite in yeast.

Sulfite tolerance of different yeast strains had obvious differences. The differences in gene and protein structures might be the reason for the different sulfite tolerance. However, few pieces of evidence about the expression levels of SSU1 and FZF1 genes in different yeast strains were shown. Codon usage profiling of the genes reveals the characteristic features of the genes like nucleotide composition, gene expressivity, and optimal codons [12]. The variation in codon usage bias affected diverse cellular processes including protein expression, regulation, and folding [13]. Thus, the investigation of the codon usage bias of SSU1 and FZF1 genes in yeast helps explain the difference in sulfite tolerance of yeast strains.

To better understand the genes involved in sulfite tolerance of S. cerevisiae, in this study, SSU1 and FZF1 genes of 49 strains of S. cerevisiae were isolated from the genome sequences by the local blast. Then, the gene structures, functional domains, and 3D structures of both SSU1 and FZF1 proteins deduced were determined. Furthermore, the codon usage bias of both SSU1 and FZF1 genes was analyzed, and the co-expression of SSU1, FZF1, and other transcription factor genes was analyzed using the DNA microarray data. In addition, the function of YPR015C which was mined from the DNA microarray data was validated.

2. Materials and Methods

2.1. Yeast Genome Sequence Data and SSU1 and FZF1 Genes

Genome sequences of S. cerevisiae strains of CEN.PK113-7D, AWRI796, P283, YJSH1, FostersB, YJM993, VL3, Vin13, Sigma1278b and EC1118 were downloaded from NCBI. Additional yeast genome sequence data were downloaded from the Saccharomyces Genome Resequencing Project (SGRP, ftp://ftp.sanger.ac.uk/pub/users/dmc/yeast/latest). Which were re-sequenced from S. cerevisiae strains contained 273614N, 322134S, 378604X, BC187, DBVPG1106, DBVPG1373, DBVPG1788, DBVPG1853, DBVPG6040, DBVPG6044, DBVPG6765, K11, L-1374, L-1528, NCYC10, NCYC361, REF, RM11-1A, Y12, Y55, Y9, YIIc17-E5, YM789, YM975, YM978, YJM981, YPS128, YPS606, YS2, YS4, YS9, S288c, SK1, UWOPS03-461-4, UWOPS05-217-3, UWOPS05-227-2, UWOPS83-787-3, UWOPS87-2421, and W303.

The genes of SSU1 and FZF1 were identified from the genome sequences using local blast with query sequences of NM_001183906.1 and AY949918.1, respectively. The results of the blast were further screened using online servers of Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/promoter.html).

2.2. Structure Analysis of SSU1 and FZF1 Proteins

The amino acid sequences of the SSU1 and FZF1 proteins were deduced by SSU1 and FZF1 genes identified using the open reading frame (ORF) finder.
The ORF locations of both SSU1 and FZF1 genes in all yeast strains surveyed were compared. Analysis of SSU1 and FZF1 proteins was conducted by the method of Zhang et al [14]. The conserved domains, coils, and transmembrane helices of SSU1 and ZFF1 proteins were analyzed using the Expert Protein Analysis System of the Swiss Institute of Bioinformatics (http://cn.expasy.org/). The 3D structure of SSU1 and FZF1 proteins were modeled using the Swiss-Model online server (http://swissmodel.expasy.org/) [15].

2.3. Codon Usage Bias Analysis of SSU1 and FZF1 Genes
The GC composition at the third sites of codons (GC3) of SSU1 and FZF1 genes were predicted using the program codonW (http://codonw.sourceforge.net/). Additionally, the effective number of codons (ENC) and codon adaptation index (CAI) of each codon of SSU1 and FZF1 genes were calculated using the previous methods [16-19].

2.4. Co-expression of SSU1, FZF1, and Other Genes in Response to Sulfite Toxic
DNA microarray data of GSE5499 and GSE35308 were downloaded from NCBI and analyzed using the program package of Bioconductor. The normalized log ratio of Cy5/Cy3 for both SSU1 and FZF1 genes was used to determine the influence of sulfite treatment upon the gene expression of SSU1, FZF1, ROX1, ADR1, YPR015C, HAP4 and GIS1. Then, a heat map was drawn to analyze the co-expression of SSU1, FZF1, ROX1, ADR1, YPR015C, HAP4, and GIS1 in response to sulfite toxic using the R software according to the median normalized log2 ratio [20]. Furthermore, the protein interaction network of these genes was constructed using the STRING9.1 (http://string-db.org/) with the medium confidence and active prediction methods of co-expression, databases, experiments, and text-mining. GO enrichment analysis of these genes was performed using the Bioconductor program of goTools in the yeast GO database (GO: 0008150).

2.5. Validation of YPR015C Function
The strain used to validate was EC118, and the primers were designed using the Saccharomyces Genome Database (SGD). The function of YPR015C was validated by the gene-deletion method (http://www.sequence.stanford.edu/group/yeast_deletion_project/deletions3.html), and the KanMX gene was used as a selectable marker. The target gene deletion strain was selected in geneticin (G418)-containing plates and confirmed by PCR using KanMX primers. The primers used for validation were listed in Table 1. The selected transformations, together with the starting strain EC118 were inoculated and grown in YEP culture medium with 15 mM sulfite (repeated three times). The fermentation was measured and analyzed according to the method described by Liu et al [21]. RT-PCR analysis was conducted using the method described by Liu et al [22].

Table 1. Primers used in the study.

| Name    | Sequences                                                                 |
|---------|---------------------------------------------------------------------------|
| YPR015C-D-F | AAC TTT TAA TAT ACA TAA ATT TTC ATC TTC AAC ACT AAT                        |
|         | TAA ATG CGT ACG CTG CAG GTC GAC                                           |
| YPR015C-D-R  | CAT CAA AAT CAC ACT GAA AAC AAA ATA TTC TAA ACA ATG                       |
|         | CTT TCA ATC GAT GAA TTC GAG CTC G                                         |
| YPR015C-F      | CTC CCTTCCGAATGTGCATA                                                     |
| YPR015C-R      | AAAATGTATCGTGCAGCAGT                                                     |
| KanMX-F        | GTA TGC GAT AGT TCC TCA CTC TTT                                         |
KanMX-R
TGTGGTGATGATCTCGCA

SSU1-R
CCATAGCGAGCAATGCGCATA

ACT1-F
5'-CTG GGA YGA YAT GTA RAA GAT-3'

ACT2-R
5'-GYT CRG CCA GGA TCT TCA T-3'

PDA1-Lq
GGTCAGGAGCGCTATTGCTGT

PDA1-Rq
GACCAGCAATGGATCGTTCTTGG

3. Results

3.1. Structure of SSU1 and FZF1 Proteins
The open reading frame (ORF) of the SSU1 gene was shown in Figure 1-A. The ORFs of SSU1 genes in all strains were from 486 to 1862, with 458 deduced amino acids. Ten sites in SSU1 protein were achieved through multiple alignments, which included sites of 9, 16, 19, 52, 90, 122, 157, 164, 291, and 344 (Figure 1-A). The domain of the transmembrane transporter was the Pfam family member of SLAC1 (Accession number: PF03595), which were located from 11 to 410 (Figure 1-A). All but the ninth variation site were presented in the domain area. Nine coils were obtained with the Watch window 14. Three variations in amino acid sites were in the coil areas: 164th site, 291st site and 344th site (Figure 1-A). The variation bases at 19 and 52 were located in the first two transmembrane helices of SSU1 protein, which were towards outside and inside respectively. According to the variational bases and their locations, the 19th, 52nd, 164th, 291st, and 344th sites might be related to the functional difference of SSU1 protein of all surveyed strains.

Figure 1. Translation, domains, coils, and transmembrane helices, and 3D structure analysis of SSU1 and FZF1 proteins.

Note: Domains, coils, and transmembrane helices of SSU1 protein were shown in Figure A. The open reading frame (ORF) of the gene was shown in the blue frame. The green and grayish-green frames meant the domains and coils in SSU1 respectively. The red and purple frames represented the
transmembrane helices towards outside and inside respectively. The red lines meant the variation in amino acid sites. Domains and coils of FZF1 protein were shown in Figure C. 3D structures of SSUI and FZF1 proteins were shown in Figure B and D respectively.

The ORFs of the FZF1 gene were located from 476 to 1375, with 299 deduced amino acids (Figure 1-B). Twelve variation sites in FZF1 protein were achieved through multiple alignments, which included sites at 3, 100, 107, 115, 120, 132, 133, 210, 213, 228, 238 and 258 (Figure 1-B). Figure 1-B showed five SMART domains of the zinc finger where the 258th variation site was located. Six coils were obtained with the Watch window 14. Four variations in amino acid sites were in the coil areas: 100th, 107th, 115th, and 120th sites (Figure 1-B). According to the variational sites and their locations, the 100th, 107th, 115th, 120th and 258th sites might be related to the functional difference of FZF1 protein of all surveyed strains.

The 3D structures of SSUI and FZF1 proteins were shown in Figures 1-C and 1-D respectively. The vital variation sites were marked on the model of the 3D structure. The 344th variation site might extend the chamber coil, and the 164th variation site might increase the coil area (Figure 1-C). The coil structure changes might be related to the functional difference of FZF1 protein of all surveyed strains. Five domains of zinc finger were modeled on the 3D structure of the FZF1 protein, which was represented by the purple balls shown in Figure 1-D.
Figure 2. Codon usage bias of SSU1 and FZF1 genes.

Note: Red and blue plots meant the SSU1 and FZF1 genes respectively. Triangle, circle and square meant the CAI, ENC and GC3 respectively. Figure A showed the CAI and GC3 of SSU1 and FZF1 genes in all strains surveyed. The left tree was drawn based on the CAI and GC3. Figure B showed the relationship between ENC and GC3. The expected curve between GC3 and ENC under random codon usage was represented.

3.2. Codon Usage Bias of SSU1 and FZF1 Genes

Forty-nine strains were divided into two groups, according to the CAI and GC3 of SSU1 and FZF1 genes (Figure 2-A). The strains of YS4, VL3, P283 and Vin13 were classed into Group 1, and the other strains were clustered to Group 2. The CAI was the vital index to measure gene expression. The higher the CAI value was, the higher the gene expression was. The results indicated that the expression levels of SSU1 and FZF1 genes in strains of EC1118, UWOPS03-461-4, UWOPS05-217-3, UWOPS05-227-2, YPS128 and YPS606 may be higher than the other strains surveyed.

The relationship between ENC and GC3 under the mutation pressure was presented in the curve of Figure 2-B. A majority of strains were below the expected curve. Thus, the evolutions of SSU1 and FZF1 genes may be pushed by the mutation pressures.
Figure 3. Co-expression of SSU1 and FZF1 genes.

Note: Effects of over-expression and deletion of transcription factors on the expression of the SSU1 gene were shown in Figure A. High and low gene expression levels were presented on the positive and negative Cy5/Cy3 value areas respectively. Red plots meant over-expression of transcription factors, and blue plots meant deletion of transcription factors. Figure B showed the influence of over-expression and deletion of transcription factors upon expression of the FZF1 gene. A heat map of co-expression of SSU1 and FZF1 genes was presented in Figure C. The protein interaction network of these genes was shown in Figure D. Different color lines represented different interaction methods.

The results of the DNA microarray analysis for GSE5499 showed that the over-expression and deletion of transcription factors had obvious effects on the expression of SSU1 and FZF1 genes. The SSU1 and FZF1 genes played important roles in regulating the sulfite tolerance. According to whether the SSU1 and FZF1 genes were activated or inhibited by the over-expression and deletion of transcription factors, the transcription factors were divided into four groups as shown in Figures 3-A and 3-B. The SSU1 gene expression could be activated by either the over-expression of the transcription factors in Groups 1 and 3 or the deletion of them in Groups 2 and 4. Although the SSU1 gene expression could be inhibited by either the over-expression or the deletion of these transcription factors (Figure 3-A), seven up-regulated genes and four down-regulated genes for the SSU1 gene expression were achieved in Groups 1 and 2 respectively.

Just like the expression of the SSU1 gene, the expression of the FZF1 gene could be activated by either the over-expression of the transcription factors in Groups 1 and 3 or the deletion of them in Groups 2 and 4. However, the FZF1 gene expression could be inhibited by either the over-expression or the deletion of these transcription factors (Figure 3-B). Eight up-regulated genes and three down-regulated genes for the FZF1 gene expression were achieved in Groups 1 and 2 respectively.

The heat map in Figure 3-C showed that the expression levels of SSU1, FZF1, ROX1,
ADRI, YPR015C, HAP4 and GIS1 genes were obviously improved after 15 min of sulfite treatment. Thus, the gene expression of SSU1 and FZF1 may be activated by the genes of ROX1, ADRI, YPR015C, HAP4 and GIS1.

The protein interaction network of these genes was shown in Figure 3-D. The sulfite pump was regulated by the SSU1 gene, which was mediated by the FZF1 and GRR1 genes. The GRR1 gene was regulated by the REG1 and RGT1 genes. RGT1 gene was controlled by four genes, including CAT8, ADRI and SNF1. The CAT8 gene was regulated by the genes of GIS1, ADRI and YPR015C. Statistic results of GO enrichment analysis showed that eight genes were involved in the cellular response to stress, and three genes in transport (Table 2).

| Genes  | Biological Process                                  |
|--------|-----------------------------------------------------|
| ADRI   | Cellular response to ethanol (GO:0071361)            |
| GRR1   | Cellular response to pheromone (GO:0071444)         |
| SNF1   | Cellular response to starvation (GO:0009267)        |
| REG1   | Cellular response to starvation (GO:0009267)        |
| CAT8   | Cellular response to stimulus (GO:0051716)          |
| ROX1   | Cellular response to stress (GO:0033554)            |
| HAP4   | Regulation of cellular respiration (GO:0043457)     |
| FZF1   | Regulation of transport (GO:0051049)                |
| RGT1   | Regulation of transport (GO:0051049)                |
| GIS1   | Response to stress (GO:0006950)                     |
| SSU1   | Transport (GO:0006810)                              |
| YPR015C| Biological process (GO:0008150)                     |

3.3. The Function of YPR015C
The results of small-scale fermentation experiments (Figure 5) showed that the fermentation abilities of the transformations were lower than the start strain EC1118 in the grape juice containing 15 mM sodium sulfite. There was a significant difference between the maximum weight loss rates of the transformations and the start strain EC1118 (P > 0.001) (Figure 4). These results indicated that the deletion of the YPR015C gene could decrease the tolerance of S. cerevisiae to sulfite, and YPR015C played a role in the metabolism of sulfite in S. cerevisiae. The results of the qPCR analysis showed that the expression levels of SSU1 and FZF1 in the EC1118-ΔYPR015C were significantly lower than those in EC1118 (P > 0.001) (Figure 5). The results in part confirmed the results of co-expression analysis of the genes.
Figure 4. The small-scale fermentation results of strains in the grape juice containing 15 mM sodium sulfite.

Note: Fermentation capabilities of the selected transformation strains compared to the start strain EC1118. Weight loss was measured daily for 168 hours, and points were the average of triplicate fermentations.

Figure 5. The expression levels of FZF1 (A) and SSU1 (B) gene in EC1118 and EC1118-ΔYPR015C.

Note: With ACT1 and PDA1 as the housekeeping genes, expression levels of genes were calculated using the 2^−ΔΔCt method. Values were mean ± SE (n = 3). **: P < .01, with one-way ANOVA.

4. Discussion

In the study, the ORFs of SSU1 and FZF1 gene in all strains were found to contain 458 and 299 deduced amino acids, respectively. Similar results were reported in other strains. The SSU1 gene with the coding region of 1377 bp and the FZF1 genes with the coding region of 900 bp were obtained from 30 S. cerevisiae strains [7]. The changes in the coding region might affect the protein structures, which would affect the protein functions directly. The ORFs of SSU1 and FZF1 genes in S. cerevisiae were conserved, which meant the coded proteins might be conserved.

The domain of transmembrane transporter located from 11 to 410 was the Pfam family member of SLAC1, where all but the ninth variation site were identified. The transmembrane transporter was the vital region to pump sulfite. Furthermore, the transmembrane helices structures were also an important area to mediate the sulfite metabolism. Coils were the important structures to regulate the protein function. Thus, the variation in the functional domains, coils and transmembrane helices might affect the protein function. The functional difference of SSU1 protein in all surveyed strains might be related to the variation sites at 19, 52, 164, 291 and 344, which were surveyed in the functional domain region. The ORFs of SSU1 and FZF1 genes in S. cerevisiae were conserved, which meant the coded proteins might be conserved.

The domain of transmembrane transporter located from 11 to 410 was the Pfam family member of SLAC1, where all but the ninth variation site were identified. The transmembrane transporter was the vital region to pump sulfite. Furthermore, the transmembrane helices structures were also an important area to mediate the sulfite metabolism. Coils were the important structures to regulate the protein function. Thus, the variation in the functional domains, coils and transmembrane helices might affect the protein function. The functional difference of SSU1 protein in all surveyed strains might be related to the variation sites at 19, 52, 164, 291 and 344, which were surveyed in the functional domain region. Similar results were reported by Peng et al. (2013) [23]. The only one site of substitution between basic amino acids (345R/K) was crucial for sulfite pump to maintain their routine function.

Just like the SSU1 protein, similar results were found in the FZF1 protein. Five SMART domains of zinc finger were identified from the FZF1 protein. Zinc finger proteins represent the largest and most diverse superfamily of nucleic acid binding proteins in eukaryotes, which participate in a variety of cellular activities, including development, differentiation, cell cycle and tumor suppression [24]. The FZF1 gene in S. cerevisiae encoded a five-zinc-finger transcription factor involved in sulfate tolerance, and was found to be a positive regulator of SSU1 transcription [6]. Thus, the zinc finger domain was a vital area for SSU1 transcription. Furthermore, the first and fourth zinc finger protein areas were found to be involved in DNA binding and DNA recognition respectively [6,25]. Consequently, the functional difference of FZF1 protein in different strains might be related to the variation sites at 100, 107, 115, 120 and 258. Especially, the 258th variation site was achieved in the fifth zinc finger area, so the fifth zinc finger domain might be another vital area for determining the expression level of the SSU1 gene.
The codon usage bias was used to compare the gene expressivity [12]. The higher the CAI value was, the higher the gene expression level was. The expression levels of SSU1 and FZF1 genes in strains of EC1118, UWOPS03-461-4, UWOPS05-217-3, UWOPS05-227-2, YPS128 and YPS606 may be higher than the other strains surveyed. The evolutions of SSU1 and FZF1 genes were pushed by the mutation pressures. A high level of polymorphism has been observed in the SSU1 gene among vineyard-isolated strains, suggesting that this transport system is important in the evolution of SO2 resistance mechanisms [5].

The SSU1 and FZF1 genes played important roles in regulating the sulfite tolerance. We first reported the function network of transcription factor genes, which regulated the expression of the SSU1 and FZF1 genes. The expression of SSU1 and FZF1 genes could be enhanced by improving the gene expression of the ROX1, GIS1, HAP4, YPR015C and ADR1.

The ROX1 gene encodes a repressor of the hypoxic functions in S. cerevisiae [26]. Transcriptional activator/repressor GIS1 was involved in the regulation of gene expression upon nutrient starvation [27,28]. Transcriptional activator HAP4 acts as a component of the CCAT-binding factor, which is a transcriptional activator and binds to the upstream activation site (UAS2) of the CYC1 gene and other genes involved in mitochondrial electron transport, and activates their expression [29]. YPR015C was recently identified as one of 100 novel, weakly expressed cell cycle-regulated genes when yeasts were grown in a fermentor using a minimum medium [24]. Here, the function of YPR015C was validated. The results showed that the deletion of YPR015C would lead to the sulfite tolerance decrease and the decreased expression levels of SSU1 and FZF1.

The sulfite resistance genes might be associated with the shift of carbon source. In S. cerevisiae, glucose is the preferred carbon source, and fermentation is the major pathway for energy production, even under aerobic conditions [30,31]. With the fermentation processing further, glucose level descended and ethanol level increased. The ethanol produced would be a substitution of the carbon source for fermentation. The ADR1 is a positive regulator of ADH2 encoding alcohol dehydrogenase, an enzyme involved in the conversion of ethanol to acetaldehyde [31,32]. Park & Hwang [33] reported the response of S. cerevisiae to sulfite using the genome-wide transcript profiling. Genes involved in carbohydrate metabolism represented the highest proportion of induced genes, which may account for the easily required resistance to sulfite. The report supported our surveyed results of up-regulated genes.

5. Conclusions
SSU1 and FZF1 genes in 49 strains of S. cerevisiae were identified. The fifth zinc finger region was identified as a vital region in regulation, similar to the first and fourth finger regions, and was the polymorphism region linked to the different expression levels of the FZF1 gene in different strains.

The functional difference of SSU1 and FZF1 proteins in all surveyed strains might be related to the variation sites in functional domains. The sulfur-tolerant capability of EC1118, UWOPS03-461-4, UWOPS05-217-3, UWOPS05-227-2, YPS128 and YPS606 strains was higher than the other strains surveyed, according to the CAI value of SSU1 and FZF1 genes.

The mutation pressures pushed the evolution of SSU1 and FZF1 genes. The sulfite tolerance of S. cerevisiae involved genes of the cellular response to stress and of the shift of carbon source. The expression of SSU1 and FZF1 genes might be up-regulated by the ROX1, ADR1, YPR015C, HAP4 and GIS1 genes. And the function of YPR015C was validated.

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