Mol Gen Genet (2000) 262: 940–948

ORIGINAL PAPER

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Extragenic suppressors that rescue defects in the heat stress response of the budding yeast mutant *tom1*

Received: 2 July 1999 / Accepted: 24 September 1999

Abstract The *TOM1* gene codes for a so-called HECT protein, a putative ubiquitin ligase, in *Saccharomyces cerevisiae*. Deletion of the entire gene (*tom1-10*) or the sequence encoding the HECT domain (*tom1-2*) causes temperature sensitivity for growth. Here we report the isolation of extragenic, recessive suppressors of *tom1-2*, which were designated *tmr* (for *tom1* revertant) mutations. These were classified into eight complementation groups and six of the genes were identified: *tmr1*/*cyr1*, *tmr2*/*sch9*, *tmr3*/*zuo1*, *tmr4*, *tmr5*/*mot1*, *tmr6*/*sse1*, *tmr7* and *tmr8*/*kre6*. These results suggested that the *tom1* phenotype can be rescued by down-regulating the cAMP/PKA pathway. It was found that the temperature sensitivity of the *tom1-2* mutant is indeed suppressed by multiple copies of *PDE2* or *BCY1*, which encode negative regulators of the cAMP/PKA pathway. The *MSN2* gene, which encodes a zinc-finger transcription factor involved in the general stress response is also a multicopy suppressor of *tom1*. It was found that induction levels of both STRE-mediated (general stress response) and HSE-mediated gene expression (heat shock response) upon shift to high temperature are reduced by more than half in the *tom1* mutant. Most of the isolated *tmr* mutations rescued one of the defects seen in both types of heat stress response in the *tom1* mutant.

Key words *tom1* revertants · cAMP/PKA pathway · Heat stress response · Msn2 transcription factor

Introduction

At elevated temperatures, various cellular functions are altered, and expression of many genes is regulated to cope with the heat stress. For example, various heat-shock proteins are expressed that help to refold heat-denatured proteins, and ubiquitin is synthesized and conjugated to damaged proteins, which are thus marked for selective degradation by the 26S proteasome (Craig 1992; Hochstrasser 1996; Hershko and Ciechanover 1998). In budding yeast, when cells are exposed to high temperatures, at least two signal transduction pathways are known to be activated – the heat shock response and the general stress response. In the heat shock response, the transcription factor Hsf1, bound to the HSE element which acts as an upstream activating sequence, activates transcription of genes encoding heat-shock proteins like Ssa3 upon shift to high temperature (Craig 1992). In the general stress response, two redundant zinc-finger transcription factors, Msn2 and Msn4, translocate to the nucleus in response to various stresses, such as starvation for carbon, osmotic and oxidative stresses, as well as heat stress. Msn2/Msn4 binds to the STRE element and activates the transcription of many genes, including *UBI4* and *HSP104* (Estruch and Carlson 1993; Martínez-Pastor et al. 1996; Moskvina et al. 1998). Recently it has been shown that cAMP-dependent protein kinase (PKA) negatively regulates the nuclear translocation of Msn2/Msn4 (Görner et al. 1998).

The ubiquitin system is known to be required for diverse cellular processes, including homeostasis, stress responses, cell cycle regulation, DNA repair, apoptosis, antigen processing, and gene expression. The main function of ubiquitin is to mark proteins for selective degradation. Multiple molecules of ubiquitin can be ligated to a protein substrate to form multi-ubiquitin chains which are then recognized and degraded by the 26S proteasome in an energy-dependent manner (Hochstrasser 1996; Hershko and Ciechanover 1998).

The *TOM1* gene of budding yeast encodes a 380-kDa protein containing a HECT domain that is homologous to a human ubiquitin ligase, E6-AP C-terminus (Scheffner et al. 1993; Huibregtse et al. 1995; Utsugi et al. 1999). At high temperatures the *tom1-1* mutant exhibits pleiotropic phenotypes. Cells arrest at the G2/M

Communicated by H. Ikeda

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boundary in the cell cycle. Simultaneously their nuclear and nucleolar structures become abnormal and poly(A)$^+$RNA accumulates in the nucleus. A deletion that affects only the HECT domain of *TOM1* (*tom1-2*) causes temperature-sensitive growth (Utsugi et al. 1999). Here we report the isolation of extragenic, recessive suppressors of the *tom1-2* mutation, called *tmr* (*tom1* revertant) mutations. Such suppressor genes could encode putative substrates of Tom1-dependent proteolysis or related proteins. Alternatively, certain pathways that function antagonistically to the Tom1-mediated pathway might be impaired in the revertants. We identified six *tmr* genes and found that one way to circumvent the phenotypic defect in the *tom1* mutant is to down-regulate the cAMP/PKA pathway, thereby activating the general stress response. Most *tmr* mutations appeared to overcome the growth defect of the *tom1* mutant at high temperatures by changing the pattern of gene expression.

Materials and methods

Yeast strains, media and genetic methods

Yeast strains used in this study are listed in Table 1. Culture media and plates containing 5-FOA (5-fluoro-orotic acid) were prepared as described in Kaiser et al. (1994). Benomyl (GL Sciences) was added to media to a final concentration of 7.5–15 μg/ml. Crosses, sporulation, tetrad dissection, yeast transformation and microscopic observations were performed according to Kaiser et al. (1994). In order to test for synthetic lethality of the *tmr7 kre6* double mutant, the strain YTS703-A (*MAT***a** *tmr7-50*) was transformed with pTS802 carrying *KRE6* and *URA3*. The transformant was then mated with YTS803-B (*MATα kre6-LEU2*) and the resulting diploid was subjected to tetrad dissection.

Plasmids and DNA manipulations

Standard DNA manipulations were performed according to Sambrook et al. (1989). Nucleotide sequences were determined by the dideoxy chain-termination method, using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). To isolate the wild-type *TMR* genes by complementation of the growth defects of the respective mutants, a genomic library constructed in YCp50 (Stinchcomb et al. 1982) was used, which was kindly provided by Dr. R. Davis (Stanford University). Plasmids pTS101, pTS201, pTS301, pTS501, pTS601 and pTS801 were isolated from the library (Fig. 3). The 5.4-kb *Sal*I fragment containing only the *KRE6* ORF from pTS801 was subcloned into the single-copy vector pRS316 (Sikorski and Hieter 1989) to generate pTS802. The YIp-type plasmid, pTS206, carrying the 3-kb *Eco*RI-*Sal*I fragment containing *SCH9* on YIp5 (Botstein et al. 1979), was digested with *Sac*I for transformation. The plasmid pTS307 contained the 3.5-kb *Eco*RI-*Sph*I fragment bearing *ZUO1* on YIp5, and was linearized with *Hin*dIII for transformation. The plasmid pTS512, containing the 10.2-kb *Xho*I-*Pst*I fragment carrying *MOT1* on pJJ282 (Jones and Prakash 1990), was digested with *Nhe*I to allow integration into the chromosome. The plasmid pTS611 carries the 4.8-kb *Cla*I-*Sal*I fragment containing *SSE1* on YIp5 and was digested with *Bgl*II. The high-copy-number plasmid pSM1 containing *BCY1* was described previously (Yamano et al. 1987). A multicopy plasmid, YEp24 (Botstein et al. 1979), containing *PDE2* was isolated in this laboratory and a single-copy plasmid carrying *RAS2*^{val19} was provided by Dr. K. Matsumoto (Nagoya University). pGM18/17 containing seven copies of the consensus STRE element upstream of a *LEU2-lacZ* reporter was a kind gift from Dr. G. Marchler (University of Vienna; Marchler et al. 1993). YIp-HSE-lacZ was constructed by deleting the 2.1-kb *Hin*dIII fragment of pSSA1HSE2-lacZ, which was provided by Dr. K. Kohno (Nara Institute of Advanced Science and Technology; Oka et al. 1997). After digestion with *Stu*I, the plasmid was integrated into the *URA3* locus. The plasmid pTOM5, carrying *TOM1* on pRS316 (Sikorski and Hieter 1989), was described previously (Utsugi et al. 1999). The plasmid pDK001, constructed by D. Kaida, contains a 3.8-kb PCR product encompassing *MSN2* on YEplac195 (Gietz and Sugino 1988).

Plasmid rescue and sequencing after transposon mutagenesis

A genomic library carrying random Tn*3*::*LEU2* gene insertions was digested with *Not*I and introduced into the strain YTS004-A (*tom1-2*::*HIS3*). The library was a kind gift from Dr. M. Snyder of Yale University (Burns et al. 1994). From 70,000 Leu$^+$ transformants, 34 phenotypic *tom1* revertants were isolated at 35° C. In order to identify the genes affected by the insertions, one of the *tmr8* mutants, YTS801-A (*tom1-2 tmr8*::*lacZ-LEU2*, Amp$^+$), was transformed with *Pvu*I-cleaved YIp5 (Botstein et al. 1979). Genomic DNA was isolated from a single Ura$^+$ integrant and digested with *Nsi*I, and the digests were then self-ligated. Ligated DNA circles were transformed into *E. coli* to select ampicillin-resistant colonies. After purifying plasmids, the DNA sequence adjacent to the transposon was determined, using a primer based on the mini-Tn*3* sequence.

Assay of the β-galactosidase activity

Cells containing the STRE-*lacZ* or HSE-*lacZ* reporter were grown to mid-log phase in 10 ml of YPD medium at 30° C or 25° C and the cultures were shifted to 39° C or 38° C for 1 h. The cells were collected by centrifugation, cell lysates were prepared and the β-galactosidase activity was assayed according to Kaiser et al. (1994).

Results

Isolation of spontaneous revertants of *tom1-2*

The mutation *tom1-2* deletes the HECT region of *TOM1*, and causes temperature-sensitive growth when introduced into both YTS003-A (W303-1A background; Sutton et al. 1991) and YTS002-A (YPH499; Sikorski and Hieter 1989), just as in the original RAY-3A background (YTS001-A; Uesono et al. 1994), as described previously (Utsugi et al. 1999). We isolated 301 spontaneous phenotypic revertants (*tmr*; *tom1* revertants) from these *tom1-2* mutants. We then selected *tmr* mutants that showed a growth defect; 84 of the revertants were cold sensitive and 31 were sensitive to benomyl. We later found that most of the cold-sensitive *tmr* mutants showed slow growth at any temperature. Each *tmr* mutant was backcrossed with its isogenic *MATα tom1-2* mutant and the resulting *tmr*/*TMR tom1-2*/*tom1-2* diploid strains were subjected to tetrad analysis. In this way, we isolated four cold-sensitive, 49 slow-growing and 13 benomyl-sensitive *tmr* mutants. All the *tmr* mutations were recessive to the wild type with respect to both suppressor activity and the additional growth defect. Finally complementation tests were performed and the *tmr* mutants were classified into seven

Table 1 Yeast strains used in this study

Strain[a]	Genotype
RAY-3A	*MAT***a** *ura3 trp1 leu2 his3* (RAY)
YPH499	*MAT***a** *ade2 ura3 trp1 leu2 his3 lys2* (YPH)
W303-1A	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,-112 his3-11,-15 can1-100 ssd1-d2*
W303-51	*MAT***a** *STRE-lacZ-URA3*
W303-61	*MAT***a** *HSE-lacZ-URA3*
YTS001-A	*MAT***a** *tom1-2*::*LEU2* (RAY)
YTS002-A	*MAT***a** *tom1-2*::*LEU2* (YPH)
YTS003-A	*MAT***a** *tom1-2*::*LEU2*
YTS003-B	*MATα tom1-2*::*LEU2*
YTS004-A	*MAT***a** *tom1-2*::*HIS3*
YTS004-C	*MAT***a** *tom1-2*::*HIS3 STRE-lacZ-URA3*
YTS004-D	*MAT***a** *tom1-2*::*HIS3 HSE-lacZ-URA3*
YTS010-A	*MAT***a** *tom1-10*::*HIS3*
YTS011-A	*MAT***a** *tom1-10*::*HIS3 STRE-lacZ-URA3*
YTS012-A	*MAT***a** *tom1-10*::*HIS3 HSE-lacZ-URA3*
YTS102-A	*MAT***a** *tmr1-60 tom1-2*::*LEU2* (RAY)
YTS105	*MAT***a**/*MATα tmr1-60*/*tmr1-60 tom1-2*::*LEU2*/*tom1-2*::*LEU2* (RAY)
YTS106	*MAT***a**/*MATα tmr1-60*/*tmr1-60* (RAY)
YTS151	*MAT***a** *tmr1-60 tom1-10*::*HIS3 STRE-lacZ-URA3* (RAY/W303)
YTS201-A	*MAT***a** *tmr2-17 tom1-2*::*LEU2*
YTS213	*MAT***a**/*MATα tmr2-1*/*tmr2-1 tom1-2*::*LEU2*/*tom1-2*::*LEU2* (YPH)
YTS251	*MAT***a** *tmr2-17 tom1-10*::*HIS3 STRE-lacZ-URA3*
YTS252	*MAT***a** *tmr2-17 tom1-10*::*HIS3 HSE-lacZ-URA3*
YTS301-A	*MAT***a** *tmr3-8 tom1-2*::*LEU2*
YTS303	*MAT***a**/*MATα tmr3-8*/*tmr3-8 tom1-2*::*LEU2*/*tom1-2*::*LEU2*
YTS351	*MAT***a** *tmr3-8 tom1-10*::*HIS3 STRE-lacZ-URA3*
YTS352	*MAT***a** *tmr3-8 tom1-10*::*HIS3 HSE-lacZ-URA3*
YTS407-A	*MAT***a** *tmr4-102 tom1-2*::*LEU2*
YTS451	*MAT***a** *tmr4-102 tom1-10*::*HIS3 STRE-lacZ-URA3*
YTS452	*MAT***a** *tmr4-102 tom1-10*::*HIS3 HSE-lacZ-URA3*
YTS501-A	*MAT***a** *tmr5-90 tom1-2*::*LEU2*
YTS504-A	*MAT***a** *tmr5-90*
YTS506	*MAT***a**/*MATα tmr5-90*/*tmr5-90 tom1-2*::*LEU2*/*tom1-2*::*LEU2*
YTS551	*MAT***a** *tmr5-90 tom1-10*::*HIS3 STRE-lacZ-URA3*
YTS552	*MAT***a** *tmr5-90 tom1-10*::*HIS3 HSE-lacZ-URA3*
YTS601-A	*MAT***a** *tmr6-11 tom1-2*::*LEU2*
YTS604	*MAT***a**/*MATα tmr6-11*/*tmr6-11 tom1-2*::*HIS3*/*tom1-2*::*LEU2*
YTS651	*MAT***a** *tmr6-11 tom1-10*::*HIS3 STRE-lacZ-URA3*
YTS652	*MAT***a** *tmr6-11 tom1-10*::*HIS3 HSE-lacZ-URA3*
YTS701-A	*MAT***a** *tmr7-50 tom1-2*::*LEU2*
YTS703-B	*MATα tmr7-50*
YTS751	*MAT***a** *tmr7-50 tom1-10*::*HIS3 STRE-lacZ-URA3*
YTS752	*MAT***a** *tmr7-50 tom1-10*::*HIS3 HSE-lacZ-URA3*
YTS780	*MAT***a**/*MATα tmr7-50* /*TMR7 tmr8-1* (*kre6*::*lacZ-LEU2*, Amp$^+$)/*KRE6*
YTS801-A	*MAT***a** *tmr8-1*(*kre6*::*lacZ-LEU2*, Amp$^+$) *tom1-2*::*HIS3*
YTS851	*MAT***a** *tmr8-1*(*kre6*::*lacZ-LEU2*, Amp$^+$) *tom1-10*::*HIS3 STRE-lacZ-URA3*
YTS852	*MAT***a** *tmr8-1*(*kre6*::*lacZ-LEU2*, Amp$^+$) *tom1-10*::*HIS3 HSE-lacZ-URA3*

[a] All the strains were isogenic to W303-1A, except where indicated

complementation groups, as described in Table 2. Three *tmr* mutants from YTS001-A fell into one complementation group, *tmr1*. In contrast, various kinds of *tmr* mutants were isolated from YTS003-A (two *tmr2*, one *tmr3*, 10 *tmr4*, two *tmr5*, one *tmr6*, and one *tmr7*) and from YTS002-A (one *tmr1*, 10 *tmr2*, and three *tmr4*).

Isolation of *tmr* mutants by transposon mutagenesis

To isolate the different types of *tmr* mutants, we used transposon mutagenesis, as described earlier (Burns et al. 1994). The strain YTS004-A (*tom1-2*::*HIS3*) was transformed with a genomic library carrying random Tn*3*::*LEU2* gene insertions. From 70,000 Leu$^+$ transformants, 34 phenotypic *tom1* revertants were isolated, which were backcrossed with *tom1-2*. All the diploid strains failed to grow at 35° C, indicating that the reversions were recessive to the wild type. They were subjected to tetrad analysis. The *LEU2* marker was linked to the suppressor in 31 *tmr* mutants. Complementation tests revealed that all 31 *tmr* mutants fell into a new complementation group which was designated *tmr8*.

Characterization of *tmr* mutants

At 35° C all the *tmr* mutations suppressed *tom1-2*, but at 37° C only *trm4* and *tmr7* partially suppressed *tom1-2* (Fig. 1). The *tmr* mutations also suppressed the phenotype of the total disruption (*tom1-10*) at 35° C (data not shown).

Table 2 Complementation analysis of *tmr* mutations

Gene	Genetic background[a]		
	RAY	YPH499	W303
tmr1/cyr1	*58*, *60*, *63*	*106*	–
tmr2/sch9	–	*1*, *3*, *4*,...[b]	*17*, *89*
tmr3/zuo1	–	–	*8*
tmr4	–	*19*, *35*, *37*	*24*, *53*, *85*, *88*, *93*, *100*, *102*, *103*, *109*, *111*
tmr5/mot1	–	–	*39*, *90*
tmr6/sse1	–	–	*11*
tmr7	–	–	*50*

[a] Background in which the mutations were isolated. The allele numbers in each complementation group are listed

[b] A total of 10 *tmr2* mutations were isolated in YPH499

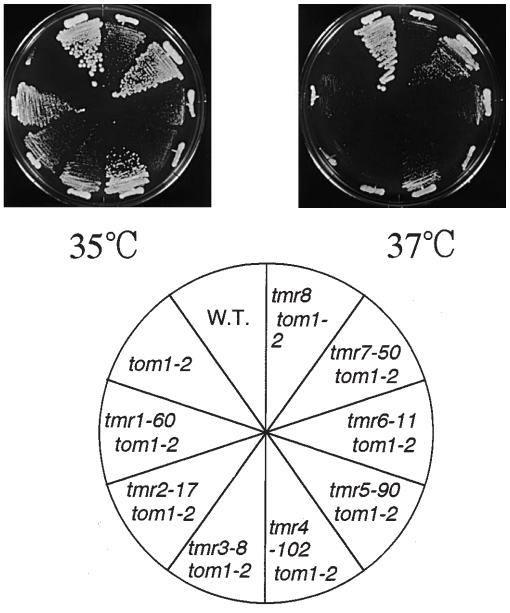


Fig. 1 Suppression of *tom1-2* by the *tmr* mutations. The following yeast strains were streaked on YPD plates and incubated at 35° C for 2 days or 37° C for 3 days: W303-1A (wild type), YTS003-A (*tom1-2*), YTS102-A (*tom1-2 tmr1-60*), YTS201-A (*tom1-2 tmr2-17*), YTS301-A (*tom1-2 tmr3-8*), YTS407-A (*tom1-2 tmr4-102*), YTS501-A (*tom1-2 tmr5-90*), YTS601-A (*tom1-2 tmr6-11*), YTS701-A (*tom1-2 tmr7-50*), and YTS801-A (*tom1-2 tmr8-1*)

Next we examined how these *tmr* mutations suppressed the morphological defects of the *tom1-2* mutant. As shown in Fig. 2, when an asynchronous culture of YST001A (*tom1-2*) was shifted to 35° C, a population of large-budded cells with a single nucleus accumulated, as described previously (Utsugi et al. 1999). Some 46% of these dumbbell-shaped *tom1-2* cells had a nucleus located away from the bud neck – a phenotype which were seldom seen in the wild-type dumbbell-shaped cells (3%). Few *tom1-2* cells were found in telophase. Also anucleate cells (13%) were observed among unbudded *tom1-2* cells. In contrast, YTS102-A (*tmr1-60 tom1-2*) cells accumulated in telophase (47%) and no anucleate unbudded cells were observed. The *tom1-2* cells carrying the other *tmr* mutations had similar phenotypes. In particular, the *tmr4* and *tmr7* mutations fully restored wild-type morphology.

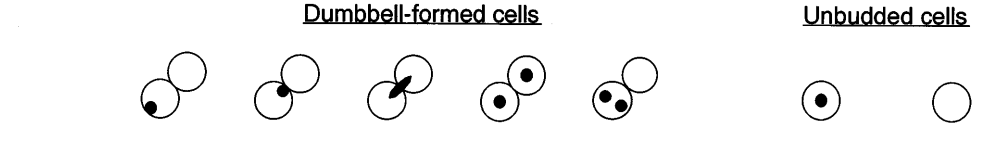
Identification of the *TMR* genes

To clone the *TMR* genes, we screened plasmids from the genomic library for the ability to complement the growth defects of the various *tmr* mutants. Secondly, we tested whether the plasmids thus recovered inhibited the growth of each *tmr tom1-2* double mutant at 35° C. By subcloning and sequence analysis, we identified the genes responsible, as shown in Fig. 3. Next we confirmed the identity of these genes by constructing YIp-type plasmids and integrating them into each *tmr tom1-2* double mutant by homologous recombination. Then tetrad analysis was performed. In this way we successfully identified *TMR1*, *TMR2*, *TMR3*, *TMR5* and *TMR6*.

TMR1 is identical to *CYR1*. A homozygous diploid strain YTS106 (*tmr1-60/tmr1-60*) was transformed with the YCp50 bank and Cs^+ transformants were selected at 15° C, from which the plasmid pTS101 was isolated. When this plasmid was introduced into YTS105 (*tmr1-60/tmr1-60 tom1-2/tom1-2*), the transformants grew at 15° C, but did not grow at 35° C. Subcloning and sequence analysis revealed that the gene responsible was *CYR1* (Fig. 3), which encodes adenylate cyclase, an effector of Ras-GTPase (Matsumoto et al. 1982).

TMR2 is identical to *SCH9*. Since YTS213 (*tmr2-1/tmr2-1 tom1-2/tom1-2*) grew slowly at 26° C, transformants that grew at a normal rate were selected at 26° C for 4 days. As shown in Fig. 3, the complementing gene turned out to be *SCH9*, which encodes a protein kinase homologous to cAMP-dependent protein kinase (PKA) (Toda et al. 1988). To confirm that *TMR2* was identical to *SCH9*, a YIp-type plasmid (pTS206) bearing *SCH9* was constructed, linearized with *Sac*I, and introduced into YTS201-A (*MAT***a** *tmr2-17 tom1-2*) by homologous integration. Normally growing and Ts^- transformants were selected and crossed with YTS003-B (*MATα tom1-2*). Tetrad analysis revealed that there were no Ts^+ or slow-growing segregants, indicating that the integrated *SCH9* was tightly linked to the *tmr2-17* mutation.

TMR3 is identical to *ZUO1*. Normally growing transformants of YTS303 (*tmr3-8/tmr3-8 tom1-2/tom1-2*) were selected at 26° C for 4 days. Subcloning and sequence analysis of yeast DNA from the isolated plasmid pTS301 revealed that the responsible gene was *ZUO1* (Fig. 3), which encodes zuotin, a tRNA-binding,



wild type	3	15	6	77	0	100	0
tom1-2	46	39	3	9	4	87	13
tmr1/cyr1 tom1-2	16	30	7	47	0	100	0

Fig. 2 Effect of *tom1* suppression on cell morphology and nuclear pattern. The strains RAY-3A (wild type), YTS001-A (*tom1-2*), and YTS102-A (*tmr1-60 tom1-2*) were grown at 26° C to logarithmic phase ($OD_{600} = 0.1$) and the cultures were then shifted to 35° C. After incubation for 6 h, cells were fixed and stained with DAPI, and their overall morphology was examined by phase-contrast microscopy; nuclear localization was determined by fluorescence microscopy. Data are presented as percentages. The total number of dumbbell-shaped cells observed was about 180 and that of unbudded cells was about 80

DnaJ-type chaperone (Zhang et al. 1992; Wilhelm et al. 1994; Yan et al. 1998). By using the YIp-type plasmid pTS307 carrying *ZUO1*, we confirmed that the integrated *ZUO1* was tightly linked to *tmr3-8*.

TMR5 is identical to *MOT1*. Normally growing transformants of YTS506 (*tmr5-90/tmr5-90 tom1-2/tom1-2*) were selected at 26° C. The plasmid pTS501 was isolated and the complementing gene was found to be an essential gene, *MOT1*, which encodes a presumptive helicase and functions as a transcriptional repressor (Fig. 3) (Davis et al. 1992). By using a YIp-type plasmid (pTS512) carrying *MOT1*, we confirmed that the integrated *MOT1* was tightly linked to *tmr5-90*.

TMR6 is identical to *SSE1*. Normally growing transformants of YTS604 (*tmr6-11/tmr6-11 tom1-2/tom1-2*) were selected at 26° C. The plasmid pTS601 was isolated and subcloning revealed that the complementing gene was *SSE1/MSI3* (Fig. 3), which encodes a member of the Hsp70 family (Mukai et al. 1993; Shirayama et al. 1993). By using a YIp-type plasmid (pTS611) carrying *SSE1*, we confirmed that the integrated *SSE1* was tightly linked to *tmr6-11*.

TMR8 is identical to *KRE6*. Genomic DNA flanking the *LEU2* insertion was isolated from YTS801-A. DNA sequencing revealed that the Tn*3* transposon was fused to *kre6*. The *KRE6* gene is involved in β-(1,6)-glucan synthesis (Roemer et al. 1991). We isolated the plasmid pTS801 by its ability to complement the slow-growth phenotype of YTS801-A. The 5.4-kb *Sal*I fragment from pTS801, containing only the *KRE6* ORF (pTS802), complemented *tmr8* (YTS801-A) (data not shown).

tmr single mutants

Each of the *tmr* mutations was isolated and the growth defects were found not to depend on the *tom1-2* mutation, except in the case of the strain YTS501-A (*mot1/tmr5-90 tom1-2*). As shown in Fig. 4A, the strain YTS504-A (*mot1/tmr5-90 TOM1*) grew much faster than YTS501-A (*mot1/tmr5-90 tom1-2*) at 26° C. Furthermore, YTS501-A carrying *TOM1* on a single-copy vector (pTOM5) grew more normally than the same strain carrying the vector alone. Thus the *mot1/tmr5-90* mutation suppresses the temperature sensitivity of *tom1–2* at 35° C, whereas the growth of the *mot1/tmr5* mutant is impaired by *tom1-2* at 26° C.

Synthetic lethality of *tmr7* and *kre6*

A large proportion of *tmr7-50* cells were large and swollen, and lysed when suspended in water (data not shown). We therefore suspected that the *tmr7-50* cells had defects in the cell wall. The *tmr8/kre6* mutation should also cause defects in cell wall integrity. A *tmr7-50* strain (YTS703-B) was crossed with YTS801-A (*kre6*::*LEU2 tom1-2*::*HIS3*), and the resulting diploid was subjected to tetrad analysis. Predicted *tmr7-50 kre6* double mutants seemed inviable or grew as microcolonies, regardless of whether the *tom1* mutation was present or not (data not shown). Thus we constructed a heterozygous diploid strain YTS780 (*tmr7-50/TMR7 kre6-LEU2/KRE6*) containing the plasmid pTS802 (YCp-*KRE6-URA3*); this strain was sporulated and subjected to tetrad dissection. About half of the Leu^+ Ura^+ clones were sensitive to 5-FOA (Fig. 4B), indicating that the *kre6 tmr7-50* double mutant was not able to grow in the absence of the *KRE6*-carrying plasmid.

Down-regulation of the cAMP/PKA pathway suppresses the *tom1* phenotype

Since *cyr1* and *sch9* were isolated as mutational suppressors of *tom1-2*, down-regulation of cAMP/PKA pathway appeared to suppress the *tom1-2* phenotype. To test this idea further, we introduced *BCY1*, which encodes a negative regulator of PKA (Toda et al. 1987), or *PDE2*, which codes for a high-affinity cAMP phosphodiesterase (Sass et al. 1986), into YTS003-A (*tom1-2*) on

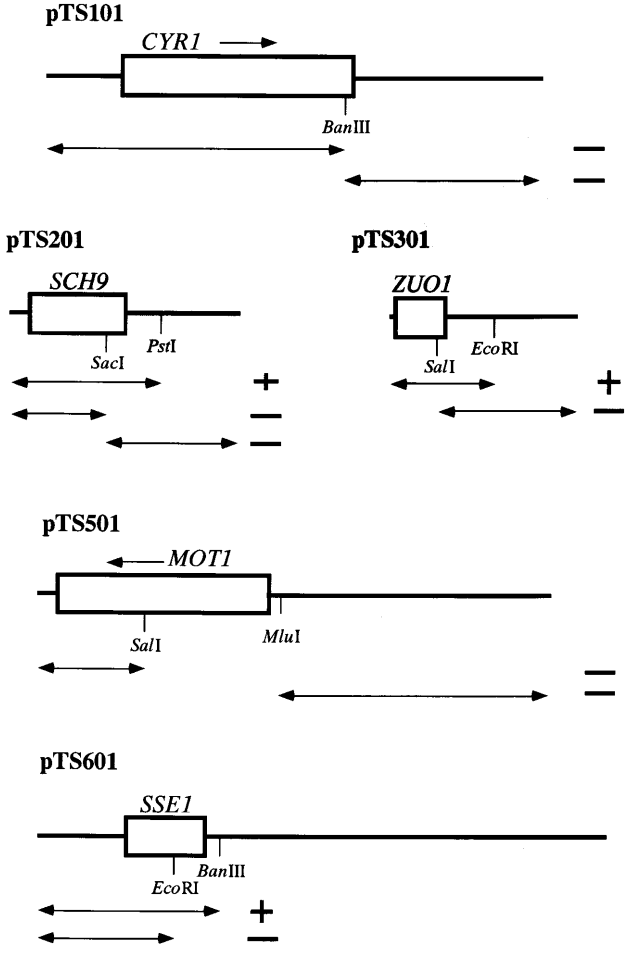


Fig. 3 Cloning and identification of the *TMR* genes. Deletions were constructed or DNA fragments were subcloned into the pRS316 vector and the resulting plasmids were examined for the ability to complement each *tmr* mutation (indicated by the *plus* signs; inability to complement is indicated by the *minus* signs). *Open boxes* represent the ORFs responsible for complementation

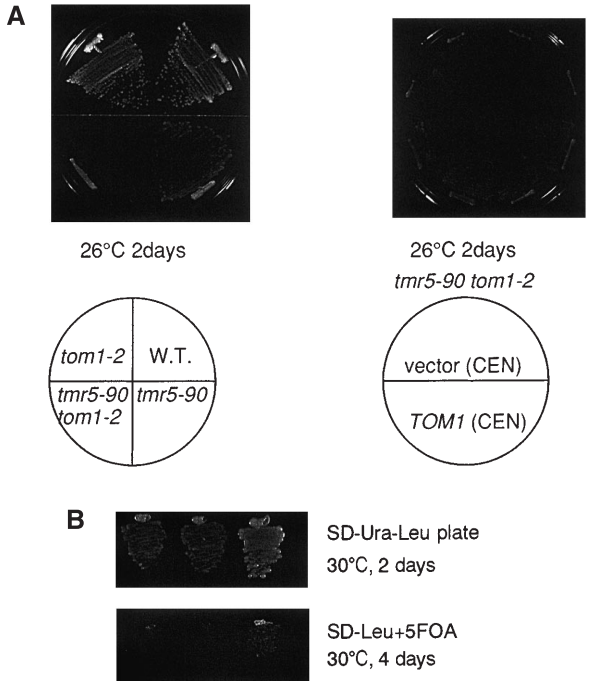


Fig. 4A, B Genetic interaction between *tmr5*/*mot1* and *tom1-2*, and between *tmr7* and *tmr8*/*kre6*. **A** The strains W303-1A (wild type), YTS003-A (*tom1-2*), YTS501-A (*mot1*/*tmr5-90 tom1-2*) and YTS504-A (*mot1*/*tmr5-90*) were streaked on a YPD plate (as indicated), which was incubated at 26° C for 2 days. Strain YTS501-A carrying the pRS316 vector or pTOM5 (pRS316-*TOM1*) was streaked (as indicated) on a SD-Ura plate, which was incubated at 26° C for 2 days **B** Synthetic lethality of *tmr7* and *kre6*. The diploid strain YTS780 (*tmr7-50*/*TMR7 kre6-LEU2*/*KRE6*) carrying a plasmid pTS802 (YCp-*KRE6-URA3*) was sporulated and subjected to tetrad dissection. Leu^+ Ura^+ transformants were selected and plated on a SD-Ura-Leu or SD-Leu + 5-FOA plate at 30° C for 2 or 5 days, respectively

a high-copy-number vector. High doses of these genes partially suppressed the growth defect of the *tom1-2* mutant at 35° C, as shown in Fig. 5A.

If down-regulation of the cAMP/PKA pathway suppresses the *tom1* mutation, hyperactivation of this pathway should have deleterious effects on the mutant. To test this, each *tmr tom1-2* double mutant was transformed with $RAS2^{Val19}$, which encodes a hyperactivate form of Ras2 (Toda et al. 1985). If certain *TMR* genes function downstream of the Ras-mediated pathway, these *tmr* mutations should attenuate the toxic effect of $RAS2^{Val19}$. As shown in Fig. 5B, $RAS2^{Val19}$ inhibited the growth of the *tom1 sch9*, *tom1 tmr4*, *tom1 mot1* and *tom1 sse1* mutants at 35° C. In contrast, $RAS2^{Val19}$ was not toxic to the *tom1 cyr1*, *tom1 zuo1* or *tom1 tmr7* mutants at 35° C. With respect to *cyr1*, this result is consistent with the fact that adenylate cyclase is the effector protein for the GTP-bound form of Ras2 (Toda et al. 1985). The other two *TMR* genes, *ZUO1* and *TMR7*, might function downstream of Ras.

Tom1 is involved in responses to heat stress

It has been shown that the cAMP/PKA pathway functions antagonistically to the general stress response (Smith et al. 1998). PKA suppresses nuclear translocation of Msn2/Msn4, zinc-finger transcription factors which act in the STRE-mediated general stress response (Görner et al. 1998). As shown in Fig. 5C, overexpression of *MSN2* on a multicopy plasmid suppresses the temperature sensitivity of *tom1-2* (YTS004-A).

To test whether Tom1 was involved in the general stress response, we measured the β-galactosidase activity of a STRE-*lacZ* reporter (Marchler et al. 1993). The induction level of the STRE-*lacZ* gene in the *tom1-2* (YTS004-C) or *tom1-10* (YTS011-A) mutant was less than half that seen in the wild type (W303-51), when the cultures were shifted to high temperature (39° C) (Fig. 6A). Furthermore, the heat shock response mediated through the HSE element was also impaired in the *tom1-2* and *tom1-10* mutants. The β-galactosidase activity of a HSE-*lacZ* reporter did not increase in either mutant (YTS004-D or YTS012-A) – unlike the case in

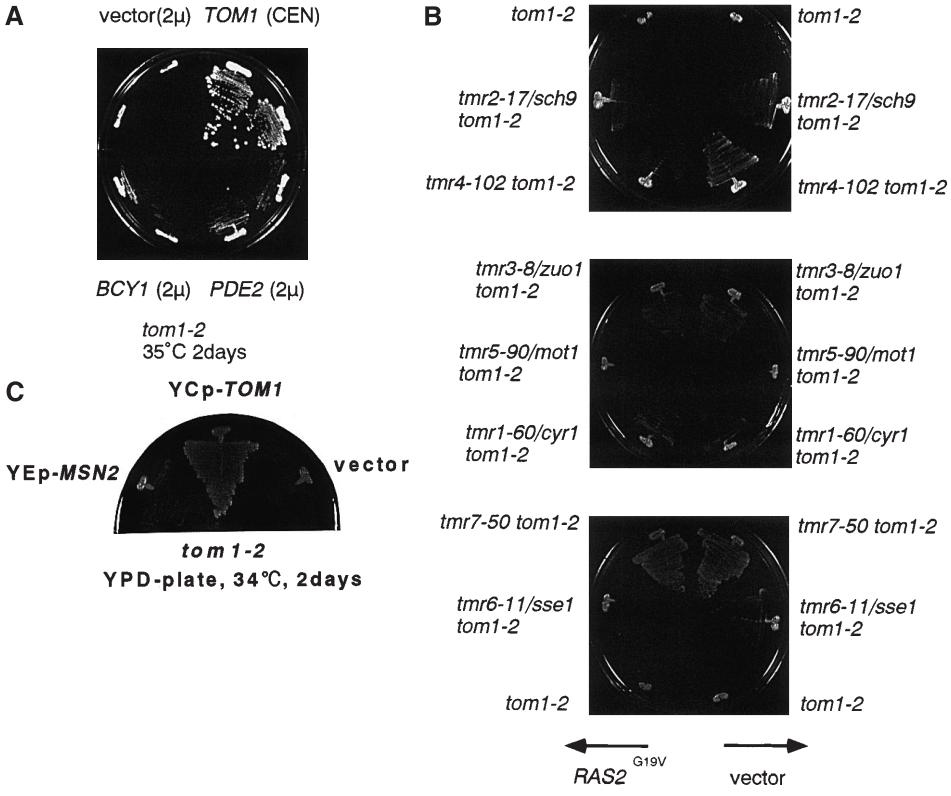


Fig. 5A–C Genetic interactions of *tom1* with the cAMP/PKA pathway. **A** High doses of *BCY1* and *PDE2* suppress *tom1-2*. Transformants of YTS003-A (*tom1-2*) containing pSM1 (YEp24-*BCY1*), YEp24-*PDE2*, YEp24 or pTOM5 (pRS316-*TOM1*) were streaked on a SD-Ura plate, which was incubated at 35° C for 2 days. **B** Effect of the hyperactive $RAS2^{Val19}$ on *tom1 tmr* mutants. The *tom1-2 tmr* mutants were transformed with a single-copy plasmid carrying $RAS2^{Val19}$ or with the pRS316 vector. The transformants were streaked on a SD-Ura plate, which was incubated at 35° C for 2 days. **C** *MSN2* is a multicopy suppressor of *tom1*. The strain YTS004-A (*tom1-2*) was transformed with pDK001 (YEplac195-*MSN2*), YEplac195 (vector) or pTOM5 (pRS316-*TOM1*), and the transformants were tested for growth on a YPD plate at 34° C for 2 days

wild-type cells (W303-61) – when the cultures were shifted to 38° C (Fig. 6B).

Most *tmr* mutations rescue the heat stress responses

Next we tested whether the general stress response of the *tom1* mutant was restored by the *tmr* mutations, by assaying the β-galactosidase activity of STRE-*lacZ* in the *tmr tom1* mutants (Fig. 6A). The activity was high even at 30° C in *tmr1*/*cyr1 tom1-10* (YTS151), *tmr2*/*sch9 tom1-10* (YTS251), *tmr3*/*zuo1 tom1-10* (YTS351) and *tmr8*/*kre6 tom1-10* (YTS851) mutants. On the other hand, the induction level remained low in the *tmr4 tom1-10* (YTS451), *tmr5*/*mot1 tom1-10* (YTS551) and *tmr7 tom1-10* (YTS751) mutants. Similar results were obtained in the *tom1-2* background (data not shown).

In contrast, the expression of HSE-*lacZ* was partially restored in the *tmr7 tom1-10* (YTS752) mutant at 38° C (Fig. 6B).

Discussion

Tom1 is a putative ubiquitin ligase. Without this protein, cells exhibit pleiotropic defects when shifted to high temperatures: cell cycle arrest at G2/M, abnormal nuclear and nucleolar structures, and accumulation of poly(A)$^+$ RNA in the nucleus (Utsugi et al. 1999). We have isolated eight different extragenic suppressors of the *tom1-2* mutation and identified six of the genes responsible: *tmr1*/*cyr1* (Matsumoto et al. 1983), *tmr2*/*sch9* (Toda et al. 1988), *tmr3*/*zuo1* (Zhang et al. 1992; Wilhelm et al. 1994; Yan et al. 1998), *tmr5*/*mot1* (Davis et al. 1992), *tmr6*/*sse1* (Mukai et al. 1993; Shirayama et al. 1993) and *tmr8*/*kre6* (Roemer et al. 1991). Although all the *tmr* mutations enabled the *tom1-2* mutant to grow at its restrictive temperature (35° C), the *cyr1*, *sch9*, *zuo1*, *mot1*, *sse1*, and *kre6* mutations were weak suppressors. On the other hand, the *tmr4* and *tmr7* mutations were strong suppressors and retained their activity even at 37° C. The degree of rescue of the morphological defects paralleled the ability to suppress the temperature sensitivity.

Down-regulation of the cAMP/PKA pathway can partially suppress the temperature sensitivity of the *tom1-2* mutant, because *cyr1* and *sch9* were isolated as mutational suppressors and *BCY1* and *PDE2* were found to be multicopy suppressors (Fig. 5A). In addition, *ZUO1* and *TMR7* may function downstream of the cAMP/PKA pathway (Fig. 5B).

Why does down-regulation of the cAMP/PKA pathway suppress the temperature sensitivity of the *tom1* mutant? As shown in Fig. 6A, Tom1 is required for full induction of STRE-mediated gene expression upon shift to high temperature. Since PKA negatively regulates the nuclear translocation of Msn2 (Görner et al. 1998), down-regulation of the cAMP/PKA pathway should facilitate the nuclear translocation of Msn2, which can then activate transcription of various stress genes, such as *UBI4* and *HSP104* (Moskvina et al. 1998). The finding that *MSN2* is a multicopy suppressor

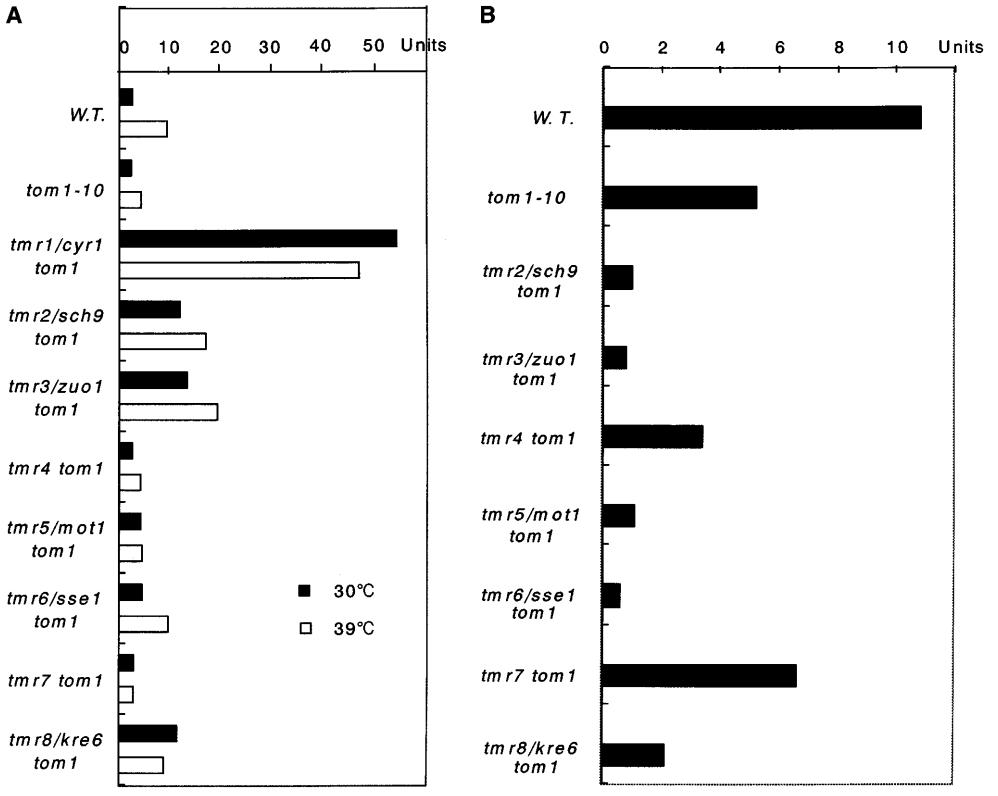


Fig. 6 Tom1 is required for the full induction of heat stress responses. **A** General stress response. The wild type (W303-51), *tom1-10* (YTS011) and various *tom1-10 tmr* mutants carrying the STRE-*lacZ* reporter (YTS151, YST251, YTS351, YTS451, YTS551, YTS651, YTS751 and YTS851) were grown to log phase at 30° C and the cultures were then shifted to 39° C. Cells were collected after 1 h, cell lysates were prepared and the β-galactosidase activity was assayed according to Kaiser et al. (1994). **B** Heat shock response. The wild type (W303-61), *tom1-10* (YTS012-A) and various *tom1-10 tmr* mutants carrying the HSE-*lacZ* reporter (YST252, YTS352, YTS452, YTS552, YTS652, YTS752 and YTS852) were grown to log phase at 25° C and the cultures were shifted to 38° C for 1 h. The β-galactosidase activity in each cell lysate was assayed according to Kaiser et al. (1994)

of the *tom1* phenotype supports this notion. It should be noted, however, that the best suppressor, *tmr7*, did not rescue induction of the STRE-mediated response (Fig. 6A), but this mutation appeared to restore the HSE-mediated heat shock response partially (Fig. 6B). The upstream region of the *UBI4* gene, for example, contains both STRE and HSE elements (Simon et al. 1999).

In the *kre6* mutant transcription of genes such as *FKS2* is known to be activated via a calcineurin-dependent response element (Kapteyn et al. 1999). Mot1 is a transcriptional repressor and functions by removing TATA-binding protein (TBP) from DNA in an ATP-dependent manner (Auble et al. 1997). This protein has also been identified as Taf170, one of the TBP-associated factors (Poon et al. 1994). The *tmr5*/*mot1* and *tmr8*/*kre6* mutations may therefore activate transcription of unknown gene(s) to suppress the *tom1* mutation. Indeed, one of the functions of Tom1 seems to be transcriptional regulation, since it has been reported that Spt7, a component of the ADA/SAGA complex, is ubiquitinated in a Tom1-dependent way (Saleh et al. 1998). Thus, most *tmr* mutations appear to change the pattern of gene expression in the *tom1* mutant, to overcome its growth defects at high temperatures.

The *TMR3*/*ZUO1* gene encodes a DnaJ-type chaperone which is reported to be a partner of Ssb1/Ssb2, a member of the Hsp70 family (Pfund et al. 1998). It is interesting to note that both *SSB1* and *SSB2* have been isolated as multicopy suppressors of *tom1* (our unpublished results), indicating that Ssb1/Ssb2 functions antagonistically to Sse1. Since mutations in *SSE1*, which codes for another Hsp70, suppressed the *tom1* mutation, Zuo1 and Sse1 may function in the same pathway, and Sse1 may compete with Ssb1/Ssb2 to form a complex with Zuo1.

TOM1 is essential for growth at elevated temperatures. The present study of suppressor mutations of *tom1* has provided valuable information on how Tom1 is needed conditions of heat stress.

Acknowledgements We would like to thank Drs. R. Davis, G. Marchler, M. Snyder, D. Kaida, K. Kohno and K. Matsumoto for plasmids, and Ms. T. Aoki for her technical assistance. This work was supported by grants from the Ministry of Education, Science, Sports and Culture to Y.K.

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