A Single Base Substitution Adjacent to the Stop Codon in the downstream of the *SMP*3 gene Affects its Post-trancriptional process in *Saccharomyces cerevisiae*

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Abstract.

The *smp3-1* mutant allele confers increased holding stability of heterologous plasmid, pSR1, and a temperature-sensitive growth defect which is remediable by the addition of 1 M sorbitol as the osmotic stabilizer. The *smp3-1* allele contains two base substitutions; one is in the open reading frame and changed the 490th CAT (encoding Histidine) to TAT (tyrosine), and the other one is an A for G substitution, at 2 bp downstream from termination codon. These base substitutions were separated each other by recombination at a *Bst*NI site located between these two substitutions. The base substitution in the 3' untranslated region was found to be lethal and the defect was unremediable by the osmotic stabilizer, while that in the open reading frame has no appreciable effect to the cell. Thus, both the base substitutions join together confer the *smp3-1* mutant phenotype. The *smp3-1* mutant cells cultivated at 37 °C in nutrient medium containing 1 M sorbitol showed similar *smp3* transcription as in the wild type. These facts suggest that *smp3-1* mutation has a defect in its post-transcriptional process.

Key word: *smp*3 mutation-temperature-sensitive mutation-osmo-remedial mutation-base substitution-post-transcriptional processing.

Introduction

Plasmid pSR1 is maintained in *Saccharomyces cerevisiae* cells less stably than in its native host, *Zygosaccharomyces rouxii* (Jearnpipatkul *et al*., 1987). To elucidate the host mechanism for stable maintenance of a plasmid, we isolated several *S. cerevisiae* mutants, *smp*, showing increased stability of pSR1 (Irie *et al*., 1991a). One such muta-

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tion, *smp3-1*, also confers a growth defect at high temperature. A cloned *SMP*3 DNA revealed that it encodes a 59.9 kDa hydrophobic protein consisting of 516 amino acid residues. Disruption of the SMP3 gene conferred a lethal phenotype to the cells. Grimme *et al*. (2001) revealed that the Smp3 protein, is responsible for processing glycosylphosphatidylinositols (GPIs) assembly which are essential for viability in yeast and have key roles in cell wall construction. A DNA fragment suppressing the growth defect of the *smp3-1* mutant at 37 °C when ligated it into a Ycp vector was isolated (Irie *et al*., 1991b; Lee and Levin 1992). An open

reading frame otf the DNA fragment, designated as the *SSP31/BCK1* gene, encodes a putative protein kinase. The other two genes, *MKK1* and *MKK2* also encoding putative protein kinases, could suppress the *smp*3-1 mutant phenotype when the DNA fragment of these genes were ligated into a multicopy vector (Irie *et al.*, 1993). Products of these suppressor genes, *SSP31/BCK1*, *MKK1*, and *MKK2*. were suggested to act in the downstream of the *PKC1* product, Pkc1p, and upstream of that *MPK1* in a signal transduction cascade which required for growth and division of cells, while the signal that stimulates Pkc1p is unknown (for a review, see Kurjan 1993).

Here we report that the *smp3-1* mutant cells are unable to maintain its integrity and lyse after prolonged incubation at 37 ᵒC, while the defect is rescued by addition of 1 M sorbitol to the growth medium. The *smp3-1* mutant DNA has two single base-substitutions, one is a codon changed from CAT (His-490) to TAT (Tyr) in the open reading frame, and the other one is an A for G substitution at 2 bp downstream from the TAA stop codon. The A for G single base substitution in the 3' untranslated region was lethal, while no appreciable effects were observed with the single Tyr for His-490 substitution in Smp3p. The *smp3-1* mutant could transcribe the *smp3* gene normally at 37 ᵒC in YPD supplemented with 1 M sorbitol.

Materials and methods
Microorganism and plasmids.
The yeast strains used are listed in Table 1. An *Escherichia coli* strain, DH5a (Sambrook et al., 1989) was used for propagation of plasmid DNAs. The principal plasmids used were pUC19 (Messing, 1983), YCp50 (Parent et al., 1985), YCplac22 (Gietz and Sugino, 1988), pSM307C (Irie *et al.*, 1991a), and pYA301 (Gallwitz and Sures, 1980).

Media.
The nutrient (YPD) and minimal (SD) media for yeast and the nutrient medium for *Escherichia coli* were described previously (Toh-e et al., 1982). For testing leucine, uracil and tryptophan auxotrophic phenotypes, Leu-test, Ura-test, and Trp-test media were prepared with SD supplemented with all appropriate nutrients but leucine, uracil or tryptophan, respectively. For osmotic support of cell growth, 1 M sorbitol was added to the media.

Genetic and biochemical methods.
Genetic analyses of *S. cerevisiae* were done by the standard method (Rose *et al.*, 1990). *S. cerevisiae* was transformed by the lithium acetate method (Ito *et al.*, 1983), and *E. coli* was transformed as described by Morrison (1977). To examine modified fragments of the *SMP3* DNA, plasmid shuffling was done as described by Sikorski and Boeke (1991). The general methods used for preparation, modification, and analysis of DNA and RNA were as described by Sambrook *et al.* (1989) and Jearnpipatkul *et al.* (1987). Total RNA was prepared according to the method of Zitomer and Hall (1976) using glass beads to disrupt the cells. Time lapse photomicroscopic analysis (Hartwell *et al.*, 1970) and alkaline phosphatase assay of yeast colonies (Paravacini *et al.*, 1992) were done as described.

Results and discussion
The smp3-1 *mutation confers cell lysis at restricted temperature*
When a cell culture of the *smp3-1* mutant in YPD medium was shifted its incubation temperature from 25 ᵒC to 37 oC, the cell growth ceased at 4 h after the temperature shift and the cell number was decreased by further incubation at 37 ᵒC (Figure 1).
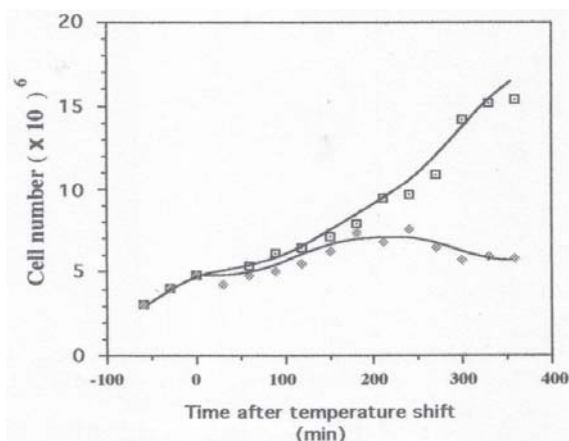
Figure 1. Cell growth of the *smp3-1* mutant. An exponentially growing cell culture of KH81 in YPD medium at 25 oC by shaking (thick line) was shifted to 37 oC (broken line). The cell growth was monitored by scoring cell number with a hemacytometer under a microscope
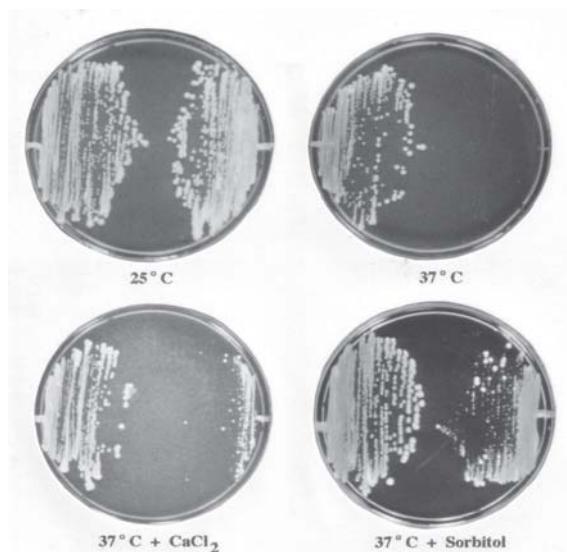

Figure 2. Osmotic stabilizers suppress the temperature-sensitive growth defect of *smp3-1* mutation. Cells of a *SMP3*+ wild-type strain (NBW5; left side of plates) and *smp3-1* mutant strain (KH82; rigth side of plates) were streaked onto YPD plates supplemented with 0.1 M of NaCl or 1 M sorbitol and the plates were incubated at 25 or 37oC for 3 days

Time lapse photomicroscopic analysis showed that the *smp3-1* mutant cells started to lyse about 4 h after the temperature shift to the restrictive temperature (data not shown). These result was consistent with assay of a vacuole enzyme, alkaline phosphatase, of the *smp3-1* mutant. The mutant colonies were developed on YPD plate at 25 ^{O}C for two days, the incubation temperature was shifted to 37 ^{O}C, and the colonies were overlaid with soft agar (1%) containing the alkaline phosphatase assay solution for 45 minute at 37 ^{O}C (Paravicini *et al.*, 1992). We observed activity of alkaline phosphatase released from the cells because the colonies turned blue, while the wild-type colonies remained white (data not shown).

Since some of temperature-sensitive cell lysis defects were rescued by osmotic stabilizer added to the culture medium (Lee and Levin, 1992; Paravicini *et al.*, 1992), we tested several compounds for the *smp3-1* mutant at 37 ^{O}C and found that the addition of 1 M sorbitol, or several the other salts, to YPD agar medium could support cell growth at 37 ^{O}C (Figure 2).

In a previous study, we, however, observed that disruption of that *SMP3* gene was lethal as the *smp3* disruptant cells could not grow on YPD plate (Irie *et al.*, 1991a). To examine the effect of 1 M sorbitol, cells of strain KHD3L with the disrupted D*smp3* genotype and harboring pSM307C plasmid bearing the *SMP3*+ and *URA3*+ genes (Table 1) were cultivated at 25 ^{O}C in YPD broth supplemented with 1 M sorbitol for 24 h. We expected that cells in some fraction of the cell population are cured for pSM307C (*URA3*+) during the cultivation, and if the D*smp3* cells can grow in the YPD containing 1 M sorbitol, then those cells might be selected on SD plates added with 1 M sorbitol supplemented with 1 mg of 5-fluoroorotic acid (5-FOA) and 50 mg uracil per ml to select Ura- clones.

The one-day culture of KHD3L was spread on YPD plates containing 1 M sorbitol and the plates were incubated at 25 ^{O}C for 2 days. Then colonies developed on the

Table 1. The *Saccharomyces cerevisiae* strains used

Strain	Genotype or characteristics	Source or reference
NBW5	*MATα pho31 ade2 his3-532 leu2-3,112 trp1-289 ura3-1,2*	Matsuzaki et al. (1990)
P-28-24C	*MATa pho3-1*	Toh-e and Oshima (1974)
KH8	*MATα smp3-1 ade2 leu2-3,112 his3-532 trp1-289 ura3-1,2*	Irie et al. (1991a)
KH81	*MATa smp3-1 leu2-3,1112 trp1-289 his4-515 ade2*	Our stock
KH82	*MATα smp3-1 leu2-3,1112 trp1-289 his4-515 ade2*	Irie et al. (1991a)
KHD3L*	*MATa leu2-3 urq3-1,2 trp1-289 his3-352 smp3::LEU2* (pSM307C = YCp50::*SMP3*)	Our stock

* Strain KHD3L has a disrupted *smp3* allele by insertion of a 2.2-kbp *Sal*I-*Xho*I fragment of the *LEU2* gene of *S. cerevisiae* in the genome, and harboring pSM307C, a plasmid constructed by insertion of a 2.5-kbp *Sal*I-*Hind*III fragment of the *SMP3* gene (see Fig. 3) into the *Hind*III-*Sal*I gap of YCp50.

plates were replicated onto each two sets of SD plates added with the same amount of 5-FOA, uracil, and sorbitol as the above. One set of replicated plates was incubated at 25 °C and the other at 37 °C for 4 days. We, however, could not detected any colonies on the replicated 5-FOA plates, irrespective of the plates whether incubated at 25 °C or 37 °C. Thus, 1 M sorbitol is not effective for supporting the cell growth of the D*smp3* cells. These results are in accordance with the observation that not more than two spores were viable in each of eight four-spored asci from D*smp3*/*SMP*3⁺ diploid when dissected (data not shown).

The SMP3 gene encodes a hydrophobic protein (Irie *et al.*, 1991a) and several lytic mutants are known to associate with a defect in synthesis or structure of cell wall or plasma membrane or both (Paravicini *et al.*, 1992; Stateva *et al.*, 1991; Maerkisch *et al.*, 1983). These facts suggest that Smp3p protein has an essential role on the cell wall or plasma membrane. Grimme *et al.* (2001) revealed that the Smp3 protein, is responsible for processing glycosylphosphatidylinositols (GPIs) assembly which are essential for viability in yeast and have key roles in cell wall construction. It is known that mutants having a defect on cell wall synthesis exhibited aberrant cell shapes, e.g., formation of an elongated bud or chain of incompletely bud-

ded cells, often on agar media (Paravicini *et al.*, 1992). However, the *smp3-1* mutant cells cultivated at 37 °C in both the liquid and solid media containing 1 M sorbitol showed similar cell morphology of the wild type cells. When cells were grown at 25 °C without osmotic stabilizer and then shifted to 37 °C, they showed two terminal phenotypes : some fraction of the cells have a tiny bud and the cells of the remaining fraction have no bud. All the cells in the both classes have only one nucleus located near to their tiny bud or one side of the cell (Figure 3).
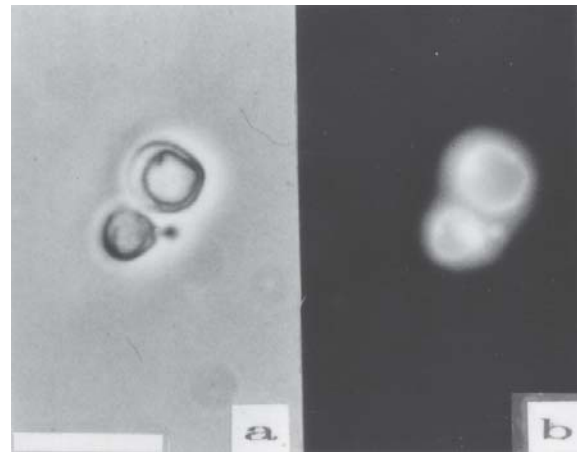

Figure 3. Terminal phenotypes by the *smp3-1* mutation. Cells of a *smp3-1* mutant, KH81, after incubating at the restrictive temperature, 37 oC, for 12 h were stained with 4',6-diamidino-2-phenylindole to observe nuclei. The cells were observed by phase-contrast microscopy (a) or by epifluorescence microscopy (b). The bar corresponds to 10 mm.

Flow cytometryc examination (Hutter and Eipel, 1979) showed that the cell population of the *smp3-1* mutant consist of two dominant cells, one arrested at G1 and the other at G2/M phase, whether they were incubated at 25 °C or 37 °C (data not shown). These evidences suggest that the Smp3p protein is involved in a function other than cell shape formation.

DNA sequence of the **smp3-1** *mutant gene*

Chromosomal DNA was prepared from the *smp3-1* mutant, KH8, digested with *Sal*I and *Hind*III and subjected to agarose gel electrophoresis. DNA fragment was purified from the fraction corresponding to the 2.5-kbp size and was cloned on pUC19 in the *E coli* host (Messing, 1983). Colonies harboring a chimeric plasmid bearing a DNA fragment of the *smp3-1* mutant gene was selected by colony hybridization (Sambrook, 1989) using a 32P-labeled 2.5-kbp *Sal*I-*Hind*III fragment of the *SMP*3 DNA prepared from pSM307C plasmid as a probe. The sequence determination of the *smp3-1* mutant DNA revealed two base substitutions, one, assigned *smp3-1a*, was located in the open reading frame of *SMP3* and changed the 490th CAT codon (encoding histidine) to TAT (tyrosine), and the other one, *smp3-1b* was A for G substitution, at 2 bp downstream from TAA termination codon (Figure 4). We confirmed that the *smp3-1b* base-substitution is in the 3'-untranslated region by repeated base-sequencing of the wild-type and mutant DNAs.

Characteristics of the **smp3-1a and smp3-1b** *mutations*

Since there are two base-substitutions in the *smp3-1* mutant allele, we attempted to elucidate which one confers the *smp3-1* mutant phenotypes, i.e., increased maintenance stability of pSR1 and thermosensitive cell growth. The 2.5-kbp *Sal*I-*Hind*III *smp3-1* DNA was restricted at the *Bst*NI site located between the two substitution sites (Figure 4). The *Sal*I-*Bst*NI and *Bst*NI-*Hind*III fragments were separated by gel electrophoresis and ligated with the similar fragment prepared from the wild type *SMP3+* DNA of P28-24C cells. Thus we obtained two hybrid fragments, bearing each one of the two base-substitutions (Figure 4).
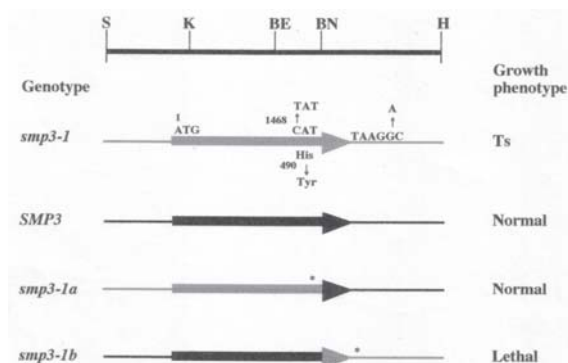

Figure 4. Structure of the wild type *SMP3* DNA and the *smp3-1* mutant DNA. The phenotypes conferred by these DNA fragments were determined by connecting them in the *Sal*I-*Hind*III or *Eco*RI-*Hind*III gaps of Ycplac22 (Gietz and Sugino, 1988), respectively and introduced into strain KDH3L as described in the text. The arrows represent the ORF of the *SMP3* gene. Shaded area represent the *smp3-1* mutant DNA, and the closed area represent the wild type *SMP3* DNA. Asterisks represent the approximate sites of base substitutions in the *smp3-1a* and *smp3-1b* mutations. The *smp3-1a* and *smp3-1b* DNAs were constructed by appropriate connection of the 2.1-kbp *Sal*I-*Bst*NI fragment of *smp3-1* or *SMP3* DNA with the 0.4-kbp *Bst*NI-*Hind*III fragment of the *SMP3* or *smp3-1* DNA respectively. S, K, BE, BN, and H represent the aproximate

To examine the contribution of these substitutions for the *smp3-1* phenotypes, plasmid shuffling method (Sikorski and Boeke, 1991) was performed. The hybrid *Eco*RI-*Hind*III fragments were ligated into the *Eco*RI-*Hind*III gap of multi-cloning site of the YCp-type plasmid, YCplac22, bearing *TRP1* gene of *S. cerevisiae* as the selection marker (Gietz and Sugino, 1988). The resultant plasmids were pGC3-a (bearing the smp3-1a fragment; Figure 4) and pGC3-b (smp3-1b). Strain KHD3L (*smp3::LEU2 leu2 ura3 trp1*) harboring pSM307C bearing *SMP3+* and *URA3+* DNAs was transformed with pGC3-a or pGC3-b plasmid marked with *TRP1*. Colonies showing the Leu⁺ Ura⁺ Trp⁺ phenotype were grown on Trp-test plates, replicated onto SD plates added with 1 mg 5-FOA and 50 mg uracil per ml (Boeke *et al.*, 1984), and incubated at 25 °C for 4 days to select cells cured with the pSM307C

plasmid. Colonies appeared on the 5-FOA plates were replica-plated onto two sets of YPD plates and incubated one set at 25 °C and the other set at 37 °C. The similar experiments were also done with the same media but supplemented with 1 M sorbitol.

The results showed that KHD3L cells harboring pGC3-a, bearing the *smp3-1a* DNA, could grow at 37 oC as well as at 25 oC, and were not distinguishable from the wild type cells. In contrast, the *smp3-1b* mutation is lethal, because KHD3L cells harboring pGC3-b never developed colonies on the 5-FOA plate with or without 1 M sorbitol both at 25 oC and 37 oC. These facts indicate that pGC3-b could not replace for pSM307C for growth of KHD3L cells and the single base substitution at 3' untranslated region of the *SMP3* gene is lethal similarly to that of the D*smp3* allele. Thus, the *smp3-1* mutant phenotype might be due to both the base substitutions. The *smp3-1b* lethal mutation is, probably, partially suppressed by the *smp3-1a* substitution.

mRNA transcription of the smp3 mutant alleles

Cells of the *smp3-1* mutant were grown in YPD broth supplemented with 1 M sorbitol at 37 oC and 25 oC to show OD_{600} = 0.9 (the mid-log phase), the cells were disrupted by using glass beads, and total RNA was prepared. The RNA was separated on a denaturing formaldehyde gel, transferred to Hybond-N nylon membrane (Amersham, Buckingham Shire, UK), and the bound RNA was hybridized at 42 oC with ^{32}P-labeled 1.2-kbp *Kpn*I-*Bst*EII fragment of *SMP*3 DNA prepared from pSM307C. For the internal marker, ^{32}P-labeled 1.0-kbp *Xho*I-*Hind*III fragment of *ACT*1 DNA was used.

The results suggested that *Smp3* transcript of the *smp3-1* mutant cells was not different from that of the wild-type cells (strain NBW5) irrespective cells were incu-

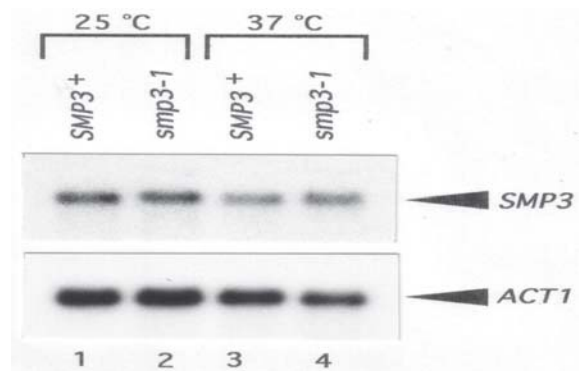bated at 25 oC or 37 oC in the sorbitol medium (Figure 5).

Figure 5. Northern analysis of total mRNA prepared from the *smp3-1* mutant cells (KH82) and the *SMP3+* wild type cells (NBW5) grown at 25 and 37 oC in YPD broth supplemented with 1M sorbitol. A ^{32}P-labeled 1.2-kbp *Kpn*I-*Bst*EII fragment of *SMP3+* prepared from pSM307C (Fig. 3) was used to detect *smp3-1* and *SMP3+* transcripts as the probe. The blot was rehybridized using a randomly ^{32}P-labeled 1.0-kbp *Xho*I-*Hind*III fragment of *ACT1* DNA to generate internal marke

The amount of both the *smp3-1* and *ACT1* mRNAs and ribosomal RNAs were quickly decreased when the cell culture in YPD (without sorbitol) was transferred at 37 OC (Figure 6), indicating the cells are dying in this condition.
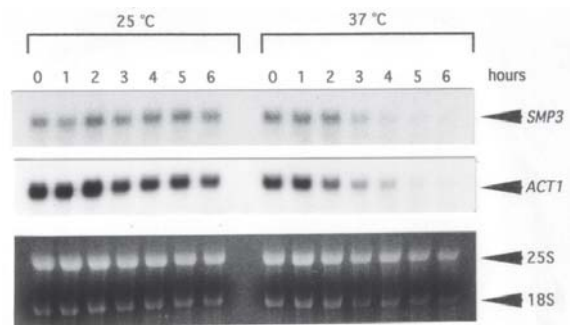

Figure 6. Time course of *smp3-1* transcript in cells of strain KH82 grown in YPD (without sorbitol) at 37 oC. The cells were precultivated by shaking in YPD at 25 oC for 16 h. Then the incubation temperature was shifted to 37 oC and the total RNA samples were prepared from the cells at indicated incubation period. The 32P-labeled probes for the *smp3-1* transcript and that of *ACT1* were the same ones used in the experiment shown in F

Thus, the most plausible explanation for the above results is that the smp3-1 mutation confers a defect on the post-transcriptional processing for the *SMP3* expression. The report stated that Smp3p protein is an enzyme (Grimme *et al.*, 2001) strongly support the above explanation.

It was described in *S. cerevisiae* that proteins bind to the 3' end of mRNAs can influence their translation (Sachs and Davis, 1989). Suppressor mutation for the mutations in poly(A)-binding protein in *S. cerevisiae* have been isolated in a gene encoding ribosomal protein. Wightman *et al.* (1991) also described that translational regulation of the tra-2 gene of *Caenorhabditis elegans* is conducted by its 3' untranslated region. The *smp3-1a* substitution may suppress the lethal effect of the *smp3-1b* mutation.

This evidence suggests two possibilities; first, tyrosine for histidine substitution at the 490th codon by the *smp3-1a* substitution increases activity of Smp3p protein which may suppress the lethal phenotype due to the reduced amount of Smp3p protein by *smp3-1b* mutation only at 25 ^{O}C but not at 37 ^{O}C. Second; the *smp3-1a* mutation does not affect structure and activity of the Smp3p protein but affect on the secondary structure of the smp3-1b mRNA which is defective in post-transcriptional processing at 37 ^{O}C. These arguments further suggest another possibility that the role of Ssp31p/Bck1p, Mkk1p, and Mkk2p protein kinases are overphosphorylating the smp3p protein produced by the smp3-1b mutant gene and increasing Smp3p activity.

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