Identification and analysis of the *Saccharomyces cerevisiae* *SYR1* gene reveals that ergosterol is involved in the action of syringomycin

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A 2.5 kb DNA fragment of the *Saccharomyces cerevisiae* *SYR1* gene was cloned by complementation of the *syr1* mutations that simultaneously lead to resistance to the phytotoxin syringomycin and sensitivity of growth to high Ca^{2+} concentrations. Sequencing of this fragment revealed a single open reading frame encoding a polypeptide of 365 amino acids. Four hydrophobic regions each separated by hydrophilic regions were present in the protein. *SYR1* was identical to *ERG3*, which is suggested to encode C-5 sterol desaturase required for ergosterol biosynthesis. The protein product of *SYR1* was identified by Western blot analysis as a protein of 40 kDa in the particulate fraction. Gene disruption experiments demonstrated that elimination of *SYR1/ERG3* is not lethal, but results in membrane C-5 desaturated sterol deficiencies, resistance to syringomycin and sensitivity to high Ca^{2+} . The *syr1* mutant cells had significantly decreased ability for syringomycin binding. The results indicated that C-5 desaturated sterols are involved in the binding of syringomycin to the cell, and the lack of the sterols in the mutant membrane results in sensitivity to high Ca^{2+} and an increased rate of cellular Ca^{2+} influx.

Keywords: *Saccharomyces cerevisiae*, ergosterol, syringomycin, *SYR1*

INTRODUCTION

The phytotoxin syringomycin is a major virulence factor produced by many strains of the bacterial plant pathogen *Pseudomonas syringae* pv. *syringae* (De Vay *et al.*, 1968; Sinden *et al.*, 1971). Syringomycin is a lipodepsipeptide with a molecular mass of 1226 Da (Segre *et al.*, 1989; Fukuchi *et al.*, 1990). In addition to contributing to plant disease, syringomycin inhibits the growth of several fungi including the yeast *Saccharomyces cerevisiae* (Zhang & Takemoto, 1986, 1987; Takemoto *et al.*, 1991). Plants and yeast appear to respond similarly to syringomycin, and the effects suggest action on membrane components. The host plasma membrane appears to be the primary site of syringomycin action. The effects on this membrane include increases in K^+ efflux, Ca^{2+} influx and membrane potential, and protein phosphorylation (Bidwai & Takemoto, 1987; Takemoto *et al.*, 1991).

To elucidate the mechanism of syringomycin action, a molecular genetic approach using syringomycin-resistant mutants of *S. cerevisiae* was undertaken (Takemoto *et al.*,

1991). About 25% of the mutants (comprising at least 12 gene complementation groups) exhibited increased sensitivity of growth to Ca^{2+} in the medium and higher rates of Ca^{2+} transport. A single recessive mutation is responsible for both the resistance to syringomycin and for the sensitivity of growth to high Ca^{2+} concentrations (Takemoto *et al.*, 1991).

In the present study, we cloned the gene *SYR1* (syringomycin resistant) that complements the phenotypes of the syringomycin-resistant mutants R4-3G and 3N-H1 (*syr1-1* and *syr1-2*, respectively). The cloned gene was identical with *ERG3*, which is suggested to be involved in the synthesis of membrane ergosterol (Arthington *et al.*, 1991). The relationship between the phenotypes of *syr1* and the alteration in membrane sterol is demonstrated.

METHODS

Strains, media and general recombinant DNA methods. The Syringomycin-resistant mutant *S. cerevisiae* R4-3G (*syr1-1*), isolated from the wild-type haploid 8A-1B (*MATa pho3 his3*

leu2), was described previously (Takemoto *et al.*, 1991). The mutant was crossed with KZ1-1D (*MATa ura3 trp1 his3 leu2*) to obtain a mutant R4-3G-12B with desired auxotrophic markers (*MATa syr1 ura3 trp1 his3 leu2*). Strain 3N-H1 (*syr1-2*) showing a similar phenotype to R4-3G was isolated from strain KZ1-1C (*MATa ura3 trp1 his3 leu2*). The two syringomycin-resistant mutants belong to the same genetic complementation group. Bacterial strains and yeast plasmid vectors were as described previously (Liu *et al.*, 1991). Genomic libraries of *S. cerevisiae* made in *Escherichia coli* using the centromere vectors YCpN1, YCp50 and the multicopy vector YEp24 were used. Standard yeast media, YPD, SD and LB, and tetrad analysis were as described previously (Liu *et al.*, 1991). Syringomycin was added to YPD agar at a concentration of $2 \mu\text{g ml}^{-1}$. Solutions of syringomycin and CaCl_2 were sterilized separately and added to YPD medium at temperatures below 60°C . Transformation was done according to the method of Ito *et al.* (1983).

Nucleotide sequence determination. DNA sequencing was performed using the dideoxynucleotide chain-termination method with M13mp18 or M13mp19 (Sanger *et al.*, 1977). Suitable clones for sequencing were produced using various restriction enzymes. DNA sequences were determined on both strands.

Disruption of *SYR1*. Plasmid pNT1 was constructed by inserting the 2.5 kb *XhoI*–*Bam*HI fragment of *SYR1* into M13mp18. Plasmid pNT2 was constructed by replacing the *SalI*–*SmaI* fragment of pNT1 with the *SalI*–*SmaI* fragment of the *HIS3* gene. The diploid *S. cerevisiae* strain RAY-3A-D (Tanaka *et al.*, 1989) was transformed with pNT2 linearized with *Pvu*II. The one-step gene disruption method was used (Rothstein, 1983).

Preparation of fusion protein and specific antibody. For the preparation of *SYR1* fusion protein, the bacterial expression vector pEX3 was used. The *SspI*–*HpaI* fragment within the open reading frame (ORF) was inserted at the *SmaI* site of the expression vector pEX3 to produce the *cro*–*lacZ*–*SYR1* gene fusion. Upon heat induction, the resulting plasmid, pNTF1, produced in *E. coli* strain pop2136 a large amount of polypeptide of about 140 kDa, consistent with the size of the predicted fusion protein. The fusion protein was purified by slab preparative SDS-PAGE. After staining the gel with Coomassie blue, the fusion protein band was excised from the gel, eluted from the gel electrophoretically and dialysed against water. Mouse antisera were prepared by first immunizing mice with $50 \mu\text{g}$ protein in complete adjuvant, and then five times with $20 \mu\text{g}$ protein in incomplete adjuvant with an interval of 10 d between injections. Antibody was purified on an affinity column of fusion protein bound to CNBr-activated Sepharose 4B. Antibody against β -galactosidase in the eluate was further eliminated by passing through a β -galactosidase–Sepharose 4B affinity column.

Protein electrophoresis and Western blot analysis. Yeast cells were grown to late-exponential-phase in 10 ml YPD medium. The cells were harvested and washed twice with 1 ml buffer 1 (50 mM imidazole/HCl, pH 7.0, 1 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM PMSF, $10 \mu\text{g ml}^{-1}$ each of leupeptin and pepstatin). Cell extracts were prepared by vortexing the cell pellet with glass beads (0.45 mm; Braun) in a microcentrifuge tube. After removing the glass beads, the lysate was centrifuged at 12000 r.p.m. for 15 min at 4°C . Equal amounts ($40 \mu\text{g}$) of protein samples (supernatant and particulate fractions) were analysed by SDS-PAGE. After electrophoresis, the proteins were transferred onto a Biodyne membrane, and Syrl protein was identified by Western blotting using antisera against Syrl. Protein samples were not heat treated in sample buffer for

solubilization, as the treatment caused loss of Syrl protein due to aggregation.

Extraction of nonsaponifiable lipids. The procedure for sterol extraction from yeast was that described by Woods (1971), using cells grown for 48 h at 28°C in YPD medium.

Binding assay for syringomycin. Syringomycin ($40 \mu\text{g}$) was added to *S. cerevisiae* cells (2×10^7) suspended in $200 \mu\text{l}$ YPD medium in a microcentrifuge tube. After incubation at 28°C for various periods of time (0–60 min), the suspension was clarified by centrifugation for 1 min at 10000 g. A $50 \mu\text{l}$ sample was taken from the supernatant fluid, and the amount of syringomycin was determined semi-quantitatively by measuring the growth inhibitory effect of the toxin using wild-type *S. cerevisiae* as assay cells.

Syringomycin purification. Syringomycin (E form) was purified as described previously (Bidwai & Takemoto, 1987).

RESULTS

SYR1 complements Ca^{2+} sensitivity of syringomycin-resistant mutants

Syringomycin-resistant mutants R4-3G and 3N-H1 were previously selected from the wild-type strains 8A-1B and KZ1-1C, respectively, on the basis of their ability to grow on YPD agar containing $2 \mu\text{g}$ syringomycin ml^{-1} . The growth of R4-3G and 3N-H1 was inhibited by 0.4 M and 0.2 M CaCl_2 , respectively. The respective wild-type parental strains were able to grow with these levels of Ca^{2+} . Both mutants showed increased rates of $^{45}\text{Ca}^{2+}$ uptake from the medium (Takemoto *et al.*, 1991). Genetic analyses of these mutants showed single recessive mutations that simultaneously led to Ca^{2+} -sensitive growth and syringomycin resistance (Takemoto *et al.*, 1991). The two mutations, designated *syr1-1* and *syr1-2*, belonged to the same complementation group. For cloning of the genes that complement the *syr1* phenotypes, we used genomic libraries of *S. cerevisiae* made with a centromere vector (YCpN1 or YCp50) and the multi-copy vector YEp24. Since R4-3G did not have appropriate auxotrophic markers for selection of these plasmids, strain R4-3G-12B (*MATa syr1-1 ura3 trp1 his3 leu2*) was obtained by crossing R4-3G with KZ1-1D. After transformation of R4-3G-12B and 3N-H1 with the genomic libraries, transformants were tested for the phenotypes to verify that the increased resistance to Ca^{2+} was due to the presence of the plasmid. The plasmids were then purified, amplified in *E. coli*, and used to transform the mutants to verify that the plasmids complemented Ca^{2+} -sensitive growth.

About 5000 and 9000 transformants of R4-3G-12B were obtained using the YCpN1- and YEp24-based libraries, respectively. From these transformants, one (clone 22) YCpN1- and four (clones 1, 3, 273 and 225) YEp24-based *syr1*-complementing clones were obtained. From about 3000 YCp50-library transformants of 3N-H1, five complementing clones were isolated (clones 28, 29, 32, 37 and 35). Restriction enzyme analysis of these clones suggested that they all contained a common DNA fragment found in the shortest clone, clone 1. The restriction maps of some of these clones are shown in Fig. 1. Southern blot analyses of genomic DNA digested with various restriction

Fig. 1. Restriction maps of the genomic clones that complement Ca^{2+} sensitivity of *syr1*. Abbreviations for restriction sites are as follows: B, *Bam*HI; RI, *Eco*RI; RV, *Eco*RV; H, *Hind*III; P, *Pst*I; S, *Sal*I; X, *Xba*I; Xh, *Xho*I.

Fig. 2. Southern blot analysis of genomic *SYR1*. Genomic yeast DNA from RAY-3D-A (10 µg) was digested with various restriction enzymes and used for hybridization with a labelled *Xho*I–*Hind*III fragment of *SYR1* as probe. Hybridization was at 42 °C. Abbreviations for restriction enzymes are the same as used in Fig. 1.

enzymes were carried out to determine if *SYR1* is a single-copy gene. One fragment with the expected size was produced with four restriction enzymes that did not cleave the *SYR1* gene, and two fragments were detected with *Eco*RI, for which a single restriction site was predicted by the cloned gene (Fig. 2). These results indicate that *SYR1* is a single-copy gene. Chromosome IV was identified as the chromosome containing *SYR1* by Southern blot analysis of the chromosome separated by CHEF gel electrophoresis (data not shown).

The *SYR1* gene encodes C-5 sterol desaturase

The 1.2 kb insert of clone 1 was sequenced. A sequence presumed to be part of an ORF was found, but a stop codon was missing at the C-terminus. Thus, the nucleotide sequence of the 2.5 kb *Xho*I–*Bam*HI fragment of clone 273 was determined (Fig. 1). In this fragment, an ORF of 1095 nucleotides that could encode a 365-amino-acid protein (42.7 kDa) was found (data not shown). The sequence of clone 1 corresponded to amino acid residues 1–322 of the ORF, indicating that the C-terminal 43 amino acids of the *Syr1* protein are not essential for restoring Ca^{2+} sensitivity of *syr1*. The hydrophobicity profile of the predicted *SYR1* gene product revealed several hydrophobic domains sufficient in length to span a membrane, suggesting that the *SYR1* gene product is a membrane protein. Comparison of the *SYR1* sequence with those in the sequence databases showed that *SYR1* is identical to yeast *ERG3*, a gene suggested to encode C-5 sterol desaturase required for ergosterol biosynthesis (Arthington *et al.*, 1991).

The *SYR1* gene is not essential for growth, and its disruption causes syringomycin resistance and Ca^{2+} sensitivity

To investigate the relationship between the *Syr1* protein and syringomycin resistance, *SYR1* was inactivated by the one-step gene disruption technique (Rothstein, 1983). The *Xho*I–*Bam*HI fragment of plasmid pNT2 was constructed by replacing a 300 bp *Sal*I–*Sma*I region within the ORF with the *Sal*I–*Sma*I fragment of *HIS3* (Fig. 3). The diploid *S. cerevisiae* strain RAY-3A-D was transformed with pNT2 linearized with *Pvu*II. Gene disruption was confirmed by Southern hybridization (Fig. 3). Tetrad analysis of the diploid was carried out upon sporulation. Viable spores were scored for genotype according to their auxotrophic requirements, and 1:1 segregation patterns of *yr1-Δ1* and *SYR1* alleles were observed. Consistent with the results of *ERG3* disruption (Arthington *et al.*, 1991), all four spores were able to grow on YPD, showing that *SYR1* is not essential for mitotic growth. However, *SYR1* disruptants (*His*⁺ colonies) were all resistant to syringomycin, and sensitive to Ca^{2+} (Fig. 4). Cells with

Fig. 3. (a) Disruption of *SYR1*. This was done as described in Methods. (b) Confirmation of gene disruption by Southern hybridization. Genomic yeast DNA (10 µg) was digested with *EcoRV* and prepared for hybridization. The probe was the same as that used in experiments shown in Fig. 2. Lanes: WT, DNA from RAY-3D-A; D, DNA from His⁺ transformed diploid (YT1).

Fig. 4. Phenotypes of the *syr1::HIS3* (*syr1-Δ1*) disrupted meiotic segregant. Tetrad analysis of the *syr1-Δ1/SYR1* diploid (YT1) was performed. Growth of selected tetrads were tested on (a) YPD medium, (b) SD without histidine, (c) YPD medium containing 2 µg syringomycin ml⁻¹ and (d) YPD containing 400 mM Ca²⁺.

the disrupted allele had an elevated rate of Ca²⁺ uptake, as previously observed with *syr1-1* mutants (Takemoto *et al.*, 1991). When the *syr1-Δ1* segregant was transformed with the YCpN1 plasmid bearing *SYR1*, both Ca²⁺-sensitive and syringomycin-resistant phenotypes were restored to wild-type levels. These results indicate that the function

Fig. 5. Western blot analysis of the Syr1 protein. Equal amounts of proteins (30 µg) were separated by electrophoresis in a 10% (w/v) polyacrylamide gel in the presence of SDS and analysed by Western blotting as described above. Total cell lysates prepared from wild-type cells of YT1-1a (lane 1) and YT1-1a harbouring *SYR1* on the YEp24 plasmid (lane 2) were immunoblotted with affinity-purified antibody against Syr1. Total cell extract (lane 3) prepared from YT1-1a harbouring *SYR1* was fractionated to soluble (lane 4) and particulate (lane 5) fractions, as described in Methods, and analysed by Western blotting. No bands were formed when total cell lysates were immunoblotted with control serum.

of *SYR1* is involved in both the action of syringomycin and in Ca²⁺ resistance of the cell.

Identification of Syr1 protein

For immunological detection of the *SYR1* product, Syr1, antiserum against LacZ–Syr1 fusion protein was prepared. Western blot analysis of whole-cell extracts prepared from a strain overexpressing *SYR1* from a multi-copy-number plasmid vector showed an immunoreactive polypeptide with a molecular mass of 40 kDa, corresponding in size to that expected from the deduced amino acid sequence (42.7 kDa). An increase in the intensity of this band was observed when *SYR1* was overexpressed from a YEp24 multi-copy-number plasmid, but only a very weak band was seen in wild-type cells (Fig. 5), implying that Syr1 is a minor protein of the cell. Syr1 was found in the particulate fraction, suggesting that it is bound to the membrane.

syr1 mutants have altered membrane sterols

Since *SYR1* encodes C-5 sterol desaturase, we measured the UV spectra of the nonsaponifiable lipid extracts to see if the levels of membrane ergosterol are altered in the *syr1* mutants (Fig. 6). Two of the sterols most abundant in *S. cerevisiae*, ergosterol and 24(28)-dehydroergosterol, are readily identified by their UV absorption spectra, but the other sterols present in the yeast membranes are not detectable from the absorption spectra (Breivik & Owades, 1957). The lipid extract prepared from YT1-a, a *SYR1* segregant of the diploid YT1 heterozygous at the

Fig. 6. UV absorption spectra of the nonsaponifiable lipid extracts from *S. cerevisiae* strains YT1-1a (*SYR1*) (A), YT1-1b harbouring YCpN1 (*SYR1*) (B), R4-3G (D) and YT1-1b (*syr1-Δ1*) (E). Ergosterol (20 $\mu\text{g ml}^{-1}$) in heptane/ethanol (1:9, v/v) is also shown (C).

SYR1 locus, showed that this strain contains ergosterol and possibly 24(28)-dehydroergosterol as the major UV-absorbing materials, as described by Bard (1972). In contrast, a *syr1-Δ1* segregant (YT1-b) did not contain detectable amounts of UV-absorbing materials in the corresponding fraction, suggesting that ergosterol is not essential for the growth of yeast cells (Fig. 6). This result is consistent with the data of Arthington *et al.* (1991), which demonstrated the lack of C-5-desaturated sterols in the mutant strains by GC-MS analysis. When *SYR1* was introduced into *syr1-Δ1* mutant cells using YCp or YEp vectors, the level of ergosterol was restored close to the wild-type level. The cells of *syr1-1* had a very low level (less than 10% of that in wild-type cells) of the UV-absorbing substances (Fig. 6).

***syr1* mutants have decreased ability for syringomycin binding**

Syringomycin presumably binds to the surface of sensitive cells as the first step of its action. To measure the syringomycin-binding ability of the cells, cell samples were incubated with syringomycin in YPD medium, and the amount of syringomycin remaining in the medium was measured. Cells were pelleted by centrifugation at

various periods of incubation, and toxin concentration in the clarified supernatant fluid was determined semi-quantitatively by bioassay using sensitive *S. cerevisiae* as indicator (Fig. 7). With wild-type cells, the amount of toxin in the supernatant rapidly decreased. In contrast, the toxin level did not appreciably decrease with *syr1-Δ1* cells, suggesting that the syringomycin-binding activity of the mutant is much lower than that of wild-type cells (Fig. 7). The decreased binding ability of *syr1-Δ1* was restored when *SYR1* was introduced by transformation with YCpN1 (*SYR1*) or YEp24 (*SYR1*) (Fig. 7). Supporting the possibility that membrane sterols are involved in the binding of syringomycin to the cell, the toxic effect of syringomycin for sensitive cells was relieved by the presence of low concentrations of sterols, such as cholesterol and ergosterol, added to the medium (data not shown).

DISCUSSION

SYR1 was cloned on the basis of its ability to complement the Ca^{2+} -sensitive phenotype of *syr1* mutants. The cloned gene also suppressed syringomycin resistance when introduced into the mutant by a centromere-based vector. The gene had interesting phenotypic and molecular features. The mutant with the *syr1* allele was resistant to syringomycin, showed Ca^{2+} -sensitive growth, and had a greatly increased Ca^{2+} -uptake rate compared to wild-type cells (Takemoto *et al.*, 1991). Since the syringomycin-resistant and Ca^{2+} -sensitive phenotypes caused by the null mutation in *SYR1* were very similar to those of *syr1* mutants, the mutations possibly resulted in loss of function of the gene product. The sequence of *SYR1* was identical with the recently reported *S. cerevisiae* gene *ERG3*, which is suggested to code for C-5 sterol desaturase, the third terminal enzyme of ergosterol synthesis (Arthington *et al.*, 1991). The *ERG3* gene was cloned by complementation of the cycloheximide sensitivity of an *erg3* mutant, which was originally identified as a mutation conferring nystatin resistance.

Western blot analyses using antibody directed against a LacZ-Syr1 fusion protein revealed a protein with an apparent size of 40 kDa in the particulate fraction. The size of the protein detected agrees with that expected from the gene. The microsome is expected to be the location of terminal sterol biosynthetic enzymes such as C-5 sterol

Fig. 7. Syringomycin binding activity of the *syr1* mutant. The decrease in the amount of syringomycin in the fluid during incubation of cells in suspensions (2×10^7 cells in 200 μl YPD medium) for 0, 10, 30 and 60 min were measured. A 50 μl sample of clarified fluid was taken, and the amount of toxin was determined semi-quantitatively by bioassay using syringomycin-sensitive *S. cerevisiae* as indicator. (a) YT1-1a (*SYR1*); (b) null mutant YT1-1b (*syr1-Δ1*); (c) YT1-1b harbouring YCpN1 (*SYR1*) plasmid. 1, Incubated without cells for 60 min; 2–5, incubated with cells for 0 (2), 10 (3), 30 (4) and 60 min (5).

desaturase (Osumi *et al.*, 1979). The predicted *SYR1* C-terminal four amino acids, Lys-Lys-X-X, are a retention signal for transmembrane endoplasmic reticulum proteins in mammalian cells (Jackson *et al.*, 1990; Shin *et al.*, 1991). However, clone 1, which contains an ORF lacking the C-terminal 43 amino acids of the Syr1 protein, complemented *syr1*, suggesting that the C-terminal portion is not essential when introduced on a multi-copy plasmid.

The availability of the gene responsible for susceptibility to syringomycin and its identification as the gene coding for an enzyme of ergosterol biosynthesis afforded an opportunity to correlate syringomycin action with sterol composition of the membrane. The *syr1* mutant cells lacked ergosterol and possibly C-5 desaturated sterols, as determined by the UV absorption spectrum of the nonsaponifiable lipid extract (Fig. 6). A similar relationship between membrane sterols and the resistance of mutants to polyene antibiotics, such as nystatin and amphotericin B, has been demonstrated (Lampen *et al.*, 1962; Woods, 1971; Bard, 1972). Ergosterol has been identified as the cellular binding site for nystatin (Lampen *et al.*, 1962). Binding experiments showed that the rate of syringomycin binding to *syr1* cells is lower than to wild-type cells (Fig. 7). These results suggest that membrane ergosterol facilitates the binding of syringomycin. The absence or severe reduction of ergosterol in the membrane results in resistance to the toxin. It is not clear, however, whether syringomycin binds directly to ergosterol, as does nystatin, or to another surface component dependent upon ergosterol biosynthesis. It was recently reported that sterol membrane composition affects the interaction of *S. cerevisiae* cells with iturin A, a lipopeptide antibiotic structurally unrelated to syringomycin (Latoud *et al.*, 1990). The involvement of ergosterol could be the common mechanism for antifungal action of lipopeptides.

The sensitivity of the *syr1* strain to high Ca^{2+} concentrations, and the increased Ca^{2+} influx of the mutant, may be ascribed to lowered membrane ergosterol levels. Some sterol mutants of yeast have altered sensitivities to pulses (e.g. 10 min) of hypertonic salt solutions (2 M), including NaCl and CaCl_2 , suggesting a change in ion permeability as a result of the alteration of the membrane sterol composition (Bard *et al.*, 1978). Also, some sterol mutants become more sensitive to cycloheximide (Gaber *et al.*, 1989; Arthington *et al.*, 1991; Ashman *et al.*, 1991). The *syr1* mutant did not exhibit increased sensitivity to other ions tested, such as KCl and MgCl_2 , but the mutant was more sensitive to NaCl than wild-type cells (data not shown). Studies of Ca^{2+} -sensitive mutants by several groups have shown that yeast has various mechanisms which allow it to grow with high Ca^{2+} concentrations. For example, vacuoles possess a potent Ca^{2+} -uptake system driven by a H^+ -ATPase and serve as an intracellular store for Ca^{2+} . Mutants of the subunit of the vacuolar H^+ -ATPase which are defective in vacuole acidification have sixfold higher cytosolic Ca^{2+} levels and were Pet^- (type IV *cls* mutants) (Ohya *et al.*, 1991). These mutants exhibit increased Ca^{2+} uptake activity and their growth is Ca^{2+} sensitive. Although the *syr1* mutants exhibited similar phenotypes with respect to Ca^{2+} sen-

sitivity and $^{45}\text{Ca}^{2+}$ uptake, the *syr1* mutants have normal levels of vacuolar ATPase activity and are not Pet^- (data not shown).

Mutants (*syr2* and *syr4*) that exhibited increased resistance to syringomycin without changes in Ca^{2+} sensitivity and had the wild-type sterol composition have been identified. Syringomycin bound to these mutants normally, but was unable to kill the cell. We are examining the defects of these mutants by cloning the genes that complement the phenotype due to the *syr* mutations. The structure of these genes may further provide information on the mechanism of syringomycin action in yeast.

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