Mechanism of action of the phytotoxin syringomycin: a resistant mutant of *Saccharomyces cerevisiae* reveals an involvement of Ca^{2+} transport

JON Y. TAKEMOTO,¹* LEI ZHANG,¹ NOBUHISA TAGUCHI,² TAMAKI TACHIKAWA²
and TOKICHI MIYAKAWA²

¹ Department of Biology and Molecular Biology Program, Utah State University, Logan, Utah 84322-5305, USA

² Department of Fermentation Technology, Faculty of Engineering, Hiroshima University, Higashi-Hiroshima 724, Japan

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The bacterial phytotoxin syringomycin affects plasma-membrane-associated functions of plants and yeast. These include increases in transmembrane K^+ , H^+ and Ca^{2+} fluxes and membrane potential. Mutants of *Saccharomyces cerevisiae* resistant to growth inhibition by syringomycin were isolated and characterized. Many of the mutant isolates were unable to grow in yeast extract/peptone/dextrose medium supplemented with 400 mM- CaCl_2 which permitted the growth of the parent strain (8A-1B). Genetic analyses of one of these mutants, strain R4-3G, showed a single recessive mutation that simultaneously led to Ca^{2+} -sensitivity and syringomycin-resistance. R4-3G had higher net $^{45}\text{Ca}^{2+}$ uptake rates than strain 8A-1B and higher intracellular Ca^{2+} levels in medium containing 1 mM- CaCl_2 . The altered $^{45}\text{Ca}^{2+}$ uptake rates of the mutant were not influenced by syringomycin and were not related to altered capabilities for Ca^{2+} efflux. R4-3G had similar syringomycin-stimulated increases in K^+ efflux but lower syringomycin-stimulated increases in membrane potential than 8A-1B. It is concluded that Ca^{2+} transport is important in the response of yeast to syringomycin and that the toxin-stimulated membrane potential increase, but not K^+ efflux, is closely associated with Ca^{2+} transport.

Introduction

The phytotoxin syringomycin is a major virulence factor in many plant diseases (Gross *et al.*, 1977; Sinden *et al.*, 1971). Many strains of the bacterial pathogen *Pseudomonas syringae* pv. *syringae* produce this toxin which is a lipodepsipeptide with an M_r of 1224 (Segre *et al.*, 1989). In addition to contributing to plant disease, syringomycin inhibits the growth of several fungi including the yeast *Saccharomyces cerevisiae* (Zhang & Takemoto, 1986, 1987).

The host plasma membrane is the primary site of action of syringomycin. Among the effects on this membrane are increases in K^+ efflux (Reidl & Takemoto, 1987), Ca^{2+} influx (Takemoto *et al.*, 1989), membrane potential (Reidl & Takemoto, 1987; Zhang & Takemoto, 1987), H^+ -ATPase activity (Bidwai *et al.*, 1987) and protein phosphorylation (Bidwai & Takemoto, 1987). Plants and yeast respond similarly (Reidl & Takemoto, 1987). The toxin appears to act on membrane components with roles in cellular signalling. For example, it closes leaf stomata in *Xanthium strumarium* and *Vicia faba*, a signalling process linked to K^+ efflux across the plasma membrane of stomatal guard cells (Mott &

Takemoto, 1989). The sequence of these responses to syringomycin is not known (Reidl & Takemoto, 1987).

The work described here is the first genetic study with *S. cerevisiae* aimed at learning more about the syringomycin response. This organism has several well-known advantages for investigating cellular phenomena, including those associated with the plasma membrane (Botstein & Fink, 1988). Molecular events of *S. cerevisiae* transmembrane signalling by mating pheromones are particularly relevant (Blinder *et al.*, 1989; Herskowitz & Marsh, 1987; Jahng *et al.*, 1988; Nakayama *et al.*, 1988). These events may be deduced by isolating and observing the phenotypes of mutants altered in the response to pheromones and identifying the impaired genes. The sequences of the signalling events are then determined by investigating the interactions of these genes and their products. In theory, the syringomycin response can be studied by an analogous approach.

Methods

Organisms and growth media. Haploid *S. cerevisiae* strains 8A-1B (*MATa his3 leu2 can1*) and Y1Y3B (*MATa his7 ura1*) were obtained from Drs A. Toh-E (University of Tokyo) and I. Yamashita

(University of Hiroshima), respectively. The Ca^{2+} -sensitive strain c1s4 was obtained from Dr Y. Ohya (University of Tokyo). These strains were maintained and grown on medium containing 1% (w/v) yeast extract, 2% (w/v) polypeptone and 2% (w/v) dextrose (YPD medium) and YPD medium containing agar (2%, w/v) at 28 °C. Mutants were maintained and selected on YPD agar medium plates containing syringomycin (25.6 units ml^{-1}) and tested for sensitivity to Ca^{2+} on YPD agar medium containing 400 mM- CaCl_2 . Solutions of syringomycin and CaCl_2 were sterilized separately and added to YPD medium at temperatures below 60 °C. For testing growth sensitivity to antibiotics, YPD agar medium was supplemented with chloramphenicol (4 mg ml^{-1}), cycloheximide (0.5 $\mu\text{g ml}^{-1}$) or tetracycline (2 mg ml^{-1}). Diploids were grown on minimal agar medium containing 0.7% yeast nitrogen base without amino acids (Difco), 2% (w/v) dextrose, and 2% (w/v) agar.

Mutant isolation. An overnight culture of strain 8A-1B in 10 ml of YPD medium was centrifuged at 1500 r.p.m. (270 g) for 10 min. The pelleted cells were suspended in distilled water, centrifuged again, and then suspended in 10 ml 0.1 M-sodium phosphate buffer, pH 8. Ethyl methanesulphonate (0.3 ml) was added and the mixture incubated for 50 min at 28 °C without shaking. The cells were then washed by three cycles of centrifugation and resuspension in distilled water. The washed cells were suspended in 2 ml of YPD medium and incubated overnight with reciprocal shaking (120 strokes min^{-1}) at 28 °C. A portion (0.5 ml) of the mutagenized culture was inoculated into 5 ml of YPD medium containing 25.6 units syringomycin ml^{-1} and incubated overnight with shaking at 28 °C. The cells were then spread onto YPD agar medium containing 25.6 units syringomycin ml^{-1} . Colonies growing within 3 d were designated syringomycin-resistant.

Genetic methods. The standard methods of Sherman *et al.* (1986) were used for mating, diploid formation, sporulation and tetrad analysis.

Growth studies. Cells grown overnight in YPD medium were used to inoculate 1 ml amounts of YPD medium (in 1.3×10.5 cm glass tubes) supplemented with various concentrations of CaCl_2 or EGTA. EGTA was added from a 1 M, pH 7.0 stock solution. The inoculation density was 10^6 cells ml^{-1} . The cultures were incubated for 15 h at 28 °C on a reciprocating shaker (120 strokes min^{-1}), and the optical density at 660 nm was determined.

Ca^{2+} uptake and efflux. Cellular Ca^{2+} uptake was measured in YPD medium or MM medium (per litre: glucose, 20 g; $(\text{NH}_4)_2\text{SO}_4$, 3 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; NaCl , 0.1 g) containing various concentrations of CaCl_2 and syringomycin. The net uptake of $^{45}\text{Ca}^{2+}$ was measured as described by Tachikawa *et al.* (1987). In all cases, the specific activity of $^{45}\text{Ca}^{2+}$ was adjusted to 4 mCi (148 MBq) per mmol of CaCl_2 . For Ca^{2+} efflux, cells were grown overnight in YPD medium (3×10^7 cells ml^{-1}), and washed twice by suspension and centrifugation with MM medium. The washed cells were resuspended in MM medium (7 ml total volume) at a concentration of 1×10^8 cells ml^{-1} and incubated at 28 °C for 30 min with gentle agitation on a reciprocal shaker. CaCl_2 (5 mM; 70 μl) containing 14 μCi $^{45}\text{CaCl}_2$ [Amersham; specific activity 40.5 Ci (1.5 TBq) per mg Ca] was added (final CaCl_2 concentration 50 μM) and the suspension incubated for an additional 60 min. The cells were centrifuged (8000 g, 2 min, 4 °C), quickly washed four times with ice-cold MM medium, and suspended in 1 ml of the same medium. The washed cells (1 ml) were mixed with 6 ml of MM medium supplemented with syringomycin and either EGTA (10 mM) or CaCl_2 (0.68 mM or 100 mM). The suspensions were incubated at 28 °C with shaking. These suspensions had the same cell densities as in the $^{45}\text{Ca}^{2+}$ uptake experiments. At designated times, the cells were centrifuged (8000 g, 1 min, 4 °C), the supernatant fractions removed, and the radioactivity was determined using a liquid scintillation counter. All Ca^{2+} uptake and efflux experiments were

done at least twice and the results were reproducible. Results presented are from representative single experiments.

K^+ efflux and [^{14}C]tetraphenylphosphonium (TPP^+) uptake. K^+ efflux rates were determined as changes in extracellular K^+ concentration. Cells were quickly suspended (0.7 mg cell dry wt ml^{-1}) in 2 mM-Tris/MES buffer, pH 6.5, and 0.1 M-glucose with or without syringomycin to an optical density at 600 nm of 1 at room temperature. At designated times, 1 ml samples were withdrawn, centrifuged in an Eppendorf microfuge for 30 sec, and the supernatant fractions were recovered. K^+ concentrations were determined by atomic absorption spectroscopy (Instrumentation Laboratories, AA/AE spectrophotometer 457). Plasma membrane potential changes were determined from the cellular uptake of [^{14}C]TPP $^+$. Measurements of [^{14}C]TPP $^+$ uptake were made as described previously (Zhang & Takemoto, 1987). All K^+ efflux and [^{14}C]TPP experiments were done twice, and similar results were obtained. Results from single representative experiments are presented.

Syringomycin. Syringomycin (E form) was purified as described previously (Bidwai *et al.*, 1987). The preparations used in this study had specific activities of either 12800 or 25600 units mg^{-1} . One unit was defined as the smallest amount of toxin applied as a 10 μl drop which completely inhibited the growth of the yeast *Rhodotorula pilimanae* spread on potato/dextrose agar plates (Zhang & Takemoto, 1987). Syringomycin was added from stock solutions (10 mg ml^{-1}) made in distilled water (pH <6.5).

Cell Ca^{2+} content. Samples (1 ml) of cells grown overnight in YPD medium were dispensed into 1.5 ml microcentrifuge tubes. Appropriate amounts of CaCl_2 were added and the cells incubated for 20 min at room temperature. The cells were then centrifuged in an Eppendorf microfuge for 15 s. The pellets were either processed directly or washed once or twice with 1 ml distilled water by suspension and centrifugation. The pellets were dissolved in 12 M-nitric acid and incubated overnight at 140 °C. The resulting ash material was dissolved in 1 M-HCl containing 1% (w/v) La_2O_3 . The Ca^{2+} levels were determined by atomic absorption spectroscopy. Solutions of CaCl_2 in distilled water were used as standards.

Results

Mutant isolation and properties

After mutagenesis of strain 8A-1B with ethyl methanesulphonate, 160 mutants resistant to 25.6 units syringomycin ml^{-1} were isolated. The growth of the parent strain, 8A-1B, was inhibited by syringomycin at levels as low as 7.5 units ml^{-1} at 28 °C. Of the resistant mutants, 38 did not grow on YPD agar medium containing 400 mM- CaCl_2 although strain 8A-1B and the other isolates grew well on this medium. Approximately 40% of these Ca^{2+} -sensitive mutants showed no or little growth with CaCl_2 levels as low as 100 mM. In addition, 33 of the 38 syringomycin-resistant, Ca^{2+} -sensitive mutants did not grow on YPD agar medium containing the calmodulin antagonist trifluoperazine (40 μM). Strain 8A-1B grew well on this medium. All the Ca^{2+} -sensitive mutants grew well on YPD agar medium supplemented with the divalent cations MgCl_2 (100 mM) or ZnCl_2

Fig. 1. Effects of CaCl_2 (●, ○) and EGTA (▲, △) on the stationary growth phase optical density of R4-3G (filled symbols) and 8A-1B (open symbols). Measurements were taken 15 h after inoculation in YPD medium.

(3 mM). These levels of MgCl_2 and ZnCl_2 are just below the levels inhibitory to yeast (Ohya *et al.*, 1986a). A representative strain, R4-3G, which was inhibited by 400 mM- CaCl_2 , was used for more detailed genetic and physiological studies.

Genetic analysis

The mutant strain R4-3G was crossed with strain Y1Y3B, of the opposite mating type, and diploids were selected on MM medium. The diploids were inhibited by syringomycin (25.6 units ml^{-1}) showing that the mutation leading to syringomycin resistance was recessive. In addition, the diploids grew on YPD medium supplemented with 400 mM- CaCl_2 . The diploids were induced to sporulate and the resulting asci, each containing four spores, were dissected and germinated. The tetrad segregation patterns were mainly 2:2 parental ditype (29 of 32 tetrads) for syringomycin-resistance and growth sensitive to 400 mM- CaCl_2 . The spore viability rate on YPD agar medium was 85%. Overall, the tetrad analyses showed that in strain R4-3G the Ca^{2+} -sensitive phenotype was due to the same single recessive mutation which gave syringomycin resistance.

Ohya *et al.* (1986b) described an *S. cerevisiae* strain, *cls4*, with a defect in bud formation and a mutation in the *CDC24* gene. Strain *cls4* is also sensitive to high levels of Ca^{2+} . To see if the syringomycin-resistance, Ca^{2+} -sensitive mutation was related to that of *cls4*, R4-3G was crossed with *cls4*. R4-3G complemented *cls4* for the Ca^{2+} -sensitive phenotype showing that the mutation differed from the *CDC24* mutation of *cls4*.

Sensitivity to Ca^{2+} and EGTA

The growth of R4-3G in YPD medium was inhibited when CaCl_2 was added at concentrations as low as 5 mM

Table 1. Cellular Ca^{2+} contents of the parent strain 8A-1B and the syringomycin-resistant mutant R4-3G

Cells were grown overnight in YPD medium and, where indicated, were incubated with 1 mM- CaCl_2 for 20 min before harvesting. Values for Ca^{2+} content are means $\pm$ SD of three separate experiments.

Strain	1 mM- Ca^{2+}	Ca^{2+} content (μg per OD_{660} unit)
8A-1B	—	0.096 $\pm$ 0.003
R4-3G	—	0.111 $\pm$ 0.03
8A-1B	+	0.133 $\pm$ 0.017
R4-3G	+	0.263 $\pm$ 0.021

(Fig. 1). Its growth was completely inhibited by addition of the Ca^{2+} chelator EGTA at 5 mM or higher concentrations. The parent strain, 8A-1B, tolerated EGTA levels as high as 10 mM.

Cellular Ca^{2+} content

The cellular Ca^{2+} contents of R4-3G and its parent were compared. The Ca^{2+} content of the mutant grown in YPD medium was similar to that of the parent (Table 1). However, when 1 mM- CaCl_2 was added 20 min before collecting and washing the cells, R4-3G and the parent showed 2.4- and 1.15-fold higher levels of cell-associated bound Ca^{2+} , respectively.

Resistance to other drugs

A mutation, *pdr1*, of *S. cerevisiae* confers cross-resistance to several drugs including chloramphenicol, tetracycline and cycloheximide (Rank *et al.*, 1975, 1977). To test if resistance to syringomycin was due to *pdr1*, R4-3G was tested for growth on YPD agar medium supplemented with chloramphenicol (4 mg ml^{-1}), tetracycline (2 mg ml^{-1}) or cycloheximide (0.5 μg ml^{-1}). The growth of R4-3G and the parent was similarly inhibited on all of these media showing that strain R4-3G did not have the *pdr1* mutation and that resistance to syringomycin was not due to this mutation.

Cellular Ca^{2+} uptake

Cells of R4-3G showed higher net $^{45}\text{Ca}^{2+}$ uptake rates than the parent, 8A-1B, in YPD medium (Fig. 2). Syringomycin at 10.2 units ml^{-1} stimulated net $^{45}\text{Ca}^{2+}$ uptake into the parent, but had no effect with R4-3G. This level of syringomycin was chosen because it was close to the minimum amount required to inhibit growth of the parent. Net $^{45}\text{Ca}^{2+}$ uptake by R4-3G was biphasic

Fig. 2. Ca^{2+} influx by R4-3G (filled symbols) and 8A-1B (open symbols) and the effects of syringomycin. Cells were grown and suspended in YPD medium (10^8 cells ml^{-1}). After incubation for 30 min at 28°C , $4 \mu\text{Ci}$ (148 kBq) $^{45}\text{Ca}^{2+}$ ml^{-1} [4 mCi (148 MBq) per mmol of CaCl_2] and 10.2 units syringomycin ml^{-1} (■, □) or no syringomycin (●, ○) were added. No carrier CaCl_2 was added. At various times, the amount of $^{45}\text{Ca}^{2+}$ accumulated by the cells was determined as described by Tachikawa *et al.* (1987).

Fig. 3

Fig. 3. Influence of CaCl_2 concentration on Ca^{2+} influx by R4-3G (filled symbols) and 8A-1B (open symbols). Cells grown in YPD medium were washed and suspended in MM medium (10^8 cells ml^{-1}) with final CaCl_2 concentrations of $50 \mu\text{M}$ (●, ○), $500 \mu\text{M}$ (▲, △) or 1 mM (■, □). The cells were incubated for 30 min at 28°C . $^{45}\text{Ca}^{2+}$ [4 mCi (148 MBq) per mmol of CaCl_2] was added (time zero), and $^{45}\text{Ca}^{2+}$ accumulation by the cells was determined as described by Tachikawa *et al.* (1987).

with a rapid (15 min) initial phase followed by a slower phase. By 60 min, the amounts of $^{45}\text{Ca}^{2+}$ incorporated by R4-3G were equivalent to those of the syringomycin-treated parent.

The influence of the extracellular Ca^{2+} concentration on $^{45}\text{Ca}^{2+}$ uptake was studied in MM medium. RF4-3G displayed higher initial uptake with elevated levels of Ca^{2+} (Fig. 3). The Ca^{2+} -dependent higher rates were not altered by exposure to syringomycin (Fig. 4a). In contrast, uptake by the parent did not change with elevated Ca^{2+} concentration for the initial 30 min of incubation. After 30 min, the rates increased slightly at the higher Ca^{2+} levels (Fig. 3). However, with syringomycin (12.8 units ml^{-1}), the parent showed dramatically increased rates with higher Ca^{2+} concentrations (Fig. 4b). With $500 \mu\text{M}$ - CaCl_2 , the syringomycin-treated parent showed 3- to 4-fold higher uptake rates than R4-3G.

Fig. 4

Fig. 4. Influence of CaCl_2 concentration on syringomycin-stimulated $^{45}\text{Ca}^{2+}$ influx by R4-3G (a) and 8A-1B (b). Cells grown in YPD medium were washed and suspended in MM medium (10^8 cells ml^{-1}) containing CaCl_2 concentrations of $50 \mu\text{M}$ (▲, △) or $500 \mu\text{M}$ (●, ○). The suspensions were incubated for 30 min at 28°C . $^{45}\text{Ca}^{2+}$ [4 mCi (148 MBq) per mmol of CaCl_2] was added with (filled symbols) or without (open symbols) 12.8 units syringomycin ml^{-1} . At various times, the amount of $^{45}\text{Ca}^{2+}$ accumulated by the cells was determined as described by Tachikawa *et al.* (1987).

Since net cellular Ca^{2+} uptake is influenced by both the inward and outward movement of this ion, Ca^{2+} efflux by cells preloaded with $^{45}\text{Ca}^{2+}$ was measured. R4-3G showed slightly higher cellular $^{45}\text{Ca}^{2+}$ efflux than the parent in MM medium (Fig. 5). Syringomycin at 12.8 units ml^{-1} did not affect $^{45}\text{Ca}^{2+}$ efflux by either R4-3G or the parent. With the parent only, a higher syringo-

Fig. 5. Effect of syringomycin on $^{45}\text{Ca}^{2+}$ efflux by R4-3G (a) and 8A-1B (b). The syringomycin concentrations were 0 ($\circ$), 12.8 ($\bullet$), 38.4 ($\blacktriangle$) or 64 ($\blacksquare$) units ml^{-1} . The total CaCl_2 concentration in the cell suspensions was $50\text{ }\mu\text{M}$. Cells were preloaded with $^{45}\text{Ca}^{2+}$, washed, and then counted for radioactivity as described in Methods.

Fig. 6

Fig. 6. Effect of syringomycin on K^+ efflux by R4-3G (filled symbols) and 8A-1B (open symbols). The syringomycin concentration was 0 ($\bullet$, $\circ$), 12.8 ($\blacksquare$, $\square$) or 25.6 ($\blacktriangle$, $\triangle$) units ml^{-1} .

Fig. 7. Effect of syringomycin on $[^{14}\text{C}]\text{TPP}^+$ uptake (membrane potential) by R4-3G (filled symbols) and 8A-1B (open symbols). Either no ($\bullet$, $\circ$) or 30.7 ($\blacksquare$, $\square$) units syringomycin ml^{-1} were added to the incubation mixture.

mycin concentration (38.4 units ml^{-1}) caused a slight inhibition, and at 64 units ml^{-1} , an initial (10 min) inhibition was followed by a significantly increased $^{45}\text{Ca}^{2+}$ efflux. For both strains, efflux was not influenced by adding EGTA (10 mM) or CaCl_2 (0.68 mM) to the measuring medium (not shown).

K^+ efflux and membrane potential.

R4-3G and the parent showed a similar K^+ efflux with syringomycin treatment (Fig. 6). In contrast, the mutant showed a lower increase in membrane potential with syringomycin than did the parent (Fig. 7). With no syringomycin addition, K^+ efflux and membrane potential were similar in both the mutant and the parent.

Discussion

Genetic and physiological analyses of strain R4-3G showed that a single recessive mutation simultaneously led to syringomycin resistance and an alteration in Ca^{2+} transport capabilities. This suggests that Ca^{2+} transport is involved in the syringomycin response of *S. cerevisiae*. A role for Ca^{2+} transport in the syringomycin response of red beet tissue has been suggested previously (Takemoto *et al.*, 1989).

Rapid initial rates of $^{45}\text{Ca}^{2+}$ uptake observed as non-zero ordinate intercepts were observed with the mutant *S. cerevisiae* strain R4-3G, but not with the parent (Figs 2–4). The relative contributions to these initial rates of Ca^{2+} influx and extracellular binding of Ca^{2+} are not known. Higher initial rates were observed when experiments were done in YPD medium as compared to MM

Fig. 7

medium. This suggests that a significant portion of the uptake was dependent on the metabolic state of the cells and therefore most likely represents transport. It seems more plausible that the mutant and parent would differ in Ca^{2+} transport capabilities rather than non-specific extracellular binding.

The reasons for the Ca^{2+} -sensitivity of mutant R4-3G are not known. One possibility is the occurrence of defective plasma membrane Ca^{2+} channels which result in abnormally high cytoplasmic Ca^{2+} concentrations. Defective Ca^{2+} channels are supported by two observations: (1) R4-3G showed higher rates of net Ca^{2+} uptake than its parent, and these rates were not affected by syringomycin treatment; (2) Ca^{2+} influx by the mutant and its Ca^{2+} content were dependent upon the external Ca^{2+} concentration (Fig. 3 and Table 1). A second possibility for the observed phenotype of R4-3G is an impaired transport system which controls the plasma membrane potential. TPP^{+} uptake was used as an indicator of membrane potential as described previously (Zhang & Takemoto, 1986, 1987). The syringomycin-stimulated increase in TPP^{+} uptake was significantly lower in R4-3G than in the parent (Fig. 2), indicating some impaired ability to control the membrane potential. Eilam & Chernikovsky (1987) suggested that Ca^{2+} influx across the plasma membrane depends on the membrane potential. The membrane potential dysfunction observed as a lowered response to syringomycin may have affected the Ca^{2+} transport capabilities of the mutant. A third possibility is a change in vacuolar membrane function. In yeast, the vacuole serves as a Ca^{2+} sink and buffer for cytoplasmic Ca^{2+} homeostasis (Eilam *et al.*, 1985; Kitamoto *et al.*, 1988). An alteration in this buffer system could account for the Ca^{2+} -sensitive properties of strain R4-3G.

Conceivably, R4-3G may have a defect in a regulatory protein which controls one or more Ca^{2+} channels. In yeast, the *YPT1* gene product (a G protein) is postulated to have a regulatory role in Ca^{2+} transport (Schmitt *et al.*, 1988). Certain mutations in this gene render cells sensitive to the calmodulin antagonist trifluoperazine, as are the mutant strains reported here. Only further studies to identify the defective gene in strain R4-3G will determine whether a similar regulatory protein is altered.

A defect in the mitochondrion is not likely. Yeast mitochondrial petite mutants and wild-type strains behave identically when treated with syringomycin, indicating that mitochondria are not directly involved in the syringomycin response (Zhang & Takemoto, 1986).

Evidently, systems responsible for yeast plasma membrane Ca^{2+} efflux such as the Ca^{2+} -ATPase and the energy-dependent K^{+} or $\text{Na}^{+}/\text{Ca}^{2+}$ antiporter (Eilam, 1982) were not affected by the mutation in R4-3G. Comparisons of the Ca^{2+} efflux properties of R4-3G and

its parent suggest that the altered net Ca^{2+} uptake rates and different Ca^{2+} contents were not due to differences in Ca^{2+} efflux. Higher Ca^{2+} uptake rates would result from lower Ca^{2+} efflux capabilities. Instead, the mutant showed slightly higher effluxes compared to the parent (Fig. 5). At high syringomycin concentrations (64 units ml^{-1}), Ca^{2+} efflux by the parent was initially (10 min) inhibited, but this was then followed by a high Ca^{2+} efflux.

The syringomycin response appears to involve the opening of Ca^{2+} channels. This notion is supported by the observation that net Ca^{2+} uptake rates by the syringomycin-treated parent strain increased with higher external Ca^{2+} concentrations (Fig. 4). Ca^{2+} channel opening with syringomycin also explains a previous finding that syringomycin inhibited the ATP-dependent accumulation of Ca^{2+} into inverted plasma membrane vesicles of red beet (Takemoto *et al.*, 1989). Open Ca^{2+} channels will not permit the energy-dependent accumulation of Ca^{2+} into such vesicles. For *S. cerevisiae*, plasma membrane Ca^{2+} channels are largely unknown. However, a mechano-sensitive plasma membrane Ca^{2+} channel having a relatively low selectivity among cations has been reported (Gustin *et al.*, 1988). At high syringomycin concentrations, the inhibition of Ca^{2+} efflux (e.g. a Ca^{2+} -ATPase) (Fig. 5) may also contribute to the higher net Ca^{2+} influx.

The enhanced net Ca^{2+} influx caused by syringomycin was not due to the toxin acting as a Ca^{2+} ionophore. Although, the Ca^{2+} ionophore A23187 allows the efflux of $^{45}\text{Ca}^{2+}$ out of preloaded artificial liposomes, syringomycin has no such effect (Takemoto *et al.*, 1989). Thus, the properties of R4-3G cannot be explained by a lowered affinity for syringomycin serving as a Ca^{2+} ionophore.

The reason for the resistance to syringomycin of R4-3G is not clear. One possibility may be that the mutation prevents a high Ca^{2+} influx during the syringomycin response. The 3- to 4-fold higher $^{45}\text{Ca}^{2+}$ influx in the syringomycin-treated parent as compared to R4-3G at high (500 μM) Ca^{2+} levels is consistent with this hypothesis. In YPD medium, which was used to isolate the mutant, the Ca^{2+} concentration is high (180 μM , Ohya *et al.*, 1986a), and syringomycin would have a similar effect. Thus, the mutant may not be subject to the detrimental effects of high Ca^{2+} influx that normally occur with exposure to syringomycin.

Resistance to syringomycin was not due to the occurrence of the multiple drug resistance mutation *pdrl* (Rank *et al.*, 1975, 1977) nor to the mutation in *CDC24* which results in a defect in bud emergence and Ca^{2+} -sensitivity (Ohya *et al.*, 1986b).

The syringomycin-induced K^{+} efflux (Reidl & Takemoto, 1987) was normal in mutant strain R4-3G. This

indicates that in the syringomycin response K^+ efflux occurs upstream or independently of Ca^{2+} influx. Furthermore, K^+ efflux alone is not sufficient to inhibit growth. In contrast, the syringomycin-induced membrane hyperpolarization as measured by TPP⁺ uptake (Zhang & Takemoto, 1986, 1987) was inhibited in the mutant. This observation demonstrates that the membrane hyperpolarization and Ca^{2+} transport are closely associated. In yeast, the membrane potential (interior negative) is largely dependent on the K^+ and H^+ concentration gradients across the plasma membrane. Since K^+ efflux is not affected in the mutant, the lowered membrane hyperpolarization may be because the mutation prevents an increase in the H^+ gradient as a result of treatment with syringomycin.

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