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Requirement of sphingolipid α-hydroxylation for fungicidal action of syringomycin E

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Abstract Syringomycin E is an antifungal cyclic lipodepsinonapeptide produced by *Pseudomonas syringae* pv. *syringae*. To understand the mechanism of action of syringomycin E, a novel resistant *Saccharomyces cerevisiae* strain, BW7, was isolated and characterized. Lipid analyses revealed that BW7 contained only the hydrophobic subspecies of sphingolipids that are normally minor components in wild type strains. This aberrant sphingolipid composition was the result of lack of α-hydroxylation of the amide-linked very long chain fatty acids, suggesting a defective sphingolipid α-hydroxylase encoded by the *FAH1* gene. A yeast strain that lacks the *FAH1* gene was resistant to syringomycin E, and failed to complement BW7. These results demonstrate that BW7 carries a mutation in the *FAH1* gene, and that the lack of α-hydroxylated very long chain fatty acids in yeast sphingolipids confers resistance to syringomycin E.

Key words: Syringomycin E; Cyclic lipodepsinonapeptide; Sphingolipid α-hydroxylation; α-Hydroxyl fatty acid; *Pseudomonas syringae*; *Saccharomyces cerevisiae*

1. Introduction

The plant bacterium *Pseudomonas syringae* pv. *syringae* produces a group of cyclic lipodepsinonapeptides (CLPs) that include syringomycin E, pseudomycin B, syringotoxin, and syringostatin [1,2]. The CLPs are considered to be plant virulence factors, and show a broad range of inhibitory activities against many fungal species including human pathogens [3,4]. Biophysical studies revealed that syringomycin E forms ion channels in artificial lipid bilayers, which may account for its biological activities [5,6]. To understand the mechanism of action of syringomycin E in biological membranes, the yeast *Saccharomyces cerevisiae* has been used as a model target organism. Characterization of syringomycin E-resistant yeast mutants has revealed at least 11 genes that, when mutated, result in a resistant phenotype [7,8]. These genes are involved in sterol biosynthesis (*SYR1*/*ERG3* and four uncharacterized genes) [7,9] or sphingolipid biosynthesis (*SYR2, SYR3*/*ELO2, ELO3, SYR4*/*IPT1, CSG1, CSG2*) [8,10]. Thus, it appears that sterols and sphingolipids facilitate the action of syringomycin E in yeast. In this study, we report the isolation of a novel syringomycin E-resistant yeast strain with a mutation in the *FAH1* gene. This gene encodes a hydroxylase that introduces an α-OH in the characteristic very long chain fatty acids (mostly hexacosanoic acid) of yeast sphingolipids [11,12]. The findings show that the antifungal action of syringomycin E requires sphingolipids with α-hydroxylated very long chain fatty acids (HFA).

2. Materials and methods

2.1. Strains and media

S. cerevisiae strains used in this study were W303C (*MAT***a** *ura3-52 his3-Δ200 lys2-801*) [10], BW7 (*MAT***a** *ura3-52 his3-Δ200 lys2-801 fah1*) (this work), BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) [13] (Research Genetics, Huntsville, AL), and BY4742 *fah1*Δ (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 fah1Δ0*) (Research Genetics, Huntsville, AL), BWBY4742 (a diploid strain made by crossing BW7 to BY4742), and BWBY *fah1*Δ (a diploid strain made by crossing BW7 to BY4742 *fah1*Δ). Yeast cells were grown in a medium with 1% yeast extract, 2% peptone, and 2% glucose (YPD medium). Syringomycin E was purified from cultures of *P. syringae* pv. *syringae* strain B301D as previously described [14].

2.2. Isolation of syringomycin E-resistant mutants

Mutants resistant to syringomycin E were generated by UV mutagenesis. W303C cells were plated on YPD medium agar plates and illuminated with UV (312 nm) for 30 s (10% survival rate). Colonies were tested for syringomycin E resistance by replica plating on YPD medium agar containing 0.5 μg/ml syringomycin E.

2.3. Lipid analyses

Ergosterol levels were measured by the method of Woods [15]. Labeling of yeast cells [8], sphingolipid extraction [16], and TLC [17] were conducted as described previously.

For very long chain fatty acid analyses, total lipids were extracted from 20 OD_{600} units of cells by the method of Hanson and Lester [16]. Fatty acids were liberated from the crude lipid extracts by alkaline treatment, and were derivatized as described in [18]. Fatty acid phenacyl derivatives were resuspended in 200 μl of acetonitrile/methanol (40:60, v/v) by sonication, and 20 μl of each sample was used for HPLC analysis. Reversed-phase HPLC was carried out using a Beckman System Gold HPLC unit equipped with a Beckman Ultrasphere XL ODS column (3 μm particles, 4.6×70 mm). The column was equilibrated with acetonitrile/methanol (40:60, v/v) at 1 ml/min. Fatty acid phenacyl derivatives were eluted with 2 ml of acetonitrile/methanol (40:60, v/v), and then with a 15 ml linear gradient to acetonitrile/methanol (25:75, v/v). UV absorption was monitored at 242 nm. Hexacosanoic acid (C26) and α-hydroxyhexacosanoic acid (C26-OH) standards were purchased from Sigma.

3. Results

3.1. Isolation of novel syringomycin E-resistant mutants

Previous analyses of syringomycin E-resistant yeast mutants, generated by nitrosoguanidine mutagenesis, identified 11 genes that are involved in susceptibility to syringomycin

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E [7–10]. To isolate additional mutants, rigorous UV irradiation mutagenesis was conducted (survival rate of 10%). From 500 colonies screened for resistance to 0.5 μg/ml syringomycin E, seven resistant strains were isolated. Among these, two strains, BW1 and BW7, were more resistant and able to grow in the presence of 0.75 μg/ml syringomycin E. Sphingolipid analyses revealed that strain BW1 was likely to be a *syr4* mutant (not shown) [8]. Strain BW7, on the other hand, did not resemble known syringomycin E-resistant strains, and it was selected for further study.

3.2. Lipid analyses of the new syringomycin E-resistant mutant

To determine whether strain BW7 was defective in sterol biosynthesis, ergosterol levels in non-saponifiable lipid extracts were measured. Strain BW7 contained ergosterol at levels similar to the wild type strain, indicating that syringomycin E resistance was not due to altered sterol biosynthesis (data not shown).

Sphingolipid analysis was conducted to determine whether strain BW7 had a defect in sphingolipid biosynthesis. Thin-layer chromatography of ^{32}P-labeled sphingolipids revealed that strain BW7 possessed the three major types of yeast sphingolipids, inositolphosphorylceramide (IPC), mannosylinositolphosphorylceramide (MIPC), and mannosyldiinositolphosphorylceramide [M(IP)$_2$C] (Fig. 1). However, the subspecies profiles of each of these sphingolipids were markedly altered in the mutant. The major wild type forms of IPC, MIPC, and M(IP)$_2$C were missing in strain BW7, and instead, more hydrophobic (higher mobility) subspecies, normally minor, were prominent. This suggests that the mutant lacked a modification that renders its sphingolipids more hydrophilic.

Two enzymes are known to introduce hydrophilic modifications to the ceramide portion of yeast sphingolipids [19]: the *SYR2* gene product catalyzes C$_4$-hydroxylation of the long chain base [20], and the *FAH1* gene product is required for the α-hydroxylation of the amide-linked very long chain fatty acids [12,21]. When strain BW7 was crossed to a strain with a *SYR2* deletion, the resulting diploid strain was sensitive to syringomycin E, demonstrating that the mutation in strain BW7 does not reside in the *SYR2* gene (data not shown).

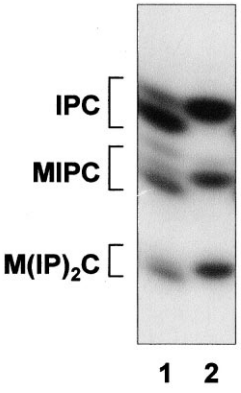

Fig. 1. Altered sphingolipid composition of strain BW7. Total lipids were extracted from [^{32}P]phosphate-labeled cells and deacylated by mild alkaline treatment. Alkaline-resistant phospholipids were resolved by TLC (solvent: CHCl$_3$/methanol/4.2 N NH$_4$OH, 9:7:2, v/v), and visualized by autoradiography. The major sphingolipid classes of yeast are indicated. Lane 1, W303C; lane 2, BW7.

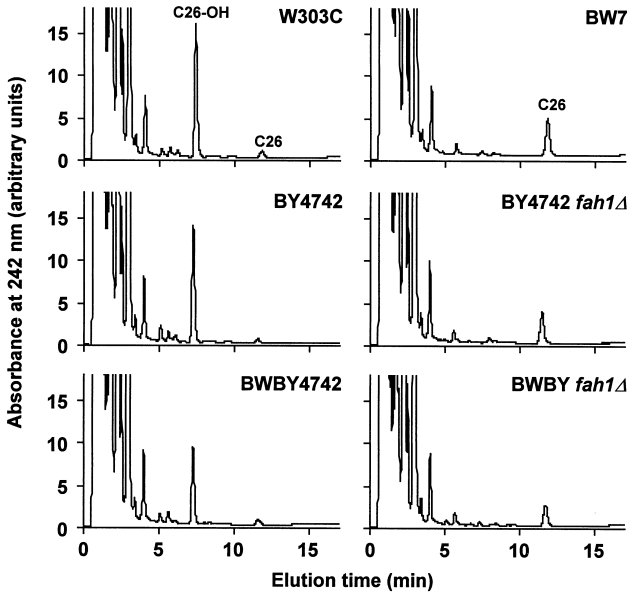

Fig. 2. HPLC analyses of very long chain fatty acids of strain BW7 and *fah1* mutants. Fatty acids were liberated from cellular lipids by alkaline treatment. UV-absorbing phenacyl derivatives were analyzed by reversed-phase HPLC. The peaks eluting between 30 s and 4 min include shorter chain fatty acid derivatives and excess derivatization reagents.

Therefore, it was speculated that the mutant lacked the HFA due to a mutation in the *FAH1* gene.

3.3. Defects in sphingolipid α-hydroxylation in strain BW7

The presence of HFA in strain BW7 was analyzed. Total cellular fatty acids were extracted and converted to UV-absorbing phenacyl derivatives for HPLC analyses. As expected, the C26 fatty acids of the wild type strain, W303C, were mostly hydroxylated (HFA) (Fig. 2) [17]. Strain BW7, however, contained non-hydroxylated C26 fatty acids (NFA) and HFA were not detected. Similarly, strain BY4742 (a *FAH1* strain) contained mostly HFA, and the same strain with a *FAH1* deletion (BY4742 *fah1*Δ) contained only NFA. Strain BY4742 *fah1*Δ showed a similar degree of resistance to syringomycin E as strain BW7 (Fig. 3). These results strongly suggest that strain BW7 carries a mutation in the *FAH1* gene. This possibility was directly tested by genetic complementation analyses between strains BW7 and BY4742 *fah1*Δ. A cross between strains BW7 and BY4742 resulted in a diploid (strain BWBY4742) that contained HFA (Fig. 2) and that displayed sensitivity to syringomycin E (Fig. 3). In contrast, a cross between strain BW7 and BY4742 *fah1*Δ resulted in a diploid strain (BWBY *fah1*Δ) that contained only NFA

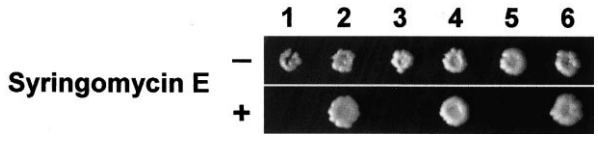

Fig. 3. Syringomycin E-resistant phenotype of *fah1* mutants. Each strain (~1×10^7 cells) was suspended in 100 μl of YPD medium, replica plated onto YPD agar plates with or without 0.75 μg/ml syringomycin E, and the plates incubated at 29°C for 48 h. 1, W303C; 2, BW7; 3, BY4742; 4, BY4742 *fah1*Δ; 5, BWBY4742; 6, BWBY *fah1*Δ.

(Fig. 2), and was resistant to syringomycin E (Fig. 3). These results demonstrate that strain BW7 carries a mutation in the *FAH1* gene, and that the lack of HFA results in resistance to syringomycin E.

With the three *fah1* strains the growth was slightly stimulated by syringomycin E (Fig. 3). Although the mechanism is not known, similar phenomena have been observed with *syr1/erg3* strains [22] and other syringomycin E-resistant strains (unpublished).

3.4. Lack of HFA and resistance to other antifungal agents

Yeast cells exhibit cross-resistance against several antifungal agents when pleiotropic drug resistance (*PDR*) genes are induced [23]. To determine whether the lack of HFA induces the *PDR* genes, susceptibility to other antifungal agents was studied. The *fah1* strains showed susceptibilities to amphotericin B, calcofluor, cycloheximide, ketoconazole, and nystatin, indicating that a *PDR* gene-related mechanism is not involved (data not shown). Interestingly, strain BW7 was more resistant to another cyclic lipodepsinonapeptide, pseudomycin B (minimal inhibitory concentration = 2.5 μg/ml), compared to the parental strain (minimal inhibitory concentration = 0.32 μg/ml). Consistent with these observations, characterization of pseudomycin B-resistant yeast mutants identified strains with *fah1* mutations (unpublished).

4. Discussion

In this study, we demonstrated that HFA in yeast sphingolipids promote sensitivity to syringomycin E. The effect appears to be specific for the cyclic lipodepsinonapeptides, since resistance to both syringomycin E and pseudomycin B, but not to other antifungal agents, was evident in *fah1* mutants. It is not yet known how HFA facilitates syringomycin E action at the plasma membrane. The HFA α-OH participates in intra- and intermolecular hydrogen bonding and influences interchain packing in membranes [24,25]. In *fah1* mutants, the lack of HFA would significantly decrease hydrogen bonding capacity and lipid–lipid interactions. One possibility is that the α-OH allows hydrogen bonding of syringomycin E directly to sphingolipids to facilitate ion channel formation in the membrane. Alternatively, α-hydroxylation may enhance formation of sterol/sphingolipid-rich membrane domains (lipid rafts), which may serve as sites for syringomycin E binding and channel formation.

S. cerevisiae contains lipid rafts that are highly enriched with ergosterol and sphingolipids, and depletion of these lipids compromises raft integrity [26]. In addition to HFA, the antifungal action of syringomycin E also requires ergosterol [7,9] and sphingolipid modifications that increase lipid–lipid interactions [8,10,20], suggesting further the involvement of lipid rafts. Association of syringomycin E to lipid rafts would increase local concentrations to facilitate formation of the oligomeric syringomycin E channels. The lack of HFA and the other sphingolipid modifications may compromise raft integrity, causing raft-associated molecules to diffuse – an effect that would decrease the ability of syringomycin E to form ion channels. Consistent with this idea, the growth of *fah1* mutants is only inhibited by syringomycin E at much higher concentrations (above 2 μg/ml), which may reflect non-raft-mediated ion channel formation. Therefore, it is of interest to examine the integrity of lipid rafts in *fah1* mutants, and to correlate it with the efficiency of ion channel formation by syringomycin E.

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