

## Effects of Sugars and Salt on the Production of Glycosphingolipids in *Mariannaea Elegans*

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### Summary

Glycosphingolipids (GSLs) are complex macromolecules in cell membranes that play important roles in various biological processes. Fungi contain several types of GSLs that are distinct from those of mammals, but little is known about their physiological functions, metabolic regulation, or biosynthetic pathways. Most fungal species that possess fungal neogala-series GSLs (FNG-GSLs) are resistant to aureobasidin A (AbA), an antifungal agent that inhibits glycosylinositolphosphoceramide (GIPC) synthesis. We have previously reported that *Mariannaea elegans* has FNG-GSLs in its cell membrane, despite the fact that it is sensitive to AbA. In this study, we demonstrated that *M. elegans* contains GIPCs, which explains its sensitivity to AbA. We also found that both cell growth and GSL composition of *M. elegans* were affected by the presence or absence of different sugars in the culture medium. In contrast, sugars had no effect on the phenotypes and GSLs of AbA-resistant *Rhizopus* sp. lacking GIPCs. Further analysis revealed that FNG-GSL production can be modulated by the addition of 600 mM NaCl in a glucose-containing medium. Finally, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of Golgi proteins from *M. elegans* showed that at least nine proteins were present exclusively in cells producing FNG-GSL, suggesting that those proteins are potential candidates for FNG-GSL synthesis.

**Abbreviations:** AbA, aureobasidin A; ER, endoplasmic reticulum; FNG-GSLs, fungal neogala-series GSLs; Gal-Cer,  $\beta$ -galactosylceramide; Glc-Cer,  $\beta$ -glucosylceramide; GSLs, glycosphingolipids; GIPCs, glycosylinositolphosphoceramides; IPC, inositolphosphoceramide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography

### Introduction

Glycosphingolipids (GSLs), which consist of a hydrophilic carbohydrate chain of variable length and structure linked to a hydrophobic ceramide moiety, are distributed in membranes of all eukaryotes and some bacteria<sup>1, 2)</sup>. In mammals, two main types of GSLs are known:  $\beta$ -glucosylceramide (Glc-Cer)-type GSLs and  $\beta$ -galactosylceramide (Gal-Cer)-type GSLs<sup>1)</sup>. Glc-Cer synthesis occurs on the cytosolic leaflet of the Golgi membrane, whereas subsequent addition of sugar moieties occurs on the luminal side of the Golgi<sup>3, 4)</sup>. Glc-Cer-type GSLs play various roles in signaling, development, and immunological responses<sup>1, 2)</sup>. In contrast to Glc-Cer-type GSLs, Gal-Cer is synthesized on the luminal side of the endoplasmic reticulum (ER). It is subsequently modified on the luminal side of the Golgi to

form sialic acid-containing GSLs<sup>4)</sup>, which are mainly distributed in neural tissues<sup>5)</sup>, and play an important role in structure, function, and stability of myelin<sup>6)</sup>.

Four distinct types of GSLs have been identified in fungi. Two of them are the main neutral monoglycosylceramides, Glc-Cer and Gal-Cer, which are essentially similar to those found in mammals, except that they have no additional sugar modification<sup>7)</sup>. Glc-Cer functions during fungal growth and dimorphism<sup>8)</sup>, and it is involved in lipid raft formation<sup>9)</sup> and virulence<sup>10)</sup>. On the other hand, little is known about the function of Gal-Cer. The third class of fungal GSLs are acidic glycosylinositolphosphoceramides (GIPCs), which are modified by the addition of mannose, galactosamine, galactose, and/or xylose<sup>11–14)</sup>. Generally, acidic GIPCs are essential membrane components for fungal growth<sup>15, 16)</sup>. The fourth class of fungal GSLs are neu-

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tral fungal neogala-series GSLs (FNG-GSLs), which have more recently been identified in certain fungal species including *Mucor hiemalis*<sup>17)</sup>, *Rhizopus oryzae*<sup>17)</sup>, *Hirsutella rhossiliensis*<sup>18)</sup>, and *Mariannaea elegans*<sup>19)</sup>. FNG-GSLs contain a characteristic core structure consisting of Gal  $\beta$  1-6Gal  $\beta$  1-Cer, with a phytosphingoid base. Complex FNG-GSLs, such as those containing five hexoses (e.g., Gal  $\alpha$  1-6Gal  $\alpha$  1-6Gal  $\alpha$  1-6Gal  $\beta$  1-6Gal  $\beta$  1-1Cer), have been identified in *M. hiemalis*<sup>17)</sup>. While FNG-GSLs are proposed to substitute GIPCs in certain of species of fungus devoid of GIPCs<sup>17, 18)</sup>, their exact role remains to be elucidated.

Fungal-specific GSLs have received considerable attention as a potential target for antifungal agents<sup>20)</sup>. Aureobasidin A (AbA) is a well-known antifungal agent, which is able to repress the growth of many species of fungus by inhibiting inositolphosphoceramide (IPC) synthase. IPC synthase catalyzes the transfer of inositol phosphate from phosphatidylinositol to phytoceramide to form IPC<sup>21-23)</sup>, which is a precursor of GIPCs, an essential membrane component<sup>15, 16)</sup>. However, AbA does not inhibit the growth of some fungal species such as *Mucor hiemalis*<sup>17)</sup>, *Rhizopus oryzae*<sup>17)</sup>, and *Hirsutella rhossiliensis*<sup>18)</sup>, because these AbA-resistant fungi have FNG-GSLs in place of GIPCs<sup>17, 18)</sup>. While the development of new antifungal agents targeted to fungal-specific GSLs is needed, their biosynthetic pathways or regulation of their production is not extensively studied.

We recently demonstrated that *M. elegans* is sensitive to AbA, despite the fact that this fungal strain possesses FNG-GSLs with two hexoses, Gal  $\beta$  1-6Gal  $\beta$  1-1Cer and Glc1-6Gal  $\beta$  1-1Cer<sup>19)</sup>. The presence of GIPCs in the fungus has been proposed, but remains to be examined. In this study, we focused on two FNG-GSL-containing fungal strains, AbA-sensitive *M. elegans* and AbA-resistant *Rhizopus* sp., and analyzed both neutral and acidic GSLs from *M. elegans*. We investigated the effect of culture medium containing various sugars and varying concentrations of salt on the composition of GSLs. Further, based on our findings, proteins potentially involved in the biosynthesis of FNG-GSLs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of Golgi fractions from *M. elegans* grown in the presence or absence of NaCl.

## Materials and Methods

### Materials

A QAE-Sephadex A-25 column and 2D Quant kit were purchased from GE Healthcare (Tokyo, Japan). Silica gel 60 thin-layer chromatography (TLC) plates were pur-

chased from Merck (Darmstadt, Germany). Yatalase was purchased from Takara Bio (Kyoto, Japan). Digitonin was purchased from Sigma-Aldrich (Tokyo, Japan), and anti-mycin A was purchased from Cosmo Bio (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Co. (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan).

### Culture of fungal strains

Ascomycota strains *M. elegans* JCM12789 and *Aspergillus oryzae* RIB40 were obtained from the Japan Collection of Microorganisms (Wako, Japan) and the National Research Institute for Brewing (Higashi-hiroshima, Japan), respectively. The *Rhizopus* sp. strain was a kind gift from Dr. Mamoru Wakayama (Ritsumeikan University, Kusatsu, Shiga, Japan). The fungal strains were cultured in 500 mL of potato medium (0.4% potato extract) containing various concentrations of sugars and NaCl at 30°C for 120 h at 100 rpm in a 2 L shaking flask. The cultured mycelia were washed with water and harvested by vacuum filtration. The harvested mycelia were stored at -80°C until use.

### Extraction and purification of GSLs

The cultured mycelia were harvested and freeze-dried. The dried powder was extracted with chloroform-methanol-water (1:2:0.8, v/v/v) at 37°C for 20 h. The extract was dried and subjected to mild alkaline hydrolysis with 0.5 M potassium hydroxide in methanol-water (95:5, v/v) at 37°C for 12 h. The hydrolysate was acidified to pH 1.0 with concentrated HCl, and then dialyzed against tap water for 2 d. Glycolipids in the dialysate were precipitated with acetone, dissolved in chloroform-methanol (2:1, v/v), and applied to a QAE-Sephadex A-25 column. Elution was carried out with 5 volumes of chloroform-methanol-water (30:60:8, v/v/v) to obtain a neutral GSL fraction. Acidic GSLs were eluted with 0.005-0.45 M ammonium acetate in methanol. The neutral and acidic GSLs were analyzed by TLC.

### TLC analysis

Silica gel 60 TLC plates were developed to a distance of 5 cm with chloroform-methanol-water (60:35:8, v/v/v). Detection of sugar moieties was performed with orcinol-H<sub>2</sub>SO<sub>4</sub> reagent<sup>24, 25)</sup>.

### Preparation of subcellular fractions

Subcellular fractions were prepared by the yeast subcellular fractionation method<sup>26)</sup> with modifications. The cultured mycelia (3 g) was resuspended in 5 mL of a di-

gestion solution consisting of 2% Yatalase, a 0.1 M maleic acid buffer (pH 5.5), and 0.5 M magnesium sulfate, and incubated for 4 h at 30°C to digest the cell wall. After centrifugation for 5 min at  $15,000 \times g$  at 4°C, the supernatant was collected as a periplasmic fraction. The pellet, which contained spheroplasts, was resuspended in a JR lysis buffer containing 20 mM HEPES (pH 7.4), 2 mM EDTA, 1 mM DTT, 50 mM potassium acetate, and 0.1 M sorbitol<sup>27)</sup>. The spheroplasts were disrupted on ice with 20 strokes from a homogenizer. The homogenate was centrifuged for 10 min at  $13,000 \times g$  at 4°C. The pellet was collected as the cell debris fraction (P13). The supernatant was further centrifuged for 60 min at  $100,000 \times g$  to obtain the P100 fraction. The supernatant was collected as the cytoplasmic fraction (S100). The P100 fraction was resuspended in a JR lysis buffer to obtain the Golgi apparatus fraction.

### Cytochrome *c* oxidase assay

Cytochrome *c* oxidase, a marker of mitochondrial enzyme activity, was assayed as described previously<sup>28, 29)</sup>. A suitable dilution of the enzyme was first prepared in a 45 mM potassium phosphate buffer (pH 7.2) containing 0.01% digitonin, and maintained at 0°C for 10 min. The reaction was initiated by the addition of 0.1 mL of 0.2 mM reduced cytochrome *c* in a 20 mM sodium hyposulfite buffer (pH 7.2). The decrease in optical density at 550 nm was measured at regular intervals for 3-10 min ( $\epsilon = 18.5 \text{ mM}^{-1} \text{ cm}^{-1}$ ), using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan).

### NADH-dependent cytochrome *c* reductase assay

NADH-dependent cytochrome *c* reductase, a marker of ER enzyme activity, was assayed as previously described<sup>30)</sup>. A suitable dilution of the enzyme was first prepared in a 20 mM potassium phosphate buffer (pH 7.2) containing 10 mM potassium cyanide, 1  $\mu\text{M}$  antimycin A, and 0.02 mM cytochrome *c*. The reaction was initiated by the addition of 0.1 mL of 2 mM NADH. The decrease in optical density at 550 nm was measured at regular intervals for 3-10 min, using a Shimadzu UV-1800 spectrophotometer.

### GDPase assay

GDPase, a marker for Golgi enzyme activity, was assayed in a volume of 0.1 mL containing 10 mM calcium chloride, 1 mg/mL Triton X-100, 2 mM GDP, and a 20 mM imidazole buffer (pH 7.6)<sup>31)</sup>. After incubating for 5 min at 30°C, the reaction was terminated by the addition of 1% SDS. To determine the amount of phosphate released, 0.2

mL of water and 0.7 mL of AMES reagent (1:6 mixture of 10% ascorbic acid and 0.42% ammonium molybdate in 1 M sulfuric acid) were added to the reaction mixture. After incubating at 45°C for 20 min, absorbance was measured at 660 nm.

### Protein determination

Protein was determined using a 2D Quant kit. For the vesicle fraction, lipids were first removed by mixing 30  $\mu\text{L}$  of the vesicle fraction with 100  $\mu\text{L}$  of ethanol and 1 mL of diethyl ether. Following centrifugation for 10 min at 10,000 rpm at 4°C, the pellet was suspended in 30  $\mu\text{L}$  of SDS sample buffer for protein determination.

### SDS-PAGE analysis

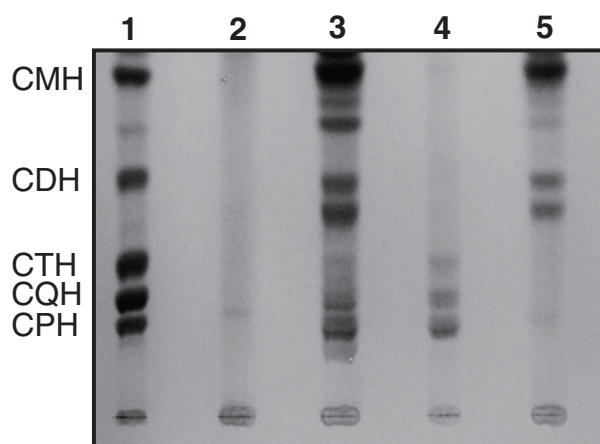
SDS-PAGE analysis was performed using a 12% polyacrylamide gel. Proteins were visualized by silver staining<sup>32)</sup>.

## Results and Discussion

### GSLs from *M. elegans*

In a previous study, we identified the neutral GSLs Gal  $\beta$ 1-6Gal  $\beta$ 1-1Cer and Glc1-6Gal  $\beta$ 1-1Cer in *M. elegans*; however, acidic GSLs were not clearly observed due to their low abundance<sup>19)</sup>. To examine the presence of acidic GSLs in the mycelia of *M. elegans*, the fungus was cultured in potato medium with 2% glucose at 30°C for 2 d. Total GSLs extracted from the mycelia were separated based on polarity into neutral and acidic fractions by ion-exchange column chromatography. Each fraction was analyzed by TLC by using a chloroform-methanol-water system. Consistent with the findings of the previous study<sup>19)</sup>, the TLC chromatogram of the neutral GSL fraction of *M. elegans* showed a major band corresponding to the monosaccharide-containing GSLs and two large bands corresponding to the disaccharide-containing GSLs (Fig. 1). The acidic GSL fraction resulted in three clearly visible bands, indicating the presence of GIPCs with different sugar modifications. AbA inhibits the growth of *M. elegans*; hence, GIPCs are likely to serve as an essential membrane component in the fungus.

Using the same TLC conditions, the neutral GSL fraction from *Rhizopus* sp. separated into five GSL bands having different numbers of sugar modifications (from mono- to pentasaccharides), and the acidic GSL fraction from *A. oryzae* showed a broad spot of GIPCs with a mobility similar to those of GIPCs from *M. elegans* (Fig. 1). Man-IPC, which is the core structure of fungal GIPCs, is generally modified with the addition of four or more sac-



**Fig. 1** TLC analysis of GSLs extracted from *M. elegans*. Lane 1, neutral GSLs from *Rhizopus* sp.; lane 2, acidic GIPCs from *A. oryzae*; lane 3, total GSLs from *M. elegans*; lane 4, acidic GSLs from *M. elegans*; lane 5, neutral GSLs from *M. elegans*. Orcinol-H<sub>2</sub>SO<sub>4</sub> reagent was used for detection. CMH, CDH, CTH, CQH, and CPH indicate the positions of GSLs with mono-, di-, tri-, tetra-, and pentasaccharides, respectively.

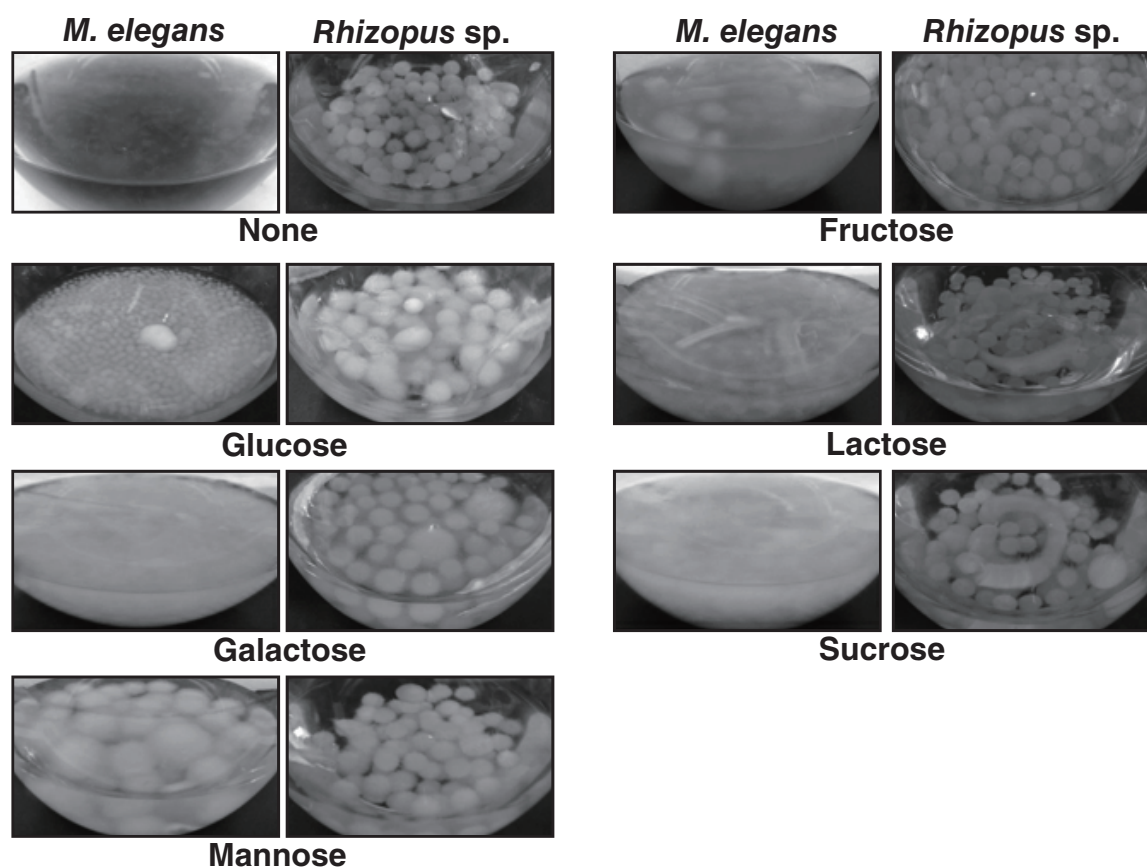
charides such as mannose, galactose, xylose, fucose, GlcN, and GlcNAc<sup>33)</sup>. The elongation of only three saccharides has been reported for GIPCs from *Histoplasma capsula-*

*tum*<sup>34)</sup> and *Paracoccidioides brasiliensis*<sup>35)</sup>. Further study is needed to clarify the structures of the GIPCs of *M. elegans*.

### Effect of various sugars on growth and synthesis of fungal GSL

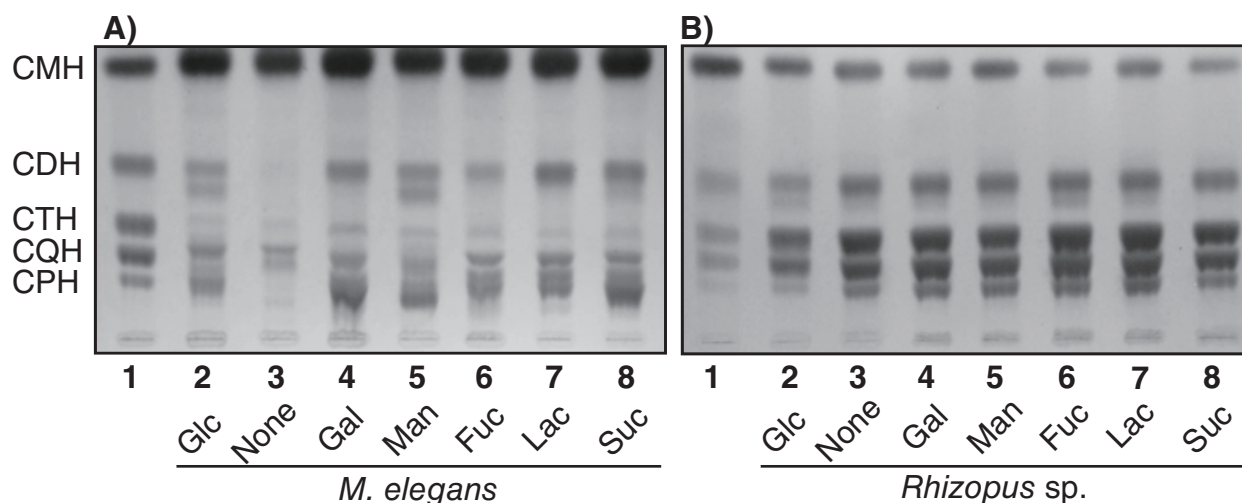
We investigated whether fungal GSL synthesis and growth were affected by the presence of different sugars in culture medium. *M. elegans* and *Rhizopus* sp. were cultured in the presence of glucose, galactose, mannose, fructose, lactose, or sucrose in the medium. Total GSLs were extracted from the mycelia and analyzed by TLC. The phenotypes of *Rhizopus* sp. were almost identical among the cultures, irrespective of the presence or absence of the different sugars (Fig. 2). The presence or absence of sugars also had little effect on the band patterns of GSLs from *Rhizopus* sp. (Fig. 3).

In contrast, *M. elegans* grown in the presence of different sugars, exhibited distinct cell morphologies (Fig. 2). A large number of small fungal cell granules were formed in the culture of *M. elegans* in the presence of glucose, galactose, and sucrose, whereas a small number of relatively large granules were seen in the presence of mannose,



**Fig. 2** Morphology of cell granules of *M. elegans* and *Rhizopus* sp. cultured in the presence or absence of various sugars. *M. elegans* (left panels) and *Rhizopus* sp. (right panels) were grown for 2 d at 30°C in a potato medium in the presence or absence of 2% of the indicated sugars.



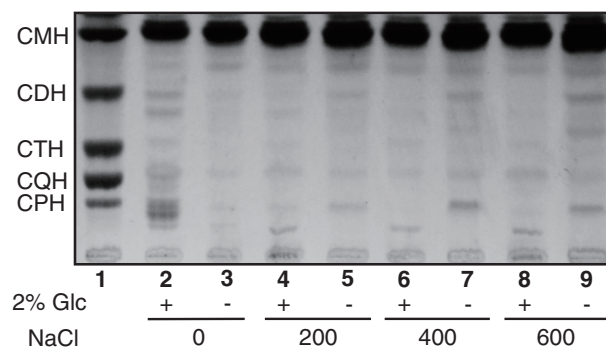


**Fig. 3** TLC analysis of total GSLs from *M. elegans* (A) and *Rhizopus* sp. (B) grown in the presence or absence of various sugars. GSLs from *Rhizopus* sp. were loaded as a control (lane 1). Cells were cultured with glucose (Glc), lane 2; no sugar, lane 3; galactose (Gal), lane 4; mannose (Man), lane 5; fructose (Fuc), lane 6; lactose (Lac), lane 7; and sucrose (Suc), lane 8. Orcinol- $\text{H}_2\text{SO}_4$  reagent was used for detection. CMH, CDH, CTH, CQH, and CPH indicate the positions of GSLs with mono-, di-, tri-, tetra-, and pentasaccharides, respectively.

fructose, and lactose. The growth of *M. elegans* was significantly retarded in the absence of sugar. TLC analysis revealed that the composition of total GSLs in *M. elegans* was significantly affected by certain sugars (Fig. 3). In the presence of glucose, mannose, and sucrose, both disaccharide-containing FNG-GSL bands were observed. However, the FNG-GSL band having the lower mobility on the TLC chromatogram was not seen in the presence of galactose, fructose, and lactose, and the two FNG-GSL bands were not observed in the absence of sugar. On the other hand, the data show that the different sugars also affected the composition of acidic GIPCs in *M. elegans* (Fig. 3). While there appeared to be no correlation between the morphological characteristics of the cell granules and the composition of GSLs, the results clearly demonstrate that both cell growth and GSL composition of *M. elegans* were affected by the presence or absence of different sugars.

#### Effect of salt concentration on fungal GSLs synthesis

To examine the effect of salt concentration on the synthesis of GSLs by *M. elegans*, the fungus was cultured in growth medium containing 2% glucose in the presence of different concentrations (0–600 mM) of NaCl. Total GSLs were extracted from the mycelia and analyzed by TLC. This analysis showed that production of FNG-GSLs was markedly repressed by increasing concentrations of NaCl in medium containing glucose (Fig. 4). In contrast, addition of NaCl induced dose-dependent synthesis of FNG-GSLs in medium without glucose. The NaCl concentration had no significant effect on the phenotype of *M. elegans*



**Fig. 4** TLC analysis of GSLs from *M. elegans* grown in the medium with different concentrations (0–600 mM) of NaCl. Neutral GSLs from *Rhizopus* sp. were loaded as a control (lane 1). Total GSLs were prepared from *M. elegans* grown in a potato medium with 2% glucose (lanes 2, 4, 6, and 8) and without glucose (lanes 3, 5, 7, and 9) in the presence of 0 mM (lanes 2 and 3), 200 mM (lanes 4 and 5), 400 mM (lanes 6 and 7), and 600 mM (lanes 8 and 9) NaCl. Orcinol- $\text{H}_2\text{SO}_4$  reagent was used for detection. CMH, CDH, CTH, CQH, and CPH indicate the positions of GSLs with mono-, di-, tri-, tetra-, and pentasaccharides, respectively.

(data not shown). These results suggest that the biosynthetic pathway of FNG-GSLs can be modulated by the addition of up to 600 mM NaCl to glucose-containing medium. Based on this assumption, we investigated the presence of proteins that are potentially involved in the biosynthesis of FNG-GSLs in *M. elegans*.

#### Comparison of Golgi proteins isolated from *M. elegans* with and without production of FNG-GSLs

In mammals, GSL synthetases are localized to the ER and Golgi apparatus<sup>4)</sup>, and most enzymes involved in saccharide chain elongation are present in the Golgi appara-

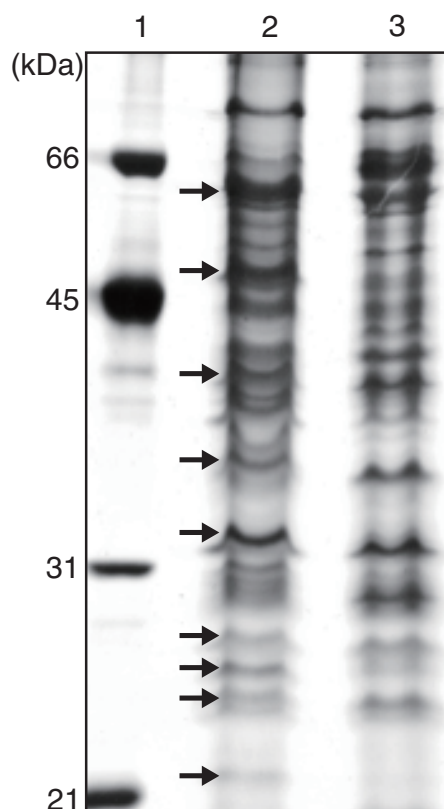
tus<sup>4)</sup>. Therefore, we focused on Golgi proteins for the investigation of proteins potentially involved in the biosynthesis of FNG-GSLs in *M. elegans*. Subcellular fractions were prepared from fungal cells cultured in glucose-containing medium in the absence or presence of 600 mM NaCl. P100 fractions enriched with Golgi apparatus were confirmed using assays for mitochondrial, ER, and Golgi enzyme activity (data not shown). The Golgi proteins in the P100 fractions were analyzed by SDS-PAGE with silver staining. At least nine additional protein bands were observed in P100 fractions from fungal cells cultured in the absence of NaCl, as compared with those from the cells cultured in the presence of 600 mM NaCl. Since production of FNG-GSLs is inhibited in the presence of 600 mM NaCl in glucose-containing medium, those distinct proteins are potential candidates in FNG-GSL synthesis.

Fungal Glc-Cer synthase has been identified in *Magnaporthe grisea*, *Candida albicans*, and *Pichia pastoris*, because of its high sequence homology to the mammalian enzyme<sup>36)</sup>. However, fungal Gal-Cer synthase remains to be identified, and there is no fungal genome-encoded protein with significant homology to its mammalian counter-

part<sup>37-39)</sup>. In addition, since FNG-GSLs specifically occur in certain fungal species, a bioinformatics approach using homologous enzyme sequences would be ineffective. In this study, we demonstrated for the first time that FNG-GSL production in *M. elegans* can be regulated by sugars and NaCl concentration, which would provide clues for studying the biosynthesis of FNG-GSLs.

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**Fig. 5** SDS-PAGE analysis of Golgi proteins in P100 fractions from *M. elegans* grown in the absence (lane 2) or presence (lane 3) of 600 mM NaCl in a glucose-containing medium. Proteins were visualized by silver staining. Marker proteins were loaded in lane 1. The arrows indicate protein bands that are present only in the P100 fractions from the fungus grown in the absence of NaCl.

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