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Involvement of lipid-translocating exporter family proteins in determination of myriocin sensitivity in budding yeast

Takahiro Kawaguchi [b], Yohei Ishibashi [c], Momoko Matsuzaki [b], Satomi Yamagata [b], Motohiro Tani [a,b,*]

[a] Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu, 501-1193, Japan
[b] Department of Chemistry, Faculty of Sciences, Kyushu University, 744, Motooka, Nishi-ku, Fukuoka, 819-0395, Japan
[c] Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 744, Motooka, Nishi-ku, Fukuoka, 819-0395, Japan

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ABSTRACT

Myriocin is an inhibitor of serine palmitoyltransferase involved in the initial biosynthetic step for sphingolipids, and causes potent growth inhibition in eukaryotic cells. In budding yeast, Rsb1, Rta1, Pug1, and Ylr046c are known as the Lipid-Translocating Exporter (LTE) family and believed to contribute to export of various cytotoxic lipophilic compounds. It was reported that Rsb1 is a transporter responsible for export of intracellularly accumulated long-chain bases, which alleviate the cytotoxicity. In this study, it was found that LTE family genes are involved in determination of myriocin sensitivity in yeast. Analyses of effects of deletion and overexpression of LTE family genes suggested that all LTEs contribute to suppression of cytotoxicity of myriocin. It was confirmed that *RSB1* overexpression suppressed reduction in complex sphingolipid levels caused by myriocin treatment, possibly exporting myriocin to outside of the cell. These results suggested that LTE family genes function as a defense mechanism against myriocin.

1. Introduction

Sphingolipids, one of the classes of membrane lipids, play pivotal roles in maintaining the function of eukaryotic biological membranes, and their depletion causes various cellular dysfunctions and ultimately cell death. Myriocin is an antibiotic derived from the entomopathogenic fungus *Isaria sinclairii* [1]. Myriocin inhibits serine palmitoyltransferase, which catalyzes the first biosynthetic step for sphingolipids [2]. In the budding yeast *Saccharomyces cerevisiae*, myriocin causes strong growth inhibition [3,4]. Through screening for multicopy suppressors that reduce myriocin-induced cell death, the kinase gene Ypk1, which regulates sphingolipid biosynthesis, and Sli1, which suppresses inhibitory activity of myriocin by acetylating it, have been identified [3,5].

Rsb1 is a seven-transmembrane protein localized in plasma membranes and the endoplasmic reticulum in budding yeast, and has been reported to be involved in the export of long-chain bases (LCBs), the backbone of all sphingolipids [6]. When phytosphingosine (PHS), the main LCB of yeast, is added to cells, it is incorporated into the cells, which is partly mediated by acyl-CoA synthases Faa1 and Faa4 [7], and then is converted to PHS 1-phosphate [6]. Abnormal accumulation of PHS 1-phosphate within cells causes strong cytotoxicity [8]; however, overexpression of *RSB1* reduces the cytotoxicity of exogenously-added PHS by excreting it from the cells [6]. Reduced cytotoxicity due to the overexpression of *RSB1* is also observed in mitochondria-mediated cell death caused by exogenously added dihydrosphingosine [9].

In budding yeast *S. cerevisiae*, proteins that exhibit sequence homology to Rsb1 are called the Lipid-translocating exporter (LTE) family or the Rta1-like family, which included Rsb1, Rta1, Pug1, Ylr046c, and Rtm1 [10] (In this study, this family will be referred to as the LTE family for convenience). Rtm1 is present in multiple copies and associated with SUC telomeric loci [11] (in several laboratory yeast strains used in this study, so it is excluded from the analysis). Overexpression of LTE family genes other than *RSB1* does not confer the resistance to abnormal accumulation of PHS 1-phosphate due to exogenously added PHS [6]. On the other hand, PHS is known to have a growth inhibitory effect on tryptophan auxotrophic cells [12], and it has also been reported that overexpression of *RTA1* confers weak resistance to PHS-induced the growth inhibition in tryptophan auxotrophic cells [13].

Since myriocin is structurally similar to LCBs [2], it is possible that LTE family proteins are related to myriocin. In this study, we found that

* Corresponding author. Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu, 501-1193, Japan.
E-mail address: tani.motohiro.i3@f.gifu-u.ac.jp (M. Tani).

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multiple deletion of LTE family genes causes hypersensitivity to myriocin whereas overexpression confers myriocin resistance. Unlike LCBs, it was found that all LTE family genes examined in this study (*RSB1*, *RTA1*, *PUG1*, and *YLR046C*) are involved in determination of myriocin sensitivity. In this study, it was shown that LTE family genes function as a defense mechanism against myriocin.

2. Materials and Methods

Yeast strains, media, and plasmids - The mutant strains of *S. cerevisiae* used are listed in Table S1. In the supplementary material, the methods for genetic manipulation of budding yeast and plasmid construction are described. The following medium were used for cell

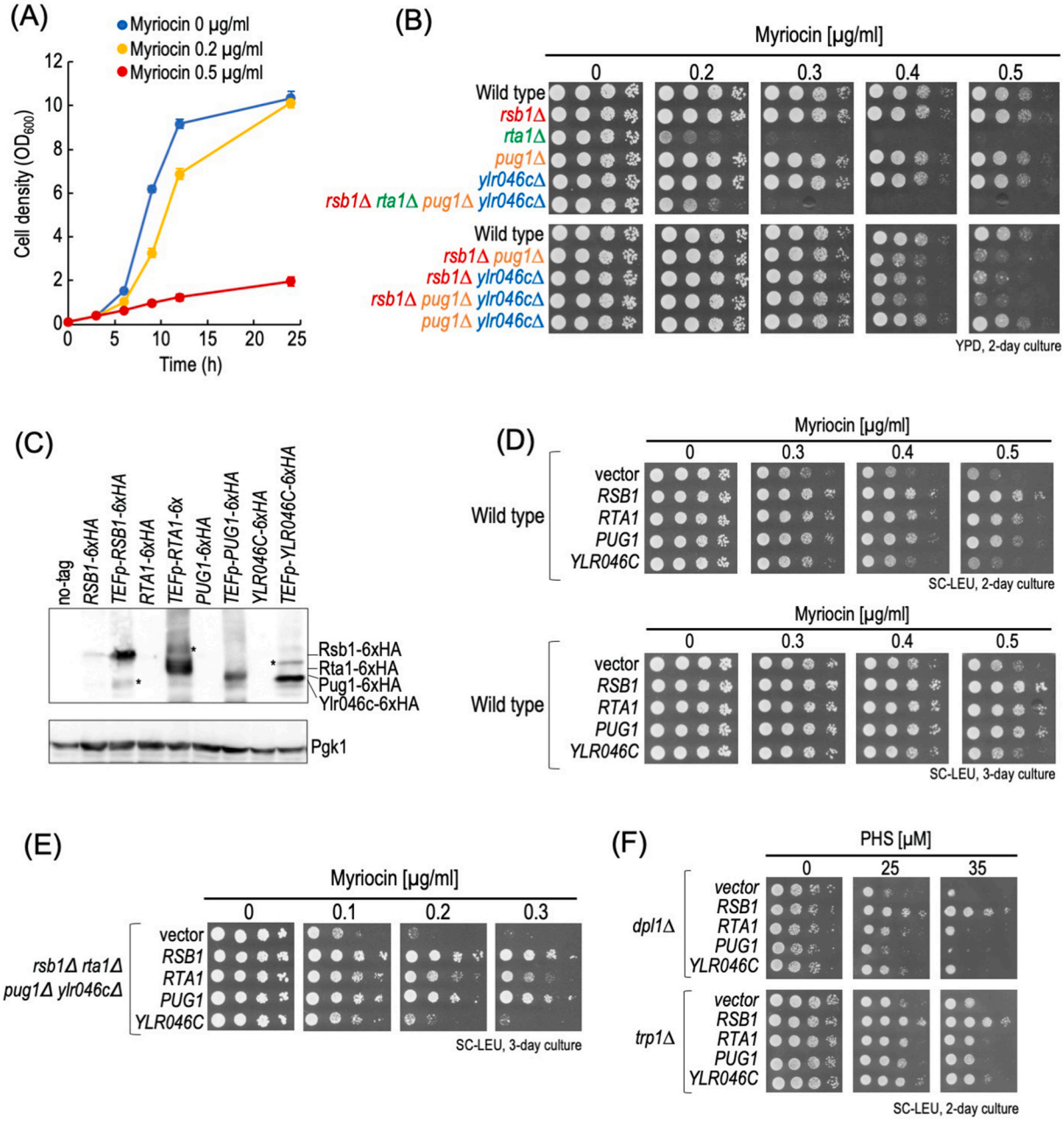

Fig. 1. Effects of deletion or overexpression of LTE family genes on myriocin sensitivity.
(A) Time course of cell growth of myriocin-treated cells. Cells were cultured overnight in YPD medium, diluted (0.1 OD_{600} units/ml) in fresh YPD medium containing 0, 0.2, or 0.5 μg/ml of myriocin, and then cultured at 30 °C. Aliquots of cell suspensions were subjected to cell density measurements (OD_{600}) at the indicated times. Data represent means ± SD from one experiment (triplicate) representative of three independent experiments. (B) Cells lacking LTE genes cultured overnight were spotted onto YPD plates with or without the indicated concentration of myriocin and then incubated at 30 °C for 2 days. (C) Western blotting analysis of overexpression of LTE proteins with the TEF promoter. Cells expressing Rsb1-6HA, Rta1-6HA, Pug1-6HA, or Ylr046c-6HA with their native promoter or TEF promoter were cultured overnight in YPD medium at 30 °C, diluted (0.3 OD_{600} units/ml) in fresh YPD medium, and then incubated for 5 h at 30 °C. Yeast cell extracts were subjected to immunoblotting using anti-HA antibody or anti-Pgk1 antibody. The asterisks indicate unidentified bands. (D and E) Wild-type (D) or *rsb1Δ rta1Δ pug1Δ ylr046cΔ* (E) cells harboring pRS415TEFp-*RSB1*, pRS415TEFp-*RTA1*, pRS415TEFp-*PUG1*, pRS415TEFp-*YLR046C*, or the empty vector were cultured overnight in SC-Leu medium, spotted onto SC-Leu plate with or without the indicated concentrations of myriocin, and then incubated at 30 °C for 2 or 3 days. (F) Effect of overexpression of LTE proteins on sensitivity to phytosphingosine (PHS). *dpl1Δ* or *trp1Δ* cells harboring the expression plasmids of LTE proteins were cultured overnight in SC-Leu medium, spotted onto SC-Leu plate with or without the indicated concentrations of PHS, and then incubated at 30 °C for 2 days.

culture; YPD (1 % yeast extract, 2 % peptone, and 2 % glucose), and SC (synthetic complete) medium (0.67 % yeast nitrogen base without amino acids (BD Difco, Heidelberg, Germany), 2 % glucose, and nutritional supplements). When adding myriocin (Cayman Chemicals, Ann Arbor, MI, USA) to the medium, ethanol (0.5 %) was included as a vehicle. When adding phytosphingosine (PHS; FOCUS Biomolecules, Plymouth Meeting, PA, USA) to the medium, ethanol (0.5 %) as a vehicle and Nonidet P-40 (0.0015 %) as a dispersant were included. The vehicle and/or the dispersant were added to the medium in control experiments.

Spot assay - Cells cultured overnight in YPD or SC lacking leucine medium at 30 °C were spotted onto YPD or SC lacking leucine plates in 10-fold serial dilutions starting with a density of 0.7 OD_{600} units/ml. After 2- or 3-day incubation at 30 °C, plates were photographed.

Lipid extraction and TLC analysis - Lipids extraction and TLC analysis were performed as described previously [14].

LC-ESI MS/MS analysis of myriocin - The culture supernatant containing myriocin (40 μl) was mixed with 160 μl of 2-propanol. Then, the mixture was centrifuged at 17,800×*g* for 5 min for removal of insoluble compounds. Myriocin in the supernatant was measured using an LC-ESI MS/MS (3200 QTRAP, SCIEX, USA) equipped with an Inert-Sustain C18 reverse-phase column (2.1 × 150 mm, 5 μm, GL Sciences, Japan) using acetonitrile/methanol/distilled water, 19:19:2 v/v/v, containing 0.1 % formic acid and 0.028 % ammonium as an isocratic mobile phase with a 200 μl/min flow rate for 6 min. Multiple reaction monitoring (MRM) analysis in the positive ion mode was performed to detect myriocin with the following combinations, 402.3(Q1) and 104.1 (Q3). MultiQuant 3.0.1 software (SCIEX) was used for analysis of the peak intensity of myriocin.

Western blotting – Extraction of protein from yeast cells, SDS–PAGE, and Western blotting were performed as described previously [15].

Flow cytometry – Propidium iodide (PI) fluorescence intensity was measured on 9000∼10,000 cells per sample using a flow rate of 10 μl/min. To generate a fluorescence threshold to identify PI-positive cells, wild-type cells treated with 70 % ethanol for 5 min were used. The threshold was set to a range that included >99 % but <100 % of 70 % ethanol-treated cells.

Statistical analysis - Statistical analysis was performed using Student's *t*-test and the *P* values obtained are indicated. The Tukey–Kramer test were also used for multiple comparisons.

3. Results

3.1. Deletion and overexpression of LTE family genes affect myriocin sensitivity

As shown in Fig. 1A, myriocin caused inhibition of yeast cell growth in a concentration-dependent manner. In addition, to obtain information on morphology of myriocin-treated cells, forward scatter (FSC) and side scatter (SCC) profiles were obtained using flow cytometry. As shown in Fig. S1, changes in profile of FSC and SCC were observed due to myriocin treatment, suggesting the alteration of morphological characteristics. To investigate whether or not LTE family genes are involved in determination of the degree of sensitivity to myriocin, the effect of myriocin on growth of mutant strains lacking any one of *RSB1, RTA1, PUG1*, and *YLR046C* was examined (Fig. 1B). *rta1Δ* cells exhibited myriocin hypersensitivity; however, *rsb1Δ, pug1Δ*, or *ylr046cΔ* had no effect. The myriocin hypersensitivity of *rta1Δ* cells was consistent with previous results of high-throughput analysis [16,17]. A double deletion mutant lacking *RSB1* and *PUG1* or *YLR046C* exhibited stronger sensitivity to myriocin as compared with wild-type cells. In contrast, double deletion of *PUG1* and *YLR046C* did not alter the sensitivity. The effect of quadruple mutation of all LTE genes is indistinguishable from that of *rta1Δ*. Thus, it was indicated that *rta1Δ* has the strongest effect on the myriocin sensitivity, but that deletion of other LTEs also affect the sensitivity (Fig. 1B). We next examined the effect of overexpression of each LTE gene by a strong and constitutive TEF promoter. To examine whether or not the expression levels of LTE genes with the TEF promoter exceed that with their native promoter, the promoter region of each LTE gene on the chromosome was replaced with the TEF promoter and each gene was tagged with a sequence encoding 6xHA. As shown in Fig. 1C, protein expression of Rta1-6HA, Pug1-6HA, or Ylr046c-6HA with the native promoter was not detected in our experimental conditions probably because of the very low expression level; however, strong expression of all 6HA-tagged LTEs was clearly observed with the TEF promoter. When wild-type cells were transformed with a single-copy plasmid containing *RSB1*, *RTA1*, or *PUG1* with the TEF promoter, resistance to myriocin was observed as compared with the empty vector transformant (Fig. 1D). These effects were also observed when plasmids were transformed into *rsb1Δ rta1Δ pug1Δ ylr046cΔ* cells, suggesting that overexpression of one LTE does not confer myriocin resistance indirectly by affecting the expression level of another LTE (Fig. 1E). It should be noted that the plasmid containing *YLR046C* did not have a clear effect on the myriocin sensitivity of wild-type cells (Fig. 1D); however, weak resistance to myriocin was observed when the plasmid was transformed into *rsb1Δ rta1Δ pug1Δ ylr046cΔ* cells (Fig. 1E). Exogenously added phytosphingosine (PHS) exhibits cytotoxicity by being converted to PHS 1-phosphate [6]. Previously, it was reported that, in *DPL1* and/or *LCB3*-deleted cells, where degradation of PHS 1-phosphate derived from PHS is reduced, overexpression of *RSB1*, but not *RTA1, PUG1,* or *YLR046C*, confers resistance to PHS, suggesting that only Rsb1 has the ability to export PHS from inside of the cells [6,18]. In our experimental conditions, we also confirmed that the overexpression of *RSB1* has the strongest effect on PHS sensitivity in *dpl1Δ* cells (Fig. 1F). Similar results were obtained for the cytotoxicity of PHS in tryptophan auxotrophic strains (*trp1Δ* cells), in which PHS exhibits strong cytotoxicity because of inhibition of incorporation of extracellular tryptophan (Fig. 1F) [12]. These results suggest that LTE proteins have distinctly different specificities for myriocin and PHS.

3.2. Overexpression of RSB1 reduces the effect of myriocin

Next, we used *RSB1*-overexpressed cells as a typical strain to investigate the effect of the overexpression on myriocin-induced inhibition of sphingolipid biosynthesis *in vivo*. We used a strain in which the promoter region of chromosomal *RSB1* was replaced with the TEF promoter (*TEFp-RSB1*) [9]. As shown in Fig. 2A, *TEFp-RSB1* cells exhibited myriocin resistance as compared to wild-type cells. Complex sphingolipid levels were reduced by myriocin treatment; however, the reduction was attenuated in *TEFp-RSB1* cells as compared with wild-type cells (Fig. 2B). Fig. 2C shows LC-ESI MS/MS analysis of the level of myriocin remaining in the medium after treating wild-type and *TEFp-RSB1* cells with myriocin for 30 min. The level of myriocin remaining in the medium was higher in *TEFp-RSB1* cells than in wild-type cells. Collectively, these results suggested that Rsb1 suppresses intracellular accumulation of myriocin.

3.3. Myriocin hypersensitivity caused by deletion of RTA1 is dependent on SLI1

Among LTE genes, the deletion of *RTA1* causes the strongest myriocin hypersensitivity (Fig. 1B); however, the effect of overexpression of *RTA1* is not stronger than that of other LTE genes (Fig. 1D and E). We noticed that the terminator region of *SLI1*, which encodes an enzyme that *N*-acetylates myriocin and suppresses its SPT inhibitory activity [3], is located immediately downstream of *RTA1* on the chromosome (Fig. 3A). Therefore, we considered the possibility that the deletion of *RTA1* may have some effect on *SLI1* expression, causing hypersensitivity to myriocin. First, we investigated the effect of deletion of LTE genes on myriocin sensitivity in the absence of *SLI1*. As reported previously, in a spot assay, the deletion of *SLI1* caused hypersensitivity to myriocin (wild-type versus *sli1Δ* cells); however, the deletion of LTE genes did not affect myriocin sensitivity in the absence of *SLI1* (Fig. 3B). Alternatively,

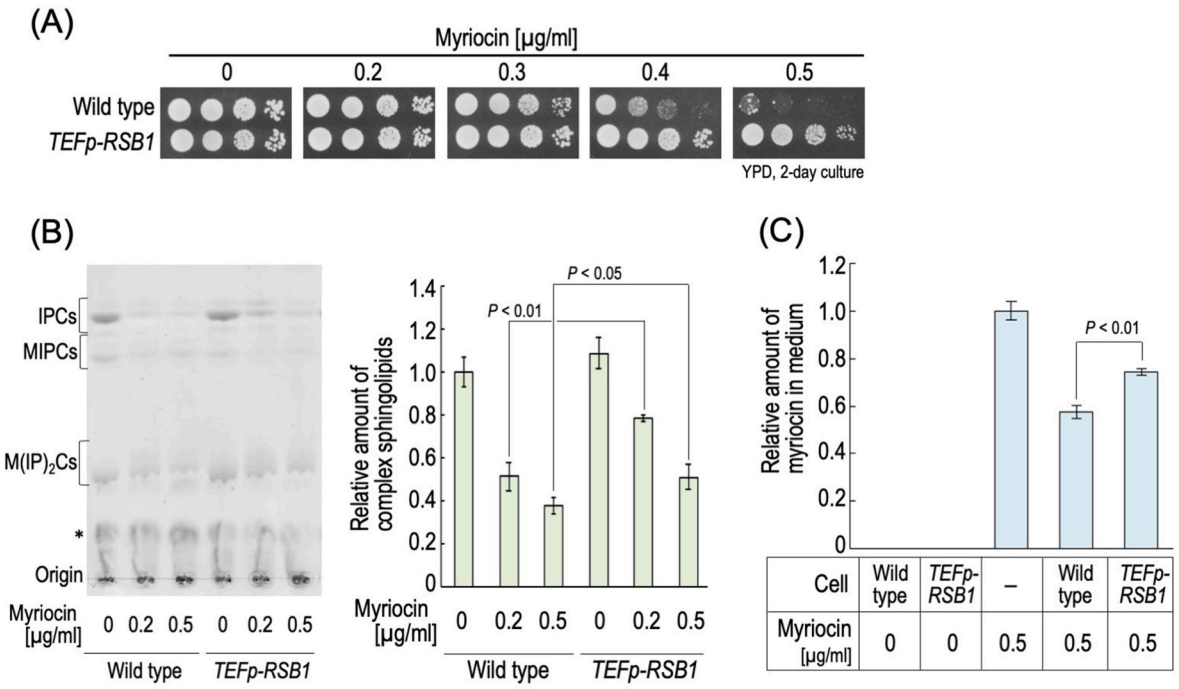

Fig. 2. Effects of overexpression of *RSB1* on exogenously added myriocin.
(A) Wild-type and *TEFp-RSB1* (TKY12) cells cultured overnight were spotted onto YPD plates with or without the indicated concentrations of myriocin and then incubated at 30 °C for 2 days. (B) Cells were cultured overnigh in YPD medium, diluted (0.5 OD_{600} units/ml) in fresh YPD medium with or without the indicated concentrations of myriocin, and then incubated for 5 h at 30°C. Lipids were extracted, and then analysed by TLC. Complex sphingolipids were quantified with IMAGEJ software (NIH). The amounts of complex sphingolipids (IPCs, MIPCs, and $M(IP)_2Cs$) in myriocin-untreated wild-type cells were taken as 1. (C) Cells were cultured overnight in YPD medium, diluted (0.3 OD_{600} units/ml) in fresh YPD medium, and then incubated for 5 h at 30°C. The cells were resuspended in fresh YPD medium containing 0 or 0.5 μg/ml of myriocin to 1.5 OD_{600} unit/ml, and then incubated for 30 min at 30°C. Myriocin in the culture supernatants was measured using LC-ESI MS/MS. The amount of myriocin in the myriocin-containing medium incubated for 30 min without cells was taken as 1. Data represent means ± SD from one experiment (triplicate) representative of three independent experiments. Statistical analysis was performed using Student's *t*-test. The details are given under Materials and Methods.

we also detected myriocin-induced cell death by propidium iodide (PI) staining (Fig. 3C). As shown in Fig. 3D, *rta1Δ*, *rsb1Δ pug1Δ ylr046cΔ*, or *rsb1Δ rta1Δ pug1Δ ylr046cΔ* cells exhibited increased myriocin-induced cell death compared to wild-type cells. When treated with myriocin, the rate of PI-positive cells was higher in *rta1Δ* cells than in *rsb1Δ pug1Δ ylr046cΔ* cells (Fig. 3D), which is consistent with the results of spot assays (Fig. 1B). In contrast, myriocin-induced cell death of *SLI1*-deleted cells was enhanced by the triple deletion of *RSB1*, *PUG1*, and *YLR046C* (*sli1Δ* versus *sli1Δ rsb1Δ pug1Δ ylr046cΔ* cells), but not by the single deletion of *RTA1* (*sli1Δ* versus *sli1Δrta1Δ* cells) (Fig. 3E). Thus, it was suggested that robust sensitivity to myriocin in *rta1Δ* cells is dependent on *SLI1*. However, it should be noted that myriocin-induced cell death in *sli1Δ rta1Δ pug1Δ ylr046cΔ* cells was higher than that in *sli1Δ* and *sli1Δ pug1Δ ylr046cΔ* cells (Fig. 3E), suggesting that endogenous Rta1 still contributes to the acquisition of resistance to myriocin even in the absence of *SLI1*. We also examined the effects of overexpression of LTE genes in the absence of *SLI1*. As shown in Fig. 3F, overexpression of any one of the LTE genes confers myriocin resistance to *sli1Δ* cells. Similar results were obtained when *sli1Δ rsb1Δ rta1Δ pug1Δ ylr046cΔ* cells were used (Fig. 3G). Next, we investigated whether or not the deletion of *RTA1* affects the expression level of Sli1. We attempted to detect Sli1 with a tag sequence expressed by the native promoter; however, it was undetectable under our experimental conditions, probably because of the very low expression level. Thus, a strain in which the ADH promoter and a sequence encoding 3xHA was inserted upstream of chromosomal *SLI1* was generated (*ADHp-3HA-SLI1*) (In this strain, the terminator region of *SLI1* in close proximity to *RTA1* is intact). *ADHp-3HA-SLI1 rta1Δ* cells exhibited myriocin hypersensitivity as compared with *ADHp-3HA-SLI1* cells (Fig. 3H), indicating that the *SLI1* dependence of myriocin hypersensitivity due to the deletion of *RTA1* is still maintained in this strain. Moreover, it was found that the deletion of *RTA1* reduces the expression level of 3HA-Sli1 (Fig. 3I). Thus, it was suggested that the deletion of *RTA1* causes myriocin hypersensitivity, possibly at least in part due to a reduced expression level of Sli1.

4. Discussion

In this study, it was indicated that all LTE proteins (Rsb1, Rta1, Pug1, and Ylr046c) contribute to suppress the effect of myriocin. Furthermore, this contribution can be observed even in the absence of *SLI1* (Fig. 3E–G), which attenuates of the cytotoxic activity of myriocin [3], indicating that acquisition of myriocin resistance by LTE proteins is a mechanism independent of Sli1; however, the strongest myriocin hypersensitivity caused by the deletion of *RTA1* is *SLI1* dependent (Fig. 1A and 3E). Myriocin-induced cell death in *sli1Δ rsb1Δ pug1Δ ylr046cΔ* cells was higher than that in *sli1Δ pug1Δ ylr046cΔ* and *sli1Δ rta1Δ pug1Δ ylr046cΔ* cells (Fig. 3E). Furthermore, deletion of *PUG1* and/or *YLR046C* causes hypersensitivity to myriocin only when combined with *RSB1* deletion (Fig. 1B). Thus, collectively, these results suggest that, among the endogenous LTE proteins, Rsb1 plays the most important role in acquiring myriocin resistance. When *RSB1*-overexpressing cells were treated with myriocin, reduction in cellular complex sphingolipid levels was suppressed compared to the wild-type cells (Fig. 2B), and the amount of myriocin remaining in the medium was increased (Fig. 2C). Thus, the most likely interpretation is that Rsb1 (and possibly other LTE proteins) releases intracellularly incorporated myriocin out of the cell.

It was reported that *RTA1* confers resistance to 7-aminocholesterol [19], and that *PUG1* contributes to heme efflux and protoporphyrin IX uptake [20]. In this study, it was found that the effect on myriocin sensitivity was observed for all LTE family proteins including Rsb1,

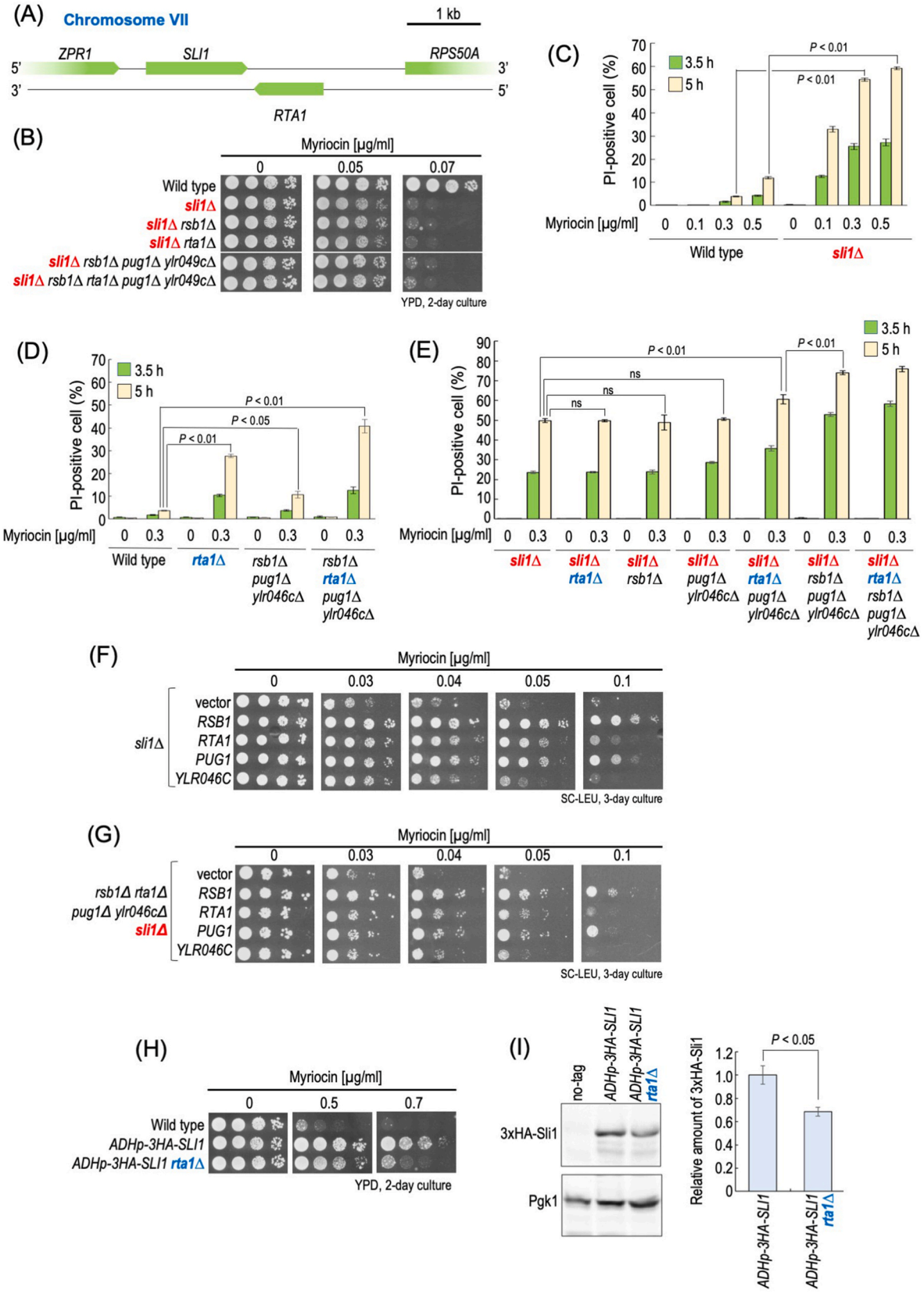

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Fig. 3. *SLI1* dependence of myriocin hypersensitivity due to the deletion of *RTA1*.
(A) Positions of *RTA1* and *SLI1* in the yeast chromosome. (B) Effect of myriocin on *SLI1*- and LTE gene-deleted cells in spot assays. (C–E) PI staining of myriocin-treated cells. Cells were cultured overnight in YPD medium, diluted (0.3 OD_{600} unit/ml) in fresh YPD medium, and then incubated for 5 h at 30 °C. The cells were resuspended in fresh YPD medium with or without the indicated concentrations of myriocin to 0.1 OD_{600} unit/ml, and then incubated for 3.5 or 5 h at 30 °C. Cells were stained with 2.5 μg/ml of PI for 20 min before flow cytometric analysis. Data represent means ± SD for one experiment (triplicate) representative of three independent experiments. Statistical analysis was performed using the Tukey–Kramer test. ns: no significant difference. (F and G) Effects of overexpression of LTE genes on sensitivity to myriocin in *sli1Δ* (F) or *rsb1Δ rta1Δ pug1Δ ylr046cΔ sli1Δ* (G) cells were examined as described in Fig. 1D and E. (H) Effect of the deletion of *RTA1* on myriocin sensitivity in cells expressing *3HA-SLI1* with the AHD promoter. (I) Protein expression level of 3HA-Sli1. *ADHp-3HA-SLI1* or *ADHp-3HA-SLI1 rta1Δ* cells cultured overnight were diluted (0.3 OD_{600} units/ml) in fresh YPD medium, and then incubated for 5 h at 30 °C. Yeast cell extracts were subjected to immunoblotiing using anti-HA antibody or anti-Pgk1 antibody. The relative amount of 3HA-Sli1/Pgk1 in *ADHp-3HA-SLI1* cells was taken as 1. Data represent means ± SD for one experiment (triplicate) representative of three independent experiments. Statistical analysis was performed using Student's *t*-test. The details are given under Materials and Methods.

Rta1, Pug1, and Ylr046c. Rsb1 has the Walker A motif involved in ATP binding and hydrolysis and the Loop 5 region that are not present in other LTEs, and these regions are important for the LCB-releasing activity of Rsb1 [6,18]. Furthermore, among LTE proteins, only Rsb1 conferred PHS resistance to *DPL1*-and *LCB3*-deleted cells [18]. From these facts, it is assumed that there is a difference in the mechanism of release between LCB and myriocin, and more detailed analysis of the molecular mechanism of release of myriocin via LTE is required.

The expression level of *RSB1* is dramatically increased through the rim101 pathway under abnormal phospholipid asymmetry at plasma membranes [21], and it has also been suggested that expression of *RTA1* is regulated by Upc2, a transcription factor involved in the regulation of sterol biosynthesis [13]. Thus, LTE expression may be affected by cell surface and intracellular lipid environment, and how LTE expression changes in the presence of sphingolipid biosynthesis inhibitor myriocin remains a future issue.

CRediT authorship contribution statement

Takahiro Kawaguchi: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Yohei Ishibashi:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Momoko Matsuzaki:** Writing – review & editing, Investigation. **Satomi Yamagata:** Writing – review & editing, Investigation. **Motohiro Tani:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101785.

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