Noda Institute for Scientific Research GRANT
2013 Research Grant

Development of efficient secretory production of ricinoleic acid, a petrochemical replacement of bioplastics, in fission yeast *Schizosaccharomyces pombe*

Hiroshi UEMURA

National Institute of Advanced Industrial Science and Technology

Research aims

Ricinoleic acid (RA, 12-hydroxy-octadeca-*cis*-9-enoic acid: C18:1-OH), an important natural raw material, is valuable as a petrochemical replacement in a variety of industrial processes. RA biosynthesis is catalyzed by the oleate Δ12-hydroxylase (*FAH12*), which adds a hydroxyl group (-OH) to the 12th carbon of oleic acid. Seeds of the castor bean plant (*Ricinus communis*) are a major source of RA, and RA constitutes approximately 90% of the total fatty acid (FA) content of the seed oil. However, castor beans are not an ideal source crop, because the production of RA from *R. communis* involves technical problems, such as the presence of toxic and allergic substances that cause health complications in workers involved in planting, harvesting, and processing; and that its cultivation is limited to tropical and sub-tropical regions. Although extensive efforts were undertaken for the heterologous production of RA in other plants or microorganisms, including yeast, a high level of RA production has never been achieved because RA is an unusual fatty acid containing a hydroxyl group (-OH) and causes toxicity in the host cells. Recently, we successfully produced RA (more than 50% of the total FA) by introducing the *Claviceps purpurea* D12-hydroxylase gene (*CpFAH12*) into *Schizosaccharomyces pombe*, despite its toxicity to *S. pombe* cells.[1, 2)] Subsequent studies have demonstrated that overexpression of *plg7*, encoding phospholipase A2, suppresses the toxic effects of RA[3)], and suggested the possibility of RA secretion into the culture media. The main goal of this project is to study the mechanisms that allow RA tolerance and its secretion in *S. pombe.*

Methods

The *S. pombe* strain ARC010-1 (*h*$^{-}$ *leu1*-32 *ura4*-D18) was used in this study. An integration plasmid containing *CpFAH12* under the *nmt1* promoter of *S. pombe*, which is repressed by thiamine, was integrated at the *ura4* locus on chromosome III, as previously described.[3)] The *S. pombe* strains were grown in EMMS or EMMS dropout media, depending on the selection pressure required to maintain the plasmids. EMMS was used for normal yeast growth, but a nitrogen-limited minimal medium (EMM-C/N3) was used for the production of RA. In EMM-C/N3, the concentration of ammonium chloride and glucose was reduced and increased to 0.1% and 10%, respectively. For the repression of gene expression under the *nmt1* promoter, 15 μM thiamine (5 μg/mL) was used. Total FA content was determined by gas chromatographic (GC) analysis.[1)] Lipid composition was analyzed by thin layer chromatography (TLC: silica gel 60 plate, Merck).[3)]

Results

Secretory production of RA by *plg7* overexpression in *S. pombe*[4)]

To produce RA in *S. pombe*, we introduced *CpFAH12* into *S. pombe*. However, RA proved toxic to *S. pombe* and the cells expressing *CpFAH12* showed poor growth at the normal growth temperature of 30°C.[1)] To explore this mechanism of toxicity in *S. pombe*, we screened an *S. pombe* cDNA library and identified *plg7*, which encodes phospholipase A2, as a suppressor that restored the growth defect without affecting the RA production. TLC analysis confirmed the reduction in RA moiety in phospholipids and a concomitant increase in free RA in the *plg7*-overexpressed strain. Since RA is synthesized at the sn-2 position of phosphatidylcholine by Fah12p, and phospholipase A2 hydrolyzes the sn-2 acyl bond of phospholipids, we speculate that *plg7* is a stress-responsive gene, and that Plg7p acts as a suppressor by removing the RA moieties from phospholipids, the major components of lipid bilayer membranes.[3)]

During the course of additional studies on *plg7*, we noticed that a large amount of free RA was secreted in the culture media when the *FAH12* integrant harboring the *plg7* multicopy plasmid was grown at 20°C, a temperature that is more favorable for RA production. We speculated that the turbidity of the culture was caused by cell lysis. However, the constant increase in turbidity of the supernatants during cultivation in 10% glucose and the highly selective composition of RA in the media indicated that the turbidity of the supernatant is due to the secretion of RA and not due to cell lysis. Besides, viability tests clearly demonstrated that there is no correlation between the viability and turbidity. In addition, TLC analysis confirmed that the major component of the turbid supernatant is free RA. Taken together, our results show that the *FAH12* integrant harboring

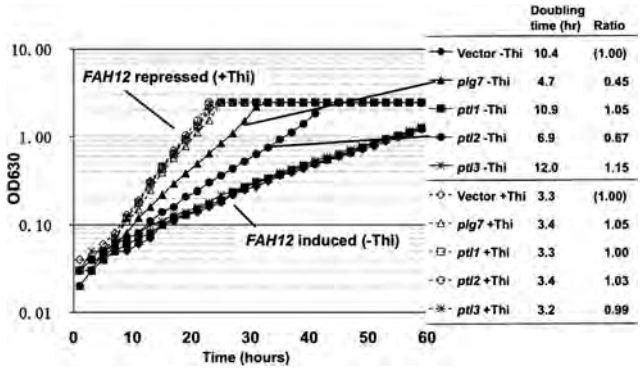

Fig. 1. Growth recovery of *CpFAH12* integrant by the overexpression of *plg7* and *ptl2* genes.
The P*nmt1-CpFAH12* integrants harboring various genomic clone plasmids were grown in EMM-C/N3 in the presence (dotted line) or absence (solid line) of thiamine at 30°C. Vector (diamonds): empty vector pAL-KS. *plg7* (triangles): genomic *plg7* plasmid. *ptl1* (squares): genomic *ptl1* plasmid. *ptl1*2 (circles): genomic *ptl2* plasmid. *ptl3* (asterisks): genomic *ptl3* plasmid. Ratio indicates doubling time ratio to that of the control strain under the same growth condition.

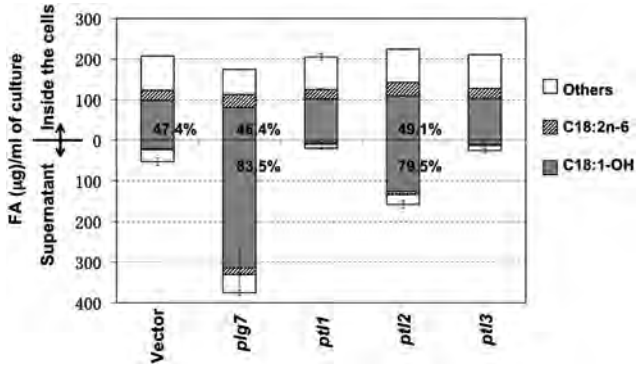

Fig. 2. Overexpression of *plg7* and *ptl2* in the *CpFAH12* integrant facilitates the secretion of RA.
The P*nmt1-CpFAH12* integrants with various plasmids were grown in EMM-C/N3 in the absence of thiamine at 20°C for 5 d starting with OD_{600}=2. Vector: empty vector pREP1. *plg7*: *plg7* expressed in pREP41. *ptl1*: *ptl1* expressed in pREP41. *ptl2*: *ptl2* expressed in pREP41. *ptl3*: *ptl3* expressed in pREP3. The upper and lower parts of the graph represent intracellular FA composition and culture supernatant, respectively. Fatty acids are categorized into three groups: (1) C18:1-OH (RA), (2) C18-2n-6 (linoleic acid, by-product of *FAH12*), and (3) others (all other original fatty acids). Values in the graph indicate the percentage of RA (out of total FA) inside the cells and culture supernatants.

the *plg7* multicopy plasmid secretes free RA into the media. The strain secretes more than 50% of the produced RA into the culture media.

Suppression of RA toxicity by *ptl2* overexpression in *S. pombe*[5)]

To further increase RA tolerance and enhance RA secretion, we extended our analysis and examined the effect of triglyceride (TG) lipase overexpression as TG metabolism is closely related to phospholipid metabolism and fatty acid remodeling. *S. pombe* has three TG lipase genes, *ptl1*, *ptl2*, and *ptl3*, which have high protein sequence similarity to each other and to the *S. cerevisiae* counterparts *TGL3*, *TGL4*, and *TGL5*, respectively.[2)] However, only *ptl2* overexpression suppressed the growth defect of RA production, and the culture grown at 20°C secreted free RA into the media. The suppression by *ptl2* was independent of *plg7* and a large amount of free RA was accumulated in the cells, along with a concomitant decrease in the RA moieties in phospholipids. Furthermore, the suppression by *ptl2* was attenuated by bromoenol lactone (BEL), a phospholipase A2-specific inhibitor, suggesting that Ptl2p has phospholipase activity. These results suggest that *ptl2* suppresses the toxicity of RA with its phospholipase activity.

Conclusions

The lipid RA has a wide range of industrial applications. Since RA is an unusual fatty acid containing a hydroxyl group (-OH), it is toxic to the host cells, and only a few plants (*R. communis* and *Lesquerella fendleri*) and fungi (*C. purpurea*) are natural producers of RA. Although RA production in *S. pombe* also shows toxicity, we circumvented this problem by isolating *plg7* as a multicopy suppressor. Our research suggests that *plg7* functions as a suppressor by removing RA moieties from phospholipids. We also discovered the secretion of RA into the growth medium when a large amount of RA was produced at low temperature. Furthermore, we found that one of the TG lipase genes, *ptl2*, is also involved in the suppression of RA toxicity. Our identification of *plg7* and *ptl2* as suppressors of RA toxicity and their analyses have opened up new avenues for the efficient production of RA without disturbing the cell growth. Since the long-term goal of our study is to produce RA in sustainable quantities using transgenic *S. pombe*, the discovery of the secretion of free RA into the media may be advantageous for the industrial application of this system.

References

1) Holic R., Yazawa H., Kumagai H., and Uemura H. (2012) Engineered high content of ricinoleic acid in fission yeast *Schizosaccharomyces pombe*. *Appl. Microbiol. Biotechnol*. **95**: 179–187.
2) Yazawa H., Kumagai H., and Uemura H. (2012) Characterization of triglyceride lipase genes of fission yeast *Schizosaccharomyces pombe*. *Appl. Microbiol. Biotechnol*. **96**: 981–991.
3) Yazawa H., Holic R., Kumagai H., and Uemura H. (2013) Toxicity of ricinoleic acid production in fission yeast *Schizosaccharomyces pombe* is suppressed by the overexpression of *plg7*, a phospholipase A2 of a platelet-activating factor (PAF) family homolog. *Appl. Microbiol. Biotechnol*. **97**: 8193–8203.
4) Yazawa H., Kumagai H., and Uemura H. (2013) Secretory production of ricinoleic acid in fission yeast *Schizosaccharomyces pombe*. *Appl. Microbiol. Biotechnol*. **97**: 8663–8671.
5) Yazawa H., Ogiso M., Kumagai H., and Uemura H. (2014) Suppression of ricinoleic acid toxicity by ptl2 overexpression in fission yeast *Schizosaccharomyces pombe*. *Appl. Microbiol. Biotechnol*. In press (DOI: 10.1007/s00253-014-6006-y).