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Application Note

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Metabolic engineering

Qualitative Analysis of Phytochemicals by High-resolution Mass Spectrometry

-Analysis for Prenylnaringenin-

Nobuyuki Okahashi*1, 2, 3, Shota Isogai*4, 5, 6, Jun Ishii*4, 5, 7, Junna Nakazono*8, Fumio Matsuda*1, 2, 3

Metabolic engineering

■ Abstract

This report introduces an example of using a high-resolution mass spectrometer for metabolic engineering research into microbes that play a role in bioproduction. A metabolically engineered yeast strain incorporating exogenous genes from plants and microbes was constructed to produce prenylnaringenin, a useful plant component. By utilizing the high resolution of the LCMS™-9030 quadrupole time-of-flight (Q-TOF) mass spectrometer and highly accurate *m/z* values, it was possible to distinguish prenylnaringenin from impurities and quickly estimate its structure. It is expected that this high-resolution mass spectrometer will contribute to accelerating bioproduction research aimed at the realization of a low-carbon society.

1. Introduction

In recent years, the importance of the bioproduction of useful compounds by microbes has been increasingly recognized. In addition to creating raw materials for biofuel and biomaterials for chemical products, bioproduction is expected to realize microbial production of useful phytochemicals, which are compounds with complex structures produced by plants. Prenylnaringenin, which is one such phytochemical, has been reported to be effective in treating cancer and diabetes. In addition, since it exhibits various bioactivities, including antioxidant, anti-inflammatory, and anti-influenza effects, it is attracting attention as a target for bioproduction.[1,2] Prenylnaringenin is biosynthesized by prenylation of naringenin which has a flavonoid skeleton. A yeast strain capable of producing naringenin has already been constructed by introducing a gene for a plant-derived enzyme into the yeast *Saccharomyces cerevisiae* using genetic engineering technology. No useful gene that can prenylate naringenin in microbes has been reported, except in some plants. Therefore this study attempts to realize microbial production of prenylnaringenin by introducing prenylation enzyme genes from various plants and microbes into *Saccharomyces cerevisiae* (Fig. 1).[3]

*1 Graduate School of Information Science and Technology, Osaka University
*2 Institute for Open and Transdisciplinary Research Initiatives (OTRI), Osaka University
*3 Osaka University Shimadzu Omics Innovation Research Laboratories
*4 Graduate School of Science, Technology and Innovation, Kobe University
*5 Technology Research Association of Highly Efficient Gene Design (TRAHED)
*6 NARA INSTITUTE of SCIENCE and TECHNOLOGY DIVISION of BIOLOGICAL SCIENCE
*7 Engineering Biology Research Center Kobe University
*8 Global Application Development Center, Analytical & Measuring Instruments Division, Shimadzu Corporation

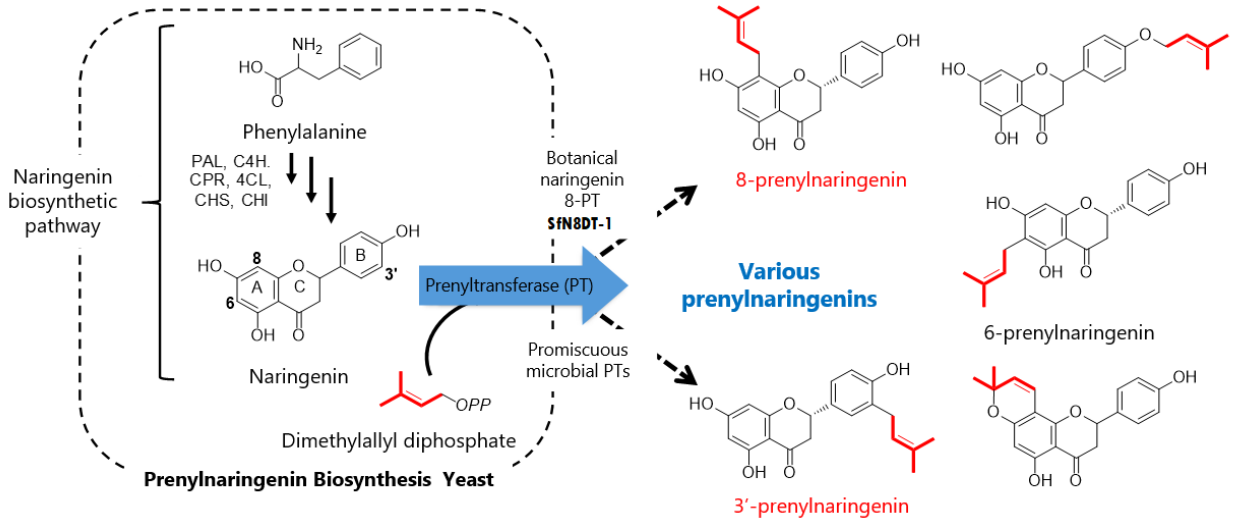

Fig. 1 Overview of metabolic engineering of prenylnaringenin biosynthesis in the yeast *Saccharomyces cerevisiae*

2. Role of High-resolution Mass Spectrometry in Bioproduction

Prenylnaringenin has multiple structural isomers with different prenylation positions (Fig. 1). There is a possibility that multiple structural isomers are simultaneously produced depending on the selectivity of the prenyltransferase (PT) gene. Compounds are generally identified by confirming the chromatographic retention time in using commercially available reference standards. However, it is possible to rapidly narrow down candidate compounds before that by using fragmentation information of product ion spectra acquired using the high-resolution LCMS-9030. Furthermore, it cannot be ruled out that unknown impurities may be produced due to the inherent metabolic functions in the yeast *Saccharomyces cerevisiae*. The high resolution of the LCMS-9030 is expected to be effective for surveying crude extracts derived from the prenylnaringenin-producing *Saccharomyces cerevisiae* and rapidly estimating the structure of the products while distinguishing them from impurities.

3. Construction and Evaluation of Prenylnaringenin-producing Yeast

The research team first constructed a pathway for synthesizing naringenin from its precursor phenylalanine in Saccharomyces cerevisiae. Six genes (*AtPAL1, AtC4H, AtCPR1, At4CL3, AtCHS3, AtCHI1*) derived from *Arabidopsis thaliana* were introduced into the yeast strain. The production of naringenin was confirmed by utilizing plant-derived genes, considering the fact that *Saccharomyces cerevisiae* belongs to the same eukaryote group as plants.

Next, we obtained 11 kinds of amino acid sequences of PTs from genome information databases of plants and microbes and introduced additional synthetic genes into the naringenin-producing yeast. For example, a metabolite was extracted from the yeast strain expressing the PT SfN8DT-1 derived from the legume, *Sophora flavescens*, and a deprotonated molecule of prenylnaringenin, $[M-H]^-$ (*m/z* 339.1), was analyzed in selective ion monitoring mode using a single quadrupole LC-MS. As a result, multiple signals were observed. There was a possibility that these included signals derived from impurities other than the desired prenylnaringenin. However, no further information was obtained.

4. Estimation of Prenylnaringenin Structure Using an LCMS-9030 Quadrupole Time-of-flight Mass Spectrometer

To obtain further information, we used a liquid chromatography-Q-TOF mass spectrometer (LCMS-9030) as a detector to acquire data in scan mode (Table 1). First, we focused on the deprotonated molecule of prenylnaringenin $[M-H]^-$ (*m/z* 339.124) and created an extracted ion chromatogram (XIC) of *m/z* 339.124±0.25 (Fig. 2(a)). As expected, multiple peaks were observed. To selectively detect prenylnaringenin, we created an XIC with an extremely narrow range of 5 ppm (*m/z* 339.124±0.002) (Fig. 2(b)). Only a signal with a retention time of 19.5 minutes was detected. On examining the data, it was determined that the peaks marked * in Fig. 2(a) were caused by an unrelated impurity with *m/z* 399.199.

Next, a product ion spectrum was acquired in product ion scan mode targeting the detected signal of Compound 1 (Fig. 3(a)). Using the obtained product ion spectrum as a query, a search on MassBank (http://www.massbank.jp/), which is a public product ion spectral database, showed that the result was very similar to 6-prenylnaringenin (ACCESSION: BS003003) and 8-prenylnaringenin (BS003083). When collated with a previously reported CID fragmentation mechanism of 8-prenylnaringenin, the *m/z* value of the predicted fragment and the measured value matched with a very small error of less than 0.001 (Fig. 3(a-c), Table 2).[4] Therefore, Compound 1 was presumed to be 6-prenylnaringenin or 8-prenylnaringenin. Although it was not possible to distinguish the structural isomers of 6-prenylnaringenin and 8-prenylnaringenin using only the exact mass information and the product ion spectra, it was possible to narrow down the candidate compounds. Finally, we confirmed the retention time using commercially available 8-prenylnaringenin and 6-prenylnaringenin reference standards (Fig. 4(b-c)). As a result, Compound 1 was identified as 8-prenylnaringenin (Fig. 4(a-b)). The same analysis was performed for *Saccharomyces cerevisiae* expressing prenylation enzymes derived from other organisms. Moreover, by using the prenylation enzyme derived from the fungus *Neosartorya fischeri*, which is generally considered to have a lower substrate selectivity than plants, we also succeeded in constructing a strain that synthesizes 3'-prenylnaringenin.[3] In the future, it is expected that bioproduction of compounds with various bioactivities will be possible by creating different structural isomers of prenylnaringenin.

Table 1 Analytical conditions

[LC]		
Instrument	:	Nexera™ X2
Column	:	COSMOSIL 5C_{18}-MSII column (2.0 mm I.D.× 150 mm, 5 mm, NACALAI TESQUE)
Flow rate	:	0.2 mL/min
Mobile phase A	:	Ultrapure Water (for QTofMS, Wako) + 0.1 % Acetic acid (Sigma-Aldrich)
Mobile phase B	:	Acetonitrile (for QTofMS, Wako) + 0.1 % Acetic acid (Sigma-Aldrich)
Time program (B conc.)	:	20 % (0 min) – 80 % (34 min) – 95 % (35-40 min) – 20 % (40.1-55 min)
Column temp.	:	30 °C
[Q-TOF-MS]		
Instrument	:	LCMS-9030
Ionization	:	ESI
Nebulizing gas flow	:	2.0 L/min
Drying gas flow	:	10 L/min
Heating gas flow	:	10 L/min
DL temp.	:	300 °C
BH temp.	:	400 °C
Interface temp.	:	300 °C
Mode	:	MS scan (0.5 s) + MS/MS scan (0.5 s, precursor ion *m/z* 339.1)
MS scan range	:	*m/z* 70-1000
MS/MS scan range	:	*m/z* 70-350
Q1 resolution	:	Low
Collision energy	:	35 V
Collision energy spread (±)	:	17 V

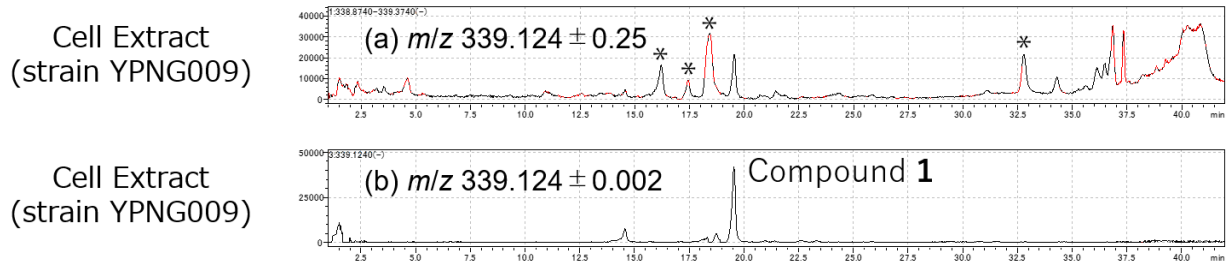

Fig. 2 Extracted ion chromatogram (XIC) of cell extract of *Saccharomyces cerevisiae*

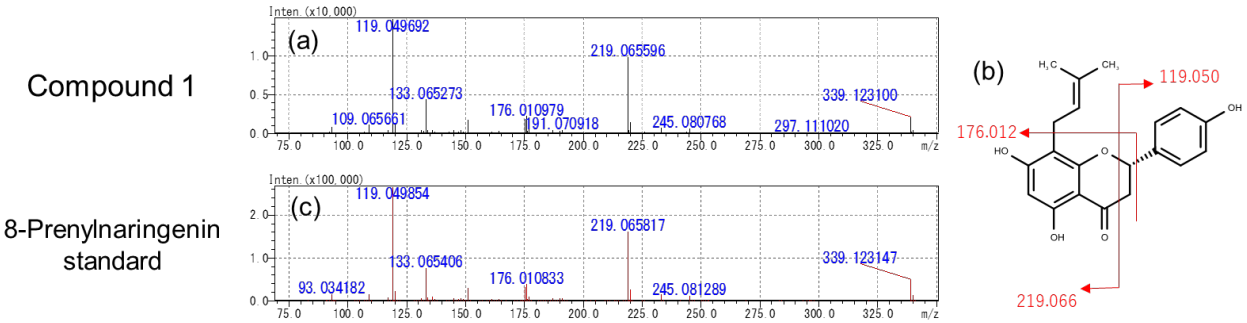

Fig. 3 Comparison of product ion spectra

Table 2 Comparison of product ion *m/z* between compound 1 and the 8-prenylnaringenin standard

	m/z		
Product ion	Theoretical value	Compound 1	8-prenylnaringenin standard
$[C_{20}H_{19}O_5]^-$	339.1232	339.1230	339.1231
$[C_{12}H_{11}O_4]^-$	219.0657	219.0656	219.0658
$[C_9H_4O_4]^-$	176.0110	176.0110	176.0108
$[C_8H_7O]^-$	119.0500	119.0500	119.0500

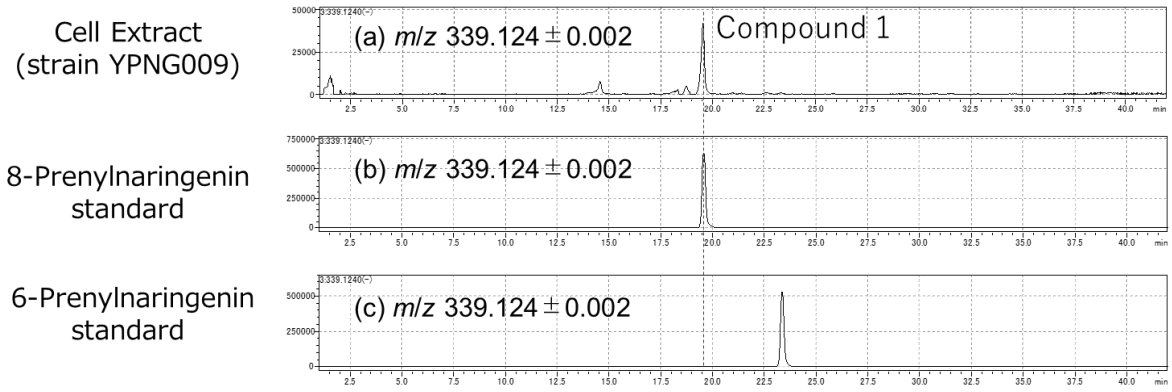

Fig. 4 Comparison of retention time between compound 1 and prenylnaringenin standards

■ Summary

Research on metabolic engineering of microbes in bioproduction has made great progress in recent years, and there is a demand for speedy evaluation of the constructed strains. By utilizing the high resolution of the LCMS-9030 and highly accurate *m/z* values, it was possible to quickly estimate the structure of compounds in the products of the metabolically engineered strains. Bioproduction of various compounds is being explored to realize a low-carbon society, and it is expected that high-resolution mass spectrometers will contribute to the rapid research and development of such compounds.

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