Accumulation of Gibberellin A₄ and the Metabolism of Gibberellin A₉ to Gibberellin A₁ in a *Phaeosphaeria* sp. L487 Culture

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Gibberellin A₄ (GA₄), which was identified as the major GA from the GA-producing fungus *Phaeosphaeria* sp. L487, was accumulated in the culture with a maltose-yeast extract medium, its amount in the culture filtrate being about 50 mg per liter after a 3-week culture. The new fungal biosynthetic pathway to GA₁ from GA₉ via GA₄ was elucidated by feeding experiments with synthetic [17⁻²H₂]GA₉ and [17⁻²H₂]GA₄.

In a continuing study on the production and metabolism of gibberellins (GAs) in a culture of the GA-producing fungus, *Phaeosphaeria* sp. L487, GA₉ (Fig. 1, 1) and GA₄ (2) were isolated from its culture filtrate,¹ and the metabolic conversion of GA₉ to GA₄ was demonstrated by feeding experiments with synthetic [17⁻²H₂]GA₉ (1)² This 3β-hydroxylation is a very important step, as well as that of GA₂₉ to GA₁, in plant GA biosyntheses because it affords physiologically active GA₄ and GA₁ (3).³ These biosynthetic pathways, however, have not been found in other GA-producing fungi, i.e., *Gibberella fujikuroi* and *Sphaeceloma manihoticola*⁴; in their GA biosynthetic pathways, 3β-hydroxylation occurs in the step from the GA₁₂-aldehyde to GA₄α-aldehyde. Our continuing study on the mass production of GAs by *Phaeosphaeria* sp. L487 showed a marked accumulation of a polar GA in a maltose-yeast extract medium. In this paper, we describe the identification and accumulation of GA₁ as the major GA in this culture, and the biosynthetic relationship among GA₉, GA₄, and GA₁ in this fungus by feeding experiments.

The fungus was cultivated in a seed medium composed of 4% maltose and 0.5% Pharmamedia in shake flasks that were reciprocally shaken for 6 days at 25–26°C. Well-grown mycelia were inoculated to 500-ml shake flasks containing each 100 ml of a GA-production medium (5% maltose, 0.4% yeast extract, 0.1% NH₄NO₃, 0.5% KH₂PO₄ and 0.1% MgSO₄·7H₂O) and the flasks were incubated on a reciprocal shaker for 15 days at 25–26°C. The culture filtrate (1.6 liter, pH 4.8) was extracted with ethyl acetate at pH 2.0. The NaHCO₃ (aqueous)-soluble acidic fraction obtained from the ethyl acetate extract was subjected to column chromatography [CQ-3 silica gel, 4:1 benzene/

acetone containing 0.2% acetic acid] to give a polar GA fraction (fractions 16–27, 44.5 mg); the GA fraction was determined by the elongation activity toward hypocotyls of Chinese cabbage seedlings.³ The GA was successively purified by preparative silica gel TLC [1:2 benzene/aceton containing 0.2% acetic acid] to afford colorless prisms having mp 255–257°C (acetone/hexane, decom.) and [x]D +36.9⁰ (c = 0.21 in EtOH). By treating with ethereal diazomethane, its methyl ester was obtained as colorless prisms [mp 235–236°C (acetone/hexane)]. EIMS m/z (%): 362 (M⁺, 32), 344 (14), 330 (100), 316 (4), 312 (18), 303 (43), and 302 (31). HR-EIMS m/z: 362.1716 (C₂₀H₂₀O₆), calc. as 362.1731.¹ H-NMR (400 MHz, CDCl₃) δ: 1.15 (s, 18-H₃), 1.27 (s, 15-H₃), 2.69 and 3.21 (ABq, J = 10.2 Hz, 6-H and 5-H), 3.72 (s, -OCH₃), 3.85 (m, 3-H), and 4.96 and 5.25 (each m, 17-H₂). From these data, the polar GA was unequivocally identified as GA₁.

This fungus produced GA₁ as the end product of its GA biosynthesis in the maltose-yeast extract medium (Fig. 2). A 3-week culture gave ca. 50 mg of GA₁ from one liter of the culture filtrate. While no GA₃ was detected by HPLC analysis, a considerable amount of GA₄ (12 mg/liter) was detected after 8 days, which then decreased rapidly to less than 1.5 mg/liter. On the other hand, GA₂₉ was scarcely detected throughout the whole course of cultivation.

Fungal GA₁ can be biosynthesized from GA₁₄ via GA₄ in *G. fujikuroi*. Thus, we examined the incorporation of [17⁻²H₂]GA₉ into GA₁ and GA₄, and of [17⁻²H₂]GA₄ (2) into GA₁ in order to determine the biosynthetic pathway to GA₁ in *Phaeosphaeria* sp. L487. The preparation of [17⁻²H₂]GA₉ from commercial GA₁₄ was described in a previous paper.⁵ For the feeding experiments, the fungus was cultured in a similar manner to that already described. When GAs (GA₁, 0.09 mg; GA₄, 0.24 mg; and GA₉, 0.14 mg; all in 100 ml) were detected in the culture filtrate at 5 days after inoculation, 25 ml of the culture containing mycelia was transferred to a 100-ml shake flask. Immediately, [17⁻²H₂]GA₉ (0.2 mg in 20 µl of EtOH) was added to the culture, and incubation was continued for another 3 days. The preparation of a strongly acidic fraction con-

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**Fig. 1. Structures of Gibberellins and Their Derivatives.**
taining GAs from the culture filtrate was done in a similar manner to that already described. From this acidic fraction (4.8 mg), GA₁- and GA₄-like compounds were separated by preparative TLC [1:1 benzene/acetone containing 0.2% acetic acid]. The GA₁-like compound (0.8 mg) was further purified by HPLC [Crestpak C8S, 15:85 CH₃CN/H₂O containing 0.2% acetic acid] and was then converted with ethereal diazomethane for an MS analysis. The GA₄-like compound (0.6 mg) was also purified by HPLC [Crestpak C18S, 35:60 CH₃CN/H₂O containing 0.2% acetic acid]. This GA₄-like compound and the methyl ester of the GA₁-like compound were determined by EIMS and HR-EIMS (Fig. 3). The MS data for the GA₄-like compound were quite similar to those obtained from the previous feeding experiments of [¹⁷²H₂]GA₄.21) The MS data for the methyl ester of the GA₁-like compound gave molecular and fragment ion peaks [m/z] 362 (M⁺), 344, 330, 316, and 312 originating from the methyl ester of natural GA₁ (4). Furthermore, the data showed the corresponding intense MS peaks [m/z] 364 (M⁺), 346, 332, 318, and 314) shifted by two mass units, indicating the presence of the [²H₃]GA₄ methyl ester ([²H₂]GA₁-Me, 4). These MS peaks of M⁺ and [M⁺−H₂O] were also confirmed by HR-EIMS: m/z 364.1836 (M⁺, C₂₀H₄₄[²H₂]O₆ calc. as 364.1856) and 346.1761 (M⁺−H₂O, C₂₀H₃₂[²H₂]O₅, 346.1750) for [²H₃]GA₁-Me: m/z 362.1757 (M⁺, C₂₀H₄₀O₆, 362.1731) and 344.1590 (M⁺−H₂O, C₂₀H₃₈O₅, 344.1625) for GA₁-Me. These observations suggested that GA₉ was metabolized to GA₄ via GA₄ in the fungus.

In order to confirm the metabolic conversion of GA₄ to GA₁, we prepared [¹⁷²H₂]GA₄ from the methyl ester of GA₄-17-norketone by a chemical transformation similar to that used in the preparation of [¹⁷²H₂]GA₉.2) The MS and ¹H-NMR data for the synthetic [¹⁷²H₂]GA₄ were as follows. EIMS m/z (%): 334 (M⁺, 2), 316 (17), 298 (9), 288 (24), and 272 (100). HR-EIMS m/z: 316.1649 (M⁺−H₂O, C₁₉H₂₀[²H₂]O₄ calc. as 316.1645). ¹H-NMR (400 MHz, acetone-d₆) δ: 1.11 (3H, s, 18-H₃), 2.60 and 3.21 (each 1H, ABq, J=11.0 Hz, 6-H and 5-H), and 3.72 (1H, m, 3-H) [signals of an exomethylene group at δ 4.97 and 4.84 (17-H₂) were not observed]. The feeding experiments with [¹⁷²H₂]GA₄ as a substrate were done in a similar manner to that used for [¹⁷²H₂]GA₉. The acidic substances extracted from the culture filtrate were separated by TLC and then purified by HPLC to give a GA₁-like compound. Its methyl ester obtained by methylation with diazomethane was determined by EIMS and HR-EIMS. The metabolic conversion of GA₄ to GA₁ was verified by the presence of the MS peaks of [²H₃]GA₁-Me: m/z 364.1862 (M⁺, 4, C₂₀H₄₀[²H₂]O₆ calc. as 364.1856), 346 (2), and 318 (4)) together with those of natural GA₁-Me: m/z 362.1746 (M⁺, 23, C₂₀H₃₈O₆ calc. as 362.1731), 344 (13), and 330 (100)).

These observations clearly indicate that GA₁ was converted from GA₉ via GA₄ in Phaeosphaeria sp. L487. This biosynthesis of GA₁ is a new pathway in fungi and is very interesting because the metabolism of GA₉ to GA₄ and of GA₄ to GA₁ is found in various higher plants.6) A large amount of GA₄ (ca. 50 mg/liter) was accumulated as

Fig. 2. Time-Course for Gibberellin (GA) Production in the Yeast Extract Medium by Phaeosphaeria sp. L487.

Each gibberellic amount in the culture filtrate in a 500-ml shake flask was determined by HPLC; GA₉ and GA₄ [Crestpak C18S, 35:60 CH₃CN/H₂O containing 0.2% acetic acid], and GA₁ [Crestpak C8S, 15:85 CH₃CN/H₂O containing 0.2% acetic acid]. Symbols: □, GA₁; ○, GA₄; ◀, GA₉.

Fig. 3. Partial EIMS Spectra of the Gibberellin A₄-like Compound and the Methyl Ester of the Gibberellin A₁-like Compound Obtained from the Culture Filtrate of Phaeosphaeria sp. L487 Fed with [¹⁷²H₂] Gibberellin A₉.

Symbol: ▼, deuterium-containing MS peaks.
the major GA in the culture of *Phaeosphaeria* sp. L487. In addition, GA$_3$ could not be detected in the culture filtrate by an HPLC analysis. These observations suggest that pure GA$_1$ for agricultural applications can be produced by this GA$_1$ fermentation.

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**References**