Note

Conversion of GA₅ to GA₆ and GA₃ in Cell-free Systems from *Phaseolus vulgaris* and *Oryza sativa*

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Gibberellin A₅ has been identified in several plants,¹⁾ and it shows activities on various bioassays.²⁾ In these bioassays, GA₅ promotes the shoot elongation of normal rice, cultivar Nihonbare, at the same level as GA₁. However, it does not promote shoot elongation of dwarf rice, cultivar Waito-C, which is a deficient mutant of 3 β -hydroxylation of gibberellins.³⁾ This suggests that GA₅ is not *per se* active in rice, but that it becomes active by conversion to an active gibberellin in rice. The conversion of GA₅ to GA₃ in intact shoots of *Zea mays*⁴⁾ and in cell-free extracts of *Marah macrocarpus*⁵⁾ has been shown. However, the metabolism of GA₅ in cell-free extracts from *Phaseolus vulgaris* (Leguminosae) and *Oryza sativa* (Gramineae) has not been confirmed. We report here the conversion of GA₅ to GA₆ or GA₃ by cell-free extracts from these plants.

In cell-free extracts of *P. vulgaris*, GA₂₀ has been converted to GA₁, GA₅ and GA₆.⁶⁾ The detection of GA₅ and GA₆ in the metabolites suggests that the extract converted GA₅ to GA₆, since GA₆ could be derived from GA₅ by an epoxidation of its double bond at C-2. Contrary to the expectation, GA₅ was not converted to GA₆ by crude enzyme extracts, probably due to the low activity of the enzyme and/or the presence of an inhibitor. In the course of a study on the substrate specificity of partially purified 3 β -hydroxylase from *P. vulgaris*, we found that the addition of cold GA₅ into the incubation mixture inhibited the conversion of [³H]GA₂₀ to [³H]GA₁.⁷⁾ This inhibitory activity of GA₅ implies that GA₅ could be a substrate for 3 β -hydroxylase as well as GA₂₀, and be epoxidized to GA₆. Partially purified gibberellin 3 β -hydroxylase from immature seeds of *P. vulgaris* was prepared by methanol

precipitation, hydrophobic interaction chromatography and ion exchange chromatography.⁸⁾ When [17-¹³C, ³H₂]GA₅ (2500 Bq) was incubated with a mixture of the enzyme preparation, 2-oxoglutarate, potassium ascorbate and Fe²⁺, a product (486 Bq) was detected at the same retention time as authentic GA₆ by HPLC. This compound was identified as [17-¹³C]GA₆ by full-scan gas chromatography-mass spectrometry (GC/MS). This result confirmed that the partially purified 3 β -hydroxylase catalyzed the epoxidation of GA₅ to GA₆. The cofactor requirement for the epoxidation of GA₅ was then studied (Table I). The amount of GA₆ produced without adding Fe²⁺ was about one third of the amount when all the other cofactors were present. The reason for the reduced activity was probably due to incomplete removal of Fe²⁺ from the enzyme solution by hydrophobic interaction chromatography and ion exchange chromatography.⁹⁾ The removal of 2-oxoglutarate or ascorbate strongly reduced the conversion of GA₅ to GA₆. Under anaerobic conditions, epoxidation of GA₅ to GA₆ was depressed by over 70%. Therefore, the epoxidation requires oxygen, Fe²⁺, 2-oxoglutarate and ascorbate, like the 3 β -hydroxylation reaction.⁸⁾ The enzyme catalyzing the epoxidation of GA₅ to GA₆ was shown to have the same characteristics as 3 β -hydroxylase. The epoxidation step was inhibited by substrates of 3 β -hydroxylase,⁷⁾ suggesting that the same enzyme might catalyze the 3 β -hydroxylation step and the epoxidation step. No conversion of GA₅ to GA₃ was observed in the extracts of *P. vulgaris*.

Recently, we have established a cell-free system from anthers of *O. sativa* that show activities to metabolize GA₉, GA₁₂, GA₂₀ and GA₅₃.¹⁰⁾ The rice system is so far the only active cell-free system prepared from Gramineae. A

Table I. COFACTOR AND OXYGEN REQUIREMENTS FOR THE EPOXIDATION OF GA₅ TO GA₆

Cofactors and conditions ^a				Relative conversion (%)
Fe ²⁺	2-Oxoglutarate	Ascorbate	Air	
+	+	+	+	100.0 ^b
—	+	+	+	36.2
+	—	+	+	6.3
+	+	—	+	10.2
+	+	+	—	25.2 ^c
Boiled enzyme with all cofactors				0

^a 0.5 mM FeSO₄, 5 mM 2-oxoglutarate and 5 mM ascorbate.

^b The conversion of GA₅ to GA₆ was 25.4% in the complete cofactor mixtures.

^c Under N₂ atmosphere. Inhibition was recovered to approximately 75% by reincubating in air.

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Fig. 1. Metabolism of GA₂₀ and GA₅ in Cell-free Systems from *Phaseolus vulgaris* and *Oryza sativa*.

study on the cofactor requirement of the enzyme reaction was not performed because the amount of the extract was not enough for preparing the partially purified enzymes needed for the study. Although NADPH was not necessary for 2-oxoglutarate-dependent dioxygenases,⁹⁾ it was added to the incubation mixture together with Fe²⁺, ascorbate and 2-oxoglutarate since it stimulated the conversion in crude rice extracts (data not shown). The extract converted [17-¹³C, ³H₂]GA₅ (767 Bq) to a product (200 Bq) which had the same retention time as GA₃ by HPLC. The compound was identified as [17-¹³C]GA₃ by full-scan GC/MS. No conversion of GA₅ to GA₆ was observed in the extracts of *O. sativa*. We have previously analyzed the metabolism of GA₂₀ in the extract, in which it was converted to GA₁, GA₈ and GA₂₉, but not to GA₃, GA₅ and GA₆.¹⁰⁾ Gibberellins A₃, A₅ and A₆ are not endogenous gibberellins in rice. This result shows that there is an enzyme catalyzing the 3 β -hydroxylation of GA₅ to GA₃ in *O. sativa*, although it lacks an enzyme to catalyze the conversion of GA₂₀ to GA₅. The shoot elongation in rice promoted by GA₅ would be due to the conversion of GA₅ to GA₃ as it is shown in intact shoots of *Zea mays*.⁴⁾ We have no information whether the enzymes catalyzing the 3 β -hydroxylation of GA₂₀ and GA₅ in rice are the same or not.

The possible metabolic pathways of GA₂₀ and GA₅ in cell-free systems are shown in Fig. 1. Gibberellin A₆ has not been isolated from any vegetative organs of higher plants,¹⁾ and the conversion of GA₅ to GA₆ might be limited to immature seeds of Phaseolae. On the other hand, the conversion of GA₅ to GA₃ appears to operate both in vegetative and reproductive organs, since GA₅ has been converted to GA₃ in maize seedlings,⁴⁾ and in cell-free systems from anthers of rice and from immature seeds of *M. macrocarpus*.⁵⁾

Experimental

Preparation of the cell-free extracts. Gibberellin 3 β -hydroxylase was purified from immature seeds of *Phaseolus vulgaris* cv. Masterpiece as described by Kwak *et al.*⁸⁾ The DEAE-purified enzyme was used for this study, being ca. 34-fold purified compared to crude enzyme extracts. Cell-free extracts were prepared from anthers of *Oryza sativa* L. cv Nihonbare as described by Kobayashi *et al.*¹⁰⁾

Incubation of [17-¹³C, ³H₂]GA₅ with the enzyme extracts and an analysis of the products by HPLC. [17-¹³C, ³H₂]GA₅ (1.51 GBq/mmol ¹³C 92.0%) was a gift from Prof. B. O. Phinney (University of California). The enzyme preparation of *P. vulgaris* (8.5 μ g of protein) was incubated at 30°C for 1 hr in a total volume of 200 μ l of K-Pi buffer (0.05 M, pH 8.0). The incubation mixture contained at the final concentration, K-2-oxoglutarate (5 mM), K-ascorbate (5 mM), FeSO₄ (0.5 mM), 2 μ l of a MeOH solution of [17-¹³C, ³H₂]GA₅ (2500 Bq), glycerol (15% v/v), sucrose (0.1 M) and DTT (1 mM). To study the cofactor requirements, [17-¹³C, ³H₂]GA₅ (500 Bq) was incubated with the same enzyme preparation for 2 hr. Anaerobic conditions were applied by passing nitrogen gas through hypodermic needles.¹¹⁾ The enzyme preparation of *O. sativa* (dialyzed 200,000 $\times g$ supernatant, 1900 μ g of protein) was incubated at 30°C for 2 hr in a total volume of 200 μ l of K-Pi buffer (0.05 M, pH 8.0). The incubation mixtures were the same as those for *P. vulgaris*, except for adding NADPH (2 mM, final conc.). After incubation, the products were purified by a Nucleosil 5C₁₈ column (100 $\times$ 4.6 mm) or Nucleosil 5N(CH₃)₂ column (50 $\times$ 4.6 mm). The column eluant was collected every 1 min, and the radioactivity of fraction was counted by a liquid scintillation analyzer.

GC/MS. GC/MS was performed on a Finnigan MAT INCOS 50 mass spectrometer coupled to a Hewlett-Packard 5890A gas chromatograph equipped with a capillary column (DB-1, 0.32 mm i.d. $\times$ 30 m). The conditions for GC were the same as those described previously.¹⁰⁾ The retention time and relative intensities of the characteristic ions of the identified gibberellins are as follows.

Authentic GA₆-Me-TMSi: 10.5 min, 432 (86), 417 (27), 373 (40), 303 (100).

[¹³C]GA₆-Me-TMSi: 10.5 min, 433 (76), 418 (11), 374 (24), 304 (100).

Authentic GA₃-TMSi: 12.5 min, 562 (100), 547 (22), 445 (75).

[¹³C]GA₃-TMSi: 12.5 min, 563 (100), 548 (40), 446 (94).

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