JARQ 24, 188–194 (1990)

A New Gibberellin Bioassay: A Proposed Method for Its Systematic Analysis

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Abstract
Sensitivity of the micro-drop assay with dwarf rice cultivars (*Oryza sativa* L., cv., Tan-ginbozu and cv. Waito-C) to gibberellins (Gas) was increased approximately thirty-fold by the use of assay plants that had been treated with uniconazole (S-3307), i.e. an inhibitor of the biosynthesis of Gas. The spectrum of detectable Gas was not changed by that treatment. In addition, the treatment with S-3307 counteracted in part the inhibition of growth of both cultivars by abscisic acid. Thus, the modified micro-drop assay is recognized to be very useful for the detection of minute amounts of Gas in plant extracts.

Discipline: Experimental apparatus and method
Additional key words: dwarf rice, modified micro-drop assay, *Oryza sativa*, uniconazole

Introduction

Among the various assays for gibberellins (GAs), the micro-drop assay, using GA-deficient mutants of dwarf rice[9] is one of the most useful methods because of the simple manipulations involved and the high degree of sensitivity of the assay to a broad spectrum of GAs. Thus, in the systematic analysis of GAs, this assay has been widely used as a valuable tool in the detection of GA-active fractions that would be suitable candidates for further analyses by gas chlomatography/mass spectrometry[14] (GC/MS). However, in this assay, the sensitivity to GAs is still lower than that of GC/MS[18], and the activity of GAs in plant extracts is occasionally masked or suppressed by some coexisting inhibitory substances, such as abscisic acid (ABA) and chlorogenic acid[8].

It was found that the sensitivity of the 'micro-drop method' to GAs was distinctively enhanced when the plants in the assay were subjected to treatment with uniconazole [S-3307; (±)-(E)-1-(4-chlorophenyl)-4, 4-dimethyl-2-(1, 2, 4-triazole-1-yl)-1-penten-3-ol], an inhibitor of the biosynthesis of GAs[4]. Furthermore, that treatment with S-3307 counteracted in part the inhibition of growth of the plants by ABA. This paper presents briefly the procedure and results of the 'modified micro-drop assay', and some examples of its practical use in plant extracts.

Procedure of modified micro-drop assay[13]

The bioassay procedure used is similar to the 'micro-drop method'[9] except for the treatment of the dwarf rice seeds with S-3307 (Fig. 1)[13]. Seeds of the dwarf rice cultivars (*Oryza sativa* L., cv. Tanginbozu and cv. Waito-C) are sterilized with Benlate [0.1% (w/v), Du Pont, Del., U.S.A.] and soaked in water that contains 20 mg/*l* of S-3307 (Sumitomo Chemical Co., Japan) for 24 hr in darkness at 30°C. The seeds are then washed with water and germinated in water under the same conditions. When the coleoptiles are ca. 4 mm in length, seven seedlings are planted in a vial (28 mm in diameter × 58 mm in height) that is filled with 0.8% (w/v) agar and incubated for 72 hr at 30°C, under continuous irradiation (77.5 $\mu mol \cdot m^{-2} \cdot sec^{-1}$, ca. 5,000 lux). The samples are dissolved in 50% (v/v) aqueous acetone. One μl of the test solution is applied with a microsyringe to the region between the coleoptile

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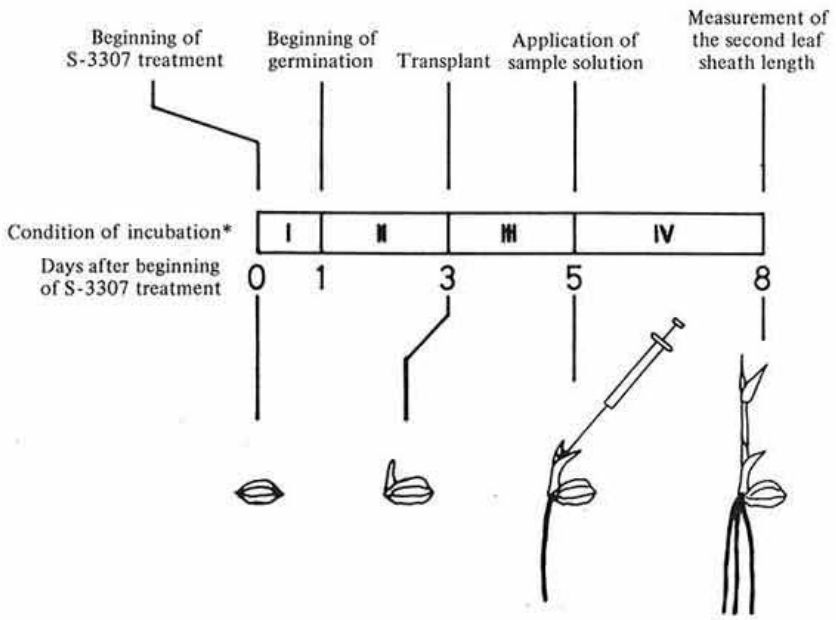

Fig. 1. Schematic diagram of procedures of the modified micro-drop assay
* Condition of incubation:
I, II; 30°C, Dark,
III, IV; 30°C, Continuous light (5,000 lux).

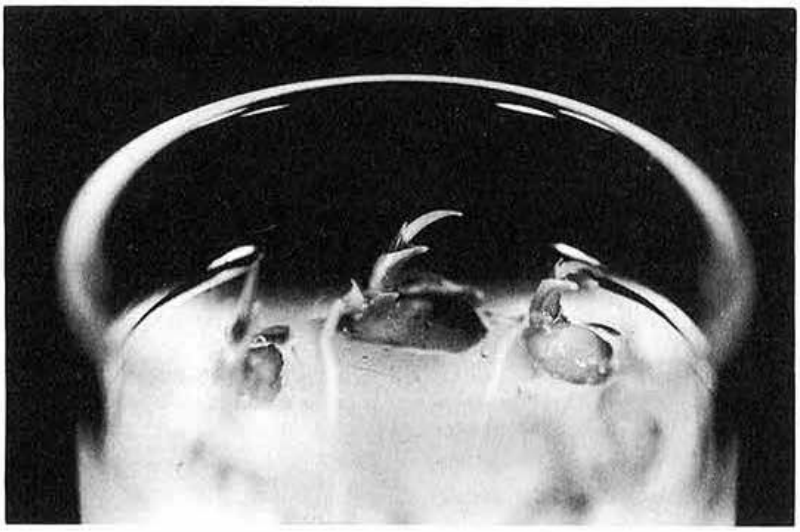

Plate 1. Seedlings of dwarf rice plants under an appropriate growth stage for applying a sample solution

and the first leaf of a seedling (also see Plate 1). Three days later, the length of the second leaf sheath was measured. The lowest quantity of a GA that significantly (t-test 0.1% level) stimulated the elongation of the second leaf sheath is regarded as the minimum detectable level of the GA.

Table 1. Minimum detectable levels and relative activity of several GAs in the usual (UA) and the modified (MA) Tan-ginbozu assays

GAs	Minimum detectable level (fmol/plant)		Relative activity* (%)	
	UA	MA	UA	MA
GA_1	1,000	30	75	70
GA_3	300	10	100	100
GA_4	3,000	30	35	50
GA_7	1,000	30	55	70
GA_8	>3,000	3,000	0	5
GA_9	3,000	100	40	50
GA_{17}	>3,000	3,000	0	3
GA_{19}	300	30	110	75
GA_{20}	300	30	85	60

* Elongations of the second leaf sheath by each GA (3×10^3 fmol/plant) are compared. Relative activities are expressed in percentages against GA_3.

Table 2. Minimum detectable levels and relative activity of several GAs in the usual (UA) and the modified (MA) Waito-C assays

GAs	Minimum detectable level (fmol/plant)		Relative activity* (%)	
	UA	MA	UA	MA
GA_1	1,000	30	85	80
GA_3	300	10	100	100
GA_4	1,000	30	75	70
GA_7	300	30	95	90
GA_8	>3,000	>3,000	0	3
GA_9	>3,000	3,000	10	20
GA_{17}	>3,000	>3,000	0	3
GA_{19}	>3,000	>3,000	0	5
GA_{20}	>3,000	>3,000	0	5

* Elongations of the second leaf sheath by each GA (3×10^3 fmol/plant) are compared. Relative activities are expressed in percentages against GA_3.

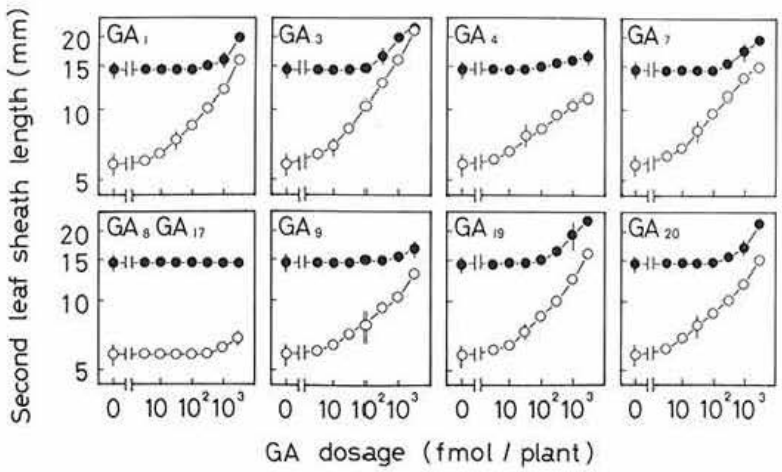

Fig. 2. Effects of GA doses on the elongation of the second leaf sheath of Tan-ginbozu plants
○: Treated with S-3307 (20 mg/l), ●: Non-treated.
Vertical bars represent SDs of the means at the minimum detectables level of GAs.

Results of the modified micro-drop assay[13]

1) Response of Tan-ginbozu and Waito-C plants treated with S-3307 to various authentic GAs

The treatment of Tan-ginbozu and Waito-C plants with S-3307 lowered the minimum detectable levels of GA_3 to 10 fmol/plant, that of gibberellins A_1, A_4, A_7, A_{19} and A_{20} to 30 fmol/plant and that of GA_9 to 100 fmol/plant (Table 1 & Fig. 2). Gibberellins A_8 and A_{17}, which had little measurable effect on non-treated Tan-ginbozu plants, were slightly active at 3,000 fmol/plant.

The C-3-hydroxy GAs which had a major effect

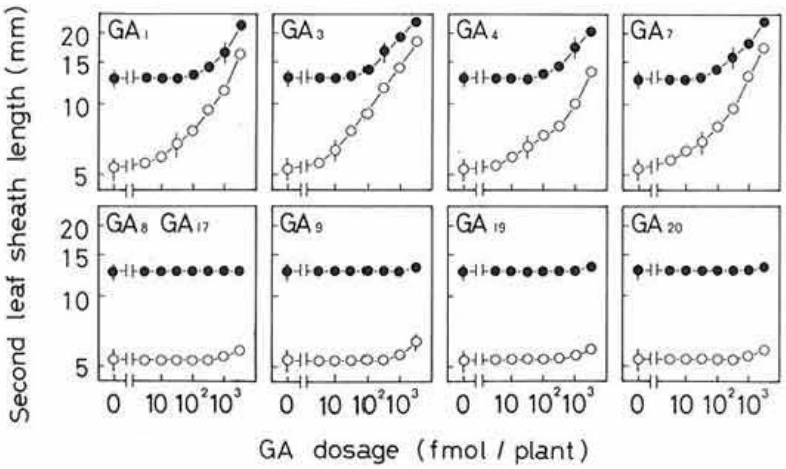

Fig. 3. Effects of GA doses on the elongation of the second leaf sheath of Waito-C plants
○: Treated with S-3307 (20 mg/l), ●: Non-treated.
Vertical bars represent SDs of the means at the minimum detectable level of GAs.

on the S-3307 treated Tan-ginbozu plants (gibberellins A_1, A_3, A_4 and A_7) also exerted the similar degree of major effects on the treated Waito-C plants (Table 2 & Fig. 3). By contrast, C-3-nonhydroxy GAs which were very active to Tan-ginbozu plants (gibberellins A_9, A_{19} and A_{20}) had little effect on the similarly treated Waito-C plants. The GAs which had little effect on the treated Tan-ginbozu plants (gibberellins A_8 and A_{17}) did not give any effect also on the treated Waito-C plants.

Tan-ginbozu[15] and Waito-C[7] are known for their having a small amount of endogenous GA each, which is active in the elongation of the second leaf sheath. The increase in the sensitivity of the Tan-ginbozu and Waito-C plants to GAs is probably based on the decrease in endogenous GAs caused by the treatment of seeds with S-3307[13].

The whole spectrum of detectable GAs is not fully identified yet in the present set of the bioassy experiments. However, it is expected that the above-mentioned modified Tan-ginbozu assay would be highly useful in detecting as broad a spectrum of GAs as the usual Tan-ginbozu bioassay. This prediction might be justifiable because S-3307 blocks the very early steps in the biosynthesis of GA (i.e., the oxidation of kaurene, kaurenol and kaurenal[5]), which occur immediately after the step that is genetically blocked in the Tan-ginbozu variant (between mevalonic acid and kaurene[10]).

On the other hand, Murakami[11] suggested that the step involved in the synthesis of gibberellin A_1 from A_{20} (hydroxylation of C-3 of GA_{20}) be blocked genetically in Waito-C and that this block render Waito-C unresponsive to C-3-nonhydroxy GAs. The observation that GA_{20} is inactive in Waito-C plants treated with S-3307[13] suggests that S-3307 do not affect the genetic blockade in the biosynthesis of GA. Thus, it is expected that Waito-C treated with S-3307 does not respond to C-3-nonhydroxy GAs in the same way as non-treated Waito-C does not.

2) Response of dwarf rice plants treated with S-3307 to authentic ABA

As mentioned above, the usual micro-drop assay has a defect that GA-activity in plant extracts is sometimes masked or suppressed by coexisting inhibitory substances. A study was undertaken to test the response of modified micro-drop assay to ABA, which is one of the common inhibitory substances in plant extract. The treatment with S-3307 counteracted in part the inhibition of growth of the Tan-ginbozu and Waito-C plants by ABA at the doses of 10 to 1,000 pmol/plant (Fig. 4).

3) Comparison of modified micro-drop assay with other GA-assays

Among the various bioassays for GAs reported, the barley α-amylase half-seed assay[6,12] and the *Rumex* leaf-senescence assay[17] are the most sensitive. In these bioassays, about 10 fmol (per m*l* of test solution) of GA_3 can be detected. The modified assay proposed here is as sensitive to GA_3 as those bioassays. Furthermore, the C-3-nonhydroxy GAs, such as GA_{19} and GA_{20}, which are almost inactive in the barley α-amylase half-seed assay and in the *Rumex* leaf-senescence assay[3,17], are extremely active in the modified Tan-ginbozu assay.

Atzorn et al.[1,2] reported that RIA and ELIZA detected at least 100 fmol and 0.5 fmol of GAs, respectively. As far as the authentic GAs that are highly active to the dwarf rices are concerned, the modified micro-drop assay has sensitivity which is comparable to that of RIA but lower than that of ELIZA. However, when inhibitory substances are contained in a sample for the modified micro-drop assay, the activity of coexisting GAs is suppressed. Therefore, it is recommendable that samples are purified consistently before testing by that bioassay.

Applications of the modified micro-drop assay to plant extract[13]

Fig. 5 shows histograms of GA-activities in fractions of the extract of taro (*Colocasia esculenta* Schott cv. Egu-imo) sprouts separated by HPLC. Under the test with the usual Tan-ginbozu assay, three fractions showed significant effect (Fig. 5A, fractions a, b and c). When the fractions were tested with the modified Tan-ginbozu assay, their activities were highly stimulated (Fig. 5B, fractions a, b and c), while with the modified assay, six additional fractions (Fig. 5B, fractions d, e, f, g, h and i) were recognized to be active. In the standard micro-drop assay, GA-activities of these six fractions had been masked by the growth inhibitors present in the extract of the taro plants.

An example of application of the modified micro-drop assay to a radish extract is shown in Fig. 6. The GAs identified by GC/MS are presented in the figure. The fractions that showed high activities in both Tan-ginbozu and Waito-C plants (fractions 3 and 4) contained C-3-hydroxy GAs ($GA_1$and GA_4).

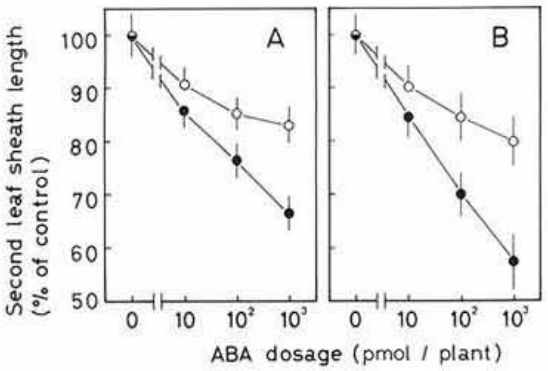

Fig. 4. Effects of ABA doses on the elongation of the second leaf sheath of Tan-ginbozu (A) and Waito-C (B) plants
○: Treated with S-3307 (20 mg/*l*),
●: Non-treated.
Vertical bars represent SDs of the means.

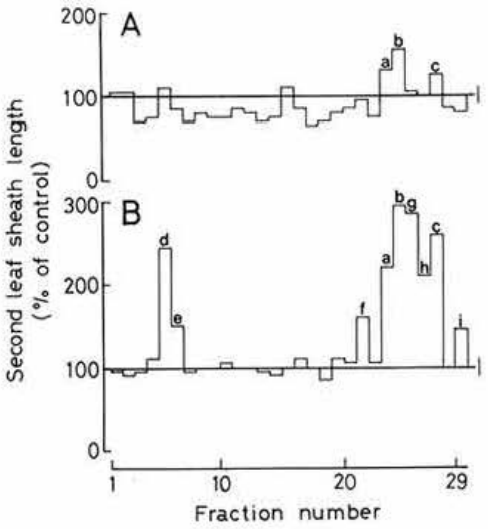

Fig. 5. GA-activity of an extract from taro sprouts tested on the usual (A) and the modified (B) Tan-ginbozu assay
Vertical bars at the right ends of the figure represent SDs of the means of the control plants.

On the other hand, the fractions that had high activities in Tan-ginbozu plants without any significant effect in Waito-C (fractions 11–12 and 24–26) contained C-3-nonhydroxy GAs. Therefore, as in the case with the usual micro-drop method, it is possible to distinguish C-3-nonhydroxy or C-3-hydroxy

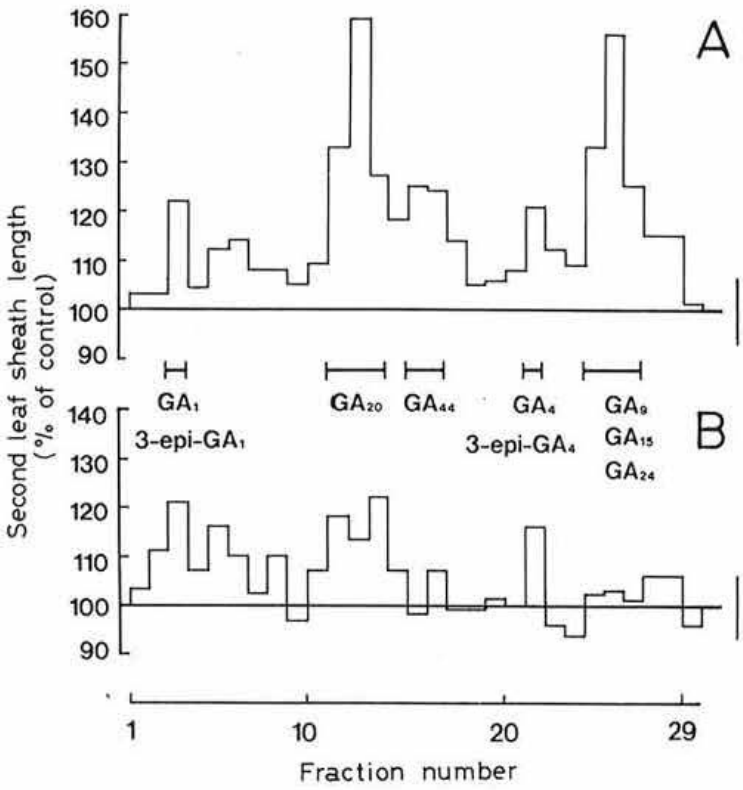

Fig. 6. GA-activity of an extract from radish leaves tested on the usual (A) and the modified (B) Tan-ginbozu assay
Vertical bars at the right ends of the figure represent SDs of the means of the control plants.

GA by the combination of S-3307-treated Tan-ginbozu and Waito-C plants in modified micro-drop assay.

Conclusion

The modified micro-drop assay proposed above proved to be an appropriate method for systematic analysis of GAs because of its simple procedure involved as well as high sensitivity to GAs exceeding that of GC/MS. In addition to the use in combination with GC/MS, the assay could be used alone as an simplified quantification method for GA-like activity because of its much higher reproducibility than that of the usual micro-drop method. In the field of agriculture and horticulture, where GC/MS is not widely used, the modified micro-drop method would be an effective tool for GA-quantification.

In the above studies, uniconazole was used as a GA-biosynthesis inhibitor. However, other inhibitors could be used if they have the same activity as uniconazole does (e.g. paclobutrazole and triapentenol).

References

1) Atzorn, R. & Weiler, E. W. (1981): The immunoassay of gibberellins. 1. Radioimmunoassay for the gibberellins A_1, A_3, A_4, A_7, A_9 and A_{20}. *Planta,* **159**, 1–6.
2) Atzorn, R. & Weiler, E. W. (1981): The immunoassay of gibberellins. 2. Quantification of GA_3, GA_4 and GA_7 by ultra-sensitive solid-phase enzyme immunoassays. *Planta,* **159**, 7–11.
3) Graebe, J. E. & Ropers, H. J. (1978): Gibberellins. *In* Phytohormones and related compounds — a comprehensive treatise, vol. 1. ed. Letham, D. S. et al., Elsevier/North Holland Biomed, Amsterdam, 107–188.

4) Izumi, K. et al. (1984): Effect of a new plant growth retardant (E)-1-(4-chlorophenyl)-4, 4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol (S-3307) on the growth and gibberellin content of rice plants. *Plant Cell Physiol.*, **25**, 611–617.
5) Izumi, K. et al. (1985): Studies of sites of action of a new plant growth retardant (E)-1-(4-chlorophenyl)-4, 4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol (S-3307) and comparative effects of its stereoisomers in a cell-free system from *Cucurbita maxima*. *Plant Cell Physiol.*, **26**, 821–827.
6) Jones, R. L. & Varner, J. E. (1967): The bioassay of gibberellins. *Planta*, **72**, 155–161.
7) Kobayashi, M. et al. (1989): Quantitative analysis of endogenous gibberellins in normal and dwarf cultivars of rice. *Plant Cell Physiol.*, **30**, 963–969.
8) Letham, D. S. et al. (1978): Phytohormones in retrospect. *In* Phytohormones and related compounds — a comprehensive treatise, vol. 1. eds. Letham, D. S. et al., Elsevier/North Holland Biomed, Amsterdam, 1–28.
9) Murakami, Y. (1968): The microdrop method, a new rice seedling test for gibberellins and its use for testing extracts of rice and morning glory. *Bot. Mag.* **79**, 33–43.
10) Murakami, Y. (1968): Gibberellin-like activity of (–)-kaurene, (–)-kauren-19-ol and (–)-kauren-19-oic acid in leaf sheath elongation of 'Tan-ginbozu' dwarf of *Oryza sativa*. *Bot. Mag.*, **81**, 100–102.
11) Murakami, Y. (1972): Dwarfing genes in rice and their relation to gibberellin biosynthesis. *In* Plant growth substances 1970. ed. Carr, D. J., Springer/Berlin, Heidelberg, New York, 166–174.
12) Nichols, P. B. & Paleg, L. G. (1963): A barley endosperm bioassay for gibberellins. *Nature*, **199**, 823–824.
13) Nishijima, T. & Katsura, N. (1989): A modified dwarf rice bioassay for detection of femtomol quantities of gibberellins. *Plant Cell Physiol.*, **30**, 623–627.
14) Oden, P. C. & Heide, O. M. (1988): Detection and identification of gibberellins in extracts of *Begonia* leaves by bioassay and gas chromatography-mass spectrometry. *Physiol. Plant.*, **73**, 445–450.
15) Suzuki, Y. et al. (1981): Seasonal changes of GA_1, GA_{19} and abscisic acid in three rice cultivars. *Plant Cell Physiol.*, **22**, 1085–1093.
16) Weiler, E. W. & Wieczorek, U. (1981): Determination of femtomol quantities of gibberellic acid by radioimmunoassay. *Planta*, **152**, 159–169.
17) Whyte, P. & Luckwill, L. C. (1966): A sensitive bioassay for gibberellins based on retardation of leaf senescence in *Rumex obstifolius* (L.). *Nature*, **210**, 1360.
18) Yokota, T., Murofushi, N. & Takahashi, N. (1980): Extraction, purification, and identification. *In* Encyclopedia of plant physiology new series, vol. 9. ed. McMillan, J., Springer Verlag, Berlin, 133–201.

(Received for publication, June 15, 1990)