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A Rice Lamina Inclination Test—A Micro-quantitative Bioassay for Brassinosteroids

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A rice lamina inclination test that is simple and specific for brassinosteroids was used as a micro-quantitative bioassay for brassinolide **1** and its 6-keto congener, castasterone **2**, in the concentration range of 5×10^{-5} μg/ml to 5×10^{-3} μg/ml, when uniform seedlings of the rice cultivars Arborio J-1 and Nihonbare were selected. A phytohormone, indole-3-acetic acid (IAA), showed similar activity in this bioassay. Its lowest effective concentration, however, was 50 μg/ml, about five orders of magnitude greater than that of brassinolide. Other phytohormones, abscisic acid (ABA) and the cytokinins kinetin and N^6-benzyladenine, inhibited the lamina inclination of rice seedlings. The addition of a cytokinin reduced the promoting effect of brassinolide. Thus, the rice lamina inclination test can be used both as a micro-quantitative bioassay for brassinosteroids and as a method for detecting antibrassinolide compounds.

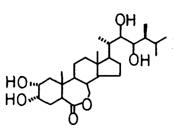
Brassinolide is a new, plant growth-promoting steroidal lactone isolated from rape pollen.[1] Our previous finding that brassinolide **1** has extremely strong activity in the rice lamina inclination test[2,3] has led to the successful isolation of new brassinosteroids from various plant sources by several Japanese research groups. Yokota *et al.*[4] have isolated castasterone **2**, a brassinolide congener with a 6-keto group instead of the ring B lactone (7-oxa-6-one) group in **1**, from insect galls of the chestnut tree. Subsequently, they found two more new brassinosteroids, dolicholide and dolichosterone, in immature seeds of *Dolichos lablab*.[5,6] Abe *et al.*[7~9] have isolated three new brassinosteroids, 28-norbrassinolide, brassinone and (24*S*)-24-ethylbrassinone, as well as brassinolide and castasterone, from immature legumes of Chinese cabbage (*Brassica campestris* var. Pekinensis). They also used the lamina inclination test coupled with micro-analytical GC-MS-SIM technique developed by Ikekawa *et al.*[10] to isolate four known brassinosteroids, castasterone, brassinone, (24*S*)-24-ethylbrassinone and brassinolide, from fresh leaves of tea. Ikeda *et al.* have isolated and identified brassinolide and castasterone from insect galls on the chestnut tree.[11]

The ubiquitousness of brassinolide and its congeners in various higher plants, as noted above, suggests that brassinosteroids, as the sixth type of phytohormone, are important in the regulation of plant growth. As only extremely small amounts of brassinosteroids have been isolated from plants (95 μg of castasterone from 40 kg of insect galls on the chestnut tree[4] and less than 1 μg of a brassinosteroid mixture from 320 kg of Chinese cabbage[7]), a micro-quantitative analytical method is needed to facilitate studies of the physiological function(s) of endogenous plant brassinosteroids.

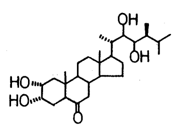
As reported elsewhere,[2] the rice lamina inclination test is extremely sensitive to brassinosteroids; therefore, this bioassay was used to establish a micro-quantitative analytical method for these compounds. We have examined the sensitivity of various rice cultivars to brassinosteroids, to determine which cultivars are suitable for this bioassay and what experimental conditions are necessary for micro-quantitative analysis with selected rice cultivars. The activity of other phytohormones in this bioassay was also examined in relation to the action of brassinosteroids.

MATERIALS AND METHODS

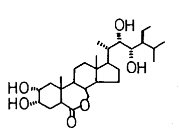
1) *Materials.* Brassinolide **1**, castasterone **2**, 22*S*,23*S*-



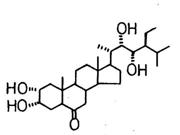
Brassinolide(1)



Castasterone (2)



22S,23S-Homobrassinolide (3)



(22S,23S,24S)-24-Ethylbrassinone (4)

homobrassinolide **3** and (22*S*,23*S*,24*S*)-24-ethylbrassinone **4** were synthesized as previously reported.[3,12] Kinetin, N^6-benzyladenine and (±)-abscisic acid (ABA) were purchased from Sigma. The sixty types of rice cultivars used, which were all harvested in the autumn of 1981, were donated by the Tokyo Metropolitan Agricultural Experiment Station, the Hokkaido Agricultural Experiment Station, and the Prefectural Agricultural Experiment Stations of Nagano, Yamagata, Chiba, Toyama and Saga Prefectures.

2) *Standard method for the micro-quantitative rice lamina inclination test.* After soaking about 1000 seeds of a rice (*Oryza sativa*) cultivar in a 0.25% aqueous solution of Benlate-T (Du Pont) for 48 hr, the seeds were cultivated at 29°C in darkness on a plastic net (17 × 25 cm), floating on distilled water in a plastic case (20 × 30, 20 cm depth). The distilled water was exchanged once on the fourth day of cultivation. Subsequent operations were carried out under the dim red light from a 20 watt fluorescent lamp covered with a red glass plate, except in the final measurement step of the leaf segment angle. Etiolated rice seedlings were grown for 7 days and uniform seedlings were then selected. Leaf segments, which consisted of the second leaf lamina (0.7 cm long) and the second lamina joint and sheath (0.7 cm long), were excised. These segments were floated on distilled water for 24 hr, after which uniformly bent segments were selected. Eight of these segments were incubated in 1 ml of 2.5 mM aqueous dipotassium maleate solution containing a finite amount of the test sample. After incubating for 48 hr at 29°C in darkness, the magnitude of the angle induced between the leaf and sheath was measured.

RESULTS

Selection of rice cultivars suitable for the micro-quantitative bioassay

The rice seedlings used in the micro-quantitative lamina inclination test must be highly sensitive to brassinolide and its related brassinosteroids and must be easy to handle in the bioassay. Therefore, desirable candidate cultivars for the bioassay were selected by two criteria; sensitivity in response to brassinolide and the height of the seedlings (the taller the seedlings, the easier the handling). A preliminary examination of sixty types of rice cultivar was made by the standard method (described in MATERIALS AND METHODS) with 22*S*, 23*S*-homobrassinolide. Eighteen of these cultivars were judged sensitive enough and were examined further with brassinolide and castasterone. The results are shown in Table I.

All eighteen cultivars showed similar responses to brassinolide and to castasterone. They could be divided into two groups based on the magnitude of the bent angle in the control segments. cultivars bent more than 70 degrees are classified in Group 1 and those with an angle of less than 70 degrees, in Group 2. Because the control segments of some cultivars bent more sharply than others, the sensitivity of leaf segments that responded to brassinolide could not be accounted for by the gross angles induced by its action. A reasonable evaluation, however, could be made from the difference between the induced angle produced by brassinolide and that found for the

TABLE I. COMPARATIVE EFFECTS OF BRASSINOLIDE AND CASTASTERONE ON THE LAMINA INCLINATION OF EXCISED SEGMENTS OF EIGHTEEN RICE CULTIVARS

The angles given in the Table are means of values for eight segments per treatment.

Rice variety	Seedlings length (cm)	Angle (degrees) between lamina and sheath							
		Sensitivity*	Control	Brassinolide concentration			Castasterone concentration		
				0.005	0.01 (μg/ml)	0.025	0.01	0.025 (μg/ml)	0.05
Group 1									
1. Arborio J-1	14.0	84	78	162	168	174	158	170	172
2. Kinmaze	6.0	50	86	136	138	151	131	129	139
3. Reiho	9.0	39	78	117	125	129	126	127	133
4. Shinanokogane	6.5	35	81	116	121	121	117	127	131
5. Misuzumochi	6.5	31	80	111	121	119	115	111	119
6. Sasanishiki	6.0	25	91	116	126	127	121	133	134
Group 2									
7. Horyu	7.0	91	43	134	137	139	132	138	138
8. Honenwase	11.0	89	26	115	111	111	118	122	125
9. Himenomochi	7.5	73	28	101	108	111	92	106	109
10. Kiyokaze	4.5	71	56	128	128	132	127	123	130
11. Todorokiwase	8.5	68	33	101	99	101	98	105	113
12. Koshijiwase	9.0	64	65	129	132	135	106	121	119
13. Nihonbare	9.0	60	48	108	111	119	98	101	114
14. Shintaishomochi	7.5	58	32	90	96	111	95	103	90
15. Tachikaze	8.0	57	56	113	114	112	115	110	116
16. Fukuhonami	5.5	55	38	93	96	108	80	93	82
17. Nishihomare	14.0	54	69	123	133	128	115	120	125
18. Shiokari	6.5	40	65	105	110	113	105	113	109

* Sensitivity is the difference between the angles induced by 0.005 μg/ml of brassinolide and the angles found for the controls.

control segments.

The rice cultivars in Table 1 are arranged in the order of the difference between the angle induced by a 0.005 μg/ml solution of brassinolide and the angle of the control. The cultivar, Horyu, had the largest value (91°), indicating that it was the most sensitive to brassinolide. In Group 1, an Italian cultivar, Arborio J-1, had a large value (84°). Moreover, seedlings of Arborio J-1 grew tallest; therefore, it was easiest to cut segments from these seedlings. Seedlings of Arborio J-1 (Group 1) and Horyu (Group 2) therefore were tested in the micro-quantitative bioassay of brassinosteroids. Other cultivars, Himenomochi, Todorokiwase, Koshijiwase and Nihonbare, were also tested further because these cultivars were not highly sensitive to brassinolide but had tall and healthy seedlings.

A 1.4 cm segment length was used for the micro-quantitative bioassay as described in MATERIALS AND METHODS, because the length of a leaf segment had to be less than 2 cm in order to dip into 1 ml of the sample solution. Healthy, straight and uniformly grown rice seedlings were selected, and leaf segments cut from them. These segments became inclined at the same angle due to the action of the brassinosteroids. Horyu, the most sensitive to brassinolide, was excluded from the experiment because it did not produce uniform seedlings. From the results of this examination, two cultivars were finally selected; Arborio J-1, the second most sensitive to brassinolide, and Nihonbare which was not highly sensitive to brassinolide but had healthy and uniform seedlings in addition to high germination.

Dose-response of brassinolide and castasterone in the rice lamina inclination test

The correlation between the induced angles of leaf segments and the concentrations of brassinolide and castasterone was determined using Arborio J-1 and Nihonbare (Fig. 1). In the tests with both cultivars, a linear correlation was obtained between 5×10^{-3} and $5 \times 10^{-5}\,\mu$/ml for each of the two brassinosteroids. The induced angles leveled off at concentrations of $5 \times 10^{-3}\,\mu$g/ml and more. These cultivars, therefore, can be used for a micro-quantitative bioassay of brassinosteroids in the appropriate concentration range.

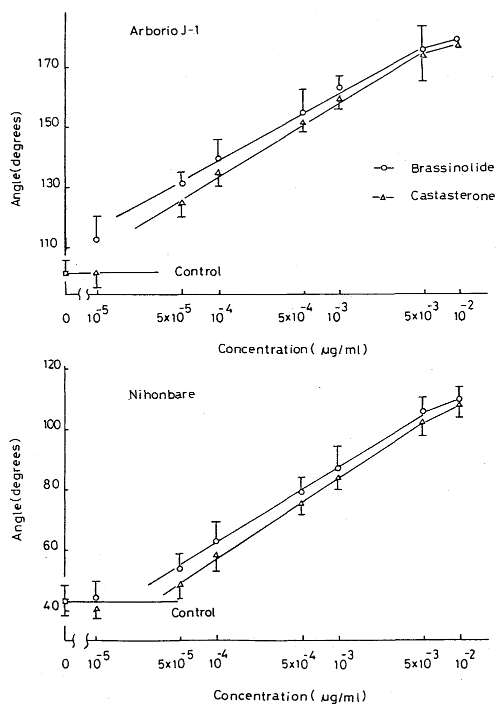


FIG. 1. Correlations between the Angle of Leaf Segments and the Concentration of Brassinolide or Castasterone in the Lamina Inclination Test with Rice Seedlings of Arborio J-1 and Nihonbare.

The mean angles of the control segments were $101° \pm 4.9$ (Arborio J-1) and $43° \pm 6.1$ (Nihonbare). The vertical bar represents the degree of standard error for the angle.

Effect of phytohormones on the lamina inclination of rice leaf segments

The effect of indole-3-acetic acid (IAA) on the rice lamina inclination of leaf segments was examined; the results are summarized in Table II. Its effect was weaker than that of brassinolide by about five orders of magnitude, and no synergistic effect was observed for the two compounds. In contrast, IAA has been reported to be synergistic to brassinolide in rice lamina inclination when intact seedlings of dwarf rice, *Oryza sativa* var. Tan-ginbozu and Waito-C, were used.[13] Gibberellin (GA_3) has been reported to have a slight effect at a concentration of 100 μg/ml.[14]

Kinetin and N^6-benzyladenine were tested in the concentration range of 0.001 to 25 μg/ml; the results are shown in Fig. 2. Both compounds had negative activity in this bioassay; weak inhibition of the lamina inclination was present at a relative high concentration (5 μg/ml), but interestingly the cytokinins reduced the promotive effect of brassinolide on lamina inclination even at a low concentration of 0.01 μg/ml in Nihonbare and 0.1 μg/ml in Arborio J-1. This suggests that kinetin and N^6-benzyladenine might act as antibrassinolide compounds in this bioassay.

Abscisic acid (ABA) had a rather strong lamina inclination–inhibitory activity at 10 and 100 μg/ml (Fig. 3). It reduced the promotive effect of brassinolide at the same

TABLE II. EFFECT OF IAA, ALONE AND IN COMBINATION WITH BRASSINOLIDE, ON THE LAMINA INCLINATION OF RICE SEEDLINGS (Nihonbare)

The angles given in the table are means of values for eight segments per treatment.

Concentration of IAA (μg/ml)	Angle (degrees) of segments (±standard error) Brassinolide (μg/ml)			
	0	0.00001	0.0001	0.001
0	41 ± 3.3	50 ± 6.3	88 ± 9.0	101 ± 5.8
1	43 ± 4.1	53 ± 7.0	83 ± 5.0	104 ± 5.4
10	46 ± 5.9	66 ± 5.4	81 ± 7.8	101 ± 5.1
50	66 ± 6.8	66 ± 4.1	83 ± 5.6	102 ± 4.3

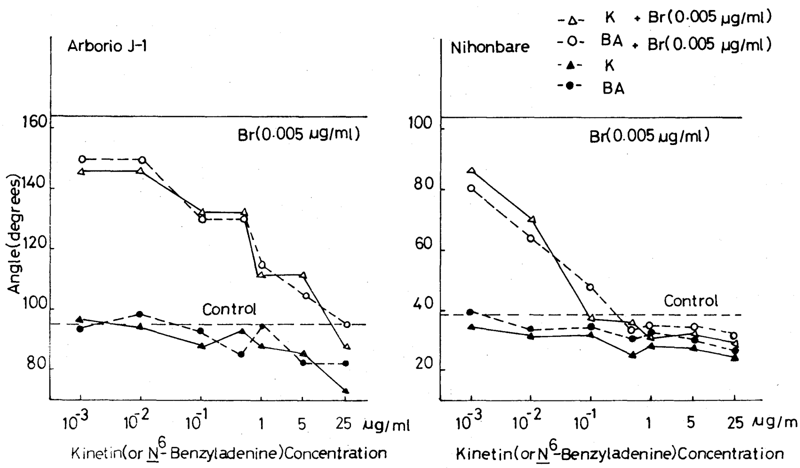


FIG. 2. Effects of Brassinolide (Br) with Kinetin (K), N^6-Benzyladenine (BA), or the Cytokinins Only, on the Lamina Inclination of Rice Leaf Segments.

The upper horizontal bar shows the angle induced by brassinolide at 0.005 μg/ml and the lower broken bar, the angle of the control.

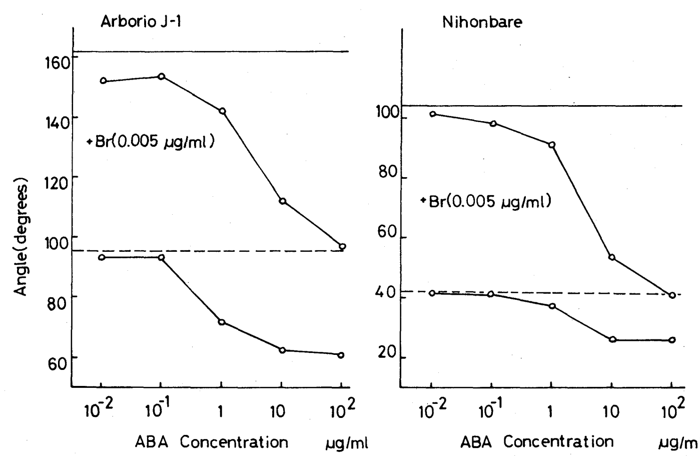


FIG. 3. Effect of Abscisic Acid (ABA) and Brassinolide Alone, or in Combination, on the Lamina Inclination of Rice Leaf Segments.

The upper horizontal bar shows the angle induced by brassinolide at 0.005 μg/ml and the lower broken bar, the angle of the control.

concentration.

Our results show that this test can be used as an antibrassinosteroid bioassay as well as a highly specific brassinosteroid bioassay.

Lamina inclination-promoting activity of castasterone and its related 6-keto-brassinosteroids

In the rice lamina inclination test, castasterone has activity similar to that of brassinolide,[4,15] and several synthetic 6-keto-brassinosteroids also have shown a rather strong activity.[15] These results raise some intriguing questions. Is castasterone a biosynthetic precursor that is converted into brassinolide in plants? And, do castasterone and its related 6-ketosteroids directly affect the active site of the rice lamina joint without being

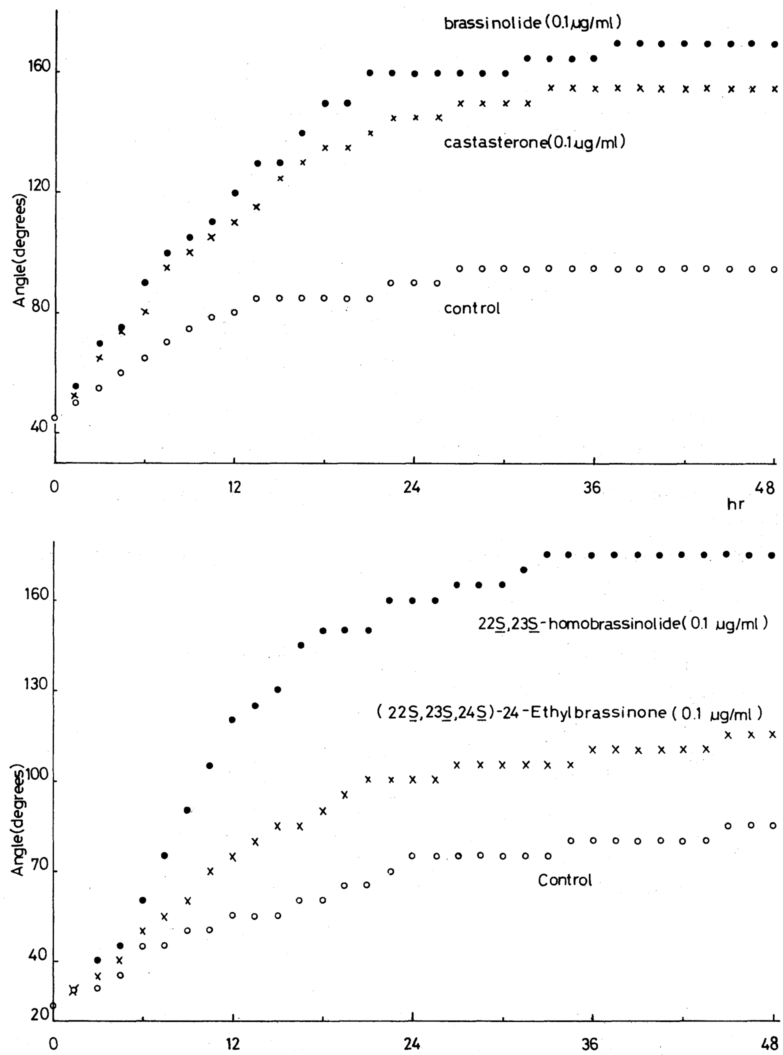


FIG. 4. Time Course of Lamina Inclination Induced by Brassinolide and Its Related Steroids.
Angles of the leaf segments (Arborio J-1) were measured every 1.5 hr.

converted to brassinolide or a corresponding lactone?

Castasterone might possibly be a biosynthetic precursor of brassinolide in plants because its 6-keto group can be converted to the corresponding lactone by a single Baeyer-Villiger oxidation. The actual main brassinosteroid contained in two plant species, the chestnut[4)] and tea,[8)] however, has been identified as castasterone, and this 6-ketosteroid itself may have one or more physiological functions in these plants.

To answer the second question, the time courses of rice lamina inclination induced by castasterone and by brassinolide were compared; the results are shown in Fig. 4. The degree of the angles induced by both compounds increased linearly during the initial 24 hr incubation, then gradually leveled off. Castasterone showed no time lapse for its activity when compared with brassinolide. The time courses of rice lamina inclination induced by (22*S*, 23*S*, 24*S*)-24-ethylbrassinone **4** and 22*S*, 23*S*-homobrassinolide **3** also were examined (Fig. 4). The angles induced by the two compounds reached maximum values (175 and 115 degrees, respectively) at the same time after a 36 hr incubation There was no delay in the manifestation of activity by the 6-keto-steroid **4** when compared with its lactone analogue. These results suggest that castasterone and its related 6-ketosteroid **4** themselves affect the lamina joint without being converted to brassinolide or compound **3**.

DISCUSSION

Brassinolide activity has been tested in a number of phytohormone bioassy systems by Mandava *et al.*[16,17] They found that responses similar to those for IAA were elicited by brassinolide in some auxin assays. They also found that brassinolide was active in gibberellin bioassays with dwarf pea epicotyl and etiolated bean hypocotyl. These bioassays, however, were neither highly sensitive nor specific to brassinolide.

The bean second internode test, used as a bioassay for the isolation of brassinolide from rape pollen by a USDA group,[1] was highly sensitive to brassinolide; characteristic swelling and splitting were induced on the second internode by the action of brassinolide, although the test was originally developed as a gibberellin bioassay. Brassinosteroids were also tested in a bean first internode bioassay for their effectiveness in stimulating auxin-induced growth and showed remarkable activity at a level of 10 ng.[18,19] Takematsu *et al.* have devised a new brassinolide bioassay, the *Raphanus* test, which is highly sensitive to brassinolide and related steroids.[20,21] This *Raphanus* test has been used for auxins. Recently, Takeno and Pharis[13] reported that brassinolide induced significant bending of the second lamina of intact seedlings of dwarf rice *Oryza sativa* var. Tan-ginbozu and Waito-C at 100 μg/plant and higher dosages.

In our test, the promotion of lamina inclination was enhanced strongly by 0.05 ng/ml (50 pg/ml) of brassinolide, none of the other phytohormones used causing promotion even at concentrations of 10,000 ng/ml. IAA has been reported to be synergistic to brassinolide in several bioassays,[13,16,18,19] but the synergistic effect was not observed in our test. A linear correlation between the angles induced and the concentrations used was obtained in a concentration range of 5×10^{-3} to 5×10^{-5} μg/ml for both brassinolide and castasterone. Thus, the rice lamina inclination test is recommended as highly specific and as a micro-quantitative bioassay for brassinosteroids. This test also can be used as an antibrassinolide bioassay as shown by the results of our experiments with kinetin, N^6-benzyladenine and abscisic acid.

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