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The current status of physiology and biochemistry of brassinosteroids

A review

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Abstract

Brassinolide, first isolated from pollen of rape as a plant growth promoting substance, has been found to be widely distributed in the plant kingdom. Over thirty endogenous analogues, called collectively brassinosteroids, have been isolated and identified. As a new class of plant hormones, brassinosteroids show not only growth promoting activity but also other physiological effects on the growth and development of plants and draw attention as promising chemicals for practical application in agriculture. This review describes the current status of the studies on the natural occurrence, analysis, physiological actions, metabolism and biosynthesis of brassinosteroids.

Abbreviations: ABA = abscisic acid; BR = brassinosteroid; GA = gibberellin; GC-MS = combined gas chromatography-mass spectrometry; GC-SIM = combined gas chromatography-selected ion monitoring; HPLC = high performance liquid chromatography; IAA = indole-3-acetic acid

1. Introduction

The discovery of brassinolide in 1979 gave a clue to the presence of steroidal compounds, having significant growth-promoting activity, in the plant kingdom. The occurrence and distribution of brassinolide and its related compounds in plants have been studied extensively since that time. This group of compounds is collectively called brassinosteroids (BRs). As results of extensive investigations, BRs were found to show characteristic physiological actions on the growth of plants in micro quantities. Therefore, BRs can be regarded as a new class of plant hormones, in addition to auxins, GAs, cytokinins, ABA and ethylene.

The historical background, chemistry and physiology of BRs have been reviewed comprehensively by Mandava [39]. Other, more recent studies on the biochemistry, physiology, practical applications, production, synthesis and entomological effects of BRs were collected in a book published in the American Chemical Society symposium series [15]. This present article mainly describes current studies on physiology and biochemistry of BRs subsequent to the above reviews.

2. Natural occurrence

The first BR isolated from pollen of rape (*Brassica napus* L.) was brassinolide (*1*). Its structure is a steroidal skeleton of 5α-cholestan, characteristic in possessing a 7-oxalactonic B-ring and two vicinal hydroxyls at the A-ring (C2α and C3α) and in the side chain (C22R and C23R).

Extensive investigations on the distribution of brassinolide-like active substances in plants led to them being found not only in pollen but also in insect galls, immature seeds, shoots and leaves of a wide variety of plants. For these studies, a bioassay using the rice lamina-inclination test (described

later), was effectively applied to detect brassinolide-like active substances, mainly by Japanese researchers. Up to now, twenty nine analogues of BRs and two conjugates, shown in Fig. 1, have been identified from monocots, dicots, gymnosperms, a fern and a green alga.

Structural features of these analogues look complicated but they may be classified into two categories. The first category is grouped by the oxidation stages of the steroidal skeleton: 7-oxalactone, 6-ketone and 6-deoxo derivatives; penta-hydroxyls at C1, C2, C3, C22 and C23, tetra-hydroxyls at C2, C3, C22 and C23, tri-hydroxyls at C3, C22 and C23. The second category is grouped by the side chain skeleton: C24S-methyl (*1–11*), C24R-methyl (*12–14*), C24-normethyl (*15*, *16*), C24-methylene (*17–19*), C24S-ethyl (*20*), C24-ethylidene (*21–23*) and C24-methylene-C25-methyl (*24–29*). The structures of the analogues in Fig. 1 were grouped at first by the side chain skeleton and analogues in each group were aligned in order of the oxidation stages. From the distribution of BRs shown in Table 1, brassinolide (*1*) and castasterone (*4*) are the most widely distributed biologically active BRs in the plant kingdom.

All of these BRs are not always biologically active. Structure-activity relationships were discussed in a review [39]. Generally, 7-oxalactone and 6-ketone forms are the most active, while 6-deoxo derivatives are almost inactive. Vicinal hydroxyls at A-ring and the side chain are important for biological activity. Thus, of the BRs identified so far, some are precursors in the biosynthetic pathway or inactivated metabolites; this is discussed in detail in the section on metabolism and biosynthesis.

The results of isolation and identification studies from plants conducted to date have shown the presence of BRs in almost every tissue or organ, such as pollen, seeds, flowers, fruits, shoots and leaves, in variable levels, though their presence in roots has not yet been confirmed. Among them, reproductive organs (pollen and immature seeds) showed higher levels than vegetative tissues. Endogenous BRs in immature seeds of *Phaseolus vulgaris* were thoroughly examined and over twenty analogues, among the twenty nine BRs shown in Fig. 1, were identified [33]. It is also noteworthy that plant cell cultures were found to produce BRs [48]. Park et al. [42] identified brassinolide (*1*) and castasterone (*4*) from the crown gall cells of *Catharanthus roseus*, their contents being at levels comparable to that in pollen or immature seeds.

3. Analysis of brassinosteroids

Detection, identification and quantification of BRs in plant tissues or organs are quite important for investigating their occurrence, biosynthesis and metabolism. Bioassays, physico-chemical analyses and immunological detections of BRs have been developed since their discovery, and microanalytical methods have been reviewed by Takatsuto [60].

3.1 Bioassays

Simple biological tests, sensitive and specific to BRs, are quite useful to detect the active principles in plants. From this point of view, the following bioassays are recommended.

The bean second-internodes test was established during the first isolation of brassinolide (*1*) from pollen of rape [22]. Cuttings of the second-internodes from seedlings of *Phaseolus vulgaris* L. cv. Pinto showed elongation, curvature, swelling, or splitting when treated with BRs. These effects depended on the amount of BRs: elongation, curvature and swelling occurred at an application of 0.01 μg of brassinolide, and splitting with 0.1 μg per plant. In this bioassay, GAs just showed an elongation effect; the other effects are specific to BRs.

The rice lamina-inclination test, originally developed as an auxin bioassay, was found to be highly responsive to BRs. From etiolated seedlings of rice, leaf segments consisting of the second leaf lamina and the second lamina joint and sheath were excised and floated on distilled water containing BRs. Bending of the lamina joint was observed proportional to the concentration of compounds applied. Brassinolide (*1*) showed activity at a concentration of 0.0005 μg/ml, but IAA showed only weak activity at 50 μg/ml [69]. Because of such great difference in dose response between BRs and IAA, this test may be considered specific to BRs and has been widely used to investigate the occurrence of BR-like active substances in plant extracts. However, the test gives inaccurate results when the extracts contain highly active auxins. For example, Park et al. [43] identified both 4-chloro-IAA methyl

ester and BRs from immature seeds of *Vicia faba* as active substances in this test. 4-Chloro-IAA methyl ester showed higher activity than IAA, while free 4-chloro-IAA was inactive in the test. Among the cultivars of rice (*Oryza sativa* L.), Arborio J-1, Nihonbare and Koshihikari are known to be sensitive in this test.

Kim et al. [34] developed a simplified rice lamina-inclination test using a dwarf mutant of rice, based on the assay reported by Takeno and Pharis [67]. This method uses intact seedlings of the dwarf rice, cv. Tan-ginbozu, which is known as a GA-deficient mutant, grown in the light. The seedlings were pretreated with IAA 1 h prior to application of BRs. This pretreatment amplified the response to BRs by synergistic effects of IAA. Kim et al. [34] detected the presence of BRs in the cambial region of Scots pine (*Pinus silverstris*) using this bioassay and identified them as brassinolide (*1*) and castasterone (*4*) by GC-MS.

The wheat leaf-unrolling test was developed by Wada et al. [68], as a sensitive bioassay of BRs. Leaf segments were excised from etiolated wheat seedlings (*Triticum aestivum* L. cv. Norin No. 61). The unrolling of the leaf segments was observed after incubating them in 2.5 mM dipotassium maleate buffer containing BRs. Brassinolide (*1*) caused unrolling of the wheat leaf at a concentration of 0.005 μg/ml, while zeatin showed the same effect at 1 μg/ml. Both ABA and IAA inhibited unrolling of the leaf segment.

3.2 GC-MS analysis

Chemical and spectrometric methods are necessary for confirmative identification and quantitative analysis of BRs. Since levels of BRs in plant tissues or organs are usually quite low, GC-MS or GC-SIM are useful analytical methods.

Microanalytical techniques by GC-MS or GC-SIM have been established for BRs [64]. The conversion of BRs into volatile derivatives by methaneboronation of vicinal hydroxyl groups, characteristic in BRs, is now effective and widely used in analyses by GC, GC-SIM and GC-MS. By treatment with methaneboronic acid and pyridine, BR analogues with two vicinal hydroxyls are derivatized to bismethaneboronates. These derivatives give characteristic fragmentation patterns on mass spectrometry, with a molecular ion and other ions resulting from fragmentation of the side chain by electron impact (EI). Table 2 shows the retention times in GC analysis and characteristic ions of bismethaneboronate of representative BRs.

Based on this mass spectrometric analysis, GC-MS or GC-SIM techniques have been applied successfully to detect and identify micro quantities of BRs in plants. GC-MS by tandem MS (GC-MS-MS) is highly sensitive and gives unambiguous identifications of BRs. Plattner et al. [44] identified brassinolide (*1*) and castasterone (*4*) in the pollen of European alder (*Alnus glutinosa*) by application of this sophisticated technique.

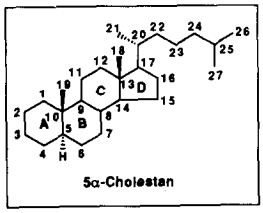
Quantitative analysis of BRs in plants requires the use of appropriate internal standards. Deutero-labelled BRs (e.g. d6-brassinolide, d6-castasterone, d6-typhasterol and d6-teasterone) have been synthesized [62] and are now available as internal standards for quantitative GC-SIM analysis.

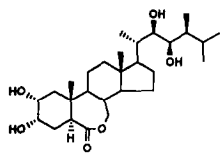
3.3 HPLC analysis

HPLC has been effectively applied to purify BRs from plant extracts. However, detection of intact BRs by HPLC in micro quantities is difficult, because BRs do not have any chromophore to give a characteristic absorption spectrum in detection systems.

Takatsuto and Gamoh developed the methods of prelabelling BRs with fluorescent reagents and tried to detect them in micro quantities by HPLC with a fluorescence detector. As prelabelling reagents, they developed boronic acid derivatives having fluorophores, such as 9-phenanthreneboronic acid [18], 1-cyanoisoindole-2-*m*-phenylboronic acid [20] and dansylaminophenylboronic acid [17]. The prelabelled BRs, using these reagents, were separated effectively by HPLC on a reversed phase column and detected using a fluorometric detector at levels of 20–50 pg. Among the reagents, dansylaminophenylboronate was the most promising, because BRs prelabelled using this reagent were detected at longer wave lengths (Ex 345 nm/Em 515 nm) than with the other derivatives.

Takatsuto et al. [63] showed the usefulness of this HPLC technique by the identification of BRs in buckwheat pollen. After the successive purification of pollen extracts using charcoal chromatography,

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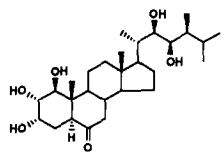
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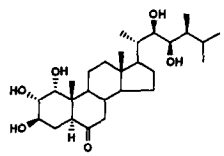
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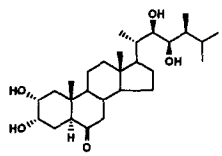
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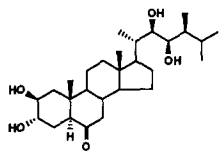
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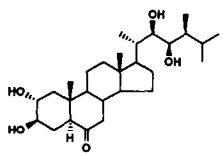
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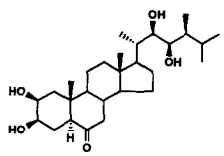
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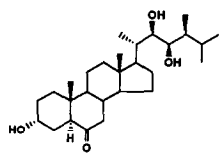
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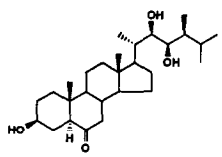
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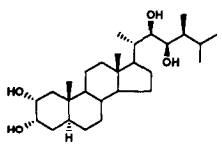
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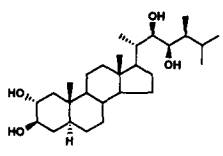
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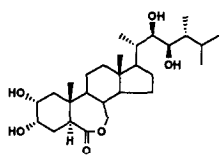
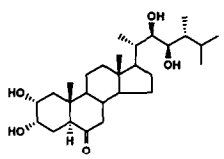

12. 24-Epibrassinolide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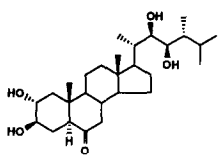
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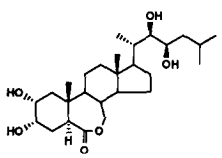
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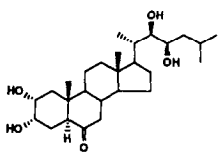
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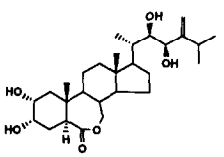
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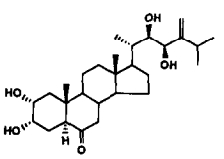
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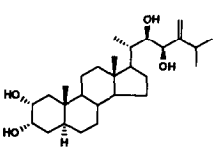
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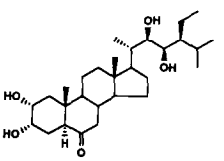
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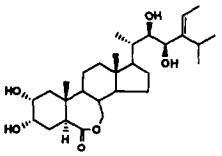
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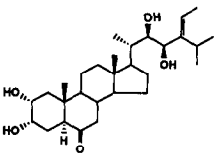
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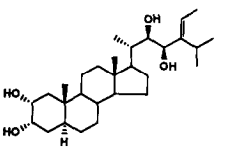
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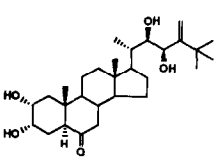
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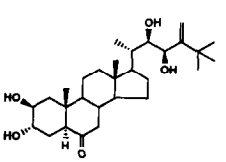
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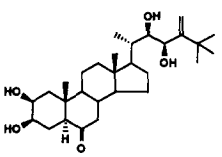
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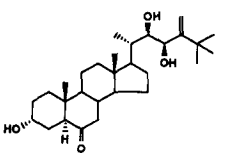
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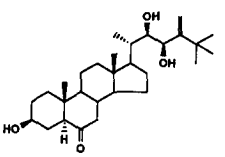
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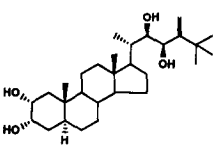
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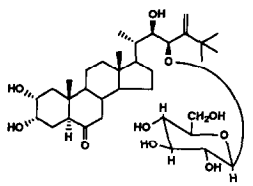
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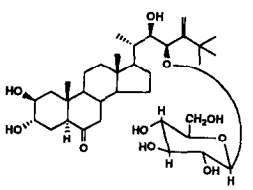
31. 23-O-β-D-Glucopyranosyl-2-epi-25-methyldolichosterone

Fig. 1. Structures of brassinosteroids found in plants.

Table 1. Distribution of brassinosteroids in the plant kingdom

Plant species	Plant parts	Brassinosteroids	References
Monocot			
Typha latifolia	pollen	*8, 9*	1, 57
Oryza sativa	shoot	*4, 18*	5
Zea mays	pollen	*4, 8, 9*	59
Lilium longiflorum	pollen	*1, 4, 8*	1
Tulipa gesneriana	pollen	*8*	1
Dicot			
Brassica napus	pollen	*1*	22
Brassica campestris	seed, sheath	*1, 4, 15, 16, 20*	2, 4
Raphanus sativus	seed	*1, 4*	56
Distylium racemosum	gall, leaf	*1, 4, 15, 16*	4, 26
Dolichos lablab	seed	*1, 4, 10, 17–19, 21, 22*	10, 73–75
Vicia faba	seed, pollen	*1, 4, 12, 16*	25, 43
Vigna radiata		*1, 4*	1
Phaseolus vulgaris	seed	*1–11, 13, 14, 17–19, 23–31*	33, 36, 78–80
Citrus unshiu	pollen	*1, 4, 8, 9*	1
Thea sinensis	leaf	*1, 4, 8, 9, 16, 20*	3, 4, 26, 41
Catharanthus roseus	cultured cell	*1, 4*	42
Pharbitis purpurea	seed	*4, 16*	58
Helianthus annuus	pollen	*1, 4, 16*	65
Castanea crenata	gall, tissue	*1, 4, 10, 16*	4, 6, 24, 70
Alnus glutinosa	pollen	*1, 4*	44
Fagopyrum esculentum	pollen	*1, 4*	63
Gymnosperm			
Pinus thunbergii	pollen	*4, 8*	72
Pinus silverstris	cambial region	*1, 4*	34
Picea sitchensis	shoot	*4, 8*	71
Green alga			
Hydrodictyon reticulatum	colony	*13, 20*	77
Fern			
Equisetum arvense	strobilus	*4, 15, 16, 18*	61

Table 2. GC retention times and EI-MS fragment ions of brassinosteroid bismethaneboronate derivatives*

Brassinosteroid	Retention time (min)**	m/z (relative intensity %)
6-Deoxodolichosterone (*19*)	11.33	496(38) 481(6) 342(13) 313(100) 273(22) 153(44) 124(93)
6-Deoxocastasterone (*10*)	11.47	498(51) 483(16) 343(7) 313(11) 288(18) 273(100) 205(22) 155(42)
Brassinone (*16*)	12.83	498(93) 483(10) 399(5) 358(21) 329(12) 287(59) 228(22) 141(100)
Dolichosterone (*18*)	13.80	510(22) 495(7) 387(13) 327(48) 207(16) 153(64) 124(93) 82(100)
Castasterone (*4*)	14.07	512(51) 441(7) 399(9) 358(22) 287(29) 207(22) 155(100) 85(57)
24-Ethylbrassinone (*20*)	15.11	526(20) 441(7) 358(17) 287(10) 228(4) 169(100) 109(34) 85(55)
Dolicholide (*17*)	15.53	526(22) 429(7) 403(22) 385(14) 343(51) 189(11) 153(68) 124(93) 82(100)
Homodolichosterone (*22*)	15.53	524(13) 481(98) 411(19) 357(31) 327(46) 287(9) 207(7) 167(100) 81(68)
Brassinolide (*1*)	15.75	528(3) 457(6) 374(32) 345(13) 288(6) 177(59) 155(100) 95(68) 85(76)
Homodolicholide (*21*)	17.48	540(16) 497(98) 427(5) 403(10) 385(16) 343(31) 189(22) 167(98) 138(100)

* From data kindly supplied by Prof. T. Yokota.

** Retention times in GC using a DB-1 column (0.258 mm × 15 m; J&W Scientific) were measured under the following conditions: Oven temperature 170 °C for the first 2 min, ramped to 270 °C at a rate of 32 °C/min, then to 290 °C at a rate of 2 °C/min, and finally held at 290 °C.

HPLC and TLC, the active fraction was prelabelled with 9-phenanthreneboronic acid or dansylaminophenylboronic acid. Brassinolide (*1*) and castasterone (*4*) were identified unambiguously by HPLC of the prelabelled derivatives.

Gamoh et al. [19] also prepared prelabelled BRs with ferroceneboronic acid, which were detected by HPLC with an electrochemical detector. The detection limit was the same as that for the fluorecent derivatives.

3.4 Radioimmunoassay

Immunological techniques of analysis have been developed for microanalysis of plant hormones, and radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs) have been applied successfully in research on plant hormones.

Yokota et al. [83] developed an RIA for BRs by preparing a polyclonal antibody of castasterone (*4*). Antiserum against castasterone was produced by immunizing rabbits with castasterone-carboxymethoxylamine oxime conjugated with bovine serum albumin. The RIA, using this antiserum, could detect 0.3 pM of castasterone and brassinolide (*1*), and showed cross-reactivity with twenty seven analogues of BRs, including synthetic ones. They applied the RIA using this antiserum to examine endogenous BRs in immature seeds and stems of *Phaseolus vulgaris*.

4. Physiological actions on plant growth

The great developments in the synthesis of BRs enabled the compounds to be distributed to investigators, not only for physiological studies but also for practical applications. Thus, intensive studies have been made on the physiological action of BRs using a wide variety of plant assay systems.

The extensive work on physiological effects of BRs, prior to 1988, was reviewed comprehensively by Mandava [39], and he summarized the growth promoting effects as follows: In many systems, BRs interact strongly with auxins, perhaps synergistically. The response of BRs and GAs appears to be both independent and additive. In systems designed to assay for cytokinins, effects of BRs vary. ABA interacts strongly with BRs and prevents BR-induced effects. BRs applied alone and in combination with auxins induce the synthesis of ethylene.

Sasse [51] discussed whether BRs could be considered as plant hormones or not, based on a detailed analysis of their physiological functions. She concluded that BRs constituted a new family of plant hormones, with an independent role in the growth regulation of plants.

In this section, a brief survey concerning current evidence on the physiological functions of BRs in plants is given.

4.1 Elongation effects

Stimulatory effects of BRs on the elongation of seedlings have been observed in many assay systems, such as normal and dwarf pea epicotyls, dwarf bean apical segments, mung bean epicotyls, cucumber hypocotyls, azuki bean epicotyls and sunflower hypocotyls [39].

Katsumi [32] investigated the BR-induced elongation of cucumber (*Cucumis sativus* L. cv. Aonagajibae and cv. Spacemaster) hypocotyls and showed that synergistic interaction between BR and IAA was clearly observed in a sequential treatment with the two hormones. The hypocotyl sections pretreated with brassinolide (*1*) for 2 h responded to IAA. The reverse order treatment is rather inhibitory. This type of interaction was the same as in the case of IAA and GA_4 which is an active GA in the cucumber hypocotyl elongation assay. From these results, both BR and GA seem to sensitize hypocotyl sections for a later response by IAA.

Sasse [50] studied the relationship of BR and auxin using segments from the hook and sub-hook zone of the stem of etiolated *Pisum sativum* L. cv. Victory Freezer seedlings. The sensitivity parameters of brassinolide (*1*) in combination with auxin were analyzed using a PEST (parameter estimation) program. As the results of the analysis show, brassinolide does not depend on auxin as a mediator in the promotion of elongation of younger tissues but it can interact in a very complex manner with auxin. In the elongation of more mature tissue, and in the bending responses, brassinolide probably accelerates the effects of auxin.

Generally, the stimulation of elongation by BRs occurs in the light but not in the dark [39]. Kamuro and Inada [30] examined the effects of light on the growth promoting effects of brassinolide (*1*) using mung bean (*Vigna radiata* L.) epicotyls. The

cuttings, with cotyledons attached, showed elongation of epicotyls in the dark, monochromatic blue light (452 nm) and far-red light (722 nm). Under these conditions, brassinolide did not exhibit any promotion of epicotyl growth. The growth of epicotyls was retarded by white light (400–700 nm) and monochromatic red light (660 nm), and the growth promoting effects of brassinolide were clearly observed under these light regimes. When the cuttings were treated by alternating irradiation with red and far-red light, final red light retarded growth of the epicotyls, and a brassinolide treatment overcame this retardation. These results indicated that the growth of epicotyls involved regulation by phytochrome and that BR action was related to phytochrome-mediated regulation of growth.

4.2 Interactions with other plant hormones

Strong interactions with auxins have been demonstrated in growth promotion by BRs, but BRs did not affect either metabolism or transport of IAA in the first-internode section of *Phaseolus vulgaris* [14].

On the other hand, BRs have been shown to stimulate ethylene production in etiolated mung been hypocotyl segments [8]. This effect was also synergistic with IAA and was confirmed to have resulted from the promotion of *de novo* synthesis of the enzyme ACC synthase, in a manner similar to that caused by IAA. On the other hand, fusicoccin, a fungal metabolite which can mimic certain effects of IAA, was found to be a specific inhibitor of BR-induced ethylene production [9].

BR-induced ethylene production is also affected by light. In contrast to effects on growth promotion, the promotion of ethylene production in etiolated hypocotyls was greatly reduced by exposure to light. Arteca et al. [7] showed that a low irradiation pretreatment for a short period of time (3.7 $mEm^{-2}s^{-2}$, for 15 min) reduced ethylene production of etiolated mung bean segments by BRs. Both IAA-induced ethylene production and synergistic effects of BRs with IAA were only slightly affected by the same preteatment. These findings are quite interesting in relation to effects of light in BRs action and synergism of BRs with IAA. Reduction of BR-induced ethylene production by exposing plants to light may be explained by reduction of endogenous levels of IAA by light, thereby reducing the ability of BR to promote ethylene formation.

Eun et al. [16] examined the effects of brassinolide (*1*) on endogenous levels of IAA and ABA, as well as on ethylene evolution in etiolated squash (*Cucurbita maxima* Duch. cv. Houkou-Aokawaamaguri) hypocotyls. The fresh weight of segments was increased by brassinolide treatments. An increase in the levels of IAA and a tendency towards a decrease in the levels of ABA were also observed in brassinolide-treated tissue. Significant promotion of ethylene evolution by brassinolide, however, was not detected in this system.

4.3 Other physiological effects on plants

Besides effects on growth promotion, many other effects of BRs on plants have been reported, such as enhancing gravitropic-induced bending in bean hypocotyls [40], and retardation of abscission of *Citrus* leaf and fruits explants [27].

Inhibitory effects of BRs on adventitious root development have been observed in mung bean hypocotyls [85] and other assays. Subsequent work reviewed by Roddick et al. [45] indicated that this inhibition might not be a primary action of BRs but rather due to differences in the optimal concentration of BR for adventitious root development and shoot elongation. When soybean (*Glycine max* L. Merr) hypocotyl segments were produced under photoperiods of 16 h light and 8 h dark, adventitious roots were induced by treatment with 24-epibrassinolide (*12*) at the very low concentration of 0.0001 μg/ml during the dark period [53]. Roddick and Ikekawa [46] examined the effects of BR on root development of seedlings and showed that lateral root elongation in wheat, mung bean and maize was reduced by treatment with 24-epibrassinolide (*12*).

Enhancement by BRs of resistance of plants to various external stresses has been evaluated with a view to finding practical applications in agriculture [66]. In maize seedlings, BRs enhanced the greening of etiolated leaves at low temperatures in light and promoted the growth recovery after a chilling treatment [23]. Schilling et al. [54] examined the effects of homobrassinolide on sugar-beet (cv. Ponemo) under drought stress. By treatment with homobrassinolide, an increase of tap-root mass under drought stress and an increase in sucrose content

and sucrose yield were observed; there was no effect under non-stress conditions. Under stress conditions of high temperatures, BRs were shown to activate total protein synthesis in wheat leaves. Kulaeva et al. [37] examined the effects of BRs on protein synthesis in wheat (cv. Saratovoskaya) under normal (23 °C) and heat-shock (40 °C) temperatures. BRs induced *de novo* polypeptide synthesis, and the proteins induced under normal temperature corresponded to heat shock proteins. BRs also stimulated the formation of heat shock granules in the cytoplasm.

Regarding the effects of BRs on the growth of plant cell cultures, variable results have been reported. In cell cultures of carrot (*Daucas carota* L.), BRs induced cell enlargement without any effect on cell division [11, 49]. On the other hand, Roth et al. [47] reported that BRs, at a concentration of 10^{-10} M, acted as a potent inhibitor of the growth of *Agrobacterium tumefaciens* transformed tobacco callus cultures. They speculated that BRs probably could be involved in modulation of endogenous auxin levels via gene regulation of the auxin-synthesis pathway, or by interfering in the catabolism of IAA.

Using experimental systems for studying xylogenesis in mesophyl cells of *Zinnia elegans* L., Iwasaki and Shibaoka [28] showed that BRs promoted tracheary-element (TE) differentiation and that it was inhibited by the synthetic plant-growth retardant, uniconazole, which is known to inhibit GA biosynthesis. The inhibition of TE was not restored by the addition of GA, but was counteracted with BRs. Since exogenously applied brassinolide (*1*) promoted TE differentiation and uniconazole has been reported to reduce the levels of endogenous BRs [81], it might inhibit TE differentiation by inhibiting the synthesis of BRs. These facts suggest that BRs possibly are involved in the induction of TE differentiation.

Using tuber explants of Jerusarem artichoke (*Helianthus tuberosus*), Clouse and Zurek [13] found that brassinolide (*1*) greatly stimulated xylem differentiation in this assay system. Normally, xylem differentiation requires 3 to 4 days after transfer to xylem-inducing medium. However, when brassinolide was added at a concentration of $6.8X10^{-9}$ M, a significant differentiation of xylem was observed after 24 h. This evidence showed the possibility that BRs are involved in cytodifferentiation of plants.

5. Molecular approach to mode of action

Inhibition of BR-induced physiological actions by various inhibitors of RNA synthesis and of protein synthesis has been investigated. The results indicate that the physiological effects of BRs, like those of auxins and GAs, depend upon the synthesis of nucleic acids and cellular proteins [39]. Treatments with BRs have been shown also to increase some enzyme activities, such as RNA and DNA polymerases in beans [29], ATPase activity of bean [12] and cucumber hypocotyls [31]. Enhancement of these enzyme activities may be the result of regulation of gene expression by BRs and may be concerned directly, or indirectly, with growth promotion induced by BRs.

Clouse and Zurek [13] examined the effects of BRs on the transcription of auxin-induced genes in light-grown soybean epicotyl sections. There was a clear difference in the expression of GH1, one of the auxin-induced genes of soybean, by treatment with brassinolide (*1*) for 17 h. This difference in expression may be due to the stability of mRNA, brought about by brassinolide, since no difference was observed 2 h after the addition of brassinolide. The results might explain the synergistic effects of brassinolide with auxin on elongation growth.

The subcellular localization of BRs was examined in pollen of *Brassica napus* and rye-grass (*Lolium perenne*) using immunocytochemical techniques with a polyclonal antibody generated in rabbit against castasterone (*4*). In germinated pollen of *B. napus*, the cytoplasm and nuclei were labelled, with more in the vegetative nucleus than the sperm cells, suggesting that there is a specific binding of BRs to nuclear components. In mature, dried pollen, heavy labelling of starch granules within the amyloplast of *L. perenne* and plastid of *B. napus* were observed. These must be storage organells for BRs. However, no specific binding of BRs to any soluble proteins was found by ELISA assay in the extracts from *L. perenne* pollen [52].

6. Metabolism

Over thirty analogues are now identified as

naturally occurring BRs, which may include biosynthetic precursors, or BR metabolites. It is important for BR studies to identify which are the physiologically active forms and which are inactive metabolites. For example, castasterone (*4*), the 6-ketone form, could be oxidized enzymatically to brassinolide (*1*), the 7-oxalactone form, and brassinolide showed higher physiological activity than castasterone in many assay systems. This fact indicates that the 7-oxalactone, as in brassinolide, is the biologically active form.

When mung bean cuttings were fed with ^{3}H-labelled castasterone (*4*), the absorbed radioactivity was distributed between unchanged castasterone and a water-soluble fraction, after incubation of 72 h. The major component in the water-soluble fraction was found to be non-glycosidic, since the radioactivity in the fraction remained unchanged by hydrolysis with pectolyase. On the other hand, when ^{3}H-labelled brassinolide (*1*) was administrated under the same conditions, 23-*O*-β-*D*-glucopyranosylbrassinolide was identified as a metabolite, together with unchanged brassinolide. The conversion of castasterone to brassinolide was not observed in mung bean [82].

Yokota et al. [76] also examined the transport and metabolism of BRs in rice seedlings. When ^{3}H-labelled brassinolide (*1*), castasterone (*4*) or 24-epibrassinolide (*12*) were fed to roots, they were taken up and translocated to shoots. Much radioactivity was recovered in the unchanged BR fraction after 6 h, and radioactivity in the water-soluble fraction was found to be not in glycosides but in sulphate ester-like compounds. Yokota et al. also examined the metabolism of ^{3}H-labelled castasterone in etiolated rice lamina explants, which were used in the rice lamina-inclination test. A part of the radioactivity was metabolized to polar non-glycosides, different from those of rice seedlings grown under light, and no conversion to brassinolide was detected.

These experiments could not confirm that plant tissue activates castasterone (*4*) by converting it to brassinolide (*1*). In the rice lamina-inclination test, 6-ketone forms such as castasterone, brassinone (*16*) and 24-ethylbrassinone (*20*) are shown to be active *per se*, since there was no difference in the time to show biological activity between those 6-ketone forms and their corresponding 7-oxalactones [1].

Schlagnhaufer and Arteca [55] investigated the uptake and metabolism of exogenously applied BR to tomato (*Lycopersicon esculentum*) plants . The plants were treated with a ^{3}H-labelled 22,23,24-triepibrassinolide, a synthetic analogue of brassinolide, for 24 h and then transferred to a brassinolide-free culture medium. The BR-induced ethylene production decreased under BR-free conditions, and polar metabolites appeared in the recovered radioactive fractions. The results indicated that the plant metabolized brassinolide (*1*) to inactive forms which remain unidentified.

7. Biosynthesis

A hypothetical biosynthetic pathway for brassinolide (*1*) has been proposed as shown in Fig. 2. It is based on biological activity in the rice lamina-inclination test, in which the activity of teasterone (*9*), typhasterol (*8*), castasterone (*4*) and brassinolide increased in this order. Campesterol or its analogues must be the first precursors in the pathway, though campesterol does not show any BR-activity. Anyway, sterols, which exist abundantly in plant tissues, will be oxidized through several steps to yield various analogues of BR.

Immature seeds of *Phaseolus vulgaris* contain various kinds of BRs, with different side chain skeletons. Kim et al. [35] examined plant sterols in the seeds of *P. vulgaris* and found them to contain 24-methylene-25-methyl cholesterol, which is closely correlated biogenetically with 25-methyldolichosterone (*24*), one of the major BRs in the immature seeds of *P. vulgaris*. Major sterols in the immature seeds, however, were compounds with a 24α-ethyl group (sitosterol and stigmasterol) and a 24-ethylidene group (isofucosterol), which together accounted for 88% of the total amount of sterols present. These findings suggest that oxidation reactions leading to BRs are selective for 24-methyl or 24-methylene sterols, which are not major sterols in the immature seeds. Nevertheless, biological conversion of plant sterols, or hypothetical precursors to BRs has not yet succeeded using plant tissues or organs, because of the minute levels of BRs found in plants.

Plant cell cultures of *Catharanthus roseus* were found to produce brassinolide (*1*) and castasterone (*4*) [42]. Their endogenous levels were comparable

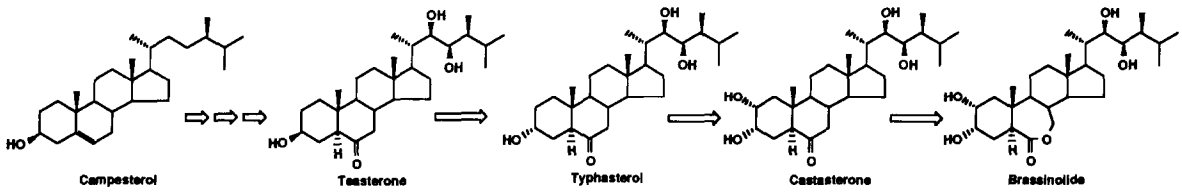

Fig. 2. Hypothetical biosynthetic pathway for brassinolide.

to those of pollen or immature seeds and sufficient for studying biosynthesis of BRs. Cell suspension cultures are suitable systems for feeding labelled compounds to pursue their metabolism, since effective uptake of the compounds may be expected without any problems of translocation.

Yokota et al. [84] confirmed the conversion of castasterone (*4*) to brassinolide (*1*) by *Catharanthus roseus* crown gall cells using ^{3}H-labelled castasterone. Subsequent work using the cell culture of *C. roseus* demonstrated the conversion of teasterone (*9*) to typhasterol (*8*) and the conversion of typhasterol to castasterone by feeding ^{2}H-labelled compounds. This is the first time the proposed biosynthetic pathway (teasterone → typhasterol → castasterone → brassinolide) shown in Fig. 2 has been established by feeding experiments using *in vitro* grown plant cells (the authors' unpublished results).

8. Concluding remarks

The majority of work on the physiological effects of BRs has been done in relation to the plant hormones auxins, GAs, cytokinins, ABA and ethylene, using assay systems for these hormones. Through these studies, evidence has been accumulated concerning their physiological action on plant growth. As a member of the plant hormone groups, BRs show characteristic effects, having strong interactions with auxin and ethylene, and through these interactions, BRs must be involved in endogenous systems which regulate the growth and development of plants.

The discovery of steroidal compounds which have the actions on plants described above really created excitement in the research field of plant growth substances. Studies on specific gene expression caused by BRs, receptors, and biosynthesis and metabolism of BRs, in relation to the other plant hormones, are expected to bring forth new approaches for understanding the endogenous regulation systems of plants at the molecular level. There is also the possibility of similar molecular mechanisms being involved in effects of steroidal compounds in plant and in animals. From this point of view, it is noteworthy that BRs showed antiecdysteroid activity in insects [38] and that they promote the growth of certain species of fungi [21].

Extensive work has been conducted concerning the practical application of BRs in agriculture. Effectiveness in increasing yields of crops and enhancement of stress resistance of crops against drought, chilling and agricultural chemicals has been reported. There are a number of patents or reports describing possible practical uses of BRs. In most cases it is difficult to evaluate their feasibility, and the field trials currently under consideration are not discussed.

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