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BRASSINOSTEROIDS: Essential Regulators of Plant Growth and Development

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ABSTRACT

Brassinosteroids (BRs) are growth-promoting natural products found at low levels in pollen, seeds, and young vegetative tissues throughout the plant kingdom. Detailed studies of BR biosynthesis and metabolism, coupled with the recent identification of BR-insensitive and BR-deficient mutants, has greatly expanded our view of steroids as signals controlling plant growth and development. This review examines the microchemical and molecular genetic analyses that have provided convincing evidence for an essential role of BRs in diverse developmental programs, including cell expansion, vascular differentiation, etiolation, and reproductive development. Recent advances relevant to the molecular mechanisms of BR-regulated gene expression and BR signal transduction are also discussed.

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INTRODUCTION

Brassinosteroid (BR) research began nearly thirty years ago when Mitchell et al (73) reported that organic extracts of *Brassica napus* pollen promoted stem elongation and cell division in plants. The announcement that the active component of these extracts was a unique steroid (46) stimulated international research interest on the chemistry and physiology of these very potent plant growth regulators, and by 1988 (when the first BR review appeared in this series; 66), over 100 publications on BR-related topics were available. Survey of this literature (66) led Mandava to conclude that "BRs may thus be regarded as a new group of plant hormones with a regulatory function in cell elongation and cell division . . .." Although the case for BRs as endogenous plant growth regulators was strong (95), there was not widespread acceptance of BRs as hormones and little or no attention was paid to these steroids in botany textbooks or general reviews of plant development.

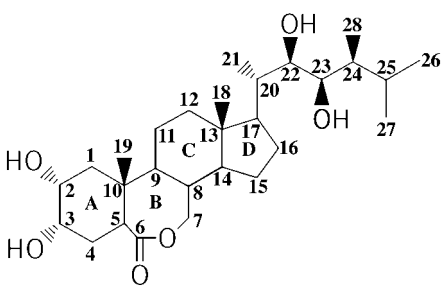
Recent application of molecular genetics to the analysis of BR biosynthesis and signal transduction (24, 56, 63, 64, 79, 107) has confirmed predictions of chemists and physiologists that BRs are essential for normal plant growth and must be considered along with auxins, cytokinins, gibberellins, abscisic acid, and ethylene in any model of plant development. A proliferation of short reviews and reports has drawn attention to these developments and prompted renewed general interest in plant steroids as signaling molecules (21, 22, 39, 52, 87, 132). BR research is moving rapidly along three convergent lines of analysis. Microchemical techniques are revealing the details of biosynthesis, distribution, and metabolism; the analysis of BR-deficient and BR-insensitive mutants is helping to clarify the physiological roles of BRs in growth and development; and the cloning of BR-regulated genes is providing insight into the molecular mechanism of plant steroid hormone action. This review focuses on recent advances in these three areas.

OCCURRENCE AND LOCALIZATION OF BRs

Distribution and Endogenous Levels

The first BR, brassinolide, (22*R*,23*R*,24*S*)-2α,3α,22,23-tetrahydroxy-24-methyl-B-homo-7-oxa-5α-cholestan-6-one (Figure 1), was isolated from pollen of *Brassica napus* (46), and over forty related compounds have now been identified in plant extracts. All are hydroxylated derivatives of cholestane and, given the possibilities of combinations of substructures in rings A and B and the side chain, the family probably has many more members. The compounds can be classified as C_{27}, C_{28}, or C_{29} BRs, depending on the alkyl-substitution pattern of the side chain (132). Their individual structures have been illustrated in several reviews, and Fujioka & Sakurai (39) include an extensive list of the families, genera, species, and plant organs from which they have been rigorously identified, and their occurrence in another five families has been summarized (96). BRs have been identified in an alga and a pteridophyte, and three families of gymnosperms; in angiosperms, they have been shown to occur in 16 families of dicots, and 5 of monocots. So BRs are probably ubiquitous in the plant kingdom, and they certainly occur in shoots and seeds of the important experimental plant *Arabidopsis thaliana* (36, 101).

Levels of endogenous BRs vary among plant tissues, and so do the suites of congeners accompanying the most active members of the family. Seeds of *Phaseolus vulgaris* contain a wide array of BRs (134), as does pollen from *Cupressus arizonica* (45) where concentrations of congeners can be ~6000-fold greater than the concentration of brassinolide. BRs occur endogenously



Brassinolide

Figure 1 The structure of brassinolide, a commonly occurring BR with high biological activity, showing *numbered positions* mentioned in the text. In natural BRs, hydroxylation can occur in *ring A* at positions 3-, and/or 2-, and/or 1-; also found are epoxidation at 2,3-, or a 3-oxo-group. In *ring B*, alternatives are 6-oxo- and 6-deoxo- forms. In the side chain methyl-, ethyl-, methylene-, ethylidene-, or *nor*- alkyl groups can occur at 24-, and the 25-methyl- series is also represented.

at quite low levels. Pollen, the original source of brassinolide, and immature seeds are the richest sources with ranges of 1–100 ng $\cdot$ g^{-1} fw, while shoots and leaves usually have lower amounts of 0.01–0.1 ng $\cdot$ g^{-1} fw (110, 137). Cultured crown gall cells of *Catharanthus roseus* have levels of brassinolide and castasterone (30 ng $\cdot$ g^{-1} fw) equivalent to that of pollen (81). In general, young growing tissues contain higher levels of BRs than mature tissues (110), which is not surprising considering that BRs show greater physiological effect on immature vs older tissue (66). While bioassay results suggest that root tissue contains BRs, and that etiolated stem tissue is also comparatively rich in them, they have yet to be identified and quantified rigorously (JM Sasse, unpublished data).

Methods of Analysis

The extraction and purification of BRs depend on solvent partitioning and subsequent chromatographic separations. The choice and sequence of these may differ, with later fractionation often guided by bioassay. The most frequently used is the rice lamina inclination test (119). Analysis of purified fractions is mostly done by GC-MS and GC-MS-SIM (reviewed in 4, 110). FAB-MS is successful with pure BRs (13, 99) but not with partially purified extracts (99). LC-MS methods are also being explored (41–43) and were found useful for the detection of epimerization (77) and identification of teasterone esters (8). Direct analysis of BRs with LC and electrospray techniques of MS is promising (60) and (PG Griffiths, JM Sasse, DW Cameron & G Currie, unpublished data).

Assay by liquid chromatography, with UV, fluorescence, or electrochemical detection of derivatives, is also a sensitive method (reviewed in 43). Fluorescence detection at long wavelengths is particularly useful for checking fractions from small samples of plant tissue. However, derivatization can be difficult in very dilute solutions, and conditions must be adjusted when very small-scale extractions and fractionations are attempted (PG Griffiths, JM Sasse & DW Cameron, unpublished data). For accurate quantitation and calculation of losses, internal standards include appropriate d-labeled BRs in MS, and selected "spikes" in LC analysis.

Identification of the conformations of BRs may be assisted by crystallographic (62) and NMR studies (reviewed in 4), and such knowledge will be useful in receptor studies, and in understanding structure/activity relationships. These relationships have been examined by several groups, and while some differences were noted between bioassays, the 7-oxalactone and 6-keto forms were generally the most active, with distinct effects of the hydroxylation patterns in ring A and the side chain, and of the alkylation pattern of the side chain (66, 84, 123, 124, 135, 140). A computer analysis of interatomic distances in energy-minimized structures of various BR structures showed that the distances

between C_{16} on the ring and the C_{22}, C_{23}, C_{24}, and C_{28} carbons as well as the O_{22} and O_{23} oxygens, were critical for optimal activity, suggesting that the overall dimensions of the side chain may be as important as the configuration at the individual chiral carbons (70).

A quantitative structure/activity study has begun (12), and the finding of high activity with inversion of the 2- and 3-hydroxyl groups together with a *cis* A/B ring junction is interesting—this is the arrangement in ecdysones. However, 20-hydroxyecdysone, alone or together with brassinolide, does not affect the rice lamina bending assay (JM Sasse, unpublished data) confirming early work with ecdysones in other assays (26, 125) and suggesting the lamina inclination assay can discriminate between patterns of substitution in the side chain, even if conformations in the ring A region permit activity.

BIOSYNTHESIS AND METABOLISM

Early and Late C_6 Oxidation Pathways

A detailed understanding of how endogenous BR levels are regulated via synthesis, breakdown, and conjugation is an essential component of a molecular model of BR action. A coordinated effort by several Japanese groups has led to rapid progress in the elucidation of the biosynthetic pathways leading to BRs in plant cell cultures and seedlings (reviewed in 38, 39, 132). Campesterol was predicted to be the plant sterol progenitor of brassinolide based on side chain structure; and the relative biological activities, co-occurrence, and molecular structure of teasterone, typhasterol, and castasterone suggested that brassinolide was synthesized from campesterol through these intermediates (136).

The reduction of campesterol to campestanol and the oxidation of campestanol to 6-oxocampestanol (Figure 2) has been demonstrated by feeding experiments (39), and the hydroxylation of 6-oxocampestanol to cathasterone is presumed, but direct demonstration of this step by feeding experiments has not been accomplished, possibly because the endogenous pool of cathasterone is 500-fold less than that of 6-oxocampestanol (37). The large difference in pool sizes suggests that this conversion may be the rate-limiting step in brassinolide biosynthesis. Conversion of cathasterone to brassinolide via the intermediates shown in Figure 3 has been demonstrated by feeding experiments (39). In the final step, some differences were seen in seedlings; castasterone was converted to brassinolide in *Catharanthus roseus*, but not in tobacco and rice (106). However, since brassinolide and castasterone co-occur as endogenous BRs in rice seedlings, it is likely the full pathway is operational in this plant as well and that the exogenous labeled castasterone used in the feeding experiments may not have reached the site of brassinolide synthesis in the rice seedlings (106). The conversion of teasterone to brassinolide did occur in lily cells (8), and

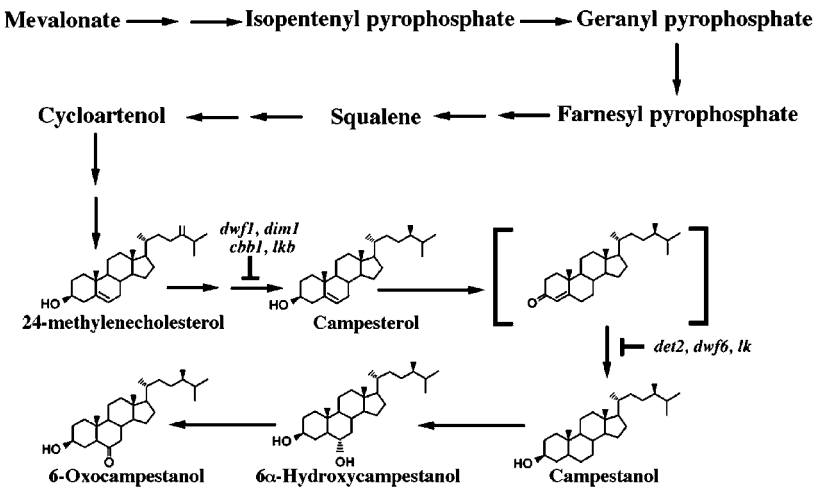


Figure 2 Biosynthesis of early members of the BR biosynthetic pathway via mevalonate and the isoprenoid pathway. Campesterol is a bulk sterol, also found in membranes, while campestanol and later derivatives are considered committed to BR biosynthesis. Proposed blocks in BR biosynthesis in Arabidopsis (*dwf*1, *dim*1, *cbb*1; *det*2, *dwf*6) and pea (*lkb*, *lk*) mutants are indicated. The structure in *brackets* is a probable intermediate based on molecular genetic and biochemical studies (62a).

the co-occurrence of teasterone, typhasterol, castasterone, and brassinolide in at least four other species (*Phaseolus vulgaris*, *Lilium elegans*, *Citrus unshiu*, and *Thea sinensis*) suggests that the complete pathway is widespread in plants (106).

C_6-oxidation is a very early step in this pathway (Figure 2). However, appreciable quantities of 6-deoxocastasterone have frequently been identified in extracts of BRs, and when other 6-deoxo-congeners were discovered, these compounds were also proposed as precursors to BRs (45, 90), and this has now been confirmed (17, 18). Thus, "early C_6-oxidation" and "late C_6-oxidation" pathways for the biosynthesis of BRs coexist in cultured cells of *Catharanthus roseus*, and in seedlings of tobacco and rice. Representatives from both pathways co-occur in many plants, e.g. Arabidopsis (36), so both could be widespread in the plant kingdom. The multiplicity of substitution patterns in the 2- and 3-positions of BRs, and in the alkyl substituents of the side chain, imply even more pathways and interconnections, and these remain to be elucidated, as does the detailed enzymology. Determining the specificity of the enzymes involved will assist our understanding, and the contribution of BR biosynthetic mutants has already been significant.

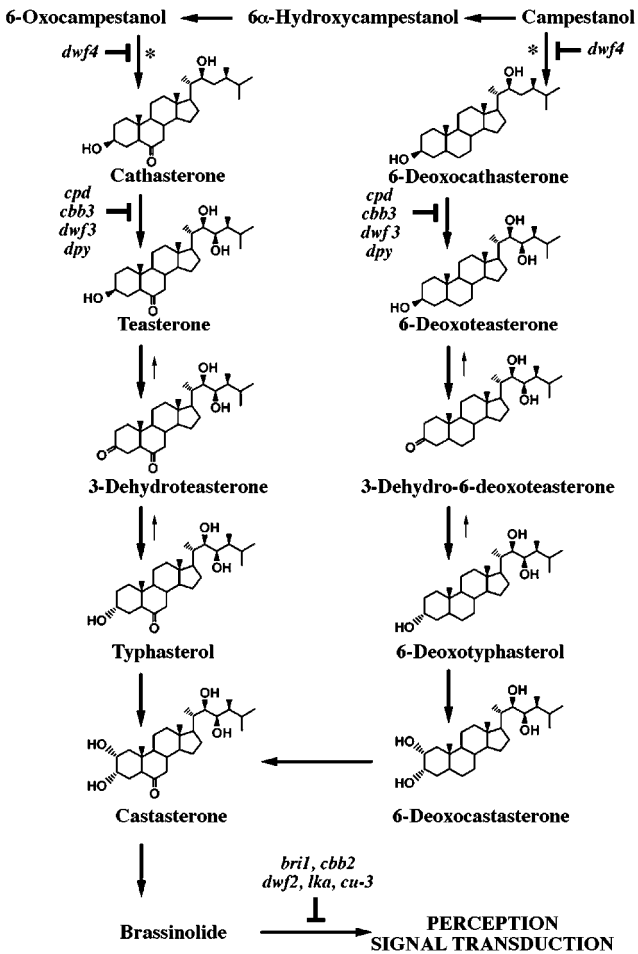


Figure 3 Biosynthesis of brassinolide via the early C_6 oxidation pathway (*left*) and the late C_6 oxidation pathway (*right*). Both pathways can occur together in the same plant. Steps marked by * have not been confirmed by feeding experiments. Putative assignments of biosynthetic and insensitive mutants in Arabidopsis (*dwf*4; *cpd*, *cbb*3, *dwf*3; *bri*1, *cbb*2, *dwf*2), pea (*lka*), and tomato (*dpy*, *cu*-3) are indicated.

BR Biosynthetic Mutants

In the past two years, several dwarf mutants, isolated during screens for physiological processes apparently unrelated to BRs, have been shown in fact to be lesions in genes encoding BR biosynthetic enzymes. Perhaps the best characterized of these is *det*2, a de-etiolated Arabidopsis mutant that was originally proposed to be a negative regulator of photomorphogenesis since it shows characteristics of light-grown plants even when grown in the dark (20). Sequence analysis of the *DET*2 gene showed considerable identity (38–42% overall, 80% in conserved regions) with mammalian steroid 5α-reductases that catalyze the reduction of 3-oxo,$\Delta^{4,5}$ steroids such as testosterone (64). A similar reduction in BR biosynthesis occurs between campesterol and campestanol, and examination of endogenous levels showed that campestanol was much reduced in the *det*2 mutant when compared with wild type (91).

Evidence that *DET*2 encodes a biosynthetic enzyme comes from the fact that BRs, but not other growth regulators, rescued the Det2 phenotype to wild type, as did the transformation of the mutant with human 5α-reductases driven by the CaMV 35S promoter (62a, 64). Rescue by the human genes did not require BR application and was prevented by specific inhibitors of the mammalian 5α-reductases (62a). Moreover, recombinant DET2 protein expressed in human embryonic kidney cells was able to reduce several 3-oxo,$\Delta^{4,5}$ mammalian steroids, including testosterone and progesterone, but failed to reduce 3β-hydroxy,$\Delta^{5,6}$ steroids such as cholesterol (62a). These experiments provide convincing evidence that the DET2 enzyme performs the same function as human steroid 5α-reductases. However, campesterol is a 3β-hydroxy,$\Delta^{5,6}$ steroid, implying that plants possess an additional enzyme that converts campesterol to its 3-oxo,$\Delta^{4,5,}$ isomer before reduction by the DET2 5α-reductase. In mammals, a membrane-bound 3β-hydroxysteroid dehydrogenase/$\Delta^{5,6}$-$\Delta^{4,5}$ isomerase performs this function (62a) and use of this gene as a probe may allow cloning of the plant homolog.

Mutants for an earlier step in the BR pathway involving a reductase have been putatively assigned. The Arabidopsis *dwf*1 (33) and its alleles *dim*1 (109) and *cbb*1 (56) are rescued to wild type by BR treatment (K Feldmann, personal communication; 56, 107). Recent examination of BR levels in *dim*1 has shown that 24-methylenecholesterol levels are elevated while campestanol and campesterol are reduced, suggesting that *dim*1 (*dwf*1, *cbb*1) is blocked in the conversion of 24-methylenecholesterol to campesterol (U Khlare, personal communication). The gene has been cloned and shows some homology to FAD-dependent oxidases (74). Since the conversion of 24-methylenecholesterol to campesterol involves a reduction, the oxidase homology is either artefactual or the oxidase functions in the reverse reaction as a reductase. Experiments with recombinant

protein and labeled substrates, as described for DET2 above, would help to resolve this question. The *lkb* mutant of pea is a BR-deficient dwarf that shows normalization of internode growth upon application of a range of BRs (79). Recent studies showed that 24-methylenecholesterol accumulated in *lkb* mutants while campesterol and campestanol were dramatically reduced, suggesting that *lkb*, like *dwf*1 in Arabidopsis, is a lesion in the gene encoding the biosynthetic enzyme responsible for 24-methylenecholesterol to campesterol conversion (T Yokota, personal communication).

Putative steroid hydroxylases have also been cloned from Arabidopsis BR-deficient dwarf mutants. Brassinolide, castasterone, typhasterol, 3-dehydroteasterone, and teasterone all rescued the *cpd* mutant to wild type phenotype in the light and the dark, while cathasterone and its precursors had no effect, implicating CPD in the C_{23} hydroxylation of cathasterone to teasterone (107). Sequence analysis of the cloned gene supports this view; CPD shows 24% identity to rat testosterone-16α-hydroxylase and 19% identity to human progesterone-21α-hydroxylase, including conserved amino acids in the steroid substrate-binding domains (107). Moreover, there is 50–90% identity with conserved domains of microsomal cytochrome P450s, and CPD has been classified as a CYP90 P450 monooxygenase (107). CPD must also serve to hydroxylate 6-deoxocathasterone to 6-deoxoteasterone, since the involvement of a different enzyme in the late C_6 oxidation pathway would allow synthesis of brassinolide in the *cpd* mutant and a dwarf phenotype should not be observed. Some direct evidence that the same hydroxylase catalyzes both early and late C_6 oxidation steps comes from the *dpy* mutant of tomato, an intermediate dwarf with severely altered leaf morphology, including the downward curling and dark-green color typical of the Arabidopsis BR mutants. We have found that teasterone and 6-deoxoteasterone, along with all subsequent intermediates, rescue *dpy* to wild type while cathasterone, 6-deoxocathasterone, and all of its precursors fail to do so (E Cerny, S Fujioka & S Clouse, unpublished data). This suggests that *DPY* is the tomato homolog of *CPD*, and cloning by transposon tagging is underway to determine the extent of sequence homology between the tomato and Arabidopsis genes (E Cerny, G Bishop & S Clouse, unpublished data). Another tomato gene, *DWARF*, was cloned by transposon tagging and shown to encode a cytochrome P450 (CYP85) with 38% identity to CPD (11). Preliminary feeding experiments suggest that DWARF is also a BR biosynthetic enzyme, but later in the pathway than CPD (E Cerny, G Bishop, S Fujioka & S Clouse, unpublished data).

The *DWF*4 gene, encoding the enzyme responsible for the C_{22} hydroxylation of 6-oxocampestanol to cathasterone (the potential rate-limiting step of BR biosynthesis), has also been cloned. Cathasterone, 6-deoxocathasterone, and

all subsequent intermediates in both the early and late C_6 oxidation pathway rescued the Arabidopsis *dwf*4 to wild type, but campestanol and 6-oxocampestanol failed to do so. Sequence analysis showed that DWF4 was another cytochrome P450 with 42% sequence identity to CPD (S-W Choe & K Feldmann, personal communication).

Conjugation and Metabolism

As discussed recently (4), conjugates of plant hormones are considered to be transport, storage, or inactivated forms. Two examples of 23-glucosyl conjugates of BRs were known (134), and reversible acyl conjugation at the 3-position has now been reported (2, 7, 8). Glucosyl, sulfate, and acyl conjugates were also detected after BRs were supplied to explants and cell cultures. The importance of the 3-β-epimerization for acylation (and also for glucosidation) has been confirmed, and reduction at C_6-, hydroxylation, and glucosidation or degradation of the side chain were also observed (4, 5, 47, 48, 77, 89, 103). Diglycosidation of exogenous 24-*epi*teasterone occurred in cell cultures of tomato, *Lycopersicon esculentum* (60), and in the same system, hydroxylation at the 25- and 26-positions of 24-*epi*brassinolide required two separate hydroxylases, with only the 25-hydroxylase sensitive to cytochrome P450 inhibitors (127). How many of these transformations occur in vivo as part of normal biosynthesis and turnover of BRs remains to be seen, but the possibility of very low levels of even more active members of the family, like those produced by fungal metabolism (48, 117, 118) is tantalizing.

PHYSIOLOGICAL RESPONSES TO BRs

Early work explored the range of effects of BRs in various bioassays and their interactions with inhibitors and promoters. The responses include effects on elongation, bending, cell division, reproductive and vascular development, membrane polarization and proton pumping, source/sink relationships, and modulation of stress. BRs also interact with environmental signals and can affect insect and fungal development (for reviews, see 3, 6, 19, 66, 67, 72, 83, 89, 94, 96). The sites for BR synthesis in planta are not yet elucidated. It may be that all tissues produce them, since BR biosynthetic and signal transduction genes are expressed in a wide range of plant organs (63), and short-distance effects (as seen in pollen, seeds, and cell cultures) can be assumed. Studies on the distribution of labeled BRs supplied exogenously suggest long-distance transport is predominately acropetal (e.g. 78, 133), but it is not yet known whether their long-distance transport is important in normal plant growth and development.

Examination of the phenotype of BR-deficient and insensitive mutants provides independent confirmation that many of the effects observed by exogenous

application of BRs to bioassay systems do in fact occur in planta. In Arabidopsis, BR mutants show extreme dwarfism (as small as one thirtieth the size of wild type) which is rescued only by BR application in the deficient but not the insensitive mutants, and microscopic examination of cell files in various organs shows that mutant cells are shorter than the corresponding wild-type cells, confirming the role of BRs in elongation (24, 56, 64, 107). In Arabidopsis and tomato, BR mutants show a de-etiolated phenotype in which dark-grown seedlings exhibit the short hypocotyl and open cotyledons characteristic of light-grown plants. In the light, these same mutants exhibit extremely altered leaf morphology (22). Thus, an important role of BRs in photomorphogenesis and leaf morphogenesis can be assumed. BR mutants generally have reduced fertility or male sterility, delayed senescence, and altered vascular development, implicating BRs in all these developmental processes (21).

Cell Expansion

BR application at nM to μM levels causes pronounced elongation of hypocotyls, epicotyls, and peduncles of dicots, as well as coleoptiles and mesocotyls of monocots (21, 66, 94). Young vegetative tissue is particularly responsive to BRs, and, if endogenous BRs are directly involved in the control of cell expansion, they must be present in such tissue. Approaches to establishing this include the analysis of levels in a BR-sensitive zone of pea stem (99) and localization of an exogenously supplied ^{125}I-BR, which accumulated in the elongating zone of mung bean epicotyls and the apex of cucumber seedlings (129).

While both BR and auxin promote elongation, their kinetics are quite different. Auxin generally shows a very short lag time of 10 to 15 min between application and the onset of elongation, with maximum rates of elongation reached within 30 to 45 min (108). In contrast, BR has lag times of at least 45 min with elongation rates continuing to increase for several hours (26, 55, 68, 140). This difference in kinetics is also seen at the level of gene expression in Arabidopsis, where auxin induces the *TCH*4 gene much more rapidly than BR (130). Other differences in the effect of auxin and BR on elongation have been observed in physiological (93, 115) and molecular studies (23, 24, 26, 140). However, synergisms between BR and auxins occur in many systems (66). Additive effects on elongation were often seen with gibberellins, and enhancement of lateral enlargement induced by cytokinins, and inhibitory effects of cytokinins, abscisic acid, and ethylene on BR-induced elongation have been described for stem tissue (55, 93 and references therein).

BR-induced expansion is accompanied by proton extrusion and hyperpolarization of cell membranes and these effects have also been observed in the asymmetric expansion of the joint pulvini of rice laminae (14) and in an alga (9) where BRs at concentrations from 10^{-15}–10^{-8} M markedly stimulated and

accelerated the growth cycle. However, some authors found little effect in vivo of 24-*epi*brassinolide on plasmalemma ATPases of wheat roots (128) and decreased activity in diploid and tetraploid buckwheat (28). Indirect modulation of ATPase activity had also been invoked to explain BR-induced effects on sucrose transport (3, 82).

Inhibitory effects of BRs on expansion have been widely reported in root tissue. In general, exogenous application of BRs inhibits primary root extension and lateral root formation, with occasional promotions of elongation or adventitious rooting seen with <pM concentrations (23, 24, 85, 97). However, there is some evidence for involvement of endogenous BRs in the control of lateral root initiation; uniconazole treatment produced many stunted lateral roots in *Lotus*, but concomitant brassinolide treatment reduced the number to the control value (57). Inhibitory effects, particularly on expansion, are often mediated via the induction of ethylene biosynthesis, and treatment with exogenous BRs increases the production of ethylene in stem tissue (summarized in 6). However, recent work on the inhibitory effects of a brief brassinolide treatment of cress seeds showed ethylene levels were not increased in the germinants, suggesting an independent inhibitory action of BR (54). It is not clear what role endogenous BRs play in the early stages of germination, but changes in levels of castasterone and brassinolide were seen after radish seeds germinated (102).

Cell Division

Reports of promotive effects on cell division in whole plants (66) proved hard to confirm in model systems, with elongation (92) or inhibition (44, 86, 126) noted instead. Furthermore, microscopic examination of BR-deficient and BR-insensitive mutants in Arabidopsis showed that the dwarf phenotype was due to reduced cell size, not cell number (56). However, in cultured parenchyma cells of *Helianthus tuberosus*, application of nanomolar concentrations of BR-stimulated cell division by at least 50% in the presence of auxin and cytokinin (25). In Chinese cabbage protoplasts, 24-*epi*brassinolide, when applied with 2,4-D and kinetin, promoted cell division in a dose-dependent manner and enhanced cluster and colony formation. The data suggested that dedifferentiation of the protoplasts was enhanced and that BR promoted or accelerated the necessary regeneration of the cell wall before division (75). Brassinolide also accelerated the rate of cell division in Petunia protoplasts in the presence of auxin and cytokinin but could not take the place of either hormone (M-H Oh & S Clouse, unpublished data). The contradictory results make the role of BRs in cell division unclear, and much further work is required to resolve this issue, including studies of the effect of BR on genes controlling cell division.

Vascular Differentiation

Auxin and cytokinin are required for the initiation of xylem development both in vivo and in vitro (40). However, evidence continues to mount that BRs may also play a significant role in vascular differentiation. In *H. tuberosus* explants, one of the major in vitro systems for studying xylem differentiation, Clouse & Zurek (25) found that nM concentrations of exogenous brassinolide increased differentiation of tracheary elements 10-fold after only 24 h. Normally, tracheary element differentiation requires 72 h in this system. In isolated mesophyll cells of *Zinnia elegans*, a second widely used model system for xylem differentiation, tracheary element formation has been divided into three stages (40). In Stage I, the mesophyll cells dedifferentiate after induction by auxin, cytokinin, and wounding, and specific transcripts are induced including the phenylpropanoid pathway members phenylalanine ammonia-lyase and cinnamate hydroxylase. During Stage II, phenylpropanoid pathway gene expression abates and three-dimensional networks of actin filaments form. In Stage III, phenylalanine ammonia-lyase and cinnamate hydroxylase gene expression again increases, the highly lignified secondary wall is formed, and programmed cell death ensues. Current evidence suggests that endogenous BRs are required for entry into Stage III (40). Uniconazole, an inhibitor of both gibberellin and BR biosynthesis, prevented differentiation of *Z. elegans* mesophyll cells into tracheary elements, and this inhibition was overcome by BR but not by gibberellin application (53). Uniconazole also inhibited Stage III–specific genes but not those specific to Stages I or II. Moreover, expression of phenylalanine ammonia-lyase and cinnamate hydroxylase was inhibited by uniconazole during Stage III but not Stage I, and this inhibition was overcome by brassinolide and several BR biosynthetic intermediates (40).

The spatial expression of *BRU*1, a BR-regulated gene encoding a xyloglucan endotransglycosylase (XET) in soybean (139), also points to a role of BRs in xylem differentiation. XETs are thought to be involved in processes requiring cell wall modification, including expansion (see below), vascular differentiation, and fruit ripening (35). In cross-sections of elongating soybean epicotyls, *BRU*1 expression was most intense in paratracheary parenchyma cells surrounding vessel elements (80) suggesting a role for BRs and XETs in xylem formation. The modification of cambial division seen in a BR-deficient mutant (56, 107) also suggests involvement of endogenous BRs in xylem differentiation in vivo, and it is relevant that BRs have been identified in cambial scrapings of *Pinus silvestris* (58) and a *Eucalyptus* species (T Yokota, unpublished work).

Pollen and Reproductive Biology

Pollen is a rich source of endogenous BRs and in vitro studies have suggested that pollen tube elongation could depend in part on BRs (51). Male sterility

of BR-insensitive mutants would support this (24, 56, 63), but the failure of the filament to elongate such that the pollen, although viable, cannot reach the stigma was suggested as an alternative mechanism of male sterility for the BR-deficient *dwf*4 mutant (S-W Choe & K Feldmann, personal communication). However, the *cpd* mutant was reported to be male sterile because the pollen itself failed to elongate during germination (107). In addition, pollination is often the initial step for the genesis of haploid plants, and in *Arabidopsis thaliana* and *Brassica juncea*, treatment with brassinolide induced the formation of haploid seeds that developed into stable plants (59). Subcellular localization of BRs was explored in pollen of *Brassica napus* and *Lolium temulentum*, using polyclonal antibodies generated against castasterone, and the data suggested BRs could be stored (or trapped) in developing starch granules and be released on imbibition (99, 114). The relative distribution of BRs in maturing pollen has also been explored chemically (8), and conjugated teasterone was present at the microspore stage. Its level decreased as the pollen developed, and levels of free BRs increased. Taken together, these data suggest that BRs have important physiological roles in the fertilization of plants.

With respect to the general effect of BRs on sex differentiation in plants, Suge (105) found that direct application of brassinolide to the staminate influorescence of *Luffa cylindrica* induced bisexual and pistillate flowers. Numerous model systems of sexual morphogenesis in plants are currently available, and application of BRs to these systems could be a profitable exercise.

Senescence

Senescence of leaf and cotyledon tissue has often been shown to be retarded in vitro by administration of cytokinins; in contrast, 24-*epi*brassinolide accelerated senescence in such systems (29, 50, 138). Altered activities of peroxidase, superoxide dismutase, and catalase and a marked increase in the level of malondialdehyde were observed, and the authors suggested BRs might regulate these effects via "activated oxygen." Delayed senescence in Arabidopsis BR mutants would tend to support the role of BRs in accelerating senescence in normal plants (24, 56, 64, 107). However, work concerned with lipid peroxidation suggests 24-epibrassinolide inhibits oxidative degradation, decreases malondialdehyde levels (31), and acts as a membrane protectant, thus delaying senescence. Examination of the effect of BR application on senescence-associated mutants of Arabidopsis and study of the expression of senescence-associated genes in the BR mutants will be necessary to help clarify the role of BRs in this process.

Modulation of Stress Responses

Since the early reports of the ameliorative effects of BR-treatment of stressed plants (49), most work has focused on chilling stress (summarized in 55, 112,

126). In rice, 24-*epi*brassinolide treatment reduced electrolyte leakage during chilling at 1–5°, reduced malondialdehyde content and slowed the decrease in activity of superoxide dismutase; while levels of ATP and, initially, proline, were enhanced. The enhanced resistance was attributed to BR-induced effects on membrane stability and osmoregulation (121). In a model system that develops both cold- and thermotolerance, treatment with 24-*epi*brassinolide enhanced both tolerances. While abscisic acid (at higher concentration) was a more effective regulator in both cases, there were interesting differences in gene expression resulting from the application of BR or abscisic acid (126). Changes in the spectrum of heat-shock proteins after BR administration, and promotion of heat shock granule formation, had also been reported in heat-stressed wheat (61).

Mild drought stress in sugar beet is ameliorated by treatment with a synthetic BR (100), and effects in other crop plants have been explored (88, 104). Improvement in salt tolerance in BR-treated rice has been confirmed (111, 112), and a protective effect on barley leaf ultrastructure after a 24-h exposure to 500 mM sodium chloride described (61). A promotive effect of 10 μM 24-*epi*brassinolide was noted on both germination rate and percentage of *Eucalyptus camaldulensis* seeds in 150 mM sodium chloride (98). BR treatment was also claimed to protect against various pathogens (49), but this may not be general, because very low concentrations of two BRs induced susceptibility of potato to *Phytophora infestans*, and the effect was long lasting (116). However, it is interesting to note that in the BR-deficient *cpd* mutant, the expression of pathogenesis related genes is greatly reduced (107). Little further work has been done on the effects of BRs as herbicide safeners (49, 112, 113), but interest in the interactions of BRs with insects (16, 83) continues, with the object of using BR-analogues as antiecdysones (65).

MOLECULAR MECHANISMS OF ACTION

Aspects of BR-Promoted Cell Elongation

The plant cell wall forms a highly cross-linked, rigid matrix that opposes cell expansion and differentiation. In order for elongation and other morphogenetic processes to occur, the cell wall must be modified, i.e. by wall relaxation or loosening and by incorporation of new polymers into the extending wall to maintain wall integrity. Several proteins with possible roles in cell wall modification processes have been identified, including glucanases, xyloglucan endotransglycosylases (XETs), and expansins (27). While the complexity of the wall has made a definitive molecular model of wall extension elusive, a plausible scenario has been presented by Cosgrove (27) in which expansins are primarily responsible for wall relaxation, but glucanases and XETs affect the extent of expansin activity by altering the viscosity of the hemi-cellulose matrix. Furthermore,

XETs may function to incorporate new xyloglucan into the growing wall, and cellulose biosynthesis would also be expected to occur. It is certain, however, that BRs alter the biophysical properties of plant cell walls (115, 122, 140) and also increase the abundance of mRNA transcripts for wall-modifying proteins such as XETs (15, 130, 139).

In elongating soybean epicotyls, BR application resulted in increased plastic extensibility of the walls within 2 h with a concomitant increase in the mRNA level of a gene named *BRU*1, which showed significant homology to numerous XETs (139). Subsequent enzyme assays with recombinant protein showed that BRU1 was indeed a functional XET (80). Moreover, increasing concentrations of applied BR during early stages of elongation lead to a linear increase in extractable XET activity in the epicotyls (80). The *BRU*1 gene was regulated specifically by BRs during early stages of elongation, and increased expression was not simply the consequence of enhanced elongation (139). Therefore, BRU1 is likely to play an important role in BR-promoted epicotyl elongation in soybean. The role of expansins in this system has yet to be examined.

BR-regulated XETs have also been identified in Arabidopsis. The *TCH*4 gene was shown to encode an XET whose expression was increased within 30 min of BR treatment, with a maximum at 2 h (130). In contrast to the soybean *BRU*1, *TCH*4 was also rapidly induced by auxin treatment (130). Environmental stimuli such as touch, darkness, and temperature also affected *TCH*4 expression. The expression of *TCH*4 was restricted to expanding tissue and organs that undergo cell wall modification such as vascular elements (130). *TCH*4 expression was greatly reduced in BR-deficient and insensitive mutants, again suggesting a role for XETs in BR-promoted elongation (56).

Cell expansion also depends on an adequate supply of wall components, and recent work (68) confirmed the inhibition of BR-induced elongation in stem tissue by inhibitors of cellulose biosynthesis or microtubule orientation (93, 94). Brassinolide, alone or in combination with auxin, enhanced the percentage of transversely oriented cortical microtubules (68). The orientation of cortical microtubules, which generally correlates with the orientation of microfibrils, follows a cyclic pattern, and phosphorylation of proteins, possibly those linking the microtubules to the plasmalemma, is an essential component in the maintenance of transversely oriented microtubules. BR-induced elongation also depends on such phosphorylation (69).

Levels of Gene Regulation by BRs

Transcriptional regulation of gene expression by BRs has been demonstrated with the *TCH*4 gene of Arabidopsis (130), using *TCH*4 promoter:*GUS* fusions lacking any 5′ or 3′ *TCH*4 transcribed sequences (W Xu & J Braam, personal communication). Subsequent work has localized the promoter region responsible for BR-regulation to within 100 bp, and linker-scanning mutagenesis is

under way to identify the specific BR response element for this gene (B Torisky, J Braam & S Clouse, unpublished data). While transcriptional regulation of gene expression by animal steroid hormones is more commonly discussed (32), posttranscriptional regulation has also been observed (76). The *BRU*1 gene of soybean represents an example of such posttranscriptional gene regulation by a plant steroid (139). The posttranscriptional regulation, which apparently involves *BRU*1 mRNA stability, is maintained in a BR-dependent manner in soybean cell suspension cultures, thus providing an excellent model system for dissecting *cis*-acting sequences responsible for this regulation (J Jiang & S Clouse, unpublished data).

BR Signal Transduction

The value of hormone-insensitive mutants in unraveling signal transduction pathways in plants has been demonstrated for ethylene (30) and abscisic acid (34), and such an approach has proven successful for BRs as well. Clouse et al (24) identified a BR-insensitive mutant in Arabidopsis by the ability of mutant plants to elongate roots in the presence of inhibitory concentrations of BR with respect to wild type. The mutant, named *bri*1, showed severe pleiotropic effects on development including dwarfism, de-etiolation, male sterility, and altered leaf morphology, which suggested that the BRI1 protein product played an important role in BR signal perception or transduction. Several alleles of *BRI*1 with identical phenotype have been isolated in independent screens (K Feldmann, personal communication; 56, 63)

In animals, there are two major paradigms of signal transduction. The first involves a membrane-bound receptor with an extracellular ligand-binding domain and an intracellular domain responsible for transducing the signal to the next member of the pathway, often a kinase or G protein. Amplification and proliferation of the signal proceeds through cascades of phosphorylation and dephosphorylation and involves second messengers such as calcium, cyclic AMP, and diacyl glycerol (131). The second pathway involves intracellular receptors that recognize steroid or steroid-like ligands to directly affect the transcription of specific genes by binding directly to the promoter of hormone-responsive genes (10). Because of the structural similarity of animal and plant steroid signaling molecules, it is reasonable to assume that plants might have members of the intracellular superfamily of steroid receptors. The *BRI*1 gene has now been cloned, and perhaps somewhat surprisingly, shows strong sequence homology not to steroid receptors but to leucine-rich receptor kinases that function at the cell surface to transduce extracellular signals (63).

The *BRI*1 gene shares homology with plant and animal receptor kinases in all conserved domains including ligand binding domain, membrane domain, and cytoplasmic kinase domain. Moreover, sequence analysis of five mutant alleles confirms that the putative ligand binding and kinase domains are essential

for in vivo function (63). On the basis of the dramatic pleiotropic phenotype of the *bri*1 mutants and the sequence homology of BRI1 to important signal transduction molecules, it is obvious that BRI1 is a critical component of the BR signal transduction pathway. However, its role as the BR receptor has not been confirmed by direct binding studies. No ligands have been identified for plant receptor kinases, and in animals, all known ligands for such receptors are polypeptides or glycoproteins (120). It is possible that the BR receptor is a distinct polypeptide that binds to BRI1 in the presence of BR, or there may be an unknown ligand that is required for BR activity. Even if BR binds directly to BRI1, it does not exclude the possibility that there are also intracellular BR receptors, since it is now known that in animals both intracellular and extracellular steroid receptors co-occur, with the intracellular receptor mediating gene expression and the extracellular receptor modulating nongenomic responses such as calcium ion flux and phosphorylation status of a variety of proteins (71).

We have recently identified a gene, *TCH4-BF*1, that may be involved in the terminal end of BR signal transduction (M-H Oh, T Altmann & SD Clouse, unpublished data). Recombinant TCH4-BF1 binds specifically to the *TCH*4 promoter in the region thought to contain the BR response element (see above), and sequence analysis shows homology to a type of zinc finger protein called the PHD finger, found in plant and Drosophila transcriptional regulators of homeodomain genes and in mammalian transcription factors (1). A hypothetical scheme that incorporates both BRI1 and TCH4-BF1 in BR signal transduction is shown in Figure 4.

CONCLUSION AND FUTURE PERSPECTIVES

Recent research on the chemistry, physiology, and molecular biology of BRs provides a convincing body of evidence that these plant steroids are essential regulators of plant growth and development. The pace of BR research is accelerating rapidly, and with the proliferation of cloned genes and advances in microchemical techniques, the range of experimental approaches to understanding BR action continues to expand. Much remains to be done, however. Determination of whether or not BRI1 is the BR receptor by measuring the binding of high-specific activity radiolabeled BRs to recombinant BRI1 protein is of top priority. Studies on the effect of phosphorylation/dephosphorylation states on BR signal transduction using available inhibitors of kinases and phosphatases will be informative; e.g. okadaic acid, an inhibitor of type I phosphatases, blocks BR-promoted elongation in soybean (S Clouse, unpublished data), suggesting a role for phosphatases in addition to the BRI1 kinases in BR action. The availability of BRI1 and TCH4-BF1 as probes will allow application of interactive cloning techniques to identify new components of the BR signal transduction

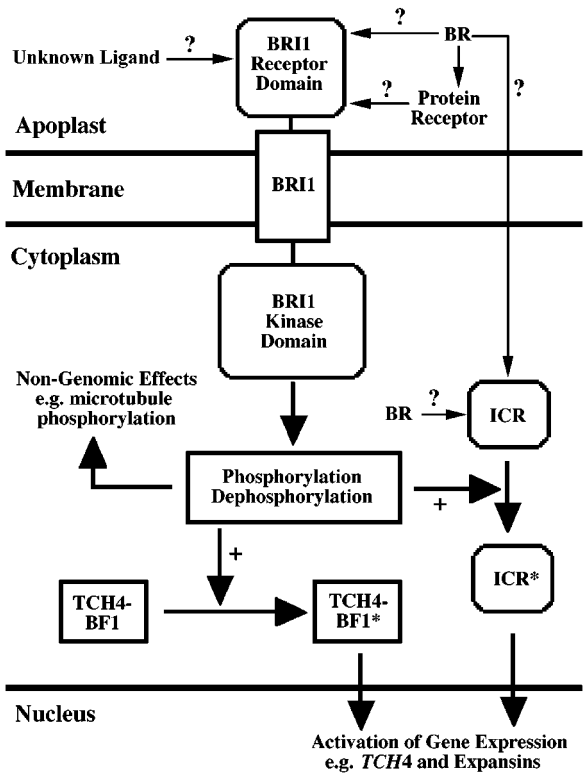


Figure 4 A hypothetical scheme for BR signal transduction in Arabidopsis. BRI1, a putative leucine-rich receptor kinase, may bind BR directly (or BR bound to a distinct protein receptor that functions at the cell surface), which causes activation of the kinase domain and subsequent phosphorylation of additional kinases and/or phosphatases. Signal transduction results in activation (*shown with* + *or* *) of transcription factors (such as TCH4-BF1 and other unknown proteins), which then bind to promoters of BR-responsive genes such as *TCH*4. It is also possible the BRI1 binds an unknown ligand, while BR binds to an intracellular receptor (ICR, similar to animal steroid receptors), which requires activation by the kinase domain of BRI1. There is currently no evidence for an intracellular receptor. Nongenomic activation by BR via BRI1-mediated phosphorylation of microtubules or metabolic enzymes, etc, is also a possibility.

pathway. The study of *BRI*1, *DET*2, *DWF*4, and *CPD* expression by in situ hybridization in different organs at different developmental stages, coupled with direct measurements of endogenous BR levels, will help verify the role of BRs in different physiological processes. Epistasis studies using BR mutants crossed with other hormone and developmental mutants should help to place BRs in the overall pattern of development, as will studies of the ectopic expression of *BRI*1 and biosynthetic genes in a variety of different transgenic plants.

By the time the next review on BRs appears in this series, our knowledge of the molecular mechanisms of BR action will have expanded dramatically, and the degree of evolutionary conservation between plant and animal steroid signaling pathways will be determined in much more detail.

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