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Physiological Actions of Brassinosteroids: An Update

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Abstract

In general, this overview covers literature from 1999 until early 2003. Topics covered include aspects of the biosynthesis and transport of brassinosteroids, their effects on cell division, expansion, and differentiation, and their effects on whole plants, including source-sink relations and other endogenous interactions. Some interactions with environmental signals are discussed, as well as results that may promise applications in future. Topics that warrant further investigation of the roles of BRs include phenotypic variability, reproductive physiology, senescence, branching, and apical dominance, whereas topics in which possible roles for BRs are relatively unexplored include lignification, phototropism, photoperiodism, and endogenous rhythms.

Key words: Brassinolide; 24-Epibrassinolide; Castasterone; Cross-talk; 28-Homobrassinolide; Plant development; Plant hormones

Introduction

Reviews on brassinosteroids (BRs) that have appeared since that of Clouse and Sasse (1998) include two books (Sakurai and others 1999; Khripach and others 1999) and articles which focus on particular aspects of their structures and biology (for example, Li and Chory 1999; Khripach and others 2000; Schumacher and Chory 2000; Müssig and Altmann 2001; Friedrichsen and Chory 2001: Bishop and Yokota 2001; Clouse 2001, 2002ab; Zullo and Adam 2002; Bajguz and Tretyn 2003; Zullo and others 2003; Fujioka and Yokota 2003). There has been impressive progress in our understanding of BR-induced signaling as well as BR-induced gene expression, whereas the roles of BR in stress and defense are receiving increasing attention. More details of the networked pathways for the biosynthesis, conjugation, and metabolism of BRs are emerging, and exploration of the value of selected or structurally modified BRs for agricultural use continues. Reports also extend to their effects on animal and human physiology. The present overview updates a chapter on the physiological actions of BRs (Sasse 1999).

Occurrence, Synthesis, and Transport of BRs

Brassinosteroids have been identified in 27 families of higher plants and three families of lower plants (Bajguz and Tretyn 2003), and they occur in all parts of higher plants, including roots. There are some species differences, as comparison of the BRs in *Arabidopsis*, pea, and tomato suggested three rate-

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limiting steps in their biosynthesis, and that the late 6-oxidation pathway a predominated in these dicots. It was the only pathway in tomato and both castasterone and the unusual BR, norcastasterone, were present in comparable amounts (Nomura and others 2001). More detailed work on the reversible conversion of 24-epiteasterone to 3-dehydro-24-epiteasterone supported the involvement of at least two enzymes, with inhibition in the dark, suggesting more mechanisms for ''fine-tuning'' of effective BR levels (Stündl and Schneider 2001).

The particular cytochrome P450 enzymes in the biosynthesis of BRs are subject to temporal and spatial in regulation, with feedback control by brassinolide concentration. In shoots and roots of young seedlings there is organ-specific distribution of transcripts, and concentrations of endogenous BRs correlate well with such expression in *Arabidopsis*, pea, and tomato (Bancos and others 2002). Another study in *Arabidopsis*, using two genes for C-6 oxidation in BR biosynthesis, *BR6ox1* and *BR6ox2*, and *DWF4*, showed expression primarily in young, actively developing organs. Endogenous levels of BRs also correlated, with the greatest expression and highest levels of endogenous BRs occurring in apical shoots, followed by siliques (Shimada and others 2003). A comprehensive review of the biosynthesis and metabolism of BRs appeared this year (Fujioka and Yokota 2003).

However, we may ask: Is long-distance transport of endogenous BRs important? It is well recognized that signaling molecules in plants and animals may act over relatively long or short distances, and long-distance transport of exogenous BRs can occur in plants, particularly from root to shoot. Although exogenous 24-epibrassinolide is not efficiently exported from leaves (Nishikawa and others 1994), it is still possible that precursors might be important for the transport of BRs in the whole plant. Localized expression in particular cells of the leaves of a gene encoding an enzyme important for the synthesis of a crucial precursor to brassinolide (Mathur and others 1998) could imply the presence of the complete biosynthetic pathway in those cells [with export of the newly synthesized active BR(s) to the sites of action] or export of precursor(s). Endogenous BRs have been quantified in cell cultures of *Zinnia elegans* (Yamamoto and others 2001), and the relative quantities of particular BRs inside and outside the cells differed (discussed in the Cytodifferentiation section).

Hydrolyzable conjugates may also be candidates for transport. Glycosidation and esterification were reported some time ago (references in Bajguz and Tretyn 2003) and reversible esterification of teasterone was reported recently (Soeno and others 2000). Sulfation was confirmed, and expression of the responsible sulfotransferase gene in *Brassica napus* was also induced by salicylic acid, suggesting that a modulation of steroid-dependent growth could be part of a plant's response to pathogens (Rouleau and others 1999). Such a dual role has also been proposed for a putative receptor in tomato, tBRI1/SR160, that recognizes both BR and systemin (Montoya and others 2002). The two signal molecules do not compete directly, and Scheer and Ryan (2002) suggest that *SR160* evolved from *BRI1*, and that systemin signaling patterns were in turn recruited as a means of storing proteins in developing potato tubers (Narvaez–Vasquez and Ryan 2002).

Another possibility for *in vivo* transport of BRs is the binding of BRs to protein carrier(s), which is also relevant for signaling. Early work using immunocytochemical techniques found no specific binding of brassinolide to any soluble protein extracted from pollen of rye grass (Smith and others 1992), but an NMR study suggested that 28-homocastasterone could bind to a cavity within an allergen from cherry (Neudecker and others 2001). The major allergen from birch pollen, Bet v 1, is part of the PR-10 family of pathogenesis-related plant proteins, and an isoform, Bet v 11, can form a complex with two deoxycholate molecules. Mass spectral analyses showed that brassinolide and 24-epicastasterone also formed complexes (Markoviç–Housley and others 2003). The authors proposed a general plant-steroid carrier function for such proteins that could be important in plant growth and development as well as defense responses. Such a role could be important even if biosynthesis of BRs close to their sites of action proves to be more important than long-distance transport.

Effects in Development: Cell Division

Restoration of leaf size after administration of brassinolide to the mutants *det2* and *dwf1* of *Arabidopsis* could not be accounted for solely by expansion, supporting a role for BRs in cell division (Nakaya and others 2002). 24-epibrassinolide can substitute for cytokinin in the culture of *Arabidopsis* callus and suspension cells, and a particular cyclin gene, *CycD3*, was upregulated. This also occurred in the BR-insensitive mutant *bri1*, reviving the possibility of an unknown, possibly intracellular, pathway for the BR signal (Hu and others 2000). Constitutive overexpression of *CYCD3*; *1* affected the anatomy of the leaf markedly, and a critical role has

been proposed for this gene in the transition from cell proliferation to differentiation (Dewitte and others 2003).

The mitotic rate increased in roots of wheat after treatment with 24-epibrassinolide, and the hexaploid *Triticum aestivum* was the most sensitive. Volumes of nucleoli were also increased, and the effects of cytokinin were similar (Fatkhutdinova and others 2002). In synchronously dividing cultures of the alga *Chlorella vulgaris*, accelerated increases in cell number and marked increases in nucleic acid and protein levels followed BR treatment (Bajguz 2000a). Significant interactions with auxin at very low concentrations of brassinolide were also observed (A. Bajguz, personal communication), so this single-cell system has potential for exploring hormone cross-talk and hormone-induced protein breakdown as well as species differences, if BRs prove to be ubiquitous in algae.

Effects in Development: Cell Expansion

Whatever the molecular signals, expansion is contingent upon transport of ions, uncharged osmotica, and water across the cell and vacuolar membranes, and upon a plastic cell wall. The possibility that BRs may control aquaporin activities was explored using the dwarf biosynthetic and sensitivity mutants *cpd* and *bri1* of *Arabidopsis*. The median water permeability of wild-type protoplasts was significantly greater than that of the mutants and covered a wider range of values. Brassinolide treatment increased osmotic permeability of hypocotyl protoplasts from the *cpd* mutant markedly, with a shift in the range of the population. As this was not a rapid response and the *bri1* mutant was not affected, Morillon and coworkers (2001) concluded that brassinolide was not likely to affect the membranes directly. However, mRNA and protein levels of aquaporin isoforms in root and shoot of radish were not affected by brassinolide treatment (Suga and others 2002).

Seedlings of another *Arabidopsis* mutant, *det3*, develop as light-grown plants in the dark and have reduced ability to respond to BR. The gene was shown to encode subunit C of the vacuolar H^+–ATPase, which has a role in the control of elongation and also in meristem activity. The mutant has an alternative method of assembly of this ATPase, and regulation of its activity by different signals could occur through different configurations of the protein complex, with a BR signal transduced via the DET3 protein (Schumacher and others 1999). Membrane permeability and selectivity in the presence of toxic metals is also affected by BR, and the metal ions can be ranked in the order in which BR treatment ameliorated their effects. Results have been reported for selected crop plants (Khripach and others 1999, 2000) and algal cells (Bajguz 2000b).

Crucial for anisotropic growth in expansion are polarization, axialization, and on-going control of the orientation of microtubules and cellulose microfibrils. Expression of β-tubulin genes correlated with brassinolide-induced growth in chickpea (Munoz and others 1998), and, in *Arabidopsis*, BR treatment restored elongation of the *bul1-1* mutant. This severe dwarf, defective in the sterol synthesis pathway that leads to campesterol and BR, has short, dissociated, and disordered microtubules (Catterou and others 2001a). Although *TUB1* expression was affected, after consideration of their data and current knowledge of the feedback relationships among microtubules, the plasma membrane, and the microfibrils of the cell wall, the authors proposed that another ''brassinosteroid-responsive pathway exists which allows microtubule nucleation/organization and cell elongation without activation of tubulin gene expression.'' They also thought some anatomical characteristics of the leaves of the *bul1-1* mutant might reflect the lack of BR-induced *TCH4* and expansin gene expression (Catterou and others 2001b). However, the possible role of actin assembly and any role for BRs there were not discussed.

Also at the plasma membrane, because expression was reduced in the *Arabidopsis* mutant *det2*, a role for BRs was suggested in the expression of *KOR*, which encodes a membrane-bound endo-1,4-β-D-glucanase unaffected by auxin, gibberellin, or ethylene (Nicol and others 1998). In rice, internodal elongation accompanies panicle formation, and, in the internode, two xyloglucan endotransglucosylase/hydrolase genes, *OsXTR1* and *OsXTR3*, were preferentially expressed in the elongating zone. These genes were upregulated by BRs and gibberellin, and Uozo and others (2000) suggested that BRs are essential for gibberellin sensitivity and that there is cross-walk between BR and gibberellin signaling. Recent reviews of the roles of membrane-bound endo-1,4-β-D-glucanases (Mølhøj and others 2002), xyloglucan endotransglucosylation and endohydrolysis (Rose and others 2002), expansins (Cosgrove and others 2002), and cellulose biosynthesis (Doblin and others 2002) in the construction and modification of the cell wall illustrate that further elucidation of the effects of BRs in the complex relationships and information flow between micro-

tubules, the plasma membrane, and the cell wall in the process of expansion will be very valuable.

Studies in other plants are also providing more insights into the roles of BRs. Modification of the conditions in mesophyll suspension cultures of *Zinnia* permitted the study of expansion only and confirmed at the cellular level that 24-epibrassinolide promoted elongation without significant radial expansion, in contrast to the effect of gibberellin. Unlike the effects of light, the effects of hormones began only after 48 h (Lee and others 2000). More extensive studies have been carried out in rice using mutants in BR biosynthesis and response. Identification of the *OsBRI1* gene provided evidence for its involvement in internode elongation, asymmetric growth in the lamina joint, and skotomorphogenesis. Internode elongation depended on the BR signal for the formation of the intercalary meristem, and internodes differed in their sensitivity. More *OsBRI1* was expressed in dark-grown rather than in light-grown seedlings, which is appreciably different than *BRI1* in *Arabidopsis* (Yamamuro and others 2000). The asymmetric expansion occurring in rice lamina inclination in response to brassinolide involves a calcium-dependent protein kinase (Yang and Komatsu 2001), which may also hint at species differences, whereas Sharma and others (2001), in a study of mitogen-activated protein kinase and calcium-dependent protein kinase in BR signaling in rice, suggested, like Hu and others (2000), that there are other BR receptor(s) besides BRI1 in plants.

Two groups have reported rice mutants with defects in BR biosynthesis, both at a late 6-oxidation step. A severe dwarf, *brd1*, had marked phenotypic changes when grown in light and constitutive photomorphogenesis in the dark. Data for roots suggested BR promotes the development of crown roots, primary and secondary branched roots, and the extension of thin primary branched roots. Study of the leaf sheaths and blades suggested effects on both expansion and division, but exogenous brassinolide could not restore reproductive development (Mori and others 2002). The other group (Hong and others 2002) described the anatomy in three allelic mutants with very similar phenotypes to that of Mori and others (2002). Introduction of the wild-type gene restored the normal phenotype, expression analysis showed the greatest intensity in the leaf sheath, and C-6 oxidase activity was confirmed. The authors consider that their data reflect an underlying disarray of the microtubules in the mutants, and also think that the lack of BRs may affect determination of sites for cell division (Hong and others 2002).

Effects in Development: Cytodifferentiation

There is steady progress in the study of vascular differentiation. Use of a potent inhibitor of BR biosynthesis, brassinazole, showed clearly that the development of secondary xylem in *Lepidum sativum* was particularly affected, with normal development restored after administration of brassinolide (Nagata and others 2001). In *Arabidopsis*, a provascular/procambial cell-specific gene, *VH1*, which marks the transition to the procambial state, has been identified. Although it encodes a leucine-rich repeat receptor kinase, it does not respond to 24-epibrassinolide (Clay and Nelson 2002). This is consistent with data from the model system of cultured *Zinnia* cells suggesting that BRs are most important in the later stages of vascular development (Yamamoto and others 1997). In *Zinnia*, endogenous BRs were shown to increase markedly before stage III of the differentiation process, where secondary wall formation and cell death occur. Seven BRs were monitored; levels of castasterone, typhasterol, their 6-deoxo-analogues, and 6-deoxo-teasterone increased, with the changes in 6-deoxocastasterone and 6-deoxo-typhasterol the most marked. The 6-deoxo-sterols were far more abundant than the 6-oxo-BRs, but the latter were preferentially excreted into the medium. The authors propose a signaling pathway whereby extracellular BRs are detected by a surface receptor like BRI1, and a signal cascade follows. The final stage of differentiation is then initiated (Yamamoto and others 2001) and the processes of lysis of the vacuole and modification of the tracheary elements follow (Kuriyama and Fukuda 2002). Any effects of BR on the process of lignification are, as yet, unknown.

In young *Zinnia* plants, mRNA transcripts of homeobox genes (analogous to those coding for Class III HD-Zip proteins in *Arabidopsis*) accumulated in procambium and immature xylem. In cultured cells, expression was suppressed by a selected inhibitor of BR biosynthesis but was restored by brassinolide. The authors concluded that the encoded proteins could be transcriptional regulators of xylogenesis, acting downstream from the BR signal. Because members of this class of genes have a sterol binding domain, they also stressed the importance of identifying such a ligand and did not exclude a BR as a candidate (Ohashi-Ito and others 2002). However, sterols other than the BR series are potential signal molecules (summarized by Clouse 2002b). The *CVP1* gene encodes a sterol methyltransferase (SMT2), which acts where BR biosynthesis diverges from the main route to sterols such as sitosterol and stigma-

sterol. In *Arabidopsis*, the mutant *cvp1* has disorganized vascular axialization and misshapen vascular cells in the cotyledons, and it accumulates early precursors to BRs, but the phenotype does not reflect high endogenous levels of the most active BRs. So Carland and others (2002) proposed that other signaling sterols control vascular cell polarization and axialization. Thus, there are promising leads for better understanding of the whole process of vascularization and the undoubtedly complex interactions between all the chemical signals involved.

Effects in Development: Seed Germination

Using exogenous applications of brassinolide and gibberellin, Leubner–Metzger (2001) found differing effects on tobacco seed germination depending on the state of dormancy, or on whether imbibition occurred in the dark or light, or on whether the seeds were inhibited by abscisic acid. The author concluded, from effects on endosperm rupture and the induction of a β-1,3-glucanase in the micropylar endosperm, that the two hormones acted in distinct pathways. He proposed that gibberellin and light act in a common pathway, whereas BR directly enhances the growth of the emerging embryo independent of gibberellin and that particular β-1,3-glucanase. In *Arabidopsis*, Steber and McCourt (2001) found 24-epibrassinolide and brassinolide treatment rescued mutants whose gibberellin biosynthesis is severely inhibited, and one which was gibberellin insensitive. They also found that germination of both a BR-biosynthetic and a BR-insensitive mutant (*det2-1* and *bri1-1*) was inhibited more strongly in the presence of abscisic acid than the wild type. They suggest that endogenous BR as well as gibberellin are needed to overcome ABA-induced dormancy and that BR acts in parallel or downstream of gibberellin.

A more developed model comes from the work of Ullah and others (2002), who considered whether coupling and cross-talk among ethylene, glucose, abscisic acid, gibberellin, and BR signals in germination involve heterotrimeric G proteins. Using *Arabidopsis* mutants *det2-1* and *bri1-5*, they attempted to distinguish direct coupling via the G-protein complex or potentiation by alteration of sensitivity to a particular signal. Regarding brassinolide and its effect on the mutant *gpa1*, they concluded that BR might be coupled via GPA1 to potentiate the gibberellin-controlled pathway for germination.

Effects in Development: Vegetative Growth

The use of inhibitors of brassinosteroid biosynthesis and exogenous application of BRs to whole plants and explants continue to provide useful leads for more detailed studies. For instance, it is well known that BR can enhance ethylene biosynthesis, and root treatment of *Arabidopsis* seedlings with brassinolide or its 24-epimer did do this, but a specific upward rather than a downward curvature of that part of the petiole nearest the leaf was observed. An ethylene-insensitive mutant responded similarly, suggesting that the effect was independent of ethylene, as well as of the particular auxins and gibberellin that were also tested (Arteca and Arteca 2001). Could this result point towards an endogenous effect of BRs in phototropism? Is there a role for BRs in the hyponastic response of *Rumex palustris* to flooding (Peelers and others 2002)?

Experiments with whole plants also interest those hoping to maximize or accelerate crop yield. For example, a spray treatment of mustard seedlings with 28-homobrassinolide led to higher fresh and dry weights, enhanced carbonic anhydrase activity and net photosynthetic rate, and enhanced pod number and seed yield per plant at harvest (Hayat and others 2000). Comparison of the effects of the optimal concentration (10 nM) with those of μM levels of indole-3-yl-acetic acid, gibberellic acid, kinetin, and abscisic acid showed that the BR was the most promotive of the regulators and that chlorophyll levels were also enhanced (Hayat and others 2001a). However, in soybean seedlings, only inhibitory effects on root and shoot length, dry weight, and nodule and lateral root numbers were observed after roots were treated with 0.1–10 μM 24-epibrassinolide (Hunter 2001). Although 24-epibrassinolide is regarded as less active than brassinolide, the concentrations for root treatments do seem relatively high; effects of lower concentrations could be of interest. Using brassinolide itself, Chon and others (2000) found that 11 representative cultivars of rice had increased leaf sheath lengths and numbers when grown in light, whereas pretreatment of seeds enhanced mesocotyl elongation in the dark. Some inhibitory effects were observed at the highest concentration used.

Wheat grown from seed treated with μM concentrations of 28-homobrassinolide had enhanced leaf number per plant, fresh and dry weight, and nitrate reductase and carbonic anhydrase activity (Hayat and others 2001b). In another study in wheat, pretreatment of seeds with 24-epibrassinolide at concentrations ranging from 0.04 nM to 40

μM led to a biphasic promotion of root length, with inhibition evident only after treatment with the highest concentration of the BR. The content of agglutinin was elevated in the most elongated roots, without a concomitant increase in abscisic acid concentration, but any role for this protein in elongation is not yet understood (Shakirova and others 2002). With rice, time of application and length of exposure to brassinolide were important. Shoot lengths of resulting seedlings were significantly promoted seven days after continuous and first day treatments of the seeds but not after treatment on days 3 and 4 of germination. Root elongation varied dramatically from enhancement to inhibition (Fujii and Saka 2001a).

Whole plants, with their need for coordination between organs and their coordinated response to their environmental conditions, challenge our concepts of control and complexity. In an opinion paper, Amzallag (2001) questioned the assumption that determination of variability in physiological studies was useful only for testing differences between means for significance. Amzallag proposed analyzing variability and using it for the investigation of redundancy and patterns of control in biological systems; connectance, coefficients of variation (CV), and differences between calculated ratios were discussed. As an example, in roots of *Sorghum bicolor* seedlings treated with 0.1–10 nM 24-epibrassinolide, highly significant increases in shoot and root fresh weight, and in the length of the sheath and blade of the fifth leaf, were observed only for the plants treated with 10 nm 24-epibrassinolide. There was no clear effect on variability, but in total plant data for those treated with the lowest concentration of BR, the CV value appeared reduced. A comparable experiment (Table 1, J. Sasse unpublished) showed no such trend, and there was a wide range of values for the CVs within the initial values where the means were not significantly different. One suspects Amzallag's aims would be better served by testing with increased replication and expert statistical input. However, the data for the highest concentration of brassinolide in Table 1 are consistent with Gregory's work (1981) where brassin treatment reduced phenotypic variability.

A more conventional approach exploited knowledge of the biosynthetic mutant *dwf4* of *Arabidopsis*. Overexpression of *DWF4* produced a dramatic increase in hypocotyl length in both light and dark and in inflorescence length at maturity. The number of branches and siliques also increased, with a concomitant increase in seed yield (Choe and others 2001). As the authors suggest, the potential for engineering of other species is considerable, and effects in woody species will be fascinating.

Effects in Development: Apical Dominance, Reproduction, and Senescence

Although disturbances in apical dominance, bolting, flowering, silique yield, and timing of senescence in *Arabidopsis* have been observed in molecular genetic studies utilizing mutants, more focused studies on BRs' effects in these areas have rarely been reported in the last few years. Recent work includes a study of embryogenesis, where BRs, although crucial for postembryonic growth, were not so for cell proliferation, but other sterols were important (Shrick and others 2002).

A study of ripening of pericarp discs from tomato showed lycopene and carbohydrate levels and ethylene production increased, whereas levels of chlorophyll and ascorbic acid decreased, after treatment with 24-epibrassinolide or 28-homobrassinolide, which is consistent with accelerated ripening (Vardhini and Rao 2002). In rice, brassinolide treatment before and at heading also accelerated ripening and significantly increased starch content in hulled grain (Fujii and Saka 2001b).

During a search for senescence-associated genes, He and others (2001) developed a preliminary model for a leaf senescence-regulating network in *Arabidopsis* where signals such as abscisic acid, jasmonic acid, ethylene, darkness, dehydration, and aging activated 147 senescence-associated enhancer trap lines. 24-epibrassinolide could activate two of these but associated genes have not yet been cloned.

Endogenous Interactions

Some progress has been made in the study of source–sink relations. Early work with exogenous BRs in bean had suggested that BRs (and auxin and gibberellin) enhance sink strength and phloem unloading (Petzold and others 1992), and Nakajima and Toyama (1999) showed treatment of cucumber roots with 24-epibrassinolide promoted transport of ^{14}C-labeled sucrose from the primary leaf to the epicotyl. Fujii and Saka (2001b) monitored the effect of brassinolide treatment on the distribution of starch and sucrose to organs of rice plants. Starch accumulated in the grains at the expense of the leaf sheaths and culms, whereas sucrose levels decreased in the culms.

Table 1. Mean Heights and Coefficients of Variation of Radish Seedlings after Root Treatment with Brassinolide[a]

BR Treatment nM	0	0.1	1	10	100
S Initial mean	7.7	7.4	7.6	7.1	7.5
Overall mean 7.46 mm					
CV	12.3	20.3	19.9	8	11.3
Overall CV 14.9					
L Initial mean	20.5	18.3	18.8	18.6	17.8
Overall mean 18.8 mm					
CV	13.8	15.9	13	9.9	7.4
Overall CV 12.9					
S Final mean	30.8	34.7	32.9	32.4	45.6*
CV	20.1	36.4	22.4	25.3	25
L Final mean	45.1	42.4	43.3	45	52*
CV	18.5	26.4	18.7	17.4	21.5

[a]*One hundred fifty 4-day-old seedlings from a single batch were divided equally into small (S), medium, and large (L) groups. Plants within the groups were randomly allocated to 5 hormone treatments. Initial mean heights within the S and L groups were not significantly different (n = 10). Seedlings were grown hydroponically for 4 days.*
**These values were not significantly different (p = 0.05).*

Extracellular invertases are important for the supply of carbohydrate to sink tissues, and in tomato cell culture, treatment with three BRs led to a specific enhancement of cell-wall-bound invertase activity, with a rapid induction of the mRNA for one isoenzyme, Lin6. As expected, sucrose uptake was also enhanced, dependent upon the enhanced invertase activity. Furthermore, in hypocotyl segments, BR-dependent growth ''correlated with a localized, tissue-specific induction of mRNA for extracellular invertase Lin6'' (Goetz and others 2000). Thus, BRs not only promote elongation but also, like other hormones, help coordinate the supply of carbohydrate necessary for that and other responses (Roitsch and others 2003).

It is well recognized that BRs influence various developmental pathways. Such effects have their own feedback controls and inevitably impinge on other processes and interact with other influences. These might be other hormones and macromolecular signals, internal conditions at the cellular and organ level such as pH, and ion and metabolite concentrations, as well as external conditions. Studies of such interactions have examined changes in endogenous levels of other hormones, described synergistic effects with exogenous treatments, and discussed modulations of sensitivities of response. For example, increased sensitivity to BRs in dark-grown rice (Yamamuro and others 2000) could reflect increased numbers of BR receptors. Quite sophisticated kinetic approaches have been used occasionally (Weyers and others 1995, and references cited therein); significant differences between response curves that reflect additive or synergistic responses with other hormones were shown for brassinolide (Sasse 1989, 1990). Such studies can point to experimental systems where more detailed investigations using current and developing techniques could provide valuable information.

A molecular genetic approach (together with robust analysis of data) was illustrated in an interaction between BR and abscisic acid in *Arabidopsis*, where a model has been developed for positive regulation of three genes, *BEE1*, *BEE2*, and *BEE3*, by BR, with negative regulation by abscisic acid. These genes encode transcription factors important in ''several developmental programs, including floral organogenesis, hormone responses, and light signaling through phytochrome'' (Friedrichsen and others 2002). BR treatment can also inhibit expression of, for example, the abscisic acid-induced gene *P5CS1*, important for the synthesis of the osmoprotectant proline (Abrahám and others 2003). Another example of an interaction between BR and abscisic acid was discussed in the Seed Germination section.

Regarding hormone levels, pretreatment of *Arabidopsis* seeds with 1 μM 24-epibrassinolide led to considerable cotyledon expansion and slight inhibition of hypocotyl elongation of dark-grown seedlings which were accompanied by significant elevations of free and bound auxin and of free abscisic acid, a slight elevation of free cytokinin, and a decrease in bound abscisic acid (Karnachuk and others 2002). Such changes could alter cellular expansion, and possibly ethylene signaling, and may

imply that BRs upregulate the biosynthesis of other hormones; for example, an effect of BR on the expression of an important enzyme for the biosynthesis of jasmonic acid has been reported (Schaller and others 2000). However, BRs might also impinge on their catabolism, and, in the case of auxin where marked synergistic effects can occur in the presence of both hormones, BRs might affect auxin-dependent targeted protein degradation (reviewed in Leyser 2001). On the other hand, as Ullah and others (2003) proposed, BRs might modulate auxin action upstream of its transcriptional control by coupling via AGB1 in a heterotrimeric G-protein complex.

Interactions with Environmental Signals and Stresses

Tropisms, particularly geotropism in stems, received some attention in the early years of BR research, and, in a more recent study, exogenously supplied brassinolide rapidly enhanced geotropic curvature of maize roots. There was a clear interaction with auxin, whose transport was essential for the response. Castasterone, although not as effective as brassinolide exogenously, was identified endogenously, and Kim and others (2000) suggest that BR increases the sensitivity of the responding tissue to auxin.

There has also been longstanding interest in the interaction of BRs with light signals, and recent advances have been reviewed (Clouse 2001). Fine tuning (Luccioni and others 2002) and species differences have been emphasized (Kang and others 2001; Symons and others 2002; Symons and Reid 2003). The effects of green light were synergistic with those of BR in wild-type *Arabidopsis* (Karnachuk and others 2002), and bioassay data suggested that endogenous BR levels are markedly increased when rice seedlings are exposed to blue light, a signal that is particularly promotive of the unrolling of etiolated leaves (Abe and others 2000). However, there is still very little information about any effects of BRs on phototropism, photoperiodism, or endogenous rhythms.

Similar responses to diverse stimuli and redundancy are concepts that are difficult to explore experimentally, but some progress is being made. The *Arabidopsis TCH4* gene, which encodes an endotransglucosylase hydrolase, is upregulated by environmental stimuli as well as hormones, and recent work suggested that responses to darkness, cold, heat, and BRs occur via shared regulatory elements, and that the environmental stimuli do not require the perception of BR by BRI1. However, a full response to auxin does require it (Iliev and others 2002).

Evidence that BR treatment can ameliorate various biotic and abiotic stresses in plants was discussed by Khripach and others (1999, 2000) and by Krishna in this issue. They and others emphasize the importance of the induction of antioxidant enzymes to protect cells, for example, in temperature stress (Mazorra and others 2002). Brassinolide treatment enhanced early seedling growth, grain ripening, and lamina inclination in chilling stress in rice (Fujii and Saka 2001ab), and Yu and others (2002) found that pretreatment with 24-epibrassinolide or abscisic acid increased tolerance and photosystem II efficiency in cucumber. Pretreatment of seeds with a formulation of synthetic BR and jasmonic acid derivatives also protected cucumber (Asao and others 2002).

In salt stress, pretreatment of rice seeds with 24-epi- or 28-homobrassinolide promoted germination in the presence of sodium chloride. Lengths, fresh and dry weights, and DNA, RNA, and soluble protein contents of the resulting seedlings were also enhanced (Anuradha and Rao 2001). The soluble protein levels were even greater than those of the water control; one hopes these can be investigated in more detail. Also, treatment with a BR analogue enhanced antioxidant enzyme activity in rice exposed to salt (M. A. T. Zullo, personal communication). However, in *Arabidopsis*, expression of a salt-induced reporter gene, *P5CS1-GUS*, was inhibited by brassinolide treatment (Abrahám and others 2003).

There is renewed interest in the protective effects of BR on plants under pathogen attack. Results from rice and tobacco plants infected with representative viral, bacterial, and fungal pathogens showed that biosynthesis of salicylic acid was not required, and acidic and basic pathogenesis-related protein synthesis was not induced in BR-induced resistance (which may also provide another example of species differences from *Arabidopsis*). The data suggest that BR-induced resistance, though ''moderately weak,'' can be distinguished from systemic acquired resistance and wound-inducible resistance, and that it might provide added protection (Nakashita and others 2003).

Other Physiological Effects

Effects of BRs on insect development, particularly molting, were reviewed by Zullo and Adam (2002). Exploration of the effects of BRs in model systems has provided insights into the catabolism of these

compounds, but prospects for their use in predation control do not seem imminent. For example, 24-epibrassinolide or 24-epicastasterone did not affect the evagination of imaginal wing discs, nor was there any effect on intact last-instar larvae of the cotton leafworm, *Spodoptera littoralis*, after oral feeding (Smagghe and others 2002). However, structurally modified BRs may prove more useful.

The low toxicity and low mutagenicity of BRs has been confirmed and protective effects in fish breeding reported. Beneficial effects of BRs for humans were also postulated; apart from our routine ingestion of ubiquitously occurring BRs in plants, the claimed health benefits of pollen may be related to the BR content (Khripach and others 1999, 2000). There are some studies of antiviral effects; analogues of 24(*S*)-ethylbrassinone were tested against herpes simplex virus type 1 and arena viruses (Wachsman and others 2000) and structure and activity were correlated against the measles virus (Wachsman and others 2002).

Towards Applications

The need for efficient crop production, land regeneration, and reafforestation in the context of global problems of salinity, drought, rising temperature, pollution, and pathogen and predator attack is obvious, so exploration of BRs' potential for applications continues. Recently, Zullo and Adam (2002) summarized prospective agricultural uses of BRs and some of their analogues.

One concern is improvement of propagation methods, not only for conventional commercial applications but also if we are to utilize our rapidly increasing number of useful transformed plants. Treatment with an analogue of 28-homoethylcastasterone enhanced the production of shoots on nodal segments from shoots of *Malus prunifolia*, although the dosage was critical. Both stem elongation and lateral branching were increased, with the secondary branching more variable than the primary (Schaefer and others 2002). Other analogues of BR enhance callus formation and shoot regeneration in lettuce in conjunction with cytokinin (M. A. T. Zullo, personal communication). Adventitious shoot regeneration in response to brassinolide itself has been reported in cauliflower hypocotyl segments, and the response was markedly enhanced in the presence of appropriate doses of cytokinins (Sasaki 2002). Levels of the secondary metabolite, artemisinin, as well as biomass, DNA, RNA, and soluble protein were increased by treatment of a hairy root culture of *Artemisia annua* with (22*S*, 23*S*)-homobrassinolide (Wang and others 2002). BR analogues were also used to accelerate vegetative bud formation in cactus pear, increasing the number of cladodes harvested as well as total fresh weight (Cortes and others 2003).

Interest in delaying sprouting of potatoes during storage also continues. The dosage of 24-epibrassinolide is important, and enhanced evolution of ethylene and increased abscisic acid content (with a change in the ratio of free to bound abscisic acid) followed exogenous treatment with the most effective concentration. The main target cells were those of the rib meristem; inhibition of their elongation is of primary importance for delaying sprouting (Korableva and others 2002). Appropriate formulation is required for the most effective use of exogenously applied BRs; wetting agents and additives that slow evaporation and assist penetration are useful, and these aspects have been discussed for crops by Khripach and others (1999, 2000). The use of inclusion complexes is also being explored. At the lowest concentrations used, a 1:1 inclusion complex between 24-epibrassinolide and β-cyclodextrin was more effective on sensitive cultivars in the rice lamina inclination bioassay than free 24-epibrassinolide (de Azevedo and others 2002; M. A. T Zullo, personal communication). *In vivo*, endogenous BR is associated with starch granules in pollen and is easily leached during aqueous fixation (Taylor and others 1993).

Triadimefon is a widely used fungicide with useful side effects of dwarfing, delay of senescence, and increased greening in plants. *Arabidopsis* plants treated with Triadimefon were rescued by combinations of gibberellin and BRs and the fungicide bound with expressed DWF4 protein. The biosynthetic pathway to active BRs was inhibited, inducing a BR deficiency. Specific inhibitors of the biosynthesis of BRs may be useful additions to the repertoire of pesticides and safeners used in agriculture and horticulture (Asami and others 2003). These data may also renew interest in the effects of BRs on fungal development (reviewed by Zullo and Adam 2002). Another idea for future work could be the protective effect of BRs on division of *Chlorella vulgaris* in the presence of toxic metal ions, and their acceleration of the early stages of endogenous phytochelatin synthesis induced by lead nitrate (Bajguz 2000b, 2002)

Other Sources

Further studies, for example, in intracellular immunolocalization of brassinolide, were reported in

the Chinese literature, and micropropagation studies utilizing BRs appeared in the Japanese and Argentinian literature. Ecotoxicological effects on representative plants and animals of a BR formulation used in agriculture were reported from Cuba, and active research groups with varied emphases continue worldwide.

Conclusion and Perspectives

Considerable progress has been made over the last few years in our understanding of BRs, and many of the questions about their roles as plant hormones (Sasse 1991) are being investigated successfully, which is very satisfying for those of us who have been intrigued by these unusual steroids for a quarter of a century or more. In the early years, interesting or novel results, especially those from outside the English-speaking world, were not widely known. Now universal awareness of their crucial role in plant development promises not only exciting results but also exciting opportunities to utilize new techniques. In this, the older literature could well be a mine worth revisiting.

We are likely to hear more details of BRs' signal perception and transduction, of their control of gene expression at several levels, of their interactions with other hormones and signals, and of their involvement in stress responses. Reports cited in this overview, other papers discussed in this issue, and literature in other languages will provide promising leads. We might expect more discussion of species differences and hope for renewed interest in their role in reproduction. We can also ask: are they involved in endogenous rhythms? Are they involved in protein processing? Do they affect transporters or their numbers?

As our understanding of BRs improves, we are also likely to see more applications since BRs appear nontoxic and environmentally friendly. Can the effects of BRs or their analogues on phenotypic variability produce a more uniform crop? Can their effects on toxic metal ion uptake be exploited? Can we synthesize our knowledge? Well designed and well analysed experiments and the contributions of statisticians, system analysts, and modelers will be crucial.

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