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Genomic Brassinosteroid Effects

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Abstract

Detailed analysis of brassinosteroid (BR)-regulated genes can provide evidence of the molecular basis of BR effects. Classical techniques (such as subtractive cDNA cloning) as well as cDNA and oligonucleotide microarrays have been applied to identify genes which are upregulated or downregulated after BR treatment or are differently expressed in BR-deficient or -insensitive mutants compared with wild type plants. Genes encoding cell-wall-modifying enzymes, enzymes of the BR biosynthetic pathway, auxin response factors, and transcription factors are subject to BR regulation. Effects on several other metabolic pathways and interactions with other phytohormones have been reported as well, although some of these effects may depend on certain environmental conditions (for example, light/dark or stress), the developmental stage of the plants, and tissue types. The identification of components of the BR signal transduction pathway revealed different modes of transcriptional control in animals and plants. Steroid signaling in plants comprises the plasma membrane receptor kinases BRI1 and BAK1 and intracellular protein phosphorylations. Thus, BR signaling in plants is reminiscent of growth factor and TGF-β signal transduction in animals. The phosphorylation cascade could be a basis of extensive signaling cross-talk and thereby explain the complexity of BR responses.

Key words: Auxin; Brassinosteroid; Cell elongation; Cell wall; De-etiolation; Gene expression; Genomic effects; Steroid hormones

Introduction

Steroid hormones (SHs) trigger genomic and nongenomic effects in animals. Nongenomic steroid effects occur rapidly within seconds to minutes and include ion fluxes, triggering of action potentials, generation of second messengers, activation of various kinases, induced exocytosis, and induced membrane fusions (Schmidt and others 2000; Watson and Gametchu 1999; Wehling 1997). Genomic effects occur within minutes to hours. The latency of genomic effects is the consequence of a series of events including signal transduction reactions followed by mRNA and protein synthesis. Therefore, genomic effects are sensitive to inhibitors of transcription or translation. SHs from animals and humans pass cell membranes by simple diffusion and bind to steroid hormone receptors (SHRs) within the target cells. Upon activation by the steroid ligand, SHRs bind to palindromic DNA sequences in the vicinity of target genes (termed hormone response elements), interact with transcriptional regulators, initiate chromatin remodeling, and activate transcription (Beato and others 1996; Beato and Klug 2000).

In plants, brassinosteroids (BRs) are required for normal growth and development and elicit various physiological responses. Major BR effects are mediated by plasma membrane receptor-like kinases (Clouse 2002). Although this finding points to the

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possibility of nongenomic BR effects, only little is known about such rapid effects. One focal point of the analysis of BR action in recent years has been the analysis of BR-regulated gene expression, thus, of genomic BR effects. The signal transduction pathway, which transfers the signal from the plasma membrane to the nucleus and to specific target genes, has also been subject to extensive analysis and more and more components are being analyzed. BR signaling to the nucleus involves phosphorylation of intracellular targets and modulation of the subcellular localization of transcriptional regulators. However, a strict differentiation between genomic and nongenomic effects of SHs and BRs may be difficult. For example, both pathways may be required [for example, for calcitriol signaling (Fleet 1999)] and might interact (for example, via second messenger-related modulation of some steroid-induced transcriptional processes).

This review focuses on BR-regulated gene expression. The use of microarrays provided global insights into BR target genes in *Arabidopsis thaliana*. In addition, classical techniques (such as subtractive hybridizations) identified BR-regulated genes in other plant species. Genes that exhibit transcriptional regulation by BRs lay the foundation for further in-depth analysis of BR responses and allow the integration of BRs into a complex signaling network in which BRs are factors that depend on and modulate other factors.

The Animal Model of Genomic Steroid Effects

SHRs from humans and animals have a modular structure, consisting of a DNA-binding domain (approximately 80 amino acids with two Cys_2–Cys_2 zinc fingers), nuclear localization signals (the number and location of which varies among SHRs), a ligand-binding domain, transcription activation functions, and regions required for receptor dimerization (Beato and others 1996; Beato and Klug 2000). Unliganded SHRs are in an inactive state and associated with a complex of chaperones, which maintain the receptors in a conformation able to bind ligands. After steroid binding SHRs gain transcriptional competence (although some receptors, such as the retinoid acid receptors, also can repress transcription in the unliganded state). SHRs bind to short palindromic DNA repeats termed hormone response elements (HREs), which are located in the vicinity of target genes and confer direct transcriptional responsiveness to adjacent genes. The binding of SHRs initiates chromatin remodeling and the regulation of gene expression. SHRs interact with the general transcription machinery, transcription intermediary factors (TIFs), sequence-specific transcription factors, corepressors, and proteins involved in chromatin remodeling. In general, the chromatin status of target genes is a major determinant of their transcriptional activity. In the two-step model for transcriptional activation, SHRs first interact with transcription factors and initiate chromatin remodeling (for example, by means of an ATP-dependent noncovalent mechanism or via histone acetylation). Then, SHRs (or interacting proteins) mediate interactions with the basal transcription machinery and activate transcription.

In plants, no genes homologous to the superfamily of nuclear receptors (which include SHRs) have been identified. Instead, BRs bind to BR receptors at the cell surface, which transfer the signal into the cytoplasm. In the cell, the phosphorylation status and nuclear localization of proteins such as BES1 and BZR1 are affected, which in turn affect gene expression patterns (see below). Thus, steroid signaling in plants is reminiscent of insulin, growth factor, or transforming growth factor β (TGF-β) signal transduction processes, which are initiated by receptor tyrosine kinases and Ser/Thr receptor kinases, respectively (Hubbard and Till 2000; Massague 1998).

BR Signal Transduction: From the Plasma Membrane to the Nucleus

Until recently, BR signal transduction analysis focused primarily on BRI1, a component of the BR receptor (Clouse 2002). A second leucine-rich repeat receptor kinase (termed BRI1-associated receptor kinase 1, BAK1), was identified that interacts with BRI1 (Li and others 2002; Nam and Li 2002). BRI1 and BAK1 may exist as inactive monomers that are in equilibrium with active dimers. BR binding promotes active dimer formation and leads to activation of both receptor kinases via transphosphorylation. The active kinases then recognize and phosphorylate downstream targets (Nam and Li 2002). Recombinant BRI1 kinase phosphorylates certain synthetic peptides, and a putative consensus sequence required for peptide-substrate recognition by BRI1 was deduced (Oh and others 2000). However, the recognition motif has not been verified *in vivo*. A plant homolog of the TGF-β receptor interacting protein (TRIP-1) contains elements of the recognition motif (Clouse 2002). TRIP-1-antisense

plants display several characteristics of BR mutants (Jiang and Clouse 2001). The (not yet demonstrated) BRI1/TRIP-1 interaction would point to parallels between BR and TGF-β signaling (Clouse 2002).

Several downstream components of the BR signal transduction pathway have been identified. *BIN2/UCU1* (*BRASSINOSTEROID-INSENSITIVE-2/ULTRACURVATA1*) encodes a GSK3/SHAGGY-like kinase and functions as a negative regulator of BR signal transduction (Li and Nam 2002; Pérez–Pérez and others 2002). Potentially, the BRI1 receptor complex inhibits BIN2/UCU1, that otherwise phosphorylates its potential substrates BZR1 and BES1. BZR1 and BES1 are closely related proteins and contain multiple consensus sites for phosphorylation by GSK3 kinases. The *bes1-D* mutation leads to a BR-hypersensitive phenotype, which does not require BRs and BRI1 (Yin and others 2002b) and causes constitutive expression of BR-regulated genes. BIN2 phosphorylates BES1 and appears to negatively affect the level of BES1 protein. Thus, phosphorylation by BIN2 might trigger the proteasome-mediated BES1 degradation and repress BR-induced gene expression. In line with these findings, unphosphorylated BES1 accumulates in the nucleus in reponse to 1 μ*M* BL treatment (Yin and others 2002b). Similar results were obtained for the *BZR1* gene, which was identified by means of the *brassinazole-resistant 1-1D* mutant (Wang and others 2002). The BZR1 protein is a positive regulator of the BR signaling pathway as well, and BIN2 phosphorylates and destabilizes it (He and others 2002). The molecular modes of action of the BES1 and BZR1 proteins are unclear. Within the nucleus, a putative topoisomerase VI is required for the occurrence of genomic BR effects. The *bin3* and *bin5* (*b*rassinosteroid *i*nsensitive *3* and *5*) mutants have identical phenotypes and display some characteristics of BR mutants (Yin and others 2002a). The affected genes represent topoisomerase VI subunits. Several BR-induced genes show impaired induction in *bin3* and *bin5* mutants (Yin and others 2002a).

BR-Regulated Genes

BR-regulated genes were identified by means of different approaches. In many cases synthetic BRs were applied to intact plants or excised tissues. However, altered transcript levels after BR treatments do not necessarily reflect normal physiological events. The rate of uptake and the degree of distribution of the applied BRs are unknown and thus is the actual dose of BRs and the tissues reached. Long-term BR treatments most likely result in secondary effects. For example, BRs may alter the levels of other phytohormones, which in turn modulate expression patterns. The application of higher concentrations of active BRs results in severe developmental alterations, endogenous regulatory pathways are overrun, and growth becomes unbalanced. Finally, responses to synthetic BRs depend on the genotype. Wild-type plants may display limited responses due to appropriate endogenous BR levels. BR-deficient plants, on the other hand, may display unusual responses due to the release from a long-term ‘‘BR starvation.’’ The sudden (and extreme) growth induction may trigger compensatory mechanisms. In summary, altered transcript levels after BR application may not only point to direct BR effects but also may indicate secondary effects (as a result of primary BR responses) or abnormal effects (as a consequence of the ectopic provision of BRs).

Conversely, the consequences of BR deficiency or lack of BR response may be studied using BR mutants in comparison to the wild type. Such analysis might be compromised by secondary effects that arise in the mutants due to a long-term block in BR biosynthesis or BR signal transduction. For example, the extreme dwarfism of some mutants and lack of organ formation in the latter may constitute secondary causes for altered gene expression patterns (Azpiroz and others 1998). Therefore, the term ‘‘BR-regulated gene’’ should be used with caution. Below we give an overview of (candidate) genes hitherto identified through a series of studies, derive potential BR functions, and integrate expression data into physiological pathways.

Cell Growth and Differentiation

BR treatment promotes vegetative growth and increases yield of crops (Ikekawa and Zhao 1991; Kamuro and Takatsuto 1999). Conversely, BR-deficient or BR-insensitive mutants display dwarfism. The reduced size of the fifth foliage leaves of *det2* and *dwf1* mutants is due to reduced cell expansion and reduced cell numbers per leaf blade. BR treatment results in the reversal of the defects in size and number of *det2* leaf cells (Nakaya and others 2002). Longitudinal sections through cotyledons and hypocotyls of *cbb1*, *cbb2*, and *cbb3* mutants (which are allelic to the *dwf1*, *bri1*, and *cpd* mutants, respectively) revealed that the reduced size of mutant seedlings is primarily due to a reduction of cell size (Kauschmann and others 1996). Thus, BR deficiency is accompanied by reduced cell elongation,

indicating an essential role of BRs in the control of cell wall expansion.

Among the first identified BR-regulated genes are a few genes from different species encoding (putative) cell-wall-modifying enzymes. Xyloglucans are seen as the polysaccharide of key importance in controlling wall expansion. Xyloglucans coat cellulose microfibrils and are long enough to span the distance between microfibrils and link them together. To expand this network, either xyloglucan molecules must be cut (potentially with insertion of additional xyloglucan, as happens during endotransglycosylation) or the xyloglucan binding to microfibrils must be weakened (Cosgrove 2000). The biochemical activity of xyloglucan endotransglucosylase/hydrolases (XTHs, previously abbreviated as XETs; Rose and others 2002) has been defined *in vitro*: XTHs generate shorter and longer polymers from a homogeneous xyloglucan mixture and they incorporate short, radiolabeled xyloglucan-derived oligosaccharides into longer xyloglucan polymers (Campbell and Braam 1999). Thus, XTHs potentially are involved in wall loosening to enable turgor-driven expansion, or they contribute to wall biogenesis and catalyze the incorporation of new cell wall material. The transcript levels of several XTHs from different plant species are upregulated after BR treatment, for example, *BRU1* from *Glycine max* (Zurek and Clouse 1994), *LeBR1* from *Lycopersicon esculentum* (Koka and others 2000), *OsXTR1* and *OsXTR3* from *Oryza sativa* (Uozu and others 2000), and *TCH4* from *Arabidopsis thaliana* (Xu and others 1995). Run-on transcription assays with isolated nuclei demonstrated that the upregulation of *BRU1* expression is posttranscriptional (Zurek and Clouse 1994). Microarray studies indicated the upregulation of several additional XTHs (Goda and others 2002; Müssig and others 2002; Yin and others 2002a, 2002b). Although BR application increases *TCH4* mRNA levels, BRs are not required for the expression of *TCH4* during morphogenesis because BR mutants such as *det2-1*, *bri1-2*, and *dwf1-6* do not display altered basal *TCH4* transcript levels (Iliev and others 2002; Müssig and others 2002).

The expression of some XTHs during fruit ripening, the usage of xyloglucans as storage polysaccharides in some species, and the sequence divergence among XTHs may point to functions not related to cell growth. In fact, some XTHs might function in cell wall degradation (for example, during fruit softening) or might perform functions that are not related to cell wall structure (Campbell and Braam 1999). These observations are consistent with a lack of BR induction of some XTHs (Müssig and others 2002; Xu and others 1996).

Expansins, which catalyze acid-induced wall extension, have been proposed to play a central role in cell wall loosening (Cosgrove 2000; Darley and others 2001). Potentially, expansins break the noncovalent bonds between cellulose and xyloglucans. In a wall under tension, this could allow movement of released hemicellulose which bonds to the microfibrils at another position. The expression of several expansin genes is stimulated by BRs (Goda and others 2002; Müssig and others 2002; Yin and others 2002a, 2002b). This finding is in line with early results that demonstrated that BRs stimulate growth by increasing wall relaxation (Wang and others 1993). In some experimental systems, acid secretion is affected by BRs (Mandava 1988), and a membrane-bound ATPase inhibitor blocks BR-induced elongation (Katsumi 1985). Thus, BR-induced growth might involve genomic and nongenomic effects, a subset of which might be secondary (for example, mediated via BR–auxin interactions).

Endo-1,4-β-D-glucanases (EGs) comprise a multigene family in higher plants. EGs hydrolyze 1,4-β linkages, and *in vitro* activity has been observed with glucan polymers such as xyloglucans (Cosgrove 2000; Darley and others 2001). The membrane-bound KORRIGAN protein might play a central role in expanding cell walls (Nicol and others 1998). The *kor* mutant displays extreme dwarfism and pronounced architectural alterations in the primary cell wall. *KOR* transcript levels are decreased in the BR-deficient *det2* mutant, indicating a further potential mechanism of growth control by BRs.

Microarray data suggest BR regulation of pectin-modifying enzymes and of structural cell wall proteins such as arabinogalactan proteins (AGPs or proteoglycans) and extensins (or hydroxyproline-rich glycoproteins) (Goda and others 2002). The amount of AGP proteins in hypocotyls of the BR-deficient *dim* mutant is lower than that in wild-type hypocotyls (Takahashi and others 1995).

The *bul1/dwf7-3* mutant is defective in the Δ^7-sterol-C5-desaturation step leading to BR biosynthesis. It displays a dwarf phenotype, which might be (partially) caused by a defect in microtubule organization. BR treatment induces cortical microtubule orientation and restores cell elongation. Microfibrils were not detected in *dim* epidermal cells (Takahashi and others 1995). *bul1* and *dim* mutant hypocotyls contain slightly lower levels of total tubulin (Catterou and others 2001), and the expression of a β-tubulin gene, *TUB1*, is reduced *in bul1* and *dim* plants (Catterou and others 2001; Takahashi and others 1995). Thus, the *bul1* and *dim* mutants (and other BR mutants as well) could be

defective in the polymerization of tubulin into microtubules. Accordingly, the expression of a β-tubulin gene from *Cicer arietinum* is promoted by exogenous BRs (Munoz and others 1998). In azuki bean epicotyl segments, BRs appear to enhance cell elongation by organizing cortical microtubules and, thereby, causing the directional deposition of cellulose microfibrils and determining the direction of cell expansion (Mayumi and Shibaoka 1995).

In addition to their role in cell elongation, BRs appear to play a role in the regulation of cell division. BRs increase division frequencies of regenerating mesophyll protoplasts, in particular under suboptimal auxin conditions (Oh and Clouse 1998). CycD3 is a mediator of plant mitogenic signals, and *CycD3* induction by BRs in *det2* suspension cultures may explain BR-driven cell division (Hu and others 2000).

Correlative evidence suggests that the growth-stimulating effect of BRs is in part due to a positive impact on primary carbon metabolism. BR treatment results in the accumulation of photosynthates and enhances CO_2 fixation (Braun and Wild 1984), whereas *CPD*-antisense plants display reduced assimilatory capacity and reduced starch content (Schlüter and others 2002). A role of BRs in source-sink regulation was demonstrated for tomato seedlings through the identification of a BR-inducible extracellular invertase (Goetz and others 2000). Correspondingly, *dwf1–6* and *CPD*-antisense plants show reduced acid invertase activity (Schlüter and others 2002).

In tomato apical meristems, *CYP85* mRNA (encoding a P450 involved in BR biosynthesis) accumulates in cells involved in the earliest visible stages of primordium formation (Pien and others 2001). Following primordium formation, *CYP85* expression is not detectable within the meristem. This finding suggests a localized synthesis of brassinolide during early leaf development. The carbohydrate metabolism is spatially regulated within the meristem. *Sus4* transcripts (encoding a sucrose synthase) show a pattern of expression similar to that of *CYP85* and accumulate throughout the meristem after brassinolide treatment, indicating a role of BRs in the regulation of carbohydrate metabolism (Pien and others 2001).

BRs play an essential role in vascular differentiation. Exogenous BRs promote xylem formation in tuber explants of *Helianthus tuberosus* and mesophyll cells from *Zinnia elegans* (Clouse and Zurek 1991; Iwasaki and Shibaoka 1991). The transdifferentiation of *Zinnia elegans* mesophyll cells can be divided into three stages. Stage 1 is a dedifferentiation, stage 2 is a restriction of developmental potential, and stage 3 comprises secondary wall synthesis and programmed cell death. The transition between stage 2 and stage 3 is irreversible and requires endogenous BRs. Uniconazole (an inhibitor of BR biosynthesis) prevented tracheary element formation and suppressed the accumulation of stage 3 transcripts (such as *ZC4H*, *ZePAL3*, and *ZCP4*). The inhibition of tracheary element differentiation and the suppression of stage 3 specific gene expression by uniconazole was overcome by the addition of BRs (Yamamoto and others 1997). Quantitative measurements of endogenous BRs in each stage showed that they increase dramatically prior to morphogenesis of tracheary elements (Yamamoto and others 2001). The BR-deficient *cpd* mutant shows extranumerary phloem cell files at the expense of xylem cells (Szekeres and others 1996), and brassinazole (an inhibitor of BR biosynthesis) treatment of *Lepidium sativum* plants likewise results in a predominance of phloem differentiation at the expense of xylem differentiation (Nagata and others 2001). Control of xylem differentiation may be mediated by three BR-induced homeobox genes (*ZeHB-10, -11,* and *-12*), which are preferentially expressed in immature xylem cells (Ohashi–Ito and others 2002).

Negative Feedback Regulation of Genes Involved in BR Biosynthesis

The expression of genes involved in BR biosynthesis is negatively controlled by BRs, indicating a negative feedback regulation of BR biosynthesis. The *CPD* gene encodes a cytochrome P450 that converts either cathasterone to teasterone or 6-deoxocathasterone to 6-deoxoteasterone and plays an essential role in BR biosynthesis (Szekeres and others 1996). The *CPD* promoter and a 5′-untranslated leader sequence was cloned upstream of an *uidA* reporter gene. Side-chain hydroxylated BRs, including brassinolide, inhibit the activity of the *CPD* promoter (Mathur and others 1998). Other phytohormones, such as indole-3-acetic acid, gibberellic acid, or 6-benzylaminopurine, did not show clear effects on the activity of the *CPD* promoter-driven *uidA* reporter gene. The expression of several other (brassino)steroidogenic P450s (such as *DWF4*, *ROT3*, *CYP85*) is downregulated by BRs as well (Bancos and others 2002; Goda and others 2002; Müssig and others 2002). Further oxygenation steps in the BR biosynthetic pathway are probably catalyzed by P450s and have yet to be characterized. Expression profiling experiments revealed some candidate

genes (Goda and others 2002). Several P450 genes involved in BR biosynthesis display differential transcript accumulation in shoots and roots of *Arabidopsis* plants, but organ-specific expression appears to be independent of BR action (Bancos and others 2002).

Plant sterols function as biosynthetic precursors of brassinosteroids. The *Arabidopsis* mutants *dwf7/ste1*, *dwf5*, and *dim/dwf1* are blocked in each successive step in the conversion of episterol to campesterol. The phenotypic alterations of these mutants are similar to BR-deficient mutants but are less severe and can be normalized by exogenous BRs. Campesterol is converted to castasterone and brassinolide, and the *DET2* gene product (a 5α-steroid reductase) catalyzes the first step in this BR-specific pathway. The *DWF7/STE1*, *DIM1/DWF1*, and *DET2* genes are not downregulated by (24-epi)brassinolide (Bancos and others 2002; Goda and others 2002; Müssig and others 2002), indicating that genes encoding enzymes of phytosterol synthesis or very early steps in BR biosynthesis are not negative feedback regulated by active BRs.

Light-Regulated Genes

Arabidopsis BR mutants display de-etiolation and de-repression of light-induced genes in the dark (Chory and others 1991; Kauschmann and others 1996; Szekeres and others 1996). The skotomorphogenetic characteristics of BR mutants (such as short hypocotyls, opened cotyledons, and the emergence of primary leaves) are in contrast to those of other dwarfed *Arabidopsis* mutants impaired in gibberellin biosynthesis and gibberellin perception (Kauschmann and others 1996). The morphological and cytological changes in brassinazole-treated *Arabidopsis* wild-type plants grown in the dark are similar to those of the *det2* mutant (Nagata and others 2000). The morphological signs of de-etiolation in mutants such as *det2* and *cpd*, as well as in brassinazole-treated wild-type plants, are accompanied by an increase in the expression of genes such as *rbcS* and *CAB* (Asami and others 2000; Chory and others 1991; Szekeres and others 1996). However, the constitutive photomorphogenic phenotype of the BR-deficient *dwf4* mutant may be a secondary effect of its reduced stature and the growth conditions, and the *dwf4* mutant does not show increased *CAB* promoter activity in the dark in comparison to wild-type plants (Azpiroz and others 1998).

Antisense *CDC2b* transgenic seedlings develop short hypocotyls in the dark (Yoshizumi and others 1999), a phenotype that is caused by impaired cell expansion. BRs induce *CDC2b* gene expression in the dark and partially restore hypocotyl cell elongation, indicating that CDC2b is acting downstream of BRs. Because BRs do not induce *CDC2b* expression in the light, there may be additional regulatory pathways.

PIF3 is a basic helix–loop–helix transcription factor required for the phytochrome regulation of photoresponsive genes (Ni and others 1998) and binds to light-regulated promoters through the G-box motif (Martinez–Garcia and others 2000). In BR-deficient *det2* plants grown under continuous light, repression of *PIF3* expression is apparent within 15 min. after BR treatment (Goda and others 2002). The promoter regions of the *Lhcb1-3* and *rcbS-1A* genes contain G boxes. The expression of both genes is repressed by BRs after a lag period of 1 h as well. Therefore, BRs may modulate light-regulated plant development by affecting *PIF3* expression. In plants exposed to cycloheximide, *PIF3* expression was not repressible by BR treatment, indicating that BRs might function upstream of a short-lived repressor of *PIF3* expression (Goda and others 2002). However, *dwf1–6* (*cbb1*) and *CPD*-antisense plants grown in soil under a 16 h light/8 h dark regime show a normal light-dependent regulation of genes such as *rcbS* and *ApS* (Schlüter and others 2002). Thus, BRs do not affect light-regulated gene expression *per se*.

The *BAS1* gene encodes a CYP450 that likely catalyzes the inactivation of BRs by means of C26 hydroxylation. *BAS1* overexpression suppresses the long hypocotyl phenotype of a weak *phyB* allele (Neff and others 1999). *BAS1* gene expression is BR-induced (Goda and others 2002) but not regulated by light (Neff and others 1999).

A novel putative link between light signaling and BR levels in pea plants has been established in the course of the identification of proteins, which interact with the small monomeric G protein Pra2. The dark-induced Pra2 protein interacts with the DDWF1 protein and stimulates its activity. The *DDWF1* (*d*ark-induced *DWF*-like protein *1*) gene encodes a P450 which catalyzes C-2 hydroxylation in BR biosynthesis (Kang and others 2001). *DDWF1* is predominantly expressed in the elongating zone of etiolated pea epicotyls and dark-induced as well. Thus, BR biosynthetic genes such as *DDWF1* are light-regulated.

However, wild-type pea plants do not show reduced endogenous levels of castasterone and brassinolide in light-grown plants in comparison with dark-grown plants. Furthermore, pea BR mutants (such as *lk* and *lkb*) are not de-etiolated and do not show altered *CAB* and *rbcS* transcript levels in the

dark. Application of brassinazole to dark-grown wild-type pea plants does not result in a de-etiolated phenotype (Symons and Reid 2003; Symons and others 2002). Thus, there is no evidence for a negative regulatory role of BRs in de-etiolation in pea.

Interactions with Other Phytohormones

Auxin and GAs

BRs elicit very clear responses in auxin bioassays (Grove and others 1979; Katsumi 1985; Takeno and Pharis 1982; Yopp and others 1981). Possibly, BRs confer increased auxin sensitivity and vice versa (Cohen and Meudt 1983; Katsumi 1985; Kim and others 1990). Genes such as *TCH4* (Xu and others 1995), *SAUR6B* (Zurek and others 1994), *IAA2*, *IAA3*, *IAA13*, *IAA19*, *IAA22*, and *SAUR-AC1* (Goda and others 2002; Müssig and others 2002) are regulated by BRs and auxin. Genes such *IAA3* and *SAUR-AC1* are not induced within 15 min after BR treatment (the time required for a significant induction by auxin treatment); they require longer induction times (Goda and others 2002). According to this observation, it is conceivable that BRs activate auxin biosynthesis, affect auxin signaling, or confer similar responses by an independent mechanism. The short-term regulation of auxin-inducible genes such as *IAA3*, *IAA19*, and *SAUR-AC1* within 1 h (Goda and others 2002; Müssig and others 2002) and the lack of induction of other genes such as *SAUR15A* (Zurek and others 1994) may point to a mechanism different from alterations of auxin levels. Correspondingly, BR treatment has been shown to reduce rather than increase free IAA levels in soybean epicotyls (Zurek and others 1994), and BRs repress *IAR3* expression suggesting reduced release of auxin from amino acid conjugates (Davies and others 1999; Müssig and others 2002).

BRs display activity in several GA bioassays (Mandava and others 1981; Yopp and others 1979). However, GA and BR additively stimulate growth (at least in mung bean epicotyls) (Gregory and Mandava 1982), indicating that both compounds stimulate growth by means of independent pathways. GA treatments fail to normalize BR mutants but elicit responses such as limited elongation of leaf petioles (Kauschmann and others 1996). Both BR and GA promote germination but appear to use distinct pathways (Leubner–Metzger 2001; Steber and McCourt 2001).

First hints of GA and BR signaling cross-talk were derived from the finding that the *bri1-201* mutant has drastically reduced transcript levels of the GA-repressed *GA5* gene and clearly elevated transcript levels of the GA-inducible *GASA1* gene. GA treatment elicited normal responses with respect to the expression of both genes. Thus, the *bri1* mutant is sensitive to GA and is not qualitatively compromised in responses to GA. BR treatment of the *cpd* mutant results in decreased *GASA1* and increased *GA5* expression (Bouquin and others 2001). BR treatment also represses *GA5* expression in plants treated with the GA biosynthesis inhibitor ancymidol and in the *cpd–ga1-1* double mutant. These findings show that BRs affect the expression of the *GA5* and *GASA1* genes antagonistically to GA, and BR and GA effects on *GA5* expression are independent from each other.

Nevertheless, BRs are not general antagonists of GA-mediated gene expression. *GA4*, *GAI*, and *RGA* mRNA levels are not significantly altered in the *bri1* mutant (Bouquin and others 2001), and expression-profiling experiments of BR-deficient mutants and BR-treated plants did not reveal major changes of transcript levels of other GA-regulated genes (Goda and others 2002; Müssig and others 2002). Potentially, there is no exclusive BR–GA interaction. In fact, there may be additive and antagonistic effects (depending on the tissue, developmental stage, or environmental conditions), and the majority of effects may be unique to either GAs or BRs.

Ethylene and Jasmonic Acid

BR treatment stimulates ethylene production, potentially via the regulation of genes involved in ethylene synthesis (Arteca and Arteca 2001; Yi and others 1999). However, other phytohormones (in particular auxins and cytokinins) have similar effects. BRs positively interact with other phytohormones (such as auxin and kinetin) with respect to ethylene production (Arteca and others 1983; Schlagnhaufer and Arteca 1983), but this positive interaction is not unique to BRs. Thus, the physiological relevance of BR–ethylene interactions remains to be clarified.

The *OPR3* gene product is involved in jasmonic acid (JA) biosynthesis (Müssig and others 2000; Schaller and others 2000). BR treatment results in elevated *OPR3* transcript levels. This finding points to a BR-stimulated JA biosynthesis. *LOX2* (and possibly *FAD7*) expression is BR induced as well within 3 h after BR treatment, but *AOS* transcript levels are not (C. Müssig, unpublished data). The basal *LOX2* and *Thi2.1* transcript levels in leaves and developing siliques of soil-grown *cbb1/dwf1-6* and *CPD*-antisense plants are not reduced, and the

OPDA and JA levels in vegetative shoot material of *CPD*-antisense plants appear not to be reduced (F. Schaller, E.W. Weiler, T. Altmann, C. Müssig, unpublished data). The *LOX2* and *Thi2.1* mRNA levels of different BR-deficient plants were analyzed following different stress treatments (such as NaCl and sorbitol application, UV-light irradiation, and wounding). In all cases, the responses of the BR-deficient plants were similar to those of wild-type plants (C. Mussig, unpublished data). Although these data do not exclude a positive effect of BRs on JA levels, they suggest normal endogenous JA levels in BR-deficient plants. Microarray analysis of BR-regulated gene expression also did not provide clear hints to a function of BRs in JA-mediated signaling (Goda and others 2002; Müssig and others 2002). In addition to the *OPR3* gene expression, *OPR1* and/or *OPR2* gene expression is BR induced as well (C. Müssig, unpublished data). Recombinant OPR1 and OPR2 proteins do not effectively convert the natural (9*S*,13*S*)-12-oxophytodienoic acid to the corresponding OPC-8:0 stereoisomer, suggesting that these proteins are not relevant for JA biosynthesis (Schaller and others 2000). Thus, the physiological relevance of BR effects on *OPR1/2* and *OPR3* gene expression is unclear.

BRs and Stress

Enhanced resistance of BR-treated plants to temperature, salt, water, phytopathogens, and other environmental stresses was reported (Khripach and others 2000; Nakashita and others 2003; Sasse 1999). However, with the exception of heat stress, systematic investigations into the underlying molecular basis have barely been undertaken. 24-Epibrassinolide-treated *Brassica napus* and tomato seedlings are more tolerant to lethal heat treatments than are control seedlings. A preconditioning heat shock was not required to observe this effect (Dhaubhadel and others 1999). BR-treated *B. napus* seedlings display higher levels of heat shock proteins (Hsps) and their corresponding mRNAs during heat stress. During recovery from heat stress, *Hsp* transcript levels are lower even though higher amounts of Hsp proteins are synthesized. Thus, Hsp synthesis is not correlated with *Hsp* transcript levels. Several translation initiation and elongation factors are present at significantly higher levels in BR-treated seedlings, suggesting that BR treatment limits the loss of essential components of the translation machinery and, thereby, allows a more rapid regeneration and higher survival rate after heat shock (Dhaubhadel and others 2002).

Conclusions and Outlook

Several groups reported the identification of BR-regulated genes, and the data reveal effects on genes involved in cell wall metabolism, vascular differentiation, auxin response, BR biosynthesis, and additional pathways (Table 1). However, some discrepancies remain and cannot be fully explained by technical or experimental differences. A major source of differences appears to be the genotype of the plants used for the analysis. BR-deficient plants (such as *cbb1/dwf1-6* and *det2*) respond in a different way to exogenous BRs than wild-type plants, and the position of the block in the BR pathway appears to be important. For example, despite their BR deficiency, the *cbb1/dwf1* mutant and other mutants of the phytosterol pathway display mild phenotypic alterations in comparison to mutants of the specific BR pathway such as *det2*, *dwf4*, and *cpd* (for hitherto unknown reasons). Expression profiles should mirror these phenotypic differences. The genetic background (for example, Col-0 or C24) may modulate BR responses, for example, with respect to environmental influences. Finally, the applied BR (for instance, brassinolide, 24-epibrassinolide, castasterone, 24-epicastasterone), its concentration, and the length of treatments are of major importance. High BR concentrations (in particular of highly active BRs) induce abnormal growth effects within a few hours and are lethal in the long term, whereas the application of precursors may allow controlled metabolic conversions and help to prevent toxic effects.

BR-responsive promoter elements have not been reported hitherto. BRs modulate the expression of transcription factors (such as *BEE1*, *BEE2*, *BEE3*, *MYB13*, and *MYB14*) (Friedrichsen and others 2002; Müssig and others 2002), which are regulated by other pathways as well and potentially bind to a diverse set of DNA motifs. In light of the phosphorylation events involved in BR signal transduction, one might expect extensive cross-talk with other signaling pathways that could be the basis for the responses of the genes to multiple signals.

Future expression analysis may be directed towards specific tissues or organs, to BR-modulated stress responses, and to BR–environment interactions. Different environmental conditions can have profound effects on gene expression patterns (Müssig and others 2002), and BR responses are likely to be adjusted to the respective situation. Thus, there may be a limited set of directly and exclusively BR-regulated genes, but numerous other genes may show BR responses under specific

Table 1. BR-Regulated Genes[a]

	Encoded protein(s)	References
Upregulated genes		
Cell wall modification	**XTHs**	
	BRU1 (*Glycine max*)	Zurek and Clouse (1994)
	LeBR1 (tomato)	Koka and others (2000)
	OsXTR1, OsXTR3 (rice)	Uozu and others (2000)
	TCH4 (*Arabidopsis*)	Xu and others (1995)
	Expansins	
	EXP5 (*Arabidopsis*)	Müssig and others (2002)
	EXP8, BRU11 (*Arabidopsis*)	Goda and others (2002)
	EG	
	KOR (*Arabidopsis*)	Nicol and others (1998)
Cell division	**Cyclin**	
	CycD3 (*Arabidopsis*)	Hu and others (2000)
Vascular differentiation	**Transcription factors**	
	ZeHB-10, -11, and -12 (*Zinnia elegans*)	Ohashi-Ito and others (2002)
	Other proteins	
	ZC4H, ZePAL3, ZCP4 (*Zinnia elegans*)	Yamamoto and others (1997)
BR response	**Transcription factors**	
	BEE1, BEE2, BEE3 (*Arabidopsis*)	Friedrichsen and others (2002)
	Other proteins	
	TRIP-1 (*Arabidopsis, Phaseolus vulgaris*)	Jiang and Clouse (2001)
Phytohormone interaction	**Auxin response**	
	SAUR6B (*Glycine max*)	Zurek and others (1994)
	IAA2, IAA3, IAA13, IAA19, IAA22, SAUR-AC1 (*Arabidopsis*)	Goda and others (2002), Müssig and others (2002)
	Gibberellin biosynthesis	
	GA5 (*Arabidopsis*)	Bouquin and others (2001)
	Ethylene biosynthesis	
	VrACS7 (*Vigna radiata*)	Yi and others (1999)
	Jasmonic acid biosynthesis	
	OPR3 (*Arabidopsis*)	Müssig and others (2000)
Carbon partitioning	**Sucrose degradation**	
	Lin6 (tomato)	Goetz and others (2000)
	Sus4 (tomato)	Pien and others (2001)
Cell rescue	**Heat shock proteins**	
	sHsps, Hsp70, Hsp90, Hsp101 (*Brassica napus*)	Dhaubhadel and others (1999), (2002)
Downregulated genes		
BR biosynthesis	**Cytochrome P450 monooxygenases**	
	CYP90A1 (CPD) (*Arabidopsis*)	Mathur and others (1998)
	CYP85A1, CYP85A2, CYP90B1 (DWF4), CYP90C1 (ROT3), CYP90D1 (*Arabidopsis*)	Bancos and others (2002)
BR signalling and response	**Miscellaneous**	
	BRI1 (*Arabidopsis*)	Goda and others (2002), Müssig and others (2002)
	BRH1 (*Arabidopsis*)	Molnár and others (2002)
Light signalling	**Transcription factor**	
	PIF3 (*Arabidopsis*)	Goda and others (2002)

[a] *Additional genes are mentioned in the text.*

conditions and constitute BR-dependent genes as well. Finally, biosynthetic intermediates could have specific functions which may be uncovered by the comparison of various mutants.

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