Journal of Experimental Botany, Vol. 55, No. 395,
Crosstalk in Plant Signal Transduction Special Issue, pp. 265–270, January 2004
DOI: 10.1093/jxb/erh024 Advanced Access publication 12 December, 2003

Journal of Experimental Botany

BRing it on: new insights into the mechanism of brassinosteroid action

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Received 24 March 2003; Accepted 8 October 2003

Abstract

Several recent breakthroughs have filled in key details of the brassinosteroid (BR) response. Identification of BAK1, a BRI1 interacting protein, the negative regulator BIN2, as well as direct targets of BIN2, BZR1 and BES1, provide a link between BR perception at the cell surface and regulation of gene expression in the nucleus. Global expression studies further defined the downstream events in this pathway, confirming the role of several factors acting in negative feedback regulation on BR levels. New links to the plant hormone, auxin, were also uncovered.

Key words: *Arabidopsis*, auxin, brassinosteroids, light.

Introduction

Hormones are at the heart of all plant growth and development, yet the mechanism by which their effects are harnessed into specific morphological outcomes are largely unknown. Hormone activity has been implicated in responses to a wide array of biotic, abiotic, and developmental stimuli. Brassinosteroids (BRs), among the newest hormones to be identified, play roles throughout the plant life cycle, including germination, root and stem elongation, seedling photomorphogenesis, vascular development, floral organ elongation, and senescence.

Brassinolide (BL), the most biologically active BR, was initially isolated in an ambitious experiment using over 200 kg of *Brassica napus* pollen as the starting material (Grove *et al*., 1979). Subsequently, BRs have been identified in all plant species examined to date. Purified BL was shown to promote cell elongation in a number of bioassays encompassing diverse species and tissue types (Mandava, 1988). A breakthrough in elucidating the crucial role of endogenous BRs came from the isolation of mutants defective in BR biosynthesis and perception (Clouse, 2002). *DET2* and *CPD* were the first genes in the biosynthesis pathway to be cloned, and have been shown to encode a steroid 5α-reductase and a C23-steroid hydroxylase, respectively (Li *et al*., 1996; Szekeres *et al*., 1996). Both mutants were originally identified for exhibiting strikingly light-grown morphology, even when grown in the dark, and so were named *constitutive photomorphogenesis* and *de-etiolation* (*cpd*) and *de-etiolated 2* (*det2*). These, and subsequently identified mutants with reduced BR levels, also show growth defects when grown in the light (Clouse, 2002). They are dark-green dwarfs with reduced internode, petiole, and leaf elongation, giving them a cabbage-like appearance. The mutants have reduced apical dominance, inflorescence stems are reduced in length, and flowers are small with reduced fertility. Roots are also shorter than those of wild-type plants.

In several different genetic screens, one loss-of-function BR-insensitive mutant, named *bri1*, was identified (Clouse *et al*., 1996; Kauschmann *et al*., 1996; Li and Chory, 1997). BRI1 is a leucine-rich repeat (LRR) receptor serine/threonine kinase expressed throughout the plant (Friedrichsen *et al*., 2000; Li and Chory, 1997; Oh *et al*., 2000). Several lines of evidence suggest that it is the BR receptor. A chimeric protein was constructed containing the N-terminus of BRI1, from extracellular through juxtamembrane domains, fused to the kinase domain of the rice gene *Xa21* involved in pathogen response (He *et al*., 2000). Application of BL to rice cell cultures

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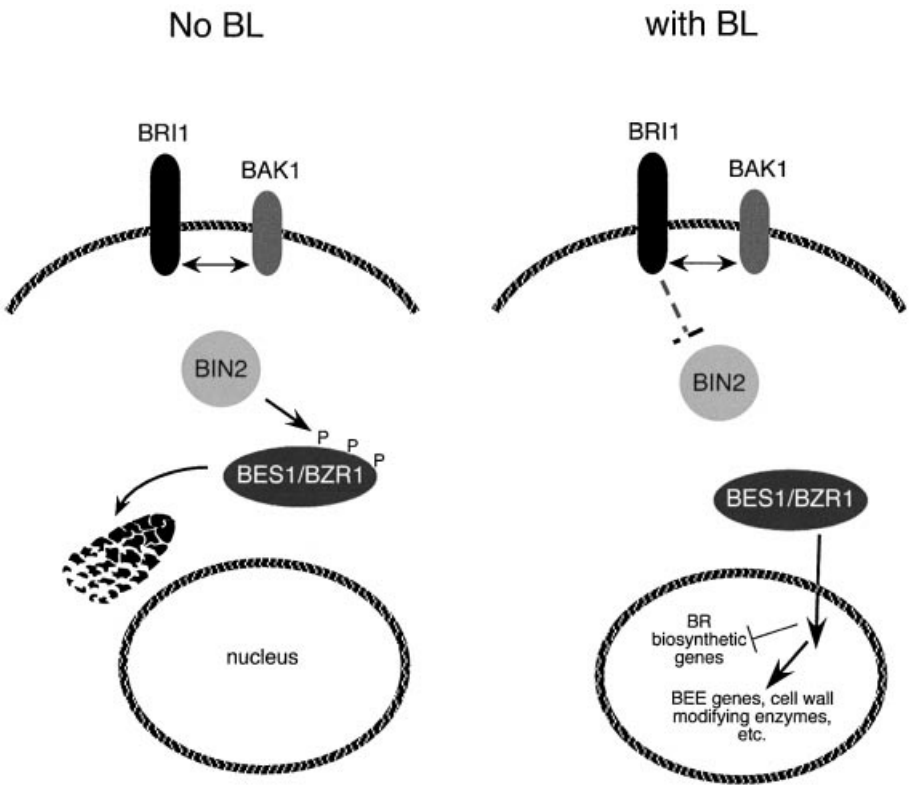

Fig. 1. In low BL conditions, BIN2, a GSK3-like kinase, phosphorylates BES1 and BZR1. This modification leads to the destabilization of BES1 and BZR1 and their degradation by the 26S proteosome. Once BL is perceived by BRI1 at the cell surface, BIN2 is inactivated by an unknown mechanism leading to the accumulation of BES1 and BZR1 in the nucleus. This activates the expression of a number of genes, including those involved in cell wall modification and growth. It also leads to the rapid repression of several BR biosynthetic genes. BAK1, like BRI1, is a transmembrane LRR kinase. Although it has been shown to interact with BRI1, its exact role in the BR response has not been shown.

expressing this chimeric protein mimicked the cell death response observed when cells expressing Xa21 were challenged with pathogen. In addition, BL-binding capacity observed in microsomal fractions of wild-type plants is lost when the extracellular domain of *BRI1* is disrupted and increased in plants overexpressing *BRI1* (Wang *et al*., 2001). In the last year, several new proteins have been described, shedding light on how the BR signal is transmitted from BRI1 at the extracellular surface into the cytoplasm and the nucleus.

BR signalling: from a lone player to a crowded field

In 2002, a number of exciting new findings were described, greatly advancing understanding of BR signalling pathways. The gene responsible for the hypermorphic BR-insensitive phenotype of *bin2* was cloned and found to encode a previously identified member of the Glycogen Synthase 3/SHAGGY family of kinases, called GSKη (Li and Nam, 2002). Recapitulation lines transformed with the *BIN2* gene carrying the *bin2-1* lesion showed a range of phenotypic severity that was correlated with transgene expression. Several lines containing the wild-type *BIN2* gene under the viral 35S promoter showed a reduction in levels of the endogenous gene and could partially suppress a weak *bri1* mutant. However, as BIN2 is part of a closely related family of proteins, it is possible that this effect is the result of reduced expression of another GSK3 or a combined effect of several. As GSK3-type kinases are known to be negatively regulated by phosphorylation in other systems, it is tempting to speculate that BRI1 might act as a direct regulator in this pathway. Although several approaches were taken to find such a connection between the two proteins, no evidence to support such a link was found.

At the same time, *ultracurvata1* (*UCU1*), which, when mutated, caused a severe dwarf phenotype, was cloned and found to encode the same gene as *BIN2* (Perez-Perez *et al*., 2002). Perez-Perez and colleagues showed that the dwarfism observed in *ucu1/bin2* mutants results from a severe defect in cell expansion, which is particularly severe on the abaxial (ventral) surface of leaves. They also showed that leaves of *ucu1/bin2* plants contain additional internal layers of cells contributing to the increased thickness of the organs. Physiological analysis of *ucu1* roots revealed an increased sensitivity to the synthetic auxin, 2,4-D and insensitivity to 24-epibrassinolide. The

close relationship of brassinosteroids and auxin was also observed in the synergistic interaction of *ucu1* with semi-dominant auxin resistant mutants, *axr2* and *shy2*.

A major breakthrough came with the cloning and characterization of *BES1* and *BZR1*, which provides a connection between the cytoplasmic BR response and the nucleus (Wang *et al*., 2002; Yin *et al*., 2002). *bes1* and *bzr1*, were identified as suppressors of *bri1* phenotypes, as well as being resistant to brassinozole, a BR biosynthesis inhibitor. *BES1* and *BZR1* encode closely related novel proteins that accumulate in the nucleus following BR treatment. Identical dominant mutations identified in both genes stabilize the respective proteins and increase their nuclear accumulation. By tracking the expression of a *BZR1* translational fusion with cyan fluorescent protein, it was possible to correlate nuclear accumulation with elongating regions of etiolated seedlings (Wang *et al*., 2002). Perhaps most importantly, BES1 and BZR1 can be phosphorylated by the negative regulator BIN2, resulting in their turnover (He *et al*., 2002; Yin *et al*., 2002). Accumulation of unphosphorylated proteins is greatly reduced in *bin2*/*BIN2* heterozygotes, consistent with their strong growth defects. These findings provided the scaffold for a new model of BR signalling (Fig. 1).

In mid-2002, a pair of papers was published describing an LRR II receptor-like protein kinase called BAK1 (Li *et al*., 2002; Nam and Li, 2002). BAK1 was uncovered in two laboratories using different approaches. In one, BAK1 was found to interact with the BRI1 cytoplasmic kinase domain in yeast two-hybrid analysis (Nam and Li, 2002). Moreover, phosphorylated products of the expected size were only observed when full-length clones of both proteins were co-expressed in yeast, suggesting transphosphorylation may be required for kinase activation of both BRI1 and BAK1. In another approach, *BAK1* was identified as an activation-tagged suppressor of a weak allele of *bri1* (Li *et al*., 2002). Interestingly, unlike *bzr1* and *bes1* mutants, overexpression of *BAK1* is not able to suppress a biosynthetic mutant or a strong *bri1* allele. Both groups showed that loss-of-function *bak1* alleles are less sensitive to exogenous BL, although the phenotype is quite subtle. This may result from some degree of redundancy among the several BAK1 homologues found in *Arabidopsis*, homologous with the SERK family in *Daucus carota*. Loss-of-function *bak1* alleles enhance a weak *bri1* phenotype, while strong *bri1* alleles are epistatic to loss of *bak1* function. When *BAK1* is overexpressed from its own promoter it shows a similar phenotype to *BRI1OX* and an increased sensitivity to exogenous BL in roots. Interestingly, when *BAK1* is expressed from the constitutive viral 35S promoter, the phenotype is less marked, and no increased sensitivity to BL is observed. Transgenic plants containing high levels of a kinase-dead bak1 protein show a strong *bri1*-like phenotype, suggesting that this mutation may create a dominant negative effect. This finding combined with data showing BRI1 and BAK1 interacting *in vivo*, led to an intriguing model where BAK1 acts near the point of BR perception, perhaps as a co-receptor with BRI1. Further analysis of BAK1's role in BL binding, the effects of BL binding on BRI1-BAK1 interactions, as well as detailed characterization of the transphosphorylation events between the two proteins should prove quite informative about the mechanism of BR perception.

Shedding light on the function of endogenous BRs

BRs have also been closely linked to the process of de-etiolation. Mutations causing decreased BR levels or decreased BR response, as well as treatment with BR biosynthesis inhibitors, cause dark-grown plants to de-etiolate (Asami and Yoshida, 1999; Li *et al*., 1996). BAS1, a steroid 26-hydroxylase involved in the regulated inactivation of BRs, provides one possible mechanistic link between brassinosteroid biosynthesis and light (Neff *et al*., 1999). Increased expression of *BAS1* results in severely reduced production of BL and is able to suppress both intermediate and null alleles of *phyB* fully in red light. Antisense lines of *bas1* are hyper-responsive to BL, and show a decreased response to white, blue, and far-red light, but no change in their red light response. Recently, brassinosteroids have been implicated in repressing some PHYA-mediated responses (Luccioni *et al*., 2002). Luccioni and colleagues performed a mutant screen, looking for plants with enhanced very low fluence responses (VLFR) by screening in hourly far-red light pulses and looking for shorter hypocotyls and opened cotyledons. One such mutant, called *eve1*, was found to be allelic to *dwf1*/*dim*, a mutant in a gene involved in BR biosynthesis. In addition to its seedling phenotypes, *eve1* also shows enhanced VLFR and reduced high irradiance responses when chlorophyll and anthocyanin accumulation are measured in either hourly pulses or continuous far-red light. This same relationship was observed in *det2* seedlings. Interestingly, when *eve1*/*dwf1* plants were germinated in sunlight, there was little difference in hypocotyl length, but when germinated in canopy shade-light, BR deficiency resulted in significantly shorter hypocotyls, suggesting a role for BRs in optimizing growth in different light environments.

Another recent paper suggests a mechanism for communication between the light receptors and BR biosynthesis (Kang *et al*., 2001). Pra2, a dark-inducible, phytochrome-repressed small G protein from pea was used as bait in a yeast two-hybrid screen. A cytochrome P_{450} hydroxylase, which they named DDWF1, was identified and shown to have an overlapping expression pattern with Pra2 in the elongating region of the etiolated pea epicotyl. In light, expression was detected only in the root.

Pra2 and DDWF1 were shown to interact *in vitro* and to co-localize on the ER membrane of onion epidermal cells. Etiolated tobacco seedlings overexpressing pea *Pra2* had short hypocotyls and are probably cosuppressed for the tobacco *Pra2* homologue. Recombinant DDWF1 was shown to catalyse the conversion of typhasterol to cathasterone and feeding experiments of cosuppressed tobacco plants suggested that *Pra2* was required for full DDWF1 activity. Although similar cosuppression experiments did not work in *Arabidopsis* for *Pra2*, overexpression of pea DDWF1 caused elongated hypocotyls. Phytochrome-mediated repression of *Pra2* transcription has been studied in some detail and these findings suggest one mechanism, operating at least in pea and tobacco, for communication between light and BR levels. However, other studies which quantified BR levels in dark- and light-grown seedlings fail to detect any change in hormone level with different light treatments (Symons and Reid, 2003). Additional genetic and biochemical studies are clearly needed to determine the molecular mechanism underlying the interaction between light and BRs; such knowledge can then be applied to the broader question of whether these mechanisms are conserved across plants adapted to different light environments.

BR effects in the nucleus

In addition to effects on BR biosynthetic enzymes, another potential site of cross-talk between different factors affecting seedling development is at the level of gene regulation. Several recent studies have shed light on the nuclear end of the BR response. Three papers using Affymetrix *Arabidopsis* oligonucleotide microarrays have yielded the first global glance at BR-mediated changes in gene expression (Goda *et al*., 2002; Mussig *et al*., 2002; Yin *et al*., 2002). Each group used quite different starting materials for RNA isolation. Plants varied in age from 7–50 d and encompassed several genetic backgrounds, including three biosynthetic mutants and *bri1*. In addition, the concentration and length of the BR treatment varied markedly from study to study. Not surprisingly, perhaps, the exact genes identified were not well matched. Despite the discrepancies between the behavior of individual genes in each experimental condition, several important trends were detected in all studies (Table 1).

Two classes of genes implicated by earlier studies on BR-regulated gene expression were confirmed in these studies. In the first case, several genes encoding cytochrome P_{450}, most notably *CPD* and *DWF4*, were strongly repressed following BR application, reflecting the tight negative feedback regulation acting on the BR biosynthetic pathway. *BAS1* was also up-regulated by BRs. Another major category of BR-regulated genes are those involved in cell-wall modification and cellular metabolism, several of which have been detected previously, and reflect the dramatic effects on growth provoked by BRs (Friedrichsen and Chory, 2001).

Table 1. *Comparison of BR-regulated genes in three recent Affymetrix oligomicroarray studies*

	Yin *et al.*	Goda *et al.*	Mussig *et al.*
Increased			
Signal transduction	7	11	9
Auxin-related	6	10	2
Cell wall	6	18	6
Other	4	10	16
Unknown	7	10	0
Total	30	59	33
Decreased			
Signal transduction	n/a	3	13
Biosynthetic enzymes	n/a	5	5
Auxin-related	n/a	0	2
Cell wall	n/a	1	4
Other	n/a	3	6
Unknown	n/a	3	0
Total	n/a	15	30

Perhaps the most surprising outcome of these studies is the modest nature of the BR response. Overall, expression changes were 2–5-fold, quite different from the 10–100-fold differences observed in the application of other phytohormones (Zhao *et al*., 2002). Importantly, where tested, all BR responsive genes required functional *BRI1*. In *bes1-D* mutants which display a dramatic constitutive BR phenotype, all of the BR-induced genes examined show either higher basal levels of expression or hyper-responsivity to exogenous BL (Yin *et al*., 2002). The small effect on gene expression observed in these studies may reflect the real strength of the BR response or, alternatively, these small changes may result from a previously unsuspected complexity in the localization of the BR response. For instance, if only a small subset of cells is fully competent to respond to increased endogenous or applied BRs then this could result in an overall dampening of the apparent changes in gene expression. A detailed analysis of distribution patterns of BR biosynthetic genes and signalling components is needed to distinguish between these possibilities.

In another approach to dissecting the nuclear response to BRs, three early BR response genes were identified and examined in greater detail (Friedrichsen *et al*., 2002). *BEE1*, *BEE2* and *BEE3* encode proteins with conserved basic helix-loop-helix motifs. Although the *BEE* genes show only a 2-fold induction by BR, plants lacking all three gene products show reduced BR responses, confirming that the small differences observed in the microarray experiments are probably relevant to BR signalling. Interestingly, these three BR early response genes are also regulated by other hormones. Most strikingly, they are all repressed by the application of abscisic acid (ABA), a known antagonist of BR signalling. Triple mutants lacking

expression of all three genes show no ABA phenotype, but roots of plants overexpressing *BEE1* show a reduced response to exogenous ABA. Taken together with the results of the microarray studies, the results with the *BEE* genes strongly suggest that small changes in gene expression are an important part of the BR response.

BR links to auxin

Another remarkable outcome from the global expression studies was the large proportion of auxin-regulated genes which also exhibited BR-responsivity. There are many examples of cross-talk between hormones in plant biology. For instance, recent elegant work has demonstrated that the growth-promotive effects of auxin in the root are largely mediated through the action of gibberellins (Fu and Harberd, 2003). Auxin and BRs have been linked to many of the same growth processes, including vascular differentiation, flower and fruit development, and root growth, in addition to their roles in seedling photomorphogenesis and shade avoidance (Mandava, 1988). In addition, auxin and BRs have synergistic effects on cell elongation in a wide variety of bioassays, including soybean and cucumber hypocotyls, azuki bean and pea epicotyls, and rice lamina joints (Katsumi, 1985; Mandava, 1988; Yalovsky *et al*., 1990; Yopp *et al*., 1981). Exogenous brassinolide has little effect on hypocotyl elongation in *Arabidopsis* mutants defective in biosynthesis or response to other hormones, with the notable exception of an auxin-response mutant *axr2* which shows a 2–3-fold increase in hypocotyl elongation (Szekeres *et al*., 1996). Also, while mutants defective in gibberellin and ethylene signalling show a normal growth response to increased auxin levels provoked by temperature increases, a mutant deficient in BR synthesis, *det2*, shows significantly reduced elongation (Gray *et al*., 1998).

Global analyses of gene expression in BR-treated plants reveal one possible mechanism for the interaction between these two hormones. Several previously identified auxin early-response genes are also up-regulated by BRs (Yin *et al*., 2002). Genes from all known classes of auxin early-response genes, *GH3*, *Aux/IAA*, *SAUR*, were represented. In addition, expression changes in putative auxin efflux carriers, auxin conjugating enzymes, and cytochrome P_{450} enzymes known to be involved in auxin synthesis were also detected in different studies. Regulation of these genes does not seem to follow a simple pattern. As described by Goda and colleagues, genes in the known auxin-responsive families fall into four classes on closer examination: those that are specifically induced by auxin, those that are induced by both BRs and auxin, those that are induced by BRs and not auxin, and those which are induced by auxin but repressed by BRs (Goda *et al*., 2002). Promoters of most auxin-responsive genes identified contain an auxin responsive element [T/A]GTCTC (Guilfoyle *et al*., 1998). A synthetic construct containing five repeats of this element, called DR5, has been shown to provide high sensitivity to auxin either *in vitro* or *in vivo* (Ulmasov *et al*., 1997). To investigate the nature of the BR:auxin connection further, the response of transgenic seedlings carrying the DR5:GUS reporter was examined. Consistent with the microarray data, the GUS staining was greatly enhanced in plants exposed to exogenous BL (J Nemhauser and J Chory, unpublished data). These findings suggest that BRs either act directly on auxin-responsive elements or are able to sensitize cells in some manner to auxin. Future studies aimed at dissecting the precise relationship between these two hormone pathways will undoubtedly shed light on the response to the individual hormones.

Future prospects

It has been a banner year for BRs. With an increasingly defined biosynthetic pathway and ever-expanding model of BR signalling, asking more sophisticated questions about BR effects are possible. It will now be possible to connect BR signalling to the cell mechanics of expansion and division, an area that remains poorly understood. A large number of putative transcription factors and proteins of unknown function are also BR-regulated and provide fertile ground for future investigations into the BR response. Moreover, it is clear that to understand the function of BRs fully, they must be placed in the context of the myriad other pathways acting throughout plant development. In particular, continued studies of how light and other hormone response pathways are integrated with BRs should provide many more interesting years ahead.

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