Hypocotyl Directional Growth in Arabidopsis: A Complex Trait[1][W][OA]

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The growth direction of the Arabidopsis (*Arabidopsis thaliana*) etiolated-seedling hypocotyl is a complex trait that is controlled by extrinsic signals such as gravity and touch as well as intrinsic signals such as hormones (brassinosteroid [BR], auxin, cytokinin, ethylene) and nutrient status (glucose [Glc], sucrose). We used a genetic approach to identify the signaling elements and their relationship underlying hypocotyl growth direction. BR randomizes etiolated-seedling growth by inhibiting negative gravitropism of the hypocotyls via modulating auxin homeostasis for which we designate as reset, not to be confused with the gravity set point angle. Cytokinin signaling antagonizes this BR reset of gravity sensing and/or tropism by affecting ethylene biosynthesis/signaling. Glc also antagonizes BR reset but acts independently of cytokinin and ethylene signaling pathways via inhibiting BR-regulated gene expression quantitatively and spatially, by altering protein degradation, and by antagonizing BR-induced changes in microtubule organization and cell patterning associated with hypocotyl agravitropism. This BR reset is reduced in the presence of the microtubule organization inhibitor oryzalin, suggesting a central role for cytoskeleton reorganization. A unifying and hierarchical model of Glc and hormone signaling interplay is proposed. The biological significance of BR-mediated changes in hypocotyl graviresponse lies in the fact that BR signaling sensitizes the dark-grown seedling hypocotyl to the presence of obstacles, overriding gravitropism, to enable efficient circumnavigation through soil.

Gravitropism, an adaptive phenomenon that collectively involves gravity perception, signal transduction, and consequently differential growth (Chen et al., 2002; Morita and Tasaka, 2004) utilizes, in part, the sedimentation of amyloplasts onto transvacuolar membranes, cytoskeleton, and the plasma membrane in specific cells of various organs (Sack, 1991; Baluška and Hasenstein, 1997; Kiss et al., 1999; Yoder et al., 2001; Saito et al., 2005; Morita et al., 2006). This mechanical signal is consequently transduced via many secondary messengers and several hormones including auxin and ethylene (Wheeler and Salisbury, 1980; Estelle, 1996; Sinclair and Trewavas, 1997; Friedman et al., 1998; Fasano et al., 2001; Kato et al., 2002; Perera et al., 2006). These secondary messengers orchestrate changes in lateral auxin transport, which in turn leads to asymmetric auxin distribution across the organ (Chen et al., 2002; Friml and Palme, 2002; Paciorek et al., 2005). All these changes manifest as differential growth.

Directional growth at most times is predominantly influenced by gravity but other signals can, and do, override gravity, such as touch (in thigmotropism) and water (in hydrotropism). Spiral growth of lianas on tree trunks and circumnavigation of obstacles by roots are good examples. One interpretation is that mechanosensing transiently resets gravity sensing or tropism to zero to bring about the appropriate directional growth. The meaning of reset here is literal and not to be confused with gravity set point angle, the angle relative to the gravity vector at which a plant organ commences gravitropism (Blancaflor and Masson, 2003). Little is known how gravity sensing becomes reset, if there is a latency period, what are the intrinsic signals, and what is their functional relationship.

There are reports of involvement of Glc in controlling root or hypocotyl directional growth in plants. Glc and indole-3-acetyl-myoinositol are asymmetrically distributed in gravistimulated *Zea mays* seedlings (Momonoki, 1988). Glc controls root gravitropism via auxin signaling (Mishra et al., 2009).

[1] This work was supported by the Department of Science and Technology, Government of India (grant no. SR/FT/LS–102/2008), by a National Institute of Plant Genome Research core grant, and by the Council of Scientific and Industrial Research, India (research fellowships to A.G. and M.S.). Research in the Jones laboratory is supported by the National Institute of General Medical Sciences (grant no. GM65989) and the National Science Foundation (grant nos. MCB0718202 and 0723515). The Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the U.S. Department of Energy (grant no. DE–FG02–05er15671 to A.M.J.) funded, in part, the genetic material used in this work.

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[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.112.195776

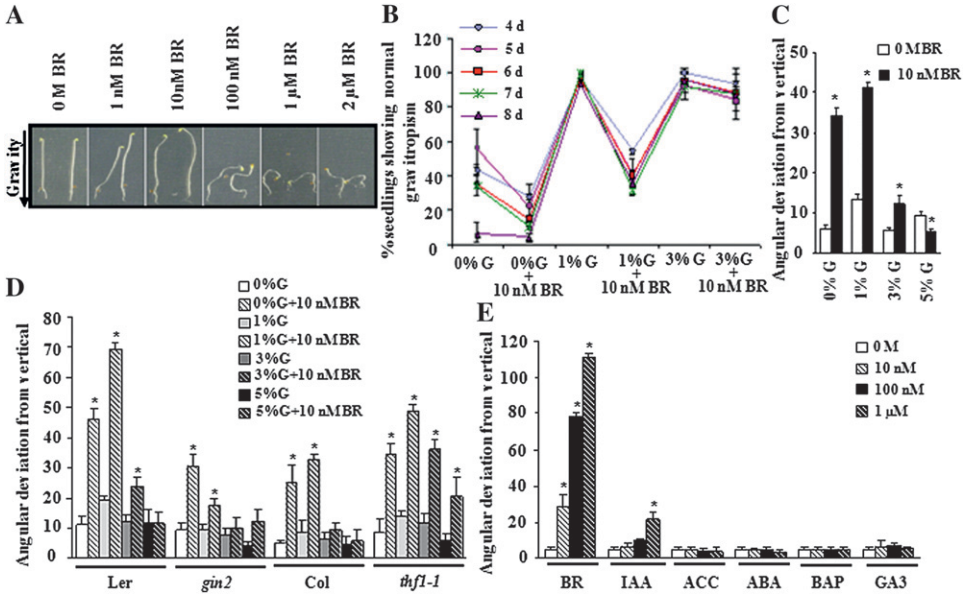

Figure 1. BR reset of hypocotyl gravitropism response in dark. A, Five-day-old, dark-grown wild-type (Col-0) seedlings on Glc-free one-half-times Murashige and Skoog medium supplemented with or without increasing BR (1 nM, 10 nM, 100 nM, 1 μM, 2 μM) as indicated. B, Gravitropic responses of wild type (Col-0) at different time points. The direction of gravity was altered by turning the plates 90° after the seedlings were grown either for 4, 5, 6, 7, or 8 d in dark. Percentages of seedlings showing normal gravitropic response were observed after 24 h. Hypocotyls do not respond to the change in direction of gravity upon exogenous BR treatment and Glc can effectively restore gravitropic response at increased concentrations (3% Glc). C, Quantification of BR reset of hypocotyl gravitropism. Five-day-old, dark-grown seedlings were transferred to the indicated concentrations of Glc and BR for 2 d then the angle of deviation of the hypocotyl from perpendicular was determined. D, A comparison of BR reset of hypocotyl gravitropism of wild-type (L*er*, Col-0), *gin2*, and *thf1-1* seedlings. The BR reset of hypocotyl gravitropism was found to be highly reduced in *gin2* while *thf1-1* shows less response toward Glc antagonism of BR reset of hypocotyl gravitropism. E, The effect of different hormones on wild-type (Col-0) seedling hypocotyls to determine their role in controlling hypocotyl directional response. Five-day-old dark-grown seedlings were transferred to different hormone-containing media for 2 d and hypocotyl deviation was quantified. The significant extent of hypocotyl randomization response was found with BR, while IAA could only bring about some randomization at a very high concentration. Data shown is the average of two representative biological replicates having at least 15 seedlings; error bars represent SE. Student's *t* test, $P < 0.001$.

Many hormones direct gravitropism, of which brassinosteroids (BRs) are the least understood. BRs control gravitropic bending in hypocotyls/shoots (Meudt, 1987; Park, 1998; Philosoph-Hadas et al., 2005; Nakamoto et al., 2006; Hála et al., 2010; Arteca and Arteca, 2011). BR acts synergistically with auxin during hypocotyl gravitropism of partially deetiolated bean (*Phaseolus vulgaris*; Meudt, 1987). Finally, BRs inhibit gravitropic responses of etiolated Arabidopsis (*Arabidopsis thaliana*) hypocotyls (Nakamoto et al., 2006) and sugar antagonizes this BR inhibition (Vandenbussche et al., 2011). Modified starch accumulation, loss of cell wall rigidity, and a faulty osmoregulation may be responsible for BR-induced loss of hypocotyl graviresponses. Collectively, these reports that individually reveal a role of different hormones or sugars in controlling Arabidopsis hypocotyl directional responses imply integration among these different signals. Here, we provide a mechanism for this signal integration and speculate on the biological significance (Vandenbussche et al., 2011).

RESULTS

BR Resets Gravitropism

Low concentrations of BR disrupt the uniform direction of hypocotyl growth in a dose-dependent manner (Fig. 1A). BR randomizes etiolated-seedling growth by inhibiting negative gravitropism of the hypocotyls for which we designate as reset. To check whether altered directional growth of hypocotyl is due to BR reset of gravitropism, BR-treated seedlings were grown in horizontally placed media plates. BR-treated etiolated seedlings when grown horizontally failed to show negative gravitropism in Glc-free medium (Supplemental Fig. S1A). The etiolated seedlings were also subjected to gravity reorientation assay by giving a 90° gravistimulation to 4- to 8-d-old vertically grown seedlings. In this assay also BR-treated seedlings could not reorient themselves to changed gravity vector in the Glc-free medium (Fig. 1B), suggesting that BR either perturbs gravity detection or response. The observed waviness in

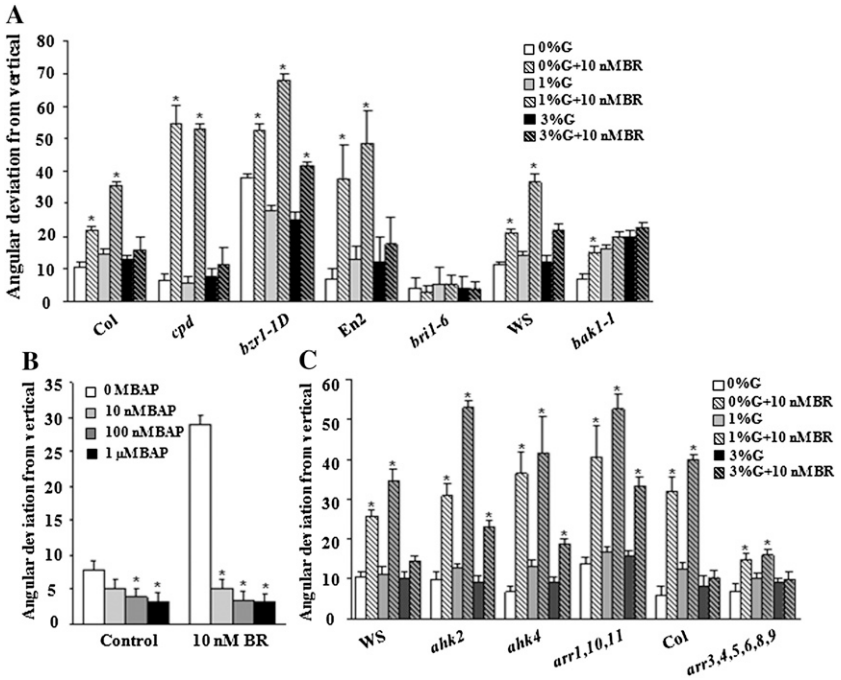

Figure 2. The role of BR and cytokinin signaling in controlling reset of hypocotyl gravitropism. A, The comparison of BR reset of hypocotyl gravitropism of Arabidopsis wild-type (Col-0) and BR biosynthesis and signaling mutants. BR reset of hypocotyl gravitropism was not found in *bri1-6* mutant and the same was highly reduced in *bak1-1* mutant. Highly exaggerated BR reset of hypocotyl gravitropism was found in *bzr1-1D* mutant that display higher hypocotyl randomization even in the absence of BR in the medium. B, Comparison of BR reset of hypocotyl gravitropism of 7-d-old wild-type (Col-0) seedlings in presence of BAP. Supplementing BAP and BR together reduced BR reset of hypocotyl gravitropism. C, Comparison of BR reset of hypocotyl gravitropism of 7-d-old wild type (Col-0, Ws), cytokinin receptors (*ahk2*, *ahk4*), type-A ARR mutant *arr3,4,5,6,8,9*, and type-B ARR mutant *arr1,10,11*. Cytokinin receptors (*ahk2*, *ahk4*) and type-B ARR mutant *arr1,10,11* show enhanced BR reset of hypocotyl gravitropism while response of type-A ARR mutant *arr3,4,5,6,8,9* was very less as compared with wild type. Data shown is the average of two representative biological replicates having at least 15 seedlings; error bars represent SE. Student's *t* test, $P < 0.001$.

the hypocotyl may be due to frequent gravity resets in the presence of BR.

The BR Reset of Gravitropism Is Affected by Glc

Normal gravitropic response was restored in the BR-treated seedlings by exogenous Glc in the gravity reorientation assay (Fig. 1B). In the vertically grown seedlings, Glc enhances the agravitropic growth behavior at lower concentrations (1%) but strongly antagonizes BR reset at higher (3%, 5%) concentrations (Fig. 1C; Supplemental Fig. S2). Exogenous Glc restored negative gravitropism in the BR-treated seedlings growing in horizontal plates (Supplemental Fig. S1A). *Phosphoglucomutase* and *endodermal-amyloplast less1* both with reduced levels of amyloplast starch were less gravitropic whereas a mutant *starch excess1* with elevated levels of starch was more gravitropic, suggesting an important role of amyloplasts in gravity sensing (Kiss et al., 1997; Fujihira et al., 2000; Vitha et al., 2007). Lugol staining did not reveal an obvious difference in starch granule accumulation between BR-treated and -nontreated seedlings growing on Glc-free medium. Ectopic accumulation of starch granules was observed in the presence of 3% Glc. Overall, our results do not support a BR mechanism that limits starch (Supplemental Fig. S1B). In yeast (*Saccharomyces cerevisiae*) and Arabidopsis, hexokinase may serve as a Glc receptor (Rolland et al., 2006). The hexokinase mutant *glucose insensitive2-1* (*gin2-1*; Moore et al., 2003) was used to investigate any physiological role of HXK-dependent Glc signaling. *gin2* showed reduced BR reset of gravitropism at both lower and higher concentrations of Glc (Fig. 1D; Supplemental Fig. S2), suggesting a direct requirement for hexokinase. However, the Glc hypersensitive mutant *thylakoid formation1-1* (*thf1-1*; Huang et al., 2006) that is associated with G-protein-coupled, HXK-independent sugar signaling mechanism was more sensitive to BR both at low and high concentrations of Glc (Fig. 1D; Supplemental Fig. S2), suggesting the involvement of a hexokinase-independent signaling pathway as well. Two other Glc signaling mutants, *regulator of g-protein signaling1* (*rgs1*) and *g-protein alpha subunit1* (*gpa1*), were less sensitive toward Glc antagonism of BR reset of gravitropism, also consistent with involvement of multiple Glc response pathways in this BR response (Supplemental Fig. S2B).

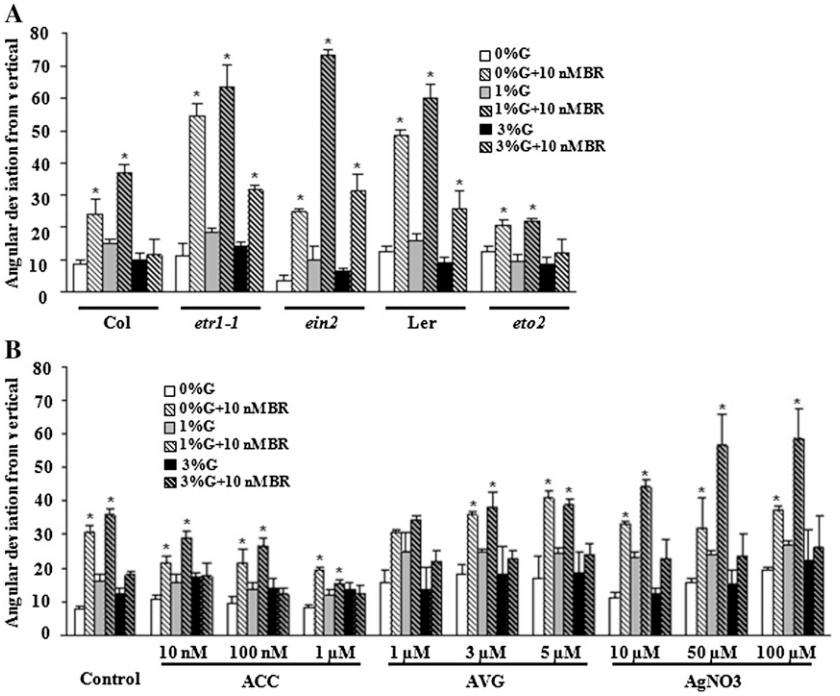

Figure 3. The role of ethylene signaling and biosynthesis in controlling BR reset of hypocotyl gravitropism. A, Comparison of BR reset of hypocotyl gravitropism of 7-d-old Col-0 and ethylene receptor and signaling mutants seedlings. Ethylene receptor mutant *etr1-1* and signaling mutant *ein2-1* show enhanced BR reset of hypocotyl gravitropism while response of *eto2* mutant was very much less as compared with wild type. B, Comparison of BR reset of hypocotyl gravitropism of wild type (Col-0) in presence of ACC, ethylene signaling inhibitor (AgNO3), and biosynthesis inhibitor (AVG) at concentrations indicated. Supplementing ACC and BR together reduced BR reset of hypocotyl gravitropism. The BR reset of hypocotyl gravitropism was highly enhanced in the presence of AgNO3, while significant induction was found in presence of AVG. Data shown is the average of two representative biological replicates having at least 15 seedlings; error bars represent SE. Student's *t* test, $P < 0.001$.

Two auxin-insensitive mutants, *nonphototropic hypocotyl4/massugu1*, and a dominant mutant, *massugu2* have defects in both gravi- and phototropism in hypocotyls (Nakamoto et al., 2006), prompting us to consider if BR is affecting a general component of tropic response pathway or gravitropism specifically. Since phototropism was not affected (or possibly even enhanced) by BR (Supplemental Fig. S3, A and B) and overall growth was not inhibited, the observed BR effect may be specific to gravitropism. The effect of BR was evident in different composition media and also in the presence of light (Supplemental Fig. S4, A and B). Wild-type seedlings grown in cytokinin, abscisic acid, 1-aminocyclopropane-carboxylic acid hydrochloride (ACC), or GA_3 containing medium did not show hypocotyl agravitropism. Apart from BR, only indole-3-acetic acid (IAA) influenced the hypocotyl growth direction but at very high concentration, suggesting that this response is regulated by BR (Fig. 1E; Supplemental Fig. S5A). Although GA_3 is known to promote hypocotyl elongation, it did not cause a change in the hypocotyl direction growth when applied alone nor did it enhance BR-induced reset of hypocotyl directional growth (Supplemental Fig. S5, B and C).

The Hypocotyl Tip Is Sufficient to Perceive the Signal and Exhibit BR Reset

To find the site of stimulus perception, seedlings were grown in one-half-times Murashige and Skoog medium for 5 d in the dark. The roots tip, whole root, hypocotyl, and hypocotyl tip along with cotyledons of the dark-grown seedlings were then excised and placed in one-half-times Murashige and Skoog medium containing different concentrations of BR and Glc. Seedlings with an excised root tip or with an intact root were agravitropic, suggesting that roots are not essential for perceiving BR in this context (Supplemental Fig. S6, A–G). Seedlings with excised hypocotyl apices did not grow. The excised hypocotyl tip with cotyledons on BR-containing media grew well and displayed BR reset of gravisensing or tropism, suggesting that the hypocotyl tip along with cotyledons alone is sufficient for this response (Supplemental Fig. S6, F and G).

Known BR Signaling Components Mediate BR Reset of Gravitropism

The BR receptor BRASSINOSTEROID INSENSITIVE1 (BRI1) heterodimerizes with BRI1 ASSOCIATED KINASE1 (BAK1) after binding BR. BRI1 and BAK1 subsequently act together to inhibit a GSK3-like kinase, BRASSINOSTEROID INSENSITIVE2 (Li et al., 2001), that, in the absence of BR, catalyzes phosphorylation of the transcription factor BRASSINAZOLE RESISTANT1 (BZR1), resulting in its inhibition of DNA binding and promoting binding to 14-3-3 proteins, leading to cytoplasmic retention or degradation (He et al., 2002; Gampala et al., 2007; Ryu et al., 2007). Signaling by BRI1/BAK1 removes this inhibition and unphosphorylated BZR1 translocates to the nucleus, where it acts together with the transcription factor BRI1-EMS-SUPPRESSOR1 to regulate expression of BR-inducible genes (Wang et al., 2002; Yin et al., 2002, 2005). BZR1 not only activates BR-induced genes and promotes cell elongation but also suppresses BR biosynthetic genes such as CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD), leading to feedback inhibition of BR biosynthesis

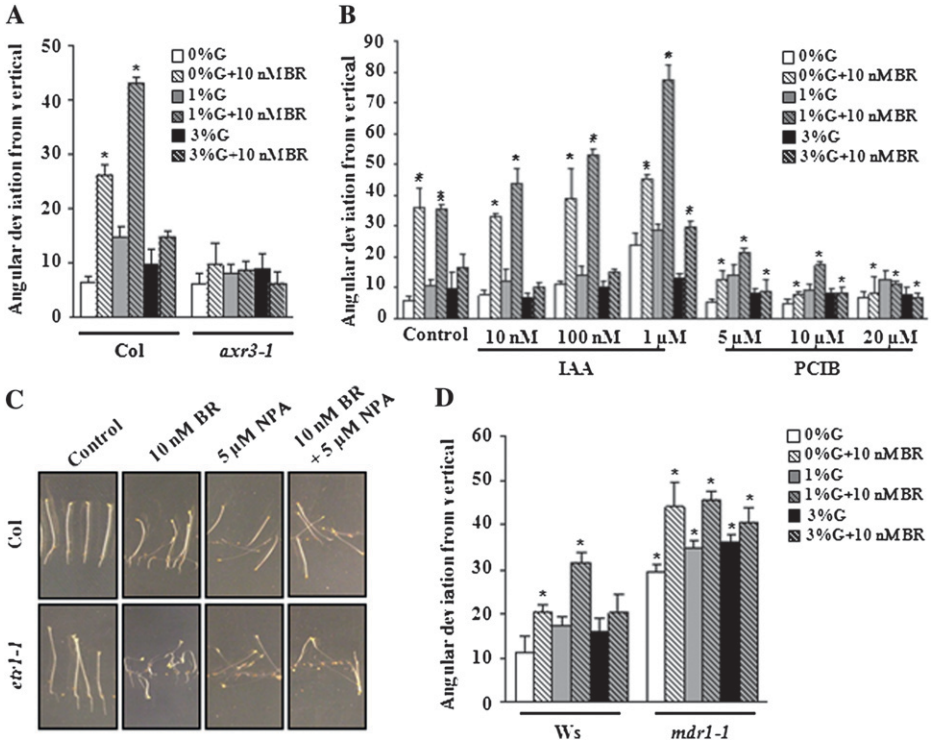

Figure 4. The role of auxin signaling and polar transport in controlling BR reset of hypocotyl gravitropism. A, The auxin signaling mutant *axr3-1* that leads to stability of auxin signaling repressor protein shows substantial reduction in BR reset of hypocotyl gravitropism. B, Wild-type (Col-0) seeds were sown on Glc-free or increasing-Glc (1%, 3%) containing one-half-times Murashige and Skoog medium supplemented with or without 10 nM BR in presence of IAA and auxin signaling inhibitor (PCIB) at concentrations indicated. IAA could increase the BR reset of hypocotyl gravitropism at higher concentration (1 μM) while application of the auxin signaling inhibitor PCIB inhibited the BR reset of hypocotyl gravitropism. C, Wild-type (Col-0) and ethylene signaling mutant *etr1-1* seeds were sown on 5 μM NPA containing Glc-free one-half-times Murashige and Skoog medium supplemented with or without 10 nM BR. NPA could effectively antagonize BR reset of hypocotyl gravitropism in both wild type and the *etr1-1* mutant. D, Lateral auxin transport mutant *mdr1-1* seeds were sown on Glc-free or increasing-Glc (1%, 3%) containing one-half-times Murashige and Skoog medium supplemented with or without 10 nM BR. The auxin transport and hypocotyl gravitropism-defective mutant *mdr1-1* displayed exaggerated BR reset of hypocotyl gravitropism. Data shown is the average of two representative biological replicates having at least 15 seedlings; error bars represent SE. Student's *t* test, $P < 0.001$.

(He et al., 2002). To investigate the involvement of various BR biosynthesis and signal transduction components, BR reset of gravitropism in an informative set of BR biosynthesis and signaling mutants was tested. While the BR biosynthesis mutant *constitutive photomorphogenesis and dwarfism* (*cpd*) was hypersensitive, the BR perception mutants *bri1-6* and *bak1-1* were resistant to BR (Fig. 2A; Supplemental Fig. S7). A dominant mutation *brassinazole resistant1-1D* (*bzr1-1D*) conferred an exaggerated BR response as evident by hypocotyl randomization even in the absence of BR (Fig. 2A; Supplemental Fig. S7). The result with the *cpd* mutant is not intuitive. We speculate that the hyperresponsiveness toward BR relative to wild type may be due to wild type being BR saturated under similar conditions (i.e. rate limiting in wild type). This was observed before; *cpd* mutant seedlings show an increase in hypocotyl elongation as compared with wild type in the presence of BR (Szekeres et al., 1996).

Cytokinin Antagonizes BR Reset via Ethylene Signaling While Glc Works Independently of Cytokinin and Ethylene

The hypocotyl directional response depends on controlled differential cell growth. In Arabidopsis, cytokinin, ethylene, and auxin signaling controls differential cell growth (Lehman et al., 1996; Nakamoto et al., 2006).

In Arabidopsis, cytokinin signaling follows a multistep phosphorelay. Cytokinin is perceived by one of three hybrid His protein kinases, ARABIDOPSIS HISTIDINE KINASE2 (AHK2), AHK3, and AHK4, in which cytokinin binding activates autophosphorylation. The phosphorylated receptors then phosphorylate His phosphotransfer proteins (AHPs) in the cytoplasm. After phosphorylation, AHPs can translocate into the nucleus where they phosphorylate type-A and type-B response regulators (ARRs). Phosphorylated type-B ARRs act as positive regulators of cytokinin signaling and induce transcription of type-A negative regulators and other

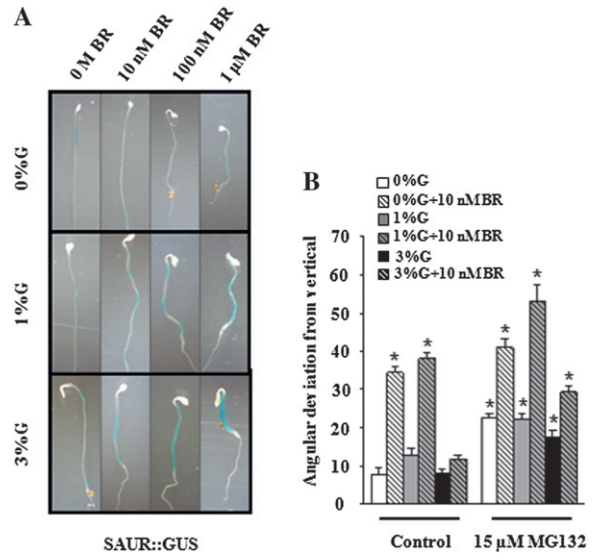

Figure 5. Glc involves changes in spatial gene expression and protein degradation pathway to affect BR reset of hypocotyl gravitropism. SAUR::GUS seedlings were directly germinated and grown for 7 d in dark on Glc-free or increasing-Glc (1%, 3%) containing one-half-times Murashige and Skoog medium supplemented with BR at the indicated concentrations. A, BR treatment causes heterogeneous/patchy SAUR::GUS expression in the hypocotyl. Higher concentrations of Glc in the medium caused accumulation of SAUR::GUS throughout the hypocotyl. B, Effect of protein degradation pathway inhibitor MG132 on Glc antagonism of BR-inhibited hypocotyl gravitropic growth of wild type (Col-0). Experiment was performed at least three times. Data shown is the average of two representative biological replicates having at least 15 seedlings; error bars represent SE. Student's *t* test, $P < 0.001$.

cytokinin early responsive genes (To and Kieber, 2008). Cytokinin and BRs act antagonistically to each other in controlling light-mediated seedling development (Chory et al., 1994). Therefore, we analyzed the effect of cytokinin (6-benzylaminopurine [BAP]) on BR reset of gravitropism. While BAP alone had no effect, BAP at a low concentration (10 nM BAP) completely abolished BR reset of gravitropism (Fig. 2B). Consistent with BAP acting through the His phosphorylation cascade, cytokinin perception mutants *ahk2*, *ahk4*, and type-B ARR triple mutant *arr1,10,11* showed an enhanced BR response while the type-A ARRs sextuple mutant *arr3,4,5,6,8,9* showed a reduced BR response (Fig. 2C; Supplemental Fig. S8, A and B). Glc antagonized BR reset of gravitropism in the cytokinin receptor and type-B mutants, suggesting that Glc acts independently of cytokinin signaling. Also BAP did not affect starch granule accumulation in BR-treated and -nontreated seedlings growing on Glc-free medium (Supplemental Fig. S8C).

Next, we investigated whether ethylene signaling is involved in BR reset of gravitropism since a number of BR-related responses are mediated by ethylene. For example, BR-induced hook formation depends on ethylene biosynthesis (De Grauwe et al., 2005). BR antagonizes the negative effects of ethylene on hypocotyl growth at a low level but, at higher levels, inhibits hypocotyl elongation through an increase in both ethylene biosynthesis and response (Deslauriers and Larsen, 2010). The ethylene receptor mutant *ethylene resistant1-1* (*etr1-1*) and the signaling mutant *ethylene insensitive2* (*ein2-1*) exhibited a high response, suggesting an antagonistic role in the ethylene signal transduction pathway. The ethylene overproducer mutant *ethylene overproducer2* (*eto2*) showed less BR reset, confirming that ethylene works antagonistically with BR to control this response (Fig. 3A; Supplemental Fig. S9, A and B). The ethylene biosynthetic inhibitor aminoethoxyvinylglycine (AVG) as well as the ethylene signaling inhibitor $AgNO_3$ enhanced the response (Fig. 3B). Glc antagonized BR inhibition of gravitropism in the ethylene receptor *etr1-1* and signaling *ein2-1* mutants (Fig. 3A; Supplemental Fig. S9, A and B). Glc also antagonized both AVG- and $AgNO_3$-induced hypocotyl randomization, suggesting Glc works independently of ethylene biosynthesis/signaling (Fig. 3B). ACC did not affect starch granule accumulation in BR-treated and -nontreated seedlings growing on Glc-free medium (Supplemental Fig. S9C).

Cytokinin antagonism of BR inhibition of hypocotyl gravitropism was abolished in ethylene-resistant *etr1-1* and *ein2-1* mutants and with exogenous application of $AgNO_3$ and AVG (Supplemental Fig. S10, A and B), suggesting cytokinin antagonizes the BR response by enhancing ethylene biosynthesis and signaling.

Auxin Signaling/Transport Is Necessary for BR Reset of Gravitropism

BR affects expression of both *AUX/IAA* gene family members as well as auxin polar transporter PIN-FORMED (PIN) proteins (Nakamura et al., 2004a), and not turnover (Nemhauser et al., 2004; Zenser et al., 2003). We, therefore, checked the involvement of auxin signaling in BR reset of gravitropism. The auxin receptor mutant *transport inhibitor response 1* (*tir1*) showed a wild-type BR response while the auxin signaling mutant *auxin resistant1-3* (*axr1-3*) was slightly hypersensitive to BR (Supplemental Fig. S11A). The gain-of-function auxin signaling mutant *axr3-1* had agravitropic hypocotyls and were not further inhibited by BR application, suggesting that proper degradation of the auxin repressor protein AXR3 is required for BR reset of gravitropism (Fig. 4A). Higher concentrations of IAA enhanced the BR response while the auxin signaling inhibitor *p*-chlorophenoxyisobutyric acid (PCIB) abrogated the BR response, suggesting auxin signaling lies downstream to BR (Fig. 4B). The auxin polar transport inhibitor 1-*N*-naphthylphthalamic acid (NPA) disrupted normal hypocotyl growth but in a different manner than BR alone since NPA-treated hypocotyls remained straight despite being insensitive to gravity (Fig. 4C). When NPA and BR were applied together, BR was unable to reset gravitropism, suggesting the mechanism is alteration of polar auxin transport. Auxin polar transport works downstream of ethylene signaling since BR reset in *etr1-1* was reduced at higher concentrations of NPA (Fig. 4C).

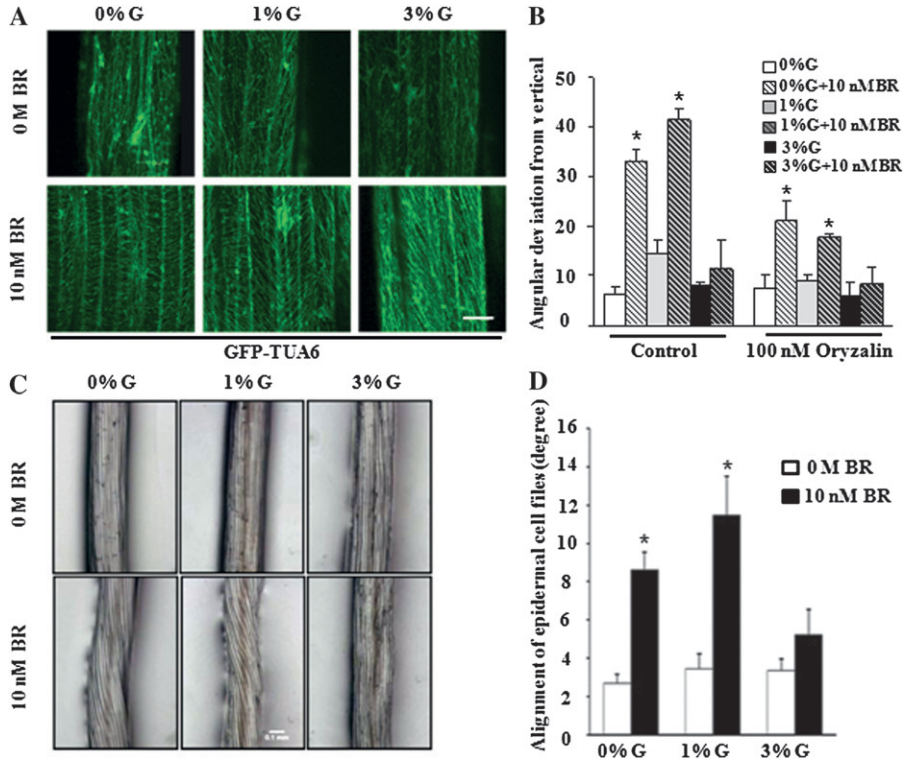

Figure 6. The cortical microtubule organization and surface view of hypocotyl epidermal cell files in dark-grown Arabidopsis wild-type (Col-0) seedlings. A, GFP-TUA6 seeds were grown for 7 d in the dark vertically on Glc-free and increasing-Glc (1%, 3%) containing one-half-times Murashige and Skoog medium supplemented with or without 10 nM BR. Confocal microscopic images reveals that the cells of hypocotyls grown on Glc-free medium displayed a network of tubulin filament organization across the hypocotyl while horizontal organization of tubulin filaments was observed in BR-treated hypocotyls. Higher concentrations of Glc along with BR caused vertical arrangement of tubulin filaments. Scale bar: 23.81 μm. B, Effect of microtubule organization inhibitor oryzalin on BR-inhibited hypocotyl agravitropic growth of wild type (Col-0). C, Stereo-Zoom (Nikon SMZ1500) microscopic images of the outer surface of cells of etiolated hypocotyls. Images denote the alignment of epidermal cell files. The hypocotyl epidermal cell patterning changes from straight profile to spiral upon BR treatment in Glc-free medium whereas higher Glc concentration can resist this change by BR. Scale bar: 0.1 mm. D, Quantification of alignment angle of epidermal cell files in etiolated hypocotyls of Arabidopsis wild-type seedlings. The angle of cells to the longitudinal axis was measured using ImageJ. Data shown is the average of two representative biological replicates having at least 15 seedlings; error bars represent SE. Student's *t* test, $P < 0.001$.

Growth of hypocotyls of the auxin polar transport mutant *multidrug resistant1-1* (*mdr1-1*) was more random (Fig. 4D) while other transport mutants *P-glycoprotein1-100* (*pgp1-100*), *pin3-4*, and *pin7-2* showed the wild-type response (Supplemental Fig. S11B). IAA did not affect starch granule accumulation in BR-treated and -nontreated seedlings growing on Glc-free medium (Supplemental Fig. S10C).

Glc Antagonizes BR-Regulated Gene Expression

To determine the global effect of Glc on BR-regulated gene profiles, whole-genome transcript profiling of 6-d-old, etiolated wild-type (Columbia-0 [Col-0]) seedlings treated with BR and/or Glc for 3 h was performed. The data were consistent with published profiles (Mishra et al., 2009; Yu et al., 2011), but it should be noted that the present and published data came from seedlings grown in liquid culture, not on solid medium, and therefore the absence of a constant gravity vector in liquid cultures may have influenced the final gene expression profile. Glc affected BR-regulated gene expression. Interestingly, only 285 genes were found to be regulated by BR in the presence of Glc as opposed to 897 genes in the absence of Glc (Supplemental Fig. S12). Only 32 genes were commonly regulated by BR in both the absence and presence of Glc, further suggesting independent signaling events taking place in either of the treatments (Supplemental Fig. S12). Glc substantially reduced expression of most of the genes annotated as BR regulated, auxin regulated, cell wall organization, and biogenesis related (Supplemental Fig. S13). These results suggest that Glc significantly affects most steps of BR signaling, predominantly by attenuation.

Glc affected the spatial expression of an auxin- and BR-inducible SAUR::GUS reporter (Gil and Green, 1997). SAUR::GUS seedlings were grown in different

concentrations of BR and Glc. GUS staining was visible in the subapical portion of etiolated hypocotyls. BR treatment caused heterogeneous/patchy GUS staining in the hypocotyl whereas Glc induced homogenous GUS staining throughout the hypocotyl (Fig. 5A).

Glc Antagonism of BR Response Involves Protein Degradation

Neither the protein biosynthesis inhibitor cycloheximide nor the actin filament organization inhibitor latrunculin B affected Glc antagonism of BR reset. However, the protease inhibitor MG132 reduced the Glc antagonism of BR reset, suggesting the involvement of protein degradation (Fig. 5B).

Glc Antagonizes BR-Induced Changes in Microtubule Organization and Cell Patterning across the Hypocotyl

BR alters the organization of cortical microtubules and increases the percentage of epidermal cells with transversely oriented cortical microtubules (Mayumi and Shibaoka, 1995). We show here that this BR-induced change in microtubule organization was attenuated by Glc. The microtubule organization of seedlings exhibiting BR reset was determined using a GFP-TUA6 transgenic line (Ueda et al., 2003). Epidermal cells of hypocotyls grown without Glc in the dark displayed a network of microtubules across the hypocotyl while horizontal organization of microtubules was observed in BR-treated hypocotyls. Application of cytokinin or high concentrations of Glc independently antagonized BR-induced microtubule rearrangement (Fig. 6A; Supplemental Fig. S14).

BR reset of hypocotyl gravitropism was reduced in the presence of the microtubule organization inhibitor oryzalin, suggesting a central role of cytoskeleton remodeling (Fig. 6B). The change in microtubule organization was correlated with cell patterning. The hypocotyls grown in 0% Glc containing one-half-times Murashige and Skoog medium in the dark displayed a straight arrangement of epidermal cells across the hypocotyl while twisting of epidermal cells in a spiral manner was observed in BR-treated hypocotyls. Higher concentrations of Glc reduced this twisting and hypocotyl agravitropism (Fig. 6, C and D). BR-induced differential cell patterning caused asymmetrical growth, leading to hypocotyl agravitropism while Glc and cytokinin antagonized this by restoring the cell files.

Adaptive Significance

The genetic evidence using loss- and gain-of-function mutations in genes encoding elements of BR, cytokinin, ethylene, and auxin signaling indicate that the hypocotyl directional growth described here integrates many signals in a hierarchical manner. However, it is not clear that this robust phenotype in the laboratory confers fitness to the plant in nature. To address this, we determined if BR reset of gravitropism positively or negatively affected adaptive responses of the skotomorphogenic hypocotyl, namely obstacle avoidance and emergence through agar in darkness.

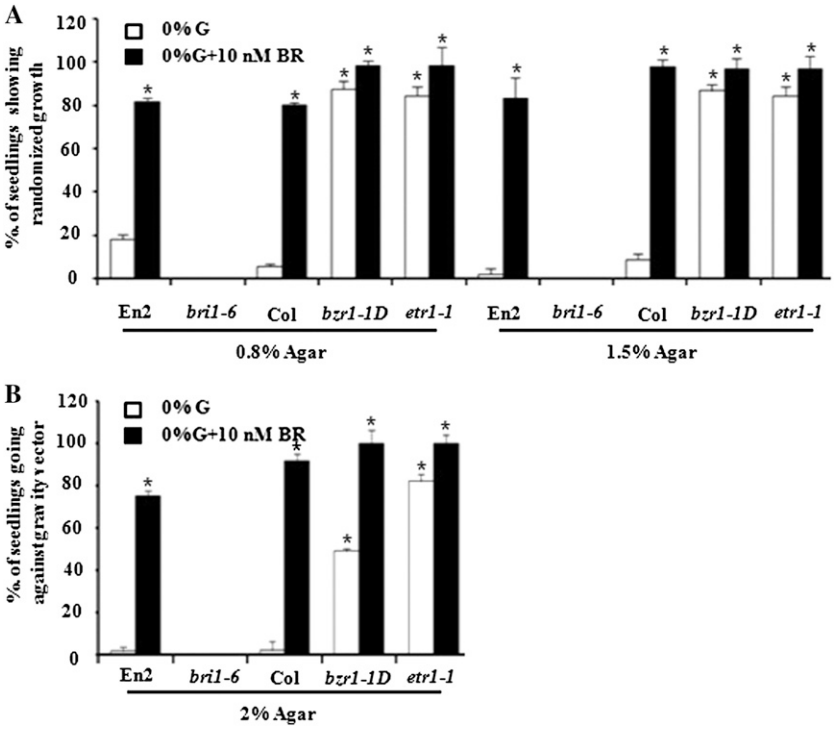

Figure 7. Quantification of seedling fitness in terms of penetrance through obstacle. Wild-type (Col-0) seedlings were grown in one-half-times Murashige and Skoog + 0.8% agar media either in presence or absence of BR. The seedlings were covered on top with 2-cm layer of the same composition media except with increasing agar concentration to challenge the seedlings with obstacle. Wild-type seedlings grew straight in 0.8%, 1.5% (A), and 2% (B) agar-containing media. Wild-type seedlings growing in BR-containing medium show randomized growth while BR receptor, *bri1-6* seedlings grew straight in the higher concentrations of agar-containing media both in the absence or presence of BR, *bzr1-1D*, and *etr1-1* mutants possessing exaggerated BR response showed reset of hypocotyl gravitropism both in the absence or presence of BR. Hypocotyls of wild-type, *bzr1-1D*, and *etr1-1* mutant seedlings could not penetrate the top medium (containing 2% agar) and grew into the basal medium (containing 0.8% agar) against the gravity vector, showing enhanced avoidance for obstacle. The data shown is the average of two representative biological replicate having at least 25 seedlings; error bars represent SE. Student's *t* test, $P < 0.001$.

Wild-type seeds were placed in Glc-free one-half-times Murashige and Skoog + 0.8% agar media either in the presence or absence of BR. The seeds were then covered with a 2-cm layer of the same composition media except with increasing agar concentrations (1.5%, 2%) to challenge the growing seedlings mechanically (obstacle) as shown in Supplemental Figure S14. Wild-type seedlings penetrated 0.8%, 1.5%, and 2% agar-containing media in the absence but not the presence of BR (Fig. 7, A and B; Supplemental Fig. S15, A and B). While the BR receptor, *bri1-6* mutant seedlings grew straight in the higher agar concentrations both in the absence or presence of BR, the *bzr1-1D* mutant displayed a random growth pattern both in the absence or presence of BR and had fewer chances to emerge less often through the higher concentration of agar-containing medium (obstacle). *etr1-1* seedlings did not penetrate the medium containing higher agar concentrations (Fig. 7, A and B; Supplemental Fig. S14, C–E). Wild-type seedlings penetrated 2% agar but not in the presence of BR. *bzr1-1D* mutant could not penetrate into the 2% agar medium even in the absence of BR whereas *bri1-6* mutant could penetrate well. To determine if encountering an obstacle enhances BR levels, wild-type seedlings were challenged with a glass coverslip in their growth path and BR levels were indirectly measured through expression levels of BR biosynthetic genes and BR-induced genes (Fig. 8A). The expression of most of the BR biosynthetic genes was increased in wild-type seedlings challenged with an impenetrable obstacle compared with controls, suggesting that mechano-stimulation leads to altered BR homeostasis in the plants. The expression levels of GUS in the BR-inducible TCH4::GUS line was more in the apical tip when the seedlings were challenged with an impenetrable obstacle (Fig. 8B), confirming more BR accumulation/response in the presence of an obstacle.

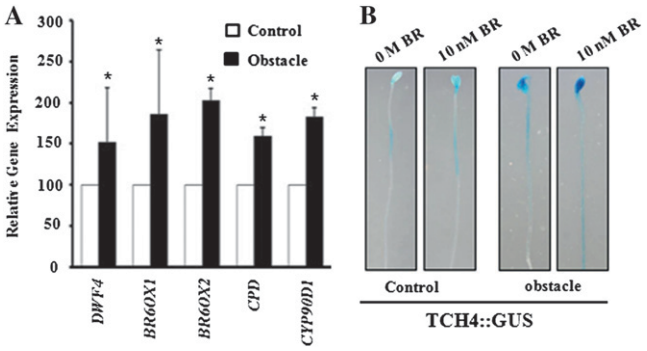

Figure 8. The effect of obstacle on expression of BR biosynthetic genes and BR-induced TCH4::GUS expression. A, The expression of genes involved in BR biosynthesis during obstacle encounter, as revealed by real-time gene expression analysis. Wild-type (Col-0) seeds were germinated and grown on horizontal, Glc-free one-half-times Murashige and Skoog medium supplemented with or without 10 nM BR, in the dark for 7 d. Germinated seeds were covered with a sterile glass coverslip to provide an impenetrable obstacle. Data shown is the average of two representative biological replicates; error bars represent SE. Student's *t* test, $P < 0.05$. B, The expression of BR-inducible TCH4::GUS upon obstacle encounter. TCH4::GUS seeds were germinated and grown on horizontal Glc-free one-half-times Murashige and Skoog medium supplemented with or without 10 nM BR, in dark for 7 d. Germinated seeds were covered with a sterile glass coverslip to provide impenetrable obstacle. Obstacle encounter caused accumulation of TCH4::GUS at the apical hook similar to BR-induced GUS expression. Experiment was performed at least three times. The data shown is of one representative biological replicate having 10 seedlings.

DISCUSSION

In nature, gravity is a major signal used to optimize the direction of organ growth, however, other signals override gravity for example to enable circumnavigation of an impenetrable obstacle. These multiple signaling pathways or elements in a signaling network must be coordinately modulated for optimal growth. Gene expression, cytoskeleton patterning, steady-state levels of signaling proteins, and hormone levels all must coordinate to bring about the efficient growth of hypocotyls in soil. We used a genetic approach to assemble the relevant signaling pathways and to assess their relationships in a complex trait, what we are calling reset of gravity sensing and/or gravitropism. We and others have shown that the plant hormone BR lies at an apical position in the signal transduction underlying this complex trait. A testable model based upon these findings and published literature is presented in Figure 9. We designated the BR-induced agravitropism of hypocotyls as gravitropism reset to zero (Fig. 1A). Reset occurs in a manner that is affected by both hexokinase-dependent, and -independent, Glc-signaling pathways (Fig. 1D). There are a number of reports of interaction of BRs with sugars. The sugar hypersensitivity of *brassinosteroid, light, sugar* mutant, is rescued on exogenous BR application (Laxmi et al., 2004). Recently, Vandenbussche and coworkers showed that an exogenous application of BR causes agravitropism in dark-grown Arabidopsis hypocotyls while sugar can antagonize this BR-inhibited gravitropism (Vandenbussche et al., 2011) that we extend here to mechanism. This BR reset of hypocotyl gravitropism response involves the BR receptor and signaling elements (Fig. 2A). Cytokinin signaling works by modulating ethylene biosynthesis and signaling to antagonize this response (Fig. 2B; Supplemental Figs. S8 and S10). Cytokinin and BRs act antagonistically to each other in controlling light-mediated seedling development (Chory et al., 1994). Cytokinin signaling mediated by ethylene signaling has also been previously shown to restore gravitropism to red-light-grown randomized Arabidopsis hypocotyls (Golan et al., 1996). A number of BR-related responses are mediated by ethylene. For example, BR-induced hook formation depends on ethylene biosynthesis (De Grauwe et al., 2005). BR antagonizes the negative effects of ethylene on hypocotyl growth at a low level but, at higher levels, inhibits hypocotyl elongation through an increase in both ethylene

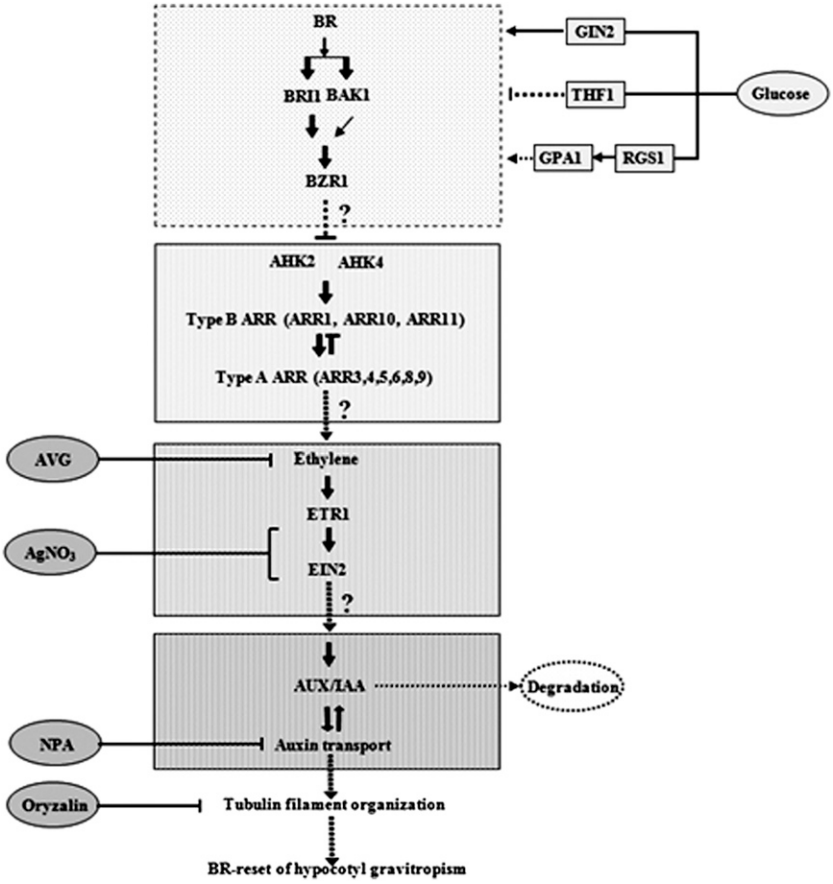

Figure 9. A testable model based on these findings and published. BR resets gravitropism by hexokinase-dependent and -independent Glc signaling. Asymmetrical exposure of BR at the hypocotyl changes cell patterning. This BR reset of hypocotyl gravitropism response involves BR receptor and signaling elements and the evidence for these elements and their relationships is provided in the discussion section. BR antagonizes cytokinin signaling and ethylene signaling to induce this response. Glc works independently of both cytokinin as well as ethylene to antagonize this response. Auxin response and transport both are involved since auxin signaling gain-of-function mutants and NPA-treated seedlings possess reduced BR reset of hypocotyl gravitropism. Differential distribution of auxin lies downstream to ethylene response mentioned above since NPA can inhibit exaggerated BR reset of hypocotyl gravitropism in ethylene signaling mutants and $AgNO_3$-treated wild-type seedlings. Glc may affect the response either via affecting BR-regulated gene expression, changing BR-regulated spatial gene expression, microtubule reorganization, or changing cell profile arrangement. Dotted arrows and question marks represent the possibility of additional routes and routes that are also consistent with the data.

biosynthesis and response (Deslauriers and Larsen, 2010). In contrast to these findings, BR randomizes hypocotyl growth by antagonizing ethylene signaling at low as well as high concentrations. Taken together, these findings suggest that the BR and ethylene interaction is tissue specific and that these hormones control different physiological responses namely, hypocotyl elongation, apical hook formation, and hypocotyl directional response in dark-grown Arabidopsis seedlings.

Glc works independently of both cytokinin as well as ethylene to antagonize BR reset of hypocotyl gravitropism (Figs. 2C and 3; Supplemental Figs. S8 and S9). Auxin may work further downstream either directly or through alternate routes since auxin signaling gain-of-function mutations and NPA treatment attenuates the BR reset (Fig. 4, A and C). Auxin and BR signaling interact in several ways and BR and auxin affect expression of both AUX/IAA gene family members as well as auxin polar transporter PIN proteins (Nakamura et al., 2004a). Our finding suggests that BR may directly or indirectly affect proteasomal degradation of members of AUX/IAA auxin signaling repressor proteins to execute BR-induced hypocotyl randomization. A differential distribution of auxin lies either directly or indirectly downstream to the ethylene response as mentioned above since NPA reduces BR reset in ethylene signaling mutants (Fig. 4C). Glc may affect the response either via affecting BR-regulated gene expression, changing BR-regulated spatial gene expression, microtubule reorganization, changing cell profile arrangement, or affecting protein degradation (Supplemental Figs. S12 and S13; Figs. 5–8). Compiling the experimental evidence, we propose the model shown in Figure 9. This model provides a foundation for testing and for discovery of additional routes available for reset of hypocotyl gravitropism.

The Relevance of BR for Optimal Hypocotyl Growth Direction

Optimal hypocotyl growth direction provides the easiest and shortest route in soil emergence for seedlings to become photoautotrophic. Changes in auxin, ethylene, gravity signaling, or alteration in cell wall properties alter hypocotyl growth direction (De Grauwe et al., 2005; Vandenbussche et al., 2011). The cytoskeleton also plays a crucial role in optimal hypocotyl direction as evident by the hypocotyl phenotypes of seedlings harboring mutations in genes encoding various microtubule-interacting proteins (Blancaflor, 2002; Bisgrove, 2008). Exogenous BR application or enhanced endogenous BR signaling compromised the ability of dark-grown seedlings to penetrate a hard medium. Our interpretation is that BR sensitizes dark-grown seedlings to the presence of an obstacle. Since hypocotyl

directional growth provides adaptive advantage during seedling growth in soil, optimal BR signaling may determine seedling fitness and survival.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotypes of Col-0, Wassilewskija (Ws), Landsberg *erecta* (L*er*), and Enkheim-2 (En-2) were used as wild-type controls. Seeds of *bzr1-1D* (AT1G75080, CS65987); *bri1-6* (AT4G39400, CS399); *bak1-1* (AT4G33430, CS6125); *tir1-1* (AT3G62980, CS3798); *axr1-3* (AT1G05180, CS3075); *axr3-1* (AT1G04250, CS57504); *etr1-1* (AT1G66340, CS237); *ein2-1* (AT5G03280, CS3071); *eto2* (AT5G65800, CS8059); *gin2-1* (AT4G29130, CS6383); GFP-TUA6 (AT4G14960, CS6551); *ahk2* (AT5G35750, CS6561); *ahk4* (AT2G01830, CS6563); *arr1,10,11* (AT3G16857/AT4G31920/AT1G67710, CS6993); and *arr3,4,5,6,8,9* (AT1G59940/AT1G10470/AT3G48100/AT5G62920/AT2G41310/AT3G57040, CS25279) were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/abrc/). Following lines are obtained from the original published source as: *cpd* (AT5G05690; Szekeres et al., 1996); *SAUR::GUS* (AT4G38850; Gil and Green, 1997); *pin3-4* (AT1G70940; Friml et al., 2002a); *pin4-3* (AT2G01420; Friml et al., 2002b); *pin7-2* (AT1G23080; Friml et al., 2003); *mdr1-1* (At3g28860; Noh et al., 2001); *pgp1-100* (At2g36910; Lin and Wang, 2005); *rgs1-1*, *rgs1-2* (AT3G26090; Chen et al., 2003); *gpa1-1*, *gpa1-2*, and *gpa1-3* (AT2G26300; Ullah et al., 2001); *thf1-1*(AT2G20890; Huang et al., 2006); and TCH4::GUS (AT5G57560; Xu et al., 1995). All mutant lines were in the Col-0 background except the following: The *bri1-6* mutant was in the En-2 background. *bak1-1*; *ahk2*; *ahk4*; *arr1,10,11*; *mdr1-1*; *gpa1-1*; and *gpa1-2* were derived from Ws background. The *gin2-1* and *eto2* were in the L*er* background. Seeds were surface sterilized and imbibed at 4°C for 48 h. Seed germination was carried out in climate-controlled growth room under long-day conditions (16 h light and 8 h darkness, 80 μmol m^{-2} s^{-1} light intensity) at 22°C ± 2°C temperature. All chemicals were purchased from Sigma except agar that is purchased from Himedia. Epibrassinolide was prepared as 10^{-2} M stock solution in 50% (v/v) ethanol. The following were prepared as 10^{-2} M stock solution in dimethyl sulfoxide: BAP, IAA, PCIB, GA_3, abscisic acid, oryzalin, and MG132 (Z-Leu-Leu-Leu-al). NPA was prepared as 10^{-2} M stock solution in 1 N NaOH. AVG, ACC, and $AgNO_3$ were prepared as sterile 10^{-2} M aqueous stock solutions. X-Gluc was prepared as 100 mg L^{-1} stock solution in N,N-dimethylformamide. All treatment concentrations for this study were chosen from previously published reports (Nakamura et al., 2004b; Deslauriers and Larsen, 2010; Kim et al., 2011; Kushwah et al., 2011; Vandenbussche et al., 2011).

Seedling Growth

Imbibed seeds were grown vertically on square (120 × 120 mm) petri plates containing one-half-times Murashige and Skoog medium supplemented with different concentrations of Glc (w/v; pH 5.7) and 0.8% agar (w/v) except where indicated otherwise. For the dark-grown seedlings, seeds on plates were first exposed to 12 h light to stimulate germination; the plates were wrapped with two layers of aluminum foil and placed in the growth chamber for all the treatments mentioned below. For experiments testing the effect of media, supplements/hormones on BR-induced hypocotyl randomization response seeds were directly sown on square petri plates containing treatment medium (one-half-times Murashige and Skoog with or without Glc and/or BR and/or other supplements) and grown vertically in climate-controlled growth room (22°C ± 2°C). For experiments testing the effect of BR on hypocotyl gravitropism, seeds were directly sown on round petri plates (100 × 20 mm) containing treatment medium (one-half-times Murashige and Skoog with or without Glc and/or BR) and grown horizontally in climate-controlled growth room (22°C ± 2°C) for 5 d. To determine the role of the root, hypocotyl, root tip, and hypocotyl tip in signal perception, wild-type seedlings were grown vertically on one-half-times Murashige and Skoog medium in dark for 5 d. The apical tip along with cotyledons, root tip, roots, and hypocotyls were excised sterilely under dim-green safe light (2 μmol m^{-2} s^{-1}). The seedlings with and without intact roots, roots tips, and hypocotyl tip (0.5 mm) were transferred to square petri plates containing one-half-times Murashige and Skoog + 10 nM BR medium containing different concentrations of Glc and 0.8% agar for 5 d. Thereafter, digital images were captured using Nikon Coolpix digital camera and angles were quantified using ImageJ (http://rsb.info.nih.gov/ij/). For all experiments, Student's *t* test with paired two-tailed distribution was used for statistical analysis. In all experiments, plates were sealed with gas-permeable tape to avoid ethylene accumulation. All end point analyses were taken on the 7th d otherwise specified though plates were observed for longer period up to 10 d.

Measurement of Hypocotyl Angular Deviation from Vertical

Five-day-old seedlings grown vertically on one-half-times Murashige and Skoog, 0.8% agar, and 1% Suc-containing medium in dark were transferred to one-half-times Murashige and Skoog, 0.8% agar-containing medium with different concentrations of Glc and BR and their hypocotyl and root tips were marked. Digital images of hypocotyl tip were captured after 2 d. All these experimental manipulations with etiolated seedlings were performed under dim-green safe light (2 μmol $m^{-2}s^{-1}$) by wrapping white fluorescent light lamp with green cellophane filter. The BR-induced hypocotyl randomization response was measured by calculating the angle of hypocotyl deviating away from the vertical axis. The angle represents the average of two independent biological replicates having at least 15 seedlings and error bars represent SE. For quantification of hypocotyl gravitropic response, direction of gravity was altered by turning the plates 90° for 48 h after the seedlings were grown for 7 d in dark. For quantification of hypocotyl phototropic curvature 5-d-old, dark-grown seedlings were exposed to unilateral blue light (7.5 μmol m^{-2} s^{-1}) for 24 h. Hypocotyl curvatures were measured using the ImageJ program from the National Institutes of Health.

Statistical Analyses

All values reported in this work are the average of two independent biological replicates having at least 15 seedlings. Error bars represent SE. Statistical differences between control and each treatment were analyzed using Student's *t* test with paired two-tailed distribution. *P*-value cutoff was taken at $P < 0.001$ except where stated otherwise.

Amyloplast Staining

Col-0 (wild-type) seeds were germinated and grown directly in Glc-free and increased-Glc (3%) containing one-half-times Murashige and Skoog medium supplemented with or without BR (10 nM, 100 nM, 1 μM) solidified with 0.8% agar in climate-controlled growth room for 5 d in the dark. Amyloplast staining was performed as previously described (Kim et al., 2011; seedlings were fixed in 5% formaldehyde, 5% ethanol, 5% acetic acid solution for 24 h at 4°C in dark). After fixation, seedlings were rinsed in 50% (v/v) ethanol once and stained in I_2-KI solution (2% [w/v] iodine, 5% [w/v] potassium iodine, and 20% [w/v] chloral hydrate) for 1 min. Samples were destained in 1:1:1 trichloroacetic acid:phenol:lactic acid for 5 min then mounted on slide for microscopic observation and the photographs were taken by Nikon Coolpix digital camera attached to a Nikon ECLIPSE E100 biological microscope. The experiment was performed three times yielding similar results.

GUS Histochemical Staining

SAUR::GUS seeds were germinated and grown directly in Glc-free and increased-Glc (1%, 3%) containing one-half-times Murashige and Skoog medium supplemented with or without BR (10 nM, 100 nM, 1 μM) solidified with 0.8% agar in climate-controlled growth room for 7 d in the dark. For TCH4::GUS expression analysis during obstacle encounter seeds were sown on Glc-free one-half-times Murashige and Skoog medium supplemented with or without 10 nM BR. Germinated seeds were then covered with a sterile glass coverslip to provide impenetrable obstacle. GUS activities were determined following the methods described previously (Kushwah et al., 2011) after 2 to 3 h for SAUR::GUS and after 4 to 6 h for TCH4::GUS. The experiment was performed three times yielding similar results. Each replicate had 10 seedlings per treatment.

Differential Cell Patterning in Hypocotyl Epidermis

Imbibed seeds were grown vertically on square (120 × 120 mm) petri plates containing one-half-times Murashige and Skoog medium supplemented with 10 nM BR, different concentrations of Glc (w/v; pH 5.7), and 0.8% agar (w/v) for 7 d in dark. The epidermal cell profile was captured using a Nikon SMZ1500 Stereo-Zoom microscope and the photographs were taken by Nikon

Coolpix digital camera attached to a Nikon SMZ1500 Stereo-Zoom microscope.

Laser Confocal Scanning Microscopy

To determine the cortical microtubule arrangement in hypocotyl epidermal cells, GFP-TUA6-expressing seeds were germinated on Glc-free and increasing-Glc (1%, 3%) containing one-half-times Murashige and Skoog medium supplemented with or without 10 nM BR for 7 d in dark. Confocal images of the hypocotyl epidermal cells below apical hook were captured using a laser confocal scanning microscope (Leica Microsystems). Three biological replicates with each replicate having 10 seedlings were performed. The laser and pinhole settings of the confocal microscope were kept identical among different treatments.

Penetrable Obstacle

For penetrable obstacle wild-type and mutant seeds were placed in sterile glass test tubes containing one-half-times Murashige and Skoog + 0.8% agar media with or without BR (10 nM). The seeds were covered on top with a 2-cm layer of the same composition media except with increasing agar concentration (0.8%, 1.5%, and 2% agar). The top layer of denser agar medium was cooled and poured just before solidification so as to avoid killing the seeds underneath. The test tubes were wrapped in two layers of aluminum foil and kept in dark for 7 d before taking observations.

Gene Expression Analysis

For global gene expression profiling, imbibed Col-0 seeds were sown on one-half-times Murashige and Skoog medium supplemented with 0.8% agar and 1% Suc. The plates were first exposed to continuous light for 12 h to stimulate germination and then wrapped with two layers of aluminum foil and placed in the growth chamber for 5 d. Once the plant material was uniformly germinated, the experimental conditions were applied. Five-day-old, dark-grown seedlings were washed seven times with sterile water followed by a wash with one-half-times Murashige and Skoog liquid medium without Suc to remove residual exogenous sugar and the plant material was kept in one-half-times Murashige and Skoog liquid without Suc in the dark for all subsequent steps. Cultures were shaken at 140 rpm at 22°C for 24 h and then treated with one-half-times Murashige and Skoog without Glc or one-half-times Murashige and Skoog supplemented with BR (100 nM), Glc (3%), or Glc (3%) + BR (100 nM) for 3 h. Seedlings were harvested after 3 h and preceded for RNA isolation and microarray analysis. RNA was prepared from frozen tissue using the RNeasy kit (Qiagen) following the manufacturer's protocol. All total RNA samples were quality assessed prior to beginning target preparation/processing steps by running out a small amount of each sample (typically 25–250 ng/well) onto a RNA Nano Chip (Caliper Technologies Corp.) that was evaluated on an Agilent Bioanalyzer 2100 (Agilent Technologies). Three biological replicates were performed. Total RNA from each sample was amplified and Cy3 labeled using Agilent's quick amp labeling kit, One Color following the manufacturer's protocols (version 6.5). After the labeling, the cRNA was cleaned and examined with the Nanodrop ND-2000. Equal amounts of Cy3-labeled cRNA (1.65 μg; for the one-color protocol) were hybridized to (4x44K) Arabidopsis microarray slides (Agilent) for 18 h at 65°C using Agilent's GE hybridization kit. Washes were conducted as recommended by the manufacturer using Agilent's gene expression wash pack. Arrays were scanned with Agilent Technologies scanner, model G2505B. Spot intensities and other quality control features were extracted with Agilent's feature extraction software version 10.7.3.1. Genespring 11.5.1 software was used for the analysis of the expression data. The raw data from the biological replicate samples was normalized using the percentile shift summarization algorithm and the signature lists of the significantly altered genes ($P \leq 0.03$, fold change ≥ 1.5) for each condition were generated using unpaired t test with Benjamini Hochberg FDR in Genespring 11.5.1. Additional microarray data presentation and manipulation were assessed using Microsoft Excel. All data are Minimum Information About a Microarray Experiment (MIAME) compliant and the raw data has been deposited in ArrayExpress database through MIAMExpress (accession no. E-MEXP-3545).

For quantitative real-time PCR analysis, the imbibed Col-0 seeds were germinated and grown on horizontal Glc-free one-half-times Murashige and Skoog medium supplemented with or without 10 nM BR, in dark for 7 d. Germinated seeds were covered with a sterile glass coverslip to provide an impenetrable obstacle. RNA isolation, reverse transcription, and PCR primer designing were performed as described previously (Kushwah et al., 2011). The values represent the average of the two biological replicates (each with three technical replicates), and error bars represent SE. For all experiments Student's t test with paired two-tailed distribution was used for statistical analysis. Primers used for PCR are described in Supplemental Figure S16.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Role of BR in gravitropism of etiolated wild-type (Col-0) seedling hypocotyls.

Supplemental Figure S2. Hypocotyl gravitropic reset growth of wild-type and Glc signaling mutants on different Glc and BR treatments.

Supplemental Figure S3. Role of BR in blue-light-mediated phototropic response of etiolated wild-type (Col-0) seedlings hypocotyl on day 6.

Supplemental Figure S4. BR reset in different medium and light condition.

Supplemental Figure S5. The effect of higher concentrations of different hormones on wild-type (Col-0) seedling hypocotyls.

Supplemental Figure S6. Identification of the stimulus perception site for BR reset.

Supplemental Figure S7. A comparison of BR reset of hypocotyl gravitropism in wild-type and BR biosynthesis and signaling mutants on different Glc and BR treatments.

Supplemental Figure S8. A comparison of BR reset of hypocotyl gravitropism in wild-type and cytokinin signaling mutants on different Glc and BR treatments.

Supplemental Figure S9. A comparison of BR reset of hypocotyl gravitropism in wild-type and ethylene signaling mutants on different Glc and BR treatments.

Supplemental Figure S10. Effect of BAP on BR reset of hypocotyl gravitropism of ethylene signaling mutants and AVG/$AgNO_3$-treated wild-type seedlings.

Supplemental Figure S11. A comparison of BR reset of hypocotyl gravitropism in wild-type, auxin signaling, and transport mutants on different Glc and BR treatments.

Supplemental Figure S12. Comparison of genes regulated in 0% Glc + BR versus 0% Glc category and 3% Glc + BR versus 3% Glc category.

Supplemental Figure S13. Effect of BR and Glc on BR, auxin, gravitropism, and cell-wall-related genes.

Supplemental Figure S14. Effect of BAP on cortical microtubule organization in dark-grown Arabidopsis GFP-TUA6 seedlings.

Supplemental Figure S15. Comparison of obstacle avoidance response in wild type (Col-0, En-2), BR signaling mutants, *bri1-6*, *bzr1-1D*, and ethylene signaling mutant *etr1-1*.

Supplemental Figure S16. List of primers used for quantitative real-time PCR.

ACKNOWLEDGMENTS

We thank the National Institute of Plant Genome Research Central Instrument Facility (real-time PCR division), Microarray Facility, and Confocal Imaging Facility for their assistance. We also thank Mr. Dhritiman Dan (Application Specialist, Imperial Life Sciences Limited) for assistance with the gene-profiling experiment.

Received February 16, 2012; accepted June 5, 2012; published June 11, 2012.

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