Running Head: Signaling interaction and hypocotyl directional growth

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Hypocotyl directional growth in Arabidopsis: a complex trait

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Abstract

The growth direction of the Arabidopsis 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, auxin, cytokinin, ethylene) and nutrient status (glucose, sucrose). We used a genetic approach to identify the signaling elements and their relationship underlying hypocotyl growth direction. Brassinosteroid (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. Glucose 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 glucose and hormone signaling interplay is proposed. The biological significance of BR-mediated changes in hypocotyl gravi-response 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.

Introduction

Gravitropism, an adaptive phenomenon which 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; Kiss and Edelmann, 1999; Baluska and Hasenstein, 1997; 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 et al., 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 in order 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 glucose in controlling root or hypocotyl directional growth in plants. Glucose and indole-3-acetyl-myoinositol are asymmetrically distributed in gravistimulated Zea mays seedlings (Momonaki et al., 1988). Glucose controls root gravitropism via auxin signaling (Mishra et al., 2009).

Many hormones direct gravitropism, of which brassinosteroids are the least understood. Brassinosteroids control gravitropic bending in hypocotyls/shoots (Park, 1998; Philosoph-Hadas et al., 2005; Nakamoto et al., 2006; Meudt, 1987; Hala et al., 2010; Arteca and Arteca, 2011). BR acts synergistically with auxin during hypocotyl gravitropism of partially de-etiolated bean (Phaseolus vulgaris) (Meudt, 1987). Finally, BRs inhibit gravitropic responses of etiolated Arabidopsis 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 gravi-responses. 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 Brassinosteroid (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 glucose-free medium (Supplemental Fig. S1A). The etiolated seedlings were also subjected to gravity reorientation assay by giving a 90° gravistimulation to 4d to 8d old vertically grown seedlings. In this assay also BR- treated seedlings could not reorient themselves to changed gravity vector in the glucose free medium (Fig 1B) suggesting that BR either perturbs gravity detection or response. The observed waviness in the hypocotyl may be due to frequent gravity ‘resets’ in the presence of BR.

**The BR reset of gravitropism is affected by glucose**

Normal gravitropic response was restored in the BR-treated seedlings by exogenous glucose in the gravity reorientation assay (Fig. 1B). In the vertically-grown seedlings, glucose 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 glucose restored negative gravitropism in the BR treated seedlings growing in horizontal plates (Supplemental Fig. S1A). *pgm* (phosphoglucomutase) and *eal1* (endodermal-amyloplast less 1) both with reduced
levels of amyloplast starch were less gravitropic whereas a mutant (sex1) 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; Vittha et al., 2007). Lugol staining did not reveal an obvious difference in starch granule accumulation between BR-treated and -non-treated seedlings growing on glucose-free medium. Ectopic accumulation of starch granules was observed in the presence of 3% glucose. Overall, our results do not support a BR mechanism that limits starch (Supplemental Fig. S1B).

In yeast and Arabidopsis, hexokinase may serve as a glucose receptor (Rolland et al., 2006). The hexokinase mutant gin2-1 (Moore et al., 2003) was used to investigate any physiological role of HXK-dependent glucose signaling. gin2 showed reduced BR-reset of gravitropism at both lower and higher concentrations of glucose (Fig. 1D; Supplemental Fig. S2) suggesting a direct requirement for hexokinase. However, the glucose hypersensitive mutant thf1-1 (Huang et al., 2006) which is associated with G protein-coupled, HXK-independent sugar signaling mechanism was more sensitive to BR both at low and high concentrations of glucose (Fig. 1D; Supplemental Fig. S2) suggesting the involvement of a hexokinase-independent signaling pathway as well. Two other glucose-signaling mutants, rgs1 and gpa1, were less sensitive towards glucose antagonism of BR-reset of gravitropism, also consistent with involvement of multiple glucose response pathways in this BR response (Supplemental Fig. S2B).

Two auxin-insensitive mutants; nonphototropic hypocotyl 4/massugu 1 (nph4/msg1), and a dominant mutant, msg2 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. S3A 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. S4A and B). WT seedlings grown in cytokinin, ABA, ACC or GA3 containing medium did not show hypocotyl agravitropism. Apart from BR, only 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 GA3 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.S5B 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 ½ X MS medium for 5 days 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 ½ X MS medium containing different concentrations of BR and glucose. 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. S6A-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. S6F and G).

Known BR signaling components mediate BR reset of gravitropism

The BR receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) heterodimerizes with BRI1 ASSOCIATED KINASE 1 (BAK1) after binding BR. BRI1 and BAK1 subsequently act together to inhibit a GSK3-like kinase, BIN2 (Li et al., 2001), that, in the absence of brassinosteroid, catalyzes phosphorylation of the transcription factor BRASSINAZOLE RESISTANT 1 (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-SUPPRESSOR 1 (BES1) to regulate expression of brassinosteroid-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 (He et al., 2002). In order 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 \textit{cpd} was hypersensitive, the BR perception mutants \textit{bri1-6} and \textit{bak1-1} were resistant to BR (Fig. 2A; Supplemental Fig. S7). A dominant mutation \textit{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 \textit{cpd} mutant is not intuitive. We speculate that the hyper-responsiveness towards BR relative to wild type may be due to WT being BR saturated under similar conditions (i.e. rate limiting in WT). This was observed before; \textit{cpd} mutant seedlings show an increase in hypocotyl elongation as compared to WT in the presence of BR (Szekeres et al 1996).

\textit{Cytokinin antagonizes BR reset via ethylene signaling while glucose 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 phosphorylation cascade. Cytokinin is perceived by one of three hybrid histidine protein kinases (AHK2, AHK3, AHK4) in which cytokinin binding activates autophosphorylation. The phosphorylated receptors then phosphorylate histidine 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 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 histidine phosphorylation cascade, cytokinin perception mutants \textit{ahk2}, \textit{ahk4} and Type B ARR triple mutant \textit{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. S8A and B). Glucose antagonized BR reset of gravitropism in the cytokinin receptor and Type B mutants suggesting that glucose acts independently of cytokinin signaling. Also BAP did not affect starch granule accumulation in BR treated and non-treated seedlings growing on glucose 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 (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 etr1-1 and the signaling mutant ein2-1 exhibited a high response suggesting an antagonistic role in the ethylene signal transduction pathway. The ethylene overproducer mutant eto2 showed less BR reset confirming that ethylene works antagonistically with BR to control this response (Fig. 3A; Supplemental Fig. S9A and B). The ethylene biosynthetic inhibitor aminoethoxyvinylglycine (AVG) as well as the ethylene signaling inhibitor AgNO3 enhanced the response (Fig. 3B). Glucose antagonized BR-inhibition of gravitropism in the ethylene receptor etr1-1 and signaling ein2-1 mutants (Fig. 3A, Supplemental Fig. S9A and B). Glucose also antagonized both AVG- and AgNO3-induced hypocotyl randomization suggesting glucose works independently of ethylene biosynthesis/signaling (Fig. 3B). ACC did not affect starch granule accumulation in BR treated and non-treated seedlings growing on glucose 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 AgNO3 and AVG (Supplemental Fig. S10A 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 proteins (Nakamura et al., 2004), and not turnover (Nemhauser et al.,
We, therefore, checked the involvement of auxin signaling in BR-reset of gravitropism. The auxin receptor mutant tir1 showed a wild type BR response while the auxin signaling mutant 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 brassinosteroid (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). Growth of hypocotyls of the auxin polar transport mutant mdr1-1 was more random (Fig. 4D) while other transport mutants pgp1-100, pin3-4, pin7-2 showed the wild type response (Supplemental Fig. S11B). IAA did not affect starch granule accumulation in BR treated and non-treated seedlings growing on glucose-free medium (Supplemental Fig. S10C).

Glucose antagonizes BR-regulated gene expression

To determine the global effect of glucose on BR-regulated gene profiles, Whole genome transcript profiling of 6-d-old, etiolated WT (Col-0) seedlings treated with BR and/or glucose for 3 h was performed. The data was 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. Glucose affected BR-regulated gene expression. Interestingly, only 285 genes were found to be regulated by BR in the presence of glucose as opposed to 897 genes in the absence of glucose, (Supplemental Fig. S12). Only 32 genes were
commonly regulated by BR in both the absence and presence of glucose, further suggesting independent signaling events taking place in either of the treatments (Supplemental Fig. S12). Glucose 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 glucose significantly affects most steps of BR signaling, predominantly by attenuation.

Glucose affected the spatial expression of an auxin- and BR-inducible SAUR::GUS reporter (Gil et al., 1997). SAUR::GUS seedlings were grown in different concentrations of BR and glucose. GUS staining was visible in the sub-apical portion of etiolated hypocotyls. BR treatment caused heterogeneous/patchy GUS staining in the hypocotyl whereas glucose induced homogeneous GUS staining throughout the hypocotyl (Fig. 5A).

**Glucose antagonism of BR response involves protein degradation**

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

**Glucose 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 et al., 1995). We show here that this BR-induced change in microtubule organization was attenuated by glucose. 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 glucose 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 glucose
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% G containing ½ X MS 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 glucose reduced this twisting and hypocotyl agravitropism (Fig. 6C, D). BR-induced differential cell patterning caused asymmetrical growth leading to hypocotyl agravitropism while glucose 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 brassinosteroid, 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.

WT seeds were placed in glucose free ½ X MS + 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%) in order to challenge the growing seedlings mechanically (obstacle) as shown in Supplemental Fig. S14. WT seedlings penetrated 0.8%, 1.5% and 2% agar containing media in the absence but not the presence of BR (Fig. 7A and B; Supplemental Fig. S15A and B). While the brassinosteroid 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.
etrl-1 seedlings did not penetrate the medium containing higher agar concentrations (Fig. 7A and B; Supplemental Fig. S14C-E). WT seedlings penetrated 2% agar but not in the presence of BR. etrl-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, WT seedlings were challenged with a glass cover slip 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 WT seedlings challenged with an impenetrable obstacle compared to 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.

Discussion

In nature, gravity is a major signal used to optimize the direction of organ growth, however, other signals over-ride 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 brassinosteroid 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, glucose-signaling pathways (Fig. 1D). There are a number of reports of interaction of BRs with sugars. The sugar hypersensitivity of bls mutant (brassinosteroid, light, sugar), is rescued on exogenous BR application (Laxmi et al.,
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) which 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 Fig. S8; Supplemental Fig. 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 (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). 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.

Glucose works independently of both cytokinin as well as ethylene to antagonize BR reset of hypocotyl gravitropism (Fig. 2C; Figure 3; Supplemental Fig. S8; Supplemental Fig. 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. 4A 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., 2004). 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). Glucose 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 Fig. S12; Supplemental Fig. S13; Fig. 5; Fig. 6; Fig. 7; Fig. 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 (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 thaliana* ecotypes of Col-0, Ws, Ler and En-2 were used as wild-type controls. Seeds of *bzr1-1D* (AT1G75080, CS65987); *brl-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, CS3251); *ahk2* (AT5G35750, CS6561); *ahk4* (AT2G01830, CS6563); *arr1,10,11* (AT3G16857/AT4G31920/AT1G67710, CS6993); *arr3,4,5,6,8,9*
(AT1G59940/AT1G10470/AT3G48100/AT5G62920/AT2G41310/AT3G57040, CS25279) were obtained from ABRC (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 (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 Col 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; gpa1-2 were derived from Ws background. The gin2-1 and eto2 was in the Ler 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 (St. Louis, MO, USA) except agar which is purchased from Himedia (Mumbai, India). Epibrassinolide (EBR) 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 (DMSO): 6-benzylaminopurine (BAP), indole-3-acetic acid (IAA), p-chlorophenoxyisobutyric acid (PCIB), gibberellic acid (GA₃), abscisic acid (ABA), oryzalin and MG132 (Z-Leu-Leu-Leu-al). 1-N-naphthylphthalamic acid (NPA) was prepared as 10⁻² M stock solution in 1 N NaOH. Aminoethoxyvinylglycine (AVG), 1-aminocyclopropane-carboxylic acid hydrochloride (ACC) and AgNO₃ were prepared as sterile 10⁻² M aqueous stock solutions. X-Gluc was prepared as 100 mgL⁻¹ stock solution in N, N-dimethylformamide (DMF). All treatment concentrations for this study were chosen from previously published reports (Nakamura et al., 2004; Deslauriers and Larsen, 2010; Kushwah et al., 2011; Vandenbussche et al, 2011; Kim et al., 2011).

Seedling growth

Imbibed seeds were grown vertically on square (120 x 120 mm) petri plates containing ½ X MS medium supplemented with different concentrations of glucose (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 (½ X MS with or without glucose 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 (100mm x 20mm) containing treatment medium (½ X MS with or without glucose 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, WT seedlings were grown vertically on ½ X MS medium in dark for 5 d. The apical tip along with cotyledons, root tip, roots and hypocotyls were excised steriley 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 ½ X MS+10 nM BR medium containing different concentrations of glucose 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 endpoint 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 ½ X MS, 0.8% agar and 1% sucrose containing medium in dark were transferred to ½ X MS, 0.8% agar containing medium with different concentrations of glucose 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 standard error (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 NIH.

**Statistical analyses**

All values reported in this work are the average of two independent biological replicates having at least 15 seedlings. Error bars represent standard error (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 (WT) seeds were germinated and grown directly in glucose free and increased glucose (3%) containing ½ X MS medium supplemented with or without BR (10 nM, 100 nM, 1µM) 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 FAA (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 I₂-KI solution [2% (w/v) iodine, 5% (w/v) potassium iodine and 20% (w/v) chloral hydrate] for 1 min. Samples were de-stained 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 glucose free and increased glucose (1%, 3%) containing ½ X MS medium supplemented with or without BR (10 nM, 100 nM, 1µM) 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 glucose free ½ X MS 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 x 120 mm) petri plates containing ½ X MS medium supplemented with 10 nM BR, different concentrations of glucose (w/v) [pH 5.7] and 0.8% agar (w/v) for 7d 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 (LCSM)**

To determine the cortical microtubule arrangement in hypocotyl epidermal cells, GFP-TUA6-expressing seeds were germinated on glucose free and increasing glucose (1%, 3%) containing ½ X MS medium supplemented with or without 10 nM BR for 7 days in dark. Confocal images of the hypocotyl epidermal cells below apical hook were captured using a Laser Confocal Scanning Microscope (Leica Microsystems, Heidelberg, Germany). 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 WT and mutant seeds were placed in sterile glass test tubes containing ½ X MS + 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 2 layers of aluminium foil and kept in dark for 7 days before taking observations.

**Gene expression analysis**

For global gene expression profiling, imbibed Col-0 seeds were sown on $\frac{1}{2}$ X MS medium supplemented with 0.8% agar and 1% sucrose. 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. 5-d-old, dark-grown seedlings were washed seven times with sterile water followed by a wash with $\frac{1}{2}$ X MS liquid medium without sucrose to remove residual exogenous sugar and the plant material was kept in $\frac{1}{2}$ X MS liquid without sucrose in the dark for all subsequent steps. Cultures were shaken at 140 rpm at 22°C for 24 h and then treated with $\frac{1}{2}$ X MS without glucose or $\frac{1}{2}$ X MS supplemented with BR (100 nM), glucose (3%), or glucose (3%) + BR (100 nM) for 3 h. Seedlings were harvested after 3h and preceded for RNA isolation and microarray analysis. RNA was prepared from frozen tissue using the RNeasy kit (Qiagen, Valencia, CA) 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., Mountain View, CA) that was evaluated on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). 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 (1.65μg) (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≤0.03, FC≥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 is MIAME compliant and the raw data has been deposited in ArrayExpress database through MIAMExpress (accession number E-MEXP-3545).

For quantitative real-time PCR (qRT-PCR) analysis, the imbibed Col seeds were germinated and grown on horizontal glucose free ½ X MS 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 previously described previously (Kushwah et al., 2011). The values represent the average of the two biological replicates (each with three technical replicates) and error bars present standard error (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 Fig. S16.

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Figure Legends

Figure 1. BR-reset of hypocotyl gravitropism response in dark.
A, 5-d-old, dark-grown WT (Col-0) seedlings on glucose-free ½ X MS medium supplemented with or without increasing BR (1 nM, 10 nM, 100 nM, 1µM, 2µM) as indicated. B, Gravitropic responses of WT (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 d, 5 d, 6 d, 7 d, 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 glucose can effectively restore gravitropic response at increased concentrations (3% G). C, Quantification of BR-reset of hypocotyl gravitropism. 5-d-old, dark-grown seedlings were transferred to the indicated concentrations of glucose 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 WT (Ler, 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 towards glucose antagonism of BR-reset of hypocotyl gravitropism. E, The effect of different hormones on WT (Col-0) seedling hypocotyls to determine their role in controlling hypocotyl directional response. 5-d-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 replicate having atleast 15 seedlings; error bars represent standard error (SE). Student’s T-test, P<0.001.

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 WT (Col-0) and BR biosynthesis and signaling mutants. BR-reset of hypocotyl gravitropism was not found in brl1-6 mutant and the same was highly reduced in bak1-1 mutant. Highly exaggerated BR-reset of hypocotyl gravitropism was found in bzrl1-ID mutant which 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 WT (Col-0) seedlings in presence of 6-benzylaminopurine (BAP). Supplementing BAP and BR together reduced BR-reset of hypocotyl gravitropism. C, Comparison of BR-reset of hypocotyl gravitropism of 7-d-old WT (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 to WT. Data shown is the average of two representative biological replicate having atleast 15 seedlings; error bars represent standard error (SE). Student’s T-test, P<0.001.

**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 less as compared to WT. B, Comparison of BR-reset of hypocotyl gravitropism of WT (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 replicate having at least 15 seedlings; error bars represent standard error (SE). Student’s T-test, P<0.001.

**Figure 4.** The role of auxin signaling and polar transport in controlling BR-reset of hypocotyl gravitropism.
A. The auxin signaling mutant *axr3-1* which leads to stability of auxin signaling repressor protein shows substantial reduction in BR-reset of hypocotyl gravitropism. B, WT (Col-0) seeds were sown on glucose free or increasing glucose (1%, 3%) containing ½ X MS 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 (1µM) while application of the auxin signaling inhibitor PCIB inhibited the BR-reset of hypocotyl gravitropism. C, WT (Col-0) and ethylene signaling mutant *etr1-1* seeds were sown on 5 µM NPA containing glucose free ½ X MS medium supplemented with or without 10 nM BR. NPA could effectively antagonize BR-reset of hypocotyl gravitropism in both WT and the *etr1-1* mutant. D, Lateral auxin transport mutant *mdr1-1* seeds were sown on glucose free or increasing glucose (1%, 3%) containing ½ X MS 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 replicate having atleast 15 seedlings; error bars represent standard error (SE). Student’s T-test, P<0.001.

**Figure 5.** Glucose 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 glucose free or increasing glucose (1%, 3%) containing ½ X MS medium supplemented with BR at the indicated concentrations. A, BR treatment causes heterogeneous/patchy SAUR::GUS expression in the hypocotyl. Higher concentrations of glucose in the medium caused accumulation of SAUR::GUS throughout the hypocotyl. B, Effect of protein degradation pathway inhibitor MG132 on glucose antagonism of BR-inhibited hypocotyl gravitropic growth of WT (Col-0). Experiment was performed atleast 3 times. Data shown is the average of two representative biological replicate having atleast 15 seedlings; error bars represent standard error (SE). Student’s T-test, P<0.001.

**Figure 6.**
The cortical microtubule organization and surface view of hypocotyl epidermal cell files in dark-grown Arabidopsis WT (Col-0) seedlings. 

A, GFP-TUA6 seeds were grown for 7 d in the dark vertically on glucose free and increasing glucose (1%, 3%) containing ½ X MS medium supplemented with or without 10 nM BR. Confocal microscopic images reveals that the cells of hypocotyls grown on glucose 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 glucose along with BR caused vertical arrangement of tubulin filaments. Scale bar: 23.81µm. B, Effect of microtubule organization inhibitor oryzalin on BR-inhibited hypocotyl agravitropic growth of WT (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 glucose free medium whereas higher glucose 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 WT seedlings. The angle of cells to the longitudinal axis was measured using ImageJ, Data shown is the average of two representative biological replicate having atleast 15 seedlings; error bars represent standard error (SE). Student’s T-test, P<0.001.

Figure 7.
Quantification of seedling fitness in terms of penetrance through obstacle. 
WT (Col-0) seedlings were grown in ½ X MS + 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. WT seedlings grew straight in A, 0.8%, 1.5% and B, 2% agar containing media. WT seedlings growing in BR containing medium show randomized growth while brassinosteroid receptor, bri1-6 seedlings grew straight in the higher concentrations of agar containing media both in the absence or presence of BR, bzrl-1D and etr1-1 mutants possessing exaggerated BR response showed reset of hypocotyl gravitropism both in the absence or presence of BR. Hypocotyls of WT, bzrl-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 atleast 25 seedlings; error bars represent standard error (SE). Student’s T-test, P<0.001.

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. WT (Col-0) seeds were germinated and grown on horizontal, glucose-free ½ X MS 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 standard error (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 glucose free ½ X MS 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 atleast 3 times. The data shown is of one representative biological replicate having 10 seedlings.

Figure 9. A testable model based on these findings and published.

BR resets gravitropism by hexokinase dependent and -independent glucose 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. Glucose 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 AgNO₃ treated WT seedlings. Glucose 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.

Supplemental Figure Legends

Supplemental Figure S1. Role of BR in gravitropism of etiolated WT (Col-0) seedling hypocotyls.
A, WT (Col-0) seedlings were grown horizontally on different concentrations of BR (10nM, 100 nM) and glucose (0%, 3%) containing ½ X MS medium in dark. Exogenous BR reduced the number of seedlings growing vertically on the glucose free medium. In the presence of 3% glucose, the BR-reset was strongly inhibited B, Whole mount Lugol staining of starch in the upper part of hypocotyls of 7-d-old WT (Col-0) grown vertically in dark on glucose free or increasing glucose (3%) containing ½ X MS medium supplemented with or without 10 nM BR. Experiment was performed at least 3 times. The data shown is of one representative biological replicate.

Supplemental Figure S2. Hypocotyl gravitropic reset growth of WT and glucose signaling mutants on different glucose and BR treatments.
A, 7d-old, dark-grown WT (Ler, Col-0), hexokinase dependent and independent glucose signaling mutants gin2-1 and thf1-1 seedlings on indicated concentrations of glucose and BR. B, A comparison of BR-Reset of hypocotyl gravitropism of WT (Col-0, Ws), rgs1-1, rgs1-2, gpa1-1, gpa1-2 and gpa1-3 seedlings. Values represent the means +/- SD from at least 10 seedlings. Student’s T-test, P<0.001.

Supplemental Figure S3. Role of BR in blue light mediated phototropic response of etiolated WT (Col-0) seedlings hypocotyl on 6th d.
A, Quantification and B, pictures of phototropic bending of WT (Col-0) seedlings hypocotyl. Seeds were sown on glucose free and increasing glucose (1%, 3%)
containing ½ X MS medium supplemented with or without 10 nM BR and grown vertically in the dark for 5d. Seedlings were exposed to unilateral low-blue light (7.5 µmol m⁻² s⁻¹) for 24 h. Phototropic response of WT was not perturbed in BR containing medium. Values represent the means +/- SD from at least 10 seedlings. Student’s T-test, P<0.001.

Supplemental Figure S4. BR-Reset in different medium and light condition.
A, Hypocotyl agravitropic growth of WT (Col-0) seedlings grown on glucose free and increasing glucose (1%, 3%) containing 1mM KNO₃ medium supplemented with or without 10 nM BR in dark. B, Hypocotyl agravitropic growth of WT (Col-0) seedlings grown on glucose free and increasing glucose (1%, 3%) containing ½ X MS medium supplemented with or without 10 nM BR for 7 d in light.

Supplemental Figure S5. The effect of higher concentrations of different hormones on WT (Col-0) seedling hypocotyls.
A, 5-d-old dark grown seedlings were transferred to different hormone (5µM and 10 µM) containing media for 2 d and the hypocotyl deviation was quantified as described. B, Pictures showing effect of GA3 on BR-reset of hypocotyl gravitropism of WT (Col-0). C, 5-d-old dark grown seedlings were transferred to different concentrations of GA3 (10 nM, 100nM, 1µM) containing ½ X MS medium supplemented with or without 10 nM BR for 2 d and hypocotyl deviation was quantified. Values represent the means +/- SD from at least 15 seedlings. Student’s T-test, P<0.001.

Supplemental Figure S6. Identification of the stimulus perception site for BR-Reset.
Arabidopsis WT (Col-0) seeds were sown on ½ X MS medium and grown vertically for 5 days in dark. The root tip, whole root, hypocotyl and hypocotyl tip of the etiolated seedlings were then excised and placed on glucose free and increasing glucose (1%, 3%) containing ½ X MS medium supplemented with or without 10 nM BR medium and grown vertically in the dark, A-G. The excised hypocotyl tip when placed in the BR containing medium could grow well and displayed BR-induced agravitropism suggesting that the hypocotyl tip alone is enough and sufficient for showing this response. Values represent the means +/- SD from at least 10 seedlings. Student’s T-test, P<0.001.
Supplemental Figure S7. A comparison of BR-reset of hypocotyl gravitropism in WT and BR biosynthesis and signaling mutants on different glucose and BR treatments. WT (Col-0, En-2, Ws) BR biosynthesis mutant cpd, BR perception mutants bri1-6, bak1-1 and BR signaling mutant bzr1-1D seeds were sown on glucose free and increasing glucose (1%, 3%) containing ½ X MS medium supplemented with or without 10 nM BR and grown vertically in the dark for 7d.

Supplemental Figure S8. A comparison of BR-reset of hypocotyl gravitropism in WT and cytokinin signaling mutants on different glucose and BR treatments. WT (Col-0, WS), cytokinin receptor mutant ahk2, ahk4 and signaling mutant arr1,10,11 (Type B ARR triple mutant) and arr3,4,5,6,8,9 (Type A ARRs sextuple mutant) seeds were sown and grown vertically for 7 d in dark on glucose free or increasing glucose (1%, 3%) containing ½ X MS medium supplemented with or without 10 nM BR. A, Cytokinin perception mutants ahk2, ahk4 and Type B ARR triple mutant arr1,10,11, showed an enhanced BR response while B, Type A ARRs sextuple mutant arr3,4,5,6,8,9, showed reduced BR response. C, Whole mount Lugol staining of starch in the upper part of hypocotyls of 7-d-old WT (Col-0) grown vertically in dark on glucose free ½ X MS medium supplemented with or without 10 nM BR and/or 1µM BAP. Experiment was performed atleast 3 times. The data shown is of one representative biological replicate.

Supplemental Figure S9. A comparison of BR-reset of hypocotyl gravitropism in WT and ethylene signaling mutants on different glucose and BR treatments. WT (Col-0, Ler), ethylene receptor mutant etr1-1 and signaling mutant ein2-1 and eto2 seeds were sown and grown vertically for 7 d in dark on glucose free or increasing glucose (1%, 3%) containing ½ X MS medium supplemented with or without 10 nM BR. A, Ethylene receptor mutant etr1-1 and signaling mutant ein2-1 show enhanced BR-reset of hypocotyl gravitropism while B, response of eto2.1 mutant was very less as compared to WT. C, Whole mount Lugol staining of starch in the upper part of hypocotyls of 7-d-old WT (Col-0) grown vertically in dark on glucose free ½ X MS
medium supplemented with or without 10 nM BR and/or 1µM ACC. Experiment was performed at least 3 times. The data shown is of one representative biological replicate.

**Supplemental Figure S10.** Effect of BAP on BR-reset of hypocotyl gravitropism of ethylene signaling mutants and AVG/AgNO₃ treated WT seedlings.

A, Comparison of BR-reset of hypocotyl gravitropism in WT (Col-0), ethylene signaling mutant *etr1-1* and *ein2-1*. Seeds were sown on 100 nM BAP containing ½ X MS medium supplemented with or without 10 nM BR. BAP could not antagonize BR-reset of hypocotyl gravitropism in *etr1-1* and *ein2-1* mutant. B, Effect of BAP on BR-reset of hypocotyl gravitropism response in AVG and AgNO₃ treated seedlings. WT (Col) seeds were sown and grown vertically on 100 nM BAP containing ½ X MS medium supplemented with or without 10 nM BR in presence of ethylene signaling inhibitor (AgNO₃) and biosynthesis inhibitor (AVG) at concentrations indicated. BAP could not antagonize BR-reset of hypocotyl gravitropism in AVG and AgNO₃ treated seedlings.

**Supplemental Figure S11.** A comparison of BR-reset of hypocotyl gravitropism in WT, auxin signaling and transport mutants on different glucose and BR treatments.

Arabidopsis WT (Col-0, Ws) A, auxin signaling mutant *tir1-1, axr1-3* and B, auxin transport mutant *pin3-4, pin4-3, pin7-2 and ppg1-100*. Seeds were sown on glucose free or increasing glucose (1%, 3%) containing ½ X MS medium supplemented with or without 10 nM BR. The BR-induced hypocotyl agravitropism response of *tir1-1, axr1-3, pin3-4, pin4-3, pin7-2 and ppg1-100* was found to be comparable to wild type. Values represent the means +/- SD from at least 10 seedlings. Student’s T-test, P<0.001.

C, Whole mount Lugol staining of starch in the upper part of hypocotyls of 7-d-old WT (Col-0) grown vertically in dark on glucose free ½ X MS medium supplemented with or without 10 nM BR and/or 1µM IAA. Experiment was performed at least 3 times. The data shown is of one representative biological replicate.

**Supplemental Figure S12.** Comparison of genes regulated in 0%G+BR Vs 0%G category and 3%G+BR Vs 3%G category. Glucose can antagonize BR regulated global gene expression. The difference in BR regulated gene expression in 0%G vs 0%G+BR
Supplemental Figure S13. Effect of BR and glucose 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.
GFP-TUA6 seeds were grown for 7 d in the dark vertically on glucose free ½ X MS medium supplemented with or without 10 nM BR and/or 100 nM BAP. Confocal microscopic images reveal that the cells of hypocotyls grown on BAP containing medium displayed a vertical alignment of microtubules. Scale bar: 25µm.

Supplemental Figure S15. Comparison of obstacle avoidance response in WT (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.
Figure 1. BR-reset of hypocotyl gravitropism response in dark.

A, 5d old, dark-grown WT (Col-0) seedlings on glucose-free ½ X MS medium supplemented with or without increasing BR (1 nM, 10 nM, 100 nM, 1µM, 2µM) as indicated.

B, Gravitropic responses of WT (Col-0) at different time points. The direction of gravity was altered by turning the plates 90° after the seedlings were grown either for 4d, 5d, 6d, 7d, or 8d 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 glucose can effectively restore gravitropic response at increased concentrations (3% G).

C, Quantification of BR-reset of hypocotyl gravitropism. 5d old, dark-grown seedlings were transferred to the indicated concentrations of glucose 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 WT (Ler, 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 towards glucose antagonism of BR-reset of hypocotyl gravitropism.

E, The effect of different hormones on WT (Col-0) seedling hypocotyls to determine their role in controlling hypocotyl directional response. 5d 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 replicate having at least 15 seedlings; error bars represent standard error (SE). Student’s T-test, P<0.001.
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 WT (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 which display higher hypocotyl randomization even in the absence of BR in the medium. B. Comparison of BR-reset of hypocotyl gravitropism of 7d old WT (Col-0) seedlings in presence of 6-benzylaminopurine (BAP). Supplementing BAP and BR together reduced BR-reset of hypocotyl gravitropism. C. Comparison of BR-reset of hypocotyl gravitropism of 7d old WT (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 to WT. Data shown is the average of two representative biological replicate having atleast 15 seedlings; error bars represent standard error (SE). Student’s T-test, P<0.001.
Figure 3

**A.** Comparison of BR-reset of hypocotyl gravitropism of 7d 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 less as compared to WT. **B.** Comparison of BR-reset of hypocotyl gravitropism of WT (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 replicate having at least 15 seedlings; error bars represent standard error (SE). Student’s T-test, P<0.001.
**Figure 4.** The role of auxin signaling and polar transport in controlling BR-reset of hypocotyl gravitropism.

A. The auxin signaling mutant *axr3-1* which leads to stability of auxin signaling repressor protein shows substantial reduction in BR-reset of hypocotyl gravitropism. B. WT (Col-0) seeds were sown on glucose free or increasing glucose (1%, 3%) containing ½ X MS 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 (1µM) while application of the auxin signaling inhibitor PCIB inhibited the BR-reset of hypocotyl gravitropism. C. WT (Col-0) and ethylene signaling mutant *etr1-1* seeds were sown on 5 µM NPA containing glucose free ½ X MS medium supplemented with or without 10 nM BR. NPA could effectively antagonize BR-reset of hypocotyl gravitropism in both WT and the *etr1-1* mutant. D. Lateral auxin transport mutant *mdr1-1* seeds were sown on glucose free or increasing glucose (1%, 3%) containing ½ X MS 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 replicate having atleast 15 seedlings; error bars represent standard error (SE). Student's T-test, P<0.001.
Figure 5. Glucose 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 7d in dark on glucose free or increasing glucose (1%, 3%) containing ½ X MS medium supplemented with BR at the indicated concentrations. A. BR treatment causes heterogeneous/patchy SAUR::GUS expression in the hypocotyl. Higher concentrations of glucose in the medium caused accumulation of SAUR::GUS throughout the hypocotyl. B. Effect of protein degradation pathway inhibitor MG132 on glucose antagonism of BR-inhibited hypocotyl gravitropic growth of WT (Col-0). Experiment was performed at least 3 times. Data shown is the average of two representative biological replicate having at least 15 seedlings; error bars represent standard error (SE). Student’s T-test, P<0.001.
Figure 6. The cortical microtubule organization and surface view of hypocotyl epidermal cell files in dark-grown Arabidopsis WT (Col-0) seedlings.

A. GFP-TUA6 seeds were grown for 7d in the dark vertically on glucose free and increasing glucose (1%, 3%) containing ½ X MS medium supplemented with or without 10 nM BR. Confocal microscopic images reveals that the cells of hypocotyls grown on glucose 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 glucose along with BR caused vertical arrangement of tubulin filaments. Scale bar: 23.81µm. B. Effect of microtubule organization inhibitor oryzalin on BR-inhibited hypocotyl agravitropic growth of WT (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 glucose free medium whereas higher glucose 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 WT seedlings. The angle of cells to the longitudinal axis was measured using ImageJ. Data shown is the average of two representative biological replicate having atleast 15 seedlings; error bars represent standard error (SE). Student’s T-test, P<0.001.
**Figure 7.** Quantification of seedling fitness in terms of penetrance through obstacle.

WT (Col-0) seedlings were grown in ½ X MS + 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. WT seedlings grew straight in A, 0.8%, 1.5% and B, 2% agar containing media. WT seedlings growing in BR containing medium show randomized growth while brassinosteroid receptor, bri1-6 seedlings grew straight in the higher concentrations of agar containing media both in the absence or presence of BR, bvr1-1D and etr1-1 mutants possessing exaggerated BR response showed reset of hypocotyl gravitropism both in the absence or presence of BR. Hypocotyls of WT, bvr1-1D 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 atleast 25 seedlings; error bars represent standard error (SE). Student’s T-test, P<0.001.
Figure 8

A

The expression of genes involved in BR biosynthesis during obstacle encounter, as revealed by Real-time gene expression analysis. WT (Col-0) seeds were germinated and grown on horizontal, glucose-free ½ X MS medium supplemented with or without 10 nM BR, in the dark for 7d. 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 standard error (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 glucose free ½ X MS 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 atleast 3 times. The data shown is of one representative biological replicate having 15 seedlings.
BR resets gravitropism by hexokinase dependent and -independent glucose 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. Glucose 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 AgNO3 treated WT seedlings. Glucose 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.