The effects of *DELLA* s on growth change with developmental stage and brassinosteroid levels

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SUMMARY

There are two stages in photomorphogenesis. First, seedlings detect light and open their cotyledons. Second, seedlings optimize their light environment by controlled elongation of the seedling stem or hypocotyl. In this study, we used time-lapse imaging to investigate the relationship between the brassinosteroid (BR) and gibberellin (GA) hormones across both stages of photomorphogenesis. During the transition between one stage and the other, growth promotion by BRs and GAs switched from an additive to a synergistic relationship. Molecular genetic analysis revealed unexpected roles for known participants in the GA pathway during this period. Members of the *DELLA* family could either repress or enhance BR growth responses, depending on developmental stage. At the transition point for seedling growth dynamics, the BR and GA pathways had opposite effects on *DELLA* protein levels. In contrast to GA-induced *DELLA* degradation, BR treatments increased the levels of REPRESSOR of ga1-3 (RGA) and mimicked the molecular effects of stabilizing *DELLA*s. In addition, *DELLA*s showed complex regulation of genes involved in BR biosynthesis, implicating them in BR homeostasis. The effects of BR, including the synergistic effects with GA, were largely independent of *PIF*s. These results point to a multi-level, dynamic relationship between the BR and GA pathways.

Keywords: brassinosteroids, gibberellins, *Arabidopsis thaliana*, photomorphogenesis, *PIFs.*

INTRODUCTION

During photomorphogenesis, seedlings use growth to optimize their light environment. The proper positioning of the embryonic leaves (cotyledons) is critical for survival, and thus the elongation of the embryonic stem (hypocotyl) is under tight regulation. A wealth of signaling pathways, including those involved in sensing light, hormones, time of day and metabolic state have been implicated in regulating photomorphogenetic growth (Arsovski et al., 2012). To further complicate this network, there is extensive feedback within pathways, as well as significant cross-regulation (Vanstraelen and Benková, 2012).

Brassinosteroid (BR) and gibberellin (GA) pathways are required for normal seedling growth. While the specifics of the signaling pathway triggered by each hormone differ, hormone-triggered activation of either pathway leads to relief of repression on downstream transcription factors. Brassinosteroids bind and activate the BRASSINOSTEROID-INSENSITIVE1 (BRI1)-associated receptor complex at the plasma membrane. A phospho-relay cascade culminates in dephosphorylated and nuclear-localized transcription factors, including BRI1-EMS-SUPPRESSOR1/BRASSINOZOLE-RESISTANT2 (BES1/BZR2) and BZR1 (Wang et al., 2012). Gibberellins bind and activate GA-INSENSITIVE-DWARF1 (GID1) receptors, triggering first binding and then turn-over of the DELLA family of repressors through interaction with the F-box protein SLEEPY1 (SLY1). In the absence of GA, the DELLA s bind and sequester transcriptional regulators, including PHYTOCHROME INTERACTING FACTOR (PIF) family members, thereby blocking GA responses (Davière et al., 2008).

The transcription factor families downstream of the BR and GA pathways are highly interconnected and integrate information across the growth network. The DELLA s regulate the function of a number of transcription factors, including BES1 and BZR1 (Bai et al., 2012; Gallego-Bartolomé et al., 2012; Li et al., 2012). BES1 and BZR1 are...
able to dimerize with each other, as well as with PIFs, further linking downstream transcriptional responses (Oh et al., 2012). DELLAs also bind to SPATULA (SPT), a close relative of the PIF family. SPATULA lacks a phytochrome-binding domain and is thought to have a DELLA-like effect by forming dimers with PIF proteins and blocking their function (Khanna et al., 2004; Josse et al., 2011; Reymond et al., 2012). Recently, SPT has also been implicated in DELLA-independent GA responses (Fuentes et al., 2012). The potential for many different transcriptional complexes may explain the extensive plasticity of seedling growth responses.

In this study, we used time-lapse imaging to analyze the dynamic relationship of BRs and GAs during photomorphogenesis, particularly focusing on the understudied early stages of seedling growth. We found that the relationship between BRs and GAs changed over developmental time, and known signaling components played unexpected roles. DELLAs and SPT, proteins previously characterized as growth repressors, were critical for normal BR pathway function. Seedlings with either loss- or gain-of-function mutations in DELLA genes had an increased BR response early in development. In the transition period when cotyledons were opening, BR treatment increased the abundance of REPRESSOR of ga1-3 (RGA) in the hypocotyl and dorns were opening, BR treatment increased the abundance early in development. In the transition period when cotyledons were opening, BR treatment increased the abundance of REPRESSOR of ga1-3 (RGA) in the hypocotyl and dorns were opening, BR treatment increased the abundance early in development.

**RESULTS**

**Brassinosteroids and GAs induce stage-specific synergistic growth**

To determine the roles of BR and GA during photomorphogenesis, we measured hypocotyl growth in seedlings exposed to each hormone alone and in combination (Figure 1). We divided photomorphogenesis into five 12-h intervals (Int. 1–5) starting at 36 h post germination (hpg) (Int. 1–5, Figure 1a). As previously described, the majority of hypocotyl elongation occurred during Int. 1–2, before the cotyledons were fully open (Figure 1b,c; Stewart et al., 2011). Brassinosteroid treatment increased growth rates substantially in every interval. In contrast, GA treatment increased growth rates only during Int. 1–3 (Figure 1b,c). The previously described synergistic growth response caused by combining BR and GA treatment (Tanaka et al., 2003) was not detected until Int. 3 (Figure 1b,c). During Int. 1–2, treatment with both hormones resulted in an essentially additive effect on growth rates. Consistent with previous studies (Bai et al., 2012; Gallego-Bartolomé et al.,

![Figure 1. Brassinosteroids (BRs) and gibberellin (GA) show stage-specific growth promotion.](image-url)

(a) Hypocotyl elongation rates were measured in 12-h intervals (Int.) spanning the 36–96 h post germination (hpg) as follows: 36–48 (Int. 1, brown), 48–60 (Int. 2, orange), 60–72 (Int. 3, yellow), 72–84 (Int. 4, light green) and 84–96 (Int. 5, dark green). Images of representative seedlings are shown for the beginning of each interval to show developmental progression.

(b) Average hypocotyl lengths (representing 12–20 seedlings per experiment) are shown for seedlings exposed to no hormone (Mock, black), brassinosteroids (BR, blue), gibberellins (GA, green) and both hormones (BR&GA, purple). Hypocotyl lengths predicted by an additive model are shown in grey.

(c) Hormone treatment of wild-type seedlings differentially promoted growth across intervals. Growth rates from each interval are shown, centered to the border between Int. 2 and Int. 3 with mock-treated seedlings in the first row (no letter). Scale bar equals 0.05 mm h^{-1}. Bar graphs are shown to highlight the differences between Int. 2 and Int. 3. Int. 3 showed the largest growth promotion by GA (green ‘g’ and green bars), while BR (blue ‘b’ and blue bars) had strong effects in both windows. Mock (black) and combined BR and GA (purple ‘bg’ and purple bars) treatments are also shown. Rates predicted by an additive model are shown by grey bars in Int. 2 and Int. 3. Growth promotion by GA was eliminated in BR-deficient det2 mutants. Error bars in (c) represent standard error and those shown are of similar magnitude with the error associated with all rate bars (Table S3). Some error bars in (b) are within the boundaries of the markers.
2012), growth promotion by GAs required BRs. Seedlings with reduced BR biosynthesis [de-etiolated2 (det2) mutants or brassinoazole (BRZ) treatment] were dramatically less sensitive to GA treatment (Figure 1c and Figure S1 in Supporting Information).

**DELLAs and SPT have interval-specific effects on BR growth promotion**

As DELLAs are a point of cross-regulation between the BR and GA pathway (Bai et al., 2012; Gallego-Bartolomé et al., 2012; Li et al., 2012), we analyzed growth rates for seedlings with altered DELLA levels. We focused on RGA and GA-INSENSITIVE (GAI), as they are known to play the largest role in seedling growth (Davière et al., 2008). Loss of RGA and GAI function resulted in increased growth primarily in Int. 2–3, consistent with the timing of strongest growth promotion by GA treatment (Figure 2a). Stabilization of GAI protein in the gai-1 mutant reduced growth rates in all intervals, with one exception (Figure 2a). Growth rates in Int. 3 were strikingly elevated in gai-1 seedlings. We also examined growth rates in spt mutants, as SPT acts alongside DELLAs in the regulation of seedling growth (Josse et al., 2011; Fuentes et al., 2012). Rather than strongly increasing growth in Int. 2–3 like the rga gai mutants, spt mutants showed the greatest increase in growth rates in Int. 1 (Figure 2a). When the function of all three repressors was lost (rga gai spt), growth rate phenotypes were largely additive (Figure 2a).

Growth analysis also revealed a stage-dependent role for DELLAs and SPT in the perception of BR. Loss of DELLA function generally increased sensitivity to BR treatment, as would be expected for growth repressors. This relationship was particularly obvious in Int. 2, where BR treatment of rga gai seedlings had the most dramatic effect on growth (Figure 2a). However, gai-1 seedlings retained BR sensitivity in several intervals (Figure 2a). During Int. 3, gai-1 mutants were actually more sensitive than wild-type seedlings to BR treatment (Figure 2a). spt seedlings had a nearly wild-type response to BR treatment, except in Int. 3 where spt mutants treated with BRs grew faster than the wild type. When all three repressors were lost (rga gai spt), no further BR sensitivity was observed beyond that observed in rga gai seedlings (Figure 2a).

We next analyzed the function of DELLAs and SPT in plants overproducing BRs endogenously. Seedlings with an activation tag inserted upstream of the BR biosynthetic gene DWF4 (DWF4ox) were approximately twice as tall as wild-type seedlings after 5 days (Figure 2b). When combined with rga gai or spt, the hypocotyl length of DWF4ox mutants was reduced (Figure S2). In quadruple DWF4ox rga gai spt mutants, the DWF4ox long-hypocotyl phenotype was completely suppressed (Figure 2b). These results were unexpected, as rga gai, spt and rga gai spt mutants had longer hypocotyls than the wild type in the

![Figure 2](image-url)
absence of DWF4ox (Figure S2). There was a striking reduction in DWF4 expression in DWF4ox rga gai seedlings (Figure 2c), providing a likely explanation for the reduction in growth. Quadruple mutants (DWF4ox rga gai spt) were sterile, precluding assessment of DWF4 expression in homozygous seedlings; however, the further suppression of the phenotype is consistent with a further reduction in DWF4 expression.

To test whether DELLA proteins affect normal expression of genes encoding BR biosynthetic enzymes, we quantified expression levels of three targets of BR negative feedback regulation: CPD, DWF4 and BR6 oxidase1 (BR6ox1) in wild-type and gai-1 mutants. Treatment with BR decreased expression of all three genes, as expected (Figure 2d). In gai-1, DWF4 expression was unchanged and CPD expression was up-regulated (Figure 2d). Surprisingly, expression of BR6ox1 was down-regulated by more than 50-fold in gai-1. While these results clearly implicate DELLA proteins in BR homeostasis, the complicated pattern of changes makes it difficult to predict the effect of GA treatment on active BR levels.

Brassinosteroids regulate the abundance and activity of RGA

Next, we measured the effect of BRs on the abundance of DELLA proteins at dawn of days 3–5 (48, 72 and 96 hpg; Figure S3a). We examined levels of RGA in the elongation zone of the hypocotyl, using plants expressing GFP-RGA fusions from the RGA native promoter (Silverstone et al., 2001). Brassinosteroids strongly increased the GFP-RGA signal, particularly at 72 hpg (Figures 3a,b and S3a,b). Treatment with GA reduced the GFP-RGA signal to background levels (Figure S3a), as expected, and combining BR and GA treatments counteracted the effect of BR (Figure S3a, b). Normal levels of BRs were not required for the accumulation of DELLA, as the abundance of GFP-RGA was largely unaffected by treatment with the BR biosynthesis inhibitor BRZ (Figure S3c). This result makes it unlikely that BRs directly antagonize GA-mediated DELLA degradation.

To investigate other potential mechanisms for BR-induced accumulation of DELLA, we analyzed the expression of DELLA family members in wild-type and gai-1 mutants. Expression of RGA, GA20ox1, GA20ox2 and GA20ox5 was well correlated with GA levels and should therefore be anticorrelated with levels of DELLA proteins (Middleton et al., 2012). In rice, inhibition of the expression of GA biosynthetic genes by BR treatment led to increases in the levels of DELLA proteins (Vleesschauwer et al., 2012). We observed exactly the opposite trend. Expression of all three GA biosynthetic genes was increased by BR treatment or overexpression of DWF4 (Figure 3c). In Arabidopsis seedlings, BRs appear to stabilize DELLA proteins without decreasing GA biosynthesis. Brassinosteroids could achieve this by facilitating interactions with DELLA partner proteins that slow degradation rates or by shifting the
timings of daily peaks of DELLA abundance (Hanano et al., 2006; Arana et al., 2011). The latter seems unlikely as treatment with BR increased the abundance of RGA similarly throughout the day (Figure S5). In either case, the increase in DELLA at dawn coincides with a time when seedlings are capable of rapid growth, depending on the status of hormone, light and metabolic pathways.

To quantify the functional impact of BR-induced accumulation of RGA, we analyzed the expression of genes induced by DELLA (Zentella et al., 2007; Josse et al., 2011). Treatment with BR induced expression of GID1a, GID1b, bHLH137 and XERICO to similar levels as observed in gai-1, and the same trend was observed in DWF4ox seedlings (Figure 3d). The effects of BR were reduced in rga gai mutant seedlings, suggesting that BRs act through DELLA to increase target gene expression (Figure S6). We tested whether SPT levels were reduced by BRs, as this is another molecular read-out of DELLA activity. Indeed, BR treatments led to a decrease in abundance of SPT (Figure 3e). This decrease was not observed in seedlings lacking RGA and GAI (Figure 3e). Brassinosteroid-induced decreases in SPT levels were similar whether seedlings were exposed to BR alone or a combined BR and GA treatment (Figure 3e). Differences in the effects of BR on SPT when examining GA treatments compared with rga gai mutants may reflect the delay in the degradation of DELLA following exogenous hormone treatment or the effects of low DELLA levels earlier in seedling development.

**Synergistic growth in response to BRs and GAs resembles skotomorphogenesis**

Increasing levels of the bZIP transcription factor ELONGATED HYPOCOTYL5 (HY5) is a convergence point for multiple light signaling pathways and leads to strong inhibition of hypocotyl growth (Arsovski et al., 2012). Seedlings treated with BR and GA resembled hy5 mutants, and growth of hy5 seedlings was largely insensitive to combined hormone treatments (Figure 4a and S7). Phytochrome interacting factors act in opposition to HY5, and mutants, and PIF1, PIF3, PIF4, PIF5 and PIF6 (pifP mutants) had substantially reduced growth rates during Int. 3–5, but retained wild-type rates before Int. 3 (Figure 4b). pifP seedlings showed very little response to GA treatment (compare Figure 1c with Figure 4b). This effect was most evident in Int. 3 where the effects of GA were most striking in wild-type seedlings. In contrast, pifP seedlings exhibited near wild-type growth responses when treated with BR alone or in combination with GA (Figure 4b). Similar hormone response trends were observed with pifQ (pif1 pif3 pif4 and pif5) and pif345 (pif3 pif4 pif5) mutants (Figures 4a and S7).

**DISCUSSION**

Cotyledon opening is coincident with a fundamental shift in hormone response and growth control, including a
striking inhibition of hypocotyl elongation (Stewart et al., 2011). In this study, we found that BRs promoted hypocotyl growth throughout seedling development, while GA growth promotion was limited primarily to early time points before the cotyledons were fully open. Both hormones applied together provoked strong growth acceleration. The application of either hormone separately. These findings, in combination with previous work (Lilley et al., 2012; Sairanen et al., 2012), suggest that the onset of photosynthesis may be a signal for rewiring of the growth control network. In further support of this hypothesis, the growth repressors DELLLAs and SPT could increase or decrease growth in a stage-specific manner. Strong growth promotion in seedlings treated with GAs and BRs did not require PIFs, contradicting expectations from recently proposed models (Wang et al., 2012, 2013), but could be mimicked by the loss of HY5. The temporal analysis of growth presented here revealed regulatory connections between the BR and GA pathways (Figure 5), highlighting the dynamics and plasticity of the growth network.

Growth promotion by the well-established growth repressors RGA, GAI and SPT was one of the most unexpected findings from the current study and probably reflects their role in modulating the strength of the BR-negative autoregulatory loop (Figure 5). Particularly during the transition period of Int. 3, seedlings with stabilized GAI had faster growth, increased BR sensitivity and altered expression of the BR biosynthetic genes CPD and BR6ox1 when compared with wild-type seedlings (Figure 2a, d). Nearly all BR biosynthetic genes are subject to negative feedback regulation, mediated by BES1 and BZR1 (Zhao and Li, 2012). As RGA and GAI can bind to both BES1 and BZR1 proteins, DELLLAs could modulate the strength of negative feedback by preventing DNA-binding of repressive transcriptional complexes containing members of the BZR1/BES1 family. Such a model predicts that reduced function of DELLA and/or SPT would enhance negative feedback, which is exactly what was observed with suppression of the DWF4ox phenotype in repressor mutant backgrounds (Figure 2b). It is worth noting that in the DWF4ox mutants used in this study, there are two characterized BR response elements associated with BZR1-mediated repression (He et al., 2005) located between the activation tag insertion site and the DWF4 transcriptional start site (Figure S8). While our study did not detect any significant change in DWF4 expression in gai-1 mutants, GAI-dependent DWF4 expression has been observed in other studies (Li et al., 2012). The induction of DELLA function by BR also modulated expression of GA biosynthetic genes, as would be expected (Figure 3c), contributing to a multi-level balancing effect between the two pathways.

The current model of interactions between GA and BR posits that GA-mediated release of DELLA repression in combination with BR-mediated increase in BES1 and BZR1 function allows for maximum PIF-mediated growth promotion (Wang et al., 2012, 2013). BZR1 and PIF4 are both required for GA-mediated growth promotion, bind to one another and share many transcriptional targets (Bai et al., 2012; Oh et al., 2012). In addition, RGA and GAI negatively regulate BES1 and BZR1 function through direct binding (Bai et al., 2012; Gallego-Bartolomé et al., 2012; Li et al., 2012). This sensible and attractive model cannot explain the growth responses described in this study, as the observed synergistic effects of BR and GA are neither consistently repressed by DELLLAs (Figure 2) nor are they PIF dependent (Figure 4). Instead, our findings suggest a dynamic growth network where the relationship between the two hormones is altered over time, potentially in response to light and resource availability.

Accessibility of transcription factor-binding sites may be essential for shaping the dynamics of hormone sensitivity during seedling development. The fact that hy5 mutants resembled seedlings exposed to BR and GA and were insensitive to hormone treatments (Figure 4a) strongly connects the combined BR and GA growth response to skotomorphogenesis and thus chromatin state. Light dramatically reconfigures the chromatin landscape (van Zanten et al., 2012). Transcriptional activation by HY5 has been recently connected to its interaction with the chromatin remodeling factor PICKLE (PKL), providing a direct link between chromatin decondensation and growth control (Jing et al., 2013). PICKLE is a known positive regulator of GA responses (Ogas et al., 1997; Henderson et al., 2004), and BR responses have also been linked to chromatin remodeling (Li, 2010; Shigeta et al., 2011). Distinct phases of chromatin states during de-etiolation may be critical for defining distinct phases of hormone sensitivity and the interaction between BR and GA.

There is tremendous combinatorial power and regulatory complexity in the modular transcription factor complexes required for hormone responses and growth
control. Repression of a given complex can be achieved through increased production of repressors like the DELLAs or through modulating the abundance and composition of other potential binding partners in the cellular population. This phenomenon is already well documented for the PIF family. For example, the atypical helix-loop-helixes PHYTOCHROME RAPIDLY REGULATED1 and LONG HYPOCHOTYL IN FR1 suppress the shade avoidance syndrome by binding and inactivating PIF4 and PIF5 (Hornitschek et al., 2009; Galstyan et al., 2011). Repressors of the jasmonate pathway interact with and titrate levels of available DELLAs to facilitate the balance between PIF-mediated growth responses and MYC2-mediated defense responses (Wild et al., 2012; Hou et al., 2013). The relative ratios of specific transcriptional complexes—ratios driven by production and degradation rates of transcription factors, subcellular localization, interaction strengths among potential partners and hormone abundance—may be critical for determining both transcriptional and growth responses in a given time and place during development.

**EXPERIMENTAL PROCEDURES**

**Plant materials and growth conditions**

The wild type is Arabidopsis thaliana ecotype Col-0. br1-116 (Li and Chory, 1997), det2-1 (Chory et al., 1991), hy5-215 (Oyama et al., 1997), pifQ (Leivar et al., 2008), RGA: GFP-RGA (Silverstone et al., 2001) and sp-12 (Ichihashi et al., 2010) are as previously described. rga-28 gai-6 and gai-1 backcrossed into Col-0 were provided by G. Choi (Oh et al., 2007). The SPT-HA transgenic line was obtained by transforming the wild type (Col-0) with the pH2GW7 binary vector carrying an engineered SPT-HA cDNA fragment under the control of the CaMV35S-promoter. DWF4ox mutants (also known as das3) were originally isolated in a suppressor screen of det2 mutants by Zhiyong Wang and Joanne Chory (Weigel and Chory, 1997), generated by crossing pif3 pif4 pif5 DWF4 pifQ1997, rgag-28 gai-t6 generated by crossing populations followed by genotyping to identify homozygous DWF4ox rga gai spt individuals. Primer sequences are listed in Table S1.

Western blot analysis was as previously described (Stewart et al., 2011). Briefly, images were captured every 12 h by a charge-coupled device camera (PL-B718F, PixelINK, http://www.pixelink.com/) equipped with a lens (NMV-25M1, Navitar, http://www.navitar.com/) and an infrared long-pass filter (LP930-35.5, Midwest Optical Systems, Inc., http://www.midopt.com/). Image capture was accompanied by a 0.5-sec flash of infrared light by a custom-built light-emitting diode (LED) infrared illuminator (512-QED234, Mouser Electronics, http://uk.mouser.com/). A custom LabVIEW (National Instruments, http://uk.ni.com/) program controlled image capture and illumination. For growth rate analysis from time-lapse photography, hypocotyl lengths from at least 12 individuals were measured using Imaged software for each time-lapse image (2200 × 3000 pixels). Hypocotyl lengths for all experiments are included in Table S3. Confocal images were captured using a Leica SP5 confocal microscope (http://www.leica-microsystems.com/) fitted with a HCX PL APO CS 63.0 × 1.2 water UV objective. Z-stacks were acquired for the uppermost two to three cell layers, and Leica AF software was used to generate a maximum projection overlay. Other fluorescent images were captured using a Leica DMI 3000B microscope fitted with a Leica long-working 10 × HCX PL FLUOTAR objective and illuminated with a Lumenkor SOLA light source (http://lumencor.com/). Images were captured using Leica LAS AF version 2.6 software and a Leica DFC345 FX camera (http://www.leica-microsystems.com/). Fluorescence was quantified from 10 nuclei from each of seven to eight seedlings for each group shown in Figure S5.

**Seeding measurements and microscopy**

Time-lapse photography was as previously described (Stewart et al., 2011). The wild type is Arabidopsis thaliana ecotype Col-0. br1-116 (Li and Chory, 1997), det2-1 (Chory et al., 1991), hy5-215 (Oyama et al., 1997), pifQ (Leivar et al., 2008), RGA: GFP-RGA (Silverstone et al., 2001) and sp-12 (Ichihashi et al., 2010) are as previously described. rga-28 gai-6 and gai-1 backcrossed into Col-0 were provided by G. Choi (Oh et al., 2007). The SPT-HA transgenic line was obtained by transforming the wild type (Col-0) with the pH2GW7 binary vector carrying an engineered SPT-HA cDNA fragment under the control of the CaMV35S-promoter. DWF4ox mutants (also known as das3) were originally isolated in a suppressor screen of det2 mutants by Zhiyong Wang and Joanne Chory (Weigel et al., 2000; Wang et al., 2001). An activation tag is integrated approximately 880 base pairs from the DWF4 transcriptional start site (Figure S8). Homozygous pifQ pif4 pif5 pif6 (pif345) and pif1 pif4 pif5 pif6 (pif5p) lines were generated by crossing pifQ to pif4-2 (Penfield et al., 2009). Homozygous lines were generated for DWF4ox rga gai, DWF4ox sp, DWF4ox GFP-RGA and rga gai SPT-HA. For rga gai sp and DWF4ox rga gai sp, growth assays were performed on segregating populations followed by genotyping to identify homozygous individuals. Primer sequences are listed in Table S1.

Seeds were sterilized (20 min in 70% ethanol, 0.01% Triton X-100, followed by a rinse in 95% ethanol), suspended in 0.1% agar (BP1423, Fisher Scientific, http://www.fisher.co.uk/), spotted on plates containing 0.5 × Linsmaier and Skoog (LS) (LS30, Caisson Laboratories, Inc., http://www.caissonlabs.com/) with 0.8% phytagar (40100072-1, Plant Media: bioWorld, http://www.plantmedia.com/), and stratified in the dark at 4°C for 3 days. Brassinosteroid (brassinolide, 101, Cenmiclones, Inc., http://www.cenmiclones.com/) and GA (GA3, 77-06-5, PhytoTechnology Laboratories, http://www.phytoechlab.com/) were suspended in 90% ethanol and diluted to 500 ng and 5 µM, respectively, directly into plate media. Brassinolide (117, Cenmiclones, Inc.) was suspended in dimethyl sulfoxide and diluted to 1 µM directly into plate medium. Plates were placed vertically at dawn in a Percival E-308 growth chamber set at 20°C in 60 µM m⁻² s⁻¹ white light with short-day conditions (8 h light, 16 h dark).

**Seeding measurements and microscopy**

Time-lapse photography was as previously described (Stewart et al., 2011). Briefly, images were captured every 12 h by a charge-coupled device camera (PL-B718F, PixelINK, http://www.pixelink.com/) equipped with a lens (NMV-25M1, Navitar, http://www.navitar.com/) and an infrared long-pass filter (LP930-35.5, Midwest Optical Systems, Inc., http://www.midopt.com/+). Image capture was accompanied by a 0.5-sec flash of infrared light by a custom-built light-emitting diode (LED) infrared illuminator (512-QED234, Mouser Electronics, http://uk.mouser.com/+). A custom LabVIEW (National Instruments, http://uk.ni.com/) program controlled image capture and illumination. For growth rate analysis from time-lapse photography, hypocotyl lengths from at least 12 individuals were measured using Imaged software for each time-lapse image (2200 × 3000 pixels). Hypocotyl lengths for all experiments are included in Table S3. Confocal images were captured using a Leica SP5 confocal microscope (http://www.leica-microsystems.com/) fitted with a HCX PL APO CS 63.0 × 1.2 water UV objective. Z-stacks were acquired for the uppermost two to three cell layers, and Leica AF software was used to generate a maximum projection overlay. Other fluorescent images were captured using a Leica DMI 3000B microscope fitted with a Leica long-working 10 × HCX PL FLUOTAR objective and illuminated with a Lumenkor SOLA light source (http://lumencor.com/). Images were captured using Leica LAS AF version 2.6 software and a Leica DFC345 FX camera (http://www.leica-microsystems.com/+). Fluorescence was quantified from 10 nuclei from each of seven to eight seedlings for each group shown in Figure S5.

**Extraction of RNA and qRT-PCR analysis**

Seeds were grown vertically on 0.5 × LS plates with 2% phytoagar. Expression analysis was performed on seedlings collected at dawn on day 4 (72 hpi). All samples were immediately frozen in liquid nitrogen and stored at −80°C until processing. Total RNA was extracted from 100 mg of whole seedling tissue using the Spectrum Plant Total RNA Kit (Sigmam, http://www.sigmaaldrich.com/), total RNA was treated with DNaseI on columns (Qiagen, http://www.qiagen.com/), and 2 µg of eluted RNA was used for complementary DNA (cDNA) synthesis using iScript (Bio-Rad, http://www.bio-rad.com/). Samples were analyzed using SYBR Green Supermix (Bio-Rad) reactions run in a CFX96 Optical Reaction Module (Bio-Rad). Expression for each gene was calculated using the formula (Pfaffl, 2001) (EFβactarget:EFβactargetcontrol-sample)/(EFβactarget:EFβactargetcontrol-sample) and normalized to a reference gene. Expression values for all experiments are included in Table S2. Primer sequences are listed in Table S1.

**Western blot analysis**

The abundance of GFP-RGA and SPT-HA was detected in extracts of whole seedlings collected at dawn on day 4 or 5. All samples were immediately frozen in liquid nitrogen and stored at −80°C until processing. Total protein was extracted from approximately 200 mg of seedling tissue expressing GFP-RGA using a previously described method (Silverstone et al., 2001), except that anti-GFP-peroxidase (ab6683, Abcam, http://www.abcam.com/) was used at a 1:10 000 dilution. Total protein was extracted from approximately 100 mg of seedling tissue expressing SPT-HA using a previously described method (Duek et al., 2004), except anti-HA-peroxidase (ab118764, Roche, http://www.roche.com/) was used at a 1:1000 dilution. Anti-ACTIN antibodies (A0480, Sigma) were used at a 1:1000 dilution and detected with anti-mouse (172-1011, Bio-Rad) used at a 1:20 000 dilution. SuperSignal West

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Femto Maximum Sensitivity Substrate (Pierce, http://www.pierce-net.com/) was used to detect signals. Blots shown are representative of at least two experiments with independent biological replicates.

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SUPPORTING INFORMATION

Additional Supporting information may be found in the online version of this article.

Figure S1. The response of gibberellin is reduced when brassinosteroid levels are low.

Figure S2. Loss of repressor function suppresses the DWF4ox long hypocotyl phenotype.

Figure S3. brassinosteroids increase DELLA abundance.

Figure S4. brassinosteroid treatment does not have large effects on repressor or SLY1 gene expression.

Figure S5. brassinosteroids increase DELLA abundance similarly throughout the day.

Figure S6. Induction of DELLA target gene expression by brassinosteroid is reduced in seedlings with reduced DELLA function.

Figure S7. Loss of PIF or HYS function has opposite effects on seedling height.

Figure S8. DWF4ox seedlings have intact brassinosteroid response elements.

Table S1. Primers used for expression analysis and genotyping.

Table S2. Gene expression analysis for all genotypes and treatments.

Table S3. Growth analysis across photomorphogenesis.

REFERENCES


