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Brassinosteroids Are Master Regulators of Gibberellin Biosynthesis in Arabidopsis

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Plant growth and development are highly regulated processes that are coordinated by hormones including the brassinosteroids (BRs), a group of steroids with structural similarity to steroid hormones of mammals. Although it is well understood how BRs are produced and how their signals are transduced, BR targets, which directly confer the hormone's growth-promoting effects, have remained largely elusive. Here, we show that BRs regulate the biosynthesis of gibberellins (GAs), another class of growth-promoting hormones, in *Arabidopsis thaliana*. We reveal that Arabidopsis mutants deficient in BR signaling are severely impaired in the production of bioactive GA, which is correlated with defective GA biosynthetic gene expression. Expression of the key GA biosynthesis gene *GA20ox1* in the BR signaling mutant *bri1-301* rescues many of its developmental defects. We provide evidence that supports a model in which the BR-regulated transcription factor BES1 binds to a regulatory element in promoters of GA biosynthesis genes in a BR-induced manner to control their expression. In summary, our study underscores a role of BRs as master regulators of GA biosynthesis and shows that this function is of major relevance for the growth and development of vascular plants.

INTRODUCTION

Brassinosteroids (BRs) are steroid hormones of plants that were identified in the 1970s because of their strong growth-promoting capacities (Mitchell et al., 1970; Grove et al., 1979). BRs regulate cell elongation, cell division, and cell differentiation and function throughout plant development in various developmental programs, including seedling development in the light and dark, adult shoot and root growth, flowering, fruit development, and senescence (Clouse, 2011). In addition, and like other hormones, BRs act to integrate stimuli perceived from the environment into endogenous developmental programs and thereby confer plants an adaptive potential to environmental factors and changes (Wang et al., 2012; Fridman and Savaldi-Goldstein, 2013).

Perhaps the most compelling phenotypes of BR-deficient plants are their dwarf growth in the light and their deetiolated phenotypes in the dark (Clouse et al., 1996; Li et al., 1996; Szekeres et al., 1996), which strongly resemble plants lacking activity of gibberellins (GAs), another class of growth-promoting hormones (Koornneef and van der Veen, 1980; Talon et al., 1990; Wilson and Somerville, 1995). Although it has long been known that BRs and GAs function redundantly in many developmental programs, the current postulation is that crosstalk of BRs and GAs in *Arabidopsis thaliana* is restricted to the signaling level, with both pathways contributing factors that interact to regulate transcription (Steber and McCourt, 2001; Bai et al., 2012; Gallego-Bartolomé et al., 2012; Bernardo-García et al., 2014).

BRs are biosynthesized from sterols and signal in a phosphorylation-dependent mode in which perception of the hormones by a receptor complex containing the receptor kinase BRASSINOSTEROID INSENSITIVE1 (BRI1) triggers a phosphorylation-dependent signal transduction cascade that leads to inactivation of Arabidopsis GSK3/shaggy-like Kinases (ASKs) of the BRASSINOSTEROID INSENSITIVE2 (BIN2) class that phosphorylate transcription factors to alter their activity in BR target gene expression (Wang et al., 2012; Fridman and Savaldi-Goldstein, 2013). The most studied members of BR-controlled transcriptional regulators are *BRI1* EMS SUPPRESSOR1 (BES1) and BRASSINAZOLE RESISTANT1 (BZR1), which are phosphorylated by BIN2 to promote their degradation and inhibit their DNA binding activity (He et al., 2002; Wang et al., 2002; Yin et al., 2002)

On the other hand, GAs are biosynthesized from *trans*-geranylgeranyl diphosphate. In the upstream GA biosynthetic pathway, *trans*-geranylgeranyl diphosphate is converted to GA_{12}-aldehyde, a branch of biosynthesis that is conserved in all plant species. The final steps to produce bioactive GAs are species specific but in most cases require activity of GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) enzymes. In Arabidopsis, GA20ox enzymes convert GA_{12} in sequential reactions to GA_9, which is then converted by GA3ox enzymes to the major bioactive GA GA_4. On the contrary, the enzyme GA2ox antagonizes GA activity by converting active GAs into inactive catabolites (Hedden and Thomas, 2012).

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The level of endogenous bioactive GA is determined by feedback regulation, where active GA suppresses the expression of *GA20ox* and *GA3ox* genes and promotes transcription of *GA2ox* genes. This feedback regulation requires GA signaling, since in mutants lacking core GA signaling components such as the GA receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1) the expression of feedback-regulated GA biosynthetic genes is highly elevated and is not repressed by exogenous GA treatment. Consequently, such mutants accumulate highly elevated levels of bioactive GA (Fujioka et al., 1988; Griffiths et al., 2006). In addition to the feedback-regulatory events, which govern GA homeostasis, GA biosynthesis is also strongly regulated by environmental factors, being sensitive to changes in light quantity, quality, or duration, as well as to abiotic stresses such as cold (Hedden and Thomas, 2012).

In this work, we provide evidence that GA biosynthesis in Arabidopsis is regulated by BRs. We show that in BR mutants the production of bioactive GA is severely compromised and the expression of genes encoding enzymes of the GA20ox and GA3ox families is reduced. Application of GA as well as reconstitution of *GA20ox* expression in the BR signaling-defective mutant *bri1-301* rescues multiple of its developmental defects. We reveal that BES1 binds to a regulatory motif present in the promoters of GA biosynthesis genes, including *GA20ox1* and *GA3ox1*, and induces their expression in a BR-promoted manner. Based on these results, and in light of a recent study that showed that BRs regulate GA biosynthetic gene expression in rice (*Oryza sativa*; Tong et al., 2014), we propose a new model for the molecular regulation of plant growth in which BRs induce GA biosynthesis to stimulate degradation of the DELLA transcriptional repressors; this releases their inhibition of BES1/BZR1 in the transcription of targets further downstream in signaling that are required to promote growth and development in different stages of the plant life cycle.

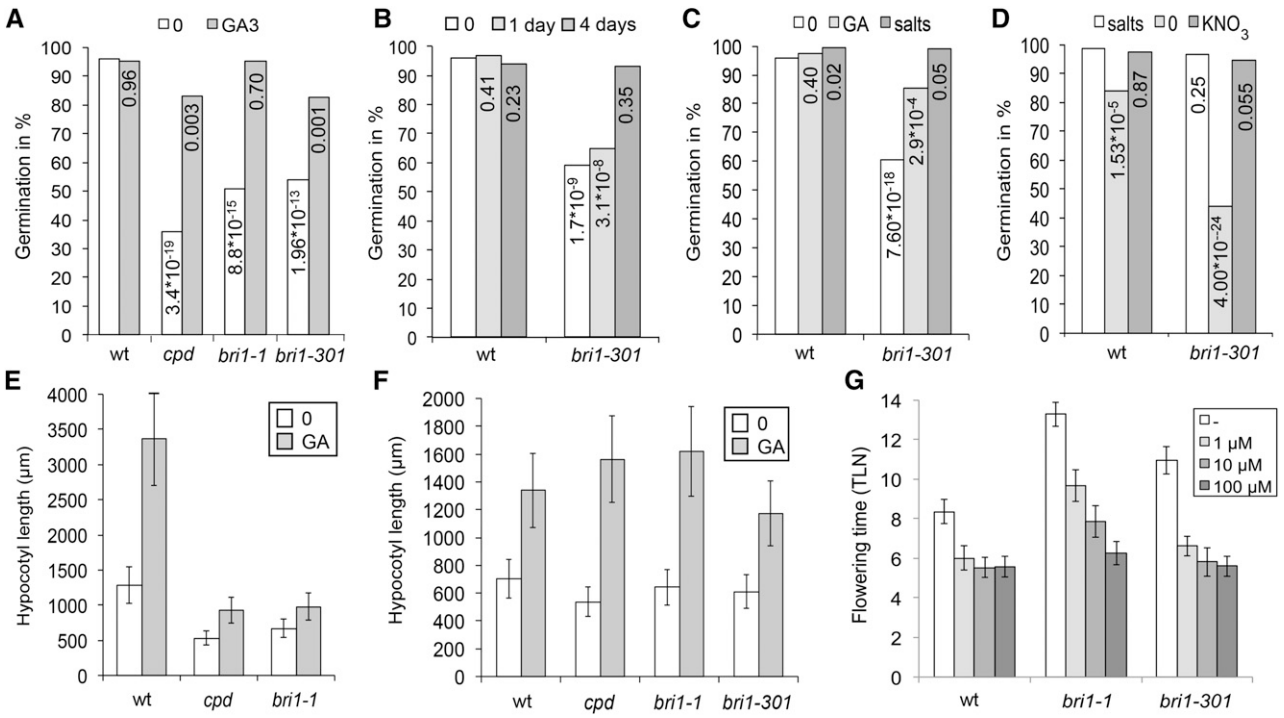


Figure 1. GA Application Rescues Growth Defects of BR Mutants.

(A) to **(D)** Germination assays. At least 100 seeds per line were plated on water agar and germination, defined as radical emergence, was scored 6 d later. Results are given in percentages. Statistical significance was calculated with the χ^2 test; the resulting P values are shown.

(A) Germination of *cpd*, *bri1-1*, and *bri1-301* seeds on water agar supplemented with 1 μM GA_4 or DMSO as a control.

(B) Germination of wild-type and *bri1-301* seeds stratified for 0, 1, or 4 d.

(C) Germination of wild-type and *bri1-301* seeds on water-agar supplemented with 1 μM GA_4 or half-strength MS salts.

(D) Germination of wild-type and *bri1-301* seeds on water-agar supplemented with half-strength MS salts or KNO_3 (same amount as in half-strength MS).

(E) Hypocotyl elongation of wild-type, *cpd*, and *bri1-1* plants grown on half-strength MS medium supplemented with 1 μM GA_4 or with DMSO as a control. Mean values and SD from 20 seedlings grown for 7 d in standard growth conditions are shown.

(F) Hypocotyl elongation of wild-type, *cpd*, *bri1-1*, and *bri1-301* plants grown on water agar supplemented with 1 μM GA_4 or with DMSO as a control. Mean values and SD from 35 seedlings grown for 7 d in standard growth conditions are shown.

(G) Flowering time of wild-type, *bri1-1*, and *bri1-301* plants grown in soil in standard conditions. The plants were sprayed three times a week with 1, 10, or 100 μM GA_{4+7} or with water as a control. Flowering time was scored as total leaf number (TLN) at bolting; the mean of at least 20 treated plants and the SD are shown.

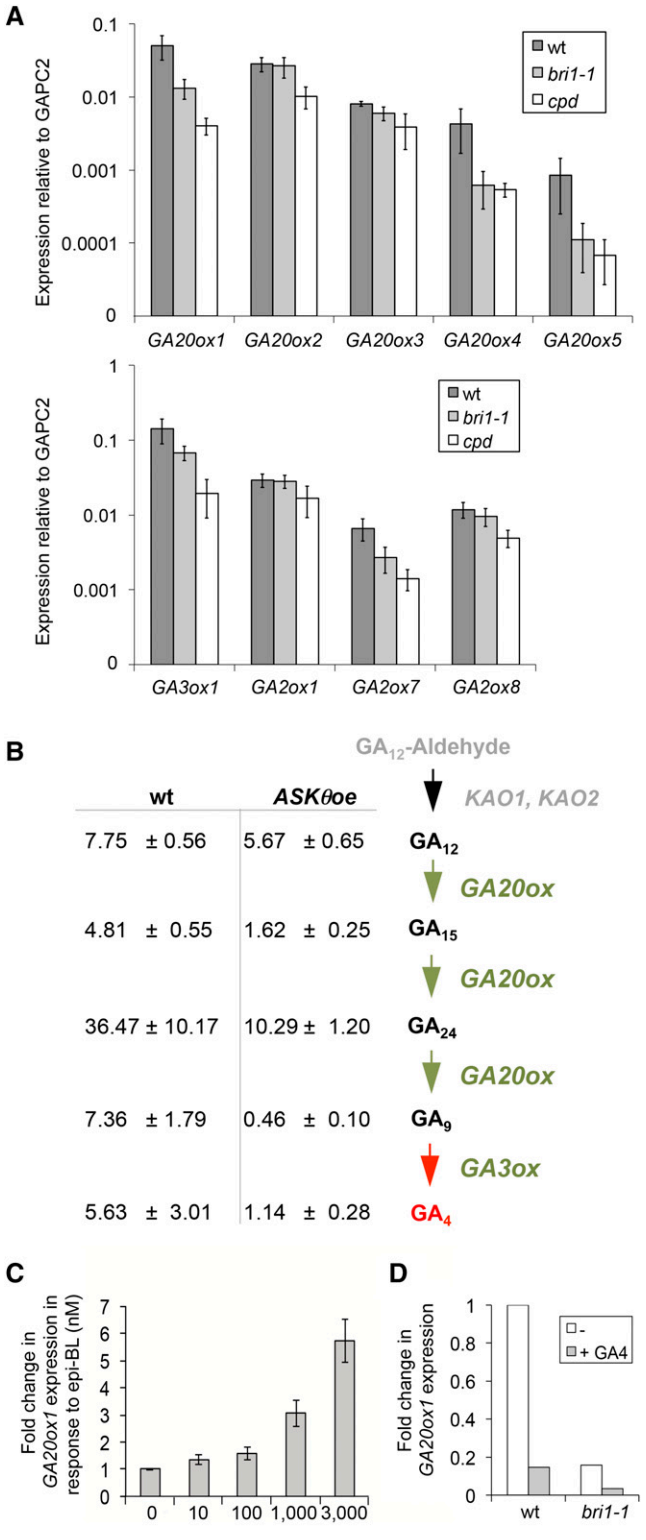


Figure 2. BRs Regulate GA Biosynthesis.

(A) qPCR analysis of the expression of GA biosynthesis genes in 10-d-old seedlings of the indicated lines. The SD was calculated from three biological replicates.

RESULTS

GA Biosynthesis Is Severely Compromised in BR-Deficient Mutants

During a recent study, we observed that treatment with voriconazole, a chemical that reduces sterol and BR contents (Rozhon et al., 2013), also reduced levels of the GA GA_{34} in wild-type Arabidopsis (results in ng/g dry weight of two measured samples: untreated control: 7.7/ 7.7; + inhibitor: 0.1/ 0.2). To investigate if a deficiency in BRs may result in reduced GA levels we measured GA_4, which is the major bioactive GA in Arabidopsis (Talon et al., 1990), in 14-d-old seedlings of the BR biosynthetic mutant *constitutive photomorphogenesis and dwarfism* (*cpd*; Szekeres et al., 1996) and the two BR signaling-deficient lines *bri1-1* (Clouse et al., 1996) and *ASKθoe* (Rozhon et al., 2010) by gas chromatography-mass spectrometry. This analysis revealed that GA_4 was reduced in all mutants (results in ng/g dry weight of two measured samples: Col-0, 2.1/2.7; *cpd*, 1.3/not detectable; *bri1-1*, 1.0/1.1; *ASKθoe*, 0.0/0.3). This was surprising, since previously it was shown that levels of bioactive GAs were not reduced in BR mutants of pea (*Pisum sativum*; Jager et al., 2005) and, more importantly, it was published several times that in Arabidopsis externally applied GA was unable to restore growth defects of BR mutant plants (Li et al., 1996; Szekeres et al., 1996; Bai et al., 2012; Gallego-Bartolomé et al., 2012).

Externally Applied GA Rescues Growth Defects of BR-Deficient Mutants

To verify these findings, we first conducted germination assays, since GAs are essential for germination in many plant species including Arabidopsis (Finkelstein et al., 2008). We harvested seeds of plants homozygous for *cpd*, *bri1-1*, and *bri1-301*, a second *bri1* null allele in the Col-0 background with milder phenotypes than *bri1-1* (Xu et al., 2008), and plated them on water-agar. The seeds were directly incubated in the light at 21°C (without a cold treatment) and germination was assessed after 6 d of incubation. As shown in Figure 1A, the germination rates of the seeds of all investigated BR mutants were strongly reduced compared with wild-type seeds. Importantly, this increased dormancy was released by external application of 1 μM GA_4, indicating that germination defects of BR mutants are caused by GA deficiency.

Previously, when germination of BR mutants was assessed, apart from an increased sensitivity to ABA, germination defects were not reported (Steber and McCourt, 2001; Xue et al., 2009). Since GA biosynthesis is highly regulated by environmental cues (Hedden and Thomas, 2012), we speculated that the choice of

(B) Measurements of GAs in 21-d-old plants of the wild type and *ASKθoe*. The values are in ng/g dry weight. The mean and SD from three biological replicates are shown.

(C) qPCR analysis of the expression of *GA20ox1* in 8-d-old *cpd* plants grown on half-strength MS medium supplemented with the indicated amounts of epi-BL (in nM). Medium + DMSO was the 0 control.

(D) qPCR analysis (performed as in **[A]**) of the expression of *GA20ox1* in 10-d-old plants of *bri1-1* and the wild type treated with GA_4 for 2 h. Fold change compared with untreated wild type is shown.

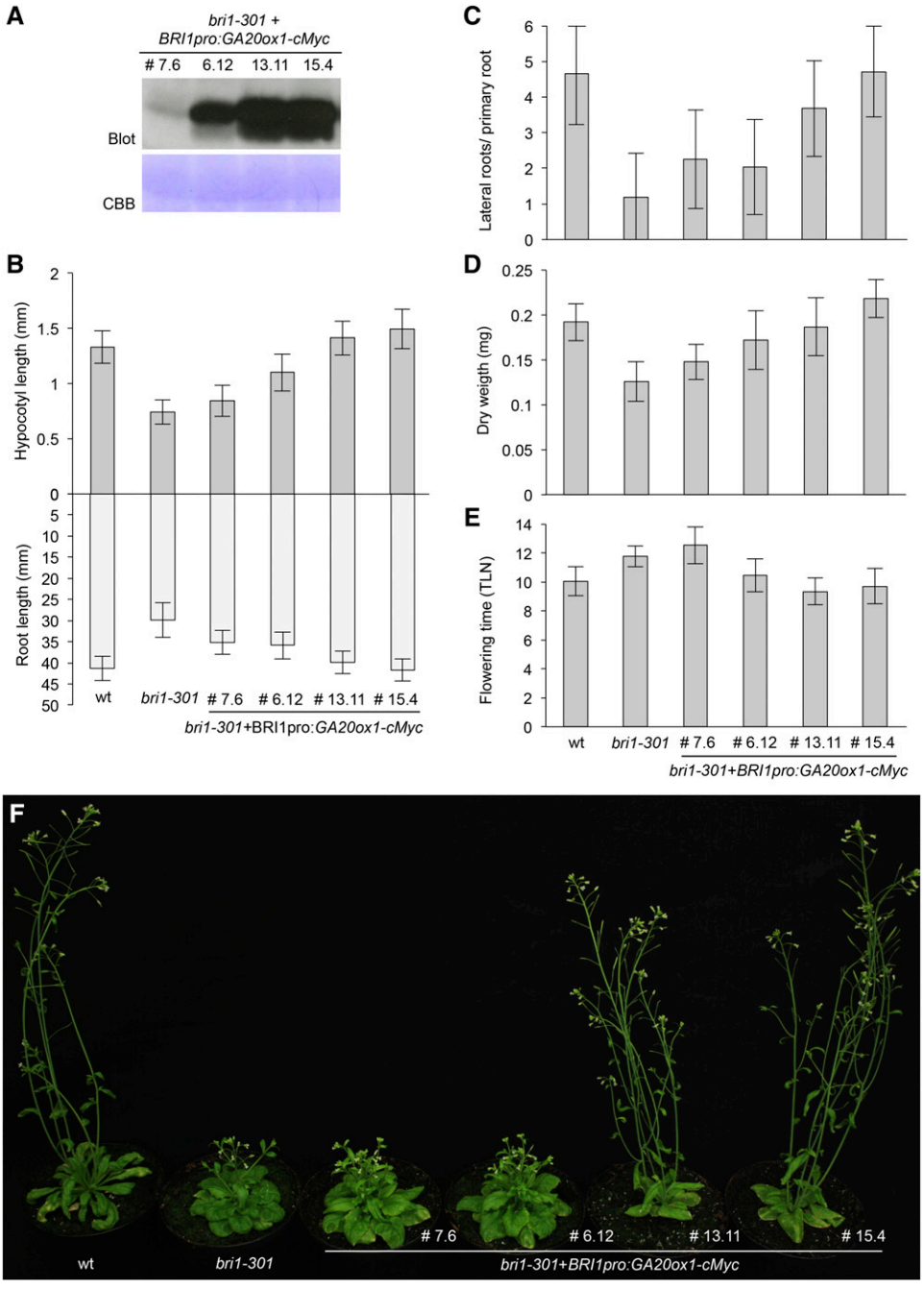


Figure 3. *GA20ox1* Expression in the *BRI1* Domains Restores *bri1-301* Growth Defects.

(A) cMyc-tagged GA20ox1 was expressed under *BRI1* promoter control in *bri1-301* and four lines with different levels of GA20ox1-cMyc abundance were selected by immunoblotting. Staining with Coomassie blue (CBB) is shown as a loading control.

(B) Hypocotyl length and primary root length of 8-d-old, light-grown seedlings of the wild type, *bri1-301*, and the four complementation lines. The mean and SD of at least 20 measured seedlings are shown.

(C) Lateral roots per primary root of 8-d-old, light-grown seedlings of the wild type, *bri1-301*, and the four complementation lines. The mean and SD of at least 25 measured primary roots are shown.

(D) Dry weight of 8-d-old seedlings of the indicated lines. The mean and SD of at least 18 seedlings are shown.

(E) Flowering time, assessed as total leaf number (TLN) at bolting, of the wild type, *bri1-301*, and the complementation lines grown in soil under long-day growth conditions. The mean and SD of at least 17 plants are shown.

(F) Adult phenotype of the complementation lines grown in long days. A representative plant of each line is shown.

experimental setups in these earlier experiments, in particular plating seeds on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and cold-treating them, may have disguised the phenotype. Since cold treatment of imbibed seeds (stratification) is known to induce GA biosynthesis and thereby facilitate germination (Yamauchi et al., 2004; Penfield et al., 2005; Finch-Savage et al., 2007), we first investigated if stratification can promote germination of BR mutant seeds. For this analysis, we picked *bri1-301* since it is less compromised in seed production than *bri1-1* (Xu et al., 2008). Four days of stratification fully restored germination of *bri1-301* seeds (Figure 1B), indicating that BRs act upstream of cold on GA biosynthesis in seeds. Moreover, half-strength MS salts (Figure 1C) and in particular nitrate (Figure 1D) fully restored *bri1-301* germination defects, confirming previous results that nitrate promotes germination (Alboresi et al., 2005; Matakiadis et al., 2009; Footitt et al., 2013) and indicating that this is a BRI1-independent effect.

We extended our analysis of the ability of GA to revert growth defects caused by BR deficiency to seedlings and measured hypocotyls of *bri1-1* and *cpd* plants grown for 7 d on half-strength MS medium. We found that GA partially rescued hypocotyl elongation defects of the tested mutants. This rescue, while subtle, was statistically significant (Figure 1E). Interestingly, when we performed the analysis on water-agar supplemented with GA, the hypocotyl elongation defects of BR mutants were less pronounced and the seedlings responded much more strongly to GA application (Figure 1F), indicating that also in seedling development MS salt components affect growth defects caused by BR deficiency.

GA application also rescued BR mutant phenotypes in the adult stage. A concentration of 10 μM applied three times a week did not significantly affect wild-type growth (Supplemental Figure 1A) but was sufficient to partially rescue *bri1-301* phenotypes; in particular, reduced plant height (Supplemental Figure 1B) and delayed flowering (Figure 1G) were fully restored by this treatment. The *bri1-1* mutant required higher concentrations of GA to respond. Whereas 10 μM GA restored only flowering time (Figure 1G), 100 μM GA also partially restored other *bri1-1* growth defects including reduced plant height and overall plant size (Supplemental Figure 1C).

In summary, these results show that external application of bioactive GA can partially release growth defects of BR signaling-deficient plants and this ability strongly depends on the developmental stages and physiological conditions applied.

BR Signaling Regulates GA Biosynthesis

Our results provided evidence that GA biosynthesis is influenced by BRs. To investigate if BR signaling regulates GA biosynthetic gene expression and to assess at which step in the pathway the regulatory effect(s) may occur, we quantified the expression of GA biosynthetic genes of Arabidopsis in 2-week-old plants of *cpd* and *bri1-1* using quantitative real-time PCR (qPCR) analysis. Whereas the expression of genes encoding enzymes upstream in the pathway (*GA1*, *GA2*, *GA3*, *KAO1*, and *KAO2*) appeared not to be significantly altered (Supplemental Figure 2), the expression of all tested *GA20ox* and *GA3ox* genes and of two of three tested *GA2ox* genes was significantly reduced in the mutants compared with the wild type (Figure 2A). In line with these results, the levels of the upstream GAs, GA_{15}, GA_{24}, and in particular GA_9, which are products

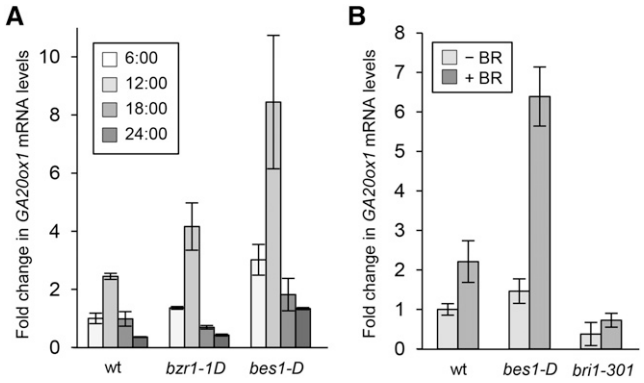


Figure 4. *GA20ox1* Induction by BRs is BRI1-Dependent and Promoted by *bes1-D*.

qPCR analysis of the expression of *GA20ox1* in the lines shown. Plants were grown for 8 d in long-day growth conditions. Samples were harvested at different times of day **(A)** or were grown on medium supplemented with 1 μM epi-BL **(B)**. Fold changes compared with the wild type at 6:00 **(A)** or untreated **(B)** are shown. The SD was calculated from three biological repeats after normalization to *GAPC2*.

of GA20ox activity (Hedden and Thomas, 2012), were strongly reduced in BR signaling-deficient plants (Figure 2B). In addition, treatment with the BR epi-brassinolide (epi-BL) strongly increased *GA20ox1* expression in *cpd* (Figure 2C).

The level of bioactive GA is determined by feedback regulation, where active GA suppresses the expression of *GA20ox* and *GA3ox* genes and promotes transcription of *GA2ox* genes (Cowling et al., 1998; Xu et al., 1999). To test if in BR mutants the feedback suppression of GA biosynthesis was active, wild-type and *bri1-1* plants were treated with GA. GA application, which repressed *GA20ox1* expression in the wild type, also further reduced *GA20ox1* expression in *bri1-1* (Figure 2D), showing that the feedback repression of GA production does not rely on BR signaling.

GA20ox1 Expression under *BRI1* Promoter Control Restores Growth Defects of *bri1-301*

GA20ox activity is highly important in GA biosynthesis (Middleton et al., 2012). In Arabidopsis, GA20ox enzymes convert GA_{12} to GA_{15}, then to GA_{24}, and finally, to GA_9 (Hedden and Thomas 2012), the last step being a rate-limiting reaction (Middleton et al., 2012). Consequently, reduced *GA20ox* expression results in strongly impaired GA biosynthesis and plant growth (Rieu et al., 2008; Plackett et al., 2012), whereas enhanced *GA20ox* expression increases GA levels (Huang et al., 1998). To investigate if defective *GA20ox* expression accounts for BR mutant phenotypes, *GA20ox1* was introduced into *bri1-301* under *BRI1* promoter control. Four independent transgenic lines expressing the recombinant protein were selected (Figure 3A) and were compared in their phenotypes to the parental line. Strikingly, in the complementation lines with strong *GA20ox1* expression, *bri1-301* phenotypes were recovered to large extents. During seedling development, hypocotyl and root elongation defects (Figure 3B) as well as lateral root development (Figure 3C) were restored. Adult complemented lines had larger, more expanded leaves (Supplemental Figure 3) and were restored

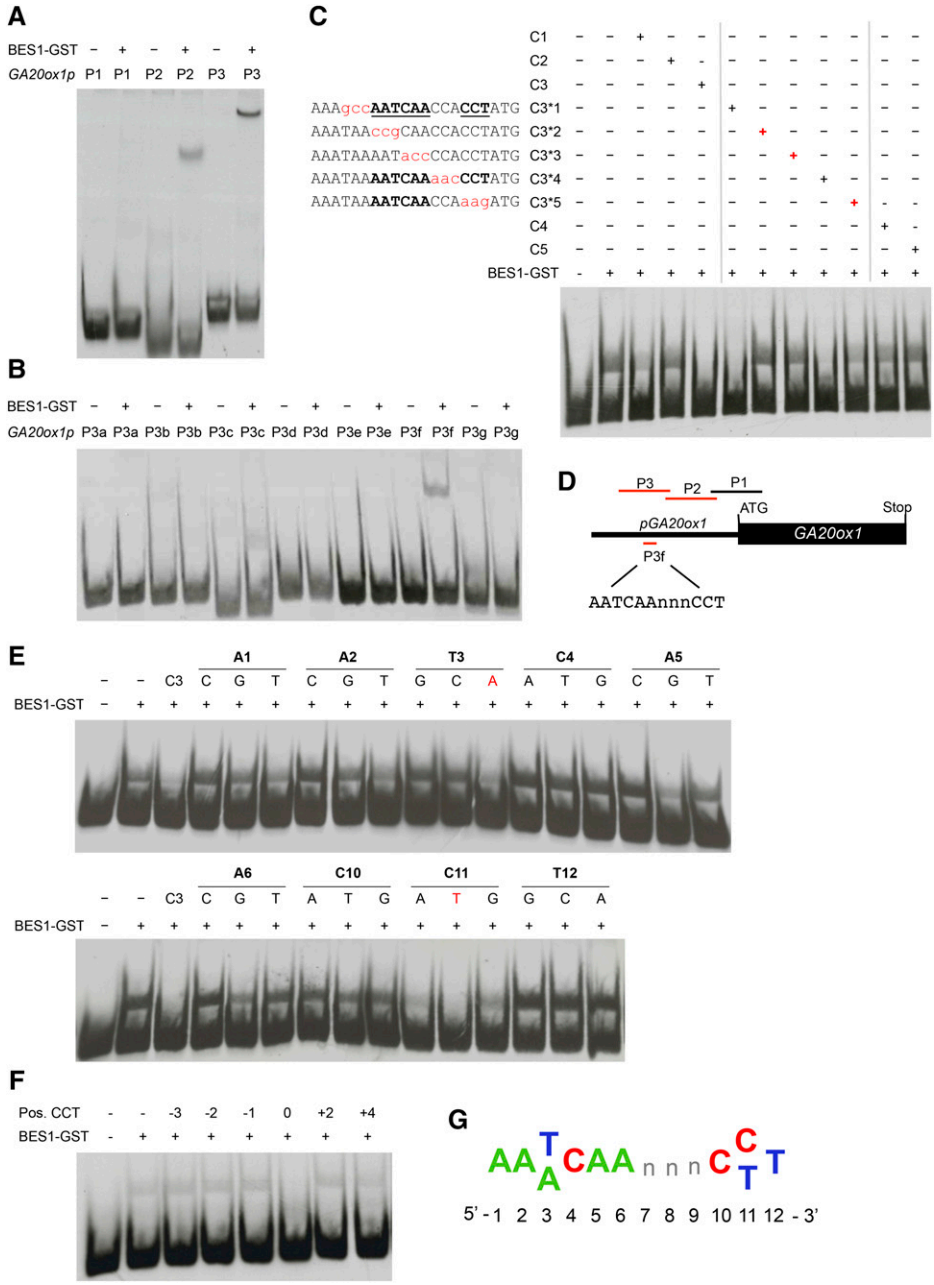


Figure 5. In Vitro Characterization of a BES1 Binding Motif.

In EMSAs with recombinant BES1-GST, in vitro DNA binding to radiolabeled fragments of the *GA20ox1* promoter was analyzed to map the BES1 binding site.

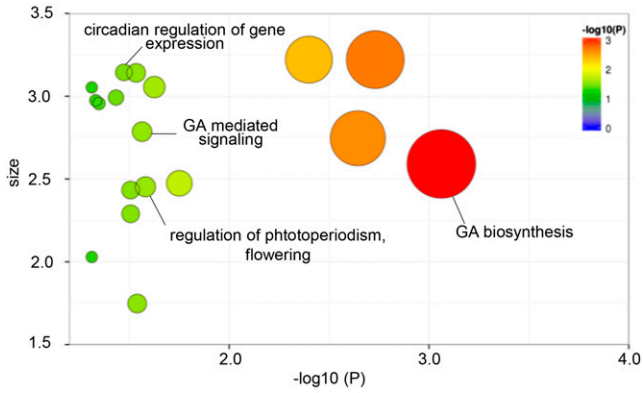
(A) EMSA shows BES1-GST binding to overlapping 250-bp DNA fragments (P1-P3) upstream of the ATG. P1 (−188 to +102); P2 (−165 to −426); P3 (−393 to −692).

(B) EMSA shows BES1-GST binding to overlapping 100-bp DNA fragments that mapped binding to region P3f. P3a (−165 to −264); P3b (−328 to −229); P3c (−393 to −294); P3d (−359 to −458); P3e (−424 to −523); P3f (−588 to −489); P3g (−554 to −653).

(C) EMSA shows BES1-GST binding to the radiolabeled P3f fragment. The addition of 100× molar excess of competitor oligonucleotides (36 bp) of C1 (–588 to –553), C2 (–570 to –535), C3 (–552 to –517), C4 (–534 to –499), or C5 (−525 to −490) and mutated versions of C3 (C*1-C*5; sequences as shown; mutations marked in red) were used to characterize the BES1-GST binding site. Mutating 5′-AATCAAnnnCCT-3′ (in C3*1, C3*2, and C3*4) inhibits the competition for DNA binding, as shown in red in the table.

(D) Illustration of the non-E-box BES1 binding site in the *GA20ox1* promoter (bound fragments in red).

A



B

GO:0009686 GA biosynthetic process

ATG no	Name	Function
AT1G12130	-	Putative function in GA biosynthesis
AT1G15550	*GA3ox1*	GA 3-beta-dioxygenase activity
AT1G50960	*GA2ox7*	GA 2-oxidase activity
AT1G66390	*MYB90*	Putative function in GA biosynthesis
AT1G80330	*GA3ox4*	GA 3-beta-dioxygenase activity
AT2G32440	*KAO2*	ent-Kaurenoate oxidase activity
AT2G41260	*ATM17*	Putative function in GA biosynthesis
AT3G54510	-	Putative function in GA biosynthesis
AT4G24150	*GRF8*	Putative function in GA biosynthesis
AT4G25420	*GA20ox1*	GA 20-oxidase activity
AT4G32980	*ATH1*	Regulates GA biosynthesis
AT4G39500	*CYP96A11*	Putative function in GA biosynthesis
AT5G06100	*MYB33*	Putative function in GA biosynthesis

Figure 6. GO Enrichment of the Non-E-Box Motif in Arabidopsis.

(A) Bubble chart depicting the enrichment of the non-E-Box motif within 750 bp upstream of the transcriptional start site in GO annotations of biological processes in Arabidopsis using Revigo software. The chart shows the −log10(P) value of the enrichment (*x* axis, size, and color) and the size of the GO term group (*y* axis) among all GO annotations.
(B) Genes of Arabidopsis involved in GO GA biosynthetic process (GO:0009686) that contain the non-E-Box motif within 750 bp upstream of the transcriptional start site.

in biomass gain (Figure 3D), flowering time (Figure 3E), fertility, and senescence (Figure 3F).

These results provided further evidence that malfunctioning *GA20ox* expression and a resulting reduced GA production accounts for major aspects of BR mutant phenotypes.

BES1 and BZR1 Bind to a Non-E-Box Motif in the *GA20ox1* Promoter

BR-responsive gene expression is mediated by different families of transcription factors, of which the BES1/BZR1 family is best studied (Wang et al., 2012). Based on microarray data, it was highlighted recently that reduced *GA20ox1* expression in *bri1-1* also correlates with elevated mRNA levels of *GA20ox1* in *bzr1-1D* (Gallego-Bartolomé et al., 2012). To investigate if BES1 and/or BZR1 may regulate *GA20ox1* expression, we employed qPCR analysis and quantified *GA20ox1* mRNA levels in the dominant *bes1-D* (Yin et al., 2002) and *bzr1-1D* (Wang et al., 2002) mutants. This showed that BR application induced *GA20ox1* expression in *cpd* (Figure 4A) and that in both *bes1-D* and *bzr1-1D GA20ox1*, expression was increased to various extents at different times of the day (Figure 4B). In addition, BR treatment further increased *GA20ox1* expression in *bes1-D* (Figure 4B), indicating that BRs promote BES1 activity in *GA20ox1* transcription. Since BR induction of *GA20ox1* expression was compromised in *bri1-301* (Figure 4B), there was evidence that it required BR signaling.

Surprisingly, the *GA20ox1* promoter did not contain any known BES1/BZR1 binding sites (Wang et al., 2012). To investigate if the proteins can directly bind to this promoter, we performed in vitro DNA binding studies, first with overlapping promoter fragments (Figures 5A and 5B) and then in the presence of competitor oligonucleotides (Figure 5C) and assessed recombinant BES1 binding using electrophoretic mobility shift assays (EMSAs). By these means, we identified a 12-bp motif, 5′-AATCAAnnnCCT-3′, located 526 bp upstream of the ATG (Figure 5D), to be required for BES1 binding in vitro. Other similar motifs located in the *GA20ox1* promoter were not bound (Supplemental Figure 4). By mutating individual bases of the motif (Figure 5E), we found little flexibility for sequence alterations. Only mutation of T in position 3 to A or mutation of C in position 11 to T was accepted. Additionally, exactly three variable nucleotides (n) in position 7-9 were found to be required for binding, since neither introducing more nor removing nucleotides maintained BES1 binding capacities (Figure 5F). This binding motif, 5′-AA(T/A)CAAnnnC(C/T)T-3′, was also bound by BZR1 in vitro (Supplemental Figure 5).

A bioinformatics analysis of representation in upstream promoter sequences of Arabidopsis revealed that this BES1/BZR1 binding site is strongly enriched in genes Gene Ontology (GO) annotated as playing a role in GA biosynthesis (Figure 6A; Supplemental Table 1) and is present also in *GA3ox1* and *GA3ox4* (Figure 6B). We verified this result and could show that, indeed, BES1 also bound to the promoters of *GA3ox1* and *GA3ox4* in vitro, whereas a promoter sequence of the *GA20ox2* promoter that contained an imperfect version of the motif (5′-AAACAAnnnCAT-3′) was not bound (Figure 7A). Analogously to *GA20ox1*, *GA3ox1* was also induced by BRs, with the induction promoted by *bes1-D* and impaired in *bri1-301* (Figures 7B and 7C).

To assess if the binding site identified in vitro was also bound by BES1 in planta, chromatin immunoprecipitation (ChIP) experiments were performed using BES1-CFP-overexpressing plants

Figure 5. (continued).

(E) EMSA to investigate BES1 binding to probe P3f in the presence of 10× molar excess of competitor oligonucleotides in which the indicated base of the binding motif was mutated to all other possible bases.
(F) EMSA to investigate BES1 binding to probe P3f in the presence of 10× molar excess of competitor oligonucleotides in which the CCT part of the motif was moved either 5′ (−) or 3′ (+) in the indicated base pair steps.
(G) Illustration of the non-E-Box BES1 binding motif identified.

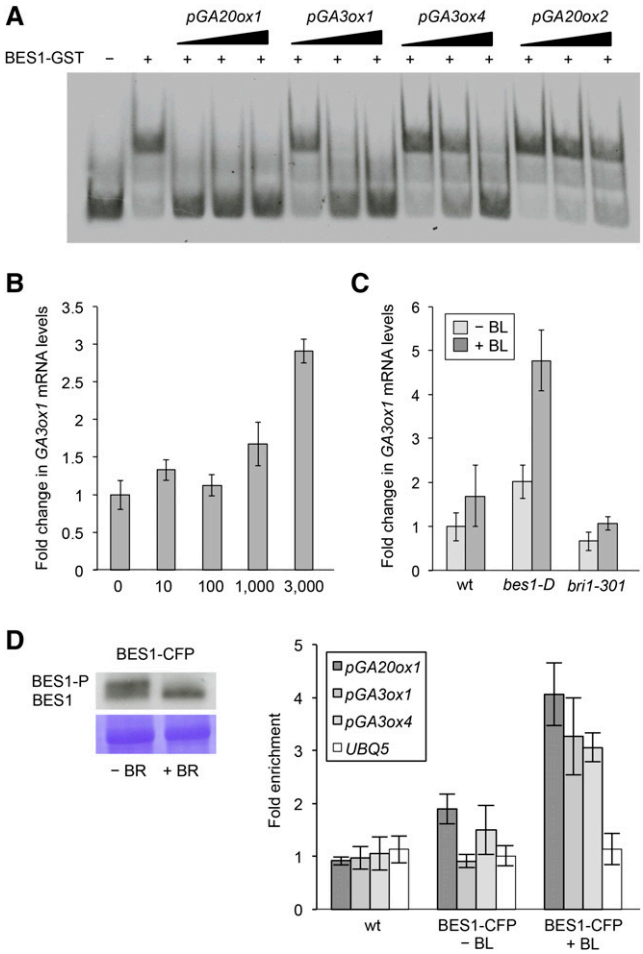


Figure 7. BES1 Binding to the Identified Motif in the Promoters of the GA Biosynthesis Genes *GA20ox1*, *GA3ox1*, and *GA3ox4* in Vivo.

(A) BES1-GST binding to a radiolabeled 100-bp DNA fragment of the *GA20ox1* promoter (same probe as in Figure 5C) in the presence of 10×, 100×, or 1000× molar excess of different competitor oligos, which were 36-bp sequences of the *GA20ox1*, *GA3ox1*, or *GA3ox4* promoters that contained the identified non E-box motif. A 36-bp sequence of the *GA20ox2* promoter that contains an imperfect version of this motif (5′-AAACAAnnnCAT-3′) was used for verification.

(B) qPCR analysis of the expression of *GA3ox1* in 8-d-old *cpd* seedlings grown on half-strength MS medium supplemented with the indicated amounts of epi-BL (in nM). Medium supplemented with DMSO was the 0 control. The SD was calculated from three biological repeats after normalization to *GAPC2*.

(C) qPCR analysis of the expression of *GA3ox1* in the lines shown. Plants were grown for 8 d on half-strength MS medium supplemented with 1 μM epiBL or DMSO as a control. The SD was calculated from three biological repeats after normalization to *GAPC2*.

(D) BL-induced enrichment of BES1-CFP on the promoters of *GA20ox1*, *GA3ox1*, and *GA3ox4*. For ChIP, 21-d-old BES1-CFP plants were sprayed with 10 μM epiBL (+ BR) or with DMSO as a control (− BR). Dephosphorylation of BES1-CFP following the treatment was verified by immunoblotting with an α-GFP antibody (left). Enrichment of BES1-CFP on fragments containing the non-E-box motif was determined by qPCR and calculating the ratio between samples without antibody and samples with antibody. Values are the mean from three biological replicates with the SE as error bars (right).

(Rozhon et al., 2010, 2014). BES1 was enriched on the identified binding site in all three promoters assessed (Figure 7D). Importantly, treatment with BL, which efficiently promoted dephosphorylation of BES1, significantly increased BES1 binding to the motif in planta (Figure 7D), providing evidence that BRs induce GA biosynthetic gene expression by increasing BES1 activity on target promoters.

DISCUSSION

When BRs were first discovered in the 1970s, their exceptional growth-promoting capacities were instantly apparent (Mitchell et al., 1970; Khripach, 2000). Since BR mutant phenotypes strongly resemble those of GA-deficient plants, the question of whether BRs may regulate growth by affecting GA biosynthesis was asked and addressed. However, since GA measurements in BR mutants of pea showed no difference in GA contents (Jager et al., 2005) and, moreover, since attempts to rescue growth defects of BR mutants with externally applied GA failed (Li et al., 1996; Szekeres et al., 1996; Bai et al., 2012; Gallego-Bartolomé et al., 2012), it was postulated that BRs do not regulate GA production. Instead a more complicated mode of crosstalk was discovered and put forward in which DELLA proteins, central regulators of GA signaling that are degraded in response to GA (Schwechheimer, 2011), inhibit BES1 and BZR1 activities whereby GAs modulate BR transcriptional outputs (Bai et al., 2012; Gallego-Bartolomé et al., 2012; Li et al., 2012).

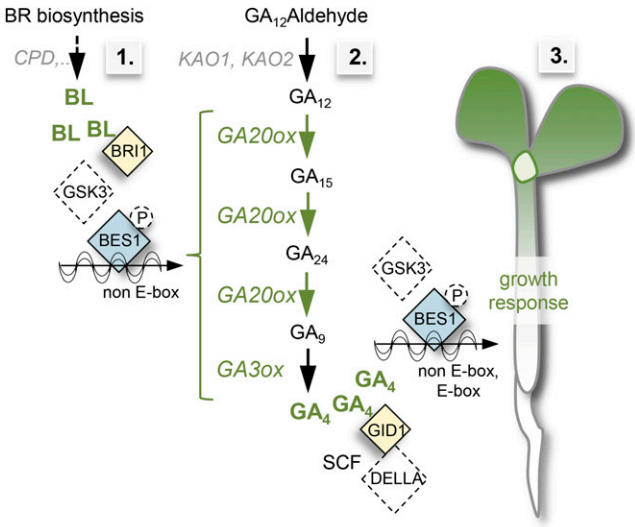


Figure 8. Model for the Control of GA-Regulated Growth by BRs.

1. Upon activation of BR signaling, BES1 accumulates in its non-phosphorylated form and induces the expression of multiple genes encoding enzymes of the GA biosynthetic pathway by binding to a non-E-box motif. 2. As a consequence, GA production is increased, which promotes DELLA degradation and releases their repressive action on BES1 in the transcription of targets further downstream in signaling. Also in this stage, BR signaling is required to maintain BES1 in its nonphosphorylated, active form. 3. This results in the promotion of growth and development and is of relevance throughout the plant life cycle.

Seemingly in contradiction, recent work has shown that BRs regulate GA biosynthesis in rice (Tong et al., 2014) and that externally applied BR induces *GA20ox* gene expression in Arabidopsis (Stewart Lilley et al., 2013). Here, we now demonstrate that also in Arabidopsis BRs are required for GA biosynthetic gene expression and thereby regulate GA production. Multiple lines of evidence support this finding. First, in BR mutants, levels of several GAs, including the bioactive GA_4, are reduced. Second, externally applied GA can restore growth defects of BR mutants. The efficiency of the rescue was dependent on the developmental stage and on the physiological conditions applied. Since GA biosynthesis is highly responsive to external cues (Hedden and Thomas, 2012) care had to be taken in the setup of the experiments. For example, cold treatment, which is known to induce *GA3ox* expression in imbibed seeds (Yamauchi et al., 2004; Penfield et al., 2005), recovered germination defects and thereby masked this phenotype. Also, nitrate, a standard medium component used for Arabidopsis phenotypic assessments, masked phenotypes of BR mutants and affected their responsiveness to GA application, which provides a possible explanation why these phenotypes had been overlooked previously. Third, multiple genes encoding enzymes of the GA20ox and GA3ox family are impaired in their expression in BR mutants. Fourth, and most importantly, multiple growth defects of the BR signaling defective mutant *bri1-301* could be restored by expression of *GA20ox1* in the *BRI1* expression domains. Since we show that BRs not only regulate *GA20ox* expression, but are also required for transcription of *GA3ox* genes, it is likely that GA production is not fully reconstituted in these lines. Nevertheless, reestablishing *GA20ox* expression in *bri1-301* recovered various growth defects of the BR signaling-deficient mutant, including reduced hypocotyl elongation, impaired primary root elongation and lateral root formation, dwarf growth, compromised leaf area expansion, and delayed flowering.

BR responses are induced by suppressing BIN2-mediated phosphorylation of BES1/BZR1 family members to enhance their activity on target promoters (He et al., 2002; Yin et al., 2002). In these promoters, BES1 and BZR1 are known to bind to E-box motifs (5′-ACnnGT-3′), a specific E-box variant called G-box (5′-CACGTG-3′), and the brassinosteroid response element BRRE 5′-GCTG(T/C) G-3′, which in two inverted copies composes a G-box (Sun et al., 2010; Yu et al., 2011). We now show that BES1 and BZR1 can also bind to a non-E-box motif, 5′-AA(A/T)CAAnnnC(C/T)T-3′, and that BES1 binding to this motif in vivo is strongly enhanced by BRs. This may explain why the sequence was previously overlooked, since in systems biology approaches that intended to identify BES1 and BZR1 in vivo binding sites on a genome-wide scale untreated plants were used (Sun et al., 2010; Yu et al., 2011). Indeed, a bioinformatic analysis of previously published data showed that the BES1 in vivo targets identified here had escaped detection with the applied ChIP-ChIP approach (Supplemental Figure 6).

How specificity in BES1/BZR1 DNA binding is conferred is not fully understood at present. However, in addition to the phosphorylation state, it is evident that conditional interactions with other proteins also determine BES1/BZR1 activity in target gene expression (Yin et al., 2005; Yu et al., 2008; Li et al., 2009, 2010; Lu et al., 2011; Oh et al., 2012). With respect to those growth processes that are regulated by both BRs and GAs, it is known that interaction with DELLAs represses BES1/BZR1 activities in the transcription of genes such as *SAUR-AC* and *PRE5* (Bai et al., 2012; Gallego-Bartolomé et al., 2012). Our work now shows that BRs also induce GA biosynthesis in Arabidopsis and do so by regulating multiple enzymes in the pathway. Since DELLAs are degraded when GA is perceived (Dill et al., 2001; Sasaki et al., 2003), an induction of GA production by BRs would provide a feed-forward means of stimulating BES1/BZR1 activity on targets further downstream in signaling (Figure 8). Because BR signaling is also needed in downstream target gene transcription to maintain BES1/BZR1 in their active forms, the rescue of severe signaling mutants by GA would not be expected to be very effective; indeed, GA did not efficiently restore *bri1-1* phenotypes.

The joint regulation of plant growth and development by GAs and BRs appears to be of relevance throughout the life cycle of Arabidopsis plants since in *bri1-301* plants in which *GA20ox* expression was reconstituted, growth repression was released in multiple development stages. However, some defects remained in these complemented lines, such as defective leaf development, which is not surprising given that GA biosynthesis is not the only BR target. It will be interesting to investigate which additional pathways contribute to the growth-promoting effects of BRs. Since our work complements and expands the recent findings that BRs also regulate GA biosynthesis in rice (Tong et al., 2014), there is evidence that the BR effects on GA production represent a general regulatory mechanism that allows for the regulation of growth and developmental processes in both monocotyledonous and dicotyledonous plant species.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana accession Col-0 was used as a background in all experiments of this study. Standard growth conditions were as follows for long day: 16 h of white fluorescent light (80 μmol m^{-2} s^{-1}/8 h dark), and 21°C on half-strength MS medium (Duchefa) with 0.7% (w/v) agar (Duchefa) and 1% (w/v) sucrose or in soil.

Germination assays were performed on water agar plates containing 0.7% (w/v) agar (Duchefa). Plates were incubated in standard conditions with or without cold treatment, and germination was scored as radicle emergence after 6 d. For analysis of early seedling development, plants were grown on the indicated medium, supplemented with GA_4 (Merck Schuchardt) when indicated, in standard conditions without a cold treatment. Hypocotyls were measured with a SZX10 stereomicroscope (Olympus). For GA treatment of adult plants, 14-d-old seedlings were grown on half-strength MS medium supplemented with 0, 1, 10, or 100 μM GA_{4+7} (Duchefa) and transferred to soil. Subsequently, plants were sprayed three times a week with 0, 1, 10, and 100 μM GA_{4+7}.

Generation of Transgenic Lines

For *bri1-301* complementation, a vector was constructed that allowed expression of *GA20ox1/GA5* (*At4g25420*) under control of the *BRI1* promoter. The *BRI1* promoter was PCR amplified from genomic DNA with the primers pbri1 fwd/rev (for primer sequences used, see Supplemental Table 2) with integrated *Xho*I and *Nco*I restriction sites and subcloned into the pGEM-T easy vector (Promega). After sequencing, *BRI1p* was cloned into the plant expression vector pGWR8 (Rozhon et al., 2010), creating pGWR8-bri1p. The *GA20ox1* open reading frame was PCR amplified from Col-0 cDNA using the gene-specific primers Ga20ox1 fwd/rev with integrated *Nco*I and *Not*I restriction sites, cloned into pGWR8-$BRI1_{pro}$, and coding sequence for a cMyc tag was added. From transformed *bri1-301* plants,

independent lines homozygous for *BRI1$_{pro}$:Ga20ox1-Myc* were selected, and using immunoblotting, two weak (#6.12 and #7.6) and two strong (#13.11 and #15.4) expression lines were chosen for further experiments.

qPCR

For qPCR ~20 mg tissue of seedlings was collected and shock frozen in liquid nitrogen. After total RNA extraction with the E.N.Z.A. Plant RNA Mini Kit (OMEGA Bio-tek), DNase I (Thermo Scientific) treatment, and cDNA synthesis with RevertAid first-strand cDNA synthesis kit (Thermo Scientific), qPCR was performed with a Eppendorf realplex^2 Mastercycler (Eppendorf) using SensiFAST SYBR Lo-ROX Mix,2x (Bioline) and specific primers for the mRNAs of interest. Data were normalized to *GAPC2* and measured in at least three technical replicates.

ChIP and EMSAs

For ChIP, *35Sp:BES1-CFP* plants (Rozhon et al., 2014) were grown for 21 d in standard growth conditions. The plants where sprayed at noon with 10 μM epi-BL and DMSO as control. Two hours after treatment ~0.6 g plant material was harvested per sample. Cross-linking and chromatin preparation were performed as described previously (Poppenberger et al., 2011). For immunoprecipitation, anti-GFP VHH agarose beads (Chromotek) and agarose beads as control were used. Washing of the beads and elution of the DNA-protein complex were performed as described previously (Kaufmann et al., 2010). DNA was purified by phenol-chloroform extraction and precipitated with 96% ethanol. For qPCR analysis, primers specific for the desired promoter region (Supplemental Table 2) were used. Purified DNA fragments were used for establishing standard curves for quantification. Data were normalized to *5S* rRNA gene.

For EMSAs, recombinant proteins of BES1-GST and BZR1-GST were expressed in *Escherichia coli* BL21 (New England Biolabs) using the expression vectors pGEX-BES1 and pGEX-BZR1 (Rozhon et al., 2010) and subsequently purified using GSH-beads (Roth). Protein elution from GSH beads was performed with elution buffer (150 mM NaCl, 5 mM DTT, 20 mM GSH reduced form, and 50 mM Tris/HCl, pH 8.0) and concentrated using Roti-spin MINI-3 columns (Roth). The DNA fragments used in EMSA as probes were PCR amplified and purified using the E.N.Z.A Gel Extraction Kit (OMEGA Bio-tek), and 1 ng DNA was used for adenosine 5′-[γ-^{33}P] triphosphate (American Radiolabeled Chemicals) labeling by PNK (Thermo Scientific) and again purified. The EMSA experiments were performed as described previously (Poppenberger et al., 2011).

Protein Immunoblotting

Immunoblot analysis was done from 12-d-old seedlings as described previously (52) using rabbit anti-cMyc antibody as the primary and alkaline phosphatase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) as the secondary antibody. Detection was performed with enhanced chemiluminescence using the CDP-Star reagent (GE Healthcare) or PhosphaGLO Reserve AP Substrat (Medac Diagnostic). For loading control, the membrane was stained with the Coomassie Brilliant Blue dye.

GA Quantification

Quantification of GAs was performed with gas chromatography-mass spectrometry as described previously (Lange et al., 2005).

Bioinformatic Analysis

Occurrences of the EMSA-derived non-E-Box motif were determined in Arabidopsis 750-bp upstream regions by direct sequence comparison. These regions were extracted starting from the transcription starting site and selecting the 750-bp upstream sequence or accordingly less, if a neighboring gene occupied the region. The conditional hypergeometric tests provided by Bioconductor GOstats package (Falcon and Gentleman, 2007) were applied to test for overrepresented biological process GO terms using the org.At.tair.db Bioconductor annotation package (Gentleman et al., 2004). Revigo (Supek et al., 2011) was used for analysis and visualization of enriched GO terms ($P < 0.05$). To test for an overrepresentation of non-E-Box genes within published sets of BR-associated genes, one-sided Fisher's exact tests were applied.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *BES1*, *AT1G19350*; *BZR1*, *AT1G75080*; *BRI1*, *AT4G39400*; *CPD*, *AT5G05690*; *GA20ox1*, *AT4G25420*; *GA20ox2*, *AT5G51810*; *GA20ox3*, *AT5G07200*; *GA20ox4*, *AT1G60980*; *GA20ox5*, *AT1G44090*; *GA3ox1*, *AT1G15550*; *GA3ox4*, *AT1G80330*; *GA2ox1*, *AT1G78440*; *GA2ox7*, *AT1G50960*; *GA2ox8*, *AT4G21200*; *KAO1*, *AT1G05160*; *KAO2*, *AT2G32440*; *GA1*, *AT4G02780*; *GA2*, *AT1G79460*; *GA3*, *AT5G25900*; *UBQ5*, *AT3G62250*; and *GAPC2*, *AT1G13440*.

Supplemental Data

Supplemental Figure 1. GA Application Rescues Growth Defects of Adult BR Mutant Plants.

Supplemental Figure 2. Expression of Upstream GA Biosynthesis Genes in BR Mutants.

Supplemental Figure 3. *GA20ox1* Expression Restores *bri1-301* Leaf Phenotypes.

Supplemental Figure 4. BES1-GST Binds Specifically to the −552 to −517 Region (from the ATG) in the *GA20ox1* Promoter.

Supplemental Figure 5. BES1 and BZR1 Bind to the Same Motif in Vitro.

Supplemental Figure 6. Enrichment of the Non-E-Box Motif in ChIP-ChIP and Microarray Data.

Supplemental Table 1. GO Enrichment of the Identified Non-E-Box Motif in Arabidopsis.

Supplemental Table 2. Primers Used in This Study.

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AUTHOR CONTRIBUTIONS

S.J.U., W.R., K.G.K., K.F.M., and B.P. designed research. S.J.U., W.R., M.P., J.K.C., T.L., and K.G.K. performed research. S.J.U., W.R., K.G.K., K.F.M, T.S., and B.P. analyzed data. B.P. wrote the article.

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