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ORIGINAL RESEARCH

BIN2/DWF12 Antagonistically Transduces Brassinosteroid and Auxin Signals in the Roots of *Arabidopsis*

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Abstract Plant growth-stimulating hormones brassinosteroids (BRs) function via interactions with other hormones. However, the mechanism of these interactions remains to be elucidated. The unique phenotypes of *brassinosteroid insensitive2/dwarf12-D* (*bin2/dwf12-D*) mutants, such as twisted inflorescences and leaves, suggested that BIN2, a negative regulator of BR signaling, may be involved in auxin signaling. Furthermore, previously, we showed that auxin stimulates *DWF4* expression. To determine the possible role of BIN2/DWF12 in Auxin signaling, we measured *DWARF4pro:GUS* activity through both GUS histochemical staining and in vivo GUS assay. We found that the GUS activity in the *bin2/dwarf12-1D* background dramatically increased relative to control. In addition, the number of lateral roots (LR) in *bin2/dwf12-1D* was greater than wild type, and the optimal concentration for auxin-mediated lateral root induction was lower in *bin2/dwf12-1D*; these findings suggest that BIN2 plays a positive role in auxin signaling. In contrast, ABA repressed both *DWF4pro:GUS* expression and lateral root development. However, the degree of repression was lower in *bin2/dwf12-1D* background, suggesting that BIN2 plays a role in ABA-mediated *DWF4pro:GUS* expression and subsequently in lateral root development, too. Therefore, it is likely that BIN2 plays a role of signal integrator for multiple hormones, such as BRs, auxin, and ABA.

Keywords Brassinosteroids · BIN2 · Auxin · ABA · DWF4 · *DWF4 pro:GUS* · Lateral root

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Introduction

Brassinosteroids (BRs), a class of polyhydroxylated plant steroidal hormones, regulate diverse processes in plant growth and development, such as cell elongation, cell division, lateral root development, vascular differentiation, and seed germination (Clouse and Sasse 1998; Choe 2004). Plant sterols serve as the precursors in the biosynthetic pathway for BRs, and the catalytic enzymes in this pathway include DWARF1 (DWF1) (Choe et al. 1999a), DWF5 (Choe et al. 2000), DWF7 (Choe et al. 1999a, b), DE-ETIOLATED2 (DET2) (Li et al. 1996), DWF4 (Choe et al. 1998), CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD) (Szekeres et al. 1996), cytochrome P450 family 90 (CYP90s) (Kim et al. 2005; Ohnishi et al. 2006), and CYP85s (Kim et al. 2008). BRs are perceived by a plasma-membrane receptor complex composed of various proteins, namely, BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Clouse et al. 1996; Li and Chory 1997), BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) (Li et al. 2002; Nam and Li 2002), BRASSINOSTEROID-SIGNALING KINASE (BSK) (Tang et al. 2008), and BRI1 KINASE INHIBITOR1 (BKI1) (Wang and Chory 2006). After the binding of BRs and the receptor complex, BRI1-5 SUPPRESSOR 1 (BSU1), a kelch-repeat protein phosphatase (Mora-Garcia et al. 2004), is activated to

dephosphorylate the tyrosine (Tyr) residues of BRASSINOSTEROID INSENSITIVE 2 (BIN2) (Kim et al. 2009a, b, c). BIN2/DWF12/UCU1 is a negative regulator in the BR signaling pathway, and its dominant gain-of-function mutant, *bin2-D*, is responsible for the characteristic BR dwarf phenotypes (Li et al. 2001; Choe et al. 2002; Li and Nam 2002; Perez-Perez et al. 2002).

BIN2 shares amino acid sequence similarity with a family of shaggy-like kinases/glycogen synthase kinases (GSKs) in animals. *Arabidopsis* possesses ten GSK3-like genes, which are divided into four classes (Jonak and Hirt 2002). Of these, class II includes BIN2/*Arabidopsis thaliana* shaggy-related kinase 21 (AtSK21), AtSK22, and AtSK23 (Yoo et al. 2006). In the BR signaling pathway, BIN2 functions redundantly with five other AtSKs belonging to groups I and II (Kim et al. 2009a; Yan et al. 2009). In addition to BR signaling, *Arabidopsis* AtSKs are involved in stress tolerance (Piao et al. 1999) and floral development (Claisse et al. 2007).

In animals, GSK3 performs multiple roles that are associated with microtubule dynamics and signaling pathways such as Wnt, Sonic Hedgehog, insulin, Notch, receptor tyrosine kinase, and fibroblast growth factors (Forde and Dale 2007; Kim et al. 2009b, c). Mammalian GSK3 exists in three isoforms: GSK3α, GSK3β, and GSK3β2 (Meijer et al. 2004). GSK3s are regulated by multiple mechanisms, which include phosphorylation at an N-terminal serine residue, autophosphorylation at Tyr216 of GSK3β and Tyr270 of GSK3α, proteolytic processing, proteasome-mediated degradation, intracellular localization, and recruitment to GSK-binding protein complexes (Jope and Johnson 2004). Some of these regulatory mechanisms, including subcellular localization, phosphorylation at Tyr200 of BIN2 and Tyr233 of AtSK12, ubiquitination, and proteasome-mediated degradation, are also found in *Arabidopsis* BIN2 (Kim et al. 2009a).

Recent studies have shown that Tyr200 of BIN2 and Tyr233 of AtSK12 are autophosphorylation sites that are subsequently dephosphorylated by BR-activated BSU1 (Kim et al. 2009a). Further, BIN2 may undergo degradation after ubiquitination in the presence of BRs (Peng et al. 2008). The activity of BIN2 may also depend on its subcellular localization since BIN2 is found in the plasma membrane and cytosol, but the mutant bin2-1D is typically located in the nucleus, wherein it phosphorylates the substrate proteins of BIN2 (Gampala et al. 2007; Ryu et al. 2007).

BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-ETHYL METHANESULFONATE-SUPPRESSOR 1 (BES1) activate BR-dependent genes; these also bind to the promoter region of the BR biosynthetic genes *CPD* and *DWF4* and inhibit their expression to form a negative feedback loop (He et al. 2005). In the absence of BRs, BIN2 phosphorylates and consequently inhibits the activities of the transcriptional factors BZR1 and BZR2 through different mechanisms, including proteasome-mediated degradation (Peng et al. 2008), nuclear export followed by 14-3-3 protein-mediated cytoplasmic retention (Gampala et al. 2007; Ryu et al. 2007), and inhibition of DNA-binding activity (Vert and Chory 2006).When phosphorylated, the transcriptional repressor BZR1 dissociates from the promoter of the *CPD* and *DWF4* genes, and the genes are de-repressed to be actively expressed in the absence of BR signaling (He et al. 2005; Tanaka et al. 2005). This mechanism is used to maintain BR homeostasis and is controlled at the transcriptional level (Tanaka et al. 2005; Kim et al. 2006). The expression levels of *CPD* and *DWF4* are used as molecular markers to understand the relation between BR signaling and BR homeostasis (Wang et al. 2002).

Similar to their functions in animal systems, GSKs may function in multiple signaling pathways in plants. This idea is supported by the fact that *bin2/dwf12-1D* mutation is responsible for the characteristic BR dwarf phenotypes and morphologies that are unique to this mutant. Examples of unique phenotypes include plants with slightly twisted organs. Since *Arabidopsis* twisted dwarf mutants are associated with the auxin transport system (Choe et al. 2002; Geisler et al. 2004), it is likely that BIN2 is also involved in auxin signaling. Previous studies have shown that BRs synergistically interact with auxins to regulate lateral root (LR) development (Bao et al. 2004), hypocotyl elongation (Nemhauser et al. 2004; Stavang et al. 2009), and radial patterning of the vascular bundle (Ibanes et al. 2009). During the combination treatment of BR and auxin, the two hormones synergistically interact and cause hypocotyl elongation through BIN2-dependent phosphorylation of AUXIN RESPONSE FACTOR 2 (ARF2) (Vert et al. 2008). In addition, a study involving the *abscisic acid deficient* mutant, *aba1*, showed that *DWF4* and *CPD* expression is induced briefly after exogenous treatment with abscisic acid (ABA) (Zhang et al. 2009). The ABA sensitivity of the *Arabidopsis* BR mutants *det2*, *bri1-5*, and *bri1-9* was higher than that of the wild type in some tests (Xue et al. 2009).

Our results support the idea that BIN2 plays an important role in both BR- and auxin-signaling pathways. We found that auxin-mediated *DWF4* expression is elevated in the *bin2/dwf12-1D* background, and the number of LRs generated after auxin treatment in the *bin2/dwf12-1D* mutant is greater than that in the wild-type or *bri1-5* background, which suggests that BIN2 is an important component of auxin signaling, especially signaling for LR development.

Results and Discussion

The Expression of the *DWF4pro:GUS* was Stronger in *Arabidopsis bin2/dwf12-1D* Background

Previously, we had reported that *DWF4 promoter-β-glucuronidase* reporter gene (*DWF4pro:GUS)* is expressed in actively growing tissues of *Arabidopsis*, and the *DWF4* expression is positively correlated with the levels of endogenous BRs (Kim et al. 2006). We also found that the *DWF4* transcript levels were high in BR-response mutants such as *bri1-5* and *bin2/dwf12* (Noguchi et al. 1999; Choe et al. 2002). To further understand the regulatory mechanism for *DWF4*, we introduced the *DWF4 pro:GUS* construct into the BR response mutant *bin2/dwf12-1D* by genetic crossing and obtained the resultant expression patterns, which are shown in Fig. 1. *DWF4pro:GUS* was expressed in the root apex in both the mutant and wild-type backgrounds; however, in the *bin2/dwf12-1D* and *bri1-5* mutants, the staining was more intense and also extended up to the elongation zone (Fig. 1a).

DWF4 expression is stimulated by exogenous treatment with auxin, and this leads to an increase in BR biosynthesis in *Arabidopsis* roots. To further understand the role of BIN2 in the regulation of *DWF4pro:GUS* expression, we treated 5-day-old seedlings with 100 nM 2,4-dichlorophenoxyacetic acid (2,4-D) for 24 h. Auxin treatment resulted in a more intense staining pattern in the roots of all the genotypes, and the stained tissues produced root hairs that were also stained (Fig. 1a).

We performed an in vivo GUS assay to quantitatively measure the GUS activity. Similar to the staining pattern, the GUS activity was the lowest in the wild type, and higher in both the *bin2/dwf12-1D* and *bri1-5* mutants: the activities in the *bin2/dwf12-1D* and *bri1-5* mutants were 3.4- and 2.8-fold higher, respectively, than that in the mock-treated wild type (Fig. 1b). Further, 2,4-D treatment elevated the GUS activity in all genotypes: the GUS activities in the wild-type plants and the *bin2/dwf12-1D* and *bri1-5* mutants were respectively 2.7-, 1.8-, and 2-fold higher than that in the mock-treated wild type. Thus, compared to the mutants, the wild type showed the best response to auxin, which suggested that *DWF4pro:GUS* expression in response to auxin treatment was maximal in these mutants. However, the total GUS activity after auxin treatment was the greatest in *bin2/dwf12-1D* mutants, followed by *bri1-5* mutants. This result confirms the opposing roles of BRs and auxins in the regulation of the BR biosynthetic gene *DWF4*: BRs repress the gene expression and auxins activate it. This finding also implies that the auxins might utilize BIN2 for auxin-dependent regulation of *DWF4*.

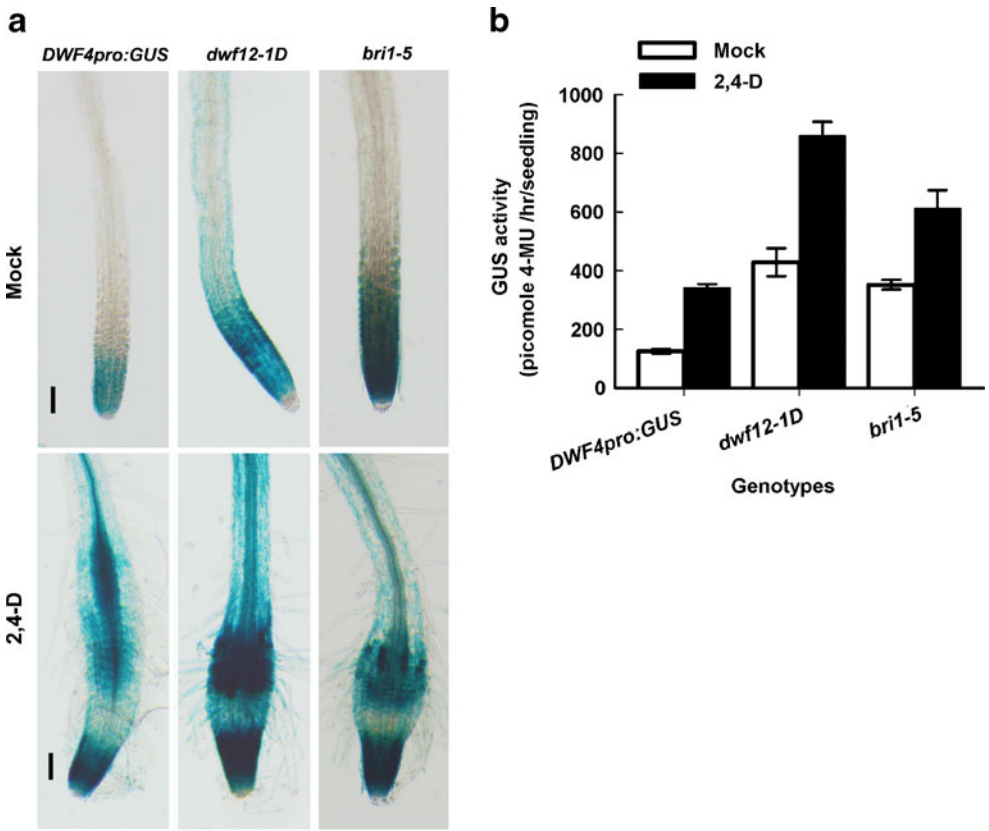


Fig. 1 An increase in the level of 2,4-D induced GUS activity in the BR response mutants. **a** GUS histochemical staining pattern of the *DWF4pro:GUS* in plants with different genetic backgrounds in the presence or absence of 100 nM 2,4-D treatment. The level of *DWF4pro:GUS* expression is indicated by *blue* staining. The staining in *bin2/dwf12-1D* mutants is more intense than that in the other two. *Scale bar*=0.1 mm. **b** Quantitative analysis of in vivo GUS activity before and after 2,4-D treatment. The *error bars* represent the standard error; n=12

Auxins Induced LR Development in BR Mutants

Auxins and BRs interact to promote LR development (Bao et al. 2004). A previous study has shown that the number of LRs is fewer in both *bri1-5* and BR-deficient *det2* mutants (Gao et al. 2008); this finding suggested that BRs have a positive role in LR development. To further understand how auxins and BRs co-regulate LR development, we examined LR development in wild-type plants as well as in the two BR mutants harboring *DWF4pro:GUS*. These lines were particularly useful because the *DWF4* promoter-driven *GUS* expressed strongly in developing LR; therefore, it was easier to count the LRs and follow their course of development.

The dose–response test results presented in Fig. 2 show that the longest primary roots were observed in the mock-treated wild-type plants. The root lengths of mock-treated *bin2/dwf12-1D* and *bri1-5* mutants were respectively 22% and 40% less than those of the wild-type plants (Fig. 2a). When auxin was administered, the root lengths of all the genotypes reduced, e.g., 5 nM auxin reduced the primary root lengths of mock-treated wild-type plants and of *bin2/dwf12-1D* and *bri1-5* mutants by 51%, 59%, and 54%, respectively.

In addition to the reduction in root lengths, the number of LRs per centimeter of primary root (NLRCM) varied among the genotypes. Among the mock-treated plants, the NLRCM of *bin2/dwf12-1D* mutants was significantly greater (12% more than that of wild type), but the NLRCM of *bri1-5* mutants was less (32% less than that of wild type; $p<0.05$; Fig. 2b), which suggested that BIN2 had a positive role in LR development. Among auxin-treated plants, the NLRCM increased in proportion to the auxin concentration in all the genotypes. To better illustrate this response, we converted the NLRCM for each genotype into a percentage value that was calculated with reference to the number of NLRCM in the corresponding mock-treated plants (Fig. 2c).The percent increase in NLRCM in all the genotypes was similar up to auxin concentration of 2.5 nM; however, in *bri1-5*, the rate of increase escalated when the auxin concentration was

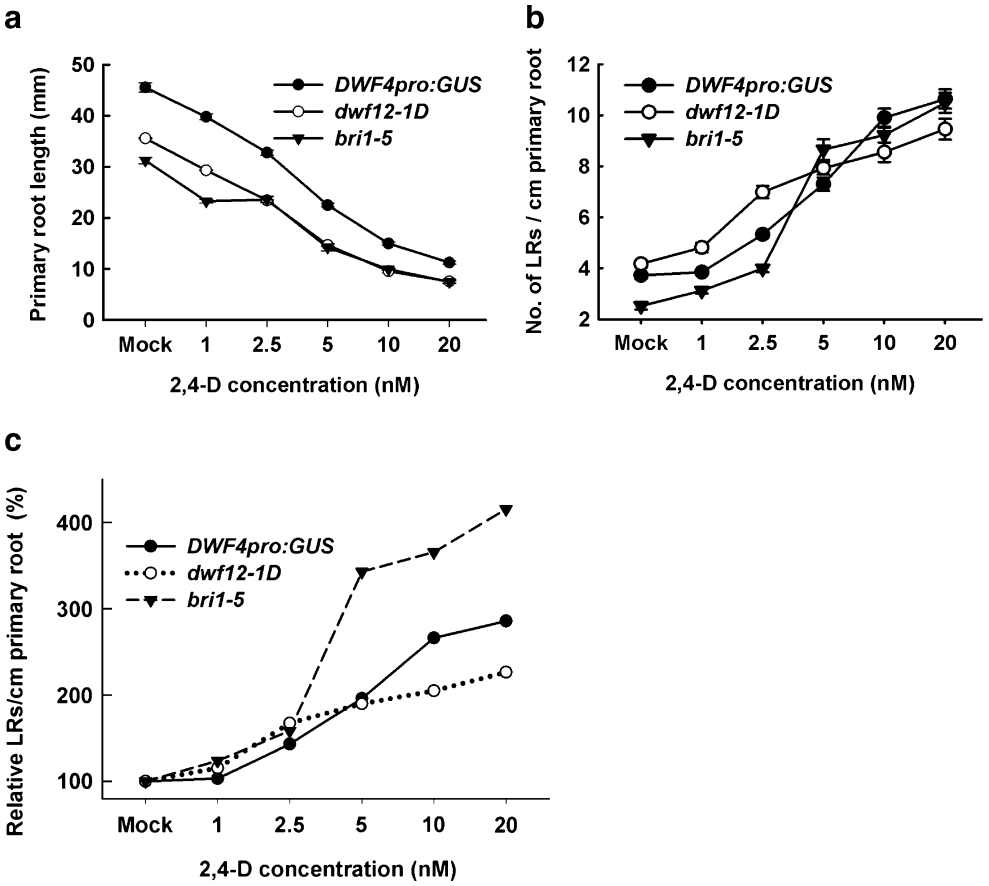
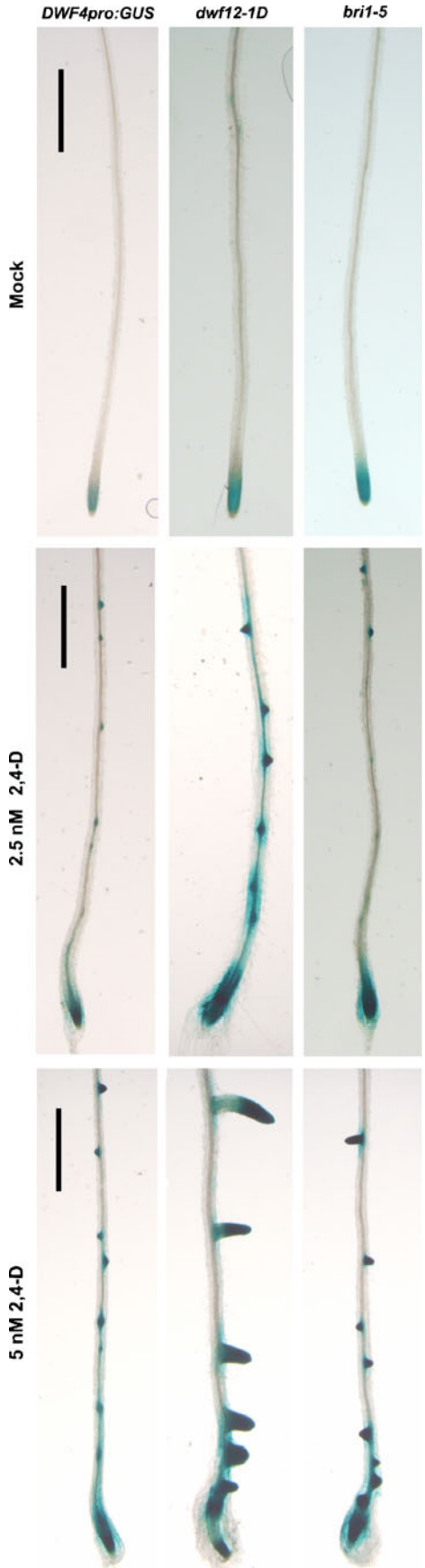


Fig. 2 Necessity of lower auxin concentrations in *bin2/dwf12-1D* mutants during auxin-induced lateral root development. **a** Auxin-dependent decrease in root growth. The primary root length linearly decreased in proportion to 2,4-D concentration, and the magnitude of increase was different for different phenotypes. **b** The average number of lateral roots per centimeter length of primary roots in plants treated with different concentrations of 2,4-D. **c** Percentage increase in the number of lateral roots per centimeter relative to that in the mock-treated plants. The percentages were calculated with reference to the number of lateral roots per centimeter of the primary root in mock-treated plants. The 3-day-old seedlings were transferred to 2,4-D media and grown for five additional days. The *error bars* represent the standard error; $n=25–50$



◀ **Fig. 3** Images of GUS-stained roots with or without auxin treatment. Development of auxin-induced lateral roots occurs after auxin treatment. Relative to the wild-type and *bin2/dwf12-1D* backgrounds, the *bri1-5* mutants show delayed lateral root development after treatment with 2.5 nM 2,4-D. At auxin concentration greater than 5 nM, auxin not only induced the development of LR but also elongated lateral roots. *Scale bar*=1 mm

greater than 5 nM. In wild-type plants, the response was greater than that of *bin2/dwf12-1D* when the auxin concentration was greater than 10 nM. Finally, treatment with 20 nM auxin resulted in NLRCMs that markedly differed from those of the mock-treated genotypes: the NLRCMs of auxin-treated *bri1-5*, wild-type, and *bin2/dwf12-1D* plants were respectively 416%, 286%, and 226% greater than those of the corresponding mock-treated plants. The contrasting responses of *bri1-5* and *bin2/dwf12-1D* also suggest that BRI1 and BIN2 play opposing roles in LR development, although mutations in both the genes are responsible for the insensitivity to BRs.

Figure 3 shows GUS-stained roots treated with or without auxin. Although the absolute NLRCM in *bin2/dwf12-1D* mutants increased slightly, the LRs in the *bin2/dwf12-1D* mutants were remarkably longer than those in the wild type (Fig. 3). This result suggests that the exogenous treatment with auxin induced elongation of the developing LRs in *bin2/dwf12-1D* mutants. As mentioned earlier, *bri1-5* was hypersensitive to 2,4-D at all concentrations. The decreased development of LRs in *bri1-5* was completely restored to the wild-type level on treatment with 2,4-D at concentrations greater than 2.5 nM (Fig. 2b–c).

BR Induced LR Development

A previous study revealed that BRs enhance LR development by promoting acropetal transport of auxin in *Arabidopsis* (Bao et al. 2004). To further understand the role of BRs in LR development, we performed dose–response tests with epi-brassinolide (BL). The tests revealed that BL significantly inhibited root elongation in the wild-type plant in a dose-dependent manner (Fig. 4a). However, *bin2/dwf12-1D* and *bri1-5* mutants were insensitive to various doses of BL (Fig. 4a) (Choe et al. 2002).

Subsequently, we examined LR development after BL treatment. At mock condition, the NLRCM in *bin2/dwf12-1D* was the highest, followed by that in the wild-type plants and *bri1-5* mutants (Fig. 4b). The NLRCM in both the wild-type plants and *bin2/dwf12-D* mutants increased gradually when the BL concentration increased up to 2.5 nM and then started to decrease gradually when the concentration increased above 10 nM. A 100 nM BL inhibited LR development in all the genotypes. Thus, this result confirms the finding that the stimulating effect of BL in LR development in the wild type occurs only at low concentrations of BL.

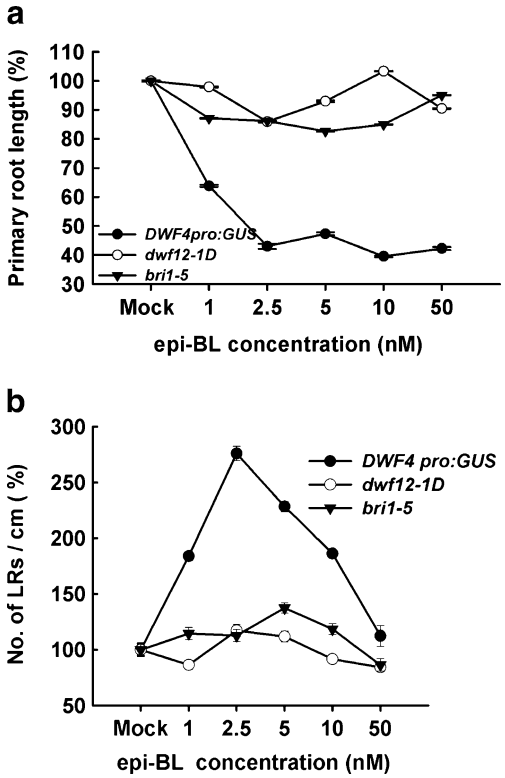


Fig. 4 Effects of epi-brassinolide (BL) on primary root elongation and lateral root development. **a** Percentile changes in primary root length in response to epi-BL treatment. The root length in mock-treated plants of each genotype was considered as 100. The primary root length linearly decreased in proportion to epi-BL concentration in the wild type. Both *bri1-5* and *bin2/dwf12-1D* were less sensitive to epi-BL. **b** The average number of lateral roots per centimeter of the primary root in all the genotypes treated with different concentrations of epi-BL. Wild-type plants showed the highest sensitivity for lateral root development in response to epi-BL. The *error bars* represent the standard error; n=25–50

ABA Repressed *DWF4pro:GUS* Expression and LR Development

ABA and BRs play antagonistic roles in several physiological processes, including germination (Steber and McCourt 2001). Further, the BR-insensitive mutant *bin2/dwf12-1D* is hypersensitive to ABA (Choe et al. 2002). To further understand the relationship between ABA and BRs, we explored the effects of ABA on *DWF4pro:GUS* expression. When 5-day-old seedlings were treated with 1 μM ABA for 24 h, GUS staining greatly diminished irrespective of the genotypic backgrounds (Fig. 5a). The GUS-staining patterns were quantified by an in vivo GUS assay (Fig. 5b). ABA-induced reduction of the GUS activity in these BR-response mutants suggests that ABA regulates the *DWF4pro:GUS* gene independent of BR signaling.

ABA treatment also affected the development of both primary root and LR (Fig. 6). Contrary to those in the wild-type plants, ABA caused reduction in the root lengths of *bin2/dwf12-1D* and *bri1-5* mutants (Fig. 6a), which suggested that the root development in these two BR mutants is hypersensitive to ABA. However, unlike the findings for the wild-type and *bri1-5* backgrounds, the NLRCM of ABA-treated *bin2/dwf12-1D* mutants was comparable with that of the mock-treated wild type. The NLRCMs in ABA-treated wild-type plants, *bin2/dwf12-1D* and *bri1-5* mutants were respectively 66%, 34%, and 56% lower than those in the corresponding mock-treated plants (Fig. 6b); this finding indicated that the ABA sensitivity of the wild type is greater than that of the two BR mutants.

Conclusions

In this article, we provided evidence that BIN2 transduces auxin signals. In addition to its role as a negative regulator in BR signaling, BIN2 plays a positive role in auxin signaling, especially in signaling for LR development. GUS histochemical analysis and in vivo activity of the *DWF4pro:GUS* line suggested that the *DWF4* expression is stimulated by auxins, and the expression level is higher in the *bin2/dwf12-1D* background. In addition, the LR phenotypes showed opposing characteristics in the 2 BR mutants *bri1-5* and *bin2/dwf12-1D*. Since the two mutants are defective in BR signaling and deficiency in BR causes a decrease in the number of LRs in *bri1-5*, an increase in the number of LRs in *bin2/dwf12-1D* mutant suggests that BIN2 must have another function that directs auxin signals towards LR development.

Currently, the mechanism of the auxin-mediated BIN2 activation for better development of LRs is unknown. Auxins might stimulate BIN2 transcription, but microarray data from the *Arabidopsis* portal (http://www.arabidopsis.org) revealed that the level of *BIN2* transcription does not increase after auxin treatment. Another possible mechanism for BIN2 activation is post-translational modification of BIN2—auxins might stimulate autophosphorylation of BIN2 for its activation or prevent degradation of this protein by suppressing the ubiquitination and proteasome-mediated degradation. However, further studies are required to determine the exact mechanism of BIN2 activation.

Materials and Methods

Plant Materials and Growth Condition

Previously, we had reported the *DWF4pro:GUS* transgenic lines in Ws-2, *bin2/dwf12-1D*, and *bri1-5* background (Kim et al. 2006). The growth conditions for the plants were as follows: surface-sterilized seeds were sprinkled on agar-solidified 1× Murashige and Skoog (MS) media supple-

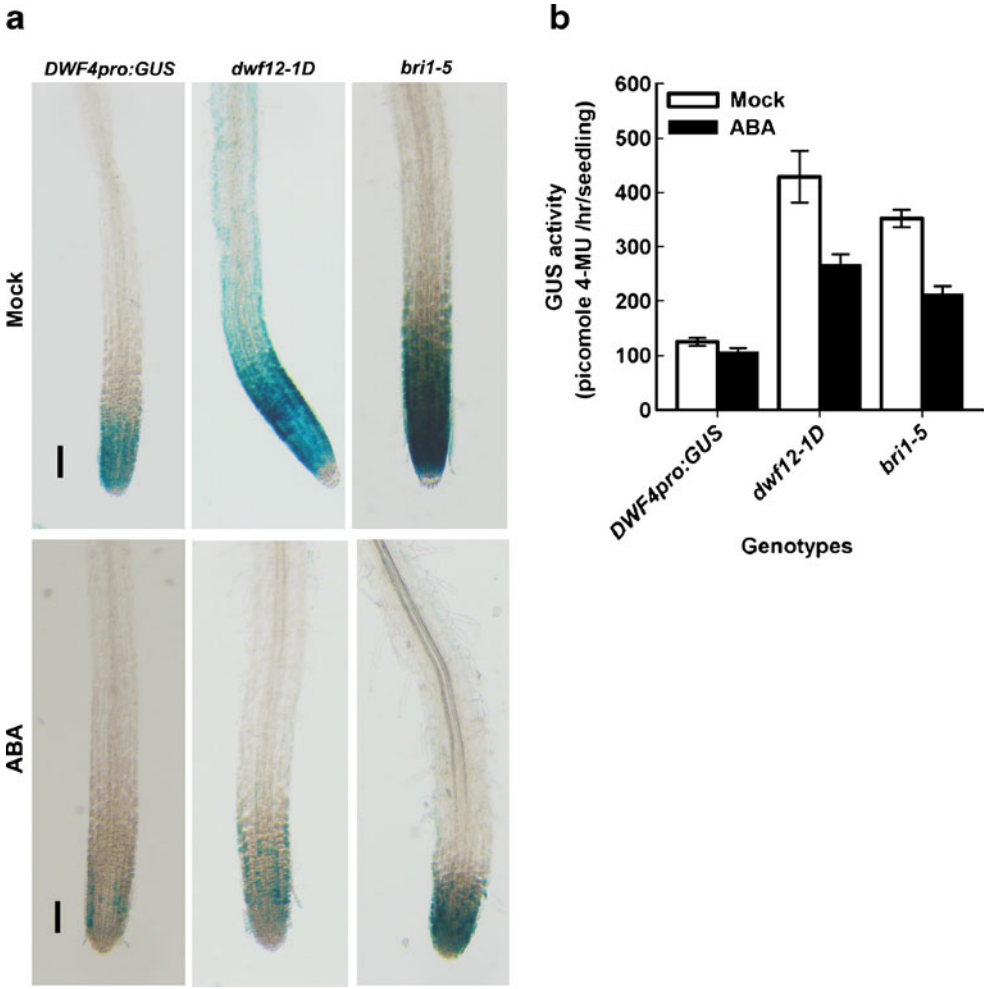


Fig. 5 Repression of *DWF4pro:GUS* expression by ABA treatment. The level of *DWF4pro:GUS* expression in the primary root is visualized by *blue* stains. **a** GUS histochemical staining pattern of *DWF4pro:GUS* in different genotypic backgrounds. Although ABA suppressed GUS staining in all the genotypes, the staining remained intense in the *bin2/dwf12-1D* and *bri1-5* backgrounds. **b** Quantitative analysis of relative activity of *DWF4 pro:GUS* after treatment with ABA. Similar to the GUS-staining pattern, GUS activity was decreased by ABA in all genotypes. The *error bars* represent the standard error; n=12; *scale bar*=0.1 mm

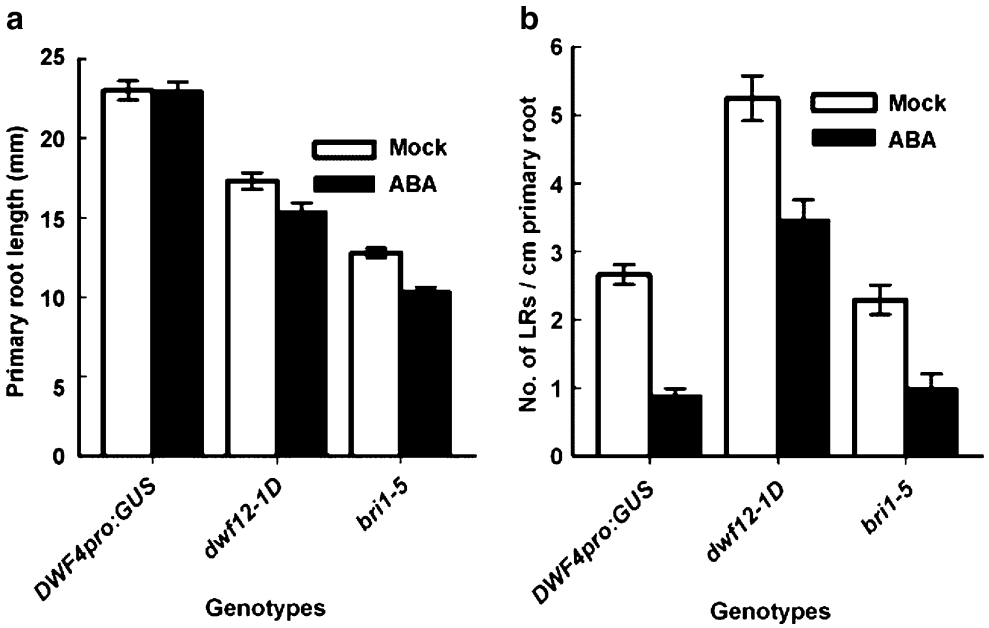


Fig. 6 Inhibition of lateral root development by ABA treatment. **a** Changes in average root lengths in response to ABA treatment. ABA caused a slight increase in the root length of the wild type, but it decreased the root length in the two BR mutants. **b** Changes in the number of lateral roots responding to ABA treatment. The *bin2/dwf12-1D* and *bri1-5* mutants were less sensitive to ABA. The number of lateral roots per centimeter of the primary root in ABA-treated *DWF4pro:GUS*, *bin2/dwf12-1D*, and *bri1-5* mutants were respectively 64%, 39%, and 26% less than the values in the mock-treated plants. The *error bars* represent the standard error; n>25

mented with 1% (*w*/*v*) sugar. The seedlings were grown at 22°C under long-day condition (16 h light and 8 h dark) for 5 days and were then transferred at the designated time period to MS media supplemented with different hormones for further growth.

Primary Root Growth and Counting of LRs

For LR growth in BL, the seedlings were grown with the plates oriented in a vertical position for 8 days. The root lengths were measured by analyzing the root images using an image analysis software (UTHSCSA image tool, version 3.0). For counting the lateral roots, the seedlings were histochemically stained, and the number of lateral root primordia and lateral roots were counted by using an optical microscope.

Histochemical and Quantitative GUS Assay

GUS staining was performed according to a standard method, with minor modifications (Jefferson et al. 1987). Briefly, seedlings were incubated at 37°C for 16 h in GUS staining buffer [1 mM 5-bromo-4-chloro-3-indoyl-β-d-GlcUA, 100 mM sodium phospahate (pH 7), 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10 mM EDTA, and 0.1% (*v*/*v*) Triton X-100]. The chlorophyll in the tissue was removed by serial treatments using 50%, 70%, and 100% ethanol, respectively, and the LRs were observed by using a stereo microscope.

Quantitative in vivo GUS assay was performed according to a previously published method (Blazquez et al. 1998). A seedling was transferred to each well of a 96-well plate pre-filled with 100 μl of a substrate solution [50 mM sodium phosphate (pH 7), 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% (*w*/*v*) SDS, 0.1% (*w*/*v*) triton X-100, 2% isopropanol, and 440 mg/L 4-methylumbelliferyl β-d-glucuronide], and the plate was incubated at 37°C for 12 h. The reaction was terminated by adding 100 μl ice-cold stop buffer (0.2 M Na_2CO_3). Fluorescent products were quantified using a fluorescence spectrophotometer (Varian, USA) with the excitation wavelength set at 360 nm and emission wavelength, 465 nm. The standard curve was obtained using a 4-methylumbelliferol solution of known concentration.

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