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Molecules and Cells

A Transcriptional Feedback Loop Modulating Signaling Crosstalks between Auxin and Brassinosteroid in *Arabidopsis*

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Auxin and brassinosteroid (BR) play essential roles in diverse aspects of growth and developmental processes in plants mainly through coordinate regulation of cell division, elongation, and differentiation. Consistent with the overlapped roles, accumulating evidence indicates that the two growth hormones act in a synergistic as well as in an interdependent manner in many cases, although the underlying molecular mechanisms are not fully understood. Here, we demonstrate that auxin and BR signaling pathways are interconnected at the transcriptional level via a negative feedback loop. An *Arabidopsis* activating tagging mutant *dlf-1D* exhibited dwarfed growth with small, dark-green leaves and reduced fertility. Hormone feeding assays revealed that the mutant phenotype is caused by the reduction of endogenous BR level. Consistent with this, a gene encoding the CYP72C1 enzyme that catabolizes BR was up-regulated. Notably, the transcript level of the ARF8 transcription factor gene, which modulates the expression of auxin-responsive genes, was significantly elevated in the mutant. In addition, the *ARF8* gene expression was significantly reduced by BR but induced by brassinazole, a BR biosynthetic inhibitor. On the other hand, two BR catabolic pathway genes, *DLF* (*CYP72C1*) and *BAS1*, were induced by auxin. Our observations indicate that at least part of auxin and BR signaling pathways are unified through a transcriptional feedback control of the *DLF* and *ARF8* genes.

INTRODUCTION

Plant hormones are small organic compounds that regulate a variety of physiological and developmental processes. Auxin and brassinosteroid (BR) are essential for modulating both cell elongation and proliferation. As the name auxin derives from the Greek 'aux-ein' meaning 'to grow' (Parry and Estelle, 2006), auxin has been implicated in virtually all plant growth and developmental processes (Vanneste and Friml, 2009). BR is closely linked with the same processes and acts synergistically with auxin (Asamie et al., 2005).

In many cases, mutants in the auxin and BR pathways show similar phenotypes, including severe growth defects (Clouse, 1996; Leyser et al., 1996). In addition, auxin and BR share a number of early responsive genes, many of which are involved in plant growth regulation (Goda et al., 2002; 2004; Nakamura et al., 2006; Nemhauser et al., 2004), supporting extensive crosstalks between the auxin and BR pathways. However, the underlying molecular schemes are poorly understood in most cases.

Critical questions are what signaling components mediate the auxin-BR interactions and how the interactions are regulated at the molecular level. Auxin signal transduction initiates at the F-box protein TRANSPORT INHIBITOR RESPONSE 1 (TIR1) and its functional homologues, which serve as auxin receptors (Dharmasiri et al., 2005; Kepinski and Leyser, 2005).

Binding of indole-3-acetic acid (IAA) to the TIR1 receptor promotes the interaction of TIR1 with the AUXIN/INDOLE ACETIC ACID (AUX/IAA) proteins (Tan et al., 2007). This interaction leads to AUX/IAA ubiquitination and subsequent degradation by the proteasome pathway. The AUX/IAA proteins bind to AUXIN RESPONSE FACTORs (ARFs), a group of transcription factors that modulate expression of auxin-responsive genes (Guilfoyle and Hagen, 2007; Tiwari et al., 2004). Therefore, auxin-mediated AUX/IAA degradation elevates the activity of ARFs and thereby induces expression of auxin-responsive genes (Guilfoyle and Hagen, 2007). Intriguingly, the regulatory sequence regions of many BR-responsive genes are frequently enriched with ARF-binding sites, indicating that the ARF transcription factors are potential molecular linkers for the auxin-BR interactions (Goda et al., 2004; Nemhauser et al., 2004).

The BR signaling pathway is more complex than the auxin signaling pathway (Belkhadir and Chory, 2006). BR is perceived by a group of plasma membrane-localized receptors, the BRASSINOSTEROID INSENSITIVE 1 (BRI1) and BRI1-like proteins (Li and Chory, 1997). Binding of BR promotes association with another membrane-bound BR receptor, BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) (Wang et al., 2005a; 2005b; Yun et al., 2009), triggering downstream events that result in phosphorylation of signaling mediators, including BR-INSENSITIVE 2 (BIN2), and transcription factors, such as

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BRI1-EMS-SUPPRESSOR 1 (BES1) (He et al., 2002; Mora-García et al., 2004; Vert and Chory, 2006; Yin et al., 2002).

Interestingly, some BR signaling components also play a role in auxin signaling. The BES1 transcription factor activates a small group of auxin-responsive genes, such as *SAUR-AC1* (Yin et al., 2005). The BR-regulated BIN2 kinase contributes to a synergistic increase in auxin-induced gene expression by direct modulation of the ARF2 transcriptional activity (Vert et al., 2008). Furthermore, BR induces a few IAA genes, such as *IAA5* and *IAA19*, as well as the *DR5-GUS* reporter, in which a synthetic auxin response element is fused to the *GUS* gene, in *Arabidopsis* (Nakamura et al., 2003a). In contrast, there have been no reports of direct regulation of BR-responsive genes by auxin signaling components.

Although recent molecular genetic studies, mostly in *Arabidopsis*, have addressed potential auxin-BR interactions, the underlying molecular mechanisms remain largely undefined. Global gene expression studies using the microarray techniques have provided insights into the auxin-BR interaction. Interestingly, many auxin-pathway genes are rapidly up-regulated in response to BR, whereas only a few BR-pathway genes are responsive to auxin (Goda et al., 2002; 2004). It has been shown that expression of the *phyB activation-tagged suppressor*1 (*BAS1*) gene was induced by IAA (Goda et al., 2004). *BAS1* is a BR catabolic pathway gene, encoding a cytochrome P450 monooxygenase (Neff et al., 1999; Turk et al., 2003). The BAS1 enzyme functions as a BR C-26 hydroxylase and plays a role in BR homeostasis. The *BAS1* induction by auxin has been a first hint towards the possibility of regulating the role of auxin in BR signaling or metabolism.

In this work, we isolated an *Arabidopsis* activation tagging mutant *dlf-1D* exhibiting dwarfed growth with low fertility. The *dlf-1D* phenotype was caused by activation of the *DLF* (*CYP72C1*) gene, which has been known to function redundantly with *BAS1* in BR catabolism (Nakamura et al., 2005; Takahashi et al., 2005; Turk et al., 2005). We also found that the transcript level of the *ARF8* gene is in inverse proportion to the endogenous BR level. In addition, two BR catabolic pathway genes, *DLF* and *BAS1*, were induced by exogenous auxin application. We therefore propose that a transcriptional feedback regulation of the *ARF8* and *DLF* genes underlies at least part of the auxin-BR crosstalks during seedling growth.

MATERIALS AND METHODS

Plant materials and growth conditions

All *Arabidopsis thaliana* lines used were in the Columbia background (Col-0). The T-DNA insertional knockout mutants, including *bas1* and *arf8*, have been previously described (Tian et al., 2004; Turk et al., 2005). Plants were grown in a controlled culture room set at 23°C with a relative humidity of 60% under long day conditions (16-h light and 8-h dark) with white light illumination (120 μmol photons/m^2s) provided by fluorescent FLR40D/A tubes (Osram, Korea). For treatments with different light wavelengths, plants were grown in complete darkness (D) or under red (R), far-red (FR), and blue (B) light (12, 8, and 15 μmol/m^2s, respectively) in a VS-940L-DUAL incubator (Vision, Korea) equipped with R, FR, or B light-emitting diodes.

Isolation of the *dlf-1D* and *dlf-1* mutants

Ecotype Col-0 was transformed with the activation tagging vector pSKI015, as previously described (Weigel et al., 2000). To select herbicide-resistant transformants, the T1 seeds were collected, sown in soil, and sprayed twice a week with a 1: 1,000 dilution (in water) of Finale (AgrEvo, USA) containing 5.78% Basta. The herbicide-resistant seeds were further selected through two additional generations, and homozygotic seeds were obtained. A morphogenic mutant *dlf-1D* having reduced growth was chosen for further analysis. The single T-DNA insertion in the *dlf-1D* mutant was verified by genomic Southern blot hybridization using the 35S enhancer sequence as a probe, followed by analysis of segregation ratios. The sequences flanking the T-DNA insertion were determined by a three-step thermal asymmetric interlaced PCR (TAIL-PCR) (Liu et al., 1995) and direct DNA sequencing

A knockout mutant *dlf-1* (SALK 120416), which has a single T-DNA insertion into the 3rd exon of the *DLF* gene, was isolated from a pool of T-DNA insertional lines deposited into the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University, USA). Absence of the *DLF* expression in the mutant was verified by RT-PCR. The knockout mutant is identical to the *cyp72c1-1* mutant that has been previously characterized by Turk et al. (2005) and Takahashi et al. (2005).

Generation of transgenic plants and *Arabidopsis* transformation

To generate transgenic *Arabidopsis* plants overproducing the *DLF* gene, a full-size *DLF* sequence covering a 1.4-kb transcriptional unit was amplified by RT-PCR using the primer set, 5′-AAAGGCGCGCCATGTTAGAGATCATTAC and 5′-AAA**TTAATTAA**CTACAGTTTTCGGATGATCA with the AscI (underlined) and PacI (underlined and bold) recognition sequences. The PCR product was double-digested with AscI and PacI and subcloned into the pBA002 binary vector that harbored the CaMV 35S promoter. *Agrobacterium*-mediated transformation of *Arabidopsis* was carried out by a modified floral dip method (Clough and Bent, 1998), and homozygotic lines were obtained through selections for three consecutive generations.

Semiquantitative RT-PCR

Total RNA was extracted from appropriate plant materials with the RNeasy Plant Mini Kit (Qiagen, USA). For semiquantitative RT-PCR, total RNA samples were pretreated extensively with RNase-free DNase I to remove any contaminating genomic DNA. The first strand cDNA was synthesized from 1-2 μg of total RNA in a 20-μL reaction volume using the Superscript II reverse transcriptase (Invitrogen, USA). RT-PCR was performed for 15-30 cycles, depending on the linear range of PCR amplification for each gene, using gene-specific primer pairs (Table 1). The Pfu Turbo polymerase (Stratagene, USA) was used for the reactions. Each cycle was performed at 94°C for 0.5 min, 60°C for 0.5 min, and 72°C for 1 min with a final cycle at 72°C for 10 min to finish polymerization. Whenever necessary, positive and negative control genes were included in the reactions to monitor the reaction conditions.

miRNA extraction and Northern blot hybridization

For miRNA Northern blot hybridization, total RNA samples were extracted from plant materials using the TRIZOL reagent (Invitrogen) according to the procedure previously described (Pfeffer et al., 2003) but with a few modifications. miRNA Northern blot hybridizations were carried out using the ULTRAHyb Oligo solution according to the procedure supplied by the manufacturer (Ambion, USA). The oligonucleotide probes were 5′ end-labeled using P^{32}-γATP and T4 polynucleotide kinase. The oligonucleotide probe used for detection of miR167 is 5′-TAGATCATGCTGGCAGCTTCA.

Growth hormone treatments

Plants were grown on Murashige and Skoog (MS)-agar plates

Table 1. Primers for RT-PCR

ARF8-F	5′-TATAGCTGCATGCAAGACAC
ARF8-R	5′-GATGAGACTAACACCGAAGC
BAS1-F	5′-ATGGAGGAAGAAAGTAGCAGCT
BAS1-R	5′-TCAATCCTCATGATTGGTCAAT
CPD-F	5′-ATGGCCTTCACCGCTTTTCTCC
CPD-R	5′-TCAAGTAGCAAAATCACGGCGC
DLF-F	5′-AAAACACGAACTCTTTCCAA
DLF-R	5′-TCTCCTCCTCTTTTGTCTCA
DWF4-F	5′-TTCTTGGTGAAACCATCGGTTATCTTAAA
DWF4-R	5′-TATGATAAGCAGTTCCTGGTAGATTT
GUS-F	5′-AAACTGTGGAATTGATCAGC
GUS-R	5′-AAAGACAGCTGAAAGCCGACA
IAA1-F	5′-ACACAAGCATTTTCAAGG
IAA1-R	5′-TCTGTTGAGTCGTTGTTC
IAA19-F	5′-AAGAAATGGAGAAGGAAGGAC
IAA19-R	5′-TCACTCGTCTACTCCTCTAGGCT
TUB-F	5′-CTCAAGAGGTTCTCAGCAGTA
TUB-R	5′-TCACCTTCTTCATCCGCAGTT
WES1-F	5′-ATGCCTGAGGCACCAAAGAAAGAA
WES1-R	5′-TTAGTTACTCCCCCACTGTTTGTGACC

containing 0.5x MS salts with vitamins, 0.5 g liter^{-1} 4-morpholineethanesulfonic acid, and 0.8% Phytagar (Duchefa, The Netherlands) for 7-10 days under long days. The plants were then incubated with gentle shaking in liquid MS media supplemented with appropriate growth hormones for the indicated time periods before harvesting plant materials. Whole plants were used for RNA extraction, unless specified otherwise.

RESULTS

The *dlf-1D* mutant exhibits dwarfed growth with reduced fertility

From a screening of a pool of activation-tagged *Arabidopsis* mutants generated by random insertion of the T-DNA enhancer element into the genome of the Col-0 ecotype, we isolated a severely dwarfed mutant *dwarfish with low fertility-1D (dlf-1D)* (Fig. 1A). The *dlf-1D* mutant also had small curled leaves with dark-green color, short petioles and internodes, and reduced apical dominance. Furthermore, seed production was significantly reduced in the mutant. Overall, the *dlf-1D* phenotypes were quite similar to those observed in BR-deficient mutants (Fujioka and Yokota, 2003), suggesting that the tagged gene might be related with BR metabolism or signaling.

Mapping the T-DNA insertional site by TAIL-PCR and gene expression analysis revealed that the *CYP72C1* gene, designated *DLF* in this work, encoding a cytochrome P450 monooxygenase, was activated by the nearby insertion of the 35S enhancer in the *dlf-1D* genome (Figs. 1B and 1C). Transgenic plants overexpressing the *DLF* gene under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter (35S:*DLF*) exhibited phenotypes with various degrees of severity in a dosage-dependent manner (Fig. 1A). Approximately 40% of the 35S:*DLF* transgenic plants was phenotypically similar to the *dlf-1D* mutant. The levels of the *DLF* gene transcripts in the transgenic plants were also similar to those in the *dlf-1D* mutant (Figs. 1A and 1C), indicating that the *DLF* gene overexpression underlies the *dlf-1D* phenotypes. In contrast, the T-DNA insertional knockout mutant *dlf-1* exhibited a slightly greater height with larger leaves, as previously observed (Fig. 1A; Takahashi et al., 2005), confirming the interconnectedness of *DLF* (*CYP72C1*) and the *dlf-1D* phenotypes.

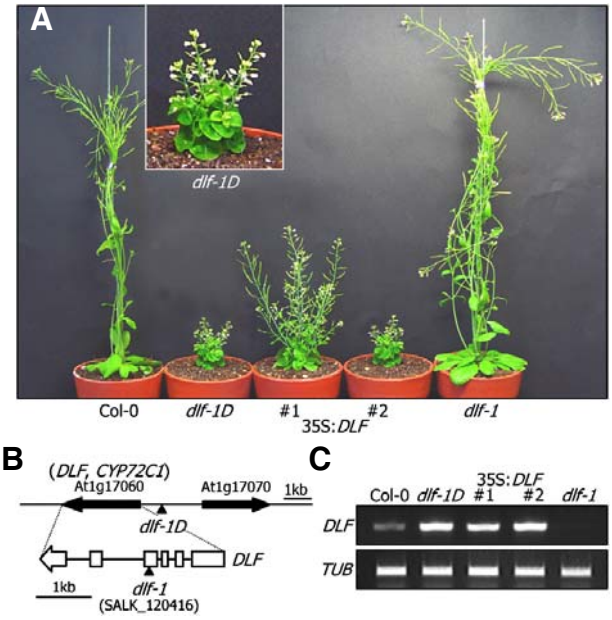

Fig. 1. Morphological and molecular characteristics of the *dlf-1D* and *dlf-1* mutants. (A) Phenotypes of the *dlf-1D* and *dlf-1* mutants. Five-week-old plants grown in soil were photographed. Transgenic plants overexpressing the *DLF* gene under control of the CaMV 35S promoter (35S:*DLF*) were included for comparison. The inset is an enlarged view of the *dlf-1D* mutant. (B) Mapping of the T-DNA insertion site in the *dlf-1D* and *dlf-1* mutants. kb = kilobase. (C) Activation of the *DLF* gene in the *dlf-1D* mutant. The 35S:*DLF* transgenic plants and the *dlf-1* mutant were also included in the assays. Transcript levels were examined by RT-PCR. A tubulin gene (*TUB*) was used as a control for RNA quantity.

The *dlf-1D* mutant is a BR-deficient mutant

The *dlf-1D* mutant turned out to be allelic to the previously reported activation-tagged *Arabidopsis* mutants, such as *chibi2* (*chi2*), *shrink1-D* (*shk1-D*), and *suppressor of phyB-4 7-D* (*sob7-D*) (Nakamura et al., 2005; Takahashi et al., 2005; Turk et al., 2005). The *DLF* (*CYP72C1*) gene is a member of the *CYP72* gene family and is homologous to the *BAS1* gene (Nelson et al., 2004). It has been shown that the BAS1 and CYP72C1 enzymes act redundantly to modulate BR accumulation by catabolizing active BRs (Takahashi et al., 2005; Turk et al., 2005). Accordingly, the dominant mutants of these genes show BR-deficient phenotypes.

To examine whether the phenotypes of *dlf-1D* mutant result from BR deficiency, a brassinolide (BL) solution of 1 μM was sprayed everyday during the *dlf-1D* mutant growth. We found that petiole elongation in the *dlf-1D* mutant was considerably reversed in a few days after BL spray (Figs. 2A and 2B). In contrast, application of IAA did not exhibit any discernible effect on the petiole elongation, indicating that the *dlf-1D* phenotype is caused by BR-deficiency.

It has been reported that the expression of *BAS1* is upregulated by exogenous BL application (Turk et al., 2003). To examine whether the *DLF* expression is affected by BL, we carried out semiquantitative RT-PCR runs using total RNAs extracted from the BL-treated, wild-type seedlings. The transcript levels of the *DLF* gene were elevated in the BL-treated seedlings (Fig. 2C), suggesting that the *DLF* gene is under the feedback control of BR, as observed with the *BAS1* gene.

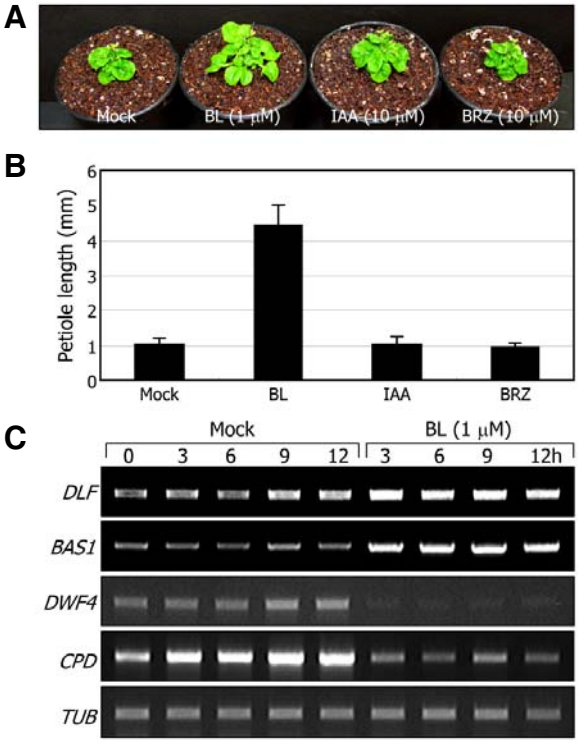

Fig. 2. Effects of BL and IAA on the *dlf-1D* phenotypes and on the *DLF* gene expression. (A) Effects of BL on petiole elongation in the *dlf-1D* mutant. Plants were grown in soil for 3 weeks. A BL solution of 1 μM was sprayed everyday during plant growth. IAA (10 μM) and brassinazole (BRZ, 10 μM) were also applied to the plants in a similar manner. (B) Comparison of petiole lengths. Twenty petioles of the 5th leaves were measured and averaged for each plant group. Bars denote standard error of the mean. (C) Effects of BL on the *DLF* gene expression. Ten-day-old, wild-type plants grown on MS-agar plates were subsequently incubated with gentle shaking in liquid MS media supplemented with 1 μM BL, and plants were harvested at the indicated time points. h = hour. Transcript levels were examined by RT-PCR. The *BAS1*, *DWF4,* and *CPD* genes were included as controls.

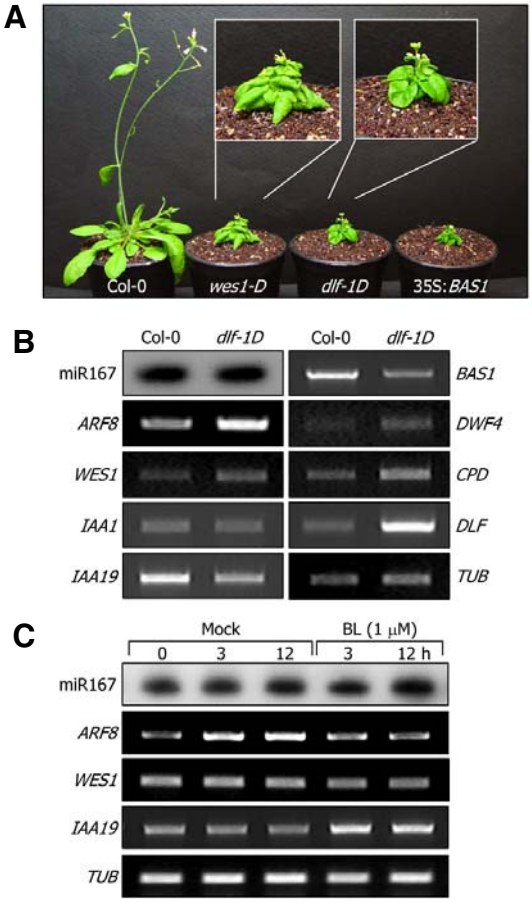

Fig. 3. Expression of auxin-related genes in the *dlf-1D* mutant. (A) Phenotypic comparison of the *dlf-1D* and *wes1-D* mutants. Transgenic plants overexpressing the *BAS1* gene under control of the CaMV 35S promoter (35S:*BAS1*) were included for comparison. (B) Expression of auxin-related genes in the *dlf-1D* mutant. Ten-day-old plants grown on MS-agar plates were used for extraction of total RNAs. (C) Effects of BL on the expression of auxin-related genes. Ten-day-old, wild-type plants grown on MS-agar plates were subsequently incubated with gentle shaking in liquid MS media supplemented with 1 μM BL, and plants were harvested at the indicated time points. h = hour. In (B) and (C), transcript levels were examined by RT-PCR. The endogenous level of miR167 was examined by miRNA Northern blot analysis.

The *DLF* gene regulates auxin signaling

The *dlf-1D* phenotype included a stunted growth with reduced apical dominance, dark-green leaves with curled leaf margins, and short petioles. Notably, these phenotypic alterations have also been observed in several auxin-metabolic mutants, such as *wes1-D*, in which constitutive expression of the *GH3.5* (*WES1*) gene encoding an IAA-conjugating enzyme resulted in reduced auxin levels (Fig. 3A; Park et al., 2007). The phenotypic similarities between auxin- and BR-deficient mutants suggested that the auxin pathway would also be disturbed in the *dlf-1D* mutant.

To explore the possible relationship between *DLF* and auxin pathway, expression patterns of a series of auxin-related genes, including *ARF8*, *WES1*, *IAA1*, and *IAA19*, were examined in the *dlf-1D* mutant. miR167, which negatively regulates the *ARF8* gene, was also included in the assays (Wu et al., 2006; Yang et al., 2006). To ensure the assay conditions, the *DWF4*, *CPD*, and *BAS1* genes that are responsive to BR deficiency were also included as controls (Goda et al., 2004; Nakamura et al., 2003a). The expression of *ARF8*, which is required for controlling an adequate level of free IAA through modulation of the *GH3* genes, significantly increased in the *dlf-1D* mutant (Fig. 3B; Tian et al., 2004; Yang et al., 2006). The *WES1* (*GH3.5*) gene was also induced slightly in the mutant.

However, the miR167 abundance was unchanged within a detectable level (Fig. 3B), indicating that induction of the *ARF8* expression did not result from post-transcriptional modification by miR167 but was attributable to the effects of auxin and BR in the *dlf-1D* mutant. It has been shown that the *IAA19* gene is induced by both auxin and BR (Nakamura et al., 2003a). On the other hand, the expression patterns of the *ARF8* and *WES1* genes in the *dlf* and *bas1* mutants were similar to those in wild-type plant (data not shown), probably because of the functional redundancy between the *DLF* and *BAS1* genes (Nakamura et al., 2005; Takahashi et al., 2005; Turk et al., 2005).

Effects of BL on the *ARF8* gene expression also indicated that the *ARF8* gene is related with BR. Consistent with the

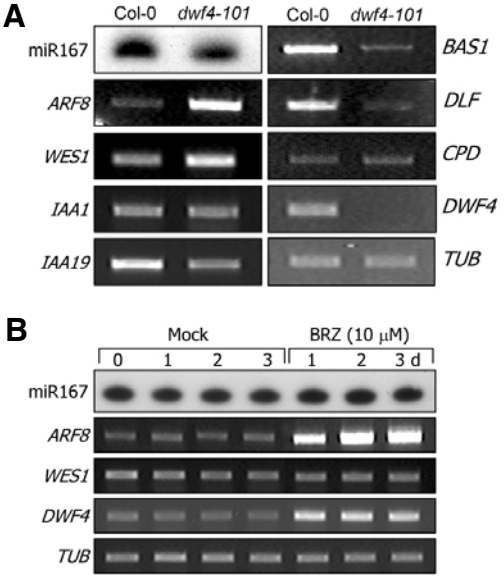

Fig. 4. Effects of BR deficiency on the expression of auxin-related genes. (A) Expression of auxin-related genes in the *dwf4-101* mutant. Ten-day-old plants grown on MS-agar plates were used for extraction of total RNAs. (B) Effects of BRZ on the expression of auxin-related genes. Ten-day-old, wild-type plants grown on MS-agar plates were subsequently incubated with gentle shaking in liquid MS media supplemented with 10 μM BRZ, and plants were harvested at the indicated time points. d = day. In (A) and (B), transcript levels were examined by RT-PCR. The endogenous level of miR167 was examined by miRNA Northern blot analysis.

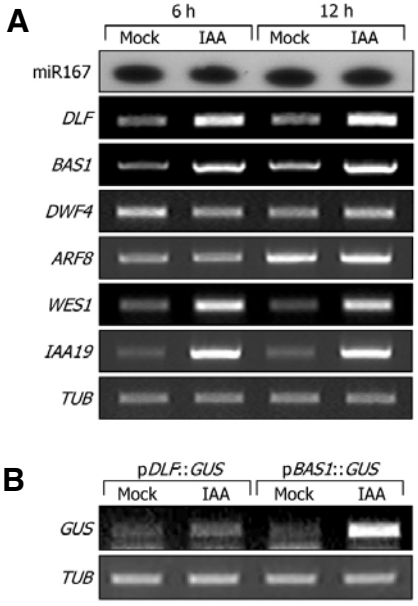

Fig. 5. Effects of IAA on the expression of BR-related genes. (A) Effects of IAA on the expression of the *DLF* and *BAS1* genes. Ten-day-old plants grown on MS-agar plates were subsequently incubated with gentle shaking in liquid MS media supplemented with 10 μM IAA for the indicated time periods. Transcript levels were examined by RT-PCR. The endogenous level of miR167 was examined by miRNA Northern blot analysis. h = hour. (B) Effects of IAA on the promoter activities of the *DLF* and *BAS1* genes. The promoter sequences, each consisting of approximately 2-kb nucleotides, were transcriptionally fused to the GUS-coding sequence, and the fusions (p*DLF-GUS* and p*BAS1-GUS*) were transformed into *Arabidopsis*. The promoter activities were examined by RT-PCR of the *GUS* gene expression. Ten-day-old transgenic plants grown on MS-agar plates were used for extraction of total RNAs.

observations in Fig. 3B, the level of the *ARF8* transcript decreased significantly, and that of the *WES1* transcript reduced slightly in plants treated with BL (Fig. 3C), suggesting that BR negatively regulates the *ARF8* and *WES1* genes.

To further investigate the link between BR and *ARF8*, expression patterns of auxin-related genes were examined in the BR-deficient *dwf4-101* mutant (Nakamoto et al., 2006). The *ARF8* and *WES1* genes were induced in the *dwf4-101* mutant (Fig. 4A), as observed in the *dlf-1D* mutant. Accordingly, the *ARF8* gene expression was significantly induced in the BRZ-treated plants (Fig. 4B), further supporting the link between BR signaling and *ARF8*.

Auxin regulates the *DLF* and *BAS1* genes

The hierarchical relationship between auxin and BR pathways is still controversial. Our data indicated that BR modulates auxin signaling or metabolism by regulating the *ARF8* gene. Therefore, the next question was whether auxin conversely regulates BR metabolism. To answer this question, expression patterns of diverse BR-metabolic pathway genes were examined in IAA-treated wild-type plants. Whereas expression of the *DLF* and *BAS1* genes was significantly induced, that of the *CPD* and *DWF4* genes functioning in the BR-biosynthetic pathway was unaltered in the IAA-treated plants (Fig. 5A), indicating that auxin interacts with BR, primarily by modulating the BR-catabolic pathway genes.

To confirm the regulation of the *DLF* and *BAS1* gene expression by auxin, we prepared the p*DLF-GUS* and p*BAS1-GUS* fusion construct, in which the promoter sequence regions of the *DLF* and *BAS1* genes, each consisting of approximately 2-kb nucleotides, were transcriptionally fused to the GUS-coding sequence. The fusion constructs were then transformed into *Arabidopsis* plants. Transgenic plants containing each fusion construct, grown for 2 weeks on MS-agar plates, were subsequently treated with auxin, and the expression of the *GUS* gene was examined by semiquantitative RT-PCR. The results showed that the transcript levels of the *GUS* gene were elevated by exogenous auxin application (Fig. 5B), further supporting the auxin regulation of the BR-catabolic enzyme genes.

BR pathway is linked with auxin pathway via a transcriptional feedback loop

Auxin and BR act redundantly to modulate *Arabidopsis* photomorphogenesis (Asamie et al., 2005; Vanneste and Friml, 2009), indicating that light is an important factor for the interactions between the two growth hormone pathways. This notion is very consistent with the previous observations showing that hypocotyl growth of the *DLF*-overexpressing mutants, such as the *sob7-D* mutant (Turk et al., 2005), responds differentially to different light wavelengths.

Our data showed that the *ARF8* gene is a molecular linker that mediates auxin-BR interactions. The *ARF8* gene is negatively regulated by miR167. However, miR167 abundance is unaffected by auxin and BR (Figs. 3C and 5A). We therefore investigated whether miR167 is regulated by light quality or not. Northern blot analysis revealed that miR167 abundance was influenced by light quality (Fig. 6A). The miR167 abundance

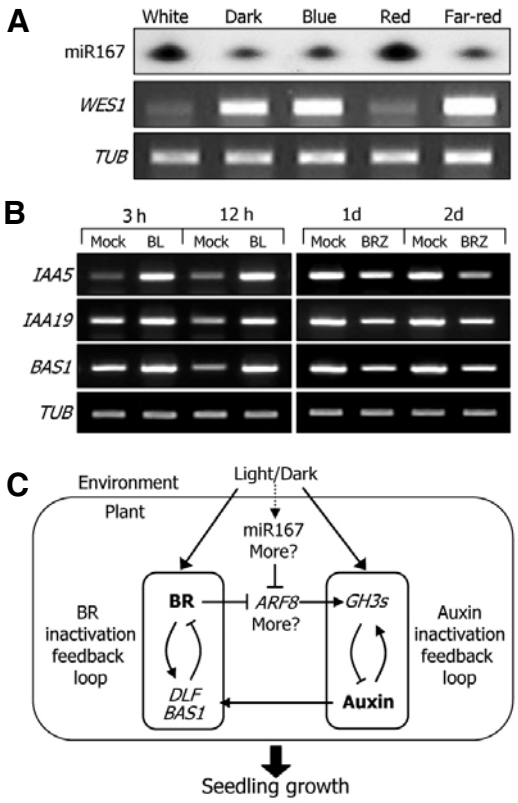

Fig. 6. A working model for the auxin-BR crosstalks. (A) miR167 abundance under different light wavelengths. Seven-day-old, wild-type plants grown on MS-agar plates in complete darkness were further grown under white light, dark, blue, red, or far-red light for one additional day before harvesting plant materials. The miR167 abundance was examined by miRNA Northern blot analysis. The *WES1* gene expression was examined by RT-PCR. (B) Effects of BL and BRZ on the expression of the *IAA5* and *IAA19* genes in the *ARF8*-deficient mutant. Ten-day-old plants grown on MS-agar plates were subsequently incubated with gentle shaking in liquid MS media supplemented either with 1 μM BL or with 10 μM BRZ for the indicated time periods. Transcript levels were examined by RT-PCR. The *BAS1* gene was included as control. d= day, h = hour. (C) A proposed working model for the auxin-BR interactions during seedling growth. The auxin and BR signaling pathways are unified through a transcriptional feedback control of the *ARF8* and *DLF* genes. It is well known that BR and auxin act redundantly to modulate *Arabidopsis* photomorphogenesis. It is therefore likely that light signals are also incorporated into the crosstalks via miR167, a negative regulator of the *ARF8* gene.

was relatively lower in darkness. However, it was significantly elevated after red light illumination but was unchanged after far-red and blue light illumination. Interestingly, the miR167 level was in reverse correlation with the *WES1* expression under different light wavelengths, which is consistent with the positive regulation of the *GH3* genes by *ARF8* (Tian et al., 2004; Yang et al., 2006). These observations support the notion that miR167 integrates light signals into the *ARF8* linker functioning in the auxin-BR interactions.

It has been reported that the *IAA5* and *IAA19* genes, but not the *ARF8* gene, are regulated by BR (Nakamura et al., 2003a). Interestingly, the degree of the *ARF8* gene induction was quite similar to the degree of the *IAA19* gene repression in the BR-deficient *dlf-1D* and *dwf4-101* mutants (Figs. 3B and 4A). A similar pattern of the *ARF8* and *IAA19* genes was also observed in the BL-treated plants (Fig. 3C), suggesting that expression of the *IAA19* gene is mediated by the *ARF8* gene. We therefore investigated the relationship between *ARF8* and *IAA19* using the *ARF8*-deficient plants treated with BL or BRZ. We found that the gene transcript levels of *IAA5* and *IAA19* were also higher in the BL-treated plants but lower in the BRZ-treated plants (Fig. 6B). These observations indicate that the link between BR and auxin signaling pathways via the *ARF8* gene is independent of the *IAA19* gene.

It has been well established that the auxin and BR hormone pathways involve inactivation mechanisms via feedback loops. The auxin-inducible GH3 enzymes inactivate IAA in auxin homeostasis, and the BR-inducible CYP72 enzymes catabolizes BL in BR homeostasis. Our data indicate that two transcriptional control mechanisms interconnect the two feedback loops (Fig. 6C). One loop is the regulation of BR-catabolic mechanism by auxin through the transcriptional activation of two BR-catabolic pathway genes, *DLF* (*CYP72C1*) and *BAS1*. The other is the BR-mediated suppression of the *ARF8* that induces expression of the *GH3* genes. In this working model, the synergistic activities of the two hormone pathways are maintained through a transcriptional feedback loop.

DISCUSSION

It is widely perceived that auxin and BR interact synergistically as well as interdependently to promote cell elongation or division in various plant growth and developmental processes. Using a variety of genomic tools combined with genetic analyses, the auxin-BR interactions have been validated mainly at the level of common target genes that integrate the two hormone pathways. At first sight, a straightforward link of auxin-BR interactions would be the modulation of the biosynthesis of one hormone by the other. However, this scheme remains to be substantiated.

As an effort to investigate the interactions between the auxin and BR pathways, we examined the molecular aspects of the *dlf-1D* mutant, a BR-deficient mutant, in which overexpression of the *DLF* gene inactivates BR through a BR-catabolic mechanism. The transcript level of the *ARF8* gene increased significantly in the *dlf-1D* mutant, as observed in the BR-deficient *dwf4-101* mutant. Additionally, the *ARF8* expression was significantly reduced in the BL-treated plants but was induced in the BRZ-treated plants. These observations indicate that the BR pathway is possibly connected with the *ARF8*-mediated auxin pathway. Interestingly, two BR catabolic pathway genes, *DLF* and *BAS1,* were induced by exogenous auxin application. Our data, as well as those from previous investigations, support the idea that plants possess a complicated network of auxin-BR interactions, which modulates the balance between the auxin and BR pathways through feedback control mechanisms.

Our proposed model is based on the phenotypic analysis of BR- and auxin-deficient mutants, hormone feeding assays, and extensive gene expression analysis. However, it should be mentioned that more extensive investigations are required to confirm the working scenario. It has not been clearly determined whether the *ARF8* gene affects auxin signaling or metabolism at the molecular level. In the BR-deficient mutants, as in the *dlf-1D* mutant, *ARF8* expression is highly elevated, but *WES1* expression is only slightly changed. The ARF8 protein has been reported to regulate positively the expression of the *GH3* genes at the transcription level (Tian et al., 2004). The GH3 enzymes conjugate free IAA with amino acids and there-

fore reduce the endogenous level of active auxin contents (Park et al., 2007; Staswick et al., 2002). It is therefore possible that the *ARF8* gene is linked with the *GH3*-mediated feedback mechanism in maintaining auxin homeostasis. However, no direct evidence has been reported yet.

Although the *ARF8* expression was suppressed by BR, the effects of BR on the *WES1* expression was not very prominent (Fig. 4A). It is possible that the ARF8 protein may regulate other *GH3* genes than *WES1*. Furthermore, additional ARF members, such as ARF6, ARF10 and ARF16, may also regulate the *GH3* genes (Mallory et al., 2005; Seo et al., 2009; Wu et al., 2006). Therefore, it will be necessary to examine the expression patterns of other *ARF* genes and *GH3* genes in response to BR. The most critical question would be whether or not endogenous auxin levels really change in the transgenic or mutant plants with altered expressions of the *ARF* genes. Because previous studies have shown that endogenous auxin levels do not change dramatically in response to BR (Bio et al., 2004; Kim et al., 2007; Nakamura et al., 2003b), precise measurement of endogenous auxin contents in various BR-related mutants, or vice versa, would be necessary to confirm the proposed transcriptional feedback mechanism modulating the auxin-BR interactions.

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