The Regulation of DWF4 Expression Is Likely a Critical Mechanism in Maintaining the Homeostasis of Bioactive Brassinosteroids in Arabidopsis

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Mutants that are defective in brassinosteroid (BR) biosynthesis or signaling display severely retarded growth patterns due to absence of growth-promoting effects by BRs. Arabidopsis (Arabidopsis thaliana) DWF4 (DWF4) catalyzes a flux-determining step in the BR biosynthetic pathways. Thus, it is hypothesized that the tissues of DWF4 expression may represent the sites of BR biosynthesis in Arabidopsis. Here we show that DWF4 transcripts accumulate in the actively growing tissues, such as root, shoot apices with floral clusters, joint tissues of root and shoot, and dark-grown seedlings. Conforming to the RNA gel-blot analysis, DWF4:β-glucuronidase (GUS) histochemical analyses more precisely define the tissues that express the DWF4 gene. Examination of the endogenous levels of BRs in six and seven different tissues of wild type and brassinosteroid insensitive1-5 (bri1-5) mutant, respectively, revealed that BRs are significantly enriched in roots, shoot tips, and joint tissues of roots and shoots. In addition, DWF4-GUS expression was negatively regulated by BRs. DWF4-GUS activity was increased by treatment with brassinazole, a BR biosynthetic inhibitor, and decreased by exogenous application of bioactive BRs. When DWF4-GUS was expressed in a different genetic background, its level was down-regulated in brassinazole resistant1-1 (bri1-1), confirming that BRASSINAZOLE RESISTANT1 acts as a negative regulator of DWF4. Interestingly, in the brassinosteroid insensitive2/dwf12-1D background, DWF4-GUS expression was intensified and delocalized to elongating zones of root, suggesting that BRASSINOSTEROID INSENSITIVE2 is an important factor that limits DWF4 expression. Thus, it is likely that the DWF4 promoter serves as a focal point in maintaining homeostasis of endogenous bioactive BR pools in specific tissues of Arabidopsis.

Plants modulate their growth and development through networked actions of phytohormones, and the flexible growth pattern of plants is of great importance especially due to their sessile nature: Plants adapt to different circumstances by adjusting their growth pattern. Of the phytohormones, brassinosteroids (BRs) are relatively recently recognized as an important hormone that promotes cell elongation in various plant organs such as hypocotyls, petioles, pedicels, filaments, leaves, and seeds (Choe, 2004). BRs are plant-originated, polyhydroxylated steroids that are involved in cell elongation, cell division, vascular system differentiation, senescence, and stress tolerance (Altmann, 1998; Clouse and Sasse, 1998; Choe, 2004). In addition, accumulating data suggest that BRs are responsible for light-dependent regulation of plant growth (Neff et al., 1999; Kang et al., 2001; Turk et al., 2003).

Brassinolide (BL), the most active BR, is synthesized from campesterol (CR) via networked biosynthetic pathways (Choe, 2004). Mutants defective in several steps in the biosynthetic pathways display characteristic growth-deficient phenotypes in hypocotyls, petioles, pedicels, inflorescences, and leaves (Choe, 2004). Arabidopsis (Arabidopsis thaliana) DWF4 (DWF4) has been proposed to be a key enzyme that determines flux in BR biosynthesis (Choe et al., 1998). When its expression is completely knocked out, it results in severe growth defects due to deficiency in bioactive BR synthesis (Choe et al., 1998). Conversely, increased flux of BR biosynthesis by 35S:DWF4 overexpression results in a complete opposite phenotype to dag1-1. All the examined organs such as petioles, pedicels, inflorescences, and leaf blades are elongated, and seed yield is noticeably increased (Choe et al., 2001). The slow rate of
the DWF4-mediated step in wild-type Arabidopsis is partly due to tight transcriptional control of this gene.

Previously, Mathur et al. (1998) showed that the expression of the β-glucuronidase (GUS; UidA) reporter gene driven by the promoter of the BR biosynthetic gene CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD; Szekeres et al., 1996), which mediates the immediate next step to DWF4, is temporally and spatially regulated. CPD expression is localized to cotyledons and the uppermost parts of hypocotyls of both dark- and light-grown seedlings (Mathur et al., 1998). In adult plants, CPD:GUS activity was strong in expanding leaf primordia and cauline leaves. In addition, when CPD:GUS plants were grown in the presence of 1 μm epi-BL, GUS activity was rarely detectable, confirming that CPD expression is negatively controlled by BR concentration. In addition, Shimada et al. (2003) showed that several BR biosynthetic genes, such as DWF4 and CYP85 are also feedback regulated by exogenous BRs.

Recently, CYP85A2, a dual function enzyme with BL synthase as well as a BR-6 oxidase activity, was shown to be expressed ubiquitously in young developing tissues including cotyledons, hypocotyls, and roots (Castle et al., 2005). It has also been shown that a BR receptor gene BRASSINOSTEROID INSENSITIVE1 (BRI1) is expressed all over the organs examined (Friedrichsen et al., 2000). However, we previously found that only parts of cells localized to shoot apices of dwf4-1 respond to exogenously applied BRs, suggesting that BR action takes place in a limited number of cells (Choe et al., 1998). To accomplish this localized BR response in Arabidopsis, localized presence of bioactive BRs rather than restricted perception by BRI1 may be required.

To better understand the DWF4-mediated regulation of BR biosynthesis, we analyzed DWF4 expression using tissue-specific RNA gel-blot analysis as well as DWF4:GUS reporter system. Furthermore, to test if the DWF4-expressing tissues are enriched with BRs, we analyzed endogenous BR contents in different tissues of wild type and the BR-accumulating mutant bri1-5.

Regulation of DWF4 expression was also tested by feeding tests and genetic crosses with mutants defective in BR responses. As previously reported by Mathur et al. (1998), DWF4 expression is curbed by exogenous application of BL, suggesting that DWF4 expression may serve as a focal point in quantitative regulation of endogenous bioactive BR levels. DWF4:GUS expression was increased where endogenous levels of BRs are high in bri1-5 mutant background. Our data strongly suggest that localized BR responses in actively growing tissues are closely related to the expression pattern of DWF4.

RESULTS

DWF4 Is Localized to the Endoplasmic Reticulum and Is Rarely Expressed in Specific Tissues

Previously, it has been shown that BR biosynthetic enzyme DWF1 is located in the endomembrane system (Klahre et al., 1998). In addition, eukaryotic cytochrome P450 enzymes are generally located in the endoplasmic reticulum (ER; Schuler, 1996). Thus it is possible that the rate-determining enzyme in BR biosynthesis is targeted to a similar location to perform steroid C-22α hydroxylation. To determine the subcellular localization of DWF4, a full-length cDNA of DWF4 was fused to green fluorescent protein (GFP) in frame and transfected into mesophyll protoplast cells of Arabidopsis and visualized using confocal laser scanning microscopy (Fig. 1A). To express the DWF4 gene in protoplasts, we first used the cauliflower mosaic virus 35S promoter. However, we could hardly see both the GFP image and proteins in western-blot analysis (data not shown). This suggests that the protein is either degraded rapidly or that the GFP-DWF4 transcript has a low translational efficiency. Thus, we switched to a known stronger promoter, CASSAVA VEIN MOSAIC VIRUS (CvMV), resulting in the signal shown in Figure 1. ER-specific localization of DWF4 was shown by examining colocalization with the ER-targeted binding protein (BiP; Koizumi, 1996; Fig. 1, C and D). Subcellular parts that emit green and red fluorescences overlapped to display yellowish color and reported that GFP-DWF4 was primarily positioned in the organelle where BiP accumulates (Fig. 1D).

Next, to examine the spatial and temporal expression pattern of DWF4, RNA gel-blot analysis with tissue-specific RNA was performed. Figure 2A illustrates that DWF4 expression is relatively stronger in tissues such as shoot apex and flower (SAF), roots, and dark-grown seedlings. Axillary buds and undifferentiated calli also showed expression (Fig. 2A). These DWF4-expressing tissues represent plant parts that participate in active growth by cell division and/or expansion. However, when compared to the immediate next-step enzyme, CPD, overall expression level is very low even in actively growing tissues (Fig. 2B).
Tissues Identified by DWF4:GUS Histochemistry Positively Correlate with RNA Gel-Blot Analysis Patterns

Tissue-specific expression of DWF4 in RNA gel-blot analysis was confirmed by the DWF4:GUS reporter system. Two different reporter constructs that include 1,132 bp or 180 bp upstream from the AUG translation start codon, respectively, were made. A select line number 6 stably and consistently expresses the UidA gene in confined tissues over several generations.

Figure 3 displays the spatial and temporal expression pattern of GUS stain in DWF4:GUS seedlings (Fig. 3, A and D). The GUS stain was detected as early as the embryo stage (Fig. 3A). When a silique was fully elongated, a mature embryo was dissected out and stained. Cotyledon margins and radicles were clearly GUS positive (Fig. 3A). To examine developmental changes, the seeds were germinated and grown for different times before being stained. Staining around the cotyledon margins remained until 1 d after germination (DAG; Fig. 3B). In addition, elongating hypocotyls and rudimentary collet tissue (joint tissues of roots and hypocotyls) were also noticeably GUS positive (Fig. 3B). The GUS stain in 3-d-old seedlings was localized to root tips, collets, and emerging leaves of shoot apices, leaving the cotyledonal margins only weakly stained (Fig. 3C).

The GUS staining patterns of the dark-grown seedlings differed from those of the light-grown ones (Fig. 3D). The GUS stain was detectable throughout the cotyledons, suggesting that light participates in controlling the spatial expression of DWF4. In addition to the root tip and collet, the elongating zone of the hypocotyls was also GUS positive in the dark (Fig. 3D; additional figures shown later).

To further examine the developmental regulation of the DWF4 gene expression, plant parts of 4-week-old adult plants were subjected to GUS histochemical assay. In general, actively growing tissues including shoot tips, joints between primary and secondary inflorescences, axillary buds, collets, root tips, and lateral root primordia were clearly stained (Figs. 3 and 4). When a GUS-stained flower was more closely examined, proximal parts of both filaments and gynoecia as well as the junction between stigma and valve were stained (Fig. 3, E–G, arrowheads). Root tips and primordia of lateral roots were all strongly stained (Fig. 3K). In addition, proximal parts of the pedicels were also GUS positive (Fig. 3, E and J, arrows) as early as they are formed.

Figure 2. RNA gel-blot analysis for tissue-specific expression of DWF4. The DWF4 expression is restricted to actively growing tissues. A, RNA gel-blot analysis using total RNA extracted from various tissues of adult plant, dark-grown (3-d-old) seedlings, and callus. Overall, DWF4 transcript level is rarely detectable, but highest in actively growing tissues such as SAF, roots, and axillary bud. Lanes are denoted with Axil for axillary bud; SAF for shoot apical meristem and flower; Siliq for silique; Pedic for pedicle; Roset for Rosette leaves; and Dark for dark-grown seedling. B, The expression level of CPD and DWF4 analyzed by a Gene Atlas tool using microarray data available at Genevestigator (http://www.genevestigator.ethz.ch/; Zimmermann et al., 2004).
in the shoot apices (Fig. 3, E and L, arrows), and remain stained until the siliques mature (Fig. 3J, arrow). GUS stain was not detectable in mature leaves and internodes (Fig. 3, L and N). The collet tissues that possess many emerging primordia of inflorescences displayed the greatest GUS stains (Fig. 3M). Emerging leaf primordia at the shoot tips were first stained throughout the blade, then the stain localized to the expanding hydathodes (Fig. 3, H and L). The axillary buds, shoot apices, roots, and dark-grown tissues that were found to possess \( DWF4 \) transcripts in the RNA gel-blot analysis (Fig. 2) were all clearly stained in the GUS histochemical analysis. This suggests that the 1,132-bp \( DWF4 \) promoter that was used in this study represents \( DWF4 \) gene expression.

The Level of \( DWF4:GUS \) Expression Reflects Exogenous and Endogenous Fluctuation of BR Levels

Previously, we found that \( DWF4 \) transcripts accumulate in both BR biosynthetic and insensitive mutants (Choe et al., 2001). To test if this is reflected in the histochemical pattern of \( DWF4:GUS \) plants, we crossed a \( DWF4:GUS \) line to a biosynthetic mutant \( cpd-388 \) and signaling mutants \( bri1-5 \) and \( bin2/dwf12-1D \). As compared to wild type (Fig. 4A), GUS staining of 7-d-old seedlings was noticeably intensified in \( cpd-388 \) (Fig. 4, A and B) and the \( bin2/dwf12-1D \) (Fig. 4C) mutant background.

Dark-grown seedlings displayed a more severely altered localization pattern. Strong GUS staining was detected in cotyledons, root tips, and hypocotyls immediately underneath the shoot apices (Figs. 5A and 6C). Furthermore, the GUS activity in the \( bin2/dwf12-1D \) was detected throughout the roots and hypocotyl (Fig. 5C) of the dark-grown seedlings. Therefore, expression of the \( DWF4 \) gene has been disrupted in the \( bin2/dwf12-1D \) mutants in the dark.

Recently, the plant-specific nuclear factor BRASSINAZOLE RESISTANT1 (BZR1) has been shown to control \( CPD \) and \( DWF4 \) expression. A gain-of-function mutation of this gene displays phenotypes of constitutive BR signaling, and lowers \( CPD \) expression levels (Wang et al., 2002). To test if the mutation also downregulates \( DWF4 \), the \( DWF4:GUS \) construct was transferred to \( bzr1-D \) by genetic crossing. As shown in Figures 4D and 5D, the overall intensity of the GUS activity is weaker (Fig. 5D) compared to the wild-type control (Fig. 5A). Exogenous application of epi-BL does

Figure 4. \( DWF4:GUS \) histochemical analysis in the light. \( DWF4:GUS \) seedlings in the different BR mutant backgrounds were grown for 7 d in the light before GUS staining. Sections A to D are mock-treated control and E to H are BL-treated seedlings. The strength of GUS stain increased in \( cpd-388 \) (B) and \( bin2/dwf12-1D \) (C), but decreased in \( bzr1-D \) (D) compared to control (A). \( DWF4:GUS \) was down-regulated in response to BL in the control (E), \( cpd-388 \) (F), and \( bzr1-D \) (H), but not in the \( dwf12-1D \) (G). Unit bar = 0.2 mm for roots, 0.5 mm for shoots.

Figure 5. \( DWF4:GUS \) histochemical analysis in darkness. Three-day-old \( DWF4:GUS \) seedlings in different BR mutant background were subjected to GUS staining. Shown are mock-treated control (A, B, C, and D) and BL-treated plants (E, F, G, and H). Intensity of GUS stain increased in \( cpd-388 \) (B) and \( bin2/dwf12-1D \) (C), and decreased in \( bzr1-D \) (D) as compared to a control (A). In the \( bin2/dwf12-1D \) background, additional tissues that are not GUS positive in the wild-type background were also stained. \( DWF4:GUS \) was down-regulated in response to BL in the control (E), \( cpd-388 \) (F), and \( bzr1-D \) (H), but not in the \( bin2/dwf12-1D \) (G). Unit bar = 0.2 mm.
not noticeably change the GUS intensity in brz1-1D background (Fig. 5H).

Furthermore, to test if the increased GUS activity in the dwarf mutants was attributable to decreased endogenous BR levels, we supplemented the plants with epi-BL before examining their GUS activity. When BL was exogenously supplied, DWF4::GUS activity in the cdp-388 was diminished relative to plants without BL treatment (Figs. 4, B and F, and 5, B and F). This GUS activity in BL-treated bin2/dof12-1D was not significantly decreased (Figs. 4, C and G, and 5, C and G), suggesting that the increased staining in these mutants is due not to shortage of endogenous BRs but to lack of feedback down-regulation of the DWF4 gene expression in this signaling mutant.

Modulation of the DWF4 expression levels by BL and Brz was confirmed by reverse transcription (RT)-PCR and RNA gel-blot analysis (Fig. 6A and B). RT-PCR analysis of DWF4 transcripts indicates that BL reduces, whereas Brz increases, the steady-state level of the DWF4 mRNA (Fig. 6A). To test if the endogenous DWF4 promoter activity is consistent with the transgenic DWF4 promoter, we performed the same RT-PCR analysis for the GUS gene. As shown in Figure 6A, similar patterns of induction and suppression were observed. In addition, we examined gene expression of DWF4 by RNA gel-blot analysis. Brz efficiently increases DWF4 transcripts in the DWF4::GUS transgenic line (Fig. 6B, left). Furthermore, to test if the Brz-mediated induction of DWF4 requires functional BRI1, we did RNA gel-blot analysis with total RNA isolated from brie-5/DWF4::GUS plants after treatment with BL or Brz (Fig. 6B, right). None of these treatments significantly changed DWF4 transcript levels in the brie-5 mutant background (Fig. 6B). The increased level of DWF4 transcripts in the brie-5 mutant background, which accumulates BRs compared to wild type, was visualized again at the tissue level. Unlike DWF4::GUS plants, DWF4::GUS staining in the brie-5 mutant background was intensified at the margin of the cotyledons, junction between the shoot and root, and root tips in the light (Fig. 6C, top). In the dark, DWF4::GUS stains in the brie-5 background were throughout the cotyledons, elongation zone of etiolated hypocotyls, and the root tips (Fig. 6C, bottom). Therefore, GUS staining was strongly intensified in the brie-5 mutant background but the expression pattern was basically maintained at the tissue level (Fig. 6C). The stronger staining in brie-5/DWF4::GUS may represent the sites of BL accumulation (Fig. 6C). As predicted based on RNA gel-blot analysis (Fig. 6B), BL-induced DWF4 feedback regulation disappeared in the brie-5 mutant background (Fig. 6C), confirming the requirement of functional BRI1 in feedback regulation of DWF4 both in the light and dark. Intensified staining in brie-5 background clarified where DWF4::GUS directs expression, which is low in wild-type background.

The Mechanism of DWF4 Transcriptional Regulation Is Distinct from That of CPD

Previously, Mathur et al. (1998) reported that transcription of CPD is repressed by BL and this process requires de novo synthesis of proteins, since BL-mediated CPD down-regulation was blocked by treatment with cyclohexamide (CHX). Interestingly, CHX treatment alone induced opposite reactions from CPD and DWF4 in that CHX treatment reduced CPD transcripts (Fig. 7, lane 1 and 2 on CPD), but increased DWF4 (Fig. 7, lane 1 and 2 on DWF4). For both CPD and DWF4, BL effectively down-regulated the transcripts, but BL + CHX attenuated the BL-mediated down-regulation. Similarly, Brz + CHX attenuated the Brz-mediated increase of CPD and DWF4 transcript levels. These data strongly suggest that CPD and DWF4 expression is differentially regulated: CPD expression is controlled primarily by transcriptional activation but DWF4 expression is controlled by strong inhibitory mechanism. However, involvement of specific posi-
reduced level of staining relative to Brz treatment alone (5). In contrast, CHX treatment combined with Brz (6) resulted in increased upon CHX treatment (2 and 4) compared to the controls (1 and 3). The effect of CHX on DWF4 expression was similar pattern as CPD is slightly attenuated by CHX (3 and 4 of CPD) and BL-mediated down-regulation of CPD is suggested that repressors are de novo synthesized to maintain relatively low level of DWF4 compared to CPD (1 and 2 of DWF4), otherwise the CHX treatment results in a similar pattern as CPD. An rRNA row is shown as a loading control. B, The effect of CHX on DWF4 expression was confirmed using DWF4-GUS transgenic lines. GUS stains in the root tip (top sections) and lateral root (bottom sections) were significantly increased upon CHX treatment (2 and 4) compared to the controls (1 and 3). In contrast, CHX treatment combined with Brz (6) resulted in reduced level of staining relative to Brz treatment alone (5).

Changes in sensitivity or concentration of phyto-hormones are two major mechanisms that trigger signal transduction pathways in specific tissues. Increased sensitivity can be achieved through production of receptor molecules or by elevating pools of bioactive signaling molecules. BRs are perceived by the plasma membrane-localized receptor BRI1 and trigger a specific signaling cascade. However, it has been shown that the expression of the BRI1 gene is not localized to specific tissues of Arabidopsis, but is ubiquitous (Friedrichsen et al., 2000). Furthermore, CYP85A2, the enzyme mediating the ultimate step in BL biosynthesis, is also broadly expressed in Arabidopsis tissues (Castle et al., 2005). In addition, CPD, an immediate next enzyme to DWF4, is also highly and ubiquitously expressed (Mathur et al., 1998; Fig. 2B). Thus, the enriched BR levels in the specific tissues may be due not to CY85A2 or CPD expression but to a localization of the rate-determining enzyme DWF4.

Previously, we found that only limited tissues in shoot tips of the dwarv4-1 inflorescences respond to applied BRs by dramatic elongation of internodes (Choe et al., 1998). This suggests that the young competent cells clustered in the shoot tip respond to BR concentration and trigger BR-dependent elongation responses. To accomplish this localized response, a pool of bioactive BRs needs to be maintained in these specific tissues.

BR content is almost quadrupled in root tissues of seedlings (Table I), and the BL level in the shoot tip scores approximately 20-fold greater than in the stem of bri1-5. This clearly indicates that the pool of bioactive BRs accumulates in specific tissues, and localized response to BRs is induced by increased concentration of bioactive BRs in these tissues. In addition, the DWF4 gene expression pattern supports that the increased pool of BRs in these tissues may originate from de novo biosynthesis. The DWF4-expressing tissues revealed by the GUS histochemical analysis of CHX effects on CPD and DWF4 transcription. After 2 h pretreatment with 100 μM of CHX, culture media were supplemented with 1 μM epi-BL or 1 μM Brz. After 6 h incubation, seedlings were harvested and used for RNA isolation. A, CHX treatment reduces the steady-state levels of the CPD transcripts (lanes 1 and 2 of CPD) and BL-mediated down-regulation of CPD is slightly attenuated by CHX (3 and 4 of CPD). Brz increases CPD level, but CHX inhibits this increase (5 and 6 of CPD). In contrast, CHX treatment increased DWF4 level (1 and 2 of DWF4), suggesting that a loading control. B, The effect of CHX on DWF4 expression was confirmed using DWF4-GUS transgenic lines. GUS stains in the root tip (top sections) and lateral root (bottom sections) were significantly increased upon CHX treatment (2 and 4) compared to the controls (1 and 3). In contrast, CHX treatment combined with Brz (6) resulted in reduced level of staining relative to Brz treatment alone (5).

### DISCUSSION

**DWF4 Is the Essential Enzyme That Determines the Tissue Specificity of BR Biosynthesis and Size of the BR Pool**

Endogenous BR Levels Are Increased in Actively Growing Tissues

DWF4 is an important flux-regulating enzyme in BR biosynthetic pathways, thus it is conceivable that its expression reflects the tissues of BR biosynthesis. To compare endogenous levels of BR biosynthetic intermediates in different tissues, rosettes, siliques, collets, stems, and shoot tips, were collected from 25-d-old Arabidopsis wild-type plants. Table I shows the endogenous levels of each of 13 biosynthetic intermediates in seven different tissues. In seedlings, roots show greater C28 sterol and BR contents than shoots. Of the five adult tissues, the shoot tip unequivocally has the greatest sterol level. However, differently from the sterol levels, collets have approximately 5-fold more BR than siliques. This enrichment of BR content is correlated with a greater level of DWF4-GUS expression in these tissues (Fig. 3M).

Successful detection of BL using gas chromatography/mass spectrometry generally requires more than 100 g of fresh tissue, which is labor intensive to collect from small tissues such as the Arabidopsis shoot tip. Thus we chose to analyze the tissues of the bri1-5 mutant to detect BL in different tissues, because it is known that bri1-5 accumulates BR biosynthetic intermediates and BL (Noguchi et al., 1999). Table II summarizes the endogenous levels of BRs in six different tissues of Arabidopsis bri1-5 mutants. The seeds and shoot tips possess significantly enriched amounts of BRs compared to other tissues.
tochemical analysis identified tissues with a greater amount of BR levels than those organs that have less DWF4 gene expression. Therefore, tissue specificity of BR biosynthesis, accumulation, and localized response of BRs is likely to be imparted by DWF4 expression.

BL-Induced Feedback Regulation of DWF4 Requires Functional BRI1

Several BR biosynthetic enzymes including DWF4, CPD, and CYP85 were shown to be feedback regulated upon BR treatment at the transcriptional level (Bancos et al., 2002; Shimada et al., 2003). Previously, feedback regulation of CYP90A, which is highly homologous to CYP85, was shown to be dependent on functional BRI1 since BL had no effect on the expression of CYP90A in the BR-insensitive cbb2 (allelic to bri1; Bancos et al., 2002). We found that DWF4::GUS was significantly up-regulated and was not down-regulated upon BL treatment in the bri1-5 and bin2/dwf12-1D mutant background (Figs. 4, 5, and 6). In addition, endogenous levels of BRs were significantly increased in bri1-5 tissues expressing high levels of DWF4 (Table II). Therefore, it is likely that BL-induced feedback regulation of DWF4 requires functional BRI1 and increased level of DWF4 expression is responsible for the increase of BR pools in this mutant.

Table I. Different levels of sterols and BRs in tissues of the Ws-2 wild type

The two rows of text in bold indicate the summed total of sterol (24-MC, CR, CN, and 6-deoxoCN) or BRs (from 6-DeoxoCT [CT] to BL), respectively. nd, Not detected; 24-MC, 24-Methylenecholesterol; CR, Campestanol; CN, 6-OxocN, 6-Oxocampestanol; 6-DeoxoCT, 6-Deoxocastasterone; 6-DeoxtET, 6-Deoxoestearosterone; 6-DeoxoTY, 6-Deoxophytosterol; 6-DeoxoCS, 6-Deoxocastasterone.

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<th>Callet</th>
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<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>BR content</td>
<td>5.04</td>
<td>21,944</td>
<td>6.44</td>
<td>30.2</td>
<td>8.0</td>
<td>8.5</td>
<td>9.3</td>
</tr>
</tbody>
</table>

Table II. BR levels in different tissues of bri1-5

Abbreviations are defined in the Table I legend. The two rows of text in bold indicate the summed total of sterol (24-MC, CR, CN, and 6-deoxoCN) or BRs (from 6-DeoxoCT [CT] to BL), respectively.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Silique</th>
<th>Shoot Tip</th>
<th>Stem</th>
<th>Rosette</th>
<th>Cauline</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-MC</td>
<td>5,170</td>
<td>6,070</td>
<td>92,800</td>
<td>3,430</td>
<td>10,600</td>
</tr>
<tr>
<td>CR</td>
<td>56,700</td>
<td>295,000</td>
<td>82,400</td>
<td>42,900</td>
<td>30,100</td>
</tr>
<tr>
<td>CN</td>
<td>4,700</td>
<td>2,590</td>
<td>2,430</td>
<td>2,440</td>
<td>1,530</td>
</tr>
<tr>
<td>6-OxocN</td>
<td>123</td>
<td>227</td>
<td>100</td>
<td>45</td>
<td>37</td>
</tr>
<tr>
<td>C28 sterol content</td>
<td>66,693</td>
<td>303,887</td>
<td>177,730</td>
<td>48,815</td>
<td>42,267</td>
</tr>
<tr>
<td>6-DeoxoCT</td>
<td>7.58</td>
<td>85.6</td>
<td>26.6</td>
<td>1.73</td>
<td>4.22</td>
</tr>
<tr>
<td>6-DeoxoET</td>
<td>0.3</td>
<td>1.34</td>
<td>3.24</td>
<td>0.43</td>
<td>0.46</td>
</tr>
<tr>
<td>6-DeoxoTY</td>
<td>5.72</td>
<td>0.99</td>
<td>8.32</td>
<td>4.66</td>
<td>2.28</td>
</tr>
<tr>
<td>6-DeoxoCS</td>
<td>6.91</td>
<td>5.81</td>
<td>18.3</td>
<td>7.81</td>
<td>4.01</td>
</tr>
<tr>
<td>CT</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>TE</td>
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<td>0.13</td>
<td>0.08</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>TY</td>
<td>3.92</td>
<td>0.7</td>
<td>5.96</td>
<td>0.77</td>
<td>0.72</td>
</tr>
<tr>
<td>CS</td>
<td>9.13</td>
<td>7.22</td>
<td>48.1</td>
<td>2.99</td>
<td>2.34</td>
</tr>
<tr>
<td>BL</td>
<td>1.79</td>
<td>4.58</td>
<td>4.29</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>BR content</td>
<td>35.4</td>
<td>106.9</td>
<td>114.9</td>
<td>18.7</td>
<td>14.5</td>
</tr>
</tbody>
</table>
BZR1 Represses DWF4 Expression

According to Wang et al. (2002), the brassinazole resistant mutation \textit{bzz1-D} effectively down-regulates \textit{CPD} transcript levels, suggesting that BZR1 plays a role as a repressor of \textit{BR} biosynthetic gene \textit{CPD}. Recently, BZR1 has been proposed as a transcriptional regulator that can bind directly to \textit{CPD} and act as repressor (He et al., 2005). Interestingly, the expression pattern of BZR1 and \textit{DWF4} is mutually exclusive. The tissues just below the apical hook are noticeably stained for \textit{DWF4:GUS} (Fig. 6C), whereas the corresponding regions in the \textit{bzz1-D} mutant are not stained (Fig. 5D; Wang et al., 2002). Therefore, the \textit{DWF4:GUS} staining pattern alternative to the BZR1 protein expression suggests that BZR1 plays an important role as a repressor of \textit{DWF4}. Previously, Wang et al. (2002) showed that the \textit{bzz1-D} protein is stable and present in these tissues where BZR1 is not. In our studies, \textit{DWF4:GUS} activity was lower than corresponding control in the light- and dark-grown seedlings of \textit{bzz1-D}. These results imply that BZR1 is a repressor of \textit{DWF4} expression.

BIN2/DWF12 Directs Localized Expression of DWF4

\textit{BIN2/DWF12} encodes a highly conserved Ser/Thr kinase that negatively regulates \textit{BR} responses by phosphorylating two positive regulators, BZR1 and BZR2/BES1, to be targeted for degradation (Li and Nam, 2002; Zhao et al., 2002). In the \textit{dwf12-1D} mutant background, \textit{DWF4:GUS} was significantly up-regulated, especially in the dark. In addition, upon BL treatment, \textit{DWF4:GUS} was not significantly down-regulated either in the light or dark. Since \textit{dwf12-1D} has increased \textit{BR} level (Choe et al., 2002), the up-regulation of \textit{DWF4:GUS} in this mutant background is probably due to the lack of feedback regulation. Unlike \textit{CPD} or \textit{bri1-5} mutant background, localization of \textit{DWF4:GUS} was disrupted severely in the roots of \textit{dwf12-1D} (Fig. 5, C and G). This is probably due to the enhanced degradation of repressor BZR1, whose phosphorylation and subsequent degradation is activated in \textit{dwf12-1D}. The mislocalized pattern of the \textit{DWF4:GUS} gene is obvious in the \textit{d wf12-1D} background. For example, GUS stain seems to change the pattern from the vasculature (Fig. 5C, mock) to epidermal cell layer (Fig. 5G, BL treated) in the dark-grown \textit{d wf12-1D} mutant seedlings. In addition, epidermal staining was also found in cotyledon margins of all four mutants tested (Figs. 5, F–H, and 6C). Taken together with more predominant ectopic expression of \textit{DWF4:GUS/ d wf12-1D} in the dark, light seems to be the key regulator involved in the regulation of \textit{DWF4} expression via regulation of \textit{BIN2/DWF12}.

Regulation of DWF4 Transcription Is Distinct from That of CPD

\textit{GUS} histochemical analysis patterns of \textit{DWF4} and \textit{CPD} are biphasic; they have overlapping expression, but also distinct tissues. Like \textit{CPD}, \textit{DWF4:GUS} activity is seen in the emerging leaf primordia and margins of developed leaves (Mathur et al., 1998). However, differently from \textit{CPD}, \textit{GUS} activity driven by the \textit{DWF4} promoter is obvious in the dark-grown cotyledons, whereas light-grown cotyledons have only their margins and hydathodes stained (Fig. 3, A and H). In addition, \textit{DWF4:GUS} expression was detected in root tips, whereas \textit{CPD:GUS} was not (Mathur et al., 1998). Regulatation of \textit{CPD} and \textit{DWF4} seems to be quite distinct since they have different tissue/organ specificity as revealed by this study and patterns identified by Mathur et al. (1998). In addition, the mechanisms of \textit{DWF4} and \textit{CPD} gene expression are quite distinct at the transcriptional level, since CHX treatment increases \textit{DWF4} transcripts, but decreases \textit{CPD} transcripts. This strongly suggests that \textit{CPD} expression is regulated mainly by transcriptional activator but \textit{DWF4} expression is by strong inhibitory mechanism. Thus, inhibition of protein synthesis by CHX treatment resulted in opposite effects on the \textit{DWF4} and \textit{CPD} gene transcription.

Involvement of BZR1 as a repressor and localized expression of the \textit{DWF4} gene was clearly demonstrated in this study. However, BZR1-mediated inhibition of \textit{DWF4} seems to be only one among various inhibition mechanisms since the transcript level of \textit{DWF4} is extremely labile; GFP fluorescence by transcription are directly controlled in this study. However, BZR1-mediated inhibition of \textit{DWF4} cannot be excluded, as implicated by differential effects of CHX on \textit{DWF4} and \textit{CPD}. Further studies should be focused on the identification of inhibitory or activating components that are involved in \textit{DWF4} transcriptional regulation.

In conclusion, unlike other genes involved in \textit{BR} biosynthesis and signal transduction pathways, such as \textit{CPD}, \textit{BL synthase} (\textit{CYP85A2}), and \textit{BRI1}, \textit{DWF4} expression is tightly regulated in specific tissues of \textit{Arabidopsis}. Its expression is feedback down-regulated by bioactive \textit{BRs}. Reduction in endogenous \textit{BR} levels in a \textit{cpd-388} mutant background or by treatment with Brz induces \textit{DWF4} expression, but exogenous application of \textit{BRs} decreases \textit{DWF4} expression. Both transcriptional and posttranscriptional regulations of \textit{DWF4} are important means that determine the level of bioactive \textit{BR}. In this study, we found that \textit{DWF4} protein is extremely labile; GFP fluorescence by transient expression in protoplasts through known strong promoter cauliflower mosaic virus 35S either at N- or C-terminal fusions are hardly detectable. We could detect GFP only by expression using a far stronger promoter, Cmv. In addition, previously we showed that the levels of \textit{DWF4} transcription are directly responsible for the hypocotyl length (Choe et al., 2001). Knock-out mutant, wild type, and 35S\textit{DWF4} gradually, in this order increased the hypocotyl length proportional to the level of \textit{DWF4} expression. More im-
portantly, we showed that the DWF4::GUS-expressing tissues are positively correlated with the organs that possess a higher level of bioactive BRs. The organs such as collets and roots express DWF4 and thus contain elevated amounts of bioactive BRs. Thus it is conceivable that Arabidopsis meets the requirement of BR-dependent growth through precise regulation of the DWF4 gene expression. Identification and functional characterization of the trans-acting factors that act on the DWF4 promoter should reveal the mechanism as to how the environmental and developmental signals regulate DWF4 gene expression.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Transgenic Plants Harboring DWF4::GUS Constructs

Two different reporter constructs were made. DNA fragments of 1,132 bp and 180 bp upstream from AUG translation start codon, respectively, were placed in front of the GUS (uidA) reporter gene in the pBI101 binary vector. These constructs were introduced into Arabidopsis (Arabidopsis thaliana) Wassilewskija-2 (Ws-2) wild-type plants in standard callus-inducing media. The conditions for PCR amplification were an 5-bromo-4-chloro-3-indolyl-β-D-GlcUA according to the method described in Jefferson et al. (1987).

Through genetic crosses, the 1,132 bp promoter-containing DWF4::GUS construct was introduced into various genotypes including cpd-388, dhsf1-2D, bri1-1D, and bri1-5. Putative DWF4::GUS lines in the different genetic backgrounds were selected from F2 populations that show both morphological phenotypes and kanamycin resistance. Doubly homozygous lines for each mutation and the DWF4::GUS transgene were further confirmed at the F3 generation. The doubly homozygous lines were plated on agar-solidified medium supplemented with 0.1 μM BL and grown for 8 (light) and 3 (dark) days before GUS staining.

Feeding Tests, RT-PCR, and RNA Gel-Blot Analysis

Conditions for plant growth and feeding tests were described previously in Kwon et al. (2005). Briefly, the Arabidopsis ecotype Ws-2 seeds were surface sterilized with solution containing 20% (v/v) Clorex and 0.1% (w/v) SDS for 10 min and washed five times with sterile distilled water. The seeds were germinated in Murashige and Skoog liquid media (1× Murashige and Skoog basal salt mixture, 1% (w/v) Suc, pH 5.8 with KOH) for 7 d in a growth chamber (16-h light/8-h dark at 23°C) with continuous shaking at 100 rpm. The seedlings were pretreated with 100 μM of CHX for 2 h and then the culture media were supplemented with 1 μM ppi-BL and 1 μM Brz for further 6 h of incubation with continuous shaking at 100 rpm. The harvested seedlings were quickly frozen in liquid nitrogen before proceeding to total RNA isolation. Total RNA was isolated using TRIzol reagent (Sigma) and further purified again using phenol-chloroform extraction. The purified total RNA (2 μg) was used for first-strand cDNA synthesis using the Superscript RNaseH- reverse transcriptase (Invitrogen). The PCR conditions for DWF4 amplification were an initial denaturation step for 5 min at 95°C, followed by 25 cycles of 15 s at 94°C, 30 s at 50°C, and 1 min at 72°C, followed by final extension for 5 min at 72°C. The primers and nucleotide sequences used for RT-PCR analysis are as follows; for DWF4, DHTFE, 5'-TCTCTTCCTGACACCATGCTGATATCTCTC-3', and for CDP, CPDRT-F1, 5'-GTTAATCTTTGCTGATGATCT-3', CPDRT-R1, 5'-ATTATATACCTT-GATCTCAACAT-3'. The amplified PCR products were separated on a 1.2% (w/v) agarose gel, transferred to a nylon membrane, and probed with a 32P-labeled probe containing a 3′-end of DWF4 and CDP genes. For staining with GUS after CHX treatment, 100 μM of CHX and 100 μM of BL and 1 μM Brz. After 24 h incubation, seedlings were stained overnight using 5-bromo-4-chloro-3-indolyl-β-D-GlcUA according to the method described in Jefferson et al. (1987).

To determine the DWF4 transcript level, 15 μg of total RNA isolated from various tissue types of mature Arabidopsis, 5-d-old dark-grown seedlings, and 3-week-old callus in standard callus-inducing media were fractionated in denaturing gel and subjected to the RNA gel-blot analysis according to the standard protocol (Sambrook et al., 1989).

Endogenous BR Analysis

For analysis of endogenous levels of BRs in different tissues, Arabidopsis plants were grown on soil (SUNSHINE MIX no. 5, SunGro) for 5 weeks under a long-day condition. Mature silique, the joint tissues of shoot and root, shoot tips including developing floral organs, stems, and rosette leaves of 5-week-old Ws-2 wild-type plants were separated to collect up to 30 g of fresh tissues. Similarly, different tissues of bri1-5 were collected from population of 7-week-old plants. To obtain shoot and root tissues of seedlings, Ws-2 seeds were plated in a row on agar-solidified medium, and the plates were placed in a vertical position to have the roots to grow on the surface of agar media. The root and shoot tissues were separated with a razor blade before freezing in liquid nitrogen and further processing for steroid purification.

BR purification and quantification were carried out according to the method described by Noguchi et al. (1999). Gas chromatography/mass spectrometry analysis was done on a mass spectrometer (QMS-AM SUN200, JEOL) connected to a gas chromatograph (6890A, Agilent Technologies) with a capillary column DB-5 (0.25 mm × 15 m, 0.25 μm film thickness, J&W Scientific).

Subcellular Localization of DWF4

The DWF4 cDNA was obtained by RT-PCR, and cloned as an N-terminal GFP fusion in a plant expression vector containing CoMV promoter (Verdaguer et al., 1998) and nopaline synthase terminator. The CoMV-GFP::DWF4 construct was cotransfected with the 3SS:B-RFP that is an ER marker protein (Kotzuma, 1996) into Arabidopsis mesophyll protoplasts by polyethylene glycol-mediated transformation as described by Hwang and Sheen (2001). GFP and red fluororescent protein (RFP) fluorescences were observed using a confocal laser scanning microscope (Carl Zeiss LSM 510). The filter sets, BP505 to 530 (excitation 488 nm, emission 505–530 nm), BP560 to 615 (excitation 543 nm, emission 560–615 nm), and LP650 (excitation 488 nm, emission 650 nm) for GFP, RFP, and the chlorophyll autofluorescence, respectively, were used.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number U12639.

ACKNOWLEDGMENTS

We thank Dr. Joanne Chory for her gift of bri1-1D, and Dr. Tadao Asami for provision of Bzr. The authors are grateful to Mi-Ok Lee, Kee-Hong Song, Hyeon Kyung Lee, Yanya Kim, Makoto Kobayashi, and Masayo Sekimoto for their technical assistance.

Received July 1, 2005; revised December 19, 2005; accepted December 19, 2005; published January 11, 2006.

LITERATURE CITED


mediates multiple 22α-hydroxylation steps in brassinosteroid biosynthesis. Plant Cell 10: 231–243


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Tissue-Specific Expression of DWARF4