Brassinosteroid Homeostasis in Arabidopsis Is Ensured by Feedback Expressions of Multiple Genes Involved in Its Metabolism

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Homeostasis of brassinosteroids (BRs) is essential for normal growth and development in higher plants. We examined responsiveness of 11 BR metabolic gene expressions to the decrease or increase of endogenous BR contents in Arabidopsis (*Arabidopsis thaliana*) to expand our knowledge of molecular mechanisms underlying BR homeostasis. Five BR-specific biosynthesis genes (*DET2*, *DWF4*, *CPD*, *BR6ox1*, and *ROT3*) and two sterol biosynthesis genes (*FK* and *DWF5*) were up-regulated in BR-depleted wild-type plants grown under brassinazole, a BR biosynthesis inhibitor. On the other hand, in BR-excessive wild-type plants that were fed with brassinolide, four BR-specific synthesis genes (*DWF4*, *CPD*, *BR6ox1*, and *ROT3*) and a sterol synthesis gene (*DWF7*) were down-regulated and a BR inactivation gene (*BAS1*) was up-regulated. However, their response to fluctuation of BR levels was highly reduced (*DWF4*) or nullified (the other eight genes) in a *bri1* mutant. Taken together, our results imply that BR homeostasis is maintained through feedback expressions of multiple genes, each of which is involved not only in BR-specific biosynthesis and inactivation, but also in sterol biosynthesis. Our results also indicate that their feedback expressions are under the control of a BRI1-mediated signaling pathway. Moreover, a weak response in the mutant suggests that *DWF4* alone is likely to be regulated in other way(s) in addition to BRI1 mediation.

Brassinosteroids (BRs) are steroid hormones that play important roles in plant growth and development activities that include stem elongation, leaf expansion, vascular differentiation, stress tolerance, and senescence (Clouse and Sasse, 1998). In the past decade, molecular geneticists have discovered and characterized many BR-related genes that function in BR biosynthesis, inactivation, and signaling (Bishop and Yokota, 2001; Clouse, 2002). Furthermore, recent transcriptome studies have identified numerous BR-responsive genes (Hu et al., 2001; Goda et al., 2002; Mussig et al., 2002). These studies have provided insight into molecular mechanisms of BR action.

It is crucial to adjust to and maintain appropriate levels of endogenous BRs for normal plant growth and development. For example, BR-deficient mutants of several plants exhibit strong dwarfism with curly and dark-green leaves in the light and deetiolation with short hypocotyls and open cotyledons in the dark (Bishop and Yokota, 2001). Abnormal growth that is apparent in the mutants is attributable to the constitutive decrease of endogenous BRs. Administration of excess amounts of bioactive BRs such as brassinolide (BL) and castasterone also causes abnormal organ growth, such as swelling and twisting of hypocotyls and roots in both light and dark (Hata et al., 1986; Wang et al., 2002; Tanaka et al., 2003). These results anticipate that endogenous BR contents are checked and balanced constantly via control of BR biosynthesis and inactivation rates in normally growing plants.

Recently, several studies using Arabidopsis (*Arabidopsis thaliana*) have shown that expressions of some BR metabolic genes are modulated at mRNA levels for BR homeostasis. For instance, the mRNAs of BR biosynthesis genes such as *DWARF4* (*DWF4*) and *CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM* (*CPD*) increase in response to lowered amounts of endogenous BRs in BR-deficient mutants as well as in wild-type Arabidopsis treated with a BR biosynthesis inhibitor, brassinazole (Brz; Noguchi et al., 2000; Asami et al., 2001; Choe et al., 2001). In contrast, mRNAs of *DWF4* and *CPD* are decreased rapidly; that of a BR inactivation gene, *phyB activation-tagged suppressor1* (*BAS1*), is increased when BL is applied to wild-type plants (Mathur et al., 1998; Goda et al., 2002). Furthermore, it has been reported that BL-induced down-regulation of *CPD* expression is annulled in BR-insensitive *bri1* mutants in which BR perception is defective (Li et al., 2001; Bancos et al., 2002), suggesting that feedback expressions of BR metabolic genes require BR-insensitive1 (BRI1) function. Indeed, accumulation of biologically active BRs and mRNA elevation of BR biosynthesis genes

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DEETIOLATED2 (*DET2*), *DWF4*, and *CPD* are observed in *bri1* mutants (Noguchi et al., 1999, 2000; Choe et al., 2001; Bancos et al., 2002). Similar results have also been reported for other plant species including pea (*Pisum sativum*), rice (*Oryza sativa*), tomato (*Lycopersicon esculentum*), and barley (*Hordeum vulgare*; Nomura et al., 1997; Yamamuro et al., 2000; Montoya et al., 2002; Chono et al., 2003).

In this study, we attempted to examine the expression of 11 BR metabolic genes in Brz-treated and BL-treated Arabidopsis seedlings to better understand molecular mechanisms underlying BR homeostasis. We report here that mRNA levels of the nine genes were altered in response to temporarily fluctuating endogenous BR contents and that intact BRI1 function is necessary for their respective feedback expressions.

RESULTS

Effect of Brz on Expression of BR Metabolic Genes in Wild-Type Arabidopsis

Phytohormones' homeostasis, including BR's, should be attained by balance of their biosynthesis and inactivation rates. Therefore, to elucidate the feedback control of BR homeostasis at mRNA levels, we examined the expression of BR metabolic genes in response to alternation of endogenous BR contents, which are involved either in sterol biosynthesis, BR-specific biosynthesis, or BR inactivation pathways.

We used Brz to reduce endogenous BR contents (Asami and Yoshida, 1999). Figure 1 shows that seedling growth was apparently retarded when Brz was given to 14-d-old Arabidopsis plants. Growth arrest of the leaf petioles was most evident; they did not elongate at all in the presence of Brz, irrespective of Brz concentrations of 1 or 5 μM (Fig. 1A). Morphological observation further disclosed dwarfism of Brz-treated seedlings accompanied by typical characteristics of BR mutants, such as curly and dark-green leaves and shortened leaf blades and petioles (Fig. 1B). These morphological aberrations became detectable 2 d after Brz administration (Fig. 1B, subsections a and c). The aberrations were apparent after 4 d (Fig. 1B, subsections b and d). Abnormality was recovered by feeding of BL (data not shown). These traits of Brz-treated seedlings resembled those of the previously reported BR-deficient mutants (Li et al., 1996; Szekeres et al., 1996). Therefore, endogenous BR contents of the Brz-treated seedlings are likely to be decreased in our condition.

We performed reverse transcription (RT)-PCR analysis to examine mRNA levels of 11 BR metabolic genes in 5 μM Brz-treated seedlings. Figure 2 shows that three BR-specific biosynthesis genes, *DET2*, *DWF4*, and *CPD*, were up-regulated in response to Brz, which is consistent with accumulation of their mRNAs in BR-deficient mutants (Noguchi et al., 2000; Choe et al., 2001). In addition, we discovered Brz-induced expressions of

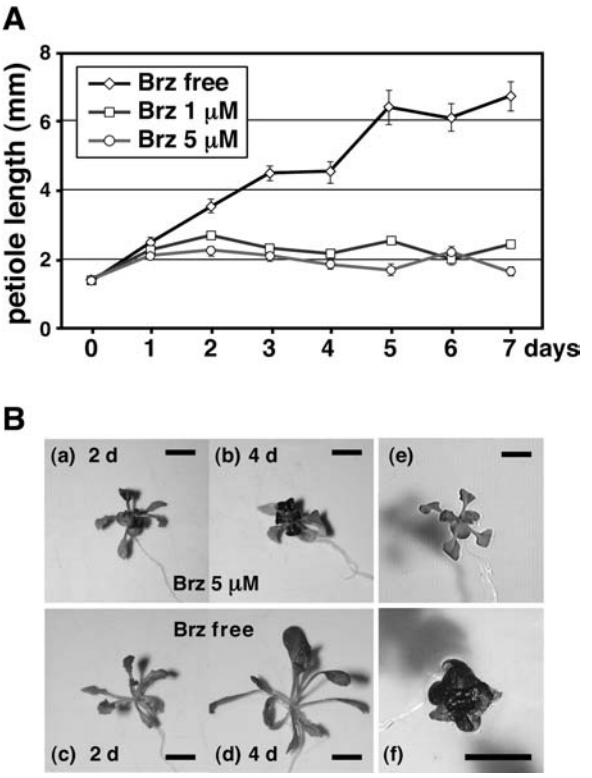

Figure 1. Effects of Brz on the growth of Arabidopsis seedlings. Wild-type seedlings (14 d old) were cultured in the presence of 1 or 5 μM Brz for the indicated days as described in "Materials and Methods." A, Lengths of the third and fourth leaf petioles. Each value represents mean of 20 seedlings with SE. B, Photographs show wild-type seedlings treated with (subsections a and b) or without (subsections c and d) Brz. Nontreated wild-type (subsection e; 14 d old) and *bri1-401* mutant seedlings (subsection f; 14 d old) are shown as controls. Scale bars indicate 5 mm.

the other two BR-specific biosynthesis genes, *BR-6-oxidase* (*BR6ox1*) and *ROTUNDFOLIA3* (*ROT3*). Interestingly, expressions of *FACKEL* (*FK*) and *DWARF5* (*DWF5*) were induced remarkably by Brz application, both of which function in sterol biosynthesis to provide precursor compounds of BR production (Schaller, 2003).

Brassinazole-induced expression differed kinetically among the seven genes (Fig. 2). These genes can be classified into at least two groups. The first group of genes includes *FK*, *DWF5*, *DET2*, *DWF4*, and *BR6ox1*, for which inductions were recognized 1 d after Brz treatment. The second group of genes, including *CPD* and *ROT3*, were induced 2 to 3 d after Brz administration. We chose *DWF4* and *CPD* as representatives of the two groups and further analyzed their expressions in the early period of Brz treatment to confirm differences in their induction modes. Figure 3 shows that *DWF4* expression was increased following 1 h lag time. It attained a plateau 8 h after Brz addition. In contrast, *CPD* expression was not up-regulated, even after 24 h.

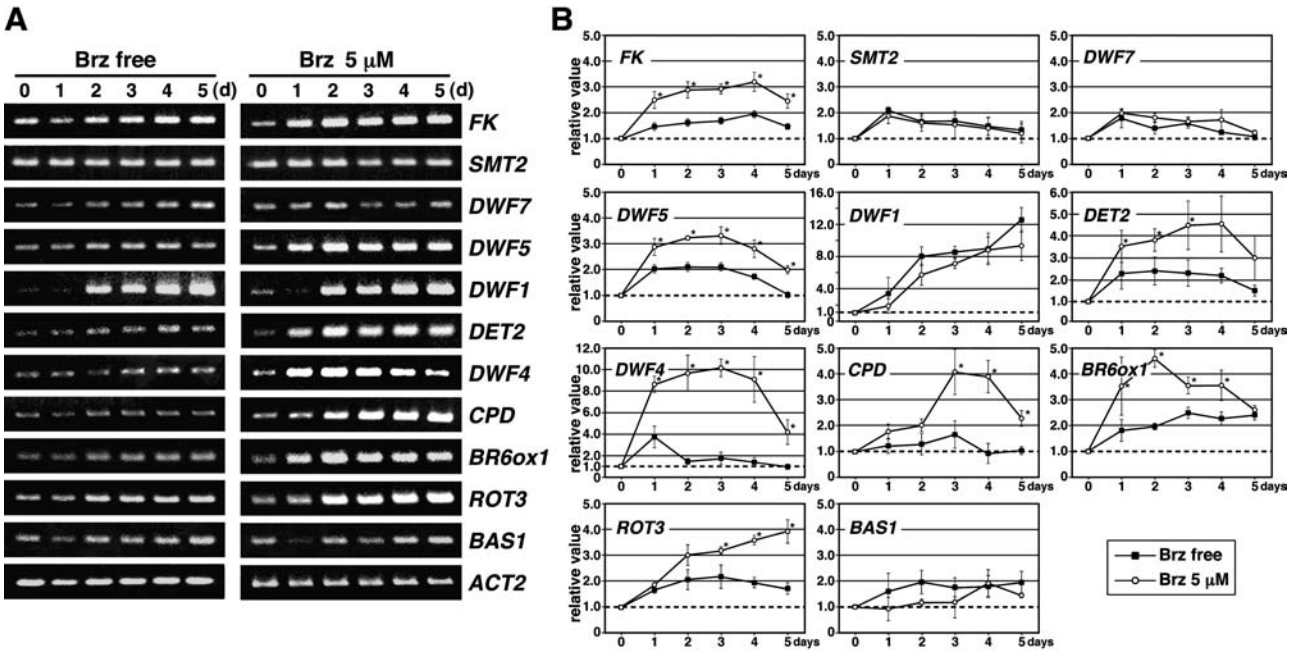

Figure 2. Expressions of BR metabolic genes in response to Brz in Arabidopsis seedlings. RT-PCR analysis was performed to examine steady-state levels of mRNAs for 11 BR metabolic genes in Brz-administered wild-type seedlings. Amplified PCR products were fractionated through an agarose gel and stained with ethidium bromide. Fluorescence on the gel was scanned with a fluoro-image analyzer. Fluorescence intensity of each band was measured and converted into a relative value [initial (0 d) = 1.0] after normalization with that of *ACT2* band used as an internal control. A, Photographs show representative gel images in three replicated experiments. B, Each value in the graphs shows mean with SE of three experiments. Asterisks indicate statistically significant differences compared with nontreated control ($*P < 0.05$).

Effect of BL on Expression of BR Metabolic Genes in Wild-Type Arabidopsis

Following examination of Brz-affected expression of BR metabolic genes, we tested the effect of exogenously applied BL on their expressions. It has long been argued that endogenous phytohormones often perturb effects of exogenous identical hormones (Mussig and Altmann, 2003). Therefore, we used Brz prior to BL application for reduction of endogenous BR contents. As mentioned before, Brz up-regulated five BR metabolic genes and caused growth aberrations of the seedlings within 2 d of Brz administration (Figs. 1 and 2), suggesting that endogenous BR contents in the seedlings were reduced sufficiently to bring about these phenomena by that time. Based on these results, we applied BL to Arabidopsis seedlings that had been cultured for 2 d in the presence of Brz.

We performed RT-PCR analysis to quantify mRNA levels of BR metabolic genes in the BL-treated seedlings described above. Figure 4 shows that expressions of four BR-specific biosynthesis genes, *DWF4*, *CPD*, *BR6ox1*, and *ROT3*, were reduced by 0.1 μM BL treatment. These repressions were apparent 1 to 2 h after BL administration and reached their lowest levels within 4 h. The *DWF4* and *CPD* expressions were decreased most remarkably: they shrank to less than 7% of their initial levels. Reductions of *BR6ox1* and *ROT3* expressions were more moderate than those of *DWF4* and *CPD*. The *DET2* expression was not altered by BL supplementation (Fig. 4), but it responded to Brz treatment (Fig. 2). On the other hand, expression of *BAS1* involved in BR inactivation was elevated rapidly 1 h after BL administration; it reached a plateau level

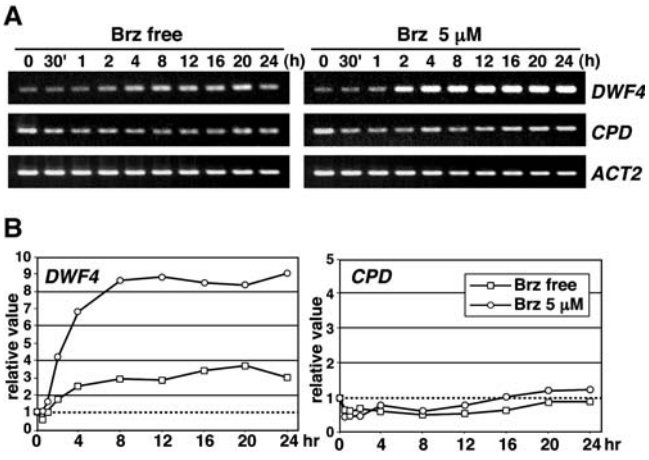

Figure 3. Kinetic difference between *DWF4* and *CPD* expressions in Brz-applied Arabidopsis seedlings. RT-PCR analysis was performed to compare the expression of *DWF4* and *CPD* in an early period of Brz treatment. Experimental procedures are identical to those in Figure 2. A, Gel photographs show amplified PCR bands with those of *ACT2*. B, Fluorescence intensity of each band is shown as a relative value [initial (0 h) = 1.0] after normalization with that of *ACT2* band. Results are representative of three independent experiments.

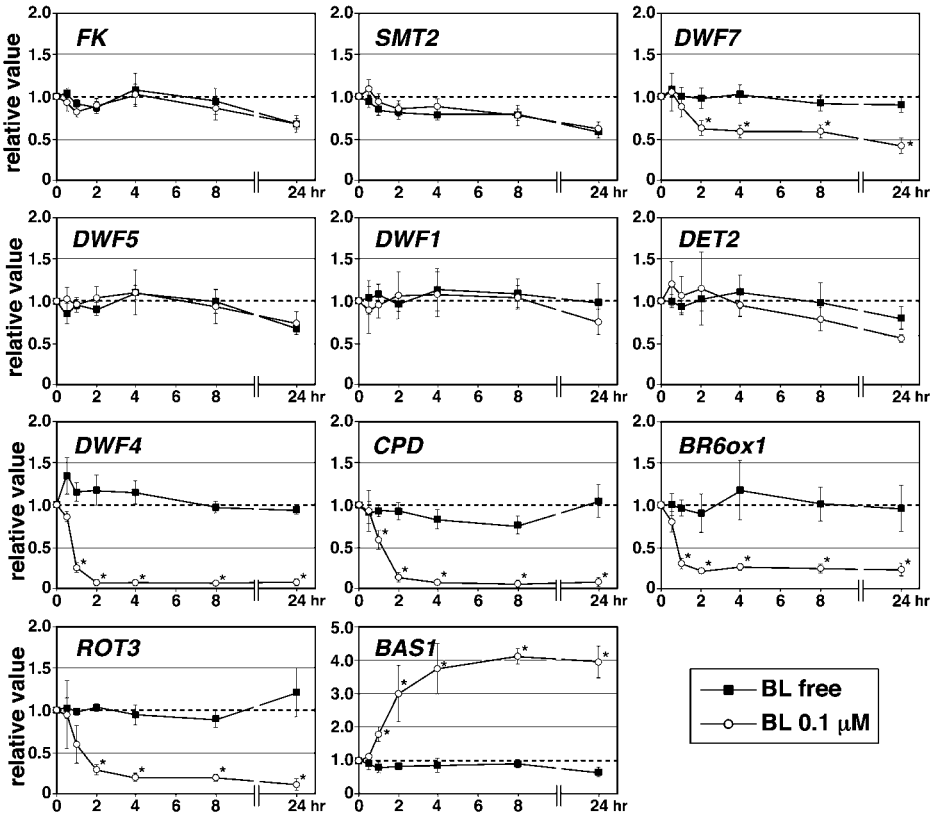

Figure 4. Expressions of BR metabolic genes in response to BL in Arabidopsis seedlings. Following 5 μM Brz treatment for 2 d, 0.1 μM BL was given to wild-type seedlings for the indicated periods. The steady-state mRNA levels of 11 BR metabolic genes were examined using RT-PCR. Presentation styles of graphs are identical to those in Figure 2.

at 8 h, at which time the transcript abundance was 4.15 times the initial value (Fig. 4). The *DWARF7* (*DWF7*) expression alone was reduced by BL supplementation among five sterol biosynthesis genes (Fig. 4). The transcript level reached its minimum level within 2 h after BL addition and was about 50% of the initial value. Expressions of *FK* and *DWF5* were little affected by exogenously applied BL (Fig. 4), whereas they were increased when the seedlings were grown in the presence of Brz (Fig. 2). Expressions of the other sterol biosynthesis genes, *STEROL METHYLTRANSFERASE2* (*SMT2*) and *DWARF1* (*DWF1*), did not respond to BL (Fig. 4) or to Brz (Fig. 2).

Based on the results described thus far, 11 BR metabolic genes were divided into four groups according to their respective responses to endogenous BR contents: the first group includes genes such as *DWF4*, *CPD*, *BR6ox1*, and *ROT3*, for which expressions were altered by both Brz and BL treatments; elements of the second (*FK*, *DWF5*, and *DET2*) and third (*DWF7* and *BAS1*) groups responded to Brz and BL, respectively; the fourth one includes genes such as *SMT2* and *DWF1*, for which expressions were unaffected by the two chemical treatments.

Expression of BR Metabolic Genes in a BR Signaling Mutant, *bri1-401*

BR-induced down-regulation of *CPD* is reportedly nullified in BR-insensitive *bri1* mutants (Li et al., 2001; Bancos et al., 2002). Therefore, we examined responsiveness of other BR metabolic genes to fluctuation of endogenous BR contents in a *bri1* mutant to clarify whether intact BRI1 function is required for their feedback expressions (Figs. 2 and 4). A newly identified *bri1* mutant was used for this experiment, which was designated as *bri1-401* with Wassilewskija (WS) background (Fig. 1B, subsection f) because we have used the WS ecotype throughout our research (Tanaka et al., 2003). DNA sequence analysis of *bri1-401* revealed two point mutations (data not shown), which caused amino acid substitutions of G895R in kinase domain I and G1194E in the C-terminal region. First, we tested expressions of seven BR-metabolic genes in Brz-treated *bri1-401* seedlings because they all increased dependently on Brz administration in wild-type plants (Fig. 2). Figure 5 shows that expressions of the other six genes except for *DWF4* (*FK*, *DWF5*, *DET2*, *CPD*, *BR6ox1*, and *ROT3*) were unaffected by Brz application in *bri1-401* mutants. *DWF4* expression alone was up-regulated by Brz application in *bri1-401* and became 3.2 times the initial level (Fig. 5). However, this level of increase was approximately one-third of that (10 times the initial level) in wild-type seedlings (Fig. 2).

Subsequently, we tested expressions of six BR metabolic genes in BL-administered *bri1-401* seedlings as they all responded to exogenous BL in wild-type plants (Fig. 4). Again, *DWF4* expression alone was altered in response to BL and down-regulated to

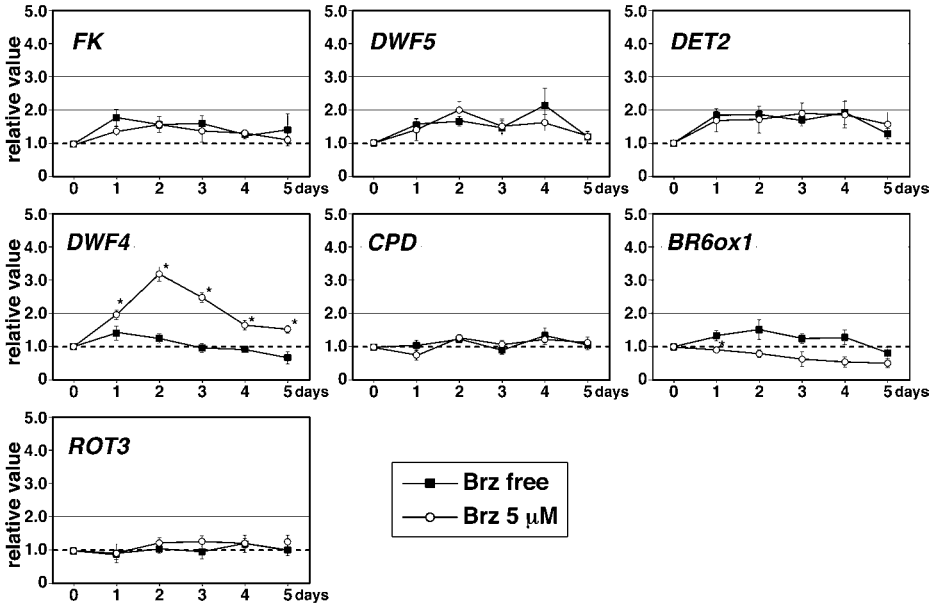

Figure 5. Expression profiles of BR metabolic genes in a BR signaling mutant fed with Brz. Five micromolar Brz were administered to *bri1-401* seedlings for the indicated days. RT-PCR was performed to examine the expressions of BR metabolic genes. The experimental procedure and graph styles are identical to those in Figure 2.

0.31-fold of the initial level in *bri1-401* seedlings (Fig. 6), but this reduction level was much less than that (0.07 times that of the initial level) in wild type (Fig. 4). Expressions of the other five genes (*DWF7*, *CPD*, *BR6ox1*, *ROT3*, and *BAS1*) were not affected in *bri1-401* by BL application (Fig. 6). In all, eight BR metabolic genes among the nine we tested did not respond to BL, Brz, or both in *bri1-401*, whereas they clearly did so in the wild type. Expression of *DWF4* alone is still affected by both chemicals in the mutant, but the responsiveness was much weaker than that in wild type.

DISCUSSION

Phytohormone homeostasis is believed to be crucial for normal growth and development in higher plants. For that reason, it is readily inferred that BR biosynthesis and inactivation can be modulated in a feedback manner to reduce elevated BR concentrations when bioactive BRs are given excessively to the plants. We observed BL-induced down-regulation of four BR-specific biosynthesis genes, *DWF4*, *CPD*, *BR6ox1*, and *ROT3*, as well as BL-induced up-regulation of a BR inactivation gene, *BAS1*, in our experimental system (Fig. 4; Table I), which are consistent with data in previous reports (Mathur et al., 1998; Bancos et al., 2002; Goda et al., 2002). Taken together, these results indicate that the increase of endogenous BR contents results in a feedback regulation at mRNA levels of these BR metabolic genes to maintain BR homeostasis. Furthermore, we found that a sterol biosynthesis gene, *DWF7*, was markedly down-regulated by BL addition (Fig. 4). Mussig et al. (2002) also observed BL-induced down-regulation of *DWF7* in DNA microarray analysis. Nevertheless, that study did not refer to this phenomenon. Our time-course experiment showed that the kinetic behavior of *DWF7* differed greatly from those of the four BR-specific biosynthesis genes described above (Fig. 4); *DWF7* mRNA level was decreased rapidly upon BL addition, but it never reached as low as the levels of BR-specific biosynthesis genes. Kinetic differences between *DWF7* and other BR-specific biosynthesis genes probably reflect that the former

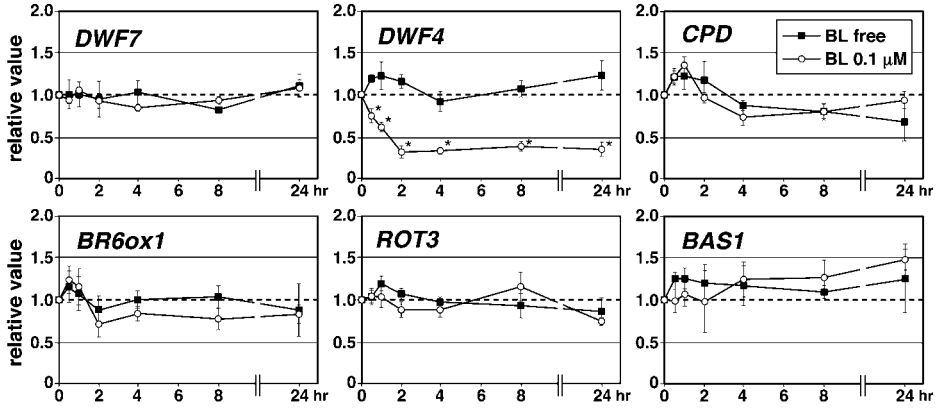

Figure 6. Expression profiles of BR metabolic genes in a BR signaling mutant fed with BL. The *bri1-401* seedlings were treated with 0.1 μM BL for the indicated periods after culture for 2 d in 5 μM Brz. Then, expressions of BR metabolic genes were examined by RT-PCR. Graph styles are identical to those in Figure 2.

Table I. *Expression profiles of BR-metabolic genes in Brz- or BL-treated Arabidopsis seedlings*

Symbols are indicated as up-regulation (↑), down-regulation (↓), or no significant change (–) of gene expression.

Category	Gene Name	Wild Type		*bri1-401*	
		+Brz	+BL	+Brz	+BL
Sterol biosynthesis	*FK*	↑	–	–	–
	SMT2	–	–	–	–
	DWF7	–	↓	–	–
	DWF5	↑	–	–	–
	DWF1	–	–	–	–
BR-specific biosynthesis	*DET2*	↑	–	–	–
	DWF4	↑	↓	↑[a]	↓[a]
	CPD	↑	↓	–	–
	BR6ox1	↑	↓	–	–
	ROT3	↑	↓	–	–
BR inactivation	*BAS1*	–	↑	–	–

[a]Responsiveness of *DWF4* expression in *bri1-401* to both chemicals was much weaker than that in wild type.

affects not only BR synthesis, but also sterol synthesis, whereas the latter influences only BR production.

Little information is available regarding responses of BR metabolic genes when endogenous BR concentrations are reduced. A few studies have shown that some BR-specific biosynthesis genes, *DET2*, *DWF4*, and *CPD*, are up-regulated in BR-deficient mutants such as *dwf1*, *dwf4*, and *cpd* (Noguchi et al., 2000; Choe et al., 2001). Our study found that *BR6ox1* and *ROT3*, in addition to the three genes described above, were up-regulated when endogenous BR concentrations were temporarily reduced by Brz (Fig. 2; Table I). All five BR-specific biosynthesis genes we tested responded to a decrease of endogenous BR contents; four of them, aside from *DET2*, also respond to their increase. These results suggest that the BR-specific biosynthesis genes play important roles in maintaining BR homeostasis. Although Choe et al. (2001) reported lower expression of *BAS1* in a *dwf4* mutant than that in wild type, we did not detect Brz-induced down-regulation of *BAS1* in our system (Fig. 2). Currently, causes of the discrepancy in *BAS1* response to the depletion of endogenous BR contents between the two laboratories remain unknown. Those different results might reflect different experimental conditions with respect to the use of either a BR-deficient mutant or a Brz-fed wild-type Arabidopsis. We observed that Brz caused up-regulation of sterol biosynthesis genes *FK* and *DWF5* (Fig. 2). *FK* expression in Brz-fed plants is consistent with results of He et al. (2003). Their study demonstrated that *FK*::*GUS* expression is higher in BR-deficient *det2* mutants than in wild type. Taken together, our results indicate that BR homeostasis is finely modulated by the feedback expressions of multiple (at least nine) BR metabolic genes, each of which is involved not only in BR-specific biosynthesis and inactivation, but also in sterol biosynthesis.

Why are sterol biosynthesis genes *FK*, *DWF5*, and *DWF7* up-regulated or down-regulated by respective administration of Brz or BL (Figs. 2 and 4; Table I)? Two plant sterols, campesterol and its epimer, are precursor compounds for BR biosynthesis (Schaller, 2003). For that reason, endogenous BR concentrations,

Table II. *Primer sequences, cycle numbers, and annealing temperatures used in the RT-PCR analysis*

Gene Name	Locus	Primer Sequences	No. of Cycles	Annealing Temperature
FK	At3g52940	5′-TCTCCTTTGAAAATCCTTCTTCTGC-3′ 5′-AATCTGAAAGGATTCATGACTTGGC-3′	31	52
SMT2	At1g20330	5′-TCTTCCTCACTCTTAACGAAAATGG-3′ 5′-TTTTCCGGTGATGTTTCCTTTCTAC-3′	31	52
DWF7	At3g02580	5′-ATCAATCCGGCGAATCTTCTTTCGT-3′ 5′-CAATATAATTGGAAAGGTTGAGAGA-3′	31	50
DWF5	At1g50430	5′-GACGAGAGAAGCAGAAGAAGAAAAT-3′ 5′-GAGAACAACAGACTTCGTTACAATC-3′	29	52
DWF1	At3g19820	5′-TTTCCTTTTTGGTTTGATGCAGTGA-3′ 5′-ACATCAAACCAAGGCTTATTATTAC-3′	35	50
DET2	At2g38050	5′-AATTCCATAACCCGAAAAATGGAAG-3′ 5′-AGGTAGTTTGGAAACAAATTGACAC-3′	29	50
DWF4	At3g50660	5′-TACCTCTTCTTCTTCTCCCATCGC-3′ 5′-CGAGAAACCCTAATAGGCAAACCG-3′	29	55
CPD	At5g05690	5′-TCCTCCTCCTCTCTTCCATCGCCG-3′ 5′-ACGGCGCTTCACGAAGATCGGGTA-3′	27	55
BR6ox1	At5g38970	5′-AAAACAGAGCAGAAAACAGAGTGAG-3′ 5′-TCTGTATCCTCTGCGTGCATAAAAT-3′	33	52
ROT3	At4g36380	5′-AAGAAACCGGTTTAACTGTTTACGT-3′ 5′-GCACAAACAGTTTTGTTCTTTGAAA-3′	31	50
BAS1	At2g26710	5′-GCTCTCCTTTTTGTGTTTTCTCTCT-3′ 5′-AGTCCGGAACAAATTTTTGACCGTT-3′	31	52
ACT2	At5g09810	5′-TTCCGCTCTTTCTTTCCAAGCTCA-3′ 5′-AAGAGGCATCAATTCGATCACTCA-3′	31	50

whether excessive or depleted, may affect expression of these sterol biosynthesis genes to decrease or increase precursor contents and thereby control the BR biosynthesis rate. However, their altered expressions should accessorily influence contents of sterols other than two precursor sterols for BR production because the three genes are also proven to function in synthesis of two major sterols: sitosterol and stigmasterol (Catterou et al., 2001; Schaller, 2003). Otherwise, both the rise and fall of endogenous BR concentrations may affect sterol contents and compositions positively. In animal systems, specialized areas called lipid rafts of cellular membranes, which are rich in both cholesterol and sphingolipids, are known to function as membrane scaffolds in signal transduction and protein sorting (Simons and Toomre, 2000). Furthermore, Mongrand et al. (2004) reported recently that tobacco plasma membranes possess such lipid rafts that are rich in sitosterol, stigmasterol, and 24-methylcholesterol as well as in cholesterol and sphingolipids. Therefore, changes of sterol contents and compositions that depend on endogenous BR concentrations may alter the properties and number of lipid rafts and then indirectly modulate BR perception on membrane-bound BR receptors. It also cannot be ruled out that endogenous BR-dependent expressions of the sterol biosynthesis genes may merely reflect crosstalk between BRs and signaling plant sterols, as in the case of interactions between BRs and other phytohormones (Tanaka et al., 2003). Increasingly numerous reports indicate that plant sterols function as signal molecules, but not as membrane constituents for plant growth and development, especially in embryogenesis (Diener et al., 2000; Jang et al., 2000; Schrick et al., 2000, 2002; Souter et al., 2002). Therefore, BRs may crosstalk with signaling plant sterols by means of the control of sterol biosynthesis. In any case, further studies are necessary to elucidate these conjectures relating to BR content-dependent expression of sterol biosynthesis genes.

Feedback regulation of endogenous BR concentrations is probably achieved through successful perception and transduction of BR signals because bioactive BRs, BL, and castasterone accumulate in BR-insensitive *bri1/cbb2* and *bin2/dwf12* mutants (Noguchi et al., 1999; Choe et al., 2002). Furthermore, a BR-specific biosynthesis gene, *CPD*, fails to be down-regulated in the same mutants fed with BL (Li et al., 2001; Bancos et al., 2002). This inference is supported strongly by our observation showing that expression of nine BR metabolic genes had a reduced or no response to fluctuation of endogenous BR contents in a *bri1-401* mutant, whereas all of them clearly responded in wild type (Table I). It is interesting to speculate why *DWF4* expression was regulated by fluctuation of endogenous BR contents in this mutant even though its responsiveness was much weaker than that in wild-type plants. One possible explanation for this phenomenon is that feedback expression of *DWF4* is mediated by other receptor(s) in addition to a BRI1 receptor. Hu et al. (2000, 2004) reported that BR-responsive genes *CycD3* and *RAV1* are regulated by epi-BL in *bri1* mutants. Furthermore, BRI1-like receptor genes have been shown to form a small gene family in the respective genomes of Arabidopsis, rice, and tomato (Yin et al., 2002; Nomura et al., 2003; Cano-Delgado et al., 2004; Zhou et al., 2004). These reports together suggest that feedback expression of *DWF4* is also under the control of either BRI1 homologs or unknown receptors without homology to BRI1. Alternatively, *DWF4* expression may be regulated directly in a feedback manner by end-products (BL and castasterone) or immediate products of DWF4-catalyzing reactions.

What molecular mechanisms are hidden under BR signaling mediated feedback expressions of its metabolic genes? Our results shed some light on those mechanisms. First, we found that seven BR metabolic genes that were up-regulated by the decrease of endogenous BR contents were classifiable into two groups with regard to their responsiveness: early response genes (*FK*, *DWF5*, *DET2*, *DWF4*, and *BR6ox1*) and late response genes (*CPD* and *ROT3*; Figs. 2 and 3). Results suggest that two independent feedback pathways modulate these two classes of genes separately when BR contents return to appropriate levels from its depletion state. Alternatively, a single pathway regulates both classes sequentially. Secondly, we clarified that nine BR metabolic genes responded to either excessive (*DWF7* and *BAS1*), depleted (*FK*, *DWF5*, and *DET2*), or both levels (*CPD*, *DWF4*, *BR6ox1*, and *ROT3*) of endogenous BRs. This result gives rise to several questions concerning the feedback expressions of BR metabolic genes as described below. How are the rise and fall of BR concentrations sensed in plant cells? Are they monitored separately by different steps in BR signaling cascade or together detected by a single step, for example, perception of BR signals? How are the differences of BR levels distinguished and transmitted as distinct messages to each BR metabolic gene if the latter is the case? What signaling components are responsible for this process? Wang et al. (2002) reported that a single component downstream of BRI1, BZR1, is involved in feedback expression of *CPD*. More recently, BZR1 has been shown to act as a novel transcriptional repressor that binds directly to the promoters of BR-specific biosynthesis genes including *CPD* and *DWF4* in BR homeostasis (He et al., 2005). Therefore, BZR1-interacting proteins and target genes of BZR1 may be good candidates for components that function in BR-signaling-mediated feedback expressions of BR metabolic genes. Based on our results, we are currently searching for missing links, including the above proteins (genes), that connect BR signaling and BR metabolism.

MATERIALS AND METHODS

Chemicals

All chemicals except those mentioned elsewhere were purchased from Wako Pure Chemical Industries, Osaka. Stock solutions of BL (Fuji Chemical,

Tokyo) and Brz were prepared as described in our previous paper (Tanaka et al., 2003).

Plants

In this study, we used both a wild-type Arabidopsis (*Arabidopsis thaliana*) WS ecotype and a newly identified *bri1* mutant with the same genetic background. A *bri1* mutant was isolated as described below. Approximately 9,000 wild-type seeds were mutagenized using ethyl methanesulfonate (Kanto Chemical, Tokyo) as described by Redei and Koncz (1992). The mutagenized seeds (M_1) were bulked in a pool of 1,000 each and propagated to obtain M_2 seeds. The BR-insensitive dwarf plants were selected in M_2 generation and applied to an allelism test with the known *bri1* mutants [Arabidopsis Biological Resource Center (ABRC) stock no. CS3723 (*bri1-1*) and CS292 (*cbb2*)]. From a mutant candidate, the coding region of a *bri1* gene was PCR-amplified with a primer set (5′-CTTCCCACACTTTTCTCTCTCACAA-3′ and 5′-GCTTTGGCTCTGTTTCTAACTCTCA-3′) using KOD-plus DNA polymerase (Toyobo, Osaka) with high fidelity and then sequenced to identify the mutation. A newly identified *bri1* mutant was designated as *bri1-401*. The *bri1-401* seeds are available upon request in a timely manner for noncommercial research purposes.

Chemical Treatments

Arabidopsis plants were cultured under growth conditions described in our previous paper (Tanaka et al., 2003). Chemical treatments were performed as follows. Twenty wild-type seedlings (10 in the case of a *bri1* mutant) that had been grown for 14 d on a solidified Murashige and Skoog medium were further cultured with shaking at 80 rpm (Recipro Shaker NR-10; Taitec, Saitama, Japan) in Murashige and Skoog liquid medium (0.5 mL/plant) supplemented with either BL or Brz. Dimethylsulfoxide, a solvent used to dissolve BL and Brz, was added into each medium, not to exceed a final concentration of 0.1%.

RNA Isolation and RT-PCR

Total RNA was isolated from Arabidopsis seedlings using the guanidine thiocyanate method (McGookin, 1984). First-strand cDNA was synthesized from 2.5 μg total RNA using Moloney murine leukemia virus reverse transcriptase (ReverScript I; Nippon Gene, Tokyo) and subjected to PCR. The PCR program was composed of initial denaturation for 3 min at 94°C, amplification by each cycle of denaturation for 30 s at 94°C, annealing for 30 s at an appropriate temperature for each gene, elongation for 1.5 min at 72°C, and final elongation for 7 min at 72°C. The number of PCR cycles was determined through preliminary experiments to ensure linearity of DNA amplification. Primer sequences, annealing temperatures, and cycle numbers for each gene are given in Table II. The PCR products were fractionated in 0.7% (w/v) agarose gels and stained with ethidium bromide. Fluorescence signals of each band were measured using a fluoro-image analyzer (FLA 2000; Fuji Photo Film, Tokyo) and analyzed by the attached program ImageGauge version 3.4.

Statistical Analysis

All experiments were replicated at least three times. Data obtained were analyzed using ANOVA followed by paired or unpaired Student's t test. Difference with $P < 0.05$ was considered significant.

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