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Regulation of Transcript Levels of the Arabidopsis Cytochrome P450 Genes Involved in Brassinosteroid Biosynthesis

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Cytochrome P450 enzymes of the closely related CYP90 and CYP85 families catalyze essential oxidative reactions in the biosynthesis of brassinosteroid (BR) hormones. Arabidopsis CYP90B1/DWF4 and CYP90A1/CPD are responsible for respective C-22 and C-23 hydroxylation of the steroid side chain and CYP85A1 catalyzes C-6 oxidation of 6-deoxo intermediates, whereas the functions of CYP90C1/ROT3, CYP90D1, and CYP85A2 are still unknown. Semi-quantitative reverse transcriptase-polymerase chain reaction analyses show that transcript levels of CYP85 and CYP90 genes are down-regulated by brassinolide, the end product of the BR biosynthesis pathway. Feedback control of the CYP90C1, CYP90D1, and CYP85A2 genes by brassinolide suggests that the corresponding enzymes might also participate in BR synthesis. CYP85 and CYP90 mRNAs show strong and transient accumulation during the 1st week of seedling development, as well as characteristic organ-specific distribution. Transcripts of CYP90A1 and CYP85A2 are preferentially represented in shoots and CYP90C1, CYP90D1, and CYP85A1 mRNAs are more abundant in roots, whereas CYP90B1 is ubiquitously expressed. Remarkably, the spatial pattern of CYP90A1 expression is maintained in the BR-insensitive cbb2 mutant, indicating the independence of organ-specific and BR-dependent regulation. Quantitative gas chromatography-mass spectrometry analysis of endogenous BRs in shoots and roots of Arabidopsis, pea (Pisum sativum), and tomato (Lycopersicon esculentum) reveal similar partitioning patterns of BR intermediates in these species. Inverse correlation between CYP90A1/CPD transcript levels and the amounts of the CYP90A1 substrate 6-deoxocathasterone in shoots and roots suggests that transcriptional regulation plays an important role in controlling BR biosynthesis.

Brassinosteroids (BRs) are plant steroid hormones that influence a wide range of important developmental processes, including germination, cell elongation, differentiation of vascular elements, photomorphogenesis, and pollen fertility (Clouse and Sasse, 1998; Steber and McCourt, 2001). The maintenance of optimal local BR concentrations by coordinated biosynthetic and inactivation mechanisms, in combination with the differential responsiveness of target cells and tissues, enables the proper regulation of these physiological functions during plant development. The pathways of BR biosynthesis have been elucidated by a series of detailed biochemical studies. brassinolide (BL), the biologically most active BR, is synthesized from campesterol via either early or late C-6 oxidation routes (Fig. 1). The BR biosynthesis pathways are conserved between Catharanthus roseus, Arabidopsis, pea (Pisum sativum), tomato (Lycopersicon esculentum), and rice (Oryza sativa), although a limitation of early C-6 oxidation has been observed in some of these species (Fujioka and Sakurai, 1997; Fujioka et al., 2000; Noguchi et al., 2000; Nomura et al., 2001).

Molecular genetic analysis of BR-deficient mutants has identified several BR biosynthesis genes that, with the exception of DET2 (DEETIOLATED2; Li et al., 1996) were all found to encode cytochrome P450 monoxygenases (for review, see Bishop and Yokota, 2001). Arabidopsis DWF4 (DWARF4), a protein classified as CYP90B1 according to the international cytochrome P450 nomenclature (Nelson et al., 1996; http:// drnelson.utmem.edu/CytochromeP450.html), was
shown by BR intermediate feeding to catalyze C-22 hydroxylation of the steroid side chain (Choe et al., 1998). Likewise, rescue of the Arabidopsis cpd (constitutive photomorphogenesis and dwarfism) mutant revealed that CPD/CYP90A1, another member of the CYP90 family, functions as C-23 steroid hydroxylase (Szekeres et al., 1996). Rescue of the dwarf phenotypes of cpd and dwf4 mutants by BR intermediates indicates that CYP90A1 and CYP90B1 are responsible for the C-23 and C-22 side chain hydroxylation reactions in both the early and late C-6 oxidation pathways of BR biosynthesis. Mutation of the Arabidopsis ROT3 (ROTUNDIFOLIA3) gene, encoding CYP90C1, results in defective cell elongation and reduced leaf expansion. Because of the apparent lack of phenotypic rescue with externally supplied BRs, the role of CYP90C1 in BR biosynthesis remained unclear (Kim et al., 1998, 1999). Similarly, due to the lack of mutants, no function has been assigned for CYP90D1, the fourth Arabidopsis gene of the CYP90 family.

C-6 oxidation of BR intermediates is catalyzed by an enzyme of the CYP85 family (Bishop et al., 1996), as was demonstrated in vitro with yeast (Saccharomyces cerevisiae)-expressed CYP85A1 of both tomato (DWARF) and Arabidopsis (Bishop et al., 1999; Shimada et al., 2001). In these assays, CYP85A1 oxidized only the late biosynthetic intermediates 6-deoxoteasterone, 3-dehydro-6-deoxoteasterone, 6-deoxotyphasterol, and 6-deoxocastasterone, but did not catalyze the C-6 oxidation of campestanol, a substrate of CYP90B1 (Fig. 1). The function of CYP85A2, the second member of the CYP85 family in Arabidopsis, is so far unclear. Two further oxidative reactions, namely C-2 hydroxylation and the formation of BL by Bayer-Villiger lactonization of the steroid B ring,
are also thought to be catalyzed by yet unidentified cytochrome P450 enzymes (Asami and Yoshida, 1999). Recently, Kang et al. (2001) have detected steroid C-2 hydroxylase activity of DDWF1 (dark-induced DWF-like protein 1), a pea P450 designated CYP92A6. Because the Arabidopsis genome does not encode any member of the CYP92 family, in this plant, the C-2 hydroxylation reaction is probably performed by a different cytochrome P450 enzyme.

The regulatory mechanisms of BR homeostasis are little understood. Noguchi et al. (1999, 2000) observed the accumulation of BL and its precursors, as well as up-regulation of the DWF4 and CPD transcripts, in the BR-insensitive bri1 mutant of Arabidopsis, suggesting a role for BRI1 in the regulation of BR biosynthesis. Furthermore, BL treatment of Arabidopsis seedlings markedly decreased the steady-state level of CPD mRNA, and this transcriptional response was shown to require de novo protein synthesis (Mathur et al., 1998). These results suggest that BR synthesis is controlled by an elaborate feedback regulation, one that shows analogy to the negative control of GA biosynthesis genes by GAs (Yamaguchi and Kamiya, 2000).

The cellular concentration of active BRs is also influenced by the catabolism of BL and/or its precursors. In feeding experiments, the activation-tagged Arabidopsis bas1-D mutant overexpressing BAS1/CYP72B1 was found to accumulate biologically inactive 26-hydroxybrassinolide (Neff et al., 1999). The dwarf phenotypes of bas1-D and chibi2, another activation-tagged Arabidopsis mutant with high CYP72C1 level (Nagatani et al., 1998; Bishop and Yokota, 2001), are very similar to those of the BR-deficient mutants. As compared with the wild type, the BR-deficient and -insensitive Arabidopsis mutants contain diminished BAS1 transcript levels, indicating that BL may induce expression of the corresponding catabolic enzyme (Choe et al., 2001).

So far, only limited information is available about the temporal and spatial control of the genes responsible for BR biosynthesis. Strong CPD expression was detected during the 1st week of seedling development, and in transgenic plants, a CPD promoter-driven GUS reporter fusion showed activity in cotyledons, leaves, and floral organs, but not in roots (Mathur et al., 1998). Similar GUS histochemical (G.J. Bishop, unpublished data) and in situ hybridization assays (Pien et al., 2001) revealed that tomato DWARF promoter activity is localized mainly in the apical and root meristem regions, whereas ROT3 is expressed in all organs and cell types of Arabidopsis seedlings (Kim et al., 1999). Thus, further studies are required to elucidate how and to what extent differential expression of particular BR biosynthesis genes affects active hormone levels and intermediate partitioning during plant development.

In this paper, we report that genes of the closely related CYP85 and CYP90 cytochrome P450 families implicated in BR biosynthesis are coordinately regulated by BL. Feedback control of the genes encoding ROT3/CYP90C1 and CYP90D1 suggests that these enzymes may also be involved in BR synthesis. Although all CYP85 and CYP90 genes are strongly expressed during the 1st week of seedling development, their transcripts have characteristically different accumulation patterns in the shoots and roots of seedlings and fully developed plants. The expression level of the CPD/CYP90A1 gene shows correlation with the spatial partitioning of the CYP90A1 substrate 6-deoxocathasterone, suggesting that transcriptional control of the CYP85 and CYP90 genes can contribute to the regulation of BR biosynthesis.

RESULTS

Cytochrome P450 Monoxygenases in BR Biosynthesis Are Evolutionarily Related

All cytochrome P450 enzymes of Arabidopsis with known function in BR biosynthesis belong to either the CYP85 or CYP90 families. Protein sequence comparison based on BLAST homology analysis (Altschul et al., 1990) revealed that these two P450 families are closely related, sharing approximately 35% amino acid sequence identity. We found that the CYP85 and CYP90 proteins also show high levels (about 30%) of sequence identity with ent-kaurenoic acid oxidases, members of the CYP88 P450 family involved in GA biosynthesis (Helliwell et al., 2001). In contrast, the two Arabidopsis CYP72 hydroxylases responsible for BR inactivation are only distantly related to the P450s of BR biosynthesis, featuring less than 20% sequence identity with any member of the CYP90 and 85 families. A phylogenetic tree generated by the ClustalW multiple alignment program (Thompson et al., 1994) shows the close relationship between BR-biosynthetic and CYP88 P450s, as compared with CYP72 proteins, in Arabidopsis (Fig. 2A).

The analysis of exon-intron organization of the same P450 genes uncovered similar relationships (Fig. 2B). Each intron of the CYP85, CYP90 and CYP88 genes was found at one of eight conserved positions, whereas CYP72B1 and CYP72C1 showed a different exon-intron pattern. The close relationship indicated by both protein and gene structure analyses, together with the similarity of enzymatic functions, suggest that during their evolution, the CYP85 and CYP90 families diverged after their specialization to steroid substrates.

Coordinated Feedback Regulation of CYP85 and CYP90 Genes

It was demonstrated previously that transcription of the CPD gene is down-regulated by BL, the end product of BR biosynthesis (Mathur et al., 1998). Therefore, we were interested in determining whether the transcript levels of other CYP90 or CYP85 tran-
scripts are similarly regulated by this phytohormone. Because of the low abundance of these P450 mRNAs, in these experiments, the steady-state transcript levels were monitored by more sensitive semiquantitative RT-PCR, rather than northern hybridization. BL treatment reduced the amount of \textit{CPD}/CYP90A1, \textit{DWF4}/CYP90B1, \textit{ROT3}/CYP90C1, \textit{CYP90D1}, \textit{CYP85A1}, and \textit{CYP85A2} transcripts to approximately 10% or less of the level detected in untreated control seedlings (Fig. 3A). These data show that in Arabidopsis all \textit{CYP85} and \textit{CYP90} gene activities are controlled by BR-dependent feedback regulation.

To confirm our results, we also analyzed the relative amounts of \textit{CYP85} and \textit{CYP90} transcripts in BL-treated and untreated Arabidopsis mutants impaired in BR biosynthesis or perception. Compared with wild-type plants, the mRNA levels of \textit{CYP85} and all four \textit{CYP90} genes were significantly higher, indicating derepressed expression, in the BR-deficient \textit{cpd} and \textit{cbb3} mutants (\textit{cbb3} being allelic to...
The amounts of CYP85 and CYP90 transcripts were reduced in these mutants upon external application of BL, but remained somewhat higher than in BL-treated wild-type plants (Fig. 3B). In the BR-insensitive cbb2 mutant (Kauschmann et al., 1996), however, BL had no effect on the expression of CPD (Fig. 3C) or any other BR-responsive CYP85 or CYP90 genes (data not shown). This result indicates that BR-mediated feedback regulation of the CYP85 and CYP90 genes is dependent on the function of the BRI1 Leu-rich repeat receptor kinase (Li and Chory, 1997), which has been inactivated in the cbb2 mutant.

Regulation of CYP85 and CYP90 mRNA Levels during Germination and Seedling Development

To gain better insight into the regulation of BR-biosynthetic P450 genes during the early stages of plant development, we determined the relative amounts of CYP85 and CYP90 transcripts by RT-PCR in seedlings and young plants throughout the first 8 d after imbibition and after 2 weeks of development (Fig. 4). At the earliest, transcripts of the CYP85A2 and ROT3 genes were already detectable from the 1st d of germination. Each CYP85 and CYP90 mRNA reached a peak level of abundance during the 1st week of seedling development but, with the exception of CPD, their levels declined to about 10% or less of the maximum values by the end of this period. Subsequently, between d 8 and 14 of the time course, only little or no change was detectable in the transcript levels. Although individual CYP85 and CYP90 genes featured different temporal expression profiles, their transient induction during the 1st week after germination suggests the requirement of BR biosynthesis enzymes during the early stages of seedling development.

Differential Regulation of CYP85 and CYP90 Transcript Levels in Shoots and Roots

Previously, we reported that CPD expression is much stronger in the aerial parts than the roots of Arabidopsis (Mathur et al., 1998). To obtain information on the organ-specific regulation of CYP85 and CYP90 genes, their transcript levels were compared by RT-PCR in roots and shoots (representing combined cotyledon and hypocotyl tissues) of 7-d-old seedlings. CPD and CYP85A2 were found preferentially expressed in cotyledons and hypocotyls, whereas the expression of CYP85A1, ROT3, and CYP90D1 was stronger in roots (Fig. 5A). Intrigu-

Figure 4. Changes in transcript levels of CYP85 and CYP90 genes during germination and seedling development. RT-PCR products prepared from total RNA of developing wild-type seedlings and young plants (1 through 8 and 14 d after imbibition). Quantitative data are plotted as percentage of the highest value measured during the experimental period.

Figure 5. Differential accumulation of BR-biosynthetic P450 mRNAs in shoots and roots. A, Transcript levels in shoots (S) and roots (R) of wild-type seedlings. B, CPD/CYP90A1 transcript levels in shoots (S) and roots (R) of BR-insensitive cbb2 seedlings. C, CPD/CYP90A1 transcript levels in roots (R) of wild-type seedlings incubated for 4 h in the presence (BL) or absence (Ctr) of 100 nM BL. RT-PCR products were obtained from total RNA of 7-d-old seedlings. UBQ10 was used as internal control.
Table I. Shoot to root ratio of transcript levels of genes involved in BR biosynthesis

<table>
<thead>
<tr>
<th>Transcript</th>
<th>7-d-Old Arabidopsis</th>
<th>20-d-Old Arabidopsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP85A1</td>
<td>0.26</td>
<td>0.60</td>
</tr>
<tr>
<td>CYP85A2</td>
<td>12.12</td>
<td>8.66</td>
</tr>
<tr>
<td>CYP90A1</td>
<td>8.33</td>
<td>3.23</td>
</tr>
<tr>
<td>CYP90B1</td>
<td>0.95</td>
<td>1.09</td>
</tr>
<tr>
<td>CYP90C1</td>
<td>0.31</td>
<td>0.32</td>
</tr>
<tr>
<td>CYP90D1</td>
<td>0.32</td>
<td>0.28</td>
</tr>
<tr>
<td>DIM1</td>
<td>1.02</td>
<td>0.98</td>
</tr>
<tr>
<td>DET2</td>
<td>0.90</td>
<td>0.95</td>
</tr>
</tbody>
</table>
CYP85A2, sharing 82% amino acid sequence identity with CYP85A1, may represent a second Arabidopsis enzyme with steroid C-6 oxidase activity. At least a partial redundancy of this function in Arabidopsis is suggested by the lack of BR-deficient dwarf mutants defective in CYP85A1. Because P450 monooxygenases of the BR pathway are known to accept multiple substrates (Choe et al., 2001; Shimada et al., 2001), these two enzymes may also differ in their substrate preferences. In vivo feeding experiments using radiolabeled precursors revealed an early and a late C-6 oxidation of campestanol and 6-deoxocastasterone, respectively (Choi et al., 1997), whereas less efficient conversion of 6-deoxotyphasterol to typhasterol was also demonstrated (Noguchi et al., 2000). In yeast expression systems, CYP85A1 of both tomato (DWARF) and Arabidopsis was shown to oxidize 6-deoxocastasterone. The Arabidopsis enzyme also utilized the upstream intermediates 6-deoxoasterone, 3-dehydro-6-deoxoasterone, and 6-deoxotyphasterol, but not campestanol and 6-deoxocastasterone (Bishop et al., 1999; Shimada et al., 2001). If the lack of campestanol conversion was not due to its limited uptake by the yeast cells, this early C-6 oxidation would require the action of another enzyme for which CYP85A2 is a likely candidate.

The possible role(s) of the closely related (53% amino acid identity) ROT3/CYP90C1 and CYP90D1 proteins in BR synthesis is unclear. The rot3 mutant phenotype (Kim et al., 1998) is much weaker than those of the other BR biosynthesis mutants, which may indicate that CYP90C1 has overlapping function with another enzyme, possibly CYP90D1. In Arabidopsis, there are two potentially P450-mediated reactions in BR synthesis for which the genes have not yet been identified, namely the C-2 hydroxylation reaction and the Bayer-Villiger lactonization step converting castasterone to BL (Asami and Yoshida, 1999). Therefore, it seems conceivable that CYP90C1 and/or CYP90D1 might participate in one of these enzymatic reactions.

Transcript levels of the CYP85 and CYP90 genes were found to change in a wide range, from about 10% of the wild-type amount in BL-treated plants to 5 times the wild-type value in BR-deficient mutants. Thus, under normal developmental conditions, BR biosynthesis can be efficiently controlled through feedback regulation of these genes because their expression is partially repressed at physiological BR concentrations. The similarity of BR response suggests that the activity of CYP85 and CYP90 genes might be controlled by the same transcriptional regulators that modulate CPD expression (Mathur et al., 1998). The BR response of CPD, and probably all other feedback-controlled CYP85 and CYP90 genes, requires an intact BR perception mechanism. Down-regulation of CPD was abolished in mutants deficient in the BRI1 BR receptor function, just as in bin2, another BR-insensitive mutant (Li et al., 2001).

Different organ-specific expression of the CYP85 and CYP90 genes may provide another means of controlling BR biosynthesis. This regulation appears to be independent of BR action because: (a) BR insensitivity does not interfere with shoot-specific accumulation of the CPD transcript, (b) low root levels of this mRNA further decrease upon BL treatment, and (c) CYP85 and CYP90 genes, displaying similar steroid responses, show different preferences for shoot- and root-specific expression. Transcripts of CYP85A1 were detected primarily in the roots, whereas those of CYP85A2 accumulated preferentially in the shoots. The differential organ specificity can be seen as further indication for the different functions of the two Arabidopsis CYP85 enzymes. In a recent microarray-based transcript analysis of 142 Arabidopsis cytochrome P450 genes, Xu et al. (2002) have shown that both CYP90A1 and CYP85A1 are preferentially expressed in the aerial portion of 30-d-old plants. In the case of the CYP85A1 transcript, the difference between the shoot versus root ratio detected by these authors and our organ specificity data is likely caused by hybridization of the array probe

- **Table II. Endogenous BR levels in shoots and roots of Arabidopsis, pea, and tomato**

<table>
<thead>
<tr>
<th>BR</th>
<th>20-d-Old Arabidopsis Shoots</th>
<th>20-d-Old Arabidopsis Roots</th>
<th>15-d-Old pea Shoots</th>
<th>15-d-Old pea Roots</th>
<th>36-d-Old tomato Shoots</th>
<th>36-d-Old tomato Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Deoxocastasterone</td>
<td>790</td>
<td>1,800</td>
<td>670</td>
<td>5,100</td>
<td>640</td>
<td>2,800</td>
</tr>
<tr>
<td>6-Deoxocatesterone</td>
<td>100</td>
<td>170</td>
<td>85</td>
<td>330</td>
<td>150</td>
<td>120</td>
</tr>
<tr>
<td>3-Dehydro-6-deoxoasterone</td>
<td>180</td>
<td>320</td>
<td>73</td>
<td>670</td>
<td>16</td>
<td>62</td>
</tr>
<tr>
<td>6-Deoxotyphasterol</td>
<td>540</td>
<td>970</td>
<td>1,700</td>
<td>4,300</td>
<td>250</td>
<td>480</td>
</tr>
<tr>
<td>6-Deoxocastasterone</td>
<td>220</td>
<td>90</td>
<td>11,700</td>
<td>630</td>
<td>330</td>
<td>180</td>
</tr>
<tr>
<td>Cathasterone</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Teasterone</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3-Dehydroteasterone</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Typhasterol</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Castasterone</td>
<td>150</td>
<td>35</td>
<td>690</td>
<td>38</td>
<td>140</td>
<td>11</td>
</tr>
<tr>
<td>Brassinolide</td>
<td>Trace</td>
<td>Trace</td>
<td>ND</td>
<td>24</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, Not detected.
with both the CYP85A1 and CYP85A2 mRNAs. These transcripts share 82% sequence homology, which is above the claimed 70% distinction limit of these microarray assays (Xu et al., 2002). In contrast to genes of the CYP85 and CYP90 families, DIM1 and DET2, encoding enzymes acting farther upstream in the BR pathway were found to be ubiquitously expressed.

In addition to the differences in transcript levels, we have also detected differential distribution of BR biosynthesis intermediates between the aerial and underground organs. We found that in Arabidopsis 6-deoxotyphasterol and earlier precursors were more abundant in the roots, whereas the level of 6-deoxocastasterone and castasterone was higher in the shoots. A similar pattern of organ-specific intermediate accumulation could be observed in pea and tomato, indicating that analogous mechanisms may regulate BR distribution in these plants. In accordance with these findings, C-27 BRs (i.e. 28-norcasterone and its precursors) have also been shown recently to be differentially partitioned between the shoots and roots of tomato (Yokota et al., 2001). The potential significance of higher early intermediate levels in roots and the accumulation of 6-deoxocastasterone in shoots is unclear, but worthy of further investigation. The distribution of CYP85 and CYP90 transcripts suggests that roots actively participate in BR synthesis. Because in several plant species root development is inhibited at sub-nanomolar BR concentrations (Clouse and Sasse, 1998), suppression of metabolic flow to biologically active BR forms might help to maintain the low hormone level in this organ. Moreover, the sensitivity of our GC-selected ion monitoring analysis, BL could only be detected in Arabidopsis and pea, but not in tomato, where castasterone is thought to be the only active BR (Yokota et al., 1997; Nomura et al., 2001). In Arabidopsis roots, the low level of CPD expression was found to coincide with the accumulation of 6-deoxocastasterone, the substrate of CPD/CYP90A1. This seems to indicate a role for transcriptional regulation in determining the abundance and activity of CYP90A1, and perhaps also other P450 enzymes of the BR pathway.

Considering the importance of BRs in regulating early developmental functions, high-level expression of the BR-biosynthetic CYP85 and CYP90 genes in germinating seeds and young seedlings implies that, in addition to BL accumulation in the seeds (Fujioka et al., 1998), efficient de novo synthesis might be required for ensuring the optimal hormone concentration. Although in the whole plant, the activity of these genes declines after the seedling stage, strong expression may be maintained in differentiating regions, as it was shown in the case of CPD (Mathur et al., 1998). Transcriptional activity of the genes involved in BR metabolism is controlled by multiple physiological factors. High level of active hormone results in the repression of biosynthetic P450 genes and the induction of BAS1 responsible for BR catabolism (Choe et al., 2001). In addition to their feedback regulation, the activities of CYP85 and CYP90 genes are also subject to organ-specific and developmental control. Furthermore, a recent DNA microarray analysis revealed that the expression of several genes required for the synthesis of early sterol intermediates in the BR pathway are down-regulated by light (Ma et al., 2001). Therefore, it is reasonable to believe that these transcriptional mechanisms are crucial for adjusting the optimal levels and maintaining the homeostasis of active BRs.

### MATERIALS AND METHODS

**Plant Material and Growth Conditions**

In vitro cultures of wild-type Arabidopsis (ecotype Columbia-0), the BR-deficient cpd (Szekeres et al., 1996), cbb3, and BR-insensitive cbb2 (Kauschmann et al., 1996) mutants were grown from surface-sterilized seeds.
on Murashige and Skoog medium supplemented with 0.5% (w/v) Suc and 0.2% (w/v) Phytagel (Sigma, St. Louis) at 22°C, under 14-h-light/10-h-dark cycles. BR treatments were carried out in the same Murashige and Skoog liquid medium supplemented with 100 nm BL (CIDTech Research Inc., Mississauga, ON, Canada), whereas hormone-free control samples received only the ethanol carried in with the BL stock solution (0.01% [v/v]). The Arabidopsis plants used for organ-specific mRNA and BR analyses were grown under similar conditions in Gamborg’s BS liquid medium (Gamborg et al., 1968) with continuous shaking at 50 rpm. Seeds of pea (Pisum sativum L. cv Torsdag) and tomato (Lycopersicon esculentum Mill. cv Sekaiichi) were sown in vermiculite and the germinated plants grown in the greenhouse under natural light (13-h day, 11-h night). Five-day-old pea seedlings and 22-d-old tomato plants were then grown hydroponically in the same greenhouse using Tanado and Tanaka (1980) liquid medium for further 10 and 14 d, respectively.

Analysis of Steady-State Transcript Levels

Steady-state mRNA levels were analyzed by semiquantitative and quantitative RT-PCR assays according to Chelly and Kahn (1994) with minor modifications. Total RNA was isolated from 1 g of fresh plant material using TRI Reagent (Sigma). After treatment with RNase-free DNasel, cDNA was prepared from 5 μg of RNA with Ready-To-Go T-Primed First-Strand Kit (Pharmacia Biotech, Piscataway, NJ). One-tenth of the cDNA obtained was PCR amplified within the linear range of accuracy by specific primers spanning 250- to 300-bp regions near the 3’ ends of the translated sequences. One percent of the RT-PCR products was labeled with [α-32P]dCTP using a single detection primer that was three nucleotides longer in the 3’ direction than one of the amplification primers. Signal intensities were detected by autoradiography after size separation on a 2% (w/v) agarose gel and quantitatively evaluated using a PhosphorImager 445 SI (Molecular Dynamics Inc., Sunnyvale, CA). The constitutively expressed UBQ10 mRNA (Sun et al., 1998) with continuous shaking at 50 rpm. Seeds of pea (Pisum sativum L. cv Torsdag) and tomato (Lycopersicon esculentum Mill. cv Sekaiichi) were sown in vermiculite and the germinated plants grown in the greenhouse under natural light (13-h day, 11-h night). Five-day-old pea seedlings and 22-d-old tomato plants were then grown hydroponically in the same greenhouse using Tanado and Tanaka (1980) liquid medium for further 10 and 14 d, respectively.

Quantitative Determination of Endogenous BR Levels

Twenty-day-old Arabidopsis, 15-d-old pea (n = 192), and 36-d-old tomato (n = 147) plants were separated into shoots (130, 289, and 241 g fresh weight, respectively) and roots (115, 293, and 78 g fresh weight, respectively). BR extraction and analysis were carried out as has been described by Nomura et al. (2001). In brief, methanol extracts of these tissues were subjected to solvent partitioning and purified by LH-20 chromatography and then reversed phase HPLC. Before LH-20 chromatography, charcoal chromatography was applied to the Arabidopsis shoot extracts; silica gel chromatography was applied to the extracts of pea shoots, pea roots, and tomato roots; and both silica gel and charcoal chromatography were applied to the tomato shoot extract. Quantitative analyses of BRs were conducted by GC-mass spectrometry/selected ion monitoring, using a JMS AX 505W instrument (JEOL, Tokyo).

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