# Organ-Specific Expression of Brassinosteroid-Biosynthetic Genes and Distribution of Endogenous Brassinosteroids in Arabidopsis

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Brassinosteroids (BRs) are steroidal plant hormones that are essential for growth and development. There is only limited information on where BRs are synthesized and used. We studied the organ specificity of BR biosynthesis in Arabidopsis, using two different approaches: We analyzed the expression of BR-related genes using real-time quantitative reverse transcriptase-polymerase chain reaction, and analyzed endogenous BRs using gas chromatography-mass spectrometry. Before starting this study, we cloned the second BR-6-oxidase (*BR6ox2*) gene from Arabidopsis and found that the encoded enzyme has the same substrate specificity as the enzyme encoded by the previously isolated 6-oxidase gene (*BR6ox1*) of Arabidopsis. Endogenous BRs and the expression of BR-related genes were detected in all organs tested. The highest level of endogenous BRs and the highest expression of the *BR6ox1*, *BR6ox2*, and *DWF4* genes were observed in apical shoots, which contain actively developing tissues. These genes are important in BR biosynthesis because they encode the rate-limiting or farthest downstream enzyme in the BR biosynthesis pathway. The second highest level of endogenous BRs and expression of *BR6ox1* and *DWF4* were observed in siliques, which contains actively developing embryos and seeds. These findings indicate that BRs are synthesized in all organs tested, but are most actively synthesized in young, actively developing organs. In contrast, synthesis was limited in mature organs. Our observations are consistent with the idea that BRs function as the growth-promoting hormone in plants.

Since the discovery of brassinolide (BL; Grove et al., 1979), more than 40 natural analogs, collectively called brassinosteroids (BRs), have been isolated and characterized (Fujioka and Sakurai, 1997a, 1997b; Yokota, 1997; Fujioka, 1999). Exogenous application of BRs to plants at nanomolar to micromolar concentrations has a wide spectrum of physiological effects, including promotion of cell elongation and division, enhancement of tracheary element differentiation, retardation of abscission, enhancement of gravitropic-induced bending, promotion of ethylene biosynthesis, and enhancement of stress resistance, as reviewed by Clouse and Sasse (1998) and Sasse (1999). A number of BR-deficient mutants have been discovered in Arabidopsis, pea (*Pisum sativum*), and tomato (*Lycopersicon esculentum*; for review, see Clouse and Feldmann, 1999; Schumacher and Chory, 2000; Bishop and Koncz, 2002). These mutants exhibit dwarfism when grown in either light or dark conditions. Many of these mutants also have dark-green leaves, reduced fertility, a prolonged lifespan, and abnormal skotomorphogenesis. BR-insensitive mutants have been identified in Arabidopsis, pea, to-mato, and rice (*Oryza sativa*; for review, see Müssig and Altmann, 2001; Bishop and Koncz, 2002).

In Arabidopsis, studies have reported BR biosynthesis and signal transduction mutants, including *det2* (Li et al., 1996; Noguchi et al., 1999a), *cpd* (Szekeres et al., 1996), *dwf4* (Choe et al., 1998), *dwf1/dim* (Klahre et al., 1998; Choe et al., 1999a), *ste1/dwf7* (Choe et al., 1999b), *sax1* (Ephritikhine et al., 1999), *dwf5* (Choe et al., 2000), *fackel* (Jang et al., 2000; Schrick et al., 2000), *bri1* (Clouse et al., 1996; Li and Chory, 1997), *brs1* (Li et al., 2001), *bin2/ucu1/dwf12* (Choe et al., 2002; Li and Nam, 2002; Perez-Perez et al., 2002), *bzr1* (Wang et al., 2002), and *bes1* (Yin et al., 2002). *BAS1/CYP72B1*, which encodes a repressor of a *phyB* mutant, catalyzes C-26 hydroxylation of BL (Neff et al., 1999). We recently showed that the *BAS1* gene is induced by exogenous BRs (Goda et al., 2002). These observations strongly suggest that the *BAS1* gene functions to maintain steady-state levels of endogenous BRs by inactivating active BRs. The *ROT3/CYP90C* gene was reported to be involved in polar cell elongation of leaf cells (Kim et al., 1998). We also demonstrated that the *ROT3* gene and its homolog, *CYP90D*, are both repressed by exogenous BRs (Goda et al., 2002). These observations, together with the fact that the *ROT3* and *CYP90D* genes are highly homologous to known BR-biosynthetic genes (*CYP85As*, *CYP90A1*, and *CYP90B1*), suggest that

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both *ROT3* and *CYP90D* genes encode putative BR-biosynthetic P450 enzymes (Goda et al., 2002).

From the analysis of mutants and wild-type plants, we identified BL, castasterone (CS), typhasterol (TY), teasterone (TE), 6-oxocampestanol (6-OxoCN), 6-deoxocastasterone (6-DeoxoCS), 6-deoxotyphasterol (6-DeoxoTY), 3-dehydro-6-deoxoteasterone (6-Deoxo3DT), 6-deoxoteasterone (6-DeoxoTE), and 6-deoxocathasterone (6-DeoxoCT) as endogenous BRs in various Arabidopsis tissues, including shoots, siliques, and seeds (Fujioka et al., 1996, 1998, 2002; Noguchi et al., 1999b, 2000; S. Fujioka, unpublished data; Fig. 1). On the other hand, we studied the metabolism of deuterium-labeled BR intermediates in Arabidopsis and demonstrated the operation of the biosynthetic sequence: campestanol (CN) → 6-DeoxoCT → 6-DeoxoTE → 6-Deoxo3DT → 6-DeoxoTY → 6-DeoxoCS → 6α-hydroxyCS (6-OHCS) → CS → BL (Noguchi et al., 2000). We also showed the operation of the biosynthetic sequence: TE → 3-dehydroteasterone (3DT) → TY → CS → BL. We have also characterized the Arabidopsis *BR6ox* and tomato *Dwarf* gene, which were expressed in yeast (*Saccharomyces cerevisiae*), and the ability of the transformed yeast cells to metabolize 6-deoxo-BRs was tested. Both enzymes catalyze multiple steps in BR biosynthesis, 6-DeoxoTE to TE, 6-Deoxo3DT to 3-DT, 6-DeoxoTY to TY, and 6-DeoxoCS to CS (Shi-

mada et al., 2001). These studies established the BR-biosynthetic pathway in Arabidopsis (Fig. 1).

In contrast to the intensive studies on the BR-metabolic pathway and BR-related genes, there is only limited information on where BRs are synthesized and function. BRs occur at very low concentrations in plants, and pollen and immature seeds are known to be the richest sources (Fujioka, 1999). The transcript abundance of downstream BR-metabolic genes, *DWF4* (Noguchi et al., 1999a; Choe et al., 2001), *ROT3* (Kim et al., 1998), *BAS1* (Neff et al., 1999), and *BR6ox* (Shimada et al., 2001) are reported at extremely low levels. The signals are too weak to be detected on RNA gel blots, except for the *CPD* gene (Szekeres et al., 1996; Mathur et al., 1998). Therefore, studies have mainly used semiquantitative reverse transcriptase (RT)-PCR. There have been histochemical studies of the *CPD* (Mathur et al., 1998), *ROT3* (Kim et al., 1999), *BRI1* (Friedrichsen et al., 2000), and tomato *dwarf/CYP85* (Pien et al., 2001) genes, but it is still unclear if the histochemical pattern of the reporter or in situ hybridization signal corresponds to the native gene expression, enzyme existence/activity, or endogenous BR accumulation.

Before starting to study organ-specific BR biosynthesis, we isolated and characterized the second *CYP85A* gene from Arabidopsis, which is homologous to the previously isolated *BR6ox/CYP85A1* gene and the tomato *Dwarf* gene (Fig. 2). The BR6ox/Dwarf enzyme catalyzes an important biosynthetic step of BRs, 6-DeoxoCS to CS via 6-OHCS. This step is the farthest downstream step in BL biosynthesis for known mutations and enzymes. A defect in the *Dwarf* gene results in CS deficiency and causes dwarfism by suppressing stem elongation and leaf expansion (Bishop et al., 1996, 1999). These studies indicate that 6-deoxo BRs have very weak biological activity. It remains to be determined whether any or all of the 6-oxo BRs, e.g. TE, 3DT, TY, or CS, are active per se, or become active after being converted to BL. Intermediates of the late C-6 oxidation pathway are predominant over those of the early C-6 oxidation pathway in many species, including Arabidopsis, tomato, and pea (Choi et al., 1997; Yokota et al., 1997; Bishop et al., 1999; Noguchi et al., 1999b; Koka et al., 2000; Nomura et al., 2001). Especially high accumulation of 6-DeoxoCS has been recorded in many plants, suggesting that C-6 oxidation is a rate-limiting step. Such regulation seems to be canceled in BR-insensitive mutants. CS accumulates at aberrant levels in pea *lka* (Nomura et al., 1997, 1999) and tomato *curl-3* (T. Nomura, T. Yokota, and G.J. Bishop, personal communication). Both CS and BL accumulate in the Arabidopsis *bri1* mutant (Noguchi et al., 1999b). These observations indicate that C-6 oxidation is controlled by a feedback mechanism in steady-state conditions in wild-type plants. Interestingly, the presence of BL has not been demonstrated in tomato, indicating that CS may be a biologically active BR. The Dwarf en-
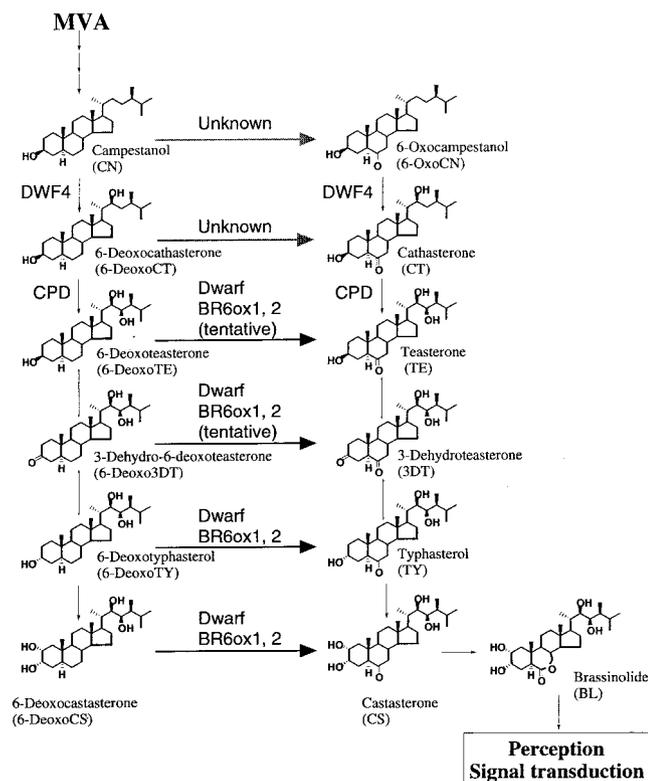


**Figure 1.** BR biosynthesis pathway in Arabidopsis. The proposed biosynthetic pathway for BL from mevalonate (MVA) is shown with identified enzymes.
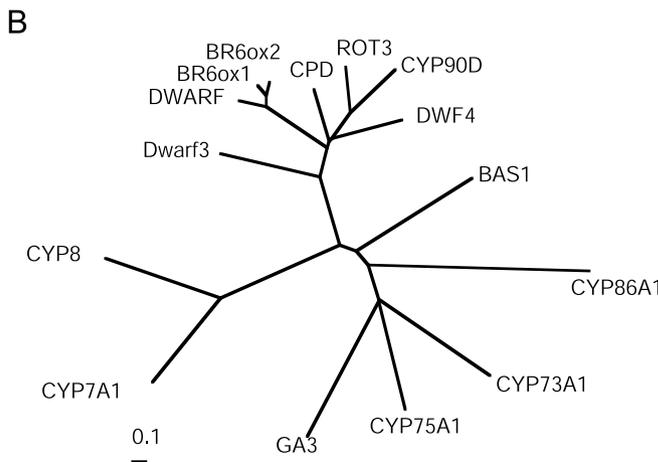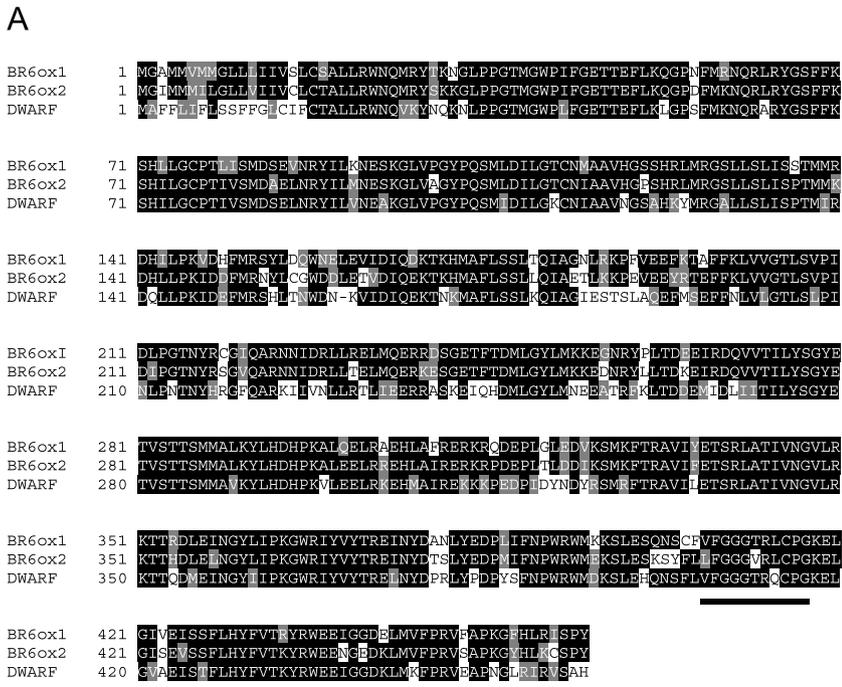
## A



## B



**Figure 2.** Sequence comparison of *BR6ox2* and related genes. A, Sequence alignment of the *BR6ox2*, *BR6ox1*, and tomato *Dwarf* genes. Reverse contrast characters highlight identical amino acid residues and hatched characters indicate conserved residues. The GenBank/EMBL/DDBJ accession numbers of the *BR6ox1* (CYP85A1) and the tomato *Dwarf* gene (CYP85A1) are AB035868 and U54770, respectively. The heme-binding signature sequence is underlined. B, Phylogenetic relationship between *BR6ox2* and selected P450 genes. *BR6ox2* belongs to a group consisting of *Dwarf* (tomato, CYP85A1), *BR6ox1* (Arabidopsis, CYP85A1), *CPD* (Arabidopsis, CYP90A1 and X87367), *ROT3* (Arabidopsis, CYP90C1 and AB008097), *DWF4* (Arabidopsis, CYP90B1 and AF044216), and *CYP90D* (Arabidopsis, AB066286). *BAS1/CYP72B1* (Arabidopsis, BAS1 and AC003105) is reported to be involved in BR inactivation. *Dwarf3* (maize [*Zea mays*], CYP88A and U32579) and *GA3* (Arabidopsis, CYP701A and AF047720) are reported to be involved in GA biosynthesis. The first P450 genes functionally identified from higher plants are *CYP73A* (*Helianthus annuus* cinnamate 4-hydroxylase, Z17369) and *CYP75A* (*Petunia hybrida* flavonoid-3′,5′-hydroxylase, D14588); both belong to the higher plant-specific group A of P450 genes. The accession numbers of the other members are D30718 (CYP8), M93133 (CYP7A), and X90458 (CYP86A).

zyme may be a key enzyme governing the physiological role of BRs in tomato. Based on these observations, it is physiologically important to study how BR-6-oxidases regulate the biosynthesis of 6-oxo BRs, including BL.

In this study, we isolated and characterized the second BR-6-oxidase gene from Arabidopsis. Then, we studied organ-specific BR biosynthesis using two different approaches. First, we studied gene expression of BR-related genes, including genes encoding the downstream BR-biosynthetic enzymes (*BR6ox1*, *BR6ox2*, *CPD* [23-hydroxylase], and *DWF4* [22-hydroxylase]), genes encoding the putative BR-biosynthetic enzymes (*ROT3* and *CYP90D*), a gene encoding a degradation enzyme (*BAS1* [26-hydroxylase]), and a gene encoding a critical component of the BR receptor (*BRI1*). We used a real-time quantitative (RTQ) RT-PCR to analyze

transcripts at extremely low levels. Because BR-biosynthetic P450 genes have sequence similarities each other, care was taken to avoid cross detection/hybridization. The other approach was the biochemical quantitation of endogenous BRs by gas chromatography-mass spectrometry (GC-MS). These studies enable us to report the organ-specific distribution of BR biosynthesis for the first time, to our knowledge.

## RESULTS

### Cloning the Second BR-6-Oxidase from Arabidopsis

Recent progress in the Arabidopsis genome sequencing project (Arabidopsis Genome Initiative, 2000) has revealed that two Arabidopsis genes (*CYP85A* genes) are putatively orthologous to the

tomato *Dwarf* gene. We previously cloned one of them, *CYP85A1/At5g38970*, and named it BR-6-oxidase (*BR6ox*; Shimada et al., 2001). Here, we isolate the second homolog, *CYP85A2/At3g30180* from Arabidopsis, and examine functional overlap and differences between the two genes. An Arabidopsis cDNA library was screened using a PCR-based strategy and two cDNA clones were isolated. The entire nucleotide sequence of the two cDNAs revealed that they contained an identical open reading frame. Because one of these inserts had an in-frame stop codon in the 5′-upstream region of the open reading frame, we concluded that these cDNAs are the full length. The entire sequence of one of these cDNA clones, pGWB28, has been deposited to the GenBank/EMBL/DNA Data Bank of Japan (DDBJ) databases (accession no. AB087801; nucleotide sequence data not shown). We designated this gene *BR6ox2* and renamed the previously reported *BR6ox/CYP85A1* gene as *BR6ox1*. The deduced amino acid sequence of *BR6ox2* has 82% identity with *BR6ox1* and 68% identity with the tomato *Dwarf* gene (Fig. 1A). It was apparent that *BR6ox2* belongs to the P450 gene superfamily because its amino acid sequence has characteristics of P450 genes, such as the heme-binding consensus sequence FxxGxxxCxG (lowercase x indicates variable amino acid residue; Nelson et al., 1996). A phylogenic relationship of P450 genes (Fig. 1B) indicates that *BR6ox2* is nearer to the *BR6ox1* than to the tomato *Dwarf* genes or any other Arabidopsis P450 genes. Comparison of the cDNA and genomic sequences revealed that the *BR6ox2* gene consists of nine exons. The exon-intron structures are completely conserved in *BR6ox1* and *BR6ox2*. These observations suggest that the *BR6ox* genes arose by recent gene duplication in this species.

### Functional Analysis of BR6ox2

The sequence similarity between *BR6ox1* and *BR6ox2* does not necessarily indicate that the BR6ox enzymes catalyze the conversion of the same substrates in the BR-biosynthetic pathway. To establish the biochemical function of the *BR6ox2* product, the gene was functionally expressed in yeast. The protein-coding region of the *BR6ox2* gene was subcloned into a yeast expression vector, pYeDP60, and expressed in the yeast strain, WAT11, which carries Arabidopsis NADPH-P450-reductase (Urban et al., 1997). In this strain, both *BR6ox2* and the P450 reductase gene were overexpressed in the presence of Gal (Pompon et al., 1996). An induced culture of the yeast transformant was incubated with 5 µg of deuterated [$^2$H$_6$]BRs. Products from the incubation were analyzed by GC-MS. The identity of the products was confirmed by a direct comparison of the relative abundance of characteristic ions of the metabolites and standard compounds (data not shown). When the yeast strain expressing *BR6ox2* was incubated

with [$^2$H$_6$]6-DeoxoCS, both [$^2$H$_6$]CS and [$^2$H$_6$]6-OHCS were identified as metabolites. Other 6-deoxo compounds were also fed to the yeast to determine whether they can also act as substrates for the BR6ox2 enzyme. It was found that [$^2$H$_6$]6-DeoxoTY, [$^2$H$_6$]6-Deoxo3DT, and [$^2$H$_6$]6-DeoxoTE were converted to [$^2$H$_6$]TY, [$^2$H$_6$]3DT, and [$^2$H$_6$]TE, respectively. However, [$^2$H$_6$]6-DeoxoCT and [$^2$H$_6$]CN were not converted to [$^2$H$_6$]cathasterone (CT) and [$^2$H$_6$]6-OxoCN, respectively. No conversion was detected in a yeast strain that was transformed only with the vector pYeDP60. From these results, we concluded that the BR6ox2 enzyme has the same substrate specificity as the BR6ox1 and tomato Dwarf enzymes, at least in our yeast expression system (summarized in Fig. 1).

### Specificity of RTQ RT-PCR to Analyze BR-Related P450 Genes

To understand the organ specificity of BR biosynthesis, we analyzed downstream BR-biosynthetic genes, putative BR-biosynthetic genes, the *BRI1*, and the *BAS1* genes. It has been technically difficult to quantify transcript abundance of BR-related genes because their transcript levels are extremely low. This study used the RTQ-RT-PCR to analyze transcript abundance. We tested the risk of cross amplification/detection before analyzing BR-related P450 genes, two *CYP85* genes, four *CYP90* genes, and one *CYP72B1* gene, which share significant sequence similarity. We compared the SYBR Green and the Taq-Man methods. DNA solutions ($10^1 \sim 10^7$ copies $\mu L^{-1}$) of cDNA clones for BR-metabolic P450 genes were used as templates to draw standard curves. DNA solutions in this range of concentration were used as templates to estimate the rate of cross detection in each method. When the SYBR Green method was used, the *CPD* gene primers cross amplified *ROT3* DNA (the efficiency of the amplification was less than $10^{-2}$ when compared with the amplification of the *CPD* DNA). This implies that if there are 100 times as many *ROT3* transcripts as *CPD* transcripts, the abundance of *CPD* transcripts will be overestimated by a factor of two. Other primers generally cross detected nonspecific templates at low rate ($<10^{-3}$) in SYBR Green method (data not shown). On the other hand, when the Taq-Man method was used for the analysis, Taq-Man probes cross detected nonspecific templates at lower rates ($<10^{-4}$) compared with the SYBR Green method (Table I). We concluded that the rate of cross detection in the Taq-Man method does not affect the analysis of BR-related transcripts. The subsequent analyses were performed with the Taq-Man method.

### Feedback Regulation at the Transcript Level

It has been reported that the *CPD* (Mathur et al., 1998; Asami et al., 2001) and *DWF4* (Noguchi et al.,

**Table I.** *Cross detection of BR-related P450 gene expression using the TaqMan method*

| Template | Primers and TaqMan Probes | | | | | | |
|---|---|---|---|---|---|---|---|
| | BR6ox1 | BR6ox2 | CPD | DWF4 | ROT3 | CYP90D | BAS1 |
| BR6ox1 | – | $<10^{-4}$ | ND[a] | ND | $<10^{-5}$ | ND | ND |
| BR6ox2 | ND | – | ND | ND | ND | ND | ND |
| CPD | $<10^{-5}$ | $<10^{-5}$ | – | ND | ND | ND | ND |
| DWF4 | $<10^{-4}$ | $<10^{-4}$ | ND | – | ND | ND | ND |
| ROT3 | ND | $<10^{-4}$ | ND | $<10^{-4}$ | – | $<10^{-4}$ | ND |
| CYP90D | ND | ND | ND | ND | $<10^{-4}$ | – | ND |
| BAS1 | ND | $<10^{-4}$ | ND | ND | ND | $<10^{-4}$ | – |

ND, Not detected.

1999a; Choe et al., 2001) genes are regulated by BRs in a feedback-regulatory manner. Here, we tested whether the *BR6ox2* gene is regulated by BL. When 7-d-old *det2* seedlings were treated with 10 nM BL, the transcript abundance of the *BR6ox2* was decreased (Fig. 3). The repression was apparent 15 min after BL treatment and peaked at 6 h, at which time the transcript abundance was less than 2% of the initial level. The wild-type plants showed similar feedback response to BL but they were less sensitive than the *det2* mutants (data not shown). We have reported recently that the *BR6ox1* gene is also feedback regulated by BL, along with all *CYP90* genes (Goda et al., 2002). The results of the RTQ-RT-PCR coincided with those of the GeneChip analysis. These observations provide good evidence that our system is reliable for analyzing rare transcripts of BR-biosynthetic genes.
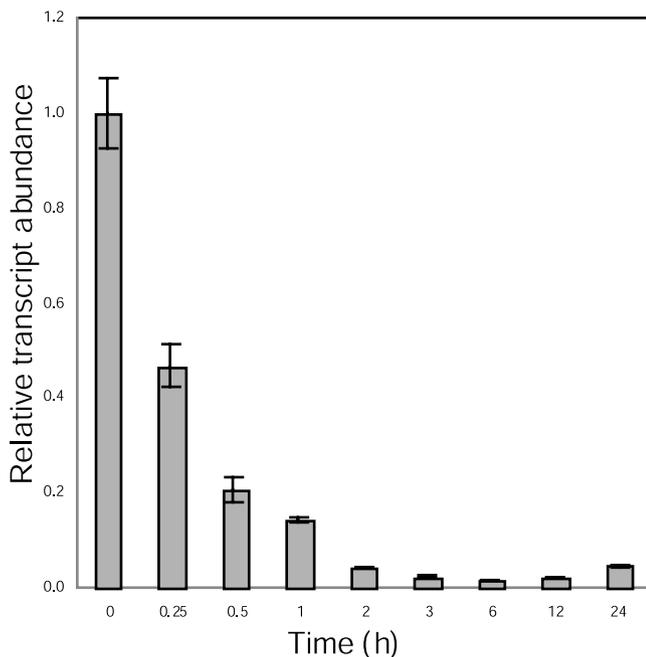
## Organ-Specific Expression of the BR-Biosynthetic Genes

Organ-specific expression of the BR-related genes was analyzed by the RTQ-RT-PCR (Fig. 4). The genes analyzed were *BR6ox1*, *BR6ox2*, *CPD* (encoding a putative 23-hydroxylase), *DWF4* (encoding a putative 22-hydroxylase), *ROT3*, *CYP90D* (encoding putative BR-biosynthetic enzymes), *BAS1* (encoding a putative 26-hydroxylase, which inactivates BRs), and *BRI1* (encoding the critical component of the BR receptor). Arabidopsis seedlings were grown in soil for 4 to 12 weeks. The aerial parts of the plants were divided into apical shoots including flower buds, inflorescent stems, rosette leaves, cauline leaves, and siliques. To compare roots and shoots, plants were germinated on agar medium for 7 d, and then cultured in liquid medium for 7 to 30 d, and divided into shoots and roots. Three independent plant samples were analyzed and the averages of transcript abundance are shown with variations (Fig. 4). The following common trends were found in gene expression, although the variations between different plant samples suggested that the expression of BR-biosynthetic genes can be influenced by factors such as age of the plant, the developmental stage of the organ, etc. In soil-grown plants, the expression of *BR6ox1* was greatest in apical shoots and relatively high in siliques. The expression of *BR6ox2* gene was greatest in apical shoots. *CPD* was expressed in all organs, except that the expression was low in siliques. *DWF4* expression was highest in apical shoots, and high in siliques. *ROT3* expression was relatively high in apical shoots. The expression of the *CYP90D* was predominant in inflorescent stems. The expression of *BRI1* and *BAS1* was similar; they were expressed ubiquitously in aerial organs, but in lesser amounts in siliques.

When roots were compared with shoots in liquid-cultured seedlings, the expression of *BR6ox1*, *ROT3*, *CYP90D*, and *BAS1* in roots was higher than in shoots. The expression of *BR6ox2*, *DWF4*, and *BRI1* in roots was comparable with that in shoots. The ex-

**Figure 3.** Kinetics of regulation of the *BR6ox2* gene by BL. Light-grown *det2* seedlings were treated with 10 nM BL for the indicated times. Transcript abundance was analyzed using the Taq-Man RTQ RT-PCR. Transcript abundance levels are given as relative values normalized to 18S ribosomal RNA levels. Data are shown as the means ± SE from three different plant samples.
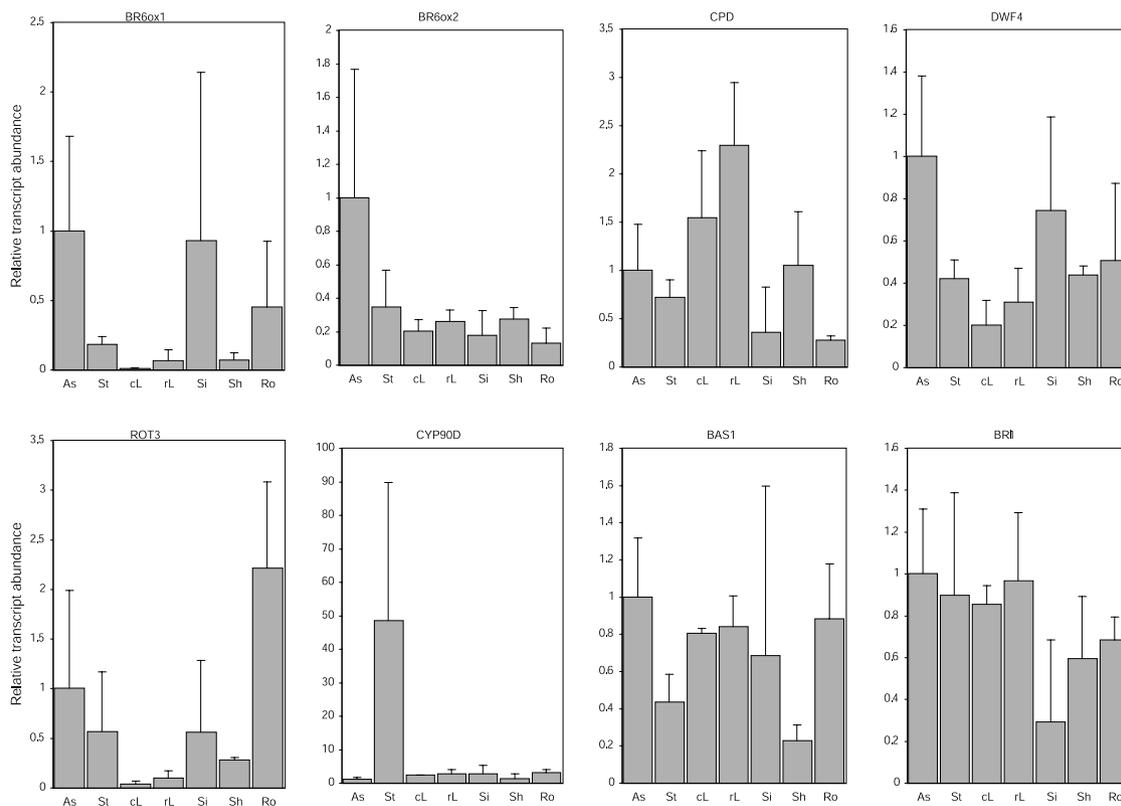
**Figure 4.** The expression of BR-related genes in various organs. Arabidopsis seedlings were grown in soil for 4 to 12 weeks. The aerial parts of the plants were divided into apical shoots (As), inflorescence stems (St), rosette leaves (rL), cauline leaves (cL), and siliques (Si). Alternatively, plants were germinated on agar medium for 7 d, then cultured in liquid medium for 7 to 30 d, and then divided into shoots (Sh) and roots (Ro). Transcript levels were analyzed by the Taq-Man RTQ RT-PCR. Transcript levels are given as relative values to As (the value of 1), after being normalized to the 18S ribosomal RNA levels. Data are shown as the means with variation bars (SD) from three different plant samples for As, St, rL, Si, Sh, and Ro, or from two different plant samples for cL.

pression of *CPD* was exceptional; the expression in roots was lower than that in shoots.

### Organ-Specific Distribution of Endogenous BRs

Arabidopsis seedlings were grown in soil for 6 weeks to compare aerial organs. Alternatively, plants were germinated on agar medium for 7 d, and then cultured in liquid medium for 13 d to compare roots and shoots. They were then divided into organs as described for the gene expression analysis. The endogenous BRs were then extracted and analyzed by GC-MS (Table II). In soil-grown plants, apical shoots contained much higher levels of BRs than other organs, especially 24-methylenecholesterol, 6-DeoxoCT, 6-DeoxoCS, and CS. Siliques also contained more endogenous BRs, especially CN, 6-DeoxoCT, 6-DeoxoCS, and CS. Inflorescent stems, rosette leaves, and cauline leaves contained relatively less BRs. When roots are compared with shoots in liquid-cultured seedlings, roots contained more BRs, especially 6-DeoxoCT, 6-DeoxoTE, and 6-DeoxoTY. In contrast, shoots contained higher lev-els of further downstream components, 6-DeoxoCS and CS. BL was not detected in any of the organs tested. Repeated experiments using different plant samples gave similar results (data not shown).

### DISCUSSION

In a previous report, we demonstrated that the BR6ox1 enzyme, expressed in yeast, catalyzed C-6 oxidation of 6-DeoxoCS, 6-DeoxoTY, 6-Deoxo3DT, and 6-DeoxoTE, whereas the enzyme did not catalyze C-6 oxidation of 6-DeoxoCT or CN (Shimada et al., 2001). We found naturally occurring 6-OxoCN in Arabidopsis (Choe et al., 2001). These two findings suggest that an unknown BR-6-oxidase catalyzing CN to 6-OxoCN (or 6-DeoxoCT to CT) exists in Arabidopsis (Shimada et al., 2001). In this manuscript, we isolated the second *BR6ox* gene, *BR6ox2*, from Arabidopsis and characterized the encoded enzyme in transgenic yeast, to test whether the isolated enzyme has the same or different substrate specificity as the BR6ox1 enzyme. The BR6ox2 enzyme catalyzed the same C-6 oxidation steps, 6-DeoxoCS to CS,

**Table II.** *Endogenous BRs in different organs*

| | Soil-Grown Plants | | | | | Liquid-Cultured Plants | |
|---|---|---|---|---|---|---|---|
| | Apical shoot (3.62 g) | Stem (44.7 g) | Cauline (56.0 g) | Rosette (11.0 g) | Silique (3.89 g) | Shoot (41.5 g) | Root (11.0 g) |
| Sterol ($\mu$g g fresh wt$^{-1}$) | | | | | | | |
| 24-MC[a] | 38.7 | 2.11 | 3.27 | 2.91 | 7.77 | 0.64 | 0.85 |
| CR[b] | 72.7 | 27.1 | 28.9 | 18.8 | 47.9 | 14.6 | 40.4 |
| CN | 1.52 | 0.90 | 0.57 | 0.50 | 5.27 | 0.28 | 0.72 |
| 6-OxoCN | 0.030 | 0.018 | 0.013 | 0.018 | 0.036 | 0.012 | 0.035 |
| 6-DeoxoBRs (ng g fresh wt$^{-1}$) | | | | | | | |
| 6-DeoxoCT | 7.93 | 0.65 | 0.63 | 0.97 | 8.89 | 0.29 | 1.71 |
| 6-DeoxoTE | 0.72 | 0.14 | 0.20 | 0.19 | 0.36 | 0.08 | 0.22 |
| 6-DeoxoTY | 1.18 | 2.24 | 2.01 | 1.08 | 2.86 | 0.69 | 1.37 |
| 6-DeoxoCS | 6.01 | 2.64 | 4.33 | 2.85 | 5.64 | 0.73 | 0.23 |
| 6-OxoBRs (ng g fresh wt$^{-1}$) | | | | | | | |
| CT | ND[c] | ND | ND | ND | ND | ND | ND |
| TE | 0.03 | ND | ND | ND | ND | ND | ND |
| TY | 0.30 | 0.22 | 0.11 | 0.06 | 0.38 | 0.01 | 0.01 |
| CS | 2.02 | 0.40 | 0.31 | 0.13 | 0.94 | 0.12 | 0.05 |
| BL | ND | ND | ND | ND | ND | ND | ND |

[a] 24-MC, 24-Methylenecholesterol.    [b] CR, Campesterol.    [c] ND, Not detected.

6-DeoxoTY to TY, 6-Deoxo3DT to 3DT, and 6-DeoxoTE to TE, but did not catalyze 6-DeoxoCT to CT and CN to 6-OxoCN. Therefore, the existence of an unknown BR-6-oxidase catalyzing CN to 6-OxoCN (or 6-DeoxoCT to CT) remains hypothetical, whereas the *BR6ox1* and *BR6ox2* genes are paralogous. The occurrence of the biosynthetic steps, 6-DeoxoCS to CS and 6-DeoxoTY to TY, has been reported in planta (Noguchi et al., 2000), but in planta conversion of 6-Deoxo3DT to 3DT and 6-DeoxoTE to TE is still tentative (Shimada et al., 2001). This is the first example of paralogs of BR-biosynthetic genes, and gene duplication may explain why no recessive mutant of the *BR6ox* genes is known in Arabidopsis, although a mutant has been identified in tomato (Bishop et al., 1999).

In the studies of aerial tissues of soil-grown seedlings, the two *BR6ox* genes showed different organ-specific expression patterns. The *BR6ox1* expression predominates in apical shoots and siliques. In contrast, *BR6ox* expression is relatively ubiquitous, although it is most abundant in apical shoots (Fig. 4). The difference in organ specificity suggests that the two *BR6ox2* genes play different physiological roles, especially in siliques. Further studies of the *BR6ox1* gene may provide a clue to BR functions in siliques, embryo, and seed development. The analysis of endogenous BRs found that CS (the main product of the BR6ox enzymes) accumulated in apical shoots and siliques. Furthermore, the ratio of 6-DeoxoCS/CS in these organs was significantly lower than in other organs. These observations are consistent with the gene expression, and suggest that BR biosynthesis is active in apical shoots and siliques, especially the C-6 oxidation step. The RTQ-RT-PCR indicated that the transcript abundance of *BR6ox1* and *BR6xo2* roughly ranged between $5 \times 10^{-6}$ to $10^{-8}$ and $5 \times 10^{-5}$ to $3 \times$ $10^{-7}$ copies, respectively, per copy of 18S ribosomal RNA.

*DWF4* had lowest transcript abundance among the analyzed BR-biosynthetic genes. The transcript abundance was roughly $2 \times 10^{-6}$ to $10^{-7}$ copies per 18S ribosomal RNA from the RTQ-RT-PCR. This is consistent with the observation that the biggest gap between endogenous levels of BR precursors is at the step of 22-hydroxylation. For example, the CN content of inflorescent stems, rosette leaves, and cauline leaves was 0.90, 0.57, and 0.50 $\mu$g g$^{-1}$ fresh weight, respectively, whereas that of 6-DeoxoCT (22-hydroxylated form of CN) was 0.65, 0.97, and 0.63 ng g$^{-1}$ fresh weight (Table II). These findings suggest that the 22-hydroxylation step is one of the rate-limiting steps in BR biosynthesis and, therefore, is important for BR biosynthesis. The organ-specific expression pattern of the *DWF4* gene was similar to that of *BR6ox* genes, but *DWF4* expression was more ubiquitous than *BR6ox1* expression and more abundant in siliques than *BR6ox2* expression. *CPD* transcripts were extraordinarily abundant, ranging between $10^{-3}$ and $3 \times 10^{-5}$ copies per the 18S ribosomal RNA. The organ-specific expression of *CPD* was also extraordinary; *CPD* was highly expressed in mature or maturing organs, inflorescence stems, cauline leaves, and rosette leaves (Fig. 4). These findings suggest that the *CPD* gene has an extraordinary function in the BR-biosynthetic pathway. For example, the CPD enzyme might have substrate specificity to sterols or upstream intermediates of BRs, which are more abundant than downstream BR intermediates. *BAS1* transcripts were the most rare of the analyzed BR-related P450 genes. It was estimated to range between $10^{-6}$ and $5 \times 10^{-8}$ copies per 18S ribosomal RNA. The organ-specific expression of *BAS1* was generally higher where the expression of BR-

**Table III.** *Primers and TaqMan probes used for RT-PCR*

| Gene Name | Forward Primer Sequence | Reverse Primer Sequence | TaqMan Probe Sequence |
|---|---|---|---|
| BR6ox1 | TGGCCAATCTTTGGCGAA | TCCCGTATCGGAGTCTTTGGT | ACCGAGTTTCTCAAACAAGGCCCCAAC |
| BR6ox2 | CAATAGTCTCAATGGACGCAGAGT | AACCGCAGCTATGTTGCATG | ACTTGTTGCCGGTTACCCGCAATCTATG |
| CPD | CCCAAACCACTTCAAAGATGCT | GGGCCTGTCGTTACCGAGTT | TCTGCCATCTCCAAGGGTTGAAAGTGC |
| DWF4 | GTGATCTCAGCCGTACATTTGGA | CACGTCGAAAAACTACCACTTCCT | CAGCAAAACAACGGAGCGTCATCG |
| ROT3 | ATTGGCGCGTTCCTCAGAT | CAAGACGCCAAAGTGAGAACAA | CTCACCTCAAAGACCGGATCACTCGAGA |
| CYP90D | CTCATTACCCTTGCCGTCAAA | CAGCTTCATGTTTTCTTCCGTTAG | CCTCTCTGATTCTCCTGCTGCCCTCAAT |
| BAS1 | TTGGCTTCATACCGTTTGGC | TTACAGCGAGTGTCAATTTGGC | CGGAGTTCGTACATGCATTGGTCAGAATC |
| BRI1 | GGTGAAACAGCACGCAAAACT | CACGCAACCGCAACTTTTAA | ACCCCGAGCTTATGAAGGAAGATCCAGC |
| 18S ribosomal RNA | CGGCTACCACATCCAAGGAA | GCTGGAATTACCGCGGCT | TGCTGGCACCAGACTTGCCCTC |

biosynthetic genes was higher, suggesting that BR catabolism is active in organs where BR biosynthesis is active. *BRI1* was expressed more ubiquitously than the BR-biosynthetic genes. This concurs with a previous GFP reporter study (Friedrichsen et al., 2000), and suggests that BR action in Arabidopsis is regulated at the level of biosynthesis rather than by the number of receptors. In contrast, the *OsBRI1* gene expression predominates in shoot apex in rice (Yamamuro et al., 2000).

Only limited information is available on the sites of biosynthesis or action of BRs at the organ, tissue, and cell levels. Exogenously applied BRs are absorbed by plants, move acropetally, and promote cell elongation (Sasse, 1999), but long-distance transport of endogenous BRs is controversial. The variegated revertant phenotype of transposon-mutagenized tomato dwarf mutants (Bishop et al., 1996) suggests that BRs are synthesized in tissues adjacent to where they function. Therefore, it is very important to determine where BRs are synthesized to understand their sites of action. This study analyzed organ specificity in the BR biosynthesis, for the first time to our knowledge, using two different approaches. BR-biosynthetic gene expression was detected in all organs tested, as was the occurrences of endogenous BRs. Our findings indicate that BRs are synthesized in all organs. When aerial tissues of soil-grown plants were compared, apical shoots contained the highest levels of endogenous BRs and the greatest expression of *BR6ox1*, *BR6ox2*, and *DWF4*, which are important genes encoding the rate-limiting or farthest downstream enzyme in the BR-biosynthetic pathway. These observations strongly suggest that BRs are most actively synthesized, and, therefore, used in apical shoots, which contain actively developing tissues. Siliques contained the second highest levels of endogenous BRs and expression of *BR6ox1* and *DWF4*. Siliques also contain developing embryos and seeds. Based on these observations, we propose that BRs are synthesized most actively in young developing organs in aerial tissues.

When roots were compared with shoots in liquid-cultured seedlings, the expression of *BR6ox1*, *ROT3*, *CYP90D*, and *BAS1* was higher in roots than in shoots. The levels of midstream BR intermediates,

6-DeoxoCT, 6-DeoxoTE, and 6-DeoxoTY, were higher in roots than in shoots, whereas the levels of the downstream intermediates, 6-DeoxoCS and CS, were higher in shoots than in roots. Yokota et al. (2001) reported a very similar accumulation patter for $C_{27}$ BRs in shoots and roots of tomato; 6-deoxo-28-norcathasterone and 6-deoxo-28-nortyphasterol levels were higher in roots than in shoots, whereas 6-deoxo-28-norcastasterone and 28-norcastasterone levels were higher in shoots than in roots. Therefore, Arabidopsis and tomato seems to share a common mechanism to maintain opposing levels of midstream and downstream BR intermediates, although the physiological or biochemical significance of this mechanism is not clear. One of our hypotheses is as follows. BR biosynthesis may be more active in roots than in shoots, as assumed from the biosynthesis gene expression pattern. On the other hand, from the *BAS1* expression pattern, BR catabolism may also be more active in roots than in shoots. If the BAS1 enzyme acts more efficiently on downstream BRs (6-DeoxoCS and CS) as hypothesized in a previous report (Neff et al., 1999), the balance of biosynthetic enzymes and degradation enzymes could explain why more midstream intermediates accumulate in roots. The greater BAS1 enzyme activity in roots would maintain the lower levels of the furthest downstream, active BRs in roots because roots are more sensitive to active BRs than shoots.

Very recently, Bancos et al. (2002) reported endogenous BR levels in shoots and roots of Arabidopsis, pea, and tomato. In these plants, the levels of midstream BR intermediates were higher in roots than in shoots and the levels of downstream intermediates were higher in shoots than in roots. Their results, therefore, coincide well with our results. They also reported differential accumulation of Arabidopsis *CYP85* and *CYP90* genes in shoots and roots. Their results generally coincide with our results except for the *BR6ox2/CYP85A2* gene, which was reported to be expressed preferentially in shoots. On the other hand, in our growth condition, the expression of *BR6ox2* gene was not always higher in shoots than in roots (Fig. 4).

## CONCLUSIONS

We isolated and characterized the second *BR6ox* gene from Arabidopsis. We then analyzed the organ specificity of BR biosynthesis using two different approaches: analyses of gene expression and analyses of endogenous BRs. BRs were synthesized in all organs tested, but were most actively synthesized in young actively developing organs. In contrast, synthesis was limited in mature organs. Our observation is consistent with the idea that BRs function as growth-promoting hormone in plants.

## MATERIALS AND METHODS

### Plant Materials

Arabidopsis ecotype Columbia was used as the wild type in this study. Wild-type seedlings were grown for 4 to 12 weeks to compare aerial organs. Alternatively, they were grown aseptically on one-half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) Suc with or without 0.8% (w/v) agar in a growth cabinet (at 22°C, continuous illumination of 100 $\mu$Em$^{-2}$ s$^{-1}$). Plants were grown under similar conditions and harvested at different times for gene expression analyses and BR quantification analyses. The growth conditions and BL treatment of *det2-1* mutants were described previously (Goda et al., 2002).

### GC-MS Analysis

GC-MS analysis was carried out on a mass spectrometer (JMS-AM SUN200, JEOL, Tokyo) connected to a gas chromatograph (6890A, Agilent Technologies, Wilmington, DE) with a capillary column DB-5 (0.25 mm × 15 m, 0.25-$\mu$m film thickness, J&W Scientific, Folsom, CA).

### Isolation of cDNA Clones

An Arabidopsis cDNA library (SUPERSCRIPT Arabidopsis cDNA Library, Invitrogen, Carlsbad, CA) was screened using a PCR-based strategy with gene-specific primers, BR6ox2–101F (5′ GGG GGA TCC ATG GGC ATA ATG ATG ATG ATT TTG3′) and BR6ox2–1509R (5′ GGG GGT ACC GAC AAA ACT AGT CAG TAA GGT GAA CAC TT3′). The isolated cDNA clone is a derivative of the pSPORT-P vector (Invitrogen).

### DNA Sequence Analyses

DNA sequences were determined using an automated DNA sequencer (model 373A DNA Sequencing System, PE-Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The nucleotide sequence was compiled and analyzed using GENETYX-Mac (Software Development Co., Ltd., Tokyo). The BLAST (Altschul et al., 1990) program was used to search for entries of homologous sequences in the DDBJ. The ClustalW program on the server at DDBJ was used to align the amino acid sequences and to draw phylogenic relationships using the neighbor-joining method (Saitou and Nei, 1987). The aligned sequences were shaded using the program Boxshade, available on the server at the European Molecular Biology Network.

### Yeast (*Saccharomyces cerevisiae*) Expression Vector

The full-length cDNA of the *BR6ox2* gene was subcloned from a cDNA clone pGWB28 to a vector, pDONR207, yielding pDONRB28 using the BP Clonase reaction of the Gateway system (Invitrogen). The cDNA was then subcloned into a yeast expression vector pYESDEST52 (Invitrogen) using the LR Clonase reaction (Invitrogen). The resulting plasmid was designated pYESB284. In this construct, the *BR6ox2* gene is controlled under the yeast *GAL1* promoter.

### Yeast Functional Assay

pYESB284 vector was transformed to yeast strain WAT11 (Pompon et al., 1996) under selection using the *URA3* gene. Yeast expression and functional assay of the *BR6ox2* gene were performed as descried previously (Bishop et al., 1999; Noguchi et al., 1999b; Shimada et al., 2001). Products from the incubations were analyzed by GC-MS. The identity of the products was confirmed using their full-scan mass spectra.

### RT-PCR Analysis

Total RNAs were extracted from Arabidopsis seedlings using the guanidine-hydrochloride method (Kawakami and Watanabe, 1988). The RNAs were then treated with DNase I. They were then converted to cDNAs with random primers using the Super Script First-Strand Synthesis System (Life Technologies/Gibco-BRL, Cleveland). Unless otherwise noted, quantitative RT-PCR was performed with the use of real-time-monitoring Taq-Man technology (Holland et al., 1991) with a model 7700 sequence detector and a Taq-Man Universal PCR Master Mix (PE-Applied Biosystems). 6-Carboxyfluorescein and 6-carboxytetramethylrhodamine were used for "Reporter" and "Quencher" to label Taq-Man primers, respectively. 6-Carboxy-X-rhodamine was used for "Passive Reference" as an internal standard to normalize the "Reporter" signal. Normalization is necessary to correct for well-to-well fluorescent fluctuations of "Reporter" because of changes in concentration or volume. The gene-specific primers were designed with care (e.g. not to be homologous to other Arabidopsis genes) using the Primer Express program (PE-Applied Biosystems) and the BLAST program. The primers and Taq-Man probes are listed in Table III. The 18S ribosomal RNA was analyzed as an internal control to monitor the efficiency of the RT reaction, and was used to normalize the transcript abundance in each sample. The 18S ribosomal RNA was chosen because it is the most abundant transcript in vivo. If the efficiency of the RT reaction of a sample was significantly lower than the efficiency of other samples, these samples were not used for further analysis. The same cDNA substrate was used for the quantifications of all genes, including the 18S ribosomal RNA. Because the RTQ-RT-PCR system is so sensitive that it can detect degradation of DNA solutions caused by freezing and thawing or absorption to tubes, the raw data of transcript abundance varies in each measurement. Therefore, we presented relative transcript abundance in results because relative values are reproducible regardless of measurements.

### Quantification of BRs in Arabidopsis Seedlings

BR purification and quantification were carried out according to the method described by Noguchi et al. (1999a).

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