Brassinosteroid-6-Oxidases from Arabidopsis and Tomato Catalyze Multiple C-6 Oxidations in Brassinosteroid Biosynthesis[1]

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Brassinosteroids (BRs) are steroidal plant hormones that are essential for growth and development. It has been proposed that BRs are synthesized via two parallel pathways, the early and late C-6 oxidation pathways according to the C-6 oxidation status. The tomato (*Lycopersicon esculentum*) *Dwarf* gene encodes a cytochrome P450 that has been shown to catalyze the C-6 oxidation of 6-deoxocastasterone to castasterone. We isolated an Arabidopsis ortholog (*AtBR6ox* gene) of the tomato *Dwarf* gene. The encoded polypeptide has characteristics of P450s and is classified into the CYP85 family. The *AtBR6ox* and tomato *Dwarf* gene were expressed in yeast and the ability of the transformed yeast cells to metabolize 6-deoxo-BRs was tested. Metabolites were analyzed by gas chromatography-mass spectrometry. Both enzymes catalyze multiple steps in BR biosynthesis: 6-deoxoteasterone to teasterone, 3-dehydro-6-deoxoteasterone to 3-dehydroteasterone, 6-deoxotyphasterol to typhasterol, and 6-deoxocastasterone to castasterone. Our results indicate that the *AtBR6ox* gene and the tomato *Dwarf* gene encode steroid-6-oxidases and that these enzymes have a broad substrate specificity. This suggests that the BR biosynthetic pathway consists of a metabolic grid rather than two separate parallel pathways.

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; Fujioka, 1999). Exogenous application of BRs to plants between nanomolar and micromolar concentrations causes 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 reviews, see Clouse and Feldmann, 1999; Schumacher and Chory, 2000). These mutants exhibit dwarfism under both light and dark conditions. Many of these mutants also have dark-green leaves, reduced fertility, a prolonged lifespan, and display abnormal skotomorphogenesis. BR-insensitive mutants have been identified in Arabidopsis, pea, tomato, and rice (*Oryza sativa*; Clouse et al., 1996; Kauschmann et al., 1996; Li and Chory 1997; Nomura et al., 1999; Koka et al., 2000; Yamamuro et al., 2000).

We have studied the biosynthetic pathways leading to BL (the most active BR) using cultured cells of *Catharanthus roseus* and proposed the two alternative biosynthetic pathways shown in Figure 1 (Fujioka and Sakurai, 1997a, 1997b). One is the early C-6 oxidation pathway, in which oxidation at C-6 occurs before the introduction of vicinal hydroxyls at C22 and C23 of the side chain (Fig. 1). The other is the late C-6 oxidation pathway in which C-6 is oxidized after the introduction of hydroxyls on the side chain and the A ring (Fig. 1). The natural occurrence of intermediates from both the early and late pathways have been shown in a variety of plants. Most of the steps in both the early and late C-6 oxidation pathways have been defined by feeding plants deuterium-labeled substrates and then identifying the metabolites using gas chromatography-mass spectrometry (GC-MS). In contrast, the physiological or biochemical relevance of two alternative pathways remained unclear.

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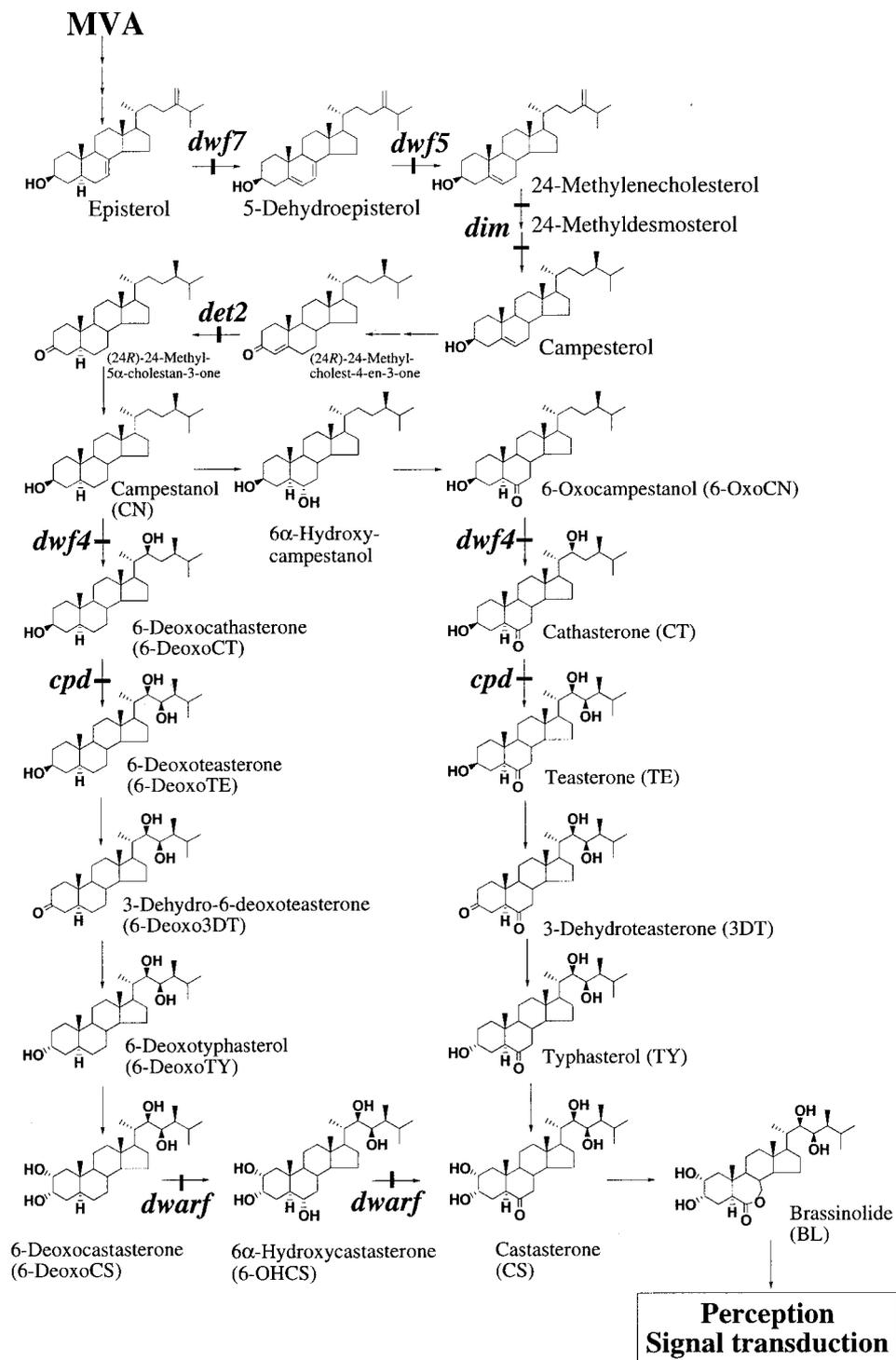
Figure 1. BR biosynthesis pathway. The proposed biosynthetic pathway for BL from mevalonate (MVA) is shown with the steps blocked in reported mutants.

A number of studies have reported BR biosynthesis and signal transduction mutants in Arabidopsis. For example, *det2* (Li et al., 1996; Noguchi et al., 1999b), *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), and *bri1* (Clouse et al., 1996; Li and Chory, 1997). From the analysis of these mutants, we identified BL, castasterone (CS), typhasterol (TY), teasterone (TE), 6-deoxocastasterone (6-DeoxoCS), 6-deoxotyphasterol (6-DeoxoTY), 6-deoxoteasterone

(6-DeoxoTE), and 6-deoxocathasterone (6-DeoxoCT) as endogenous BRs in various Arabidopsis tissues, such as shoots, siliques, and seeds (Fujioka et al., 1996, 1998; Noguchi et al., 1999a, 2000). All BRs identified in Arabidopsis are important components of either the early or late C-6-oxidation pathways, indicating that both pathways are functional in this species. We studied the metabolism of deuterium-labeled BR intermediates in Arabidopsis very recently and demonstrated the operation of the biosynthetic sequence: campestanol (CN) → 6-DeoxoCT → 6-DeoxoTE → 3-dehydro-6-deoxoteasterone (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. These studies established that the previously determined biosynthetic pathway is also present in Arabidopsis.

The tomato *Dwarf* gene was isolated by transposon tagging (Bishop et al., 1996) and shown to encode a cytochrome P450 enzyme that converts 6-DeoxoCS to CS via 6-OHCS, a step where the early C-6 oxidation and the late C-6 oxidation pathways are connected (Bishop et al., 1999). This step is the furthest downstream step in BL biosynthesis among those known for mutations and enzymes. A defect in the *Dwarf* gene results in deficiency of CS and causes dwarfism with stem elongation and leaf expansion being suppressed (Bishop et al., 1996; Bishop et al., 1999). These studies demonstrated that 6-deoxo BRs exhibit very weak biological activity and 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. Therefore, it is physiologically important to understand the biosynthesis of 6-oxo BRs including BL with the C-6 oxidation step potentially being one of the key regulatory steps in BR biosynthesis. Furthermore, 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., 1999a; Koka et al., 2000; Nomura et al., 2000).

Especially high accumulation of 6-DeoxoCS has often been recorded for many plants, suggesting that the C-6 oxidation is a rate-limiting step. Such regulation seems to be cancelled in BR-insensitive mutants because 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, unpublished data). In Arabidopsis, both CS and BL accumulate in the *bri1* mutant (Noguchi et al., 1999a), indicating that C-6 oxidation is controlled by a feedback mechanism in steady-state conditions of wild-type plants. It is interesting that in tomato, the presence of BL has not been demonstrated, suggesting that CS is a biologically active BR. Therefore, the Dwarf enzyme

may be a key enzyme governing the physiological role of BRs in tomato.

Dwarf enzyme functionally expressed in yeast cells can convert 6-DeoxoCS to CS but cannot oxidize CN to 6-oxocampestanol (6-oxoCN; Bishop et al., 1999). However, there is no information on whether the Dwarf enzyme catalyzes the conversion of other intermediates of the late C-6 oxidation pathway. Arabidopsis seedlings recently were found to oxidize the C-6 of both 6-DeoxoTY and 6-DeoxoCS (Noguchi et al., 2000). This finding has raised a question: In Arabidopsis, is such C-6 oxidation regulated by a single *Dwarf* ortholog or by multiple genes? In the present work, we report the isolation of a *Dwarf* gene ortholog termed *AtBR6ox* from Arabidopsis and the functional analysis of both *AtBR6ox* and *Dwarf*.

RESULTS

Cloning of an Arabidopsis Homologue for the Tomato *Dwarf* Gene

Databases were searched for homologs of the tomato *Dwarf* gene (Bishop et al., 1996) and three expressed sequence tag (EST) clones were found to have the highest sequence similarities; they are F5F3, 224F7, and 313B11 (the respective GenBank/EMBL/DNA Data Bank of Japan [DDBJ] accession nos. are AA713019, N65267, and AA394869). Based on partial DNA sequencing and restriction mapping, it was found that the clones corresponded to the same gene, but only F5F3 had sufficient length to contain the full-length cDNA (data not shown). When the entire nucleotide sequence of the cDNA insert of F5F3 was determined and compared with the *Dwarf* gene, it was apparent that the first intron remained un-spliced with the consensus splice sites at the putative exon-intron boundaries in the cDNA. Therefore, we re-isolated cDNA clones by reverse transcriptase (RT)-PCR. The isolated clones contained the same open reading frame (ORF) sequence, except that each of these clones had base substitutions in independent positions that are likely to be derived from PCR errors (data not shown).

The complete ORF cDNA sequence encodes a polypeptide of 466 amino acid residues. The deduced amino acid sequence of the cDNA is shown in Figure 2A. We designated the corresponding gene *AtBR6ox*. The deduced amino acid sequence had characteristics of P450s. For example, the heme-binding consensus sequence FxxGxxxCxG (lowercase x indicates variable amino acid residues; Nelson et al., 1996) is conserved (Fig. 2A). The N terminus retains a hydrophobic region that likely functions as an anchor to the endoplasmic reticulum membrane (data not shown). *AtBR6ox* nucleotide sequence has 71% homology with that of the tomato *Dwarf* gene and the deduced amino acid sequence has 81% similarity and 68%
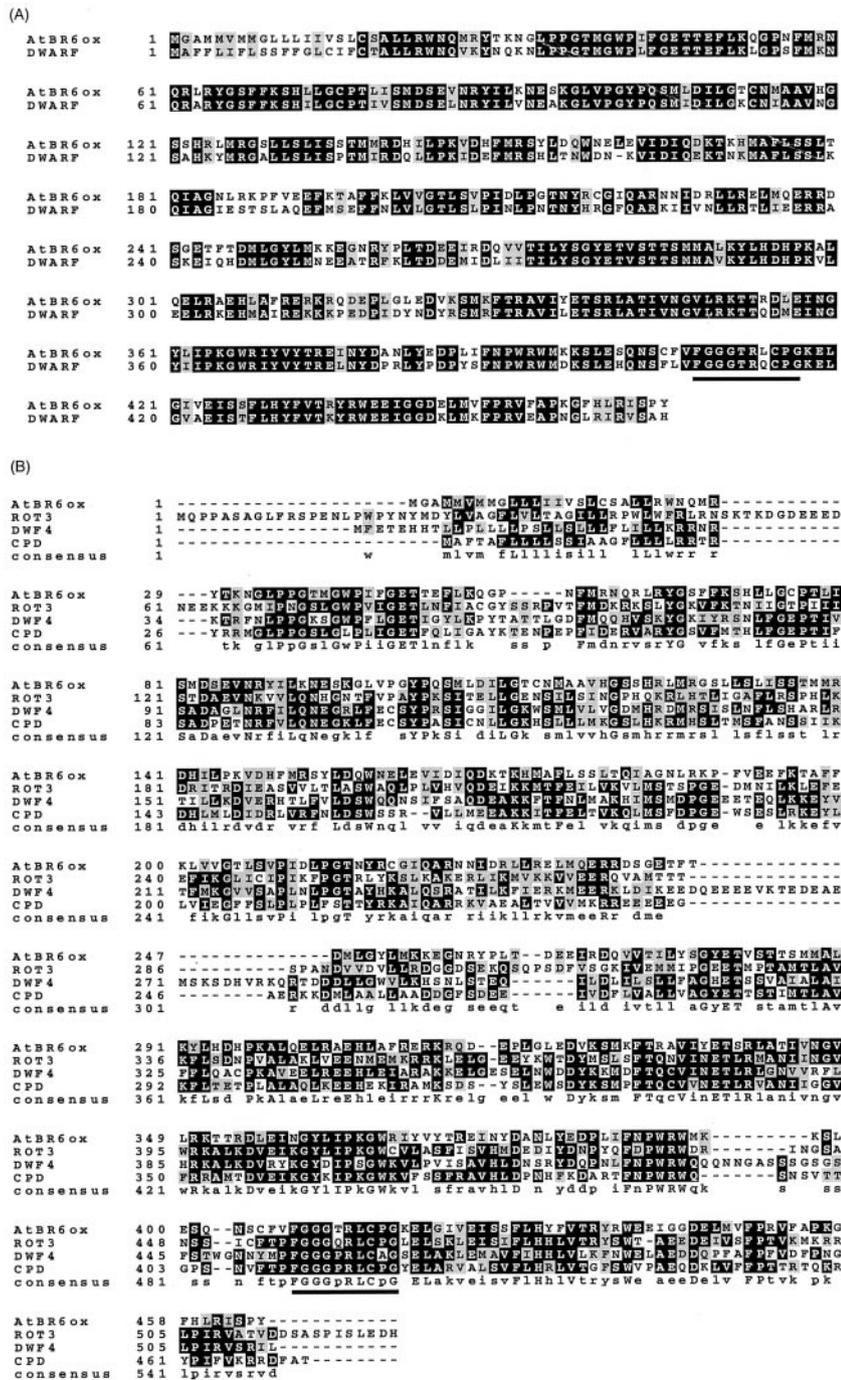
Figure 2. Sequence alignment of *AtBR6ox* and related genes. A, Sequence alignment of *AtBR6ox* and the tomato *Dwarf* gene. Reverse contrast characters highlight identical amino acid residues and conserved ones are indicated by hatched characters. Gaps introduced to improve the alignment are shown by hyphens. The GenBank/EMBL/DDBJ accession nos. of the *AtBR6ox* gene (CYP85) and the tomato *Dwarf* gene (CYP85) are AB035868 and U54770, respectively. The heme-binding signature sequence is underlined. B, Sequence alignment of Arabidopsis P450s that are related to BR biosynthesis. In the consensus line, capital letters indicate identical amino acid residues in all genes and small letters indicate the most commonly conserved amino acid residues. The GenBank/EMBL/DDBJ accession nos. of *ROT3* (CYP90C), *DWF4* (CYP90B), and *CPD* (CYP90A) are AB008097, AF044216, and X87363, respectively. The heme-binding signature sequence is underlined.

identity (Fig. 2A). It also has high sequence similarity to members of CYP90 from Arabidopsis, namely with *CPD*, *DWF4*, and *ROT3* at the amino acid level (Fig. 2B). A phylogenic relationship was calculated and is shown in Figure 3. This indicates that *AtBR6ox* is nearer to the tomato *Dwarf* than to Arabidopsis P450 genes in the CYP90 family, some of which are thought to be involved in BR biosynthesis. *AtBR6ox* is also designated CYP85A1 according to the nomenclature of the P450 superfamily (Nelson et al., 1996).

Genome Analysis

We also searched for genomic clones of the *AtBR6ox* gene in the databases. The nucleotide sequence of the cDNA clone, F5F3 completely matched to sequences from an Arabidopsis genomic clone, K15E6, sequenced by the Arabidopsis genome project (Sato et al., 1998; the GenBank, EMBL, and DDBJ accession no. is AB009048). Comparison of the cDNA and the genomic clone revealed that the *AtBR6ox* gene consists of nine exons (Fig. 4A). All the exon-
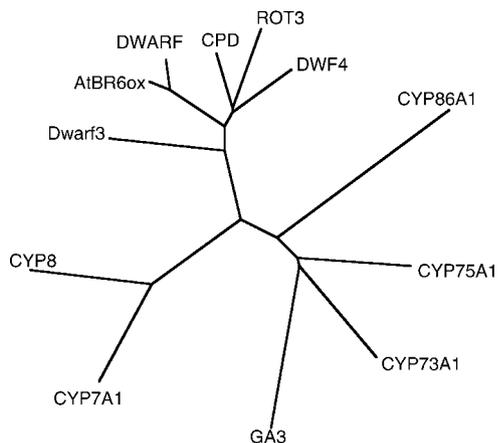
Figure 3. Phylogenetic relationship between *AtBR6ox* and selected P450 genes. *AtBR6ox* belongs to the group consisting of *Dwarf* (Tomato, CYP85), *CPD* (Arabidopsis, CYP90A), *ROT3* (Arabidopsis, CYP90C), and *DWF4* (Arabidopsis, CYP90B). All of these genes are thought to be involved in BR biosynthesis, except *ROT3*. The GenBank/EMBL/DDBJ accession nos. of *Dwarf3* (Maize, CYP88A) and *GA3* (Arabidopsis, CYP701A) are U32579 and AF047720, respectively; both are suggested to be involved in gibberellin (GA) biosynthesis. The first P450 genes functionally identified from higher plants are CYP73A (*Helianthus* cinnamate 4-hydroxylase, Z17369) and CYP75A (*Petunia* flavonoid-3′, 5′-hydroxylase, D14588); both belong to the higher plant-specific Group A of P450 genes. The accession nos. of the other members are D30718 (CYP8), M93133 (CYP7A), and X90458 (CYP86A).

intron boundaries consist of the conserved sequences of consensus splice sites. Clone K15E6 maps to chromosome 5 (Sato et al., 1998). To investigate the number of related genes in Arabidopsis, a genomic DNA gel blot was hybridized with an RNA probe from cDNA clone F5F3. The result after low-stringency washes is shown in Figure 4B. The result indicates that there are no other genes that cross-hybridized to the *AtBR6ox* gene according to the restriction map of the genomic sequence. The band pattern was the same after high-stringency washes (not shown).

Functional Analysis

The sequence similarity between *AtBR6ox* and *Dwarf* does not necessarily imply that the *AtBR6ox* enzyme catalyzes the conversion of the same substrates in Arabidopsis as the *Dwarf* enzyme does in tomato. To establish the biochemical function of the *AtBR6ox* product, the gene was functionally expressed in yeast. The protein-coding region of the complete sequence was synthesized by fusing the 5′-coding region of PCR clone pCRCSS12 and the 3′ region of the EST clone, F5F3. A cDNA sequence of the protein-coding region was then sub-cloned into a yeast expression vector, pYeDP60, and was expressed in the yeast strain, WAT11, which carries Arabidopsis NADPH-P450-reductase (Urban et al., 1997). In this strain, both *AtBR6ox* and P450 reductase were over expressed 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. Confirmation of the identity of the products was provided by a direct comparison of the relative abundance of characteristic ions of the metabolites and standard compounds (Table I). Conversion rates obtained by GC-selected ion monitoring for AtBR6ox and Dwarf are summarized in Tables II and III, respectively.

When a yeast strain expressing *AtBR6ox* was incubated with [^{2}H$_6$]6-DeoxoCS, both [^{2}H$_6$]CS and [^{2}H$_6$]6-OHCS were identified as metabolites (Tables I and II), whereas BL was not identified in this incubation. From this result, we concluded that *AtBR6ox* encodes a steroid-6-oxidase. Other 6-deoxo compounds were also fed to the yeast to know whether these can be substrates for the AtBR6ox 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 (Tables I and II). No conversion was detected in a
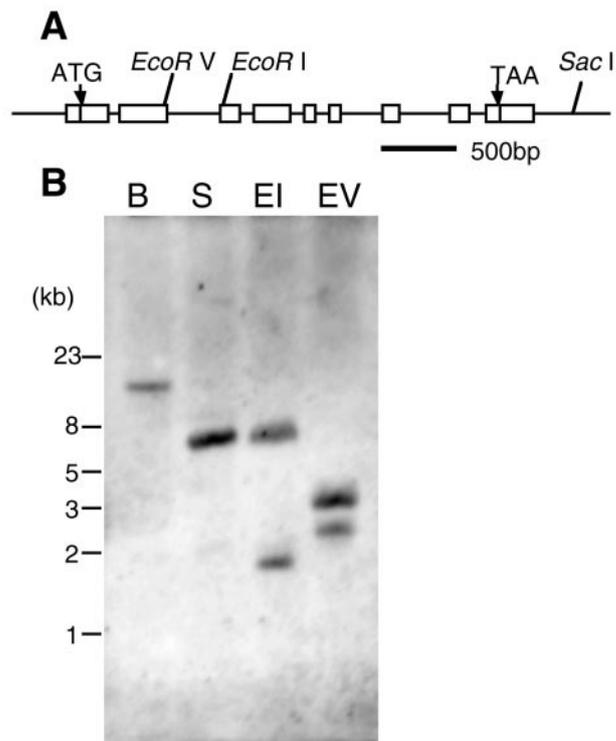

Figure 4. Analysis of the *AtBR6ox* gene. A, The physical structure of the *AtBR6ox* gene in Arabidopsis. White boxes represent exons. Slanting bars indicate restriction sites. There are no *Bam*HI sites in the genome region presented. Start and stop codons are indicated by arrows. B, Genomic Southern analysis. The blot contains 2 μg genomic DNA that was digested with *Bam*HI (B), *Sac*I (S), *Eco*RI (EI), or *Eco*RV (EV), and was hybridized with a digoxigenin-labeled RNA probe derived from the F5F3 clone. The blot was washed under low-stringency condition.

Table I. *GC-MS identification of [$^2H_6$]-labeled BRs in yeast cultures expressing AtBR6ox*

M$^+$, Molecular ion; *m/z*, mass-charge ratio.

Compound	Retention Time on GC	Characteristic Ions (Relative Intensity Percentage)
	min	*m/z*
[$^2H_6$]CS[a]		
Standard	11.82	518 [M$^+$] (33), 399 (5), 358 (11), 287 (23), 161 (100)
Metabolite	11.83	518 [M$^+$] (33), 399 (5), 358 (12), 287 (22), 161 (100)
[$^2H_6$]6-OHCS[b]		
Standard	11.40	592 [M$^+$] (2), 577 (22), 502 (64), 271 (66), 161 (100)
Metabolite	11.42	592 [M$^+$] (2), 577 (23), 502 (72), 271 (63), 161 (100)
[$^2H_6$]TY[b]		
Standard	11.23	550 [M$^+$] (81), 535 (50), 521 (100), 460 (38), 161 (58)
Metabolite	11.23	550 [M$^+$] (71), 535 (46), 521 (100), 460 (46), 161 (66)
[$^2H_6$]3DT[a]		
Standard	11.92	476 [M$^+$] (12), 316 (9), 287 (7), 245 (13), 161 (100)
Metabolite	11.92	476 [M$^+$] (11), 316 (8), 287 (6), 245 (13), 161 (100)
[$^2H_6$]TE[b]		
Standard	11.95	550 [M$^+$] (25), 535 (66), 521 (100), 460 (2), 161 (25)
Metabolite	11.97	550 [M$^+$] (25), 535 (65), 521 (100), 460 (3), 161 (21)

[a] Methaneboronate derivative.　　[b] Methaneboronate-trimethylsilyl derivative.

yeast strain that was transformed only with the vector, pYeDP60.

A yeast strain expressing the tomato *Dwarf* gene was also examined for its substrate specificity using the same 6-deoxo BRs, revealing that the transformant had the same substrate specificity as the *AtBR6ox*-expressing yeast (Table III).

DISCUSSION

We isolated the *AtBR6ox* gene, an Arabidopsis ortholog of the tomato *Dwarf* gene. The primary structure of the isolated *AtBR6ox* gene suggests that it belongs to the P450 superfamily. Phylogenic sequence comparison in Figure 3 revealed that it is most similar to the tomato *Dwarf* gene, among known P450s. *AtBR6ox* and its homologs form a family with *CPD* and *DWF4*, BR-related P450 genes, in a phylogenic tree. The only exception is the maize (*Zea mays*) *Dwarf3* gene that was reported to be involved in gibberellin biosynthesis (Winkler and Helentjaris

1995). The present work demonstrated that *AtBR6ox* and tomato *Dwarf* are involved in C-6 oxidation, and hence the corresponding proteins were designated BR-6-oxidases. Sequence comparison in Figure 2A revealed the existence of consensus amino acid sequences for BR-6-oxidases. On the other hand, sequence comparison in Figure 2B revealed consensus sequences in Arabidopsis P450 mono-oxygenases in CYP85 and CYP90 families suggesting specified protein structures. Most members of these families are involved in BR biosynthesis.

This study revealed that both Arabidopsis AtBR6ox and tomato Dwarf enzymes catalyze identical multiple reactions in which the C-6 position of 6-DeoxoCS, 6-DeoxoTY, 6-Deoxo3DT, and 6-DeoxoTE are oxidized. In a number of plants including Arabidopsis, both of these substrates and products seem to occur naturally, suggesting that these reactions may be operative in planta. In Arabidopsis seedlings, TY has been recovered as a metabolite of 6-DeoxoTY (Noguchi et al., 2000), supporting the presence of the path-

Table II. *C6 oxidation of BRs by AtBR6ox expressed in yeast*

Substrate	Product	Conversion Rate[a]		
		First experiment	Second experiment	Vector
		%		
[$^2H_6$]6-DeoxoCS	[$^2H_6$]CS	35	42	N.D.[b]
[$^2H_6$]6-DeoxoCS	[$^2H_6$]6-OHCS	16	13	N.D.
[$^2H_6$]6-DeoxoTY	[$^2H_6$]TY	23	–	N.D.
[$^2H_6$]6-Deoxo3DT	[$^2H_6$]3DT	36	38	N.D.
[$^2H_6$]6-DeoxoTE	[$^2H_6$]TE	13	21	N.D.
[$^2H_6$]6-DeoxoCT	[$^2H_6$]CT	N.D.	N.D.	N.D.
[$^2H_6$]CN	[$^2H_6$]6-OxoCN	N.D.	N.D.	N.D.

[a] The conversion rate was calculated roughly as a percentage of the amount of each metabolite versus the amount of substrate added to the culture.　[b] N.D., Not detected.

Table III. *C6 oxidation of BRs by Dwarf expressed in yeast*

Substrate	Product	Conversion Rate[a]	
		First experiment	Vector
		%	
[²H₆]6-DeoxoCS	[²H₆]CS	62	N.D.[b]
[²H₆]6-DeoxoCS	[²H₆]6-OHCS	30	N.D.
[²H₆]6-DeoxoTY	[²H₆]TY	66	N.D.
[²H₆]6-Deoxo3DT	[²H₆]3DT	96	N.D.
[²H₆]6-DeoxoTE	[²H₆]TE	50	N.D.
[²H₆]6-DeoxoCT	[²H₆]CT	N.D.	N.D.
[²H₆]CN	[²H₆]6-OxoCN	N.D.	N.D.

[a] The conversion rate was calculated as a percentage of the amount of each metabolite versus the amount of substrate added to the culture. Non-labeled BRs were added to the media before extraction and used as internal standards. [b] N.D., Not detected.

way from 6-DeoxoTY to TY in planta. In contrast, neither 3DT nor TE was recovered from 6-Deoxo3DT and 6-DeoxoTE (Noguchi et al., 2000). A number of possibilities can account for this discrepancy between in yeast and in planta metabolism so far. For example, in planta metabolic flux from 6-DeoxoTE to TE and 6-Deoxo3DT to3DT cannot be detected because the 6-oxo BRs are turned over very rapidly to regulate their endogenous levels at low levels. Another possibility is that in planta metabolic flows from 6-DeoxoTE to TE and 6-Deoxo3DT to3DT are minor or even absent; flows from 6-DeoxoTY to TY and flows from 6-DeoxoCS to CS are major.

Although tomato Dwarf oxidizes the same substrates as AtBR6ox, no 6-oxo BRs such as 6-OHCN, 6-oxoCN, CT, TE, 3DT, and TY have been identified as endogenous BRs (Yokota et al., 1997, Bishop et al., 1999, Koka et al., 2000). Therefore, conversion of 6-DeoxoCS to CS seems to be the only major C-6 oxidation pathway in tomato tissues. Further biochemical and metabolic studies of BR-6-oxidases will be needed to clarify the C-6 oxidation mechanism and its difference between Arabidopsis and tomato.

Yeasts transformed with *AtBR6ox* and *Dwarf* could not catalyze C-6 oxidation of [²H₆]6-DeoxoCT and [²H₆]CN (Tables I and II). The possibility is not likely that these substrates cannot be incorporated into yeast cells because [²H₆]6-DeoxoCT and [²H₆]CT were metabolized to [²H₆]6-DeoxoTE and [²H₆]6-OxoCN when fed to *C. roseus* cells and Arabidopsis seedlings (Noguchi et al., 2000; Suzuki et al., 1995). We recently found the natural occurrence of 6-OxoCN in Arabidopsis (S. Fujioka, unpublished data), which may suggest that an unknown BR-6-oxidase catalyzing CN to 6-OxoCN exists in Arabidopsis. However, genomic DNA gel-blot analysis did not support the existence of such an Arabidopsis gene that can hybridize with the *AtBR6ox* gene (Fig. 4). Further efforts to isolate a putative unknown BR-6-oxidase are in progress. In an RNA gel-blot analysis using the N-terminal region of the *AtBR6ox* gene as a probe, we observed no signal (data not shown). When using full-length *AtBR6ox* cDNA, a single band

was observed, but it was significantly smaller than the expected mature mRNA (data not shown) and also than the ORF sizes of the five cDNA clones derived from RT-PCR. We concluded that the in vivo level of *AtBR6ox* transcription is extremely low and further approaches, such as RT-PCR, are needed to study the expression and the regulatory function of the *AtBR6ox* gene.

Altogether, pathways from 6-DeoxoTY to TY, from 6-Deoxo3DT to 3DT, and from 6-DeoxoTE to TE are postulated to be present in Arabidopsis and possibly other plants, although the latter two remain tentative (Fig. 5). However, occurrence of these pathways in tomato seems less likely. It is also indicated that these C-6 oxidation steps as well as the conversion of 6-DeoxoCS to CS are catalyzed by a single BR-6-oxidase. The BR biosynthetic pathway seems to consist of a metabolic grid similar to that in GA biosynthesis, rather than of two independent parallel pathways, the early and late C-6-oxidation pathways.

MATERIALS AND METHODS

Plant Materials

Arabidopsis ecotype Colombia (Col-0) was used as wild type in this study. Seedlings were grown aseptically on one-half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1.5% (w/v) Suc and 0.8% (w/v) agar in a growth cabinet (at 22°C, continuous illumination of 100 μmol mm^{-2} s^{-1}).

Isolation of cDNA Clones

Arabidopsis EST clones were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). Total
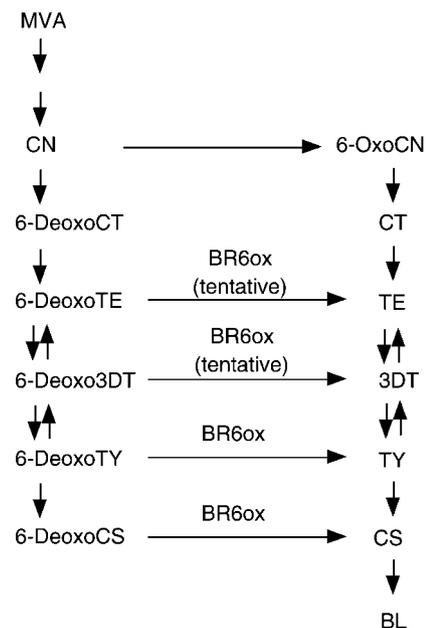

Figure 5. BR biosynthesis pathway. BR-6-oxidase (BR6ox) converts 6-DeoxoTE to TE, 6-Deoxo3DT to 3DT, 6-DeoxoTY to TY, and 6-DeoxoCS to CS.

RNAs were extracted from 10-d-old Arabidopsis seedlings grown on one-half-strength Murashige and Skoog agar medium in light by the guanidine-hydrochloride method (Kawakami and Watanabe 1988). cDNA was synthesized with a Ready To Go T-Primed First Strand Kit (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK) according to the manufacturer's instructions. The full-length cDNA fragment of *AtBR6ox* was amplified by PCR with the cDNA, LA *Taq* Polymerase (Takara Shuzo, Kyoto), and primers complementary to regions of the putative start and stop codons: CSS-F2 (5′ CAG AGC AGA AAA CAG AGT GAG ATG G 3′) and CSS-R1 (5′ TAC GTC TTC TGT ATC CTC TGC GTG C 3′). The fragments were cloned into the pCR2 vector using a TOPO TA Cloning Kit (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. They were designated pCRCSS12, 19, 93, 94, 98, and 99. The nucleotide sequences of these clones were determined and it was found that the first intron was properly spliced out, except for clone pCRCSS99.

DNA Sequence Analyses

DNA sequences were determined using an automated DNA sequencer (model 373A and model 310A DNA Sequencing System, PE Biosystems, Foster City, CA) according to the manufacturer's instructions. The nucleotide sequence was compiled and analyzed with 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 databases at 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 Boxshade program, available on the server at the European Molecular Biology Network.

Southern Analysis

Genomic DNA was isolated from Arabidopsis using Nucleon PhytoPure Plant and Fungal DNA Extraction Kits (Amersham International PLC) according to the manufacturer's instructions. The DNA (2 μg) was digested, fractionated in 0.8% (w/v) agarose-Tris-EDTA gel, and transferred to positively charged nylon membranes (Roche, Mannheim, Germany) according to Sambrook et al. (1989). For probe preparation, the cDNA clone F5F3 was digested with *Bam*HI. The full-length antisense RNA was labeled with digoxigenin using a DIG RNA Labeling Kit (Roche) according to the manufacturer's instructions. The probe was hybridized to the blot at 50°C overnight and washed four times with 1 × SSPE (Sambrook et al., 1989), 0.1% (w/v) SDS for low-stringency washes, or washed two times with 1 × SSPE, 0.1% (w/v) SDS and then an additional two times with 0.1 × SSPE, 0.1% (w/v) SDS at 50°C for high-stringency washes. Washed blots were reacted with Anti-Digoxigenin-AP and CPD-Star (Roche) according to the manufacturer's instructions, and then detected with a luminescent image analyzer (LAS 1000, Fujifilm, Tokyo).

Yeast Expression Vector

An EST clone of the *AtBR6ox* gene, F5F3, contained the first intron, which was not spliced out properly. Each of the full-length cDNA clones isolated by RT-PCR had base substitutions, derived from PCR artifacts, in different positions. Therefore, the full-length cDNA clone corresponding to the complete sequence was made by fusing the 5′-coding region of clone pCRCSS12 and the 3′ region of the EST clone, F5F3, as follows. F5F3 was completely digested with *Bam*HI then digested partially with *Cla*I. The band consisting of the 3′ region of the cDNA and the vector, pBluescriptII SK(-), was purified from a gel. The 5′ region of pCRCSS12 was amplified by PCR with a cDNA clone, pCRCSS12, and primers: CSS-F-bam primer (5′ GGG GAT CCA TGG GAG CAA TGA TGG TG 3′) and CSS-R2-cla primer (5′ AAG CAT CGA TTG TGG GTA ACC AG 3′). The amplified DNA was digested with *Bam*HI and *Cla*I, and then ligated to the 3′ region of the cDNA derived from F5F3. The clones were sequenced to verify the sequence accuracy. A cDNA clone of the full-length sequence was identified and designated pBCSS4. The full-length cDNA was excised from pBCSS4 with *Bam*HI and *Kpn*I, and then sub-cloned into a yeast expression vector, pYeDP60 (Pompon et al., 1996). The resulting plasmid was designated pYCS41.

Yeast Functional Assay

Yeast expression of the tomato (*Lycopersicon esculentum*) *Dwarf* gene was performed as descried previously (Bishop et al., 1999). For the Arabidopsis gene, pYCS41 and pYeDP60 were transformed to yeast strain WAT11 (Pompon et al., 1996) using a Frozen-EZ Yeast Transformation Kit (ZYMO Research, Orange, CA). Transformants were selected on SGI-agar medium (Pompon et al., 1996). Isolated colonies were cultured in 3 mL of SGI medium overnight at 30°C. Yeast cells were collected by centrifugation, and then washed twice with sterile distilled water. They were then inoculated into 30 mL of YPL medium (Pompon et al., 1996) to induce expression of the *AtBR6ox* gene and an Arabidopsis NADPH-P450-reductase gene (Urban et al., 1997). After 6 h of cultivation, substrate solutions (5 μg/5 μL ethanol) of $[^2H_6]$6-DeoxoCS, $[^2H_6]$6-DeoxoTY, $[^2H_6]$6-Deoxo3DT, $[^2H_6]$6-DeoxoTE, $[^2H_6]$6-DeoxoCT, and $[^2H_6]$CN were added to the culture as substrates for the AtBR6ox protein. After 16 h of incubation, the BRs were extracted and analyzed as described previously (Bishop et al., 1999; Noguchi et al., 1999a). Products from the incubations were analyzed by GC-MS/selected ion monitoring. Confirmation of the identity of the products was provided by their full-scan mass spectra. Quantification was based on calibration curves constructed using $[^2H_6]$-labeled and non-labeled BRs. The conversion rate was roughly calculated as a percentage of the amount of each metabolite versus the amount of substrate added to the culture for Arabidopsis. For the tomato experiment, non-labeled BRs were added to the media before extraction and used as internal standards to calculate the conversion rate.

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