

The *DWF4* Gene of *Arabidopsis* Encodes a Cytochrome P450 That Mediates Multiple 22 α -Hydroxylation Steps in Brassinosteroid Biosynthesis

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dwarf4 (*dwf4*) mutants of *Arabidopsis* display a dwarfed phenotype due to a lack of cell elongation. Dwarfism could be rescued by the application of brassinolide, suggesting that *DWF4* plays a role in brassinosteroid (BR) biosynthesis. The *DWF4* locus is defined by four mutant alleles. One of these is the result of a T-DNA insertion. Plant DNA flanking the insertion site was cloned and used as a probe to isolate the entire *DWF4* gene. Sequence analysis revealed that *DWF4* encodes a cytochrome P450 monooxygenase with 43% identity to the putative *Arabidopsis* steroid hydroxylating enzyme CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM. Sequence analysis of two other mutant alleles revealed deletions or a premature stop codon, confirming that *DWF4* had been cloned. This sequence similarity suggests that *DWF4* functions in specific hydroxylation steps during BR biosynthesis. In fact, feeding studies utilizing BR intermediates showed that only 22 α -hydroxylated BRs rescued the *dwf4* phenotype, confirming that *DWF4* acts as a 22 α -hydroxylase.

INTRODUCTION

The sessile nature of plants requires that they make fine but responsive adjustments in growth to survive harsh environmental conditions and to optimize their use of limited resources (Trewavas, 1986). Plant growth in response to environmental factors is modulated by plant hormones acting alone or in concert (Evans, 1984), and growth depends on regulated cellular events, such as division, elongation, and differentiation. In addition to the classic hormones, such as auxin and gibberellic acid (GA), brassinosteroids (BRs) have been discovered to be important in growth promotion (reviewed in Clouse, 1996). This conclusion is based on the results of experiments in which a BR was applied to a series of *Arabidopsis dwarf* (*dwf*) mutants. Several types of dwarf or dwarflike mutants have been described in *Arabidopsis*. Among these are the dwarfs that are rescued by GA (Koornneef and Van der Veen, 1980) as well as a collection of *dwf* mutants that are rescued by BRs (Kauschmann et al., 1996; Li et al., 1996; Szekeres et al., 1996; Azpiroz et al., 1998). There are additional dwarfs that are insensitive to one of these hormones, such as *bri* (*brassinosteroid insensitive*;

Clouse et al., 1996; Li and Chory, 1997), *gai* (*gibberellic acid insensitive*; Koornneef et al., 1985), and *axr2* (*auxin resistant2*; Timpote et al., 1994). We are characterizing a large collection of BR-rescued *dwf* mutants as an approach to understanding the mechanisms involved in the biosynthesis of these compounds.

The term BRs collectively refers to the growth-promoting steroids found in plants (Grove et al., 1979). They are structurally very similar to the molting hormones of insects, ecdysteroids (Richter and Koolman, 1991), but active BRs have unique structural features. As shown in Figure 1, a 6-oxolactone or 7-oxalactone in the B ring, 5 α hydrogen, and multiple hydroxylations at four different positions with specific stereochemistry have been proposed as an essential configuration for BRs (reviewed in Marquardt and Adam, 1991). Among >40 naturally occurring BRs, brassinolide (BL; 2 α , 3 α , 22(R), 23(R)-tetrahydroxy-24(S)-methyl-B-homo-7-oxa-5 α -cholestan-6-one) has been shown to be the most biologically active (reviewed in Mandava, 1988). As a major biological effect, BRs stimulate longitudinal growth of young tissues via cell elongation and cell division (reviewed in Clouse, 1996; Fujioka and Sakurai, 1997a).

Elucidating the BR biosynthetic pathways has been a major area of recent interest. Biochemical analyses have been used to elucidate the BR biosynthetic pathway (Fujioka et

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al., 1996; Choi et al., 1997), and mutational analyses are being used to confirm this pathway. Similar to the biosynthetic pathways of the human steroid hormones and insect ecdysteroids (Rees, 1985; Granner, 1996), BRs are synthesized via multiple parallel pathways (Fujioka et al., 1996; Choi et al., 1997). Starting from the initial precursor, campesterol (CR), the BR intermediates undergo a series of hydroxylations, reductions, an epimerization, and a Baeyer-Villiger-type oxidation leading to the most oxidized form, BL (Fujioka and Sakurai, 1997b; Figure 1). Castasterone (CS) oxidation, the last step in BR biosynthesis, is not found in some species, such as mung bean. In that case, CS plays a role as the major BR rather than BL (Yokota et al., 1991).

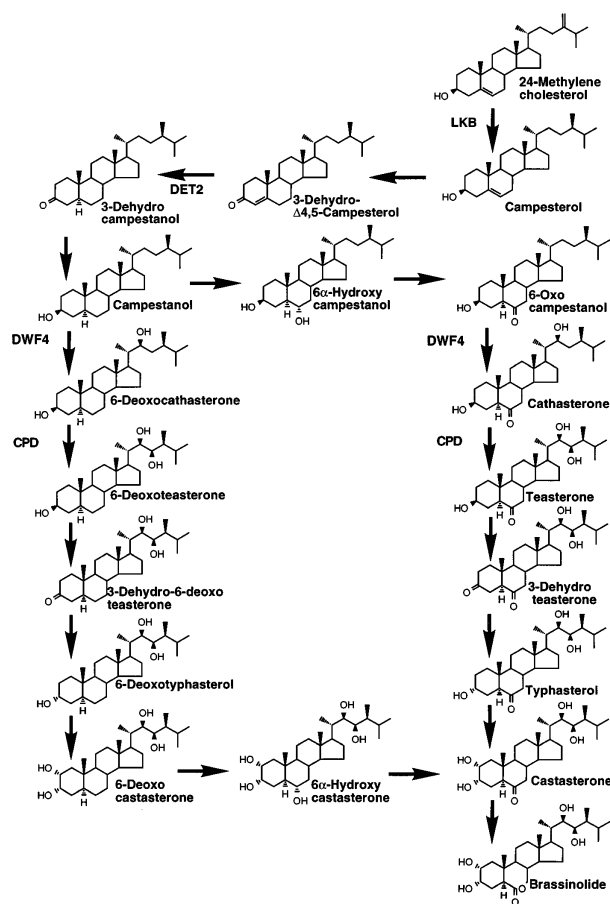


Figure 1. Proposed Biosynthetic Pathway for BL.

CR goes through at least two different pathways, referred to as the early C-6 oxidation (right column) and late C-6 oxidation (left column) pathways. Steps mediated by DWF4, CPD (Szekeres et al., 1996), DET2 (Fujioka and Sakurai, 1997a; Li et al., 1997), and LKB (Yokota et al., 1997) are indicated. This diagram is adopted from Fujioka and Sakurai (1997a).

Traditionally, BR biosynthetic pathways have been elucidated by feeding deuterio-labeled intermediates to BR-producing cell lines of Madagascar periwinkle (Sakurai and Fujioka, 1996). The present model, including parallel branched pathways and early and late C-6 oxidation pathways, was established using these feeding studies (Fujioka and Sakurai, 1997a, 1997b; Sakurai and Fujioka, 1997).

Genetic, molecular, and biochemical studies with *Arabidopsis* dwarf mutants have begun to confirm the steps of the proposed BR biosynthetic pathway. Of a growing number of BR-related genes that have been identified, several have been cloned. *DWF1* (Feldmann et al., 1989) was cloned first (GenBank accession number U12400; B.P. Dilkes and K.A. Feldmann, unpublished data). Takahashi et al. (1995) hypothesized that *DWF1*, which they isolated with an allele of *dwf1*, referred to as *diminuto1* (*dim1*), contains a potential nuclear targeting signal, which may confer a regulatory function to the protein. However, Mushegian and Koonin (1995) and our unpublished data (B.P. Dilkes and K.A. Feldmann) indicated that *DWF1* displays limited homology with flavin adenine dinucleotide (FAD)-dependent oxidoreductase, suggesting an enzymatic function in BR biosynthesis. According to Kauschmann et al. (1996; *dwf1-6* described as *cabbage1* [*cbb1*]) and our unpublished data (B.P. Dilkes, S. Choe, and K.A. Feldmann), *dwf1* mutants were rescued by exogenous application of BRs. The second and third genes cloned were *DEETIOLATED2* (*DET2*) and *CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM* (*CPD*). *DET2* was shown to encode a putative steroid 5 α -reductase, mediating an early step in BR biosynthesis (Li et al., 1996, 1997; Fujioka et al., 1997; Figure 1), and *CPD* has been proposed to be a novel cytochrome P450 (CYP90A1; Szekeres et al., 1996), encoding a putative 23 α -hydroxylase that acts in BR biosynthesis. In addition to these BR-responsive dwarfs, Clouse et al. (1996) isolated *bri* by screening ethyl methanesulfonate-mutagenized populations for mutants whose root growth is not retarded at inhibitory concentrations of BR. Thus, the *BRI* protein is proposed to be involved in BR signal perception or transduction (Clouse, 1996). Kauschmann et al. (1996) described a phenotypically similar mutant *cbb2* that maps to the same location. In addition, our *dwf2* alleles possess a phenotype similar to *bri* and map to the same region (Feldmann and Azpiroz, 1994; data not shown). It seems likely that all of the BR-insensitive dwarf mutants described to date are allelic. Recently, *BRI* has been cloned and shown to encode a leucine-rich-repeat receptor kinase, suggesting a role in the BR signal transduction pathway (Li and Chory, 1997).

Mutants defective in BR biosynthesis have also been isolated in other plant species. Bishop et al. (1996) isolated a tomato *dwarf* mutant by transposon tagging. The tomato *Dwarf* gene encodes a pioneering member of the CYP85 family, and it appears to be involved in BR biosynthesis. In addition, Nomura et al. (1997) reported that the *Ika* and *Ikb* mutants in garden pea are deficient in BR biosynthesis (*Ikb*) or perception (*Ika*).

Currently, we have defined eight BR dwarf loci in Arabidopsis, *dwf1* to *dwf8*. Six *dwf* loci are defined by two to 10 alleles each, whereas for two loci (*dwf7* and *dwf8*), only a single mutant allele has been identified. As mentioned, *dwf1* is allelic to *cbb1* and *dim1*; *dwf2* maps to the same region as *bri* and *cbb2*; *dwf3* is allelic to *cpd* and *cbb3*; *dwf6* is allelic to *det2*; and *dwf4*, *dwf5*, *dwf7*, and *dwf8* are new genes. All of our *dwf* mutants, except for *dwf2* alleles, are highly responsive to exogenously supplied BRs. In this article, we continue our genetic and phenotypic studies with *dwf4* (Azpiroz et al., 1998) and describe the molecular analysis of *DWF4* and the physiological characteristics of *dwf4* mutants.

RESULTS

Molecular Cloning of *DWF4*

The *dwf4-1* mutation was identified originally in a screen of 14,000 transformants of Arabidopsis, resulting in a dwarfed phenotype similar to *dwf1* and *det2* (Azpiroz et al., 1998). *dwf4-1* segregated for a single kanamycin resistance marker, and gel blot analysis with DNA from single plants of this family confirmed that the pattern is consistent with a single insert. Cosegregation analysis of the dwarf phenotype and the kanamycin resistance trait indicated tight linkage. Partial sequencing of a *dwf4-1* genomic clone containing the T-DNA left border revealed a portion of an open reading frame encoding a cytochrome P450 steroid hydroxylase (Azpiroz et al., 1998). Simultaneously, we used plasmid rescue from the left border (as described in Dilkes and Feldmann, 1998) to isolate a larger stretch of plant DNA flanking the T-DNA in *dwf4-1*. Two different plant DNAs of 1.1 and 5.6 kb were identified. This result suggested that the T-DNA insert in *dwf4-1* was flanked by two left border regions. This was confirmed by gel blot analysis with genomic DNA, using the putative plant flanking DNAs as probes (see Methods).

A wild-type genomic library was screened using the 5.6-kb fragment as a probe. Of 12 λ clones identified, the restriction pattern of one, D4G12-1 (13-kb insert), indicated that it contained the complete 5.6-kb sequence (data not shown). Gel blot analysis and partial sequencing of the plant flanking DNA suggested that a 4.8-kb fragment of D4G12-1 delimited by EcoRI-HindIII spans the entire *DWF4* locus (Figure 2A; see below). The wild-type DNA was completely sequenced.

Analysis of the complete genomic sequence, starting at the EcoRI site, with the promoter prediction by neural network (NNPP) package (<http://www-hgc.lbl.gov/projects/promoter.html>), indicated that the gene included a putative promoter (TATAT is found in the putative promoter region between nucleotides -143 to -78) and polyadenylation signal sequences (AATAA near a position at 3238 bp and a putative GU-rich signature from 3283 to 3290 bp).

Unsuccessful attempts to detect mRNA by tissue-specific RNA gel blot analysis, using the 4.8-kb fragment as a probe, suggested that *DWF4* encoded a rare message. In addition, there were no matching expressed sequence tags in the Arabidopsis database. Therefore, we screened two different cDNA libraries made with either normalized mRNA from different tissues or RNA from floral tissues, using the 4.8-kb fragment as a probe (see Methods; ABRC stock numbers CD4-7 and CD4-6, respectively). After finding no positives in 10^9 clones screened, we chose to directly amplify *DWF4* cDNA from total RNA made from 5-day-old seedlings, using reverse transcriptase-polymerase chain reaction (RT-PCR). Whereas RNA from both light-grown and dark-grown seedlings yielded the expected RT-PCR products, RNA from dark-grown seedlings generated significantly more (data not shown). The bands were gel purified and sequenced. Alignment of the genomic and cDNA sequences indicated that the *DWF4* gene was composed of eight exons and seven introns (Figure 2A).

Sequence analysis of the *dwf4-1* allele revealed that the T-DNA was inserted in the 5' end of intron 7 (Figure 2A). In addition, sequence analysis of the left border plant junctions indicated that at one junction (5'), 75 bp of unknown DNA was inserted, whereas at the other junction (3'), 24 bp of left border and 19 bp of plant DNA were deleted. To prove that *DWF4* had been cloned, two other *dwf4* alleles (*dwf4-2* and *dwf4-3*) were sequenced to identify possible lesions. As shown in Figure 2B, *dwf4-2* contained a deletion of three conserved amino acids (324 to 326) caused by a 9-bp deletion, and *dwf4-3* contained a premature stop codon (289) caused by changing a tryptophan codon (UGG) to a nonsense codon (UGA). Due to a premature stop codon, translation is predicted to be terminated before the heme binding domain, which is essential for cytochrome P450 function (Poulos et al., 1985). Because T-DNA-generated alleles *dwf4-1* and *dwf4-2* and an additional mutant allele all possess loss-of-function mutations affecting the same protein, we conclude that we have cloned the *DWF4* gene. Additional evidence is presented below.

The *DWF4* Gene Encodes a Cytochrome P450

The open reading frame of *DWF4* encodes a protein composed of 513 amino acids. BLAST database searches (Altschul et al., 1990) for similar sequences yielded a superfamily of cytochrome P450 proteins as significant high-scoring segment pairs. Typically, microsomal cytochrome P450 enzymes can be identified by their characteristic signature sequences, including the heme binding domain, domain A (also referred to as dioxygen binding), domain B (steroid binding), and domain C (Nebert and Gonzalez, 1987; Kalb and Loper, 1988). All of these signature sequences were found in *DWF4*; the relative positions of the domains are indicated in Figure 2B.

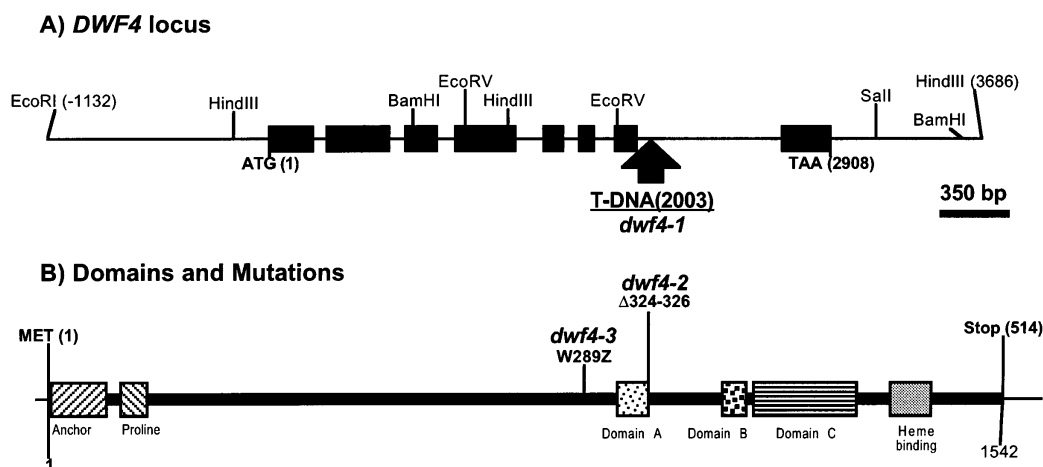


Figure 2. Schematic Representation of the *DWF4* Gene and Protein.

(A) The *DWF4* coding sequence (1542 bp) consists of eight exons and seven introns. The exons and introns ranged in length from 93 to 604 and 84 to 754 bp, respectively. All of the introns were bordered by typical consensus splice junctions, 5'-GU and AG-3'. Closed rectangles indicate exons. The T-DNA position in *dwarf4-1* is marked with an arrow.

(B) Relative positions of the major domains in DWF4 cytochrome P450 are shown. All of the major domains found in the cytochrome P450 superfamily are conserved in DWF4. The estimated molecular mass and isoelectric point of the *DWF4* protein were 58 kD and 7.28, respectively. Hydropathy plotting and protein localization prediction by the PSORT software package (Nakai and Kanehisa, 1992) suggested that the protein may reside in a membrane of the endoplasmic reticulum as an integral protein. Mutations identified in the other *dwarf4* alleles are indicated. Both figures are drawn to scale.

The most similar protein to DWF4 is the Arabidopsis CPD protein. A mutation in *CPD* also caused dwarfism (Szekeres et al., 1996; CYP90A1, GenBank accession number X87368). DWF4 and CPD share 43% identity and 66% similarity. Conforming to the recommended nomenclature for cytochrome P450 enzymes, DWF4 and CPD (CYP90A1) are grouped into the same family within different subgroups (Durst and Nelson, 1995). As such, DWF4 represents a second member of the CYP90 family and is designated CYP90B1. Sequence similarity between the two proteins occurs throughout their length, with the greatest similarity in the classically conserved domains. Residues conserved between DWF4 and CYP90A are boxed and italicized in Figure 3. The second most similar protein is the tomato CYP85 (Bishop et al., 1996; GenBank accession number U54770). A mutation in this gene also results in dwarfism. DWF4 and CYP85 share 35% identity and 59% similarity in their overall protein sequences.

Six cytochrome P450 sequences with the greatest homology to DWF4, CYP90A1, CYP85, CYP88 (Winkler and Helentjaris, 1995; GenBank accession number U32579), cyanobacteria CYP120 (Kaneko et al., 1996; GenBank accession number D64003), human CYP3A3X (Molowa et al., 1986; GenBank accession number M13785), and zebrafish

CYP26 (White et al., 1996; GenBank accession number U68234), were chosen for multiple sequence alignment. Putative domains defined by Kalb and Loper (1988) are boxed and labeled in Figure 3. First, the heme binding domain pFGGfRpRICpGkel matches completely the sequence defined previously. Uppercase letters in the domain indicate amino acids conserved at all seven sequences in the alignment, and lower-case letters represent residues conserved in at least half of the proteins. Of the amino acids conserved in the heme binding domain, the function of the cysteinyl is established as a thiolate ligand to the heme (Poulos et al., 1985).

Domain A is defined by xllfaGhEttsxlxxa. Lowercase x's indicate variable amino acids. An invariant glutamate (E) preceded threonine (T) at position 314, T314, which is believed to bind dioxygen, was conserved in all proteins compared except CYP88 of maize. The second signature sequence, domain B, is also conserved in DWF4 with significant similarity. A valine at position 370 is conserved in all of the proteins, but it does not appear in Kalb and Loper's classic report (1988) on conserved domains. Again, DWF4 matches the domain C consensus sequence. Finally, the anchoring domain in the N-terminal end was distinguished by a repeat of the hydrophobic residue leucine. In addition, in DWF4, two acidic (glutamate) and two basic (histidine) residues

precede the repeated leucine in the N-terminal leader sequence. These charged residues may add more stability to the membrane topology of the protein as a strong start-stop transfer peptide (von Heijne, 1988).
Phylogenetic analyses of these seven proteins with cyto-

chrome P450s unique to plants (group A; Durst and Nelson, 1995) indicate that DWF4 does not cluster with these cytochrome P450s (Figure 4). Rather, DWF4 clustered with cytochrome P450s from other organisms such as bacteria, rat, and human.

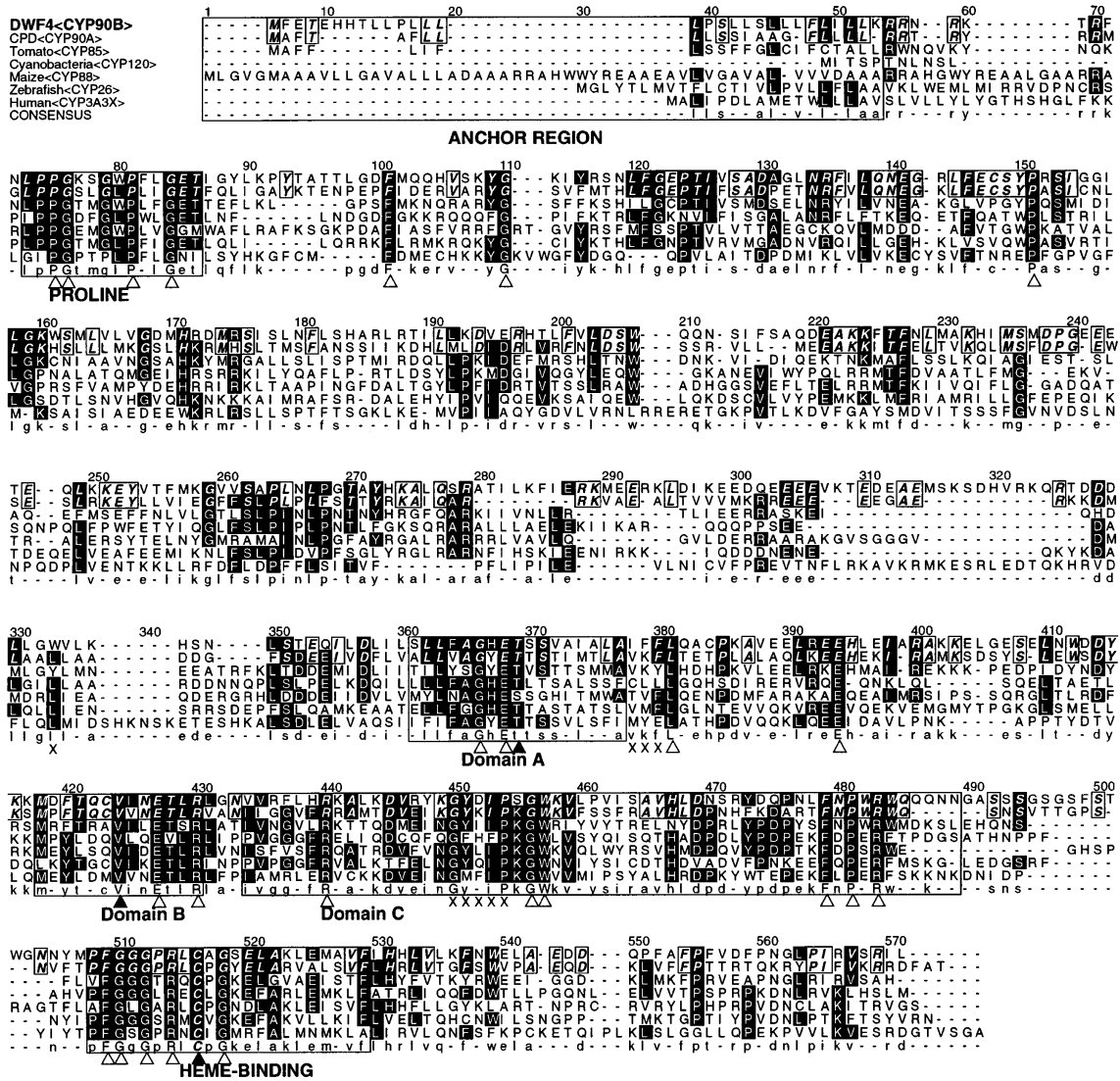


Figure 3. Multiple Sequence Alignment of Cytochrome P450s Most Similar to DWF4 (from BLAST Searches).

GenBank accession numbers are AF044216 (DWF4; CYP90B), X87368 (CPD; CYP90A), U54770 (tomato; CYP85), D64003 (cyanobacteria; CYP120), U32579 (maize; CYP88), U68234 (zebrafish; CYP26), and M13785 (human; CYP3A3X). Dashes indicate gaps introduced to maximize alignment. Domains indicated in Figure 2B are highlighted in a box. Amino acid residues that are conserved >50% between the compared sequences are highlighted by a reverse font, and identical residues between DWF4 and CPD are boxed and italicized. Open triangles are placed under the 100% conserved residues. Closed triangles locate functionally important amino acid residues, for example, threonine (T) at 369, which is thought to bind molecular oxygen, and cysteine (C) at 516, which links to a heme prosthetic group by a thiolate bond. X's indicate mutated residues in *dwf4* alleles. Multiple sequence alignment was performed using PILEUP in the Genetics Computer Group package, and box shading was made possible by the ALSRIPT package (Barton, 1993).

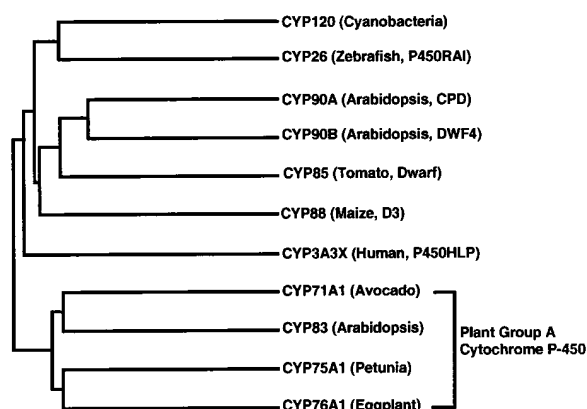


Figure 4. Phylogenetic Relationship between DWF4 and Selected Cytochrome P450s.

DWF4 did not cluster with the group A plant cytochrome P450s that are known to mediate plant-specific reactions (Durst and Nelson, 1995). CYP90A, CYP85, and DWF4, which are thought to be involved in BR metabolism, branched from CYP88, which mediates GA biosynthesis. GenBank accession numbers for the group A cytochrome P450s are M32885 (avocado; CYP71A1), P48421 (Arabidopsis; CYP83), P48418 (petunia; CYP75A1), and X71658 (eggplant; CYP76A1). The DISTANCE utility in the Genetics Computer Group software package was employed to calculate the relationships.

Feeding Experiments with BR Biosynthetic Intermediates

Because *dwf4* was previously observed to be a BL-responsive dwarf (Azpiroz et al., 1998) and the DWF4 protein sequence is similar to the BR biosynthetic enzyme CPD, we hypothesized that DWF4 mediates one or more of several steroid hydroxylation steps in the BR biosynthetic pathway. To test this, *dwf4* was grown on all of the available biosynthetic intermediates in the BR biosynthetic pathways and examined to ascertain which intermediates could rescue the dwarf phenotype. In addition to the intermediates belonging to the early C-6 oxidation and late C-6 oxidation pathways (Choi et al., 1997), 22 α -hydroxycampesterol (22-OHCR), 6 α -hydroxycathasterone (6-OHCT) (Takatsuto et al., 1997), and 6 α -hydroxycastasterone (6-OHCS) (S. Takatsuto, T. Watanabe, T. Noguchi, and S. Fujioka, unpublished data) were synthesized and tested.

Germinated seedlings were transferred to media supplemented with one of the intermediates or BL to pinpoint the step catalyzed by DWF4. Figure 5A shows that cathasterone (CT; early C-6 oxidation pathway), 6-OHCT, 6-deoxocathasterone (6-deoxoCT; late C-6 oxidation pathway), and 22-OHCR, and all of the downstream compounds belonging to each branch, rescued the light-grown *dwf4* phenotype, whereas the known precursors failed to cause an elongation re-

sponse. Rescued seedlings exhibited greatly elongated cotyledonary petioles and expanded cotyledons, moderately elongated hypocotyls, and leaves that were larger and not as curled compared with nonrescued dwarfs as shown in Figure 5A. In addition, the rescued seedlings were less green than the dwarfs. These experiments were conducted in liquid media. Feeding experiments performed in the dark yielded similar results (data not shown).

Dose-response tests on the putative substrates and products of DWF4 were performed. Figure 5B shows the response of light-grown *dwf4* seedlings. *dwf4* seedlings failed to respond to 6-oxocampestanol (6-oxoCN) even at high concentrations (3×10^{-6} M). However, on CT the overall morphology of *dwf4* was essentially rescued to wild-type phenotype at 3×10^{-7} M and higher, whereas with 6-deoxoCT, rescue occurred with as little as 10^{-7} M and may have even been inhibitory at higher concentrations. Of particular interest is the more dramatic response of the epicotyls versus the smaller response of the hypocotyls to CT (Figure 5B). This same phenomenon was true for seedlings treated with $>10^{-7}$ M 6-deoxoCT. At concentrations $>10^{-7}$ M, the seedlings displayed an inhibition in hypocotyl and root elongation as well as cotyledon and leaf expansion. The seedlings shown in Figure 5B were tested on agar-solidified medium.

Figure 5C shows the result of the dose-response experiment in the dark. Again, the seedlings failed to respond to 6-oxoCN (10^{-8} to 3×10^{-6} M). A higher concentration of CT for dark-grown seedlings, compared with light-grown seedlings, 3×10^{-6} M (Figure 5B), was required to convert the hypocotyl to a length similar to that of the wild type. High concentrations of 6-deoxoCT caused dramatic elongation but were less effective at rescuing *dwf4* hypocotyls to wild-type phenotype (Figure 5C).

To determine whether the results of the seedling feeding experiments could be applicable to soil-grown mature plants, 6-week-old *dwf4* plants were treated with BR intermediates and BL. Concentrations of applied intermediates were adjusted empirically to optimize responses. Consistent with the results obtained from the seedling experiments, Figure 5D shows that only 22 α -hydroxylated compounds can rescue the *dwf4* phenotype. The elongation response was only observed in the young tissues of the inflorescence, regardless of whether the BRs were applied locally or sprayed over the entire plant. In contrast to the striking elongation of the peduncles and pedicels, fertility was not restored by BR treatment. The sterility in *dwf4* is hypothesized to be mechanical, which means that the filaments are shorter than the carpels such that the pollen is shed onto the ovary walls rather than onto the stigmatic surface (Azpiroz et al., 1998). In fact, if *dwf4* plants are hand pollinated using *dwf4* pollen, fertility increases (data not shown). Studies on the underlying mechanism of filament growth are under way.

Pedicels displayed a more consistent response to exogenously applied BRs than did internodes, which led us to quantify the sensitivity of pedicels to these compounds. As

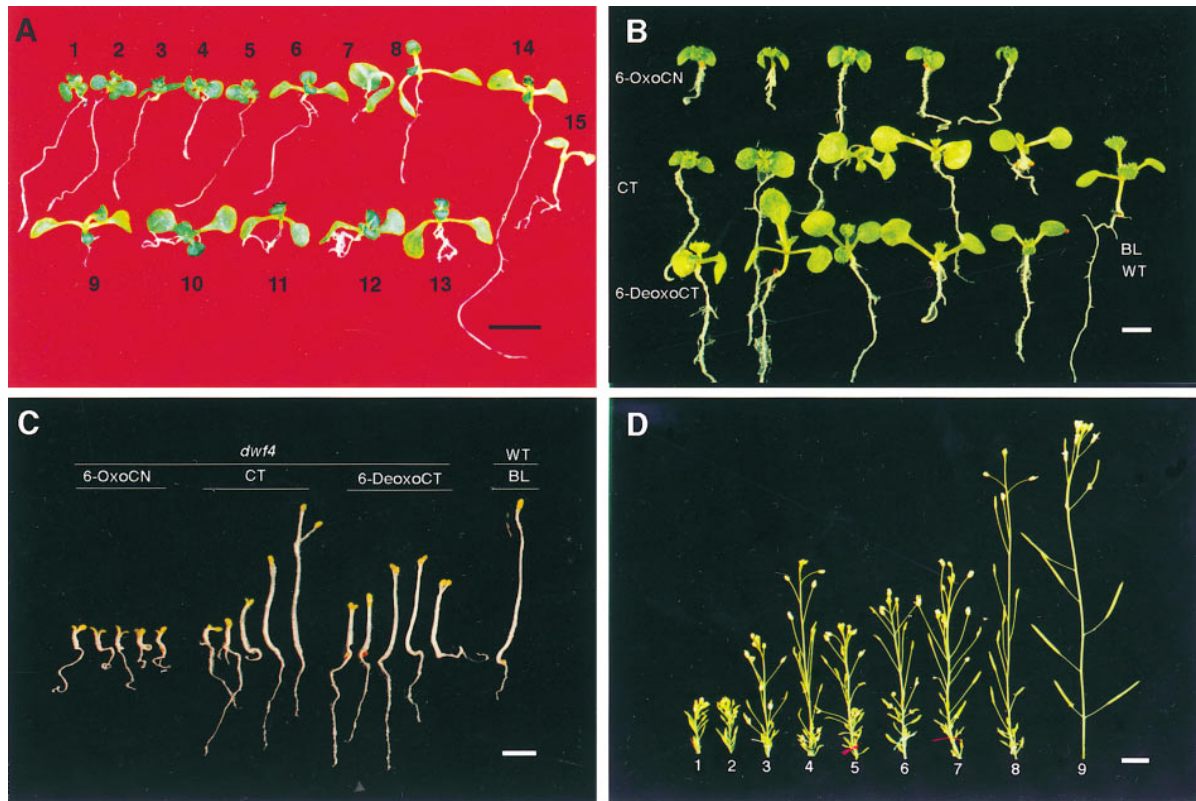


Figure 5. Results of Feeding BR Intermediates to *dwf4* Plants.

(A) *dwf4-3* seedlings grown on BR-supplemented liquid media. Intermediates used for each seedling are numbered in a biosynthetic order: (1) control, (2) CR, (3) campestanol (CN), (4) 6-OHCN, (5) 6-oxoCN, (6) CT, (7) 6-OHCT, (8) 6-deoxoCT, (9) 22-OHCR, (10) CS, (11) 6-OHCS, (12) deoxocastasterone (6-deoxoCS), and (13) BL for *dwf4-3* mutants. (14) and (15) are wild-type seedlings treated with control and BL, respectively. Only the intermediates hydroxylated at C22, such as CT, 6-OHCT, and 6-deoxoCT, caused a response in the *dwf4* seedlings. Responsive plants displayed elongated petioles, lighter green leaves, and higher length/width ratios for leaves. The closer the BR intermediate was to the end product BL, the more effective it was at inducing elongation and inhibiting root growth. Seedlings were grown on BR-supplemented liquid media (10^{-7} M) for 5 days. Each seedling shown is representative of at least 15 seedlings for each treatment. Bar = 0.5 cm.

(B) Dose response of *dwf4-3* to BR in the light. The seedlings were grown on agar-solidified medium supplemented from left to right with 10^{-8} , 10^{-7} , 3×10^{-7} , 10^{-6} , and 3×10^{-6} M of 6-oxoCN (top row), CT (middle row), and 6-deoxoCT (bottom row). The seedling at far right is a wild-type (WT) control. Bar = 0.5 cm.

(C) *dwf4-3* dose response to 6-oxoCN (left), CT (middle), and 6-deoxoCT (right) in the dark (concentrations are the same as listed in **[B]**). The seedling at far right was grown on 10^{-7} M BL. Bar = 1 cm.

(D) Mature plant feeding experiment. Six-week-old *dwf4-1* plants were treated with the BR intermediates noted and the end product BL. Five microliters of (1) control, (2) 10^{-5} M 6-oxoCN, (3) 10^{-5} M CT, (4) 10^{-6} M 6-deoxoCT, (5) 10^{-5} M TE, (6) 10^{-6} M 6-deoxoTE, (7) 10^{-7} M BL, and (8) 10^{-5} M 22-OHCR were applied directly to the inflorescence tip daily for 1 week. (9) indicates wild-type inflorescence without BR treatment; the concentration of BR intermediates was varied to optimize the response. Bar = 1 cm.

shown in Figure 6, *dwf4* pedicels were more sensitive to BR intermediates belonging to the late C-6 oxidation pathway, 6-deoxoCT (10^{-6} M) and 6-deoxoteasterone (6-deoxoTE; 10^{-6} M), compared with CT (10^{-5} M) and teasterone (TE; 10^{-5} M) of the early C-6 oxidation pathway. The end product of the BR pathway, BL (10^{-7} M), possessed the highest bio-activity. This concentration induced approximately the same degree of response as its precursor compounds at 10^{-6} M.

Finally, application of 22-OHCR (10^{-5} M) also resulted in a dramatic elongation response (Figure 6).

Rescue of *dwf4* by 22 α -hydroxylated steroids confirms that the missing step in *dwf4* is hydroxylation at the C-22 position. In fact, we found that the chemically synthesized 22-OHCR was also effective in rescuing *dwf4* (Figures 5A, 5B, and 6). This compound is structurally similar to its presumed precursor CR except for the hydroxylation at C-22.

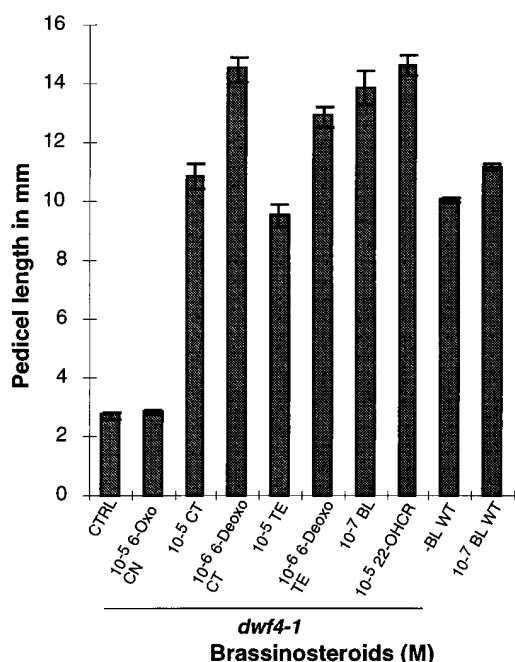


Figure 6. Pedicel Elongation of *dwf4* Mature Plants Responding to Exogenous Application of BR.

Measurements were performed with the BR-fed plants shown in Figure 5D. *dwf4-1* plants were more sensitive to intermediates belonging to the late C-6 oxidation pathway (10^{-6} M 6-deoxoCT and 10^{-6} M 6-deoxoTE) compared with compounds in the early C-6 pathway (10^{-5} M CT and 10^{-5} M TE). BL (10^{-7} M) induced almost the same amount of elongation with one-tenth the concentration of its precursors. Rescue by 22-OHCR (10^{-5} M), which is structurally similar to the presumed precursor CR, except for a 22α -hydroxyl functional group, shows that the only defect in *dwf4* is the C-22 hydroxylation reaction. Complementing intermediates and BL induced dramatic elongation in the elongating zone of the inflorescence and pedicel, but fertility was not increased. Data represent the means \pm SE of 15 to 20 pedicels. CTRL, control; WT, wild type.

These results indicate that there is no defect other than 22α -hydroxylation in *dwf4* plants.

DISCUSSION

In this study, we show that the BR-responsive mutant *dwf4* is defective in BR biosynthesis, specifically in steroid 22α -hydroxylation, which is a key regulatory step (discussed later) in BR synthesis. Two independent approaches led to this conclusion: molecular characterization of the *DWF4* gene and the biochemical complementation studies of the mutant. Genetically, the *DWF4* locus was defined by four independent mutant alleles. We have cloned the *DWF4* gene

by plasmid rescue from a T-DNA-tagged allele *dwf4-1*. We have also identified the mutations in *dwf4-2* and *dwf4-3* and shown that these dwarf phenotypes are likely also the result of null mutations. Database searches revealed that *DWF4* encodes a cytochrome P450 monooxygenase that displays significant homology with the BR biosynthetic enzyme CPD (Szekeres et al., 1996).

DWF4 Encodes a Cytochrome P450 Monooxygenase

Cytochrome P450s are heme-thiolate enzymes. They display a characteristic Soret absorption peak at 450 nm when the substrate-bound, reduced form is exposed to the light (Jefcoate, 1978). Typical microsomal cytochrome P450s hydroxylate various substrates via their monooxygenase activity, which utilizes molecular oxygen and reducing equivalents from NAD(P)H. In addition to the hydroxylation, other activities of cytochrome P450 enzymes, such as oxidation, dealkylation, deamination, dehalogenation, and sulfoxide formation, are involved in a variety of biological events in catabolism, anabolism, and xenobiotic metabolism in plants as well as animals (reviewed in West, 1980; Nebert and Gonzalez, 1987; Guengerich, 1990, 1993; Durst, 1991; Bolwell et al., 1994; Durst and Nelson, 1995; Schuler, 1996). Evolutionarily, cytochrome P450s have been found in a broad spectrum of living organisms, and they share significant homology at the amino acid sequence level. Thus, it has been proposed that all known cytochrome P450s were derived from a common ancestor (Nelson and Strobel, 1987).

Typical cytochrome P450s contain four characteristic domains as defined by Kalb and Loper (1988). Of the four domains, A, B, C, and D, at least two of them have been assigned specific functions. Domain A binds a substrate and molecular oxygen, and domain D has been shown to bind heme-prosthetic groups via a thiolate bond (Poulos et al., 1985). The deduced sequence of *DWF4* contains all four domains and shows significant homology in these domains.

Durst and Nelson (1995) classified plant cytochrome P450s into two distinct groups based on their clustering nature in a phylogenetic tree. All of the group A families cluster and are assumed to originate from a common plant P450 ancestor. The group A cytochrome P450s conform to the characteristic consensus sequences (A/G)GX(D/E)T(T/S) in domain A (also called helix I) and PFG(A/S/V)GRRXC(P/A/V)G of the heme binding domain (D) with only a few exceptions. Group A cytochrome P450s appear to catalyze plant-specific reactions such as lignin biosynthesis (Figure 4; GenBank accession number P48421). By contrast, P450s that do not belong to group A (non-A P450s) are scattered in the phylogenetic tree. They share more amino acid identity/similarity with P450s found in animals, microbes, and fungi than with those found in plants. The non-A P450s possess functions, such as steroid metabolism, that are not limited to plants. Generally, non-A P450s have limited homology with known domains described for group A. *DWF4* clusters with

these latter P450s: cyanobacteria (CYP120), rat (CYP3A2), human (CYP3A3X), and plants (CYP90, CYP85, and CYP88). DWF4 also deviates from the consensus sequence in the group A heme binding domain in that it possesses a PFGGGPRL-CAG sequence in which arginine (R) is substituted for proline (P). However, domain A of DWF4, AGHETS, fits the consensus of domain A of group A. These characteristics suggest that DWF4 is a monooxygenase, similar to P450s of group A, that utilizes molecular oxygen as a source of the hydroxyl group, but it mediates some reaction(s) that are not necessarily specific for plants, for instance, steroid hormone biosynthesis, which is a critical event for animals. In fact, the similarity of DWF4 to the rat testosterone 6 β -hydroxylase (34%; GenBank accession number 631895) or glucocorticoid-inducible hydroxylase (31%; Molowa et al., 1986; GenBank accession number M13785) supports this idea. Further, the similarity that DWF4 shares with CYP90A and CYP85, 66 and 59%, respectively, is additional proof that it is involved in plant steroid biosynthesis (Bishop et al., 1996; Szekeres et al., 1996).

DWF4 Monooxygenase Mediates 22 α -Hydroxylation Reactions in BR Biosynthesis

In BR biosynthesis, Fujioka and Sakurai (1997b) have demonstrated that there are at least two branched biochemical pathways to the end product BL (Figure 1; Fujioka and Sakurai, 1997a, 1997b; Sakurai and Fujioka, 1997). Depending on the oxidation state of C-6, they are referred to as the early or late C-6 oxidation pathways. In the early pathway, the C-6 is oxidized to a ketone at campestanol (CN), whereas in the late pathway it is oxidized at 6-deoxocastasterone (6-deoxoCS). Otherwise, the two pathways share equivalent reactions. Our results from the experiments with the available BR intermediates clearly demonstrate that *dwf4* is defective in the 22 α -hydroxylation steps in each of the pathways. Application of all 22 α -hydroxylated intermediates in these pathways, such as CT and 6-deoxoCT, cause dramatic elongation of *dwf4* plants, but compounds not hydroxylated at C-22 had no effect. This result also suggests that DWF4 recognizes at least two substrates: CN and 6-oxoCN. It seems reasonable to hypothesize that the same result will be found for *CPD*, a 23 α -hydroxylase; that is, it will use 6-deoxoCT as well as CT as substrate.

The rescue of *dwf4* by 22-OHCR is an important observation. First, it confirms DWF4 as a 22 α -hydroxylase. Second, this result suggests that 22-OHCR was metabolized to induce the same responses as other complementing BRs. This is not just a general effect because our unpublished data show that another dwarf mutant that we have identified in our screens, *dwf8-1*, is not rescued by this compound. Finally, these feeding experiments suggest that the metabolism of 22-OHCR may represent a new subpathway in the BR biosynthetic pathway. If this compound also exists in vivo and constitutes the first step in a separate subpathway,

by analogy to the chemical structure, the C-6 hydroxylated BRs, for example, 6-OHCT, 6-hydroxyteasterone, and so on, may be possible intermediates in this network. If so, the intermediates in this pathway may play a role as bridging molecules between the early and late C-6 oxidation pathways. Alternatively, it might be possible that 22-OHCR merges into one of the two pathways to be metabolized. In this case, the late C-6 oxidation pathway is the best candidate; our unpublished data show that 22-OHCR is more effective in the light in rescuing the *dwf4* phenotype, which is true for all of the intermediates in the late C-6 oxidation pathway (discussed below).

Currently, biochemical feeding studies suggest that the two pathways merge to produce BL or CS (Yokota et al., 1991; Figure 1). How could the seemingly redundant pathways be utilized to respond to environmental or developmental signals? First, the pathways could respond to specific signals. For instance, it is possible that various cues such as light, dark, or developmental signals play a role in regulating these subpathways. Our feeding experiments consistently showed that BRs in the late C-6 oxidation pathway are more effective at promoting cell elongation in light-grown plants (*dwf4* and wild type; Figures 5A, 5B, and 6) and that the BRs belonging to the early C-6 oxidation pathway are more active in dark-grown seedlings (Figure 5D). Thus, it may be possible that the late C-6 oxidation pathway operates in the light and that the early C-6 oxidation pathway functions primarily in the dark. Second, rather than a simple merger of branched pathways to BL as an end product, each intermediate may have nascent bioactivity. The in vivo ratio or composition of BRs at different oxidation states may result in different responses. Noticeably distinctive phenotypes for the various BR dwarfs, defective in different biosynthetic steps, support this idea. Third, the biosynthetic rate of each pathway toward production of the end product may differ. In this case, the biosynthetic rate could be modulated by controlling the level of gene expression or the activity of participating enzymes. Certain signals, requiring different rates of BR biosynthesis, may induce one of the subpathways, which would then affect the concentration of the intermediates in one pathway relative to the other.

Of the steps in BR biosynthesis in Madagascar periwinkle, the 22 α -hydroxylation reaction has been suggested to be the rate-limiting step (Fujioka et al., 1995a). In periwinkle, the endogenous level of CT was as low as one-twenty thousandth of CR; however, CT was almost 500 times more active than 6-oxoCN in the rice-lamina inclination assay (Fujioka et al., 1995b). Based on these results, we propose that the step encoded by *DWF4* serves as the rate-limiting reaction and that once past this step, the intermediates are easily converted to the end product. Although biochemical studies on DWF4 need to be performed to ascertain whether it mediates the rate-limiting step, *DWF4* seems to be greatly downregulated compared with *CPD*, the next enzyme in the pathway; RT-PCR revealed that the *DWF4* transcript is much less abundant than the *CPD* transcript (data not shown).

METHODS

Arabidopsis Mutants and Growth Conditions

The *dwf4-1* and *dwf4-2* mutations are in the Wassilewskija (*Ws-2*) background; the *dwf4-3* and *dwf4-4* mutations are in the Enkheim (*En-2*) background. Seeds for brassinosteroid (BR) feeding studies were sown on 0.8% agar-solidified medium containing 1 × Murashige and Skoog (1962) salts and 0.5% sucrose (*w/v*) and cold treated (4°C) for 2 days in the dark before transfer to the light (24 hr light; 80 μmol m⁻² sec⁻¹). The plates were sealed with Parafilm (American National Can Co., Chicago, IL) for the entire experiment. For nucleic acid extraction, genetic analysis, and other experiments in which mature plants were required, seeds were sown on Metromix 350 (Grace Sierra, Milpitas, CA) presoaked with distilled water. The pots were covered with plastic wrap and cold treated (4°C) for two days before transfer to a growth chamber (16:8, light [240 μmol m⁻² sec⁻¹]; dark; 22 and 21°C, respectively, and 75 to 90% humidity). The plastic wrap was removed 5 days after germination, and the pots were subirrigated in distilled water as required.

Isolation of the *DWF4* Gene

Standard molecular techniques were performed as described previously (Sambrook et al., 1989). The plant DNA flanking the T-DNA was cloned using the plasmid rescue technique as described by Dilkes and Feldmann (1998). Briefly, *dwf4-1* genomic DNA was digested with EcoRI (for the right border) or Sall (for the left border), ligated under conditions to maximize intramolecular events, and introduced into competent *Escherichia coli* cells. The resulting colonies were screened on ampicillin. Five colonies from the left border transformation contained plant DNA flanking the insertion site. The restriction pattern displayed two different types of plant DNA. Three contained a 5.6-kb insert, whereas the other two contained a 1.1-kb insert. This result suggested that the T-DNA insert in *dwf4-1* was flanked by two left border sequences. To confirm this orientation, gel blot analysis was performed on genomic DNA by using the putative plant flanking DNAs as a probe. A single wild-type EcoRI fragment was split into two fragments in *dwf4-1*.

Wild-type genomic clones were isolated from a library made from *Ws-2* DNA by using the 5.6-kb fragment as a probe. The library was constructed using λ DASH-II arms (Stratagene, La Jolla, CA). Approximately 10,000 primary plaques were screened. Duplicate-filter screening resulted in 12 positives. Restriction mapping of the secondary clones revealed that some contained part of the *DWF4* locus. In fact, one of the clones, D4G12-1, contained an intact 13-kb DNA spanning the T-DNA insertion site. The 13-kb insert in D4G12-1 was subcloned into pBluescript SK– (Stratagene). Subclones were sequenced from each end of the insert by using the universal primers in the plasmid. DNA sequencing was performed using an ABI 377 (Perkin-Elmer, Norwalk, CT) automated sequencer at the Arizona Research Laboratories (Tucson, AZ).

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to isolate a cDNA clone. RNA was isolated from 5-day-old dark- and light-grown seedlings. Superscript II reverse transcriptase (BRL, Gaithersburg, MD) was used for the cDNA synthesis, according to the manufacturer's protocol. Briefly, 7 μg of total RNA was mixed with the reverse primer, D4R3. To the heat-denatured RNA-primer mix, the RT mixture was added and incubated for 1 hr at 43°C.

Two microliters of RT product was used for PCR amplification by using different primers sets intended to cover all of the putative coding region. RT-PCR products were fractionated on an 0.8% agarose gel (Sambrook et al., 1989); the expected bands were purified using a GeneClean kit (BIO 101, Inc., Vista, CA), further amplified, and sequenced to determine the coding region.

Sequencing of Mutant Alleles

dwf4-2 was isolated from a T-DNA mutant population as an untagged allele, whereas *dwf4-3* and *dwf4-4* were obtained from the Nottingham Arabidopsis Stock Centre (University of Nottingham, UK; stock nos. N365 and N374); the mutagenesis method for these two lines is not known. Based on the DNA sequence of wild-type genomic DNA, pairs of primers were designed to amplify ~1-kb stretches of genomic DNA. Oligonucleotide sequences are shown 5' to 3'. The numbers shown correspond to positions in the genomic sequence, with the adenine base in the translation initiation codon set as position 1. D4OVERF, 1-ATGTTCCGAAACAGAGCATCATACT-24; D4PRM, (-1)-CCTCGATCAAAGAGAGAGAGA(-21); D4RTF, 143-TTCTTGGTGAAACCATCGTTATCTTAAA-171; D4RTR, 853-TATGATAAGCAGTTCCCTGGTAGATTT-828; D4F1, (-242)-CGAGGCAACAAAAGTAATGAA(-222); D4R1, 689-GTTAGAACTCTAAAGATTCA-669; D4F2, 576-GATTCTTGGCAACAAAACCTAT-598; D4R2, 1685-CCGAACATCTTTGAGTGCTT-1666; D4F3, 1606-GTGTGAAGGTTA-TAAATGAACTCTT-1631; D4R3, 3156-GGTTTAAATAGTGTGACACTAATA-3132; D4F4, 2316-CCGATGACTTGTACGTGCGTTA-2337; D4F5, 730-GCGAAGCATATAATGAGTATGGAT-753; and D4R5, 1876-GTTGGTCATAACGAGAATTATCCAAA-1851. Because the two stock center lines were in a different genetic background than the wild-type gene that we had sequenced (*WS*), primers were based primarily on the exon sequence to avoid sequence variation between introns. Genomic DNA isolated from the mutants was subjected to PCR, using these primer sets. The amplified DNA fragments were fractionated on 0.8% TAE agarose gel (Sambrook et al., 1989), purified using GeneClean (BIO 101, Inc.) or Qiaquick columns (Qiagen Inc., Chatsworth, CA), and sequenced. Putative mutations were identified by comparing the mutant DNA sequence with the wild-type sequence. The sequence was confirmed by sequencing independently amplified fragments at least three times for each mutation to eliminate PCR misincorporation.

Sequence Analysis

Annotations in multiple sequence alignment were performed using the ALSCRIPT package provided by G.J. Barton (1993). Searches for similar protein sequence were performed with the BLAST program (Altschul et al., 1990). In addition, useful packages, available on the internet, such as promoter, protein targeting, polyadenylation site, and splice site, have been employed to characterize the DNA and protein sequence (consolidated in the search launcher, Baylor College of Medicine, Baylor, TX). All other sequence analysis was performed using the Genetics Computer Group (Madison, WI) software package.

BR Feeding Experiments

All of the biosynthetic intermediates in the biosynthetic network shown in Figure 1 were tested. The BR intermediates were chemi-

cally synthesized (Fujioka et al., 1997). The BR intermediates were dissolved in 95% ethanol. For the dark-grown seedlings, prechilled plates containing the seeds were illuminated for 3 hr and then wrapped with three layers of aluminum foil. For light-grown seedlings, seeds were plated on agar-solidified medium. After 3 days of cold treatment, 10 seedlings were transferred to 1.5-mL of BR-supplemented liquid medium in a single cell of a 24 Cell-Wells plate (Corning, Inc., Corning, NY). The culture medium was the same as that used for germination except that BR biosynthetic intermediates were added. Plates were sealed with porous tape (stock No. 2-0300; 3M, St. Paul, MN) and grown in the dark for 5 days on a platform shaker (220 rpm). For the control, an equivalent amount of alcohol (95%) was added to the medium. After 5 days, seedlings from each test well were placed on agar plates and measured to the nearest unit by using the ocular micrometer on the dissecting microscope.

For the feeding experiments with soil-grown plants, *dwf4* and wild-type plants were grown on soil for 6 or 3 weeks, respectively. To examine the effects of BR on soil-grown plants, BR intermediate feeding experiments were performed. Inflorescence apices were marked by tying a string around the plant to tell the portion of BR-induced growth from untreated growth. Five microliters of 6-oxoCN (10^{-5} M), CT (10^{-5} M), TE (10^{-5} M), 6-deoxoCT (10^{-6} M), BL (10^{-7} M), and 22-OHCR (10^{-5} M) were applied to the tip of the shoot every day for 1 week. After the treatment, the three most elongated pedicels were chosen from five inflorescences tested for each BR, and the length was measured to the nearest millimeter. Inflorescences tested were cut near the string mark and photographed.

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The *DWF4* Gene of *Arabidopsis* Encodes a Cytochrome P450 That Mediates Multiple 22 α -Hydroxylation Steps in Brassinosteroid Biosynthesis
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