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# The Arabidopsis *dwarf1* Mutant is Defective in the Conversion of 24-Methylenecholesterol to Campesterol in Brassinosteroid Biosynthesis

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At the time of this publication, Dr. Gregory was affiliated with the University of Arizona, but he is now a faculty member at the University of Pennsylvania.

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# The Arabidopsis *dwarf1* Mutant is Defective in the Conversion of 24-Methylenecholesterol to Campesterol in Brassinosteroid Biosynthesis

## Abstract

Since the isolation and characterization of *dwarf1-1* (*dwf1-1*) from a T-DNA insertion mutant population, phenotypically similar mutants, including *deetiolated2* (*det2*), *constitutive photomorphogenesis and dwarfism* (*cpd*), *brassinosteroid insensitive1* (*bri1*), and *dwf4*, have been reported to be defective in either the biosynthesis or the perception of brassinosteroids. We present further characterization of *dwf1-1* and additional *dwf1* alleles. Feeding tests with brassinosteroid-biosynthetic intermediates revealed that *dwf1* can be rescued by 22 $\alpha$ -hydroxycampesterol and downstream intermediates in the brassinosteroid pathway. Analysis of the endogenous levels of brassinosteroid intermediates showed that 24-methylenecholesterol in *dwf1* accumulates to 12 times the level of the wild type, whereas the level of campesterol is greatly diminished, indicating that the defective step is in C-24 reduction. Furthermore, the deduced amino acid sequence of DWF1 shows significant similarity to a flavin adenine dinucleotide-binding domain conserved in various oxidoreductases, suggesting an enzymatic role for DWF1. In support of this, 7 of 10 *dwf1* mutations directly affected the flavin adenine dinucleotide-binding domain. Our molecular characterization of *dwf1* alleles, together with our biochemical data, suggest that the biosynthetic defect in *dwf1* results in reduced synthesis of bioactive brassinosteroids, causing dwarfism.

## Disciplines

Amino Acids, Peptides, and Proteins | Biology | Nucleic Acids, Nucleotides, and Nucleosides | Plant Sciences

## Comments

At the time of this publication, Dr. Gregory was affiliated with the University of Arizona, but he is now a faculty member at the University of Pennsylvania.

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# The Arabidopsis *dwarf1* Mutant Is Defective in the Conversion of 24-Methylenecholesterol to Campesterol in Brassinosteroid Biosynthesis<sup>1</sup>

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Since the isolation and characterization of *dwarf1-1* (*dwf1-1*) from a T-DNA insertion mutant population, phenotypically similar mutants, including *deetiolated2* (*det2*), *constitutive photomorphogenesis and dwarfism* (*cpd*), *brassinosteroid insensitive1* (*bri1*), and *dwf4*, have been reported to be defective in either the biosynthesis or the perception of brassinosteroids. We present further characterization of *dwf1-1* and additional *dwf1* alleles. Feeding tests with brassinosteroid-biosynthetic intermediates revealed that *dwf1* can be rescued by 22 $\alpha$ -hydroxycampesterol and downstream intermediates in the brassinosteroid pathway. Analysis of the endogenous levels of brassinosteroid intermediates showed that 24-methylenecholesterol in *dwf1* accumulates to 12 times the level of the wild type, whereas the level of campesterol is greatly diminished, indicating that the defective step is in C-24 reduction. Furthermore, the deduced amino acid sequence of DWF1 shows significant similarity to a flavin adenine dinucleotide-binding domain conserved in various oxidoreductases, suggesting an enzymatic role for DWF1. In support of this, 7 of 10 *dwf1* mutations directly affected the flavin adenine dinucleotide-binding domain. Our molecular characterization of *dwf1* alleles, together with our biochemical data, suggest that the biosynthetic defect in *dwf1* results in reduced synthesis of bioactive brassinosteroids, causing dwarfism.

T-DNA-insertion mutagenesis has proven to be useful for the isolation of many important genes controlling plant growth and development (Choe and Feldmann, 1998). The Arabidopsis *dwarf1* (*dwf1*) mutant was originally isolated from a T-DNA mutant population, and was the first mutant shown to cosegregate with the selectable marker in the T-DNA (Feldmann et al., 1989). The *dwf1* mutant was identified because of its short stature, dark-green leaves, reduced fertility, and robust stems when grown in the light. Physiologically, *dwf1* was not rescued by any of the known growth-promoting phytohormones such as GA<sub>3</sub> or auxin

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(Feldmann et al., 1989). Using the plant DNA flanking the T-DNA as a probe, DWF1 was cloned and sequenced (accession no. U12400).

Independently, Takahashi et al. (1995) isolated a morphologically similar mutant, *diminuto* (*dim*), from a different T-DNA mutant collection. Cloning and sequencing revealed that *dim* is disrupted in the DWF1 sequence, indicating that it is an allele of *dwf1*. One year later, Kauschmann et al. (1996) isolated another allele of DWF1 from a transposon-tagged population. They identified three tiny mutants named *cabbage1*, *cabbage2*, and *cabbage3* (*cbb1*, *cbb2*, and *cbb3*). Altmann et al. (1995) found the sequence of genomic DNA flanking the transposon in *cbb1* to be identical to that of DWF1. Kauschmann et al. (1996) originally found that *cbb1* (*dwf1-6*) could be rescued by exogenous application of brassinosteroids, suggesting that *cbb1* (*dwf1-6*) is defective in brassinosteroid biosynthesis. They also analyzed the expression of genes known to be involved in cell elongation, such as  $\gamma$ -*tonoplast intrinsic protein* ( $\gamma$ -TIP) (Höfte et al., 1992; Phillips and Huttly, 1994), and cell wall-modifying enzymes such as xyloglucan endotransglycosylases, including TOUCH4 (TCH4) (Xu et al., 1995) and MERI5 (Medford et al., 1991). The steady-state mRNA levels of TCH4 and MERI5 were lowered, whereas the expression of  $\gamma$ -TIP was increased in the *cbb* mutants. Based on this, they proposed that a defect in brassinosteroid biosynthesis in *cbb1* (*dwf1-6*) leads to failure in cell elongation, which requires partial activity of the cell wall-modifying enzymes TCH4 and MERI5.

Brassinolide, the proposed end product of the brassinosteroid-biosynthetic pathway, is synthesized from sterol substrates. Therefore, plants defective in the biosynthetic steps leading from mevalonic acid to sterol, as well as in steps modifying sterols to brassinolide, could display the characteristic *dwf* phenotype. Currently, *dwf7* is reported to be defective in a step of sterol biosynthesis (Choe et al., 1999), and three mutants, *deetiolated2* (*det2*) (Li et al.,

Abbreviations: EMS, ethyl methanesulfate; EST, expressed sequence tag; SIM, selective ion monitoring; SSLP, simple sequence length polymorphism.

1996), *dwf4* (Choe et al., 1998), and *constitutive photomorphogenesis and dwarfism (cpd)* (Szekeres et al., 1996), are defective in the brassinosteroid-specific biosynthetic steps, from campesterol to brassinolide. Specifically, *dwf7* is blocked in the sterol C-5 desaturation step, which is the most upstream step identified in *dwf* mutants thus far (Choe et al., 1999). Fujioka et al. (1997) have shown *det2* to be blocked in the 5 $\alpha$ -reduction step converting campesterol to campestanol. Choe et al. (1998) have proposed that *dwf4* is disrupted in the 22 $\alpha$ -hydroxylation step, which is hypothesized to be the rate-limiting step in brassinosteroid biosynthesis. Finally, Szekeres et al. (1996) have found *cpd* to be defective in the 23 $\alpha$ -hydroxylation step following *dwf4*. Both DWF4 and CPD have been assigned to the same group of Cyt P450 proteins (CYP90) because they share more than 40% identity. In addition to these biosynthetic mutants, Clouse et al. (1996) have also identified *brassinosteroid insensitive1 (bri1)*, a mutant insensitive to brassinosteroids. Recently, *BRI1* was cloned and was shown to encode a Leu-rich repeat receptor kinase, suggesting a role in brassinosteroid signal perception and transduction (Li and Chory, 1997). All of the brassinosteroid dwarf mutants share characteristic phenotypes in the light, as described above, as well as abnormal skotomorphogenesis in the dark, including short hypocotyls and expanded cotyledons.

Recent characterization of these *dwf* mutants provides compelling evidence that brassinosteroids are essential modulators for proper growth and development in plants. To understand all of the roles assigned to brassinosteroids in plants, the identification of the components of the brassinosteroid pathway and the regulation of endogenous brassinosteroid biosynthesis is critical. The proposed brassinosteroid-biosynthetic pathway predicts that there are at least 20 genes involved in brassinolide synthesis, which begins with squalene (Choe et al., 1999). To identify mutants in each biosynthetic step, we are characterizing a large collection of Arabidopsis dwarfs with the characteristic brassinosteroid dwarf phenotype. Currently, we have identified 12 different brassinosteroid loci. Six of these mutants, *bri1* (*dwf2*) (Clouse et al., 1996; Li and Chory, 1997), *cpd* (*dwf3*) (Szekeres et al., 1996), *dwf4* (Choe et al., 1998), *det2* (*dwf6*) (Li et al., 1996), *dwf7* (Choe et al., 1999), and *dwf1* (Feldmann et al., 1989; Takahashi et al., 1995; Kauschmann et al., 1996), have been characterized.

Here we report further studies on *dwf1*. Because Kauschmann et al. (1996) have shown that *dwf1* can be rescued by exogenous application of brassinosteroids, we have used different methods to pinpoint the specific biosynthetic step that is defective in *dwf1*. First, we applied biosynthetic intermediates to *dwf1* plants to identify compounds that rescued *dwf1* phenotypes. In addition, we analyzed the endogenous brassinosteroid levels using GC-SIM to identify accumulated compounds. Based on this biochemical analysis, we found that a C-24 reduction step converting 24-methylenecholesterol to campesterol was blocked in *dwf1*. Coupled with sequence analyses of *DWF1* and identification of the site of mutation in eight *dwf1* alleles, we propose that DWF1 acts as a biosynthetic enzyme, catalyzing C-24 reduction in sterol biosynthesis.

## MATERIALS AND METHODS

### Mutant Isolation

The isolation of *dwf1-1* and the cosegregation of the T-DNA with the dwarf phenotype are described by Feldmann et al. (1989). *dwf1-3*, *dwf1-4*, and *dwf1-5* were isolated by screening dwarf mutants of the Enkheim-2 (En-2) ecotype obtained from the Nottingham Arabidopsis Stock Center (University of Nottingham, UK). These mutants were generously donated by Albert Kranz. Genetic-complementation tests were employed to determine allelism to *dwf1-1*. Three lines, 318, 355, and 356, were shown to be new alleles of *dwf1*. Moreover, it has been shown that *dim* (Takahashi et al., 1995) and *cb1* (Kauschmann et al., 1996) contain insertions in the *DWF1* gene (Altmann et al., 1995). For consistency with Kauschmann et al. (1996), we will refer to *dim* and *cb1* as *dwf1-2* and *dwf1-6*, respectively. In addition, we have isolated 43 new *dwf* mutants in a screen of approximately 50,000 M<sub>2</sub> lines from an EMS-mutagenized population (ecotype Wassilewskija-2 [Ws-2]).

Dwarf mutants resembling *dwf1* in both phenotype and brassinosteroid-feeding response were outcrossed to plants of the Columbia ecotype to test for linkage to markers near *dwf1*. We isolated DNA from individual F<sub>2</sub> dwarfs, and tested the genetic linkage of the new mutants to *dwf1*, using SSLP markers as described by Bell and Ecker (1994). Previously, SSLP mapping of *dwf1-1* showed linkage of *dwf1* to *nga162*; the meiotic recombination ratio was 1 out of 40 chromosomes tested. Five mutants resembling *dwf1* (WM1-7, WM3-1, WM5-5, WM9-3, and WM12-1) were also closely linked to *nga162*. Molecular characterization showed that these contained mutations in the *DWF1* gene and, as such, were renamed *dwf1-7* (WM1-7), *dwf1-8* (WM3-1), *dwf1-9* (WM5-5), *dwf1-10* (WM9-3), and *dwf1-11* (WM12-1) (see Table I).

### Growth Conditions and Feeding Tests

For plant growth in soil, seeds were sown in 5.5-cm pots filled with wet Metromix (Grace Sierra, Milpitas, CA). Pots were cold treated for 2 d before transfer to incubators (Percival, Boone, IA) set at 22°C with long-day conditions (16 h of light [240  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ] at 22°C and 75% RH; 8 h of dark at 21°C and 90% RH). Plants were subirrigated with a modified Hoagland solution (Feldmann and Marks, 1987) diluted 1:1 with deionized water as necessary. For brassinolide-dose experiments, seeds were surface-sterilized and sprinkled on agar-solidified medium containing Murashige-Skoog salts (GIBCO-BRL) supplemented with 0.5% Suc and 0.8% agar (Difco, Detroit, MI). Plates were cold treated for 2 d at 4°C before germination. After germination for 3 d, two to three seedlings at a similar growth stage were transferred to a single cell of a 24-well plate (Corning Inc., Corning, NY) prefilled with 1.5 mL of brassinosteroid-supplemented liquid Murashige-Skoog medium. Plates were sealed with porous tape (3M) and grown on a platform shaker (230 rpm) under continuous light (240  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). As a control, an equivalent amount of ethanol (95%) was added to the medium, because ethanol was used as the solvent for brassinolide.



After 7 d of growth, seedlings from each well were placed in 0.05% toluidine blue in phosphate buffer, pH 4.4, and transferred to agar plates to measure the hypocotyl length using an ocular micrometer on a dissecting microscope. For inflorescence feeding experiments, *dwf1-1* and *Ws-2* wild-type plants were grown on soil until the inflorescence reached 1 cm in length. Inflorescence apices were marked by tying a string just below the unopened flowers to distinguish the portion of brassinosteroid-induced growth from untreated growth. Brassinosteroid-biosynthetic intermediates were diluted to the desired concentration with water containing 0.01% Tween 20,  $10^{-6}$  M cathasterone, 6-deoxocathasterone,  $22\alpha$ -hydroxycampesterol, and  $10^{-7}$  M brassinolide. Two microliters of each brassinosteroid solution was applied daily to the shoot tips of plants using a micropipette (Gilson P-20, Rainin Instrument, Emeryville, CA). After 1 week of treatment the pedicels and inflorescence above the string were measured to the nearest millimeter ( $n = 15$ ). Choe et al. (1999) have described the methods used for the detection of endogenous brassinosteroid levels.

### Molecular Cloning and Characterization of Mutations

Plant DNA flanking the T-DNA borders in *dwf1-1* was isolated as described by Dilkes and Feldmann (1998). We used DNA from a right-border rescue as a probe to isolate corresponding cDNA clones from a  $\lambda$ PRL-2 cDNA library constructed with  $\lambda$ ZipLox2 (GIBCO-BRL). The Arabidopsis Biological Resource Center (Ohio State University, Columbus) provided the library (stock no. CD4-7). In addition, genomic DNA clones were isolated from a  $\lambda$ DASH-II (Stratagene) genomic library constructed using genomic DNA from the *Ws-2* ecotype. To confirm the identity of the clone, a 7-kb restriction fragment from two genomic clones predicted to span the T-DNA insertion site was sequenced and compared with the sequences of the right-border-rescued fragment and cDNA (accession no. U12400). DNA sequencing was carried out using an automated sequencer (model ABI373, Perkin Elmer, Norwalk, CT) at Arizona Research Laboratories (University of Arizona, Tucson). We performed searches for similar sequences using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al., 1997) at the National Center for Biotechnology Information. We aligned similar sequences identified from the BLAST search using the PileUp program of the software package from the Genetics Computer Group (GCG, Madison, WI). Boxing and highlighting of aligned sequences was performed using ALSRIPT software provided by Barton (1993). We used the PSORT package (Nakai and Kanehisa, 1992; <http://psort.nibb.ac.jp:8800/>) to predict subcellular targeting.

We identified the mutations in the *dwf1* alleles by sequencing PCR-amplified DNA. Oligonucleotide sequences of the primers from 5' to 3' are: D1F1, GTTTGATGCAGT-GAGGA; D1F2, TGAGGCCCAAGAGGAAGAAG; D1R5, ACGCCCCGAGAGAACATCAG; D1F3, ACAAGGAGA-AGATGACTGC; D1R4, GGAACGCTGGTGCCCTAACG; D1F4, TACATTGATTCTTTTGCTCC; D1R3, GGAACA-CACGGACACCATCA; D1R2, TGGCGCATGACTCCGA-

CCTT; D1F5, TGAATTGTATGAGGAGTGC; and D1R1, AAGTATCCGTTTAGGTTTTTC. Genosys Biotechnologies (The Woodlands, TX) provided the PCR primers and Boehringer Mannheim supplied the *Taq* polymerase. We followed the manufacturer's method for PCR amplification. To obtain the longest PCR product spanning the entire coding region, we used the D1F1 and D1R1 primer pair. The PCR-amplified DNA fragments were gel purified (Prep-A-Gene DNA purification system, Bio-Rad) and subjected to sequencing using the primers described above. Using the BestFit program of the GCG software package, we compared DNA sequences from the mutant allele with the sequence of the wild type to identify base changes. Putative base changes in the mutant allele were confirmed by repeated sequencing of multiple PCR reactions (at least twice for each strand) to eliminate possible PCR artifacts.

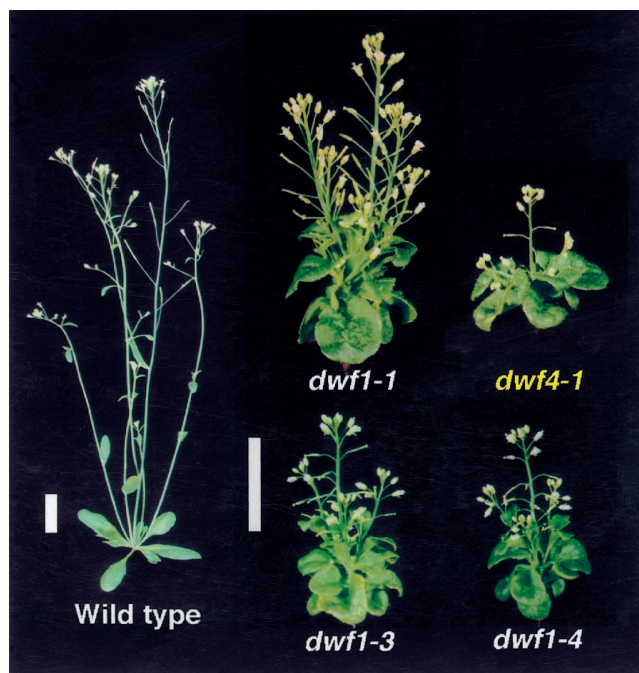
## RESULTS

### Altered Development of Arabidopsis *dwf1* Plants

*dwf1-1* was originally isolated from the first population of 100 T-DNA lines generated by seed infection (Feldmann et al., 1989). To identify more alleles, 14,000 additional lines representing 21,000 insertion events were screened for dwarfs phenotypically similar to *dwf1-1*. Genetic-complementation tests showed that none of the 13 additional dwarfs isolated from the screen were allelic to *dwf1-1*. Next, we obtained all of the dwarf mutants maintained by the Nottingham Arabidopsis Stock Center and screened them for brassinosteroid-responsive dwarfs. Among 55 mutants analyzed, 3 were allelic to *dwf1-1* (stock numbers 318, 355, and 356) and were therefore renamed *dwf1-3*, *dwf1-4*, and *dwf1-5*, respectively.

Figure 1 shows a comparison of the *Ws-2* wild type with three *dwf1* alleles at 35 d of age. The two *En-2* alleles of *dwf1* (*dwf1-3* and *dwf1-4*) were morphologically distinct from the known null mutant *dwf1-1* (described below). Unlike *dwf1-1*, they possess a shorter stature, a more tightly bunched rosette, and a more highly branched inflorescence (Fig. 1). These differences are probably due to ecotypic backgrounds. As evidence for this, when *dwf1-5* plants were outcrossed twice into the *Ws-2* ecotype, they looked more like *dwf1-1* (data not shown). Finally, we isolated 43 *dwf* mutants from a screen of 50,000  $M_2$  lines of an EMS-mutagenized population (ecotype *Ws-2*). Five mutants resembled *dwf1* in phenotype and brassinosteroid-feeding response. Mapping using SSLP markers (Bell and Ecker, 1994) indicated that these five mutants are linked to *dwf1-1*. Allelism to *dwf1* was further suggested by the identification of mutations in the *DWF1* gene in these five lines (see below). Table I summarizes the *dwf1* alleles and their origins.

Morphological differences between *dwf1-1* and wild-type plants are shown in Tables II and III. Measurements were taken from 35- and 111-d-old (mature) organs of *dwf1-1* and *Ws-2* wild-type plants. A comparison of their heights at 35 d indicates that *dwf1-1* plants were 4-fold shorter than the wild type. The decrease in organ size caused by the *dwf1-1* mutation was not uniform. Inflorescence length was



**Figure 1.** Comparison of 5-week-old Arabidopsis wild type, *dwarf1*, and *dwarf4-1* plants. Bar = 2 cm. The characteristic phenotype of brassinosteroid dwarfs includes a short, robust inflorescence; round, dark-green leaves; and reduced fertility. *dwarf1-1* (Ws-2 background) is taller than *dwarf1-3* and *dwarf1-4* (En-2). Compared with *dwarf4*, *dwarf1* alleles have a less-severe phenotype.

the most affected. The internode distance was reduced to approximately 25% of that of the wild type (Table II; Fig. 1). Leaf length was one-half that of the wild type, although leaf width was not changed significantly (Table II), resulting in a round shape. The observation that leaf tissues between veins buckled suggests that the elongation of veins was more affected than the expansion of areoles (Fig. 1). Similarly, petiole length was 40% of that of the wild type. The total number of organs from the wild type and *dwarf1-1* was recorded. *dwarf1-1* plants produced more rosette leaves than did the wild type at 35 d (Table II). Furthermore, the total number of siliques, as well as the number of siliques on the primary inflorescence, was increased 4-fold compared with wild type at maturity. As previously de-

scribed for the *dwarf7* mutant (Choe et al., 1999), *dwarf1* plants are not completely sterile. Although the defect in cell elongation affected the stamens more than the gynoecium, some stamens were long enough to reach the stigmatic surface for successful fertilization (data not shown; Choe et al., 1999).

Arabidopsis inflorescences are indeterminate (Bowman, 1994); however, wild-type plants typically cease flowering and senesce by 57 d after germination (Table III). All *dwarf1* mutants continued to grow and flower up to 110 d under controlled-growth conditions (Table III). Although *dwarf1-1* plants bolted and flowered later than wild-type plants, the primary cause of the expanded life cycle of *dwarf1* was due to a prolonged generative phase. *dwarf1-1* plants flowered for more than twice as long as wild-type plants. It is this extension of the generative phase that allows the *dwarf1-1* plants to overproduce siliques and eventually reach one-half the height of wild type at maturity (Table III).

#### Biochemical Characterization of the Brassinosteroid-Biosynthesis Defect in *dwarf1* Plants

Clouse et al. (1996) have shown phenotypically similar dwarf mutants of Arabidopsis to be insensitive, whereas Li et al. (1996), Szekeres et al. (1996), and Choe et al. (1998) have reported others to be rescued by the exogenous application of brassinosteroids. Previously, Kauschmann et al. (1996) had shown that *ccb1* (*dwarf1-6*) was biochemically complemented by exogenous application of 24-epibrassinosteroids. To further examine this response in our *dwarf1* alleles, we applied different concentrations of brassinolide. Figure 2 summarizes the effect of different concentrations of brassinolide on the elongation of hypocotyls of Ws-2, En-2, *dwarf1-1*, and *dwarf1-3* grown for 10 d in the light. The two ecotypes responded differently to increasing concentrations of brassinolide. Although En-2 showed a small increase after the application of  $10^{-10}$  M brassinolide, elongation was not enhanced with higher concentrations. In contrast, Ws-2 hypocotyls displayed no increase at  $10^{-10}$  M, but appeared to respond to higher concentrations of brassinolide. *dwarf1-1*, in the Ws-2 background, showed an increase in length at  $10^{-10}$  M and was comparable with the wild type at  $10^{-9}$  M and higher (Fig. 2). *dwarf1-3*, in the En-2 background, did not show a significant increase in length until the application of a  $10^{-9}$  M

**Table I.** Alleles of the Arabidopsis *dwarf1* mutant

Allele	Previous Name	Mutagen	Ecotype	Reference
<i>dwarf1-1</i>	T-31	T-DNA	Ws-2	Feldmann et al. (1989)
<i>dwarf1-2</i>	<i>dim</i>	T-DNA	Columbia	Takahashi et al. (1995)
<i>dwarf1-3</i>	318	Unknown	En-2	NASC <sup>a</sup> stock no. 318
<i>dwarf1-4</i>	355	Unknown	En-2	NASC stock no. 355
<i>dwarf1-5</i>	356	Unknown	En-2	NASC stock no. 356
<i>dwarf1-6</i>	<i>ccb1</i>	Ac/Ds <sup>b</sup>	C-24	Kauschmann et al. (1996)
<i>dwarf1-7</i>	WM1-7	EMS	Ws-2	This paper
<i>dwarf1-8</i>	WM3-1	EMS	Ws-2	This paper
<i>dwarf1-9</i>	WM5-5	EMS	Ws-2	This paper
<i>dwarf1-10</i>	WM9-3	EMS	Ws-2	This paper
<i>dwarf1-11</i>	WM12-1	EMS	Ws-2	This paper

<sup>a</sup> Nottingham Arabidopsis Stock Center.

<sup>b</sup> Ac/Ds, Activator/dissociation transposable element.

**Table II.** Morphometric analysis of *Ws-2* wild-type and *dwf1-1* plants

The sizes of various organs were measured to the nearest millimeter, and the number of organs was counted in *dwf1-1* and wild-type plants grown individually in 5.5-cm pots. Each value represents the mean of 10 or more plants  $\pm$  SE.

Parameter	At 35 d		At Maturity <sup>a</sup>	
	<i>Ws-2</i>	<i>dwf1-1</i>	<i>Ws-2</i>	<i>dwf1-1</i>
<i>mm</i>				
Length				
Inflorescence	25.80 $\pm$ 0.83	5.47 $\pm$ 0.40	26.99 $\pm$ 0.85	14.72 $\pm$ 0.88
Internode	nd <sup>b</sup>	nd	1.10 $\pm$ 0.03	0.27 $\pm$ 0.01
Petiole <sup>c</sup>	0.57 $\pm$ 0.03	0.22 $\pm$ 0.01	nd	nd
Leaf blade <sup>c</sup>	1.72 $\pm$ 0.02	0.80 $\pm$ 0.03	nd	nd
Leaf blade width <sup>c</sup>	0.77 $\pm$ 0.02	0.84 $\pm$ 0.03	nd	nd
<i>no.</i>				
Number of organs				
Rosette leaves	7.1 $\pm$ 0.3	10.9 $\pm$ 0.6	nd	nd
Rosette bolts	3.6 $\pm$ 0.2	3.7 $\pm$ 0.3	6.4 $\pm$ 0.2	5.0 $\pm$ 0.2
Branches <sup>d</sup>	1.8 $\pm$ 0.1	3.1 $\pm$ 0.2	2.8 $\pm$ 0.1	4.6 $\pm$ 0.2
Siliques <sup>d</sup>	49.8 $\pm$ 4.0	38.0 $\pm$ 5.0	118.9 $\pm$ 12.1	492.6 $\pm$ 28.9
Siliques <sup>e</sup>	101.7 $\pm$ 11.3	72.4 $\pm$ 7.5	336.5 $\pm$ 28.6	1303.5 $\pm$ 52.1
Seeds/silique	nd	nd	37.7 $\pm$ 0.7	3.2 $\pm$ 1.0

<sup>a</sup> Maturity is considered to be the cessation of new flower development. <sup>b</sup> nd, Not determined. <sup>c</sup> Measured from the second leaf pair. <sup>d</sup> Number from the primary inflorescence. <sup>e</sup> Number from the entire plant.

concentration of brassinolide, and then continued to increase with concentrations up to  $10^{-7}$  M. In addition to increasing the length of the hypocotyls, brassinolide application increased the size of *dwf1-1* cotyledons and first leaves (data not shown). At concentrations of more than  $10^{-9}$  M brassinolide, these organs were indistinguishable from those of the wild type, although cotyledon and hypocotyl morphology became distorted with higher brassinolide concentrations (data not shown).

Rescue of *dwf1-1* by exogenous application of brassinolide suggests that *dwf1* is defective in brassinolide biosynthesis. Therefore, we wanted to pinpoint the exact step that is defective in *dwf1*. To this end, we examined the effectiveness of some of the brassinosteroid-biosynthetic intermediates in restoring the growth of *dwf1* pedicels. *dwf1* responds to cathasterone, 6-deoxocathasterone, 22 $\alpha$ -hydroxycampesterol, and brassinolide with increased growth of pedicels (Fig. 3). The synthetic compound 22 $\alpha$ -hydroxycampesterol has been used in brassinosteroid-feeding studies to overcome problems with the undetectable bioactivity of early biosynthetic intermediates in this

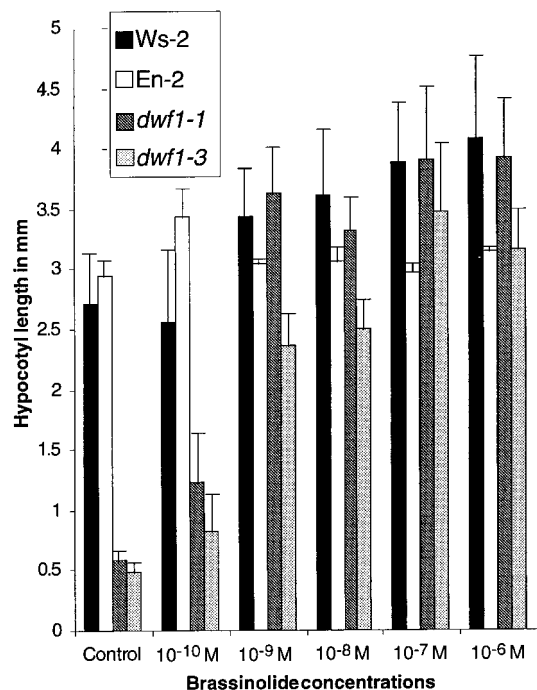
bioassay, and rescue by 22 $\alpha$ -hydroxycampesterol is interpreted as being complemented by campesterol (Choe et al., 1998). Therefore, complementation of *dwf1* by 22 $\alpha$ -hydroxycampesterol and downstream intermediates suggests that the biosynthetic defect in *dwf1* resided before campesterol.

**Table III.** Timing of phase transitions

Anatomical characteristics associated with growth-phase changes were scored, and the days after germination (DAG) were recorded. Each number represents the mean of 10 or more plants.

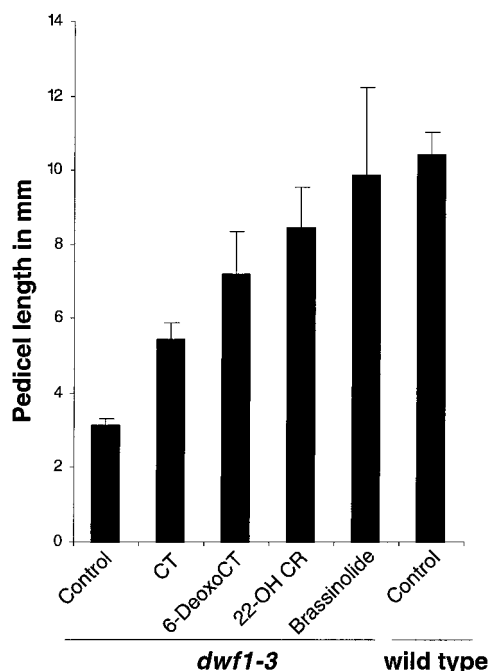
Characteristic	<i>Ws-2</i>	<i>dwf1-1</i>
	<i>DAG</i>	
Bolt	16.9	19.8
Anthesis of first flower	21.8	24.9
First silique senescence	34.6	44.6
Termination of flowering <sup>a</sup>	57	102

<sup>a</sup> Considered to be the senescence of the last flower.



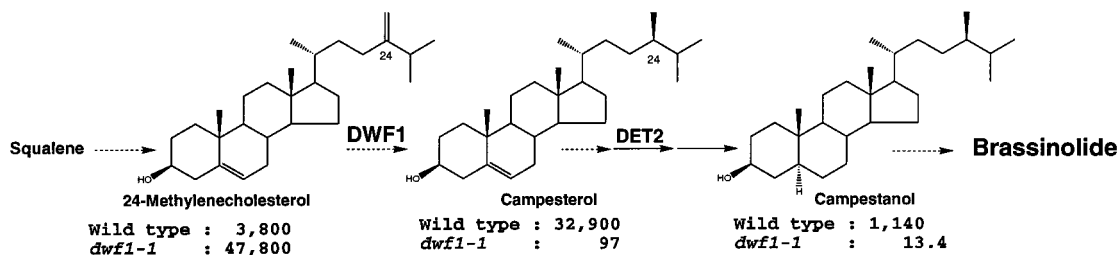
**Figure 2.** Response of Arabidopsis wild type (*Ws-2* and *En-2*) and *dwf1* alleles to different concentrations of exogenously applied brassinolide. The length of 15 or more hypocotyls grown for 10 d in liquid medium was measured. Bars =  $\pm$  SD.





**Figure 3.** Feeding tests with brassinosteroid-biosynthetic intermediates. Inflorescences of 4-week-old *dwf1-3* plants were treated with specified biosynthetic intermediates for 1 week. Pedicel length ( $n = 15$ ) was measured. All of the compounds tested induced a significant response from the pedicels compared with controls. In particular, 22 $\alpha$ -hydroxycampesterol and brassinolide completely rescued the short pedicel length to the normal wild-type length. Bars =  $\pm$ SD.

To further delimit the biosynthetic defect to a single step, we analyzed the endogenous levels of brassinosteroid-biosynthetic intermediates in *dwf1-1* and the wild type using a GC-SIM assay. Figure 4 shows the endogenous amount of selected intermediates with their chemical structures. Compared with the wild type, 24-methylenecholesterol accumulates to a 12-fold higher level in *dwf1-1*, whereas the level of the downstream compound, campesterol, is decreased to 0.3% of the wild type. The level of the next compound, campestanol, is accordingly decreased (most likely due to a shortage of the substrate compound campesterol). Together, the accumulation of 24-methylenecholesterol with the simultaneous decrease of campesterol in *dwf1-1* suggests that the C-24 reduction step is deficient in *dwf1*.



**Figure 4.** Brassinosteroid-biosynthetic step found to be defective in *dwf1* plants. The aerial parts of 5-week-old wild-type and *dwf1* plants were subjected to analysis of endogenous brassinosteroid levels using GC-SIM. Accumulation of 24-methylenecholesterol with a simultaneous reduction in the campesterol level suggests that a specific step, C-24 reduction, was blocked in *dwf1* mutants. Campesterol is known to serve as substrate for brassinolide biosynthesis.

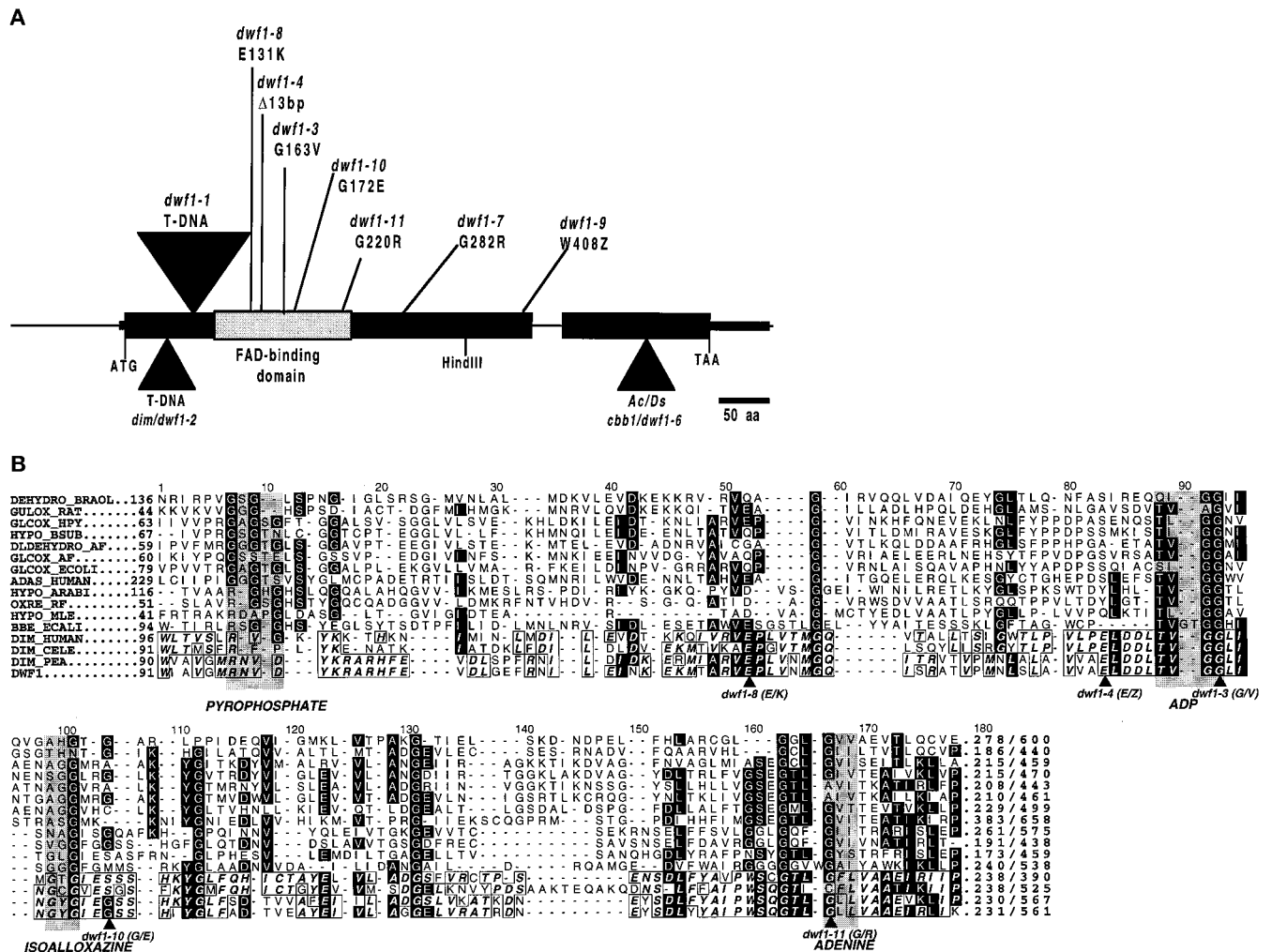
### Molecular Characterization of *dwf1* Alleles

Feldmann et al. (1989) demonstrated that the *dwf1-1* phenotype cosegregated with the kanamycin marker derived from an inserted T-DNA. Plant DNA flanking the T-DNA was isolated by plasmid rescue, as described by Dilkes and Feldmann (1998). The rescued clone was found to contain 300 bp of DNA flanking an intact right border. In Southern-blot analyses with genomic DNA from *dwf1-1* and wild-type plants, restriction fragment length polymorphism was detected in the T-DNA line when the plant-flanking DNA was used as a probe. The plant-flanking DNA was used to isolate a cDNA clone. Plasmid-rescued DNA, the corresponding cDNA, and genomic clones spanning the entire coding region were sequenced. The cDNA sequence was deposited in GenBank (accession no. U12400). Takahashi et al. (1995) subsequently isolated the identical gene and named it *DIMINUTO*. As previously described by Takahashi et al. (1995), the *DWF1* coding sequence is 1686 bp in length and is disrupted by one 92-bp intron 1282 bp downstream from the start codon.

The primary structure of the *DWF1* protein (561 amino acids) was subject to analyses using PSORT software (Nakai and Kanehisa, 1992), which revealed that the protein is likely to be targeted to endomembrane systems such as the ER, Golgi apparatus, and mitochondria as an integral protein, with amino acids 27 to 43 serving as the membrane-spanning domain. Based on the criteria set by Hicks and Raikhel (1995) and Robbins et al. (1991), the NUCDISC (discrimination of nuclear-localization signals) subprogram of the PSORT package did not find any nuclear-localization signals. To test the expression of the gene, Arabidopsis EST database searches for sequences identical to *DWF1* were performed. Fourteen EST clones were identified (K3F6TP, 106C3T7, 137P3T7, E4F9T7, 128K1T7, G10C7T7, E1D10T7, G9E12T7, 94O7XP, 176H17T7, 94O7T7, VBV10-12592, G5D11T7, and VCVDH10). As predicted by the abundance of EST clones, the steady-state level of the *DWF1* transcript was present in all organs and at all developmental stages tested (data not shown; Takahashi et al., 1995).

Sequencing the genomic DNA from the various mutants and comparing it with the wild type showed that there were single mutations in all of the mutant alleles. Figure 5A





**Figure 5.** Schematic representation of Arabidopsis *DWF1* locus with characterized mutations (A) and aligned amino acid sequences of proposed FAD-binding domains from various oxidoreductases (B). Elucidation of *DWF1* organization resulted from comparison of cDNA with genomic DNA. Sites of the FAD-binding domain (Mushegian and Koonin, 1996; Fraaije et al., 1998) and mutations identified from *dwf1* alleles, including *dim* (*dwf1-2*; Takahashi et al., 1995) and *cbb1* (*dwf1-6*; Kauschmann et al., 1996), are indicated. Sequences corresponding to the region of the FAD-binding domain depicted in A are aligned to show conserved residues and relative positions of mutations. Accession numbers for the following are given in parentheses: DEHYD\_BRAOL (2760543), GULOX\_RAT (625202), GLCOX\_HPY (2313619), HYPO\_BSUB (1770026), DLDEHYD\_AF (2650235), GLCOX\_AF (2649802), GLCOX\_ECOLI (1707917), ADAS\_HUMAN (2498106), HYPO\_ARABI (2618686), OXRE\_RF (1169648), HYPO\_MLE (3150105), BBE\_ECALI (400972), DIM\_HUMAN (3182980), DIM\_CELE (3182979), DIM\_PEA (3182981), DWF1 (U12400). Similar sequences were identified using gapped BLAST (Altschul et al., 1997), followed by alignment using PileUp software (GCG). Box shading was carried out using the ALSCRIPT package developed by Barton (1993).

schematically represents *DWF1*, including the positions of various mutations. Most mutations (7 of 10) were located in the amino-terminal half of the gene. The T-DNA is inserted 200 bp downstream from the start codon in *dwf1-1*. To determine whether the T-DNA insertion disrupted *DWF1* gene expression, we performed northern-blot analysis with total RNA from *dwf1-1* and from the wild type. When the blot was probed with *DWF1* cDNA, we detected no *DWF1* RNA in the *dwf1-1* sample, although this RNA was easily detected in the wild-type sample (data not shown). This indicated that steady-state RNA levels in *dwf1-1* were be-

low the threshold for detection. Along with the position of the T-DNA, this suggests that *dwf1-1* is a null mutation. *dwf1-4* carries a 13-bp deletion from bases 437 to 449, causing a frame shift that generates a stop codon eight amino acids downstream of the deletion, indicating that it is also a null allele. Sequencing of *dwf1-5* revealed that it contained the same mutation as *dwf1-4*, suggesting that these two alleles arose from the same mutational event. Moreover, the EMS-induced allele *dwf1-9* contains a transition mutation from guanine to adenine at 1279, changing Trp into a stop codon. Four of the five remaining alleles

contained substitution mutations that changed Gly into a charged amino acid (*dwf1-7*, *dwf1-10*, and *dwf1-11*) or Val (*dwf1-3*). Finally, *dwf1-8* caused a change from a negatively charged Glu to a positively charged Lys.

To better understand DWF1 function, we performed database searches with the DWF1 cDNA and its deduced protein sequence. BLAST searches (Altschul et al., 1997) produced two categories of proteins; one displayed global similarity with DWF1. This group included DIM-like proteins isolated from pea (88% similarity; Shimizu and Mori, 1996; accession no. 3182981), *Caenorhabditis elegans* (53%; accession no. 3182979), and human (60%; Nomura et al., 1994; accession no. 3182980). The other group of proteins showed similarities to only a part of the DWF1 protein from amino acids 91 to 231. Figure 5B illustrates the results of the local sequence alignment. The second group of proteins is represented by sequences from bacteria through plants and animals, and the functions of many of these proteins are unknown.

Proteins with known functions include L-gulonolactone oxidase of rat (GULOX\_RAT; Koshizaka et al., 1988), L-galactono-1,4-lactone dehydrogenase of *Brassica oleracea* (DEHYD\_BRAOL; Ostergaard et al., 1997), alkyldihydroxyacetonephosphate synthase of human (ADAS\_HUMAN; de Vet et al., 1997), berberine-bridge-forming enzyme of *Eschscholzia californica* (BBE\_ECALI; Dittrich and Kutchan, 1991), glycolate oxidase subunit of *Helicobacter pylori* (GLCOX\_HPY; Tomb et al., 1997), and D-lactate dehydrogenase of *Archaeoglobus fulgidus* (DLDEHYD\_AF; Klenk et al., 1997). Mushegian and Koonin (1995) initially proposed that the domain illustrated in Figure 5B is conserved among a broad spectrum of oxidoreductases requiring FAD as a prosthetic group. In the FAD-binding domain shown in Figure 5B, 46 of 140 residues (91–231) in DWF1 are conserved. Ten of the 49 conserved residues are Gly. In support of the importance of the conserved domain, mutations in our *dwf1* alleles correspond well to the conserved residues. Among eight *dwf1* mutations, six are located in or before this FAD-binding domain. Four mutations are located in highly conserved residues (*dwf1-2*, *dwf1-8*, *dwf1-10*, and *dwf1-11*), and one that creates a premature stop codon (*dwf1-3*) is predicted to delete the FAD-binding domain.

## DISCUSSION

### Comparison of Different *dwf* Mutants

Brassinosteroids have long been known to be involved in many different developmental events throughout the life cycle of plants (Mandava, 1988). However, definitive evidence for the role of brassinosteroids during plant growth has remained unclear until the recent characterization of mutants defective in brassinosteroid biosynthesis or perception. Brassinosteroid dwarf mutants, including *dwf1*, are characterized by multiple phenotypes: reduced height, robust stems, reduced fertility, prolonged life cycle, dark-green color, round and curled leaves, and, when grown in the dark, short hypocotyls and expanded cotyledons. This phenotype has been reported for several brassinosteroid dwarfs, including *bri1* (*dwf2*) (Clouse et al., 1996), *cpd* (*dwf3*)

(Szekeres et al., 1996), *dwf4* (Choe et al., 1998), *det2* (*dwf6*) (Li et al., 1996), and *dwf7* (Choe et al., 1999). Therefore, it is worth comparing *dwf1* with other dwarf loci.

At 35 d of age, *dwf1-1* was 5.47 cm tall (Table I), whereas *dwf4-1* grew to only half of this height, or 2.8 cm (Azpiroz et al., 1998). In addition, the heights of *dwf2-1* (*bri1*), *dwf3-1* (*cpd*), and *dwf6-1* (*det2*) were 1.4, 1.92, and 5.1 cm, respectively (B.P. Dilkes, B. Schulz, S. Choe, R. Azpiroz, and K.A. Feldmann, unpublished data). The length of dark-grown hypocotyls for these mutants was proportional to the severity of their phenotype in the light (data not shown). Thus, based on their severity, the *dwf* loci can be divided into two groups: standard and small dwarfs. The standard dwarfs include *dwf1* and *dwf6* (*det2*), whereas the small dwarfs include *dwf2* (*bri1*), *dwf3* (*cpd*), and *dwf4*. The earlier the biosynthetic defect, the less severe the phenotype. In other words, biosynthetic defects in the standard dwarfs reside before the DWF4 step, the putative rate-limiting step in brassinosteroid biosynthesis (Fujioka et al., 1995; Choe et al., 1998), whereas small dwarfs correspond to the DWF4-mediated steps and steps following them.

This relationship is reversed in GA-biosynthetic dwarfs such as *ga1* to *ga5* (Ross et al., 1997). Mutants blocked in the early reactions of GA biosynthesis, such as *ga1*, *ga2*, and *ga3* (defective in steps before GA<sub>12</sub>), display extreme dwarfism compared with *ga4* and *ga5*, which are defective in later biosynthetic steps (Koornneef and van der Veen, 1980; Finkelstein and Zeevaart, 1994). It is thought that later biosynthetic steps in GA biosynthesis are mainly modification reactions conferring increased bioactivity to GAs through network reactions of many redundant isozymes (Finkelstein and Zeevaart, 1994). Thus, GA-biosynthetic mutants could still synthesize a limited number of active GAs (Finkelstein and Zeevaart, 1994). It is also possible that brassinosteroid-biosynthetic reactions downstream of the DWF4-mediated step are more critical to conferring functionality to brassinosteroids than upstream reactions.

One difference between the GA- and brassinosteroid-biosynthetic pathways may be that the genes involved in the reactions upstream of the DWF4 step are genetically redundant. In support of this, we have detected lightly hybridizing bands with DWF1 and DWF7 probes in northern and Southern blots (A. Tanaka, B.P. Dilkes, S. Choe, F.E. Tax, and K.A. Feldmann, unpublished data). A DWF7 homolog has been isolated and is being characterized (A. Tanaka, S. Choe, F.E. Tax, and K.A. Feldmann, unpublished data). In addition, the possibility of a second gene for DET2 was reported by Fujioka et al. (1997). Further analysis of these cross-hybridizing genes in the upstream reactions through cloning, sequencing, and expression studies, including temporal and spatial localization studies, will provide us with in-depth knowledge about the regulation of brassinosteroid biosynthesis in relation to the phenotypic severity of these dwarf mutants.

### Delayed Senescence in *dwf* Mutants

*dwf1* mutants and other brassinosteroid dwarf mutants show an expanded life span. Dwarf plants both maintain green leaves and produce flowers for a longer period of

time than the wild type. Bolting, first flower anthesis, first silique senescence, and termination of flowering are all delayed in *dwarf1* (Table III). Similarly, Azpiroz et al. (1998) reported that completion of one generation takes 98 d for *dwarf4-1*, compared with 57 d for the wild type. The cause of the extended life span for brassinosteroid *dwarf* mutants is not known. However, one possible explanation is that a long life cycle is associated with reduced fertility. Guarente et al. (1998) and Nooden (1988) have shown that developing seeds in need of mobilized nutrients trigger the onset of senescence as part of a global nutrient-recycling program.

Nooden (1988) showed that removal of the developing seed pods of soybean greatly delayed senescence of leaves and whole plants because of an increased quantity of cytokinins transported from the roots. Similarly, it is possible that the failure of seed development in dwarf mutants to produce signals for the onset of senescence could be the reason that *dwarf1* displayed an expanded life span. In support of this, the generative phase was significantly expanded in *dwarf1*. It took 82 d for *dwarf1-1* to progress from bolting to the termination of flowering, whereas 40 d were required for the wild type. However, the vegetative phase of *dwarf1* was not delayed significantly (Table III). Furthermore, there are data (B. Dilkes, R. Azpiroz, S. Choe, and K. Feldmann, unpublished data) suggesting that the generation time was proportional to the fertility of brassinosteroid *dwarf* mutants: the less fertile (i.e. *dwarf4* and *bri1* [*dwarf2*]) the longer the generation time. Despite the delayed senescence, we do not know whether intrinsic, age-dependent senescence was also delayed or if it was unaffected. Hensel et al. (1993) have suggested that age-dependent senescence can be uncoupled from reproductive development. Recently, Park et al. (1998) and Weaver et al. (1998) have cloned and tested many senescence-related genes in relation to various internal and external signals. Exploring the expression of the senescence-related genes in *dwarf1* plants could provide more detailed information about the mechanism of delayed senescence in *dwarf1* and about the role of brassinosteroids in the senescence process.

### Possible Function of DWF1 Protein

In addition to the morphological analysis of *dwarf1*, we characterized the molecular basis of several *dwarf1* alleles, and showed that *dwarf1* was defective in the conversion of 24-methylenecholesterol to campesterol in the sterol-biosynthetic pathway leading to brassinosteroid biosynthesis. Analysis of the amino acid sequence of DWF1 indicated that it probably resides in the endomembrane system, most likely in the ER. It has been shown that most of the steroid-biosynthetic enzymes need to be located in membranes for proper functioning (for review, see Bach and Benveniste, 1997). In addition, part of the DWF1 protein sequence showed significant similarity to oxidases from many different organisms, including bacteria and humans (Fig. 5B). Mushegian and Koonin (1995) initially found that this putative domain was shared by many oxidases that require FAD as a coenzyme.

More recently, based on the crystal structure of an  $8\alpha$ -( $N^3$ -histidyl)-FAD-containing flavoprotein, vanillyl-alcohol

oxidase, Fraaije et al. (1998) reported that there were two major domains: a FAD-binding domain and a substrate-binding domain. The FAD-binding domain was characterized by subdomains for the binding of pyrophosphate, ADP, isoalloxazine, and adenine (Fraaije et al., 1998; Fig. 5B). According to their findings, the residues in the FAD-binding domain were highly conserved among enzymes involved in a diverse range of redox reactions. The absence of residues for isoalloxazine-ring linkages such as His, Cys, or Tyr in the pyrophosphate subdomain in DWF1 (underlined as pyrophosphate in Fig. 5B) suggests that DWF1 binds to its coenzyme, FAD, not by a covalent bond but by a dissociable bond, which is common for many FAD-dependent enzymes (Mewies et al., 1998). Furthermore, conservation of the subdomains in DWF1 indicated that it belonged to this novel group of oxidases. Six of eight *dwarf1* mutations reported in this paper interfered with this domain.

The null allele, *dwarf1-1*, contained an insertion upstream of the FAD-binding domain, and *dwarf1-4* contained a stop codon in the middle of the domain. In addition, in *dwarf1-3* and *dwarf1-11*, residues belonging to the ADP- and the adenine-contacting regions, respectively, were altered (Fig. 5B). The severity of *dwarf1* alleles was correlated with the location of the mutations relative to the FAD-binding domain. The mutants in the Ws-2 background, *dwarf1-1*, *dwarf1-8*, *dwarf1-10*, and *dwarf1-11*, which are located at or before the FAD-binding domain, displayed more severe phenotypes than *dwarf1-7* and *dwarf1-9*, in which the mutations were found after the FAD-binding domain (data not shown). With the addition of *dwarf1-2* (*dim*) (T-DNA insertion upstream of FAD-binding domain [Takahashi et al., 1995]) and *dwarf1-6* (*cbb1*) (transposon insertion 515 amino acids downstream of the start codon [Kauschmann et al., 1996]), 7 of 10 mutations were localized in or before the FAD-binding domain. This suggests that the conserved residues in the FAD-binding domain are critical for DWF1 function, perhaps through binding of the coenzyme FAD to the DWF1 apoenzyme leading to redox reactions catalyzed by the flavoenzyme.

Aside from the putative FAD-binding domains, we can speculate on the role assigned to the rest of the protein. First, as Fraaije et al. (1998) have pointed out, one part of the protein may play the role of the substrate-binding domain, and this binding domain may be variable in sequence between diverse redox enzymes. Second, the rest of the protein sequence may confer an enzymatic function other than that of an oxidase. For instance, it has been proposed that campesterol is produced via isomerization of  $\Delta^{24(28)}$  to  $\Delta^{24(25)}$  as an intermediate before the double bond is saturated by the reductase activity. Thus, the isomerization and reduction of the  $\Delta^{24(25)}$  double bond could be performed by the rest of the protein in this multifunctional enzyme.

In conclusion, loss of the enzyme that converts 24-methylenecholesterol to campesterol in *dwarf1* plants caused a dramatic reduction in campesterol biosynthesis (Fig. 4) to approximately 0.3% of wild-type levels. The reduction of campesterol resulted in reduced biosynthesis of brassinolide, which seemed to be the direct cause of altered devel-



opment in *dwf1* plants. In addition to *dwf7*, *dwf1* is now a second sterol-specific biosynthetic mutant showing a characteristic *dwarf* phenotype that can be rescued to wild type by the exogenous application of brassinosteroids.

#### NOTE ADDED IN PROOF

While this manuscript was in the review process, Klahre et al. (1998) also showed that *diminuto* (*dwf1-2*) was defective in the conversion of 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis. In addition, the authors provided evidence that DIM/DWF1 is associated with the endomembrane system rather than with the nucleus.

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