

The Arabidopsis *DIMINUTO/DWARF1* Gene Encodes a Protein Involved in Steroid Synthesis

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We have identified the function of the Arabidopsis *DIMINUTO/DWARF1* (*DIM/DWF1*) gene by analyzing the *dim* mutant, a severe dwarf with greatly reduced fertility. Both the mutant phenotype and gene expression could be rescued by the addition of exogenous brassinolide. Analysis of endogenous sterols demonstrated that *dim* accumulates 24-methylenecholesterol but is deficient in campesterol, an early precursor of brassinolide. In addition, we show that *dim* is deficient in brassinosteroids as well. Feeding experiments using deuterium-labeled 24-methylenecholesterol and 24-methyldesterol confirmed that *DIM/DWF1* is involved in both the isomerization and reduction of the $\Delta^{24(28)}$ bond. This conversion is not required in cholesterol biosynthesis in animals but is a key step in the biosynthesis of plant sterols. Transient expression of a green fluorescent protein–*DIM/DWF1* fusion protein and biochemical experiments showed that *DIM/DWF1* is an integral membrane protein that most probably is associated with the endoplasmic reticulum.

INTRODUCTION

Plant growth is regulated by a large number of environmental stimuli and endogenous factors. Among the latter are well-known plant growth regulators such as auxins, gibberellins, cytokinins, ethylene, and abscisic acid. Recently, it has been found, however, that plants contain steroid compounds that are as active at similarly low concentrations (nanomolar to picomolar) as animal steroid hormones. Although a role for plant sterols has been postulated for almost 20 years (Grove et al., 1979), their importance in plant growth and development has only recently gained acceptance, mainly due to the isolation of Arabidopsis and pea mutants that are defective in steroid biosynthesis (Clouse, 1996; Creelman and Mullet, 1997; Nomura et al., 1997; Aziproz et al., 1998).

Sterols are used by many organisms as modulators of membrane elasticity and as precursors for steroid hormones. Whereas the basic structure of four rings and an alkyl chain is similar in all sterols, there are many structural variations in the alkyl side chain and the degree of saturation of the ring bonds. Animals mainly synthesize cholesterol, er-

gosterol is the predominant sterol in yeasts, and sitosterol, stigmasterol, and campesterol are the most abundant sterols in plants. In mammalian cells, cholesterol serves as the precursor for steroid hormones, which are characterized by reduced complexity caused by the removal of most of the side chain. Plants use campesterol as a precursor for brassinosteroid (BR) biosynthesis and do not substantially shorten the side chain to form active hormones but rather employ a series of reduction and hydroxylation steps to do so. Interestingly, animals that cannot synthesize sterols use dietary sterols, which may be of animal or plant origin, as precursors.

The biological activity of BRs has been generally accepted since their isolation from vast amounts of rape pollen in 1979 (Grove et al., 1979). However, great differences in effects seen with assay systems and variations observed among plant species have made it difficult to assess the general importance of plant steroid hormones. Numerous articles have described the various effects of BRs, in particular brassinolide, on a variety of plant tissues and species (Adam et al., 1991; Sakurai and Fujioka, 1993). Recent research using Arabidopsis and pea as model systems has clarified the important role of BRs in plant growth and development. During the past 2 years, it was discovered that the

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phenotype of certain Arabidopsis and pea mutants can be restored to that of the wild type by the application of BRs (Kauschmann et al., 1996; Li et al., 1996; Szekeres et al., 1996; Fujioka et al., 1997; Nomura et al., 1997; Azpiroz et al., 1998; Choe et al., 1998). In particular, Szekeres et al. (1996) have found that the phenotype of several pleiotropic mutants, including *deetiolated* (*det*), *constitutive photomorphogenic* (*cop*), *fusca* (*fus*), as well as *diminuto* (*dim*), can be restored to that of the wild type by adding brassinolide to the culture medium. In Arabidopsis, two classes of mutants have been isolated: those that cannot synthesize BRs (*det2*, *constitutive photomorphogenesis and dwarfism* [*cpd*], and *dwarf4* [*dwf4*]; Li et al., 1996; Szekeres et al., 1996; Fujioka et al., 1997; Azpiroz et al., 1998; Choe et al., 1998) and those that are insensitive to the exogenous application of these compounds (*bri1*, *cabbage2* [*cbb2*]; Clouse et al., 1996; Kauschmann et al., 1996). Arabidopsis mutants that accumulate reduced endogenous amounts of BRs grow as dwarfs, and their fertility is greatly impaired. As expected, BR-insensitive mutants show a very similar phenotype. So far, four genes, *CPD*, *DET2*, *DWF4*, and *BRI1*, have been identified; they encode cytochrome P450-like enzymes (*CPD*, Szekeres et al., 1996; *DWF4*, Choe et al., 1998), a steroid hormone 5 α -reductase with high similarity to mammalian enzymes (*DET2*, Li et al., 1996), and a transmembrane receptor kinase (*BRI1*, Li and Chory, 1997), respectively.

Exogenous application of nanomolar concentrations of brassinolide on wild-type Arabidopsis plants leads to a substantial reduction of root elongation in an auxin-independent manner and an increase in peduncle elongation (Clouse et al., 1993). Although altered gene expression has been observed in response to the application of BRs (Clouse et al., 1993; Zurek and Clouse, 1994), only one group of genes, the xyloglucan endotransferases (XETs), has been demonstrated to be regulated directly by BRs (Zurek and Clouse, 1994; Xu et al., 1995, 1996; Kauschmann et al., 1996).

The *dim* mutant was initially isolated as a slowly growing dwarf. The *DIM* gene was cloned, and genomic analysis and gene expression studies strongly suggest that *dim* does not produce any DIM protein (Takahashi et al., 1995). The *DIM* gene has also been referred to as *DWF1* (Feldmann, 1991) or *CBB1* (Kauschmann et al., 1996). Analysis of the DIM protein sequence, however, did not reveal homology to any known proteins in the database, except for reported homology to a domain found in FAD-dependent oxidoreductases (Mushegian and Koonin, 1995). Rescue experiments using BRs on dwarfed mutants such as *det2*, *cpd*, and *dim* (Kauschmann et al. 1996; Szekeres et al., 1996; and see below) indicate that *dim* may be deficient in BRs.

In this report, we show that the morphological as well as molecular phenotypes of *dim* can be complemented by the exogenous application of BRs. Measurements of endogenous sterol levels and feeding experiments demonstrate that the DIM/DWF1 protein is involved in the conversion of an early precursor of BRs, 24-methylenecholesterol to campesterol. Furthermore, our data show that the DIM/DWF1 pro-

tein is an integral membrane protein that is most likely localized to the endoplasmic reticulum.

RESULTS

The *dim* Mutant Can Be Rescued by the Application of Brassinolide

The biosynthesis pathway for brassinolide, by far the most active BR found in plants to date, has been established in transformed cell cultures of *Catharanthus roseus* (Sakurai and Fujioka, 1997; Yokota, 1997). To localize the possible lesion in brassinolide biosynthesis in the *dim* mutant, we provided each mutant plant with brassinolide and its immediate precursors, teasterone, typhasterol, and castasterone. Figure 1 shows that the phenotype of young *dim* seedlings can be restored to that of wild type by low concentrations of brassinolide and its precursors. However, the precursors were less active the farther they were upstream in the biosynthesis of brassinolide. Figure 2 shows quantitatively that the addition of both castasterone and brassinolide can successfully restore the hypocotyl growth of *dim* seedlings to that of wild-type levels. This is true for plants grown in the light (Figure 2A) and in the dark (Figure 2B). Quantification of the results demonstrated that brassinolide and castasterone were highly active, whereas precursors earlier in the pathway were gradually less efficient in rescuing the phenotype

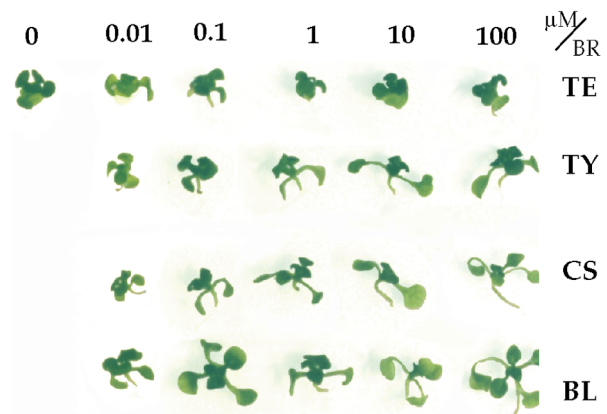


Figure 1. Phenotypic Restoration of *dim* by BRs.

A biochemical complementation analysis of *dim* plants is shown. *dim* seedlings were germinated and grown in constant white light at 22°C in the absence of BRs for 3 days, after which 1 μ L of brassinolide (BL) or its biosynthetic precursors teasterone (TE), typhasterol (TY), and castasterone (CS) in the indicated concentration was placed on the seedlings. The photograph was taken after an additional 5 days of growth in continuous white light. Complementation could be observed with brassinolide at all concentrations, whereas precursors become gradually less active.

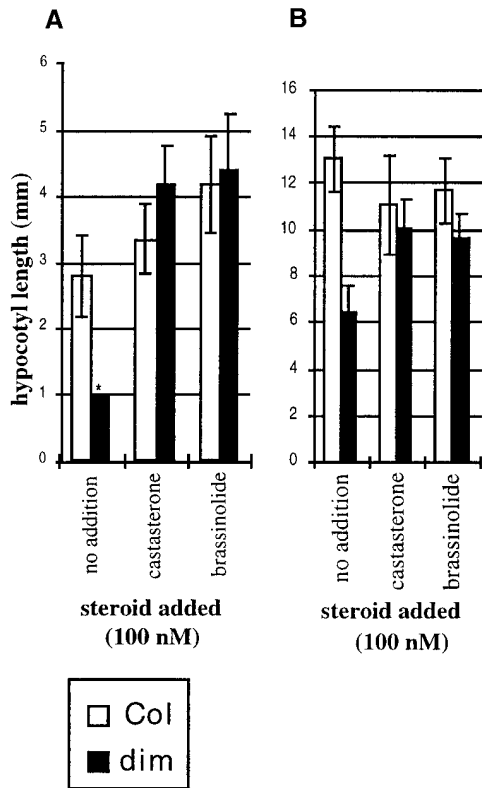


Figure 2. Quantification of Complementation Analysis.

Seedlings were grown on MS medium without sucrose containing the indicated concentrations of BRs, and hypocotyls were measured.

(A) Seedlings grown for 14 days in continuous white light at 22°C.

(B) Seedlings grown for 6 days in the dark at 22°C.

The asterisk indicates that untreated seedlings of *dim* were too small to be reliably measured without dissection. However, none of the hypocotyls of these seedlings was >1 mm. Col, Arabidopsis ecotype Columbia.

(quantification not shown). The phenotypic restoration of the *dim* phenotype was complete when young seedlings were used; however, the phenotypic restoration was less effective in the case of adult plants. The low fertility of the mutant could not be overcome by the simple addition of BRs.

Because earlier precursors of the brassinolide biosynthetic pathway also become gradually less active in other systems, as has been reported previously (Fujioka et al., 1995), it was difficult to determine the biosynthetic lesion by the exogenous application of the precursors only. Even high concentrations of the earlier precursors led to a relatively small effect on hypocotyl elongation, whereas substantial root shortening was observed (data not shown). The reduced activity seen with these precursors was not due to an uptake problem, because labeled precursors were readily taken up and metabolized (see below).

BR-Regulated Genes Are Expressed to a Lower Level in *dim* Plants

We also wanted to determine whether the restoration to the wild-type phenotype was paralleled by a similar restoration of the wild-type gene expression pattern. In an effort to isolate genes that are regulated by brassinolide, we used a differential display approach (Liang and Pardee, 1992; see also Methods) and identified genes that are induced by BR application. We isolated, among others, one gene that is upregulated by brassinolide by a mechanism that is independent of protein synthesis. This gene, called *S10*, encodes a protein that is identical in sequence to a member of the XET family of proteins (Okazawa et al., 1993; GenBank accession number D16454). Figure 3 shows that *S10* was upregulated severalfold by BR in both the wild type and the *dim* mutant. The kinetics of the induction were the same in the wild-type and *dim* plants, but the basal level of expression was much lower in the mutant (Figure 3). Therefore, we conclude that the mutant is capable of responding to the exogenous application of BRs with respect to the level of gene expression. The lower initial steady state mRNA level in *dim* most likely reflects a low level of induction by endogenous BRs, indicating that the mutant is probably defective in BR biosynthesis.

The *dim* Mutant Accumulates 24-Methylenecholesterol and Isofucosterol

Because the *dim* mutant phenotype can be rescued by many of the downstream precursors of brassinolide, we compared the endogenous levels of upstream precursors of brassinosteroids (sterols) in *dim* and wild-type shoots. Table 1 shows that 24-methylenecholesterol and isofucosterol

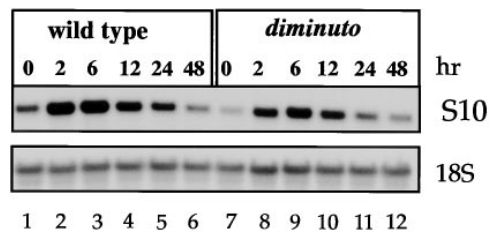


Figure 3. BRs Can Rescue the *dim* Mutant with Respect to mRNA Expression.

Plants were grown for 6 days in constant white light at 22°C, and brassinolide was sprayed onto the plants at a concentration of 100 nM. Samples were collected for RNA gel blot analysis at the indicated time points. Ten micrograms of total RNA was loaded per lane. The blot was probed with the *S10* DNA fragment. Note that the *S10* RNA accumulated in both the wild type and the *dim* mutant with similar kinetics but that the initial levels were lower in the mutant. The *S10* fragment is homologous to a fragment of the previously identified XET cDNA (Okazawa et al., 1993). The blot was reprobed with an 18S rDNA fragment as a loading control (18S).

Table 1. Analysis of Endogenous Sterols in the Wild Type and the *dim* Mutant

Sterol	WT ^a	<i>dim</i> ^a
24-Methylenecholesterol	Trace	30.7
Campesterol	47.2	4.1
Campestanol	0.8	0.1
Isofucoesterol	11.6	614.8
Sitosterol	312.9	20.2
Stigmasterol	4.9	1.5
Sitostanol	4.6	ND ^b
Cholesterol	8.6	3.7
Total sterols	390.6	675.1
End pathway sterols ^c	373.6	29.5

^aMicrograms of sterols per gram fresh weight of wild-type (WT) *Arabidopsis* ecotype Columbia and *dim* tissue.

^bND, not detectable.

^cEnd pathway sterols denote the sum of cholesterol, campesterol, sitosterol, and stigmasterol.

accumulated in *dim*, whereas the levels of campesterol and sitosterol were greatly reduced. The results suggest that the DIM/DWF1 protein is important for the conversion of 24-methylenecholesterol to campesterol as well as the conversion of isofucoesterol to sitosterol. Figure 4 describes the steps affected in the *dim* mutant. In the conversion of 24-methylenecholesterol to campesterol, the $\Delta^{24(28)}$ bond is first isomerized to yield 24-methylidestosterol, followed by a reduction of the resulting double bond. Similar isomerization and reduction reactions are also implicated in the conversion of isofucoesterol to sitosterol via $\Delta^{24(25)}$ sitosterol. Therefore, it appears that DIM/DWF1 can perform both steps. However, DIM/DWF1 is not likely to be involved in the conversion of desmosterol to cholesterol, because cholesterol levels are only marginally lower in the mutant (see Table 1).

To ascertain that the defect in the *dim* mutant is critical for the biosynthesis of BRs, we analyzed the endogenous BRs in wild-type and mutant plants grown aseptically in liquid medium. As expected, we found lower amounts of BRs in *dim* than in the wild-type plants (castasterone, 0.04 ng/g fresh weight; typhasterol, 0.12 ng/g fresh weight; 6-deoxoteasterone, 0.26 ng/g fresh weight in the wild type; all of these BRs were below detectable levels in the mutant). These results indicate that the DIM/DWF1 protein is required for the maintenance of appropriate levels of BRs.

dim Cannot Convert 24-Methylenecholesterol to Campesterol

Because campesterol has been identified as a key precursor of brassinolide (Fujioka and Sakurai, 1997a, 1997b), we concluded that the stunted phenotype of the *dim* mutant is caused by the blocked biosynthesis of brassinolide. However, application of campesterol to the *dim* mutant cannot

rescue the phenotype (data not shown), presumably because the conversion of exogenously supplied campesterol to brassinolide was inefficient or ineffective. A very similar situation was found in the *lkb* mutant of pea, where 24-methylenecholesterol and isofucoesterol accumulate to levels comparable to those in *dim*, suggesting that the *LKB* gene is an ortholog of *DIM/DWF1*. Also, in the case of the pea mutant, the phenotype could not be rescued by the exogenous application of campesterol (T. Nomura, Y. Kitisaka, S. Takatsuto, J.B. Reid, and T. Yokota, unpublished data).

These results show that the *dim* mutant has a severely altered sterol composition. The amounts of the sterols at the end of the pathway (see Table 1) were drastically reduced and the immediate precursors accumulated. These changes could possibly result in a membrane defect in the *dim* mutant. Plants, however, do vary substantially in their sterol composition; some species contain >80% sitosterol, whereas others have lower sitosterol levels but contain higher levels of 24-methylenecholesterol and isofucoesterol (Schiff and Feldlaufer, 1996). These observations suggest that the altered sterol composition in *dim* is very likely not responsible for the severe growth defect seen in the mutant. Moreover, *Arabidopsis* mutants with defects in later steps of brassinosteroid biosynthesis (e.g., *det2*, *cpd*, and *dwf4*; Li et al., 1996; Szekeres et al., 1996; Fujioka et al., 1997; Azpiroz et al., 1998; Choe et al., 1998) have a phenotype very similar to that of *dim*. This notion is supported by the finding that *dim* seedlings can be rescued by the addition of low amounts of brassinolide. Therefore, we postulate that the majority of the phenotypic defect in *dim* is due to the lack of BRs rather than sterols.

Because campesterol is the most important precursor for brassinolide, the biologically most active BR identified, we analyzed the conversion of 24-methylenecholesterol to campesterol in greater detail. Specifically, we wanted to clarify whether the DIM/DWF1 protein is required for both the isomerization and reduction steps in the conversion of 24-methylenecholesterol to campesterol. To this end, we incubated seedling cultures of *dim* plants and wild-type plants with 25,26,27-²H₇-labeled 24-methylenecholesterol for 4 days before gas chromatography and mass spectrometry analysis of the resulting metabolites (see Methods). Table 2 shows that the mutant was unable to convert the precursor into campesterol, whereas both campesterol and campestanol in their deuterated forms were abundant in the wild type. This result provides evidence that DIM/DWF1 is necessary for the biosynthesis of campesterol from 24-methylenecholesterol. In addition, ²H₆-labeled campesterol and ²H₆-labeled campestanol were detected instead of ²H₇-labeled compounds. This indicated that the reduction of the $\Delta^{24(28)}$ bond does not occur directly and that the isomerization ($\Delta^{24(28)}$ to $\Delta^{24(25)}$) takes place before the reduction. Therefore, we also provided *dim* plants with the intermediate 24-methylidestosterol in a labeled form. Table 3 shows the analysis of the metabolites. Because 24-methylidestosterol did not accumulate endogenously in the wild type, it seems likely

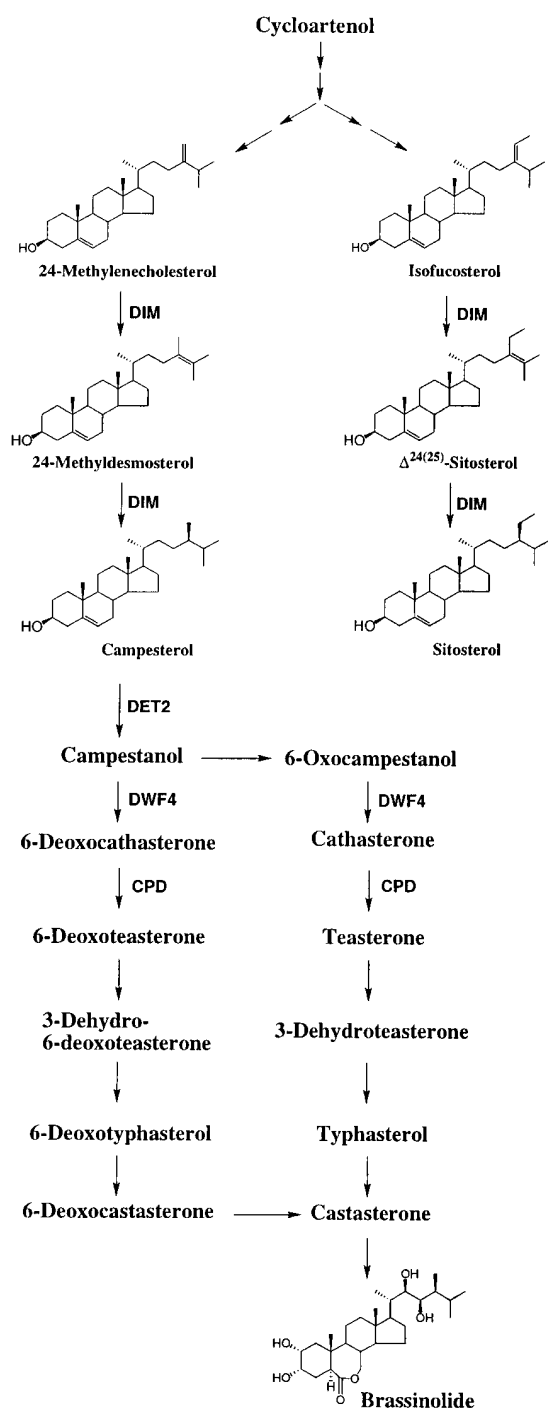


Figure 4. Biosynthetic Pathway for Brassinolide.

Shown is a schematic diagram of the part of the brassinolide biosynthesis pathway affected in the *dim* mutant. This mutant is unable to isomerize the $\Delta^{24(28)}$ bond into a $\Delta^{24(25)}$ bond and subsequently to reduce the double bond. 24-Methylenecholesterol is formed from cycloartenol. The product of the reaction, campesterol, is ultimately converted to brassinolide, the active steroid. A similar reaction is

that this intermediate has a very short half-life in campesterol biosynthesis. By contrast, the precursor was not converted to campesterol in *dim* plants (Table 3). Taken together, these experiments show that DIM/DWF1 is necessary for both the isomerization and reduction of 24-methylenecholesterol.

We have also generated transgenic Arabidopsis plant lines (DIMOE) that overexpress the *DIM/DWF1* gene. To our surprise, these transgenic lines did not display any clearly visible phenotype (data not shown), suggesting that DIM/DWF1 does not catalyze a rate-limiting step in brassinolide biosynthesis. Nevertheless, the overexpression of DIM/DWF1 in Arabidopsis led to a faster conversion of labeled precursors to campesterol and campestanol when compared with the wild type (Tables 1 and 2). Moreover, the endogenous levels of campesterol and campestanol were significantly higher in the overexpressing plants (two- to fourfold; Tables 2 and 3). These findings are in good agreement with the role of DIM/DWF1 proposed above.

During the analysis of these feeding experiments, we noted that in *dim*, the deuterium-labeled 24-methylenecholesterol precursor was converted into 24-methylenecholest-4-en-3-one and 24-methylene-5 α -cholestan-3 β -ol rather than campesterol. Recently, Fujioka et al. (1997) showed that campesterol was converted to campestanol via (24*R*)-24-methylcholest-4-en-3-one and (24*R*)-24-methyl-5 α -cholestan-3-one in the *det2* mutant. By analogy, 24-methylenecholesterol is obviously converted to 24-methylene-5 α -cholestan-3 β -ol via 24-methylenecholest-4-en-3-one in *dim* plants. A bypath from 24-methylenecholesterol is presented in Figure 5. 24-Methylenecholest-4-en-3-one also could be found endogenously at 20 times higher levels in *dim* plants as compared with wild-type or DIMOE plants. It is likely that this branch of the biosynthetic pathway is activated in *dim* because of the accumulation of endogenous 24-methylenecholesterol.

DIM/DWF1 Is a Membrane-Associated Protein

In a previous analysis of the DIM amino acid sequence (Takahashi et al., 1995), putative nuclear localization signals were found. Therefore, it was possible that the DIM/DWF1 protein might perform a regulatory function in the nucleus rather than a catalytic role in BR biosynthesis. We had previously attempted to overexpress the DIM/DWF1 protein in bacteria as well as in yeast, but in neither case were we able to obtain enough material for antibody preparation or for in

catalyzed in the conversion of isofucosterol to sitosterol via $\Delta^{24(25)}$ sitosterol. The reactions that are catalyzed by other enzymes of BR biosynthesis are indicated. DWF4 (Azizproz et al., 1998; Choe et al., 1998), DET2 (Li et al., 1996; Fujioka et al., 1997), and CPD (Szekeres et al., 1996) are shown.

Table 2. Measurement of Endogenous Sterols and Metabolites of Exogenously Added, Deuterium-Labeled 24-Methylenecholesterol^a

Sterol	Precursor	WT ^b	<i>dim</i> ^b	DIMOE ^b
24-Methylene-cholesterol	d7 ^c	12.3	3.01	7.05
	Endogenous	1.48	24.0	0.299
Campesterol	d6 ^c	0.222	ND ^d	0.172
	Endogenous	24.5	0.144	41.4
Campestanol	d6 ^c	0.118	ND	0.696
	Endogenous	0.353	0.093	0.772

^a 24-Methylenecholesterol was also converted into the putative metabolic compound 24-methylenecholest-4-en-3-one and the usually not found 24-methylene-5 α -cholestan-3 β -ol (see also Figure 4).

^b Micrograms of sterols per gram fresh weight of wild-type (WT) *Arabidopsis* ecotype Columbia, *dim*, and DIMOE (transgenic line carrying a 35S-*DIM/DWF1* transgene [see Methods]) tissue.

^c d7 and d6 denote labeled precursors extracted from plants that were fed deuterium-labeled 24-methylenecholesterol and incubated for 4 days in liquid culture.

^d ND, not detectable.

in vitro studies (data not shown). To detect the protein within the cell and in cell extracts, we therefore generated transgenic plants that express the DIM/DWF1 protein with a T7 tag located at the C terminus. Monoclonal antibodies generated against the T7 tag are commercially available (see Methods).

Because sterols as well as steroid hormones are relatively hydrophobic moieties, one would expect that BR synthesis may occur on or near membranes. In animal cells, cholesterol is mainly synthesized in the membranes of the smooth endoplasmic reticulum (ER) and in peroxisomes. Sterols are then transported to mitochondria, where steroids are produced by specific cleavage of the cholesterol precursor. To corroborate our results indicating that DIM/DWF1 is a protein directly involved in the biosynthesis of sterols and BRs, we performed cell fractionation studies to localize the DIM/DWF1 protein. DIM/DWF1 was found at low levels in the fraction containing nuclei (P0.2; data not shown). Figure 6A shows that the majority of the DIM/DWF1 protein sediments at high-speed centrifugation, which is used to isolate membrane-derived microsomes (P100). No protein was detected in the supernatant (S100), indicating that DIM/DWF1 is a membrane-associated protein.

Calreticulin (Denecke et al., 1995), a luminal ER protein, is present mainly in the pellet fractions (Figure 6A and data not shown). Unlike DIM/DWF1, however, calreticulin is also present in the high-speed supernatant, which suggests that during homogenization of plant materials, some of the protein was released, because it is not an integral membrane protein. A precise association of the DIM/DWF1 protein with a subcellular compartment cannot be postulated based on the results presented in Figure 6A. By analogy to animals, sterol biosynthetic enzymes are expected to be present in

the ER or in peroxisomes. It is possible that DIM/DWF1 might also be present in peroxisomes, but sequence analysis of DIM/DWF1 does not reveal any peroxisomal-targeting sequence (Mullen et al., 1997).

To test whether DIM/DWF1 is a peripheral or an integral membrane protein, we treated the microsomal fraction with various destabilizing agents. Figure 6B shows that whereas urea, NaCl, and high pH did not release DIM/DWF1 from the microsomes, the dissolution of the membranes by Triton X-100 led to the solubilization of the protein, indicating that this protein is an integral membrane protein.

As shown in Figure 7A, hydropathy prediction of the protein identifies one N-terminal domain as a potential membrane anchor. This prediction suggests that the protein has only one membrane-spanning domain, which would orient the bulk of the protein on either side of the membrane. To clarify whether the majority of the protein is facing the lumen or the cytosol, we treated the membrane fraction with thermolysin, a protease that does not penetrate membranes and digests only proteins that are externally disposed (Joyard et al., 1983). Figure 6C shows that when digested with different concentrations of protease, the majority of the DIM/DWF1 protein is degraded, indicating that the protein is anchored with its N terminus into the membrane, leaving the C terminus accessible to protease digestion. Because the T7 tag is attached at the very C terminus of the protein, no degradation products could be observed. Because small amounts of undigested protein remained, we surmised that during microsome preparation, a fraction of the membranes are reassembled as inside-out vesicles. The residual amount of DIM/DWF1 trapped in these vesicles can only be digested after dissolution of the membranes by Triton X-100. Unlike DIM/DWF1, the luminal ER protein calreticulin (Denecke et al., 1995) is not degraded by the thermolysin treatment, unless the membranes were rendered permeable by a detergent (Figure 6C). These data strongly suggest that the DIM/DWF1

Table 3. Measurement of Endogenous Sterols and Metabolites of Exogenously Added, Deuterium-Labeled 24-Methyl-desmosterol

Sterol	Precursor	WT ^a	<i>dim</i> ^a	DIMOE ^a
24-Methyl-desmosterol	d6 ^b	ND ^c	0.077	ND
	Endogenous	ND	ND	ND
Campesterol	d6	0.114	ND	0.110
	Endogenous	10.3	0.118	30.8
Campestanol	d6	0.182	ND	2.050
	Endogenous	0.582	0.180	2.090

^a Micrograms of sterols per gram fresh weight of wild-type (WT) *Arabidopsis* ecotype Columbia, *dim*, and DIMOE (transgenic line carrying a 35S-*DIM/DWF1* transgene [see Methods]) tissue.

^b d6 denotes a labeled precursor extracted from plants that were fed deuterium-labeled 24-methyl-desmosterol and incubated for 4 days in liquid culture.

^c ND, not detectable.

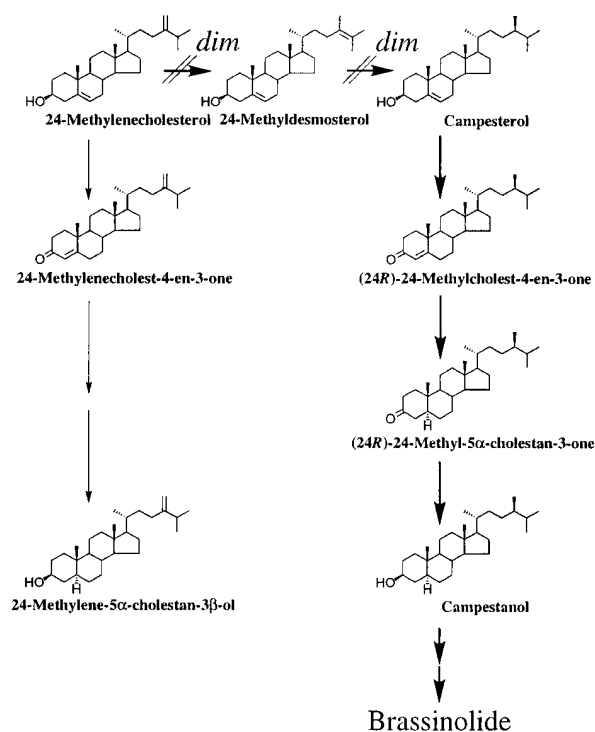


Figure 5. Parallel Pathway Originating from 24-Methylenecholesterol.

Shown is a schematic diagram of a parallel pathway originating from 24-methylenecholesterol. Both 24-methylenecholest-4-en-3-one and 24-methylene-5 α -cholestan-3 β -ol accumulate in the *dim* mutant. Presumably, 24-methylene-5 α -cholestan-3 β -ol will be converted into campestanol by DIM/DWF1.

protein is anchored on the cytoplasmic side of a membranous compartment.

It is difficult to localize a protein to a specific subcellular compartment by using cell fractionation studies alone. Therefore, we constructed plasmids encoding a DIM/DWF1-green fluorescent protein (GFP) fusion protein for subcellular localization studies (see Methods). Because BRs were first isolated from pollen (Grove et al., 1979), we used biolistic bombardment of pollen to investigate the intracellular localization of the DIM/DWF1-GFP fusion protein. The *DIM/DWF1-GFP* transgene was transcribed from a *LAT52* promoter, which is highly active in pollen (Twell et al., 1990). Figure 8 shows that the DIM/DWF1-GFP protein is associated with speckled structures within the cytoplasm (Figure 8A). When compared with the localization of GFP alone (Figure 8C), a markedly different expression pattern was observed. Importantly, unlike the GFP protein alone, the DIM/DWF1 protein does not localize to the nucleus (cf. Figures 8A and 8C, respectively). Reference images (Figures 8B and 8D) show that pollen tubes grew normally upon transfection

with both constructs. Similar results were obtained when tobacco BY2 cells were transfected with constructs containing either a cauliflower mosaic virus 35S-GFP or a 35S-GFP-DIM/DWF1 transgene (data not shown; see Methods). These data show that (1) the DIM/DWF1 protein is not localized to the nucleus and thus is likely to be a catalytic enzyme rather than a regulatory protein, and (2) DIM/DWF1 is localized in speckled structures within the cytoplasm.

DISCUSSION

Our results demonstrate that the *dim* mutant is defective in the synthesis of campesterol and compounds located downstream of it in the steroid biosynthesis pathway, including BRs. Feeding experiments clearly show that *dim* is unable to convert 24-methylenecholesterol to campesterol. The mutant is capable of responding to BRs, and the seedling phenotype can be restored to that of the wild type by the addition of brassinolide. Cell fractionation shows that the protein is found on the cytoplasmic side of microsomal membranes. In germinating pollen tubes, a DIM/DWF1-GFP fusion protein was localized to a compartment that appears in a speckled pattern in the cytosol, most likely the ER.

Restoration of the Mutant Phenotype by the Application of BRs

Because the *dim* mutant phenotype can be rescued by the application of brassinolide, the slow growth of mutant plants is likely due to a lack of this hormone. The *dim* mutant is a T-DNA-tagged null mutant and does not express any detectable amounts of *DIM* mRNA (Takahashi et al., 1995); yet, low amounts of campesterol and campestanol accumulate. This argues for the existence of alternate pathways for the synthesis of campesterol and ultimately brassinolide. The reaction that is catalyzed by the DIM/DWF1 protein represents an early step in brassinolide biosynthesis but is also important for the conversion of abundant sterols. As in animal cells, a network of parallel pathways to synthesize sterols and steroids most likely exists in plant cells, and campesterol could be synthesized from cycloartenol in a pathway that does not include 24-methylenecholesterol (Yokota, 1997). However, the regulation of substrate availability by rate-limiting steps early in the pathway (Choe et al., 1998) provides control mechanisms that are clearly important, as illustrated by the phenotype of BR-deficient mutants and the observation of a drastic drop in the abundance of earlier precursors (Fujioka et al., 1995). This means that despite the existence of parallel pathways, later steps in the pathway cannot compensate (e.g., by a feedback mechanism) for the loss of function brought about by the *dim* mutation.

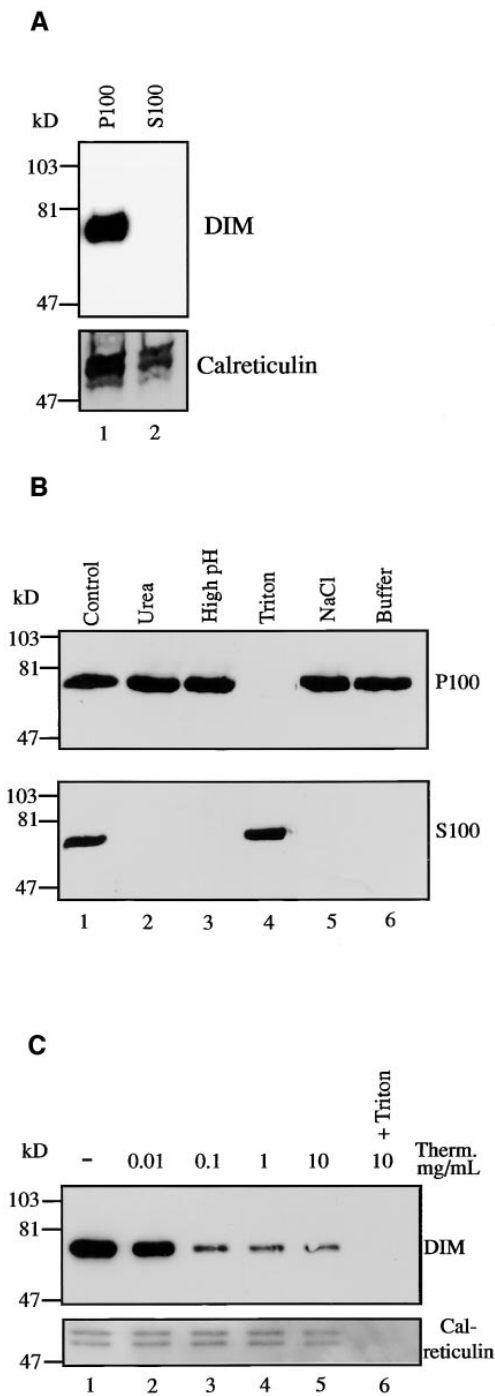


Figure 6. Immunoblot Analysis of DIM/DWF1 and Calreticulin in Cell Fractions.

(A) An Arabidopsis extract was fractionated into a high-speed pellet fraction containing membrane-derived microsomes (P100, lane 1) and a high-speed supernatant fraction containing cytosolic proteins (S100, lane 2). Approximately 50 μ g of protein from each fraction was separated by SDS-PAGE. The DIM/DWF1 protein was detected by a monoclonal antibody (Novagen) generated against its C-terminus.

It would be interesting to know whether brassinolide is the only active BR in Arabidopsis. In certain species, brassinolide could not be detected but castasterone was present, suggesting that the latter has biological activity itself (see Yokota, 1997). It is conceivable that other active steroid compounds exist in plants and that they are also synthesized from campesterol (or isofucosterol). Our observation that *dim* plants cannot be completely rescued at the adult stage, especially with respect to fertility, by the addition of brassinolide alone might indicate that other active compounds are also needed. Alternatively, the uptake of the compound could be reduced at this stage, or the lack of an appropriate amount of sterols and their metabolites might be more important for plant fertility.

Gene Expression in Response to BR Addition Is Normal in the *dim* Mutant

The only group of genes identified so far that are significantly regulated directly by BRs are the XETs (Zurek and Clouse, 1994; Xu et al., 1995, 1996; Kauschmann et al., 1996). It is possible that BRs do not act mainly via transcriptional regulators but rather control translation and protein activities, such as those of kinases. The cloning of a putative receptor kinase gene, whose gene product might be a possible sensor for brassinolide, BRI1 (Clouse et al., 1996; Li and Chory, 1997), is consistent with such a mechanism. In animal cells, all known steroid signals are mediated by nu-

nal T7 tag (see Methods). The blot was reprobbed with an antibody generated against the luminal endoplasmic reticulum protein calreticulin (Dennecke et al., 1995). Numbers at left indicate the positions of marker proteins with the indicated molecular masses.

(B) Immunoblot of microsomal fractions treated with chaotropic agents. Fractions were untreated (lane 1) or treated with 1 M urea (lane 2), 100 mM sodium carbonate, pH 11 (lane 3), 1% (v/v) Triton X-100 (lane 4), 1 M sodium chloride (lane 5), or lysis buffer (lane 6; see Methods). Note that the DIM/DWF1 protein is released into the soluble fraction (S100) only by Triton X-100, which solubilizes the membranes (lane 4). Numbers at left indicate the positions of marker proteins with the indicated molecular masses.

(C) Immunoblot of the microsomal fraction treated with thermolysin (Therm.), a protease that cannot penetrate membranes (Joyard et al., 1983). Microsomes were treated with thermolysin buffer alone (lane 1) or with the indicated concentrations of thermolysin (lanes 2 to 6). The fraction in lane 6 was also treated with Triton X-100 to dissolve the microsomal membranes (+Triton). The blot was probed with the monoclonal antibody against the T7 tag (DIM) and an antibody against the luminal endoplasmic reticulum protein calreticulin. The C terminus of the DIM/DWF1 protein is accessible to the protease, indicating that this region faces the cytoplasmic side of a membranous compartment. On the other hand, calreticulin is not degraded by the treatment, demonstrating that the protease cannot enter the microsomal lumen. Numbers at left indicate the positions of marker proteins with the indicated molecular masses.



Figure 7. Structure Prediction for DIM/DWF1.

(A) Hydropathy prediction of the DIM/DWF1 protein. Predictions were done using the TMpred program (Hofmann and Stoffel, 1993). An N-terminal hydrophobic stretch is clearly discernible; this stretch probably anchors the protein on the cytosolic face of a membrane. The program predicts transmembrane α helices in two possible sce-

clear receptors that directly regulate transcription; however, extracellular receptors might have been hitherto overlooked because of the assumption that steroids can readily cross the plasma membrane (Picard, 1998).

Using a differential display approach, we have identified a gene that is clearly upregulated by exogenous brassinolide. Because the response of this gene to brassinolide is normal in the *dim* mutant, it is unlikely that the latter is insensitive to BRs.

The Reaction Impaired in *dim* Mutants Is Probably Unique to Plants

Using labeled precursors, the reaction catalyzed by DIM/DWF1 was not detected in mammalian cells (Nes et al., 1973). This reaction is probably not important in animal cells, with the possible exception of the degradation of dietary sterols. In this regard, the existence of an as yet uncharacterized cDNA encoding a human protein with high amino acid sequence homology to portions of DIM/DWF1 is therefore surprising (GenBank accession number D13643; see Figure 7B). A gene encoding a very similar protein has been identified recently in the genome sequencing project of *Caenorhabditis elegans* (GenBank accession number AF026214). Compared with the plant protein, the two animal proteins are shorter at their C termini. This portion of the protein could be responsible for the specificity of the plant enzyme's function. It is possible that similar proteins are present in other organisms, in which they might have related yet distinct functions.

The reaction impaired in *dim* presumably does not occur in yeast either, because the analogous reduction of the $\Delta^{24(28)}$ bond in ergosta-5,7,22,24(28)-tetraen-3 β -ol to yield ergosterol is known to be catalyzed by an enzyme unrelated to DIM/DWF1. Moreover, this reaction precedes the reduction of the $\Delta^{22(23)}$ bond, which might change the structure of

narios for both orientations of the protein, inside out and outside in, as indicated (i->o and o->i).

(B) Alignment of proteins from human (H.s.; GenBank accession number D13643) and *C. elegans* (C.e.; Genbank accession number AF026214) that share substantial sequence homology with DIM/DWF1 from Arabidopsis (A.t.), especially in the N-terminal half (56% similarity over 361 amino acids and 51% similarity over 431 amino acids, respectively, as determined by the BESTFIT program [Genetics Computer Group, Madison, WI]). Whereas the core sequence is highly conserved, the most N-terminal sequence varies substantially between DIM/DWF1 and the animal proteins. Furthermore, the C terminus of the DIM/DWF1 protein is extended, which might be important for the specificity of the enzyme. The putative transmembrane domain is boxed, and the underlined part indicates sequence homology to FAD-dependent oxidases (Mushegian and Koonin, 1995). Apostrophes below amino acids indicate identity in all three proteins.

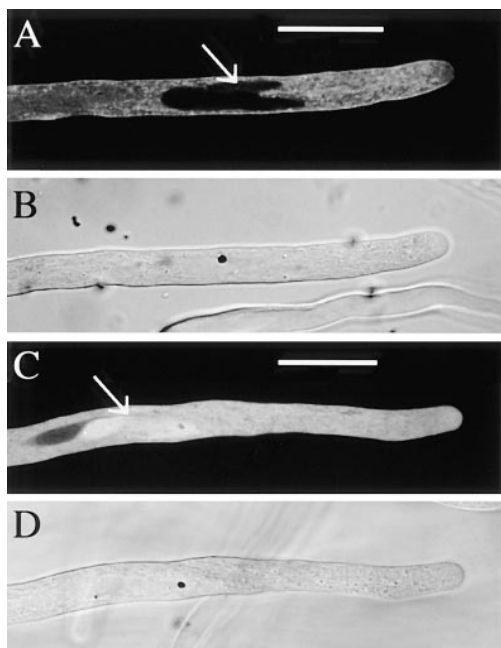


Figure 8. Detection of a GFP-DIM/DWF1 Fusion Protein in Pollen Tubes.

Pollen grains were bombarded with a *LAT52-GFP-DIM/DWF1* construct or a *LAT52-GFP* construct (see Methods). After an overnight incubation, pictures were taken with a confocal microscope.

(A) Pollen tube expressing a GFP-DIM/DWF1 fusion protein.

(B) Light microscopic reference image of (A).

(C) Pollen tube expressing GFP alone.

(D) Light microscopic reference image of (C).

Note that GFP is found in the nucleus and throughout the cytoplasm, whereas GFP-DIM/DWF1 is exclusively localized to a compartment, probably the endoplasmic reticulum, that appears as speckles suspended in the cytoplasm. Arrows indicate the positions of the nuclei. Bars in (A) and (C) = 25 μm for (A) to (D).

the substrate molecule substantially. However, the substrate specificities have not been determined for either the yeast enzyme or DIM/DWF1. It would be interesting, therefore, to analyze the effect of a DIM/DWF1 expression in yeast. It seems noteworthy that only *C. elegans* and certain phytophagous insects, which rely on plant sterols for their own sterol synthesis, have been shown to be able to reduce 24-methylenecholesterol (Lozano et al., 1985; Svoboda et al., 1995; Schiff and Feldlaufer, 1996).

The Biosynthesis of BRs Does Not Follow a Linear Pathway

As seen in other organisms, such as yeast and mammals, the pathway adopted by plants to synthesize steroid hormones is probably not linear for most steps. Recently, sev-

eral studies have confirmed that an early oxidation pathway exists that runs parallel to the pathway established earlier in *C. roseus* (Choi et al., 1997; Fujioka and Sakurai, 1997b; Choe et al., 1998). Here, we present data to suggest that side branches exist even earlier in the pathway. In the *dim* mutant, the conversion of 24-methylenecholesterol to campestanol does not take place, resulting in the accumulation of the former precursor. This accumulation probably leads to the activation of a minor pathway utilized by *dim* to reduce the double bond at C-5 (mediated by DET2; Fujioka et al., 1997) before the isomerization and reduction at C-24 brought about by the DIM/DWF1 protein. In wild-type *Arabidopsis*, the levels of 24-methylenecholesterol are not very high (see Table 1). Therefore, it is probable that the conversion of this compound into 24-methylidestosterone is much faster than the conversion into 24-methylenecholesterol-4-en-3-one and cannot be detected. Whereas this branch of the pathway may not be a major route for BR biosynthesis, it confirms the general notion that enzyme specificities are probably relaxed, leading to a meshwork of alternate pathways.

DIM/DWF1 Is Very Likely a Catalytic Enzyme and Not a Regulatory Protein

We previously reported that the DIM/DWF1 protein sequence contains putative nuclear localization signals and therefore assumed a likely regulatory role for the protein in the nucleus (Takahashi et al., 1995). The DIM/DWF1 protein shows very little sequence homology to other proteins, and a regulatory role in the BR metabolism or biosynthesis seemed possible.

However, for three main reasons, the results presented here make it likely that the DIM/DWF1 protein is directly involved in the biosynthesis of BRs. (1) A severely altered sterol profile in the *dim* mutant indicates that a biosynthetic enzyme is defective (Table 1). (2) The analysis of the DIM/DWF1 amino acid sequence (Figure 7A) predicts a trans-membrane domain at the N terminus of the protein. (3) Cell fractionation studies (Figure 6) show that DIM/DWF1 is an integral membrane protein. Because sterols are hydrophobic molecules, such subcellular localization is in good agreement with the proposed role for the DIM/DWF1 protein as a biosynthetic enzyme.

In animal cells, sterol biosynthesis occurs in peroxisomes and the smooth ER. In specialized cells, such as steroid hormone-secreting cells, there is a proliferation of smooth ER. In analogy to animal cells, we would expect that campesterol is synthesized in the ER. Furthermore, transiently expressed GFP-DIM/DWF1 fusion protein was localized to speckled structures in the cytoplasm both in pollen tubes and BY2 cells. Similar signals were observed upon expression of an ER protein-GFP fusion protein (B. Kost and N.-H. Chua, unpublished results). These data suggest that DIM/DWF1 is associated with the ER rather than the plasma membrane, the tonoplast membrane, or the membrane of

chloroplasts or mitochondria. However, a precise subcellular localization of the protein would require antibodies specific to DIM/DWF1, because wild-type expression levels might be critical for an appropriate localization.

The fact that a protease treatment of microsomes degrades the DIM/DWF1 protein makes it likely that campesterol synthesis occurs on the cytoplasmic face of the ER (or a similar structure within the cytoplasm). If the later steps in BR biosynthesis occur at the same subcellular location, it is likely that a different mechanism for the release of steroids exists in plants. It would be interesting to determine the site of synthesis, as well as action, of plant steroids, which tend to be more hydrophilic than their animal counterparts. Furthermore, no specialized plant cells have been identified that are responsible for the secretion of BRs, and the biosynthetic enzymes seem to be expressed throughout the plant. These data raise the questions of whether BRs act cell autonomously and whether hormone transport is important for cell growth and development.

METHODS

Plant Growth and Measurements

Plants (*Arabidopsis thaliana*) were grown on Murashige and Skoog (MS) medium (JRH Biosciences, Lenexa, KS) under constant white fluorescent light at 22°C for the indicated periods of time. The addition of sucrose (3%) or kanamycin (50 µg/mL) is described in the text. For measurements of root length, plants were grown on vertically oriented plates for various lengths of time, as indicated. For hypocotyl measurements, plants were grown on MS plates and aligned on fresh MS plates for photography and subsequent measurements.

Differential Display

Suspension cultures of *Arabidopsis* (ecotype Columbia) or *diminuto* (*dim*) were initiated by adding root sections to liquid medium, as described in Mathur et al. (1998). RNA was isolated using RNA Matrix (Bio-101 Inc., Richmond, CA) according to the manufacturer's instructions. Differential display was performed as described by Liang and Pardee (1992). *Arabidopsis* (ecotype Columbia) or *dim* suspension-cultured cells were grown on B5 medium (Sigma) supplemented with 3% sucrose, 0.5 mg/L 2,4-D, 2 mg/L indole-3-acetic acid, and 0.5 mg/L benzylaminopurine. Cycloheximide was added to a concentration of 70 µM 1 hr before the addition of brassinolide.

RNA Gel Blot Analysis

RNA was isolated from plant tissues (Kuhlemeier et al., 1988), and RNA gel blotting was performed according to Barnes et al. (1996). Ten micrograms of RNA was used per sample, and loading was monitored by using ethidium bromide and reprobing the blots with 18S rDNA or a cDNA encoding actin (expressed sequence tag 179M16).

DNA Manipulations

A full-length cDNA encoding *DIM/DWARF1* (*DWF1*) was reconstituted from a partial cDNA clone by using polymerase chain reaction (PCR) and standard cloning methods. The cDNA was cloned into VIP26 (van der Krol and Chua, 1991), which contains a cauliflower mosaic virus 35S promoter and a ribulose carboxylase small subunit E9 polyadenylation sequence, to yield plasmid B1. The T7-tagged version of *DIM/DWARF1* (plasmid B2) was constructed by PCR amplification of the C terminus of *DIM/DWARF1*, and the fragment was used to replace the corresponding fragment in plasmid B1. The nucleotide sequences were verified by sequencing.

Plasmids for green fluorescent protein (GFP) fusions were constructed as follows. The coding region of *DIM/DWARF1* was amplified by PCR using oligonucleotides U99 (5'-GCACTCGAGATGTCGG-ATCTTCAG-3') and U100 (5'-GATCTCGAGATCTGCCTCGGGATA-AGG-3'), and the product was digested with XhoI. The *DIM/DWARF1* cDNA fragment was ligated into pGFP2 (P. Spielhofer and N.-H. Chua, unpublished data), which contains the 35S promoter and the GFP coding region (Clontech, Palo Alto, CA) flanked at the 3' end by the nopaline synthase (*Nos*) polyadenylation sequence. The *DIM/DWARF1*-GFP-encoding fragment (XhoI-SpeI) was subcloned into pBK25 (B. Kost and N.-H. Chua, unpublished data), which contains a *LAT52* promoter (Twell et al., 1990) instead of the 35S promoter. All constructs were verified by DNA sequencing.

Plant Transformation

Plants were transformed by cocultivation of agrobacteria with root explants, as described previously (Valvekens et al., 1988). Plasmid B1 (see DNA manipulations) was transformed into *Arabidopsis* (ecotype C24), and plasmid B2 was transformed in *Arabidopsis* (ecotype Landsberg *erecta*).

Sterol Analysis

Shoots of 46-day-old plants grown at 22°C under continuous fluorescent light (3000 lux) were harvested. Sterols derived from 100 mg fresh weight tissue were extracted, spiked with 1 µg of ²H₆-labeled campestanol as an internal standard, and then saponified with 1 N sodium hydroxide in methanol. The unsaponifiable fraction was purified by using a silica gel column, trimethylsilylated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, and subjected to gas chromatography-selected ion monitoring (GC-SIM). The levels of endogenous sterols were determined on the basis of calibration curves constructed from the ratios of the M+ peak area of ²H₆-labeled campestanol. GC-SIM was performed using a JEOL (Tokyo, Japan) JMS AX 505 instrument equipped with a DB-5 capillary column (0.25 µm × 15 m; 0.25-mm film thickness; J & W Scientific, Folsom, CA) in an electron impact mode (70 eV). The carrier gas was He at a flow rate of 1 mL min⁻¹, the injection port temperature was 260°C, and the samples were introduced by splitless injection. The column oven temperature was programmed at 170°C for 1.5 min before being elevated to 280°C at 37°C min⁻¹ and then to 300°C at 1.5°C min⁻¹.

Metabolism of Deuterium-Labeled Precursors

Seedling cultures were started by adding 7-day-old seedlings to liquid B5 medium (Sigma). Cultures were grown under constant light at

25°C with gentle agitation on a platform shaker. After 10 days, deuterium-labeled precursors of 24-methylenecholesterol or 24-methylsterol were added, and the cultures were grown under the same conditions for an additional 4 days. Plants were harvested by draining off all of the liquid. Ten volumes of methanol was added, and tissues were homogenized in a Waring blender. The material was filtered through Miracloth (Calbiochem, La Jolla, CA), and the filtrate was lyophilized to <0.1 of the volume. The aqueous phase was extracted three times with an equal amount of chloroform, and the fractions were pooled. The chloroform-soluble fraction was purified with a cartridge of silica gel (Sep-Pak Vac 2g; Waters, Milford, MA), which was eluted with 20 mL of chloroform. The chloroform fraction was dissolved in methanol and passed through a cartridge of ODS (Sep-Pak Plus C18; Waters), which was eluted with 20 mL of methanol. The eluate was subjected to HPLC on an ODS column as follows: the column was a Senshu Pak ODS-4150-N (150 × 10 mm; Senshu Scientific Co., Ltd., Tokyo, Japan); the solvent was methanol; the flow rate was 2 mL min⁻¹; detection was at a wavelength of 205 nm. The fractions were collected every 30 sec (retention time of 12 to 18 min). Each fraction was subjected to gas chromatography and mass spectrometry analysis after derivatization. Gas chromatography and mass spectrometry analysis were performed according to the method described in Fujioka et al. (1997).

Deuterium-labeled 24-methylenecholesterol and its related steroids were synthesized as described by Takatsuto et al. (1998).

Cell Fractionation and Protease Treatment

Plant extracts were prepared as described by Ahmed et al. (1997), with minor modifications. Five grams of tissue from transgenic seedlings carrying a 35S-*DIM/DWF1* transgene (see DNA manipulations) grown in liquid B5 medium was ground in liquid nitrogen. The homogenate was resuspended in a lysis buffer (10 mM Hepes-KOH, pH 7.1, 12.5% [w/v] sucrose, 10 mM potassium acetate, and 3 mM MgCl₂), supplemented with the protease inhibitor mix Complete (Boehringer Mannheim), and filtered through Miracloth (Calbiochem). Subsequent manipulations were performed at 4°C. Fractions were collected by centrifugation for 10 min at 200g (1500 rpm in a JS13 rotor [Beckman Instruments, Palo Alto, CA], yielding fraction P0.2), for 20 min at 10,000g (8000 rpm in a JS13 rotor, yielding fraction P10), and for 100 min at 100,000g (33,000 rpm in a SW40 swing-out rotor [Beckman Instruments], yielding fractions P100 [pellet] and S100 [supernatant]). Total amounts per fraction were ~10 mg (P0.2), 7.5 mg (P10), 5 mg (P100), and 400 mg (S100). For treatment with various chaotropic agents, 100 μg of protein from the P100 fraction was taken up in the indicated solutions (1 M urea, 100 mM sodium carbonate, pH 11, 1% Triton X-100, and 1 M NaCl, all in lysis buffer, except for the carbonate buffer). The samples were incubated for 30 min on ice and then centrifuged for 40 min in a SW55Ti rotor (Beckman Instruments) at 33,000 rpm. Supernatants and pellets were separated on a 10% SDS-polyacrylamide gel, blotted onto a nitrocellulose membrane (BA85; Schleicher & Schuell), and probed with a T7 monoclonal antibody (Novagen, Madison, WI).

For the protease treatment, 100 μg of protein from the P100 fraction was sedimented again for 30 min at 100,000g (see above), and the pellet was taken up in 400 μL of thermolysin buffer (10 mM Tricine-NaOH, pH 7.8, 0.3 M sucrose, and 1 mM CaCl₂) containing the indicated concentration of thermolysin. After a 30-min incubation on ice, 10 μL of 0.5 M EDTA was added to inactivate the protease, and the samples were centrifuged at 100,000g, as described above. Pro-

teins were separated on 10% SDS-polyacrylamide gels for gel blot analysis.

SDS-PAGE and Immunoblotting

Protein concentrations were measured by using a Bradford assay kit (Bio-Rad) and by Coomassie Brilliant Blue R 250 staining of protein gels. Gel electrophoresis was performed as described by Sambrook et al. (1989). Gels were run on a Bio-Rad minigel apparatus at 100 V. Proteins were transferred onto nitrocellulose (BA85) by using a semi-dry blotter (No. EBU-4000; C.B.S. Scientific Co., Del Mar, CA) at 60 V at 4°C for 90 min. Blocking was performed in PBS containing 3% milk powder (ALBA, high calcium; Heintz, Pittsburgh, PA). Blots were incubated with primary antibody in PBS containing 3% milk powder for 4 to 16 hr at 4°C. Anti-T7 tag antibodies (Novagen) were diluted 1:10,000, and anti-calreticulin antibodies (Denecke et al., 1995) were diluted 1:5000. Blots were washed four times with PBS, incubated with secondary antibodies (Amersham; diluted 1:3000 in 3% milk in PBS) at room temperature for 1 hr, washed four times with PBS, and assayed using enhanced chemiluminescence (ECL) reagents as described (Amersham ECL kit; model RPN 2108). X-ray films (X-OMAT-AR) were from Kodak.

Biolistic Bombardment of Pollen Tubes and Confocal Microscopy

Tobacco pollen grains were harvested from mature flowers and placed on germination medium (B. Kost and N.-H. Chua, unpublished data). The sample was bombarded without preincubation by using a PDS-1000/He biolistic particle delivery system (Bio-Rad). DNA loading on gold particles and the delivery (1100 psi) were done according to the manufacturer's instructions. After bombardment, the samples were incubated overnight on the same medium and then analyzed on a confocal microscope (model LSM410; Zeiss, Thornwood, NY), with excitation at 488 nm and emission at 515 to 565 nm.

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