

Arabidopsis Brassinosteroid-Insensitive *dwarf12* Mutants Are Semidominant and Defective in a Glycogen Synthase Kinase 3 β -Like Kinase¹

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Mutants defective in the biosynthesis or signaling of brassinosteroids (BRs), plant steroid hormones, display dwarfism. Loss-of-function mutants for the gene encoding the plasma membrane-located BR receptor BRI1 are resistant to exogenous application of BRs, and characterization of this protein has contributed significantly to the understanding of BR signaling. We have isolated two new BR-insensitive mutants (*dwarf12-1D* and *dwarf12-2D*) after screening Arabidopsis ethyl methane-sulfonate mutant populations. *dwarf12* mutants displayed the characteristic morphology of previously reported BR dwarfs including short stature, short round leaves, infertility, and abnormal de-etiolation. In addition, *dwarf12* mutants exhibited several unique phenotypes, including severe downward curling of the leaves. Genetic analysis indicates that the two mutations are semidominant in that heterozygous plants show a semidwarf phenotype whose height is intermediate between wild-type and homozygous mutant plants. Unlike BR biosynthetic mutants, *dwarf12* plants were not rescued by high doses of exogenously applied BRs. Like *bri1* mutants, *dwarf12* plants accumulated castasterone and brassinolide, 43- and 15-fold higher, respectively, providing further evidence that DWF12 is a component of the BR signaling pathway that includes BRI1. Map-based cloning of the *DWF12* gene revealed that *DWF12* belongs to a member of the glycogen synthase kinase 3 β family. Unlike human glycogen synthase kinase 3 β , DWF12 lacks the conserved serine-9 residue in the auto-inhibitory N terminus. In addition, *dwarf12-1D* and *dwarf12-2D* encode changes in consecutive glutamate residues in a highly conserved TREE domain. Together with previous reports that both *bin2* and *ucu1* mutants contain mutations in this TREE domain, this provides evidence that the TREE domain is of critical importance for proper function of DWF12/BIN2/UCU1 in BR signal transduction pathways.

Brassinosteroids (BRs) are poly-hydroxylated plant steroids structurally similar to animal steroid hormones such as ecdysone. Essential roles for BRs in plant growth and development have been demonstrated by the dwarf phenotype displayed in mutants defective in BR biosynthetic or signaling pathways in Arabidopsis, rice (*Oryza sativa*), tomato (*Lycopersicon esculentum*), and pea (*Pisum sativum*). Phenotypes of the light-grown BR dwarf mutants include short stature, dark-green and round leaves, reduced fertility, and a prolonged life cycle, as well as altered skotomorphogenesis in dark-grown plants. Arabidopsis

dwarf mutants defective in six genes encoding BR biosynthetic enzymes are rescued by exogenous application of BRs (Li et al., 1996; Szekeres et al., 1996; Choe et al., 1998, 1999a, 1999b, 2000), whereas mutants in signaling components are morphologically similar but insensitive to applied BRs. BR-insensitive mutants in a gene known as *bri1* (*brassinosteroid insensitive1*) were previously isolated based on the phenotype of derepressed root-growth inhibition on media containing BRs (Clouse et al., 1996). Thus far, all BR-insensitive recessive mutants where the affected gene is known are BRI1 alleles (Li and Chory, 1997; Noguchi et al., 1999; Friedrichsen et al., 2000). The *BRI1* gene was isolated and shown to encode a Leu-rich repeat receptor protein kinase (Li and Chory, 1997). Evidence from kinase domain swapping experiments with a similar Leu-rich repeat receptor protein kinase encoded by the rice *Xa21* gene (He et al., 2000), the cellular localization of BRI1::GFP translational fusion protein to the plasma membrane (Friedrichsen et al., 2000), and in vitro BR-binding assays using an epitope-tagged BRI1 protein (Wang

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et al., 2001) have shown that BRI1 is a component of a BR receptor located in the plasma membrane.

The isolation and characterization of mutants insensitive or resistant to plant hormones has greatly enhanced our understanding of their signaling pathways (Gray and Estelle, 2000). However, the scarcity of BR-insensitive mutants has delayed unraveling BR-mediated signaling pathways. It has been hypothesized that loss-of-function mutations in additional BR signaling genes may result in lethality because of their essential roles or they may have unnoticeable physiological or morphological phenotypes because of gene redundancy. We have extensively screened both ethyl methanesulfonate (EMS)-induced and T-DNA mutant populations to obtain additional mutants, and recovered two semidominant mutants in a gene we call *dwf12* (*dwarf12*). The semidominant *dwf12* mutants are insensitive to exogenously applied BRs and display an extreme dwarf phenotype both in the light and dark. We also show that *dwf12-1D* plants, like *bri1*, accumulate significant amounts of BRs. The phenotypic similarity among *dwf12* and previously reported BR mutants, the brassinolide (BL) insensitivity of *dwf12-1D* and *dwf12-2D*, and the mapping of *dwf12* to a chromosomal location distinct from *BRI1* indicate that *dwf12* mutants define a new gene in the BR signal transduction pathway. Recently, two different groups have independently reported new BR-insensitive mutants. Li et al. (2001) identified two alleles of *bin2* (*brassinosteroid insensitive 2*), and Perez-Perez et al. (2002) found three alleles of *ucu1* (*ultracurvata 1*). The *bin2* and *ucu1* mutants were shown to have mutations in an Arabidopsis glycogen synthase kinase (GSK) 3 β -like kinase gene (Li and Nam, 2002; Perez-Perez et al., 2002), the same gene that is mutant in our *dwf12* alleles.

GSKs are a family of cytoplasmic kinases that belong to the mitogen-activated protein kinase superfamily and are found in animals, fungi, and plants. Diverse roles for GSK3s include dorsal/ventral polarity determination in Wnt/Wg signaling in *Drosophila melanogaster* and *Xenopus laevis*, endoderm/mesoderm formation in *Caenorhabditis elegans*, and prespore/prestalk fate determination in *Dictyostelium discoideum* (Kim and Kimmel, 2000). In mammals, they are involved in insulin-dependent Glc homeostasis, β -catenin-mediated cell signaling, and development of tau-associated Alzheimer's disease (Bienz and Clevers, 2000; Harwood, 2000). In the animal model systems studied thus far, differentially spliced transcripts arise from only one or two GSK3 genes, whereas in plants, GSK3 genes consist of multigene families; currently, the GenBank database contains three genes for alfalfa (*Medicago sativa*; Pay et al., 1993), five for tobacco (*Nicotiana tabacum*), three for rice, four for petunia (*Petunia hybrida*; Decroocq-Ferrant et al., 1995), and 10 for Arabidopsis (Bianchi et al., 1994). Dornelas et al. (1999) reported that the

Arabidopsis genes show different temporal and spatial expression patterns. More recently, Dornelas et al. (2000) also showed that antisense down-regulation of the two GSK3-like genes, AtSK11 and AtSK12, results in altered floral development, including increased number of perianth organs and abnormal apical-basal patterning in the gynoecium. Here, we present additional evidence demonstrating that one of the Arabidopsis GSK3-like kinases, previously named ASK η (*etha*), plays a crucial role in BR signaling.

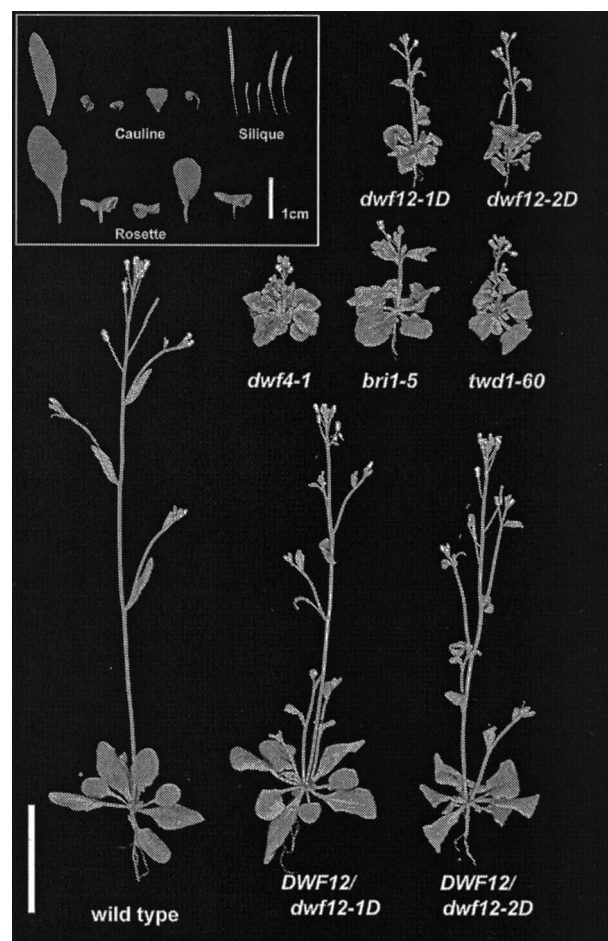


Figure 1. Phenotypic comparison of the BR biosynthetic mutant *dwf4-1*, and the insensitive mutants *bri1-5*, *twisted1-60*, and *dwf12*. Plants shown are 25 d old. *dwf12* exhibits characteristic BR dwarf phenotypes including short inflorescences, infertility of homozygous mutants, short round leaves, and abnormal de-etiolation. In addition, *dwf12* leaves show more severe downward curling than *dwf4* and *bri1*. This phenotype and the mild twisting of inflorescences appear to be weaker version of *twd1* (B. Schulz and K. Feldmann, unpublished data). The height of *dwf12* heterozygous plants is intermediate between the homozygote and wild type, suggesting that the mutation is semidominant. Unit bar = 2 cm. In the inset, one set each of the adaxial side of a cauline leaf, a rosette leaf, and a silique of Ws-2 wild type, *dwf12-1D*, *dwf12-2D*, *dwf12-1D/+*, and *dwf12-2D/+* are shown. Note the extreme downward curling of the leaves. Siliques of the homozygous *dwf12* mutants are almost completely sterile and contain few seeds.

RESULTS

dwf12-1D and *dwf12-2D* Are New BR-Insensitive Dwarf Mutants

To identify additional players in the BR signaling pathway, we have screened EMS-induced mutant populations (>50,000) for characteristic BR dwarfs (Fig. 1), and identified 43 new dwarf mutants. Most of these 43 new dwarf mutants were rescued to a wild-type phenotype when BRs were topically applied. However, two mutants, *wm1-1* and *wm5-1*, were insensitive to BRs, and phenotypically and genetically distinct from the other mutants. The phenotypes of the two mutants were typical of BR dwarfs, but with slightly twisted inflorescences, and leaves that are severely curled and rolled downward (Fig. 1). Interestingly, the progeny of the two dwarf mutants segregated for discrete groups of wild-type, severe, and weak dwarf plants (Fig. 1), suggesting that the parental dwarf mutants were heterozygous for a semidominant mutation that causes dwarfism. To determine whether *dwf12* is allelic to *bri1*, we chose to map the mutations rather than doing genetic complementation tests because the semidominance of *dwf12* may preclude reliable results in a complementation test. We mapped both *dwf12* mutations to the middle of chromosome 4 to a position distinct from *BRI1*, which is linked to the DHS1 marker located on the bottom of chromosome 4 (see "Materials and Methods"). Thus, we designated the two novel BR-insensitive mutants, *wm1-1* and *wm5-1*, *dwarf12-1* dominant (*dwf12-1D*) and *dwf12-2D*, respectively. The demonstration of allelism comes from sequencing, which is discussed below.

The phenotypes of the two alleles were further characterized by morphometric analysis. The total height of aerial parts and the length of pedicels and siliques were all measured and found to be greatly decreased (Table I). Interestingly, the length of these plant organs was noticeably shorter in *dwf12-2D* mutants, suggesting that *dwf12-2D* is a more severe allele compared with *dwf12-1D*. Apical dominance, judging by the number of inflorescences, is increased at 4 weeks of age, but during its prolonged development, *dwf12* mutants produced additional inflorescences as a result of decreased apical dominance.

To rule out the possibility that the lack of rescue of *dwf12* mutants in our initial BR application experiment was because of a lower BL dose than required, we tested three different concentrations of *epi*-BL. The wild-type seedlings responded dramatically to both 10^{-8} and 10^{-7} M *epi*-BL in that the hypocotyls and petioles elongate, whereas root growth was stunted (Fig. 2A). Similarly, seedlings of the BR-deficient mutant *dwf4-1* were rescued to wild-type phenotype with 10^{-8} and 10^{-7} M *epi*-BL. In contrast, both *dwf12-1D* and *bri1-5* (a weak *bri1* allele) failed to show noticeable responses to *epi*-BL, even at the highest concentration of 10^{-7} M. These results indicate that the *dwf12* mutants are defective in a step of the BR signal transduction pathway.

To quantitatively understand the responsiveness of *dwf12* mutants to *epi*-BL and other phytohormones, we measured the root growth after treating the seedlings with *epi*-BL, ABA, and auxin (2,4-dichlorophenoxyacetic acid [2,4-D]). As shown in Figure 2B, the root growth inhibition in wild type was decreased linearly and proportionally to the *epi*-BL concentration. In contrast, both *dwf12-1D* and *bri1-5* mutants are significantly less sensitive to different *epi*-BL concentrations. In response to all the concentrations tested, root growth inhibition in *bri1-5* was not noticeable (Fig. 2). However, *dwf12-1D* was sensitive to this treatment in that root growth was inhibited about 10% in response to 10^{-8} and 10^{-7} M *epi*-BL, and the root length was approximately one-half of the control size at 1 μ M. By definition, the control size is the length of roots grown on media that contain the same amount of 95% (v/v) ethyl alcohol used to dilute BL from a 4 mM stock solution. It has been shown previously that root growth of BR mutants is hypersensitive to inhibition by exogenous application of ABA (Ephritikhine et al., 1999). Similarly, the root growth of wild type, *bri1-5*, and *dwf12-1D* was unchanged, 23% decreased, and 57% decreased, respectively, compared with their controls in response to 0.5 μ M ABA. The inhibition by ABA was more pronounced in *bri1-5* than in *dwf12-1D*. When the two insensitive mutants were treated with a synthetic auxin 2,4-D (0.5 μ M), their root growth was greatly inhibited like wild-type seedlings (Fig. 2B); however, it is noteworthy that *dwf12-1D* and *bri1-5* are slightly

Table I. Morphometric analysis of the key characteristics of the *dwf12* mutants relative to the *Ws-2* wild type

All the characteristics were measured at 4 weeks of age. An average (>15) is shown with the SD. na, Not analyzed.

Parameter	Wild Type	<i>dwf12-1D</i>	<i>dwf12-2D</i>	<i>dwf12-2D/DWF12</i>
			mm	
Height	399.9 $\pm$ 20.6	88.6 $\pm$ 10.4	57.5 $\pm$ 11.3	252.5 $\pm$ 27.3
Pedicel	12.2 $\pm$ 1.4	3.9 $\pm$ 0.6	3.7 $\pm$ 0.8	9.1 $\pm$ 1.6
Silique	12.6 $\pm$ 2.6	2.9 $\pm$ 0.3	2.5 $\pm$ 0.5	6.9 $\pm$ 2.8
Petiole	20.0 $\pm$ 1.5	na	na	6.0 $\pm$ 1.1
Leaf blade length	10.0 $\pm$ 0.9	na	na	10.6 $\pm$ 2.1
Leaf blade width	11.6 $\pm$ 1.0	na	na	11.0 $\pm$ 1.1
No. of inflorescences	3.8 $\pm$ 0.8	1.6 $\pm$ 0.8	1.8 $\pm$ 0.7	3.3 $\pm$ 0.9
Branches on a 1° inflorescence	2.3 $\pm$ 0.6	2.6 $\pm$ 0.5	1.9 $\pm$ 0.6	3.0 $\pm$ 0.6

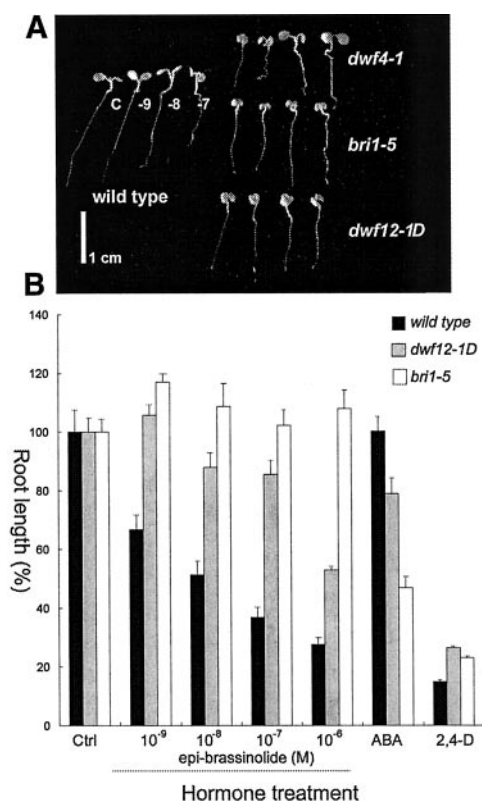


Figure 2. A, Dose response of wild type, *dwf4-1*, *bri1-5*, and *dwf12-1D* to *epi*-BL. Different doses of *epi*-BL are C, control; -9, 10⁻⁹ M; -8, 10⁻⁸ M; and -7, 10⁻⁷ M. A wild-type seedling at 10⁻⁷ M shows typical BR responses, such as elongated hypocotyls and petioles as well as shortened root length. In addition, the BR biosynthetic mutant *dwf4-1* is fully rescued to a wild-type phenotype at a concentration as low as 10⁻⁸ M, showing expanded cotyledons and an elongated hypocotyl. However, *dwf12-1D* seedlings did not respond to *epi*-BL regardless of the concentrations, confirming that *dwf12-1D* is defective in sensing or downstream signaling. Similar insensitivity is shown also in the *bri1-5* mutant. Unit bar = 1 cm. B, Quantitative analysis of the hormone dose response tests. Root growth inhibition of wild type is decreased linearly and proportionally to the *epi*-BL concentration. In contrast, both *dwf12-1D* and *bri1-5* are significantly insensitive to *epi*-BL at all concentrations tested. The two signaling mutants also show altered responses to abscisic acid (ABA) and auxin in that they are more sensitive to ABA but slightly resistant to auxin. Percent root length represents the ratio of the root length grown on BL-supplemented media over the root length grown on the control media containing the same amount of 95% (v/v) ethyl alcohol used to dilute BL from a 4 mM stock solution.

resistant to the auxin treatment as compared with wild type.

dwf12 Is Defective in Feedback Regulation of BR Biosynthesis

Previously, we have shown that BRs accumulate relative to wild type in direct correlation with the severity of the *bri1* allele (Noguchi et al., 1999). The inability to perceive BRs in *bri1* mutants also results in increased steady-state levels of BR biosynthetic gene transcripts (Noguchi et al., 1999). These and

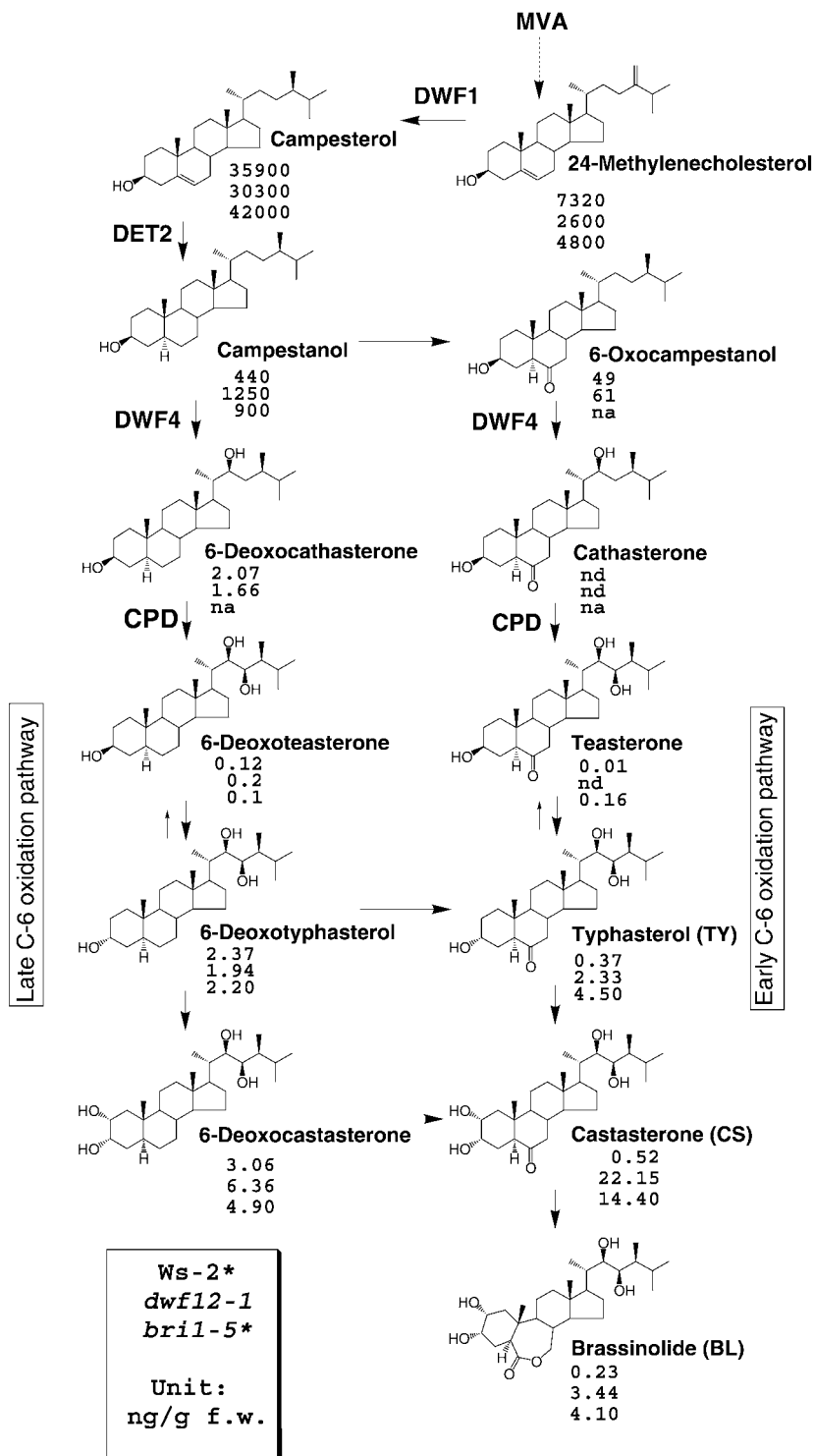
other data (Mathur et al., 1998) led us to propose a model in which a transcriptional feedback loop downstream of BRI1 regulates BR levels. If DWF12 is a component of the BR signaling pathway, *dwf12* mutants might also be defective in feedback regulation and accumulate BRs. We collected tissue from *dwf12-1D* and analyzed the BR levels and found that *dwf12-1D* plants accumulate significant quantities of BRs (Fig. 3). The degree of accumulation is more pronounced in downstream compounds in the biosynthetic pathway. The levels of the end product BL and the penultimate compound CS were 15- and 43-fold higher as compared with wild-type levels, respectively. The *bri1-5* mutant, which shows similar inflorescence height to *dwf12*, displayed an 18- and 27-fold increase of BL and CS levels, respectively (Noguchi et al., 1999; Fig. 3).

DWF12 Encodes a Member of the GSK3 Gene Family

To understand the role of DWF12 in BR signaling, we isolated the *DWF12* gene using conventional map-based cloning techniques. First, we found that *dwf12* mutations are located between the AG and CH42 markers in the middle of chromosome 4 (Fig. 4, A–D). The Arabidopsis Genome Initiative has sequenced and annotated this region; thus, we took advantage of this information in selecting candidate genes for *dwf12*. The gene in which we found the G to A transition mutations, typical of EMS-induced changes, was a GSK/shaggy-like kinase (F28A21.120). Both *dwf12-1D* and *dwf12-2D* carried base changes in this gene (Fig. 4D). Interestingly, the two mutations were only 3 bp apart: *dwf12-2D* possessed a G to A transition at nucleotide position 986, whereas *dwf12-1D* had a G to A change at nucleotide position 989 (Fig. 4D). These two mutations resulted in substitution of an acidic Glu (GAA) to a basic Lys (AAA) residue in two adjacent amino acids (263 [*dwf12-2D*] and 264 [*dwf12-1D*], respectively; Fig. 4D).

To learn more about the role of DWF12 as well as the importance of the changes in these two amino acids, we conducted protein sequence analysis. First, we searched the GenBank and SwissProt databases, and found a group of kinases belonging to the GSK3 family as the most similar sequences to DWF12. Multiple sequence alignments revealed that the DWF12 protein sequence could be subdivided into three discrete domains: (a) N-terminal variable domain (1–39), (b) conserved kinase domain (40–324), and (c) C-terminal domain (325–380). When these domains were individually compared with the corresponding domains of human GSK3 β , the sequence identity was 44%, 71%, and 49%, respectively. The lower sequence identity at the N-terminal domain was attributable partly to the short length of this domain in DWF12, only 39 amino acids, but 53 in the human GSK3 β (Fig. 5). Arabidopsis has 10 copies of the GSK3-like genes, named AtSKs (Arabidopsis shaggy-like kinases) af-

Figure 3. Endogenous levels of BRs in wild-type (*Ws-2*), *dwf12-1D*, and *bri1-5* plants. Similar to *Arabidopsis bri1* plants, *dwf12-1D* accumulates significant amounts of BRs, especially typhasterol (TY), castasterone (CS), and BL. BR biosynthetic pathways with major intermediates and enzyme names are shown with their chemical structures. nd, Not detected; na, not analyzed; unit = ng g fresh weight⁻¹.



ter their similarity to *D. melanogaster* shaggy-like kinase (Dornelas et al., 1998). The *Arabidopsis* AtSK sequences deviate substantially in the length of their N-terminal domains. The total number of amino acids ranged from 380 (DWF12) to as long as 472 (KGSQ_ARATH). The length of the DWF12 N-terminal domain is the shortest when compared

with GSK3 from other organisms including human, *D. melanogaster*, and *D. discoideum* (Fig. 5).

DISCUSSION

The phenotypes of the *dwf12* mutants can be summarized by the statement that all organs are reduced

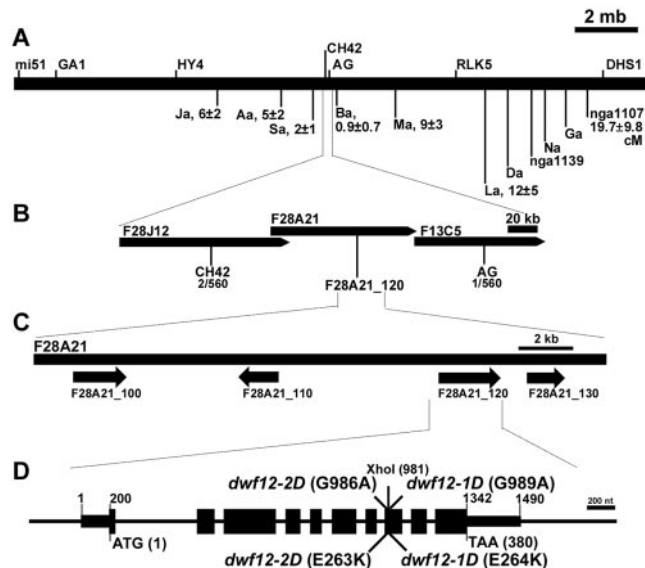


Figure 4. Map-based cloning of *DWF12*. A, Chromosome 4 with major genetic markers. In addition to previously available markers, we have developed novel simple sequence length polymorphism (SSLP) markers, including Ga, Na, Da, La, Ma, Ba, Sa, Aa, and Ja (Table III). B, Localization of the mutation between CH42 and AG markers. The number of recombinants found with CH42 and AG and *dwf12-1D* is two and one of 560 examined chromatids, respectively. C, Geography around the *DWF12* gene annotated as F28A21.120 by the Arabidopsis Genome Initiative. D, The schematic of *DWF12* with mutations. The gene consists of 10 exons and nine introns. The two *dwf12* mutations are in exon 8, changing consecutive Glu residues to Lys. The *dwf12-2D* mutation abolishes an *Xho*I site (CTCGAG to CTCGAA). Schematics are drawn to the scales that are depicted on the top right of each diagram.

in size. The specific characteristics examined in this research, such as plant height and the length of pedicels, siliques, petioles, hypocotyls, and roots, all are significantly shorter than wild type (Table I; Figs. 1 and 2). These morphological alterations are typical of BR biosynthetic or signaling mutants (Figs. 1 and 2). Thus, it is likely that the *dwf12* mutants are defective in BR biosynthesis or signaling. One interesting exception in *dwf12* is a typical leaf curling in the abaxial direction. Downward curling leaves are often found in mutants that are defective in auxin signaling, such as *axr1* and *axr2* (Lincoln et al., 1990; Timpte et al., 1992). These shared phenotypes between *dwf12* and auxin mutants suggest that DWF12 also plays a role in auxin signaling in specific cell types.

Two lines of evidence strongly suggest that the *dwf12* mutants are BR insensitive. First, a BR biosynthetic mutant *dwf4-1* was rescued to wild-type phenotype at 10^{-8} and 10^{-7} M *epi*-BL (Fig. 2A), but these concentrations did not induce noticeable responses such as elongating hypocotyls and petioles from *dwf12-1D* and *bri1-5*. In addition, root growth inhibition by concentrated *epi*-BL in the *dwf12-1D* mutants was significantly less sensitive compared with wild type. Second, previously we have shown that *bri1* mutants accumulate significant amounts of BRs

(Noguchi et al., 1999). Similarly, here we reported that BRs also accumulate in *dwf12-1D* plants. Because increased BR signaling often accompanies decreased levels of BL through negative feedback regulation (Choe et al., 2001), it is likely that the mechanisms controlling endogenous BR levels act downstream of the two signaling components BRI1 and DWF12.

Recent structural determination of the human GSK3 β revealed that the N-terminal domain (55 amino acids) plays a key role as an intramolecular inhibitory domain: Once Ser at position 9 from the N terminus (Ser-9) is phosphorylated, this domain occupies the active site and prevents access to substrates (Dajani et al., 2001; ter Haar et al., 2001). However, in DWF12, the first 15 amino acids, corresponding to the self-inhibitory domain of human GSK3 β , are missing, suggesting that the auto-inhibitory role of the N-terminal region may be lacking in DWF12. Protein kinase B, which inactivates GSK3 by phosphorylation at Ser-9, has also not been found in plants. In addition, human GSK3 β was found to be in an active kinase conformation if Ser-9 is not phosphorylated: The structure of the human GSK3 β activation segment is completely superimposed with that of the activated kinase ERK2-P₂ (Dajani et al., 2001), and the active site is buried when two monomers form a dimer. Thus, dissociation into a monomer is required for activity of this protein. We found that the residues participating in dimerization are well conserved in DWF12 (white squares in Fig. 5). Based on these findings, we postulate that DWF12, which is lacking the inhibitory N-terminal domain, is a permanently active kinase that does not require prior activation, and dimerization and dissociation may affect the activity of this protein.

A search for possible protein modification sites in DWF12 using ScanProsite utility (<http://www.expasy.ch/prosite/>) revealed that the two acidic Glu residues at 263 and 264 could help the phosphorylation of the adjacent Thr residue at 261 (Thr-261), possibly by casein kinase II, because these amino acids are most similar to the CKII consensus phosphorylation site (<http://www.expasy.org/cgi-bin/get-prodoc-entry?PDOC00006>). Because this region, including Thr-261 to Glu-264 (TREE domain), is highly conserved in GSK3s, it likely plays a critical role. Interestingly, structural determination of the human GSK3 β protein revealed that the TREE domain is exposed at dimerization, implying that access by another modifier like CKII is possible. Assuming that phosphorylation of Thr-261 is essential in the regulation of DWF12, the *dwf12* alleles may have negative effects on phosphorylation at Thr-261, leading to the dominant nature of DWF12 mutant protein. Thus, phosphorylation of the TREE domain in DWF12 could replace the protein kinase B phosphorylation at Ser-9 as a negative regulatory event. Alternatively, the TREE domain may define an interaction domain with itself or with another protein, with the

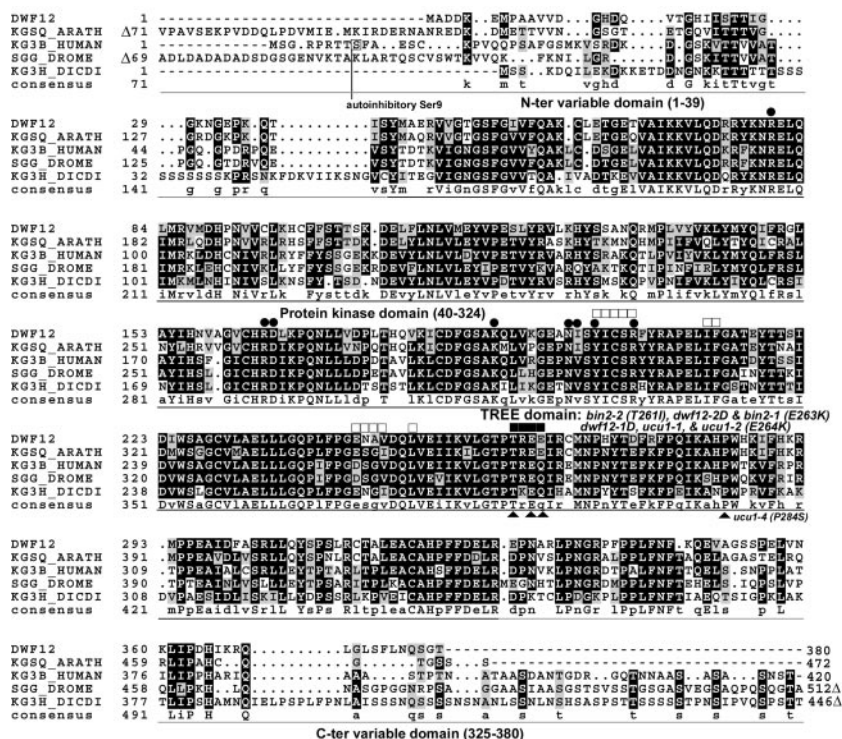


Figure 5. Multiple sequence alignment of two Arabidopsis GSK3-like protein and GSK3s from human, *D. melanogaster*, and *D. discoideum*. DWF12 (GenBank accession no. AY157149) and KGSQ_ARATH (Q96287), possessing the longest amino acid sequence among 10 Arabidopsis GSK3-like proteins, were compared with GSK3 β proteins from human (P49841), *D. melanogaster* (SGG_DROME, P18431), and *D. discoideum* (KGS3H_DICDI, P51136). Alignment was performed using the PileUp program of the Genetics Computer Group software with a gap creation penalty of 3 and a gap extension penalty of 1. The aligned sequences were further annotated using BOXSHADE and Photoshop programs. Annotations are based on structural characterization described by Dajani et al. (2001). Black circles indicate the residues involved in active site formation. White squares identify the residues that are involved in homodimerization. The TREE domain was identified to be a putative Thr phosphorylation site by casein kinase II, and the two *dwf12* mutations were located in this domain. Thick lighter bars delimit an N- or C-terminal variable domain, and a darker bar spans the protein kinase domain. Numbers in parentheses are based on the DWF12 protein sequence. Dashes and dots indicate gaps introduced to maximize alignment. Δ , Truncation of amino acid sequences for better comparison.

dwf12 mutations causing either stronger or reduced binding, resulting in a dominant phenotype. Our mapping and sequence data indicate that our *dwf12* mutants are allelic with the recently identified BR-insensitive mutants *bin2* (Li and Nam, 2002) and *ucu1* (Perez-Perez et al., 2002). Remarkably, the six semi-dominant alleles are all missense mutations in the TREE domain (Table II), further emphasizing the importance of this domain in the regulation of BIN2/UCU1/DWF12.

To address the role of BIN2/UCU1/DWF12 in BR signaling, Li and Nam (2002) purified both a wild-type kinase and a kinase with the *bin2-1* mutation, and found that the mutant kinase phosphorylated a substrate approximately 30% more than the wild-type kinase. These authors provide further support for the model that the BR insensitivity of the *bin2/ucu1/dwf12* mutants is caused by an overly active kinase by generating plants expressing the *bin2-1* mutant kinase under the control of its own promoter

Table II. Alleles of Arabidopsis BIN2/UCU1/DWE12

Allele	DNA Sequence Change	Amino Acid Change	Ecotype	Reference
<i>bin2-1</i>	G989A (GAA→AAA)	E263K (= <i>dwf12-2D</i>)	Columbia (Col-0)	Li and Nam (2002)
<i>bin2-2</i>	C981T (ACT→ATT)	T261I	Col-0	Li and Nam (2002)
<i>ucu1-1</i>	G986A (GAA→AAA)	E264K (= <i>ucu1-2</i> , <i>dwf12-1D</i>)	Landsburg <i>erecta</i> (Ler)	Perez-Perez et al. (2002)
<i>ucu1-2</i>	G986A (GAA→AAA)	E264K (= <i>ucu1-1</i> , <i>dwf12-1D</i>)	Ler	–
<i>ucu1-3</i>	C1049T (CCC→TCC)	P248S	Ler	–
<i>dwf12-1D</i>	G986A (GAA→AAA)	E264K (= <i>ucu1-1</i> , <i>ucu1-2</i>)	Wassilewskija-2 (Ws-2)	This paper
<i>dwf12-2D</i>	G989A (GAA→AAA)	E263K (= <i>bin2-1</i>)	Ws-2	This paper

Table III. SSLP markers used in cloning of DWF12

Name	Orientation	Sequence (5' to 3')	Bacterial Artificial Chromosome ^a	Size ^b
				bp
Ga	GaF	ctccaactgttgaaccgct	AP22	226
	GaR	aacaatctaactattgatgtcttt		
Na	NaF	gggattttcacgatgtatttaattggt	F23E12	304
	NaR	agggttttaggtggcttacac		
Da	DaF	gcaagtaattaatacattatcaactc	F8B4	107
	DaR	gcgttaatagtgtgtatgtgttaa		
La	LaF	aagaagctcaagcgggtggaat	T10C21	287
	LaR	agaatgtccagggcgcccat		
Ma	MaF	gggtcagtgttactatgtaccaag	F16G20	270
	MaR	gagaccagtggcctaagtagat		
Ba	BaF	caaaatcgtggatgcacttacag	T18B16	295
	BaR	gtcttaccataaatggttctctcat		
Sa	SaF	gtgccttattctgactatagta	FCA9	461
	SaR	gttacaggtagcataatgtataggta		
Aa	AaF	cgtcgtcttataatttgatggatt	FCA6	426
	AsR	tccattttagcatttccattaggt		
Ja	JaF	caccacttttaagggcactaca	F8L21	358
	JaR	gatacatcaacatgctgtagaatccat		

^a The BAC clone name where markers are located.^b PCR product size of the Col-0 ecotype.

in addition to the two endogenous BIN2(+) copies. In these plants, there was a good correlation between RNA levels and the severity of the dwarf phenotype (Li and Nam, 2002). Reducing the levels of the BIN2/UCU1/DWF12 RNA can suppress the phenotype of a weak *bri1* allele, providing further evidence that this gene encodes a negative regulator that acts downstream of BRI1 (Li and Nam, 2002).

These results indicate that BIN2/UCU1/DWF12 is a kinase of the GSK3 family with a role in BR signaling. Our evidence includes the observations that *dwf12* mutants share characteristic BR dwarf phenotypes, including short stature, insensitivity to BRs, and the accumulation of BRs. Characterization of the DWF12 gene indicates that the encoded protein shows >70% sequence identity to the human GSK3 β protein, except that DWF12 lacks the N-terminal auto-inhibitory domain containing the Ser-9 that is phosphorylated in GSK3 β . Our model is that DWF12 is a naturally active kinase that functions as a repressor of BR signaling pathways. Because BIN2/UCU1/DWF12 has no targeting sequences, it is most likely located in the cytoplasm. This location predicts that BIN2/UCU1/DWF12 operates downstream of BRI1, but there is no evidence yet that BRI1 interacts with or phosphorylates BIN2/UCU1/DWF12. The TREE domain does not match a phosphorylation site consensus derived from in vitro studies of BRI1 (Oh et al., 2000).

Recently, Yin et al. (2002) provided evidence that BIN2 physically interacts with a novel type of nuclear protein named BES1 (*bri1*-EMS-suppressor). Furthermore, Wang et al. (2002) also showed that a BES1-like nuclear protein BZR1 (brassinazole resistant) acts downstream of BIN2 and plays a role as a transcriptional inhibitor of BR biosynthetic genes. In the fu-

ture, it will be important to elucidate the signaling components that regulate BIN2, and to identify additional components downstream of BIN2 that lead to the transcription of BR biosynthetic and BR response genes.

MATERIALS AND METHODS

Plant Materials and Endogenous BR Analysis

More than 50,000 M2 seeds of Arabidopsis ecotype Ws-2 ecotype were planted on presoaked Metromix (350, Grace Sierra, Milpitas, CA). The seeds were cold treated (4°C) for 3 d and transferred to a 16-8-h-light (200 μ mol m⁻² s⁻¹);dark cycle (22°C and 21°C, and 70%–90% humidity) until the plants reached maturity. Forty-three mutants were selected based on their characteristic dwarf phenotype. BR treatment and genetic mapping of these dwarfs showed that most of these were found to be alleles of existing loci, and have been reported previously (Choe et al., 1999a). However, among these mutants, five were not rescued to the wild-type phenotype after topical application of BR, and were designated as insensitive mutants. Three of the five were alleles of *bri1* (Noguchi et al., 1999). Two additional mutants were out-crossed to Ws-2 wild type several times to dilute out background mutations, and also crossed to the Col-0 ecotype to obtain a mapping population. The mapping population consisted of 280 homozygous F₂ dwarf mutants.

BR dose response tests were performed according to the method described in Choe et al. (1998). In brief, cold-treated seeds (3 d) of Ws-2 wild type, *dwf4-1*, *bri1-5*, and *dwf12-1D* were cocultured in 1 $\times$ liquid Murashige and Skoog media supplemented with designated concentrations of *epi*-BL:0 (control), 10⁻⁹, 10⁻⁸, and 10⁻⁷ M. After incubation for 3 d, the seedlings were recovered from the culture media, their root and hypocotyl lengths were measured, and photographs were taken using representative seedlings for each concentration.

In the analysis of root growth inhibition in response to *epi*-BL, ABA (Gibco-BRL, Cleveland), and 2,4-D, seeds of wild type, *dwf12-1D*, and *bri1-5* were germinated on Murashige and Skoog solid media for 4 d, and transferred to plates supplemented with 10⁻⁹, 10⁻⁸, 10⁻⁷, and 10⁻⁶ molar concentrations of *epi*-BL, 0.5 μ M ABA, and 2,4-D. Seedlings on the agar plates were grown vertically for 7 more d in the light and their root length was measured.

Endogenous levels of BRs in 4-week-old *dwf12-1D* mutant plants were determined using gas chromatography/mass spectrometry. Procedures for

gas chromatography/mass spectrometry were described previously (Choe et al., 1999b), and the endogenous BR levels of *bri1-5* were taken for comparison from Noguchi et al. (1999).

Map-Based Cloning

The approximate location of *dwf12* in the Arabidopsis genome was determined by testing genomic DNAs from 24 F₂ homozygous *dwf12* plants from a mapping population with SSLP markers distributed on the five Arabidopsis chromosomes. Markers included nga59, nga280, and nga111 from chromosome 1, nga1145 and nga168 from chromosome 2, nga172, nga162, and nga6 from chromosome 3, nga8, nga1139, and nga1107 from chromosome 4, and nga151, nga76, and nga129 from chromosome 5. PCR was performed as described by Bell and Ecker (1994). In the course of fine mapping *dwf12*, we developed new sets of SSLP markers. The novel SSLP markers were developed based on the prediction available from the Arabidopsis sequence table (<http://www.Arabidopsis.org/cgi-bin/maps/Seqtable.pl?chr=4>). The new SSLP markers are shown in Table III. The names of the markers, orientation, sequences, corresponding bacterial artificial chromosome clones, and the size of the PCR products amplified using Col-0 DNA are included in the table. PCR products were run on 4% (w/v) agarose gels for >2 h for maximum separation of the polymorphic fragments.

The mutations in the two *dwf12* alleles were detected and confirmed by sequencing genomic DNA from *dwf12* homozygous mutants amplified using PCR. Primers used in the amplification of the genomic DNA and sequencing are, from 5' to 3', D12F2, gaggggtttgagttctgagc; D12F3, gccaa-cattttcatctgct; D12F4, tttttctgctttgtttct; D12F5, tggctacaaaatcctcactg; D12R2, ggaagatcatacaataaaggagtaag; D12R3, gtacatggcggagcgaggt; D12R4, caagatagaagatacaagaaccgagaact; D12-OVF1, gtcgaattcgccatgctgatgataag; and D12-OVR1, gtctctagacccttttaagtccagattgattc.

The structure of the *DWF12* gene was confirmed by comparing the cDNA sequence with that of the genomic DNA. cDNA was synthesized using D12-OVR1 as a primer for reverse transcriptase-PCR. Ten exons and nine introns were delimited by local alignment of the two sequences, and the schematic diagrams shown in Figure 4 were prepared using BestFit (Genetics Computer Group, Madison, WI), Vector NTI (InforMax, Bethesda, MD), and Photoshop (Adobe, San Jose, CA) software. Multiple sequence alignment was performed using the protein sequences available in Swiss-Prot protein knowledge bases. The His at 350 of the KG3B_HUMAN sequence was corrected to Leu according to Dajani et al. (2001) before comparison. The KGSQ_ARATH was chosen from 10 Arabidopsis GSK-like sequences because it has the longest total amino acid sequence. The five sequences were subjected to multiple sequence alignment using the PileUp program of GCG package. The conserved sequences were shaded using the BOXSHADE program (http://www.ch.embnet.org/software/BOX_form.html), and further annotation was carried out using Photoshop.

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