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DFL1, an auxin-responsive *GH3* gene homologue, negatively regulates shoot cell elongation and lateral root formation, and positively regulates the light response of hypocotyl length

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Summary

A novel dominant mutant designated 'dwarf in light 1' (*dfl1-D*) was isolated from screening around 1200 Arabidopsis activation-tagged lines. *dfl1-D* has a shorter hypocotyl under blue, red and far-red light, but not in darkness. Inhibition of cell elongation in shoots caused an exaggerated dwarf phenotype in the adult plant. The lateral root growth of *dfl1-D* was inhibited without any reduction of primary root length. The genomic DNA adjacent to the right border of T-DNA was cloned by plasmid rescue. The rescued genomic DNA contained a gene encoding a *GH3* homologue. The transcript was highly accumulated in the *dfl1-D*. The *dfl1-D* phenotype was confirmed by over-expression of the gene in the wild-type plant. The *dfl1-D* showed resistance to exogenous auxin treatment. Moreover, over-expression of antisense *DFL1* resulted in larger shoots and an increase in the number of lateral roots. These results indicate that the gene product of *DFL1* is involved in auxin signal transduction, and inhibits shoot and hypocotyl cell elongation and lateral root cell differentiation in light.

Keywords: *Arabidopsis*, auxin, hypocotyl elongation, light, lateral root formation.

Introduction

Hypocotyl elongation is the most important process at the beginning of germination in dicotyledenous plants. Hypocotyl elongation enables the emergence of the cotyledons from the soil, allowing exposure to light and the initiation of the photosynthetic machinery. A plant can proceed via either of two developmental pathways: photomorphogenesis in the light and skotomorphogenesis in the dark. However, under normal environmental conditions these pathways may overlap (von Arnim and Deng, 1996). The choice between these two pathways is crucial for the plant's development to ensure survival in its environment. To start on the appropriate pathway, plants possess several photoreceptors to detect the different qualities and quantities of light (Fankhauser and Chory, 1997). At the same time several plant hormones, such as auxin, cytokinin, gibberellin and brassinosteroid, also influence hypocotyl elongation (Moller and Chua, 1999). Recent physiological experiments indicate that auxin is the major plant hormone closely connected with light signal transduction (Neff *et al.*, 1999; Steindler *et al.*, 1999). In addition to hypocotyl elongation, auxin affects numerous aspects of plant growth and development such as gravitropism, lateral root differentiation and apical dominance (Estelle and Klee, 1994; Hobbie, 1998). Auxin has different effects on different tissues: it stimulates cell elongation in stems and hypocotyls, whereas in roots it stimulates cell division for lateral root formation (Estelle and Klee, 1994; Hobbie, 1998).

Auxin is also known to induce the expression of several genes. Various groups of genes are induced very early

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after auxin induction. These include the *IAA/AUX*, the *SAUR* and the *GH3* gene families (Abel and Theologis, 1996). *GH3* was first isolated by differential screening from *Glycine max* as an early auxin-inducible gene (Hagen *et al.*, 1984). Its expression is induced within 5 min of auxin application (Hagen and Guilfoyle, 1985; Roux and Perrot-Rechenmann, 1997). This *G. max GH3* gene contains an auxin-responsive element in its promoter (Liu *et al.*, 1994). Extensive studies have been made using this promoter to identify other auxin-responsive elements (Liu *et al.*, 1994; Ulmasov *et al.*, 1999). GH3 does not have any similarity to known functional motifs, and there has been no report on its function (Gee *et al.*, 1991; Hagen *et al.*, 1991).

To understand the mechanism controlling hypocotyl elongation, we screened activation-tagged lines under various monochromatic light conditions. We found one mutant that showed short hypocotyls in all light conditions but showed normal morphology in darkness. The corresponding gene for this mutant was found to be a member of the *GH3* gene family. The gene, designated *DFL1*, is the member of the *GH3* gene family having a function in light-dependent hypocotyl elongation.

Results

dfl1-D *is a novel dominant mutant that shows short hypocotyl in light*

To screen for hypocotyl length mutants, we analysed the T_2 generation of ≈1200 activation-tagged lines under monochromatic light conditions. We isolated a dominant mutant that showed short hypocotyls under continuous red, far-red and blue light conditions, but hypocotyls of the same length as wild-type in darkness (Figure 1a; Table 1). We designated this mutant *dfl1-D* (dwarf in light 1) based on its short hypocotyl phenotype under light conditions. Three quarters of the T_2 progeny harboured the hygromycin-resistance gene located on the T-DNA, and also showed the *dfl1-D* phenotype. Southern blotting data indicated that there was only one T-DNA insertion in the mutant genome (data not shown). This observation, and the co-segregation of the mutant phenotype with the T-DNA, indicated that the dominant phenotype may be caused by a single T-DNA insertion locus. All these short-hypocotyl seedlings grew with epinastic cotyledons and true leaves (Figure 1b,c). In the later stages of development of *dfl1-D*, an exaggerated dwarf phenotype was observed. Homozygous *dfl1-D* plants had shorter stems, smaller leaves and fewer lateral roots than wild-type (Figure 1d). There were almost no differences in primary root length (Table 1). Plant height and leaf size of heterozygous *dfl1-D* plants were intermediate between those of the homozygous *dfl1-D* plants and wild-type plants, as shown in Figure 1(d). The timing of bolting and flowering of *dfl1-D* were the same as the wild type, and there was no difference in the structure of flowers (data not shown). We did not observe differences in apical dominance.

In Arabidopsis it is known that there is no difference between plants grown in the light and those grown in the dark in the number of cells making up the hypocotyl cell file. The hypocotyl cell file is composed of ≈22 cells in both dark-grown and light-grown seedlings (Gendreau *et al.*, 1997; Gendreau *et al.*, 1998). We examined the hypocotyl of *dfl1-D* under an electron microscope and found no difference in the number of cells in the hypocotyl cell file (data not shown). We concluded that the short-hypocotyl phenotype of the *dfl1-D* was due to a reduction in hypocotyl cell length, and not to a reduction in cell number.

DFL1 *encodes a* GH3 *homologous gene*

We cloned the genomic DNA fragments adjacent to the T-DNA by plasmid rescue. Using the BLAST program, we searched the GenBank database for sequences homologous to the DNA sequence adjacent to the left border of the T-DNA. The sequence was perfectly matched to the P1 genomic clone F24B18, which maps to the bottom of chromosome 5. The T-DNA insertion site was at 50 429 nucleotides from the top of F24B18. There were predicted open reading frames on both sides of the T-DNA insertion site (Figure 2a). The cauliflower mosaic virus (CaMV) 35S enhancers on the T-DNA were located downstream of both open reading frames (Figure 2a). The open reading frame near the left border of the T-DNA encoded a putative tryptophan repressor binding protein. The distance between the CaMV 35S enhancers and the predicted translation start site of this protein was ≈9.2 kb. This transcript was not detected by Northern blot analysis in either the wild type or *dfl1-D* (data not shown). The open reading frame near the right border of the T-DNA encoded a homologue of the auxin-responsive gene *GH3* (Figure 2b). The distance between the CaMV 35S enhancers and the predicted translation start site was about 4.0 kb. This transcript was slightly accumulated in the wild type, and highly accumulated in the *dfl1-D* (Figure 2c), indicating that this *GH3* homologous gene might be responsible for the *dfl1-D*.

To confirm whether this *GH3* homologous gene causes the short-hypocotyl and dwarf phenotype of the *dfl1-D*, we introduced the gene under the control of the glucocorticoid-inducible promoter in sense and antisense orientations. We introduced the constructs into Arabidopsis by *Agrobacterium* transformation. We analysed 15 sense and 15 antisense transgenic plants. In the absence of gluco-

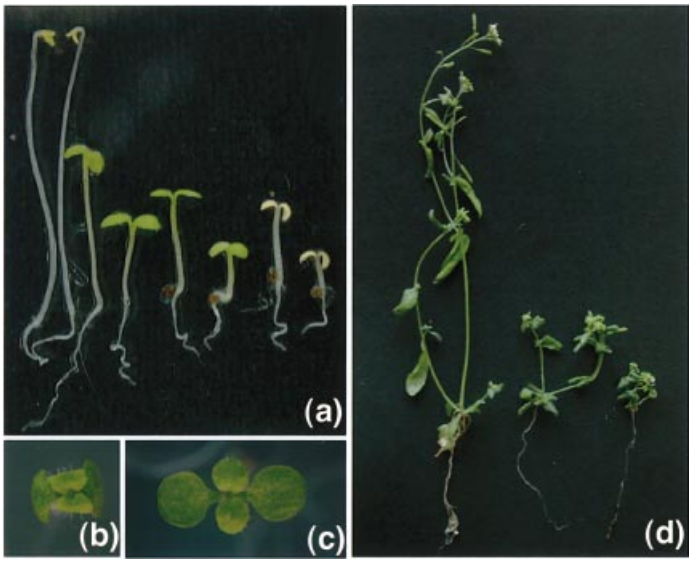


Figure 1. Phenotypic comparison between *dfl1-D* and wild type.
(a) Five-day-old seedlings grown under various light conditions. From left to right: wild type in darkness; *dfl1-D* in darkness; wild type under blue light; *dfl1-D* under blue light; wild type under red light; *dfl1-D* under red light; wild type under far-red light; *dfl1-D* under far-red light. Blue, red and far-red lights were irradiated at 11, 16 and 18 μmol sec^{-1} m^{-2}, respectively.
(b) Top view of a 10-day-old *dfl1-D* seedling grown in white light.
(c) Top view of a 10-day-old wild-type seedling grown in white light.
(d) Adult phenotype of *dfl1-D* and wild type. From left to right: a wild-type plant; a *dfl1-D* heterozygous plant; a *dfl1-D* homozygous plant grown under continuous white light for 4 weeks.

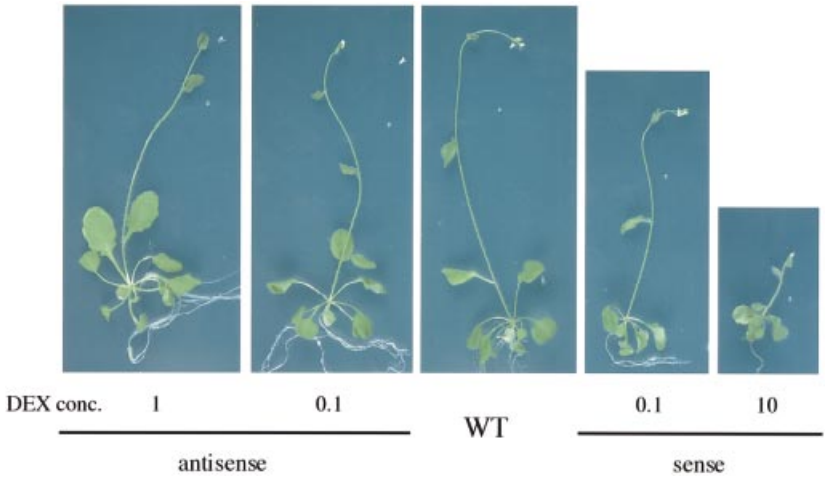


Figure 3. *DFL1* sense and antisense transgenic seedlings exhibit altered shoot and root phenotype.
Plants were grown on DEX-containing medium at the concentrations indicated. All plants were grown under continuous white light for 4 weeks. The left two panels show *DFL1* antisense transgenic plants. WT, wild-type plant without DEX. Similar morphology was observed using sense or antisense plants without DEX treatment. The right two panels show *DFL1* sense transgenic plants. DEX concentration is indicated in μM.

corticoid induction, no phenotype was observed in either the sense or antisense transgenic plants. When these transgenic plants were grown on plates containing various concentrations of the glucocorticoid dexamethasone (DEX), 10 out of 15 sense plants showed epinastic leaves and dwarf phenotype, and both of these characteristics increased in severity with increasing concentration of DEX (Figure 3). At 10 μM DEX the degree of the dwarf phenotype was more pronounced than in the *dfl1-D*, while no significant phenotype was observed in wild-type plants grown on plates containing the same concentration of DEX. When antisense plants were grown on plates containing various concentrations of DEX, 9 out of 15 transgenic plants showed larger leaves than the wild-type (Figure 3). From these results, we confirmed that the *dfl1-D* phenotype is caused by over-expression of the *GH3* homologue or *DFL1* gene.

DFL1 *is a member of the* GH3 *gene family*

By searching the GenBank database using the predicted DFL1 protein sequence, we found at least 10 *GH3* homologous genes in the Arabidopsis genome (Figure 2b). The database revealed that the deduced amino acid sequence of DFL1 shares between 32.4 and 90.4% homology with other members of the *GH3* gene family. The most homologous sequence to DFL1 (gi 3269287) has 90.4% amino acid identity and is located on chromosome 4 (the second line in Figure 2b). The identity between DFL1 and *G. max* GH3 was 59.1%. We could not find any

significant motifs or domains in the deduced amino acid sequence of DFL1. The PSORT program predicted that the DFL1 protein, and other members of the *GH3* gene family, locate in the cytoplasm.

DFL1 *expression levels altered the lateral root formation*

The *dfl1-D* has shorter hypocotyls in all monochromatic light conditions, but the primary root length was almost the same as the wild type (Table 1). However, while observing the *dfl1-D* and *DFL1* sense and antisense transgenic plants, we found there were obvious differences in the formation and development of lateral roots. The number of lateral roots of the *dfl1-D* was about one-fifth that of the wild type (Figure 4). The *DFL1* sense transgenic plants also had fewer lateral roots with increasing concentration of DEX (Figure 3). At 10 μM DEX, the root phenotype was more pronounced than in the original mutant. On the other hand, the *DFL1* antisense transgenic plants when grown on DEX-containing plates had more lateral roots than the wild type (Figure 3). These results suggested that, although it may not regulate primary root elongation, the expression level of the *DFL1* gene regulates the formation and development of lateral roots.

DFL1 *expression is induced by auxin*

Glycine max GH3 gene expression is rapidly induced by the addition of auxin (Hagen *et al.*, 1984). The promoter region of this *GH3* gene has been reported to contain multiple auxin-responsive elements, 5′-GTGCTC-3′ (Liu *et al.*, 1994). In the promoter region of the *DFL1* gene there is also a potential auxin-responsive element, 5′-GTGCAC-3′. To determine whether *DFL1* gene expression is also induced by auxin, we compared the accumulation of the transcript in the wild type with and without auxin treatment. Northern blot analysis showed that the *DFL1* transcript was induced by auxin treatment in wild-type seedlings (Figure 2d). This result indicated that the *DFL1* gene was inducible by auxin. We also examined whether the expression of the *DFL1* gene was altered by light. Comparison between dark-grown plants, light-grown plants and end-of-day far-red treated plants showed the same expression level of the *DFL1* gene (data not shown). This result suggested that the expression of the *DFL1* gene is not controlled by light.

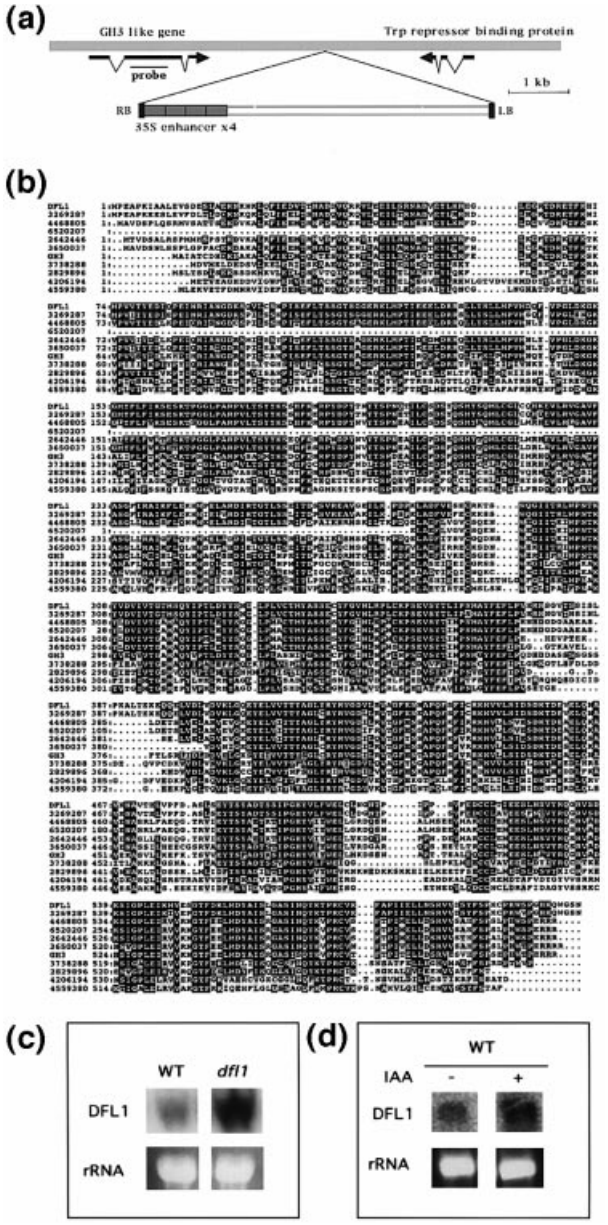


Figure 2. DFL1 is an auxin-inducible GH3 gene family.
(a) Map of the T-DNA insertion site. The grey bar shows genomic DNA. Solid arrows indicate predicted genes. Black boxes, right and left borders of T-DNA. Grey boxes, CaMV 35S enhancers.
(b) Amino acid sequence alignment of DFL1 and its homologues in Arabidopsis and *Glycine max* GH3. The number of each sequence is its GenBank Gen Info Identifier number. *Glycine max* GH3 is shown as GH3. Black boxes with white characters are identical amino acid residues in all sequences. Grey boxes are functionally similar amino acid residues.
(c) Northern analysis of the *DFL1* gene in mutant and wild type. Total RNA was extracted from 10-day-old wild-type and *dfl1-D* seedlings grown on germination medium (GM) under continuous white light, and 10 μg total RNA was loaded on each lane. The position of the *DFL1* gene-specific PCR fragment used as a probe is shown in Figure 2(a). Ethidium bromide staining pattern of ribosomal RNAs shows equal loading.
(d) IAA-dependent expression of *DFL1*. Northern analysis of the *DFL1* gene in the wild type with or without IAA treatment. Wild-type seedlings were grown on GM plates for 10 days under continuous white light and transferred to distilled water with or without 0.1 mM IAA. After incubation for 1 h, total RNA was extracted. Total RNA (10 μg) was loaded in each lane. *DFL1* PCR fragment was used as a probe as in (c).

dfl1-D *is resistant to exogenous auxin*

Auxin is involved in root elongation and differentiation. As mentioned above, the *G. max GH3* gene was originally isolated as an auxin-inducible gene, and the *DFL1* gene has also been shown to be auxin inducible. To better characterize any role for DFL1 in auxin signalling, we examined the effect of exogenously applied auxin on the root elongation of *dfl1-D*. When plants were grown on a medium containing a low concentration of IAA (10^{-10} and 10^{-9} M), no significant differences in primary root elongation were observed between *dfl1-D* and wild type (Figure 5). At a relatively high concentration (10^{-7} M), while the growth of wild-type roots was strongly inhibited, the degree of inhibition was not so severe in *dfl1-D* (Figure 5). At a high concentration of IAA (10^{-5} M), the growth of both *dfl1-D* and wild-type roots were strongly inhibited and formation of adventitious roots was observed in both (data not shown). These results indicated that *dfl1-D* has weaker sensitivity towards the exogenously applied auxin than wild-type plants. The *dfl1-D* has a shorter hypocotyl than the wild type in white light. We also observed the effect of exogenously applied auxin on hypocotyl elongation. In our experimental conditions (10^{-10} to 10^{-5} M IAA), we could not observe significant effects of exogenously applied auxin in hypocotyl elongation both *dfl1-D* and wild type (data not shown).

DFL1 *locates downstream of light signal transduction*

We have shown that *dfl1-D* had shorter hypocotyls than the wild type under all light conditions except in darkness (Figure 1a). This result suggests that DFL1 is a molecule that acts downstream of light signal transduction and modulates hypocotyl elongation. We analysed the relationship between light fluence rate and hypocotyl elongation in *dfl1-D* under various light conditions. At all light fluence rates and wavelengths investigated, *dfl1-D* showed shorter hypocotyls than those of the wild type (Figure 6a–c). The inhibition of hypocotyl elongation of *dfl1-D* increased in severity with increasing fluence of blue, red and far-red light (Figure 6a–c). Both *dfl1-D* and the wild type started greening in blue light at 0.1 μmol sec^{-1} m^{-2}. At this fluence, *dfl1-D* had fully opened cotyledons whereas the wild type still had closed cotyledons (data not shown). We could not observe any differences in cotyledon opening between *dfl1-D* and wild type at the fluence rates used for red and far-red light. These results indicate that *dfl1-D* is more sensitive than the wild type in all light conditions studied, especially to blue light.

Discussion

The activation-tagging technique was developed in order to isolate a new spectrum of dominant mutants that were not formerly isolated from the pool of knock-out type mutants (Hayashi *et al.*, 1992). Using this technique we isolated a novel short hypocotyl mutant, *dfl1-D*, which showed enhanced sensitivity to all light conditions and reduced sensitivity to exogenous auxin.

DFL1 regulates hypocotyl elongation and lateral root formation

The phenotypes of sense and antisense *DFL1* transgenic plants suggest that DFL1 negatively regulates shoot and hypocotyl cell elongation and lateral root formation. The short-hypocotyl phenotype of sense over-expressors and *dfl1-D* is caused by a reduction in the length of individual cells in the hypocotyl, and not by a reduction in the number of cells making up the hypocotyl cell files. The lateral roots emerge from the primary root by a series of divisions in the pericycle cells (Malamy and Benfey, 1997). The *dfl1-D and DFL1* sense over-expressors had a reduced number of lateral roots, while the *DFL1* antisense plants grew more lateral roots than the wild type. This result indicated that the DFL1 protein

Table 1. Comparison of hypocotyl and root length of 4-day-old *dfl1-D* with the wild type

	Dark		Blue[a]		Red[a]		Far-red[a]	
	dfl1-D	Wild type	*dfl1-D*	Wild type	*dfl1-D*	Wild type	*dfl1-D*	Wild type
Hypocotyl length (mm)	10.53 ± 0.46	11.39 ± 0.65	2.06 ± 0.40	3.52 ± 0.80	3.50 ± 0.43	5.54 ± 0.86	1.44 ± 0.26	4.88 ± 0.33
Root length (mm)	7.31 ± 0.45	7.30 ± 0.48	9.06 ± 1.26	9.05 ± 0.82	8.05 ± 0.57	8.27 ± 0.50	8.85 ± 1.05	9.90 ± 0.081

Hypocotyl length and root length were measured using the NIH image program. For each light condition, images of ≈30 seedlings were scanned into a computer. Images were magnified with a length standard and calculated to represent the mean length with standard error.
[a]Fluence rate, 10 μmol sec^{-1} m^{-2}.

might inhibit lateral root formation by suppressing the cell division from pericycle cells.

Both hypocotyl cell elongation and lateral root formation are known to be controlled by auxin (Estelle and Klee, 1994; Hobbie, 1998). These results indicate that DFL1 is located in auxin signal transduction. This conclusion was also supported by the observation that the primary root growth of the *dfl1-D* was resistant to exogenous auxin.

DFL1 *belongs to auxin-inducible gene family*

The *DFL1* gene showed homology to *GH3*, which was originally isolated from *G. max* by differential screening as an early auxin-inducible gene (Hagen *et al.*, 1984). We could not find any motif in DFL1 using the GenBank motif search program. It is reported that the *G. max GH3* gene is expressed in the lower side of the shoot stimulated by gravity, and in the dark side of the shoot stimulated by unilateral light (Li *et al.*, 1999). A phototropic mutant, *nph4*, exhibited a reduced induction of the *GH3* gene family by auxin (Liscum and Briggs, 1996). These results may suggest that the *G. max* GH3 protein has a function in the process of shoot or hypocotyl cell elongation. We examined the gravitropism and phototropism of *dfl1-D*. Preliminary results indicated that *dfl1-D* showed normal responses to both stimulations.

As is shown in Figure 2(b), the *GH3* gene is a member of a small gene family of at least 10 genes in the Arabidopsis genome. This kind of genetic redundancy has also been observed in another auxin-inducible gene family, the *IAA*s. This may imply that a mutation in this kind of gene would be difficult to isolate by the usual knock-out type-screening method. This is also supported by several reports that *IAA* mutants were isolated as gain-of-function mutations and not as recessive mutations. Examples include the mutations in the *SHY2/IAA3*, *IAR2/IAA28*, *AXR2/IAA7* and *AXR3/IAA17* genes (Kim *et al.*, 1996; Leyser *et al.*, 1996; Rogg *et al.*, 1999; Wilson *et al.*, 1990). These mutations have related but distinct morphology. *shy2* and *iar2* have few lateral roots whereas *axr2 has* more than the wild type (Rogg *et al.*, 1999; Tian and Reed, 1999; Wilson *et al.*, 1990); *axr2*, *axr3* and *shy2* show decreased gravitropism (Wilson *et al.*, 1990; Leyser *et al.*, 1996; Tian and Reed, 1999); and *axr2*, *shy2* and *axr3* have a short hypocotyl and have leaves in darkness (Kim *et al.*, 1996; Leyser *et al.*, 1996; Wilson *et al.*, 1990). These observations indicate that auxin may induce a group of genes whose functions partially overlap each other but also have distinct functions. Drawing an analogy from the *IAA* gene family, GH3

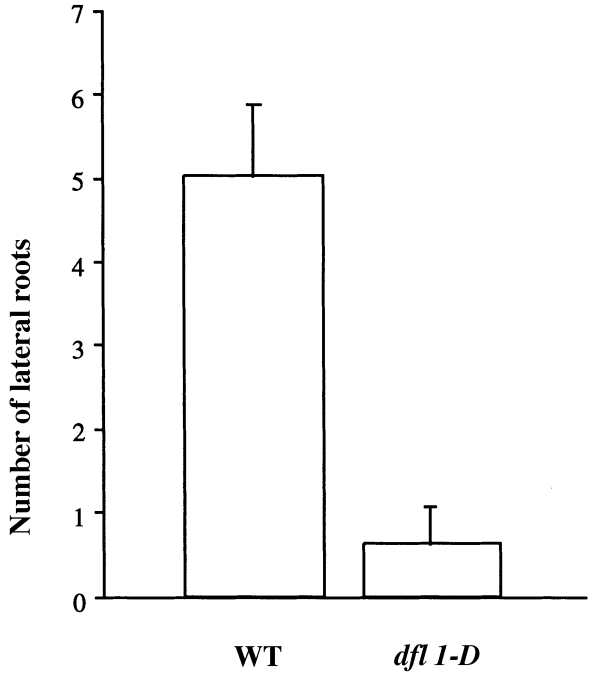


Figure 4. Lateral root formation in wild type and *dfl1-D*.
The number of lateral roots was counted in 10-day-old seedlings. Plants were grown on germination medium plates placed in a vertical position under white light. The lateral roots of ≈20 seedlings were counted. Graph shows average lateral root number with standard error.

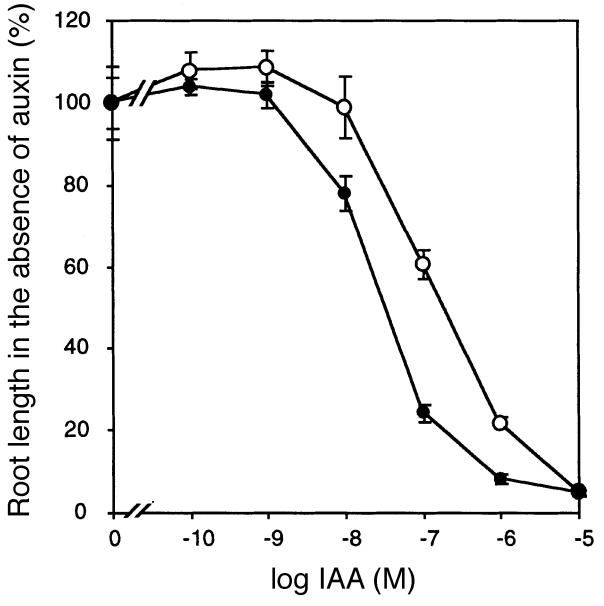
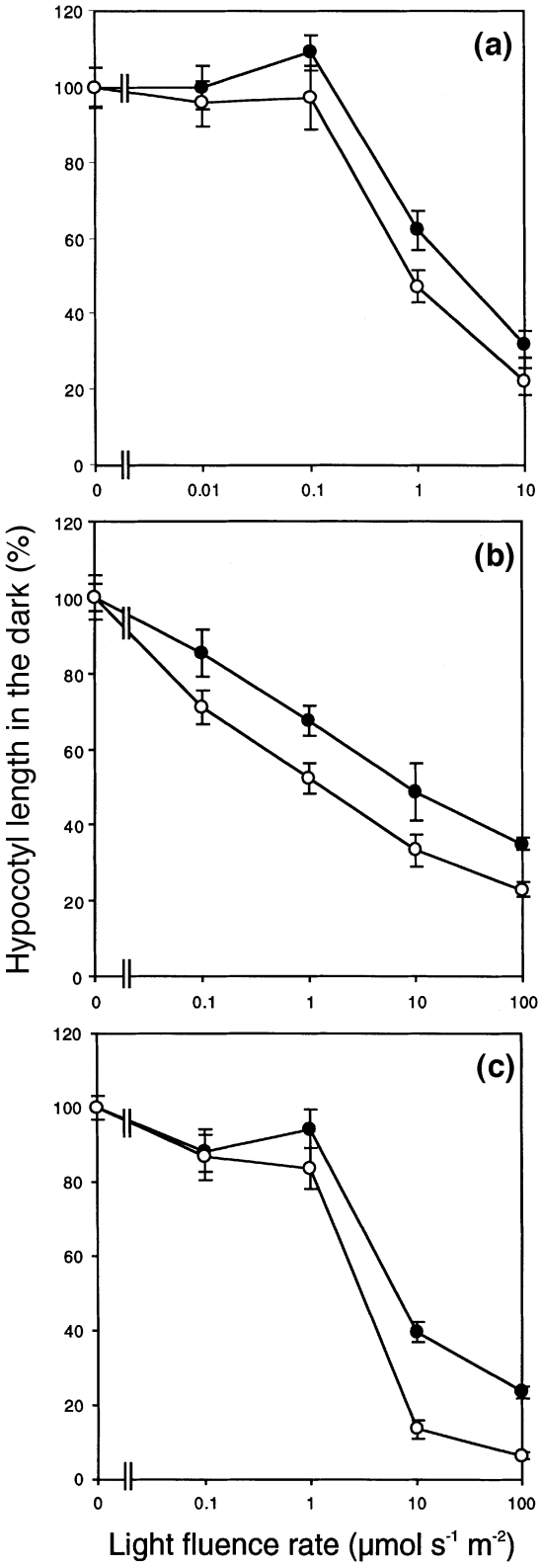


Figure 5. Primary root growth inhibition in response to exogenous IAA.
Seedlings of wild type and *dfl1-D* were grown for 10 days on various concentrations of IAA-containing germination medium plates under continuous white light. ●, wild-type seedlings; ○, *dfl1-D*. About 20 seedlings were measured for primary root length. Root length was represented as percentage of the length in the absence of auxin. Primary root length of *dfl1-D* and wild type in the absence of auxin is 44.21 ± 2.74 and 47.17 ± 4.19 mm, respectively (± indicates standard error).

homologous proteins in Arabidopsis may have some overlapping functions, but also have distinct functions. It would be interesting to know the functions of individual *GH3* gene family members.



dfl1-D *is dependent on light*

dfl1-D has a similar morphology to *shy2* in that it has fewer lateral roots (Tian and Reed, 1999). It also has a similar morphology to *axr3* and *shy2* in that it has curled leaves (Kim *et al.*, 1996; Rouse *et al.*, 1998). Although *dfl1-D* shows similarity to these mutants, it is distinct from them in that it has almost the same hypocotyl length as the wild type in darkness. *dfl1-D* has a longer hypocotyl and closed cotyledons under dark conditions, implying that this mutation is light-dependent. *dfl1-D* is also different from *IAA* mutants because it has almost the same primary root length as the wild type.

Despite the observation that the short-hypocotyl phenotype of *dfl1-D* has specificity to light, the expression of *DFL1* is not controlled by light. We observed almost the same level of expression of the *DFL1* gene in both light-grown and dark-grown plants (data not shown). This light dependency may be explained as follows. DFL1 controls hypocotyl elongation with one or more partners, synthesized only under light conditions. In darkness, even in the presence of a high level of the DFL1 protein, it cannot interact with this partner or partners and therefore the phenotype of the *dfl1-D* remains normal. On interaction with the light-inducible partner(s), the DFL1 protein inhibits hypocotyl length in a light-dependent manner. Identification of such partners may elucidate the complex nature between light signal transduction and plant hormone function.

Recently, the *FIN219* gene was isolated as a suppressor of the *cop1* mutation that encodes a protein similar to the GH3 family (Hsieh *et al.*, 2000). The *fin219* mutant shows long hypocotyl only under continuous far-red light. FIN219 is thought to be a component of PHYA signalling which is involved in light inactivation of COP1 activity. This observation suggested that FIN219 is a molecule that links PHYA and auxin signalling. Although DFL1 is distinct from FIN219 in light dependency, both GH3 members are involved in hypocotyl elongation in light conditions. The observation suggested that light signalling transduction

Figure 6. Sensitivity to light is increased in the *dfl1-D*.
Fluence rate-response curves for hypocotyl growth in continuous (a) blue; (b) red; (c) far-red light. Seedlings were irradiated at various photon fluence rates for 4 days after germination. ○, *dfl1-D*; ●, wild type. Relative hypocotyl length is represented as percentage of the dark control in each genotype. Hypocotyl lengths in the dark are (a) wild type, 10.98 ± 0.56 mm, *dfl1-D*, 9.25 ± 0.49 mm; (b) wild type, 11.39 ± 0.65 mm, *dfl1-D*, 10.53 ± 0.46 mm; (c) wild type, 12.33 ± 0.80 mm, *dfl1-D*, 10.85 ± 0.65, respectively (± indicates standard error).

has close connections with auxin signal transduction through the GH3 family.

From analyses of DFL1 protein expression, its interacting partner(s), and other member of the GH3 family, it may be possible to connect these two signal transduction pathways.

Experimental procedures

Mutant screening

Arabidopsis thaliana (L*er*) was transformed using *Agrobacterium tumefaciens* GV3101 possessing the binary activation-tagging plasmid pPCVICEn4HPT (Hayashi *et al.*, 1992), which contains the *ColE1* origin of replication, an ampicillin resistance marker for plasmid rescue and a hygromycin resistance gene as the plant transformation marker. Hygromycin resistance T_1 plants were transferred onto soil and T_2 seeds were harvested. T_2 seeds were surface sterilized using chloride vapour (Clough and Bent, 1998), and each line was sown on three germination medium (GM) plates (Valvekens *et al.*, 1988). Plates were cold-treated at 4°C for 4 days, then moved into monochromatic light chambers and incubated at 22°C for 5 days. In the case of the far-red light treatment, plates were pre-incubated for 8 h in white light at 22°C to enhance germination. After incubation, hypocotyl length and seedling phenotypes were observed. All light conditions except the intensity were according to Peters *et al.* (1998).

Cloning of DFL1 *gene*

To isolate the T-DNA boundary genomic sequences, plant genomic DNA was isolated from rosette leaves (Watson and Thompson, 1986). Plant DNA (2 μg) was digested with *Eco*RI, and after heat inactivation of enzyme at 65°C for 10 min, genomic DNA was self-ligated with T4 DNA ligase (Life Technologies Inc., Rockville, MD, USA) at 15°C for 15 h. The total ligated solution was electroporated into XL1-Blue electrocompetent cells. The genomic fragments containing the T-DNA were rescued by spreading on Luria-Bertani agar plates containing ampicillin. Three plasmids isolated from 30 colonies were sequenced with the primer 5′-CTGCGGACTGGCTTTCTACG-3′ which corresponds to the partial sequence of the promoter region of the nopaline synthase gene of T-DNA, and all three plasmids possessed the same genomic sequence.

Construction of the inducible DFL1 *plasmid*

The PCR primers *Xho*9264 (5′-GGGCTCGAGAAACACAAAACC-TAAACGAT-3′) and *Spe*1529 (5′-GGGACTAGTCTCTTTAGTTAC-TCCCCCAT-3′) were used to amplify the sense *DFL1* gene from a cDNA library prepared from 5-day-old light-grown seedlings, while *Spe*9264 (5′-GGGACTAGTAAACACAAAACCTAAACGAT-3′) and *Xho*11529 (5′-GGGCTCGAGCTCTTTAGTTACTCCCCCAT-3′) were used to amplify *DFL1* antisense cDNA from the same cDNA library. These PCR products were digested with *Xho*I and *Spe*I, and introduced into a T-DNA vector pTA7002 which contains the glucocorticoid inducible promoter (Aoyama and Chua, 1997). These plasmids were introduced into *A. tumefaciens* GV3101 by electroporation and transformed into *A. thaliana* (Col-0) by vacuum infiltration (Bechtold *et al.*, 1993). Dexamethasone (DEX) treatment was used to induce sense and antisense *DFL1* RNA as described previously (Yoshizumi *et al.*, 1999).

Northern blot analysis

Extraction of total RNA and RNA gel blotting were performed as described previously (Yoshizumi *et al.*, 1999). To detect *DFL1* mRNA, a cDNA fragment was amplified using the PCR primers 447 U (5′-CAAAGGCAAAGGGATGTATT-3′) and 1506 l (5′-GCTCCCAGAATAAGACATAG-3′) and used as the template for making the probe.

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