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DFL2, a New Member of the *Arabidopsis GH3* Gene Family, is Involved in Red Light-Specific Hypocotyl Elongation

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A new *GH3*-related gene, designated *DFL2*, causes a short hypocotyl phenotype when overexpressed under red and blue light and a long hypocotyl when antisensed under red light conditions. Higher expression of this gene was observed in continuous white, blue and far-red light but the expression level was low in red light and darkness. *DFL2* gene expression was induced transiently with red light pulse treatment. *DFL2* transgenic plants exhibited a normal root phenotype including primary root elongation and lateral root formation, although primary root elongation was inhibited in antisense transgenic plants only under red light. The adult phenotypes of sense and antisense transgenic plants were not different from that of wild type. *DFL2* promoter activity was observed in the hypocotyl. Our results suggest that DFL2 is located downstream of red light signal transduction and determines the degree of hypocotyl elongation.

Keywords: *Arabidopsis* — GH3 — Light signal transduction — Red light.

Abbreviations: cB, continuous blue; cFR, continuous far-red; cR, continuous red; cW, continuous white; GUS, β-glucronidase; phyA, phytochrome A; phyB, phytochrome B; phot, phototropin.

Introduction

Plants perceive light not only as an energy source but also as an environmental stimulus that modulates plant growth and development (Kendrick and Kronenberg 1994). Environmental light signals are perceived by several photoreceptors. Five phytochromes have been characterized in *Arabidopsis* for red and far-red light perception. Two cryptochromes and two phototropic receptors (Phot1 and Phot2) have been identified for blue light perception and their molecular functions characterized in detail (Briggs and Huala 1999, Briggs and Christie 2002). The light signals perceived by these photoreceptors are transduced to the cell nucleus and cause induction of several light regulated genes (Sakamoto and Nagatani 1996, Nagy and Schafer 2002, Quail 2002). Several components that connect to these photoreceptors directly or indirectly were characterized and specific binding to the *cis*-regulatory elements of light-inducible genes has been reported (Martinez-Garcia et al. 2000). Besides photoreceptors, phytohormones are known to be involved in cell division and differentiation. Hypocotyl elongation is controlled by ethylene, gibberellin and auxin (Moller and Chua 1999, Swarup et al. 2002). Auxin in particular is a major phytohormone involved in the control of hypocotyl elongation.

Auxin is known to regulate a variety of cellular processes, including cell division, cell extension and cell differentiation (Estelle and Klee 1994, Hobbie 1998). Auxin regulates some cellular responses through direct effects on the membrane or cytoskeleton, but it also regulates the expression of many genes (Reed 2001, Hagen and Guilfoyle 2002, Tian et al. 2002). Several groups of genes have been identified as early auxin-responsive genes, including *AUX/IAA*, *SAUR* (small auxin up-regulated), and *GH3* (Abel and Theologis 1996, Sitbon and Perrot-Rechenmann 1997). Several *IAA* mutants have been isolated (Wilson et al. 1990, Timpte et al. 1994, Leyser et al. 1996, Kim et al. 1998, Reed et al. 1998, Rouse et al. 1998, Tian and Reed 1999, Nagpal et al. 2000, Fukaki et al. 2002). Most of these were gain-of-function mutants and the mutation in domain II of the IAA proteins was involved in the stabilization of the corresponding proteins. Some of these mutants exhibited hypocotyl phenotypes. For example, the gain-of-function mutation in the *SHY2/IAA3* gene caused a short hypocotyl not only in light but also in darkness (Kim et al. 1998, Reed et al. 1998, Tian and Reed 1999). The function of SAUR proteins is still unknown. *GH3* was first isolated from *Glycine max* by differential screening after treatment with auxin (Hagen et al. 1984, Hagen and Guilfoyle 1985). In the *Arabidopsis* genome, there are 20 *GH3*-related sequences (Table 1), and the expression of several *GH3*-related genes is regulated by both the light and auxin signaling pathways. The expression of one *GH3*-related gene of *Arabidopsis* is regulated by *NPH4/ARF7/MSG1* (Stowe-Evans et al. 1998). It is reported from a DNA-microarray study that two *GH3*-related genes of *Arabidopsis* are regulated by phyA (Tepperman et al. 2001). Recently, another *GH3*-related gene, *AtGH3a*, was isolated from *Arabidopsis* promoter-trap lines that exhibited light-dependent reporter gene expression (Tanaka et al. 2002). The *AtGH3a* gene is expressed in response to end-of-day far-red light treatment and this expression is regulated by phyB.

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Table 1 *Arabidopsis GH3* genes

GH3 gene	Gene code	Synonymous
GH3-1	At2g14960 / T26I20.12	
GH3-2	At4g37390 / F6G17.40	
GH3-3	At2g23170 / T20D16.20	
GH3-4	At1g59500 / T30E16.2	
GH3-5	At4g27260 / M4I22.70	*AtGH3a*
GH3-6	At5g54510 / F24B18.13	*DFL1*
GH3-7	At1g23160 / T26J12.7	
GH3-8	At5g51470 / K17N15.2	
GH3-9	At2g47750 / F17A22.14	
GH3-10	At4g03400 / F9H3.1	*DFL2*
GH3-11	At2g46370 / F11C10.6	*FIN219*/*JAR1*
GH3-12	At5g13320 / T31B5.140	
GH3-13	At5g13350 / T31B5.170	
GH3-14	At5g13360 / T22N19.10	
GH3-15	At5g13370 / T22N19.20	
GH3-16	At5g13380 / T22N19.30	
GH3-17	At1g28130 / F3H9.21	
GH3-18	At1g48670 / F11I4.14	
GH3-19	At1g48660 / F11I4.15	
GH3-20	At1g48690 / F11I4.13	

GH3 numbers (*GH3-1* etc) were assigned by Hagen and Guilfoyle (Hagen and Guilfoyle 2002). Gene codes (chromosome and BAC open reading frame numbers) of each *GH3* gene were obtained at the Munich Information Center for Protein Sequences *Arabidopsis thaliana* Database (MATDB) (http://www.mips.biochem.mpg.de/proj/thal/db/index.html).

FIN219 is also a member of the *Arabidopsis GH3* gene family. It was isolated as a suppressor mutation of *cop1* (Hsieh et al. 2000). The monogenic mutation of *fin219* showed a longer hypocotyl than wild type under continuous far-red (cFR) light conditions. This indicated that FIN219 is a component or a molecule that associates with light signal transduction from the phyA photoreceptor. *DFL1* is also in the *GH3* gene family of *Arabidopsis* and overexpression of this gene by an activation tagged mutation caused shorter hypocotyls under continuous red (cR), continuous blue (cB) and cFR light conditions (Nakazawa et al. 2001). This activation-tagged mutant showed normal hypocotyl length in darkness. It was suggested that DFL1 controls hypocotyl elongation in a light-dependent manner or it requires the presence of another molecule that is controlled by light in order to function. The expression of *FIN219* and *DFL1* are also induced by exogenously applied auxin (Hsieh et al. 2000, Nakazawa et al. 2001). These results suggest that some of the *Arabidopsis GH3* genes are involved in hypocotyl elongation that is controlled by light and that there are differences in the GH3-related proteins utilized depending on the light conditions. Recently it has been revealed that some of the *Arabidopsis* GH3-related proteins in vitro exhibited the capacity to adenylate plant hormones including IAA (Staswick et al. 2002). GH3-related proteins are classified into three groups by sequence similarity. This classification is consistent with substrate specificities of adenylation activities. In group I, the JAR1/FIN219 protein shows the capacity to adenylate jasmonic acid (JA). The DFL1 and AtGH3a proteins belong to group II and some of the group II GH3-related proteins including DFL1 and AtGH3a can adenylate IAA. The GH3-related proteins of group III did not show any capacity to adenylate phytohormones in vitro.

To understand the functional differences between GH3-related proteins, we constructed overexpressing and antisense lines for eight *Arabidopsis GH3*-related genes. This approach, especially the overexpression of individual *GH3*-related genes, helped to dissect the overlapping functions of *GH3*-related genes. We will present here the characterization of one of the transgenic lines that showed a light-specific phenotype by overexpression of one of these genes. The line overexpressing *At4g03400*, designated as *DFL2*, showed a shorter hypocotyl under cR and cB light conditions but a normal hypocotyl length under cFR light and in the dark. In contrast, the antisense line exhibited an elongated hypocotyl under cR light.

Results

The transgenic lines of At4g03400 showed a light-specific hypocotyl phenotype

We previously isolated *DFL1*, an *Arabidopsis GH3*-related gene, from an activation tagged line as the gene responsible for a light-specific dwarf phenotype (Nakazawa et al. 2001). This mutant had a short hypocotyl under cR, cB and cFR light but showed normal hypocotyl length in darkness. *FIN219* is another *GH3*-related gene and it controls hypocotyl elongation through phyA (Hsieh et al. 2000). The *AtGH3a* gene was isolated from a promoter trap line and its expression is controlled by phyB (Tanaka et al. 2002). There are 20 *GH3*-related genes in *Arabidopsis* (Table 1). They are localized on all chromosomes except chromosome 3 and show good conservation in the coding region.

To understand the function of individual members of *GH3*-related gene family, we constructed overexpressing and antisense lines for several of the genes and examined seedling phenotypes under monochromatic light conditions. We could observe clear phenotypes for the overexpressing and antisense lines of *At4g03400*. The overexpressors of this gene showed shorter hypocotyls under white light conditions. In contrast, antisense lines showed longer hypocotyls under white light compared with wild type (data not shown). These phenotypes became more obvious when the transgenic lines were grown under low white light (data not shown). We characterized these transgenic lines under monochromatic light. *At4g03400* overexpressors showed the hypocotyl phenotype under cR and low cB light conditions but not under cFR light (Fig. 1). They did not show the phenotype in the dark (Fig. 1). In contrast, the antisense lines showed longer hypocotyls in cR light. These results indicate that the At4g03400 protein is involved in

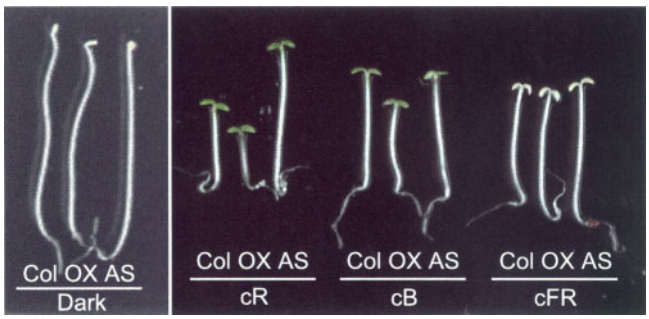

Fig. 1 Hypocotyl phenotype of *DFL2* transgenic lines. Five-day-old seedlings of wild type (Col), *DFL2* overexpressor (OX) and antisense (AS) lines grown under various light conditions. cR, cB or cFR light was irradiated at 0.286, 0.156 or 0.080 W m^{-2} respectively.

hypocotyl elongation but that it is dependent on the light conditions. We designated this gene as *DFL2* (*DwarF in Light 2*). The *dfl1-D* mutant showed not only the hypocotyl phenotype but also a dwarf phenotype including shorter stems, smaller leaves and fewer lateral roots in adult plants. However, no obvious phenotype was observed in the adult stage in *DFL2* transgenic lines compared with wild type (data not shown). Moreover, *DFL2* transgenic plants exhibited normal root formation including primary root elongation and lateral root formation in cW light (data not shown).

DFL2 transgenic plants showed red-light dependent hypocotyl length

To understand the light dependency of the *DFL2* transgenic lines, we selected homozygous lines for the transgene. We grew these transgenic lines under various fluences of monochromatic light. The *DFL2* overexpressors showed shorter hypocotyls under cR light (Fig. 2A) and also under cB light (Fig. 2B). They did not show any differences compared with wild type under cFR light conditions (Fig. 2C). The *DFL2* antisense lines had a longer hypocotyl than wild type under cR light (Fig. 2A). The *DFL2* antisense lines did not show any differences compared with wild type under cB and cFR light at any of the fluence rates examined (Fig. 2B, C). The *DFL2* transgenic lines also showed a root phenotype under red light conditions. There were almost no differences between wild type and *DFL2* overexpressing lines, while the *DFL2* antisense lines exhibited shorter primary roots than wild type under cR (Table 2).

Hypocotyl phenotype is caused by change of cell size

In *Arabidopsis*, hypocotyl cell files are composed of 22–25 cells and it is reported that there is no difference in this

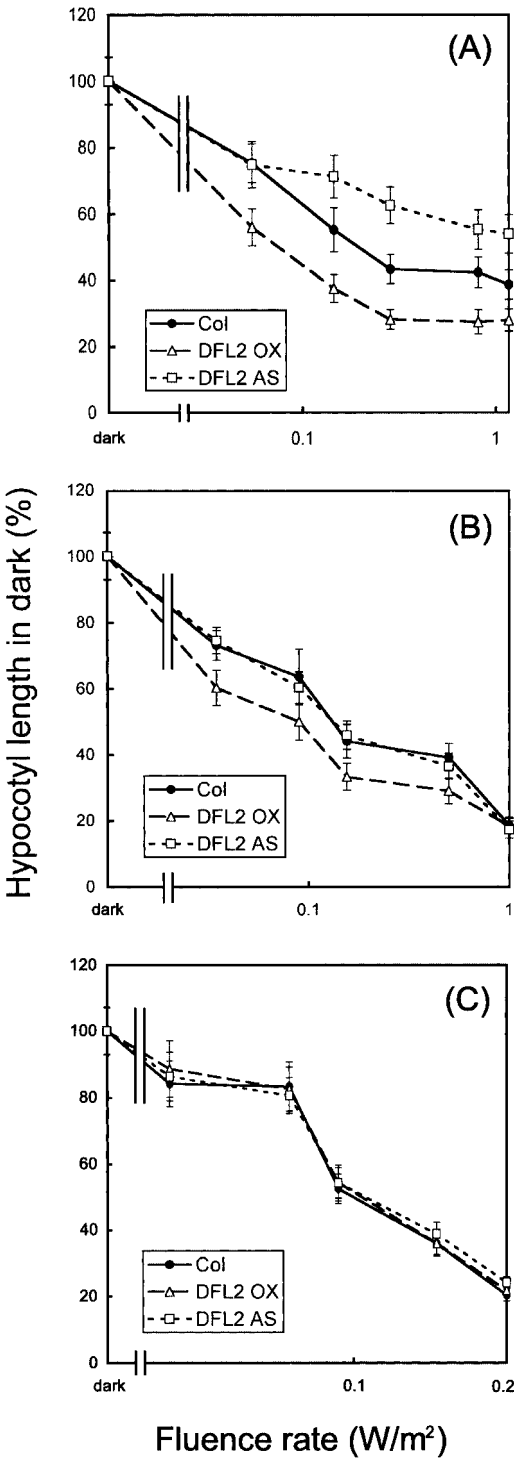

Fig. 2 The *DFL2* transgenic lines show the hypocotyl phenotype under red light and low blue light. Fluence rate-response curves for hypocotyl length in cR (A), cB (B) and cFR (C) light. Seedlings were grown on germination (GM) plates for 5 d under monochromatic light with various fluence rates. Filled circles, wild type (ecotype Columbia); open triangles, *DFL2* overexpressor; open squares, *DFL2* antisense line. Relative hypocotyl length is represented as a percentage of the dark control in each genotype. Each point represents the mean hypocotyl length of approximately 40 seedlings. Hypocotyl lengths in the dark are wild type (ecotype Columbia), 13.73±0.96 mm; *DFL2* overexpressor, 13.54±0.97 mm; *DFL2* antisense line, 13.93±1.01 mm; respectively (± indicates standard error).

Table 2 Comparison of root length of wild type and *DFL2* transgenic lines

	Root length (mm)			
	Dark	cR	cB	cFR
Wild type	3.25±0.46	10.07±1.29	3.6±0.64	4.28±0.76
DFL2 OX	3.06±0.38	10.26±1.05	3.30±0.57	4.57±0.78
DFL2 AS	3.59±0.44	4.76±0.68	2.99±0.50	4.55±0.57

Seedlings of wild type (ecotype Columbia) and the *DFL2* transgenic line were incubated on a GM plate for 5 d under cR, cB and cFR and in darkness. Red, blue or far-red light was irradiated at fluence rates of 10, 0.05 or 1.05 W m^{-2}, respectively. Root lengths of approximately 40 seedlings were measured using NIH image software (± indicates standard error).

number in seedlings grown in light and those grown in darkness (Gendreau et al. 1997, Gendreau et al. 1998). This means that the long hypocotyl phenotype of dark-grown plants is caused by longitudinal elongation of hypocotyl cells. To investigate whether the hypocotyl phenotype of the *DFL2* transgenic lines under cR light is caused by a change in the number of cells or in cell size, we scanned hypocotyls using an electron microscope (Fig. 3). The *DFL2* antisense lines showed elongated hypocotyl cells and the *DFL2* overexpressors had smaller cells in the hypocotyl cell file under cR light. These observations indicated that the hypocotyl phenotype of the transgenic lines is caused by a change of cell size rather than cell number.

Expression level of DFL2 controls the sensitivity of light-dependent hypocotyl elongation

As shown in Fig. 2, the *DFL2* antisense lines showed lower sensitivity to red light. In contrast, the *DFL2* overexpressors showed higher sensitivity to red and blue light. To examine whether the expression level of the transgene correlated with hypocotyl length, we used RT-PCR to examine the expression level of the *DFL2* gene in transgenic lines showing varying hypocotyl lengths (Fig. 4A, B). In the *DFL2* overexpressors, there seemed to be a close inverse correlation between the *DFL2* expression level and hypocotyl length. One overexpressing line, OX2, showed strong inhibition of hypocotyl elongation under cR light and the expression level of *DFL2* is the highest of the four overexpressors examined. In the *DFL2* antisense lines, we were unable to detect *DFL2* mRNA by RT-PCR. This may be explained by specific degradation of *DFL2* mRNA by expression of antisense RNA. In most cases, expression of *DFL2* antisense RNA is sufficient to suppress endogenous *DFL2* mRNA and cause long hypocotyl phenotypes in cR light. These results showed good correlation of transgene expression with hypocotyl length especially in the sense overexpressors and may indicate that the level of *DFL2* expression determines the hypocotyl length in red light.

DFL2 expression is controlled by light but not by auxin

Using microarray techniques, it has been reported that the expression of *At4g03400* or *DFL2* is induced by cFR light irradiation and regulated by phyA (Tepperman et al. 2001). There are some *cis*-regulatory elements including a putative auxin-responsive element in the promoter region of *DFL2* (data not

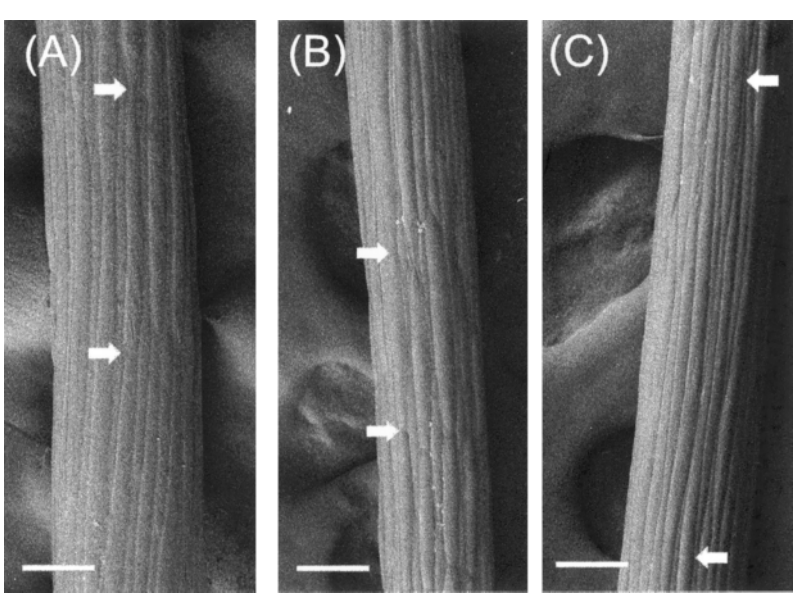

Fig. 3 Scanning electron microscopy of hypocotyl cells of wild type and *DFL2* transgenic seedlings. Wild type (A, ecotype Columbia) and *DFL2* transgenic seedlings (B, overexpressor; C, antisense line) were grown on GM plates for 5 d under cR light. After fixation, the middle part of the hypocotyl was examined with a scanning electron microscope. Arrows indicate the representative cell junctions. Bar = 100 μm.

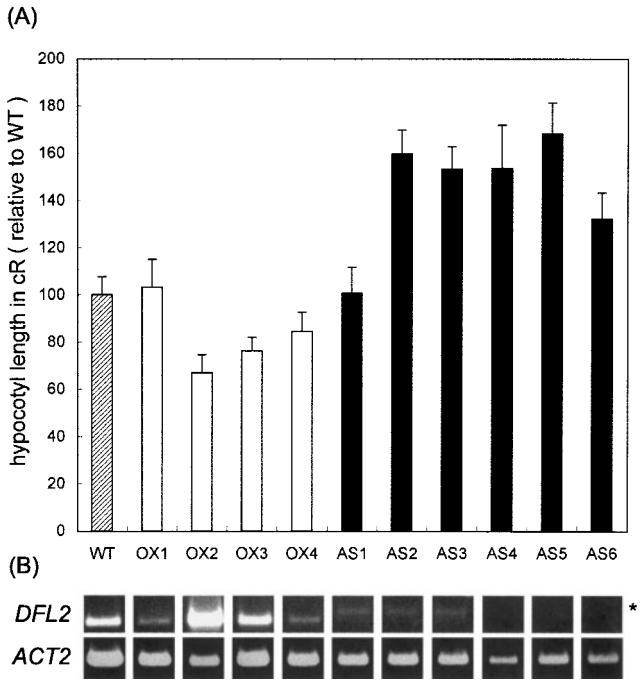

Fig. 4 The *DFL2* gene expression and hypocotyl length of transgenic lines showed correlation. (A) Hypocotyl length of 5-day-old seedlings under cR light at a fluence rate of 1 W m^{-2}. Relative hypocotyl length of four *DFL2* overexpressor lines (OX, open column) and six *DFL2* antisense lines (AS, closed column) are represented as a percentage of the wild-type (ecotype Columbia, hatched column) control. Hypocotyl length of wild type is 7.11±0.54 mm (± indicates standard error). The hypocotyl lengths of approximately 40 seedlings were measured. (B) RT-PCR analysis of *DFL2* gene expression in wild type and *DFL2* transgenic lines. Total RNA was extracted from 10-day-old seedlings grown on a GM plate under continuous white light. The *actin2* primers were used as an internal control (Li et al. 2001). 10 µl of PCR product were analyzed on a 0.8% agarose gel. Asterisk indicates *DFL2* antisense RNA that contained an intron sequence.

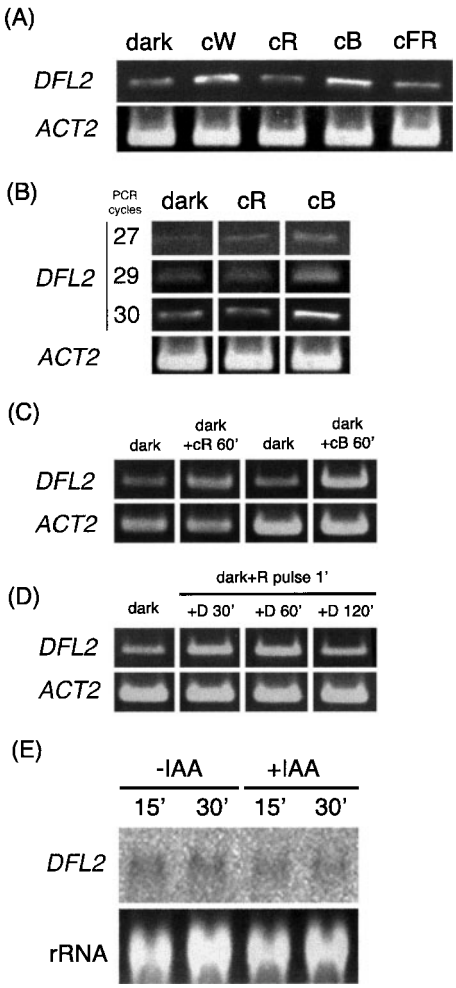

Fig. 5

shown). To understand the control of *DFL2* gene expression, we examined its expression under various light conditions and after exogenous application of auxin. We grew wild-type seedlings under cW and cB light and in darkness. Using RT-PCR, we were able to detect expression of the *DFL2* gene in the seedlings grown under cW, cB and cFR light (Fig. 5A). Taking into account that hypocotyl length is correlated with *DFL2* expression (Fig. 4), we examined the expression of *DFL2* at a rate of fluence that gave 50% hypocotyl inhibition for individual monochromatic lights. We observed very low expression under cR light and in darkness (Fig. 5A). We observed a slight increase in expression in cFR light compared to darkness (Fig. 5A). Close examination of the *DFL2* level using semi-quantitative PCR did not reveal significant differences when we compared the expression level of *DFL2* in cR and in darkness, although high expression of *DFL2* was observed under cB light (Fig. 5B).

To further understand the *DFL2* gene induction by light, we examined the *DFL2* gene expression when the dark-grown seedlings were transferred to red or blue light. RT-PCR revealed that *DFL2* gene was strongly induced by blue light treatment for 1 h (Fig. 5C). The expression level of *DFL2* gene was also increased by red light treatment for 1 h, although there was no significant difference in the level of *DFL2* gene expression between in cR and in darkness (Fig. 5B, C). This *DFL2* gene induction was also observed by 1-min red light pulse treatment, and this increased expression level was maintained at least for 1 h and decreased after 2 h incubation in dark (Fig. 5D). Therefore, our results indicated that the expression of *DFL2* is controlled both red and blue light.

To examine the regulation of the *DFL2* gene by auxin, we immersed light-grown seedlings in a solution containing 0.1 mM IAA and examined *DFL2* gene expression. As shown in Fig. 5E, we could not observe any significant difference in the *DFL2* gene expression after auxin treatment for 15 or 30 min. *DFL2* gene expression was also not induced by a short

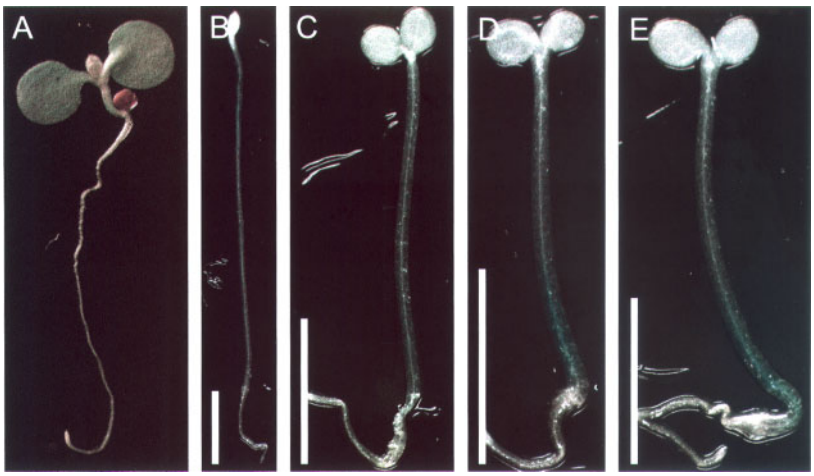

Fig. 6 *DFL2* gene was expressed in hypocotyl. Histochemical analysis of *GUS* expression in *DFL2* gene promoter-*GUS* transgenic plants. *pDFL2::GUS* transgenic seedlings were grown on GM plates in (A) cW, (B) dark, (C) cR, (D) cB and (E) cFR for 5 d. cR, cB or cFR light was irradiated at 0.03, 0.187 or 0.087 W m^{-2} respectively. Seedlings were incubated with 1 mM X-Gluc for (A) 2 h and (B to E) 4 h. Each bar indicates 2 mm length.

application of auxin (5 min treatment). The same result was obtained from RT-PCR analysis (data not shown). These results indicated that *DFL2* expression is induced by light, and not affected by auxin treatment.

DFL2 gene was expressed in hypocotyls and cotyledons

DFL2 transgenic plants mainly exhibited the hypocotyl phenotype in light. Therefore, we examined GUS activity in transgenic plants containing the *DFL2* promoter region fused to the β-glucronidase (*GUS*) gene. *pDFL2*::*GUS* contained the 1.9 kb region upstream of the *DFL2* start codon and the first nine codons of the *DFL2* gene. We examined more than ten independent lines and the *GUS*-reporter gene expression pattern was almost same for each transgenic line. After 2 h staining, *DFL2* promoter activity was observed in cotyledons and in the lower part of the hypocotyl in transgenic seedlings grown in cW light (Fig. 6A). We also checked the *DFL2* promoter activity in monochromatic light conditions and in darkness. However, GUS activity was only detected in the hypocotyl under any of the light conditions or darkness after 4 h staining (Fig. 6B–E). Strong *DFL2* promoter activity was observed in transgenic seedlings grown in cB and cFR and slightly in those grown in cR and darkness (Fig. 6B–E), consistent with the RT-PCR result (Fig. 5A). There was no GUS activity in roots in any light conditions or in darkness. But *DFL2* gene expression was observed in roots using RT-PCR (data not shown). Further observation revealed that *DFL2* promoter activity was detected in the stipules (data not shown) and was not affected by auxin treatment, as shown by Northern analysis (data not shown).

Fig. 5 Analysis of *DFL2* gene expression. (A) *DFL2* gene expression under various light conditions was analyzed by RT-PCR. Wild-type (ecotype Columbia) seedlings were grown on GM plates under cW, cR, cB, cFR light or in darkness (Dark) for 5 d. cR, cB or cFR light was irradiated at 0.140, 0.156 or 0.087 W m^{-2} respectively. Total RNA extracted from aerial parts of seedlings was used for RT-PCR analysis. The *actin2* primers were used as an internal control for RT-PCR. (B) The *DFL2* gene expression was upregulated by cB light but not by cR light. For *DFL2*, PCR amplification was conducted at 94°C for 2 min (denature), at 55°C for 1 min (annealing), and at 72°C for 2 min (synthesis) for 27, 29 or 30 cycles. After PCR, 10 μl of PCR product were analyzed on a 0.8% agarose gel. (C) *DFL2* gene expression was induced by light treatment. Wild-type seedlings were grown on GM plates in darkness for 5 d. After dark incubation, seedlings were transferred to red light (0.720 W m^{-2}) or blue light (0.191 W m^{-2}) for 1 h. Total RNAs were extracted from whole seedlings and used for RT-PCR. PCR cycle was 32 for amplification of *DFL2*. (D) Transient expression of *DFL2* gene by red light. Five-day-old dark-grown wild-type seedlings were treated with a red light pulse (0.753 W m^{-2}) for 1 min. After red light treatment, seedlings were additionally incubated in dark for 30 (D+30′), 60 (D+60′) or 120 (D+120′) min. Total RNAs were extracted from whole seedlings and were used for RT-PCR. PCR cycle was 32 for amplification of *DFL2*. (E) *DFL2* gene was not induced by auxin treatment. Wild-type (ecotype Columbia) seedlings were grown on a GM plate for 10 d under cW light and transferred to distilled water with or without 0.1 mM IAA. After incubation for 1 h, total RNA was extracted. 10 μg of total RNA were used for Northern blot analysis. Total RNA was loaded on each lane and separated by electrophoresis. After transfer to a nylon membrane filter (Hybond N+, Amersham, Tokyo, Japan), the membrane was hybridized with gene-specific probes. Ethidium bromide staining pattern of ribosomal RNAs shows equal loading.

Discussion

DFL2 is involved in red light signal transduction

In *Arabidopsis*, there are 20 *GH3*-related genes (Table 1). Some of these genes, including *FIN219* and *DFL1*, function in light signal transduction but they are involved in different aspects of the pathway (Hsieh et al. 2000, Nakazawa et al. 2001). To understand the functions of individual members of this gene family, we constructed overexpressing and antisense lines for several *GH3*-related genes. We found one member, *At4g03400/DFL2*, showed a very obvious phenotype under cW light and characterized it in detail. *DFL2* showed a shorter hypocotyl phenotype when overexpressed. These lines showed shorter hypocotyls under cR and cB light but normal hypocotyl length under cFR light (Fig. 1). *DFL2* antisense lines had long hypocotyls under cR light conditions (Fig. 2A). Our observa-

tion showed that *DFL2* transgenic plants exhibited hypocotyl phenotype only under red and blue light, although one *GH3* dominant mutant, *dfl1-D*, has short hypocotyls in various light conditions including cR, cB and cFR (Nakazawa et al. 2001). Therefore, DFL2 seems to have similar but clearly different function compared with DFL1. *DFL1* sense transgenic plants and *dfl1-D* mutants have few lateral roots compared with wild type although there are almost no differences in primary root length (Nakazawa et al. 2001). In contrast, *DFL2* transgenic plants growing in cW light did not exhibit any obvious root phenotype including primary root elongation and lateral root formation (data not shown), although we observed inhibition of the primary root in *DFL2* antisense plants that were grown under cR light conditions (Table 2). These results indicate that DFL2 is in the red light signaling pathway and is involved in hypocotyl and root elongation controlled by red light. Since *DFL2* gene was highly accumulated by blue light irradiation (Fig. 5B, C, 6D), *DFL2* level might also affect hypocotyl length under cB light. Our observation that *DFL2* antisense lines show normal hypocotyl elongation under cB light might be caused by overlapping functions of other members of the GH3-related proteins. Other transgenic lines harboring the *GH3*-related genes, *At2g14960*, *At5g13320* and *At5g13370*, did not show any obvious hypocotyl phenotypes under cW light and in darkness (data not shown). However, the antisense line of *At5g13320* seems to exhibit a phenotype under low white light (data not shown).

DFL2 expression is controlled by light

It has been reported that some *GH3* gene expression is controlled by light and regulated by phytochromes (Tepperman et al. 2001, Tanaka et al. 2002). For example, gene expression of *AtGH3a* was induced by end-of-day far-red light and regulated by phyB (Tanaka et al. 2002). To understand the control of expression of the *DFL2* gene, we examined its expression under various light conditions (Fig. 5A, B). It has been reported using DNA microarray experiments that the expression of *At4g03400*/*DFL2* is induced rapidly by cFR light (Tepperman et al. 2001). Northern blot analysis revealed that the expression of *DFL2* is induced by cFR light (data not shown), similar to the DNA microarray result. Additionally, the *DFL2* gene was upregulated by cB and cW light but the expression level was very low in the dark and in cR light (Fig. 5A, B, 6A–D) and hardly detectable by Northern analysis (data not shown). However, further analysis of light treatment of dark-grown seedlings showed the expression of *DFL2* gene was induced by red and blue light (Fig. 5C, D). These results suggested that *DFL2* gene expression was controlled by light.

Several *Arabidopsis GH3* genes including *DFL1* and *AtGH3a* have been confirmed as having auxin-inducible expression. Contrary to expectation, *DFL2* expression was not induced by exogenously applied auxin (Fig. 5E), although there are putative auxin responsive elements in the promoter region of the gene (data not shown). The auxin-responsive element (ARE) found in the promoter may not function as a true responsive element or other physiological conditions may be required to observe auxin induction of *DFL2*. Indeed, strong induction by application of auxin was not observed for some *GH3* genes (Hagen and Guilfoyle 2002, Tian et al. 2002). For example, the expression level of the *GH3-17* gene (*At1g28130*) was not elevated by auxin treatment, although the *GH3-17* gene was expressed in the absence of auxin treatment (Hagen and Guilfoyle 2002). *IAA28* gene expression was slightly repressed by exogenously applied auxin (Rogg et al. 2001), although there is one ARE located 1.5 kb upstream from the transcriptional start site of the gene (searched by PLACE; http://www.dna.affrc.go.jp/htdocs/PLACE).

DFL2 expression levels determine the hypocotyl length in red light

As shown in Fig. 3, *DFL2* overexpressing plants have shorter hypocotyl cells than wild type. On the other hand *DFL2* antisense lines have longer hypocotyl cells in cR light. There were no differences in the number of cells comprising the hypocotyl cell files in either *DFL2* sense or antisense seedlings. These results suggest that *DFL2* might regulate hypocotyl length by changing the hypocotyl cell length but not the cell number. We observed a close connection between the expression level of *DFL2* and the hypocotyl length under cR light (Fig. 4). This means that the level of *DFL2* may determine the hypocotyl length, at least under cR light. To determine whether *DFL2* gene expression is upregulated by red light, we examined the expression in darkness and in red light in detail (Fig. 5B–D). Obvious *DFL2* gene induction was observed when dark-grown seedlings were transferred to red light, although we could not observe significant differences in the levels using semi-quantitative RT-PCR in cR and in the dark. Our data showed that *DFL2* gene expression was controlled by red light, and suggested that DFL2 may regulate hypocotyl elongation by its expression level. Transient induction of *DFL2* by a red light pulse might be sufficient to regulate hypocotyl elongation.

Histochemical GUS analysis using the *DFL2* promoter region fused to the *GUS* gene revealed that *DFL2* was expressed in the hypocotyl but not in the root under light conditions and in darkness (Fig. 6). This result shows good correlation between the *DFL2* gene expression patterns and the phenotypes of *DFL2* transgenic plants. *DFL2* gene expression was observed in roots using RT-PCR (data not shown), but not by histochemical GUS analysis (Fig. 6). This expression pattern also correlated with short primary root phenotype in *DFL2* overexpressors under cR light (Table 2).

DFL2 may regulate hypocotyl elongation through specific modification of auxin by light

A biochemical approach has revealed that GH3-related proteins show the capacity to adenylate several phytohormones such as JA, IAA and salicylic acid (SA) (Staswick et al. 2002).

GH3-related proteins are classified into three groups by sequence homology, and the DFL2 protein is in group II, as is JAR1/FIN219, the protein that is most homologous to it (Staswick et al. 2002). The *JAR1* gene was isolated from a JA-responsive mutant and found to be identical to *FIN219*. Because of the high amino acid similarity between DFL2 and JAR1 (47% identities), we examined the hypocotyl phenotype of the *jar1* mutant in cR, cB and cFR light. In spite of the sequence similarity, three *jar1* alleles, *jar1-1*, a biochemically inactive mutant (Staswick personal communication), *jar1-8* (frame-sift mutant) and *jar1-9* (nonsense mutant) did not show any obvious light-dependent hypocotyl phenotype (data not shown). *fin219* was reported with elongated hypocotyls in cFR light. The difference in response to cFR between *fin219* and *jar1* mutants could be explained by the fact that mutation in *fin219* is an epigenetic mutation due to an altered methylation pattern (Hsieh et al. 2000). Elongated hypocotyls of *fin219* might be caused by misregulation of the *JAR1*/*FIN219* gene. Another possibility is that the *fin219* mutation might affect some other gene. Our result suggested that the *DFL2* antisense phenotype in cR light is not caused by suppression of a close member of the *GH3*-related gene family. There is still a possibility that overexpression of *DFL2* in the antisense orientation might suppress some other member of the *GH3* gene family, in addition to suppressing the *DFL2* gene itself. The hypocotyl phenotype of the *DFL2* antisense line might be the sum of such suppressions, but we could not observe a cR light-specific hypocotyl phenotype in transgenic plants of three members of the *GH3* family (*At2g14960*, *At5g13320* and *At5g13370*). Another possibility is that DFL2 and JAR1/FIN219 are functionally different molecules. It was reported that DFL2 did not show the capacity to adenylate any phytohormones, whereas JAR1/FIN219 showed adenylation of JA in vitro (Staswick et al. 2002). This observation indicated that there is a functional difference between DFL2 and JAR1 even though they have high amino acid similarity.

There are several possibilities as to how DFL2 regulates hypocotyl elongation. One possibility is that DFL2 might be involved in the adenylation of phytohormones even though activities are low in biochemical assays or that other protein(s) produced by light are required for the function of DFL2. DFL2 action might alter the activities of phytohormones, resulting in hypocotyl elongation under light conditions. Another possibility is DFL2 might have another function in hormone response, other than adenylation. Indeed, several plant hormones, such as cytokinin, gibberellin and brassinosteroid are known to influence hypocotyl elongation (Moller and Chua 1999).

As DFL2 shows a red light-specific phenotype we may speculate that several *GH3*-related genes in *Arabidopsis* may have specific functions depending on light signal transductions. Characterization of transgenic plants of other *GH3*-related genes may help to elucidate the functions of other members of this small gene family.

Materials and Methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* were surface sterilized and plated on GM medium (Valvekens et al. 1988) supplemented with 0.8% Bactoagar (Difco, Detroit, U.S.A.). Plates were cold treated at 4°C for 7 d then transferred to continuous white light at 22°C for 8 h to induce germination. After induction of germination, the plates were transferred to monochromatic light chambers and incubated at 22°C for 5 d. Fluence rates were measured with a radiometer (model LI-189, Li-Cor, Lincoln, NE, U.S.A.). After incubation, the seedlings were placed on plates and photographed using a digital camera. The hypocotyl and root length of seedlings were measured with National Institute of Health imaging software (see: http://rsb.info.nih.gov/nih-image/). All light conditions except the intensity were according to Peters et al. (1998).

Construction of GH3 transgenic lines

The PCR primers 4206194F (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGG AAA CTG TAG AAG CGG G-3′) and 4206194R (5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TAG TCA TAA GCT GAA CTG C-3′) were used to amplify the *At4g03400* gene. Other primers used were 3650037F/R (*At2g14960*, 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGG CGG TAG ACT CCA ACC T-3′ and 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TAT CTT CTC CTT CTC TCC G-3′), 7529287F/R (*At5g13320*, 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGA AGC CAA TCT TCG ATA T-3′ and 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT CAA ATA CTG AAG AAT TTG G-3′) and 7543904F/R (*At5g13370*, 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGT TAC CAA AGT TCG ATC C-3′ and 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TAT CTG CCG GAG TGT AAC T-3′). The template was genomic DNA from *A. thaliana* ecotype Columbia. These PCR products were subcloned into the pBluescriptGWP1 entry vector that contains *attp*1 and *att*P2 sites by BP reaction using the GATEWAY cloning system (Invitrogen Corp., Carlsbad, CA, U.S.A.). After the BP reaction, the LR reaction was performed to make an expression vector using the GATEWAY cloning system (Invitrogen Corp., Carlsbad, CA, U.S.A). The pBIDAVLGWR1 binary vector was used to make a sense construct, and the pBIDAVLGWR2 vector was used to make an antisense construct. These two plasmids consisted of *att*R1 and *att*R2, the chloramphenicol resistance gene, the *ccdB2* selection marker and DAVL sequence that is recognized by an anti-phyA monoclonal antibody. For the *DFL2* promoter-GUS constructs, *pDFL2::GUS*, PGUS11U (5′-GGG TCG ACA GTT GCT TCG AGT CCT ACC G-3′) and PGUS11L (5′-CCG GAT CCA TCA TCA TGC CCC GCT TCT AC-3′) were used to amplify the 1.9 kb upstream region of the *DFL2* gene. This PCR product was digested with *Bam*HI and *Sal*I, and subcloned into the pBI101 vector. All constructs were introduced into *Agrobacterium tumefaciens* GV3101 by electroporation and transformed into *A. thaliana* (Col-0) by vacuum infiltration (Bechtold et al. 1993).

Electron microscopy

Five-day-old seedlings grown under red light were used for scanning electron microscopy. Seedlings were fixed with 4% (w/v) paraformaldehyde for 1 d. Fixed seedlings were observed using low vacuum scanning electron microscopy (JSM-5600LV, JEOL).

RNA analysis

Extraction of total RNA was performed as described previously (Yoshizumi et al. 1999). The amount of *DFL2* mRNA was determined

by quantitative RT-PCR (Sambrook et al. 1989). The PCR primers RTU11 (5′-GGA TGG AAA CTG TAG AAG CGG GG-3′) and RTL11 (5′-GGC TAG TCA TAA GCT GAA CTG CG-3′) were used to amplify the *DFL2* gene. Equal amounts of total RNA (500 ng) were subjected to RT-PCR analysis using Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA, U.S.A.). After PCR, products were separated by 0.8% agarose gel electrophoresis. The *actin2* gene was used as an internal control for RT-PCR, and the primers for *actin2* were (5′-CTA GGA TCC AAA ATG GCC GAT GGT GAG G-3′) and (5′-GAA ACT CAC CAC CAC GAA CCA G-3′) described by Li et al. (2001).

Northern analysis was performed as described previously (Yoshizumi et al. 1999). To detect *DFL2* mRNA, a cDNA fragment was amplified using the primers RTU11 and RTL11. This PCR product was used as a template for making a probe.

Histochemical GUS analysis

Transgenic plants harboring the *pDFL2::GUS* construct were stained for GUS activity in a solution containing 100 mM sodium phosphate buffer at pH 7.0, 10 mM EDTA, 0.1% Triton X-100 and 1 mM of X-Gluc (Nakalai Tesque, Inc., Kyoto, Japan). Staining was carried out at 37°C for between 2 and 4 h. After staining, the plants were washed several times to remove chlorophyll using a graded ethanol series and then stored in 95% (v/v) ethanol.

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