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ydk1-D, an auxin-responsive *GH3* mutant that is involved in hypocotyl and root elongation

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Summary

To study the *GH3* gene family of *Arabidopsis*, we investigated a flanking sequence database of *Arabidopsis* activation-tagged lines. We found a dwarf mutant, named *yadokari 1-D* (*ydk1-D*), that had a T-DNA insertion proximal to a *GH3* gene. *ydk1-D* is dominant and has a short hypocotyl not only in light but also in darkness. Moreover, *ydk1-D* has a short primary root, a reduced lateral root number, and reduced apical dominance. A *GH3* gene, named *YDK1*, was upregulated in *ydk1-D*, and *YDK1* transgenic plants showed the *ydk1-D* phenotype. *YDK1* gene expression was induced by exogenously applied auxin and regulated by auxin-response factor (ARF)7. In addition, *YDK1* gene expression was downregulated by blue and far-red (FR) lights. Strong promoter activity of *YDK1* was observed in roots and flowers. These results suggest that YDK1 may function as a negative component in auxin signaling by regulating auxin activity.

Keywords: *Arabidopsis*, GH3, auxin, root elongation, hypocotyl elongation, light.

Introduction

Auxin is a plant hormone that regulates growth and development, including cell elongation and differentiation, tropism, apical dominance, and root initiation (Estelle and Klee, 1994; Hobbie, 1998). Auxin also regulates a variety of genes, including those of the *Aux/IAA*, *SAUR*, and *GH3* gene families (Abel and Theologis, 1996; Sitbon and Perrot-Rechenmann, 1997). Most of the genes in these families are induced rapidly by exogenously applied auxin and are called early auxin-responsive genes. In the promoter region, there are auxin-responsive elements (AREs) in which the consensus sequence 5′-TGTCTC-3′ can be found (Hagen and Guilfoyle, 2002). Auxin-response factors (ARFs) are transcriptional factors that bind to AREs and regulate the expression of auxin-responsive genes. There are 23 *ARF* genes and 29 *Aux/IAA* genes in the *Arabidopsis* genome (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). ARFs have a DNA-binding domain at the N-terminus and bind to AREs as homodimers or heterodimers with *Aux/IAA* (Kim *et al.*, 1997; Ouellet *et al.*, 2001; Tiwari *et al.*, 2003; Ulmasov *et al.*, 1997). Several mutations of *ARF* and *Aux/IAA* genes cause auxin-related developmental phenotypes, suggesting that ARF and *Aux/IAA* proteins have important functions in auxin signaling by regulation of auxin-responsive gene expression (reviewed by Liscum and Reed, 2002). For example, the loss-of-function mutation in *ARF7* caused the lack of phototropic and gravitropic responses of hypocotyls, suggesting that the ARF7 protein plays a central role in auxin-mediated differential growth (Stowe-Evans *et al.*, 1998; Watahiki and Yamamoto, 1997).

The *GH3* gene was first isolated from *Glycine max* as an early auxin-responsive gene (Hagen and Guilfoyle, 1985; Hagen *et al.*, 1984). In the *Arabidopsis* genome, *GH3* genes are presented as a multigene family consisting of 20 members. Many of *GH3* genes in *Arabidopsis* are induced by exogenously applied auxin, and some of them are also regulated by light signals (Hagen and Guilfoyle, 2002; Nakazawa *et al.*, 2001; Tanaka *et al.*, 2002; Tepperman *et al.*, 2001; Tian *et al.*, 2002). Experiments using the microarray

technique have revealed that the expression levels of some *GH3* genes are changed by far-red (FR) treatment and regulated by phytochrome A (phyA; Tepperman *et al.*, 2001). Furthermore, analysis of promoter trap lines indicates that gene expression of *AtGH3a* is induced by end-of-day FR light treatment and regulated by phytochrome B (phyB; Tanaka *et al.*, 2002).

The *fin219* gene was isolated as a suppressor of the *cop1* mutation, and this mutant exhibited longer hypocotyls under continuous FR (cFR) light (Hsieh *et al.*, 2000). However, the *jar1* mutant, allelic to *fin219*, did not show a hypocotyl phenotype under cFR (Staswick *et al.*, 2002). This discrepancy could be explained by the fact that the mutation in *fin219* is epigenetic because of an altered methylation pattern (Hsieh *et al.*, 2000). A dominant mutant of the *GH3* gene, *dfl1-D*, was isolated from activation-tagged lines as a short hypocotyl mutant in light (Nakazawa *et al.*, 2001). The *dfl1-D* seedlings also had reduced numbers of lateral roots and had epinastic leaves and cotyledons. Adult plants of *dfl1-D* exhibited a dwarf phenotype. Recently, several GH3 proteins have been reported to adenylate plant hormones *in vitro* (Staswick *et al.*, 2002). Based on the protein structure and substrate specificity, *Arabidopsis* GH3 proteins are classified into three groups. Group I is composed of two members, including JAR1/FIN219. JAR1 adenylates jasmonic acid (JA) *in vitro*. DFL1 and AtGH3a belong to group II, and most of the members of this group adenylate indole-3-acetic acid (IAA) *in vitro*. No function is known for the group III proteins, and the members of this family do not adenylate IAA, JA, or salicylic acid (SA). FIN219 and soybean GH3 proteins are located in the cytoplasm but not in the nucleus (Hsieh *et al.*, 2000; Wright *et al.*, 1987).

Activation tagging is a powerful tool to examine the function of genes that are members of a gene family (Nakazawa *et al.*, 2003). To further understand the function of *GH3* genes, we searched the T-DNA flanking sequences of activation-tagged lines for the presence of *GH3* genes. Here, we present a new dominant mutant of a *GH3* gene that showed a dwarf phenotype. This mutant, designated *yadokari 1-D* (*ydk1-D*), exhibited a short hypocotyl not only under light conditions but also in darkness. The adult plant of *ydk1-D* exhibited epinastic and small rosette leaves and reduced apical dominance. *YDK1* gene expression was regulated by auxin and light. Our results suggest that YDK1 plays a role in the inhibition of hypocotyl and root elongation by regulation of auxin activity.

Results

ydk1-D *is a dominant mutant that shows a dwarf and hypocotyl phenotype*

We have previously characterized a dominant mutant of *GH3* gene, *dfl1-D*, that had a short hypocotyl only under light conditions (Nakazawa *et al.*, 2001) and exhibited a dwarf phenotype. To understand more about the *GH3* family of *Arabidopsis*, we searched the flanking sequence database of activation-tagged lines (Nakazawa *et al.*, 2003). We found one dominant mutant that had a T-DNA proximal to a *GH3* gene that has been assigned as *GH3-2* (*At4g37390*). This mutant had small, epinastic leaves compared with the wild type, had short stems, and showed reduced apical dominance (Figure 1a,b). We designated this mutant *ydk1-D*, as the rosette phenotype showed similarity to Yadokari (a hermit crab). The heterozygous plants of *ydk1-D* showed an intermediate dwarf phenotype between homozygous and wild-type plants (data not shown). The *ydk1-D* seedlings had epinastic leaves and cotyledons (Figure 1c,d). Furthermore, the *ydk1-D* seedlings exhibited a short hypocotyl phenotype under continuous red (cR), continuous blue (cB), and cFR light conditions at all the fluence rates examined (Figure 2a,b). In contrast to *dfl1-D*, the *ydk1-D* mutant had a short hypocotyl and open cotyledons in darkness (Figure 2a,b).

Co-segregation study of ydk1-D *mutant*

Hygromycin resistance of the T_2 progeny of *ydk1-D* indicated that there were at least two T-DNA insertions in the genome of this mutant. One flanking genomic DNA sequence isolated by plasmid rescue indicated that the T-DNA was inserted on chromosome 4, and through the database search, one of the candidate genes was shown to be an *Arabidopsis GH3* gene family member (*At4g37390*). The distance between this *GH3* gene and the cauliflower mosaic virus (CaMV) 35S enhancer repeats located near the right border of the T-DNA was approximately 4.7 kbp (Figure 3a). On the other side of this T-DNA, there was a hypothetical gene (*At4g37380*), and the distance between the left border of the T-DNA and the predicted start codon of this gene was approximately 7.3 kbp. Another T-DNA was inserted on chromosome 3 in the second intron of a gene encoding a putative DNA-binding protein (*At3g11580*; data not shown).

To confirm which T-DNA insertion was responsible for the mutant phenotype, we examined the segregation of the mutant phenotype with the T-DNA insertions by genomic PCR. We checked the T-DNA insertions in 54 T_2 progeny of the *ydk1-D* mutant and isolated 21 mutant plants that showed a dwarf phenotype with one T-DNA insertion on chromosome 4. This insertion co-segregated with the dwarf phenotype (data not shown). This result strongly suggested that this T-DNA might be responsible for the *ydk1-D* mutant. All the progeny of *ydk1-D* that had a T-DNA insertion on chromosome 4 showed the short hypocotyls in the T_3 generation (data not shown). In addition, six T_2 progeny that only had the T-DNA insertion on chromosome 3 did not show any obvious phenotype (data not shown). These

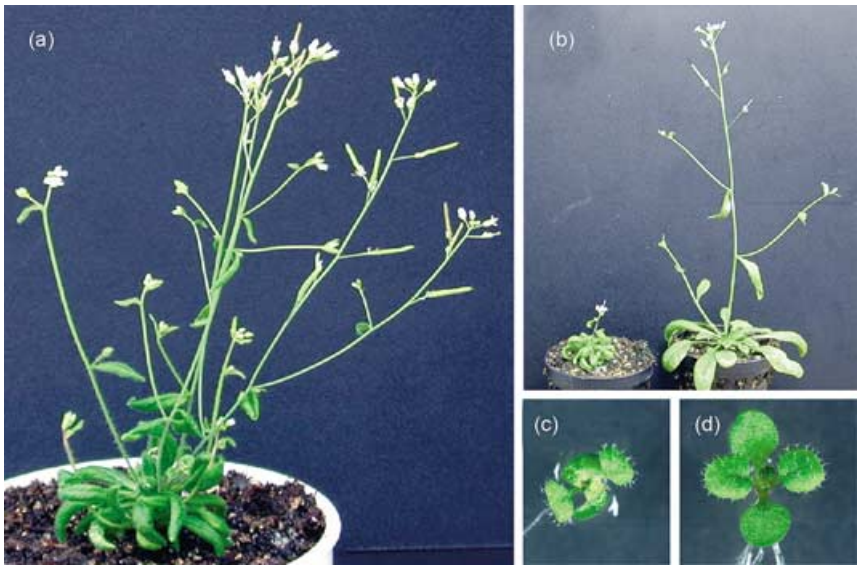

Figure 1. The *ydk1-D* mutant showed a dwarf phenotype.
(a) Adult phenotype of the *ydk1-D* mutant. A *ydk1-D* plant was grown on soil for 34 days under white light.
(b) Comparison of adult phenotype of *ydk1-D* (left) with the wild type (ecotype Col-0; right).
(c, d) Leaf phenotypes of *ydk1-D* (c) and wild-type (d) seedlings grown under white light for 10 days.

results indicated that the dwarf and short hypocotyl phenotype of *ydk1-D* mutant was caused by the T-DNA insertion on chromosome 4.

We examined the expression level of *At4g37390* in the *ydk1-D* mutant by RT-PCR. We observed increased expression in *ydk1-D* seedlings and could not detect amplified products in the aerial parts of wild-type seedlings (Figure 3b). This result suggested that increased expression of *At4g37390* was responsible for the *ydk1-D* mutation.

Overexpression of At4g37390 *recapitulates the* ydk1-D *phenotype*

To confirm whether overexpression of the *At4g37390* gene causes the *ydk1-D* phenotype, we made transgenic plants

Figure 2. *ydk1-D* showed a short hypocotyl not only under monochromatic light but also in darkness.
(a) Hypocotyl phenotype of *ydk1-D* and the wild type under monochromatic light and in darkness. *ydk1-D* (right) and wild-type (left) seedlings were incubated on GM plates under monochromatic light or in darkness for 5 days. cR, cB, and cFR lights were irradiated at 0.050, 0.160, and 0.070 W m^{-2}, respectively.
(b) Fluence rate–response curves for hypocotyl length in cR, cB, and cFR lights. Filled circles, wild type; open squares, *ydk1-D*. Each point represents the mean hypocotyl length of approximately 60 seedlings. Error bar represents SE.

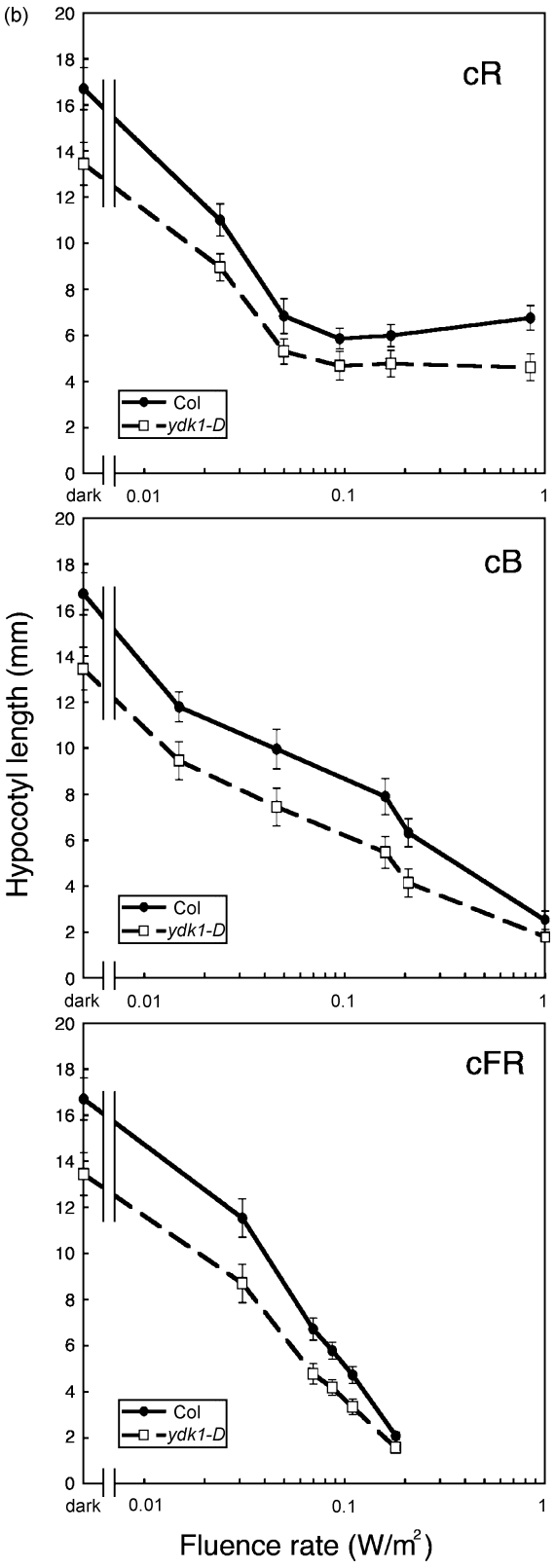

Figure 2. continued

by introducing the *YDK1* cDNA under the control of CaMV 35S promoter. We examined over 100 transgenic plants in the T_1 generation. Some of the transgenic plants showed epinastic leaves (Figure 4a), and these plants showed the dwarf phenotype and reduced apical dominance (Figure 4b,c). The frequency of dwarf transgenic plants was as low as 8 out of 100 plants. On the other hand, approximately 80% of transgenic plants had a short hypocotyl as observed in the *ydk1-D* mutant (Figure 4d). Homozygous transgenic seedlings showed an obviously short hypocotyl compared with wild-type seedlings (Figure 4d). Heterozygous transgenic seedlings had an intermediate hypocotyl length under monochromatic light conditions (data not shown). Dark-grown homozygous transgenic seedlings exhibited not only short hypocotyls but also open and expanded cotyledons (Figure 4d). These results indicated that overexpression of *At4g37390* caused the short hypocotyl and dwarf phenotype of the *ydk1-D* mutant. We designated *At4g37390* as *YDK1*, the gene responsible for the *ydk1-D* mutation.

To further confirm whether the *YDK1* gene expression level correlates with hypocotyl length, we examined the expression level of *YDK1* in transgenic lines showing various hypocotyl lengths (Figure 5a,b). Two transgenic plants, OX3 and OX4, had short hypocotyls and open cotyledons in darkness (Figure 4d for OX3). These plants had strong expression of the *YDK1* gene, although *YDK1* mRNA accumulation was not detected in aerial parts of wild-type seedlings (Figure 5b). OX7 showed moderate inhibition of hypocotyl elongation in darkness and had a moderate increase in *YDK1* gene expression compared to OX3 and OX4 (Figure 5b; see 30 and 32 PCR cycles). OX5 had a normal hypocotyl in darkness, although OX5 showed a slight increase in *YDK1* gene expression (Figure 5a,b). These results showed good correlation between the expression level of the transgene and the short hypocotyl phenotype.

ydk1-D *and* YDK1 *overexpressors cause de-etiolated phenotype*

Two photomorphogenic mutants, *cop1* and *det1*, have short hypocotyls and open cotyledons in darkness (Chory *et al.*, 1989; Deng *et al.*, 1991). Partial chloroplast development of *cop1* and *det1* mutants causes a block in the greening process when dark-grown seedlings are transferred to light. In addition, some gain-of-function mutations of *Aux/IAA* genes cause inhibition of hypocotyl elongation and partial chloroplast development in darkness (Kim *et al.*, 1998; Leyser *et al.*, 1996; Nagpal *et al.*, 2000; Reed *et al.*, 1998; Tian and Reed, 1999; Timpte *et al.*, 1994). We examined the greening process in dark-grown *ydk1-D* seedlings and in transgenic seedlings. Wild-type seedlings started greening when dark-grown etiolated seedlings were

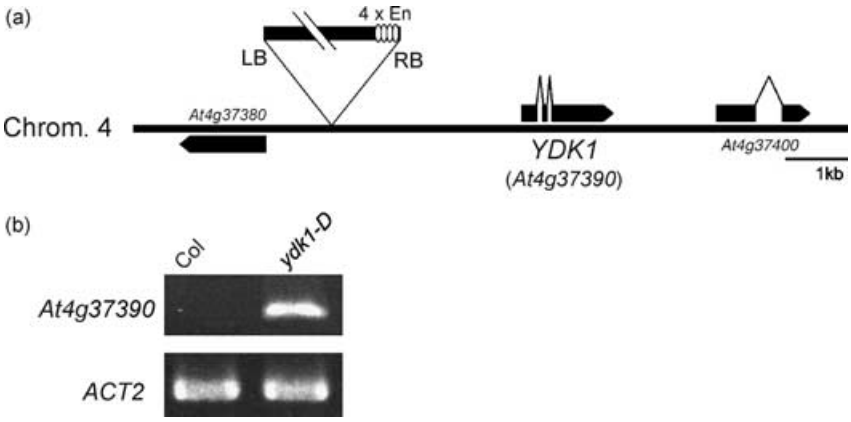

Figure 3. *YDK1* is a *GH3* gene.
(a) T-DNA insertion site in *ydk1-D*. Black bars indicate predicted genes. The four white circles show CaMV 35S enhancer repeats. The predicted genes around the T-DNA insertion are *YDK1* (*At4g37390*), *At4g37380* encoding a putative protein and *At4g37400* encoding a cytochrome P450 monooxygenase-like protein on chromosome 4.
(b) *YDK1* gene expression in *ydk1-D* and the wild type. T_3 *ydk1-D* and wild-type seedlings were grown on GM plates under white light for 7 days. Total RNA extracted from the aerial part of these seedlings was used for RT-PCR analysis. The *actin2* (*ACT2*) primers were used as an internal control for the RT-PCR. Ten microliters of PCR product was analyzed on a 0.8% agarose gel.

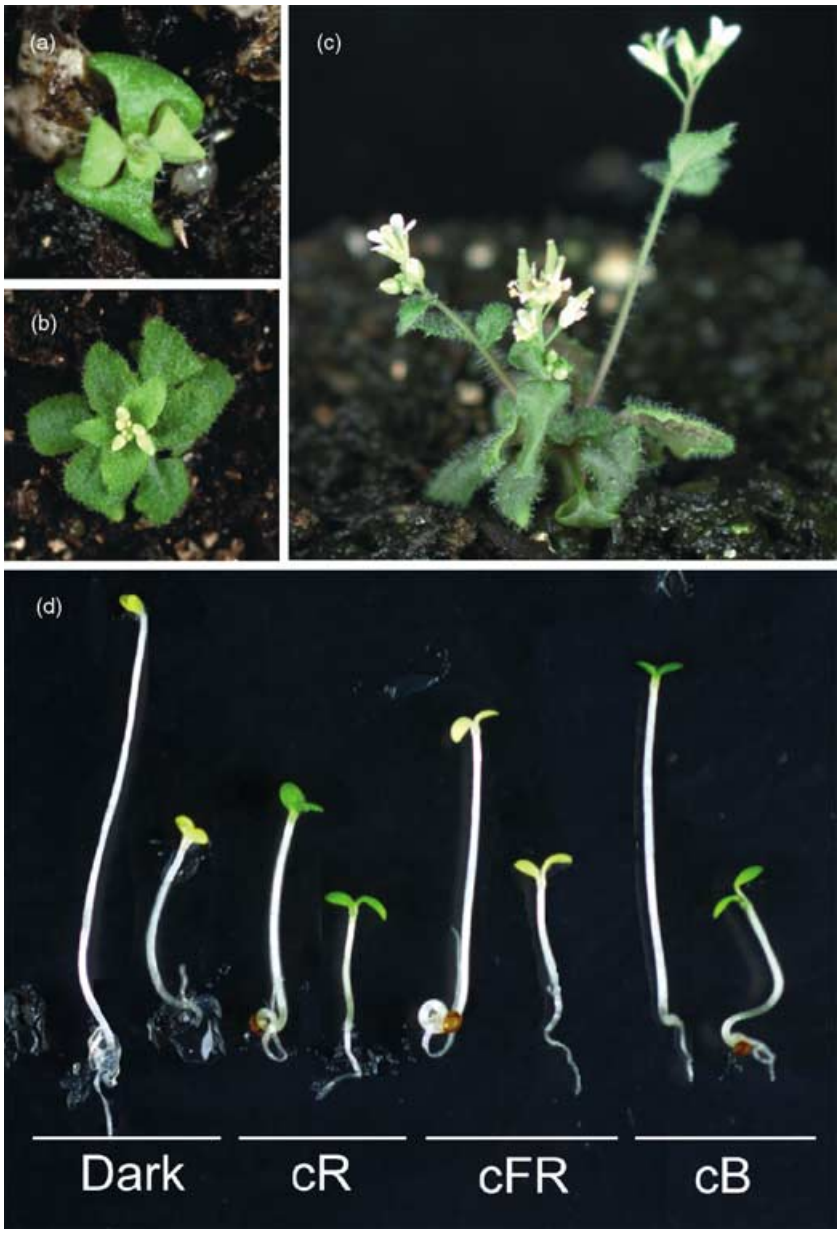

Figure 4. *YDK1* transgenic seedlings showed dwarf and hypocotyl phenotypes.
(a) Epinastic leaves of a *YDK1* transgenic plant. It was grown on a GM plate for 11 days under white light.
(b) Top view of 21-day-old *YDK1* transgenic plant grown under white light.
(c) Severe dwarf phenotype of a *YDK1* transgenic plant grown under white light for 35 days.
(d) Hypocotyl phenotype of *YDK1* transgenic seedlings and the wild type under monochromatic light and in darkness. Wild-type (left) and T_2 *YDK1* transgenic (OX3; right) seedlings were incubated on GM plates under monochromatic light or in darkness for 5 days. cR, cFR, and cB lights were irradiated at 0.051, 0.080, and 0.083 W m^{-2}, respectively.

transferred to light (Table 1). However, we observed that 7% of *ydk1-D* seedlings and approximately 60% of OX3 seedlings failed to green when moved into the light (Table 1). The expression level of the transgene was correlated with a block in the greening of cotyledons (Table 1; Figure 5b). This result indicated that overexpressing the *YDK1* gene by activation tagging or by the CaMV 35S promoter causes a block in greening and this might be the result of chloroplast differentiation in darkness.

ydk1-D *and* YDK1 *transgenic seedlings have fewer lateral roots and reduced primary root length*

As shown in Table 2, reduction of lateral root formation was observed in *ydk1-D* and *YDK1* transgenic seedlings. Furthermore, these seedlings had short primary roots compared to the wild type under various light conditions (Table 2). In the other *GH3* mutant, *dfl1-D*, primary root elongation is resistant to exogenous auxin and it also has fewer lateral roots (Nakazawa *et al.*, 2001). However, we could not observe a significant difference in sensitivity to exogenously applied auxin on primary root elongation, although the *ydk1-D* seedlings exhibited shorter primary roots than the wild type at all concentrations of auxin tested (data not shown).

YDK1 *gene expression is regulated by auxin and light*

Expression levels of several *Arabidopsis GH3* genes are increased by exogenously applied auxin (Hagen and Guilfoyle, 2002; Nakazawa *et al.*, 2001; Tanaka *et al.*, 2002; Tian *et al.*, 2002). RT-PCR analysis showed that the *YDK1* gene was strongly induced by 100 μM naphthalene acetic acid (NAA) treatment for 1 h (Figure 6a). This induction was also observed with the application of IAA, another biologically active auxin compound. The induction was as early as 15 min after treatment, suggesting *YDK1* gene is an early auxin-responsive gene (Figure 6b). ARF7 is a member of the ARF family, and a mutation in this gene causes reduced expression of auxin-responsive genes such as *SAUR*, *GH3*, and *Aux/IAA* (Stowe-Evans *et al.*, 1998). We examined whether the gene is also regulated by ARF7. *msg1-2* (*nph4-102*) is a mutant of *ARF7.* With or without NAA treatment for 1 h, *YDK1* gene expression level in *msg1-2* was low compared to that in the wild type (Figure 6a). This

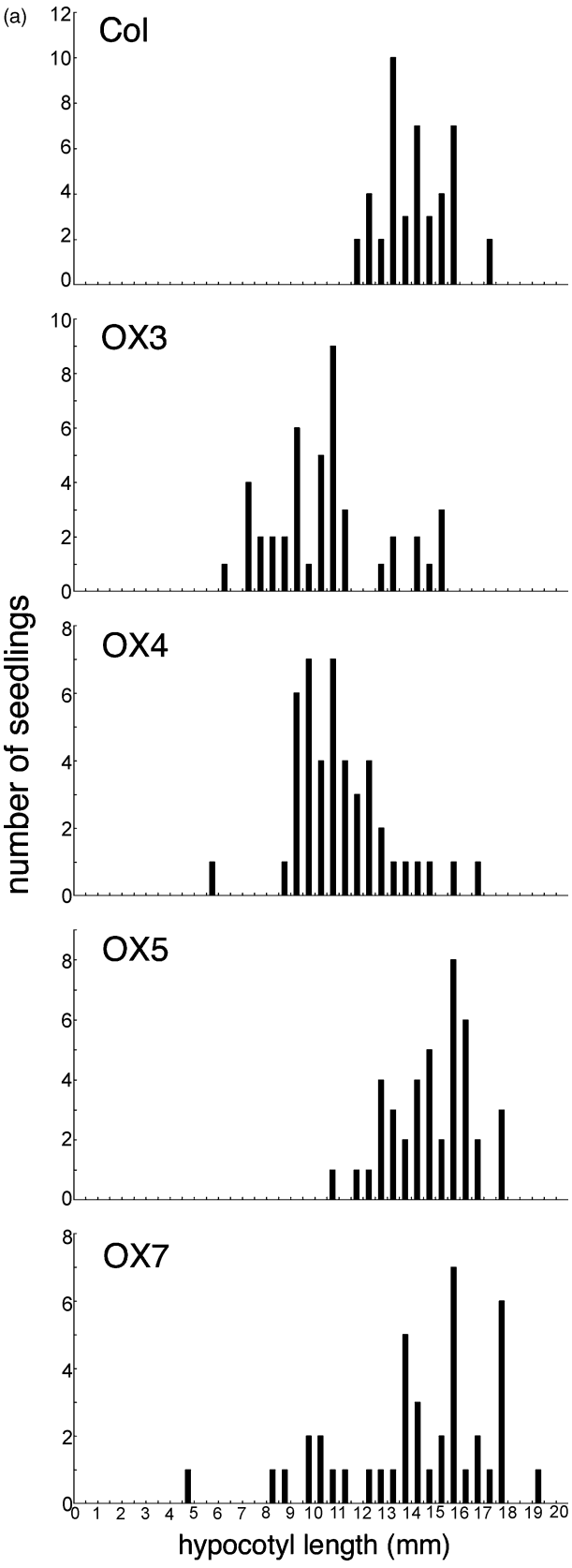

Figure 5. The *YDK1* gene expression level was correlated with a short hypocotyl phenotype in darkness.
(a) Histograms of hypocotyl length of wild-type and *YDK1* transgenic seedlings. Wild-type (Col) and *YDK1* transgenic (OX3, OX4, OX5, and OX7) seedlings were incubated on GM plates in darkness for 5 days.
(b) *YDK1* gene expression in wild-type and *YDK1* transgenic seedlings. Wild-type and *YDK1* transgenic seedlings were grown on GM plates under white light for 10 days. Total RNAs extracted from aerial parts of these seedlings were used for RT-PCR analysis.

Figure 5. continued

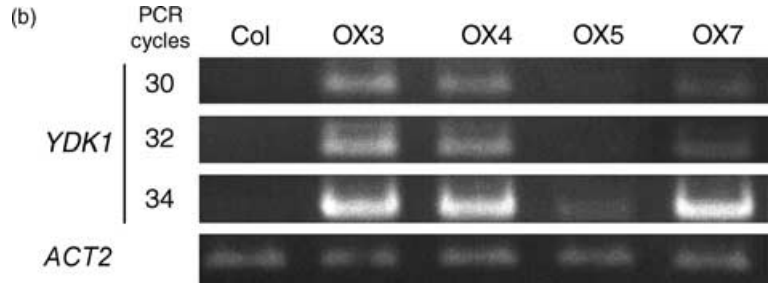

Table 1 Dark-grown *ydk1-D* and *YDK1* transgenic seedlings have been blocked in greening of cotyledons

	G	Total	Greening (%)
Col	50	50	100.0
ydk1-D	41	44	93.2
YDK1 OX3	19	44	43.2
YDK1 OX4	40	45	88.9
YDK1 OX5	42	42	100.0
YDK1 OX7	39	41	95.1

Wild-type, T_3 *ydk1-D* homozygote, and T_2 *YDK1* transgenic seedlings (OX3, OX4, OX5, and OX7) were grown on GM plates in darkness for 5 days. Each plate was transferred to white light to examine greening of cotyledons. G, the number of seedlings that had green cotyledons after transfer to light; Total, the total number of seedlings that were examined.

result may indicate that *YDK1* gene expression is mainly regulated by ARF7.

We examined the expression level of the *YDK1* gene under various light conditions. Higher *YDK1* gene expression was observed under cR light and in darkness, and it was decreased under continuous white (cW), cB, and cFR light treatment (Figure 6c). To further understand whether it was downregulated by blue or FR light, we examined *YDK1* expression in dark-grown seedlings after transfer to blue or FR light (Figure 6c). RT-PCR results indicated that *YDK1* gene expression was decreased after transfer to blue or FR light (Figure 6c). These results suggested that *YDK1* gene expression might be downregulated by light-signaling pathways, at least by blue and FR lights.

YDK1 *gene was expressed mainly in roots and flowers*

We examined tissue specificity of *YDK1* gene expression by RT-PCR. Strong *YDK1* expression was observed in roots and relatively strong expression in flowers (Figure 6d). We could not detect expression in rosettes, stems, cauline leaves, and siliques, although *ydk1-D* exhibited epinastic leaves and cotyledons, and short stems (Figures 1a and 6d).

To examine the expression of *YDK1* and *DFL1* histochemically, we made transgenic plants with the promoter region fused to the β-glucuronidase (*GUS*) gene. *pYDK1::GUS* contained a 3-kbp region upstream of the *YDK1* start codon, and *pDFL1::GUS* contained a 2.8-kbp region upstream of the *DFL1* start codon. The *pYDK1::GUS* construct included the first nine codons of *YDK1*, and the *pDFL1::GUS* construct had two codons of *DFL1*. We analyzed more than 10 independent transgenic lines for each transgene. The *GUS* reporter gene expression patterns were almost the same for each transgenic line. As shown in Figure 7, GUS activity was detected in lateral root branches in both types of transgenic seedlings (Figure 7a–e,g–i,l,m). Strong *YDK1* promoter activity was observed in these regions as soon as 15 min after staining (data not shown), and it was detected around lateral root primodia but only after 1 h staining (Figure 7c–e). Spotted *YDK1* promoter activity was observed in almost all parts of the root except for the primary root tip (Figure 7a,m). In contrast, strong *GUS* expression was observed in both lateral root primordia and lateral root tips of *pDFL1::GUS* transgenic plants with overnight staining (Figure 7g–i,l). Moreover, *GUS* activity was also detected in the primary

Table 2 *ydk1-D* and *YDK1* transgenic seedlings showed short primary roots and fewer lateral roots

	Primary root (mm)					
	cW	Dark	cR	cB	cFR	Lateral root (n) (cW)
Col	16.78 ± 1.73	2.91 ± 0.33	5.79 ± 0.66	4.76 ± 0.82	7.18 ± 0.96	2.3 ± 0.5
ydk1-D	8.89 ± 1.25	2.14 ± 0.46	3.14 ± 0.59	2.95 ± 0.57	3.80 ± 0.72	0.1 ± 0.1
YDK1 OX	9.99 ± 1.11	n.t.	n.t.	n.t.	n.t.	no

Wild-type, T_3 *ydk1-D* homozygote, and *YDK1* transgenic seedlings were grown on GM plates placed in a vertical position under various lights for 10 days. cW, cR, cB, and cFR lights were irradiated at 15.311, 0.142, 0.155, and 0.087 W m^{-2}, respectively. Primary root length and the number of lateral roots represent the mean of approximately 50 seedlings with SE. no, no lateral roots were observed; n.t., not tested.

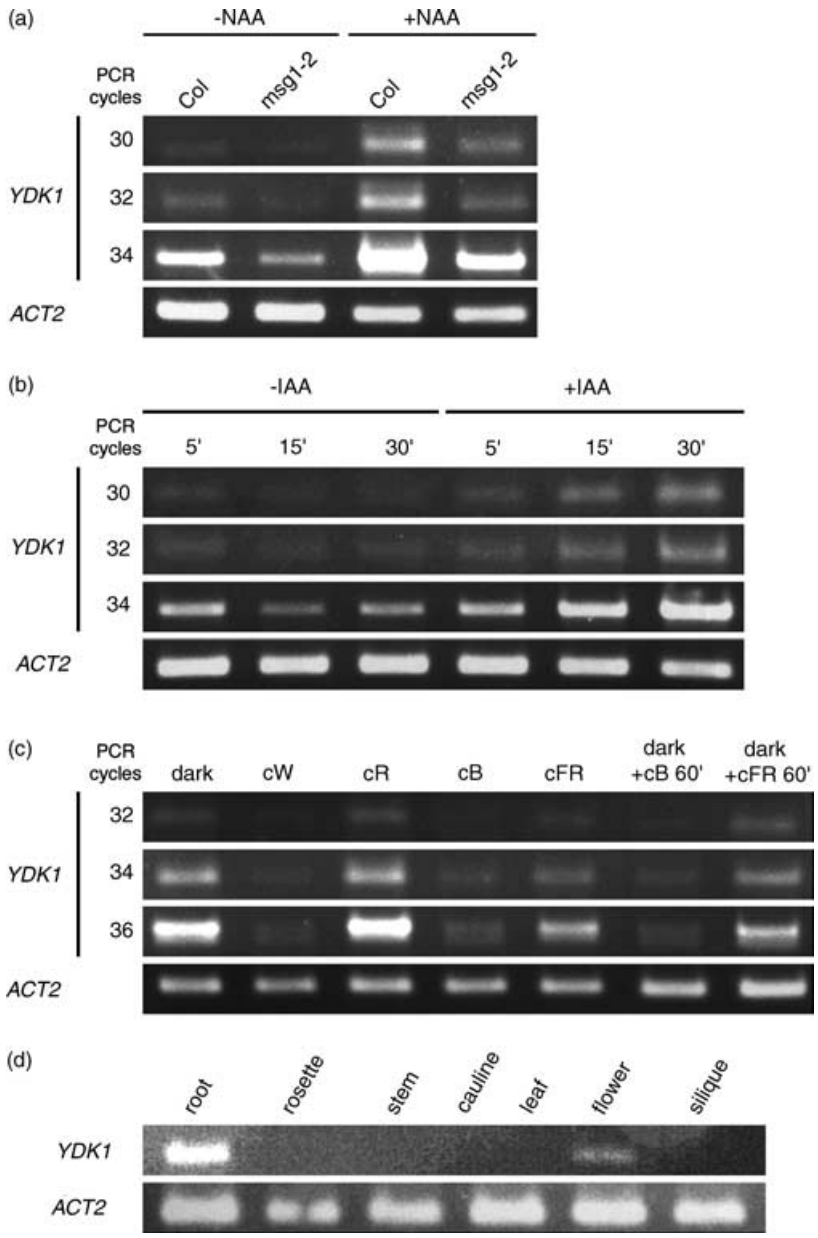

Figure 6. Analysis of *YDK1* gene expression by RT-PCR.
(a) *YDK1* gene expression was induced by exogenously applied auxin and regulated by ARF7. *YDK1* gene expression in wild type and *msg1-2* mutant with or without NAA treatment was analyzed by RT-PCR. Wild-type and *msg1-2* seedlings were grown on GM plates under white light for 10 days and transferred to distilled water with or without 0.1 mM NAA. After incubation for 1 h, total RNA was extracted from whole seedlings.
(b) Time course of *YDK1* gene expression after application of IAA. Wild-type seedlings were grown on a GM plate under white light for 10 days and transferred to distilled water with or without 0.1 mM IAA for 5, 15, and 30 min. After auxin treatments, RNA was immediately extracted from whole seedlings.
(c) *YDK1* gene expression was regulated by light. Wild-type seedlings were grown on GM plates under cW, cR, cB, and cFR lights and in darkness for 5 days. cW, cR, cB, and cFR lights were irradiated at 15.311, 0.140, 0.156, and 0.087 W m^{-2}, respectively. In addition, 5-day-old dark-grown seedlings were transferred to blue light (0.156 W m^{-2}) or FR light (0.087 W m^{-2}) for 1 h. After light treatment, total RNAs were extracted from whole seedlings.
(d) Tissue specificity of *YDK1* gene expression. Roots, rosette leaves, stems, cauline leaves, flowers, and siliques were harvested from wild-type plants.

root tips in *pDFL1::GUS* transgenic plants, although there was no *YDK1* promoter activity in the primary root tips with overnight staining (Figure 7f,j). *YDK1* promoter activity was also detected in the regions where root hairs are formed (Figure 7n). Both *GH3* genes showed promoter activity in the stipules (Figure 7n–p). Stipules are known to produce free auxin and might be involved in leaf morphogenesis (Aloni *et al.*, 2003). We could detect *YDK1* promoter activity in cotyledons, true leaves, and hypocotyls after overnight staining (Figure 7k).

The RT-PCR results indicated that the *YDK1* gene is expressed in flowers (Figure 6d). Therefore, we examined GUS activity in flowers of both types of transgenic plants. In the floral organs, strong *YDK1* promoter activity was observed in pollen, although *DFL1* promoter activity was not detected in flowers (Figure 7q,r,t). We could not observe obvious morphological differences in the flowers of *ydk1-D* compared to those of the wild type. It is unclear what function YDK1 has in pollen. GUS activity was detected in the abscission zones of flowers in both types of promoter-*GUS* transgenic plants (Figure 7s,t). Progressive changes of *YDK1* promoter activity in the abscission zones were observed (compare Figure 7r,s). Formation of these zones is inhibited by auxin (Roberts *et al.*, 2002). *GUS* expression patterns in these transgenic plants might suggest that YDK1 and DFL1 function in abscission by regulating auxin activity.

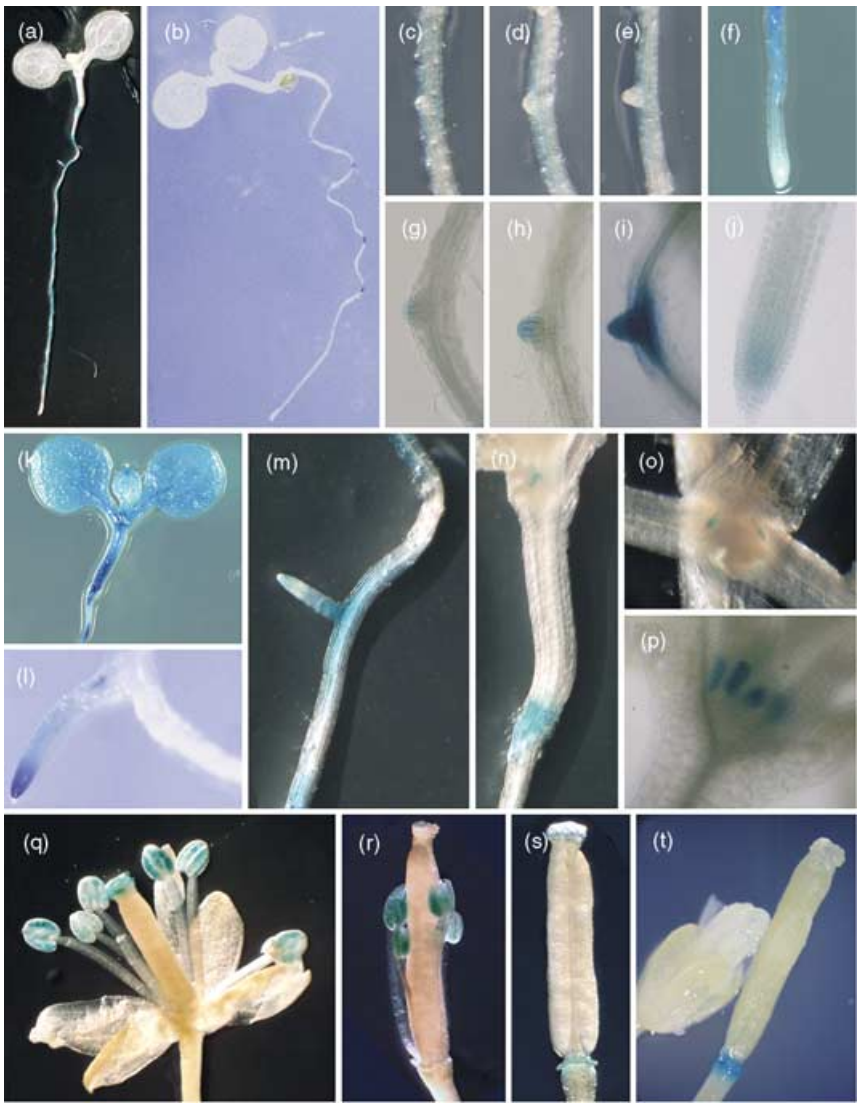

Figure 7. Histochemical analysis of *GUS* expression in *GH3* gene promoter-*GUS* transgenic plants.
(a,b), GUS activity in 7-day-old seedling; (c–e,g–i,l,m), branching point of lateral root; (f,j), primary root tip; (n), site of root hair formation; (k), aerial part of seedling; (n–p), stipules; and (q–t), flower organs and abscission zone. (a,c–f,k,m–o,q–s) GUS activities in *Arabidopsis* transgenic plants carrying *YDK1* promoter::*GUS* reporter constructs. (b,g–j,l,p,t) GUS activity in *Arabidopsis* transgenic plants carrying *DFL1* promoter::*GUS* reporter constructs. Each seedling or flower organ of transgenic lines was incubated with 1 mM X-Gluc for 30 min (c–e,o,q–s), 1 h (a,m,n), or overnight (b,f–l,p,t). (q) *YDK1* promoter activity was detected in the stigma; however, this activity was caused by staining of pollen that was attached to the stigma.

Discussion

YDK1 *is a new member of* GH3 *gene family*

We have identified a dominant dwarf mutant designated as *ydk1-D*. *ydk1-D* exhibits a short hypocotyl not only in light but also in darkness. The corresponding gene, *YDK1* (*At4g37390*), is a member of the *GH3* gene family. In *Arabidopsis*, there are 20 *GH3* gene homologs, and some of the GH3 proteins function as enzymes to adenylate plant hormones *in vitro* (Staswick *et al*., 2002). YDK1 belongs to group II, and most of the members of this group adenylate IAA *in vitro*. It is known that regulation of IAA conjugate formation is an important component in the maintenance of IAA levels (Normanly, 1997). Thus, some of the group II GH3 proteins, including YDK1, might function as negative components in auxin signaling by controlling the level of the active form of auxin. We obtained a knockout mutant of the *YDK1* gene from *Ds* transposon-tagged lines (Parinov *et al*., 1999). A *Ds* transposon was inserted in the coding region of the gene (data not shown). However, we could not find any obvious phenotypes even in hypocotyl length or root morphology compared with the wild type (data not shown). These observations may indicate that the some GH3 proteins that belong to group II might have overlapping functions. We have previously isolated *dfl1-D*, a dominant activation-tagged mutant of a *GH3* gene belonging to group II, that has a short hypocotyl only under light conditions (Nakazawa *et al*., 2001). In addition to DFL1, AtGH3a is also a member of group II and both DFL1 and AtGH3a proteins have been reported to adenylate IAA *in vitro* (Staswick *et al*., 2002). Such group II GH3 proteins may compensate YDK1 function in the inhibition of hypocotyl and root elongation in the *ydk1* knockout mutant.

YDK1 *may function in shoot and root development*

Both *ydk1-D* and *YDK1* transgenic seedlings had short hypocotyls not only in light but also in darkness, and the expression level of the *YDK1* transgene was correlated with hypocotyl length in darkness (Figure 5a,b). These observations suggested that the level of YDK1 might affect the hypocotyl length. Despite the fact that *ydk1-D* and *YDK1* transgenic seedlings showed epinastic leaves and cotyledons and a dwarf phenotype, expression of *YDK1* mRNA was not detected by RT-PCR in the aerial parts of the plant apart from that in the flowers (Figure 6d). A possible explanation of this discrepancy may be the stability of the *YDK1* mRNA. The downstream element (DST) is a highly conserved sequence in the 3′ untranslated regions (UTRs) of *SAUR* genes and is responsible for the instability of these transcripts (Newman *et al*., 1993; Sullivan and Green, 1996). There is a possible DST element in the 3′ UTR of the *YDK1* gene (data not shown). The presence of a DST element may cause rapid degradation of *YDK1* mRNA. Indeed, *YDK1* promoter activity was observed in the aerial parts of *pYDK1::GUS* seedlings (Figure 7k). In the *YDK1* promoter–*GUS* reporter construct, the *YDK1* gene, including 3′ UTR, sequence was replaced by the *GUS* reporter gene. Absence of the possible DST element in the reporter construct may result in detectable GUS activity in the aerial parts of the seedling.

The *ydk1-D* and *YDK1* transgenic seedlings showed short primary roots and reduced numbers of lateral roots in addition to short hypocotyls (Table 2). Auxin is known to regulate the elongation of primary roots and the formation of lateral roots (Estelle and Klee, 1994; Hobbie, 1998). Recently, it has been revealed that auxin regulates primary root growth by modulating the gibberellin response (Fu and Harberd, 2003). *YDK1* mRNA is highly expressed in roots (Figure 6d), and we observed *GUS* reporter expression around the region where new lateral roots emerge (Figure 7). These results may indicate that the function of *YDK1* is as a repressor of primary root elongation and lateral root formation, and this function may be achieved by specific adenylation of auxin at the site where auxin functions. The *dfl1-D* mutant also showed fewer lateral roots, but the primary root length was almost the same as that of the wild type (Nakazawa *et al*., 2001). The differences in root morphology of *ydk1-D* and *dfl1-D* might correlate with different expression patterns of these genes in roots. *DFL1*-promoter::*GUS* expression was observed mainly in the lateral root primordia and root tips (Figure 7g–j,l).

Histochemical analysis using the *YDK1* promoter region showed good correlation between expression patterns and phenotypes that were observed in *ydk1-D* and *YDK1* transgenic seedlings. This result suggested that YDK1 might play a role in root formation and shoot development. It is possible that the aerial and root phenotypes in *ydk1-D* and *YDK1* transgenic seedlings may not reflect its actual function, or that overexpression of the *YDK1* gene may activate another homologous GH3 pathway. It had been reported that overexpression of the soybean *GH3* gene in *Arabidopsis* caused a dwarf phenotype and de-etiolation in darkness (Hagen and Guilfoyle, 2002). However, *dfl1-D* and *FIN219* overexpressors did not show de-etiolated phenotypes in darkness (Hsieh *et al*., 2000; Nakazawa *et al*., 2001), suggesting that there are some non-redundant functions in the *GH3* gene family.

YDK1 *expression is controlled by auxin and light*

The *ARF7* gene is a member of the *ARF* gene family, and the protein plays a role in the auxin-dependent differential growth response (Harper *et al*., 2000; Stowe-Evans *et al*., 1998; Watahiki and Yamamoto, 1997). It has been reported that the expression levels of several auxin-responsive genes in *arf7* mutants was not increased by exogenously applied auxin (Stowe-Evans *et al*., 1998). This indicated that ARF7 was the major protein involved in activation of these genes in such conditions. In the promoter region of *YDK1* we identified one ARE approximately 250 bp upstream of the start codon (data not shown), and RT-PCR results indicated that the *YDK1* gene is an auxin-responsive gene and its expression is regulated by ARF7 (Figure 6a,b). The mutation in *msg1-2* caused the disruption of a splice site in the *ARF7* gene, and partial function of ARF7 remained (Stowe-Evans *et al*., 1998). It is possible that residual ARF7 function may cause partial auxin-responsive *YDK1* expression in *msg1-2* seedlings (Figure 6a) or that other ARFs or transcription factors might also regulate *YDK1* gene expression. There are several possible ARFs that could function as transcriptional activators (Tiwari *et al*., 2003; Ulmasov *et al*., 1999).

Several *GH3* genes are regulated by light (Tanaka *et al*., 2002; Tepperman *et al*., 2001). For example, the *AtGH3a* gene was induced by end-of-day FR light treatment and regulated by phyB (Tanaka *et al*., 2002). *YDK1* gene expression was also regulated by light (Figure 6c). However, the *YDK1* expression level does not seem to correlate with hypocotyl length under various light conditions. Despite the fact that overexpression of the *YDK1* transgene caused inhibition of hypocotyl elongation in darkness, the expression level of the *YDK1* gene was decreased by blue and FR light irradiations (Figures 5 and 6c). However, we could not detect *YDK1* mRNA expression in aerial parts of seedlings by RT-PCR (Figures 5b and 6d), and its promoter activity was low in the hypocotyls of *pYDK1::GUS* transgenic plants under various light conditions (data not shown). Thus, the RT-PCR results in Figure 6(c) might be highly reflective of the expression level of *YDK1* in roots. The YDK1 level that was regulated by light may not affect hypocotyl elongation

as its expression might be low in the hypocotyl. In contrast, there seems to be some correlation between regulation of the *YDK1* gene by light and root length. Wild-type seedlings had elongated primary roots in cW conditions compared to darkness (Table 2). In addition, *ydk1-D* had a short primary root under various light conditions, consistent with the idea that YDK1 has a role in inhibition of primary root elongation. Decrease in the *YDK1* expression level may result in an elongation of primary root that does not occur in darkness, at least in cW, cB, and cFR lights (Table 2). The expression level of *YDK1* in cR light was almost the same as that in darkness, although wild-type seedlings had elongated primary roots in cR light compared to darkness. A possible explanation is that red light might change the sensitivity of the plant to auxin. It is reported that phyA could directly interact with and phosphorylate several *Aux/IAA* proteins *in vitro* (Colon-Carmona *et al.*, 2000). In addition, *nph4* seedlings showed recovery of phototropic responses controlled by auxin with red light irradiation and the *phyA* mutation diminishes this response. This may suggest that phyA activates another ARF system apart from ARF7 (Stowe-Evans *et al.*, 2001). Altered auxin sensitivity to the auxin-signaling pathway by red light may cause the elongation of the primary root, even when expression is high under cR light.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana (Columbia (Col-0)) seeds were surface-sterilized and plated on GM medium (Valvekens *et al.*, 1988) supplemented with 0.8% Bactoagar (Difco, Detroit, USA). For measurement of hypocotyl length, plates were cold-treated at 4°C for 7 days, and then transferred to cW light at 22°C for 8 h to induce germination. After induction of germination, the plates were transferred to monochromatic light conditions or to darkness and incubated at 22°C for 5 days. Red, FR, and blue lights were generated by light emission diodes at 660, 750, and 450 nm, respectively (NK system, Japan). Fluence rates were measured with a radiometer (model LI-189, Li-Cor, Lincoln, NE, USA). For observation of root phenotypes, 1.5% agar plates were cultured vertically. After incubation, the seedlings were placed on plates and photographed using a scanning camera (KAISER Scan Do, Germany). The hypocotyl and primary root lengths of seedlings were measured with National Institute of Health imaging software (see http://rsb.info.nih.gov/hih-image/).

Co-segregation analysis of ydk1-D

We searched a T-DNA flanking sequence database of *Arabidopsis* activation-tagged lines for a mutant that had a T-DNA insertion proximal to a *GH3* gene (Nakazawa *et al.*, 2003). To check the co-segregation of the *ydk1-D* mutant phenotype and the T-DNA insertion, genomic PCR was performed. Preparation of plant genomic DNA for PCR was described previously by Klimyuk *et al.* (1993). Five primers were used for PCR to check the T-DNA insertion. LB100 primer (5′-GCCATATTGACCATCATACTCATTG-3′) matched to the left-border sequence of the T-DNA. Z066834U4 (5′-CCGTATTAATGTTTAAAAAAACGTATA-3′) and Z066834L4 (5′-CCCAAATTAACAACTCGACCTATCT-3′) were used to amplify the flanking genomic fragment of the T-DNA located on chromosome 4. Z066834U3 (5′-CCTAATTTTCATCTGTCTCGTCTC-3′) and Z066834L3 (5′-GCGTGTCTTGACGGTGGAAAAAA-3′) were used to check the T-DNA insertion on chromosome 3.

Generation of YDK1 *transgenic plants*

pBlueScript-SK(+) was digested with *Sma*I and ligated with the *Hpa*I–*Nru*I fragment from pDONR201 (Invitrogen Corp., Carlsbad, CA, USA). The resulting vector pBSKGWR2 was used to subclone the PCR products. To make the binary vector, pBI121 was digested with *Sal*I and *Sac*I and ligated with the DAVL cassette. This cassette contains an epitope tag that is recognized by an anti-phyA monoclonal antibody (Nakazawa *et al.*, unpublished data). The resulting vector, pBIDAVL, was digested with *Sma*I and ligated with the reading frame C cassette (Invitrogen Corp.). This final vector pBIDAVLGWR1 consisted of *att*R1, *att*R2, the chloramphenicol resistance gene, the *ccd*B2 selection marker, and the DAVL sequence.

To generate a *YDK1* overexpression construct, the PCR primers GWUAt4g37390 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCCGTTGATTCACCTCTTC-3′) and GWLAt4g37390 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTAACGACGTCGTTCTGGTGAC-3′) were used to amplify the *YDK1* gene. cDNAs prepared from mRNAs of light-grown Col-0 seedlings were used as PCR templates. These PCR products were subcloned into the pBSKGWP2 entry vector by BP reaction using the GATEWAY cloning system (Invitrogen Corp.). After the BP reaction, *YDK1* cDNA was transferred to the binary vector by LR reaction. For the promoter-GUS constructs, UPGUSZGH3 (5′-GGGTCGACGCTGTCGAAATTATTGGCGGAA-3′) and LPGUSZGH3 (5′-CCGGATCCCCGAGATTGAAGAGGTGAATCA-3′) were used to amplify the 3-kbp upstream region of the *YDK1* gene, and UPGUSDFL1 (5′-GGGTCGACGAAACTGGAGCCAAAAAATCAAC-3′) and LPGUSDFL1 (5′-CCGGGATCCAGGCATCGTTTAGGTTTTGTGTTTAA-3′) were used to amplify the 2.8-kbp upstream region of the *DFL1* gene. These PCR products were digested with *Bam*HI and *Sal*I, and subcloned into the pBI101 vector. These constructs were introduced into *Agrobacterium tumefaciens* GV3101 by electroporation and transferred to *A. thaliana* (Col-0 or Landsberg *erecta* (Ler)) by vacuum infiltration (Bechtold *et al.*, 1993).

RNA analysis

Extraction of total RNA was performed as described previously by Yoshizumi *et al.* (1999). The expression of the *YDK1* gene was examined by RT-PCR (Sambrook *et al.*, 1989). The PCR primers UAt4g37390 (5′-GGATGGCCGTTGATTCACCTCTT-3′) and LAt4g37390 (5′-GGCTAACGACGTCGTTCTGGTGA-3′) were used to amplify the *YDK1* cDNA. Equal amounts of total RNA (500 ng) were subjected to PCR analysis using Superscript II reverse transcriptase (Invitrogen Corp.). PCR products were analyzed on a 0.8% agarose gel. The *actin2* gene was used as an internal control for the RT-PCR, and the primers (5′-CTAGGATCCAAAATGGCCGATGGTGAGG-3′ and 5′-GAAACTCACCACCACGAACCAG-3′) were used as described by Li *et al.* (2001).

Histochemical GUS analysis

To visualize GUS activity, transgenic lines bearing the promoter–GUS fusion constructs were stained according to the following

method. Seedlings or flowers of transgenic lines were incubated with GUS staining solution (100 mM sodium phosphate buffer at pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mM of X-Gluc (Nakalai Tesque Inc., Kyoto, Japan)) for between 15 min and overnight at 37°C. After GUS staining, samples were washed several times to extract chlorophyll by using a graded ethanol series and then stored in 95% (v/v) ethanol.

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