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KIDARI, encoding a non-DNA binding bHLH protein, represses light signal transduction in *Arabidopsis thaliana*

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Abstract

Through activation tagging mutagenesis, we isolated a *kidari-D* (*kdr-D*) mutant, which exhibited a defect in blue and far-red light mediated photomorphogenesis. Under continuous blue light, the *kdr-D* mutant showed long hypocotyl phenotype, whereas it showed normal cotyledon opening and expansion. In addition, the *kdr-D* showed slightly longer hypocotyl under continuous far-red light, suggesting that *KDR* functions in a branch of cry signaling and mediates a cross-talk between cry and phyA. In the *kdr-D* mutant, a gene encoding a putative basic/Helix-Loop-Helix (bHLH) protein was overexpressed by the insertion of 35S enhancer into 10 kb upstream of the gene. Consistently, overexpression of this gene recapitulated the phenotype of *kdr-D*. KDR is composed of 94 amino acids with non-DNA binding HLH domain, a structure found in human Inhibitor of DNA binding 1 (Id-1) which functions as a negative regulator of bHLH proteins through heterodimerization with them. The KDR specifically interacted with HFR1, a bHLH protein regulating photomorphogenesis, in yeast two hybrid assay and the *kdr-D* was epistatic to *35S::HFR1* in the hypocotyl phenotype. Thus, it shows that KDR functions as a negative regulator of HFR1, similar to Id-1 in human. The *KDR* exhibited circadian expression pattern with an increase during the day. Taken together, our results suggest that *KDR* attenuates light mediated responses in day light condition through inhibition of the activity of bHLH proteins involved in light signaling.

Introduction

Throughout the life cycle of higher plants, light is a crucial environmental factor for their growth and development. As well as providing energy source for photosynthesis, light controls various developmental programs such as germination of seeds, deetiolation of seedlings, and transition to flowering (Kendrick and Kronenberg, 1994). To optimize their fitness to changing light conditions like intensity, wavelength, direction, and duration of light, plants have adopted several photoreceptors such as phytochromes (phy), cryptochromes (cry) and phototropins (Furuya, 1993; Fankhauser and Chory, 1997; Huala *et al.*, 1997; Cashmore *et al.*, 1999).

In *Arabidopsis*, two of photolyase-like blue light receptor, cry1 and cry2, have been characterized (Koornneef *et al.*, 1980; Ahmad and Cashmore, 1993; Lin *et al.*, 1998; Guo *et al.*, 1999). Both *cry1/hy4* and *cry2/fha* mutants had a defect in perceiving the blue light, thus exhibited long hypocotyl and unopened cotyledon under blue light condition. In contrast, the overexpression of *CRY1* and *CRY2* cause shorter hypocotyl and hypersensitivity to blue light, suggesting both cry1 and cry2 can mediate blue light signal (Lin *et al.*, 1998). However, the activity of cry2 is limited in

low intensity blue light most likely due to light mediated degradation of cry2 protein (Guo *et al.*, 1999). Moreover, it has also been suggested that cry1 and cry2 possess partially independent roles in blue light signal transduction, because the more severe defect of *cry1 cry2* double mutant was shown compared to either of *cry1* or *cry2* in blue light responses (Mockler *et al.*, 1999; Mazzella and Casal, 2001).

In various studies, it has been reported that cry functionally interacts with red/far-red light photoreceptor phy in diverse light mediated developmental processes (Guo *et al.*, 1998; Más *et al.*, 2000). Direct protein–protein interaction between each photoreceptor has been suggested as a possible explanation for these phenomena. Indeed, it was reported that cry1 interacted with phyA in yeast two-hybrid experiment, and recombinant oat phyA phosphorylated cry1 *in vitro* (Ahmad *et al.*, 1998). The interaction between phyB and cry2 was shown by both yeast two hybrid assay and coimmunoprecipitation analysis (Más *et al.*, 2000). This direct interaction of phyB–cry2 is known to be involved in inhibition of hypocotyl growth, regulation of circadian clock, and control of flowering time (Más *et al.*, 2000). However, the functional implication of these interactions is still unclear.

Modulation of common signaling component is another possible mechanism for the functional interaction of photoreceptors. Recently, various genetic screens have identified mutants with a defect in both blue and red/far-red light responses. The *LONG HYPOCOTYL IN FAR-RED 1* (*HFR1*), also referred as *REP1* and *RSF1*, was first identified as a positive component in phyA signaling pathway (Fairchild *et al.*, 2000; Fankhauser and Chory, 2000; Soh *et al.*, 2000). However, it was lately found that *hfr1* also had a defect in blue light signaling (Duek and Fankhauser, 2003). HFR1 encodes a bHLH transcription factor, and the *hfr1* null mutant exhibited reduced photomorphogenesis in both blue and far-red light conditions. Therefore, it has been suggested that HFR1 functions in branching point of cry and phyA signaling cascades.

The *SHORT UNDER BLUE LIGHT 1* (*SUB1*) is another example of common downstream intermediate of cry and phyA (Guo *et al.*, 2001). *SUB1* encodes a calcium binding protein and its deficiency results in short hypocotyl phenotype in both blue and far-red light condition. Interestingly, *sub1* was only epistatic to *crys*, meanwhile the *sub1 phyA* double mutant showed the same phenotype with *phyA*. This result suggested that the SUB1 is a downstream component of cry signaling cascade, but only modulates phyA activity in far-red light signal transduction.

Through activation tagging mutagenesis, we have identified *KIDARI (KDR)* as a new repressor of cry signaling and a modulator of crosstalk between cry and phyA. *KDR* encodes a homolog of human Inhibitor of DNA binding 1 (Id-1), which inhibits DNA binding of bHLH proteins by heterodimerization (Ohtani *et al.*, 2001; Toledo-Ortiz *et al.*, 2003). The overexpression of *KDR* suppressed blue light dependent inhibition of hypocotyl elongation, and such negative effect of *KDR* was also weakly found in far-red light condition. The KDR interacted with HFR1 in yeast two hybrid assay, suggesting the possible negative regulation of HFR1 activity by KDR. Consistent with these results, the *kdr-D* was epistatic to the *35S::HFR1*. Moreover, we observed a circadian clock regulated expression of *KDR* that was peaked at noon. Taken together, our results suggest that *KDR* attenuates the overinduction of light responses in day light condition probably through an inhibition of the activity of bHLH proteins involved in light signaling.

Materials and method

Plant materials and growth condition

All plants that used in this study were Col-0 background. Seeds were sterilized by incubation in freshly prepared 70% ethanol plus 0.05% (v/v) Triton X-100 for 10 min and then washed two times with 100% ethanol. The surface sterilized seeds were sown on MS plates and were cold treated for 3 days at 4 °C. The plates then were transferred to 16 h light/8 h dark cycles at 22 °C. At 7 DAG (days after germination) stage, seedlings were transplanted on soil. For measuring flowering time, the number of rosette leaves at bolting was counted. For circadian rhythm experiment, the plates were placed in the growth room with 12 h light/12 h dark cycles for 8 days and then transferred to continuous light.

Light response test

The seeds were sown on MS minus sucrose media and the plates were kept 3 days under dark at 4 °C, then illuminated with white light for 4 h before transfer to monochromatic light chamber. Far-red, red, and blue light was supplied by LED light sources (Good Feeling, Korea) for 3 days, with fluent rates of ~0.5, 30 and 20 μmol/m^{2}s, respectively. White light was supplied by cool-white fluorescent lamps. Data were collected from 40% of the longest seedlings to minimize variation in hypocotyl lengths. For phototropism test, 4 days dark grown seedlings were used. Unidirectional blue light was subjected to the etiolated seedlings for 2 days and the curvature of hypocotyl was measured.

Confirmation of T-DNA insertion and cloning of flanking region

Single copy of T-DNA insertion was confirmed by Southern blot hybridization analysis. Genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Total of 2 μg genomic DNA was digested with each restriction enzyme, and was separated on 1% (w/v) agarose gel. Separated digestion products were then transferred to Hybond™-N+ membrane (Amersham Biosciences, UK). Randomly ^{32}P-labeled *Basta^R* gene of T-DNA was used as a probe. T-DNA insertion locus was identified by TAIL-PCR amplification. All procedures in these experiments were performed as previously described (Liu *et al.*, 1995). The T-DNA left border derived primers were AtLB1 (5′-ATACGACGGATCGTAATTTGTC-3′), AtLB2 (5′-TAATAACGCTGCGGACATCTAC-3′), and AtLB3 (5′-TTGACCATCATACTCATTGCTG-3′). After PCR amplification, all positive products were directly sequenced.

Gene expression analysis

In all experiments, total RNA was extracted from 10 days old seedlings using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Through RT-PCR amplification, the expression level of each gene was analyzed. The sequences of primers used were HYB11F (5′-ATGTTCGAAGGTCGTCCTCG-3′)/HYB11R (5′-TCAACAACTCATCACGGCGC-3′) for *At1G26930*, HYB12F (5′-ATGGGAATCACGAGAAACTTG-3′)/HYB12R (5′-TCACGGGAAGCACTTCATTC-3′) for *At1G26940*, HYB13F (5′-ATGTCTAGCAGAAGATCATC-3′)/HYB13R (5′-GCAAGCTCCTAATGATGGCTG-3′) for *At1G26945*, and HYB14F (5′-ATGTTTGCGAGAACAAGCC-3′)/HYB14R (5′-TTACATGGGCACATGTCTAG-3′) for *At1G26950*. For Northern blot analysis, 10 μg of total RNA was loaded in each lane. The *KDR* cDNA was used as a probe.

Generation of KDR *overexpressing plant*

To make *35S::KDR* construct, the genomic *KDR* region was amplified by PCR from Col-0 DNA, with primers HYB25F (5′-GCGCGGATCCATGTCTAGCAGAAGATCA-3′) and HYB25R (5′-GCGCGGATCCTTAATAATTAAGCAAGCT-3′) that have *BamH*I sites. The binary vector that contains CaMV 35S promoter was constructed by the modification of pCAMBIA-1303 plasmid (accession number: AF234299). At first, the GUS-GFP fragment of pCAMBIA-1303 was removed by restriction enzyme cut with *Bgl*II and *BstE*II, then oligonucleotide linker sequence (5′-AGATCTCCACTAGTGGTGACCA-3′) was added to *Bgl*II and *BstE*II site. The amplified *KDR* genomic fragment was directly digested using *BamH*I restriction enzyme, and was subcloned into *Bgl*II site of the modified binary vector. After sequence analysis, the clones without any sequence errors were used in plant transformation. Transgenic plants were generated using the *Agrobacterium tumefaciens*-mediated floral-dip method (Clough and Bent, 1998).

Yeast two hybrid assay

The Matchmaker Two-Hybrid System (Clontech) was used for the yeast two hybrid assay. For making DNA binding domain and transcription activation domain fusion construct, pGBKT7 and pGADT7 plasmid were used, respectively. The open reading frames of analyzed genes were amplified by PCR from RT product of 10 DAG Col-0 seedlings. The sequences of primers used were HYB97F (5′-CGGAATTCATGTCTAGCAGAAGATCATCAC-3′)/HYB97R (5′-CGGAATTCTTAATAATTAAGCAAGCTCCTA-3′) for *KDR*, HYB98F (5′-CGGAATTCATGTCGAATAATCAAGCTTTCA-3′)/HYB98R (5′-CGG

AATTCTCATAGTCTTCTCATCGCATGG-3′) for *HFR1*, HYB99F (5′-CGGGATCCCGATGG AAGCAAAACCCTTAGC-3′)/HYB99R (5′-CG GGATCCTTAGTTTGGCGAGCGATAATAA-3′) for *PIL1*, HYB100F (5′-CGGAATTCATGGA TCCTCAGCAGCAACCTT-3′)/HYB100R (5′-CGGAATTCTTAACCTGTTGTGTGGTTTCC G-3′) for *PIL5*, and HYB104F (5′-GGATCCCGG ATGAATCGGAGGAGTTTACA-3′)/HYB104R (5′-GGATCCCTACACCCCCACTGTTGACAT CAT-3′) for bHLH domain of *HFR1*. Each PCR product directly inserted to the pGBKT7 and GADT7 plasmid after digestion with appropriate restriction enzyme (for *KDR* and *HFR1*:*Eco*RI, for *PIL1*, *PIL5*, and bHLH domain of *HFR1*:*BamH*I). After sequences analysis, the clones without any errors were used in the assay. Plate assays (-His) was performed according to the manufacturer's protocol.

Results

Screening of light signaling deficient mutant

To isolate new negative components of light signal transduction, we performed activation tagging mutagenesis using Col-0 ecotypes (Weigel *et al.*, 2000). Because most of the photoreceptor mutants generally showed long hypocotyls and petioles in white light condition, we focused on the long petiole phenotype in this screening. As a result, we have isolated 10 mutant candidates from 4000 primary T1 transformants and one mutant showed stably inherited phenotype through generations. This mutant exhibited long petiole, slightly pale green leaves and early flowering phenotype under white light (Figure 1A and B, and data not shown). Because the early flowering phenotype was consistently cosegregated with the long petiole and pale green phenotype, we concluded that a mutation of single gene caused all of the phenotypes. This mutant was named as *kidari-D* (*kdr-D*), a word that means a tall man in Korean.

Activation tagging mutagenesis generally causes overexpression of a gene which is closely linked to the inserted T-DNA that includes four copies of CaMV 35S enhancer (Figure 1C). Therefore, the phenotype of activation tagging mutant is dominantly inherited, and cosegregates with T-DNA insertional marker such as antibiotics resistance. To test whether the *kdr-D* mutant possesses these features, we backcrossed *kdr-D* to wild type Col-0 plant. All of the resulting F1 plants showed long petiole, pale green leaves and early flowering phenotype. Especially, the phenotypic severity of F1 plant was not different to that of *kdr-D*. Subsequent F2 populations exhibited approximately 3:1 segregation ratio for mutant to wild type phenotype (56 mutant: 24 wild type, $\chi^2 = 1.6$, $p < 0.1$). These results strongly suggested that a dominant single gene mutation occurred in the *kdr-D* mutant. To further examine if the mutant phenotype cosegregates with T-DNA insertion, we have harvested progenies of all F2 plants independently and tested their Basta resistance. All of the 56 mutants from F2 population produced Basta resistant progenies, whereas 24 wild type plants produced only Basta sensitive progenies. Taken together, our results showed that single locus was inserted by T-DNA in *kdr-D* genome and it caused the dominant long petiole, pale green leaves and early flowering phenotype.

Phenotypic characterization of the kdr-D *mutant*

To identify which light signaling cascade is disrupted in *kdr-D*, we analyzed various developmental responses of the *kdr-D* under diverse monochromatic light condition. The most conspicuous phenotype was observed in continuous blue light condition (Figure 2A). It was reported that the biological function of *CRY2* is limited in low intensity (<10 μmol/m^{2}s) blue light (Lin *et al.*, 1998; Guo *et al.*, 1999). Consistent with this, the *cry2* mutant showed weaker phenotype in our higher fluence (20 μmol/m^{2}s) blue light condition. Therefore, it is likely that the signaling cascade of *CRY2* was in less active state in our condition and the long hypocotyl phenotype of *kdr-D* is mainly due to the defect in *CRY1* signal transduction. Although the *kdr-D* mutant showed normal inhibition of hypocotyl elongation under continuous red light, it showed slightly longer hypocotyl than wild type but shorter hypocotyl than *phyA* mutant under continuous far-red light (Figure 2A). Taken together, our results suggests that the *KDR* plays a role in both blue and far-red light

signaling, while its role is more prominent in blue light signal transduction.

In addition to the inhibition of hypocotyl elongation, cry also mediates the blue light dependent cotyledon opening/expansion of *Arabidopsis* seedlings (Casal, 2000; Lin, 2002). Consistent with this, the *cry1* mutant exhibited unopened cotyledons in our blue light condition (Figure 2B), while cotyledons of *cry2* were more expanded than those of *cry1*. However, it was found that cotyledon opening/expansion was normally occurred in *kdr-D* (Figure 2B).

Additionally, we analyzed phototropic response of *kdr-D*. Although phototropism is also induced by blue light, another blue/UV-A light photoreceptor, phototropin, mainly participates in this response (Huala *et al*., 1997; Lin, 2002). To test whether the *kdr-D* mutant is also defective in phototropin signaling cascade, unidirectional blue light was subjected to etiolated seedlings of *kdr-D*. As shown in Figure 2C, the *kdr-D* mutant did not show a defect in phototropic response. This result shows that the phototropin signal transduction is not disrupted in the *kdr-D* mutant.

Molecular cloning of the KDR *gene*

As described above, we have predicted a single insertion of T-DNA in *kdr-D*. However, it is possible that multi-copies of T-DNA were inserted in very tightly linked chromosomal region, and segregated together. Therefore, we confirmed a single copy T-DNA insertion by Southern blot analysis before the identification of T-DNA flanking locus. As a result, a single copy T-DNA was detected in genomic DNA fraction of *kdr-D* (Figure 3B). Based on this result, we finally ncluded that there is only a single copy T-DNA insertion in the *kdr-D* genome.

To identify the flanking sequence of T-DNA insertion, we performed high throughput thermal asymmetric interlaced PCR (TAIL-PCR) (Liu *et al*., 1995). With primers derived from the T-DNA left border, we amplified three positive

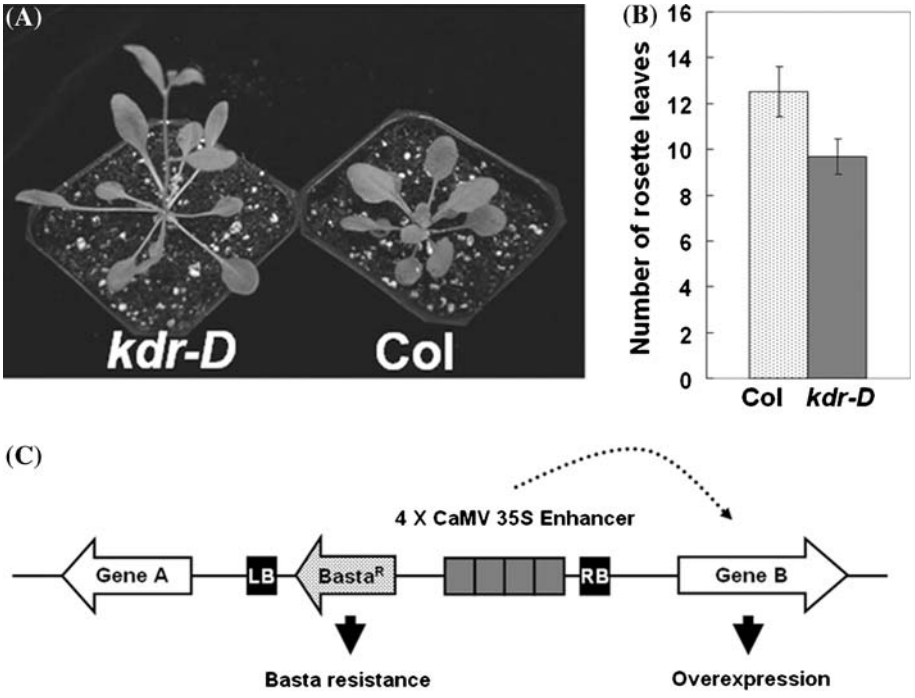

Figure 1. The genetic isolation of light signaling suppressor mutant. (A) Morphology of the *kdr-D* at adult stage. Longer petiole and earlier flowering time than wild type are observed. Both plants were grown for 30 days in 16 h light/8 h dark cycle long day photoperiod. (B) Flowering time of the *kdr-D*. Flowering time is presented as the number of rosette leaves produced when flowering. The *kdr-D* exhibits early flowering phenotype. (C) Schematic diagram of activation tagging mutagenesis. Activation tagging mutagenesis causes overexpression of a gene by inserted four copies of CaMV 35S enhancers (dotted arrow). Basta resistance gene (Basta^R) of T-DNA makes transgenic plants survive in Basta containing media. LB and RB mean left and right borders of T-DNA, respectively.

TAIL-PCR products. Subsequent sequence analysis of amplified products revealed that the T-DNA was inserted in the intergenic region between *At1G26940* and *At1G26945* on chromosome 1 (Figure 3A). The 35S enhancer was inserted about 5 kb apart from the start codon of *At1G26940* which was annotated as an expressed protein, and about 10 kb apart from the start codon of *At1G26945* which encodes a putative bHLH protein. Through RT-PCR analysis, we confirmed that the *At1G26945* was overexpressed in the *kdr-D* mutant, whereas expressions of other nearby genes were not changed (Figure 3C). To confirm whether the overexpression of *At1G26945* indeed caused phenotypes of the *kdr-D* mutant, we introduced the transgene that contains *At1G26945* genomic fragment under the control of 35S promoter into the wild type Col-0 plant. After *Agrobacterium*-mediated *Arabidopsis* transformation, total of 10 primary transformants were obtained. Although the phenotypic severity was variable in the transgenic plants, all of transformants recapitulated the *kdr-D* mutant phenotypes as long petiole (Figure 4A) and long hypocotyls under blue and far-red light (Figure 4B). In addition, we confirmed the stable overexpression of *At1G26945* in two

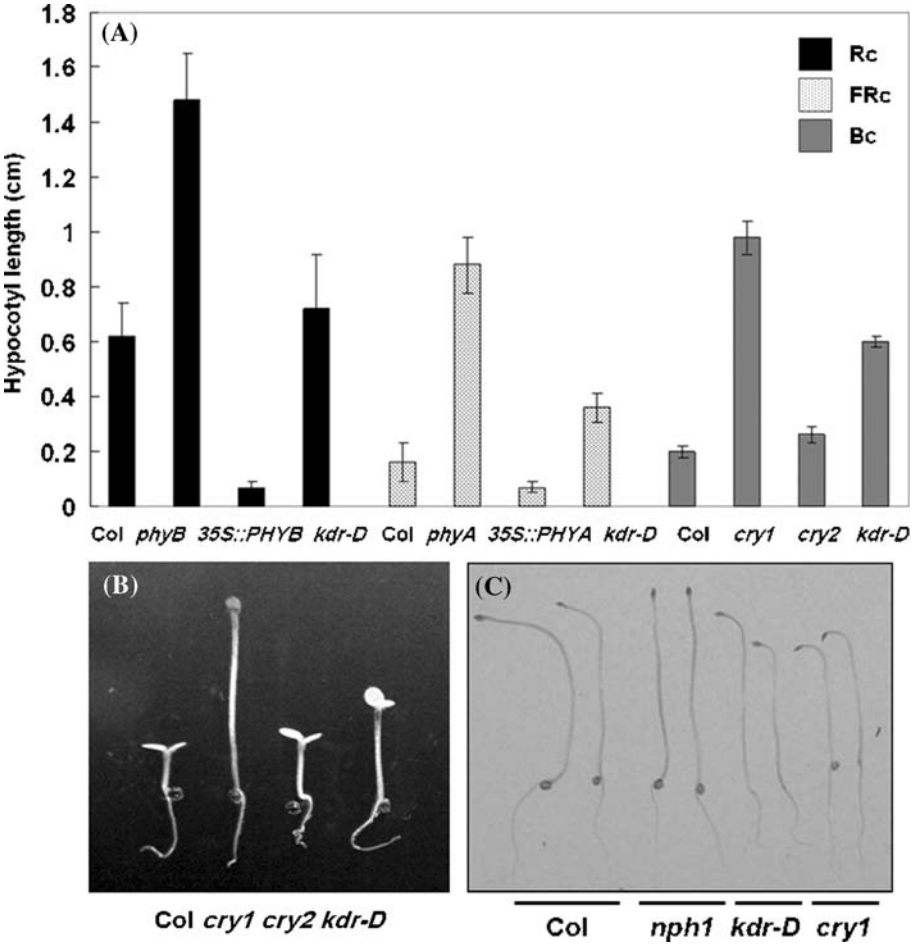

Figure 2. Phenotypic characterization of the *kdr-D* mutant. (A) Hypocotyl length of the *kdr-D* grown in monochromatic light. The *kdr-D* exhibits long hypocotyl phenotype in both continuous blue (Bc) and far-red (FRc) light condition, but phenotypic severity under far-red light is weaker than that of blue light. In continuous red (Rc) light, the *kdr-D* exhibits similar phenotype with wild type. (B) Cotyledon opening and expansion of the *kdr-D* in continuous blue light condition. Cotyledon opening/expansion is normally occurred in *kdr-D*. (C) Phototropic response to unilaterally subjected blue light. While *nph1* (*non-phototropic hypocotyl protein 1*) null mutant shows defect in phototropic response to blue light, the *kdr-D* shows normal phototropic response. The blue light was subjected from left.

representative transgenic plants that show the same severe phenotype as *kdr-D* (Figure 4A). Hence, we concluded that *At1G26945* is indeed the *KDR* gene.

KDR *protein structure and functional relatives of the* KDR

The *KDR* gene is composed of two exons and one intron (Figure 5A). The open reading frame of *KDR* encodes 94 amino acids. The resulting polypeptide contains Helix-Loop-Helix (HLH) domain in its central region. However, except for this HLH domain, there are no known functional domains in KDR (Figure 5B). Through BLAST searches, we have found eight additional proteins that showed amino acid sequence homology with KDR, five homologs from the *Arabidopsis* and three homologs from the rice. All of these proteins contained ~100 amino acids, and showed high structural similarity with KDR (Figure 5C). Especially, *Arabidopsis* homolog proteins shared more than 90% of amino acid sequence identity with KDR protein. Thus, it is very likely that KDR family proteins play redundant roles in diverse genetic pathways. As expected, the loss of function *kdr* mutant (SALK-143732 that has a T-DNA insertion in the first exon) did not show any visible defects in light response (data not shown).

Interestingly, KDR family proteins also shared homology with *Homo sapience* Inhibitor of DNA binding 1 (Id-1) (Figure 5C). As the name of Id-1 implies, the Id-1 inhibits binding of other bHLH transcription factors to their target DNA element

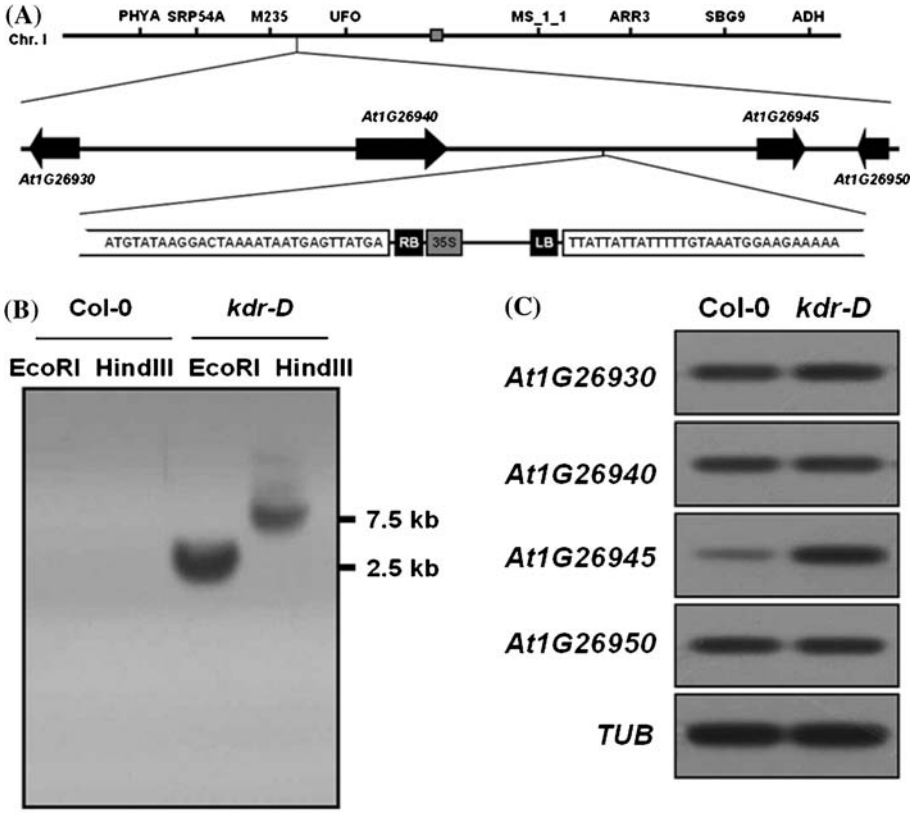

Figure 3. Molecular cloning of the *KDR* gene. (A) Schematic diagram of T-DNA flanking locus in the *kdr-D* mutant. T-DNA is inserted intergenic region between *At1G26940* and *At1G26945*. RB, 35S and LB mean right border, CaMV 35S enhancer and left border of T-DNA, respectively. (B) Confirmation of single copy T-DNA insertion in the *kdr-D* by Southern blot analysis. The Basta resistance gene of T-DNA was used as probe. Single copy of T-DNA signal is detected in 2.5 and 7.5 kb of EcoRI and HindIII digestion product of the *kdr-D* genomic DNA, respectively. (C) Identification of the *KDR* gene by RT-PCR analysis. Using gene specific primers, RT-PCR analysis was performed. The *At1G26945* is overexpressed specifically in the *kdr-D*. Total RNA was extracted from 10 days old seedlings grown in long day photoperiod condition, and *TUB* gene was used as control for the quantification.

(Ohtani *et al*., 2001; Toledo-Ortiz *et al*., 2003). Generally, common bHLH proteins form either homo- or heterodimers through their HLH domains, meanwhile the basic motif is responsible for binding of the dimers to their target DNA element. However, heterodimers resulting from the interaction between Id-1 and other bHLH protein have lost the DNA binding ability because the Id-1 lacks this basic motif in its bHLH domain. In a previous study, total of 147 bHLH proteins have been found in *Arabidopsis* genome by database searches (Toledo-Ortiz *et al*., 2003). Among them, 27 proteins were categorized to the non-DNA binding AtbHLH (called simply as HLH proteins), and predicted to function as Id-1 like transcription factors. These proteins have an average of 3.8

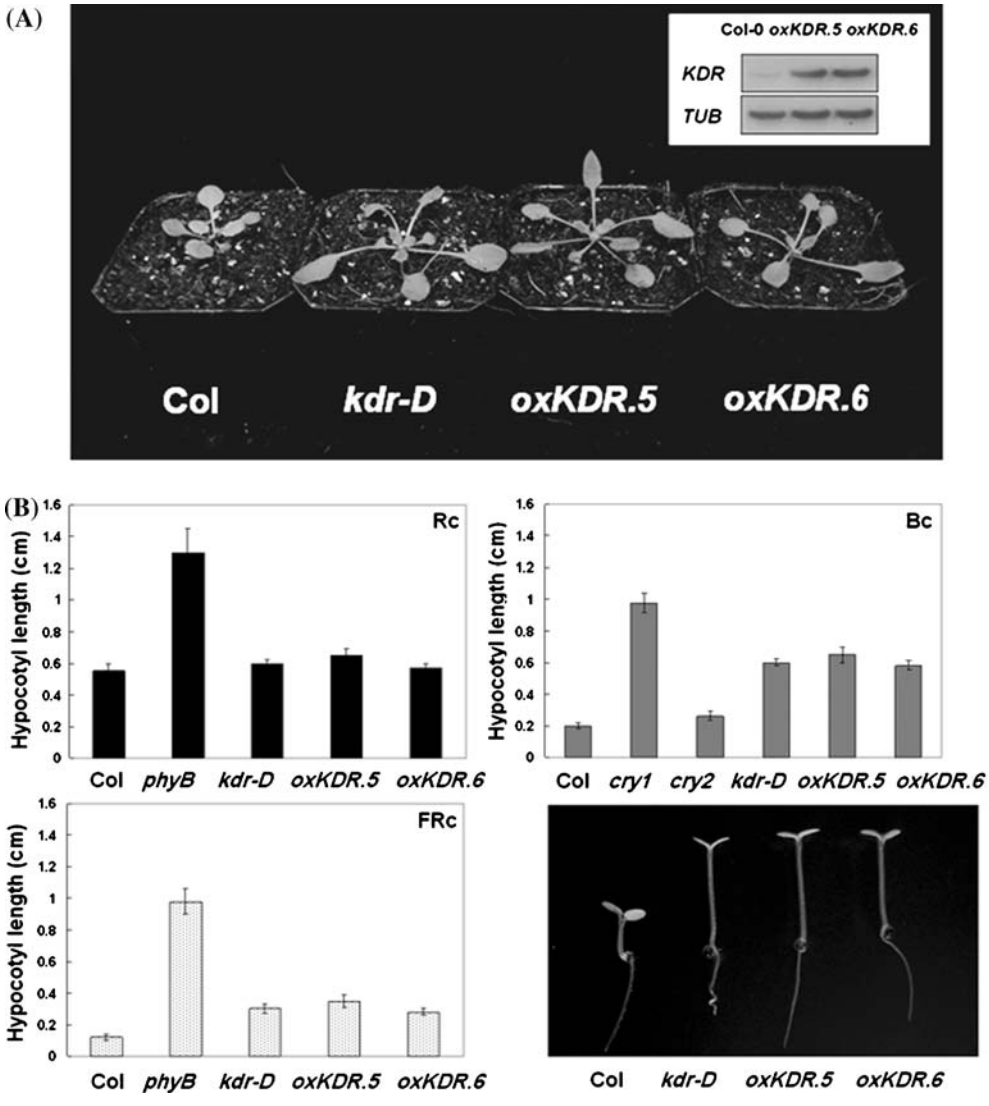

Figure 4. Phenotype confirmation of the *KDR* overexpressing transgenic plant. (A) Morphology of the *35S::KDR* transgenic plant at adult stage. Representative *35S::KDR* (*oxKDR.5*, *oxKDR.6*) plants exhibit long petiole phenotype similar to the *kdr-D*. All of plants in the figure were grown for 30 days in long day photoperiod condition. The transgenic plants were also exhibited early flowering phenotype, but the data is not shown. Overexpression of introduced *KDR* gene was confirmed by Northern blot analysis (inset). Total RNA was extracted from 10 days old seedlings, and *TUB* gene was used as control for the quantification. (B) Light responses of *35S::KDR* transgenic plants in diverse monochromatic light condition. Same as the *kdr-D*, *35S::KDR* transgenic plants show long hypocotyl phenotype in both blue (Bc) and far-red (FRc) light condition. In blue light condition, normal cotyledon opening/expansion could be also observed (bottom-right panel).

basic amino acid residues in their basic motif, whereas other typical DNA binding bHLH proteins have an average of six basic residues. In addition, those HLH proteins lack the Glu-13/Arg-17 amino acid residues in the basic motif, which are essential for DNA binding ability of bHLH proteins. *Arabidopsis* KDR family proteins showed typical characteristics of non-DNA binding HLH proteins (Figure 5C). They have less than three basic amino acid residues and lack the Glu-13/Arg-17 in their basic motif. Such structure of KDR family suggests that they act as a negative regulator of bHLH proteins through the formation of heterodimers that have lost the capacity to bind DNA.

Functional interaction of the KDR *with* HFR1

One of bHLH proteins, HFR1 has been characterized as a mediator of blue/far-red light signaling (Fairchild *et al.*, 2000; Fankhauser and Chory, 2000; Soh *et al.*, 2000). The *hfr1* mutant exhibited

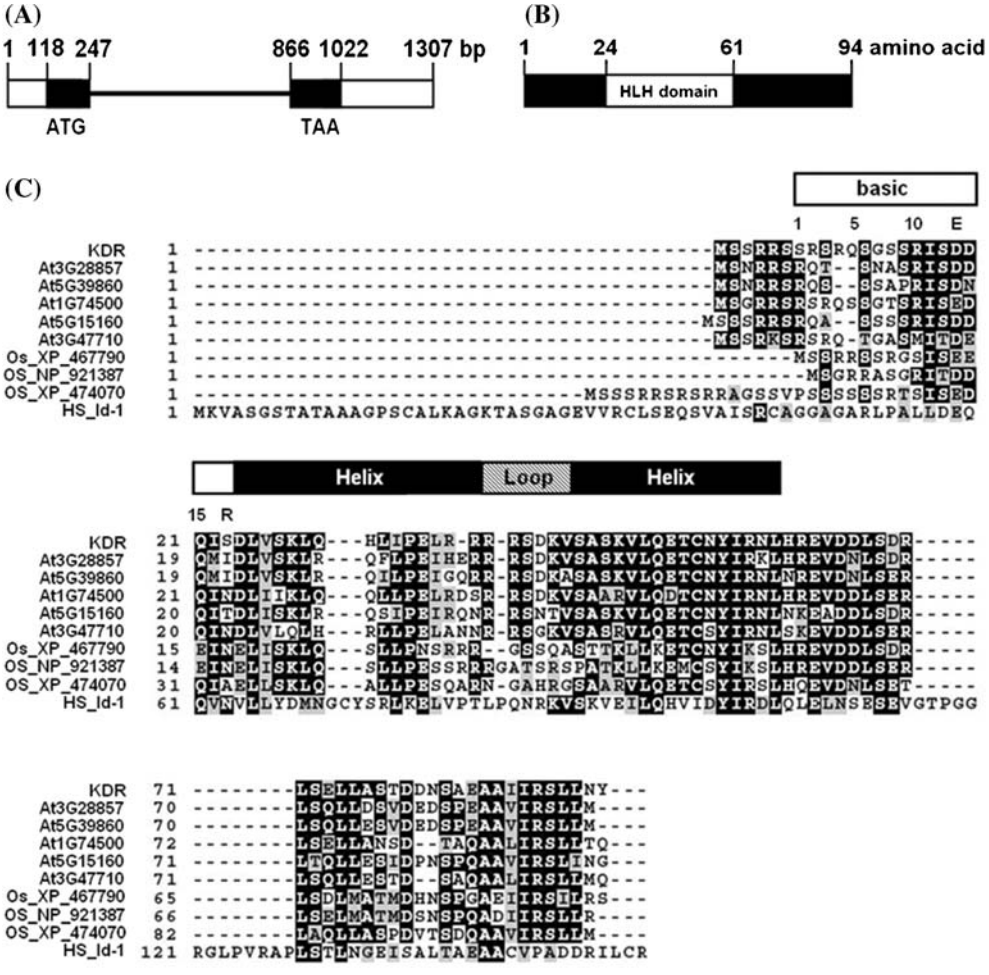

Figure 5. Structures of *KDR* gene and KDR protein. (A) Genomic structure of the *KDR* gene. *KDR* is composed of two exons (black squares) and one intron (narrow line). The white square means 5′ and 3′ untranslated regions (UTRs). (B) Domain structure of the KDR protein. KDR protein possesses HLH domain in its central region. Any other functional domains are not found in KDR. (C) Amino acid sequence alignment of the KDR family. The bHLH domain is represented above related amino acid residues. Position number of amino acid residue in basic domain is also marked on alignment result. The Glu-13/Arg-17 amino acid residues which are essential for DNA binding are marked on their related position as a single letter amino acid code. KDR family proteins do not possess these residues in their basic motifs. KDR, At3G28857, At5G39860, At1G74500, At5G15160, and At3G47710 are from *Arabidopsis* proteins. Os_XP_467790, Os_NP_921387, and Os_XP_474070 are from rice proteins. Hs_Id-1 means *Homo sapiens* Id-1.

long hypocotyl phenotype both in blue and far-red light condition. This phenotypic similarity with the *kdr-D* strongly suggested the possibility that HFR1 is the target of negative regulation by KDR. Because Id-1 inhibits the activity of target protein through heterodimerization, we analyzed the interaction between KDR and HFR1 using yeast two hybrid assay (Figure 6A). As a result, it was shown that KDR specifically interacted with HFR1. Moreover, KDR more strongly interacted with bHLH domain of HFR1. This result is consistent with the previous data that bHLH proteins interact each other through their bHLH domains (Ohtani *et al.*, 2001; Toledo-Ortiz *et al.*, 2003). We have additionally tested interaction of KDR with other bHLH proteins, PIL1 and PIL5, that also regulate light responses (Salter *et al.*, 2003; Toledo-Ortiz *et al.*, 2003; Huq *et al.*, 2004; Khanna *et al.*, 2004; Oh *et al.*, 2004). However, no positive interactions were found in these cases (Figure 6B).

To address whether this interaction between KDR and HFR1 actually occurs *in planta*, we have crossed the *kdr-D* with *35S::HFR1 hfr1* transgenic plant and the light response of resulted F1 progenies were analyzed under blue light condition (Figure 6B). As a result, all of *kdr-D 35S::HFR1* double mutant plants exhibited the same hypocotyl length with the *kdr-D* (Figure 6B), although *35S::HFR1 hfr1* transgenic plants showed wild type-like or shorter hypocotyl phenotypes (Figure 6C). This result clearly shows that KDR suppresses the activity of HFR1 *in vivo*.

Circadian rhythm and tissue specific expression of KDR *at seedling stage*

To further analyze the biological function of *KDR*, we examined the expression pattern of *KDR* during long day photoperiod, composed of 16 h light/8 h dark cycle. Interestingly, the *KDR* exhibited diurnal expression pattern (Figure 6A). The expression of *KDR* begins to increase at dawn, and showed peak of expression at noon. Thereafter, the expression level was decreasing, and was nearly absent during the night. To test whether circadian clock regulates the expression, we analyzed *KDR* expression in continuous light condition that was previously entrained by 12 h light/12 h dark cycle (Figure 6B). Even in this condition, *KDR* exhibited the same expression pattern, indicating that the expression of *KDR* is regulated by circadian clock.

Additionally, we also examined the spatial expression pattern of the *KDR* at seedling stage because the *kdr-D* exhibited a defect in the inhibition of hypocotyl elongation but normal cotyledon opening (Figures 2B and 6C). For this, we separated the seedlings into upper (cotyledon + SAM) and lower part (hypocotyl + root) for RNA extraction. The wild type showed similar expression levels of *KDR* in upper and lower parts although the expression of lower part was slightly weaker than that of upper part. In contrast, in *kdr-D* mutant, the *KDR* gene overexpression was mainly occurred in lower part that including hypocotyl tissue but not in upper part that including cotyledon (Figure 6C). Such a preferential overexpression of *KDR* in lower part may explain why *kdr-D* showed normal cotyledon opening but a defect in the inhibition of hypocotyl elongation.

Combined the negative role of *KDR* in blue and far-red light signaling, diurnal expression pattern, and functional interaction with HFR1, our results suggest that the KDR attenuates the induction of blue/far-red light response genes by inhibiting the activity of bHLH transcription factor involved in the light signaling cascade.

Discussion

The alteration of gene expression profile is a fundamental mechanism of diverse developmental processes. In several studies, it has been demonstrated that cry induced a change in whole genome-wide expression profile to evoke their signal responses (Jiao *et al.*, 2003; Chen *et al.*, 2004). It is very likely that various classes of transcription factors are involved in these biological phenomena. As described in this study, KDR possesses common features typically found in non-DNA binding bHLH proteins, like human Id-1. The basic motif of KDR is less basic than that of DNA binding bHLH proteins. Amino acid residues essential for DNA binding capacity are also absent in the KDR protein (Figure 5C). Based on these features, we suggest that KDR acts like Id-1, inhibiting the activity of bHLH protein through the direct protein–protein interaction. Especially, because the expression of *KDR*

was increased during the day time (Figure 7A and B), it seems that bHLH proteins that mediate light signal transduction are the targets of KDR protein.

In *Arabidopsis*, there are several bHLH proteins known as a mediator of light signal transduction, including PIF3, PIF4, HFR1, PIF1/PIL5, and PIL1 (Fairchild *et al*., 2000; Fankhauser and Chory, 2000; Soh *et al*., 2000; Huq and Quail, 2002; Salter *et al*., 2003; Toledo-Ortiz *et al*., 2003; Yamashino *et al*., 2003; Huq *et al*., 2004; Khanna *et al*., 2004; Oh *et al*., 2004). Among them, HFR1 is the most noteworthy for the putative binding target of KDR. The *hfr1* mutant showed defects in the inhibition of hypocotyl elongation under both blue and far-red light condition (Duek and Fankhauser, 2003). Such phenotype of *hfr1* is very similar to that of the *kdr-D* mutant. The overexpression of *KDR* also caused significantly reduced blue light responses, as well as a minor defect in phyA signal transduction under far-red light (Figure 2A). This result suggests that the *KDR* plays its major role in cry signaling, and has a minor function in phyA signal transduction. This functional similarity between *HFR1* and *KDR* supports the possibility that the KDR protein is a negative regulator of HFR1. Indeed, we found specific interaction between KDR and HFR1 using yeast two hybrid assay (Figure 6A) and the suppression of *35S::HFR1* by *KDR* overexpression (Figure 6B). In addition to HFR1, it is possible that the other bHLH proteins in *Arabidopsis*

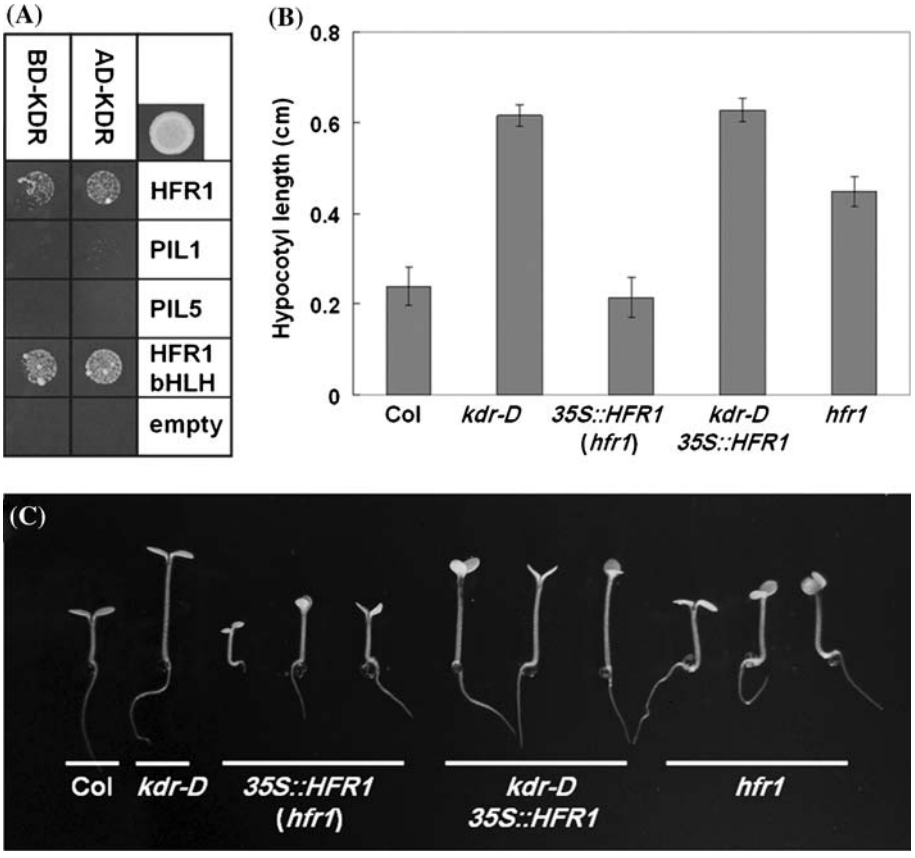

Figure 6. Functional interaction of KDR with HFR1. (A) Protein–protein interaction analysis using yeast two hybrid assay. KDR specifically interacts with HFR1 and bHLH domain of HFR1. The transformed yeast cells were grown on His minus media for analysis of interaction between introduced proteins. Positive interaction control, AD-T7 and BD-p53 fusion proteins, is spotted in upper-right panel. (B) Epistatic interaction of the *kdr-D* with *35S::HFR1*. From the F1 progenies of cross between *kdr-D* and *35S::HFR1*, the double mutant *kdr-D 35S::HFR1* were selected. The double mutants exhibit the same hypocotyl length with the *kdr-D* in blue light condition. (C) Cotyledon opening and expansion of the *kdr-D 35S::HFR1* in continuous blue light condition. The double mutant plants exhibit opened cotyledon phenotype in blue light condition.

act as the targets of KDR because *kdr-D* mutant shows additional phenotypes such as long petiole and early flowering which were not observed in *cry1* or *cry2* mutant. It indicates that the functions of the other bHLH proteins, which are not involved in cry signaling, are negatively regulated by KDR. In that sense, it might be interesting to search KDR interacting partners. Although many

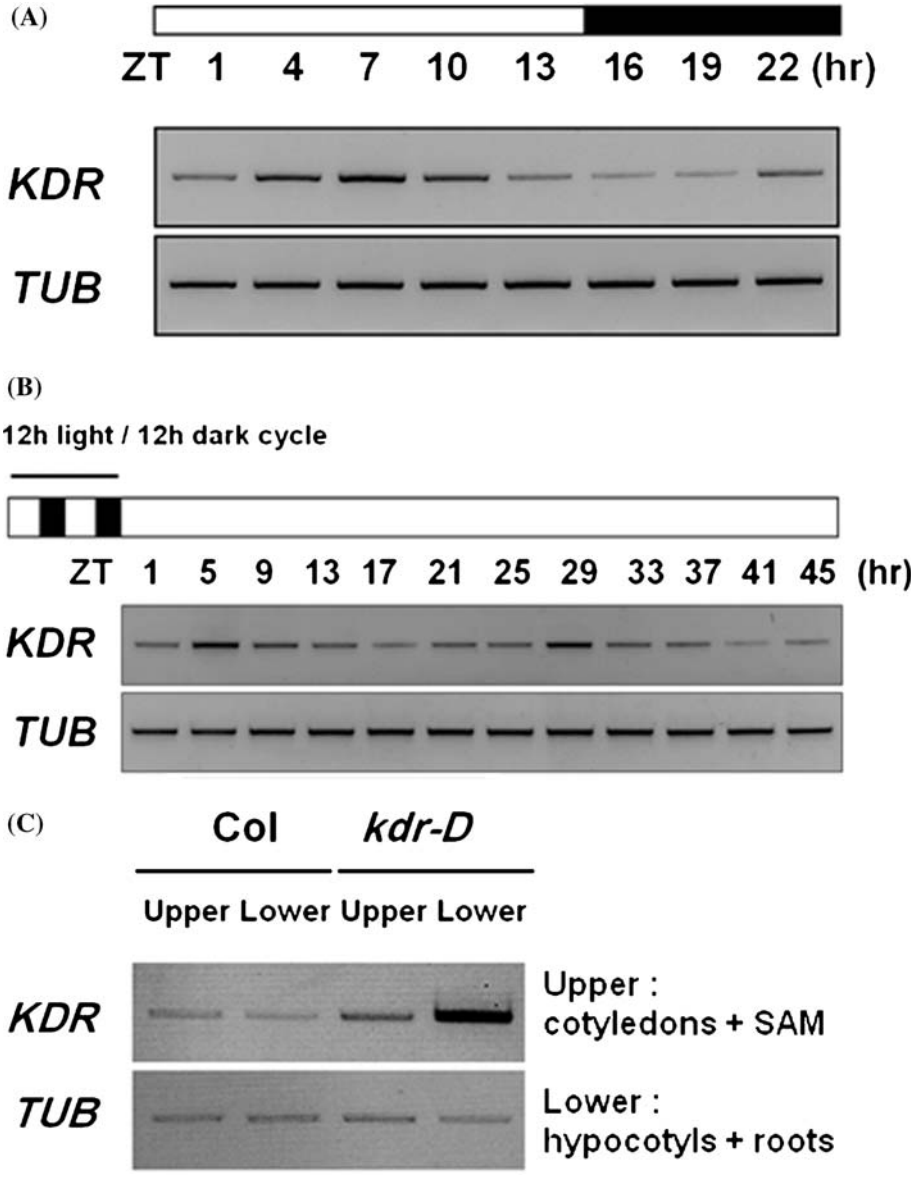

Figure 7. Expression pattern of the *KDR* gene. (A) Diurnal expression pattern of the *KDR* gene during long day photoperiod. The expression of *KDR* is analyzed using RT-PCR. White and dark bars mean light and dark period, respectively. Total RNA was extracted from 10 days old seedlings grown in 16 h light/8 h dark cycle long day photoperiod condition. The *TUB* gene was used as control for the quantification. (B) Circadian clock-regulated expression of *KDR*. Even in continuous light condition (white bar) after the 8 days entrainment in 12 h light/12 h dark cycle (striped bar), the *KDR* is still exhibited diurnal expression rhythm. The expression was analyzed by RT-PCR. Total RNA was extracted from 10 days old seedlings, and *TUB* gene was used as quantificational control. (C) Tissue specific expression of the *KDR* at seedling stage. Upper part (cotyledons and SAM) and lower part (hypocotyls and roots) of 4 days old seedlings were harvested separately and used for total RNA extraction. Total RNA was extracted at ZT12 h in long day condition (16 h light/8 h dark cycle). The *TUB* gene was used as control for the quantification. This experiment was repeated three times and the representative result is shown.

loss-of-function mutants of genes encoding Phytochrome Interacting factor Like (PIL) bHLH transcription factors were studied (Salter *et al.*, 2003; Huq *et al.*, 2004; Khanna *et al.*, 2004; Oh *et al.*, 2004), few are analyzed for blue light response, which is utmost interesting.

The circadian clock also has a role in deetiolation of seedlings and control of flowering time, by crosstalk with cry and phy signaling cascade (Somers *et al.*, 1998; Jarillo *et al.*, 2001; Lin, 2002; Yanovsky and Kay, 2002; Más *et al.*, 2003). Through this process, the light signal could be translated into the circadian clock mediated responses. Recently, it was reported that TOC1, a central oscillator of circadian clock, interacts with several PIL bHLH transcription factors (Yamashino *et al.*, 2003; Salter *et al.*, 2003; Fujimori *et al.*, 2004). In the *kdr-D* mutant, we could also find the accelerated phase transition to flowering (Figure 1B). Although phyA controls the flowering, the *phyA* deficient mutant exhibited late flowering phenotype in white light (Franklin and Whitelam, 2004). Therefore, the early flowering phenotype of *kdr-D* is not directly due to the defect in phyA signal transduction. Instead, the overexpression of *KDR* may induce repressed circadian clock responses through an inhibition of circadian clock-regulated bHLH protein's activity, which results in the early flowering phenotype. Further analysis is required to confirm this possibility.

Interestingly, the effect of *kdr-D* mutation on *KDR* expression was different among tissues as a slight increase in upper part whereas strong overexpression in lower part (Figure 7C). Most likely, due to such difference, the *kdr-D* showed strong phenotype in hypocotyl elongation but normal in cotyledon opening. It is possible that the T-DNA including 35S enhancer intervened between cotyledon specific cis-element and hypocotyl specific cis-element in the *kdr-D* mutant and it caused the block of the activity of cotyledon specific cis-element but enhancement of the activity of hypocotyl specific cis-element. Because it has been reported that the activation tagged gene was overexpressed following its native expression pattern (Weigel *et al*, 2000), it is probable that the activity of hypocotyl specific cis-element and that of CaMV 35S enhancer synergistically increased *KDR* expression in hypocotyl. However, the *35S::KDR* transgenic plants also exhibited normal cotyledon opening in blue light (Figure 4B). Because the transgenes must be randomly inserted in *Arabidopsis* genome, it is not conceivable that the phenotype of *35S::KDR* is due to the positional effect of T-DNA insertion. Thus, it is more likely that the activity of 35S promoter for *KDR* is suppressed in cotyledon. Currently, we do not know the mechanism how KDR overexpression is suppressed in cotyledon.

The precise control of light response is crucial for adaptation to environment in *Arabidopsis*. In this study, we have demonstrated the negative roles of *KDR* in blue and far-red light signal transduction. We propose that this negative function of *KDR* attenuates the light responses during day time and using this regulatory mechanism, plants possibly control the precise light responses.

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