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Overexpression of *LSH1*, a member of an uncharacterised gene family, causes enhanced light regulation of seedling development

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Summary

Light regulates plant growth and development through a network of endogenous factors. By screening *Arabidopsis* activation-tagged lines, we isolated a dominant mutant (light-dependent short hypocotyls 1-D (*lsh1-D*)) that showed hypersensitive responses to continuous red (cR), far-red (cFR) and blue (cB) light and cloned the corresponding gene, *LSH1*. *LSH1* encodes a nuclear protein of a novel gene family that has homologues in *Arabidopsis* and rice. The effects of the *lsh1-D* mutation were tested in a series of photoreceptor mutant backgrounds. The hypersensitivity to cFR and cB light conferred by *lsh1-D* was abolished in a *phyA* null background (*phyA-201*), and the hypersensitivity to cR and cFR light conferred by *lsh1-D* was much reduced in the phytochrome-chromophore synthetic mutant, *hy1-1* (long hypocotyl 1). These results indicate that LSH1 is functionally dependent on phytochrome to mediate light regulation of seedling development.

Keywords: *Arabidopsis*, activation tagging, light, LSH1, new gene family, photoreceptors.

Introduction

As an important environmental factor, light regulates various aspects of plant development from germination to flowering. In *Arabidopsis*, de-etiolation events such as hypocotyl growth inhibition and cotyledon expansion are controlled by blue (B), red (R) and far-red (FR) light. Plants perceive light information through different types of photoreceptors including the R/FR-absorbing phytochromes, the B/UV-A-absorbing cryptochromes, and unknown UV-B photoreceptors (Briggs and Olney, 2001; Kendrick and Kronenberg, 1994).

The phytochromes are the best characterised photoreceptors. Phytochromes are able to absorb R and FR light via a tetrapyrrole chromophore covalently attached to the phytochrome polypeptide. They are synthesised in darkness as an R-light-absorbing form (Pr) and are converted to the FR-light-absorbing form (Pfr) upon R light absorption; the conversion from Pr to Pfr initiates phytochrome signalling (Quail *et al.*, 1995). In *Arabidopsis*, among the five members of the phytochrome protein family (phyA–phyE; Sharrock and Quail, 1989), it has been revealed that phyA is uniquely responsible for continuous FR (cFR) light-mediated inhibition of hypocotyl elongation (Nagatani *et al.*, 1993; Whitelam *et al.*, 1993), whereas phyB is the primary photoreceptor for R light-mediated inhibition of hypocotyl elongation (Reed *et al.*, 1993), although they also have partially overlapping functions (Neff and Chory, 1998; Reed *et al.*, 1994).

Genetic and molecular analyses of mutants with altered light responses have identified many components involved in light signalling. These components can be divided into three categories. The first class includes *COP* (constitutively

photomorphogenic)/*DET* (de-etiolated)/*FUS* (fsuca) gene products. *cop/det/fus* mutants show photomorphogenic development in darkness as if subjected to light, indicating that their gene products are pleiotropic negative regulators of photomorphogenesis (Kwok *et al*., 1996; Wei and Deng, 1996). The second class of mutants exhibit altered light responses mediated by different photoreceptors but no phenotype when grown in darkness. The best characterised example is *hy5* that shows reduced sensitivity to R, FR and B light-mediated inhibition of hypocotyl elongation. HY5 has been revealed to be a bZIP transcriptional factor, and it interacts with COP1 (Ang *et al*., 1998; Hardtke *et al*., 2000; Oyama *et al*., 1997). The third class includes components involved in specific photoreceptor signalling pathways. Very impressively, a number of genes have recently been identified as being involved in phyA signal transduction including *FAR1* (Far-red-impaired response 1) (Hudson *et al*., 1999), *PAT1* (phytochrome A signal transduction 1) (Bolle *et al*., 2000), *FIN219* (far-red-insensitive 219) (Hsieh *et al*., 2000), *HFR1* (long hypocotyl in far-red 1) (Fairchild *et al*., 2000), *LAF1* (Long after far-red light 1) (Ballesteros *et al*., 2001), *LAF6* (Moller *et al*., 2001), *FHY1* (Far-red elongated hypocotyl) (Barnes *et al*., 1996; Desnos *et al*., 2001), *FHY3* (Wang and Deng, 2002), *SPA1* (suppressor of *phyA105* 1) (Hoecker *et al*., 1998; Hoecker *et al*., 1999) and *EID1* (empfindlicher in donkelroten licht 1) (Buche *et al*., 2000; Dieterle *et al*., 2001). Also, a few loci that appear to function selectively in phyB signalling have been identified, including *RED1* (red elongated 1) (Wagner *et al*., 1997), *PIF4* (phytochrome interacting factor 4) (Huq and Quail, 2002) and *SRR1* (sensitive to red light reduced 1) (Staiger *et al*., 2003). *SUB1* (short under blue light 1), a mutation that causes hypersensitive responses to B and FR light, was suggested to be involved in cryptochrome and phytochrome co-action (Guo *et al*., 2001). Recently, PP7, a Ser/Thr protein phosphatase, was indicated to act as a positive regulator of cryptochrome signalling in *Arabidopsis* (Moller *et al*. 2003).

All the components mentioned above were identified through loss-of-function mutations. Using a gain-of-function approach can be a powerful method to identify gene members that may have functional redundancy (Nakazawa *et al*., 2003). By such a method, it has been revealed that *DFL1* (Dwarf in light 1) (Nakazawa *et al*., 2001) and *COG1* (cogwheel in light signal pathway 1) (Park *et al*., 2003), both of which belong to gene families, act in the regulation of light signalling.

In this paper, we report the isolation of a dominant mutant *lsh1-D* from activation-tagged lines. The corresponding gene for this mutant, designated *LSH1*, was found to be a member of an uncharacterised gene family, and the protein is localised in nucleus. Analysis of *lsh1-D* in photoreceptor mutant backgrounds revealed that its effect is dependent on phytochromes.

Results

Identification of a dominant mutant lsh1-D *that shows hypersensitive light responses from activation-tagged lines*

We transformed the Columbia (Col) ecotype of *Arabidopsis* with a binary vector, pPCVICEn4HPT (Hayashi *et al*., 1992), to generate an activation T-DNA tagging population. The T-DNA region of the vector contains four enhancers of the cauliflower mosaic virus (CaMV) 35S promoter tandemly arranged near the right border (RB), a hygromycin resistance gene (*HPT*, Hygromycin phosphotransferase) as the plant transformation selection marker near the left border (LB) and an ampicillin resistance marker for plasmid rescue. We screened approximately 5000 independent T_2 lines for hypocotyl length mutants. One line that showed dominant short hypocotyls and large cotyledons in light was isolated. DNA gel blot analysis using T-DNA sequence as the probe revealed that there were two T-DNA insertions in the line (data not shown). The mutant was backcrossed with the wild type. Similar to the T_2 generation in mutant screening, in the backcrossed F_2 generation the segregation ratio of mutant to wild type was 3 : 1, confirming that the mutation is dominant. DNA gel blot analysis further demonstrated that one of the T-DNA insertions co-segregated with the mutant phenotype (data not shown).

The purified homozygous line with only the co-segregating T-DNA obtained from the F_3 generation of backcrossing was named as *lsh1-D* (for light-dependent short hypocotyls 1-D) and used for all the analyses reported in this paper. As shown in Figure 1(a–d), *lsh1-D* exhibited shorter hypocotyls and larger cotyledons than those of the wild type in continuous white (cW), red (cR), blue (cB) and far-red (cFR) light. In addition, *lsh1-D* also exhibited shorter rosette petioles compared with the wild type (Figure 1e).

Further comparison of *lsh1-D* and the wild type in a range of monochromatic fluence rates as well as in the dark revealed that *lsh1-D* exhibited significantly shorter hypocotyls in all the fluence rates tested. However, it exhibited a hypocotyl length indistinguishable from that of the wild type when grown in darkness (Figure 2). At very high fluence rates when light intensity approaches near saturation for hypocotyl growth inhibition, the differences became smaller (Figure 2). Taken together, these data showed that *lsh1-D* is a dominant light-dependent mutant exhibiting hypersensitive responses to cFR, cR and cB light-mediated inhibition of hypocotyl elongation.

In *Arabidopsis*, it is reported that there is no difference in the number of cells making up the hypocotyl cell file between plants grown in the light and those grown in the dark (Gendreau *et al*., 1997). We examined the hypocotyls of *lsh1-D* and the wild type grown in light under an electron microscope and found no difference in cell num-

Figure 1. Phenotypes of *lsh1-D* and *35S:LSH1* transgenic seedlings.
Seven-day-old seedlings grown in cW light (a), cB light (b), cR light (c) and cFR light (d). (e) Three-week-old seedlings grown in cW light. The light intensities for (a–e) are 13, 3, 10, 10, 20 μmol m^{-2} sec^{-1}, respectively. In all panels, seedlings were arranged from left to right: Col wild type, *lsh1-D*, *35S:LSH1-oe11* and *35S:LSH1-oe25*. The triangle indicates the junction of hypocotyl and root. Bars in all the panels indicate 2 mm.

ber. We also found that the hypocotyl cell length of *lsh1-D* was much shorter than that of the wild type (data not shown), suggesting that the short-hypocotyl phenotype of *lsh1-D* was not because of a reduction in cell number, but a result of a reduction in cell length.

Cloning of the T-DNA insertion locus in lsh1-D

To identify the gene responsible for the mutant phenotype of *lsh1-D*, the T-DNA insertion site in *lsh1-D* was determined by plasmid rescue and thermal asymmetric interlaced (TAIL)-PCR (see Experimental procedures). Sequencing of the rescued plasmids revealed that the LB was flanked by a region of approximately 700 bp of Ti plasmid vector sequence, indicating that this region had not excised properly and was integrated into the plant genome. Next to the Ti vector sequence was a fragment of approximately 10.5 kbp of sequence unrelated to the Ti vector. Using the NCBI BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/), this sequence was perfectly matched to a genomic clone F24J2 that maps to chromosome V of *Arabidopsis* (Figure 3a), and the T-DNA insertion site in the genome was

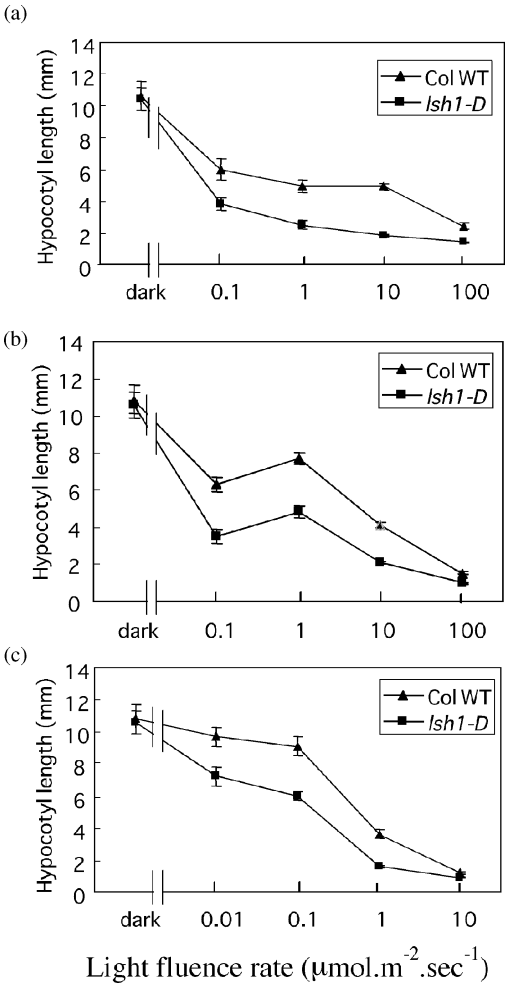

Figure 2. Fluence rate response curves for hypocotyl growth of Col wild type and *lsh1-D*.
Seedlings were grown under cR (a), cFR (b) and cB (c) at various photon fluence rates for 5 days. The mean length of 30–40 seedlings is displayed with SD.

predicted to be between 68008 and 68009 nucleotides (nt) from the top of F24J2.

By TAIL-PCR, we also obtained a sequence flanking the LB that was different from the Ti vector sequence flanking the LB revealed by plasmid rescue. A BLAST search revealed that it also matched the genomic clone F24J2, but upstream of the region recovered by plasmid rescue. The results from the plasmid rescue and TAIL-PCR indicated that the organisation of the inserted T-DNA was unusual and it might have two LB regions. To clarify the organisation of the inserted T-DNA, we amplified a fragment of approximately 1.2 kbp upstream of the predicted T-DNA insertion site by PCR. Sequence of this PCR product revealed that a fragment of approximately 300 bp including the LB of one T-DNA had invertedly integrated next to the RB of a second T-DNA (Figure 3a).

The expression of At5g28490/LSH1 *is highly enhanced in* lsh1-D

One candidate gene, *At5g28490,* that we named *LSH1* was located proximal to the T-DNA insertion site (Figure 3a). The distance between the putative translational start codon of *LSH1* and the 35S enhancers of the T-DNA was approximately 2.1 kbp. No other genes were predicted within 16 kbp of the left of the enhancers, and no gene was annotated within 20 kbp of the right of the enhancers. Using the predicted coding region as a probe, we performed RNA gel blot analysis using total RNA prepared from *lsh1-D* and the wild type. Strong expression of *LSH1* was detected in *lsh1-D*, but not in the wild type (Figure 3b), indicating that the endogenous expression of *LSH1* may be too low to be detected by the usual RNA gel blot method while in the activation-tagged mutant its expression is much enhanced. The transcript detected in *lsh1-D* was approximately 1 kbp.

As no cDNA exactly matching *LSH1* has been reported in the *Arabidopsis* databases used for the search, we cloned a partial sequence of the *LSH1* cDNA by 3′ rapid amplification of cDNA ends (RACE) using poly(A)+ RNA isolated from 7-day-old light-grown *Arabidopsis* seedlings. It was found that the 3′ non-coding region of the *LSH1* transcript was 170 nt long including a 22-nt poly(A) tail (data not shown). The fact that the *LSH1* gene could be obtained by 3′ RACE verified that it is a naturally expressed gene at the young seedling stage.

Overexpression of LSH1 *recapitulates the* lsh1-D *mutant phenotype*

To confirm that the mutant phenotype of *lsh1-D* is indeed caused by enhanced expression of *LSH1*, we generated transgenic lines with the gene under the control of the CaMV 35S promoter (named as *35S:LSH1* lines). In the T_2 generation, 16 of the 30 independent lines analysed showed a dominant phenotype of shorter hypocotyls and shorter rosette petioles. Homozygous lines were obtained in the T_3 generation for further analysis. The phenotypes of two representative lines (*oe11* and *oe25*) are shown in Figure 1 as compared with the wild type and *lsh1-D*. Obviously, both the lines showed shorter hypocotyls and larger cotyledons than those of the wild type in all light conditions (Figure 1a–d) and shorter rosette petioles under white light (Figure 1e), and *oe25* exhibited a phenotype stronger than that of *oe11*.

As the expression level of *LSH1* in young seedlings of the wild type seemed too low to be detected by RNA gel blot analysis (Figure 3b), semiquantitative RT-PCR analysis was used to determine its expression level in some of the *oe* lines in comparison with the wild type and *lsh1-D*. It was revealed that in all the selected *oe* lines that exhibited

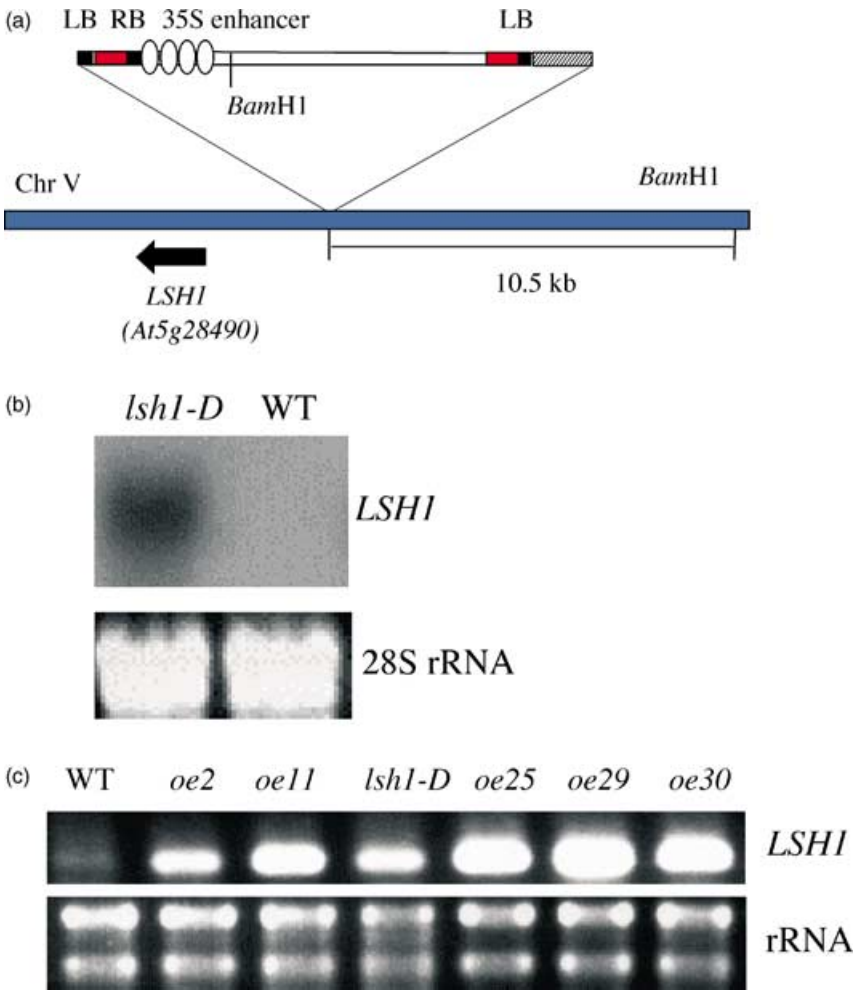

Figure 3. *LSH1* is the corresponding gene conferring mutant phenotypes.
(a) Map of the T-DNA insertion site in *lsh1-D*. T-DNAs were inserted on chromosome V in a complex manner. Four copies of the CaMV 35S enhancers are indicated by ellipses. RB and LB of the T-DNA are indicated by black boxes. A fragment of approximately 300 bp of the LB region (indicated by the red box) of one T-DNA was connected to the RB of a second T-DNA. Approximately 700 bp of Ti plasmid sequence (indicated by the striped box) was integrated into the plant genome.
(b) RNA gel blot analysis of *LSH1* gene expression in *lsh1-D* and the wild type (WT). Total RNA was extracted from 7-day-old Col WT and *lsh1-D* seedlings grown under cW light, and 10 μg of total RNA was loaded in each lane. Ethidium bromide staining pattern of ribosomal RNAs shows equal loading.
(c) RT-PCR analysis of the *LSH1* expression level in Col WT, *lsh1-D* and *35S:LSH1* plants (*oe2*, *oe11*, *oe25*, *oe29* and *oe30*).

hypersensitive responses to light, the expression of *LSH1* was much higher than that in the wild type (Figure 3c; *oe29* and *oe30* were two lines that also showed phenotypes stronger than those of *lsh1-D*). Consistent with the phenotypes, the *LSH1* expression level is higher in the *oe25*, *oe29* and *oe30* lines than in *lsh1-D* (Figure 3c). From this result, we conclude that overexpression of *LSH1* recapitulates the *lsh1-D* phenotype and that it is the gene responsible for the mutant phenotypes.

Transgenic plants harbouring *LSH1* in the antisense orientation under the control of the 35S promoter were also generated and analysed. No significant difference in the hypocotyl length between the antisense transgenic lines and the wild type was observed under the different light conditions examined (data not shown).

LSH1 *is a member of a novel gene family*

From the cDNA sequence, it was predicted that *LSH1* encodes a protein of 190 amino acids (aa). Database searches by NCBI BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/) revealed that it defined a new gene family with 10 homologous members in *Arabidopsis* and at least two in rice. The 10 *Arabidopsis* members were named LSH1 to LSH10 (Figure 4). All the members are highly conserved in the middle region but are diverse near the N- and C-terminals. LSH1 is most homologous to LSH2/At3g04510, and their N- and C-terminals are also conserved. From protein motif prediction programs, it was predicted that LSH1 has no known protein motif. The conserved region of this family has been given the coding number DUF640 in the PFAM database (http://www.sanger.ac.uk/cgi-bin/Pfam).

LSH1 was predicted to have a nuclear localisation signal (NLS), KKRK, from a protein localisation prediction program (http://psort.nibb.ac.jp), and this signal is conserved in all LSH members (Figure 4).

LSH1 *is expressed in hypocotyls, shoot apices and lateral root primordia*

In our experiments, *LSH1* expression could not be detected by RNA gel blotting in young seedlings grown in light or

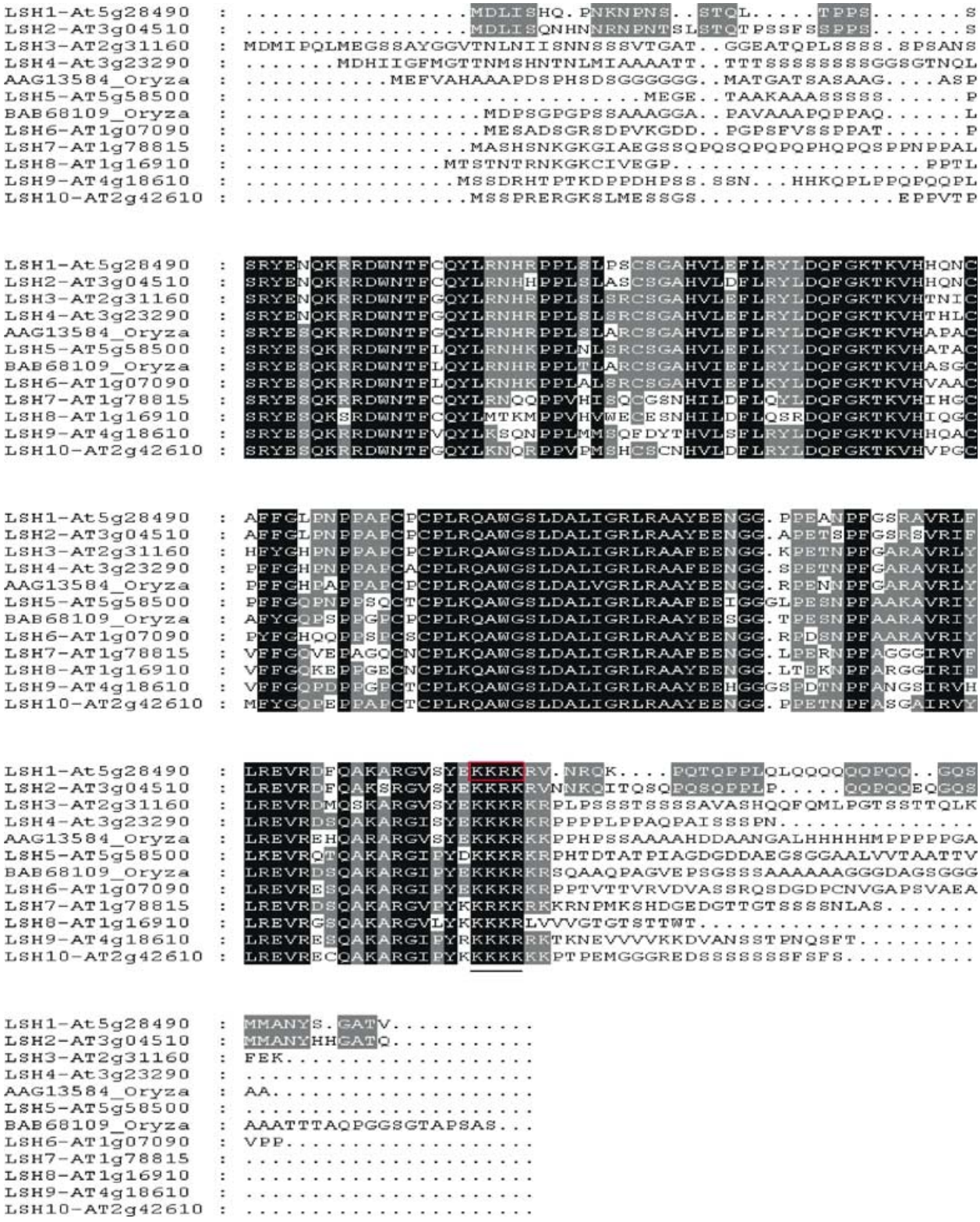

Figure 4. Amino acid sequence alignment of LSH1 and its homologues in *Arabidopsis* and rice.
The residues identical or similar among all the family members are indicated by black shading, and the residues conserved by at least half of the family members as well as the residues conserved by LSH1 and LSH2 at the N- and C-terminals are indicated by grey shading. The 10 members from *Arabidopsis* are named LSH1 to LSH10, and their corresponding TAIR identification numbers are also shown. The two members from rice (*Oryza sativa*) are shown by the NCBI Accession numbers. Gaps are introduced to achieve maximal similarity. A predicted NLS for LSH1 is indicated by the red square, and the underlining indicates that the signal is conserved for all the family members.

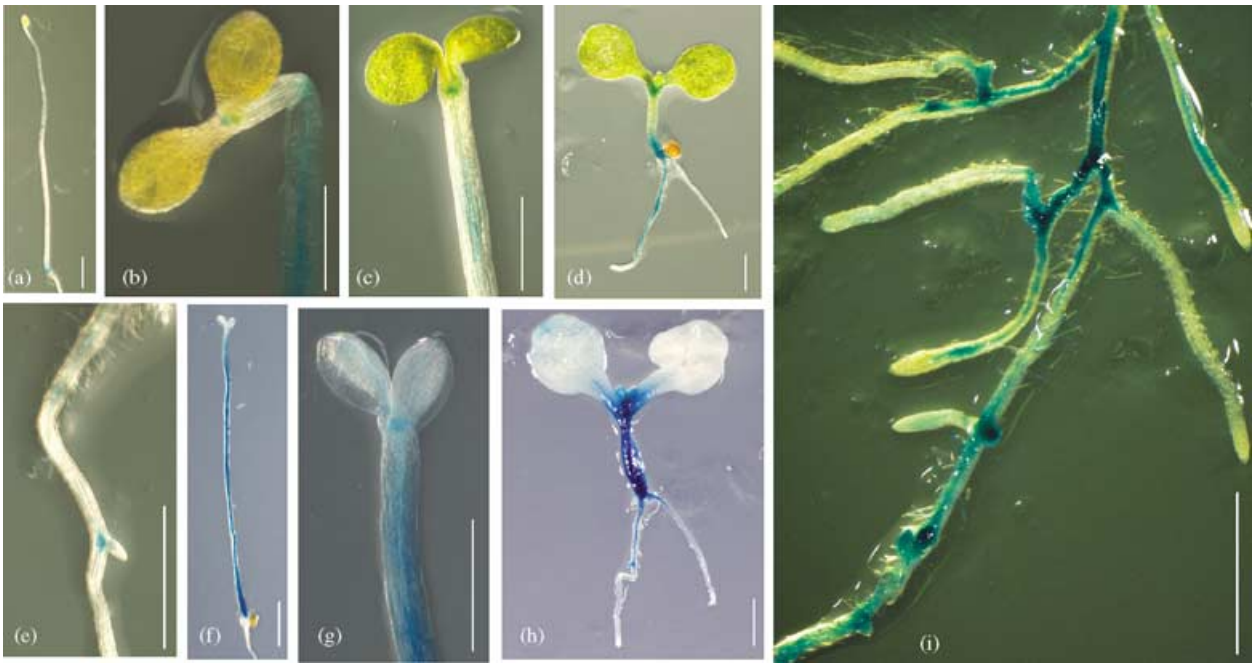

Figure 5. *LSH1:GUS* expression in seedlings grown in different light conditions.
Seedlings were stained for different lengths to clarify the strongly stained regions. (a–e) Short staining (4 h); after staining, the seedlings were not treated by ethanol to remove the chlorophyll.
(f–i) Long staining (12 h); after staining, the seedlings were treated with ethanol to remove the chlorophyll. Seedlings were grown in the dark (a,e,f), cFR (b), cB (c) and cW (d,h) light for 6 days. (e) Primary root and lateral root of dark-grown seedlings. (g) Close observation of a dark-grown seedling shown in (f). (i) Primary and lateral roots of 15-day-old seedlings grown under cW light.
Bars in all the panels indicate 1 mm.

dark (Figure 3b; data not shown), although the result of the 3′ RACE experiment demonstrated that it is naturally expressed in young seedlings. To determine the tissue specificity of *LSH1* expression and whether its expression is regulated by light, we generated transgenic lines expressing the β-glucuronidase (*GUS*) gene under the control of the *LSH1* promoter (named as *LSH1:GUS* lines). We examined the transgenic seedlings grown under monochromatic light conditions and in darkness. Shorter staining of the *LSH1:GUS* seedlings grown in the dark, cFR, cB, cW or cR (data not shown) light showed that the staining was always detected first in the shoot apices and hypocotyls (Figure 5a–d). After longer staining, GUS activity could be observed in the cotyledons and more strongly in the hypocotyls (Figure 5f–h; data not shown). These observations indicate that *LSH1* is expressed in shoot apices and hypocotyls irrespective of light conditions.

In roots, GUS staining was detected in the lateral root primordia (Figure 5e,i) and weakly in the vascular tissues while it was not detected in both primary and lateral root tips (Figure 5d,e,h,i).

LSH1 is localised in the nucleus

The presence of a putative NLS signal in LSH1 (Figure 4) suggests that LSH1 might be a nuclear protein. To explore the subcellular localisation of LSH1, we generated transgenic plants carrying a modified green fluorescence protein (*GFP*) gene fused in-frame to the N-terminal end of the full-length LSH1 protein under the control of the 35S promoter (named as *35S:GFP-LSH1*). Although GFP on its own was observed all over the cell (data not shown), the GFP-LSH1 fusion protein was observed only in the nuclei of hypocotyl cells (Figure 6a–c) and root cells (Figure 6d,e). Alteration of the light conditions did not affect this nuclear localisation (data not shown), indicating that the localisation of LSH1 is not regulated by light. It should be noted that some of the *35S:GFP-LSH1* lines showed significantly shorter hypocotyls than those of the wild type when grown in light (data not shown), demonstrating that the fusion protein is functional.

The effect of lsh1-D *in photoreceptor mutant backgrounds*

To determine whether overexpression of *LSH1* intrinsically promotes responses to light or whether the effects are dependent on multiple photoreceptors, we generated double mutants carrying *lsh1-D* and the photoreceptor mutations *phyA201*, *phyB-1*, *hy1-1* and *hy4-2.23N*. If the effect of *lsh1-D* is dependent on a photoreceptor, *lsh1-D* should not produce hypersensitive responses to light in a

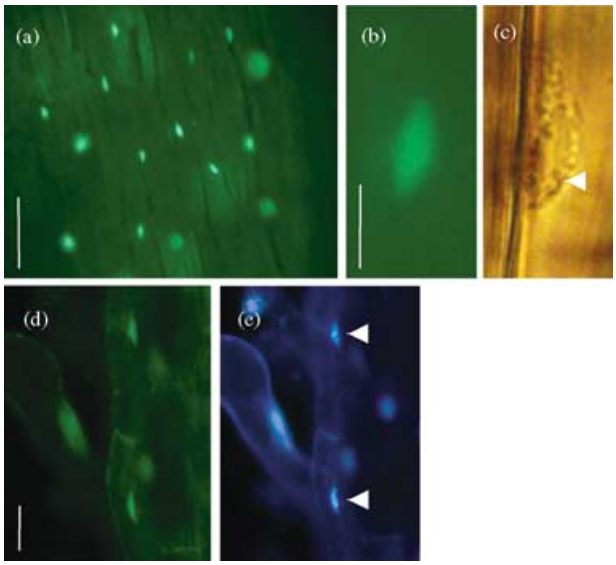

Figure 6. LSH1 is localised in nucleus.
Transgenic *Arabidopsis* (*35S:GFP-LSH1*) seedlings expressing the GFP-LSH1 fusion protein were examined under a fluorescent microscope.
(a) Fluorescence image of *35S:GFP-LSH1* hypocotyl cells.
(b) Fluorescence image of a *35S:GFP-LSH1* hypocotyl cell.
(c) Optical image of the same cell as shown in (b) to show the nucleus location (indicated by the triangle).
(d) Fluorescence image of *35S:GFP-LSH1* root cells.
(e) DAPI staining to show the nucleus' location (indicated by the triangle) in (d).
Bar in: (a), 50 μm; (b), 10 μm for (b,c); (d), 20 μm for (d,e).

mutant of that photoreceptor. If the effect of *lsh1-D* does not depend on a photoreceptor, the double mutant should show similar responses to *lsh1-D* or an intermediate phenotype between that of *lsh1-D* and that of the photoreceptor mutant resulting from the opposite effects of the mutations. However, if the effect of *lsh1-D* is dependent on more than one photoreceptor, the double mutant may also show an intermediate phenotype.

phyA-201, a null mutant allele of phyA, is blind to cFR-mediated inhibition of hypocotyl elongation and cotyledon expansion. The *lsh1-D phyA-201* mutant exhibited a phenotype similar to that of *lsh1-D* when grown in cR light and darkness (data not shown), but was similar to that of *phyA-201* in cFR light (Figure 7a), indicating that *lsh1-D* requires phyA for hypersensitive responses to cFR light. When exposed to cB light, *phyA-201* exhibited longer hypocotyls and smaller cotyledons than those of its wild type L*er* (Landsberg *erecta*) (Figure 7b). This is in agreement with the report that phyA is also involved in the hypocotyl-growth response in B light (Neff and Chory, 1998). Significantly, it was observed that the *lsh1-D phyA-201* mutant exhibited nearly the same hypocotyl length as that of *phyA201* (Figure 7b), indicating that *lsh1-D* also requires phyA for hypersensitive responses to cB light. It was observed that *lsh1-D phyA-201* seedlings exhibited different cotyledon sizes (Figure 7b). This may be because the double mutant was generated by crossing plants of two different ecotypes (Col has larger cotyledons than L*er* under cB light, Figure 7b).

phyB-1, a severe mutant allele of phyB, exhibits much reduced sensitivity to cR light. The *lsh1-D phyB-1* mutant exhibited almost the same phenotype as that of *lsh1-D* when grown under cFR and cB light (data not shown) and a phenotype intermediate between *lsh1-D* and *phyB-1* in respect of both cotyledon size and hypocotyl length under cR light (Figure 7c).

hy1-1 is defective in responses to both cR and cFR light as the mutation causes blocking of phytochrome-chromophore biosynthesis (Davis *et al.*, 1999; Muramoto *et al.*, 1999). The *lsh1-D hy1-1* mutant was similar to *hy1-1* under cR light (Figure 7d) and cFR light (Figure 7e), while it showed a slightly shorter hypocotyl than that of *hy1-1* under cR and cFR light (Figure 7d,e; data not shown). A possible explanation may be that the *hy1-1* mutation does not completely block the synthesis of the phytochrome-chromophore, and therefore there is still a very low level of phytochrome activity in *hy1-1*. Consequently, in the double mutant, signals from the low level of phytochrome activity could be increasingly transduced by enhanced expression of *LSH1* and thus causes more inhibition of hypocotyl elongation than in *hy1-1*.

hy4-2.23N has a null mutation in the B-light photoreceptor, CRY1 (cryptochrome 1), and shows reduced sensitivity to cB light (Ahmad and Cashmore, 1993). The *lsh1-D hy4-2.23N* mutant exhibited a phenotype similar to that of *lsh1-D* under cR and cFR light but an intermediate hypocotyl length under cB light (data not shown).

Discussion

From activation-tagged lines, the dominant mutant *lsh1-D* was identified as showing hypersensitive responses to light regulation of hypocotyl elongation and cotyledon expansion. We showed that consistent with the enhanced responses to light, *LSH1* expression is much enhanced in *lsh1-D* and *35S:LSH1* lines compared to the wild type, which provides evidence that LSH1 is positively involved in light signalling.

Analysis of the *lsh1-D phyA-201* double mutant revealed that *lsh1-D* requires phyA to effect hypersensitive responses to cFR light, and therefore rules out the possibility that *lsh1-D* has intrinsic effects in promoting responses to light. The fact that the *lsh1-D hy1-1* double mutant exhibited a phenotype similar to that of *hy1-1* under cR light indicates that *lsh1-D* requires phytochromes to mediate responses to cR light. phyB might not be the only R-light photoreceptor that *lsh1-D* depends on as the *lsh1-D phyB-1* double mutant exhibited significantly increased responses to R light compared to *phyB-1*. Some studies have suggested that phytochromes other than phyB might also be involved in

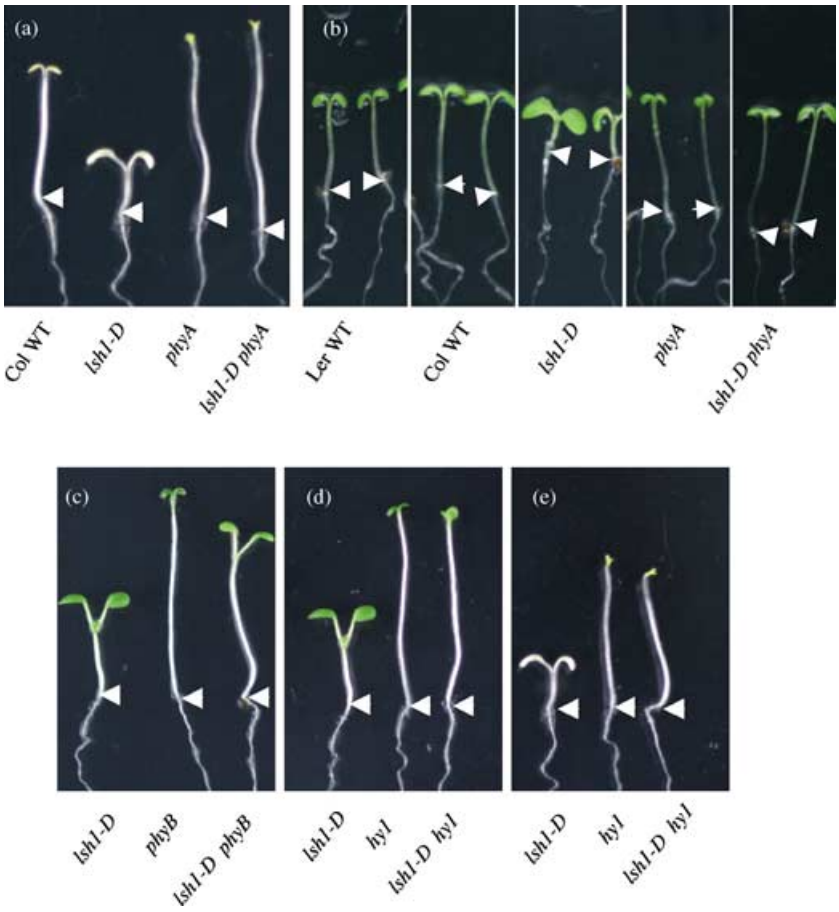

Figure 7. The phenotypes of *lsh1-D* in phytochrome mutant backgrounds.
Six-day-old seedlings grown under cFR light (a,e), cB light (b) and cR light (c,d). The light intensities for cFR, cB, cR are 1.2, 1, 30 μmol m^{-2} sec^{-1}, respectively.

mediating R-light responses (Hennig *et al.*, 1999; Qin *et al.*, 1997). Thus, it is possible that *lsh1-D* confers hypersensitive responses to cR light not only through phyB but also through other phytochromes. Under cB light, the *lsh1-D phyA-201* double mutant exhibited a hypocotyl length nearly identical to that of *phyA-201*, indicating that *lsh1-D* is mainly dependent on phyA to mediate its hypersensitive response to cB light. The fact that the *lsh1-D hy4-2.23N* double mutant showed an intermediate hypocotyl length between *lsh1-D* and *hy4-2.23N* might have alternative explanations. One possibility might be that *lsh1-D* is not only dependent on CRY1 but also dependent on other B-light photoreceptors such as CRY2 and phyA for its hypersensitive responses to cB light. If *lsh1-D* is indeed dependent on the photoreceptors CRY1 and CRY2 for responses to cB light, the *lsh1-D phyA-201* double mutant should also have shown an intermediate phenotype between *lsh1-D* and *phyA-201* under cB light. However, our result illustrated that this double mutant exhibited a hypocotyl length nearly identical to that of *phyA-201*. Therefore, we prefer to conclude that *lsh1-D* is mainly dependent on phyA for its hypersensitive responses to cB light. Anyway, without the analysis of *lsh1-D* in *cry1cry2* null background, we cannot rule out the possibility that LSH1 is dependent on phytochrome and cryptochrome coaction.

The fact that the hypersensitive responses to light conferred by overexpression of LSH1 are dependent on phytochromes suggests that the function of LSH1 as a light-signalling component is phytochrome dependent. Recent advances suggest a model for phytochrome signalling, whereby phytochromes perceive light, enter the nucleus, interact with transcriptional factors such as PIF3, and thus regulate gene transcription (Martinez-Garcia *et al.*, 2000). LSH1 was shown to be in the nucleus but contains no known DNA-binding motifs. It might be imagined that LSH1 is integrated into phytochrome signalling by interacting with transcriptional complexes, in the manner of a co-activator. In this case, LSH1 might be a rate-limiting factor for the transcriptional complexes as the effects conferred by LSH1 are correlated with its expression level. Alternatively, LSH1 may bind DNA via an unidentified motif after somehow being activated by phytochrome signalling (such as via phosphorylation). Interestingly, a homology search of the *Arabidopsis* databases (http://mips.gsf.de/cgi-bin/proj/thal/thaliana) revealed that LSH1 has significant homology with the following four proteins that have some

known motifs: At4g19500, a disease resistance protein homologue with two TIR (Transport inhibitor response) domains and two NB-ARC domains; At2g45430, a putative AT-hook DNA-binding protein; At1g69010, a putative HLH DNA-binding protein; and At2g28610, a putative homeodomain transcriptional factor (data not shown). The homologous parts of these proteins are not within the known motif regions or only slightly overlap with the identified motifs, but such homologies may indicate that LSH1 has the potential to function as a transcriptional regulatory protein.

The phenotypes of a gain-of-function mutant could be the result of mimicking or disturbances of the naturally functional genes and does not necessarily reflect a gene's role in wild-type plants. Thus, our present data cannot rule out the possibility that overexpression of *LSH1* actually reflects the natural function of other members of the gene family. As we could not obtain a knock-out mutant for the *LSH1* gene, we generated *LSH1* antisense transgenic lines to evaluate the role of LSH1 in light signalling. However, the antisense lines showed no significant difference from the wild type in response to light (data not shown). It should be noted that *LSH1* is not homologous with other LSH family members in nucleotide sequence except for *LSH2/At3g04510*. Therefore, the function of other LSH members apart from LSH1 and LSH2 might not be disturbed by *LSH1* antisense expression. As LSH1 belongs to a gene family in which the members are quite conserved at the amino acid level (Figure 4), functional redundancy might be a problem in the analysis of the family using loss-of-function methods. Generation and analysis of single-, double- and multiple-knockout or antisense transgenic mutants of the members may be helpful in further disclosing their function.

Experimental procedures

Mutant screening

Arabidopsis thaliana (Col) was transformed using *Agrobacterium tumefaciens* GV3101 possessing the binary activation tagging plasmid, pPCVICEn4HPT (Hayashi *et al*., 1992). Hygromycin-resistant T_1 plants were moved to soil, and T_2 seeds were harvested. Screening conditions for hypocotyl elongation mutants were according to Nakazawa *et al*. (2001).

Seedling growth and light sources

For all analyses, seeds were sterilised by soaking for 3 min in 70% ethanol followed by 10 min in 7% bleach with 0.05% Triton X-100 and then washed five times in sterilised distilled water. Seeds were sown on germination medium (GM; Valvekens and Van Lijsebettns, 1988) plates with 1% sucrose and then cold-treated at 4°C in darkness for 5 days. Plates were pre-treated with white light for 7 h before being transferred to the different light conditions.

The monochromatic light conditions (except the intensity) for Figures 1 and 2 were according to Nakazawa *et al*. (2001), while for all the other figures, the monochromic light sources were obtained by a light emission diodes (LED) facility (NK system, Japan). The source for the white light was always cool-white fluorescent lamps.

Cloning of the T-DNA insertion locus

For cloning by plasmid rescue, 2 μg of genomic DNA isolated from *lsh1-D* seedlings was digested with *Bam*H1 and self-ligated using T4 DNA ligase (Life Technologies Inc., Rockville, MD, USA) at 15°C for 15 h. One-tenth of the ligation solution was used to transform DH10B electro-competent cells (Gibco-BRL, Gainthersburg, MD, USA) by electroporation. The colonies containing rescued plasmids were obtained by spreading the transformants on LB agar plates containing ampicillin and cultured overnight at 37°C. The rescued plasmids were sequenced.

The T-DNA flanking sequence was also determined by thermal asymmetrical interlaced (TAIL)-PCR (Liu *et al*., 1995) using genomic DNA isolated from *lsh1-D* seedlings as the template and nested specific primers corresponding to the LB region of the T-DNA in combination with the common primers used for TAIL-PCR. The PCR products were sequenced.

A region upstream of the T-DNA insertion was amplified by PCR using genomic DNA extracted from *lsh1-D* seedlings as the template and the primer RB1 (5′- ATCTAGATCCGAAACTATCAGTG-3′) that corresponds to a region in the RB of the T-DNA and the primer (5′-GATGCCGTTAGTGTCCGTGCCCCAT-3′) that corresponds to a region 822 nt upstream of the T-DNA insertion site. A fragment of approximately 1.2 kbp was amplified and sequenced.

3′ RACE

3′ rapid amplification of cDNA ends was performed according to the protocol of the kit (3′ RACE system, Gibco-BRL). Briefly, 1 μg of polyadenylated RNA from 7-day-old wild-type seedlings was used as a template for first-strand cDNA synthesis. The first, second and third PCRs were performed using the same adaptor primer (AUAP) in combination with a set of *LSH1* gene-specific primers F2 (5′-ATGGATTTGATCTCTCACCAACCA-3′), F3 (5′-CAAGAACCCTAATTCCTCAACAC-3′) and F4 (5′-AACTAACACCACCTTCCTCAAGC-3′), respectively. The F2, F3 and F4 primers correspond to the nucleotides 1–24, 27–50, and 52–75 of the *LSH1* gene coding region, respectively. The resulting 3′ RACE-PCR product was cloned and sequenced.

RNA gel blot analysis

Total RNA from *Arabidopsis* seedlings was isolated as described by Yoshizumi *et al*. (1999), and for each sample, 10 μg was electrophoresed and blotted onto Hybond N+ membranes. A DNA fragment corresponding to the *LSH1* coding region was amplified from *Arabidopsis* genomic DNA by PCR using the gene-specific primers F2 (5′-ATGGATTTGATCTCTCACCAACCA-3′) and R1 (5′-TACTGTTGCACCCGAGTAATTAGC-3′) and labelled for use as a probe. The probe labelling, hybridisation and detection were performed according to the protocol of the kit RPN3690 (Amersham Pharmacia Biotech, Uppsala, Sweden).

Reverse transcription-based PCR (RT-PCR)

Total RNA was extracted from the inflorescences of 40-day-old plants grown in soil under continuous white light. The primers

used for amplification of *LSH1* were F2 and R1 as described above. Equal amounts of total RNA (500 ng) were subjected to RT-PCR analysis using Superscript II reverse transcriptase (Life Technology). The PCR samples were collected after 25 cycles.

Generation of 35S:LSH1 *sense and antisense transgenic plants*

To generate *35S:LSH1* sense and antisense transgenic lines, Gateway cloning techniques (http://www.lifetech.com/gateway) were used to make the constructs. The coding region of the *LSH1* gene was generated by PCR amplification of genomic DNA using the primers EntryF1 (5′-**GGGGACAAGTTTGTACAAAAAAGCAGGCT**ATGGATTTGATCTCTCACCAACCA-3′) and EntryR1 (5′-**GGGGACCACTTTGTACAAGAAAGCTGGGT**TCATACTGTTGCACCCGAGTAATTAGC) (attB sites for recombination cloning are shown in boldface, and the sequence corresponding to LSH1 is underlined). For making *LSH1* overexpression lines, the PCR product was introduced into the vector pBIDAVL-GWR1, and to make *LSH1* antisense lines, the same PCR product was introduced into the vector pBIDAVL-GWR2. pBIDAVL-GWR1 and pBIDAVL-GWR2 are vectors modified from pBI121 for Gateway cloning by our laboratory. pBIDAVL-GWR2 is the same as pBIDAVL-GWR1, except that it has opposite recombination direction.

The inserts in the constructs were sequenced to confirm the orientation and sequence. The construct was electroporated into *A. tumefaciens* strain GV3101, and transformed into *Arabidopsis* (Col) by the floral dip method (Clough and Bent, 1998).

Construction and expression studies of LSH1:GUS

The region of the *LSH1* gene from −1742 to +21 bp relative to the translation start site was generated by PCR amplification of genomic DNA using the primers 5′-TC**AAGCTT**TCGTCGTTCTGTTACCATTAAGGC-3′ and 5′-GC**TCTAGA**TGGTTGGTGAGAGATCAAATCCATG-3′ (restriction sites are shown in boldface, and the sequence corresponding to *LSH1* is underlined). The PCR product was digested by *Hin*dIII and *Xba*I and ligated into the *Hin*dIII and *Xba*I sites of the binary vector pBI101, and the resulting *LSH1:GUS* construct was transferred into *Arabidopsis* (Col).

For the GUS staining, seedlings were immersed in 50 mM sodium phosphate buffer (pH 7.2) with 1 mM 5-bromo-4-chloro-3-indolyl-D-glucuronic acid and incubated at 37°C for 3–12 h. Chlorophyll was extracted by passing through increasing concentrations of ethanol. Fifteen independent T_2 transgenic lines were analysed, and commonly observed staining patterns were reported.

Subcellular localisation of the GFP-LSH1 fusion protein

To construct the *GFP-LSH1* fusion, the coding region of the *LSH1* gene was obtained by PCR amplification of genomic DNA using the gene-specific primers 5′-GC**GGATCC**ATGGATTTGATCTCTCACCAACCA-3′ and 5′-TT**GAGCTC**TCATACTGTTGCACCCGAGTAAT-3′ (restriction sites are shown in boldface, and the sequence corresponding to *LSH1* is underlined). The PCR product was digested by *Bam*HI and *Sac*I and ligated into the *Bam*HI and *Sac*I sites of the binary vector yy217 (Yamamoto *et al*., 2001), which contains a GFP gene under the control of 35S promoter, and the resulting GFP-LSH1 fusion (*35S:GFP-LSH1*) was transferred into *Arabidopsis*. T_2 seedlings of independent lines were inspected for intracellular localisation of the GFP-LSH1 fusion protein under a fluorescence microscope. The yy217-transformed seedlings (provided by Dr Yamamoto) were also checked as controls (data not shown). Seedlings were also stained with DAPI (4,6-diamidino-2-phenyindole) to visualise the location of the nucleus.

Construction and determination of double mutants

The *phyA-201*, *hy1-1*, *phyB-1* and *hy4-2.23N* mutants are all in the L*er* ecotype. *lsh1-D* pollen was used for crossing. The resulting F_1 seeds were sown on GM medium with hygromycin (25 μg ml^{-1}), and only the hygromycin-resistant seedlings were moved to soil to harvest F_2 seeds. F_2 seedlings were grown to maturity to obtain independent F_3 lines. The F_3 seeds (40–50 seeds for each line) were sown on GM medium with hygromycin and grown under cW light to determine their genotypes for the *lsh1-D* locus. The F_3 lines homozygous for the *lsh1-D* locus were sown on GM medium and grown under various monochromic light conditions. The double mutants were confirmed as showing a phenotype similar to that of the corresponding photoreceptor mutants or different from that of *lsh1-D* under some monochromatic light conditions.

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