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LAF1, a MYB transcription activator for phytochrome A signaling

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The photoreceptor phytochrome (phy) A has a well-defined role in regulating gene expression in response to specific light signals. Here, we describe a new *Arabidopsis* mutant, *laf1* (long after far-red light 1) that has an elongated hypocotyl specifically under far-red light. Gene expression studies showed that *laf1* has reduced responsiveness to continuous far-red light but retains wild-type responses to other light wavelengths. As far-red light is only perceived by phyA, our results suggest that LAF1 is specifically involved in phyA signal transduction. Further analyses revealed that *laf1* is affected in a subset of phyA-dependent responses and the phenotype is more severe at low far-red fluence rates. *LAF1* encodes a nuclear protein with strong homology with the R2R3–MYB family of DNA-binding proteins. Experiments using yeast cells identified a transactivation domain in the C-terminal portion of the protein. LAF1 is constitutively targeted to the nucleus by signals in its N-terminal portion, and the full-length protein accumulates in distinct nuclear speckles. This accumulation in speckles is abolished by a point mutation in a lysine residue (K258R), which might serve as a modification site by a small ubiquitin-like protein (SUMO).

[*Key Words*: Signal transduction; phytochrome A; *Arabidopsis*; MYB; transcription factor; nuclear speckles]

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Plants are sessile organisms that depend on light not only as their source of energy but also for the timing of important developmental processes such as seed germination, stem elongation, and the transition to reproductive growth. To monitor variations in the wavelength, intensity, direction, and period of light plants have evolved different photoreceptors, among which the phytochromes are probably the best studied photoreceptors (Fankhauser 2001). Phytochromes have the capacity to reversibly convert from a red-light-absorbing form, Pr, to a far-red-light-absorbing form, Pfr, through the absorption of either red (R) or far-red (FR) light. This mechanism allows phytochromes to sense different R:FR light ratios and to act as a light-responsive developmental switch (Furuya 1993; Quail et al. 1995; Smith 2000). Among the five members of the *Arabidopsis* phytochrome protein family (phyA–phyE; Clack et al. 1994), phyA is the only one that can perceive FR light, and it is involved mainly in the regulation of de-etiolation (Whitelam et al. 1993; Reed et al. 1994). Recent findings have shown that phyA can be transported to the nucleus in a FR light–dependent manner (Kircher et al. 1999; Hisada et al. 2000; Kim et al. 2000). After import into the nucleus on FR light illumination, the phyA–GFP forms numerous speckles that dissolve after transfer to darkness (Nagy et al. 2001).

Various approaches have been taken to understand how the FR light signal is transduced by phyA to activate gene expression. Two-hybrid screens using phytochrome as a bait have led to the identification of three proteins, PIF3, PKS1, and nucleoside diphosphate kinase 2 (NDPK2) that interact with both phyA and phyB (Ni et al. 1998; Choi et al. 1999; Fankhauser et al. 1999). Genetic analyses have yielded several *Arabidopsis* mutants that are specifically disrupted in phyA signal transduction. The mutants *fhy1*, *fhy3*, *fin2*, *fin219*, *far1*, *pat1*, *rsf1*/*hfr1*/*rep1*, and *laf6* (Whitelam et al. 1993; Soh et al. 1998, 2000; Hudson et al. 1999; Bolle et al. 2000; Fairchild et al. 2000; Fankhauser and Chory 2000; Hsieh et al. 2000; Møller et al. 2001) show reduced responses under FR light conditions, whereas *spa1* and *eid1* (Hoecker et al. 1998; Büche et al. 2000) show exaggerated responses.

The identified genetic components of phyA signaling

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fall into three classes according to the cellular locations of their encoded products: PKS1, NDPK2, PAT1, and FIN219 localize preferentially in the cytoplasm, LAF6 is imported into plastids, and FAR1, SPA1, PIF3, RSF1/HFR1/REP1, and EID1 are nuclear (Ni et al. 1998; Choi et al. 1999; Fankhauser et al. 1999; Hoecker et al. 1999; Hudson et al. 1999; Bolle et al. 2000; Fairchild et al. 2000; Hsieh et al. 2000; Soh et al. 2000; Dieterle et al. 2001; Møller et al. 2001). The functions of most of these proteins are not obvious from their sequences and remain to be determined. FAR1 and PSK1 are novel proteins with no sequence similarities to other proteins in the database, SPA1 is a WD-40 repeat protein, and PAT1 is a member of the plant-specific GRAS (GAI, RGA, and Scarecrow) protein family, some members of which regulate gibberellin response or root development. LAF6 shows homology with ABC transporters, FIN219 is a member of the GH3 protein family, which is involved in auxin signaling, and EID1 is a F-box containing protein. PIF3 and RSF1/HFR1/REP1 show homology with the basic helix–loop–helix (bHLH) class of transcription factors. This array of proteins and their diverse intracellular localization reflect the complexity of phyA responses and suggest that FR light-activated phyA initiates a signaling cascade with several branches.

Many physiological modifications observed after activation of phyA signal transduction are caused by changes in gene expression (Kuno and Furuya 2000). Although some sequences necessary for light responsiveness have been defined, no single consensus motif nor a single transcription factor has been identified for such regulation and no *cis*-element has yet been linked to a single photoreceptor. Among the transcription factors implicated in light-regulated transcription in plants, most have been isolated by their ability to bind to specific promoter sequences. These include the MYB protein CCA1, several leucine zipper proteins (bZIPs such as ATHB-2/4, CPRF and GBF1) and GT-1 (Weisshaar et al. 1991; Gilmartin et al. 1992; Schindler et al. 1992; Carabelli et al. 1993; Wang et al. 1997). Nevertheless, genetic evidence for the functional involvement of most of the DNA binding proteins in light-regulated gene expression and photomorphogenesis remains to be shown.

Another approach to investigating the function of specific transcription factors is to analyze mutants disrupted in genes encoding these proteins and to characterize their light responses. Examples are the bZIP HY5, whose deficiency in plants resulted in elongated hypocotyls under all light conditions, the MYB protein LHY1, which is involved in circadian rhythm, and the bHLH proteins PIF3 and RSF1/HFR1/REP1 (Oyama et al. 1997; Schaffer et al. 1998; Halliday et al. 1999; Fairchild et al. 2000; Soh et al. 2000; Spiegelman et al. 2000).

Here, we report the isolation of the *laf1* mutant, which is specifically impaired in phytochrome A signal transduction. Our results show that LAF1 belongs to the R2R3–MYB transcription family of proteins and likely functions as a positive component of phyA signaling. This transcription activator may be responsible for regulating the expression of a specific subset of phyA target genes. Moreover, we show that a LAF1–GFP fusion can localize to subnuclear speckles, and this localization is abolished by the K258R point mutation in LAF1.

Results

Mutant screening and isolation of laf *mutants*

To identify genes involved in the phyA signal transduction pathway, we screened a collection of independent *Arabidopsis* gene trap lines, which were generated using the *Ds*-based system of Sundaresan et al. (1995). Mutants with elongated hypocotyls under FR light and/or resistance to FR light-induced killing were selected (Barnes et al. 1996a; Bolle et al. 2000; Møller et al. 2001). The progeny of putative mutants was tested under continuous FR and red (R) light conditions, and only mutants with long hypocotyls under FR light were considered to be specifically impaired in phyA signaling. Here, we describe the isolation and characterization of one such mutant, called *laf1* (long after far-red light 1).

Physiological characterization of the laf1 *mutation*

The mutant *laf1* is defective in several seedling responses when grown under continuous FR light. FR suppresses hypocotyl elongation in both the wild-type (WT) and the *laf1* mutant, but this response was reduced significantly in the latter (Fig. 1). In contrast, a phyA photoreceptor mutant (*phyA*) is completely blind to FR, resulting in long hypocotyls under these conditions (Fig. 1; Whitelam et al. 1993; Shinomura et al. 2000). The suppression of hypocotyl elongation in R (Fig. 1A,B), white (W; Fig. 1A), and blue (B) light (data not shown) was not altered in *laf1*. Mutant seedlings also showed a normal etiolated phenotype when grown in the dark (D; Fig. 1A,B).

The ability to green in white light after a prolonged FR light treatment is a specific feature of mutants defective in the phyA photoreceptor or blocked in phyA signaling (Barnes et al. 1996a). We tested *laf1* for its ability to green after exposure to different fluencies of FR light. At FR fluencies lower than 2 µmole/m^2 sec, *laf1* seedlings were resistant to the FR-induced killing, whereas under fluencies higher than 2 µmole/m^2 sec they were sensitive and died. This is in contrast with WT seedlings, which were sensitive to FR fluencies even lower than 2 µmole/m^2 sec (data not shown). Under FR light, WT plants synthesize anthocyanin, whereas phyA mutants are blocked in this process (Barnes et al. 1996b). We tested for anthocyanin accumulation in *laf1* seedlings grown under three different FR fluencies (1.5, 3, and 6 µmole/m^2 sec). Although *laf1* was able to synthesize anthocyanin under all three light conditions, the levels were reduced by 40%–50% compared with WT (data not shown).

We also examined other seedling responses triggered specifically by phyA, such as FR-dependent apical hook opening, cotyledon unfolding and expansion, and gravitropism (for review, see Neff et al. 2000; Smith 2000).

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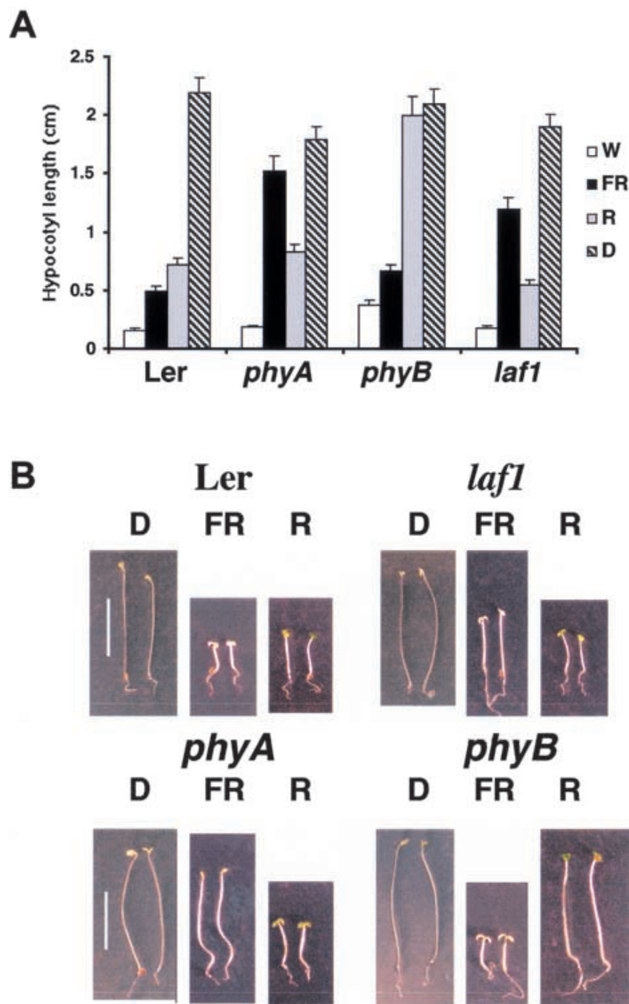

Figure 1. *laf1* seedlings are specifically impaired in FR-induced de-etiolation. (*A*) Hypocotyl lengths of *laf1* compared with WT (L*er*), *phyA*, and *phyB* under white (W; 15 µmole/m^2 sec), far-red light (FR; 3 µmole/m^2 sec), red light (R; 35 µmole/m^2 sec) conditions and darkness (D). Error bars show standard deviations. (*B*) Phenotypes of *laf1*. Seedlings of WT (L*er*), *laf1*, *phyA*, and *phyB* were grown for 4 d either in complete darkness under FR (3 µmole/m^2 sec) or under R light (35 µmole/m^2 sec). Bar, 10 mm.

These responses were not altered in *laf1* in comparison with WT over a range of fluencies. Furthermore, there was no obvious adult phenotype in *laf1* plants, other than the fact that mutant plants appear to have a slightly shorter inflorescence than WT. This did not affect the flowering time of *laf1*, which was very similar to that of the WT when measured under long day (16 h day/8 h night) conditions.

The loss of responsiveness to FR observed in *laf1* could, in principle, result from a reduction in phyA levels or spectral activity. No difference in phyA protein levels between WT and *laf1* was observed in etiolated seedlings (data not shown), and the mutation is not linked to the phyA locus. These results, together with the fact that *laf1* is impaired only in some FR light/phyA specific responses, indicate that the mutant is not compromised in the perception of FR light but rather in the transduction of its signal.

Genetic characterization of laf1

Genetic analysis showed that the mutant phenotype (i.e., elongated hypocotyls in FR light and partial resistance to FR-induced killing) cosegregated with the kanamycin resistance marker present on the single *Ds* insertion (Sundaresan et al. 1995). Backcrosses established that the *laf1* mutant phenotype was recessive. The mutation was located on chromosome IV and does not correspond to any other mutation that produces a FR-specific long-hypocotyl phenotype (*fhy1*, *fhy3*, *fin2*, *far1*, *pat1*, *rsf1/hfr1/rep1*, *fin219*, *laf6*).

Cloning of the LAF1 *gene*

We used the *Ds* tag in *laf1* to clone the *LAF1* locus. The nucleotide sequence flanking the insertion was identical to that of a region on chromosome IV containing an ORF that corresponds to a *MYB* gene previously identified as *AtMYB18* (GenBank accession no. Z95744; Kranz et al. 1998). After isolation of the cDNA by RT–PCR and its comparison to the genomic sequence deposited in the database by the *Arabidopsis* Genome Initiative, we determined that the *LAF1* gene contained three exons and two introns (Fig. 2A). The *Ds* element was inserted in the third exon at nucleotide position 506 from the translation start point.

The *LAF1* gene encodes a protein of 283 amino acids with two MYB domains located at the N-terminal region. Each MYB domain consists of an ~50-amino-acid helix–turn–helix motif, and each contains tryptophan residues in characteristic positions (Fig. 2B). In these respects, LAF1 is similar to the two-repeat (R2R3-type) MYB proteins, more than 130 members of which so far have been identified in the *Arabidopsis* genome (Martin and Paz-Ares 1997; Riechmann et al. 2000). Overall, LAF1 is most similar to AtMYB19 from *Arabidopsis* whose function is unknown (Fig. 2B; Kranz et al. 1998).

Expression pattern of LAF1

The steady state level of the *LAF1* transcript is very low. Reverse Northern analysis described by Kranz and coworkers (1998) indicated no strong induction of AtMYB18/LAF1 expression after treatment with hormones or elicitors or exposure to abiotic stresses. The only tissues in which weak expression could be detected were cauline leaves. Using Northern blot hybridization with poly(A) RNA from cauline leaves of WT plants, we could detect a single band of ~850 nucleotides, which corresponds to the appropriate size of the *LAF1* cDNA. No *LAF1* transcript could be detected in *laf1* mutant plants even with RT–PCR suggesting that *laf1* is a true null mutant (data not shown).

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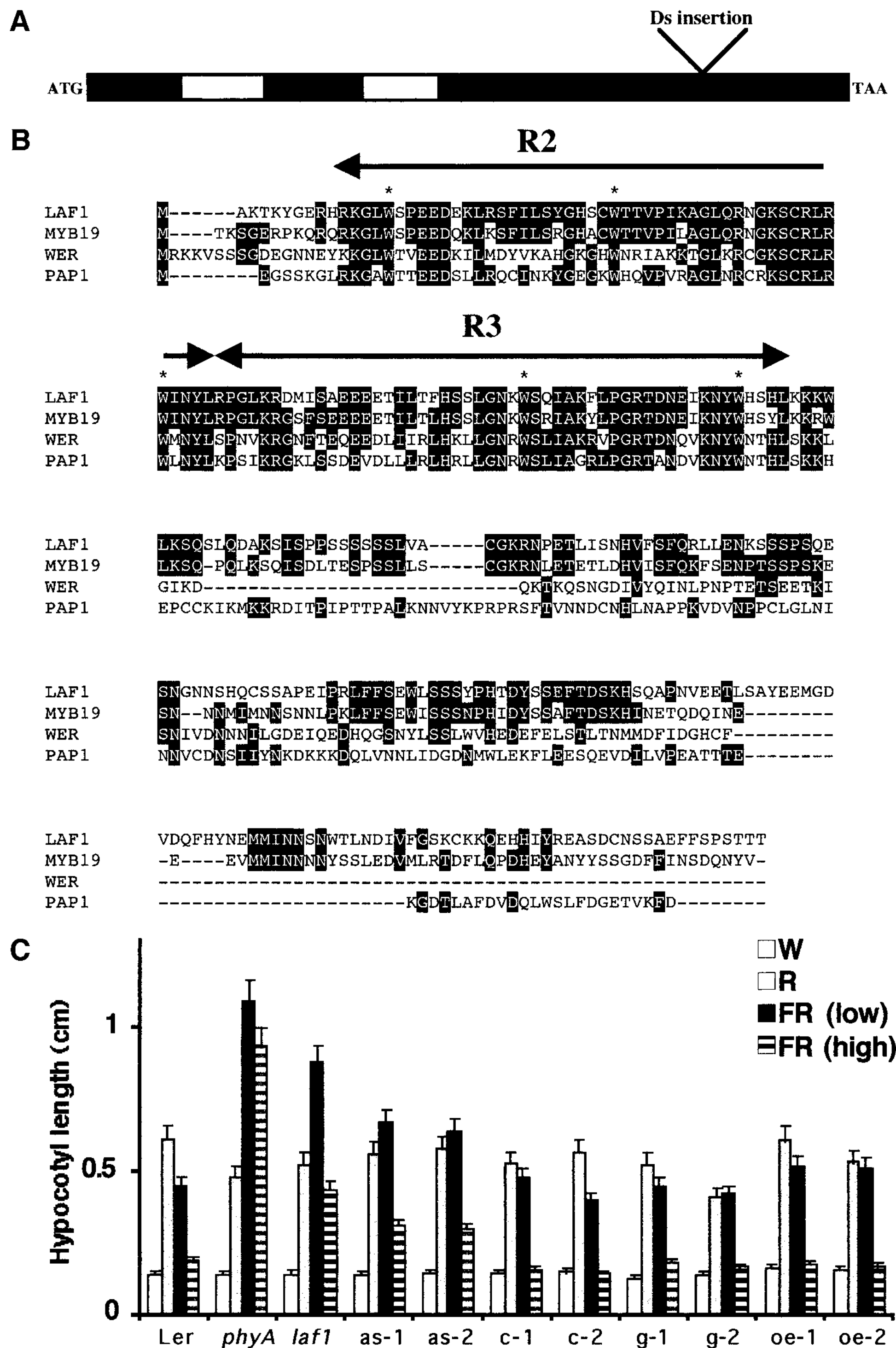

Figure 2. *LAF1* encodes a protein with homology with R2R3–MYB proteins. (*A*) A schematic representation of the genomic organization of *laf1*. The exon (black)/ intron (white) structure and the insertion site of the *Ds* element (506 bp from the ATG start codon) are shown. (*B*) Sequence comparison of LAF1 with other members of the R2R3–MYB protein family in *Arabidopsis*: AtMYB 19 (MYB19; GenBank accession no. Z95745), WEREWOLF (WER; Lee and Schiefelbein 1999), and Production of Anthocyanin Pigment 1 (PAP1; Borevitz et al. 2000). Conserved amino acid residues are highlighted in black. (Arrows) MYB domains (R2 and R3) ; (asterisks) conserved tryptophan residues within these domains. (*C*) Hypocotyl lengths of *laf1* complemented with the *35S–LAF1* cDNA (c-1, c-2) or the *35S–LAF1* genomic transgene (g-1, g-2) and transgenic WT lines with the *LAF1* antisense construct (as-1, as-2) or the *35S–LAF1* cDNA overexpressed (oe-1, oe-2). In comparison, the hypocotyl lengths of WT (L*er*), *phyA*, and *laf1* are shown. Light conditions are W (15 µmole/m^2 sec), R (5 µmole/m^2 sec), FR light at low fluence (1.5 µmole/m^2 sec), and FR light at high fluence (6 µmole/m^2 sec). Error bars show standard deviations.

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Genetic complementation of laf1 *and analyses of transgenic antisense plants*

To test for complementation, we transformed *laf1* with either a *LAF1* genomic DNA (1018 bp long) or a *LAF1* cDNA (852 bp long) under the control of a 35S promoter. Seven independent transgenic lines from each transformation experiment were characterized. Figure 2C shows representative transgenic lines, which display WT hypocotyl lengths under low and high FR fluencies, showing complementation of the mutant phenotype. Interestingly, plants overexpressing the *LAF1* gene do not show any clear phenotype under any analyzed light conditions, although the transcript levels are elevated (Fig. 2C).

The third exon of the *LAF1* gene, which shows no high sequence homology with any other gene in the databank, was cloned in the antisense orientation under the control of a 35S promoter, and this expression cassette was transferred into WT plants. Four independent *LAF1* antisense transgenic lines showed hypocotyl lengths 1.5-fold longer than WT under FR light, but WT hypocotyl lengths under white, R and B light, and in darkness (Fig. 2C). Together, these results confirm that indeed the disruption of the *LAF1* gene is responsible for the *laf1* mutant phenotype.

Altered expression of several phyA-regulated genes

Regulation of developmental processes requires fine tuning of gene expression regulating cell elongation and cell differentiation. Previous pharmacological experiments in tomato and soybean cells have indicated that phyA regulates gene expression by at least three pathways: a cGMP-dependent pathway mediating chalcon synthase (*CHS*) gene expression, a Ca^{2+}/calmodulin-dependent pathway that is necessary for chlorophyll a/b-binding protein (*CAB*) and ribulosebisphosphate carboxylase small subunit (*RBCS*) gene expression, and a third pathway, which requires both cGMP and Ca^{2+}/calmodulin to induce plastocyanin (*PET E*) and ferredoxin:$NADP^{(+)}$ oxidoreductase (*PET H*) gene expression (Neuhaus et al. 1993; Bowler et al. 1994). To locate the site of action of LAF1, we performed Northern blot hybridizations with *CHS*, *CAB*, and *PET E* probes by using *laf1* seedlings grown in the dark followed by exposure to different FR fluencies for 18 h. Figure 3 shows that the expression levels of all three target genes were reduced in the *laf1* mutant compared with WT. However, the reduction was not as severe as that of the *phyA* mutant, which was used as a control. These results confirm the morphological phenotype under FR light, showing that *laf1* is deficient in phyA signaling over a wide range of fluencies.

We also analyzed the expression of *CAB*, *CHS*, and *XTR7* induced by either R or FR light (Fig. 4). *CAB* gene expression is reduced under FR light conditions in the *phyA* mutant and under R light conditions in the *phyB* mutant. In *laf1*, a reduction in *CAB* levels can be observed only under FR light. *CHS* gene expression is dependent on a functional phyA signaling pathway under R and FR light conditions (Barnes et al. 1996b), and accordingly it is reduced under R and FR light conditions in *laf1*. *XTR7*, which is involved in cell elongation as it encodes a xyloglucan endotransglucosylase-related protein (Xu et al. 1996), is negatively regulated by phyA and phyB in FR and R light, respectively (Kuno et al. 2000). Expression of *XTR7* in the *laf1* mutant was reduced in R light similar to WT, but less reduced in FR light. These data confirm the specificity of LAF1 for phyA-specific pathways. Note that the effects on FR-dependent gene regulation of *CAB* and *XTR7* in the *laf1* mutant are not strong after 2 h but very pronounced after 18 h.

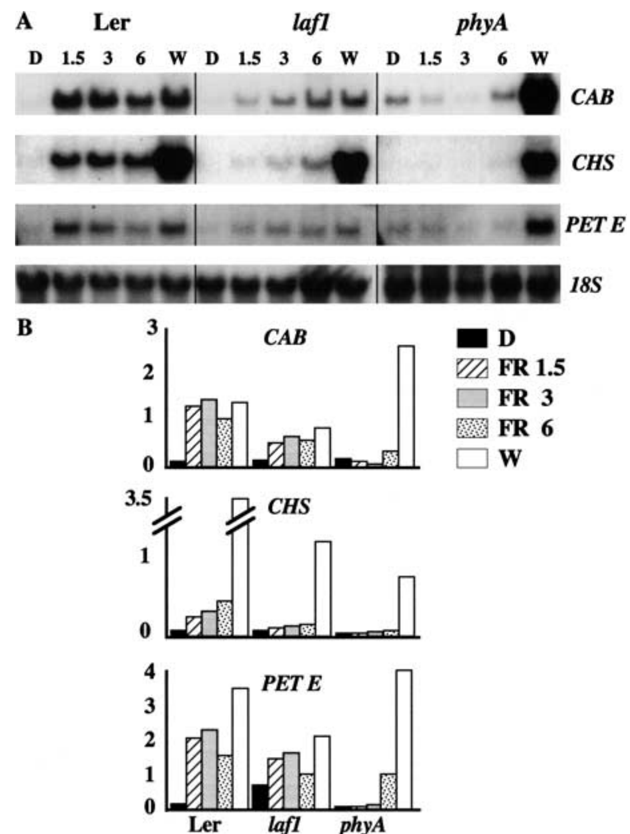

Figure 3. Expression of *CAB*, *CHS* , and *PET E* in *laf1*. Four-day-old seedlings were grown either in the dark under different FR light fluencies (1.5, 3, and 6 µmole/m^2 sec) or in W light for 18 h. Each lane contained 10 µg of total RNA. *CAB*, *CHS*, and *PET E* were used as probes. (*A*) Representative Northern blots. The hybridization with the 18S rDNA probe was used as a loading control. (*B*) Quantitative expression levels of *CAB*, *CHS*, and *PET E* in WT (Ler), *laf1*, and *phyA*. Northern blots were quantified with a PhosphoImager, and the expression levels were normalized with respect to the 18S rRNA level.

LAF1 acts as a transcriptional activator

All MYB transcription factors contain one to three conserved MYB domains at the N terminus where the DNA binding domain resides (Rosinski and Atchley 1998; Rabinowicz et al. 1999). The C terminus, on the other hand, shows a high degree of variability among the vari-

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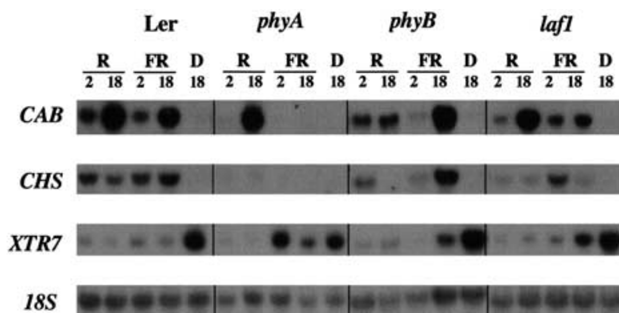

Figure 4. The *laf1* mutant is specifically disrupted in phyA-dependent gene regulation. Four-day-old seedlings (L*er*, *phyA*, *phyB*, and *laf1*) were grown in darkness and then, where indicated, transferred to R (35 µmole/m^2 sec) or FR light (3 µmole/m^2 sec) for 2 or 18 h. Each lane contained 10 µg of total RNA. *CAB*, *CHS*, and *XTR7* were used as probes. The 18S rRNA band was used as a loading control.

ous MYB proteins and is suggested to be important for protein–protein interaction. To determine whether LAF1 is a transcriptional activator and to identify the region of this protein responsible for this function, we fused the full-length *LAF1* cDNA and *LAF1* deletion mutants to sequences encoding the *GAL4* DNA binding domain. These DNA fusion constructs were tested in yeast for their ability to promote expression of the *lacZ* and *HIS3* reporter genes by binding to upstream activation regions. This allows assessment of growth on medium without histidine and measurement of β-galactosidase activity. Figure 5A shows that the full-length GAL4–LAF1 protein has low levels of transcriptional activity, but the C-terminal fragment GAL4–LAF1/113–283 (Ct) showed high levels of activation as measured by growth without histidine and in presence of 30 mM 3-AT. The LAF1 N-terminal fragment (Nt; GAL4–LAF1/1–128) and a C-terminal fragment (CtA; GAL4–LAF1/113–177) alone were unable to transactivate the reporter gene and prevented growth in the absence of histidine. However, GAL4–LAF1/164–232 (CtB) and GAL4–LAF1/217–283 (CtC) promoted better growth without histidine than the full-length LAF1. Only CtC, however, could promote growth in the presence of 30 mM 3-AT, which increased the stringency of the experiment. These results were confirmed by β-galactosidase assays (Fig. 5B). These data show that the C-terminal domain (amino acids 164–283) of LAF1 contains sequences with transactivation function. Furthermore, the results suggest the existence of two possibly distinct transactivation regions, one between amino acid residues 164 and 232 and the other, which shows six times higher β-galactosidase activity, between amino acid residue 217 and the C terminus. As

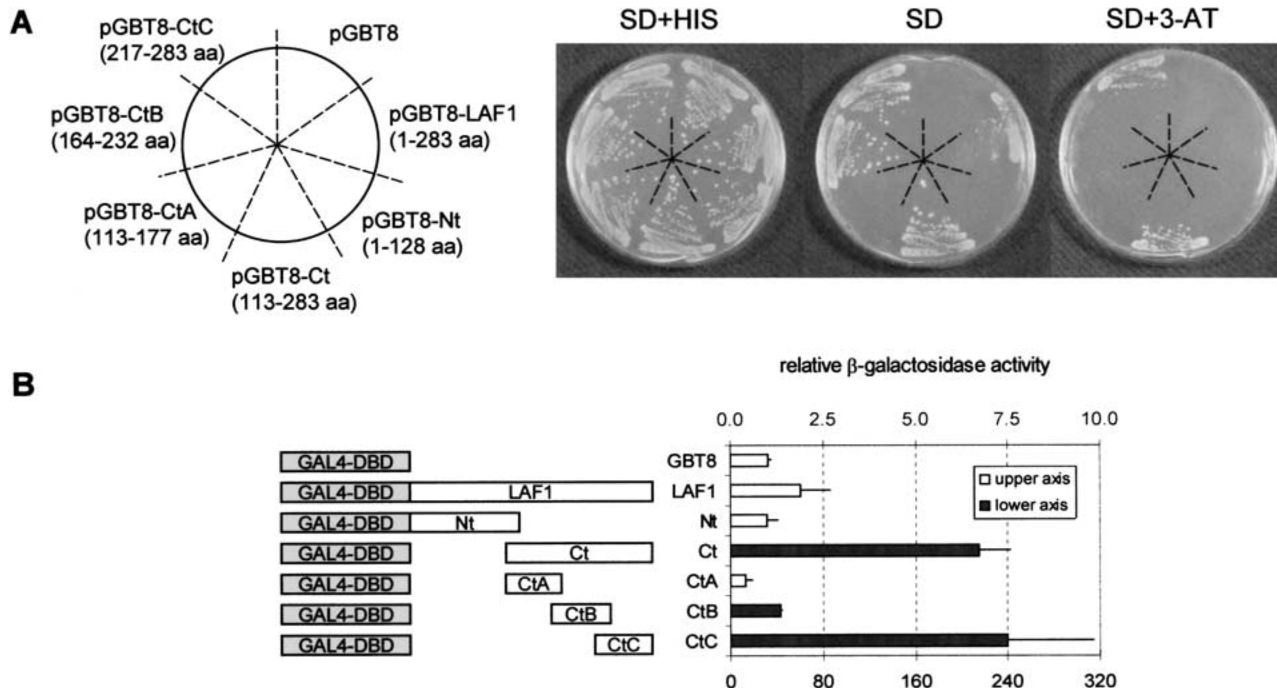

Figure 5. LAF1 acts as a transcriptional activator. (*A*) Transactivation analysis of LAF1 in yeast. Different *LAF1* cDNA fragments were fused to sequences encoding the *Gal4* DNA-binding domain in the yeast vector pGBT8 and transformed into yeast strain HF7c. Transformants were plated onto SD plates with histidine and incubated for 3 d. Three different colonies were transferred to SD plates with histidine (SD + HIS), without histidine (SD) and without histidine plus 30 mM 3-AT (SD + 3-AT), and cell growth ability was analyzed after incubation for 4 d. (aa) Amino acid. (*B*) β-Galactosidase liquid culture assay from yeast carrying a pGBT8 vector either empty or with different *LAF1* cDNA fragments by using ONPG as a substrate. The obtained β-galactosidase activity obtained from each construct was depicted relative to the basal levels obtained from the pGBT8 vector (=1). The data represent average values from three independent experiments in which several colonies were used to initiate the cultures.

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the full-length GAL4–LAF1 fusion shows less activity than the C-terminal fragment alone (~100-fold reduction in β-galactosidase activity), the results suggest a negative regulatory function associated with the N-terminal part of the protein, which contains the DNA-binding domain.

Nuclear localization of LAF1

The predicted LAF1 protein contains three potential monopartite nuclear localization signals (Fig. 6). The presence of these signals and the fact that LAF1 is a MYB-type protein with transcriptional activity suggest that LAF1 might function in the nucleus. We used a LAF1–GFP fusion protein to assay for the subcellular localization of the protein. A 35S–*LAF1–GFP* construct was transfected into onion epidermis cells by particle bombardment, and the treated onion peels were incubated in the dark or in W light. In contrast with the cytoplasmic/nuclear distribution of the GFP protein, which was used as a control, we found that the LAF1–GFP protein is localized in the nucleus irrespective of the light conditions (Figs. 6,7). Moreover, we observed that LAF1–GFP localizes to subnuclear foci (speckles), which are distributed throughout the nucleus.

This formation of speckles is time dependent. Four to six hours after bombardment of the onion cells with the full-length *LAF1–GFP* construct, GFP staining was evenly distributed throughout the nucleus (Fig. 7C). Nuclear speckle formation was observed only after ~8–10 h (Fig. 7D,E). At this time point, in ~90% of the nuclei examined, the GFP signal was detected exclusively in these foci with no significant background signal elsewhere in the nucleus. Four to eight h later, usually no GFP staining was visible. These results suggest that the formation of speckles might precede degradation of the protein.

To test the functions of the putative NLS and the signals that direct the LAF1–GFP fusion protein to nuclear speckles, we made deletion constructs of *LAF1* and fused them to the *GFP* coding sequence. An N-terminal fragment of LAF1 (LAF1/1–70) containing only the R2 domain was sufficient to direct nuclear localization, but the fusion protein did not accumulate in nuclear speckles (Figs. 6,7F). The same result was obtained for LAF1/1–113, LAF1/1–121, LAF1/1–161, and LAF1/1–175 (Fig. 7G). Only the deletion construct LAF1/1–262 again was able to localize to nuclear speckles (Fig. 6).

The C terminus of LAF1 (amino acids 162–283) fused to GFP was no longer able to localize to the nucleus (Figs. 6,7H), and its distribution throughout the cell resembled the localization of the GFP protein alone (Fig. 7K). The most conserved putative nuclear localization

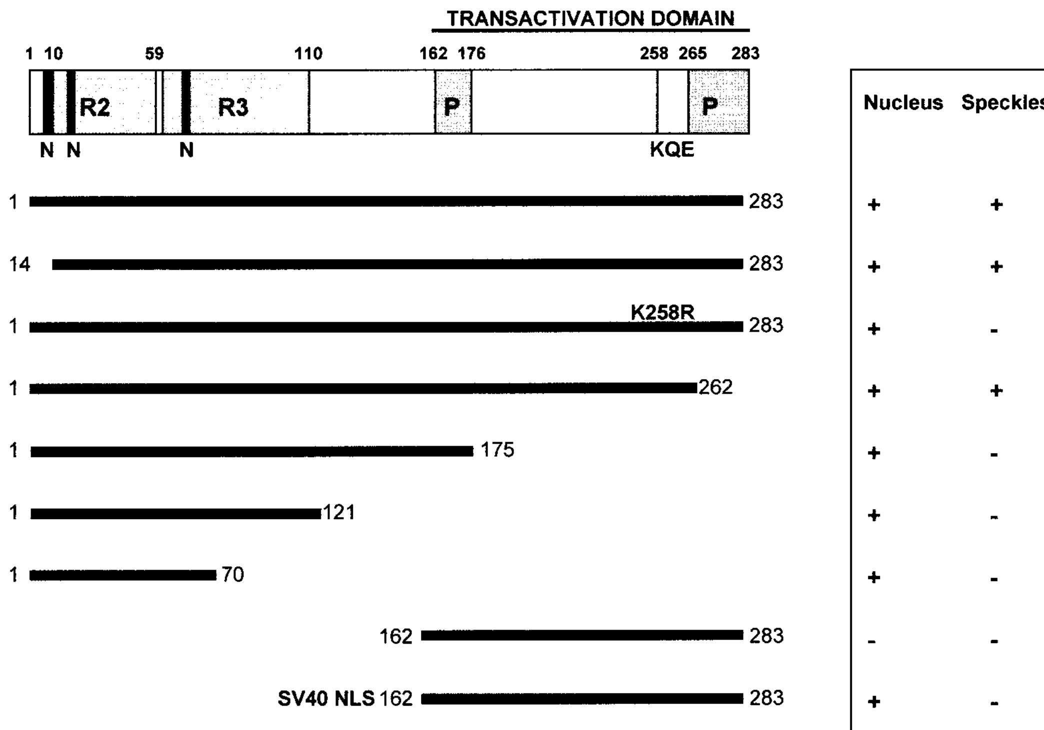

Figure 6. Structure of LAF1–GFP fusion proteins and their respective localization to the nucleus and to nuclear speckles. A schematic diagram of the structure of the LAF1 protein. The MYB domain (R2, R3), the PEST domains (P), the nuclear localization signals (N), the putative sumoylation site (KQE), and the transactivation domain are indicated at the top (numbers indicate amino acid residues). The LAF1 fragments that were fused to the N terminus of GFP are illustrated by bars. SV40-NLS indicates a fusion with the SV40 nuclear localization signal. For analysis of the localization of fusion proteins at least 50 transformed cells were examined in at least three independent transient expression assays by using onion epidermal cells. The table on the right shows the localization of the various GFP fusion proteins (+) positive; (–) negative.

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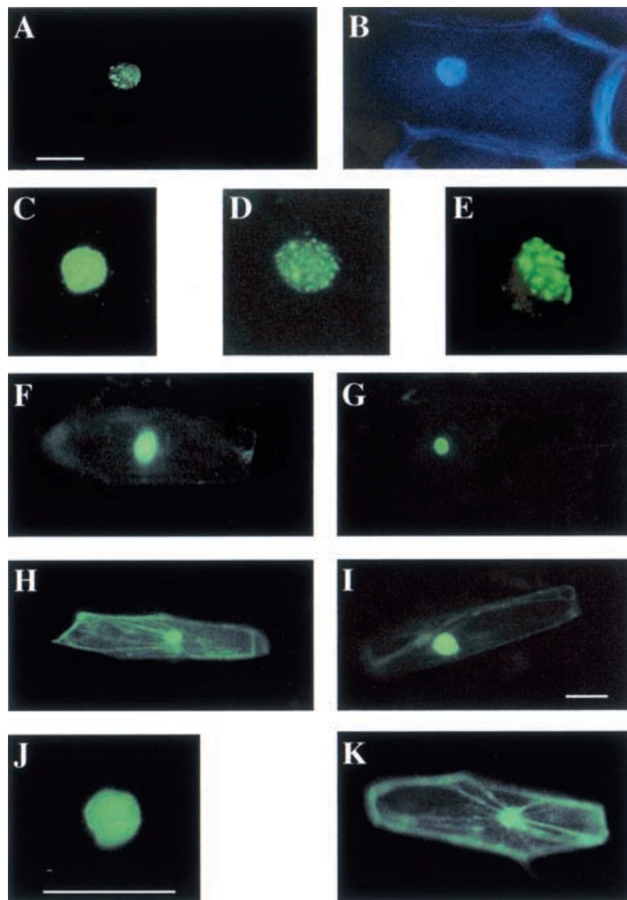

Figure 7. Representative images of subcellular location of LAF1–GFP fusions in transiently transformed onion epidermal cells. All experiments, except (*D*) were assayed 6–12 h after bombardment. (*A*) LAF1/1–283–GFP. (*B*) The same cell as in (*A*) stained with DAPI to show the location of the nucleus. (*C*) LAF1/1–283 3–6 h after bombardment. (*D*,*E*) LAF1/1–283 6–12 h after bombardment. (*F*) LAF1/1–70, (*G*) LAF1/1–175, (*H*) LAF1/162–283, (*I*) SV40-LAF1/162–283, (*J*) LAF1/1–283 K258R, (*K*) A control cell expressing GFP alone. Bars in *A*, *I*, and *J*, 50 µm.

signal (NLS) occurs between amino acids 9 and 12 (RHRK). However, an N-terminal deletion of LAF1 (LAF1/14–283) still was able to localize to the nucleus and to speckles (Fig. 6). Furthermore, this putative NLS (ERHRKG; amino acids 8–13) alone could not direct GFP to the nucleus efficiently (data not shown). Therefore, nuclear localization signals other than the one between amino acids 9–12 are needed to direct LAF1 to the nucleus, and these are located between amino acids 14–70, within the MYB domain R2.

Results from our deletion experiments suggest that sequences between amino acids 176 and 260 are responsible for localization to nuclear speckles. Nonetheless, certain sequences at the N terminus of LAF1 are, in addition, necessary for subnuclear localization, as fusion of an NLS from SV40 (APKKKRKVG; van der Krol and Chua 1991) to LAF1/162–283 caused accumulation in the nucleus but not in speckles (Figs. 6,7I).

Studies in mammalian cells have identified several proteins that localize to nuclear speckles (for review, see Lamond and Earnshaw 1998). In a few cases, for example, PML, it has been shown that these proteins accumulate in speckles only when they are conjugated with a ubiquitin-like protein called SUMO (small ubiquitin-like modifier; Müller et al. 1998). SUMO has been implicated in directing proteins to specific nuclear structures and stabilizing them rather than being involved in protein degradation as is ubiquitin (Melchior 2000; Müller et al. 2001).

We observed a consensus motif for sumoylation (ΨKXE; Melchior 2000; Rodriguez et al. 2001) at amino acid residues 257–260 (KKQE), without the hydrophobic amino acid (Ψ) preceding the target lysine (K). A glycine residue, which is usually in the vicinity of a sumoylation signal, can be found five amino acids upstream of this site. As SUMO usually is conjugated to a lysine residue, we mutated K258 to an arginine in the context of the full-length LAF1 protein fused to GFP. The LAF1 (K258R) mutant localized to the nucleus but not to speckles, suggesting that sumoylation plays a role in the localization of LAF1 to nuclear speckles (Figs. 6,7J).

Discussion

Although phyA is one of the best characterized photoreceptors in higher plants, the protein components involved in the signal transduction pathway are just beginning to emerge (Deng and Quail 1999; Smith 2000; Neff et al. 2000; Fankhauser 2001; Nagy et al. 2001). Isolation and characterization of mutants, especially from *Arabidopsis*, has proven to be one of the most powerful tools in dissecting the phyA signaling pathway.

laf1 *is a novel mutant specific for the phytochrome A signal transduction pathway*

Here, we report the genetic identification of a *LAF1*, which is involved in phyA signal transduction. The most obvious phenotype of *laf1* is the reduced inhibition of hypocotyl elongation when germinated under FR light. LAF1 is a specific phyA signaling intermediate as the hypocotyl length is not affected under any other light condition. This is underscored by the molecular analysis, which showed that expression of *CHS*, *CAB*, *PET E*, and *XTR7* is only affected in a phyA-dependent way.

The genetic analysis performed here indicates that *LAF1* is not allelic to photomorphogenic mutants reported previously. The genetic evidence also indicates that the *laf1* mutation is recessive, and that the insertion of a *Ds* element into the third exon of the gene caused a complete loss-of-function mutation, as no *LAF1* transcript can be detected in the mutant.

Nevertheless, *laf1* is not completely blocked in phyA signaling, because the hypocotyl elongation is still responsive to FR light and even under low FR light the hypocotyl is not as long as that of a *phyA* mutant. Under higher FR light fluencies *laf1* seedlings are also sensitive to the FR-killing effect, which they are not under low fluencies (<2 µmole/m^2 sec). This fluence dependency is

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confirmed by hypocotyl length analysis (Fig. 2C) and the CAB gene expression pattern (Fig. 3). Furthermore, the *laf1* mutant is affected only in a subset of phyA-mediated responses, as under FR light hook opening is not impaired, cotyledons are completely unfolded and expanded (Fig. 1B), and the seedlings show no loss of FR-dependent gravitropism.

As the loss of LAF1 in this null mutant leads to a reduction in phyA signaling, LAF1 must be either an integral component of the transduction pathway or a positive regulator of it. The results suggest that LAF1 could be an element of a pathway that contributes only to some aspects of de-etiolation and needs to act in concert with other pathways for full effect. On the other hand, the partial block of signaling can be explained by the fact that other proteins might have overlapping functions with LAF1; therefore, the loss of LAF1 has only a mild effect on physiological responses. LAF1 appears to be more important at lower FR fluencies compared with high fluencies. LAF1 could be rate limiting at low FR light intensities, but at higher light intensities other factors might be able to substitute. This also might explain why the phenotype is not as strong as in the *phyA* mutant.

LAF1, a R2R3–MYB protein, acts as a transcriptional activator

The *LAF1* gene encodes a protein with sequence homology with the large R2R3–MYB protein family (Romero et al. 1998), corresponding to AtMYB18, previously named by Kranz et al. (1998). The constitutive nuclear localization of transiently expressed LAF1 in onion epidermal cells and the presence of the putative DNA binding domain suggest that LAF1 might act as a transcription factor. We tested whether LAF1 can transactivate a reporter gene when fused to the DNA binding domain of GAL4. The results showed that the domain necessary for transactivation is located in the C terminus of the protein (amino acids 164–283) and especially between amino acid 217 and the C terminus. In other MYB transcription factors, this region also has been shown to be important for transactivation, although no sequence homology is apparent (Lee and Schiefelbein 1999). These results suggest that LAF1 might be involved in the fine tuning of a certain subset of genes as a transcription factor.

Although we understand well how MYB factors bind to target DNA (Rosisnsky and Atchley 1998), little is known about their functions. In vertebrates, for example, many MYB proteins have been found to play an essential regulatory role in cell proliferation and differentiation (Thompson and Ramsay 1995). The functions of *MYB* genes in plants appear to be far more diverse. Several members have been implicated in the regulation of secondary metabolism, cellular morphogenesis or the control of cell differentiation and cell cycle, signal transduction in plant growth, and responses to hormones, stress, and defense (Jin and Martin 1999).

To our knowledge, this report describes the first R2R3–MYB transcription factor shown to be involved in light signal transduction. So far, few transcription factors have been shown to function in light signaling in *Arabidopsis*. PIF3 and RSF1/HFR1/REP1 each contain a bHLH motif (Ni et al. 1998; Fairchild et al. 2000; Soh et al. 2000; Spiegelman et al. 2000), CCA1 and LHY1 each contain a single MYB domain (Wang et al. 1997; Schaffer et al. 1998), and HY5 is a bZIP (Oyama et al. 1997). PIF3 was identified as a phytochrome-interacting factor in a yeast two-hybrid screen. Bound to a DNA target site, PIF3 can interact directly with phytochrome in the Pfr form in vitro, suggesting that one mode of phytochrome signal transduction is the direct transcriptional regulation of target genes (Martinez-Garcia et al. 2000). Although PIF3 originally was identified as a protein interacting with both phyA and phyB, more recent biochemical and physiological data suggest that PIF3 plays a more prominent role in phyB signaling than in phyA signaling (Halliday et al. 1999; Zhu et al. 2000). In contrast, based on their respective loss-of-function phenotypes, RSF1/HFR1/REP1 and LAF1 are implicated in phyA signaling, but not phyB signaling.

The effect of the *laf1* mutation on *CAB*, *PET E*, *CHS*, and *XTR7* gene expression does not allow the distinction whether LAF1 has a direct or indirect impact on the transcription of these genes. The difference in the gene expression pattern of *laf1* as compared with WT is more pronounced after 18 h than after 2 h incubation in FR. This suggests that LAF1 might control the sustained transcription of these genes under FR light. A similar observation has been made in the *rep1* mutant, where at the 3-h time point the induction of *CAB* gene expression is similar to WT, but after 6 h the induction is strongly reduced (Soh et al. 2000).

LAF1 localizes to nuclear speckles

Recent studies have shown that besides transcriptional regulation, post-translational modifications such as phosphorylation or protein degradation also play critical roles in the regulation of plant transcription factors (Callis and Vierstra 2000; Hardtke and Deng 2000). One indication that LAF1 accumulation or activity might be regulated is that transgenic plants overexpressing a *35S–LAF1* transgene do not show any obvious phenotype nor any hypersensitivity to FR light, although the *LAF1* transcript levels are elevated (Fig. 2C).

One interesting observation is that the LAF1 protein not only localizes to the nucleus, but is directed to distinct nuclear speckles in a time-dependent manner. Nuclear speckles are formed for different reasons. One class of speckles is localized within the interchromatin space and enriched in splicing factors (Lewis and Tollervey 2000). Nuclear speckles also have been implicated in protein modifications caused by SUMO, a small ubiquitin-like modifier, which is conjugated to target proteins by an isopeptide bond (Melchior 2000; Müller et al. 2001). In contrast with ubiquitination, however, the covalent attachment of SUMO does not lead to protein degradation. Only a few SUMO target proteins have been identified, and so far, to our knowledge, none in plants. The exact function of SUMO modification, or sumoyla-

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tion, is not known. In some cases (e.g., IκBα and p53), conjugation of SUMO could lead to protein stabilization and protection from degradation, whereas in other cases (PML, SP100, RanGAP1, and HIPK2), SUMO conjugation could lead to a different subcellular localization of the modified protein, especially to nuclear speckles (summarized in Melchior 2000; Müller et al. 2001). Surprisingly, many SUMO targets, such as RanGAP1, PML, and HIPK2, contain PEST sequences, which are stretches of at least 12 amino acids rich in P, E, D, S, or T but lacking positively charged amino acids (Rechsteiner and Rogers 1996). In LAF1, two putative PEST sequences can be identified (Fig. 5).

The covalent modification of a target protein by SUMO has been shown to occur at a lysine residue within a minimal consensus sequence, ΨKX(E,D) (Melchior 2000; Rodriguez et al. 2001). LAF1 contains the sequence KKQE (257–260), which is a good match with the consensus sequence, although lacking the hydrophobic amino acid. On changing K258 to R258, thereby disrupting the putative SUMO conjugation site, we observed diffuse nuclear staining and rare speckle formation. These results suggest that recruitment of LAF1 to nuclear speckles requires K258, and this lysine might act as a modification site for SUMO. The domain that is important for nuclear speckle localization is distinct from the nuclear localization signals, which are in the N-terminal portion. However, the C-terminal sequence, containing the domain with the putative sumoylation signal is not sufficient for targeting to nuclear speckles. An SV40 NLS fused to LAF1/171–283 directed the protein to the nucleus but not to speckles. It appears that a functional DNA-binding domain is needed for localization of LAF1 to nuclear speckles.

So far, the only plant proteins that have been identified to localize to speckles are COP1, all phytochromes, CRY2, a blue-light photoreceptor, and RPN6, a component of the proteasome (von Arnim et al. 1998; Mas et al. 2000; Nagy et al. 2001; Peng et al. 2001). COP1 is a RING-finger protein with WD-40 repeats acting as a negative regulator of photomorphogenic development. The COP1 protein has been compared with PML because of their conserved domain structure and similar localization to speckles (Reyes 2001). Analysis of COP1 deletion mutants identified a 50-amino-acid long domain (SNLS) that is necessary for the localization in speckles (Stacey and von Arnim 1999). Although this domain shows no obvious homology with LAF1, it contains a putative sumoylation signal, RKME.

In summary, we present molecular and genetic evidence that LAF1, a nuclear protein containing two MYB motifs, is necessary for a branch of phyA signaling that regulates various photoresponses, including inhibition of hypocotyl elongation as well as *CAB*, *PET E*, *XTR7*, and *CHS* gene expression. The localization of LAF1 suggests an evolving theme that transcription factors are regulated on the level of protein stability and/or partitioning. Further analysis of the genes that are regulated by LAF1, and the factors that interact with LAF1 should provide important clues for identifying molecular intermediates, which lead from phytochrome photoconversion to alterations in gene expression.

Materials and methods

Plant material, growth conditions, and light sources

A collection of ~4000 *Ds* insertion mutants of *Arabidopsis thaliana* (L.) Heynh. var. Landsberg *erecta* (generated as described by Sundaresan et al. 1995), was used for genetic screens. The *laf1* mutant corresponds to line GT1968. Null mutants of *phyA* (*phyA-201*) and *phyB* (*phyB-1*) in L*er* were used as controls (provided by the *Arabidopsis* Biological Resource Center). Growth conditions and light sources were described in Bolle et al. (2000). Unless otherwise indicated fluence rates were as follows: FR, 3 µmole/m^2 sec; R, 35 µmole/m^2 sec; W, 15 µmole/m^2 sec. Individual lines were screened as described in Møller et al. (2001). For hypocotyl length measurements, experiments were repeated at least three times, each time measuring more than 20 seedlings per genotype. For Northern blot analysis, 5-day-old etiolated seedlings were either kept in continuous D or transferred into R or FR light for 2 or 18 h.

Genetic analysis

The *laf1* mutant was crossed with WT (L*er*), and the F_2 progeny was analyzed for kanamycin resistance and the *laf* (long after FR light) phenotype. Cosegregation of the two traits was observed among 120 F_2 seedlings, indicating a close linkage of a single *Ds* element insertion and the *laf1* mutation.

Extraction of DNA and RNA

Plant genomic DNA was isolated using the Genomic-tip-100 Kit according to the manufacturer's protocol (QIAGEN). Total RNA was extracted using the RNeasy Plant minikit (QIAGEN), and 10 µg of total RNA was used to isolate poly(A) RNA with the Oligotex kit (QIAGEN).

Isolation of LAF1 *cDNA and sequence analysis*

DNA sequences flanking the left border of the *Ds* element were obtained by inverse PCR of genomic DNA from *laf1* mutant plants, using the primers and restriction enzymes described in Sundaresan et al. (1995). PCR was performed with Takara LA Taq (Panvera) as recommended by the supplier. DNA samples were amplified using 35 cycles (94 °C for 20 sec, 60 °C for 30 sec, 68 °C for 8 min) followed by elongation at 68 °C for 10 min. A resulting 400-bp long fragment was cloned into pGEM-Teasy vector (Promega) and sequenced. Database searches were performed at the U.S. National Center for Biotechnology Information or *Arabidopsis* Information Resource (TAIR) with the BLAST program (Altschul et al. 1990) and showed that the *Ds* element was inserted into the third exon of a MYB transcription factor on chromosome IV (GenBank accession no. Z95744).

A 1018-bp genomic clone and a 852-bp cDNA were amplified from a genomic DNA or a cDNA library, respectively, using primers flanking the ORF predicted in the genome database. The cDNA library was made from *Arabidopsis* seedlings grown under either FR or W light by using the protocol described by the manufacturer for the Marathon cDNA Amplification Kit (Clontech). RT–PCR using this cDNA library confirmed the correct annotation of the predicted ORF in the genebank. Amino acid alignments were performed using the ClustalW program (DNAstar).

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Northern blot analysis

Northern blot analyses were performed as described in Bolle et al. (2000). The bands were quantified by a PhosphorImager (Molecular Dynamics) using 18S RNA as internal standard. As a probe for *LAF1*, the sequence of the third exon was used as a probe. *CHS*, *CAB*, and *PET E* probes were described in Barnes et al. (1996b). A 300-bp fragment from the 3′ end of the *XTR7* cDNA was used to ensure specificity (Xu et al. 1996).

Constructs for complementation and antisense gene

A *35S–LAF1–NOS* gene cassette in a binary vector containing a basta-resistance gene (Kost et al. 1998) was used for complementation. Both full-length *LAF1* genomic DNA and cDNA were used. Both fragments were generated by using primers to amplify the coding region of *LAF1* from the ATG start codon, adding a *Xho*I site, to the end of the coding region, adding a *Spe*I site. For the antisense construct, the third exon of the *LAF1* gene was expressed in the reverse orientation by using the cauliflower mosaic virus (CaMV) 35S promoter in a binary vector containing a kanamycin-resistance gene (van der Krol and Chua 1991). The constructs were verified by sequencing. The binary vectors were used to transform WT L*er* and *laf1* plants by vacuum infiltration (after Clough and Bent 1998). T1 transformants were selected on either basta or kanamycin-containing medium, grown to maturity, and selfed. Ten independent transgenic lines were generated with the construct containing the genomic DNA, seven with the cDNA and 10 with the antisense sequence. The presence of the transgene transcript was verified by Northern blot hybridizations. Homozygous T3 seedlings were analyzed for physiological responses.

Transactivation experiments

Full-length *LAF1* cDNA and deletion mutants were amplified by PCR, and appropriate restriction sites (5′ *Bam*HI, 3′ *Nhe*I) were introduced. PCR was performed using *Pfu* DNA polymerase (Stratagene) and appropriate primers under the conditions described by the manufacturer. The oligonucleotides used for LAF1/1–283 were (5.1) 5′-CTCTGGATCCATGGCGAAGAC GAAATATGG-3′ and (3.1) 5′-GACCGCTAGCTTACGTCGT TGTTGATGGAG-3′, for Nt (LAF1/1–128) (5.1) and 5′-GACC GCTAGCGGGAAATAGATTTTGCATC-3′, for Ct (LAF1/ 113–283) (5.2) 5′-CTCTGGATCCAGAAATGGCTCAAGTC TC-3′ and (3.1), for CtA (LAF1/113–177) (5.2) and 5′-GACCGC TAGCATTGATGGCTGTTATTTCCG-3′, for CtB (LAF1/164–232) 5′-CTCTGGATCCCTTCATCTCCCTCACAAGAAAG 3′ and 5′-GACCGCTAGCCTGATCAACATCACCCATTTC 3′, and for CtC (LAF1/217–283) 5′-CTCTGGATCCTGTC GAAGAGACTCTCTCAG-3′ and (3.1). Inserts were fused inframe to sequences encoding the *Gal4* DNA binding domain by cloning them into pGBT8 (Clontech).

We used the yeast strain HF7c, which contains the *LacZ* and *His3* reporter genes under the control of *GAL4* 17 mers (×3) or *GAL1* UAS, respectively (Feilotter et al. 1994). Yeast LiAc-mediated transformation and β-galactosidase liquid culture assays using *o*-nitrophenyl β-D-galactopyranoside (ONPG) were performed as described in the Clontech Yeast Protocols Handbook (Palo Alto, CA). The synthetic dropout (SD) medium was used either alone or with addition of 40 mg/L histidine (HIS) or 30 mM 3-AT.

Transient expression of GFP fusions in onion cells

The *GFP* coding sequence (Kost et al. 1998) was fused in-frame to the 3′ end of the *LAF1* cDNA. *LAF1* cDNA was generated by using primers to amplify the coding region from the ATG start codon (LAF1–ATG Xho 5′-CTCGAGATGGCGAAGACGAAA TATGG-3′) with an additional *Xho*I site, to the TAA stop codon (LAF1–TGA Kpn 5′-GGTACCCGTCGTTGTTGATGGAG-3′) adding a *Kpn*I site whereas deleting the stop codon. C-terminal deletions of *LAF1* were generated by the same method using the LAF1–ATG Xho primer and variable reverse primers: LAF1/1–262 (5′-GGTACCATGATGCTCCTGCTTCTTACATTTGG-3′), LAF1/1–175 (5′-GGTACCGCTGTTATTTCCGTTGCTTTC-3′), and LAF1/1–161 (5′-GGTACCGTTCTCTAGAAGTCTCTGG-3′). To amplify the constructs LAF1/1–70 and LAF1/1–121, we used a 5′ primer that contained an *Xba*I site instead of an *Xho*I site, and the 3′ primers were 5′-CTCGAGTGCACTAATCAT ATCCCTCTTTAACC-3′ and 5′-CTCGAGTTGTAAGCTCT GAGACTTGAGC-3′, respectively, introducing an *Xho*I site. For the N-terminal deletions, variable 5′ primers were used together with LAF1–TGA Kpn: LAF1/14–283 (5′-CTCGAGAT GTTATGGTCACCTGAAGAAGACG-3′) and LAF1/162–283 (5′-CTCGAGATGAAATCTTCATCTCCCTCACAAGAAAGC-3′). To generate the LAF1/8–13 fragment, we annealed two oligonucleotides (5′-CTAGATGGAGAGACATAGGAAAGGGC 3′ and 5′-TCGAGCCCTTTCCTATGTCTCTCCAT-3′), encoding the amino acid residues ME<u>RHRK</u>G. To generate the SV40-NLS, we annealed the following oligonucleotides: 5′-CTAGAA CAATGGCTCCCAAGAAGAAGAGAAAGGTAC-3′ and 5′-TCGAGTACCTTTCTCTTCTTCTTGGGAGCCATTGTT-3′, encoding the amino acid residues MAPKKKRKVG (van der Krol and Chua 1991). The two NLSs, LAF1/1–70 and LAF1/1–120, were cloned either into a vector containing only the *GFP* gene (Kost et al. 1998) or upstream of the LAF1/162–283–GFP construct utilizing the *Xba*I and *Xho*I restriction sites. The K258R mutation in the context of LAF1/1–283 was generated by using a primer (5′-ATGATGCTCCTGCCT<u>CTT</u>ACATTTGGAACC-3′) that introduced the point mutation in the appropriate position and the GeneEditor system (Promega).

Onion epidermal cells were transfected with DNA constructs containing the different *LAF1–GFP* fusions by using a helium biolistic gun (Kost et al. 1998). Unless stated otherwise, treated epidermal cells were kept in the dark for 6–16 h until the GFP localization was evaluated using an Axioskop microscope (Carl Zeiss).

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