PIF4, a phytochrome-interacting bHLH factor, functions as a negative regulator of phytochrome B signaling in *Arabidopsis*

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Plants sense and respond to red and far-red light using the phytochrome (phy) family of photoreceptors. However, the mechanism of light signal transduction is not well defined. Here, we report the identification of a new mutant *Arabidopsis* locus, *srl2* (short under red-light 2), which confers selective hypersensitivity to continuous red, but not far-red, light. This hypersensitivity is eliminated in *srl2phyB*, but not *srl2phyA*, double mutants, indicating that this locus functions selectively and negatively in phyB signaling. The *SRL2* gene encodes a bHLH factor, designated PIF4 (phytochrome-interacting factor 4), which binds selectively to the biologically active Pfr form of phyB, but has little affinity for phyA. Despite its hypersensitive morphological phenotype, the *srl2* mutant displays no perturbation of light-induced expression of marker genes for chloroplast development. These data suggest that PIF4 may function specifically in a branch of the phyB signaling network that regulates a subset of genes involved in cell expansion. Consistent with this proposal, PIF4 localizes to the nucleus and can bind to a G-box DNA sequence motif found in various light-regulated promoters.

Keywords: bHLH transcription factor/G-box DNA motif/light signaling/phytochrome/T-DNA tagging

Introduction

Being sessile organisms, plants have developed an enormous plasticity to adapt to the constantly changing environment. To keep track of time and spatial position, and to optimize growth and development throughout their life cycle, plants have at least three types of informational photoreceptors: an unknown UV-B receptor, UV-A/blue-light receptors, and phytochromes (phys), which sense the red (R)/far-red (FR) region of the spectrum (Kendrick and Kronenberg, 1994; Smith, 2000; Sakai *et al.*, 2001). Phys are soluble chromoproteins of ~125 kDa encoded by a small gene family (*PHYA*–*PHYE*) in *Arabidopsis* (Mathews and Sharrock, 1997). Phys can exist in two spectral forms. When synthesized in the inactive Pr form, absorption of red light converts the molecule to its biologically active Pfr form, which can be converted back to the Pr form upon absorption of far-red light. This reversible switching between the two forms is critical for

its photosensory and biological function (Quail, 1997, 2002; Smith, 2000).

A phy molecule consists of two domains: an N-terminal globular domain, which anchors the bilin chromophore and is responsible for photosensory function; and a C-terminal domain, which is thought to be responsible for signal transfer (Quail, 1997). Rapid progress has been made in recent years in identifying phy signaling intermediates, as well as in characterizing the biochemical function of phys (Deng and Quail, 1999; Hudson, 2000; Fankhauser, 2001; Quail, 2002). However, definition of the primary biochemical or molecular mechanism linking the absorption of light to activation of gene expression is still lacking.

A number of approaches have been used to investigate phy signal transduction. Biochemical approaches have provided evidence that recombinant oat phyA possesses serine/threonine protein kinase activity (Yeh and Lagarias, 1998). Pharmacological and microinjection approaches have implicated G-proteins, cGMP and Ca²⁺/calmodulin in phy signaling (Bowler *et al.*, 1994). Genetic approaches have identified a number of mutants that are either chromophore-biosynthetic or photoreceptor mutants, or signaling mutants (Hudson, 2000). Analyses of photoreceptor mutants, especially *phyA* and *phyB*, suggest that phyA is solely responsible for sensing continuous far-red light, and phyB is the major photoreceptor for red light in seedling de-etiolation (Quail *et al.*, 1995). Analyses of early signaling mutants indicate that they are either specific to each phy or responsive to both phys (Hudson, 2000). In addition, a large group of pleiotropic mutants (*cop1det/fus* group) have been characterized whose relevant loci encode proteins that function as general repressors of phy signaling as well as other pathways (Schwechheimer and Deng, 2000). These results suggest that each photoreceptor may have distinct genetically separable pathways, which might converge later in the signal transduction cascade (Deng and Quail, 1999; Hudson, 2000; Fankhauser, 2001; Quail, 2002). A number of genetic loci have been cloned recently and most of them are nuclear proteins (Fankhauser, 2001). In addition, phys are also translocated into the nucleus upon light activation (Nagy *et al.*, 2000), indicating that a major phy signal transduction event occurs in the nucleus.

Yeast two-hybrid screening coupled with reverse genetic approaches have identified a small group of apparently functionally unrelated proteins that are involved in phy signaling (Quail, 2000). In addition, targeted yeast two-hybrid assays showed that CRY1 can interact with phyA (Ahmad *et al.*, 1998), and *in vivo* FRET assays showed interaction between CRY2 and phyB (Mas *et al.*, 2000), suggesting cross-talk between phy and cryptochrome signaling. The C-terminal domain of phyB has also been shown to interact with ZTL/ADO1 and COP1

(Jarillo *et al.*, 2001; Yang *et al.*, 2001). Among the phy interacting factors, phytochrome kinase substrate 1 (PKS1), a cytosolic protein, interacts with the C-terminal domain of both phyA and phyB, and might be involved in retention of phy in the cytosol (Fankhauser *et al.*, 1999). NDPK2 is also a cytosolic enzyme, which interacts with phyA (Choi *et al.*, 1999). ELF3 interacts with phyB (Liu *et al.*, 2001), and is involved in the circadian clock (Cavington *et al.*, 2001). PIF3 is a bHLH protein that interacts with the biologically active Pfr form of both phyA and phyB (Ni *et al.*, 1998, 1999; Zhu *et al.*, 2000). In addition, G-box promoter element-bound PIF3 can interact with the Pfr form of phyB, suggesting a direct pathway for controlling gene expression by phys in response to light (Martinez-Garcia *et al.*, 2000).

Although a number of mutants are available that are selectively defective in photoresponsiveness to red light (Hudson, 2000; Fankhauser, 2001), the phyB signaling pathway does not seem to have been screened to saturation. Previously characterized components in this pathway include both positively and negatively acting factors (Fankhauser, 2001). Three of the positively acting loci, *POC1/PIF3*, *GI* and *ELF3*, have been molecularly cloned (Ni *et al.*, 1998; Fowler *et al.*, 1999; Halliday *et al.*, 1999; Park *et al.*, 1999; Huq *et al.*, 2000b; Hicks *et al.*, 2001; Liu *et al.*, 2001), and all three are nuclear proteins. In addition, two negatively acting components, PKS1 (Fankhauser *et al.*, 1999) and ATHB2 (Steindler *et al.*, 1999), have been molecularly characterized. PKS1 is a cytosolic protein that interacts with both phyA and phyB (Fankhauser *et al.*, 1999), and ATHB2 is involved in shade avoidance. However, thus far, no mutants have been reported for these or any other molecularly characterized, negatively acting component in the phyB pathway. Here, we have sought to identify early signaling components specific to the phyB-mediated de-etiolation process. Using a genetic approach, we previously isolated mutants that are affected in the phyB signaling pathway and characterized two of them, *srl1* and *gi-100* (Huq *et al.*, 2000a,b). A third mutant isolated from this screen, *srl2* (short under red-light 2), is described here. We have cloned the *SRL2* gene, and show that *SRL2* encodes a protein designated PIF4 (phytochrome-interacting factor 4), which acts as a negative regulator specific to the phyB signaling pathway.

Results

SRL2 encodes PIF4, a phytochrome-interacting bHLH factor

We previously performed genetic screens under continuous red light (Rc) to isolate mutants specific to this wavelength (Huq *et al.*, 2000a,b). One previously uncharacterized hypersensitive mutant isolated in this screen is *srl2*. This mutant, isolated from a T-DNA-mutagenized population, maps to the bottom of chromosome 2, >25 cM south of *srl1*, another Rc-specific hypersensitive mutant we reported previously (Huq *et al.*, 2000a). *srl2* is a monogenic, semi-dominant mutation, which co-segregates with the kanamycin resistance marker in the T-DNA insert in a 3:1 resistant-to-sensitive ratio. We amplified flanking sequences of the *srl2* locus using the T-DNA tag. Sequence analysis of these fragments showed that the T-DNA is inserted within the third intron

of the *SRL2* gene (Figure 1A). We have also isolated and sequenced a partial cDNA clone. This clone was truncated at the 5'-end, based on the fact that an expressed sequence tag (EST) clone (36H2T7) was longer in the 5'-end, and the transcript size is ~1.7 kb on a northern blot (Figure 1B). Sequencing of the EST clone showed that the EST sequence has a mutation (C to T) introducing a stop codon at amino acid 281. The EST and the partial cDNA clones were spliced together to obtain a 1687 bp full-length cDNA. This full-length cDNA sequence has two in-frame stop codons in the 5'-untranslated region, and a 1290 bp open reading frame (ORF) encoding a predicted 430 amino acid protein designated PIF4 (see below). The PIF4 protein corresponds to the AAD22130 ORF on chromosome 2, which has been misannotated in the database as a 423 amino acid unknown protein.

A database search revealed that PIF4 contains a region with strong homology to the bHLH superfamily of transcription factors (Littlewood and Evan, 1998; Atchley *et al.*, 1999). Sequence comparison of the bHLH domain from both animal and other plant species showed that the residues that define the HLH domain (Atchley *et al.*, 1999) are extensively conserved in the PIF4 sequence (Figure 1C). Moreover, PIF4 has some sequence similarity to PIF3 (Ni *et al.*, 1998) outside the bHLH domain (Figure 1E; data not shown). PIF4 has a classical bipartite nuclear localization signal (NLS) (Dehesh *et al.*, 1995; Jans and Hübner, 1996) (Figure 1C) as well as another putative monopartite NLS (RKRK; amino acids 224–227). PIF4 does not contain the canonical PAS (Per-Arnt-Sim-like domain) domain (Kay, 1997; Dunlap, 1998). However, it has a region similar to the putative PAS-like domain of PIF3 located close to the bHLH region (Figure 1D and E).

We have investigated the expression of *PIF4* mRNA from wild type and *srl2* mutant under different light conditions using northern blot analysis. The results show that *srl2* expresses a truncated version of the *PIF4* mRNA compared with wild type (WS) (Figure 1B), suggesting that this insertional disruption is likely to be responsible for the *srl2* phenotype. The truncated *PIF4* mRNA has the potential to express a C-terminally deleted 242 amino acid PIF4 protein lacking the bHLH domain (Figure 1A). Thus, if synthesized, this truncated protein would not be expected to bind to any DNA target site, and might therefore either be inactive or have the potential to act in a dominant-negative fashion. *PIF4* gene expression also appears to be induced ~5- to 6-fold by both Rc and FRc light, and ~1.5 fold by pulses of R and FR light (Figure 1B). The absence or very low abundance of *PIF4* mRNA in the dark and subsequent induction by light indicate that this induction might be involved in PIF4 function. However, because both R and FR induce expression to about the same extent, this induction appears unlikely to be responsible for the Rc-specific activity of the *SRL2* gene.

srl2 is specifically hypersensitive to Rc

Since *srl2* was isolated as a short hypocotyl mutant under Rc, we investigated the specificity of this response under a range of Rc and FRc-light fluence rates. Figure 2A shows that *srl2* is hypersensitive over a broad range of Rc fluence rates compared with the wild type, whereas it is similar to the wild type under all fluence rates of FRc light. Since we

Fig. 1. *SRL2* encodes PIF4, which is a bHLH protein. (A and B) A T-DNA insertion in the light-regulated *SRL2* gene is responsible for the hypersensitive phenotype of *srl2*. (A) *srl2* has a T-DNA insertion in the *SRL2* gene. The exon–intron structure of the *SRL2* gene is shown as black and white rectangles on a thin line (top). The triangle indicates the position where the T-DNA is inserted. Truncated form of PIF4 protein potentially expressed in *srl2* (bottom). Exons, black (coding) and white (non-coding) rectangles; introns, line between exons. (B) *PIF4* expression is induced by R and FR light. Left panel: *PIF4* mRNA levels in WS and *srl2* seedlings grown for 3 days under Rc (20 $\mu\text{mol/m}^2/\text{s}$), FRC (15 $\mu\text{mol/m}^2/\text{s}$) or dark (Dk). Right panel: pulse experiment. *PIF4* mRNA levels in 4-day-old dark-grown seedlings that were exposed to 5 min of R light only (20 $\mu\text{mol/m}^2/\text{s}$) (Rp), 5 min of FR light only (20 $\mu\text{mol/m}^2/\text{s}$) (FRp) or 5 min of FR light immediately after 5 min of R light (Rp/FRp), followed by return to darkness for 1 h before RNA extraction. Full-length *PIF4* ORF from the cDNA was used as a probe. mRNA sizes are shown on the right side. 18S rDNA was used as a control to show the amount of RNA loaded in each lane. *PIF4* mRNA signals in the wild type (WS) were quantified using a phosphorImager and expressed as the relative expression level by dividing by the WS dark value after normalizing with 18S probe. Rp, red light pulses; FRp, far-red light pulses; Rp/FRp, red light pulses followed by far-red light pulses. (C) Sequence similarity of the bHLH domain of PIF4 to other plant and animal bHLH proteins. Identical residues are shown in reverse contrast. The basic region is marked by a solid line and the helix–loop–helix domain is indicated by the wavy and dashed thin lines below. The putative bipartite NLS is shown by the thick dashed line above. DDBJ/EMBL/GenBank accession Nos and amino acid numbers (in parentheses) are: PIF3, AF100166 (337–397); HFR1, AF324245 (127–188); SPT, AF319540 (191–251); RAP1, X99548 (406–466); BPERU, X57276 (378–435); R-Lc, P13526 (409–466); MyoD, CAA40000 (103–165); Arnt, P41739 (83–147); Ahr, P30561 (19–84). (D) The overall domain structures of PIF4 and PIF3. The putative PAS-like domain, NLS and the bHLH domain of PIF3 are indicated. (E) Sequence similarity of the putative PAS-like domain of PIF3 with a region of PIF4. The asterisk indicates the glycine residue whose conversion to valine significantly reduced the binding of PIF3 to the active Pfr form of phyB (Zhu *et al.*, 2000). The downward arrow indicates the position where the PIF4 protein is truncated in the *srl2* mutant. Multiple sequence alignments (C and E) were performed using the PILEUP program of the GCG package.

had only a single allele of *srl2*, we also performed reverse-genetic experiments to test for functional involvement of PIF4 in phy signaling. Figure 2D and E shows that transgenic antisense *PIF4* lines display a short hypocotyl phenotype under Rc similar to that of *srl2* (only two out of >10 lines are shown). In contrast, *PIF4* sense-over-expressing lines show longer hypocotyls under Rc (Figure 2D and E). In addition, the hypocotyl lengths of both sense and antisense transgenic lines are similar to the wild type under FRc (Figure 2D and E), as in the case of the *srl2* mutant (Figure 2A and D). The hypocotyl lengths of *srl2* and *PIF4* transgenic lines are also similar to their respective wild types in the dark (Figure 2A, D and E). Thus, both genetic and reverse-genetic data are mutually consistent, and indicate that PIF4 acts negatively in Rc signaling.

We have also measured the cotyledon area of the *srl2* mutant and compared this to that of the wild type (WS). Figure 2B shows that *srl2* has more expanded cotyledons

than the wild type (WS), similar to a phyB overexpresser line (ABO) (Wagner *et al.*, 1991), and in contrast to the *phyB* mutant compared with its wild type (No-O). Western blot analysis of phyB protein level using monoclonal antibodies against phyB (Hirschfeld *et al.*, 1998) showed no significant difference between *srl2* and the wild type (WS) (Figure 2C), suggesting that the *srl2* phenotype is not due to overexpression of the phyB photoreceptor. These results confirm that *srl2* is an Rc-specific hypersensitive mutant potentially specific to the phyB pathway.

phyB is required for the hypersensitive phenotype of *srl2*

Since *srl2* showed a hypersensitive phenotype only under Rc, we constructed *srl2phyA* and *srl2phyB* double mutants to investigate whether phyA and phyB are required for the phenotype of *srl2*. As shown in Figure 3A, the *srl2phyA* double mutant is still hypersensitive to a range of Rc compared with its *phyA* sibling, suggesting either that

Fig. 2. PIF4 acts as a negative regulator of phyB-mediated responses. (A) Fluence-rate response curves of mean hypocotyl lengths of wild type (WS) and *srl2* mutant grown for 3 days under either Rc (left) or FRc (right). (B) *srl2* has more expanded cotyledons than wild type in Rc. Cotyledon areas of WS and *srl2* as well as No-O, ABO(No-O) and *phyB*(No-O) grown under Rc (20 $\mu\text{mol}/\text{m}^2/\text{s}$) for 4 days. (C) *phyB* protein level is unaltered in the *srl2* background compared with the wild type (WS). Total protein was isolated from 3-day-old dark-grown or Rc-grown (20 $\mu\text{mol}/\text{m}^2/\text{s}$) seedlings. Total protein was separated by 8% SDS-PAGE, transferred to PVDF membrane and probed with B1-B7 monoclonal antibodies (Hirschfeld *et al.*, 1998). (D) Photographs of seedlings grown under Rc (30 $\mu\text{mol}/\text{m}^2/\text{s}$), FRc (7 $\mu\text{mol}/\text{m}^2/\text{s}$) or Dk for 3 days. Col, Columbia wild type; As1, antisense *PIF4* line 1; As2, antisense *PIF4* line 2; Ox1, overexpressed *PIF4* line 1; Ox2, overexpressed *PIF4* line 2. (E) Mean hypocotyl lengths of the transgenic antisense and overexpression lines of *PIF4* along with the wild-type Col under dark (Dk), red (Rc; 30 $\mu\text{mol}/\text{m}^2/\text{s}$) and far-red light (FRc; 7 $\mu\text{mol}/\text{m}^2/\text{s}$). The *PIF4* and *PIF3* message levels in both the sense and antisense lines grown under Rc are shown in the inset. 18S rDNA was used as a control to show the amount of RNA loaded in each lane.

phyA is not required or plays a minor role in the hypersensitive phenotype of the *srl2* mutant. Although it appears that *srl2phyA* has a somewhat reduced hypersensitive response compared with *srl2*, this effect is paralleled by the slightly reduced sensitivity of the *phyA* single mutant compared with the WS wild type. It is possible that this difference is due to ecotypic differences, since the *srl2phyA* double mutant and its *phyA* sibling are in a WS-RLD hybrid background.

In contrast, the hypocotyl lengths of the *srl2phyB* double mutant are very similar to those of the *phyB* sibling, suggesting that *phyB* plays the major role in conferring the hypersensitive phenotype of the *srl2* mutant in Rc (Figure 3A). In addition, the cotyledon phenotype of the

Fig. 3. *phyB* is required for the hypersensitive phenotype of *srl2*. (A) Fluence-rate response curves of mean hypocotyl lengths of wild type (WS), *srl2* mutant, *srl2phyA* double mutant, *phyA* sibling, *srl2phyB* double mutant and *phyB* sibling grown for 3 days under Rc. (B) Visual phenotype of seedlings showing similar cotyledon size for *srl2phyB* and *phyB* siblings grown under Rc (30 $\mu\text{mol}/\text{m}^2/\text{s}$) and Dk for 3 days.

srl2phyB double mutant is indistinguishable from that of the *phyB* siblings (Figure 3B). These results also suggest that the other phytochromes (*phyC*, *D* and *E*) play a minor, if any, role in mediating the hypersensitive phenotype of the *srl2* mutant.

Light-regulated gene expression is not affected in *srl2*

To investigate the molecular phenotype of *srl2* and *PIF4* transgenic lines, we performed northern blot analyses on several selected marker genes involved in chloroplast development and circadian rhythms, including *CAB*, *RBCS*, *CCA1* and *LHY* (Tepperman *et al.*, 2001). However, the results did not show any significant difference between wild type and *srl2* or *PIF4* transgenic lines in response to light (data not shown). This is consistent with the chlorophyll content of the *srl2* and *PIF4* transgenic lines, which are also similar to that of the wild type (data not shown). Since *srl2* and *PIF4* transgenic lines have light-dependent, visible morphological phenotypes (Figures 2 and 3), it is possible that *PIF4* selectively controls other genes, such as those that are involved in cell expansion processes.

PIF4 interacts preferentially with *phyB*

Because *PIF4* is related to the phytochrome-interacting factor *PIF3* (Ni *et al.*, 1998), we investigated whether *PIF4* also interacts with *phys* using *in vitro* co-immunoprecipitation assays (Fairchild *et al.*, 2000). As shown in Figure 4B and C, *PIF4* interacts preferentially with the Pfr form of *phyB* and only very weakly with *phyA*. This is similar to *PIF3*, which interacts strongly with the Pfr form of *phyB* and comparatively weakly with *phyA* (Figure 4B and C; Zhu *et al.*, 2000). We have also investigated this interaction using the missense mutant versions of *phyA* and *phyB* (Figure 4A) that have been shown to have reduced capacity for signal transfer *in vivo* without affecting light perception ability (Quail *et al.*, 1995). Both mutant versions of *phyB* have reduced affinity for

Fig. 4. PIF4 interacts selectively with the Pfr form of phyB *in vitro*. (A) Schematic representation of the point mutations in phyA (above) and phyB (below) that showed reduced sensitivity to far-red and red light *in vivo*, respectively (Quail, 1995). Amino acid substitutions tested for interaction here are shown in reverse contrast. (B) Autoradiographs showing interactions of PIF4 with the wild type and two mutant forms of phyA and phyB, respectively. PIF4:GAD, GAD:PIF3 and GAD alone were used as baits to co-immunoprecipitate either wild-type phyA and phyB, or mutant versions of phyA and phyB (phy), either as the apoprotein (without chromophore attached), or as the holoprotein in Pr and Pfr forms. PIF4GAD, PIF4 fused to the Gal4 activation domain at its C-terminus; GADPIF3, PIF3 fused to the Gal4 activation domain at its N-terminus; GAD, Gal4 activation domain. (C) Quantitative analysis of the data obtained in (B). The percentage of input prey bound to bait is shown on the y-axis for each of the prey molecules indicated on the x-axis. (D) Autoradiographs showing the increased binding with increasing amounts of phyA or phyB in the Pfr form with either PIF4:GAD, or GAD:PIF3, or GAD alone used as bait. Approximately the same amount of bait (~12 fmol) was used for each construct in each tube. PIF4GAD, GADPIF3 and GAD are described as in (A). (E) Quantitative analysis of the data obtained in (D). The amount of each bait and prey used was calculated from a standard curve using a known amount of [³⁵S]methionine. The femtomoles of prey/femtomoles of bait are plotted against increasing amount of prey used.

PIF4, as observed for PIF3 (Figure 4B and C; Ni *et al.*, 1999), suggesting that this *in vitro* interaction might be biologically significant. We have also investigated interactions with phyC, D and E, and found no significant interaction with PIF4 (data not shown). Therefore, we conclude that PIF4 interacts selectively with the biologically active Pfr form of phyB.

We have also compared the apparent binding affinities of PIF4 and PIF3 for phyA and phyB by using increasing amounts of phyA and phyB as prey with a constant amount of PIF4:GAD and GAD:PIF3 as baits. PIF4 has about half the apparent affinity for phyB compared with PIF3 (Figure 4D and E). However, the ratios of binding between phyB and phyA are similar for both PIF3 and PIF4 (Figure 4E), respectively. The data also show that PIF4

interaction with phyB was saturated with the highest amount of phyB input (14 fmol), while PIF3 interaction was still increasing (Figure 4D and E), indicating that PIF3 has a higher interaction capacity for phyB than PIF4. Both proteins interacted with phyA very weakly under the present experimental conditions, although PIF3 showed slightly higher affinity (Figure 4D and E) as reported previously (Zhu *et al.*, 2000).

PIF4 binds to a G-box DNA motif

As PIF4 is a member of the well-characterized bHLH family of DNA binding proteins, we investigated whether PIF4 can bind to the G-box DNA motif found in many light-regulated promoters using gel-shift assays (Terzaghi and Cashmore, 1995; Chattopadhyay *et al.*, 1998; Ishige

Fig. 5. PIF4 binds to the G-box DNA motif present in various light-regulated promoters. (A) PIF4 binds to the G-box presumably as a homodimer in a sequence-specific manner. One microliter of the TnT-expressed PIF4 was used in each lane indicated (+). A total of 30 000 c.p.m. of labeled probe were used in each lane. The binding conditions were as described by Martinez-Garcia *et al.* (2000). pLUC control plasmid was translated in the TnT and used as the TnT-only control. The samples were separated on a 5% gel. FP, free probe. (B) G-box-bound PIF4 does not bind to phyB. The experiment was performed according to Martinez-Garcia *et al.* (2000). Briefly, TnT-expressed PIF4 or PIF3, phyB and labeled probe were mixed together in 1× binding buffer and exposed to either 10 min of R (88 μmol/m/s) or 5 min of FR (88 μmol/m/s). After light treatment, samples were incubated on ice for 2 h in the dark before running on a 4% gel. The gels were dried and exposed to a phosphorImager or X-ray film for analysis. Numbers on the top are microliters of each component added in each lane.

et al., 1999; Martinez-Garcia *et al.*, 2000). The data show that PIF4 can indeed bind to this motif under these conditions (Figure 5A). This binding can be competed by the addition of excess amounts of the cold G-wt probe, but not with a single base (T to G) mutated version of the G-mut probe (Martinez-Garcia *et al.*, 2000; Figure 5A). Thus, PIF4 can bind to the G-box motif presumably as a homodimer, which is characteristic of this family of proteins (Littlewood and Evan, 1998).

G-box-bound PIF4 does not bind to phyB

We have previously shown that G-box-bound PIF3 can interact with the Pfr form of phyB (Martinez-Garcia *et al.*,

Fig. 6. PIF4 is a nuclear protein. Schematic representations of GUS-only and GUS-PIF4 fusion constructs used in transient assays in onion epidermal cells are shown at the top. The left pair of panels show GUS staining for GUS-PIF4 and GUS-only control. The middle panels show DAPI staining for nuclei in the left panels. The right panels show superimposition of the GUS and DAPI signals. GUS-PIF4, β-glucuronidase fused to the N-terminus of the truncated PIF4 (amino acids 59–430).

2000). Since PIF4 can bind to the G-box as well as the Pfr form of phyB separately, we investigated whether the G-box-bound PIF4 can interact with the Pfr form of phyB. In contrast to PIF3, G-box-bound PIF4 does not bind detectably to the Pfr form of phyB even with a 4-fold higher amount of phyB than that used for PIF3 binding (Figure 5B). Under the same conditions, G-box-bound PIF3 can interact with the 4-fold lower amount of phyB and can form a supershifted complex (Figure 5B), as reported previously (Martinez-Garcia *et al.*, 2000). Thus, PIF4 cannot bind to both the G-box and phyB simultaneously under these experimental conditions.

PIF4 is localized in the nucleus

Since PIF4 has a putative NLS (Figure 1C), we investigated the subcellular localization of a GUS-PIF4 fusion protein in a transient transfection assay using onion epidermal cells (Huq *et al.*, 2000b). As shown in Figure 6, the GUS-PIF4 fusion protein is localized to the nucleus as compared with the GUS-only control, which is distributed throughout the cell. This result shows that PIF4 is a nuclear protein. We did not test whether this nuclear localization is affected by light in our transient assay. However, in previous investigations, both PIF3 and HFR1 were found to be constitutively nuclear, independent of light treatment (Ni *et al.*, 1998; Fairchild *et al.*, 2000).

Discussion

The data presented here provide genetic, photobiological and molecular evidence that PIF4 functions as a nuclear localized, negatively acting component potentially specific to the phyB signaling pathway. Compared with the wild type, both *srl2*-mutant and *PIF4*-antisense lines display short hypocotyls, whereas, conversely, the *PIF4*-overexpression lines show long hypocotyls under Rc (Figure 2A, D and E). The hypocotyl lengths of all these lines are similar in the dark, establishing that this phenotype is light dependent (Figure 2A, D and E). Moreover, all these lines also show similar hypocotyl lengths under all fluence rates of FRc (Figure 2A, D and

E), suggesting lack of involvement of PIF4 in phyA signaling. Since phyB is the major photoreceptor for Rc-light responses in seedling de-etiolation (Quail *et al.*, 1995), these photobiological experiments suggest the conclusion that PIF4 is involved selectively or specifically in the phyB signaling pathway. This conclusion is verified by the results of double mutant analyses, which show that phyB plays the major role in the hypersensitive phenotype of the *srl2* mutant (Figure 3). The alternative possibilities that the Rc-specific phenotype of *srl2*-mutant and *PIF4*-antisense lines is an indirect consequence of phyB overexpression (Wagner *et al.*, 1991), or a hyperactive phyB mutant molecule (Kretsch *et al.*, 2000), appear unlikely. This is because western blot analysis shows that the phyB level is similar in *srl2* and the wild type (Figure 2C), and the *SRL2* gene maps to a locus on chromosome 2, >35 cM south of *PHYB*.

Importantly, the concomitant Rc enhancement of cotyledon expansion and inhibition of hypocotyl elongation observed in the *srl2* mutant compared with the wild type (Figure 2B) establishes that the *srl2* mutation enhances normal light-induced photomorphogenic development, as opposed to causing a general defect in seedling growth and development. This conclusion is based on the well-established reciprocal effects of light on cell expansion in hypocotyls and cotyledons during de-etiolation, whereby hypocotyls are inhibited and cotyledons are stimulated by light (Kendrick and Kronenberg, 1994; Quail, 2002). These opposite, organ-specific effects provide a robust phenotypic method of distinguishing between mutants that globally inhibit general cell expansion and those specifically affecting light-regulated cell expansion.

The similar hypersensitive phenotype of the antisense lines and the T-DNA insertion mutant argues for the conclusion that *srl2* is a loss-of-function mutant and against the possibility that the *srl2* phenotype is due to a dominant-negative effect of the potential truncated PIF4 protein (Figure 1A and B). This, in combination with the converse hyposensitive phenotype of the transgenic overexpression lines (Figure 2), indicates strongly that PIF4 acts as a negative regulatory component in the signaling pathway.

A number of previous studies have reported the identification of other loci that appear to function selectively in Rc signaling in seedling de-etiolation. These include *pef2* and *pef3* (Ahmad and Cashmore, 1996), *red1* (Wagner *et al.*, 1997), *poc1* (Halliday *et al.*, 1999), *gi* (Huq *et al.*, 2000b) and *elf3* (Liu *et al.*, 2001), which act as positive regulators, and *PKS1* (Fankhauser *et al.*, 1999), *ATHB2* (Steindler *et al.*, 1999) and *srl1* (Huq *et al.*, 2000a), which act as negative regulators. Of the positively acting loci, only *POC1/PIF3* (Ni *et al.*, 1998), *GI* and *ELF3* have thus far been molecularly cloned, and all three have been reported to encode nuclear proteins. Of the negatively acting components, *PKS1* has been shown to be a cytosolic protein that interacts with both phyA and phyB, and *ATHB2* has been implicated in shade avoidance. However, no mutant has been reported for these latter two genes. Thus *SRL2* (encoding PIF4) is the first genetically identified locus that acts negatively in phyB-specific signaling.

Although PIF4 and PIF3 are closely related bHLH proteins, both involved in implementing phy-regulated

Fig. 7. Hypothetical model depicting a possible functional role of PIF4 in phyB-regulated seedling de-etiolation. PIF4 is proposed to act in a branch of phyB-regulated photomorphogenesis that controls cell expansion. The model indicates, in simplified fashion, that phyB and phyA regulate multiple aspects of seedling de-etiolation, including cell expansion and plastid development in response to Rc and FRc, respectively, via PIF3. PIF4, in contrast, is proposed to act specifically in the phyB pathway to regulate a subset of genes involved in cell expansion.

seedling de-etiolation, there appear to be important differences in the functional roles played by each in this process. PIF3 appears to function in both phyA and phyB pathways, and has been shown to be involved in regulating not only the cell expansion responses underlying the visible hypocotyl and cotyledon growth phenotypes, but also the expression of key regulatory genes, such as *CCA1* and *LHY*, which are involved in controlling chloroplast biogenesis and circadian rhythms (Ni *et al.*, 1998; Martinez-Garcia *et al.*, 2000). PIF4, by contrast, appears to be specific to the phyB pathway, and, while clearly being involved in the growth responses that constitute the visible phenotype (Figures 2 and 3), does not appear to regulate *CCA1*, *LHY* or other frequently used marker genes of plastid development (data not shown). These observations suggest a possible bifurcation in the phyB signaling pathway, whereby PIF4 may act specifically in phyB-regulated expression of a subset of genes involved in cell expansion, whereas PIF3 may act more globally in controlling multiple facets of seedling de-etiolation (Figure 7). It will be necessary to identify phyB-controlled genes that regulate the cell expansion process in response to light in order to investigate this possibility further.

The cellular and molecular mechanisms by which PIF4 exerts its regulatory function remain to be fully elucidated. At one level, the light-induced increase in *PIF4* expression (Figure 1B) raises the possibility that the implied increase in PIF4 protein abundance might be involved in its function. However, because both R and FR light are about equally effective in enhancing *PIF4* expression, it is unlikely that this effect provides the apparent specificity of PIF4 in phyB signaling. At another level, the apparent constitutive nuclear localization of PIF4 (Figure 6) and its capacity to bind to the G-box DNA-sequence motif (Figure 5) indicate strongly that PIF4 is likely to function in regulating gene expression in seedling de-etiolation, consistent with the established role of bHLH factors as transcriptional regulators. However, the apparent lack of involvement of PIF4 in regulating previously characterized G-box-containing genes, such as *CCA1* and *LHY*, which are targets of PIF3 (Martinez-Garcia *et al.*, 2000), suggests that nucleotides outside the core G-box hexanucleotide sequence might provide *cis*-element specificity

enabling different members of the bHLH family to discriminate between target promoters in the living plant cell, as is known for other systems (Littlewood and Evan, 1998). Based on these considerations, it might be anticipated that PIF4 will be selectively targeted, at least in part, to promoters of genes involved in regulating cell expansion.

The role of the physical interaction we have observed here between PIF4 and phyB in phyB signaling also remains unclear. On the one hand, the molecular specificity of the interaction of phyB with PIF4 is similar to that of PIF3 in *in vitro* pull-down assays. PIF4 binds with strong selectivity for the Pfr form of phyB, and with reduced affinity for signaling-mutant derivatives of phyB (Figure 4). These data, along with the phyB-dependent phenotype of the *srl2* mutant, are consistent with the notion that this direct interaction is functionally significant to phyB-specific signaling. However, in contrast to PIF3, PIF4 does not appear to retain the capacity to bind to phyB once bound to DNA (Figure 5). Thus, in principle, there appear to be at least two hypothetical mechanisms whereby a phyB–PIF4 interaction might operate negatively in phyB signaling: (i) PIF4 might sequester the active form of phyB in a non-productive complex, thereby reducing the photoreceptor's effective activity elsewhere; (ii) similarly, but conversely, this complex might effectively inhibit the biochemical function of PIF4, counteracting its negative activity. However, there are currently insufficient data to assess the relative contribution, if any, of either of these or other possible mechanisms to the function of PIF4. Thus, despite the compelling evidence that PIF4 is involved in phyB-regulated de-etiolation, the molecular mechanism by which this putative transcriptional regulator participates in this process requires further investigation.

Materials and methods

Seedling growth, mutant screening, measurements, mapping and western blot analysis

Seeds were surface sterilized and plated on growth medium (GM) without sucrose as described by Huq *et al.* (2000a). The red and far-red light sources were described by Wagner *et al.* (1991). Fluence rates of various lights were measured by a spectroradiometer (model LI-1800; LiCor, NE). The T-DNA-tagged seed pools were obtained from the *Arabidopsis* Biological Resource Center (Stock no. CS5600). These lines were generated in the WS ecotype at INRA using a promoter-trap construct (Bouché *et al.*, 1993). The screening was described by Huq *et al.* (2000b). Hypocotyl length measurements, mapping of the *srl2* locus and western blot analysis of phyB protein level using B1–B7 monoclonal antibodies (Hirschfeld *et al.*, 1998) were as described by Huq *et al.* (2000a).

Cloning of the *SRL2* locus and isolation of *PIF4* cDNA

The flanking sequence at the *srl2* locus was cloned using the genome walker kit from Clontech. An EST clone (36H2T7) corresponding to the flanking sequence was obtained from the ABRC stock center. A partial *PIF4* cDNA was also isolated and sequenced.

Transformation of *Arabidopsis* and transgenic plant analysis

The ORF from the *PIF4* cDNA was amplified using PFU Turbo polymerase (Stratagene) with forward and reverse primers that included restriction sites for cloning. The resulting fragments were cloned into the pKF111 vector (Ni *et al.*, 1998) in both orientations and sequenced. The resulting constructs were introduced into GV3101 (MP90) *Agrobacterium* and used for transformation of wild-type Col by the floral dip method (Clough and Bent, 1998). Transgenic seeds were plated on GM-Suc plates containing 5 µg/ml glufosinate-ammonium

(Riedel-de Haen, Germany), and the resistant seedlings were transplanted to soil and grown in the greenhouse.

Construction of *srl2phyA* and *srl2phyB* double mutants

Since *srl2* co-segregates with the kanamycin resistance marker, kanamycin selection was used to isolate homozygous *srl2* mutants. For the *srl2phyA* double mutant, homozygous *srl2* was crossed to *phyA101* (*hy8-1*, RLD ecotype) (Dehesh *et al.*, 1993) mutant. The resulting F₁ seedlings were selfed, and homozygous *phyA101* mutant-like seedlings were selected from the F₂ population under FRc (15 µmol/m/s). These seedlings were selfed and the F₃ seeds were plated on GM media containing kanamycin (100 µg/ml). Homozygous kanamycin-resistant and -sensitive lines were chosen as *srl2phyA* double mutant and *phyA* sibling, respectively. Construction of *srl2phyB* and *phyB* siblings is almost the same as described for *srl2phyA* and *phyA* siblings, except that selection of the F₂ population was performed under Rc (20 µmol/m/s). *phyB-1* (Reed *et al.*, 1993) introgressed into No-O background was used as the *phyB* mutant for constructing the *srl2phyB* double mutant. All the double mutants and single mutant siblings were confirmed by PCR markers.

RNA isolation and northern blotting

Total RNA was isolated from seedlings of different backgrounds as described by Huq *et al.* (2000a). Full-length *PIF4* ORF was used as a probe to detect message levels in the *srl2* mutant background and the overexpression lines. A 250 bp fragment from the 3'-UTR was amplified by PCR and used as a probe to detect the *PIF4* mRNA in the antisense lines. Full-length *PIF3* cDNA was used to detect the level of *PIF3* mRNA in the *PIF4* transgenic lines.

In vitro co-immunoprecipitation assay

In vitro co-immunoprecipitation experiments were performed as described by Fairchild *et al.* (2000). All proteins were expressed from T7 promoters in the TnT *in vitro* transcription/translation system (Promega) in the presence of [³⁵S]methionine. The construct and procedure for expressing full-length holophytochrome A were described by Fairchild *et al.* (2000), and holophytochrome B and GAD:PIF3 by Ni *et al.* (1999). The PIF4:GAD vector was constructed by PCR amplification of GAD and PIF4 separately, followed by three fragment ligation into a pET17b vector (Invitrogen), and confirmed by sequencing. The binding buffer used contained 1× PBS pH 7.2, 0.1% (v/v) Tergitol NP-40 (Sigma), 0.1% BSA and 1× Complete protease inhibitor (Roche). The same buffer was used for the first wash of the pellet, and the final wash was performed with the same buffer without BSA.

Electrophoresis mobility shift assays (EMSAs)

EMSAs were performed according to Martinez-Garcia *et al.* (2000). All the proteins were synthesized using the TnT system (Promega). Full-length PIF3 was described by Martinez-Garcia *et al.* (2000). The PIF4 ORF was cloned into pET17b to produce naked PIF4 protein. GAD:PIF4 was as described above. pLUC control plasmid was translated in TnT and used as TnT control. A total of 30 000 c.p.m. of labeled probe were used in each lane.

Subcellular localization of *PIF4*

For subcellular localization, an *XhoI* fragment from the original PIF4 cDNA clone was fused to the C-terminus of GUS in the vector TEX3 digested with *SalI* (Hoecker *et al.*, 1999). This PIF4 fragment contains amino acids 59–430 of the PIF4 protein. The resulting GUS–PIF4 fusion construct was transiently transfected into onion epidermal cells and stained for GUS activity according to Ni *et al.* (1998).

PIF4 accession number

The PIF4 cDNA sequence has been submitted to the DDBJ/EMBL/GenBank database. The accession No. is AJ440755.

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