

# Interaction of shade avoidance and auxin responses: a role for two novel atypical bHLH proteins

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Plants sense the presence of potentially competing nearby individuals as a reduction in the red to far-red ratio of the incoming light. In anticipation of eventual shading, a set of plant responses known as the shade avoidance syndrome (SAS) is initiated soon after detection of this signal by the phytochrome photoreceptors. Here we analyze the function of *PHYTOCHROME RAPIDLY REGULATED1* (*PAR1*) and *PAR2*, two *Arabidopsis thaliana* genes rapidly upregulated after simulated shade perception. These genes encode two closely related atypical basic helix–loop–helix proteins with no previously assigned function in plant development. Using reverse genetic approaches, we show that *PAR1* and *PAR2* act in the nucleus to broadly control plant development, acting as negative regulators of a variety of SAS responses, including seedling elongation and photosynthetic pigment accumulation. Molecularly, *PAR1* and *PAR2* act as direct transcriptional repressors of two auxin-responsive genes, *SMALL AUXIN UPREGULATED15* (*SAUR15*) and *SAUR68*. Additional results support that *PAR1* and *PAR2* function in integrating shade and hormone transcriptional networks, rapidly connecting phytochrome-sensed light changes with auxin responsiveness.

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## Introduction

The shade avoidance syndrome (SAS) refers to a set of plant responses aimed to adapt growth and development to environments of high plant density, like those found in both natural (e.g., forests) and agricultural (e.g., orchards) communities. Presence of nearby plants results in a reduction in the red (R) to far-red (FR) ratio (R:FR) caused by a specific enrichment in FR light reflected from the surface of neighboring leaves. The R:FR changes are perceived by the phytochrome photoreceptors (Smith, 1982; Smith and Whitelam, 1997). The phytochromes detect the R and FR parts of the spectrum and have a major role in controlling adaptive responses such as seed germination, stem elongation, leaf expansion and flowering time. In *Arabidopsis thaliana*, a small gene family of five members encodes the phytochromes (*PHYA-PHYE*) (Quail, 2002; Chen *et al*, 2004). Although phyB is the major phytochrome controlling SAS, genetic and physiological analyses have shown that other phytochromes act redundantly with phyB in the control of some aspects of SAS-driven development, like flowering time (phyD, phyE), petiole elongation (phyD, phyE) and internode elongation between rosette leaves (phyE) (Smith and Whitelam 1997; Devlin *et al*, 1998, 1999).

Downstream of R:FR perception by phytochromes, expression of several genes has been shown to rapidly and reversibly change in response to simulated shade (Devlin *et al*, 2003; Salter *et al*, 2003). Although it is unclear as to what extent the changes in the expression of these *PHYTOCHROME RAPIDLY REGULATED* (*PAR*) genes are instrumental for implementing the morphological and physiological SAS responses, it is likely that these photoresponses are a consequence of the regulation of a complex transcriptional network by phytochromes, as postulated for seedling de-etiolation (Quail, 2002; Jiao *et al*, 2007). Indeed, genetic approaches have demonstrated roles in SAS for some *PAR* genes encoding transcription factors, including *ATHB2*, *HFR1* and *PIL1*. A positive role for *ATHB2* has been proposed based on overexpression studies (Steindler *et al*, 1999), whereas a negative role for *HFR1* and *PIL1* was deduced based on the elongation responses of mutant hypocotyls to shade (Sessa *et al*, 2005; Roig-Villanova *et al*, 2006). The low R:FR perception might therefore rapidly change the balance of positive and negative factors, resulting in the appropriate SAS responses. Genetic analyses have recently identified *CSA1* as an SAS component that regulates the expression of *ATHB2* and *HFR1* (Faigón-Soverna *et al*, 2006), participating by unknown mechanisms in the shade-modulated transcriptional network. Eventually, this transcriptional network intersects with those of the major plant hormones, which regulate cell division and expansion changes needed for the specific photoresponses, that is, stem elongation and/or other changes to overgrow nearby competition. Indeed, several studies have established multiple links that connect auxin, brassinosteroid (BR), ethylene and

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gibberellins in the regulation of different photomorphogenic responses and specifically in the SAS responses (Tian and Reed, 2001; Devlin *et al*, 2003; Halliday and Fankhauser, 2003). Nonetheless, the precise molecular links for the interaction between SAS and hormonal transcriptional networks are mostly unknown.

Based on de-etiolation studies, it has been postulated that nuclear-localized phytochromes can potentially access G-box regulatory motifs located in the promoter regions of master regulatory genes by directly interacting with phytochrome-interacting factors (PIFs) (Martínez-García *et al*, 2000). One of them, PIF3, was the founder of a subgroup of basic helix-loop-helix (bHLH) proteins that act as regulators of seedling de-etiolation (Ni *et al*, 1998; Fairchild *et al*, 2000; Huq and Quail, 2002; Kim *et al*, 2003; Salter *et al*, 2003; Huq *et al*, 2004). This subfamily, known as group VII or subfamily 15 (Heim *et al*, 2003; Toledo-Ortiz *et al*, 2003), includes PIFs (i.e., PIF1, PIF4) and PIF3-like proteins (PILs), which lack the ability to directly interact with the phytochromes (i.e., HFR1, PIL1), but have been shown or proposed to heterodimerize with PIFs, potentially modulating the bHLH network activity. All the known bHLHs (PIFs and PILs) involved in phytochrome-mediated light signaling belong to this subfamily. The bHLH domain that defines the bHLH class of transcription factors encompasses ca. 60 amino acids arranged in two subdomains: a basic N-terminal stretch of 15–20 residues and an HLH domain composed of two amphipathic alpha helices separated by a variable loop region. The basic domain is involved in DNA binding, whereas the HLH domain is required for protein–protein interaction (i.e., dimerization). Comparison of animal bHLH sequences led to a hypothetical consensus motif of 19 conserved residues that define the bHLH domain (Atchley *et al*, 1999). Different groups of plant bHLHs fit this consensus (Buck and Atchley, 2003; Heim *et al*, 2003; Toledo-Ortiz *et al*, 2003). Several subfamilies of *Arabidopsis* bHLH proteins show conserved motifs outside the bHLH domain that might provide additional DNA-binding ability and specificity and/or protein interaction activities (Heim *et al*, 2003; Toledo-Ortiz *et al*, 2003). For instance, several members of the *Arabidopsis* bHLH subfamily 15 have an active phytochrome-binding motif shown to be necessary for PIF4 function in phyB signaling (Khanna *et al*, 2004).

We previously identified *PAR1*, a direct target gene of phytochrome action, whose expression is rapidly upregulated by shade (Roig-Villanova *et al*, 2006). In this work we show that *PAR1* and its close relative *PAR2* are atypical bHLH proteins that negatively control growth and metabolic SAS responses. In addition, we found that they act in the nucleus, impairing the auxin-regulated expression of two *SMALL AUXIN UPREGULATED (SAUR)* genes.

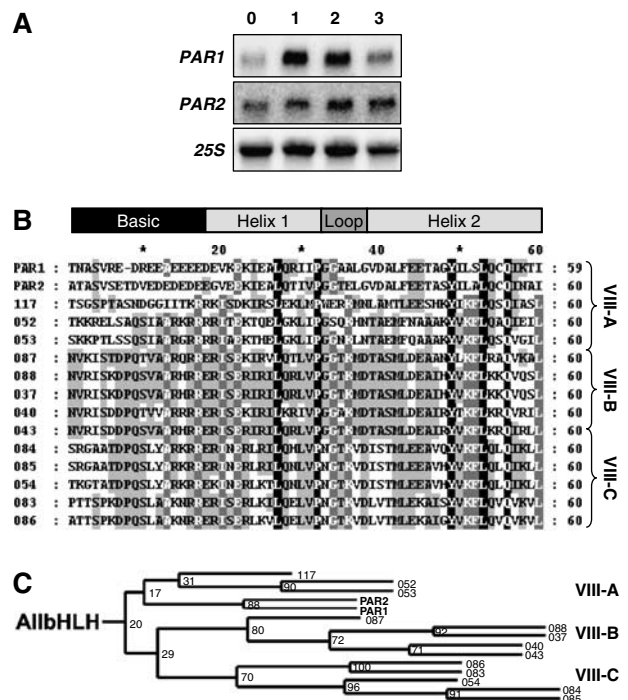
## Results

### *PAR1* and *PAR2* are novel bHLH-like proteins

The *PAR1* gene (At2g42870), previously identified to be a primary target of phytochrome signaling (Roig-Villanova *et al*, 2006), encodes a short protein of 118 residues of unknown function. *PAR1* is closely related in sequence to another *Arabidopsis* gene that we have named *PAR2* (At3g58850), which encodes a protein of the same size and with 72% similarity (64% identity) to *PAR1* (Supplementary Figure S1). Based on the existence of ESTs and our own data

(www.arabidopsis.org; Roig-Villanova *et al*, 2006), we concluded that both *PAR1* and *PAR2* are expressed genes with no introns and short 5' and 3' UTRs. As shown in Figure 1A, the expression of *PAR1* and *PAR2* was rapidly upregulated after a simulated shade treatment of FR-enriched white light (W + FR), consistent with their classification as *PAR* genes (Roig-Villanova *et al*, 2006). *PAR2* upregulation, however, was slower and weaker compared to that of *PAR1* (Figure 1A).

As a first step to investigate the functional identity of *PAR1* and *PAR2* proteins, InterProScan searches (www.ebi.ac.uk/InterProScan/) were performed, identifying a region corresponding to a bHLH domain (IPR011598). When *PAR1* and *PAR2* were used as queries in PSI-BLAST searches, only sequence hits corresponding to *Arabidopsis* proteins of the bHLH group were retrieved with significant scores. *PAR1* and *PAR2* are most similar to these transcription factors within the HLH region (Figure 1B; Supplementary Figure S2A), sharing most of the conserved sites and fitting well to the hypothetical, predictive consensus motif previously proposed (Atchley *et al*, 1999; Toledo-Ortiz *et al*, 2003). By contrast, *PAR1* and *PAR2* sequences diverged in the basic region, lacking the H/K9-E13-R17 motif characterized as critical for



**Figure 1** Expression and sequence analyses of *PAR1* and *PAR2*. (A) RNA blot analysis of *PAR1* and *PAR2* expression in 7-day-old W-grown wild-type seedlings treated with W + FR for 0, 1, 2 and 3 h. 25S rRNA levels are shown as a loading control. (B) Multiple sequence alignment of bHLH domains from *Arabidopsis* group VIII proteins (Heim *et al*, 2003), *PAR1* and *PAR2*. Identical residues are boxed in black. Gray boxes mark partially conserved residues. The position of the basic, helix and loop regions is indicated. (C) Neighbor joining unrooted phylogenetic tree based on the alignment shown in Supplementary Figure S2. Only the monophyletic clade grouping *PAR1*, *PAR2* and group VIII bHLHs is shown. Branch lengths are not proportional to the distance between sequences. Numbers in nodes correspond to bootstrap support values indicated as percentages.

proper DNA binding (Ferre-D'Amare *et al*, 1993; Atchley *et al*, 1999; Toledo-Ortiz *et al*, 2003).

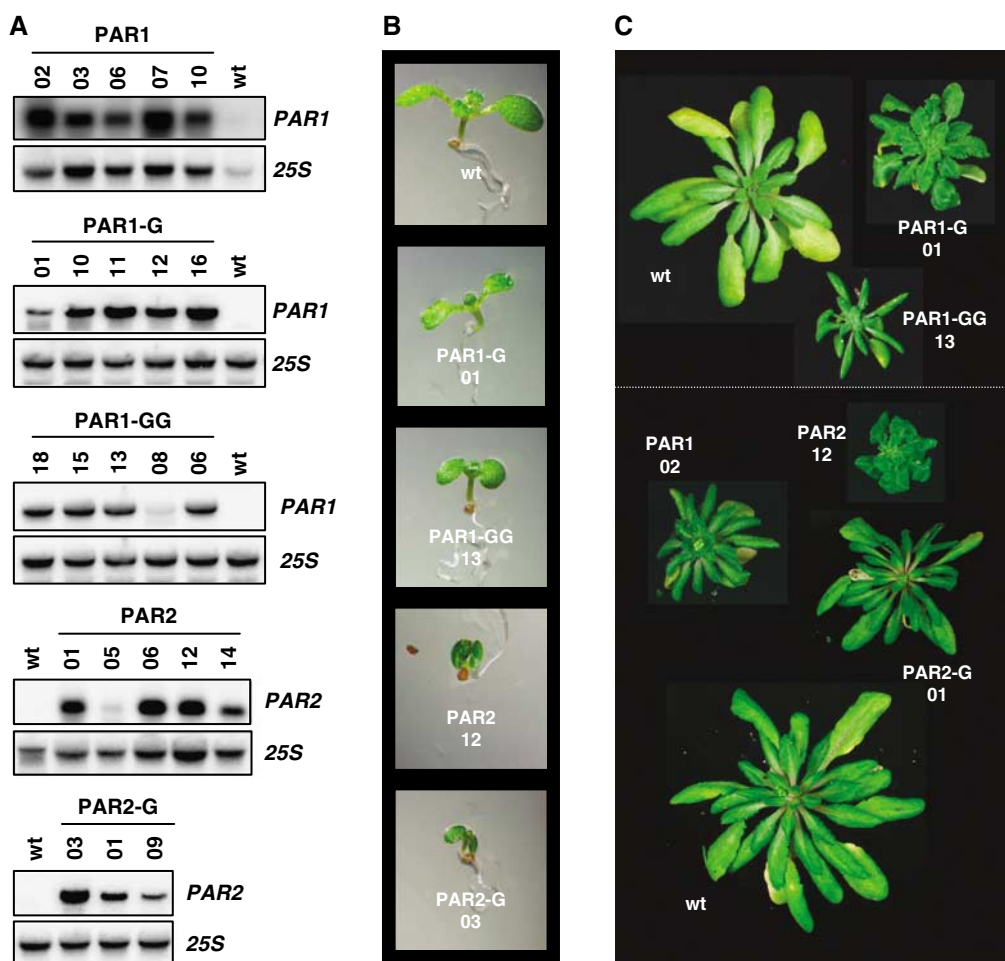
The predicted bHLH-like domains of PAR1, PAR2 and 133 putative *Arabidopsis* bHLHs were aligned (Supplementary Figure S2), generating a phylogenetic tree with a similar topology to that previously reported (Heim *et al*, 2003). As shown in Figure 1C, PAR1 and PAR2 are included within subgroup VIII-A (Heim *et al*, 2003), which corresponds to subfamilies 19 and 20 from a different classification (Toledo-Ortiz *et al*, 2003). No function has been proposed for any of the members of this subgroup. An MEME analysis (<http://bioweb.pasteur.fr/seqanal/motif/meme>) to search for highly conserved regions outside the bHLH-like domain within group VIII showed no conserved motifs (Supplementary Figure S3).

### Overexpression of PAR1 and PAR2 results in dwarf dark-green plants

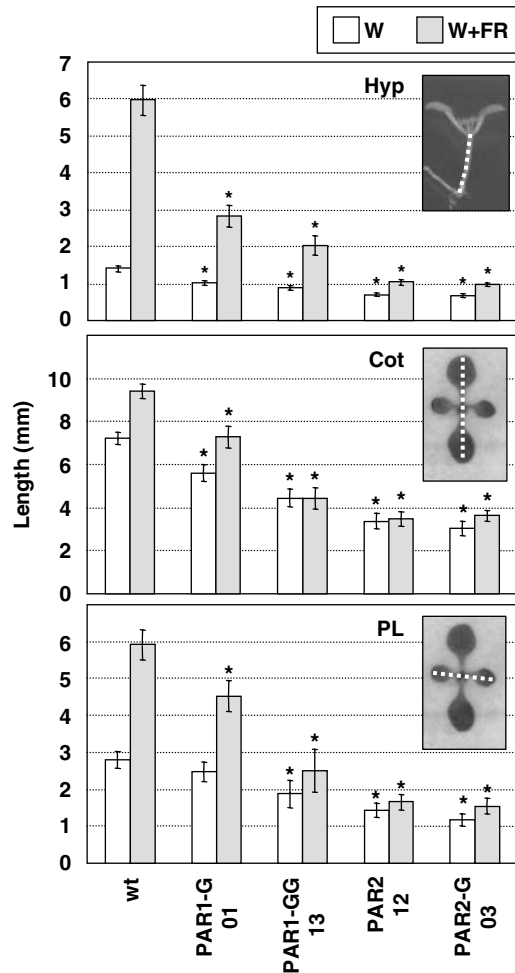
To investigate the role of PAR1 *in planta*, we generated plants constitutively overexpressing PAR1 alone, or as an N-terminal fusion with the green fluorescent protein (GFP) and the GFP-GUS double reporter (P<sub>35S</sub>:PAR1, P<sub>35S</sub>:PAR1-G and P<sub>35S</sub>:PAR1-GG lines, respectively) (Figure 2). Transgenic plants presented a characteristic dwarf phenotype with compact rosettes and inflorescences, epinastic leaves, shorter flowering stems and siliques and a general dark-green color (Figure 2). This phenotype was most frequent when the untagged PAR1 protein was used (data not shown). The most severely affected lines grew slowly and their short siliques resulted in reduced seed production, complicating the isolation of homozygous lines. All the lines overexpressing the transgene displayed a dwarf dark-green phenotype, supporting that these traits were caused by PAR1 overexpression. Similar results were obtained with P<sub>35S</sub>:PAR2 and P<sub>35S</sub>:PAR2-G plants overexpressing PAR2 alone, or as an N-terminal fusion with GFP (Figure 2). These data suggest that both PAR1 and PAR2 might play a similar role in plant development.

**Phenotypic traits are oppositely affected by PAR1 or PAR2 overexpression and simulated shade**

The most obvious phenotypes of PAR1-overexpressing seedlings grown under continuous white light (W) were a short hypocotyl length and a strongly reduced cotyledon and primary leaf longitudinal expansion (Figures 2B and 3),



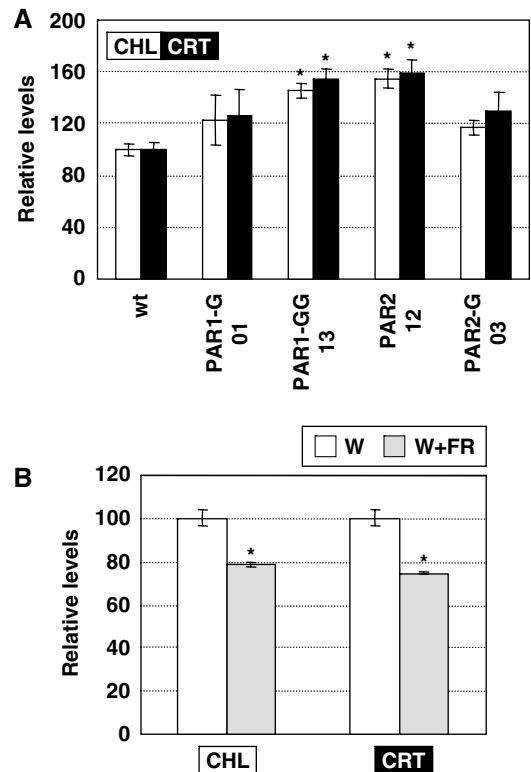
**Figure 2** Phenotype of plants overexpressing PAR1 or PAR2. (A) Molecular characterization of PAR1 and PAR2 overexpressing plants. RNA extracted from 7-day-old W-grown wild-type (wt) or transgenic seedlings was used for RNA blot analysis of PAR1 and PAR2 expression levels. Each RNA sample was extracted from a pool of seedlings corresponding to a segregating population. 25S rRNA levels are shown as a loading control. (B) Representative 7-day-old W-grown wt and transgenic seedlings overexpressing the indicated version of PAR1 or PAR2. (C) Adult wt and transgenic plants overexpressing the indicated version of PAR1 or PAR2 grown for 6 (upper panel) or 8 weeks (lower panel) under short-day conditions. In each section panels are to the same scale.



**Figure 3** Morphological phenotype of seedlings overexpressing *PAR1* or *PAR2*. wt and transgenic seedlings were germinated and grown for 2 days under W and then either kept in W (white bars) or transferred to W + FR (gray bars) for 5 more days. At least 15 seedlings for each treatment were used to measure the length of their hypocotyls (Hyp), cotyledons (Cot) and primary leaves (PL). Columns represent the mean and bars represent twice the standard error of the mean ( $2 \times \text{s.e.}$ ) of the data. Asterisks indicate highly significant differences ( $P < 0.01$ ) relative to the corresponding wt controls.

which ultimately resulted in the dwarf phenotype observed. The dark-green phenotype of these lines suggested that the levels of photosynthetic pigments might also be altered in transgenic plants. As predicted, measurements of chlorophyll and carotenoid levels in W-grown seedlings demonstrated that transgenic seedlings accumulated more chlorophylls and carotenoids than wild-type plants (Figure 4A). All these phenotypes were typically enhanced in the lines overexpressing *PAR2* (Figures 3 and 4).

The observed effects of *PAR1* and *PAR2* overexpression on these aspects of *Arabidopsis* development and metabolism led us to analyze whether these traits were associated with the response of wild-type plants to simulated shade. We observed that besides promoting hypocotyl elongation, simulated shade clearly induced cotyledon and primary leaf longitudinal expansion in wild-type seedlings (Figure 3). Chlorophyll accumulation has also been reported to be affected by shade (Smith and Whitelam, 1997). We noticed that *Arabidopsis* seedlings became paler after prolonged

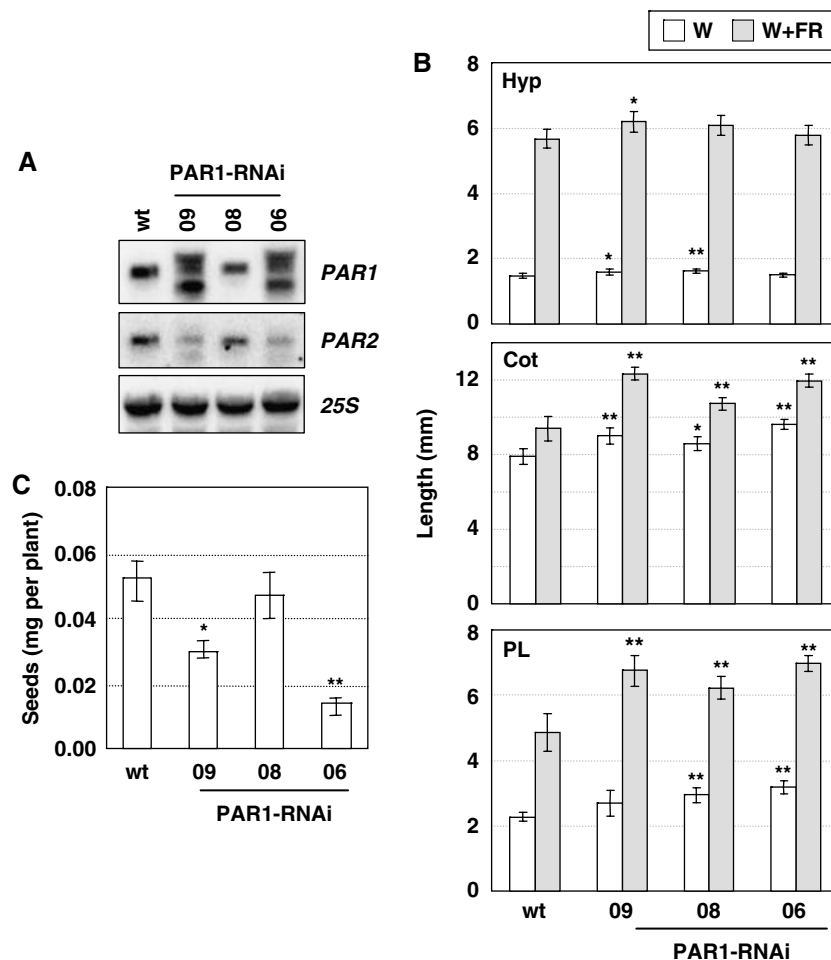


**Figure 4** Photosynthetic pigment levels in seedlings overexpressing *PAR1* or *PAR2*. (A) Chlorophyll (CHL) and carotenoid (CRT) levels of 7-day-old W-grown wt and transgenic seedlings. (B) CHL and CRT levels of wt seedlings grown as described in Figure 3. Values are means and s.e. of three (A) and four (B) independent samples. Values for W-grown wt seedlings were taken as 100. Asterisks indicate significant differences ( $P < 0.05$ ) relative to the corresponding controls.

exposure to W + FR. Consistently, a significant decrease in the amount of chlorophylls and carotenoids was observed in wild-type seedlings after simulated shade treatment compared to W-grown controls (Figure 4B). These data indicate that simulated shade and *PAR1* or *PAR2* overexpression have opposite effects in SAS-related responses in *Arabidopsis* seedlings. Furthermore, the response of transgenic seedlings to W + FR in terms of hypocotyl, cotyledon and primary leaf elongation was clearly attenuated compared to the wild type (Figure 3), suggesting that *PAR1* and *PAR2* may act as negative regulators of SAS.

#### Decreased *PAR1* and *PAR2* transcript levels result in enhanced SAS responses

To analyze the consequence of reduced *PAR1* and *PAR2* levels in *Arabidopsis*, we initially targeted the *PAR1* gene for silencing, using an RNAi approach. Seven *PAR1*-RNAi lines with a single T-DNA insertion were generated. To select for silenced lines among them, *PAR1* transcript levels were evaluated by RNA blot analyses in seedlings treated with W + FR for 1 h. The level of endogenous *PAR1* transcripts was mildly reduced in three *PAR1*-RNAi lines tested compared to the wild type (Figure 5A). Two extra bands recognized by the *PAR1* probe were also observed in some lines (Figure 5A), likely corresponding to the expression of the *PAR1*-RNAi construct. Analysis of *PAR2* expression in the same lines also showed

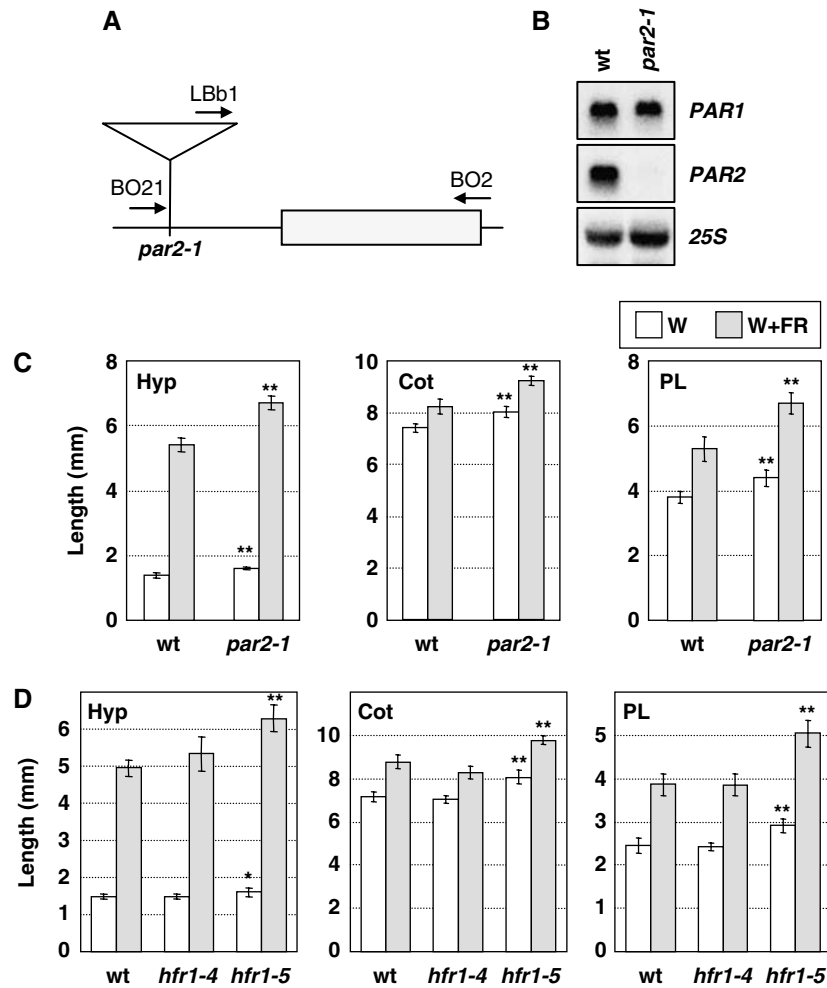


**Figure 5** Characterization of PAR1-RNAi lines. (A) RNA blot analysis of *PAR1* and *PAR2* expression in 7-day-old W-grown seedlings treated with W + FR for 1 h. 25S rRNA levels are shown as a loading control. (B) Length of hypocotyls (Hyp), cotyledons (Cot) and primary leaves (PL) of wt and independent PAR1-RNAi lines grown as indicated in Figure 3. Mean and  $2 \times$  s.e. of at least 15 seedlings for each treatment are shown. (C) Seed production of wt and independent PAR1-RNAi lines grown under greenhouse conditions. Mean and s.e. of at least seven plants for each line are shown. Asterisks indicate significant ( $*P < 0.05$ ) or highly significant ( $**P < 0.01$ ) differences relative to the corresponding wt plants.

reduced transcript levels (Figure 5A), indicating that the PAR1-RNAi construct partially silenced both *PAR1* and *PAR2* genes. The most apparent phenotype of adult PAR1-RNAi plants grown under our greenhouse conditions was reduced fertility, that is, seed production (Figure 5C), and, as described for sterile mutants (Hensel *et al*, 1994), increased branching (data not shown). In seedlings, reduced *PAR1* and *PAR2* levels resulted in only slightly longer hypocotyls compared to wild-type controls (Figure 5B). Cotyledon and primary leaf longitudinal expansion phenotypes, however, were more strongly affected in PAR1-RNAi lines, as shown for overexpression lines. Under W, transgenic seedlings had longer cotyledons and primary leaves than the wild type, a phenotype similar to that induced by simulated shade. These differences were clearly increased under W + FR (Figure 5B), supporting the fact that reduced *PAR1* and *PAR2* transcript levels resemble an enhanced response to W + FR treatment.

In the course of this study, we found a SALK line with a T-DNA insertion in the promoter region of *PAR2*, about 350 nucleotides upstream of the ATG codon (Figure 6A). Northern blot analysis showed no detectable *PAR2* expression, whereas *PAR1* transcript levels were unaffected (Figure 6B). These

results indicate that this line, which we named *par2-1*, is probably a null mutant for *PAR2*. Adult *par2-1* plants grown in the greenhouse showed no reduction in fertility (data not shown), suggesting that this trait is either redundantly regulated by both *PAR1* and *PAR2*, or is due to off-target effects of the PAR1-RNAi construct. Under W, *par2-1* seedlings showed slightly but significantly longer hypocotyls, cotyledons and primary leaves compared with wild-type controls. Also, as shown for PAR1-RNAi lines, these differences were clearly increased under W + FR (Figure 6C), arguing in favor of a specific negative role of *PAR1* and *PAR2* in the SAS-related traits analyzed. Two-way ANOVA tests indicated a significant ( $P < 0.05$ ) interaction between low levels of *PAR1* and/or *PAR2* and simulated shade treatments (Figures 5B and 6C) in the case of cotyledon and primary leaf elongation responses. Hypocotyl elongation under W + FR was significantly different to that under W in *par2-1* seedlings, but not in PAR1-RNAi lines, when compared with the wild type. This statistical analysis also confirmed a highly significant interaction between increased levels of *PAR1* or *PAR2* and light treatments in all the three traits analyzed (Figure 3). Together, our data show that altered levels of *PAR1* and/or *PAR2*



**Figure 6** Characterization of *par2* and *hfr1* mutant seedlings. (A) Schematic representation of *PAR2* (At3g58850) genomic sequence and T-DNA insertion in *par2-1*. The intronless coding sequence (light-gray box) and the position of oligonucleotides used for genotyping are shown. (B) RNA blot analysis of *PAR1* and *PAR2* expression in 7-day-old W-grown wt and *par2-1* seedlings after W + FR treatment for 4 h. 25S rRNA levels are shown as a loading control. (C, D) Length of hypocotyls (Hyp), cotyledons (Cot) and primary leaves (PL) of mutant *par2-1* (C) and *hfr1* seedlings (D) grown as indicated in Figure 3. Mean and  $2 \times$  s.e. of at least 15 seedlings for each treatment are shown. Asterisks indicate significant ( $*P < 0.05$ ) or highly significant ( $**P < 0.01$ ) differences relative to the corresponding wt plants.

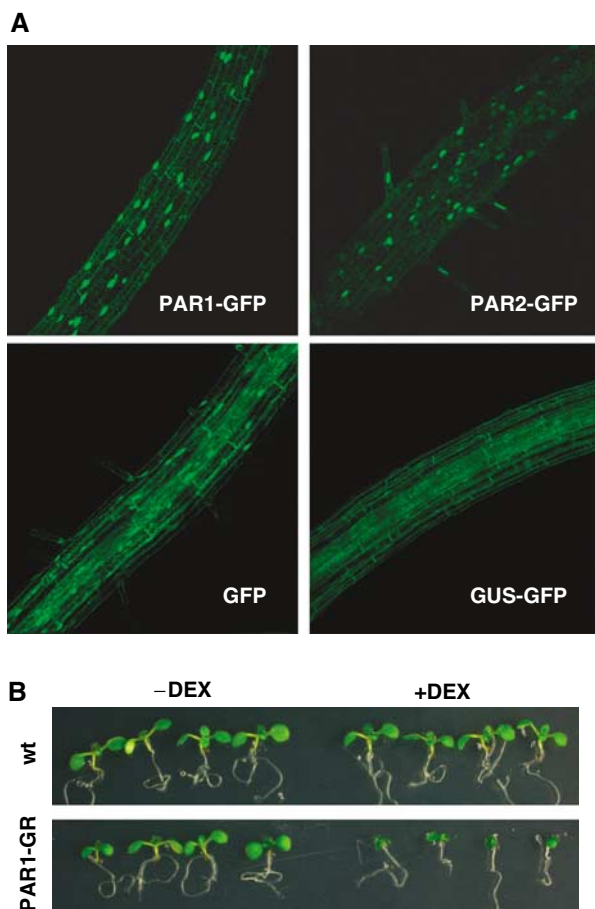
significantly affect seedling responsiveness to simulated shade.

To compare the phenotypic effect of reduced *PAR1* and *PAR2* levels with that of known negative regulators of SAS, we analyzed hypocotyl, cotyledon and primary leaf length in *hfr1-4* and *hfr1-5* mutant seedlings grown under our specific experimental (light) conditions. Under W, the null *hfr1-5* mutant (Sessa *et al*, 2005) showed slightly longer hypocotyls, cotyledons and primary leaves compared with wild-type controls, whereas seedlings of the *hfr1-4* mutant (which shows low levels of *HFR1* transcripts; Sessa *et al*, 2005) were indistinguishable from the wild type. Under W + FR, these traits were significantly enhanced only in *hfr1-5* seedlings (Figure 6D). These data indicate that after simulated shade treatments, loss of *HFR1* function results in SAS phenotypes qualitatively and quantitatively similar to those displayed by seedlings with reduced *PAR1* and/or *PAR2* expression. In summary, the results with transgenic lines in which *PAR1* and *PAR2* levels are altered support a role for these atypical bHLH proteins as negative regulators of SAS responses in *Arabidopsis* seedlings.

#### ***PAR1* and *PAR2* proteins function in the nucleus**

Because of the sequence similarity of *PAR1* and *PAR2* to members of the bHLH family of transcription factors, we hypothesized that these two proteins could regulate development by modulating gene transcription. We initially investigated whether *PAR1* and *PAR2* were nuclear proteins. Taking advantage of our transgenic lines overexpressing functional chimeras of *PAR1* and *PAR2* fused to GFP, we examined the subcellular localization of the fusion proteins in the roots of *P<sub>35S</sub>:PAR1-G* and *P<sub>35S</sub>:PAR2-G* plants with a dwarf phenotype. As controls, we used transgenic *P<sub>35S</sub>:GFP* and *P<sub>35S</sub>:GUS-GFP* plants. As expected, cytoplasmic and nuclear localization were observed for GFP, whereas the larger size of the GUS-GFP fusion prevented its diffusion into the nucleus (Figure 7A). Both *PAR1-GFP* and *PAR2-GFP* proteins were mainly localized in the nuclei (Figure 7A). Nuclear localization of the fusion proteins was also observed in shoot tissues (Supplementary Figure S4) and it was not affected by light conditions (data not shown).

To confirm whether nuclear localization was required for *PAR1* function, a translational fusion between *PAR1* and the



**Figure 7** Subcellular localization and function of tagged versions of PAR1 and PAR2. **(A)** GFP fluorescence in roots from 7-day-old W-grown transgenic seedlings expressing the indicated GFP-tagged proteins. Panels are to the same scale. **(B)** Phenotype of 7-day-old W-grown wt and transgenic seedlings overexpressing *PAR1-GR* germinated and grown on medium either supplemented (+) or not (-) with 5  $\mu$ M DEX.

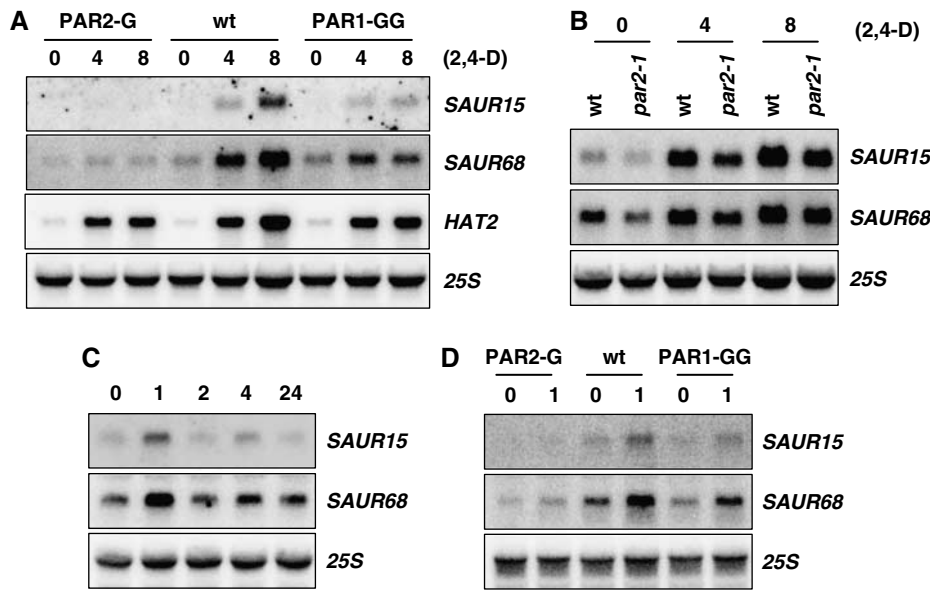
glucocorticoid receptor (GR) domain was constitutively expressed in plants ( $P_{35S}$ :*PAR1-GR* lines). The GR domain typically retains a nuclear factor in the cytoplasm in the absence of a steroid ligand, but nuclear localization is restored in the presence of the synthetic glucocorticoid dexamethasone (DEX). In the absence of DEX, most transgenic  $P_{35S}$ :*PAR1-GR* seedlings were similar to wild-type seedlings (Figure 7B), suggesting that accumulation of cytosolic *PAR1-GR* did not have a visible effect on plant development or pigment accumulation. By contrast, DEX treatment only induced a severe dwarf dark-green phenotype in  $P_{35S}$ :*PAR1-GR* seedlings (Figure 7B). From a total of 10 independent transgenic lines with a single T-DNA insertion isolated, five clearly showed a dwarf dark-green phenotype upon DEX treatment. In some lines we observed a mild phenotype in the absence of DEX, an effect attributed to the production of truncated, constitutively active versions of the fusion protein (Sablowski and Meyerowitz, 1998). In these lines, however, DEX treatment dramatically enhanced the transgenic phenotype (data not shown). Together, these results demonstrate that PAR1 is active only when localized in the nucleus, suggesting a role for both PAR1 and PAR2 as transcriptional regulators.

### ***PAR1* and *PAR2* repress hormone-mediated upregulation of *SAUR* genes**

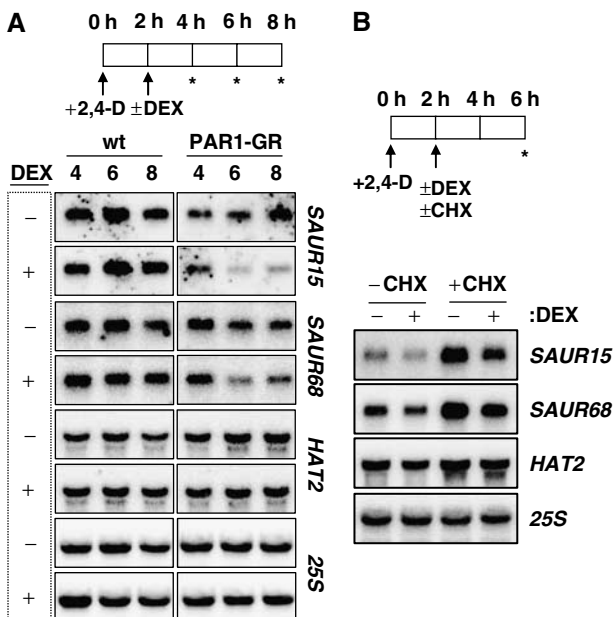
To look for potential targets of PAR1 action, we analyzed global transcript profiles in W-grown wild-type and dwarf  $P_{35S}$ :*PAR1-GG* seedlings (a transgenic line already available at that time) to screen for differentially expressed genes. From the 120 genes identified as being misregulated in the  $P_{35S}$ :*PAR1-GG* seedlings (Supplementary Table S1), 34 (28%) had been previously identified by other authors as regulated by auxin and/or BR (Nemhauser *et al*, 2004, 2006; Supplementary Table S2). Most of these (30 genes) belong to the subgroup of 70 genes downregulated in  $P_{35S}$ :*PAR1-GG* seedlings; from these, 22 genes are upregulated by auxins and/or BR with almost half of them (10) upregulated by both the hormones (Supplementary Figure S5). Most strikingly, seven of these 10 genes belong to the *SAUR* family (McClure and Guilfoyle, 1987; Supplementary Table S2). For further experiments we focused on *SAUR15* (also named *SAUR-AC1*; At4g38850) and *SAUR68* (At1g29510), for which a 30–40% reduction in their expression level was detected in the microarray (Supplementary Table S1). Because the low expression levels of these genes did not allow us to conclusively validate changes between wild-type and *PAR1*-overexpressing seedlings by RNA blot analysis, we tested whether the auxin-dependent induction of these *SAUR* genes was affected in transgenic lines. As shown in Figure 8A, the upregulation of both *SAUR15* and *SAUR68* in response to treatment with 2,4-D (a synthetic auxin) was clearly attenuated when *PAR1* or *PAR2* was overexpressed. Similar results were observed after treatment with brassinolide (data not shown). By contrast, *PAR1* or *PAR2* overexpression did not affect the auxin-mediated upregulation of *HAT2* (Figure 8A), another gene, which is rapidly induced by auxin (Sawa *et al*, 2002). Transcript levels for *SAUR15* and *SAUR68* were also lower in *par2-1* line compared with the wild type, both before and after 2,4-D treatments (Figure 8B). Altogether, our results indicate that a certain level of PAR1 and/or PAR2 is required for the normal response of a subset of auxin-regulated genes (including *SAUR15* and *SAUR68* but not *HAT2*) to increased 2,4-D levels.

Interestingly, the expression of *SAUR15* and *SAUR68* is also rapidly but transiently induced by simulated shade (Figure 8C), which indicates that these two *SAUR* genes are also authentic *PAR* genes. The acute *SAUR15* and *SAUR68* expression response to simulated shade was clearly attenuated in *PAR1*- and *PAR2*-overexpressing lines (Figure 8D), which suggests that the observed PAR1- and PAR2-mediated downregulation of *SAUR* expression is meaningful for the regulation of SAS responses.

To address whether the observed negative role of PAR1 on *SAUR* gene expression was an early (direct) or late (indirect) effect on transcription, *SAUR15* and *SAUR68* transcript levels were monitored, following the targeting of *PAR1-GR* to the nucleus by DEX treatment of  $P_{35S}$ :*PAR1-GR* seedlings. Transgenic and wild-type seedlings were first treated with 2,4-D to induce *SAUR* gene expression and 2 h later they were either treated or not with DEX (Figure 9A). In wild-type seedlings, auxin-induced expression of *SAUR15*, *SAUR68* and *HAT2* was unaffected by DEX application. By contrast, expression of *SAUR15* and *SAUR68* was reduced 4 h after DEX treatment in  $P_{35S}$ :*PAR1-GR* seedlings (time point 6 h), whereas that of *HAT2* was unaffected (Figure 9A). A similar



**Figure 8** Effect of PAR1, PAR2, auxin and simulated shade on *SAUR* expression. Seven-day-old W-grown seedlings were treated with either 50  $\mu$ M 2,4-D (A, B) or W + FR (C, D) for the indicated times (in h). (A) RNA blot analysis of *SAUR15*, *SAUR68* and *HAT2* expression in auxin-treated wt and transgenic seedlings overexpressing *PAR1-GG* (line 13) and *PAR2-G* (line 03). (B) RNA blot analysis of *SAUR15* and *SAUR68* expression in auxin-treated wt and *par2-1* seedlings. (C) RNA blot analysis of *SAUR15* and *SAUR68* expression in wt seedlings after simulated shade (W + FR). (D) RNA blot analysis of *SAUR15* and *SAUR68* expression in seedlings from the lines described in panel A treated with W + FR. 25S rRNA levels are shown as a loading control.



**Figure 9** Identification of the primary targets of PAR1. (A) RNA blot analysis of the effect of DEX-dependent nuclear translocation of PAR1 on 2,4-D-induced expression of *SAUR15*, *SAUR68* and *HAT2* in 7-day-old W-grown wt and *PAR1-GR* overexpressing seedlings treated with 50  $\mu$ M 2,4-D, incubated for 2 h and then either treated (+) or not (-) with 5  $\mu$ M DEX. (B) RNA blot analysis of the effect of CHX on DEX-dependent repression of *SAUR15* and *SAUR68* in 7-day-old W-grown *PAR1-GR* overexpressing seedlings treated with 10  $\mu$ M 2,4-D, incubated for 2 h and then either treated (+) or not (-) with 5  $\mu$ M DEX in the absence (-CHX) or presence (+CHX) of 50  $\mu$ M CHX. Plant material for RNA extraction was harvested at the time points indicated with asterisks. 25S rRNA levels are shown as a loading control.

experiment was performed in the presence of the protein synthesis inhibitor cycloheximide (CHX). Transgenic seedlings were first treated with 2,4-D to induce *SAUR* expression,

and 2 h later they were either treated or not with DEX and/or CHX (Figure 9B). In the absence of CHX, addition of DEX only reduced the expression of *SAUR15* and *SAUR68*, as expected. In the presence of CHX, expression of all three genes (*SAUR15*, *SAUR68* and *HAT2*) was increased, as previously reported (Zimmermann *et al*, 2004). Addition of both DEX and CHX only repressed *SAUR15* and *SAUR68* expression (Figure 9B). We concluded that the DEX-dependent repression of *SAUR15* and *SAUR68* does not require *de novo* protein synthesis, consistent with these two *SAUR* genes being direct targets of PAR1 action.

## Discussion

Despite the importance of SAS for plant survival, we know relatively little about the genetic components involved in its control. In contrast with the information available for other photomorphogenic responses, like seedling de-etiolation, genetic and molecular approaches have identified few SAS regulators, including *ATHB2* (Steindler *et al*, 1999), *HFR1* (Sessa *et al*, 2005) and *PIL1* (Salter *et al*, 2003; Roig-Villanova *et al*, 2006). These proteins belong to two different families of transcription factors: homeodomain (*ATHB2*) and bHLH (*HFR1* and *PIL1*). In this paper, we report the characterization of PAR1 and PAR2, two atypical bHLH-like proteins localized within the nucleus, with a role in the integration of light and auxin signaling.

The bHLH proteins represent one of the largest transcription factor families found in nature. They are widely distributed in all the eukaryotic kingdoms and control a great diversity of biological processes. In plants, the best characterized group is probably group VII (Heim *et al*, 2003) or subfamily 15 (Toledo-Ortiz *et al*, 2003), which includes *HFR1*, *PIL1* and all the other PIFs and PILs involved in phytochrome signaling. PAR1 and PAR2 are classified here as part of group VIII-A (Figure 1C), whose members have a single exon

encoding the bHLH domain. *PAR1* and *PAR2* are localized in regions of chromosomes 2 and 3, respectively, which have been subjected to segmental duplications (Blanc *et al*, 2000; Vision *et al*, 2000), suggesting that they are the result of a recent gene duplication event. To our knowledge, there is no previous functional information about the members of group VIII.

*HFR1* and *PIL1* act as negative regulators of SAS responses, whereas a positive role has been proposed for *ATHB2* (Steindler *et al*, 1999; Sessa *et al*, 2005; Roig-Villanova *et al*, 2006). All these factors, as well as *PAR1*, were first identified based on their very rapid response to shade in terms of gene expression (Carabelli *et al*, 1996; Salter *et al*, 2003; Sessa *et al*, 2005; Roig-Villanova *et al*, 2006). All but *HFR1* were demonstrated to be direct targets of phytochrome signaling (Roig-Villanova *et al*, 2006). *PAR2*, the closest *PAR1* relative in the *Arabidopsis* genome, is also induced by shade, although more slowly than *PAR1* (Figure 1). Alteration of *PAR1* and *PAR2* levels, however, similarly affects plant development, with a particularly clear effect on elongation and pigmentation responses. The traits affected by *PAR1* or *PAR2* overexpression in transgenic seedlings are also influenced by prolonged simulated shade treatments but in opposite ways; whereas simulated shade-treated plants are typically longer and paler, those overexpressing *PAR1* or *PAR2* are shorter and darker than the wild type (Figures 2–4). In addition, reduction of *PAR1* and/or *PAR2* levels results in enhanced elongation phenotypes, suggestive of a mild constitutively active SAS (Figures 5 and 6). Reduced *PAR1* and *PAR2* transcript levels also result in a severe reduction in seed production (Figure 5C), a trait reported to be similarly affected by simulated shade treatments (Smith and Whitelam, 1997). The negative correlation between *PAR1* and *PAR2* levels, and the developmental and metabolic responses to simulated shade suggest that *PAR1* and *PAR2* are negative regulators of SAS.

On the other hand, some of the phenotypes of *PAR1* and *PAR2* overexpression lines resemble those of auxin, BR or gibberellin mutants (reviewed in Halliday and Fankhauser, 2003). Both gain- and loss-of-function *PAR1* and *PAR2* plants display altered phenotypes under both W and W + FR (Figures 3, 5 and 6), suggesting that such phenotypes might not be strictly photomorphogenic. Actually, *PAR1* and *PAR2* may not be considered photomorphogenic components since they are not participating in events from light perception to the first changes in gene expression elicited by the shade signal (i.e., pretranscriptional events). *PAR1* and *PAR2* are, instead, early components of the shade-regulated transcriptional network, together with *ATHB2*, *HFR1* and *PIL1*. In this sense, we claim that these genes are components of shade signaling. Simulated shade not only affects the expression of *PAR1* and *PAR2*, but also of a wide diversity of both negative and positive regulators of SAS and, in this networked context, mutants deficient in a single early or primary target of phytochrome action, such as those knocked down in this report, are expected to have a mild effect on the studied phenotypes. When analyzing the role of early components of the transcriptional network initiated by the phytochromes during seedling de-etiolation, similar mild effects were reported (Khanna *et al*, 2006).

*HFR1*, a reported master negative regulator of SAS responses, was shown to have a very strong and clear effect

under shade conditions that reduced both R:FR ratio and photosynthetic active radiation (amount of light in the 400–700 nm range; Sessa *et al*, 2005), mimicking natural situations when canopy closure occurs. Our simulated shade conditions, by contrast, only reduce R:FR ratio, without significantly affecting photosynthetic active radiation, mimicking plant proximity detection before actual canopy shading occurs. Under our conditions, the loss-of-function *hfr1-5* mutant displays a moderate phenotype, which is qualitatively and quantitatively very similar to that displayed by lines with reduced *PAR1* and *PAR2* levels (Figures 5 and 6), supporting the fact that all these factors are negative regulators of SAS. The phenotypes resulting from *PAR1* and *PAR2* overexpression, however, are different from those described in plants overexpressing *HFR1*, which show clear effects on several light-regulated traits, displaying shorter hypocotyls and higher anthocyanin accumulation than wild-type seedlings after de-etiolation (Yang *et al*, 2003, 2005; Duek *et al*, 2004). By contrast, overexpression of *PIL1*, another negative regulator of SAS, has not been reported to constitutively inhibit elongation responses or pigment accumulation (Salter *et al*, 2003). The distinct effect of increased levels of *HFR1*, *PIL1*, *PAR1* or *PAR2* on plant development suggests that these factors might control distinct circuits of the shade-regulated transcriptional network involved in implementing SAS responses.

Although no nuclear localization signals in *PAR1* or *PAR2* could be identified by any web-based program, fusing *PAR1* and *PAR2* to reporter proteins showed that both are nuclear proteins (Figure 7A). In addition, the characteristic dwarf phenotype of *PAR1*-overexpressing lines required nuclear targeting of the protein (Figure 7B). These data demonstrate that nuclear localization is required for *PAR1* activity, consistent with a role for these atypical bHLHs as transcriptional regulators. As shown here, *PAR1* and *PAR2* seem to regulate *in vivo* the transcription of a subset of auxin-regulated genes, including *SAUR15* and *SAUR68* but not *HAT2* (Figure 8). The large proportion of genes misregulated in *P<sub>35S</sub>:PAR1-GG* seedlings that are also differentially expressed in wild-type plants upon treatment with auxin and/or BR hormones (Supplementary Figure S5) suggest a broad role for *PAR1* (and likely *PAR2*) in integrating light and hormone signaling networks during SAS. The repressor effect of increased *PAR1* levels on auxin-induced *SAUR* expression is very likely direct, since it is CHX-independent and was observed only 4 h after DEX application (Figure 9B). This time of action is consistent with that reported for other transcription factor-GR fusions over their direct targets (Sablowski and Meyerowitz, 1998; Ohgishi *et al*, 2001; Roig-Villanova *et al*, 2006). On the other hand, additional factors might have a more prominent role in the observed decrease in *SAUR* response to 2,4-D treatment when *PAR1* and/or *PAR2* levels are constitutively reduced (Figure 8B, data not shown).

Auxin- and BR-signaling pathways converge at the level of transcriptional regulation of target genes with common regulatory elements (reviewed in Halliday, 2004), two of these dual targets being *SAUR15* and *SAUR68* (Supplementary Table S2). Analysis of only auxin-regulated, and common auxin- and BR-regulated promoters identified G-box and E-box elements, respectively (Nemhauser *et al*, 2004), both of them recognized by at least some bHLH members. Consistent with *SAUR15* being a direct target of *PAR1*, it has been shown that three E-box sequences present in *SAUR15* promoter are

necessary for its activation by members of the bHLH family (Yin *et al*, 2005). Although there is no molecular or biochemical characterization of any member of bHLH group VIII (to which PAR1 and PAR2 belong), they have been suggested to lack the ability to bind DNA, and to form heterodimers, acting as negative regulators of other transcription factors, particularly bHLH proteins (Atchley and Fitch, 1997; Heim *et al*, 2003; Toledo-Ortiz *et al*, 2003). It is therefore tempting to speculate that the molecular mechanism behind this repressor effect is the ability of PAR1 and PAR2 to inhibit the DNA-binding activity of transcription activators, most likely belonging to the bHLH family, after heterodimerizing with them. This would make PAR1 and PAR2 transcription cofactors, with the ability to regulate transcription but lacking a DNA-binding domain (Wray *et al*, 2003).

After simulated shade perception, light signaling networks intersect with those of plant hormones. Auxins are known to play a role in plant responses to changes in light quality (Steindler *et al*, 1999; Tian and Reed, 2001; Halliday and Fankhauser, 2003). Following auxin transport and accumulation, auxin responsive genes (including *Aux/IAA*, *GH3* and *SAUR* genes) are induced, triggering signaling pathways (transcriptional cascades) that ultimately lead to cell expansion. Interestingly, simulated shade also affects rapidly but transiently the expression of *SAUR15* and *SAUR68*. Changes in the expression of *SAUR* genes in response to auxin can be a marker of auxin-sensitivity to exogenous and, by extension, to endogenous auxin. Our data showing a decreased response to 2,4-D treatment when PAR1 or PAR2 levels are altered can therefore be interpreted as a fine modulation of auxin sensitivity by PAR1 and PAR2. The direct control of *PAR* gene expression by phytochromes after SAS induction could therefore represent a mechanism to rapidly modulate some auxin responses (*SAUR* expression) and to integrate shade perception and hormone signaling pathways.

## Materials and methods

### Plant material and growth conditions

*Arabidopsis* (*A. thaliana*) transgenic lines were generated in the Col-0 background. The SALK\_109270 line was named *par2-1*. The mutant lines *hfr1-4* and *hfr1-5*, also generated by the SALK collection (Alonso *et al*, 2003), have been described previously (Sessa *et al*, 2005). Details of the genotyping of *hfr1* and *par2-1* mutant plants are given in the Supplementary data. Adult plants were either grown under short-day photoperiodic conditions (Figure 2A), or in the greenhouse (Figure 5C), as described (Martínez-García *et al*, 2002). All the other experiments were performed with seedlings grown in plates, as detailed below. Seeds were germinated on Petri dishes with solid growth medium (without sucrose, GM-) as described (Roig-Villanova *et al*, 2006). Plates were incubated in a I-36VL growth chamber (Percival Scientific Inc., Perry, IA, USA) at 22°C under two different light conditions: (i) W, which was provided by four cool-white vertical fluorescent tubes ( $80 \mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation; R:FR ratio of 3.2–4.5) and (ii) simulated shade (W + FR), which was generated by enriching W with supplementary FR provided by QB1310CS-670-735 LED hybrid lamps (Quantum Devices Inc., Barneveld, WI, USA) ( $80 \mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation; R:FR ratio of 0.05). Fluence rates were measured using an EPP2000 spectrometer (StellarNet Inc., Tampa, FL, USA).

### Construction of transgenic lines

Details of the generation of the constructs used to obtain the described transgenic lines are provided in the Supplementary data. *Arabidopsis* plants were transformed with the obtained binary

vectors as described (Roig-Villanova *et al*, 2006). The presence of the transgene in the selected T<sub>1</sub> plants was verified by PCR analysis. Only lines with a single T-DNA insertion (as estimated from the segregation of the marker gene in T<sub>2</sub> populations) were eventually selected.

### RNA blot analysis

Total RNA was isolated from seedlings, electrophoresed and blotted as described (Roig-Villanova *et al*, 2006). Hybridization probes for *PAR1*, *HAT2* and *25S* rRNA were prepared as described (Roig-Villanova *et al*, 2006). Probes for *PAR2*, *SAUR15* and *SAUR68* were made from the respective full-length fragments cloned in pJB3, pAG3 and pAG2, by PCR using specific primers (see Supplementary data). Expression levels were normalized with the *25S* rRNA signal. Hybridization, washes, exposure and quantification of radioactive signals were carried out as described (Martínez-García *et al*, 2002). These experiments were conducted at least twice.

### Physiological measurements

The National Institutes of Health ImageJ software (Bethesda, MD, USA) was used on digital images to quantify hypocotyl length (after laying out seedlings flat on agar plates) and cotyledon and primary leaf longitudinal expansion (after rolling seedlings flat on a transparent self-adhesive sheet). At least 15 seedlings were used for each treatment and experiments were repeated 2–5 times. Data shown in Figures 3, 5B, 6C and D, correspond to a representative experiment. Error bars represent twice the standard error of the mean ( $2 \times \text{s.e.}$ ), which corresponds to 95% confidence intervals (Cumming *et al*, 2007). Photosynthetic pigments were extracted, separated by HPLC and quantified as indicated (Rodríguez-Concepción *et al*, 2004). These experiments were repeated twice and a representative experiment is shown (Figure 4). Statistical analysis of the data was performed using the Simple Interactive Statistical Analysis (SISA) T-test available online (<http://home.clara.net/sisa/t-test.htm>). Two-way ANOVA tests were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

### CHX, DEX and 2,4-D treatments

CHX (Sigma-Aldrich) was dissolved in 50% (v/v) ethanol at 50 mM; DEX and 2,4-D (Sigma-Aldrich) were dissolved in 100% ethanol (v/v) at 5 and 250 mM, respectively. These stock solutions were kept at  $-20^\circ\text{C}$  until use. Working solutions were prepared in water prior to the treatments. Treatments were performed using 7-day-old seedlings grown on filter-paper circles, as described (Roig-Villanova *et al*, 2006).

### Sequence and phylogenetic analysis

Multiple sequence alignments were performed using the CLUSTALX 1.8 program (Thompson *et al*, 1997). Alignments were edited with GeneDoc ([www.psc.edu/biomed/genedoc](http://www.psc.edu/biomed/genedoc)). Limits of the bHLH domains were taken according to the proposed consensus motif (Ferre-D'Amare *et al*, 1993; Atchley *et al*, 1999; Toledo-Ortiz *et al*, 2003). Neighbor joining and 50% majority-rule consensus trees were constructed using NEIGHBOR and CONSENSUS, respectively from the PHYLIP package ([evolution.gs.washington.edu/phylip.html](http://evolution.gs.washington.edu/phylip.html)). To provide statistical confidence on the retrieved topology, a bootstrap analysis of 100 replicates was performed through the SEQBOOT application. The trees were represented using the TreeView v1.6.6. software (Page, 1996).

### Subcellular localization analysis

Transgenic seedlings were mounted in water on glass slides. GFP fluorescence was inspected with a Leica TCS SP confocal microscope using a 488 nm argon laser-line (Leica Microsystems, Heidelberg, Germany). At least two independent transgenic lines were examined for each construct.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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