New Phytologist

Research

Convergence of CONSTITUTIVE PHOTOMORPHOGENESIS 1 and PHYTOCHROME INTERACTING FACTOR signalling during shade avoidance

Manuel Pacín[1], Mariana Semmoloni[1], Martina Legris[2], Scott A. Finlayson[3,4] and Jorge J. Casal[1,2]

[1]IFEVA, Facultad de Agronomía, Universidad de Buenos Aires and CONICET, Av. San Martın 4453, 1417 Buenos Aires, Argentina; [2]Fundación Instituto Leloir, Instituto de Investigaciones Bioquímicas de Buenos Aires–CONICET, 1405 Buenos Aires, Argentina; [3]Department of Soil and Crop Sciences, Texas A & M University, College Station, TX 77843, USA; [4]Faculty of Molecular and Environmental Plant Sciences, Texas A&M University, College Station, TX 77843, USA

Author for correspondence:
Jorge J. Casal
Tel: +54 11 4524 8000
Email: casal@ifeva.edu.ar

Received: *16 January 2016*
Accepted: *4 March 2016*

New Phytologist (2016)
doi: 10.1111/nph.13965

Key words: *Arabidopsis thaliana*, auxin, CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1), ELONGATED HYPOCOTYL 5 (HY5), LONG HYPOCOTYL IN FAR-RED (HFR1), PHYTOCHROME INTERACTING FACTOR (PIF), shade avoidance.

Summary

- Shade-avoidance responses require CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) but the mechanisms of action of COP1 under shade have not been elucidated.
- Using simulated shade and control conditions, we analysed: the transcriptome and the auxin levels of *cop1* and *phytochrome interacting factor 1* (*pif1*) *pif3 pif4 pif5* (*pifq*) mutants; the dynamics of ELONGATED HYPOCOTYL 5 (HY5) and LONG HYPOCOTYL IN FAR-RED (HFR1) proteins; and the epistatic relationships between *cop1* and *pif3*, *pif4*, *pif5*, *hy5* and *hfr1* mutations in *Arabidopsis thaliana*.
- Despite severely impaired shade-avoidance responses, only a few genes that responded to shade in the wild-type failed to do so in *cop1*. Shade enhanced the convergence between *cop1* and *pifq* transcriptomes, mainly on shade-avoidance marker genes. Shade failed to increase auxin levels in *cop1*. Residual shade avoidance in *cop1* was not further reduced by the *pif3*, *pif4* or *pif5* mutations, suggesting convergent pathways. HFR1 stability decreased under shade in a COP1-dependent manner but shade increased HY5 stability.
- The *cop1* mutant retains responses to shade and is more specifically impaired in shade avoidance. COP1 promotes the degradation of HFR1 under shade, thus increasing the ability of PIFs to control gene expression, increase auxin levels and promote stem growth.

Introduction

The presence of neighbouring vegetation modifies the light environment experienced by plants. Shade conditions involve reductions in overall irradiance and altered spectral distribution, including low red to far-red (R : FR) ratios of the light. These signals initiate shade-avoidance responses, such as enhanced stem growth, and acclimation responses, which tend to reduce the adverse impact of extreme conditions that cannot be avoided (Franklin, 2008; Martínez-García *et al.*, 2010; Casal, 2013).

Shade signals are perceived mainly by phytochrome B (phyB), a photoreceptor that bears a linear tetrapyrrole chromophore and presents two photo-interconvertible forms: the active Pfr form and the inactive Pr form (Vierstra & Zhang, 2011). In unshaded places phyB is predominantly in its Pfr form in the nucleus, forming relatively large bodies. In response to the low R : FR ratios and irradiance of shade conditions that reduce Pfr levels, new small phyB nuclear bodies appear (Trupkin *et al.*, 2014). Shade also reduces cryptochrome activity (Sellaro *et al.*, 2010; Keller *et al.*, 2011).

Active phyB binds to several PHYTOCHROME-INTERACTING FACTORS (PIFs), which belong to a subfamily of the basic helix-loop-helix (bHLH) transcription factor superfamily (Leivar & Quail, 2011). Full shade-avoidance responses require PIF3, PIF4, PIF5 and PIF7 (Lorrain *et al.*, 2008; Leivar *et al.*, 2012a,b; Li *et al.*, 2012; Sellaro *et al.*, 2012). Although PIF1 does not appear to be required for the stem-growth response (Leivar *et al.*, 2012a,b), it plays a very significant role in gene expression responses to shade signals (Zhang *et al.*, 2013). As a result of the direct interaction with active phyB, PIF1 (Oh *et al.*, 2006; Shen *et al.*, 2008), PIF3 (Bauer *et al.*, 2004; Park *et al.*, 2004; Al-Sady *et al.*, 2006), PIF4 (Lorrain *et al.*, 2008) and PIF5 (Shen *et al.*, 2007; Lorrain *et al.*, 2008) become phosphorylated and degraded via the ubiquitin–proteasome system, whereas PIF7 becomes phosphorylated but not strongly degraded (Leivar *et al.*, 2008a; Li *et al.*, 2012). In addition, phyB reduces the ability of PIF7 (Li *et al.*, 2012) and PIF3 (Park *et al.*, 2012) to bind their target promoters. Therefore, when plants are transferred from high to low R : FR ratios that reduce phyB activity, PIF3 (Leivar *et al.*, 2012a,b) and PIF5 (Lorrain *et al.*, 2008) protein levels increase rapidly, and the binding of (at least) PIF7 to its target gene promoters is enhanced (Li *et al.*, 2012). Direct targets of PIFs include cell wall-loosening genes

and members of the *YUCCA* genes involved in auxin synthesis (De Lucas *et al.*, 2008; Hornitschek *et al.*, 2012; Leivar *et al.*, 2012b; Li *et al.*, 2012). PIFs promote stem growth by increasing auxin levels (Hornitschek *et al.*, 2012; Li *et al.*, 2012). Cryptochromes also bind PIF4 and PIF5 reducing their activity (Pedmale *et al.*, 2016).

Shade-avoidance responses also require the complex formed by CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) (Lau & Deng, 2012), SUPRESSOR OF PHYTOCHROME A1 (SPA1) (Zhu *et al.*, 2008) and other SPA proteins (McNellis *et al.*, 1994; Crocco *et al.*, 2010; Rolauffs *et al.*, 2012; Pacín *et al.*, 2013). COP1 accumulation in the nucleus increases in response to shade (Pacín *et al.*, 2013). Several protein targets of COP1 E3 ubiquitin ligase activity have been identified in seedlings that have never been exposed to light. They include ELONGATED HYPOCOTYL 5 (HY5), HY5-HOMOLOG (HYH), LONG AFTER FAR-RED LIGHT 1 (LAF1) and LONG HYPOCOTYL IN FAR-RED (HFR1) (Osterlund *et al.*, 2000; Holm *et al.*, 2002; Saijo *et al.*, 2003; Seo *et al.*, 2003; Duek *et al.*, 2004; Yang *et al.*, 2005). Conversely, no targets of COP1 during shade avoidance have been defined. In the absence of specific proof, the COP1 targets defined in dark-grown seedlings cannot be regarded as COP1 targets during shade avoidance. COP1-mediated degradation of HY5, HYH, HFR1 and LAF1 is reduced under FR light (Osterlund *et al.*, 2000; Duek *et al.*, 2004; Yang *et al.*, 2005), and shade contains a substantial proportion of FR light that could be enough to release these targets from COP1 action.

In order to investigate the molecular mechanisms of COP1 action during shade avoidance, we analysed the transcriptome of *cop1* mutant plants in response to shade. To place the action of COP1 in the context of the current model for the mechanisms of shade avoidance, which is largely based on the action of PIFs ((Hornitschek *et al.*, 2012; Li *et al.*, 2012), we compared the *cop1* phenotype not only to that of the wild-type but also to the quadruple mutant *pif1 pif3 pif4 pif5* (*pifq*). This analysis revealed that shade significantly enhances the similarity of the transcriptional phenotypes of the *cop1* and *pifq* mutants. We established HFR1 and auxin levels as points of signalling convergence between COP1 and PIFs during the responses to shade.

Materials and Methods

Plant material and light treatments

All the experiments were done with plants of *Arabidopsis thaliana* (L.) Heynh. The mutants and transgenic lines *cop1–4*, *cop1–6* (McNellis *et al.*, 1994), Pro_{MSG2}*:GUS* (Kami *et al.*, 2014), *hfr1-201* (Kim *et al.*, 2002), *hfr1-101* (Duek *et al.*, 2004), *cop1–4 hfr1-201*, *cop1–6 hfr1-201*, Pro_{35S}*:GFP-HFR1*, *cop1–6*/Pro_{35S}*:GFP-HFR1* (Yang *et al.*, 2005), *hy5-211*, Pro_{35S}*:HY5-myc* fusion (Shin *et al.*, 2007), *pif3-3* (Monte *et al.*, 2004), *pif4-101*, *pif5-3*, *pif4 pif5* (Lorrain *et al.*, 2008), *hfr1 pif4 pif5* (Hornitschek *et al.*, 2009) and *pifq* (Leivar *et al.*, 2008b) and the Columbia (Col-0) wild-type (WT) were used in this study. The double mutants *cop1-4 pif3-3*, *cop1-6 pif3-3*, *cop1-4 pif4-101*, *cop1-6 pif4-101* and *cop1-4 hy5-211*, and the *cop1-6* mutant bearing Pro_{MSG2}*:GUS* and Pro_{35S}*:HY5-myc*, were obtained by simple crossing and genotyped by PCR or sequencing (*cop1-4, cop1-6* and *hy5-211*). Primers are described in Supporting Information Table S1.

Seeds were sown on 20 ml of 0.8% agar in clear plastic boxes (4.5 × 8.0 × 2.0 cm height).The boxes were incubated in darkness at 5°C for 5 d and given 8 h of red light followed by 16 h of darkness (22°C).Then, the seedlings were grown under white light photoperiods (10 h) under white light provided by a mixture of fluorescent and halogen lamps (100 μmol m^{-2} s^{-1} between 400 and 700 nm, red : far-red (R : FR) ratio 1.1), and transferred to simulated shade provided by the same light sources in combination with two green acetate filters (no. 089; LEE Filters, Hampshire, UK) to reduce blue and red light and the R : FR ratio (10 μmol m^{-2} s^{-1} between 400 and 700 nm, R : FR ratio 0.1), 1 h after the beginning of Day 3 (the controls remained under white light), as described in Fig. S1(a) and Pacín *et al.* (2013), unless stated otherwise. The temperature was held at 22°C.

Hypocotyl growth rate

Fifteen seeds per genotype were sown in each replicate box. To calculate the growth rate, the seedlings were photographed using a digital camera 1 and 10 h after the beginning of Day 3 (PowerShot; Canon, Tokyo, Japan) and hypocotyl length was determined at both time points using image processing software (Sellaro *et al.*, 2009). The difference in length was divided by the elapsed time.

Chlorophyll and anthocyanin levels

One hundred seedlings per replicate were harvested at the end of Day 5 (i.e. 57 h after the beginning of the shade treatment). Chlorophyll and anthocyanin levels relative to FW were measured as described (Cagnola *et al.*, 2012; Maier *et al.*, 2013).

Indole-3-acetic acid abundance

Four biological samples of each genotype and treatment combination were harvested in liquid Nitrogen 2 h after the beginning of Day 3 (1 h of shade treatment). Indole-3-acetic acid (IAA) was quantified by the stable isotope dilution method on an Agilent 7890A/5975C XL GC-MS operated in selected ion monitoring mode, equipped with a 0.25 mm × 30 m DB-5MS column (0.25 m film) using pulsed splitless injection as described (Cagnola *et al.*, 2012).

β-glucuronidase staining

β-glucuronidase (GUS) expressing seedlings were harvested at the end of Day 3, incubated overnight in X-Gluc solution and transferred for 1 d to 70% ethanol. Seedlings were photographed under a Wide Field Zoom Stereo Microscope Luxeo 4D (Labomed, Los Angeles, CA, USA).

Confocal microscopy

Confocal fluorescence images were taken with an LSM 510 Meta (Zeiss) laser scanning microscope with a Plan-Neofluar 40×/1.3 oil objective lens. For chloroplast visualization, probes were excited with a He-Ne laser (λ = 543 nm) and fluorescence was detected using an LP560 filter. For GFP-HFR1 fusion protein visualization, probes were excited with an Argon laser (λ = 488 nm) and fluorescence was detected using a BP 505-530 filter.

Protein blots

Protein extracts of Arabidopsis seedlings were prepared in extraction buffer (50 mM Tris/HCl pH 7.5, 100 mM EDTA, 1 mM 1,4-dithiothreitol, 0.1% TritonX100 and Protease Inhibitor Cocktail, Roche). Total protein was quantified using Bio-Rad Protein Assay (Bio-Rad) and β-mercaptoethanol was added just before loading. Aliquots from each sample containing equal amounts of protein were subjected to polyacrylamide gel electrophoresis. Immunodetection of HY5-myc and GFP-HFR1 was performed by using anti-myc (Life Technologies, Carlsbad, CA, USA) and anti-green fluorescent protein (Roche) primary antibodies, respectively. Anti-mouse-HRP was used as secondary antibody (Invitrogen) and Amersham ECL Prime Western Blotting Detection Reagent kit (GE Healthcare, Little Chalfont, UK) was used for detection. Ponceau staining was used to visually check the loading uniformity.

Quantitative reverse transcriptase-PCR

Three biological samples of each genotype and treatment were harvested in liquid Nitrogen 5 h after the beginning of Day 3 (4 h of shade treatment); total RNA was extracted with Spectrum Plant Total RNA Kit (Sigma-Aldrich) and subjected to a DNAse treatment with RQ1 RNase-Free DNase (Promega). cDNA derived from this RNA was synthesized using Invitrogen SuperScript III and an oligo-dT primer. The synthesized cDNAs were amplified with FastStart Universal SYBRGreen Master (Roche) using the 7500 Real Time PCR System (Applied Biosystems, available from Invitrogen) cycler. The *UBIQUITIN-CONJUGATING ENZYME 2* (*UBC2*) gene was used as the normalization control (Czechowski *et al.*, 2005). The primers used for *PIL1*, *ATHB2*, *XTR7*, *IAA29*, *YUC8*, *IAA19* and *UBC2* are described in Table S2.

Microarrays

Three biological samples of each genotype and treatment combination were harvested in liquid Nitrogen 5 h after the beginning of Day 3 (4 h of shade treatment), and total RNA was extracted with the Spectrum Plant Total RNA Kit (Sigma-Aldrich). cDNA and cRNA synthesis and hybridization to ATH1 Affymetrix Arabidopsis Gene Chips were performed in accordance with Affymetrix instructions. For microarray experiments, expression data were normalized, restricted by presence criteria (Sellaro *et al.*, 2011), and used for ANOVA to identify the genes showing significant effects of treatments ($P < 0.043$, $q < 0.050$) (Storey & Tibshirani, 2003) (Table S3).

Multiple linear regression analyses

For all of the physiological outputs, protein blot, fluorescence images and real-time PCR data, we used multiple linear regression analysis. The basic linear model was generated by using stepwise linear regression involving four terms: 'Shade' (White light = 0, Shade = 1), *'COP1'* (*cop1* mutants = 0, *COP1* WT allele = 1), *'PIFq'* (*pifq* quadruple mutant = 0, *pif3, pif4* and *pif5* single mutants = 0.75, *pif4 pif5* double mutant = 0.5, *PIFq* WT alleles = 1) and the 'Shade × *COP1* × *PIFq'* interaction (product of the values for each one of the three variables). In Figs 2(a,c,e) and 5(a) (see later) we indicate the terms of this model that are statistically significant. Additional terms were added to address specific questions. In Fig. 4 (see later), we included the term Shade × *cop1* × *PIFq* interaction (the *cop1* mutant under shade = 1, *cop1 pif3*, *cop1 pif4* and *cop1 pif5* under shade = 0.75, all the other conditions = 0), to quantify the effect of *PIF3*, *PIF4* and *PIF5* in the *cop1* background and compare it to the effect in the WT *COP1* background (provided by term 'Shade × *COP1* × *PIFq'* of the basic model). In Fig. 6 (see later) only the term 'Shade' was included in the model because no *cop1* or *pif* mutants are included. In Figs 8(a) and 9 (see later), *pif* mutants were not included and therefore the terms '*PIFq*' and 'Shade × *COP1* × *PIFq'* were not used in the model, whereas the terms 'Shade × *COP1* × *hfr1'* interaction (*hfr1* mutant under shade = 1, all other conditions = 0) and 'Shade × *cop1* × *hfr1'* interaction (*hfr1 cop1* double mutant under shade = 1, all other conditions = 0) were added to compare the effect of the *hfr1* mutation under shade in the *COP1* vs the *cop1* background. In Fig. 7 (see later), *pif* mutants were not included and therefore the terms '*PIFq*' and 'Shade × *COP1* × *PIFq'* were not used. In Fig. 7(a) (see later) the terms 'Time' (rather than 'Shade') and 'Time × *COP1'* interaction were included to represent the effects of shade of different duration and their interaction with COP1. In Fig. 8(b) (see later), *cop1* mutants were not included and therefore the terms 'COP1' and 'Shade × *COP1* × *PIFq'* were not used in the model, whereas the terms Shade × *PIFq* × *hfr1* (*hfr1* mutant under shade = 1, *pif4 pif5 hfr1* triple mutant under shade = 0.5, all other conditions = 0) and Shade × *pifq* × *hfr1* (*pif4 pif5 hfr1* under shade = 0.5, all other conditions = 0) were added to compare the effects of *hfr1* in the *PIF4 PIF5* vs the *pif4 pif5* background. In Fig. S3 (see later), only the term 'Shade' was included in the model. In Fig. S4 (see later), the terms 'Shade', '*cop*1', '*hy5*' (*hy5* mutants = 1, *HY5* WT allele = 0), 'Shade × *COP1'* interaction, 'Shade × *COP1* × *hy5'* interaction (*hy5* mutant under shade = 1, all other conditions = 0) and 'Shade × *cop1* × *hy5'* interaction (*hy5 cop1* double mutant under shade = 1, all other conditions = 0) were added to compare the effect of the *hy5* mutation under shade in the *COP1* vs the *cop1* background. The slope of each term provides a proxy for the effect of the variables involved in that term and selected slopes are represented for comparative purposes in Figs 4, 8 and 9 (see later).

Results

Despite its severe shade-avoidance phenotype, *cop1-4* retains significant gene expression responses to shade

In order to investigate the molecular phenotype of the *cop1-4* mutant during the response to shade, we performed a transcriptome analysis of plants of the Columbia WT and the *cop1-4* mutant. The quadruple *pifq* mutant was included for comparative purposes. Seedlings were grown under white light photoperiods (10 h), transferred to simulated shade 1 h after the beginning of Day 3 (the controls remained under white light; Fig. S1a), and harvested 4 h later. The 3465 genes that showed significant effects of treatments ($P < 0.043$, $q < 0.050$) were grouped according to the significance of the main effects (shade, genotype) and their interaction, and cluster analysis (dChip, Li & Hung Wong, 2001) was conducted within each statistical group (Table S3).

Despite the major differences in experimental conditions, there is a remarkable similarity between the patterns reported here and those observed in previous studies that analysed rapid genome-wide transcriptional responses to lowering the R : FR ratio (Leivar *et al.*, 2012b) and natural shade compared to sunlight (Sellaro *et al.*, 2011) (Fig. S2).The latter is also true for the impact of the *pifq* mutant (Leivar *et al.*, 2012b). Therefore, gene expression responses to shade signals are robust. The only exception is cluster 6, which showed stronger responses in the WT in previous studies than those reported here.

It is noteworthy that, despite the severely impaired shade-avoidance responses of the *cop1* mutants (McNellis *et al.*, 1994; Crocco *et al.*, 2010; Rolauffs *et al.*, 2012; Pacín *et al.*, 2013), most genes showing responses to shade in the WT (1828 genes) also responded in the *cop1-4* mutant (1648 genes, 92.1%, clusters 1, 2, 3, 4 and 7) (Fig. 1).

In clusters 1 and 2, expression was decreased or increased (respectively) by simulated shade without significant effects of genotype or interaction (Fig. 1). These genes would be related to the processes that adjust plant form and function to shade conditions independently of COP1. In clusters 3 and 4, expression was decreased or increased (respectively) by simulated shade and affected in the opposite direction by the *cop1-4* mutation without significant interaction between genotype and shade condition (i.e. the response to shade was not affected by the mutations). In cluster 7, only the *pifq* mutant failed to respond to shade and these genes showed a significant interaction between simulated shade treatment and genotype.

The genes included in clusters 1, 3, and 7 showed nearly WT reductions of expression under shade in the *cop1-4* mutant (Fig. 1). The gene ontology terms over-represented among these

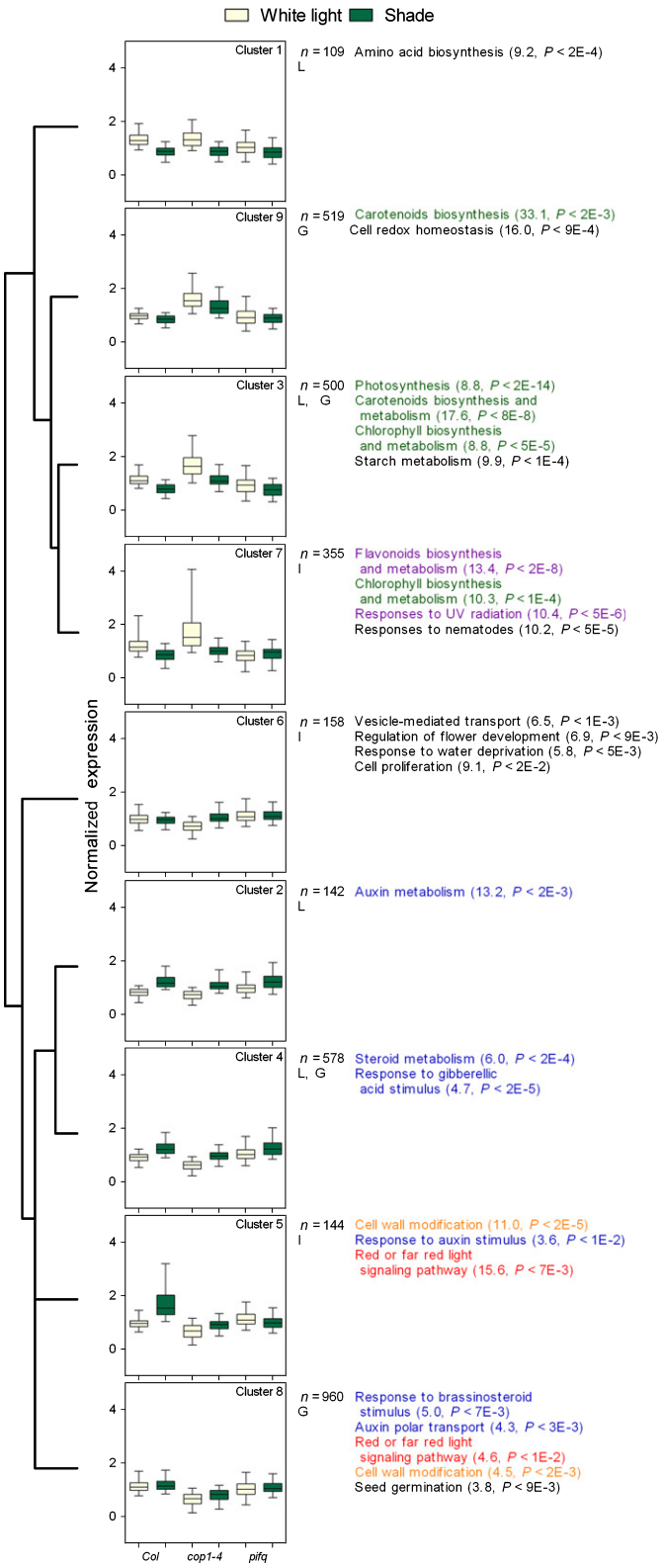

Fig. 1 Many genes retain expression responses to shade in the *cop1-4* mutant of Arabidopsis. The 3465 genes that showed significant effects of treatments in ANOVA ($P < 0.043$, $q < 0.050$) were grouped according to the significance of the main effects (L, light condition; G, genotype) and their interaction (I). Cluster analysis was conducted within each statistical group by using dChip (Li & Wong, 2003). Gene expression was normalized to the median of each gene. Box-plots show median, 1–3 interquartile range and 95% confidence interval of normalized values. The clusters are ordered by similarity of their average expression patterns. The number of genes, the significant terms of ANOVA (L and/or G, or I) and the enriched functions (including their fold enrichment and *P*-value) are indicated. Related GO terms share the same colour. Protocol in Supporting Information Fig. S1(a).

genes (Vandepoele *et al.*, 2009) include amino acid biosynthesis ($P<$ 2E-4) in cluster 1, photosynthesis ($P<$ 2E-14), carotenoid biosynthesis and metabolism ($P<$ 8E-8) and starch metabolism ($P<$ 1E-4) in cluster 3, and flavonoid biosynthesis and metabolism ($P<$ 2E-8), responses to UV radiation ($P<$ 5E-6) and responses to nematodes ($P<$ 5E-5) in cluster 7. Chlorophyll biosynthesis and metabolism ($P<$ 5E-8) was over-represented in clusters 3 and 7.

The genes included in clusters 2 and 4, showed nearly WT promotion of expression under shade in the *cop1-4* mutant (Fig. 1). Several hormone-related genes are present in these clusters, including two auxin metabolism-related genes in cluster 2 (*YDK1* and *RGLG1,* $P<$ 2E-3), seven steroid metabolism genes ($P<$ 2E-4) and 10 response to gibberellic acid stimulus genes (including six genes encoding MYB-domain proteins, *GA INSENSITIVE* and *EXPANSIN A3,* $P<$ 2E-5) in cluster 4.

Genes that failed to respond to shade in the *cop1-4* mutant

The response to shade was impaired only by *cop1* for the genes included in cluster 5 (144 genes, 4.2%), which showed a significant interaction between light/shade condition and genotype due to the promotion observed in the WT and not in the mutants (Fig. 1). These genes are likely to represent the consequence of increased COP1 nuclear activity under shade (Pacín *et al.*, 2013).

Cluster 5 includes numerous cell-wall modification associated genes ($P<$ 2E-5), such as *EXPANSIN-LIKE A2*, *EXPANSIN A8* and *EXPANSIN A11*. Cluster 5 also includes five response to auxin stimulus genes (*SMALL AUXIN UP RNA 23*, *33* and *68*, and *INDOLE-3-ACETIC ACID 6* and *19*, $P<$ 1E-2), two red or FR light signalling pathway genes (*PHYA* and *ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 2*, $P<$ 7E-3) and *HFR1*.

Genes affected in the *cop1-4* mutant even in the absence of shade

The *cop1-4* mutation affected the background level of expression in the absence of shade of a significant proportion of the genes (3070 genes, 88.6%, clusters 3, 4, 6, 7, 8, 9) (Fig. 1). Clusters 3, 4 and 7 have already been described among those where the *cop1* mutant retains responses to shade. In clusters 8 and 9, gene expression was decreased or increased (respectively) by the *cop1-4* mutation, but these genes did not respond significantly to shade. Cluster 8 is enriched in hormone-related genes, including four response to brassinosteroid stimulus genes ($P<$ 7E-3), six auxin polar transport genes (including *PIN-FORMED 4* and *7*, and *PIN-LIKE 2* and 5, $P<$ 3E-3). Four red or FR light signalling pathway genes (*PHYB, PROTEIN PHOSPHATASE 5*, *HY5* and *PIF3*, $P<$ 1E-2), cell wall modification ($P<$ 2E-3) and seed germination ($P<$ 9E-3) are also present in cluster 8. Cluster 9 is enriched in carotenoid biosynthesis ($P<$ 2E-3) and cell redox homeostasis ($P<$ 9E-4). Cluster 6, where expression is decreased by *cop1* only in the absence of shade, is enriched in vesicle-mediated transport ($P<$ 1E-3), regulation of flower development ($P<$ 9E-3), response to water deprivation ($P<$ 5E-3) and cell proliferation ($P<$ 2E-2).

Clusters 4, 8 and 6, where expression is reduced by *cop1* in the absence of shade, contain growth-related gene-ontology (GO) terms (e.g. genes related to hormones that promote growth). We searched for the functional information available in the literature for all the genes within these categories present in the latter clusters, to investigate whether their reduced expression could help to account for the reduced hypocotyl growth of *cop1*. Some of these genes do promote hypocotyl growth (*EER1*, *APM1i SAV1/DWF4*, *GPA1*, *MYB30*, *HMG1*, *CPD/CBB3/DWF3*) but others actually inhibit hypocotyl growth (e.g. *PILS5*, *MIF1*, *GAI*, *GASA1*, *CYP72C1*, *WES1/GH3.5*) (Table S4).

cop1-4 and *cop1-6* mutants retain selected physiological responses to shade

The analysis of the transcriptome suggests that the *cop1* mutant might retain selected physiological responses to shade. The GO terms over-represented within the clusters where the *cop1* mutant showed strong gene expression responses to shade include flavonoid biosynthesis and metabolism, and chlorophyll biosynthesis. The normalized expression of all the genes within these two categories that showed significant effects of treatments were averaged to summarize the overall trend, and compared with anthocyanin and chlorophyll responses. For comparative purposes we also included stem growth and all of the cell-wall loosening genes showing significant effects of treatments.

As expected, the shade-avoidance response involving enhanced stem growth was severely impaired in the *cop1* mutants (Fig. 2a). This pattern was paralleled by the average response of cell-wall loosening genes and by the response of individual genes such as *EXPANSIN A11* (Fig. 2b; Table S5). Conversely, the shade-induced reductions of anthocyanin and chlorophyll levels were at least as intense in the *cop1* mutants as in the WT (Fig. 2c,e). Again, the average patterns of expression of flavonoid biosynthetic process genes and chlorophyll biosynthetic process genes, as well as the patterns of expression of individual genes within these groups such as *CHALCONE SYNTHASE* (*CHS*) and *CONDITIONAL CHLORINA* (*CCH*), resemble the physiological responses (Fig. 2d,f; Table S5). It has been shown that COP1/SPA complex controls the protein stability of the MYB transcriptions factors PAP1 and PAP2 involved in anthocyanin accumulation (Maier *et al.*, 2013).

Shade increases the convergence between *cop1* and *pifq* transcriptome responses

In order to investigate the degree of convergence between the phenotypes of the *cop1-4* and *pifq* mutants their gene expression differences with the WT were evaluated by Student's *t*-test. The analysis was restricted to those genes showing significant effects of treatments in the ANOVA. We found 2514 genes with significant changes in expression in the *cop1-4* mutant and 1076 in the *pifq* mutant. The proportion of genes with expression either reduced or enhanced by both *cop1-4* and *pifq* mutants (i.e. the convergence), increased significantly under shade compared with white light conditions (Fig. 3). The enrichment of *cop1-4-pifq*

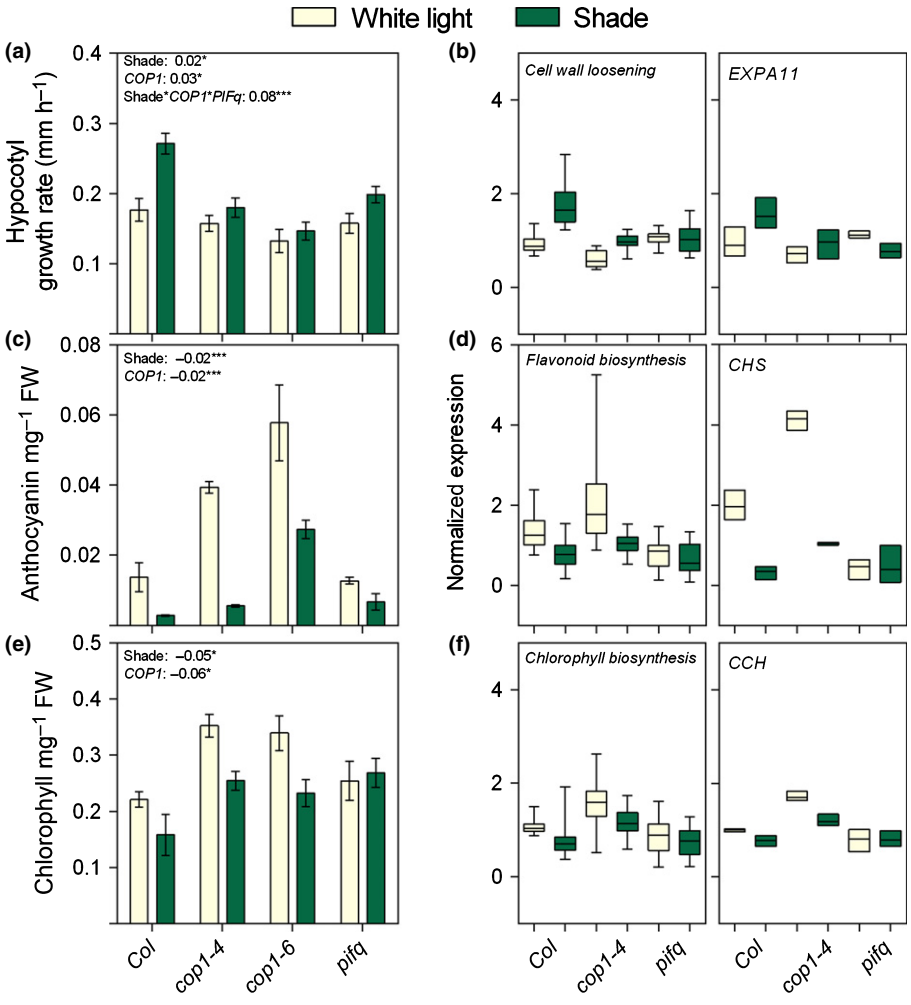

Fig. 2 Despite their severe shade-avoidance phenotype, the *cop1-4* and *cop1-6* mutants retain pigment responses to shade in Arabidopsis. (a) Hypocotyl growth rate, (c) anthocyanin content relative to FW, (e) chlorophyll content relative to FW. Data are means ± SE of (a, e) eight or (c) four replicate boxes. For each physiological response statistically significant terms are indicated (*, $P < 0.05$; ***, $P < 0.0001$). (b, d, f), Box-plots (median, 1–3 interquartile range and 95% confidence interval) of normalized expression of all the genes that showed significant effects of treatments within a gene ontology (GO) term related to each physiological process (left) and normalized expression of one representative gene of the group (right): (b) cell-wall loosening genes (5 genes), (d) flavonoid biosynthetic process genes (32 genes), (f) chlorophyll biosynthetic process genes (32 genes). Protocol in Supporting Information Fig. S1(a).

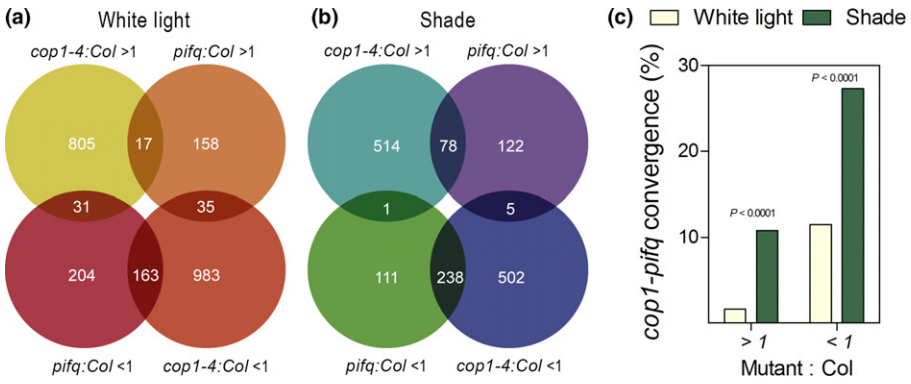

Fig. 3 Shade enhances the convergence of the *cop1* and *pifq* transcriptional phenotypes in Arabidopsis. Venn diagram for genes with increased or decreased expression in *cop1-4* and/or *pifq* mutants, compared with the wild-type (*t*-test), under (a) white light or (b) shade, and (c) *cop1-pifq* convergence under each conditions. Contingency data were analysed by Fisher's exact test and the significance is indicated ($P < 0.0001$). Protocol in Supporting Information Fig. S1(a).

convergence was also significantly higher for the genes that increase their expression than for those that reduce their expression as a result of the mutations ($P < 0.0001$) (Fig. 3).

The group of genes that decreased their expression compared with the WT in both mutants under with light is enriched in flavonoid biosynthesis (13.1-fold enrichment, $P < 3E\text{-}4$). The group of genes that decreased their expression compared with the WT in both mutants under shade is enriched cell wall modification (14.9-fold enrichment, $P < 2E\text{-}5$) and red or FR light signalling pathway (13.8-fold enrichment, $P < 2E\text{-}3$).

The shade-avoidance phenotypes of *cop1* and *pifq* are not additive

Because gene expression data shows that *cop1-4-pifq* convergence is increased by shade, especially for shade-avoidance related genes, we studied the hypocotyl growth response in the *cop1-4 pif3*, *cop1-6 pif3*, *cop1-4 pif4* and *cop1-6 pif4* double mutants. As expected, shade-avoidance responses were reduced in both *cop1* mutant alleles (McNellis *et al.*, 1994; Crocco *et al.*, 2010; Rolauffs *et al.*, 2012; Pacín *et al.*, 2013), *pif3* (Leivar *et al.*,

2012a,b; Sellaro *et al.*, 2012) *pif4* and *pif5* (Lorrain *et al.*, 2008; Leivar *et al.*, 2012a; Sellaro *et al.*, 2012) mutants. However, the effects of the *cop1* and *pif3, pif4* or *pif5* mutations were not additive; that is, the action of *PIFs* under shade is largely *COP1* dependent (Fig. 4). These results are different from the case of etiolated seedlings, where the *pif1 cop1* double mutants are shorter than their single mutants (Xu *et al.*, 2014), and suggests that PIFs and COP1 might share common signalling pathways in shade avoidance.

Auxin levels do not increase in *cop1* and *pifq* in response to shade

Shade avoidance responses have been associated with increased levels of active auxins (IAA) (Tao *et al.*, 2008), a response that involves PIF-mediated promotion of the expression of *YUCCA* auxin synthesis genes (Hornitschek *et al.*, 2012). Given the convergence of the *cop1* and *pifq* mutant phenotypes in terms of patterns of expression of shade-avoidance related genes and stem growth in response to shade, we examined IAA levels in the WT and in *cop1-4*, *cop1-6* and *pifq* mutants (Fig. 5a). The results confirm the increased IAA levels in response to shade in the WT and that this response requires PIFs. Additionally, we show that the *cop1* mutant is also impaired in the ability to increase IAA levels in response to shade.

Noteworthy, *pifq* mutant shows high background levels of IAA in both conditions (comparable to the WT under shade). This might reflect multiple regulatory actions of PIFs as suggested by the observation that both mutation and overexpression of PIF genes can reduce IAA levels (Hornitschek *et al.*, 2012). IAA levels correlated with the normalized expression of the *YUCCA* genes showing the highest expression (Fig. 5b), which are promoted by shade (Stepanova *et al.*, 2011; Won *et al.*, 2011).

Pro_{MSG2}*::GUS* was used as reporter to analyse auxin signalling (Kami *et al.*, 2014), in the WT and *cop1-6* background. High staining was observed in the WT hypocotyl under shade, but not in the white light control or in the *cop1* background in both conditions (Fig. 5c). These observations indicate that *COP1* is necessary for IAA accumulation during shade avoidance.

COP1 enhances HFR1 degradation under shade

The gene expression and genetic data are consistent with signalling convergence between COP1 and PIFs. This convergence could occur at multiple levels but here we focused on the analysis of two proteins that are able to physically interact with COP1 and as a result of this interaction become targeted for degradation in the proteasome in dark-grown seedlings: HY5 and HFR1 (Hardtke *et al.*, 2000; Saijo *et al.*, 2003; Duek *et al.*, 2004; Jang *et al.*, 2005; Yang *et al.*, 2005). At least under certain conditions, these proteins can interfere with signalling by PIFs (Hornitschek *et al.*, 2009; Toledo-Ortiz *et al.*, 2014) and could therefore account for the proposed convergence. The hypothesis is that the enhanced nuclear abundance of COP1 under shade favours degradation of HY5 and/or HFR1 and this in turn facilitates the activity of PIFs. This hypothesis has three predictions: HY5 and/or HFR1 should reduce their abundance under shade compared with white light conditions; this reduction should be COP1-dependent; and the mutations *hy5* and/or *hfr1* should at least partially rescue the *cop1* shade-avoidance phenotype.

Figure 6 shows that shade does not reduce HY5 stability – it actually increases its abundance. Therefore, HY5 does not fulfil the first prediction of the hypothesis. Conversely, shade does reduce HFR1 abundance (protocol detailed in Fig. S1b).

In more detailed experiments, we observed that transfer from white light to shade conditions caused an exponential decay of nuclear GFP-HFR1 levels, whereas in the *cop1* background GFP-HFR1 levels were only slightly reduced under shade (Fig. 7). The significant interaction between time under shade and presence or absence of COP1 indicates that the shade-induced decay of nuclear HFR1 depends on COP1. The slight decay of HFR1 under shade in *cop1* could be caused by residual COP1 activity or to the action of other degradation mechanisms. The enhanced abundance of HY5 under shade was observed even in the *cop1* mutant background (Fig. S3). The *cop1* mutant retained higher levels of HY5 than the WT independently of shade, suggesting that the levels of COP1 activity present under white light might be enough to control HY5 abundance.

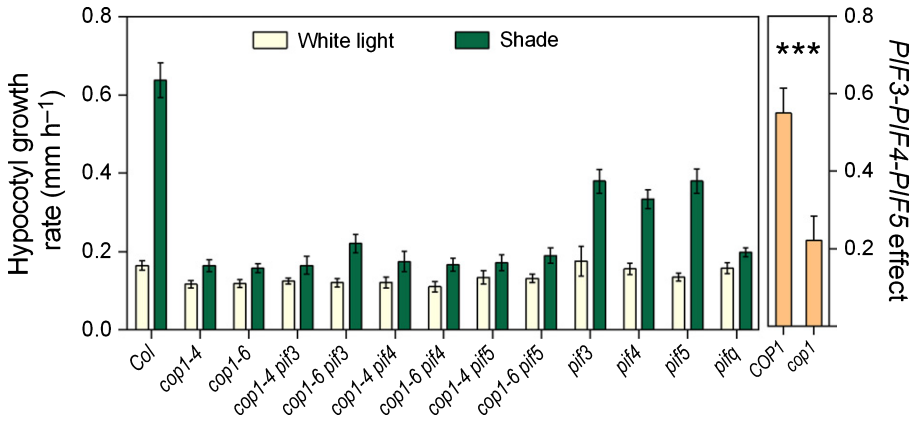

Fig. 4 COP1 enhances the effect of PIF3, PIF4 and PIF5 on shade avoidance in Arabidopsis. Hypocotyl growth-rate data are means ± SE of 4–12 replicate boxes. The effect of *PIF3*, *PIF4* and *PIF5* under shade in the *COP1* vs the *cop1* background and their SE are shown and the significance of a *t*-test is indicated (right) (***, $P < 0.0001$). Protocol in Supporting Information Fig. S1(a).

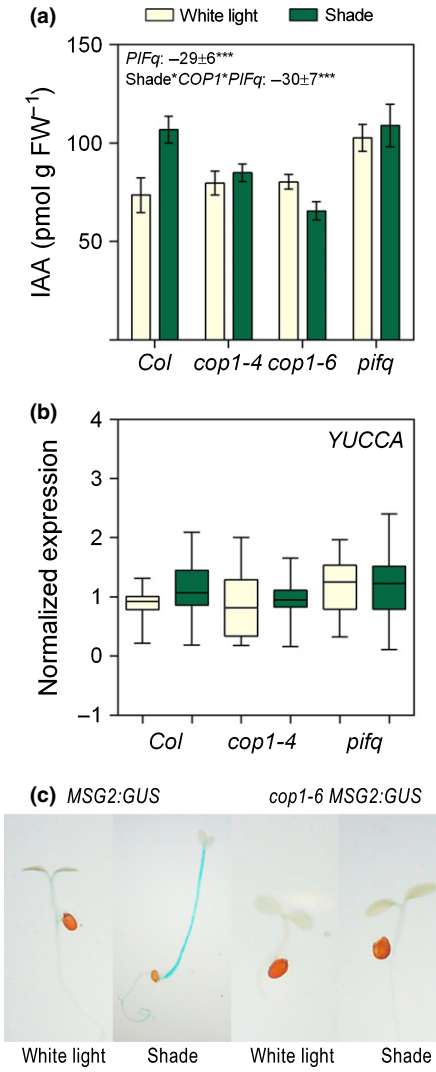

Fig. 5 Impaired auxin response to shade in the *cop1* mutants of Arabidopsis. (a) INDOLEACETIC ACID (IAA) content relative to FW. Data are means ± SE of four replicate boxes. The statistically significant terms are indicated (***, $P < 0.0001$). (b) Box-plot (median, 1–3 interquartile range and 95% confidence interval) of normalized expression of *YUCCA 2, 3, 5, 7, 8* and *9* genes (microarray data) resembles the IAA level pattern. (c) Representative images of Pro_{MSG2}::*GUS* and *cop1*–6/Pro_{MSG2}::*GUS* seedlings after β-glucuronidase (GUS) staining. Protocol (a, b) in Supporting Information Fig. S1(a), or (c) S1(b).

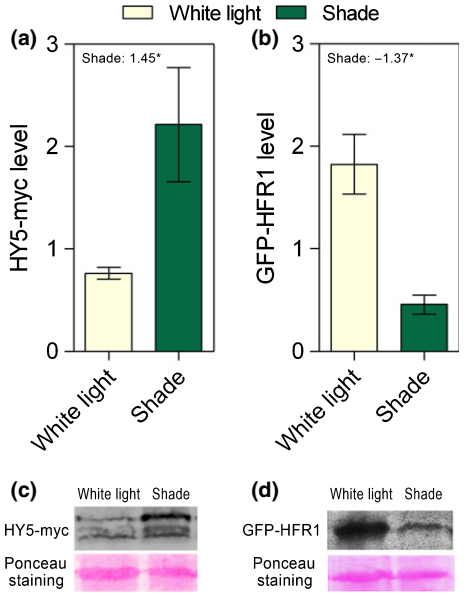

Fig. 6 Shade increases the stability of HY5 and reduces the stability of HFR1 in Arabidopsis. Seedlings were grown under white light or shade conditions and harvested at midday of Day 3. A representative protein blot and the mean ± SE of three independent samples is shown for (a, c) HY5 and (b, d) HFR1. The statistically significant term is indicated (*, $P < 0.05$). Protocol in Supporting Information Fig. S1(b).

hfr1 mutation rescues shade avoidance responses in *cop1*

In order to test the third prediction of the hypothesis for HFR1, we evaluated the shade-avoidance phenotype of *cop1*, *hfr1* and *cop1 hfr1* double mutants. The impaired growth response to shade observed in *cop1* mutants was rescued in the *cop1 hfr1* double mutants (Fig. 8a).This observation is consistent with previous reports showing that the *hfr1* mutation rescues the response to low R : FR ratios in the *spa1 spa3 spa4* triple mutant background (Rolauffs *et al.*, 2012) and hypocotyl growth in etiolated *cop1* seedlings (Kim *et al.*, 2002). As expected, the hypocotyl growth promotion induced by shade in the WT was reduced in the *pif4 pif5* double mutant (Lorrain *et al.*, 2008) and increased in the single *hfr1* mutant (Hornitschek *et al.*, 2009).

The effect of the *hfr1* mutation under shade was significantly higher in the *cop1* mutant than in the *COP1* background (Fig. 8a) and in the *PIF4 PIF5* than in the *pif4 pif5* background (Fig. 8b).

In order to test the third prediction of the hypothesis for HY5, we evaluated the shade-avoidance phenotype of *cop1,hy5* and *cop1 hy5* double mutants. The impaired growth response to shade observed in *cop1* mutants was not rescued in the *cop1 hy5* double mutants (Fig. S4) (Rolauffs *et al.*, 2012). Noteworthy, *hfr1* rescued the shade-avoidance response of *cop1* without increasing its stem growth rate under white light (Fig. 8a), whereas *hy5* increased stem growth under white light but failed to rescue shade avoidance (Fig. S4). No enhanced growth of *hy5* had been observed in previous reports (Rolauffs *et al.*, 2012) likely because they involved the use of continuous light, where other light signalling components might compensate for the absence of HY5.

In order to further investigate whether the COP1-PIF convergence is mediated by HFR1, we analysed the expression of four early targets of PIFs during shade avoidance (Hornitschek *et al.*, 2009) in *cop1*, *hfr1* and *cop1 hfr1* mutants. The expression of *XYLOGLUCAN ENDOTRANSGLYCOSYLASE 7* (*XTR7*), *PHYTOCHROME INTERACTING FACTOR 3-LIKE 1* (*PIL1*), *ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 2* (*ATHB2*) and *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*) was severely reduced in the *cop1* mutants under shade (Pacín *et al.*, 2013). Compared with the WT, the *hfr1* mutation caused, at most, a weak increase in expression under shade (Fig. 9). However, for the four genes, the *hfr1* mutation significantly rescued (in some cases fully rescued) the gene expression response of these PIF target genes in the *cop1* mutant background

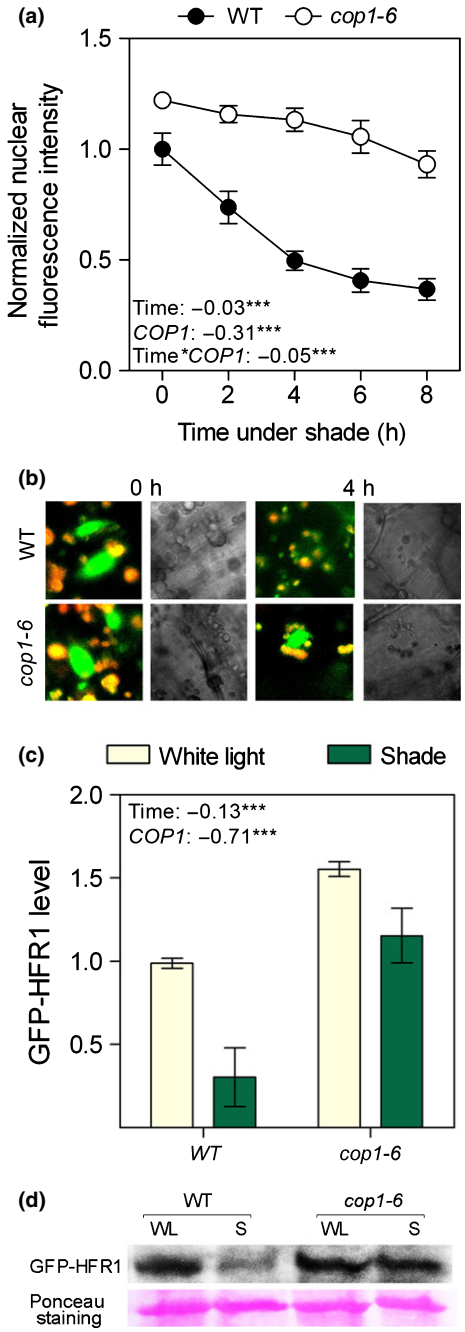

Fig. 7 Shade reduces HFR1 nuclear abundance in a COP1-dependent manner in Arabidopsis. (a) Nuclear fluorescence intensity in wild-type (WT) and *cop1*-mutant plants bearing the *Pro*$_{35S}$*:GFP-HFR1* fusion, plotted against the time under shade (seedlings were harvested at 10 h of Day 3). Data are means ± SE of 10–12 seedlings. (b) Representative nuclei of *Pro*$_{35S}$*:GFP-HFR1* and *cop1–6*/*Pro*$_{35S}$*:GFP-HFR1* seedlings 0 or 4 h after transferring to shade conditions. (d) A representative protein blot and (c) the mean and SE of three independent samples is shown for HFR1. Statistically significant terms are indicated (***, $P < 0.0001$).

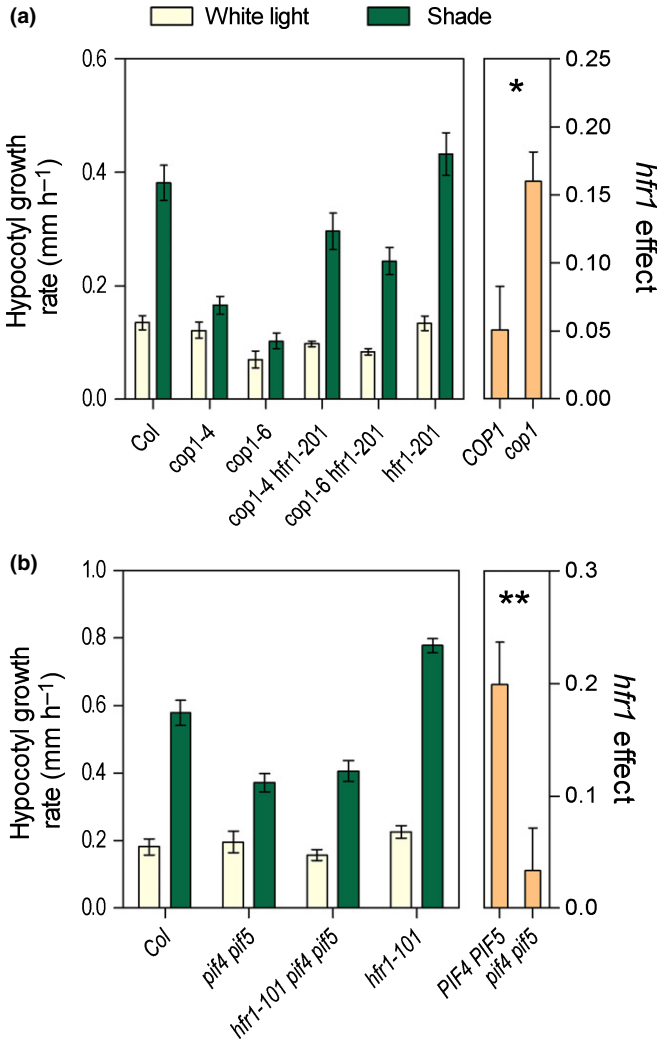

Fig. 8 The effect of the *hfr1* mutation is reduced by (a) COP1 and by (b) the *pif4 pif5* mutations in Arabidopsis. Data are means ±SE of 4–12 replicate boxes. The effects of the *hfr1* mutation under shade in (a) the *COP1* vs the *cop1* background and in (b) the *PIF4 PIF5* vs the *pif4 pif5* background and their SE are shown (right) and the significance of a *t*-test is indicated: *, $P < 0.05$; **, $P < 0.01$. Protocol in Supporting Information Fig. S1(a).

(Fig. 9).The expression of *YUCCA 8* (YUC8) and *INDOLE-3-ACETIC ACID INDUCIBLE 19* (*IAA19*) genes under shade was also impaired in *cop1* mutants and rescued in the *cop1 hfr1* double mutants (Rolauffs *et al.*, 2012; Fig. 9). For all of the genes investigated here, the impact of the *hfr1* mutation was significantly larger in the *cop1* than in the *COP1* background.

Discussion

Despite the severe shade-avoidance phenotype of *cop1* (McNellis *et al.*, 1994; Crocco *et al.*, 2010; Rolauffs *et al.*, 2012; Pacín *et al.*, 2013), most genes that responded to shade in the wild-type (WT) also responded to shade in *cop1*. The latter genes include many involved in chlorophyll, carotenoid and flavonoid biosynthesis and metabolism (Fig. 2). The expression patterns of these genes correlated with chlorophyll and anthocyanin levels across genotypes and light/shade conditions (Fig. 2). We used weak *cop1* alleles to obtain a severe shade-avoidance phenotype while minimizing unrelated defects (null alleles are seedling-lethal; McNellis

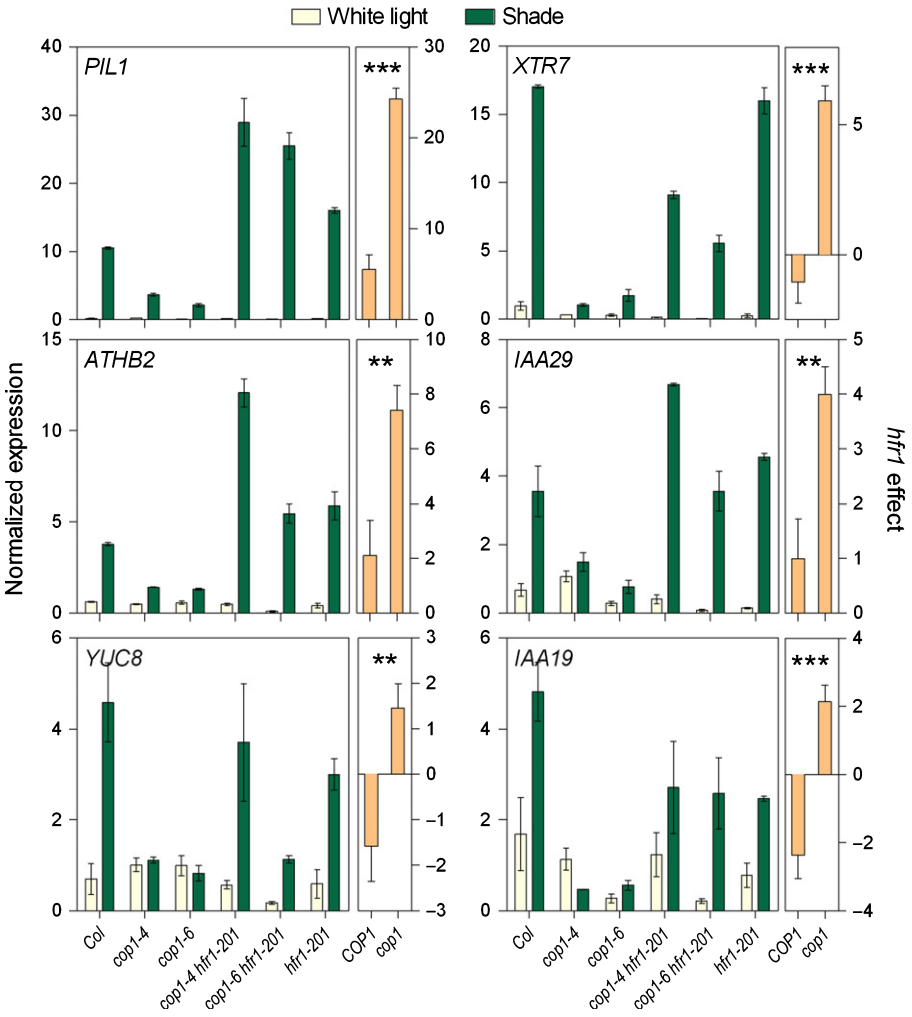

Fig. 9 The *hfr1* mutation significantly rescues gene expression responses to shade in the *cop1* mutant of Arabidopsis. Normalized expression of *PHYTOCHROME INTERACTING FACTOR 3-LIKE 1* (*PIL1*), *XYLOGLUCAN ENDOTRANSGLYCOSYLASE 7* (*XTR7*), *ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 2* (*ATHB2*), *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*), *YUCCA8* (*YUC8*) and *INDOLE-3-ACETIC ACID INDUCIBLE 19* (*IAA19*). Data are means ± SE of three replicate boxes. The effects of the *hfr1* mutation under shade in the *COP1* vs the *cop1* background and their SE are shown (right) and the significance of a *t*-test is indicated: **, $P < 0.01$; ***, $P < 0.0001$. Protocol in Supporting Information Fig. S1(a).

et al., 1994), which could hinder the identification of specific molecular mechanisms of COP1 action during shade avoidance. Therefore, we cannot rule out the possibility that the observed pigment and associated gene responses are mediated by the residual COP1 activity in the weak alleles. However, it is clear that these pigment responses (i.e. acclimation responses) require a much lower threshold of COP1 activity than shade-avoidance responses.

Among the mutants with impaired shade avoidance, *cop1* was one of the first reported (McNellis *et al.*, 1994) and one of the most severe (Casal, 2013). In our conditions, the *hy5* mutation enhanced hypocotyl growth in the *cop1* background without rescuing shade-avoidance responses (Fig. S4), indicating that the impaired shade avoidance of *cop1* is not simply the result of a general growth restriction. Furthermore, the *cop1* mutation affected the expression of many genes in the absence of shade but the functional analysis of these genes does not provide a strong indication of a general growth restriction (Table S4). The levels of auxin were not affected by *cop1* in the absence of shade (Fig. 5a). These observations argue against the idea that COP1 affects shade-avoidance responses indirectly, only by setting the molecular conditions for these responses to operate. Instead, they favour the alternative interpretation; that is, that COP1 is intrinsically involved in the mechanisms of shade avoidance.

Both the *cop1* and *pifq* mutations affected the expression of many genes in the absence of shade, but the degree of coincidence was relatively poor (13%; Fig. 3). Shade increased the convergence of the effects three-fold (38%; Fig. 3), which is consistent with the enhanced activity of PIFs (Lorrain *et al.*, 2008; Leivar *et al.*, 2012a,b; Li *et al.*, 2012) and COP1 (Pacín *et al.*, 2013) in response to shade. Only one gene cluster (cluster 5) showed impaired response to shade in *cop1*; and this cluster was enriched in shade-avoidance markers. The *pifq* mutation similarly affected the expression pattern of these genes (Fig. 1). Both *cop1* and *pifq* mutations impaired the increment of auxin levels caused by shade signals (Fig. 5a) as previously reported for the *pif4 pif5* (Hornitschek *et al.*, 2012) and the *pif7* (Li *et al.*, 2012) mutants. The *pif3*, *pif4* and *pif5* mutations had no significant effects on hypocotyl growth in the *cop1* mutant background (Fig. 3), which contrasts with the observations in dark-grown seedlings (Xu *et al.*, 2014). These observations are consistent with the occurrence of signalling convergence between COP1 and PIFs during shade avoidance.

Here, we show that shade enhances the degradation of HFR1 in a COP1-dependent manner (Fig. 7). HFR1 is a direct target of COP1 in darkness (Duek *et al.*, 2004; Jang *et al.*, 2005; Yang *et al.*, 2005). Because HFR1 negatively regulates shade-avoidance responses by forming dimers with PIFs so they are not able to bind their target promoters (Hornitschek *et al.*, 2009), convergence between COP1 and PIFs signalling during shade avoidance could occur via an enhanced activity of PIFs caused by COP1-dependent degradation of HFR1. In accordance with this interpretation, *hfr1* rescued both shade-avoidance responses and PIF-target gene expression responses to shade in the *cop1* background without having much effect in the *COP1* background (Figs 8a, 9). Furthermore, the effect of PIFs depended on the presence of COP1 (Fig. 4) and the impact of the *hfr1* mutation was higher in the presence of *PIF4* and *PIF5* (Fig. 8b). In dark-grown seedlings, PIF1 enhances COP1 E3 ligase activity (Xu *et al.*, 2014) and COP1 enhances PIF3 accumulation (Bauer *et al.*, 2004). These points of synergistic interaction between COP1 and PIF signalling could also be important during shade avoidance.

In dark-grown seedlings, COP1 targets HY5 and HFR1 for degradation (among several other proteins) via direct physical interaction (Hardtke *et al.*, 2000; Saijo *et al.*, 2003; Duek *et al.*, 2004; Jang *et al.*, 2005; Yang *et al.*, 2005). When dark-grown seedlings are exposed to light, COP1 activity decreases, leading to increased HY5 and HFR1 abundance. However, the fates of HY5 and HFR1 diverge when light-grown seedlings are transferred to shade. In fact, shade reduced HFR1 abundance but increased HY5 abundance (Fig. 6). This indicates that not all nuclear targets of COP1 in darkness become destabilized by the enhanced COP1 nuclear abundance under shade.

In conclusion, we propose that COP1 is intrinsically involved in the mechanisms of shade avoidance. COP1 nuclear abundance increases under shade as a result of reduced red light perceived by phytochrome B and blue light perceived by cryptochromes (Pacín *et al.*, 2013), and this enhances the degradation of HFR1 (Fig. 7), which is a nuclear-localized target of COP1 (Duek *et al.*, 2004; Jang *et al.*, 2005; Yang *et al.*, 2005). In turn, reduced HFR1 levels would increase the activity of PIFs (Figs 8, 9) and hence the strength of shade-avoidance responses. Therefore, shade avoidance operates via two convergent pathways involving the action of both phytochromes and cryptochromes on PIFs (Lorrain *et al.*, 2008; Pedmale *et al.*, 2016) and on COP1 (Pacín *et al.*, 2013).

Acknowledgements

We thank Jianping Yang (Institute of Crop Sciences, Chinese Academy of Agricultural Sciences) for his kind provision of *cop1–4 hfr1*, *cop1–6 hfr1*, *Pro$_{35S}$:GFP-HFR1*, *cop1–6/Pro$_{35S}$:GFP-HFR1* mutants and lines, and Christian Fankhauser (University of Lausanne) for the *hfr1 pif4 pif5* mutant. This work was supported by grants from the University of Buenos Aires (grant no. 20020100100437) and Agencia Nacional de Promoción Científica yTecnologica (grant nos. PICT 2012-1396 and PICT 2013-1444).

Author contributions

M.P., S.A.F. and J.J.C. planned and designed the research, M.P., M.S., M.L. and S.A.F., performed experiments, M.P. and J.J. analysed data and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1 Light protocols.

Fig. S2 Clusters of normalized gene expression data compared to previous studies.

Fig. S3 The enhanced abundance of HY5 under shade is observed even in the *cop1* mutant background.

Fig. S4 The impaired growth response to shade observed in *cop1* mutants is not rescued in the *cop1 hy5* double mutants despite its enhanced growth.

Table S1 Primers used for genotyping

Table S2 Primers used for real-time RT-PCR

Table S3 Gene expression of Col, *cop1* and *pifq* under simulated sunlight and shade conditions

Table S4 Phenotypes of mutants of growth related genes with expression reduced in *cop1*

Table S5 List of cell wall loosening, chlorophyll biosynthesis and flavonoid biosynthesis genes

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