UNIVERSITAT POLITÈCNICA DE VALÈNCIA

ibmcp Instituto de Biología Molecular y Celular de Plantas

MASTER THESIS

Master's degree in Plant Molecular and Cellular Biotechnology

Comparative analyses between shade-avoider and shade-tolerant species: the role of phytochrome B.

Ángela Sánchez García

January 2022

Acknowledgments

I would like to express my gratitude to all the IBMCP staff for allowing me to complete this project. Specially, I would like to thank the members of the Light and shade mediated regulation of plant development group, who have helped me and taught me a lot during these months. Finally, my most sincere gratitude to Jaume Martínez García, my master thesis tutor, for his guidance, support and help throughout this project.

INDEX

Abstract

Light perception by plants allows them to gather information about the surrounding environment and acclimate accordingly their development. One example of this is the effect that light filtration and reflexion by proximity vegetation has on the quantity and quality of the incoming sunlight. Detecting these changes enables plants to notice nearby competitors and direct vegetation shade that might cause light starvation. Plants have evolved into adopting two different strategies to cope with vegetation shade and proximity: avoidance and tolerance. Studying the molecular mechanisms that support these different strategies has been possible due to comparative analyses between closely related species with different shade-induced responses: *Arabidopsis thaliana* (shade avoider) and *Cardamine hirsuta* (shade tolerant). In this work we aimed to deepen into the role of the main photoreceptor involved in perceiving vegetation proximity, phytochrome B (phyB), in shade tolerance. To do so, we followed several analyses that involved phenotypical characterization of lines with altered phyB activity in both species. Our results have reinforced the hypothesis that a reduced phyB signalling pathway activity in *C. hirsuta* is partially responsible for its shade-tolerant phenotype.

Keywords: SAS, shade tolerance, *Arabidopsis thaliana*, *Cardamine hirsuta*, phytochrome B.

1. Introduction

Throughout evolution plants have developed mechanisms to perceive environment signals and respond consequently by acclimating their growth, development and metabolism. Among these signals, light is of upmost importance for photosynthetic plants, as it can limit their growth and development. In plants, light perception is achieved by several photoreceptors, which are specific for certain wavelength ranges of the solar light (sunlight) spectrum: phytochromes [far-red (FR) and red light (R) detection]; phototropins, cryptochromes and F-box containing Flavin binding proteins [UV-A and blue light (B) detection], and UVB-RESISTANCE 8 (UVR8, detection of UV-B light) (Paik & Huq, 2019).

Light-shade perception and vegetation proximity signal

Both in natural and agricultural environments, light perceived by plants is altered by the proximity of other plants, especially in crowded vegetation environments. Plants detect vegetation proximity as changes in the R to FR ratio (R:FR) of the incoming light. The R:FR of sunlight is about 1.2-1.5. This ratio can be affected by the proximity of vegetation because of the selective absorption and reflection of light by photosynthetic pigments in plants. FR is reflected by plant tissues, which enriches sunlight and lowers the R:FR of incoming light perceived by nearby vegetation. Moreover, plants absorb light from the photosynthetic active radiation region (PAR, 400-700nm) while transmitting through FR. This selective depletion of the R causes a very low R:FR ratio in the sunlight filtered by leaves (Roig-Villanova & Martínez-García, 2016).

These two ways of lowering R:FR of incoming light caused by nearby vegetation generate two possible situations regarding plant shading: proximity and canopy shade. Proximity shade is caused by nearby vegetation which reflects FR and mildly reduces R:FR of the incoming light (low R:FR). Canopy shade, however, occurs in highly dense areas in which plants grow under plant canopies. In this second scenery not only FR is reflected by nearby vegetation and transmitted through, but also B and R is strongly absorbed by the plant canopy and R:FR decreases significantly more (very low R:FR).

All these informative signals are detected and integrated by the phytochrome photoreceptors, which in turn elicit molecular responses that help plants to acclimate to these situations. In relation to these proximity and canopy signals, two different plant adaptive strategies have been characterized so far, which divide plants in two groups:

shade avoider and shade tolerant (Martínez-García et al., 2014; Roig-Villanova et al., 2019; Wang et al., 2020).

Shade avoidance: molecular mechanisms

Responses to low R:FR signals in shade-avoider plants are referred to as shade-avoidance syndrome (SAS), which has been studied using *Arabidopsis thaliana* as a model. This strategy promotes responses to overcome neighbouring vegetation by "escaping" from shade. SAS includes a set of responses: promotion of plant elongation, an upward leaf movement (hyponasty) and apical dominance (reduced branching) to overgrow nearby vegetation and reach better light conditions, promotion of flowering to ensure reproduction and reduction in photosynthetic pigments to adjust the photosynthetic rate. This redistribution of the resources in shade has an impact on crop yield, favouring elongation in detriment of other desirable functions, such as defence or biomass generation (Ballaré & Pierik, 2017; Roig-Villanova et al., 2019). Among these changes, hypocotyl elongation in shade has been widely used to study SAS elongation responses at seedling stage.

At the molecular level, SAS responses are triggered by phytochrome-mediated perception of shade (Figure 1A). This is so because phytochromes exist in two photoconvertible forms: an inactive R-absorbing form (Pr) and an active FR-absorbing form (Pfr). And changes on the R:FR alter the Pfr to total phytochrome (P_T) ratio (Pfr/P_T). A high R:FR displaces the photoequilibrium towards the active Pfr form ($Pfr/P_T \geq 0.7$), which in turn represses SAS. On the contrary, the low R:FR associated to shade conditions displaces the photoequilibrium towards the inactive Pr form ($Pfr/P_T \leq 0.6$), which in turn induces SAS (Roig-Villanova & Martínez-García, 2016).

In *A. thaliana* phytochromes are encoded by a gene family of 5 members: *PHYA* to *PHYE.* PhyA is the only Type I phytochrome in *A. thaliana*, that is light labile and allows germination and de-etiolation at low Pfr/P_T ratios such as those found in monochromatic FR (or even under very low R:FR conditions). Type II phytochromes (phyB-phyE) are light stable but require a higher Pfr/P_T ratio to be active, repressing SAS in environments with a high R:FR (Legris et al., 2019). PhyB has been described as the main type II phytochrome involved in shade perception. This different action spectrum between phyA and phyB allows for a more complex regulation of shade responses. In high R:FR (direct sunlight) the light-labile phyA is rapidly degraded and the light-stable phyB gets activated, which represses SAS (and hypocotyl elongation). In proximity shade (low R:FR), phyB is inactivated while phyA is still degraded, and therefore SAS responses

occur. Finally, in canopy shade conditions (very low R:FR), although phyB remains inactivated, phyA is not degraded and accumulates to enough levels to inhibit SAS response. Therefore, the phyA-mediated repression in very low R:FR conditions prevents an excessive SAS response caused by phyB inactivation. The different action spectra of phyA and phyB allows the plant to distinguish between proximity and canopy shade (Figure 1B) (Martínez-García et al., 2014).

PHYTOCHROME INTERACTING FACTORs (PIFs) are key downstream elements of the SAS signalling cascade known to interact with active phytochromes. PIFs are basic-helix-loop-helix (bHLH) transcription factors that directly modulate gene expression by DNA binding. Under high R:FR, active phytochromes migrate to the nucleus and inhibit PIFs function. When exposed to shade (low R:FR), phytochrome inactivation removes the inhibition imposed in PIFs that are then able to induce the expression of auxin-synthesis genes and other growth-related genes which in turn elicit SAS responses (Casal, 2013; Wang et al., 2020). Apart from PIFs, other regulatory elements are known to participate in SAS signalling pathway, such as HY5 or HFR1 (Figure 1A).

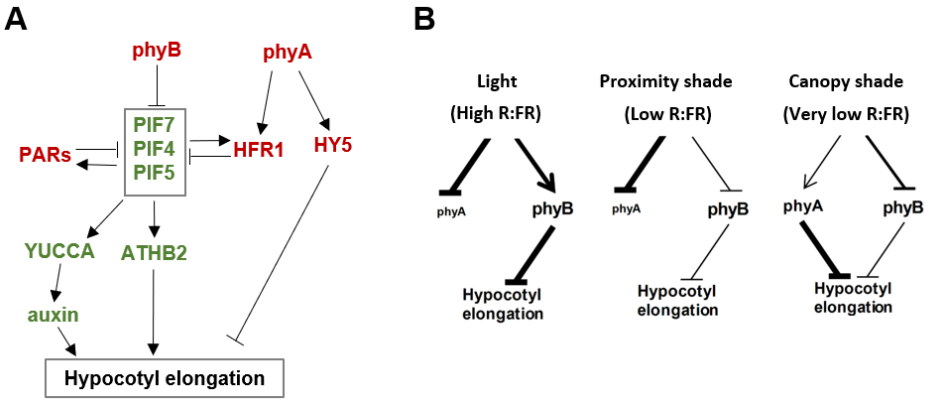

Figure 1. Schematic representation of the main molecular components involved in shade-induced hypocotyl elongation and their function. (A) Model depicting the signalling pathway involved in shade-induced hypocotyl elongation. The positive regulators of hypocotyl elongation are marked with green colour and the negative ones are marked with red colour. **(B)** Model depicting the different action spectra of phyA and phyB on the shade-induced hypocotyl elongation. Wider arrows represent a stronger activating/repressing effect. Bigger sizes of phyA/phyB labels represent a higher contribution to the hypocotyl elongation repression. Adapted from Martínez-García et al., 2014.

Shade tolerance: *Cardamine hirsuta* as a model

Shade tolerance is an adaptive strategy adopted by plants that complete their life cycle in shaded areas such as forest understories in which both R:FR and light quantity are reduced compared to open sunlight conditions. These species supress elongation in

shade and focus their resources on adapting to low light conditions and optimizing their carbon gain. This is achieved by optimization of the photosynthesis, higher leaf area, high physical defence levels (stress tolerance) and little shade-induced elongation and plasticity (Gommers et al., 2013; Paulišić et al., 2021; Valladares & Niinemets, 2008). *C. hirsuta* and *A. thaliana* are close relative species with different responses to shade: while *A. thaliana* is a shade-avoider plant, *C. hirsuta* is considered a shade-tolerant plant with no or little shade-induced hypocotyl elongation (Figure 2A) (Molina-Contreras et al., 2019).

The few comparative analyses of these two species have determined that they share some genetic elements for shade-related signalling. However, the different shade-induced response of the two species seems to be achieved by differences in the activity of these components: negative regulators would have a stronger activity in *C. hirsuta* whereas positive regulators activity would be reduced, resulting in a more effective suppression of SAS response. Indeed, *C. hirsuta* phyA seems to have a major role in shade tolerance: it has a stronger intrinsic activity than *A. thaliana* phyA, which allows for a stronger growth suppression in low and very low R:FR. *C. hirsuta* also shows a stronger HFR1-mediated repression, due to higher gene expression and protein stability, and an attenuated PIF7 activity (Figure 2B) (Molina-Contreras et al., 2019; Paulišić et al., 2021). Moreover, in Molina-Contreras et al. (2019) it has been pointed out that additional components, such as phyB, may have a role in shade tolerance in *C. hirsuta*.

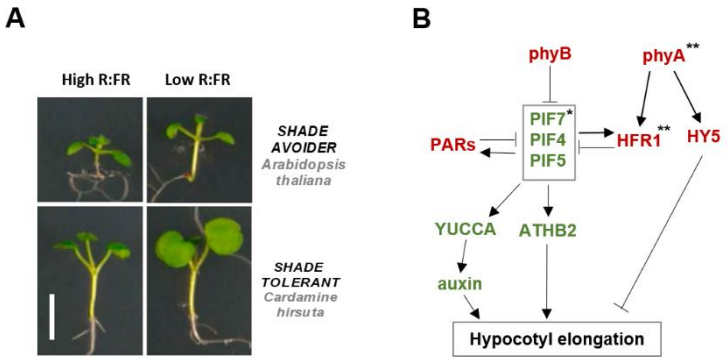

Figure 2. Schematic representation of shade-tolerance phenotype and molecular mechanisms involved. (A) Image showing the shade-induced hypocotyl elongation in shade-avoider and shade-tolerant plants. Adapted from Molina-Contreras et al., 2019. **(B)** Model depicting the signaling pathway involved in shade tolerance in *C. hirsuta*. The positive regulators of hypocotyl elongation are marked with green color and the negative ones with red color. Elements with enhanced activity in *C. hirsuta* compared with *A. thaliana* are marked with two asterisks, and elements with reduced activity are marked with one asterisk.

Mimicking vegetation shade in the laboratory

Studying responses to vegetation proximity in a laboratory requires growth conditions that mimic vegetation shade. These are called simulated shade conditions. Whereas sunlight is mimicked in growth chambers by tubes emitting white light (W), simulated shade is achieved supplementing W with FR provided by LED lamps. This supplementation lowers the R:FR ratio while maintaining the same amount of PAR, which allows us to study shade responses triggered by phytochromes rather than changes caused by a reduced photosynthesis (Roig-Villanova & Martínez-García, 2016; Wang et al., 2020). In this work, we employ two treatments that mimic either proximity or canopy shade. Proximity shade is simulated by growing plants in FR pulses conditions: 1-hour light cycles of 18 min of W+FR (R:FR = 0.05) + 42 min of W (R:FR = 3.55). These pulses have been proved to elicit physiological changes equivalent to those achieved with a constant R:FR = 0.5-0.3 that do not involve the action of phyA. Canopy shade is achieved by growing plants in W enriched continuously with FR (R:FR = 0.05).

2. Objectives

In order to unveil the molecular differences between shade-avoider and shade-tolerant plants, different elements known to be involved in shade-avoidance signalling pathways have been studied in shade-tolerant plants, using *C. hirsuta* as a model. Phytochrome B (phyB), being one of the main photoreceptors involved in shade perception, is hypothesized to have a role in shade tolerance. Hereby, the general objective of this project is to study the role of phyB in the regulation of shade tolerance in *C. hirsuta*. To do so, we aim to attain some specific objectives:

1) Phenotypically characterize *A. thaliana* lines with altered phyB activity grown in shade and *C. hirsuta* grown in different monochromatic lights. These observations will help us to establish a working hypothesis for phyB role in *C. hirsuta*'s shade tolerance.
2) Compare the amino acidic sequence of *A. thaliana* PHYB (AtPHYB) and *C. hirsuta* PHYB (ChPHYB) by sequence alignment. This bioinformatic approach might expose any divergent region(s) responsible for a different intrinsic activity of ChPHYB.
3) Generate *C. hirsuta* mutants deficient in *PHYB* by CRISPR-Cas gene editing system and mutant screening and characterize them phenotypically and molecularly. When available, mutant lines deficient in this photoreceptor will help us to understand better the phyB role in the shade-tolerant phenotype of *C. hirsuta* and test our working hypotheses.
4) Generate and characterize *C. hirsuta* lines overexpressing *AtPHYB* both molecularly and phenotypically. These lines offer the possibility to study the effect of an enhanced phyB activity in *C. hirsuta*'s shade response.

3. Materials and methods

Plant material and plant growth conditions

Plants of *Arabidopsis thaliana* accession Columbia-0 (Col-0, At^{WT}), *phyB-9*, *phyA-211*, *cry1-304 cry2-1* (*cry1cry2* from now on), 35S:AtPHYB-GFP (AtPBG) transgenic line (all in Col-0 background) (Lascève et al., 1999; Nagatani et al., 1993; Ortiz-Alcaide et al., 2019; Reed et al., 1993), as well as *Landsberg erecta* (Ler-0) accession, *phyB-1* and *phyB-4* (both phyB-deficient lines in Ler-0 background) (Reed et al., 1993) have been used in this work. Plants of *Cardamine hirsuta* accession Oxford (OX, Ch^{WT}) were also used in this project (Hay et al., 2014).

Seeds were surface-sterilized before being sown in Petri dishes containing solid growth medium without sucrose (0.5x GM-) [0.8% (w/v) agar, 0.22% (w/v) Murashige & Skoog (MS) salt mixture with vitamins, 0.025% (w/v) MES, pH 5.8]. After stratification (3-5 days at 4ºC in darkness), seeds were moved to growth chambers to induce germination and perform the experiments (see below).

Shade experiments were performed by growing seedlings in normal and two types of simulated shade conditions (proximity shade and canopy shade) at a constant temperature of 22ºC. Normal light condition refers to continuous W emitted by white horizontal LED tubes (PAR = 55 $\mu mol\ m^{-2}\ s^{-1}$, R:FR = 3.55). The two types of simulated shade conditions were generated by enriching W with FR provided by LED lamps (R:FR = 0.05): (1) proximity shade was mimicked by growing plants under 18 min of FR pulses in 1-hour cycles of W (W+FRp); and (2) canopy shade was mimicked by growing plants under W enriched continuously with FR (W+FRc).

De-etiolation experiments were performed by growing seedlings at 22ºC under different LED lamps producing B, R or FR (light intensities are indicated in each experiment). Fluence rates were measured with a Spectrosense2 meter associated with a four-channel sensor (Skye Instruments), which measures PAR (400-700 nm) and 10-nm windows in the B (464-473 nm), R (664-673 nm), and FR (725-734 nm) regions.

For gene expression analysis, seeds were sown on a sterilized nylon membrane placed on top of the solid 0.5x GM-. After the stratification, they were kept in W and samples were harvested on day 7.

Hypocotyl length measurements

Twenty-five *A. thaliana* seeds or 30 *C. hirsuta* seeds were sown per genotype in each plate. On the final day of the experiment, seedlings were laid on the agar plates and photographed. Hypocotyl measurements were done using The National Institutes of Health ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018) on digital images. These experiments were repeated at least 3 times and the significance was evaluated by statistical Student´s *t*-test.

Protein sequence comparison

The sequence of *A. thaliana* PHYB (AtPHYB) protein (AT2G18790) was obtained in The Arabidopsis Information Resource (TAIR), on www.arabidopsis.org, August 2021. Using this AtPHYB sequence, we used the BLAST tool in *Cardamine hirsuta* Genetic and genomic resource (http://chi.mpipz.mpg.de/) to obtain *C. hirsuta* PHYB (ChPHYB) protein sequence (CARHR092300.1). Finally, we performed a Pairwise Sequence Alignment of these two sequences with The European Bioinformatics Institute (EMBL-EBI) EMBOSS needle tool (Needleman et al., 1970).

Generation of mutant lines

We searched to obtain *C. hirsuta phyB* mutants by using the CRISPR-Cas9 gene editing system. To do so, a gene block containing the guide RNA targeting *ChPHYB* (5'-GTCGGGGACGAAATCGCTTA-3', $gRNA_{ChPHYB}$) under the *A. thaliana* U6 promoter ($pU6:gRNA_{ChPHYB}$) flanked by the Gateway attB1 and attB2 recombination sites (attB1< $pU6:gRNA_{ChPHYB}$<attB2) was synthesized (Integrated DNA Technologies, IDT). A BP recombination reaction using the Gateway BP Clonase II (Invitrogen) allowed us to introduce this block into the Gateway donor vector pDONR207 (Invitrogen). This led to the entry vector pAS5 ($pU6:gRNA_{ChPHYB}$ flanked by attL1 and attL2 recombination sites, attL1<$pU6:gRNA_{ChPHYB}$<attL2). A following recombination performed with the Gateway LR Clonase II combined pAS5 with the binary vector pDE-Cas9 (Invitrogen), creating pAS7, which contains a spectinomycin resistance gene (selection marker in bacteria) and a PPT resistance gene (selection marker in plants).

Agrobacterium tumefaciens strain GV3101 was transformed by electroporation with pAS7. Colonies were selected on solid LB medium with antibiotics [gentamicin (30 µg/mL), rifampicin (100 µg/mL) and spectinomycin (100 µg/mL)] and used for Ch^{WT}

transformation by floral dipping (Zhang et al., 2006). Once the seeds were harvested, transgenic plants were selected by growing them either in 0.5x GM- containing PPT (30 µg/mL) or by treating soil-grown seedlings with a 1:2000 dilution of BASTA® (0.757M glufosinate-ammonium). In the second selection method, seeds were first placed over wet soil and stratified for 3-5 days at 4ºC. Then they were moved to a long-day photoperiod growth chamber (16 h light and 8 h dark) to induce germination and sprayed with the Basta solution on days 3, 4, 5 and 6 after being moved to the growth chamber.

Selection of stable transgenic lines

Seeds of the T2 generation of *C. hirsuta* transgenic lines containing 35S:AtPHYB-GFP were available in the group. Selection of homozygous transgenic lines with 1 T-DNA insertion was performed by growing the T2 generation seeds in 0.5x GM- containing hygromycin (25 µg/mL). The resulting lines were used in the experiments described.

Gene expression analyses

Gene expression analyses were performed *on C. hirsuta* whole seedlings grown for 7 days in W, as indicated hereabove. Three biological replicates of about 30 seedlings each replica were analysed. Total RNA was extracted from seedling samples using a commercial kit (PureLink™ RNA Mini Kit, Invitrogen). Then, 1 µg of RNA was reverse-transcribed with NZY First-Strand cDNA Synthesis Kit (NZYTech) and real-time qPCR analysis was performed. Normalization of the results was carried out with *C. hirsuta ELONGATION FACTOR 1α (EF1α)* gene. Primers SPO102 and SPO103 (*AtEF1α* and *ChEF1α*) have been described before (Molina-Contreras et al., 2019).

Gene	Forward primer (5’ → 3’)	Reverse primer (5’ → 3’)
GFP	SPO2, TCATATGAAGCGGCACGACTT	SPO3, TAGTTCCCGTCGTCCTTGAAGA
EF1α	SPO102, ATGATTACTGGTACCTCCCAGGC	SPO103, CTCACGGGTCTGACCATCCT

Table 1 - Primers used in real-time qPCR analysis

4. Results and discussion

Characterization of *A. thaliana* lines with altered phyB activity

To deepen into the role of phyB in SAS regulation, we first performed a phenotypical characterization of several lines with altered phyB activities of *A. thaliana*, our reference species, grown in two simulated shade conditions. These lines include *phyB-1* (a null mutant) and *phyB-4* (an hypomorphic mutant with attenuated phyB activity), both in Ler-0 background, *phyB-9* (a null mutant in Col-0 background), and a transgenic line overexpressing the *A. thaliana PHYB* gene (*AtPHYB*) fused to the *GFP* (35S:AtPHYB-GFP) in Col-0 background (AtPBG). We have measured the hypocotyl elongation of one week-old seedlings of these lines grown under W (high R:FR), W+FRp (proximity shade) and W+FRc (canopy shade) conditions (Figure 3A).

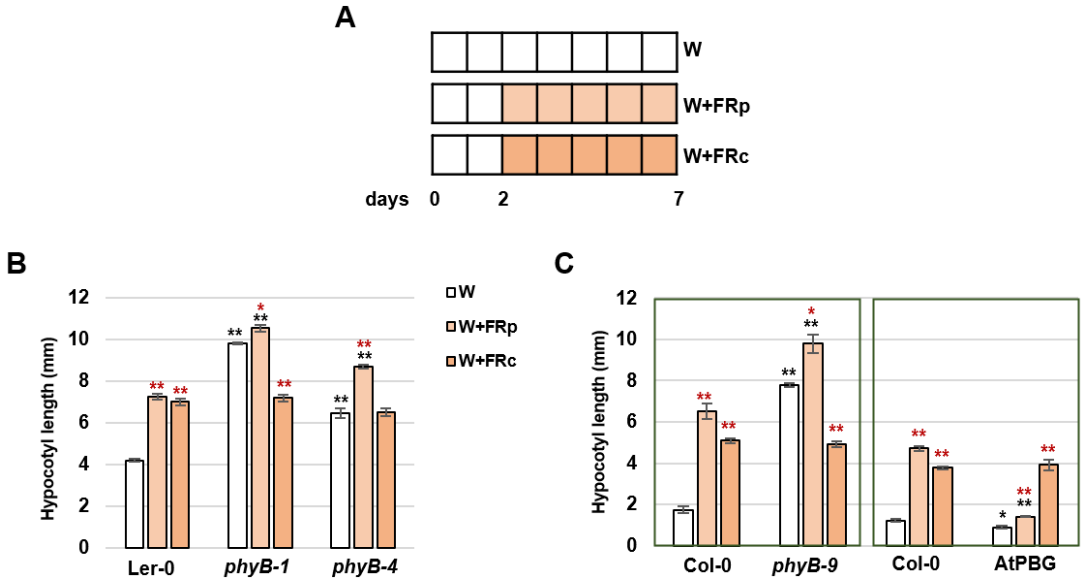

Figure 3. Variation in phyB activity affects hypocotyl elongation in response to simulated shade in *A. thaliana*. (A) Cartoon representing the design of the experiment to study the response of *A. thaliana* hypocotyls to simulated shade. After stratification, seedlings were grown in W for 2 days and then kept either under W or transferred to two types of simulated shade: W+FRp and W+FRc. On day 7 pictures were taken and hypocotyl length was measured. **(B)** Hypocotyl length of wild-type Landsberg erecta (Ler-0), *phyB-1* and *phyB-4* seedlings grown as indicated in **A**. **(C)** Hypocotyl length of wild-type Columbia (Col-0), *phyB-9* and a line overexpressing *AtPHYB-GFP* (AtPBG) seedlings grown as indicated in **A**. Two wild-type seed stocks were used to have a contemporary pool of seeds for each mutant or transgenic line. Values are the mean and standard error (SE) of biological triplicates (n≈25 seedlings per replica). In **B** and **C**, black asterisks represent significant differences between the mutant or transgenic lines and the corresponding wild type grown in the same light conditions; red asterisks represent significant differences between W+FRp or W+FRc and W conditions within the same genotype. Student t-test: * $p<0.05$ and ** $p<0.01$.

Wild-type Ler-0 hypocotyls in W+FRp and W+FRc were significantly longer than in W, with no significant differences between the two simulated shade conditions tested. W-grown *phyB-1* seedlings were longer than the wild-type (Ler-0) ones, and further elongated their hypocotyls under W+FRp, whereas under W+FRc they elongated less, reaching a similar length than Ler-0 seedlings. *phyB-4* seedlings presented a similar elongation response, although they did not elongate as much in W and W+FRp as *phyB-1* did (Figure 3B). Wild-type Col-0 seedlings elongated in simulated shade but, in contrast with Ler-0, hypocotyls were longer in W+FRp than in W+FRc. Seedlings of the *phyB-9* null mutant showed a similar pattern of elongation as *phyB-1* when compared to its Col-0 (Figure 3C). As reported, loss of phyB activity results in longer hypocotyls in W due to the lack of growth inhibition by this photoreceptor in these conditions. In proximity shade (W+FRp), phyB inactivation promotes hypocotyl elongation of wild-type seedlings; in *phyB-1* and *phyB-9* seedlings, W+FRp further inactivates other photostable phytochromes (e.g., phyD, phyE) which produces a more drastic elongation phenotype. However, in canopy shade (W+FRc), phyA can accumulate to enough levels to repress the elongation promoted by phyB/D/E inactivation, which attenuates the elongation of wild-type Col-0, *phyB-1* and *phyB-9* seedlings. These responses are also seen in *phyB-4*, although more attenuated due to the partial phyB activity in this line. As canopy shade (W+FRc) did not affect Ler-0 as much as it did Col-0 elongation, these differences likely reflect a relatively higher phyA activity in Col-0 than in Ler-0 responsible for a stronger growth repression in W+FRc.

The transgenic AtPBG seedlings had slightly and significantly shorter hypocotyls than its Col-0 in W and W+FRp. *PHYB* overexpression results in a stronger growth repression in W that is maintained in W+FRp despite phyB inactivation by FR, likely because the W intervals in between the shade pulses (W+FRp) allow the activation of the overexpressed phyB which, in turn, inhibits growth. By contrast, AtPBG hypocotyl elongation in W+FRc was not significantly different from that of the Col-0 in the same conditions. This indicates that in canopy shade (W+FRc) the continuous low R:FR inactivates both the endogenous and the overexpressed phyB, and hypocotyl growth inhibition is phyA-mediated, as in Col-0.

Taken together, these results led us to conclude that *A. thaliana* lines with disrupted phyB activity show longer hypocotyls in W than those of the corresponding wild type grown in the same conditions. These lines further elongate their hypocotyls in W+FRp, although this elongation is more subtle than the one observed in the wild-type line seedlings. Finally, W+FRc grown seedlings show shorter hypocotyls that are similar in length to the corresponding wild type. On the contrary, overexpression of *AtPHYB* in *A.*

thaliana causes shorter hypocotyls in W and W+FRp-grown seedlings, while in W+FRc hypocotyl length is similar to that of the wild type in the same light conditions. This evidences the role of phyB in growth repression in W and the role of phyA in growth repression in W+FRc. It also reveals that enhancing phyB activity in *A. thaliana* produces a clear effect in hypocotyl elongation of seedlings grown in W and W+FRp, but not in W+FRc.

C. hirsuta is hyposensitive to red light

We aimed to study if photoreceptor activities of *C. hirsuta* were substantially different to those of *A. thaliana*, our reference species. To do so, we compared the de-etiolation response to monochromatic lights of *C. hirsuta* and *A. thaliana* seedlings. Whereas de-etiolation under Bc is mostly regulated by the B-receptors cryptochrome 1 (cry1) and cry2, phyB is the major photoreceptor mediating de-etiolation in response to Rc and phyA is the only photoreceptor mediating de-etiolation under FRc. Therefore, these experiments will inform us about differences in the activities of these three light-perception pathways between both species. To do so, we used data already available in the group (partly published in Molina-Contreras et al., 2019) in which wild-type *A. thaliana* Col-0 (At^{WT}) and *C. hirsuta* OX (Ch^{WT}) were grown under different intensities of monochromatic Bc, Rc or FRc and their hypocotyls were measured (Figure 4). As At^{WT} and Ch^{WT} are two species with different sizes, it is not straightforward to compare their hypocotyl length using absolute values of elongation. Therefore, the graphs represent the hypocotyl elongation of these genotypes in the given light conditions relative to their growth in darkness (maximum possible hypocotyl elongation) that was taken as 100.

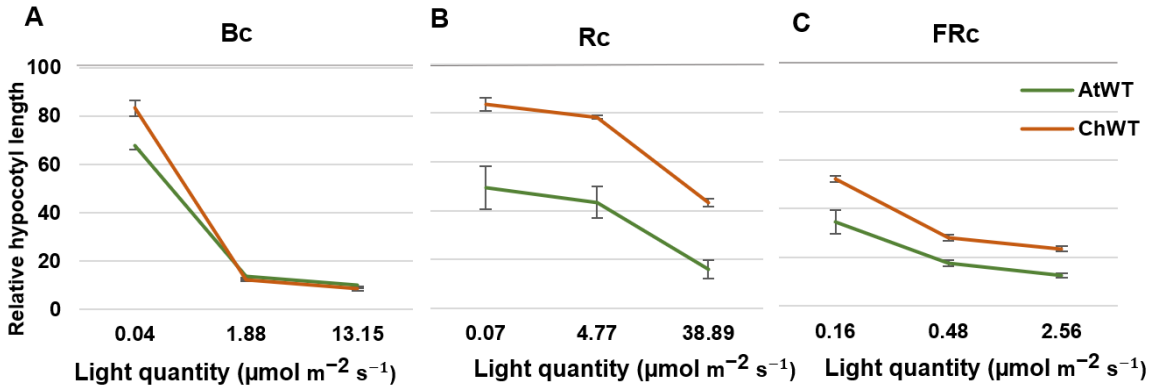

Figure 4. *Cardamine hirsuta* is hyposensitive to Rc. Hypocotyl length of At^{WT} and Ch^{WT} grown under Rc, FRc and Bc. Seedlings were grown for 4 days in darkness or under monochromatic Bc **(A)**, Rc **(B)** or FRc **(C)** (as detailed in Figure 7A). In each experiment seedlings were grown in the dark and under the 3 different light intensities indicated. On day 4, pictures were taken and hypocotyl length was measured. Mean and standard error (SE) of biological triplicates (n≈25 seedlings per replica) was calculated. Shown values in each light condition are relative to the hypocotyl length reached in darkness (maximum growth) that was taken as 100.

Even though Ch^{WT} hypocotyls grew slightly more in some of the three light conditions when compared to At^{WT} (15 % more in 0.04 *µmol* m^{-2} s^{-1} of Bc and 18 % more in 0.16 *µmol* m^{-2} s^{-1} of FRc), their different response to R (reduced in Ch^{WT}) is remarkable. Whereas At^{WT} reached 49 % of its length in darkness when exposed to 0.07 *µmol* m^{-2} s^{-1} of Rc, Ch^{WT} grew 84 % in the same conditions, which means that *C. hirsuta* elongated 35 % more than At^{WT} in the same light conditions (Figure 4B).

Given that light perception by photoreceptors inhibits plant growth, these results indicate that Ch^{WT} is similarly sensitive to B and FR but hyposensitive to R when compared to At^{WT}. PhyB, the main R receptor, is activated by R in an intensity-dependent manner and represses growth in turn. Thus, our results indicate that phyB activity in Ch^{WT} is attenuated compared to *A. thaliana*. We hypothesize that the R hyposensitivity of Ch^{WT} can contribute to partially explain the lack of shade-induced hypocotyl elongation in this species. Ch^{WT} being hyposensitive to R leads to less growth inhibition by phyB pathway in W (high R:FR ratio). Therefore, when Ch^{WT} is exposed to simulated shade, the low R:FR treatment makes a small impact on ChphyB activity and the phenotypical differences between *C. hirsuta* grown under high (W) or low (shade) R:FR conditions are smaller than the ones in *A. thaliana*. Consistently with this hypothesis, we showed in here that a reduction in phyB activity in the shade-avoider *A. thaliana* leads to an attenuated shade-induced hypocotyl elongation (phenotype of *phyB-4* line, Figure 3B), further supporting our working hypothesis that *C. hirsuta* has an attenuated phyB activity. We reasoned that the phyB hyposensitivity could be caused by various non-exclusive reasons: a) less amount of photoreceptor in Ch^{WT}, b) less intrinsic activity of the endogenous *C. hirsuta* phyB (ChphyB) or c) reduction in some elements downstream of phyB action in Ch^{WT} compared to At^{WT}. Currently, we have no evidence supporting any of these possibilities.

AtPHYB and ChPHYB protein sequence comparison

In order to unveil the molecular differences between ChPHYB and AtPHYB proteins, we compared their amino acidic sequence. We performed a Pairwise Sequence Alignment with EMBOSS Needle tool [European Bioinformatics Institute (EMBL-EBI)], which is presented in Figure 5. This figure also illustrates defined AtPHYB domains and their

distribution in the primary structure. Amino acidic substitutions (highlighted in yellow) are evenly present throughout the protein.

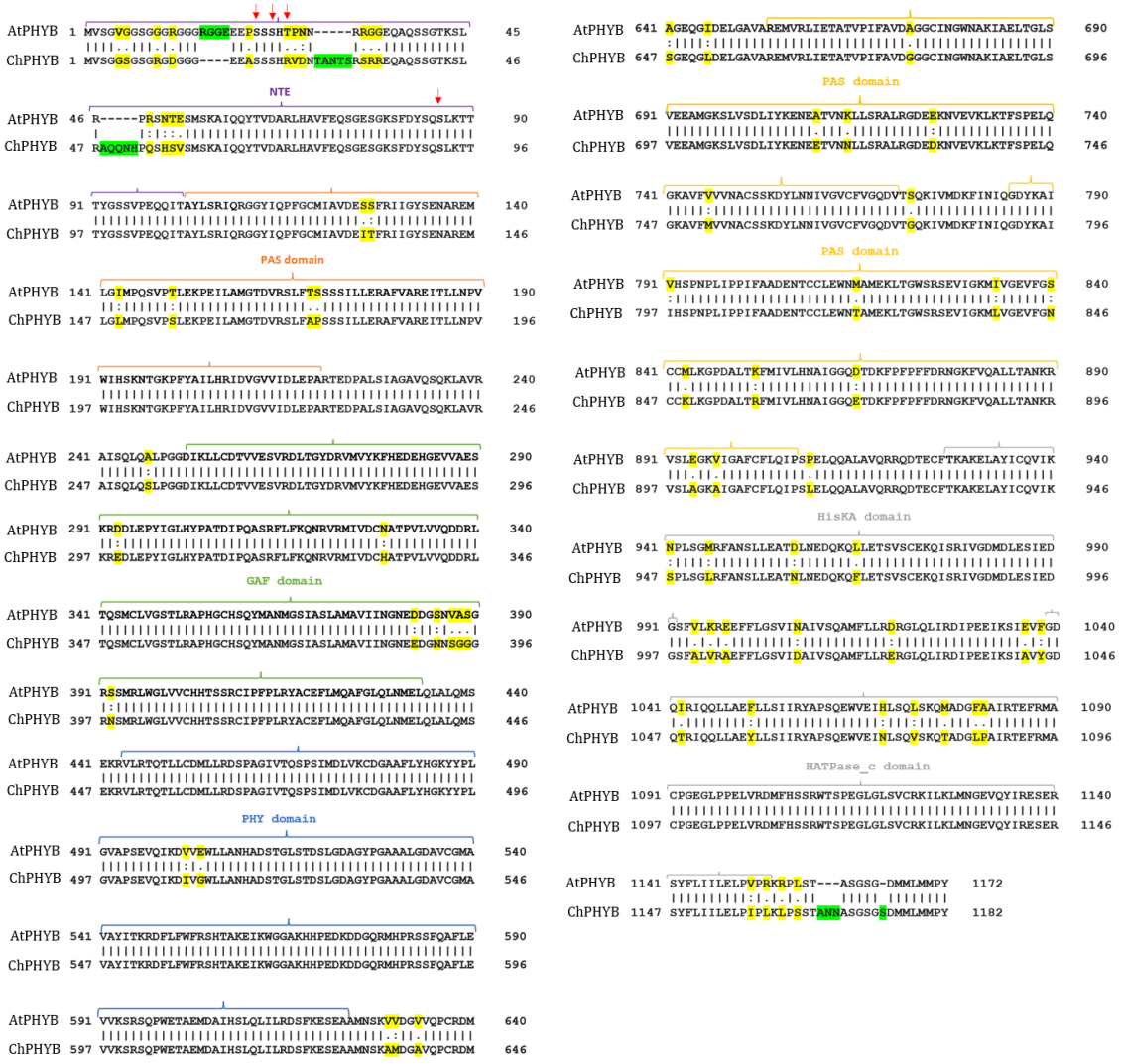

Figure 5. Alignment of AtPHYB and ChPHYB protein sequences does not point to any specific residue responsible for different intrinsic activity. Global alignment of AtPHYB and ChPHYB proteins. Distribution of identified domains of AtPHYB are marked above its sequence. NTE, N-terminal extension domain. HisKA domain, Histidine kinase (phospho-acceptor) domain. HATPase_c, Histidine kinase-like ATPase domain. Amino acidic substitutions are highlighted in yellow, and gaps are highlighted in green. Light and temperature regulated phosphorylation sites in AtPHYB NTE are marked with red arrows.

There is a relatively high gap and substitution density in the N-terminal region of both proteins. In *A. thaliana*, the N-terminal extension domain of PHYB (NTE) has a major role in phyB light-dependent stability and accumulation and it is involved in the control of thermal relaxation. Hence, differences in this region between both species could cause different phyB intrinsic activities. In this respect, it is known that NTE phosphorylation regulates these functions (Legris et al., 2019). The dynamic phosphorylation of AtPHYB NTE has been studied in Viczián et al. (2020), where several phosphorylation sites have been found to be light and temperature regulated: S23/S25/T27 and S86. Although S86

is conserved in both species, ChPHYB lacks T27 [T27R]. This difference could have an impact on light and temperature mediated regulation of phyB activity in *C. hirsuta*. However, given the high number of mismatches between these two proteins we can not determine a clear region or amino acid responsible for any difference in phyB intrinsic activity.

To sum up, we have performed an alignment with AtPHYB and ChPHYB sequences to analyse any divergent region between them that could account for the observed differences in phyB activity between these two proteins. Although some differences in phosphorylated residues of the NTE region between AtPHYB and ChPHYB could affect ChPHYB protein stability, the high number of mismatches evenly present throughout both proteins prevent us from reaching any clear conclusion.

Creating *C. hirsuta* mutant lines deficient in phyB

To further study phyB role in shade tolerance we aimed to obtain *phyB* mutants in *C. hirsuta*. We used CRISPR-Cas9 technology to generate mutations in the endogenous *ChPHYB* that lead to phyB-deficient lines. After designing the guide RNA (gRNA), we cloned it in an appropriate vector that contained all the needed components for plant transformation by *Agrobacterium tumefaciens* and CRISPR-Cas9-based genome editing. We named this vector as pAS7 (Figure 6).

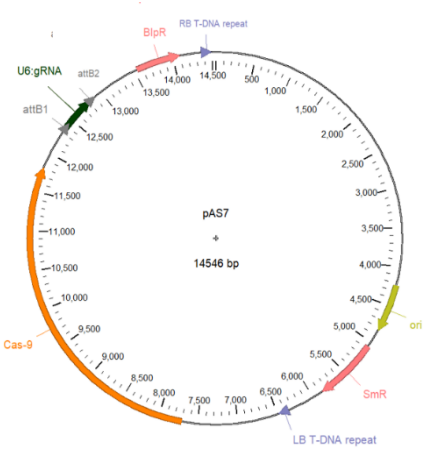

Figure 6. Design of the plasmid used to generate phyB-deficient mutants in *C. hirsuta* by CRISPR-Cas9 technology. Cartoon showing pAS7 plasmid and the distribution of some of its elements: ori (high-copy-number origin of replication), SmR (selection marker in bacteria, confers spectinomycin and streptomycin resistance), LB and RB T-DNA repeats (required for T-DNA insertion in plant genome by *A. tumefaciens*), Cas9 enzyme, attB1 and attB2 (recombination sites for Gateway® reactions), U6:gRNA (backbone containing U6 promoter and the gRNA designed to bind a *ChPHYB* region) and BIpR (selection marker in plants, confers PPT resistance).

After having transformed Ch^{WT} plants by floral dipping we obtained seeds (T1 generation) and proceeded to select them by phosphinothricin (PPT) treatment. PPT is an herbicide that inhibits glutamine synthase (GS), a key enzyme of the nitrogen metabolism involved in the incorporation of ammonium into the amino acid glutamine. Inhibition of this enzyme causes a build-up of phytotoxic ammonia in plants which disrupts cell membranes. The

first selection approach consisted of growing sterilized T1 seeds in medium containing PPT. Nevertheless, the constant appearance of fungi contamination in the plates impeded the selection. As a result, we followed a different protocol and performed the selection in non-sterile conditions by germinating and growing T1 seeds directly in soil. Seedlings were then sprayed with a dilution of Basta, a commercial herbicide containing PPT. This method allowed us to obtain 11 putative transgenic plants that showed Basta resistance. These candidate plants will be characterized in the future by PCR-based genotyping and *phyB* mutants will be confirmed by further sequencing of *ChPHYB*.

Screening of *C. hirsuta* mutants with a "slender in white light" phenotype

We also performed a screening of previously selected (but not characterized) *C. hirsuta* mutant lines (already available in the group) searching for putative *phyB* mutants. These mutant lines were selected from an Ethyl Methane Sulfonate- (EMS-) mutagenized population of *C. hirsuta* that was challenged with simulated shade (W+FRc) and individuals with long hypocotyls were selected (Molina-Contreras et al., 2019). We next screened the seedlings of the available mutant lines in W conditions and selected the ones with a long hypocotyl phenotype, given that this response could be due to lack of phyB activity. Among the 15 lines tested, 4 of them were obviously longer than Ch^{WT}: m70.1, m126.2, m133.1 and m20.20. They were named as "*slender in white light*" (*siw*) and will be referred as *siw* from now on. Unfortunately, we could not continue studying m20.20 line due to viability problems.

A more detailed phenotypical characterization of m70.1, m126.2 and m133.1 seedlings was carried out by analysing the de-etiolation of these lines under different monochromatic lights (Bc, Rc and FRc) and measuring their hypocotyl length afterwards (as done before in Fig. 4). As controls, *A. thaliana* mutant lines *cry1cry2* [deficient in both cry1 and cry2 that was expected to have a longer hypocotyl than At^{WT} (Col-0) when grown in Bc], *phyA-211* (expected to be blind to FRc compared to At^{WT}) and *phyB-9* (expected to have a longer hypocotyl than At^{WT} when grown in Rc) were also grown in

the same conditions. These genotypes were useful to corroborate that those light conditions were suitable for identifying light-sensing defective mutants.

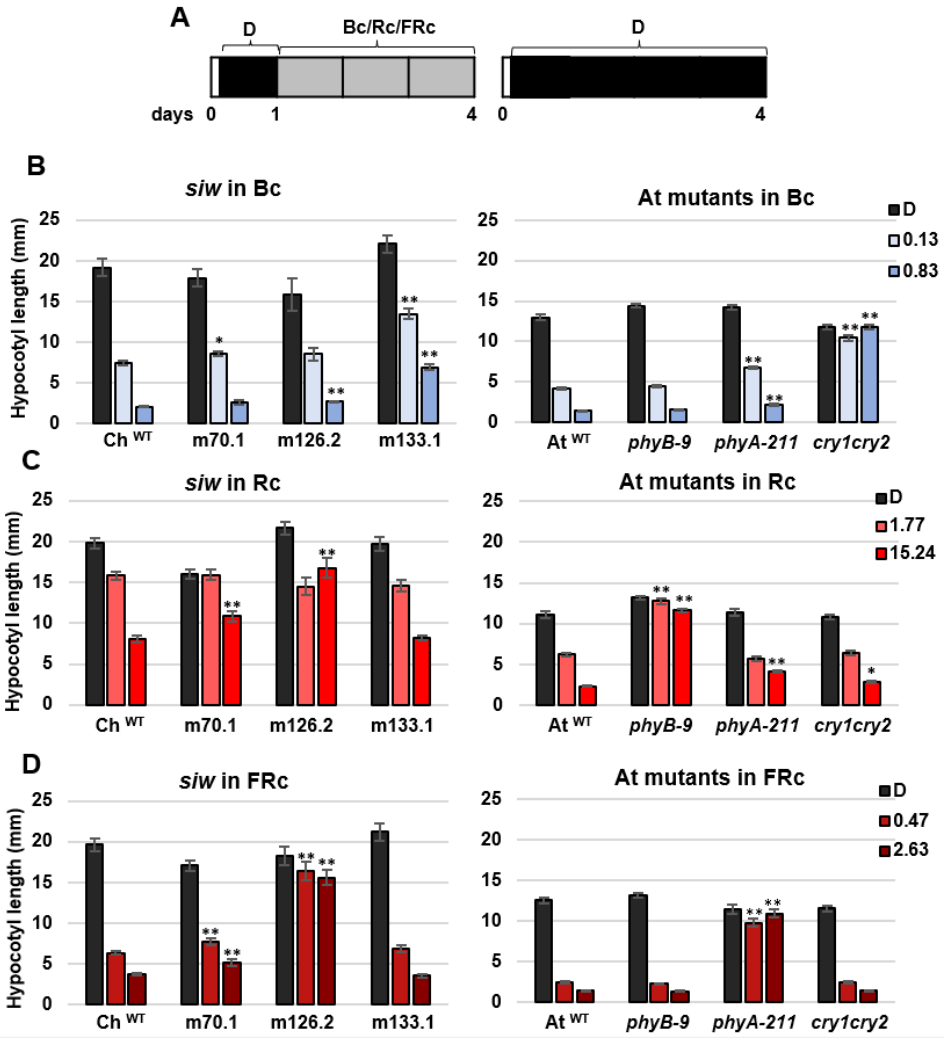

Figure 7. Phenotypic analyses of *siw* lines under different monochromatic lights indicates disruption of light-sensing pathways. Hypocotyl length of Ch^{WT} and *siw* lines m70.1, m126.2 and m133.1, and At^{WT} and *phyB-9*, *phyA-211* and *cry1cry2* mutants in darkness (D) and two different intensities ($\mu mol\ m^{-2}\ s^{-1}$) of the indicated monochromatic lights. **(A)** Cartoon representing the design of the experiments. After stratification, seeds were exposed to W for 2 hours to induce germination. Afterwards they were either grown in darkness (D) for 4 days or kept in D for 22 hours and then transferred to the corresponding monochromatic light for 3 additional days. On day 4 pictures were taken and hypocotyl length was measured. Hypocotyl length of plants grown in D or two different intensities of Bc **(B),** Rc **(C)** or FRc **(D)**, as indicated in **A**. Values are the mean and SE of n≈20 seedlings. In **B**, **C** and **D** asterisks represent significant differences between mutant lines and the corresponding WT grown in the same light conditions. Student t-test: * p<0.05 and ** p<0.01.

As it is shown in Figure 7, m133.1 line had a response that resembled that of *cry1cry2*: it was hyposensitive to Bc (it was longer than Ch^{WT}) whereas it was similar to Ch^{WT} both in Rc and FRc. Therefore, we hypothesized that m133.1 is affected in the cryptochrome pathway, given that cryptochromes are B photoreceptors. Line m126.2 appeared to be wild type to Bc but hyposensitive to Rc and FRc; this phenotype could be explained by a mutation in a phytochrome-related element, as both phyA and phyB-mediated growth

repression are impaired (e.g., a component involved in the biosynthesis of the phytochrome chromophore). Lastly, m70.1 was also significantly different to Ch^{WT} in Rc and FRc. However, it seemed to be a greater length difference between them in Rc. This lack of R sensitivity could be related to phyB deficiency. Nevertheless, to better characterize this line it would be useful to measure m70.1 hypocotyl length in simulated shade conditions and check if it behaves like a putative *phyB* mutant (based on what we have observed for the *phyB* mutants of *A. thaliana*, Figure 3). If these results point out to phyB deficiency, the next step would be to sequence *ChPHYB* gene in m70.1 line in search of sequence alterations.

In essence, we have characterized 3 selected mutants with long hypocotyls in W. We performed de-etiolation experiments in 3 monochromatic lights to determine putative light-sensing elements affected in those lines: m133.1 is believed to have an impaired B sensitivity because of a disruption in an element involved in cryptochrome-related pathways; m126.2, however, is clearly hyposensitive to both R and FR, which indicates that phytochrome biosynthesis or signalling is affected; lastly, m70.1 is longer than Ch^{WT} both in R and FR. Despite not showing the same phenotype as *phyB-9*, the differences between the dark-grown phenotype of m70.1 and its clear hyposensivity to R make it a good candidate for being affected in the phyB signalling pathway. Further characterization of this line is necessary to determine which light-sensing related element is disrupted.

Characterization of *C. hirsuta* lines overexpressing *AtPHYB* (ChPBG)

As the shade-tolerant *C. hirsuta* seems to be hyposensitive to R (compared to the shade-avoider *A. thaliana*) because of its reduced phyB activity, we hypothesized that an increase in phyB activity might contribute to recover the shade-avoidance phenotype of this species. To test this possibility, we next performed a characterization of several independent *C. hirsuta* transgenic lines overexpressing *AtPHYB-GFP* (the same transgene as in AtPBG line, shown in Figure 3C) that we named ChPBG. Several ChPBG T2 generation seeds were available in the group. We first identified those lines with 1 T-DNA insertion by growing T2 generation seedlings in medium containing hygromycin and selecting the lines that had a resistant:sensitive seedling ratio of 3:1. Next, we transferred hygromycin resistant to pots to produce the T3 generation seeds. Homozygous plants in the T3 generation were identified by growing them in medium containing hygromycin and selecting the lines that had no sensitive seedlings (i.e., the

resistant trait was not segregating). The final seeds used in the following experiments were homozygous T3 seeds of #02, #03, #05, #07 and #08 lines.

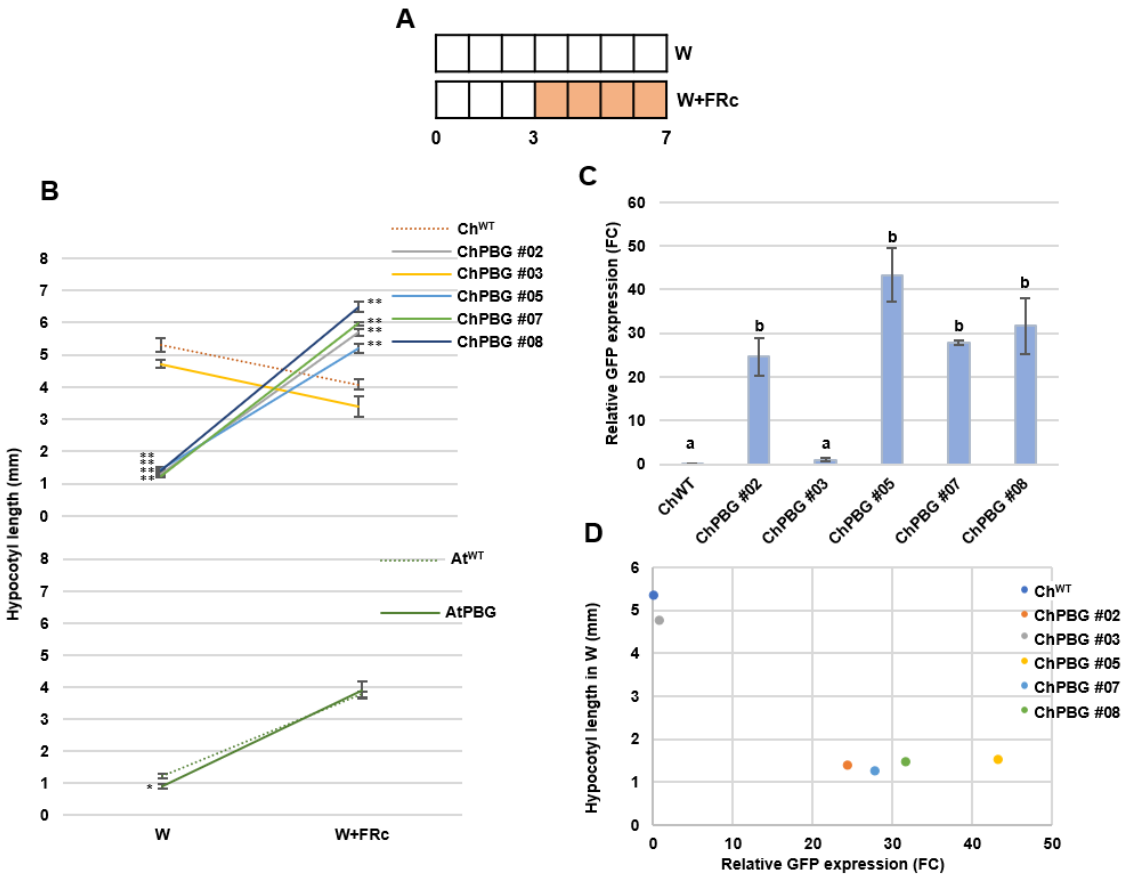

Figure 8. *C. hirsuta* lines overexpressing *AtPHYB* display an At^{WT}-like phenotype in simulated shade. (A) Cartoon representing the design of the experiment to study the response of *C. hirsuta* hypocotyls to simulated shade. *C. hirsuta* seedlings were grown in W for 3 days and then they were kept either under W or transferred to canopy shade (W+FRc). On day 7 pictures were taken and hypocotyl length was measured. **(B)** Hypocotyl length of Ch^{WT} and lines expressing 35S:AtPHYB-GFP (ChPBG): #02, #03, #05, #07 and #08; grown as indicated in **A**. Values are the mean and SE of biological triplicates (n≈30 seedlings per replica). Hypocotyl length of At^{WT} (Col-0) and an *A. thaliana* line expressing 35S:AtPHYB-GFP (AtPBG) in W and W+FRc. Data correspond to data shown in **Figure 3C**. Asterisks represent significant differences between AtPBG/ChPBG lines and the corresponding wild type grown in the same light conditions. Student t-test: * p<0.05 and ** p<0.01. **(C)** Relative *GFP* expression value in Ch^{WT} and ChPBG lines. Expression was analyzed in 7-day old W-grown seedlings. Transcript abundance was normalized to *EF1α* levels. Values are the mean and SE of biological triplicates relative to ChPBG 035 value. Uneven letters represent significant differences between genotypes (ANOVA and Tuckey p<0.05). **(D)** Hypocotyl length in W vs relative GFP expression of Ch^{WT} and ChPBG lines. Values correspond to those represented in **B** and **C**.

We first performed a phenotypical characterization of their shade-induced hypocotyl elongation by measuring their hypocotyl length in W and W+FRc conditions (Figure 8B). Whereas hypocotyls of #03 line elongated as those of Ch^{WT} (with no significant differences in any of the conditions tested), hypocotyls of #02, #05, #07 and #08 lines were much and significantly shorter in W and significantly longer in W+FRc than Ch^{WT}.

To complement this phenotypical characterization, we checked the transgene expression levels in all five ChPBG lines by quantifying *GFP* transcript in a real time q-PCR (RT-qPCR) analysis (Figure 8C). The correlation between transgene expression and phenotypical response (indicated as hypocotyl elongation in W) suggested that the Ch^{WT}-like hypocotyl length of ChPBG #03 in W (and W+FRc) is caused by the low expression levels of the transgene. The rest of the lines (ChPBG #02, #05, #07 and #08) showed higher expression levels and a greater growth repression in W (Figure 8D). Hence, overexpressing *AtPHYB* in *C. hirsuta* causes a greater growth repression in W compared to Ch^{WT}; a much weaker but similar effect was observed in the AtPBG line under the same W conditions (Figure 8B). This is consistent with the well-known effect of high R:FR (e.g., W) on active phyB formation, that represses elongation (Legris et al., 2019).

Overexpression of *AtPHYB* in *A. thaliana* (AtPBG line) and *C. hirsuta* (ChPBG lines with higher transgene expression levels) also display a similar phenotype when exposed to simulated shade (Figure 8B). However, overexpression of *AtPHYB* has a different effect in *A. thaliana* than in *C. hirsuta*. Whereas AtPBG and At^{WT} lines showed no differences in W+FRc, ChPBG lines exhibit a greater elongation in W+FRc than Ch^{WT}. The different effect of the same transgene on the shade-induced elongation in the two species suggests that *AtPHYB* overexpression has a negative impact on the strong phyA-mediated hypocotyl growth inhibition in simulated shade that operates in *C. hirsuta* (Molina-Contreras et al., 2019). The antagonistic interaction between phyB and phyA has been reported before in *A. thaliana* lines overexpressing *PHYB* only when sucrose is present in the media (Short, 1999). Although currently we do not understand these differences, it would be interesting to continue researching about this phenomenon in *C. hirsuta*, the reason behind the crosstalk between these two pathways and the dependency on sucrose.

Summarizing, the phenotypic characterization of ChPBG lines and its correlation with GFP expression levels has been useful to understand the impact of increasing phyB activity (by overexpressing *AtPHYB*) in *C. hirsuta*. It results in a greater growth repression in W that allows for a recovery of the shade-induced elongation phenotype, as hypothesized before. The fact that a stronger phyB activity in *C. hirsuta* causes this response supports our early hypothesis of an attenuated ChphyB activity partially responsible for its shade tolerance. Moreover, the longer hypocotyls of ChPBG in W+FRc when compared to Ch^{WT} suggest an antagonistic interaction between phyA and phyB pathways in which phyB negatively impacts phyA-mediated growth repression in that light condition.

5. Conclusions

The main ideas that can be gathered from this work are summarized below:

I. The hyposensivity of *C. hirsuta* hypocotyl to R suggests an attenuated ChphyB signalling-pathway activity that could contribute to its lack of shade-induced elongation. This is supported by the phenotypes observed in *A. thaliana* lines with attenuated phyB activities, which show an attenuated growth repression in W (high R:FR) and a reduction of the shade-induced hypocotyl elongation. Although some residues in the N-terminal region potentially can contribute to these differences, comparison between AtPHYB and ChPHYB sequence does not point out to any specific amino acid responsible for a different intrinsic activity of ChPHYB.

II. Generation of *phyB* mutants in *C. hirsuta* would be useful to further study phyB role in shade tolerance. Both CRISPR-Cas9 gene editing technology and mutant screening are useful tools to generate those mutants in the future.

III. *C. hirsuta* lines overexpressing *AtPHYB* (with stronger phyB activity) showed a recovered shade-induced elongation, which supports our working hypothesis of an attenuated phyB signalling-pathway activity in *C. hirsuta*. The phenotype of these lines in simulated shade also shows a negative effect of *AtPHYB* overexpression in phyA-mediated growth repression.

6. References

Ballaré, C. L., & Pierik, R. (2017). The shade-avoidance syndrome: Multiple signals and ecological consequences. *Plant Cell and Environment*, *40*(11), 2530–2543. https://doi.org/10.1111/pce.12914

Casal, J. J. (2013). Photoreceptor signaling networks in plant responses to shade. *Annual Review of Plant Biology*, *64*, 403–427. https://doi.org/10.1146/annurev-arplant-050312-120221

Gommers, C. M. M., Visser, E. J. W., Onge, K. R. S., Voesenek, L. A. C. J., & Pierik, R. (2013). Shade tolerance: When growing tall is not an option. *Trends in Plant Science*, *18*(2), 65–71. https://doi.org/10.1016/j.tplants.2012.09.008

Hay, A. S., Pieper, B., Cooke, E., Mandáková, T., Cartolano, M., Tattersall, A. D., Ioio, R. D., McGowan, S. J., Barkoulas, M., Galinha, C., Rast, M. I., Hofhuis, H., Then, C., Plieske, J., Ganal, M., Mott, R., Martinez-Garcia, J. F., Carine, M. A., Scotland, R. W., … Tsiantis, M. (2014). Cardamine hirsuta: A versatile genetic system for comparative studies. *Plant Journal*, *78*(1), 1–15. https://doi.org/10.1111/tpj.12447

Lascève, G., Leymarie, J., Olney, M. A., Liscum, E., Christie, J. M., Vavasseur, A., & Briggs, W. R. (1999). Arabidopsis contains at least four independent blue-light-activated signal transduction pathways. *Plant Physiology*, *120*(2), 605–614. https://doi.org/10.1104/pp.120.2.605

Legris, M., Ince, Y. Ç., & Fankhauser, C. (2019). Molecular mechanisms underlying phytochrome-controlled morphogenesis in plants. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-019-13045-0

Martínez-García, J. F., Gallemí, M., Molina-Contreras, M. J., Llorente, B., Bevilaqua, M. R. R., & Quail, P. H. (2014). The shade avoidance syndrome in Arabidopsis: The antagonistic role of phytochrome A and B differentiates vegetation proximity and canopy shade. *PLoS ONE*, *9*(10). https://doi.org/10.1371/journal.pone.0109275

Molina-Contreras, M. J., Paulišić, S., Then, C., Moreno-Romero, J., Pastor-Andreu, P., Morelli, L., Roig-Villanova, I., Jenkins, H., Hallab, A., Gan, X., Gomez-Cadenas, A., Tsiantis, M., Rodríguez-Concepción, M., & Martínez-García, J. F. (2019). Photoreceptor Activity Contributes to Contrasting Responses to Shade in Cardamine and Arabidopsis Seedlings. In *The Plant cell* (Vol. 31, Issue 11). https://doi.org/10.1105/tpc.19.00275

Nagatani, A., Reed, J. W., & Chory, J. (1993). Isolation and initial characterization of Arabidopsis mutants that are deficient in phytochrome A. *Plant Physiology*, *102*(1), 269–277. https://doi.org/10.1104/pp.102.1.269

Needleman, S.B., Wunsch, C.D., (1970). A general method applicable to the search for similarities in the amino acid sequence of two proteins, *Journal of Molecular Biology,* 48(3), 443-453.https://doi.org/10.1016/0022-2836(70)90057-4.

Ortiz-Alcaide, M., Llamas, E., Gomez-Cadenas, A., Nagatani, A., Martínez-García, J. F., & Rodríguez-Concepción, M. (2019). Chloroplasts modulate elongation responses to canopy shade by retrograde pathways involving hy5 and abscisic acid. *Plant Cell*, *31*(2), 384–398. https://doi.org/10.1105/tpc.18.00617

Paik, I., & Huq, E. (2019). Plant photoreceptors: Multi-functional sensory proteins and their signaling networks. *Seminars in Cell and Developmental Biology*, *92*(April 2018), 114–121. https://doi.org/10.1016/j.semcdb.2019.03.007

Paulišić, S., Qin, W., Arora Verasztó, H., Then, C., Alary, B., Nogue, F., Tsiantis, M., Hothorn, M., & Martínez-García, J. F. (2021). Adjustment of the PIF7-HFR1 transcriptional module activity controls plant shade adaptation. *The EMBO Journal*, *40*(1), 1–16. https://doi.org/10.15252/embj.2019104273

Reed, J. W., Nagpal, P., Poole, D. S., Furuya, M., & Chory, J. (1993). Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout arabidopsis development. *Plant Cell*, *5*(2), 147–157. https://doi.org/10.1105/tpc.5.2.147

Roig-Villanova, I., & Martínez-García, J. F. (2016). Plant responses to vegetation proximity: A whole life avoiding shade. *Frontiers in Plant Science*, *7*(FEB2016). https://doi.org/10.3389/fpls.2016.00236

Roig-Villanova, I., Paulišić, S., & Martinez-Garcia, J. F. (2019). Shade Avoidance and Neighbor Detection. *Methods in Molecular Biology*, *2026*, 157–168. https://doi.org/10.1007/978-1-4939-9612-4_13

Short, T. W. (1999). Overexpression of arabidopsis phytochrome B inhibits phytochrome A function in the presence of sucrose. *Plant Physiology*, *119*(4), 1497–1505. https://doi.org/10.1104/pp.119.4.1497

Valladares, F., & Niinemets, Ü. (2008). Shade tolerance, a key plant feature of complex nature and consequences. *Annual Review of Ecology, Evolution, and Systematics*, *39*, 237–257. https://doi.org/10.1146/annurev.ecolsys.39.110707.173506

Viczián, A., Ádám, É., Staudt, A. M., Lambert, D., Klement, E., Romero Montepaone, S., Hiltbrunner, A., Casal, J., Schäfer, E., Nagy, F., & Klose, C. (2020). Differential phosphorylation of the N-terminal extension regulates phytochrome B signaling. *New Phytologist*, *225*(4), 1635–1650. https://doi.org/10.1111/nph.16243

Wang, X., Gao, X., Liu, Y., Fan, S., & Ma, Q. (2020). Progress of Research on the Regulatory Pathway of the Plant Shade-Avoidance Syndrome. *Frontiers in Plant Science*, *11*(April), 1–12. https://doi.org/10.3389/fpls.2020.00439

Zhang, X., Henriques, R., Lin, SS. *et al.* (2006). *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat Protoc,* 1, 641–646. https://doi.org/10.1038/nprot.2006.97