UNIVERSITAT POLITÈCNICA DE VALÈNCIA

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Plant Molecular and Cellular Biology Joint Research Institute (IBMCP)

Comparative studies between plant systems to understand divergent strategies to vegetation shade: the role of HY5 transcription factor.

Master's Thesis

Master's Degree in Plant Molecular and Cellular Biotechnology

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Abstract

In many plant ecosystems, close vegetation proximity and high plant density can affect the quantity and quality of the incoming sunlight, limiting the availability of light for photosynthesis. To deal with this situation, plants have adopted two divergent strategies: avoiding or tolerating plant shade. When detecting vegetation proximity, shade-avoider species activate a set of responses that impact plant development, such as promoting stem elongation and flowering, or adjusting photosynthetic metabolism to grow in low light levels. In contrast, shade-tolerant species lack the characteristic stem elongation response to "escape" the unfavorable shade conditions and are well adapted to growing in low light environments. Whereas the regulation of shade-avoidance is quite well understood, much less is known about the regulation of shade-tolerance at the molecular and genetic level. Recent comparative studies between the shade-avoidance *Arabidopsis thaliana* and the shade-tolerant *Cardamine hirsuta* have indicated that shade tolerance in *C. hirsuta* is implemented by components also known to regulate hypocotyl elongation in the shade-avoiding species *A. thaliana*.

In this work, we aimed to study the role of the bZIP transcription factor *ELONGATED HYPOCOTYL 5* (*HY5*) in the development of a shade tolerant habit in *C. hirsuta*. *HY5* has been previously described as a negative regulator of shade avoidance responses, but its precise role is still unclear. Through the phenotypical characterization of lines with altered *HY5* activity we hypothesize that HY5 activity is higher in *C. hirsuta* than in *A. thaliana*. In addition, using a genetic approach, we put forward the existence of a synergistic effect between *HY5* and *PHYB.*

Keywords: *Arabidopsis thaliana*, *Cardamine hirsuta*, genetic regulation, HY5, phytochromes, PIFs, plant shade.

Resumen

En muchos ecosistemas vegetales la proximidad de la vegetación y la alta densidad de plantas puede afectar a la cantidad y calidad de la luz solar percibida, limitando la disponibilidad de luz a la hora de realizar la fotosíntesis. Para afrontar esta situación, las plantas han adoptado dos estrategias divergentes: evitar o tolerar la sombra vegetal. Las especies que evitan la sombra se caracterizan por activar un conjunto de respuestas de aclimatación con un fuerte impacto en el desarrollo, como el fomento del alargamiento del tallo y la floración, o el ajuste del metabolismo fotosintético para crecer bajo niveles reducidos de luz. Por el contrario, las especies tolerantes a la sombra carecen de la característica respuesta de elongación del tallo para "escapar" de las condiciones de sombra desfavorables y están bien adaptadas a crecer en ambientes con poca luz. Mientras que la regulación de la evitación de la sombra se comprende bastante bien, se sabe mucho menos sobre la regulación a nivel molecular y genético de la tolerancia a sombra. Recientes estudios comparativos entre especies emparentadas filogenéticamente que divergen en su respuesta a sombra, como la especie modelo *Arabidopsis thaliana* y *Cardamine hirsuta*, han indicado que la tolerancia a sombra en *C. hirsuta* se implementa empleando componentes que se sabe que también regulan el alargamiento del hipocótilo en la especie que huye de la sombra *A. thaliana*.

El objetivo del presente trabajo es estudiar el papel del factor de transcripción bZIP codificado por *ELONGATED HYPOCOTYL 5* (*HY5*) en el desarrollo de un hábito tolerante a la sombra en *C. hirsuta*. *HY5* ha sido descrito previamente como un regulador negativo de las respuestas de huida de la sombra, pero su función exacta aún no está clara. A través de la caracterización fenotípica de líneas con actividad *HY5* alterada demostramos que *HY5* inhibe el alargamiento del hipocótilo y reforzamos la hipótesis de que la actividad de este factor es mayor en *C. hirsuta* que en *A. thaliana*. Además, utilizando un enfoque genético, evidenciamos la existencia de un efecto sinérgico entre *HY5* y *PHYB*.

Palabras clave: *Arabidopsis thaliana*, *Cardamine hirsuta*, regulación genética, HY5, fitocromos, PIFs, sombra vegetal.

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Introduction

To efficiently adapt and survive in the changing environment, plants have developed sophisticated ways of detecting external cues and translating them into internal signaling pathways (Paik & Huq, 2019). Among the perceived signals, light is of utmost importance for the growth and development of these sessile and photoautotrophic organisms. Light is not only the energy source for photosynthesis, but also an environmental cue that provides crucial information of ecological value (Jang *et al.*, 2013). As light conditions fluctuate during the day in quantity (photon amount or intensity) and quality (wavelengths or colors), they provide information to plants about the position that they occupy in relation to their surroundings, allowing them to accordingly adjust and adapt their physiological responses (Martinez-Garcia & Rodriguez-Concepcion, 2023). Examples of light-mediated responses include seed germination, stem elongation, phototropism of leaves and stems, development of leaves and chloroplasts, stomatal opening, and flowering (Reed *et al.*, 1993).

Light perception and vegetation proximity signal

In both natural and agricultural settings, it is common to find plants in close proximity with others from the same or different species, reaching in some cases very high densities (e.g., forests, prairies, monocultures). In these environments, light can become a limiting resource that compromises plant development, favoring the competition between individuals which might result in overgrowing and eventual overshading by neighbors (Roig-Villanova & Martinez-Garcia, 2022).

Light quality is an important signal that informs plants of potential competitors (Ortiz-Alcaide *et al.*, 2019). Although the solar irradiation that reaches the ground, known as daylight or sunlight, can vary in quantity (e.g., due to clouds) during the day, its quality is usually rather constant. The spectrum of sunlight used for photosynthesis is known as photosynthetic active radiation (PAR) and shows a good correspondence to that visible to the human eye (from 400 to 700 nm). When sunlight impacts on plant tissues, photosynthetic pigments (chlorophylls and carotenoids) absorb most of the light in the PAR region, including blue (B, c. 400 - 500 nm) and red light (R, c. 600 nm), whereas far-red light (FR, c. 725 nm) is reflected or transmitted through plant tissues. Direct sunlight has a natural high ratio of R to FR (high R:FR > 1.2) but, when neighboring plants grow and get closer without overshading, it mixes with the FR reflected by the nearby plants, resulting in a reduction in the R:FR of the incident light (intermediate or low R:FR between 0.8 and 0.1) without lowering light intensity. This is known as **vegetation proximity** and acts as an

early warning of the proximity of potentially competing vegetation. When growing in an understory, however, the incoming light is filtered through the plant canopy and hence not only is characterized by a strongly reduced R:FR (very low R:FR < 0.1) but also by a lower light intensity in the PAR region since B and R are used for canopy photosynthesis (**Figure 1**). Consequently, **plant shade** is a warning that photosynthesis might be already compromised (Martinez-Garcia & Rodriguez-Concepcion, 2023).

To deal with nearby vegetation or shade, plants have evolved two main strategies: avoidance and tolerance. Shade-avoider (sun loving) species, such as *Arabidopsis thaliana* or *Solanum lycopersicum* (tomato), invest energy into readjusting their growth and development to escape eventual shade conditions. By contrast, shade-tolerant species, such as *Cardamine hirsuta* or some *Geranium* species, adapt to grow and survive under low light levels adopting a conservative growth strategy (Molina-Contreras *et al*., 2019).

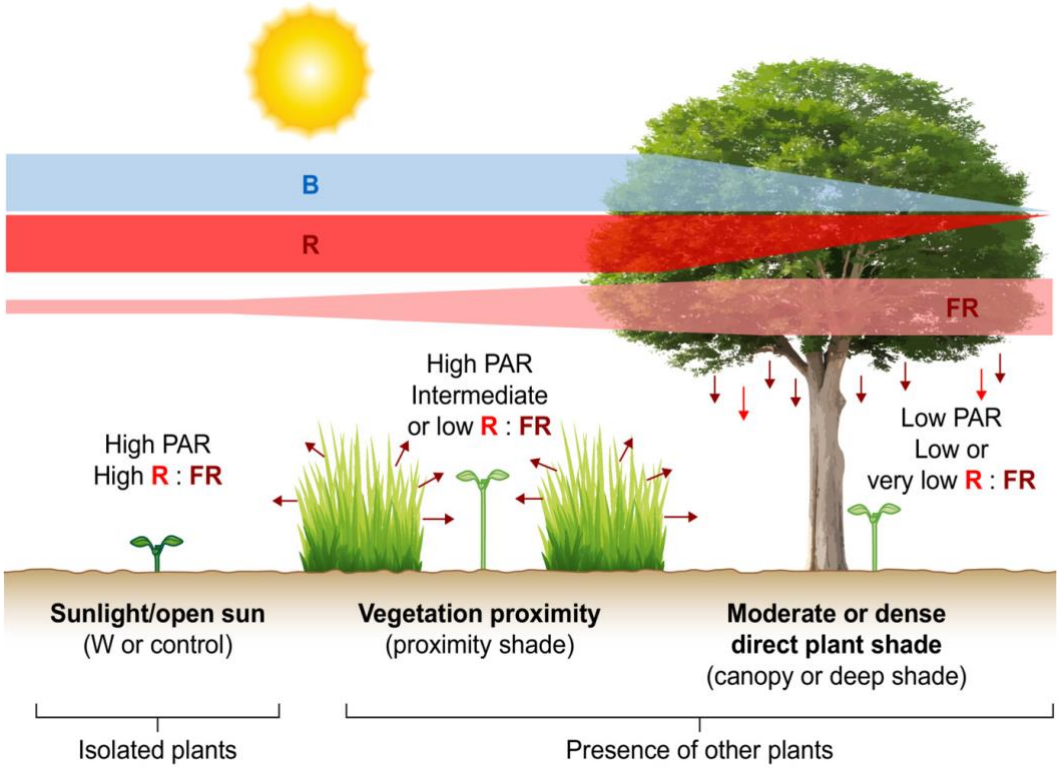

Figure 1. Light characteristics of sunlight, vegetation proximity, and moderate or dense direct plant shade. Isolated plants receive sunlight that contains high amount of photosynthetic active radiation (PAR) of a high red (R) to far- red light (FR) ratio (R:FR). When vegetation density increases, sunlight is not filtered and PAR intensity (blue, B; and R) is unaffected; however, FR is reflected by neighboring vegetation, which results in an intermediate or low R:FR. We name these conditions as vegetation proximity. Under the shade of other plants, sunlight is filtered by the leaves, which lowers PAR; FR can be transmitted, which results in a low or very low R:FR. We name these conditions as moderate or dense direct plant shade. Parenthesis indicates the name of the laboratory treatments that mimic the corresponding natural condition. Red arrows, R filtered by a plant canopy; dark-red arrows, FR reflected or transmitted by vegetation. Adapted from Martinez-Garcia & Rodriguez-Concepcion, 2023.

Mimicking vegetation shade in the laboratory with light treatments

Experimental conditions that mimic vegetation proximity or direct plant shade are generally known as simulated shade. In the laboratory, signals informing about the presence of nearby plants can be reproduced by enriching a fixed intensity of white light (W) with FR provided by LED lamps. By changing the intensity of the applied FR, R:FR levels can be adjusted while maintaining essentially the same amount of PAR thus, allowing the study of responses triggered by photoreceptors rather than caused by a limited generation of photoassimilates under shade conditions. In consequence, intermediate R:FR values (0.8 - 0.5) result in proximity shade treatments that mimic vegetation proximity, whereas low (0.5 - 0.1) or very low (< 0.1) R:FR levels result in canopy shade treatments that mimic moderate or dense direct plant shade (**Figure 1**) (Roig-Villanova & Martinez-Garcia, 2016).

Shade avoidance: molecular mechanisms

In shade-avoider species, perception of plant proximity triggers a set of responses collectively known as the shade avoidance syndrome (SAS) that strongly affects plant development and metabolism. Among them, one of the best studied and characterized response is the promotion of the hypocotyl elongation in seedlings of the well-known model system *A. thaliana* (Roig-Villanova & Martinez-Garcia, 2022).

The R:FR changes indicative of plant proximity or shade are detected by the phytochrome (phy) photoreceptors, which in *A. thaliana* are encoded by a small gene family of five members (*PHYA* to *PHYE*) that possess overlapping but distinct functions (Shin *et al.*, 2007). Whereas *PHYA* encodes the only photolabile phytochrome, phyA, the other *PHY* genes encode photostable phytochromes, phyB-phyE. Phytochromes exist as two photoconvertible isomers, the inactive R-absorbing Pr form and the active FR-absorbing Pfr form. Consequently, in the light, the relative abundance of both forms depends on the R:FR conditions. Under high R:FR (i.e., low vegetation density) the photoequilibrium is displaced toward the active Pfr form and SAS responses are suppressed. On the contrary, under low R:FR the photoequilibrium is displaced toward the inactive Pr form and SAS responses are induced (Roig-Villanova & Martinez-Garcia, 2016). phyB is the major phy controlling seedlings' responses to shade, whereas phyD and phyE act redundantly with it to control some aspects of SAS-driven development such as petiole elongation or flowering time. In contrast to the other phys, phyA has an antagonistic negative role in the SAS hypocotyl response (Martinez-Garcia *et al.*, 2014). Under high R:FR conditions, while most phyA is degraded, active phyB represses SAS responses. Alternatively, under very low R:FR phyA accumulates to enough levels to antagonize and prevent the excessive elongation

responses triggered by the deactivation of phyB that would, otherwise, require a lot of energy and compromise survival (**Figure 2**) (Martinez-Garcia & Rodriguez-Concepcion, 2023). The absorption of light by phytochromes is followed by nuclear translocation. There, the active form of phys interact with many different transcription factors to initiate light-signaling cascades (Gangappa & Botto, 2016; Shin *et al.*, 2007).

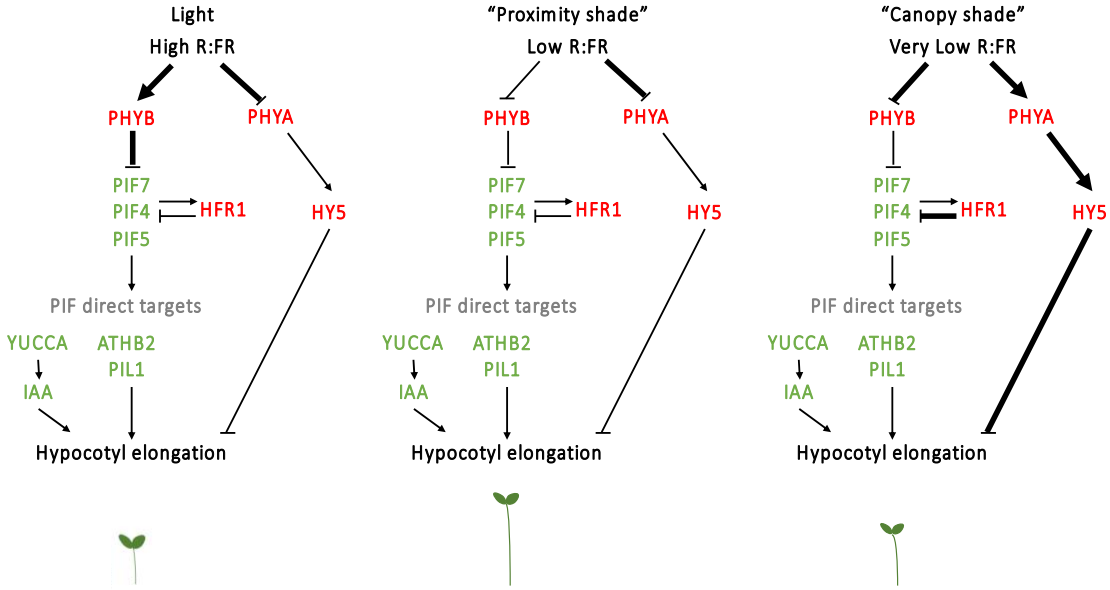

Figure 2. Schematic representation of the main molecular components involved in shade- induced hypocotyl elongation and their function. The model depicts the signaling pathway involved in shade-induced hypocotyl elongation. The positive regulators of hypocotyl elongation are marked in green color and the negative ones are marked in red color. Wider arrows represent a stronger activating/repressing effect. Modified from Martinez-Garcia et al., 2014.

Analyses of the hypocotyl elongation responses to simulated shade in *A. thaliana* led to conclude that the SAS induction is in part regulated by the interaction of active phys with PHYTOCHROME INTERACTING FACTORs (PIFs); mainly PIF7 and, to a lower extent PIF4 and PIF5. Active phyB interacts with these members of the basic helix-loop-helix (bHLH) family of transcription factors to trigger their degradation and/or inhibit their transcriptional activity. Subsequently, inactivation of phyB by decreased R:FR allows PIFs to recover their capacity to bind to their target genes, like *YUCCAs* (*YUCs*) and *PHYTOCHROME RAPIDLY REGULATED* (*PAR*). *YUCs* encode flavin monooxygenases enzymes involved in the production of the auxin indole-3-acetic acid (IAA) required for hypocotyl growth. On the other hand, *PAR* genes encode (i) atypical non-DNA binding bHLH proteins, such as *PAR1*, *PAR2* and *LONG HYPOCOTYL IN FAR-RED LIGHT 1* (*HFR1*) or (ii) true transcription factors such as *ATHB2*, *ATHB4* and *B-BOX-CONTAINING* (*BBX*) genes. Overall, these proteins are reported to act as either positive or negative SAS regulators (Martinez-Garcia & Rodriguez-Concepcion, 2023).

Another described regulator of shade-induced hypocotyl elongation is the basic leucine zipper (bZIP) transcription factor *ELONGATED HYPOCOTYL 5* (*HY5*). Extensive genetic and biochemical studies have established that this phyA-dependent (Ciolfi *et al.*, 2013) protein inhibits hypocotyl and root growth in a light-dependent manner in *A. thaliana* (Gangappa & Botto, 2016). However, its precise role in the current SAS working model is still unclear. At the protein level, low and very low R:FR treatments stabilize HY5 and promote the accumulation of this SAS repressor presumably to prevent seedlings from exhibiting excessive elongation (Martinez-Garcia & Rodriguez-Concepcion, 2023). Whereas in darkness, *CONSTITUTIVELY PHOTOMORPHOGENIC 1* (*COP1*) and *SUPPRESSOR OF PHYTOCHROME A-105* (*SPA*) E3 ligase complex directly targets HY5 protein for polyubiquitination and degradation (Xiao *et al.*, 2022).

Shade tolerance: *Cardamine hirsuta* as a model for comparative studies

Shade tolerance is an ecological concept that refers to the capacity of some plant species to live and thrive under low light conditions associated with high plant density environments (e.g., in the understory of forests and woodlands) (Valladares & Niinemets, 2008). Tolerance to shade can be achieved through photosynthesis optimization and maximization of abiotic and biotic stress resistance (Paulišić *et al.,* 2021). As in shade avoidance, a large number of physiological, biochemical, and morphological traits are associated with shade tolerance. However, little is known about its molecular and genetic basis.

Recently, comparative analyses of related plants displaying divergent elongation responses when exposed to shade-inducing light signals have proven to be a useful strategy for the identification of genes that cause phenotypic diversity (Martinez-Garcia & Rodriguez-Concepcion, 2023). In this regard, *C. hirsuta*, a shade-tolerant and close relative of the reference plant and shade-avoider *A. thaliana*, has emerged as an ideal genetic system for carrying out comparative studies due to the many desirable traits that it shares with *A. thaliana*: it is a diploid and self-compatible annual plant with an abundant seed set, has an 8-week seed-to-seed generation time and displays a small rosette growth habit that is amenable to large-scale cultivation (Hay *et al.*, 2014).

Shade tolerance: differences and similarities with shade avoidance

At present, very little is known about the mechanistic and regulatory adjustments made in shade-tolerant species light signaling to allow them to live under low light environments. However, the latest published comparative studies between two mustard shade-divergent species, *A. thaliana* and *C. hirsuta*, indicate that shade tolerance in *C. hirsuta* is implemented by

components also known to regulate hypocotyl elongation in the shade-avoiding species *A. thaliana* (Martinez-Garcia & Rodriguez-Concepcion, 2023).

Despite the lack of elongation in response to shade exhibited by *C. hirsuta* seedlings, the presence of phytochrome photoreceptors in its genome, together with its ability to induce the expression of typical gene markers of shade perception (e.g., *C. hirsuta ATHB2* gene*, ChATHB2*) indicate that *C. hirsuta* is able to sense and respond to changes in the R:FR signal. Moreover, a recently performed genetic screening using an ethyl methane sulfonate-mutagenized population of *C. hirsuta* seeds identified mutants that displayed long hypocotyls under simulated shade (*slender in shade*, *sis*, mutants), demonstrating that *C. hirsuta* shade perception is also able to trigger the promotion of hypocotyl elongation (Molina-Contreras *et al.*, 2019). Taken together, these findings suggest that the molecular components that explain the differences in elongation between shade avoider and shade-tolerant species do not involve blindness to the shade signal, but instead stronger mechanisms to repress the shade-induced elongation (Martinez-Garcia & Rodriguez-Concepcion, 2023).

Recent work has identified that *SIS1* encodes the phyA photoreceptor, that hence is as a major contributor to the suppression of hypocotyl elongation of *C. hirsuta* seedlings in response to shade. While *A. thaliana phyA* mutant seedlings only show a distinct phenotype under canopy shade conditions (very low R:FR), phyA-defective *C. hirsuta* seedlings display a mutant phenotype also under proximity shade (intermediate and low R:FR) thus, suggesting that *C. hirsuta* phyA (ChphyA) has a stronger activity than AtphyA. Higher levels of *ChPHYA* expression and ChphyA protein abundance could be confirmed through RT-qPCR and immunoblot analyses respectively, while complementation of *A. thaliana phyA* mutant plants with the *ChPHYA* or *AtPHYA* genes supported the conclusion that ChphyA also has a higher intrinsic activity than AtphyA. Collectively, these results described an enhanced phyA activity in *C. hirsuta*, which would sustain the enhanced shade-induced suppression activity required for the implementation of a shade tolerant habit in this species. By contrast, phyB photoreceptor, known for repressing hypocotyl elongation under high R:FR conditions in *A. thaliana* seems to have an attenuated activity in *C. hirsuta* (ChphyB). Consistent with this hypothesis, weak *A. thaliana phyB-4* mutant seedlings display a reduced (though not abolished) shade-induced hypocotyl elongation (Sánchez-García, 2022; Molina-Contreras *et al.*, 2019). Nonetheless, at the moment there is no available data about the molecular activity of ChphyB nor its functional role in shade tolerance (Martinez-Garcia & Rodriguez-Concepcion, 2023).

In addition, *C. hirsuta* plants deficient in phytochrome downstream transducers, such as the negative SAS regulator HFR1, also gain the capacity to elongate in response to shade. Similar to

phyA, the repressor role of HFR1 in shade-unresponsive *C. hirsuta* hypocotyls relies on an increased expression of the *ChHFR1* gene and a much-enhanced ChHFR1 protein stability in comparison with AtHFR1, which combined produce a higher total activity of this atypical non-DNA binding bHLH protein in this species. The increased protein stability was established to be the result of ChHFR1 lower binding affinity to COP1, a transcriptional regulator that targets HFR1 for degradation through ubiquitination. HFR1 central activity is to interact with PIFs to prevent them from binding to the DNA and altering gene expression, acting as transcriptional cofactor that modulates SAS responses in a PIF-dependent manner and thus, forming the PIF-HFR1 transcriptional regulatory module. As a consequence of the enhanced HFR1 activity in *C. hirsuta*, PIF activity would decrease, subsequently provoking the absence of a shade-induced hypocotyl elongation as well as an attenuation of other PIF-mediated responses, such as thermal-induced morphogenesis (TIM) and dark-induced senescence (DIS), in this species (Paulišić *et al.*, 2021).

Dark-induced senescence (DIS)

Leaf senescence, the final stage of leaf development, is a highly controlled developmental process accompanied by massive transcriptional and metabolic changes that destabilize intracellular organelles (mainly chloroplast) and provoke translocation of nutrients into developing tissues to achieve reproductive success (Sakuraba, 2021). Although leaf senescence is mainly driven by developmental age, it is also affected by a range of internal factors, such as phytohormones, as well as external signals, including light environment. Reduced light intensities and alterations in its composition strongly contribute to the onset of senescence for shaded plants (Li *et al.*, 2023).

Numerous studies have demonstrated that phyB can mediate a light signaling-dependent retardation of senescence. In addition, PIFs, specifically PIF4 and PIF5, have been shown to have prominent roles in promoting DIS in *A. thaliana*. Under prolonged darkness PIFs expression is induced in a phyB-dependent manner, causing an acceleration of DIS symptoms. By contrast, their mutant combinations display a delay in this phenomenon (Liebsch & Keech, 2016).

A trait associated with shade tolerance appears to be a delay in DIS, as light deprivation caused by the dark can be considered an extreme case of shading. Indeed, the shade-avoiding *A. thaliana* is known to rapidly enter in senescence when transferred to shade or dark conditions. By contrast, DIS is delayed in the shade-tolerant *C. hirsuta* (Paulišić *et al.*, 2021).

Thermal-induced morphogenesis (TIM)

Light and temperature are prominent cues that signify seasonal and climatic change, as well as the phase of the daily light/dark cycle. The ability to sense and integrate these external signals is essential for plant life cycle progression and ultimately, survival (Toledo-Ortiz *et al.*, 2014). Multiple light signaling components have been demonstrated to be also involved in temperature-mediated development, suggesting that both light and temperature signaling work in concert to control plant growth and development (Bian *et al.*, 2022).

Mild warm temperatures (below the heat stress range) result in plant acclimation responses collectively known as TIM, which include the elongation of hypocotyls, stems, petioles and roots, leaf hyponasty and a reduction in leaf blade size (Delker *et al.*, 2022). Recent studies have revealed that the R photoreceptor phyB, its interacting partner PIF7, and the core component of the plant circadian clock EARLY FLOWERING 3 (ELF3), enable perception of higher ambient temperature (Bian *et al.*, 2022). Downstream of these thermosensors, PIF4 emerges as a central signaling hub controlling growth and development through the activation of genes involved in auxin biosynthesis and signaling. Other key light signaling components reported to define photomorphogenesis include DET1 and COP1, which have been proposed to control hypocotyl elongation by regulating *PIF4* expression and stability, as well as *HY5*, which would negatively regulate PIF4-mediated elongation growth through competitively binding to the PIF4 targets gene promoters (Gangappa & Botto, 2016).

Objectives

To cope with the light gradients produced by the presence of nearby vegetation, plants have evolved two main strategies: avoidance and tolerance. While many aspects of the molecular regulation of the shade avoidance has been elucidated studying the mustard *A. thaliana*, little is known about the molecular and genetic basis of shade tolerance. Comparative studies between *A. thaliana* and the shade tolerant *Cardamine hirsuta*, a relative of *A. thaliana*, indicate that tolerance mechanisms are implemented by components also known to regulate hypocotyl elongation in shade-avoiding species. Among them is HY5, a bZIP type transcription factor, known to play a key role in the regulation of light-dependent development. The general objective of this work is to study the role of HY5 in the regulation of shade tolerance using *A. thaliana* and *C. hirsuta* as our model systems. For this purpose, we propose the following specific objectives:

1. Phenotypic characterization of *A. thaliana* and *C. hirsuta* lines with altered *HY5* activity under different light and temperature conditions. These observations will allow us to establish a working hypothesis for the role of HY5 in the development of a shade tolerant habit in *C. hirsuta*.

2. Determination of the genetic relationship between *HY5* and *PHYB*. The reduced activity of phyB in *C. hirsuta* cannot fully explain the lack of shade-induced hypocotyl elongation in this species, needing the intervention of other components. To address if HY5 could be one of them we will explore the possible functional relationship between HY5 and the main R receptor, phyB.

3. Comparative molecular analyses of protein abundance and stability between ChHY5 and AtHY5. We will explore whether protein sequence affects ChHY5 and AtHY5 protein abundance or stability in heterologous system and thus, explain the observed differences in their biological activities.

4. In vivo analysis of HY5 interaction with other SAS-regulatory proteins. HY5 activity is achieved through physical interaction with other light-signaling components. The discovery of new protein-protein interactions would help to elucidate HY5 mechanism of action in the regulation of seedling responses to shade.

Materials and methods

Plant material and growth conditions

Plants of *A. thaliana* accession Columbia-0 (Col-0), *hy5-2* (in Col-0 background) (Bou-Torrent *et al.*, 2015) and accession Landsberg *erecta* (L*er*-0), *hy5-1*, *phyB-1* and *phyB-4* (all in L*er*-0 background) (Roig-Villanova *et al.*, 2006; Reed *et al.*, 1993), as well as plants of *C. hirsuta* accession Oxford (OX), *chy5-1*, *chy5-2*, *chy5-3*, *chy5-4*, *chy5-5* and *chy5-6* (all in OX background) (Qin, 2022; Hay *et al.*, 2014) were used in this work.

Seeds were surface-sterilized [20 min in 0.1% (v/v) Tween-20 and 10 min in 10% (v/v) bleach plus 0.1% (v/v) Tween-20] 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] at a density of about 2 seeds·cm^{-2}. After stratification (3 – 4 days at 4°C in darkness), plates were incubated in growth chambers at 22°C under continuous white light (W) to induce seed germination prior to applying a treatment.

Shade-induced hypocotyl elongation experiments. W was provided by horizontal white LED tubes [55 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR); R:FR of 3.55]. Fluence rates were measured with a Spectrosense2 meter associated with a four-channel sensor (Skye Instruments Ltd.), 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.

The different simulated shade treatments were produced by supplementing W with FR (W + FR) emitted from a LED FR module (R:FR of 0.05). Proximity shade was mimicked by exposing plants to 18 min of W+FR under one-hour cycles of W (W + FRp), whereas canopy shade was mimicked by growing plants under continuous W + FR (W + FRc).

Thermal-induced morphogenesis (TIM) experiments. Petri dishes with the germinating seeds were placed under continuous W (PAR = 52 - 58 µmol m^{-2} s^{-1}; R:FR between 5 and 5.6) provided by fluorescent tubes at constant temperature in matching growth chambers (IBERCEX) set to 22°C (control conditions) and 28°C (warm conditions), respectively.

Dark-induced senescence (DIS) experiments. Seedlings were grown in Petri dishes for 7 days at 22°C under continuous W before being transferred to darkness (D). The in vivo fluorescence measurements were taken on the day of the transfer (0DD) as well as after 2, 4 and 7 days in D (2DD, 4DD and 7DD respectively).

A. thaliana and *C. hirsuta* plants were grown in the greenhouse under long-day photoperiods (16h light and 8h dark) to produce seeds. For transient expression assays, *Nicotiana benthamiana* plants were also grown in the greenhouse under long-day photoperiods for about 3-4 weeks.

Measurement of hypocotyl length

The ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/) was used on digital images to measure hypocotyl length after laying out the seedlings flat on the agar plates. At least 20 seedlings were measured for each condition and experiments were repeated three times. The significance was evaluated by statistical Student's t-test. Additionally, two-way ANOVA statistical analyses were performed using GraphPad PRISM v.8.00.

Measurement of chlorophyll fluorescence

In vivo fluorescence measurements were performed at room temperature using a Handy FluorCam FC 1000-H/GFP (Photon Systems Instruments) on seedling cotyledons. After applying blue light (0.5 µmol m^{-2} s^{-1}, 450 nm) for 5s to record the basal fluorescence (F_0), samples were illuminated for 800 ms with saturating white light (2700 µmol m^{-2} s^{-1}) to obtain the maximum fluorescence (F_m), and then exposed to continuous actinic light (800 µmol m^{-2} s^{-1}) for 90s to drive photosynthetic activity. The maximum quantum yield of photosystem II (QY_{max}) (F_v/F_m) was calculated for each cotyledon using the FluorCam7 software.

Genetic crosses and genotyping

The mutants *phyB-1* (loss of phyB function) and *phyB-4* (attenuated phyB activity) were crossed with *hy5-1* (loss of function) mutants to generate the double mutants *phyB-1 hy5-1* and *phyB-4 hy5-1*. To achieve this aim, flowering plants were manually cross-pollinated. After 17-19 days, the seeds from the cross (F1) were harvested. Three to five F1 seeds were sown in 0.5x GM-medium, transferred to individual pots and grown to maturity to produce seeds (F2 generation). Then, the segregating F2 seeds were germinated and grown under W for 7 days in order to carry out a genotype selection of *phyB* mutant seedlings based on the long hypocotyls phenotype exhibited under W. Pre-selected F2 plants were genotyped using PCR and specific primers designed for this project (**Table S1**). For that purpose, plant genomic DNA was extracted from basal leaves, as described elsewhere (Edwards *et al.*, 1991). The PCR was performed in a final volume of 20 µL which contained the DNA template (2 µL), 5µM of each primer (2 µL) and the

NZYTaq II 2x Green Master Mix (10 µL) (NZYTech), using a standard three-step protocol. The optimal annealing temperature for each primer pair use was identified by a set of gradient-PCR reactions. The PCR temperature profile consisted of an initial denaturation at 94°C (3 min), followed by 30 cycles each of 94°C (30 sec), annealing (30 sec) (for temperatures see **Figure 6B**) and 72°C (1 min), before a final extension at 72°C (3 min). The amplified fragments were visualized in a 1% agarose gel stained with ethidium bromide.

Transient expression of HY5 in *N. benthamiana* leaves

To transiently overexpress *AtHY5* and *ChHY5* in *N. benthamiana* plants, *Agrobacterium tumefaciens* (strain GV3101, that is resistant to rifampicin and gentamycin) was transformed by heat shock (Gold Biotechnology, 2021) with the following binary vectors: pWQ21 (35S:attB1<*AtHY5-3xHA*<attB2, 35S:*mGFP5*) and pWQ22 (35S:attB1<*ChHY5-3xHA*<attB2, 35S:*mGFP5*) (Qin, 2022), both conferring resistance to kanamycin.

A colony of *A. tumefaciens* transformed with either pWQ21 or pWQ22 was then used to inoculate 3 mL of Lysogeny Broth (LB) medium [10% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, pH 7.5] supplemented with the appropriate antibiotics (100 µg/mL rifampicin, 50µg/mL kanamycin and 30 µg/mL gentamycin) and the culture was grown overnight at 28°C. Next, 200 µL of the overnight culture were employed to inoculate 20 mL of LB (supplemented with the indicated antibiotics) and grown overnight at the same temperature. The day of the agroinfiltration, the optical density of the cultures was measured at 600 nm (OD_{600}) and adjusted to a value of 0.5. The cultures were then centrifuged, and the cell pellets were resuspended and incubated for 2 hours at 28°C with shaking in infiltration solution [10 mM Mg_2Cl, 10 mM MES pH 7, 150 µM acetosyringone].

Leaves of 3 – 4 week-old *N. benthamiana* plants were infected with a mixture (10:1, v/v) of a culture of *A. tumefaciens* transformed with pWQ21 or pWQ22 and a culture of the same strain expressing the P19 protein to inhibit plant silencing (Garabagi *et al.*, 2012). The agroinfiltration was carried out using 1 mL syringes on the abaxial part of the leaves, which were previously injured with a needle to aid the entry of the bacteria into the plant tissue. After the agroinfiltration, *N. benthamiana* plants were kept in the greenhouse under a long-day photoperiod. Samples (6 leaf circles obtained from infiltrated areas) were collected using a punch (Ø = 80 mm) 3 days after the agroinfiltration and immediately frozen in liquid nitrogen until processed. Each biological sample consisted of approximately 75 mg of tissue from the same leaf. Four biological replicates were harvested per each construct.

Protein extraction and immunoblot analyses

Frozen plant material (50 - 75 mg) was ground to powder, and total proteins were extracted using an SDS-containing Extraction Buffer (EB) [40 mM Tris-HCL pH 6.8, 4% (w/v) SDS, 5% glycerol, 1x proteases inhibitor cocktail (Roche cOmplete EDTA-free); 4 µL of EB per mg of fresh weight]. Protein concentration was determined using the PierceTM BCA Protein Assay Kit (Thermo Scientific). Protein extracts were diluted taking as a reference the sample with the lowest concentration in order to reach the same concentration in all of them, 4x Loading Buffer [4x Laemmli Buffer, 4% (v/v) B-mercaptoethanol; 1:9 (v/v)] was added to each one and they were boiled at 95°C for 5 min. Electrophoresis was carried out in a 12% SDS-PAGE gel, where 50 µg of proteins were loaded per lane. Resolved proteins were transferred to a PVDF membrane and immunoblotted in TBST buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20] containing 0.5% (w/v) milk protein with a 1:2500 dilution of rat monoclonal anti-HA or a 1:2000 dilution of rabbit polyclonal anti-GFP. Anti-rat (1:5000 dilution) or anti-rabbit (1:10000 dilution) horseradish peroxidase-conjugated antibodies were used as secondary antibodies. An ECL Prime Western Blotting Detection Reagent RPN 2235 (GE Healthcare) was used for the development of the blot and the signal was visualized using the Amershan™ ImageQuant™ 800 (GE Healthcare).

Generation of constructs for Yeast two Hybrid (Y2H) assays

The *A. thaliana HY5* (*AtHY5*) CDS was PCR-amplified using the plasmid pWQ8 as the DNA template and the primers MVTO3 and GHO8, which added the attB1 and attB2 sequences. The resulting PCR product (attB1<AtHY5<attB2) was then recombined with pDONR™207 using Gateway BP Clonase II to obtain pGH1 (attL1<*AtHY5*<attL2). The insert was sequenced to confirm its identity. Finally, the *AtHY5* CDS was subcloned in frame with the DNA-binding domain (BD) and transcription activation domain (AD) of the yeast GAL4 protein after a LR clonase reaction between the pGH1 entry clone and the destination vectors pGBKT7-GW (BD<attR1<*ccdB*<attR2) and pGADT7-GW (AD<attR1<*ccdB*<attR2) (Chini *et al.*, 2009), resulting in pGH2 (BD<attB1<*AtHY5*<attB2) and pGH3 (AD<attB1<*AtHY5*<attB2). These plasmids allowed the production of the fusion proteins BD-AtHY5 and AD-AtHY5 respectively, under the *ADH1* promoter in yeast.

Y2H directed assays

For Y2H assays, pGH2 (BD-HY5) and pGH3 (AD-HY5) constructs were generated in this project (see above). Plasmids pGBKT7 (empty vector; BD-Ø), pJB37 (BD-HFR1-3xHA), pGADT7 (empty

vector; AD-Ø), pSP118 (AD-HFR1-3xHA), pBA11 (AD-PIF7-3xHA) and pJB62 (AD-PIF1-3xHA) were already available in the laboratory (Paulišić *et al.*, 2021; Bou-Torrent *et al.*, 2015).

To perform Y2H assays we employed a cell mating system in order to introduce two different plasmids into the same host cell (Takara Bio, 2013; Clontech, 1997). First, haploid yeast cells of the tryptophan (W) auxotroph pJ694α strain were transformed with the BD derivative constructs, and haploid yeast cells of the leucine (L) auxotroph YM4271a strain were transformed with the AD derivative constructs **(Table S2)**. To perform the transformations, frozen competent yeast cells were centrifuged 2 min at 4000 rpm and incubated at 30°C in the following solution: 50% (v/v) polyethylene glycol (PEG^{3350}), 0.1 mM lithium acetate, 100 µg salmon sperm DNA (carrier DNA) and 0.5 µg plasmid DNA. After 30 min, 10% (v/v) DMSO was added and cells were incubated at 42°C for 15 min. Finally, cells were centrifuged and the pellet was resuspended in 200 µL of sterile water. Independent transformed yeast cell colonies were selected on synthetic dropout (SD) minimal medium [2% (w/v) bacto-agar DIFCO, 2% (w/v) D-glucose, 0.85% (v/v) dropout (DO) supplement, 0.67% (w/v) yeast nitrogen base without amino acids, pH 5.8] lacking either leucine (SD-L) or tryptophan (SD-W). For the mating, the selected positive colonies were grown 24 h with shaking at 28°C in liquid yeast peptone dextrose adenine (YPDA) medium [2% (w/v) agar, 2% (w/v) peptone, 2% (w/v) D-glucose, 1% (w/v) yeast extract, 0.3% adenine hemisulfate, pH 5.8] and then allowed to mate by mixing equal volumes (500 µL) of the two types of transformed yeast cells. After 48 h at 28°C without agitation, diploid cells were selected on liquid SD-LW before being plated on SD-LW solid medium, to confirm the mating, and on SD lacking adenine and histidine aside from leucine and tryptophan (SD-AHLW), to test protein-protein interactions. Different dilutions were used for testing the interactions (1:1, 1:10 and 1:100) (Gallemí *et al.*, 2017).

Results and discussion

Characterization of *C. hirsuta* lines with altered HY5 activity

To deepen our knowledge on the role of HY5 in the regulation of the shade seedling responses, we first performed a phenotypic characterization of several lines with altered *HY5* activity under different light and temperature conditions. These lines included the shade-avoider *A. thaliana hy5-1* (null mutant in Ler-0 background) and *hy5-2* (null mutant in Col-0 background), as well as the shade-tolerant *C. hirsuta chy5-1*, *chy5-2*, *chy5-3*, *chy5-4*, *chy5-5* and *chy5-6* (all null mutants in OX background).

HY5 contributes to the *C. hirsuta* shade tolerance habit

Hypocotyl elongation of one week-old seedlings grown under W (high R:FR) and canopy shade conditions (W + FRc; very low R:FR) was measured in order to detect differential responses of plant growth to shade in the studied genotypes.

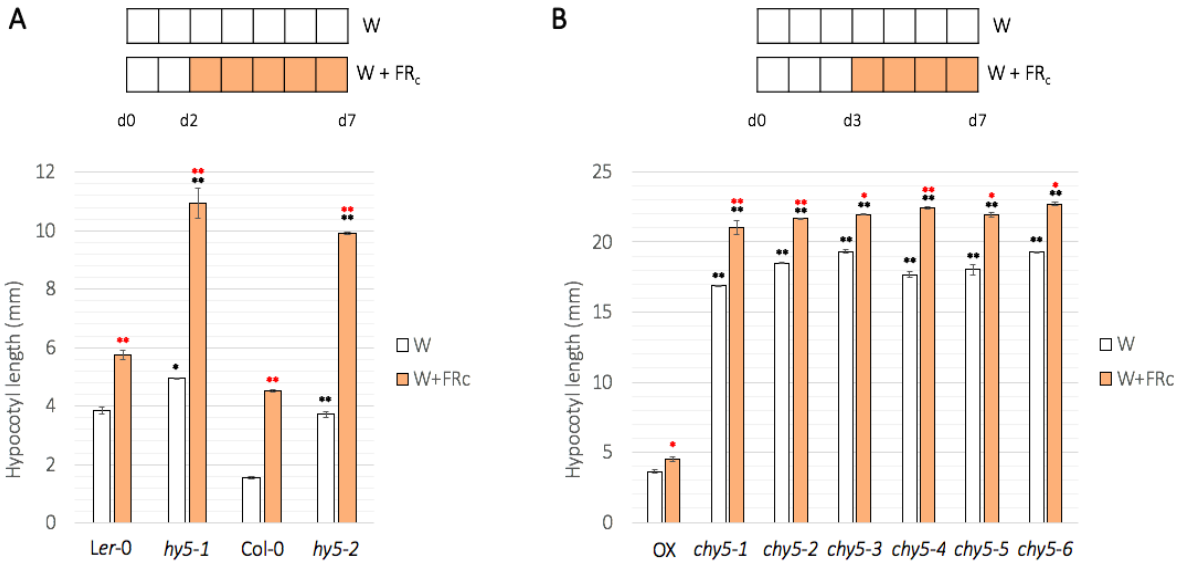

Figure 3. Loss of *HY5* activity in *A. thaliana* and *C. hirsuta* affects hypocotyl elongation in response to canopy shade. (A) Hypocotyl length of *A. thaliana* wild-types (L*er*-0 and Col-0) and their respective *hy5* mutants (*hy5-1* and *hy5-2*). Seedlings were grown in W for 2 days and transferred to canopy shade (W + FRc) for 5 days, as represented at the top. **(B)** Hypocotyl length of *C. hirsuta* wild-type (OX) and six null mutant lines (*chy5-1*, *chy5-2*, *chy5-3*, *chy5-4*, *chy5-5* and *chy5-6*). Seedlings were grown in W for 3 days and transferred to W + FRc for 4 days, as represented at the top. In **A** and **B**, values are mean and standard error (SE) of biological triplicates (n≈25 seedlings per replica). Black asterisks represent significant differences between the mutant lines and the corresponding wild-type grown in the same light conditions; red asterisks represent significant differences between W + FRc and W treatments within the same genotype. Student t-test: * p<0.05 and ** p<0.01.

When grown under W + FRc, L*er*-0 and Col-0 (At^{WT}) seedlings displayed significantly longer hypocotyls than those grown in W. Mutant *hy5-1* seedlings were significantly longer than the wild-type (L*er*-0) under both W and simulated shade, though in W+FRc they were much longer

than L*er*-0. Mutant *hy5-2* seedlings were much longer than the wild-type (Col-0) in both light conditions (**Figure 3A**). By contrast, OX (Ch^{WT}) seedlings responded much less to W + FRc compared to W treatment, whereas all the *chy5* mutant lines showed very long hypocotyls under W and a stronger elongation response to W + FRc (**Figure 3B**). Two-way ANOVA statistical analyses further confirmed the complete recovery of the shade-induced hypocotyl elongation in all of the *chy5* mutants except for the *chy5-3* allele.

Previous studies with *A. thaliana* lines have shown that loss of HY5 activity (*hy5-1* mutant) enhances hypocotyl elongation in seedlings grown in the light (Oyama *et al.*, 1997) or under simulated canopy shade conditions (Jang *et al.*, 2013). The same has been reported for the *hy5-2* mutant seedlings, that displayed longer hypocotyls than wild-type seedlings when grown under light of a lower R:FR reminiscent of canopy shade (Ortiz-Alcaide *et al.*, 2019; van Gelderen *et al.*, 2018). The available information indicates that HY5 represses hypocotyl elongation in seedlings exposed to shade conditions, although the precise role of this transcription factor in the current SAS working model is unclear. It is hypothesized that HY5 may inhibit elongation by negatively regulating gibberellin (GA) signaling via DELLA proteins (Roig-Villanova & García-Martínez, 2022). In low R:FR environments, GA promotes hypocotyl elongation by triggering the degradation of DELLAs, growth-repressing proteins that physically interact with PIFs and prevent them from binding to their DNA targets. A key mechanism controlling bioactive GA levels in *A. thaliana* involves 2β-hydroxylation of GA to an inactive form by GA2-oxidases (encoded by *GA2OX* genes). It has been described that HY5 is required for the expression promotion of *GA2OX* in seedlings exposed to UV-B light plus either W or W + FR. Under these light conditions, the subsequent increase in GA catabolism would contribute to an increased DELLA protein stability, which suppresses PIF activity, thus inhibiting the shade-triggered hypocotyl elongation response (Hayes *et al.*, 2014; Djokovic-Petrovic *et al.*, 2007).

In this work we showed that loss of *HY5* function in the shade-tolerant species *C. hirsuta* (*chy5* lines) results in a very exaggerated long hypocotyl phenotype in W compared to the milder long hypocotyl of the *hy5* mutant seedlings in *A. thaliana*. This fact suggests that HY5 activity is higher in *C. hirsuta* than in *A. thaliana,* hence, that *ChHY5* is more active in repressing elongation growth than *AtHY5.* The difference in activity may be caused by several interdependent and non-excluding factors, such as the different biological functions encoded by these genes or the post-translational regulation which affects protein stability and degradation (Paulišić *et al.,* 2021). In addition, the recovery of the response to simulated canopy shade of the *chy5* mutant lines in comparison to the Ch^{WT} evidence the key role of this transcription factor in preventing the hypocotyl elongation in response to W + FRc, and hence its participation in the development of

a shade tolerant habit in *C. hirsuta*. Taken together, these results differ from what has been observed in *A. thaliana* seedlings, where the hypocotyl elongation is more noticeable when exposed to simulated shade conditions than when kept in W.

hy5 mutants display a delayed DIS phenotype

Dark-induced senescence (DIS) drops chlorophyll levels in light-grown tissues. To quantify and compare the impact of HY5 in the senescence induced by prolonged exposure to darkness, alterations in photosystem II (PSII) function were assessed through chlorophyll fluorescence (CF) *in vivo* measurements of one week-old light-grown seedlings transferred to darkness at four different time points: 0, 2, 4 and 7 DD (**Figure 4A**).

CF is an effective, non-invasive and highly popular technique used in plant physiology due to the ease with which the user can gain detailed information on the photosynthetic fluxes at a relatively low cost (Ni *et al.*, 2019; Kalaji *et al.*, 2014; Fernandez-Jaramillo *et al.*, 2012; Henriques, 2009). Since the first experiments carried out in 1931 by Kautsky and Hirsch this method has progressed quickly, being the chlorophyll fluorescence imaging (CFI) technique that has been used in this work one of the latest innovations developed. CFI allows multiple plants to be monitored at the same time under identical conditions, providing an ideal screening platform. At the same time, it delivers a detailed spatial representation of the measured parameter, allowing the assessment of sample heterogeneity (Gorbe & Calatayud, 2012).

The principle underlying CF analysis is relatively simple. Light energy absorbed by chlorophyll molecules can drive photosynthesis (photochemistry), be re-emitted as heat or be re-emitted as infrared light (CF). These three processes do not exist in isolation but rather in competition with each other, such that any increase in the efficiency of one will result in an inversely proportional decrease in the yield of the other two. For this reason, the CF signal can be used as a probe for photosynthetic activity and heat dissipation as well as to monitor regulatory processes affecting the PSII antenna (Kalaji *et al.*, 2017). Once light is applied to a leaf after a period of darkness, the special chlorophyll of PSII, P_{680}, ejects an electron derived from water splitting to the electron acceptor Q_A (a bound quinone) via the initial acceptor pheophytin. As Q_A is reduced, it is not able to accept another electron until it has passed the first onto a subsequent electron carrier (Q_B). In this state, the reaction center is considered to be “closed”, inevitably causing a decline in quantum efficiency of PSII and so, a corresponding increase in fluorescence. Following this initial rise, the fluorescence signal declines after a few minutes due to a phenomenon termed “quenching”. Quenching arises from the combination of two processes: (i) the increase in the rate at which electrons are transported away from PSII due to

the light-induced activation of enzymes involved in Calvin cycle and the opening of stomata (photochemical quenching), and (ii) the increase in the efficiency with which energy is converted to heat to prevent the formation of damaging free radicals (non-photochemical quenching, NPQ) (Murchie & Lawson, 2013).

In order to estimate the CF yield, it is necessary to (i) be able to distinguish between the photochemical and non-photochemical contributions to quenching and (ii) switch off one of them. This is accomplished through the exposure of fully dark-adapted leaves to a short high intensity flash of light that transiently reduces the photochemical quenching to zero. When a leaf is kept in the dark, Q_A becomes maximally oxidized and the PSII reaction centers are open. During the flash, all PSII reaction centers close, allowing the fluorescence yield to reach a value equivalent to that which would be attained in the absence of any photochemical quenching, the maximum fluorescence (F_m). If this value is compared with the minimum value for chlorophyll fluorescence (F_o), drawn out by exposure to light of an intensity too low to induce electron transport through PSII, it can give information about the efficiency of photochemical quenching and by extension, the performance of PSII. The difference between F_o and F_m is defined as the variable fluorescence (F_v). Theoretically and empirically, it has been shown that the ratio of F_v/F_m gives a robust indicator of the maximum quantum yield of PSII (QY_{max}) chemistry (Baker, 2008; Maxwell & Johnson, 2000). We used this F_v/F_m parameter to estimate the senescence advancement of light-grown seedlings transferred to darkness for a few days.

Seedlings of 0DD exhibited highly consistent and very similar QY_{max} values (0.79 - 0.81) in all the studied genotypes. But once plants were exposed to darkness, a marked decrease in this parameter was observed at each subsequent time point. Although at 2DD no obvious differences could be detected, a turning point was observed after 4DD. At this time point L*er*-0 (At^{WT}) QY_{max} values had already dropped to 0.10, while *hy5-1* mutants maintained moderately higher QY_{max} values (0.30). Similarly, *hy5-2* seedling also presented slightly higher QY_{max} values than the wild-type (Col-0; At^{WT}) ones (**Figure 4B**). Furthermore, the same effect was observed in *C. hirsuta* genotypes: *chy5* seedlings exhibited a slightly enhanced delay in the DIS compared to the wild-type (OX; Ch^{WT}) ones. After 10DD, PSII activity had drastically dropped to below 10% in all A. *thaliana* genotypes, while *C. hirsuta* genotypes experienced a gradual decrease in theirs (QY_{max} values around 0.40) (**Figure 2C**), in agreement with the reported delay in DIS of *C. hirsuta* compared to the shade-avoider *A. thaliana* (Paulišić *et al.,* 2021).

Phenotypic analysis showed that *hy5* mutants senesced slower than wild-type plants under dark incubation conditions in both *A. thaliana* and *C. hirsuta*, thus indicating that HY5 is a positive regulator of senescence. Other authors have reported that HY5 accumulation in response to

light provokes premature leaf senescence by upregulating the expression of several genes involved in the uptake and assimilation of nitrogen, phosphate, sulfate and copper. This enhanced nutrient assimilation would speed up the hydrolysis and translocation of nutrients from the mature leaves to the emerging tissues or storage organs as a way to achieve reproductive success thus, accelerating the process of leaf senescence (Sakuraba, 2021). In shade it has also been reported that this phyA-dependent transcription factor regulates the brassinosteroids (BRs) signaling pathway to positively impact leaf senescence. Whereas in darkness, the inactivation of phyB and CRYs would result in an enhanced activity of the E3 ligase complex COP1/SPA, which controls the ubiquitination and subsequent degradation of HY5 (Li *et al.*, 2023). Hence, no significant effect of this protein would be expected in the absence of light. To sum it up, we can conclude that HY5 plays a critical role in the light signaling-mediated regulation of leaf senescence also in the shade-tolerant *C. hirsuta*.

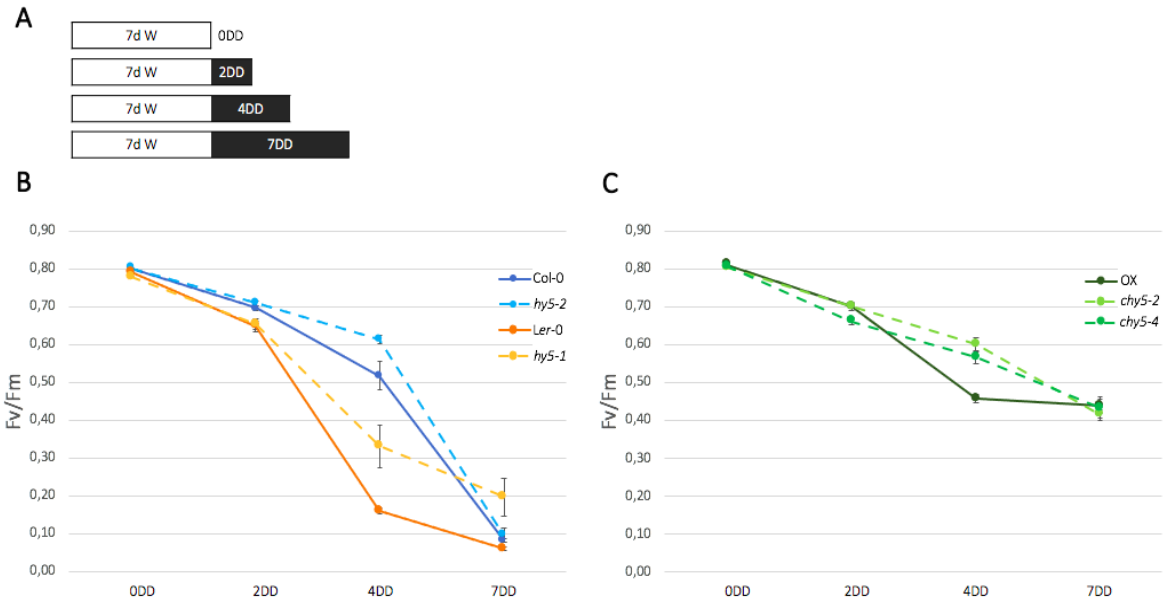

Figure 4. Loss of *HY5* activity in *A. thaliana* and *C. hirsuta* delays dark-induced senescence (DIS). (A) Seedlings were grown in W for 7 days and transferred to darkness for 2, 4 and 7 days. **(B)** Maximum quantum yield of photosystem II (QY_{max}; F_v/F_m) of *A. thaliana* wild-types Ler-0 and Col-0 and their respective *hy5* mutants, *hy5-1* and *hy5-2*. **(C)** F_v/F_m of *C. hirsuta* wild-type OX and two null mutant lines, *chy5-2* and *chy5-4*. Values represent mean and standard error (SE) of n≈12 seedlings per treatment.

HY5 seems to promote senescence yet, *C. hirsuta* genotypes display a delayed DIS phenotype. These results seem to contradict our initial hypothesis, which stated that HY5 activity might be higher in the shade-tolerant species. A possible explanation for the observed discrepancy could be that DIS might be more dependent on other intervening factors, such as PIF activity. Recent analyses have demonstrated that PIFs, mainly PIF4 and PIF5, orchestrate DIS by targeting chloroplast maintenance, chlorophyll metabolism, hormone signaling and production, and the expression of senescence master regulators (Liebsch & Keech, 2016). Consistently, the *pif*

quadruple mutant (*pif1, pif3, pif4,* and *pif5*) *pifQ* is reported to senesce slower than At^{WT}. The described connection between DIS and PIF activity and the similar delayed decline in QY_{max} values displayed by Ch^{WT} in comparison with *pifQ*, suggests that the main cause for the extend survival upon total darkness in shade-tolerant plants might be a reduced PIF activity (Paulišić *et al.*, 2021). In addition, previous research done in the laboratory with a HY5 overexpressing line (*HY5ox*) showed that the pattern of its photosynthetic-related responses did not decrease as fast as in At^{WT} but was also not as gradual as in Ch^{WT}, indicating that HY5 activity in the absence of light is very low (Antón-Sales, 2020). Taken together, these results indicate that HY5 may play a minor role in modulating the senescence response in darkness, in contraposition to PIFs, which assume major roles in it.

HY5 plays a minor role in TIM

Hypocotyl elongation of one week-old seedlings grown at 22°C (control conditions) or at 28°C (warm conditions) (**Figure 5A**) was measured in order to analyze the impact of HY5 on the response of plant growth to warm temperature in the studied genotypes.

When grown at 28°C, L*er*-0 and Col-0 (At^{WT}) seedlings displayed significantly longer hypocotyls than those grown at 22°C. Mutant *hy5-1* and *hy5-2* seedlings were considerably longer than At^{WT} under both normal and warm temperatures. However, only *hy5-2* showed an exaggerated hypocotyl response at 28°C. Seedlings of OX (Ch^{WT}) responded much less to warm temperatures. In addition, the two *chy5* alleles analyzed (*chy5-2* and *chy5-4*) displayed very long hypocotyls at 22°C and a slight temperature-induced hypocotyl growth at 28°C (**Figure 5B**). Two-way ANOVA statistical analyses indicated that HY5 activity impacted the elongation response to warm temperature in Col-0 (*hy5-2* allele) and L*er* (*hy5-1* allele) backgrounds, as well as in OX (c*hy5-4* allele), though not in the *chy5-2* allele.

Recent studies have revealed that the collective genetic activity of *PHYB*, *PIFs*, *HY5*, *COP1* and *DE-ETIOLATED 1* (*DET1*) define an intertwined regulatory hub that acts at the interface between light and temperature signaling (Gaillochet *et al.*, 2020). As part of it, DET1 and COP1, together with HY5, would coordinate to regulate PIFs function and consequently control plant responsiveness to warm temperatures (Bian *et al.*, 2022). Downstream of the thermosensors, PIF transcription factors, and particularly PIF4, mediates shoot growth by binding to the promoters and positively regulating the expression of genes involved in auxin biosynthesis (e.g., *YUC8* and *TAA1*) and signaling (Gaillochet *et al.*, 2020; Casal & Balasubramanian, 2019). Protein levels of this key element of plant thermomorphogenesis are controlled by diverse mechanisms, including phyB activity. In addition to sensing specific wavelengths of the light spectrum, phyB

has also been identified as a thermosensor. This photoreceptor is a known repressor of PIFs activity by promoting its degradation via phosphorylation under light conditions. Warm temperatures are known to trigger the conversion of active phyB (Pfr) to its inactive configuration (Pr), subsequently preventing sequestration and degradation of PIFs and thus, allowing its accumulation (Delker *et al.*, 2022). This regulatory circuit integrates inputs from DET1/COP1 and HY5 as well. Warm temperature increases nuclear accumulation of COP1. In turn, DET1 and COP1 positively control PIF4 at both the transcriptional and protein levels. Furthermore, they also promote thermal-induced shoot elongation in part, by targeting HY5 for proteasomal degradation in darkness. Conversely, HY5 acts antagonistically to PIF4 by competing for PIF binding sites (i.e., G-boxes) in target gene promoters potentially involved in growth responses to temperature (e.g., *YUC8*) and repressing their expression. Modulation of HY5 function could, therefore, fine-tune PIF4-mediated elongation growth (Gangappa & Kumar, 2017).

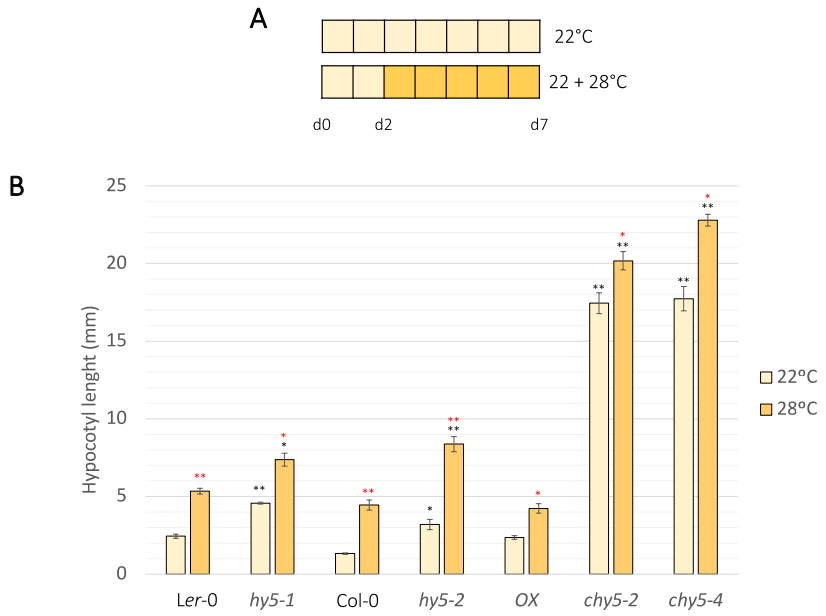

Figure 5. HY5 has a minor role in promoting the *C. hirsuta* hypocotyl elongation response to warm temperature. (A) Seedlings were grown for 7 days in W at 22°C (control conditions) or germinated at 22°C and then transferred at day 2 (d2) to 28°C for the remaining 5 days (22°C + 28°C). **(B)** Hypocotyl length of *A. thaliana* Ler-0 and Col-0 (At^{WT}) and their respective *HY5* null mutants (*hy5-1* and *hy5-2*) as well as C. hirsuta OX (Ch^{WT}) and two null mutant lines (*chy5-2* and *chy5-4*) grown as represented in *A*. Values are means and standard error (SE) of biological triplicates (n≈25 seedlings per replica). Black asterisks represent significant differences between the mutant lines and the corresponding wild-type grown in the same temperature conditions; red asterisks represent significant differences between 22 and 28°C within the same genotype. Student t-test: * p<0.05 and ** p<0.01

In these experiments we showed that *C. hirsuta* seedlings elongated less when exposed to warm temperatures than *A. thaliana* ones. Furthermore, it appeared that HY5 had a bigger impact in the control of hypocotyl elongation in the shade-tolerant species. A possible explanation for the observed differences between species could be the differences in HY5 activity, that is higher in

C. hirsuta. As explained above, HY5 is a negative regulator of thermal-induced hypocotyl elongation so, higher activity of this transcription factor would hinder PIF4 access to its target gene promoters and thus, reduce thermal-induced morphogenesis responses. In addition, *HY5* loss of function mutants for both species showed only a slight hypocotyl elongation at higher temperatures in comparison to control conditions. This observation leads us to conclude that HY5 plays a minor role in the control of thermomorphogenesis, probably because other components of the temperature signaling hub, such as PIF4, are more important for its regulation. At warm temperatures, HY5 protein becomes less abundant (Toledo-Ortiz *et al.*, 2014), likely through increased COP1 activity leading to removal of repression. This coincides with elevated PIF4 accumulation and binding for target gene activation, consequently leading to a significant reduction of the competitive inhibition driven by HY5 under warm temperatures.

phyB hy5 double mutants exhibit a synergistic phenotype

Previous research done in the laboratory indicates that Ch^{WT} seedlings are hyposensitive to monochromatic R in comparison to At^{WT} (Sánchez García, 2022; Molina-Contreras *et al.*, 2019). The photostable photoreceptor phyB is known as the main R receptor and the major phy controlling shade perception. Genetic analyses in *A. thaliana* have shown that phyB-deficient mutants (e.g., *phyB-1*) display long hypocotyls under high R:FR, whereas hypomorphic mutations (partial loss of function, e.g., *phyB-4*) results in an attenuated shade-induced hypocotyl elongation under the same light conditions (Sánchez García, 2022). Although there are no available data about the molecular activity of ChphyB nor its functional role for the shade tolerance implementation in *C. hirsuta*, Ch^{WT} seedlings' response to high R:FR appears to be similar to the *phyB-4* mutant, exhibiting substantially longer hypocotyls than those of At^{WT}. Therefore, suggesting that phyB activity might be attenuated in the shade-tolerant species (Martinez-Garcia & Rodriguez-Concepcion, 2023). Nonetheless, the hyposensitivity to R demonstrated by Ch^{WT} is not enough to fully suppress the shade-induced hypocotyl elongation in this species, requiring the intervention of additional components (Molina-Contreras *et al.*, 2019). Based on the results obtained from our phenotypic characterization of *hy5* mutant lines, we think that HY5 could be participating together with phyB in the establishment of a shade-tolerant habit in *C. hirsuta*.

To investigate the possible functional relationship between phyB and HY5, *phyB-1* and *phyB-4* plants were crossed with *hy5-1* plants to generate double mutants. The F2 progeny was screened for the characteristic long hypocotyl phenotype in W associated with the phyB

mutation and the pre-selected individuals were PCR-genotyped to identify homozygous mutants for *phyB* and *hy5*.

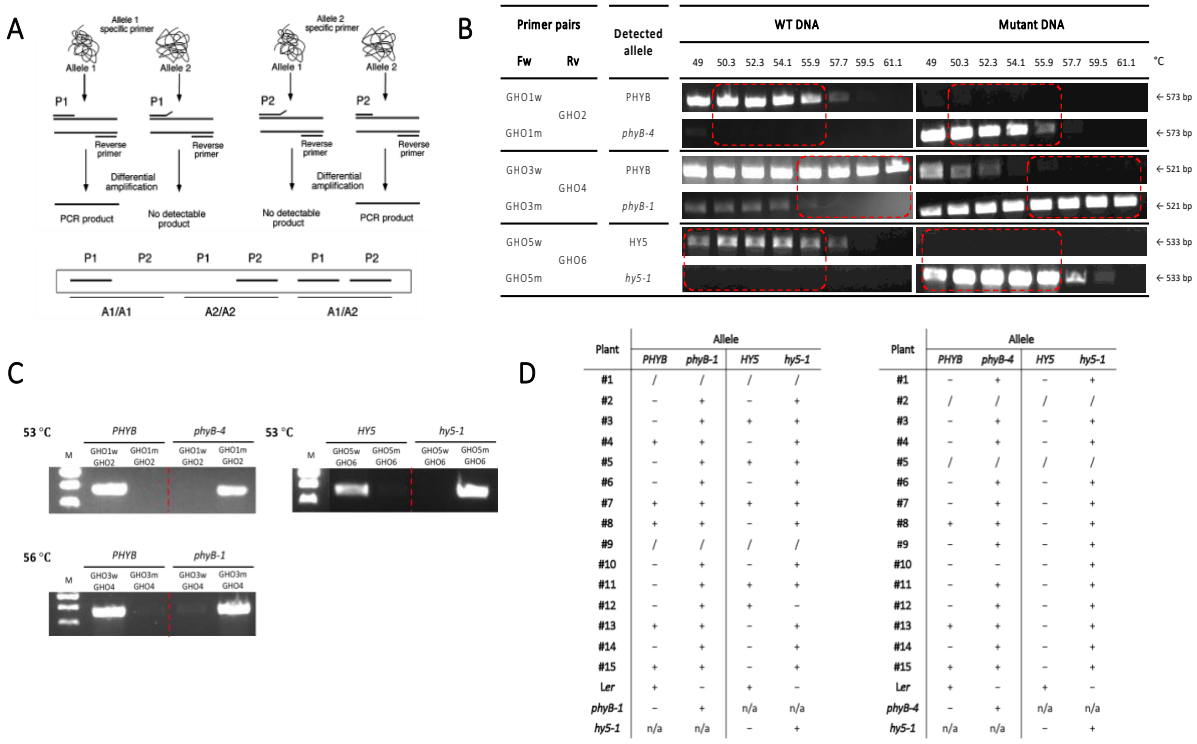

Figure 6. Genotyping *phyB hy5* double mutants. (A) Schematic representation of the allele-specific PCR strategy. Primers with 3' nucleotides that correspond to an SNP site are used to preferentially amplify specific alleles. Primer P1 forms a perfect match with allele 1 but forms a mismatch at the 3' terminus with the DNA sequence of allele 2. Primer P2 forms a perfect match with allele 2 and a 3' terminus mismatch with allele 1. Below is represented the agarose gel analysis showing the expected outcome for the amplification of organisms homozygous and heterozygous for both alleles using primers P1 and P2. P1 = Primer 1; P2 = primer 2; A1 = allele 1; A2 = allele 2. **(B)** Analysis of specificity for designed SNAP-PCR primer pairs (left) using a temperature gradient from 49°C to 61.1°C. PCR reactions in lanes 1 to 8 were performed using L*er*-0 DNA, whereas the reactions in lanes 9 to 16 were performed using the corresponding mutant line DNA. Red boxes marked the temperature range which allows the distinction of mutant alleles from their respective wild-types. (**C**) Analysis of specificity for designed SNAP-PCR primer pairs using the selected optimal annealing temperatures (53°C for both *phyB-4* and *hy5-1*, and 56°C for *phyB-1*) for conducting the screening of *phyB hy5* double mutants. (**D**) Results of gradient PCR screening of *phyB-1 hy5-1* and *phyB-4 hy5-1* double mutant plants. The products amplified by PCR were analyzed by agarose gel electrophoresis: – = absence of band; + = presence of band; / = not assessed due to plant death, and n/a = not applicable.

The only difference between *phyB-1*, *phyB-4* and *hy5-1* mutant alleles and their respective wild-type alleles is just one nucleotide. To overcome this difficult for the PCR-genotyping, we employed a modified allele-specific PCR procedure for assaying single nucleotide polymorphisms (SNPs) known as SNAP (for single-nucleotide amplified polymorphisms). For that purpose, allele-specific primers were designed such that the 3' nucleotide corresponds to the site of the SNP and an extra mismatch is added within the last four bases of the 3' end. Because mismatched 3' termini are extended by DNA polymerases with much lower efficiency than correctly matched termini, the allele-specific primer would preferentially amplify the specific allele (e.g., *PHYB*) over the nonspecific one (e.g., *phyB-1*) (**Figure 6A**). The additional introduced

base pair change has been demonstrated to produce a significant increase in the specificity of the primer by dramatically reducing the PCR product yield of the nonspecific allele (Drenkard *et al.*, 2000).

After designing the primers, our next step was the determination of the experimental conditions that would ensure primer specificity. To achieve this, eight PCR reactions were carried out using L*er*-0, *phyB-1*, *phyB-4* and *hy5-1* genomic DNA as the DNA templates. In order to observe the amplification of each primer pair as they approached their optimal melting temperature (T_m), the standard PCR temperature profile was modified to create a gradient from 49°C to 61°C during the annealing phase. This gradient reaction allowed us to select the optimal annealing temperature that permitted the distinction of mutant alleles from their corresponding wild-type allele (**Figure 6B**). After various test runs, the selected annealing temperatures for conducting the genotyping were: 53°C for both *phyB-4* and *hy5-1*, and 56°C for *phyB-1* (**Figure 6C**). Out of the 15 pre-selected F2 plants for each cross, 4 were homozygous for *hy5-1* and *phyB-1*, and 9 were homozygous for *hy5-1* and *phyB-1* (**Figure 6D**). The hypocotyl response of the obtained double mutants was then analyzed in W conditions.

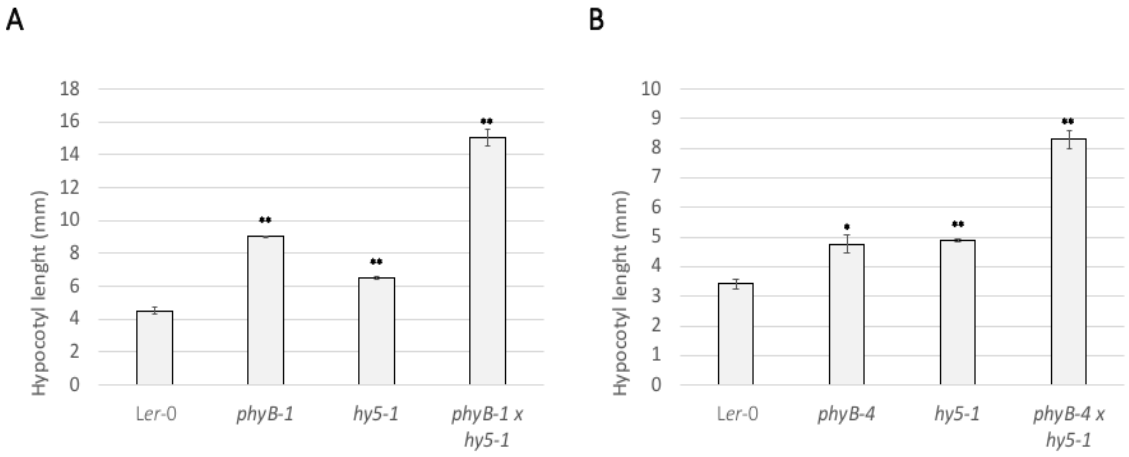

Figure 7. *hy5 phyB* double mutants display a synergistic phenotype. (**A**) Hypocotyl length of L*er*-0, *phyB-1*, *hy5-1* and *phyB-1 hy5-1* seedlings. (**B**) Hypocotyl length of L*er*-0, *phyB-4*, *hy5-1* and *phyB-4 hy5-1* seedlings. Seedlings were grown for 7 days in W. In **A** and **B**, values are mean and standard error (SE) of biological triplicates (n≈25 seedlings per replica). Black asterisks represent significant differences between the mutant lines and the corresponding wild-type. Student t-test: * p<0.05 and ** p<0.01.

When grown under W, *phyB-1* and *hy5-1* had longer hypocotyls in comparison with wild-type (L*er*-0), although *hy5-1* did not elongate as much as *phyB-1* did. Double mutant *hy5-1 phB-1* seedlings were much longer than those of the *hy5-1* and *phyB-1* single mutants (**Figure 7A**). W-grown *phyB-4* and *hy5-1* seedlings were longer than wild-type ones, both showing a similar elongation response. The hypocotyl length of the *hy5-1 phyB-4* double mutant greatly surpassed that of *hy5-1* and *phyB-4* single mutants (**Figure 7B**).

Phenotypic analysis showed that the joint contribution of *phyB* and *hy5* mutations to the hypocotyl elongation of a double mutant is greater than the sum of their individual effects. These results indicate that there might be a synergistic effect between *PHYB* and *HY5*, which would explain the additional hypocotyl elongation of the double mutant under W. Two-way ANOVA statistical analyses further revealed an interaction between both genes, therefore indicating that they might contribute to the repression of hypocotyl elongation in high R:FR environments through different pathways that converge at a given node. These results might also help to explain the enhanced elongation under W observed in the *chy5* mutants compared to its wild-type (Ch^{WT}), in contrast to the mild elongation observed in the *hy5* mutant seedlings compared to their wild-type lines (At^{WT}) (**Figure 3**).

Differences in protein abundance or stability between AtHY5 and ChHY5 couldn't be detected

As mentioned above (**Figure 3**), we hypothesized that ChHY5 activity may be higher than that of its ortholog, AtHY5. Differences in protein activity could be the result of (i) differential gene expression, (ii) post-translational regulation and/or (iii) intrinsic protein activity differences, which in turn would lead to a higher specific activity of the HY5 protein in the shade-tolerant species. Results of the laboratory indicated that *HY5* expression levels were comparable between both species (Vilarmau-Marsinyach, 2018), which argued against differential gene expression as the cause of the observed differences in protein activity.

Predicted AtHY5 and ChHY5 primary structure is very similar; both proteins have the same number of residues (168) and there are only three conservative replacements throughout the entire sequence: V51A, S76T and R133K (**Figure 8A**). Still, it is not known whether these molecular differences could be reflected in changes in protein abundance or stability and, thus, be responsible for the observed differences in biological activities between ChHY5 and AtHY5. To explore this possibility, we agroinfiltrated *N. benthamiana* leaves with constructs that overexpress either *AtHY5* or *ChHY5* fused to a 3xHA tag, as well as *mGFP5* (**Figure 8B**). GFP was used as a loading control to normalize obtained relative levels of HY5 protein (Paulišić *et al.*, 2021). Samples were collected three days after the agroinfiltration, and protein extracts were prepared to quantify HY5 abundance through immunoblotting using commercial anti-HA antibodies.

One minute of blot exposure was enough to confirm the presence of target bands. Nonetheless, increasing exposure time to 15 min helped to detect them more clearly. Even though the predicted size of HY5-3xHA fusion protein is 23 kDa, we detected its signal near 50 kDa on our blots. The cause of this size disparity is unclear at present. On the other hand, GFP, whose predicted size is 27 kDa, was accurately detected between 25 and 35 kDa (**Figure 8C**). The observed variation between biological replicates prevented the precise detection of biological relevant effects in independent samples, thus obstructing AtHY5 and ChHY5 comparison. In addition, the perceived variability in the GFP signal hindered the protein quantification altogether since, without normalization, an apparent difference in target abundance on a Western blot cannot be accurately interpreted (Pillai-Kastoori *et al.*, 2020). Therefore, quantitative immunoblot analyses were deemed non-viable due to the various problems encountered in regard to reproducibility.

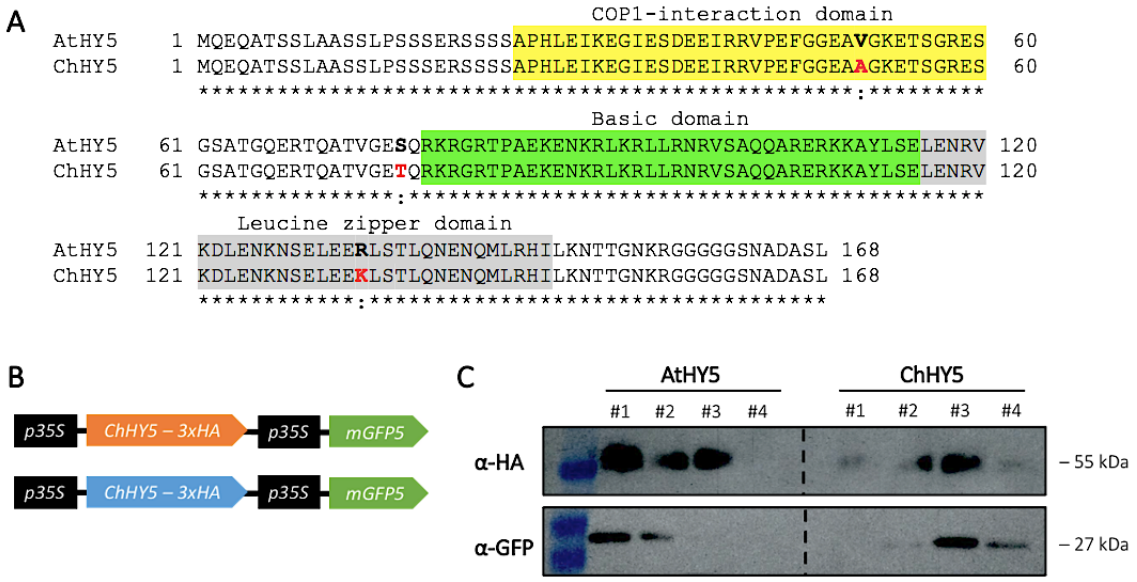

Figure 8. Comparative analysis of HY5 stability between *A. thaliana* and *C. hirsuta*. (A) Global alignment of AtHY5 and ChHy5 proteins. Distribution of identified domains of AtHY5 are highlighted in different colors: Yellow = COP1 interaction domain, Green = Basic domain, and Grey = Leucine zipper domain. Amino acidic substitutions in ChHY5 are marked in red color. **(B)** Cartoon of constructs containing AtHY5 or ChHY5 under the 35S promoter used for transient expression of transgenes in *N. benthamiana* leaves. **(C)** Representative immunoblots of AtHY5 and ChHY5 separated on a 12% SDS-PAGE are shown. Upper panel: α-HA was used to detect AtHY5 and ChHY5. Lower panel: α-GFP was used as a loading control. Samples were collected from *N. benthamiana* leaves 3 days after the agroinfiltration. **A** and **B** are modified from Quin, 2022.

To sum up, from the three possibilities considered to explain the enhanced activity of ChHY5 compared to AtHY5 [(i) differential gene expression, (ii) post-translational regulation and/or (iii) intrinsic protein activity differences], we could only discard the first. To circumvent the encountered problems when addressing the second possibility, we decided to use a different approach: stable genetic transformation. *A. thaliana* wild-type plants were transformed using

floral dipping with the same constructs used for transient expression in *N. benthamiana* leaves. With this technique we expect to at least reduce the observed variability between biological replicas. Currently, we are in the process of selecting successfully transformed individuals to continue on with the next steps of the process.

HY5 interaction with other light-signaling components using a Y2H approach

HY5 promotes photomorphogenesis by physically interacting with other light-signaling components, including both regulatory proteins (e.g., COP1) and transcriptional regulators factors (e.g., HFR1, HYH, BBX) (Gangappa & Botto, 2016). These interactions are achieved thanks to the leucine zipper domain located in the C-terminal region of this bZIP protein, which confers the ability to form either homo- (Yoon *et al.*, 2007) or heterodimers with other bZIP proteins, or via the VP motif in its N-terminal end, with proteins such as COP1 (Zhao *et al.*, 2020; Chen *et al.*, 2013; Jang *et al.*, 2013; Holm *et al.*, 2002).

Other authors have shown by in vitro pull-down and in vivo assays that HY5 can also associate with HFR1 (Jang *et al.*, 2013). Since both proteins are targets of the COP1 E3 ligase, it is presumed that they dimerize as a way to modulate their stability, similarly as what has already been documented for HFR1 interactions with other transcription factors (Jang *et al.*, 2013). Additionally, it has been demonstrated through several in vitro and in vivo assays that HY5 can form heterodimers with PIF1 and PIF3 to antagonistically regulate their activities (Chen *et al.*, 2013). As HFR1, PIF1 and PIF3 all are bHLH proteins, these previously confirmed interactions led us to think that HY5 could also physically and directly interact with PIF7, the main AtPIF promoting hypocotyl elongation in response to shade. Considering that HFR1, another negative regulator of SAS, interacts with several PIFs in *A. thaliana* and sequesters them to form nonfunctional heterodimers (Paulišić *et al.*, 2021), we speculate that HY5 could act in a similar way to antagonize PIF7 activity and contribute to the development of *C. hirsuta* shade tolerance habit. Therefore, we performed a Y2H directed assay in order to (i) test whether we can replicate the already described HY5 interactions with itself and other light-signaling components in our laboratory conditions, and (ii) explore a possible interaction between HY5 and PIF7.

Yeast mating was verified for all the studied combinations by plating yeast liquid cultures on SD-LW solid medium, whereas the protein-protein interactions were confirmed by examining cell growth on SD-AHLW solid medium. The red color exhibited by yeast colonies growing on SD-LW served as another means to further confirm protein-protein interactions. If fusion proteins do not interact, the GAL4 BD cannot activate transcription of downstream reporter genes. This

includes *ADE2*, a gene which encodes an enzyme involved in the *de novo* purine nucleotide biosynthetic pathway. Consequently, when *ade2* mutant diploid yeast cells are plated on a medium low in adenine and there is no association between the produced fusion proteins, they are not able to biosynthesize this nucleotide due to the lack of *ADE2* expression. In its place the cells accumulate an adenine intermediate known as 5-aminoimidazole ribotide that, when oxidized, confers pink-red color to the colonies.

Negative controls were performed by setting up yeast mating of transformants containing the plasmids we wished to test in combination with transformants containing the corresponding control plasmids (BD- or AD-Ø). Diploid yeast cells should be able to grow on the SD-LW, thus indicating that the mating occurred successfully; while on SD-AHLW, they should not be able to survive, showing that the produced hybrid proteins cannot autoactivate the reporter genes. As positive controls we incorporated two interactions already confirmed under our laboratory conditions: HFR1 with PIF1 and PIF7 (Paulišić *et al.*, 2021; Bou-Torrent *et al.*, 2015).

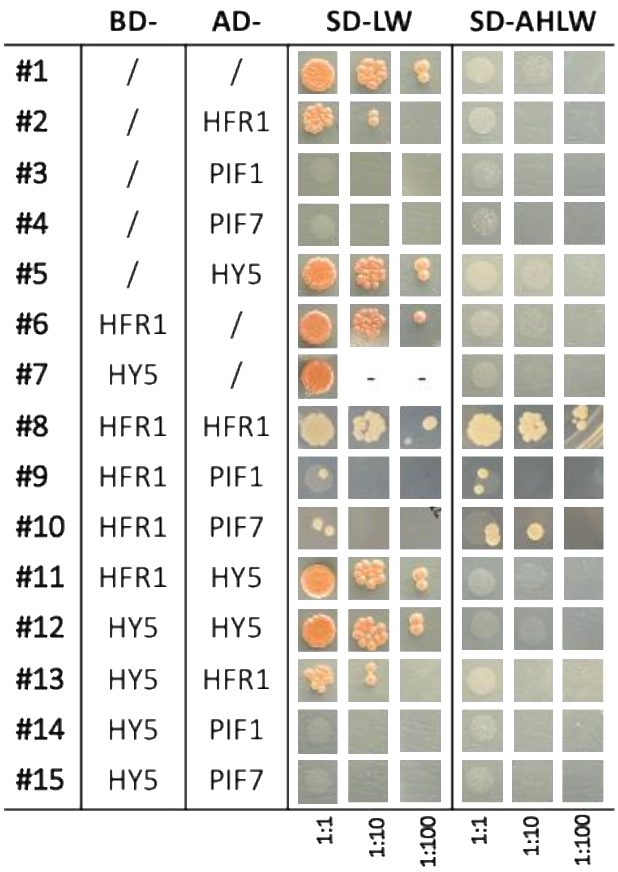

Figure 9. Y2H growth assay to detect HY5 partners. The BD- and the AD- derivative constructs used in the assay are shown on the left side of the panel. SD-LW and SD-AHLW refer to the selective medium indicative of transformed cells or interaction between the hybrid proteins, respectively. SD-LW yeast liquid cultures were plated as drops in dilutions of 1, 1:10 and 1:100. HFR1 (BD-fused) and PIF1 and PIF7 (both AD-fused), known to interact, were used as a positive control. Empty vectors (/) were used as negative controls.

After four days of incubation, several colonies grew on SD-LW, confirming that the mating worked properly in all cases. The only exception were diploid yeast cells producing either AD-PIF1 or AD-PIF7 in combination with BD-Ø and HY5-BD. Previously, we had already observed that haploid yeast cells producing these PIFs hybrid proteins grew remarkably slower and were significantly smaller in comparison to yeast cells producing other fusion proteins. This could be an indication that these transcription factors are toxic to the yeast cells so, to overcome this problem we may have to switch to a vector that has a lower level of expression, such as pGBT9 (Takara Bio, 2013). Aside from that, all the other negative control combinations grew as expected on SD-LW and not on SD-AHLW. Regarding the positive control pairs, the observed HFR1 homodimerization indicated that its HLH domain, indispensable for the dimerization ability of this bHLH protein, was functional in this assay. Consequently, we could confirm its interaction with PIF1 and PIF7, although the PIFs negative controls couldn't be validated. Conversely, we failed to detect a direct interaction between HY5 and any other given protein. The cause of this negative result is unclear at present (**Figure 9**). To summarize, although we were able to use the Y2H approach to confirm some previous interactions (e.g., HFR1 homodimerization), we were not able to corroborate HY5 homodimerization or interactions with other light-signaling components, including PIF7. Consequently, no clear conclusions could be reached from this experiment.

Conclusions

1. The phenotypes displayed by *A. thaliana* and *C. hirsuta* HY5-deficient mutants in a range of light, dark and temperature treatments indicate that this bZIP transcription factor participates in the development of a shade tolerant habit in *C. hirsuta*. Our results also evidence the key role that HY5 plays in repressing hypocotyl elongation in shade conditions, while having minor contributions to the regulation of DIS and thermomorphogenesis. In addition, the lack of shade-induced hypocotyl elongation of *C. hirsuta* wild-type plants support our working hypothesis of HY5 activity being higher in *C. hirsuta* than in *A. thaliana*. Conversely, although the lack of thermal-induced hypocotyl elongation and the delay in this DIS exhibited by them are not consistent with this hypothesis, they can be reconciled with it due to the secondary role that HY5 assumes in the regulation of these processes.

2. The synergistic effect between *PHYB* and *HY5* suggested that these two genes might cooperate to suppress the shade-induced hypocotyl elongation in *C. hirsuta*.

3. Protein sequence similarities and previous information of the lab lead to the rejection of the premise of differential gene expression as the cause of the enhanced activity of ChHY5 compared to AtHY5. Out of the three possibilities considered to explain the discrepancies in protein activity, post-translational regulation and intrinsic protein activity differences remain to be addressed.

4. The discovery of more protein-protein interactions might enable a better understanding of the regulatory complexity underlying HY5 function in response to environmental cues. A Y2H approach could be helpful for confirming still unknown interactions.

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Supplementary information

Table S1. Primers used for cloning and genotyping.

Gene	Primer name	**Sequence (5′ → 3′)**	Description
PHYB	GHO1w	TAAGTTTCATGAAGATGTGC	Forward primer for genotyping and discriminating *PHYB* from *phyB-4* mutant allele
	GHO1m	TAAGTTTCATGAAGATGTGT	
	GHO2	CTCTGTGTAACAATTCCAGC	Reverse primer for GHO1w/m
	GHO3w	GAAACGCGTTTTGAGAAGGC	Forward primer for genotyping and discriminating *PHYB* from *phyB-1* mutant allele
	GHO3m	GAAACGCGTTTTGAGAAGGT	
	GHO4	*TCAGAATAAGCTGGAGCGAG*	Reverse primer for GHO3w/m
HY5	GHO5w	GAGTAAGAAAAATGCAGGTAC	Forward primer for genotyping and discriminating *HY5* from *hy5-1* mutant allele
	GHO5m	GAGTAAGAAAAATGCAGGTAT	
	GHO6	GAATAGAGAAAAAGACACCTC	Reverse primer for GHO5w/m
	GHO8	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAAAGGCTTGCATCAGC	Reverse primer to clone *AtHY5* flanked by attB1 and attB2
	MVTO3	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGCAGGAACAAGCGACT	Forward primer to clone *AtHY5* flanked by attB1 and attB2
	JRO13	TCATCAAGCTCTGCTCCAC	Forward primer for *HY5*
	HY5Rv	GCTGAGCTGAAACTCTGTTC	Reverse primer for *HY5*
-	MJO27	GGGGACAAGTTTGTACAAAAAAGCAGGCT	Primer for attB1
	MJO28	GGGGACCACTTTGTACAAGAAAGCTGGGT	Primer for attB2
	SEO2	GTAACATCAGAGATTTTGAGACAC	Forward primer for PDONR207

Table S2. Plasmids used for transient expression of *HY5* in *Nicotiana benthamiana* leaves and Yeast two Hybrid directed assays.

Name	Vector	Insert	Description	Resistance
pGH1	pDNOR207	*AtHY5*	*HY5* flanked by attL1 and attL2 in a GW entry vector	Gentamycin
pGH2	pGBKT7-GW	*AtHY5*	BD-HY5 construct for Y2H	Kanamycin
pGH3	pGADT7-GW	*AtHY5*	AD-HY5 construct for Y2H	Carbenicillin
pWQ8	PCRII-TOPO	*AtHY5*	*HY5* cloned into a TOPO-TA vector	Kanamycin, Carbenicillin
pWQ21	pSP135	*AtHY5-3xHA*	Construct for transient expression of *AtHY5* in *N. benthamiana*	Kanamycin, Hygromycin
PWQ22	pSP135	*ChHY5-3xHA*	Construct for transient expression of *ChHY5* in *N. benthamiana*	Kanamycin, Hygromycin
pGBKT7	pGBKT7-GW	-	Negative control for Y2H	Kanamycin
pGADT7	pGADT7-GW	-	Negative control for Y2H	Carbenicillin
pJB37	pGBKT7-GW	*AtHFR1-3xHA*	BD-HFR1 construct for Y2H	Kanamycin
pSP118	pGADT7-GW	*AtHFR1-3xHA*	AD-HFR1 construct for Y2H	Carbenicillin
pBA11	pGADT7-GW	*AtPIF7-3xHA*	AD-PIF7 construct for Y2H	Carbenicillin
pJB62	pGADT7-GW	*AtPIF1-3xHA*	AD-PIF1 construct for Y2H	Carbenicillin

Table S3. Yeast two Hybrid cloning vectors.

Vector	Description	Selection on SD Medium
pGBKT7-GW	*GAL4* DNA-BD, *TRP1*, kan^r	-Trp
pGADT7-GW	*GAL4* AD, *LEU2*, amp^r	-Leu

Table S4. Yeast strains used in Yeast two Hybrid.

Strain	System	**Genotype**	Transformation marker	**Reporter(s)**
pJ694α	GAL4 2H	*MATα, trp1-901, leu2-3,112, his3-200, ura3-52, gal4Δ, gal80Δ, LYS2::GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ*	*trp1*	*HIS3, ADE2, LacZ*
YM4271a	GAL4 2H	*MATa, trp1-901, leu2-3, 112, his3-200, lys2-801, ura3-52, ade2-101, ade5, tyr1-501, gal4Δ, gal80Δ, ade5::hisG*	*leu2*	-