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Investigation into temperature effects on the plant light signalling pathways.

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Declarations

This thesis contains original work based on the following publication:

Light receptor action is critical for maintaining plant biomass at warm ambient temperatures. Plant J. 2011 Feb;65(3):441-52.

* indicates joint first authorship

I hereby declare that this thesis is my own work except where explicitly stated. No part of this thesis has been submitted for a professional qualification or a degree at the University of Edinburgh or any other university.

Âke Henrik Johansson
April, 2013
Abstract

The ability to withstand environmental temperature variation is essential for plant survival. Formative studies in Arabidopsis have revealed that light signalling pathways has a potentially unique role in shielding plant growth and development from seasonal and daily fluctuations in temperature. In this thesis we further investigate the integration of the light signalling networks and temperature signalling on the molecular level in Arabidopsis. First, we identified the transcript of the bHLH transcription factor LONG HYPOCHOTYL IN FAR-RED 1 (HFR1) to be highly dependent on the ambient temperature and under strong control of the red light photoreceptor PHYTOCHROME B (phyB). We found that the long hypocotyl phenotype of the hfr1 mutant was exaggerated in warm conditions, specifically in blue light, downstream of cryptochrome 1. We further show that HFR1 acts in the warm by suppressing the function of PHYTOCHROME INTERACTING FACTOR 4 and 5 (PIF4, PIF5). PIF4 appears to act as a master regulator of several temperature responses and is directly regulated by the phytochromes. Thus, we define a molecular network where red light and blue light signals together with temperature merge on the regulation of PIF4.

In the second part of this thesis, we investigate the relationship between temperature and the fluence rate of light on the inhibition of hypocotyl elongation in Arabidopsis. We find that the response to increasing fluence rates of light is highly dependent on the ambient temperature and that PIF4 and PIF5 acting downstream of the major red light photoreceptor, phyB, are essential for this response. In addition, we provide evidence that in cool conditions, PIF activity is under strong suppression by the gibberellin and HY5 pathways specifically at high fluence rates of red light.

The collected work of this thesis highlights the importance of the PIF proteins as integrators of temperature and light signals and furthermore, demonstrates that the response to temperature is highly dependent on both the quality and quantity of light.
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Chapter 1 - Introduction

1.1. Light perception in Arabidopsis

Plants are sessile organisms and must therefore constantly adapt to the surrounding environment in order to optimize development and growth. Since light is the main source of energy for most plants, this external signal could be regarded as the most important for plants to monitor and respond to. Consequently, plants are able to monitor the quality, quantity, duration and direction of the incoming light [1]. Great amounts of research have been conducted to reveal how plants perceive light and how this signal is transduced leading to the physiological plant response. Most of this work has been carried out using the model organism Arabidopsis thaliana.

As an external signal, light has been shown to control many aspects of plant growth and development throughout the plant lifecycle. In nature, a seed is likely to end up under a layer of soil or litter, where there is no or little light available. Upon germination (a process regulated by light [2]), the seedling will undergo etiolated or skotomorphogenic development resulting in an elongated hypocotyl with an apical hook protecting the undeveloped cotyledons in the pursuit of more favourable light conditions. If this strategy is successful, light activation of the photoreceptors will result in de-etiolation and the start of photomorphogenesis, where the elongation of the hypocotyl is inhibited, the apical hook unfolds and the cotyledons develop in
order to harvest energy from the surrounding light [3]. As the growing plant obtains photosynthetic capacity, it starts to compete with the neighbouring plants for the light. By measuring the quality of the surrounding light, the plant can monitor the immediate foliage and will if necessary initiate the shade avoidance response in order to grow out of an overhanging canopy [4]. Furthermore, as for most organisms, light also acts as the major input to the circadian clock [5]. This allows for the plant to keep track of the day length over the seasons, and as a facultative long day plant, Arabidopsis flowers early in long day conditions [6]. Thus, light as an external signal affects the plant throughout development and therefore it is not surprising that plants have evolved a battery of photoreceptors to facilitate these responses.

![Figure 1.1. Absorption spectra of the Arabidopsis light receptors](image)

**Figure 1.1. Absorption spectra of the Arabidopsis light receptors**

uvr8 acts as a UV-B photoreceptor while PHOT1-2 and CRY1-2 perceive light in the UV-A and blue range of the spectra. PHYA-E acts as receptors of the red to far-red light spectra.

The perception of red and far-red light in Arabidopsis is through a group of proteins consisting of five members called Phytochromes (PhyA-E) (further discussed in 1.1.1) (Figure 1.1). Blue light is perceived by the two Cryptochromes (Cry1-2) (further discussed in 1.1.2) and the two Phototropins (Phot1-2) (Figure 1.1). Both phot1 and phot2 have been shown to regulate phototropic response, stomatal opening...
and leaf movements. In addition, phot1 mediates rapid inhibition of hypocotyl elongation while phot2 regulates chloroplast movements [7]. Furthermore, the recently identified UVR8 acts as a photoreceptor in the UV-B range of the light spectrum regulating a range of light specific responses (Figure 1.1) [8-11]. However, in this thesis, we will focus on red light as perceived by the phytochromes and blue light perceived by the cryptochromes and their regulation of seedling development.

1.1.1. Phytochromes as red/far-red reversible switches

In plants, the phytochromes constitute a small family of proteins with an ancient evolutionary origin [12, 13]. Phytochromes are produced as apo-proteins and only after covalent binding to phytochromobilin, the holo-protein will be able to perceive light and act as a photoreceptor [14]. In darkness the phytochromes are in their Pr conformation which is the inactive ground state. The Pr state is stable and has an absorption maximum of 660nm. Upon red light radiation, the dimerized holo-proteins undergo a conformational change from the Pr to the Pfr (active) form which preferentially absorbs light in the far-red spectra (730nm). In addition to far-red absorption, a slower thermal relaxation of the Pfr to Pr form named dark reversion acts to promote the inactive Pr form [15]. These processes ultimately allows for the establishment of a Pr/Pfr ratio which is highly dependent on the relative levels of red and far-red light allowing the plant to sense and respond changes in the spectral composition [4]. Provision of an end-of-day (EOD) far-red pulse, that rapidly converts Pfr to Pr, has been used extensively as an experimental tool to reduce the persistence of Pfr in darkness [16].
In Arabidopsis, the phytochrome family consists of five members (phyA-E) with both overlapping and distinct functions [17, 18]. Synthesised in their inactive (Pr) form they are localised to the cytosol and rapidly translocated to the nucleus upon light activation [19] [20] [21]. While phyB-phyE works as red/far-red reversible switches, phyA is functionally distinct, signalling in response to very low fluencies of light or continuous far-red irradiation to regulate a wide array of responses, from germination to de-etiolation and flowering [22-28]. Furthermore, although all phytochromes are light labile to some extent, phyA is rapidly degraded in response to red light [29-31]. However, phyA has been reported to be stabilised under very high fluence rates of red light >100µmol/m²/s acting to promote de-etiolation in the phyB background [32].

Phenotypic analysis of phyB mutants suggests that phyB is the major photoreceptor in continuous red light exhibiting a severely elongated hypocotyl, small cotyledons and reduced chlorophyll synthesis [33, 34]. Additionally, phyB acts to suppress CONSTANS (CO) protein levels, which acts directly on the FLOWERING LOCUS T (FT) promoter to regulate flowering time [35]. Analysis of phyC, phyD and phyE have revealed minor effects in red light suggesting that they also contribute to photomorphogenic development in Arabidopsis [36-38].

Possibly the most dramatic light response is the de-etiolation when a dark grown seedling with an elongated hypocotyl and undeveloped cotyledons is exposed to light which results in rapid inhibition of hypocotyl elongation, opening of the apical hook and expansion of the cotyledons. This transition is accompanied with a massive transcriptional response, affecting up to one third of the genome [39]. In accordance with their rapid light induced nuclear localisation, the phytochromes have been found
to promote and suppress the accumulation of negative and positive factors of photomorphogenesis respectively, many of which are transcription factors (Figure 1.2) [40]. Most notably are the inhibition of the E3 ubiquitin ligase, CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) and the degradation of the PHYTOCHROME INTERACTING FACTORS (PIFs) bHLH transcription factors (discussed below).

1.1.2. The cryptochromes perceive light in the blue range of the visible spectra

The cryptochromes are photolyase-like flavoproteins with important roles in seedling de-etiolation and photoperiodic control of flowering [41]. In Arabidopsis there are 3 cryptochromes (cry1-3) where the function of cry1 and cry2 in the nucleus are well studied while less is known about cry3 that is localised to the organelles [42]. Mutants of both cry1 and cry2 was initially identified in screens as having a long hypocotyl specifically when grown in blue light[43, 44]. Both cry1 and cry2 are activated and phosphorylated upon blue light irradiation [45, 46]. However, while cry1 appears to move between the cytosol and the nucleus, cry2 is exclusively localised in the nucleus [47-49]. cry1 have been shown to act at high fluencies to promote photomorphogenesis whereas cry2 acts at low fluencies of blue light [43, 50], which is consistent with the observation that cry2 is a light labile and cry1 is a light stable protein [50]. However, cry1cry2 double mutant seedlings grown in blue light are largely etiolated, exhibiting a stronger phenotype than both cry1 and cry2 indicative of significant functional redundancy [51, 52] and demonstrating that these
two proteins are the main photoreceptors in blue light to promote photomorphogenic growth [53]. As for the phytochromes, the cryptochromes control seedling de-etiolation at least partly by deactivating COP1, resulting in the accumulation of positive factors of photomorphogenesis (Figure 1.2) [54-60]

1.2 Light signalling networks

The perception of light by the photoreceptors results in a dramatic reprogramming of the transcriptome [39]. At this point, there is no evidence that any of the photoreceptors can bind directly to DNA to regulate transcription. Instead, the active photoreceptors seem to act on a limited set of key factors to relay the light signal to the transcript level. In particular, both the cryptochromes and phytochromes act to suppress the activity of the E3 ubiquitin ligase COP1 either directly or indirectly [56, 61]. Furthermore, phyA and phyB interacts with and degrades the PIF transcription factors that are central regulators of photomorphogenesis [62, 63].

1.2.1. COP1 is a central negative regulator of photomorphogenesis

Forward genetic screens identified a group of proteins referred to as COP/DET/FUS [64]. These mutants displayed photomorphogenic growth in darkness, and their recessive nature indicated that they act to suppress de-etiolation in the dark. Of these, the E3 ubiquitin ligase, COP1 is extensively characterised, acting as a master regulator of the transition from etiolated to de-etiolated development. A null cop1 mutant is lethal at the seedling stage, however, characterisation of weak alleles show pleotropic effects throughout the plant life cycle, including high anthocyanine
accumulation, dwarf stature and early flowering [65, 66]. Consistent with a role as a key repressor of photomorphogenic development, transcriptome analysis of dark grown cop1 seedlings compared to light grown wild type seedlings show a remarkable overlap [67]. Moreover, when grown on sucrose supplemented media, the cop1-6 mutant is able to achieve reproductive growth, suggesting that also other developmental transitions are de-repressed in the cop1 mutant [65]. Thus, photoreceptor dependent inactivation of COP1 is both sufficient and necessary to initiate photomorphogenic growth.

COP1 is localized to the nucleus in the dark [68], where it acts to target a range of positive regulators of photomorphogenesis for degradation via the 26S proteasome. Upon transfer to light, COP1 activity is inhibited by photoreceptor dependent exclusion from the nucleus [61]. Although the mechanism for this translocation is unknown, nuclear exclusion is evident in response to red, far-red and blue light indicating that both phytochromes and cryptochromes are regulating this response (Figure 1.2) [61]. Additionally, the activity of COP1 has been shown to be directly inhibited by light activation of the cryptochromes [56, 69, 70]. The resulting inhibition of COP1 leads to accumulation of factors that promote photomorphogenic development such as LONG HYPOCOTYL5 (HY5), HY5 HOMOLOG (HYH), and LONG HYPOCOTYL IN FAR-RED1 (HFR1) among others [58-60].

1.2.2. HY5 promotes photomorphogenesis downstream of COP1

Possibly the best characterised transcription factor acting downstream of the photoreceptors is the bZIP transcription factor HY5. Mutants of hy5 exhibit a long hypocotyl phenotype in all light conditions suggesting that HY5 acts downstream of
the phytochromes, cryptochromes and uvr8 [71-73]. Additionally, mutational analysis has shown that HY5 promotes chlorophyll and anthocyanine accumulation as well as having a role in both auxin and cytokinin signalling pathways [59, 73-76]. HY5 is a target of COP1, and as such, protein levels are depleted in darkness and accumulate after transition to light [58]. Consistently, hy5 is able to completely suppress the dark phenotype of the cop1 mutant which is indicative of its importance in de-etiolation [77]. As a transcription factor, HY5 has been shown to bind to more than 9000 genes and affecting the expression of over 1000 transcripts [78] including many light-regulated genes as well as genes encoding transcription factors [79].

1.2.3. HFR1 acts in blue and far-red light downstream of COP1

HFR1 is a PIF-like (PIL) bHLH transcription factor, residing in subfamily 15 of the bHLH transcription factor family. However, in contrast to several other members (PIFs) promoting elongated growth, HFR1 is a positive regulator of photomorphogenesis and is lacking any apparent phyA or phyB binding domain [63, 80]. hfr1 was initially identified as a mutant hyposensitive to far-red light acting downstream of phyA [81-83]. Further studies demonstrated that HFR1 also acts as a positive regulator of photomorphogenesis under blue light, mainly dependent on the blue light photoreceptor cry1 but also acting downstream of phyA under lower fluence rates [84]. As HY5, HFR1 is targeted for degradation by COP1 and accumulates in response to light treatment [60, 85]. Although overexpressed HFR1 has been shown to accumulate in response to all light conditions [86], as would be expected for a COP1 target. HFR1 transcription is strongly suppressed by red light, consistent with the lack of a red light phenotype in the hfr1 mutant [81]. HFR1 was
also identified as a transcript highly upregulated in response to a shade. Analysis of the mutant in these conditions suggested that HFR1 acts as a negative regulator of the shade avoidance syndrome in order to restrain an exaggerated response [87]. Under these conditions, it was recently shown that HFR1 can form heterodimers with the related bHLH transcription factors PIF4 and PIF5, inhibiting their transcriptional activity [88, 89]. Thus, under low R:FR light, which elevates the positive regulators of shade responses PIF4 and PIF5, HFR1 appears to moderate the activity of these growth promoters. As \textit{pif4pif5} appears largely epistatic to \textit{hfr1} also in monochromatic far-red light, inhibition of PIFs is likely to constitute the main mechanism by which HFR1 is regulating light responses (Figure 1.2) [88].

\subsection{1.2.4. Phytochrome interacting factors promote elongated growth}

In addition to inactivation of COP1, light activated phytochromes (Pfr) have been found to act directly on a group of bHLH transcription factors named \textit{PHYTOCHROME INTERACTING FACTORs}. As HFR1, the PIFs belong to subfamily 15 of the bHLH superfamily [80, 90]. As the founding member, PIF3 was first identified in a yeast two hybrid screen using phyB as bait, and was later shown to interact specifically with the Pfr form of both phyA and phyB [91]. The binding sites for phyA and phyB were identified as two discrete domains named Active Phytochrome A-binding (APA) and Active Phytochrome B-binding (APB) [92, 93] and while the APB domain is present in all PIFs the APA is only found in PIF1 and PIF3 [80]. As transcription factors, PIF1, PIF3, PIF4, PIF5 and PIF7 have been found to bind the G-box motif to regulate transcription[89, 94-97] and ChIP-chip analysis performed in different conditions have shown that PIF1, PIF4 and PIF5 can
bind directly to the promoters of ~700, ~4000 and ~1000 genes respectively [96, 98, 99]. This suggests that the PIFs have a high hierarchical position in the light signal transduction, a notion further supported by microarray experiments using *pif* mutants showing their large effect on the transcriptome [100, 101].

Interaction with the Pfr form of phyA and/or phyB results in phosphorylation of PIF1, PIF3, PIF4 and PIF5 and their subsequent degradation [93, 102, 103]. PIF7 appears to be more stable in the light than the other PIFs, and was recently shown to be dephosphorylated when transferred from shaded light to white light [30, 97].

All *pif* mutant seedlings display light hypersensitivity to various degrees and PIFs are now generally thought to promote skotomorphogenic growth [30, 88, 101, 104-106]. However, the lack of a phenotype in darkness in single and double mutants, early prompted an alternative hypothesis. It was noted that in multiple *pif* mutants, phyB levels were elevated when grown in prolonged light, suggesting that the PIFs are responsible for phytochrome degradation [29, 30]. Additionally, it has been shown that phyB levels in the seedling correlate with light hypersensitivity [30, 106, 107]. Finally, it was also shown that overexpressed PIF3 mutated in its DNA binding domain was able to promote elongated growth, while PIF3 mutated in the APA and the APB domain could not, thus suggesting that PIF3 acts mainly by regulating phytochrome levels [107]. Although the PIFs now are known to regulate phytochrome levels through a COP1 mechanism [29], analysis of the *pif1pif3pif4pif5* quintuple mutant (*pifQ*) revealed a constitutively photomorphogenic phenotype in darkness, supporting the notion that the PIFs acts as transcription factors promoting skotomorphogenesis [101, 108]. However, PIFs have also been shown to promote the light induced translocation of phyB from the cytosol to the nucleus [109],
highlighting the complexity of the reciprocal regulation between the phytochromes and the PIFs.

Although the PIFs appear to have overlapping and redundant functions [101, 108] in some responses the action of a PIF appears distinct. Most notably is PIF1 which by itself acts to suppress seedling germination [110] and has a more important role in chlorophyll and carotenoid synthesis compared to the other PIFs [111, 112].

### 1.2.5. PIFs regulates responses to shade

In response to shaded light conditions, the reduction in Pr to Pfr ratio of the phytochromes initiates the Shade Avoidance Syndrome (SAS) [4]. As a consequence of the reduced phyB activity in these conditions, the stability of PIF4 and PIF5 was shown to be promoted [103]. The pif4pif5 mutant showed a reduced response to shade and consistently, overexpression of either PIF4 or PIF5 resulted in exaggerated elongation in white light similar to the wt seedlings grown under shade [103]. Furthermore, consistent with a role as positive regulators of the SAS, PIF4 and PIF5 has been shown to bind directly to the promoters of several shade induced genes to promote their transcription [89, 96, 103]. Perhaps most notably, PIF4 and PIF5 have been shown to bind the promoter of YUC8 encoding a key enzyme in the auxin biosynthetic pathway [96, 113]. As auxin signalling and transport is elevated and possibly indirectly causing many aspects of the SAS, this potentially puts PIF4 and PIF5 as master regulators of the SAS [96, 103, 114]. In shaded light conditions HFR1 was shown to form non-functional heterodimers with PIF4, PIF5 and most likely PIF3 that inhibits their DNA binding and thus their transcriptional activity, explaining the exaggerated shade avoidance response in the hfr1 mutant [81, 87, 89].
Interestingly, it was recently shown that PIF7 also plays an important role regulating the SAS. Detailed analysis of the hypocotyl response to shade revealed that pif7 largely lacked an immediate response, while the pif4pif5 double mutant partially retained a response. It was further shown that PIF7, as PIF4 and PIF5, is able to bind the G-box of the YUC8 promoter to regulate its transcription [97].

1.2.6. External coincidence model

Entrained in diurnal light/dark conditions, the hypocotyl elongation of young Arabidopsis seedlings were shown to be under circadian regulation with the peak rate of elongation at the subjective dusk [115]. However, it was later shown that in diurnal short day conditions (SD) the rate of elongation peaked at the end of the night which was rapidly inhibited when entering the light phase [116]. With a clever microarray approach the authors of this study [116] identified the transcript levels of PIF4 and PIF5 to be correlated with growth and largely responsible for rhythmic elongation of the hypocotyl. The transcription of both PIF4 and PIF5 are under strong circadian regulation [117] and peaks during the day in SD. However, during the day, light activated phyB acts as a strong suppressor of PIF4/5 protein levels [89]. Thus, an external coincidence model was proposed where increasing transcript levels of PIF4/5 during the night translate into increasing PIF4/5 protein levels to promote elongation. However, upon dawn, phyB dependent degradation of PIF4/5 negates the high transcript levels resulting in decreased elongation rate of the hypocotyl [116]. This model was further supported by the demonstration that the pif4pif5 mutant exhibited a stronger phenotype with decreasing day length [118].
1.2.7. Gibberellins act to promote PIF activity through the DELLAs

The gibberellin (GA) phytohormone has been shown to regulate many aspects of morphogenesis throughout plant development [119]. GA is perceived by the three orthologous GA receptors GIBBERELLIN INSENSITIVE DWARF1 (GID1A-C) [120]. Upon their activation, they interact with and degrade the five DELLA proteins (RGA, GAI, RGL1, RGL2 and RGL3) [121] which ultimately results in transcriptional changes in GA responsive genes [122]. However, as the DELLA proteins are lacking any obvious DNA binding domain it was proposed that they act by regulating the activity of other transcription factors. By the use of yeast two hybrid screens PIF3 and PIF4 were first found to interact with RGA but PIF3 was also shown to interact with all five DELLA proteins [121, 123]. Interestingly, it was further shown that the DELLA interaction with these PIFs resulted reduced G-box binding by the PIFs and altered transcription of PIF targets. Thus, through a sequestering mechanism, GA acts to promote PIF activity by binding to GID1A-C promoting degradation of the DELLA proteins, resulting in the release of PIFs [121] [123].

1.3. From light perception to gene regulation

Here, we have discussed the major early events of light signal transduction. phyB-phyE acts as red/far-red reversible switches and enters the nucleus when active. phyA is functionally distinct acting in very low fluence rates and in continuous far-red light. In the nucleus, the active phytochromes act to promote nuclear exclusion of COP1 and interact directly with the PIF transcription factors to promote their degradation and consequently promote photomorphogenesis. Reciprocally the PIFs act to degrade the phytochromes through a COP1 mechanism. The cryptochromes
perceive blue light and act in the nucleus to promote nuclear exclusion of COP1 as well as directly interacting with COP1 to suppress its activity. In darkness, COP1 acts to degrade positive factors of photomorphogenesis, such as HY5 and HFR1. Thus light inactivation of COP1 results in the accumulation of HY5 that acts directly as a transcription factor and HFR1 that inactivates the PIFs by forming non-DNA binding heterodimers. In conclusion, the perception of light results in the inactivation and promotion of negative and positive factors of photomorphogenesis respectively, in a highly interconnected network that relays the external light signal to transcriptional regulation (Figure 1.2).

**Figure 1.2. Simplified schematic of the light signalling pathways.**

Red light perception activates the phytochromes resulting in their translocation to the nucleus. In the nucleus, they act directly on the PIFs to promote their degradation and acts to promote nuclear exclusion of COP1. The PIFs act as transcription factors promoting elongated growth but is also promoting the degradation of the phytochromes via COP1. Cry1 is activated by blue light and acts directly to suppress COP1 activity in the nucleus but also to promote its nuclear exclusion. In darkness, COP1 acts to degrade positive factors of photomorphogenesis, including HFR1 and HY5. HY5 binds the promoters of a vast number of genes promoting de-etiolation while HFR1 acts by inhibiting the action of PIFs.
1.4. Temperature signalling in Arabidopsis

With changes in the ambient temperature, the rate of all chemical reactions in a biological system is expected to be affected. It is therefore likely that a change in the ambient temperature has global effects on all processes in the plant cells. Remarkably, plants are able to tolerate and survive a broad range of temperatures [124], suggesting that there are mechanisms available to deal with temperature fluctuations. Indeed, as for most organisms, the free running period of the circadian clock is well buffered against changes in the ambient temperature [125] and recent advances in Arabidopsis research have found that several core components of the clock are important for this [126, 127].

In contrast to buffering against temperature alterations, several distinct responses to a relatively small increase in the ambient temperature have been revealed in plants, including hypocotyl and petiol elongation, leaf hyponasty and the promotion of flowering in short days [128-132]. Here we will introduce what is known about temperature signalling, with emphasis on the interaction with the light signalling pathways.

1.4.1. A role for light receptors in temperature responses

Previous work has demonstrated that failure of photoreceptor activity results in striking temperature dependent alterations in growth and development. In particular, while the phyB mutant was shown to flower with an equal number of rosette leaves as the wt in 16°C, a small increase in the ambient temperature revealed an early
flowering time phenotype in the mutant [133]. Further removal of phyA and phyD (phyAphyB phyD) resulted in extremely early flowering only in the warm, suggesting that the phytochromes buffer the effects of a small increase of the ambient temperatures [133]. cry2 has also been implicated in the regulation of flowering time, acting to promote flowering specifically at the cooler temperatures [132]. In addition, although Arabidopsis plants normally lack any elongation of the internodes, mutants of phyB, phyAphyB phyE and phyB cry1 have been shown to exhibit extreme internode elongation [36, 134]. Consistent with the temperature effects of flowering time, the internode elongation is only observed in higher temperatures, thus providing additional support for the necessity of photoreceptor action to regulate responses at specific temperatures.

1.4.2. PIF4 acts as a major regulator of temperature responses

Seedlings grown in warmer temperatures display increased leaf hyponasty, petiole and hypocotyl elongation [114, 128, 129, 135]. Interestingly, all these phenotypic responses to increased ambient temperatures resemble the morphological changes of plants grown in shade conditions [4]. Based on the similarities between the two responses, Koini and collaborators [129] identified the PIF4, previously identified as a potent regulator of the SAS [103], as a major regulator of high temperature responses in Arabidopsis. Mutants of pif4 but not pif3 or pif5 showed a dramatic reduction in temperature induced hypocotyl elongation indicating that PIF4 has a unique role in temperature signalling compared to the other PIFs. However, in slightly different conditions, a later study also implicated PIF5 in the same response.
Chemically blocking the transport of the phytohormone auxin with N-1-naphthylphthalamic acid (NPA) has been shown to largely block the hypocotyl temperature response [128, 135]. Furthermore, mutants of TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (taa1), an early enzyme in auxin biosynthesis, have a severely reduced temperature response. Thus, auxin signalling appears imperative for temperature responses [114]. Interestingly, PIF4 was shown to bind to the promoter region of TAA1 to positively regulate its transcription in response to warm temperatures, locating PIF4 upstream of auxin signalling [136]. Potentially even more relevant, PIF4 was also shown to bind to the promoter of YUC8 to promote its expression in response to warm temperatures and shaded conditions [96, 113]. The YUCCA family of flavin containing monooxygenases are thought to constitute a rate limiting step in IAA biosynthesis as their overexpression, but not the overexpression of TAA1, results in high auxin levels and/or auxin related phenotypes in the plant [114, 137]. As a direct target of phyB and a major regulator of light responses, the role of PIF4 in temperature responses provides another link between photoreceptor action and temperature signalling.

1.4.3. H2A.Z occupancy is temperature dependent

The transcription rates of HEAT SHOCK PROTEIN 70 (HSP70), has been shown to correlate with the ambient temperatures [126, 130, 138]. Using the promoter of HSP70 as a temperature readout in a forward genetic screen, arp6 was identified as having constitutively high HSP70 expression and therefore potentially an important
regulator of temperature responses [138]. ARP6 and its homologue PIE1 act in the SWR1 complex that is essential for incorporating the H2A.Z histone variant into the nucleosomes [139, 140]. Consistent with the fact that the arp6 mutant displays constitutively warm grown phenotypes while being deficient in H2A.Z nucleosome incorporation, H2A.Z promoter occupancy was shown to be severely reduced in the warm compared to cool conditions. Therefore, a model was proposed, were warm temperatures result in reduced H2A.Z promoter occupancy, making the promoter region available for binding of transcription factors to regulate genes on the genome wide scale [138].

Mutants of arp6 have previously been shown to flower early in short days [140-142]. Furthermore, the promoter region of FT has been shown to be occupied by H2A.Z nucleasomes [143, 144], thus thermal induction of flowering could be directly linked to H2A.Z occupancy on the promoter. Although pif4 have been shown to not affect flowering time [129], a recent study showed that the thermal induction of flowering in short days is largely inhibited in the pif4 mutant in SD [144]. It was further shown that PIF4 can bind directly to the FT promoter and that the binding increases with the ambient temperature correlating negatively with H2A.Z occupancy [144]. Thus, a model emerges suggesting that PIF4 activity on the promoter of FT is increased in warm temperatures as a direct result of reduced H2A.Z occupancy [144].

1.4.4. HY5 acts in the cool to target light responsive genes

The bZIP transcription factor HY5 has classically been described as a positive regulator of photomorphogenesis, targeting and promoting the transcription of light induced genes as RBCS-1A and CHS [71]. Interestingly, the anthocyanine levels and
the transcript levels of CAB1, was shown to dramatically increase in response to low temperature treatments (4°C) in wt seedlings [145, 146] but to a lesser extent in a hy5 mutant suggesting that the transcriptional activity of HY5 is increased in the cool [146, 147]. In these conditions, the degradation rate of HY5 is reduced, and consistent with being a target of COP1, dark dependent translocation of COP1 into the nucleus is severely impaired in the cool [147]. Thus, HY5 activity is increased at low temperatures, possible due to reduced negative regulation by COP1, resulting in the promotion of light induced targets like CAB1 and CHS.

In conclusion, there appears to be ample evidence of crosstalk between temperature dependent development and responses and the light signalling pathways in the literature. Both members of the blue and red light photoreceptors are clearly implicated, as well as downstream signalling factors as PIF4 and HY5. However, the details and mechanisms of the light/temperature crosstalk are currently largely lacking.

**1.5 Aims of thesis**

The work presented in this thesis aims to further our knowledge about how temperature affects the light signalling pathways. In chapter 3, we identify the transcript of HFRI as highly dependent on the ambient temperature. Analysis of the mutant revealed a blue light specific temperature effect suggesting that HFRI function is increased in warm temperatures. We further analyse HFRI in the context of PIF4 and phyB to define a network where red light (via phyB) and blue light (cry1
via HFR1) signals merge on the regulation of PIF4 action to control temperature dependent growth and development [148].

In chapter 4 we aim to gain further understanding of the temperature dependence of the red light photoreceptors. Based on the fact that a temperature induced hypocotyl elongation only is observed under high illumination and not in darkness [128] we investigate the effect of increasing temperatures over a wide array of fluence rates of red light. Surprisingly, while we observe a classical fluence rate dependent inhibition of hypocotyl elongation in cooler conditions, at the high temperatures we observed a biphasic response. By mutational analysis, we determined that phyB, PIF4 and PIF5 action is necessary for the biphasic response in the warm, and we provide evidence for a previously unidentified positive regulation of red light on PIF4 and PIF5.

Finally in chapter 5, we investigate the role of the phytohormone GA and the bZIP transcription factor HY5 in relation to PIF4 and PIF5 activity over a fluence rate of red light at different ambient temperatures. Our results suggest that the PIFs are under strong DELLA suppression in the cool at high fluence rates of red light. In addition, we provide data suggesting that also HY5 acts to suppress PIF action at high fluence rates of light in the cool.
Chapter 2 - Experimental Procedures

2.1. Plant material

Single and multiple mutants in the Columbia ecotype, phyA-211 [27], phyB-9 [34], phyC [38], phyD4-1 [149], cry1-304 [84], pif1-1 [112], pif3-3 [150], pif4-101 [103], pif4-2 [30], pif5-3 [106], pif7-1 [150], hy5-215 [73], cop1-4 [65], spt-11 [151], hfr1-101 [84], hfr1-2 [83], prr7-3 [152] and prr9-1 [152] have been described before. The della4 mutant is in the Ler ecotype, pie1-1 in the Ws and the ABO in the No-0 ecotype [153-155]. 35S::HFR1-HA, 35S::PIF5-HA, 35S::PIF4-HA and 35S::HY5-HA have been described previously [60, 79, 116]. phyB-9hfr1-101 and phyB-9hfr1-101pif4-101 were obtained by crossing hfr1-101 and pif4-101 mutants with phyB-9 and pif4-101hy5-215 by crossing pif4-101 with hy5-215 and the resultant F2 populations were genotyped by PCR.

2.2 Growth conditions

For all experiments in chapter 3, seeds were surface-sterilized, sown on Gilroy-agar media and stratified in darkness for 3 days at 4°C. The seeds were then given a 2h white light pulse to induce germination and kept in dark for 22h at 20°C. The plates were moved to the appropriate light regime at 20°C for an additional 24h (to ensure equal germination for the two temperatures), then shifted to the indicated experimental temperature. Unless stated otherwise, light intensities used were 13
µmol m$^{-2}$ s$^{-1}$ blue, 40 µmol m$^{-2}$ s$^{-1}$ red or 1 µmol m$^{-2}$ s$^{-1}$ far-red light. For all experiments in chapter 4 and chapter 5, seeds were surface-sterilized, sown on half strength MS-agar media (MURASHIGE & SKOOG MEDIUM, Duchefa Biochemies) and stratified in darkness for 3 days at 4°C. The seeds were then moved to 20°C and given a 2 hour light and kept an additional 22h in darkness before moved directly into the experimental light and temperature condition.

2.3. Light sources and filters

Monochromatic blue light was provided by custom made LED arrays with a peak at 464nm ($\lambda_{\text{max}}$=464) and a width of 24nm at half intensity ($\lambda_{0.5} \pm 12$nm) (Figure 2.1 A). For experiments in chapter 3, custom made LED arrays were used for red light treatments ($\lambda_{\text{max}}$=652, $\lambda_{0.5} \pm 12$nm) and far-red EOD treatments ($\lambda_{\text{max}}$=745, $\lambda_{0.5} \pm 15$nm) (Figure 2.1 A). For all other monochromatic light experiments SNIJDERS cabinets (Model EB2-NE-PB) were used emitting Red ($\lambda_{\text{max}}$=658, $\lambda_{0.5} \pm 10$nm) and far-red light ($\lambda_{\text{max}}$=739, $\lambda_{0.5} \pm 13$nm) (Figure 2.1 B).

Figure 2.1. Spectral energy distribution of the LED light sources used for experiments.

For fluence response curves, neutral density filters (LEE filters) was put on top of plates in order to obtain specific fluence rates of light, while the bottom and sides were covered by black paper and black tape to prevent additional light leakage.

2.4. Hypocotyl and cotyledon measurements

For hypocotyl length measurements, seedlings were flattened on their agar plates to reveal the full extent of their hypocotyl phenotype, and images were taken using a digital camera. For cotyledon size measurements, the cotyledons were removed from the seedling and flattened out on a agar plate before images were taken with a digital camera. Hypocotyl length and cotyledon size was then measured using the ImageJ software (NIHimage, http://www.rsb.info.nih.gov/nih-image/).

2.5. Hormone treatment

For the NPA and GA treatments, seeds were sown as above on plates supplemented with 1, and 10µM of NPA or 1 and 10µM of GA$_3$. For control plates in the NPA and GA experiment an equivalent amount of DMSO and Ethanol was added to correspond to the treatment plates respectively. Hypocotyl lengths were then measured as above.
2.6. Adult phenotypes and dry weight

For adult plant analysis in chapter 3, seeds were sown directly on soil and stratified in darkness for 3 days at 4°C. The trays were then transferred to diurnal (12:12) white light (100 µmol m\(^{-2}\) s\(^{-1}\)) at 21°C for 24 hours, then moved to 28°C or kept in 21°C. For dry weight measurements, 4 week old plants were harvested and the root and florescent stem removed. The remaining rosette material was dried for 3 days at 93°C before weighing on a precision balance. Representative plants were photographed after 5 weeks.

2.7. Immunoblotting and phosphatase treatment

For western blotting, samples were generally grown for 6 days before harvesting unless stating otherwise. Samples were harvested into liquid nitrogen and grinded using a pestle or a tissue lyser (Retsch MM 300). Total protein was then extracted in \(2^*(\text{tissue weight/0.00142})\) µL of extraction buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Na deoxycholate, 0.5% Triton X-100, 1 mM DTT, 50µM of MG132, MG115, ALLN, PS1 and 1x Complete protease inhibitor cocktail, EDTA-free (Roche)). After 10 minute centrifugation at 14000 rpm in 4°C, the supernatant was moved to a new tube and the samples were kept in -80°C for later use. For immunoblotting, the samples were mixed 3:1 with 4x sample buffer (0.25 M Tris pH6.8, 8% SDS, 40 % glycerol, 0.04% bromophenol blue and 10% b-mercaptoethanol). The samples were run on a 10% SDS-PAGE gel, followed by a wet transfer to nitrocellulose or PVDF membrane. The HA tag was detected by probing the membrane with a rat anti-HA-HRP antibody (3F10, Roche) at a dilution
of 1:1000. Loading was controlled by directly re-probing membranes using a goat anti-UGPase antibody (AGRISERA) at a dilution of 1:1000 followed by a HRP-conjugated sheep anti-goat (Bio-Rad) at a dilution of 1:5000. Signals were detected using the Amersham ECL kit as per instructed by the manufacturer.

For phosphatase treatment the samples were extracted as above and 400 units of lambda protein phosphatase (NEB) was added to a total volume of 50 µl. For control samples, the phosphatase buffer was used alone and phosphatase with the addition of an inhibitor (50 mM EDTA). The samples were then incubated in 30°C for 30 min and the reaction was stopped by adding 25 µl of 4x sample buffer before analysed by immunoblotting as above.

### 2.8. Quantitative PCR

For qPCR experiments, seedlings were grown as previously described in three biological repeats and harvested in RNAlater (SIGMA). RNA was then extracted using a RNeasy Plant Mini Kit (QIAGEN) with on-column DNase digestion. cDNA synthesis was performed using SuperScript VILO cDNA Synthesis Kit (Invitrogen) as described by the manufacturer. Primers used are listed in Table 2.1. The qPCR was set up as 10 µl reactions using SYBR Green (Roche) in a 384 well plate with a liquid-handling robot (Tecan Freedom EVO, http://www.tecan.com/) and qPCR performed with a Lightcycler 480 system (Roche) and the results were analysed using the LightCycler 480 Software (1.5.0).
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
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</tr>
<tr>
<td>JF17-ACT7-R</td>
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</tr>
<tr>
<td>JF143-XTR7-F</td>
<td>ACACATCATATTCTTTGTTGGAC</td>
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<td>JF781-YUC8-R</td>
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Table 2.1. List of primers used in this study for qPCR.
2.9. Luciferace assay

For protein quantification using Luciferace, ~100mg of 6 day old seedlings (PHYB:phyB-Luc, 35S:PIF4-luc) were harvested into liquid nitrogen. The samples were then grinded in liquid nitrogen and proteins were extracted in 200 µL Promega Cell Culture Lysis Reagent complemented with 50µM of MG132, MG115, ALLN, PS1 and 1x Complete protease inhibitor cocktail, EDTA-free (Roche). The samples were then centrifuged at 14000 rpm for 10 min at 4°C to remove debris. 50 µL of each sample was then added to a 96 well plate and analysed using MicroLumatPlus LB96V Microplate Luminometer with the Luciferin based Reporter Lysis Buffer (Promega). In order to determine the total protein content of the samples, 50 µL of each sample was first washed using the Compat-Able Protein Assay Preparation Reagent Set (Thermo) to remove the detergent according to the manufacturer’s instructions. Total protein was then assayed using Pierce BCA kit (Thermo) according to the manufactures instructions and absorbance was measured at 562nm. A dilution series using BSA was used as a reference to determine the sample protein content.
Chapter 3 - Light receptor action is critical for maintaining plant viability at warm temperatures

(Parts of this work have been published in The Plant Journal [148] in which sections of the data were acquired by J. Foreman (Table S1 and Figure S5) and P. Hornitschek (Figure 3B and Figure S2). These results appear in Appendix A, and will be referred to in the main text.)

3.1 Introduction

As sessile organisms, plants must continuously monitor the environment in order to appropriately respond to and cope with any changes. Dependent on light as the source of energy, this environmental signal is without doubt the most important for plants to monitor. Accordingly, plants have evolved an array of photoreceptors in order to measure the intensity, wavelength, duration and direction of the incoming light [3]. Apart from light, the ability to withstand environmental temperature variation is essential for plant survival [156, 157]. Interestingly, previous work has demonstrated that failure of photoreceptor activity results in striking temperature dependent alterations in growth or development suggesting that the photoreceptors
also plays a role in the temperature signalling pathways [36, 132-134, 158, 159]. However, the molecular events underlying this apparent interaction between the light signalling pathways and temperature signalling is not known.

In this chapter we describe the molecular circuitry through which the photoreceptors phyB and cry1 regulate growth at warm temperatures. By the use of microarrays, we identify *HFR1* as a potential molecular junction of light and temperature signalling. We show that HFR1 acts in a warm branch of the cry1 pathway to inhibit the action of PIF4 and PIF5. We further demonstrate that the light dependent accumulation of HFR1 is highly temperature dependent, resulting in increased HFR1 levels in the warm. Furthermore, we show that HFR1 acts at warm temperatures to restrain the activity of PIF4, which protein levels and activity also is increased in the warm. The dual activity of blue (cry1 via HFR1) and red (phyB) light in regulating PIF4 and PIF5 activity is critical to control growth as temperatures rise, and we show that the loss of this light mediated control has dramatic consequences for adult plant development in the warm.
3.2 Results

3.2.1. Light and temperature converge on \textit{HFR1} transcription

Previous studies have indicated that the phytochromes play a vital role in buffering the effects of temperature in regards to flowering time [36, 133]. Therefore, an Arabidopsis Affymetrix microarray analysis was performed in photoreceptor null \textit{phyB} plants and wild type plants in order to identify molecular components with altered temperature regulation [148] (Table 1 Appendix A). \textit{HFR1} emerged as a strong candidate as it exhibited strong temperature dependent regulation in addition to being highly regulated by phyB. Additionally, to our encouragement, HFR1 had previously been characterised as a major regulator of light signalling [81, 84, 87, 160], making this a good potential target for investigating the interconnection of light and temperature signalling.

In order to confirm the microarray data, we grew WT and \textit{phyB-9} seedlings for 6 days at 12°C and 27°C and measured \textit{HFR1} transcript levels by qPCR. In agreement with the microarray, \textit{HFR1} was highly upregulated in the warm temperatures (28 fold, 27/12°C) (Figure 3.1). Furthermore, depletion of phyB led to an increase of \textit{HFR1} transcript levels at both temperatures, but a reduced response to elevated temperatures (4.5 fold, 27/12°C). These data illustrated that \textit{HFR1} was subject to strong regulation by both temperature and phyB, suggesting that \textit{HFR1} may be an important molecular junction for light and temperature signals.
3.2. HFR1 control of hypocotyl growth is temperature dependent

The strong temperature dependent regulation of HFR1 transcript levels suggests that HFR1 might have a temperature dependent role. HFR1 has been characterised as a positive regulator of photomorphogenic growth, partly due to the long hypocotyl of the hfr1 mutant observed in blue and far-red light [82, 84]. In order to establish whether HFR1 function was temperature-dependent we examined the hypocotyl phenotype of a hfr1-101 loss-of-function mutant seedling in darkness or in diurnal 12/12 monochromatic light/dark photo-cycles of red, far-red and blue light at 15 and 25°C. Consistent with previous studies, we did not observe any phenotype of hfr1 compared to the WT in red light [81] regardless of the ambient temperature (Figure 3.2 A). However, the WT (and hfr1 mutant) seedlings showed a strong response to the increased temperature in red light (3.8 fold, 25/15°C). In agreement with the published literature, we observed only a minor effect on hypocotyl elongation in darkness [128] and hfr1-101 was indistinguishable from the wild type.
at both 15 and 25°C (Figure 3.2 A). In far-red photoperiods, as expected, hfr1-101 seedlings had longer hypocotyls than the wild type, and the impact of the mutation was comparable at both temperatures (Figure 3.2 A). Contrasting with this, when grown under blue photoperiods, hfr1-101 hypocotyls were far more elongated at 25°C (hfr1-101/WT=2.34) than at 15°C (hfr1-101/WT=1.56) (Figure 3.2A and B) suggesting that the function of HFR1 is more important in the warm to suppress hypocotyl elongation, but only in blue light.

**Figure 3.2.** hfr1 hyposensitivity to blue light is temperature dependent.

(A) Hypocotyl measurements of 6 day old WT and hfr1-101 seedlings grown at 15°C or 25°C under 12:12 diurnal photo-cycles in red, far-red or blue monochromatic lights, or in...
darkness. Error bars represent SE, \( n \geq 24 \). (B) Picture of representative 6 day old WT and \( hfr1-101 \) seedlings grown at 15°C or 25°C under 12:12 diurnal blue photo-cycles. Scale bar = 1mm. (C) Hypocotyl measurements of 6 day old WT, \( hfr1-201 \) and \( 35S::HFR1-HA \) seedlings grown in blue light as in (A). Error bars represent SE, \( n \geq 25 \).

Although \( hfr1-101 \) is reported to be a loss of function allele of \( HFR1 \), to confirm that the observed effect is due to the loss of \( HFR1 \) function and not specific to this allele, we obtained \( hfr1-201 \) [161] as a second allele as well as a line over expressing \( HFR1-HA \). Analysis of the second allele of \( hfr1 \) showed similar responses as \( hfr1-101 \) demonstrating that the temperature dependent phenotype is likely to be caused by the loss of \( HFR1 \) (Figure 3.2 C). Accordingly, seedlings expressing 35S-driven \( HFR1 \) exhibited a markedly reduced warm response (Figure 3.2 C).

In conclusion, these data indicate that \( HFR1 \) action is not only dependent on light quality as previously thought but is also temperature regulated, validating the microarray approach taken to find key players in the junction of light and temperature signalling. Furthermore, the suggested enhanced action of \( HFR1 \) at 25°C is specific to blue light, therefore appears to require blue light photoreceptor action.

### 3.2.3. HFR1 operates in a warm branch of the cry1 pathway

In blue light, HFR1 has previously been shown to mainly operate in the cry1 photoreceptor pathway to control hypocotyl growth. However, at lower fluence rates \( HFR1 \) functions partly downstream of the light receptor, phyA [84]. To establish whether the temperature phenotype of \( hfr1 \) is dependent upon cry1 or phyA action, we analysed \( phyA-211hfr1-101 \) and \( cry1-304hfr1-101 \) seedlings in blue photoperiods. Our data show complete cry1 epistasis over hfr1 and additive phenotypes between
hfr1 and phyA alleles at both 15°C and 25°C, indicating that the temperature-specific effect of hfr1 requires cry1 presence but not phyA (Figure 3.3). Interestingly, as seen previously in white light, the cry1 phenotype itself was not temperature dependent, as cry1 hypocotyls were 3.8 fold longer than the wt at both 15°C and 25°C [134]. Our results therefore suggest that cry1 operates over a broad range of temperatures to control hypocotyl elongation, and that HFR1 predominantly resides in a warm branch of the cry1 pathway.

![Figure 3.3. HFR1 acts downstream of cry1](image)


### 3.2.4. HFR1 protein levels are highly temperature-regulated

In darkness HFR1 is degraded in a COP1 and SPA dependent manner and exposure to light leads to the accumulation and phosphorylation of HFR1 [60, 85, 161-163]. As HFR1 is most effective in restraining hypocotyl growth in the warm under blue light, we set out to establish whether HFR1 protein levels were subject to regulation
under these conditions. As previous studies have generally assessed HFR1 protein levels during de-etiolation [60], we first grew seedlings expressing 35S::HFR1-HA for 4 days in darkness at 15 and 25°C, followed by exposure to blue light. In darkness we observed low levels of a single band in both temperatures (Figure 3.4 A). When exposed to blue light HFR1 accumulates over time in both temperatures, and we observe the appearance of a second band that is likely to be a phosphorylated form of HFR1 [60]. Interestingly, and in agreement with our phenotypic data, suggesting increased importance of HFR1 in the warm, higher levels of HFR1-HA are detected at the warmer temperatures (Figure 3.4 A). However, the ratio between the two forms of HFR1 does not appear to be regulated by the temperature.

As our mutant analysis was performed in 12:12 photocycles we continued to examine the post-translational regulation of HFR1-HA in diurnal conditions over the course of a day. These results illustrates that at 15°C, HFR1-HA levels rise gradually during the light phase, with a modest peak at 8h post-dawn (Figure 1 Appendix A). In 25°C-grown seedlings, HFR1 accumulates more rapidly and to higher levels after transfer to light. Following a peak, detected 1h post-dawn in our assay, HFR1 levels gradually fall during the course of the photoperiod. These data indicate that temperature alters both the timing and the abundance of HFR1 protein levels. The elevated levels of HFR1 protein in the warm are consistent with our phenotypic data that suggest a more prominent role for HFR1 under warm conditions. Consistent with this proposition, 35S-driven HFR1 lines preferentially suppressed hypocotyl elongation at the warmer temperature (Figure 3.2 C). It therefore appears that cry1 regulation of HFR1 is highly dependent on ambient environmental temperature as
HFR1 accumulates to high levels only under warmer conditions, where it restricts plant growth.

Figure 3.4. HFR1 accumulates in the light and acts during the day.

(A) Immunoblot of HFR1-HA protein levels in 35S::HFR1-HA hfr1 seedlings grown for 4 days in darkness at 15 and 25°C before exposure to 30 µmol m$^{-2}$ s$^{-1}$ of blue light for the indicated amount of time. Anti-HA antibodies were used to visualise HFR1. UGPase is shown as loading control. (B) Hypocotyl measurements of 6 day old WT and hfr1-101 seedlings grown in diurnal 12:12/Light:Dark blue light at constant 15°C and 25°C or 15°C:25°C, 25°C:15°C thermo-cycles. Error bars represent SE, n≥ 27.

The post-dawn HFR1 peak and persistence of HFR1 protein through the day suggested to us that, at 25°C, HFR1 may act primarily during the photoperiod. This was tested by subjecting seedlings to 25°C/15°C or 15°C/25°C blue/dark thermo-
cycles. Here the full impact of the \textit{hfr1-101} mutation was only evident in seedlings exposed to 25°C during the daytime (Figure 3.4 B). This indicates that in warmer conditions HFR1 does indeed operate during daylight hours to control hypocotyl elongation.

\textbf{3.2.5. In blue/dark photocycles loss of PIF4 attenuates the \textit{hfr1} phenotype}

It was recently shown that, under low red:far-red ratio light, HFR1 operates by suppressing the growth promoting activity the two close homologues PIF4 and PIF5 [88, 89, 103]. Under low red:far-red ratio, light, which elevates PIF4 and PIF5 protein levels [103], HFR1 prevents excessive hypocotyl elongation and PIF4 and PIF5 controlled gene expression though direct interaction [89]. As \textit{pif4} was recently shown to have a temperature-dependent phenotype in white light [129, 135] and \textit{pif4} has a subtle blue hypocotyl phenotype [164, 165], we reasoned that PIF4 would be a likely HFR1 target at warm temperatures in blue photoperiods. Analysis of the \textit{hfr1-101pif4-101} mutant supports this notion. In our conditions, \textit{pif4-101} had no impact on hypocotyl elongation in an otherwise wild type background, but did perturb hypocotyl elongation in the presence of \textit{hfr1-101} (Figure 3.5 A and B). In addition, analysis of the \textit{hfr1-101pif4-101pif5-1} triple mutant suggests that PIF4 and PIF5 are largely responsible for the \textit{hfr1-101} phenotype in blue light (Figure 3.5 B). In the \textit{pif4pif5} double mutant we do also observe a short, albeit small, hypocotyl phenotype, that is only observed in the warm temperatures.

At the protein level we observe a small increase of PIF4-HA at warmer temperatures in seedlings expressing \textit{35S::PIF4-HA} [116] with the appearance of slower migrating
bands (Figure 2 Appendix A). These data indicate that PIF4 action is inhibited by HFR1, which can fully suppress PIF4 activity in wild type seedlings grown in blue light (Figure 3.5 A and B) as was suggested under shade and far-red light [88, 89].

Figure 3.5. HFR1 acts to suppress PIF4 and PIF5 in the warm


(B) Hypocotyl measurements of 6 day old WT, hfr1-101, pif4-101 and pif5-1 single, double and triple mutant seedlings grown in diurnal 12:12 blue light at constant 15°C and 25°C. Error bars represent SE, n ≥ 18.
3.2.6. In red light, PIF4 action is highly dependent on the ambient temperatures

As we did not observe a strong temperature dependent effect of *pif4-101* in blue light (Figure 3A and 3B) contrary to published data from white light experiment [129, 135] we decided to investigate the role of *pif4* in far-red and red light, as PIF4 has been characterised as having a major role in these light conditions [88, 104]. In far-red light, *pif4-101* shows a hypersensitive response to the incoming light at both 15 and 25°C resulting in a short hypocotyl compared to the WT seedlings (Figure 3.6 A). However, as for the *hfr1-101* mutant (Figure 3.2A, 3.6A) no obvious temperature dependence was observed in the *pif4* mutant. The fact that the *hfr1-101:pif4-101* double mutant showed intermediate phenotype at both temperatures is consistent with HFR1 negatively acting on PIF4 action, if the action of PIF5 (and possibly other PIFs) are taken into account (Figure 3.6 A) [88, 89]. Nevertheless, analysing the impact of *pif4-101* in the *hfr1-101* background at the end point measurement reveals an increased importance of PIF4 in the warmer temperatures (*hfr1-101/hfr1-101:pif4-101* = 1.15 and 1.34 for 15 and 25°C respectively), similar to our observations in blue light (Figure 3.5). In contrast, red light grown *pif4-101* was effective in perturbing elongation at 25°C, but not at 15°C (Figure 3.6 B and C) thus showing strong dependence on the ambient temperature similar to what has been recently reported in the literature under white light conditions [113, 129, 135]. In red, the *hfr1-101* mutant behaved like the WT seedlings, and did not cause any additional effects in the *pif4-101* background (Figure 3.6 B), consistent with HFR1 not having a major function in red light [104].
Figure 3.6. The \textit{pif4} phenotype in under red light is temperature regulated

(A-B) Hypocotyl growth curves of WT, \textit{pif4-101}, \textit{hfr1-101} and \textit{hfr1-101pif4-101} seedlings grown at 15°C or 25°C in 12:12 diurnal far-red (A) or red (B) photo-cycles. Error bars
represent (A) SE, \( n \geq 25 \) (B) SE, \( n \geq 23 \). (C) Picture of representative 6 day old WT and \( pif4-101 \) seedlings grown as in (B). Scale bar = 1mm.

Taken together, the analysis of \( pif4-101 \) in blue, far-red and red light (Figure 3.5 and 3.6) suggests that the previously reported temperature dependency of PIF4 action in white light is mainly dependent on red light signalling pathways, and consequently under the control of red light photoreceptor activity, while minor temperature effects in blue and far-red light is masked by HFR1 action.

### 3.2.7. PIF4 accumulates in the warm

As the physiological data suggests a strongly temperature dependent activity of PIF4 in red light, we wanted to investigate if PIF4 protein levels correlate with these results. Immunoblotting of seedlings expressing \( 35S::PIF4-HA \) grown in diurnal red light showed that PIF4 levels was consistently higher throughout the day at 25°C compared to 15°C, where PIF4 was barely detectable (Figure 3.7 A). Surprisingly, we did not observe a major light dependent degradation of PIF4 during the day, as have been suggested in diurnal white light (160min light : 320min dark photoperiods) [116] and in etiolated seedlings exposed to red light [103]. Therefore, in order to gain confidence in this data set, the red light dependent degradation of PIF4 in de-etiolated seedlings was examined in similar conditions (with the exception of the ambient temperature) as previously published. Again, we observe a strong temperature dependent accumulation of PIF4-HA in the warm, which appears to be independent on light (Figure 3.7 B). Importantly, this data using etiolated
seedlings do match the published literature, suggesting that the stability observed during the light phase (Figure 3.7 A) is not due to unexpected modifications of the 35S::PIF4-HA line used. Thus, these data show a strong temperature dependent accumulation of PIF4 in red diurnal conditions, which correlates with the phenotypic analysis of the pif4 mutant.

**Figure 3.7. PIF4-HA stability is increased in the warm**

(A) Immunoblot of PIF4 protein levels in 6 day old 35S::PIF4-HA seedlings grown in diurnal 12:12 red photo-cycles at 15°C or 25°C. EON= End of night, 15h = 3h into following night. (B) Immunoblots of PIF4 in 5 day old 35S::PIF4-HA etiolated seedlings exposed to red light for indicated amount of time. Seedlings were grown at 15 or 25°C. Anti-HA antibodies were used to visualise PIF4. UGPase is shown as loading control.
3.2.8. End-of-day far-red pulse affects PIF levels only in the cool

In our conditions, the WT hypocotyl response increasing temperatures appears most potent under red light illumination (Figure 3.2 A). We have shown that the action of PIF4 is largely required for this response especially in red light (Figure 3.6 B and C). In addition, epitope tagged PIF4-HA protein levels under the regulation of a 35S promoter increases in the warm to some extent in blue light (Figure 2 Appendix A), but manifold in red (Figure 3.7 A). Taken together, this data strongly implicates phytochrome regulation of the temperature dependency of PIF4. To gain further support for this notion, we determined the impact of an end-of-day (EOD) FR light pulse, which depletes active phytochrome (Pfr) levels at the beginning of night, on PIF4 levels. When seedlings were grown at 15°C the EOD far-red pulse resulted in a moderate rise in PIF4 levels during the night and a subsequent fall in PIF4 levels at 2h post dawn (Figure 3.8). However, at 25°C, PIF4 levels were constitutively elevated and EOD FR treatment had no detectable impact on PIF4-HA levels suggesting that the phytochrome regulation of PIF4 protein levels are indeed temperature dependent.
Figure 3.8. PIF4 levels are under phytochrome regulation during the night in cool temperatures

Immunoblot of PIF4 in 35S::PIF4-HA seedlings grown at 15°C or 25°C in diurnal 12:12 red light. Samples were collected at the end of day 6 (EOD), midnight (MN), at the end of the night (EON) or 2 hours into day 7. The seedlings were either transferred directly into darkness at the end of day 6 or given a 5 minute far-red pulse prior to transfer. Anti-HA antibodies were used to visualise PIF4. UGPase is shown as loading control. Two different exposures are shown for clarity.

3.2.9. Warm temperatures promotes accumulation of phosphorylated forms of PIF4

The stability of PIF4 is known to be regulated post-translationally by phosphorylation modifications [103, 116]. PIF4 is phosphorylated in the dark and exposure to light induces the appearance of additional phosphorylation events that destabilises the protein [103]. Upon close examination of our immunoblots, warmer temperatures appeared to correlate with an increase in slower migrating isoforms of PIF4-HA (Figure 2 Appendix A, Figure 3.7 and 3.8). Samples from seedlings expressing PIF4-HA were therefore harvested at 3 time points over the dark to light
transition in diurnally grown seedlings at 15 and 25°C. These samples were treated with lambda phosphatase to confirm the presence of phosphorylation by western blotting. Due to the low levels of PIF4-HA in the cooler conditions the loading of the 15°C samples was increased relative to 25°C to facilitate detection at this temperature. The phosphatase treatment led to a depletion of the slower migrating forms at 25°C, but had no impact at 15°C, suggesting that warm temperatures promote the accumulation of phosphorylated forms of PIF4 (Figure 3.9 A).

The fact that we could detect phosphorylated isoforms of PIF4 at the end of the night period, where the majority of phytochromes are expected to be in their inactive Pr form, suggested that this temperature dependent phosphorylation event could be light independent. To test this hypothesis, seedlings were grown at a range of temperatures in constant darkness for 5 days, before being analysed by western blotting. This revealed a gradual increase in slower migrating bands, increasing with the temperature from 14.5 to approximately 24.5°C where saturation appeared reached (Figure 3.9 B). We further confirmed that these bands corresponded to phosphorylations also in etiolated seedlings by treating samples with phosphatase (Figure 3.9 C).

In conclusion, we have discovered that PIF4 is phosphorylated in response to increasing temperatures. However, this phosphorylation event is likely to be independent on photoreceptor activity due to the fact that it can be observed in etiolated seedlings. Interestingly, this phosphorylation correlates with increased levels of PIF4, in contrast to the previously described light dependent phosphorylation of PIF4 that precedes its degradation [103].
**Figure 3.9. PIF4 is hyper phosphorylated in the warm**

(A) Phosphatase treatment of PIF4 protein in 6 day old 35S::PIF4-HA seedlings grown in diurnal 12:12 red photcycles at 15°C or 25°C. Samples were collected at the end of the night (EON), 10 minutes or 4 hours into the day. Total protein was treated with phosphatase alone, phosphatase inhibitor alone, or phosphatase and inhibitor. More total protein was loaded from seedlings grown in 15 compared to 25°C. (B) Immunoblot of PIF4 in 35S::PIF4-HA seedlings grown in darkness for 6 days at indicated temperature. (C) Phosphatase treatment of PIF4 protein in 5 day old 35S::PIF4-HA seedlings grown in darkness at 15 or 25°C. Total protein was treated as in (A). (A-C) Proteins were analysed by immunoblot using an anti-HA antibody and UGPase is shown as loading control.
3.2.10. Temperature dependence of pif4 in red light requires functional phyB

The red light dependency of the pif4 temperature phenotype in addition to our EOD far-red treatment and the published links between PIF4 and phyB [103, 116, 166, 167] prompted us to investigate their genetic relationship under different temperatures. As previously shown, the pif4-101 mutant does not show a significant phenotype at 15°C but has a severely impaired elongation response in 25°C (Figure 3.10). Being the major photoreceptor in red light, the phyB-9 mutant displayed a long hypocotyl at both temperatures. However, the mutant still retains a temperature response (1.5 fold, 25/15°C). Consistent with the results from the EOD far-red treatment, removing PIF4 in the phyB-9 background reveals a function of PIF4 in the cool, suggesting that PIF4 is under the control of phyB in these temperatures. In addition, comparing the impact of pif4-101 in the WT and phyB-9 background reveals that phyB action is necessary for the temperature specificity of PIF4 action on hypocotyl elongation (Figure 3.10).
Figure 3.10. Loss of phyB renders pif4-101 temperature independent
Hypocotyl growth curves of WT, pif4-101, phyB-9 and phyB-9pif4-101 seedlings grown at 15°C or 25°C in 12:12 diurnal red photo-cycles. Error bars represent SE, n ≥ 19.

To further investigate PIF4 activity in red light, we measured the transcript levels of HFR1, a direct target of PIF4 and PIF5 [103]. In these conditions hfr1 mutants does not display any phenotype, thus we assume that HFR1 is unable to form heterodimers with, and suppress the activity of PIF4 and PIF5. Here, we measured the transcript levels 1 hour before dawn where PIF4 and PIF5 activity is expected to be high [116, 118] in addition to the middle of the light period (Midday). Consistent with the physiological data, HFR1 transcripts are up-regulated in warm temperatures at both timepoints, fully dependent on PIF4 indicating that PIF4 transcriptional activity is increased in the warm (Figure 3.11 A). In the phyB-9 background, HFR1 transcript levels are high, consistent with its presumed negative action on PIF levels, indicating that in wild-type seedlings phyB suppresses the accumulation of HFR1 over this temperature range. In addition, the midday samples show that in the phyB-9
background, *pif4-101* has an impact at both temperatures. However, *HFR1* transcription was still high in the *phyB-9pif4-101* double mutant suggesting that other phyB regulated transcription factors are acting on *HFR1*. As PIF5 is a likely candidate [89], we investigated the *HFR1* transcription in a *phyB-9pif4-101pif5-1* triple mutant, which indeed largely attenuated the *HFR1* levels at both time points (Figure 3.11 B). Thus, the genetic relationship between PIF4 and phyB observed by the phenotypic analysis suggests that phyB action is sufficient to completely suppress PIF4 at the cooler temperatures (Figure 3.10). However, although the transcriptional data is consistent with this notion it does additionally suggest that phyB is able to moderate the action of both PIF4 and PIF5 at the two temperatures.
Figure 3.11. PIF4 regulates HFR1 transcription downstream of phyB

(A) Expression of HFR1 in WT, pif4-101, phyB-9 and phyB-9pif4-101 seedlings grown for 6 days in 12-h light:12-h dark diurnal red photocycles and harvested 1 hour before dawn and at midday. HFR1 expression was measured using quantitative PCR with ACT7 acting as a control. Error bars represent SE, n = 3. (B) Expression of HFR1 in WT, pif4-101, phyB-9, phyB-9pif4-101pif5-1 seedlings grown and analysed as in (A) except in 12 and 27°C. Error bars represent SE, n = 3.
3.2.11. The dual action of HFR1 and phyB is essential for maintaining growth at warm ambient temperatures.

We have shown that both HFR1 and PIF4 are strongly temperature-regulated acting mainly in Blue and Red light respectively. At high temperatures PIF4 accumulates stimulating hypocotyl growth. This response is tempered by phyB, as well as cry1 that trigger HFR1 suppression of PIF4 activity. This suggests that PIF4 acts as a convergence point between the red and blue light signalling pathways and that phyB and particularly cry1 (via HFR1) plays an important role in buffering the effects of warm temperatures on molecular activity and plant growth. We therefore hypothesised that when grown in white light, removal of both the red and blue light regulation of PIF4 action should have severe consequences on plant growth particularly at the warmer temperatures (Figure 3.15). To test this we grew seedlings mutated in the red (phyB) and blue (cry1, hfr1) light dependent regulation of PIF4 activity on soil in white light at high temperatures to analyse hypocotyl and petiole elongation, leaf size and total biomass at different time points of development.

Hypocotyl measurements after 10 days in white light shows a clear temperature dependent elongation in the WT seedlings, and removal of HFR1 exaggerates this response (3 fold and 4 fold, 28/21°C for WT and hfr1-101 respectively) while the response in the pif4-101 mutant is severely attenuated (1.8 fold) (Figure 3.12 A and B).

Interestingly, under these conditions pif4-101 appears completely epistatic to hfr1-101. The phyB-9hfr1-101 is extremely elongated especially at the warm temperatures consistent with our model; however, the phyB-9hfr1-101pif4-101 triple mutant does
not completely abolish the effect of the *hfr1-101* mutation suggesting that the removal of phyB results in the increased activity of other factors, such as PIF5. Surprisingly, although HFR1 has been shown to mediate more than 70% of cry1 signalling the *phyb-9cry1-304* mutant is less elongated in the warm than the *phyB-9hfr1-101* mutant [168]. However, this mutant is also severely elongated in the cool, resulting in an impaired temperature response (1.1 fold) (Figure 3.12). This is likely to reflect the fact that loss of both phyB and cry1 as major photoreceptors leaves the plant largely blind to light, thus the temperature response goes towards that of a dark grown seedling [128].

**Figure 3.12. pif4 is epistatic to hfr1 in white light**

(A) Hypocotyl measurements of WT and mutant seedlings grown on soil in diurnal (12:12) white light for 10 days. Error bars represent SE, *n*≥26. (B) Picture of representative seedlings. Scale bar = 10mm.
Figure 3.13. Red and blue light regulation of PIF4 is vital for controlling leaf size in the warm

(A) Petiole measurements of the first true leaf from WT and mutant plants grown on soil in diurnal (12:12) white light for 4 weeks at 21 or 28°C. Error bars represent SE, n>10. (B) Measurements of the leaf area from plants grown as in (A). Error bars represent SE, n=10. (C) Picture of representative leafs. Scale bar = 10mm.
Analysis of the petiole elongation on older plants revealed very similar results to the hypocotyl measurements (Figure 3.13 A). Interestingly, the leaf size appears to be well buffered against changes in temperature in the WT, *pif4-101* and *hfr1-101* mutants. However, removing phyB and HFR1 results in extremely small leaves especially in the warm, suggesting that phyB and HFR1 are vital to buffer the effects temperature in this response (Figure 3.13 B and C). Further removal of PIF4, in the *phyB-9hfr1-101pif4-101* partially restores the size of the leaf particularly in the warm, consistent with PIF4 acting downstream of phyB and HFR1. In contrast to the regulation of the hypocotyl and petiole, the leaf size of *phyb-9cry1-304* is highly dependent on the ambient temperature, showing a response similar to *phyB-9hfr1-101* (Figure 3.13 B and C).

Finally, measurements of the biomass showed that the combined loss of phyB and HFR1 has severe consequences for the adult plant, particularly at high temperatures were development was severely impaired (Figure 3.14 A and B) as was indicated by leaf size measurements (Figure 3.13 B). In accordance with HFR1 negatively regulating PIF4, the *pif4-101* null allele partially restored the biomass of the *phyB-9hfr1-101*. Furthermore, the *phyB-9cry1-304* shows severely retarded growth similar to *phyB-9hfr1-101* highlighting the importance of the dual action of red and blue light in maintaining growth in warm conditions.
Figure 3.14. phyB and HFR1 are critical for plant growth at warm temperatures

(A) Rosette dry weight of 4-week-old and mutant plants grown on soil in diurnal (12:12) white light for 4 weeks at 21 or 28°C. Error bars represent SE, n=8. (B) Picture of representative leafs. Scale bar = 10mm.
3.3. Discussion

In the natural environment, plants are able to withstand daily and seasonal temperature fluctuations, yet relatively little is known about how this is achieved. Previous studies have shown that mutants of both red and blue light photoreceptors are impaired in specific temperature responses, suggesting that photoreceptor pathways have a unique role in reacting to changes in the environmental temperature [132, 133]. In this chapter, we show that phyB and cry1 act over a wide temperature range to inhibit hypocotyl elongation. However, the light-regulated downstream factors HFR1 and PIF4 are temperature dependent. We show that HFR1 accumulates in the warm under blue light. Furthermore, we have shown that also PIF4 abundance increases with the temperature, and provide genetic data suggesting that HFR1 strongly suppresses PIF4 activity in these conditions. The importance of the combined negative regulation of PIF4 by phyB and HFR1 is highlighted by mutant analysis, showing that plants deficient in both regulators exhibit severely retarded growth, particularly at warm ambient temperatures.

3.3.1. HFR1 operates at warm temperatures

Earlier work have shown that the phytochromes and cryptochrome 1 plays important roles in regulating flowering time at warm temperatures [36, 132, 133]. However, the retention of other light responses over a temperature range suggested that the temperature signal might regulate downstream components rather than the photoreceptors themselves [133, 134]. For this reason, we performed a microarray in order to identify downstream factors as putative targets of light and temperature.
(Table 1 Appendix A). Using this approach, we found that HFR1, a bHLH transcription factor previously implicated in blue and far-red light signalling, acting downstream of cry1 and phyA respectively [81, 84, 160], emerged as a strong candidate whose transcript levels were strongly dependent on the ambient temperature as well as under the control of phyB. Analysis of a mutant deficient in HFR1 revealed that the long hypocotyl phenotype of hfr1 was highly temperature dependent, specifically in blue light and required cry1 (Figure 3.3). As our data, and that of others, showed that cry1 acts over a wide temperature range, suggests that HFR1 resides in a warm branch of cry1 signalling [134]. The broad temperature spectrum which cry1 works within could therefore be achieved through multiple signalling components with different functional temperature optima. Indeed, although HFR1 has been shown to relay more than 70% of cry1 signalling [168], retention of the hypocotyl inhibition in the hfr1 mutant compared to the cry1hfr1 suggests that other signalling factors reside in the warm branch of cry1 signalling (Figure 3.3).

3.3.2. Light dependent stabilisation of HFR1 is temperature dependent

In darkness, phosphorylated forms of HFR1 are actively degraded by the COP1-SPAI E3 ubiquitin ligase complex [60, 85, 161, 162]. Light activation of the cryptochrome 1 and cryptochrome 2 directly results in the activation of this complex in addition to the nuclear exclusion of COP1 [68-70]. This does in turn result in accumulation of phosphorylated forms of HFR1 [60, 163]. We show that the blue light dependent accumulation of HFR1 is highly dependent on the ambient temperature and that HFR1 accumulate to a higher extent in the warm (Figure 3.4 A).
Recent work demonstrated that HY5, another target of COP1 dependent degradation, accumulates in cold conditions (4°C) to promote anthocyanine accumulation [146]. Interestingly, it was shown that HY5 protein accumulation was due to the inability of COP1 to enter the nucleus in these conditions. The fact that we observe HFR1 accumulation in the warm illustrates the complexity of COP1 action and target selection and suggests that COP1 activity is regulated both at low and high temperatures. In accordance with increased HFR1 levels, we demonstrated that HFR1 acts in the warm specifically during the daytime when the protein levels are high (Figure 3.4 B). Interestingly, it was recently shown that another bHLH transcription factor, SPT, also acts during the day to suppress growth [169]. However, in contrast to HFR1, SPT protein levels are boosted by cool ambient temperatures. The ability to switch between different homologues genes tuned to different temperatures could provide a mechanism for maintaining growth over a temperature range.

3.3.3. HFR1 acts upstream of PIF4 and PIF5 in blue light

HFR1 was originally found to act in far-red light to suppress hypocotyl elongation and further work identified HFR1 transcript levels to be highly upregulated under shade conditions acting as a part of a negative feedback, inhibiting an exaggerated SAS response [87, 89]. Later work has shown that far-red light and shade promotes the accumulation of Phytochrome Interacting Factors, for example PIF4 and PIF5, which drive the transcription of HFR1 [103]. Furthermore, it was recently shown that HFR1 acts by forming non-DNA-binding heterodimers with PIF4 and PIF5, thus completing the negative feedback loop [88, 89]. As PIF4 was identified as a major
promoter of temperature dependent elongation, we reasoned that HFR1 acts in the warm to suppress PIF4 and PIF5 activity. This was confirmed by our mutant analysis, suggesting that the *hfr1-101* phenotype observed under blue light in the warm was largely due to increased PIF4 and PIF5 action (Figure 3.5), suggesting that HFR1 acts by antagonising the activity of PIF4 and PIF5 in blue light.

### 3.3.4. PhyB and warm temperatures have opposite effects on PIF4 protein abundance

PIF4 has previously been implicated in growth promotion in response to warm temperatures [129, 135]. More recent studies have demonstrated that PIF4 is achieving this through direct transcriptional regulation of key enzymes in the auxin synthetic pathways [113, 136]. Furthermore, thermal induction of flowering was later shown to be dependent on PIF4 and it was suggested that temperature dependent H2A.Z occupancy on the *FT* promoter resulted in increased PIF4 binding and transcription of *FT* [138, 144]. Several of these studies performed in white light conditions suggest that, although *PIF4* transcript levels are temperature regulated, protein levels remain largely temperature stable. In contrast, using conditions that is not directly comparable, our observations in diurnal red light suggests that PIF4 protein levels are highly temperature regulated (Figure 3.7). This observation was recently supported in another study performed in white light [99]. We further demonstrated that warmer temperatures promote the accumulation of phosphorylated forms of PIF4 (Figure 3.9). However, this was observed in etiolated as well as light-grown seedlings, indicating that this effect was mediated independently of light.
PhyB has previously been shown to trigger PIF4 phosphorylation resulting in the subsequent degradation by the proteasome machinery [103, 116]. The warm temperature dependent phosphorylations might therefore target other/additional sites of PIF4 resulting in increased abundance. Alternatively, warm temperatures prevent the degradation of phosphorylated forms of PIF4 resulting in its accumulation. In any case, our data suggests that warm temperatures and light acts antagonistically to regulate PIF4 levels. Several observations are supporting this proposal. First, we showed that EOD far-red induced accumulation of PIF4 was attenuated in warm temperatures (Figure 3.8). Second, the phyb-9 hypocotyl phenotype was proportionally reduced compared to WT at warm temperatures (Figure 3.10). Lastly, the well-known short hypocotyl of pif4-101 [104] was less dependent on phyB at the warmer temperatures than in the cool (Figure 3.10). Therefore, our data suggests that phyB and warm temperatures have antagonistic roles in the control of PIF4 levels.

3.3.5. A warm temperature-regulated negative feedback loop

PIF4 has been shown to bind the G-box regions in the promoter of PIL1 and other shade induced genes, to promote transcription [89]. One of its targets is HFR1 which acts as a suppressor of PIF4 activity, thus forming a negative feedback loop. As our work, and that of others, have shown that PIF4 action is increased in warm temperatures, explains why we initially picked up the transcript of HFR1 in our microarray as highly temperature regulated (Table 1 Appendix A). Our subsequent transcript data shows that PIF4 and PIF5 indeed are largely responsible for the temperature dependent regulation of HFR1 transcription (Figure 3.11). Furthermore, we showed that the HFR1 abundance is diurnally regulated with the peak abundance
at dawn, consistent with the proposed diurnal regulation of PIF4 and PIF5 activity [116, 118]. Thus, PIF4 and PIF5 regulated HFR1 transcription is timed to coincide with dawn, where HFR1 is stabilised by light and further elevated by warm temperatures (Figure 3 Appendix A). This molecular module enables an HFR1 mediated feedback loop to suppress PIF4 and PIF5 activity during the day in warm temperatures (Figure 3.15).

**Figure 3. 15. Schematic of the warm growth module.**
In blue light HFR1 acts downstream of cry1 to inhibit PIF4 and constrain growth. Warm temperatures enhance levels of PIF4 protein, and to restrict growth, levels of HFR1 protein also increase. In red light PHYB inhibits PIF4 to confine growth. HFR1 negatively regulates its own transcription through PIF4.

Mutants of HFR1 are not known to have a flowering phenotype in white light conditions. However, in the light of the recent finding positioning PIF4 as the major driver of the thermal induction of flowering [144], it is possible that an hfr1 mutant would promote flowering under similar conditions, as predicted by our model (Figure 3.15).
Our data suggests that HFR1 is a major suppressor of PIF4 and PIF5 at warm temperatures. We also show that in the warm, PIF4 and PIF5 is a convergence point of the blue (cry1 via HFR1) and red (via phyB) light signalling pathways. The importance of this dual regulation was illustrated by our analysis of the phyB-9hfr1-101 mutant in white light, which displayed severely retarded growth as shown by leaf size and biomass accumulation at warm temperatures (Figure 3.13 and 3.14).

In conclusion, this study illustrates that light and temperature converge on the regulation of the important growth regulators HFR1 and PIF4. Warm temperatures promote PIF4 and HFR1 activity that acts in a diurnally driven negative feedback loop (Figure 3.15). This complex molecular coordination is essential to optimise plant growth and development in an environment that is subject to fluctuating and at times extreme temperatures.
Chapter 4 - A biphasic fluence response curve in the warm, explained by light dependent activation of PIFs

4.1. Introduction

Photoreceptor activation by light results in a dramatic reprogramming of the transcriptome in Arabidopsis [39] ultimately resulting in the transition from etiolated to de-etiolated growth in the young seedling [3]. Naturally, this developmental transition is not an “on/off” response but is dependent on the fluence rate of the incoming light [170]. In red light, this can in part be explained by light independent reversion of the active (Pfr) form of the phytochromes to the inactive (Pr) form which allows for fluence rate dependent adjustment of the Pr/Pfr ratio [15]. Thus, more phytochrome activity is expected in higher fluence rates of red light due to a high proportion of Pfr. As a well studied response to light, inhibition of hypocotyl elongation correlates with, and is dependent on, fluence rate of light. As discussed in Chapter 1, the transduction of the light signal from the initial perception by the photoreceptor to transcriptional regulation is complex. However, photoreceptor
dependent inhibition of COP1 activity results in fluence rate dependent accumulation of the positive regulator of photomorphogenesis HY5 [58]. Additionally, in a seemingly unrelated pathway, light activation of the phytochromes directly targets the PIF transcription factors for degradation, which when active promote etiolated growth [103]. However, as central regulators of seedling growth, both HY5 and the PIF proteins are additionally regulated through the interaction of a substantial number of other proteins [59, 89, 99, 121, 123, 171-173].

In contrast to increasing fluence rate of light, an increase in the ambient temperature promotes elongation of the hypocotyl [128, 129]. Intriguingly, it was noted that this does not happen in etiolated seedlings indicating that light is a prerequisite for the response [128]. Additionally, we and others have shown that PIF4 is a major positive regulator of temperature dependent elongation especially in red light conditions (Chapter 3) [129, 135, 148], providing a second strong link between photoreceptor activity and the response to temperatures.

In order to gain further insights into the light regulation of temperature responses in plants, in this chapter we investigate the fluence rate dependency of temperature responses in seedlings.

Our results confirm a fluence rate dependent inhibition of the hypocotyl length in 17°C, consistent with the view that increased photoreceptor activity promotes de- etiolation. In stark contrast, we discovered that in warm temperatures, the classical hypocotyl response to an increase in fluence rate is disrupted, resulting in a biphasic response curve. This implies that, at warm temperatures, high intensities of light can promote hypocotyl elongation. We show that this response is dependent on PIF4 and PIF5 acting downstream of phyB. Specifically, we show that the transcriptional
activity of PIF4/5 are increased in high fluence rates of light especially at the warm temperatures and that PIF4 binding of promoters are promoted by red light. Thus, we provide evidence for a positive light dependent regulation of PIF4/5 transcriptional activity which becomes increasingly important as temperatures rise.
4.2 Results

4.2.1. Fluence rate dependent inhibition of hypocotyl elongation is highly dependent on the ambient temperature.

In order to investigate the relationship between the fluence rate of light and the ambient temperature, we grew WT seedlings at 17, 22 and 27°C for 7 days in constant monochromatic red light and measured the hypocotyl length. Red light was chosen due to the fact that in this wavelength increasing temperatures were more potent in promoting hypocotyl elongation compared to blue and far-red light (Figure 3.2 A). Seedlings grown at 17°C display a classical fluence rate response, where increasing intensities of light are associated with an increased inhibition of hypocotyl elongation (Figure 4.1 A, C). However, while at 27°C, light increasingly inhibited the hypocotyl elongation in the low fluence rate range (<1 µm/m2/s) similar to 17 and 22°C, surprisingly, fluence rates above this did not invoke further inhibition, instead we observed a fluence rate dependent promotion of elongation (Figure 4.1 A, C). Thus, the fluence rate dependent inhibition of hypocotyl elongation that has been classically described is abolished at warm temperatures. Instead a biphasic response is observed.

In line with previous studies demonstrating that the temperature induced hypocotyl elongation is light dependent, we have here shown that this response is explicitly dependent on the fluence rate of light. Surprisingly, and in stark contrast to the
current view, increasing fluence rates of red light above a threshold acts to promote hypocotyl elongation at warm temperatures.

Figure 4.1. Warm temperatures results in a biphasic hypocotyl fluence response curve, whereas cotyledon size is unaffected.

(A) Hypocotyl measurements of WT seedlings grown in constant red light for 7 days at indicated temperatures and fluence rate. Error bars represent SE, n ≥ 17. (B) Cotyledon measurements of seedlings grown as in (A). Error bars represent SE, n = 20. (C) Photograph of representative seedlings. Scale bar = 10mm.
Measuring the fluence rate dependent inhibition of hypocotyl elongation has historically been used as a way to investigate the de-etiolation process. Mutants with an altered response will either be denoted a positive or negative regulator of photomorphogenesis depending on whether they promote or suppress the fluence rate response. Hence, the fluence rate dependent increase of the hypocotyl elongation observed in the warm could be indicative of a loss of photomorphogenic growth or promotion of skotomorphogenesis, possibly by a fluence rate and temperature dependent inactivation of the photoreceptors.

To examine this possibility, cotyledon size was also measured in response to the fluence rate at the three temperatures. In contrast to the hypocotyl data, the cotyledon size of the seedlings increases with increasing fluence rate of red light independently of the temperatures (Figure 4.1 B). This suggests that although the inhibition of the hypocotyl fails in the warm, the photoreceptors are still fully functional to promote cotyledon expansion across a temperature range.

In order to show that the biphasic response at warm temperatures is not limited to the Col-0 accession, Ler and WS was also grown in 17 and 27°C at the relevant fluence rates of light. A qualitatively similar biphasic fluence response curve (FRC) is observed in the warm for all accessions tested, while all show the classical response in the cooler temperatures (Figure 4.2 A-B). Therefore, the observed behaviour is not limited to Col-0.
Figure 4.2. A biphasic hypocotyl fluence rate response at warm temperatures is observed in several accessions.

(A-B) Hypocotyl measurements, shown as relative to dark, of Col-0, Ler and WS seedlings grown in constant red light for 7 days at 17°C (A) and 27°C (B) at indicated fluence rate. Inlay (A-B) represents length (mm) of dark grown seedlings. Error bars represent SE, n ≥ 20.

The fact that we observed a strong fluence rate dependency of the temperature dependent hypocotyl elongation suggests that the phytochromes are regulating, and necessary for the response.

In order to see if the biphasic FRC is exclusive to red light, seedlings were grown at different fluence rates of white and blue light in 17 and 27°C, conditions where both phytochromes and cryptochromes are active. Consistent with the observations in red light, the largest effect of the temperature increase is detected in the higher fluence rates of both white and blue light (Figure 4.3 A-B). However, in neither condition is a biphasic response seen, suggesting that activation of the blue light receptors is able to suppress a part of the phytochrome dependent temperature response.
Figure 4.3. Fluence rate dependent temperature induced hypocotyl elongation in white and blue light.

(A-B) Hypocotyl measurements of Col-0 grown in constant white (A) and blue (B) light for 7 days at 17 and 27°C, at indicated fluence rate. Error bars represent SE, (A) n ≥ 26 (B) n ≥ 23.

Taken together, we have shown that the extensively characterised fluence rate dependent inhibition of hypocotyl elongation [32, 170, 174] is severely altered in warm temperatures, resulting in a biphasic FRC in red light. Furthermore, the fluence rate dependency of this behaviour suggests that the red light receptors are regulating the response. However, this temperature dependent behaviour is not observed in the size of the cotyledons, suggesting that the photoreceptors are fully functional at the warmer temperatures.
4.2.2. Genetic analysis implicates phyB in temperature regulation of hypocotyl elongation

As the temperature induced hypocotyl elongation is highly dependent on the fluence rate of light, and the biphasic FRC appears to be specific to red light, it is highly likely that these responses are acting downstream of one or several of the phytochromes. As the major regulator of hypocotyl elongation in red light, phyB is likely to be the most important also at different temperatures. However, in order to investigate the contribution of phyB and the other phytochromes, phyA, phyB, phyC and phyD single and phyAphyB double mutants were grown in 17 and 27°C in both high (40µm/m²/s) and medium (1.4µm/m²/s) fluence rate and compared to the WT for altered fluence and temperature dependent phenotypes (Figure 4.4 A-B). As previously seen (Figure 4.1), hypocotyl elongation is inhibited in 17°C and promoted at 27°C by increasing fluence rate of light (Figure 4.4 A-B). The phyA mutant is slightly shorter than Col-0 which previously has been explained by phyA’s negative regulation of phyB [175] (Figure 4.4 A). However, the response to increased light levels by phyA is similar to the wt at both temperatures, suggesting that phyA plays a minor role in temperature regulation of hypocotyl growth.

Consistent with previous reports that phyB has the most prominent role, the hypocotyl length of phyB mutant was continuously elongated in all conditions, suggesting that phyB is necessary for responding to both the fluence rate of light as well as temperature (Figure 4.4 A). This data is strongly implicating that phyB is the principle photoreceptor modulating both the cool and warm FRCs. The phyAphyB
the double mutant is slightly longer than *phyB*, especially at the high fluence rate at both temperatures which is consistent with a reported function of phyA in high intensities of red light (Figure 4.4. A) [32].

**Figure 4.4. phyB is the major photoreceptor in red light independent of temperature.**

(A-B) Hypocotyl measurements of Col-0, *phyA*, *phyB* and *phyAphyB*, *phyC* and *phyD* mutants grown constant red light (1.4 and 40µm/m²/s) for 7 days at 17 and 27°C. Error bars represent SE, (A) n ≥16 (B) n ≥19.

The *phyC* and *phyD* mutants show a similar response to the WT indicating that they are not largely involved in temperature dependent elongation (Figure 4.4 B).

Taken together, these results clearly illustrates that *phyB* is the main photoreceptor regulating hypocotyl elongation in our conditions. Since the *phyB* mutant is lacking a response to temperature, this data also suggests that *phyB* function is a requirement for the WT temperature response.
4.2.3. PIF4 and PIF5 promote hypocotyl elongation in a temperature and fluence rate dependent manner

As PIF4 previously has been shown to be important for promotion of elongation during high temperatures [129, 135, 148] and is regulated by phyB directly by interaction, we postulated that PIF4 and possibly its close homologue, PIF5 are required for the biphasic FRC in the warm. To investigate this possibility, \textit{pif4} and \textit{pif5} mutants were grown under the same four conditions that elicit the response in the WT. As PIF4 and PIF5 shows sequence similarities [80] and have at least some redundant functions with other PIFs [30, 101, 176], \textit{pif1}, \textit{pif3} and \textit{pif7} was also included in this analysis.

Although PIF4 has been suggested to act specifically in the warm, we observe a clear phenotype of the \textit{pif4} mutant at 17°C (Figure 4.5 A) [129]. At high fluence rates, \textit{pif4} shows a reduced response to increasing temperature compared to the WT (~1.9 fold vs ~2.4 fold respectively), while \textit{pif5} shows a response similar to the WT (Figure 4.5 A). However, when comparing across the two fluence rates, both \textit{pif4} and \textit{pif5} appears have largely lost the promotion of elongation in the warm. The \textit{pif4pif5} double mutant shows a strong reduction in hypocotyl elongation with higher fluence rate at both temperatures suggesting that PIF4 and PIF5 are largely responsible for the biphasic FRC in the WT seen in the warm (Figure 4.1 A). The \textit{pif3pif4pif5} triple mutant shows a almost complete lack of temperature response and do get shorter with increasing fluence, however, the relative response to increasing fluence is lower in this mutant compared to the \textit{pif4pif5} double mutant due to the fact that the triple mutant is shorter in at the lower fluence rates at both temperatures.
Figure 4.5. Loss of one or several PIFs restores a classical FRC in the warm.

(A-C) Hypocotyl measurements of Col-0, pif1, pif3, pif4, pif5 and pif7, single double and triple mutants grown constant red light (1.4 and 40 µmol/m²/s) for 7 days at 17 and 27°C. Error bars represent SE, (A) n ≥ 18, (B) n ≥ 16, (C) n ≥ 22.

The pif3 mutant is shorter than the WT in all conditions grown, consistent with published data suggesting that PIF3 acts as a negative regulator of photomorphogenesis [105]. However, the response to increasing fluence rates is similar to the WT at both temperatures (Figure 4.5 B). In contrast, the pif1pif3 double mutant does get shorter with increased fluence rate of light at both temperatures, suggesting that, at least PIF1 affects light responses in a temperature dependent
manner. The *pif1pif3pif4* triple mutant is hypersensitive to light at all conditions and shows a strongly reduced temperature response, suggesting that PIF4 is more influential than PIF1 and PIF3 at warm temperatures (Figure 4.5 B).

Lastly, *pif7* does show hypersensitivity to red light at all four conditions and does not respond to increasing fluence in the warm, suggesting that PIF7 is in part responsible for the biphasic FRC seen in the WT.

Although this analysis supports previous studies showing that PIF4 has a major function in temperature dependent regulation [129, 135, 148] it is clear that PIF4 functions at both temperatures. Furthermore, our data shows that PIF4 action also is dependent on the fluence rate of light especially in the warm. The analysis of other *pif* mutants provides clear evidence for the involvement of PIF5 in temperature responses as well as minor roles for PIF1, PIF7 and possibly PIF3.

### 4.2.4. Selective screening identifies light signalling pathway components as temperature response modulators

To identify other factors involved in the fluence dependent temperature response, mutants known to affect the red light signalling pathways were grown at 17 and 27°C in both high (40µm/m²/s) and medium (1.4µm/m²/s) fluence rate.

In contrast to the PIFs, PRR7 and PRR9 have been shown to inhibit hypocotyl elongation [177, 178]. In addition, these proteins have been shown to be necessary for the thermal input in the circadian clock [152, 179] where they act to suppress the transcription of *CCA1* and *LHY* [180, 181]. Therefore, we wanted to investigate the impact of *prr7* and *prr9* in our conditions.
Although the \textit{prr9} and \textit{prr7prr9} single and double mutant show a similar response to increasing fluence as WT, the \textit{prr7} single mutant appears hyposensitive to light, especially at the cooler temperatures. Thus, while PRR7 and PRR9 are not required for the biphasic FRC in the warm, PRR7 might play a minor role in retaining the classical FRC in the cool (Appendix B).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{HY5 act in the cool to suppress hypocotyl elongation at high fluence rates.}
\end{figure}

(A-B) Hypocotyl measurements of Col-0, hy5, cop1, spt, hfr1 and 35S::PIF4-luc grown constant red light (1.4 and 40\textmu mol/m\textsuperscript{2}/s) for 7 days at 17 and 27°C. Error bars represent SE, (A) \( n \geq 25 \), (B) \( n \geq 16 \).

HY5 and COP1 have previously been characterised as major positive and negative factors of light signalling respectively [58, 66, 77]. As such, both have been reported to exhibit hypocotyl phenotypes particularly under red light illumination [67]. Furthermore, HY5 was recently shown to promote anthocyanin accumulation in response to a cold treatment, as a response to reduced nuclear COP1 activity [147], suggesting that these two factors play a role in temperature dependent signalling.
events. To investigate their possible role in temperature dependent hypocotyl elongation over a fluence rate we obtained and grew the hy5 null mutant, hy5-215, and a weak allele of cop1, cop1-4. The hy5-215 mutant shows a similar relative response to increasing fluence rate as the wt at 27°C indicating that warm temperatures does not affect HY5 activity. However, in cooler conditions the hy5 mutant clearly exhibit a stronger phenotype than in the warm when compared to the wt, that is most pronounced at the higher fluence rates. This suggests that HY5 acts to suppress hypocotyl elongation at cooler temperatures at the higher fluence rates. Consistently, in the cop1-4 mutant where HY5 levels are expected to be constantly elevated [58], both a temperature response and fluence rate response is lacking, indicating that COP1 action is necessary for these responses (Figure 4.6. B).

Lastly we also investigated the responses in a SPATULA mutant, spt-11, and hfr1-101 which previously has been shown to regulate hypocotyl elongation in red light and in a temperature dependent manner respectively [148, 182]. The spt-11 mutant responded to fluence rate and temperature similar to the wt seedlings in our conditions, and as expected for hfr1, no red light dependent phenotype was visible at either temperature (Figure 4.6 C).

Taken together, these experiments confirm that phyB is the major photoreceptor under red light in regulation of hypocotyl elongation and operates across a temperature range. In addition, although several of the phytochrome interacting factors show altered light and temperature responses, PIF4 and PIF5 show the largest effects and are likely required for the biphasic response in the warm. In contrast, HY5 was also identified, acting seemingly opposite to PIF4 and PIF5, suppressing
elongated growth especially in the cooler temperatures at the high fluence rates of light. Therefore, our following studies will focus on phyB, PIF4, PIF5 and HY5.

4.2.5. *pif4pif5* restores a classical fluence response curve in the warm.

As both PIF4 and PIF5 appeared to be major regulators of the fluence rate dependent temperature response, we wanted to characterise the single and double mutants over a broader range of fluence rates. At 17°C, the *pif4-101* and *pif5-3* mutants exhibited a shorter hypocotyl than the wt at all fluence rates and the *pif4-101pif5-3* double mutant showed additive effects (Figure 4.7. A). In the warm however, while the single and double mutants exhibited similar effects as in 17°C at the lower fluence rates of red light, at 40µm/m²/s the relative phenotypes compared to the wt increased drastically (Figure 4.7. B). In agreement with previous studies, *pif4* here shows a stronger phenotype than *pif5* [129, 135]. However, in these conditions over the whole range of fluence rates, the *pif4-101pif5-3* double mutant completely abolished the wt biphasic response restoring a classical fluence rate dependent inhibition of the hypocotyl elongation.

Thus, these results are consistent with the results presented above (Figure 4.5 A) suggesting that PIF4 and PIF5 are largely responsible for the biphasic response observed in the wt at warm temperatures. In addition, it suggests that, at least in warmer temperatures, increased fluence of red light acts to increase the importance of these proteins, in contrast to the generally held belief that red light activation of the phytochromes degrades and inactivates PIF4 and PIF5 [116].
Figure 4.7. PIF4 and PIF5 plays a major role in promoting elongation in the warm at high fluence rates of light.

(A-B) Hypocotyl measurements of Col-0, pif4, pif5 and pif4pif5 mutant seedlings grown in constant red light for 7 days in 17°C (A) and 27°C (B) at indicated fluence rate. Error bars represent SE, (A) n ≥ 20, (B) n ≥ 21.

4.2.6. PhyB is essential for temperature and fluence rate regulation of PIF4 and PIF5.

Our initial data suggested that out of the phytochromes tested, the phyB mutant clearly lacked a response to both increasing fluence rate of light and temperature. Interestingly, it was shown that the phyBDE mutant retains a strong temperature dependent hypocotyl response when grown in white light [129] suggesting that in those conditions, other photoreceptors play an active role in the temperature response. In order to get a more detailed view of the impact of phyB over a fluence range at two temperatures, the phyB mutant was reanalysed at additional fluence rates at 17 and 27°C (Figure 4.8 A-B). In general, these results confirm that the phyB mutant is largely unresponsive to light in regards to inhibition of hypocotyl elongation.
Furthermore, in accordance with the lack of a temperature response in darkness, the largely etiolated *phyB* mutant shows a severely reduced response to temperatures (Figure 4.8 A-B). However, especially in the warmer conditions, the *phyB* mutant shows a small inhibition of the hypocotyl in the lower fluence rates, which is abrogated at the highest light intensity (Figure 4.8. B). This is consistent with the presumed action of phyA at low intensities of red light, and the red light dependent degradation of phyA at high fluence rates [170]. Consequently, our analysis of the *phyB* mutant suggests that phyB is largely responsible for the hypocotyl fluence rate response, and in addition required for warm temperatures to promote elongation.

As one of the key mechanisms of phytochrome signal transduction is thought to be through degradation of PIFs [93, 103, 116, 183], and both phyB and PIF4/PIF5 appear necessary for the biphasic response, we went on to investigate the genetic relationship between *phyB* and *pif4pif5*. Interestingly, removing PIF4 and PIF5 in the *phyB-9* background results in a similar relative shortening of the hypocotyl at all light intensities at both temperatures, with the exception of darkness (Figure 4.8 A-B). This suggest that the increased activity of PIF4 and PIF5, as measured by *pif4-101pif5-3* mutant phenotype observed at the high fluence rates in the warm, is largely dependent on the presence of phyB.
Figure 4.8. *phyB* is largely insensitive to both light and temperature and is necessary for the temperature dependence of PIF4 and PIF5.

(A-B) Hypocotyl measurements of Col-0, *phyB*, *pif4pif5* and *phyBpif4pif5* mutant seedlings grown in constant red light for 7 days in 17°C (A) and 27°C (B) at indicated fluence rate. Error bars represent SE, (A) \(n \geq 20\), (B) \(n \geq 22\).

Analysis of *phyB* function in the *pif4-101pif5-3* mutant reveals that, as in the wt, *phyB* has little to no effect in darkness and the lowest light intensity tested. However, with increasing fluence rate of light, at both temperatures, loss of *phyB* becomes increasingly important (Figure 4.8. A-B). This result is in agreement with the measurements of cotyledon size suggesting that *phyB* is able to act over a fluence rate independently of the ambient temperature (Figure 4.1. B).

In conclusion, the genetic analysis of *phyB* and *pif4pif5* shows that *phyB* is necessary for the increased action of PIF4 and PIF5 in the warm at high fluence rates. Furthermore, this data clearly illustrates that both *phyB* and PIF4/5 has a function in regulating hypocotyl elongation at a range of fluence rates in both cool and warm conditions.
4.2.7. *PIF4* and *PIF5* transcript levels do not correlate with phenotypic data.

Transcription of *PIF4* and *PIF5* has previously been shown to be upregulated by red light [106, 117]. Furthermore, the transcript of *PIF4* has been shown to be positively regulated by an increase in the ambient temperature [129, 135, 184]. A simple explanation for our results could therefore be that the transcript levels of *PIF4* and *PIF5* are highly upregulated specifically in the warm at high fluence rates of light. This would in turn result in higher protein levels and consequently a larger phenotype of the *pif4-101pif5-3* mutant specifically in these conditions.

To test this hypothesis, we measured the levels of the native *PIF4* and *PIF5* transcripts in wt seedlings grown in the same conditions as the phenotypic analyses. Consistent with previous reports, both *PIF4* and *PIF5* are highly regulated by light, but also regulated by an increase in the temperature (Figure 4.9 A-B). However, the response to light saturates at the lowest intensity of light used our conditions, and the temperature dependent increase in transcript levels appeared to be completely independent of light.
Figure 4.9. Transcriptional regulation of PIF4 and PIF5 does not correlate with increased PIF action at high fluences.

(A-B) Transcript levels of PIF4 (A) and PIF5 (B) shown as relative to IPP2 in WT seedlings grown at indicated fluence rates of light and temperature. Error bars represent SE n = 3. (C-D) Hypocotyl measurements of Col-0, and PIF4, PIF5 over-expressing lines grown constant red light for 7 days at 17°C (C) and 27°C (D). Error bars represent SE, n ≥ 18.

To further investigate the potential importance of transcriptional regulation of PIF4 and PIF5 grew seedlings expressing PIF4-LUC and PIF5-HA fusion proteins under the control of the 35S promoter. With the exception of the low intensities of light and
darkness, where PIF5 is known to affect ethylene pathways [106], the PIF4 and PIF5 overexpressor lines are consistently taller than the wt seedlings (Figure 4.9. C-D). Interestingly, at 17°C, high fluence rates of light are able to suppress the hypocotyl of both overexpressor lines comparable to the wt. Conversely, at 27°C, these lines are slightly more elongated in the high fluence rates compared to the lower intensities. This therefore suggests that the wt behaviour is retained in seedlings missexpressing \textit{PIF4} and \textit{PIF5}. Taken together, we conclude that transcriptional regulation of \textit{PIF4} and \textit{PIF5} is not a major regulatory factor resulting in a biphasic fluence response curve in the wt seedling, measuring hypocotyl length.

### 4.2.8. \textit{phyB} regulated PIF protein proteolysis occurs at 17 and 27°C

Ruling out transcriptional regulation of \textit{PIF4} and \textit{PIF5} causing the biphasic fluence rate curve, suggests that light and temperature regulation of PIF4 and PIF5 may operate at the post-transcriptional level. Indeed, the literature contains many examples of post-translational regulation of the Phytochrome Interacting Factors including both sequestering [121, 123, 173, 185] [173] and degradation [103, 116]. Therefore, we hypothesised that the increased action of PIF4 and PIF5 in high fluence rates compared to the lower fluence rates, in the warm, could be the result of increased PIF4 and PIF5 protein levels, possibly due to altered phyB activity.

In order to test this, we quantified the protein levels of PIF4 in lines over expressing both PIF4-HA and PIF4-luc over a fluence rate at of light at several temperatures by immunoblotting and luciferace activity assays (Figure 4.10). As previously shown in 22°C [103], analysis of PIF4-HA by western blotting at 17, 22 and 27°C revealed that PIF4 is degraded by red light in all temperatures (Figure 4.10. A-B).
Furthermore, especially in the dark, as we and others have shown previously [99, 148], higher levels of the recombinant protein is observed in the warmer temperatures than at 17°C. However, in all temperatures, the general trend suggests that PIF4 is increasingly degraded with higher fluence rates of light (Figure 4.10 A-B) indicating that the biphasic FRC seen in the warm is not due to increased PIF4 stability.

We further analysed the line used in the phenotypic analysis expressing PIF4 coupled Luciferase driven by a 35S promoter by measuring luminescence. Consistent with the results obtained from the HA-tagged line, PIF-luc levels degraded in red light compared to darkness, and here, the levels are consistently higher in the warm at both fluence rates used as well as in darkness (Figure 4.10 C). This clearly suggests that PIF4 protein levels do not correlate with the observed action of PIF4 over a fluence rate in the warm and that PIF4, and likely PIF5, are activated by some other mechanism. Further analysis of the PIF4-HA immunoblots reveal no apparent difference in the temperature dependent phosphorylation over the fluence rate in the warm, as expected due to the previously shown light independency of this phosphorylation event (Figure 3.9 C). Therefore, although this temperature dependent phosphorylation might result in overall higher protein levels, it is probably not involved in the fluence rate dependent activation of PIF4.
Figure 4.10. PIF4 protein levels accumulates to higher levels in the warm, but is degraded by increasing fluence rate of light independently of the ambient temperature.

(A) Western blot of 6 day old 35S::PIF4-HA seedlings grown in indicated temperature and fluence rate of red light using an HA specific antibody for detection of PIF4-HA and anti-UGPase as loading control. (B) Quantification of PIF4-HA relative to UGPase. Errorbars represents SE from 3 biological replicas. (C) Quantification of luciferace activity shown as relative to total protein from 35S:PIF4-luc seedlings grown as in A. Errorbars represent SE, n = 3.
4.2.9. Elevated phyB levels completely suppress the warm hypocotyl response

Although phyB is known to degrade several of the PIF proteins, it has been shown that the PIFs also are able to promote the degradation of phyB [29, 30]. It is therefore possible that increased PIF4/5 activity at the high fluence rates results in a stronger degradation of phyB at warm temperatures, resulting in a lengthening of the hypocotyl. Consequently, in the pif4pif5 mutant, phyB levels could be stabilised in the warm resulting in the restoration of a classical fluence response curve. In order to investigate the regulation of phyB in our conditions, we analysed a phyB over expressing line and measured phyB protein levels. Analysis of the ABO line, over expressing phyB in the No-0 accession, revealed a wt phenotype in darkness, but an extreme hypersensitivity to light at the higher light intensities at both temperatures (Figure 4.11 A). This suggests that controlling phyB levels are essential for fluence rate dependent inhibition of hypocotyl elongation, and in addition a biphasic response in the warm. However, it is possible that excessive levels of the photoreceptor results in total degradation of the PIFs, thus the ABO mutant would partly mimic a multiple PIF mutant at the high fluence rates. This conclusion is supported by the very short and temperature insensitive hypocotyl of the pif3pif4pif5 triple mutant (Figure 4.5. A) and the pifq mutant [173]. To look at the effects of fluence rate on phyB protein levels we grew phyB-9 mutant seedlings harbouring a PHYB::PHYB-LUC construct and measured luminescence in samples from 6 day old seedlings. As previously reported phyB levels are high in darkness and decreased under red light illumination (Figure 4.11 B) [29, 186].
Figure 4.11. Over-expression of PhyB saturates the inhibition of hypocotyl elongation independently of the temperature.

(A) Hypocotyl measurements of No-0 and ABO seedlings grown in constant red light for 7 days in 17 and 27°C at indicated fluence rate. Error bars represent SE, n ≥ 26. (B) Quantification of luciferase activity shown as relative to total protein from PHYB::PHYB-luc seedlings grown as in A. Errorbars represent SE, n = 3.

Additionally, in the high fluence rates of light, which is where we observe a hypocotyl temperature response, changes in the ambient temperature does not appear to have an effect on phyB levels. Contrary, it is in the lower fluence rates of light a temperature effect is observed on phyB levels where higher temperatures are correlated to higher phyB levels (Figure 4.11 B). Therefore, it is unlikely that increased PIF dependent phyB degradation at high fluence rates of light in the warm is responsible for the biphasic fluence rate curve and we conclude that although PIF4 and PIF5 are more active in the warm, this does not directly affect phyB levels. In addition, this conclusion is supported by the cotyledon measurements over a fluence
rate at different temperatures suggesting that the light receptors are fully functional across a temperature range (Figure 4.1 B).

4.2.10. Red light promotion of hypocotyl elongation at 27°C requires auxin

The temperature dependent hypocotyl response has been shown to require auxin signalling [128]. Further is was shown that PIF4 promotes the temperature response by directly binding and positively regulating the transcription of TAA1, CYP79B2 and YUC8, all key enzymes in auxin synthesis pathways, which ultimately results in higher auxin levels in the warm [113, 129, 135, 136]. Additionally, both PIF4 and PIF5 are known to regulate several known downstream targets in the network to modulate auxin signalling [96, 187]. It is therefore likely that auxin signalling is required for the biphasic response, and in particular the fluence rate dependent increase of the hypocotyl growth in the warm. To confirm this hypothesis we grew wt seedlings at different light intensities of red light with the addition of 1 and 10µmol NPA, an inhibitor of auxin transport, at 17 and 27°C (Figure 4.12 A-C).
Figure 4.12. NPA inhibits elongation in the warm at high fluence rates of light

(A-B) Hypocotyl measurements of WT seedlings grown in constant red light for 7 days in 17°C and 27°C at indicated fluence rate on media supplemented with 0, 1 and 50µM of NPA. Error bars represent SE, (A) n ≥ 28, (B) n ≥ 33. (C) Photo of representative seedlings. Scale bar represents 10 mm.

In 17°C, the addition of 1µm NPA results in a shortening of the hypocotyl at all fluence rates of light with the exception of darkness, consistent with previously published data [188]. Addition of higher concentrations does not result in additional
shortening (Figure 4.12 A). In contrast, and in agreement of the temperature response being more pronounced at higher fluence rates in the warm, elevated NPA treatment had an increased effect on hypocotyl elongation, restoring a classical fluence response in the warm (Figure 4.12 B-C). Hence, as expected, this data suggests that auxin transport is necessary for the temperature dependent hypocotyl response. However, in addition, NPA blocks the biphasic response, indicating that auxin signalling is promoted by increased rate of fluence in the warm. This is consistent with the idea that PIF4, and possibly PIF5, are activated in the warm at high fluence rates of light to promote the transcription of key enzymes of the auxin signalling pathway.

4.2.11. Temperature regulation of Auxin genes by PIFs is light dependent

Our genetic analysis indicated that the biphasic fluence response curve in the warm is due to increased PIF4 and PIF5 action at the high light intensities. However, PIF4/5 transcript levels and PIF4/5 protein levels did not correlate with the phenotype of the pif4-101pif5-3 mutant. Instead we hypothesise that PIF4 and PIF5 becomes more active under these conditions not reflected by protein levels. As the results obtained from the NPA treatment suggested that auxin signalling is required for the biphasic fluence response curve in addition to the published literature showing that PIF4 can bind to and activate the transcription of TAA1, CYP79B2 and YUC8, we decided to look at PIF4 and PIF5 transcriptional activity by the means of transcript levels of these targets [96, 113, 136]. In order to do this, we grew wt and pif4-101pif5-3 mutant seedlings in darkness, low, medium and high fluence rate of light at 17 and 27°C correlating to the physiology experiments.
Figure 4.13. *YUC8* transcription levels are highly dependent on PIFs in high fluence rates of light

(A-C) Transcript levels of *TAA1* (A), *CYP79B2* (B) and *YUC8* (C) shown as relative to *IPP2* in seedlings grown in constant red light at indicated fluence rates, or dark for 6 days in 17 and 27°C. Error bars represent SE, n = 3.

Although both *TAA1* and *CYP79B2* has been reported to be upregulated in the warm [136], dependent on PIF4, in our conditions do not observe any major temperature or PIF4/5 dependents on these transcripts. Noteworthy however is that the transcript of
CYP79B2 appears to increase with the fluence rate of light, at both temperatures (Figure 4.13 A-B). In contrast and consistent with published literature [113], the transcript of YUC8 appears to be largely regulated by PIF4 and PIF5 and shows strong temperature dependence specifically at the high fluence rates of light. Thus, the transcriptional regulation of YUC8 in the wt correlates well with the observed biphasic fluence response curve seen in hypocotyl measurements. Furthermore the pif4-101pif5-3 mutant shows a strong phenotype especially in the high light intensities at 27°C (Figure 4.13 C). Taking these results into consideration with the NPA treatment, we hypothesise that increased PIF4/5 activity on the YUC8 promoter plays an important role in establishing the biphasic fluence rate response by increasing auxin levels.

PIF dependent regulation of auxin signalling is likely to be more complicated than regulation of key biosynthetic genes as PIF4 and/or PIF5 have been shown to bind the promoters of several auxin related genes, as IAA19, IAA29, SAUR23 and ATHB2 [96, 99, 189, 190]. With the exception of ATHB2 these targets have also been shown to be temperature regulated [99] [129] [136]. We therefore continued our transcript analysis by looking at the transcript levels of these targets (Figure 4.14 A-D).
Figure 4.14. Transcriptional analysis of known PIF4 and PIF5 targets.

(A-D) Transcript levels of IAA19 (A), IAA29 (B), ATHB2 (C) and SAUR23 (D) shown as relative to IPP2 in seedlings grown in constant red light at indicated fluence rates, or dark for 7 days in 17 and 27°C. Error bars represent SE, n = 3.

All these targets appeared to be negatively regulated by increasing light intensity at both temperatures and are dependent on PIF4/5 (Figure 4.14 A-D). Additionally, these four targets show a temperature dependent upregulation specifically in the high fluence rate consistent with being both auxin and PIF4/PIF5 regulated.
Figure 4.15. Transcriptional analysis of known PIF4 and PIF5 targets.

(A-C) Transcript levels of HFR1 (A), XTR7 (B) and FHL (C) shown as relative to IPP2 in seedlings grown in constant red light at indicated fluence rates, or dark for 7 days in 17 and 27°C. Error bars represent SE, n = 3.

We further looked at the transcript levels of HFR1, XTR7 and FHL, previously shown to be directly regulated by PIF4 and PIF5 [89, 96]. In our conditions, these genes are clearly regulated by PIF4/5 (Figure 4.15. A-B). Moreover, as we previously seen (Figure 3.11), HFR1 is temperature regulated in the wt, but not in the pif4pif5 double mutant. However, in contrast to YUC8, IAA19, IAA29 and SAUR23, HFR1, XTR7 and FHL appears to constitute a separate group of targets as these does not suggest increased PIF4/5 activity with higher fluence rate of light at any temperature (Figure 4.15 A-C). Thus, the biphasic fluence response curve correlates
with PIF4 and PIF5 transcriptional activity on a specific group of target. Among the transcripts analysed in this study, these targets all relate to auxin signalling.

4.2.12. PIF4 binding to promoters increases with fluence rate of light in the warm

PIF4 dependent transcription has previously been shown to increase with the ambient temperatures and furthermore correlate with binding to promoter regions [136, 144]. We therefore hypothesised that increased PIF4 binding to promoters in the warm is dependent on the fluence rate of light, thus correlating with our transcriptional analysis.

To investigate this, we performed ChIP using 35S driven PIF4-HA grown in darkness, 1.4 and 40 µmol/m²/s at 17 and 27°C. Our preliminary data confirms that PIF4 can bind to promoter regions of YUC8, IAA19 and XTR7 containing the G-box region (Appendix B). Furthermore, in agreement with YUC8 and IAA19 transcript levels in the warm, PIF4-HA binding to their promoter region is induced by increasing fluence rate of light (Appendix B). However, this fluence rate dependent increase appears to be largely lacking at 17°C consistent with the transcript data showing the largest temperature effect in high fluence rates. Interestingly, although the transcriptional behaviour of XTR7 differed from YUC8 and IAA19, the binding of PIF4 to the promoters showed a similar dependence on temperature and light for all three targets indicating that XTR7 is under additional regulation.

These results suggest that PIF4 promoter binding is, in addition to temperature dependent, highly dependent on and promoted by light, which could explain why PIF4 and PIF5 appears more active at high fluence rates in the warm.
4.2.13. The *pie1* mutant retains a classical FRC in the cool

It was recently shown that H2A.Z nucleosome occupancy on DNA is highly temperature dependent allowing for temperature dependent transcriptional regulation [132, 138]. Consistently, it was further shown that the thermal induction of flowering requires PIF4 and is regulated by H2A.Z occupancy on the *FT* promoter [144]. Therefore, at least in the case of flowering time in short day conditions, PIF4 appears more active in the warm due to increased accessibility of the *FT* promoter. If H2A.Z occupancy additionally is light and fluence rate dependent this could potentially increased binding of PIF4 at high fluence rates as well. As *HSP70* was shown to be largely dependent on H2A.Z occupancy [138], we further analysed the transcript levels of *HSP70* in our conditions (Figure 4.16 A). As previously shown, *HSP70* is upregulated in the warm and PIF4 and PIF5 does not alter its transcription [138]. In addition, our results suggests that light appears to have some effect on this temperature response as the largest temperature effect is observed at the highest fluence rates of light (Figure 4.16 A). To gain further insights we analysed the hypocotyl phenotype of the *pie1*-1 mutant which is required for H2A.Z deposition, at several fluence rates of light at 17 and 27°C [140]. The *pie1* mutant largely retained a classical and biphasic FRC in the cool and in the warm respectively (Figure 4.16 B). However, the relatively larger phenotype in the lower fluence rates of light in the warm compared to the high fluence rate suggests that H2A.Z nucleosome occupancy might be fluence rate dependent. Thus, although the *pie1* mutant clearly still responds to an increase in the ambient temperature, it might be a contributing factor of the biphasic FRC in the warm.
Figure 4. 16. Analysis of HSP70 transcript levels and the pie1-1 mutant

(A) Transcript levels of HSP70 shown as relative to IPP2 in seedlings grown in constant red light at indicated fluence rates, or dark for 7 days in 17 and 27°C. Error bars represent SE, n = 3. (B) Hypocotyl measurements of Ws, and pie1-1 mutant seedlings grown in constant red light for 7 days in 17 and 27°C at indicated fluence rate. Error bars represent SE, n ≥ 23.
4.3 Discussion

4.3.1. Interaction of light and temperature pathways

The photoreceptors have previously been implicated in temperature signalling in plants. It has been shown that multiple phytochrome mutants flower with similar number of rosette leaves in 16°C as the wt, but when increasing the temperature to 22°C they flower much earlier, suggesting that the phytochromes are important for buffering the effects of temperature in this response [133]. Likewise, it was shown that a phyAphyB mutant exhibits a dramatic internode elongation at warmer temperature but not in the cool [134], again suggesting that in the vegetative state, phytochromes are needed to buffer the effect of warmth. In contrast, at the seedling level warm temperatures seem to trigger a response causing promotion of hypocotyl elongation [128]. This response has been shown to require light, and furthermore the presence the Phytochrome Interacting Factor 4, PIF4 [129, 135, 148]. In this chapter, we have investigated the relationship between light and temperature, by looking at the fluence rate dependency of light on the hypocotyl temperature response.

4.3.2. A phytochrome dependent biphasic fluence response curve in the warm

As light is required for hypocotyl elongation in response to warmer temperatures we hypothesised that the fluence rate of light is likely to be an important regulating factor for the response. Our data collected at three temperatures over a wide range of fluence rates of red light strongly suggests that this indeed is the case as the
temperature response increases with the fluence (Figure 4.1). Surprisingly, at 27°C this results in a biphasic fluence response curve. Thus, in contrast to the classical fluence rate response, in the warm, increasing light can act to promote elongation. Although such a dual opposite regulation is non-intuitive, similar response has been shown in Physcomitrella phototropism response to increasing fluence rate of red light [191]. Furthermore, mutants of eidl exhibit a biphasic fluence rate curve in red light [192, 193] as well as the double sublcryl mutant in blue light [194]. These results have however been shown be dependent on phyA in the case of eidl and a complex fluence rate dependent epistatic relationship between subl and cryl. In any case, as our response is fluence rate dependent, we conclude that the actions of photoreceptors are required. Indeed, over the range of fluencies tested, the hypocotyl of the phyB mutant largely fails to respond to both light and temperature (Figure 4.8). However, or data suggests that cotyledon size is unaffected by the ambient temperature, at all temperatures. The fact that the phytochromes are known to also regulate this light response suggests that they are still fully functional in the warm [195].

4.3.3. PIF4 and PIF5 action is promoted by light in the warm

The phytochrome interacting factors, PIFs, residing in the subfamily 15 of the bHLH transcription factor family have recently emerged as major negative regulators of several light responses [62] [63]. As negative regulators of photomorphogenesis they are degraded subsequent to the light activation of phyA and/or phyB [93, 102, 103, 196] resulting in inhibition of hypocotyl elongation. On the other hand, PIF4 specifically have been shown to promote elongation in response to warm
temperatures [129, 148]. Our data shows that this promotion requires light due to the lack of a phenotype of the mutant in darkness (Figure 4.7) and it is therefore not surprising that this response is regulated by the fluence rate of light. Indeed, the relative hypocotyl length of the pif4pif5 mutant in the warm was ~70% and ~30% of the wt at 1.4 and 40 \( \mu \text{m}/\text{m}^2/\text{s} \) respectively, suggesting that light can promote their activity in a fluence rate dependent manner. However, in a phyB mutant, the phenotype of pif4pif5 does not appear to be dependent on fluence rate where the phyBpif4pif5 mutant in the warm was ~75% and ~78% of the phyB at 1.4 and 40 \( \mu \text{m}/\text{m}^2/\text{s} \) respectively (Figure 4.8). Therefore, in addition to the well characterised phyB dependent degradation of PIF4/5, phyB appears necessary for the fluence rate dependent activation of PIF4 and PIF5 in the warm. It is interesting to note that PIF4 and PIF5 recently been shown to act in lower fluencies of far-red and white light [88, 96]. However, in these conditions HFR1 is known to be active, and as a target of COP1, high intensities of light would presumably increase HFR1 levels, resulting in sequestering of PIF4 and PIF5 activity [60, 89]. This is probably not the case in monochromatic red light as hfr1 is lacking a phenotype in these conditions (Figure 4.6 C).

4.3.4. High intensities of red light promote transcription of auxin related genes through PIF4 and PIF5.

Although we observe elevated PIF4 levels in the warm, over a fluence rate of light, protein levels or transcript levels did not correlate with the observed hypocotyl length (Figure 4.10). Furthermore, as the phyB levels at the high fluence rates were identical in both temperatures tested, it is unlikely that the long hypocotyl in the
warm is due to PIF dependent degradation of phyB as have been suggested before [29]. Instead we decided to investigate the transcriptional activity of PIF4 and PIF5 on some of their known targets. Several studies have shown that auxin signalling is required for the temperature response, and moreover that PIF4 can bind to and regulate the transcription of several auxin related genes [96, 113, 136]. Our NPA treatment confirms the role of auxin signalling in temperature dependent growth and the response to NPA is largest at the high fluence rates consistent with a previous report (Figure 4.12) [188]. Analysing the transcript levels of TAA1, CYP79B2 and YUC8 revealed that only YUC8 showed a temperature dependency in our conditions (Figure 4.13). Moreover, YUC8 transcription levels were dependent on the fluence rate of light and pif4pif5, therefore correlating well with the phenotypic analysis (Figure 4.13 C). Importantly, in contrast to TAA1, the YUCCA family of flavin containing monooxygenases are thought to constitute a rate limiting step in IAA biosynthesis as their overexpression results in high IAA levels [114, 137]. YUC8 but not TAA1 or CYP79B2 were also found to be upregulated in response to shade conditions dependent on PIF4 and PIF5 in a different study [96]. In any case, the fluence rate dependency of the temperature regulation of YUC8 are in agreement with the physiological data and does suggest that PIF4 and PIF5 activity can be promoted by high intensities of light.

In concordance with increased YUC8 transcription, the auxin responsive genes IAA19, IAA29 and SAUR23 showed a similar response as YUC8 (Figure 4.14). Additionally, these transcripts also showed a strong dependency of pif4pif5 which is consistent with their promoters being bound by PIF4 and/or PIF5 [96, 99, 189]. This set of data
does however not allow us to differentiate between direct PIF4/5 regulation and indirect regulation via YUC8 and auxin levels.

### 4.3.5. G-box binding by PIF4 is promoted by light independently of the ambient temperature

Transcriptional regulation by the PIFs have previously been shown to correlate with their increased binding to promoter regions of target genes [121, 123, 136, 144]. In shaded conditions this increase is likely to be due to increased protein stability levels as a result of increased Pr:Pfr ratio of phyB [103]. Furthermore, there are several examples of interacting proteins that bind to and sequester the transcriptional activity of the PIFs, including HFR1, PAR1, phyB and the DELLA proteins [89, 121, 123, 173, 185]. We performed ChIP using a PIF4-HA overexpressing line to analyse the binding of PIF4 to several promoter regions of already confirmed targets of PIF4 and/or PIF5 at different intensities of light at two temperatures. Confirming our other results showing increased importance of PIF4 and PIF5 in the warm with increasing light intensity, the ChIP results demonstrated increased binding to the promoter regions (Appendix B). To our knowledge, this dataset represents the first comparison of PIF4-DNA binding at different intensities of light and it is intriguing that we observe higher binding of PIF4 at conditions were we would expect lower PIF4 protein levels, and vice versa. However, although a correlation between the protein levels of PIF4/5 and transcript levels of confirmed targets in response to shade has been reported, direct evidence for increased DNA binding in this response is lacking. This might be relevant as our results suggest that red light (possibly through phyB action) can promote PIF4 binding, consequently leading to the direct assumption that
inactivation by shade would have the opposite effect. However, this does not have to be the case as our experiments are performed in monochromatic red light and are therefore not directly comparable to any other studies.

4.3.6. Conclusions

In this chapter we have investigated the dependency of the fluence rate of red light on temperature responses. Intriguingly, we found that warm temperatures disrupt the classically described fluence rate dependent inhibition of hypocotyl elongation resulting in a biphasic fluence response curve. Contrary to the published wealth of knowledge, these results directly suggested that high intensities of light can act to promote hypocotyl elongation. Further, we have shown that the bHLH transcription factors, PIF4 and PIF5, are required for this response in the warm, suggesting that their activity is promoted in these conditions. This suggestion was supported by transcriptional analysis of several known PIF4/5 targets. Although *PIF4* and *PIF5* transcript nor protein levels correlated with the increased activity at high fluence rates of light in the warm, PIF4 DNA binding was increased in a fluence dependent manner, suggesting that red light can promote PIF activity. Although these results are not sufficient to fully explain the behaviour of PIF4 and PIF5 over a fluence rate of light at different temperatures, this set of data clearly demonstrates a previously unknown light dependent promotion of PIF activity.
Chapter 5 - Effects of HY5 and GA on PIF4.

5.1 Introduction

The Phytochrome Interacting Factors (PIFs) have emerged as playing a major role in a wide variety of processes throughout plant development [97, 101, 111, 116, 144, 197]. For this reason, it would be expected that the regulation of their activity is tightly controlled. The literature certainly supports this view as a large amount of mechanisms have been described, including phosphorylation, degradation, and sequestering of the proteins activity [88, 93, 106] [121, 123, 185].

In the previous chapter (Chapter 4), we demonstrated by phenotypic analysis that high light intensities of red light promote PIF4 and PIF5 action in warm temperatures (Figure 4.7). This increased action did not correlate with PIF4/5 transcript or PIF4 protein levels (Figure 4.9 and 4.10). Furthermore, as phyB protein levels are the same at 17 and 27°C in high light conditions, altered PIF4/5 dependent degradation of phyB is likely not a major cause for the biphasic fluence response in the warm (Figure 4.11 B and 4.1 A). However, other mechanisms of PIF regulation have previously been demonstrated which entail sequestering of the active protein [96, 121, 123, 173]. A sequestering mechanism presents an attractive model since the
protein levels of PIF4, as observed by immunoblotting (Figure 4.10), does not correlate with expected action.

Alternatively, other transcription factors with an opposite function to the PIFs in light signalling could potentially act independently of PIF4/5 on the same promoters and consequently suppress the effect of PIF4 and PIF5.

In this chapter we investigate the role of GA signalling and the DELLA proteins on PIF4 and PIF5 activity over a fluence rate in cool and warm temperatures. Our findings suggest that PIF activity is under strong DELLA regulation in the cool temperatures especially at high fluence rates of light. Additionally, based on the selective screen performed in chapter 4, we investigate the role of the bZIP transcription factor HY5 and its relation to PIF4 over a temperature and fluence rate range.
5.2 Results

5.2.1. GA promotes elongation in the cool

Relatively recently it was reported that the DELLA proteins, that are negatively regulated by the phytohormone GA, can interact with, and inhibit PIF3 and PIF4 binding to DNA [121, 123, 198]. Interestingly, it has been shown that RGA accumulates in the hypocotyl in response to high light intensities, potentially suppressing PIF action [153]. In addition, RGA levels were shown to decrease in response to high temperatures [135]. We therefore hypothesise that the temperature dependent shift in PIF4/5 function, is due to their suppression by DELLAs in the cool, especially at high fluence rates of light. To test this idea, we grew wt and pif4-101pif5-3 seedlings on media supplemented with GA at 17 and 27°C at high and low fluence rate of red light (40 and 1.6µmol/m²/s). On control plates, the hypocotyl length of both the wt and mutant seedlings shorten in 17°C in response to increasing fluence rates, however at 27°C the wt elongates while the pif4pif5 mutant does not, as previously shown (Figure 5.1 A-B and 4.7 A-B). As predicted the GA treatment had little effect at the warm temperatures in the wt seedlings, but at 17°C, GA strongly promotes elongation specifically at the high fluence rates of light resulting in a light response similar to non GA treated seedlings grown in the warm (Figure 5.1. A-B). Consistent with DELLA action through PIFs, the same response to GA treatment is not observed in the pif4pif5 double mutant (Figure 5.1. A-B).
Figure 5.1. GA suppress PIF activity in the cool at high fluence rates

(A-B) Hypocotyl measurements of wt and pif4pif5 mutant seedlings grown for 7 days in low and high fluence rate of red light at (A) 17 and (B) 27°C with or without the supplement of exogenous GA. (C-D) Hypocotyl measurements of Ler, and della4 mutant seedlings grown in constant red light for 7 days in 17°C (C) and 27°C (D) at indicated fluence rate. Error bars represent SE, (C) n ≥ 24, (D) n ≥ 22.

The data for Figure 5.1 A-B was obtained and analysed by Douglas Pyott.
We also grew the *della4* mutant [153] in our conditions and measured the hypocotyl length of the mutant and the wt. The mutant exhibited an elongated hypocotyl at the high fluence rates of light in both temperatures. However, consistent with the GA treatment, the relative phenotype of the *della4* mutant compared to the wt suggests that the DELLA proteins are more active in the cooler conditions than in the warm (1.34 fold vs 1.21 fold respectively) (Figure 5.1 C-D).

We therefore conclude, that GA regulation of the PIFs through the DELLAs are not a major factor in the warm, but in the cool low GA levels are inhibiting PIF4 and PIF5 especially at the high fluence rates of light. The fact that the *della4* mutant behaves differently than the GA treatment is likely to be due to RGL3, the fifth DELLA protein in Arabidopsis still present in the *della4* mutant.

### 5.2.2. HY5 suppresses hypocotyl elongation in the cool.

We have demonstrated that warm temperatures severely alter the seedlings response to light by the measurement of hypocotyl lengths (Figure 4.1 A). Specifically, increasing fluence rates of light can promote hypocotyl elongation in the warm. Phenotypic analysis revealed that PIF4 and PIF5 are essential for this response and furthermore implied that PIF4/5 can be activated by high light intensities. Although PIF4 protein levels were lower at high fluence rates of light, we found that a fluence rate dependent increase of *YUC8* and *IAA19* in the warm correlated with increased binding of PIF4 to their promoters. However, while in the cooler temperatures increased fluence rate of light suppressed *YUC8* and *IAA19* transcript levels, a decrease in PIF4 binding to their promoters was not observed, suggesting that other factors are acting on these promoters (Appendix B).
In our initial screen of known pathway mutants with altered response to increasing fluence rate, *hy5-215* and its negative regulator *cop1-4*, were identified as mutants of potential interest (Figure 4.6 B). To further characterise their phenotypes we grew the mutants under a broader range of fluence rates of red light at 17 and 27°C. As reported previously, the *cop1-4* mutant exhibit photomorphogenic growth in darkness [65]. Similar to the wt, the *cop1-4* mutant does not respond to increasing temperatures in darkness, indicating that de-etiolation is not sufficient for the hypocotyl temperature response. Our data further suggests that the *cop1-4* mutant retains a minor response to light, however light does not appear to have any effect on the temperature induced hypocotyl elongation (Figure 5.2 A-C). This data is consistent with COP1 as a major negative regulator of photomorphogenesis [67] but does also suggests that COP1 function is necessary for temperature dependent hypocotyl elongation. However, as COP1 is known to act as an E3 ubiquitin ligase, degrading several positive factors of photomorphogenesis [58, 59, 199, 200] it is possible that the lack of a temperature response is due to an over accumulation of these factors, saturating the response.
Figure 5.2. HY5 acts mainly in high fluence rates of light at cooler temperatures.

(A-B) Hypocotyl measurements of Col-0, hy5 and cop1 seedlings grown in constant red light for 7 days at 17°C (A) and 27°C (B) at indicated fluence rate. Error bars represent SE, n ≥ 20.

(C) Photograph of representative seedlings. Scale bar = 10mm.

Being one of the more prominent targets of COP1, HY5 have been extensively characterised as a positive regulator of photomorphogenesis [58]. Consistent with being degraded by COP1 in darkness, in 17°C the impact of the hy5 mutation
increases with the fluence rate of light (Figure 5.2 A). Interestingly, the fluence rate response of the hy5 mutant shows a tendency towards a biphasic response in the cool similar to the WT in the warm. In the warm, the impact of the hy5 mutant relatively small, showing a slightly elongated hypocotyl in the higher fluence rates (Figure 5.2 B). Thus, in contrast to PIF4 and PIF5, these results suggest that HY5 is most important in the cooler conditions specifically at the high fluence rates of light to inhibit hypocotyl elongation (Figure 5.2 A-C).

### 5.2.3 HY5-HA accumulates faster in the cool.

As HY5 previously was shown to be stabilised at cool conditions, due to reduced COP1 nuclear localisation [147] we wanted to investigate if we could observe a temperature difference in HY5 accumulation at our temperatures. To do this, we grew seedlings over-expressing HY5 tagged to HA [79] in darkness at 17 and 27°C to subsequently expose the seedlings to 40µmol/m²/s of red light for 1 and 6 hours (Figure 5.3 A-B). Consistent with HY5 being targeted by COP1 we observed very low levels of HY5-HA in the dark (Figure 5.3 A-B). After 1 and 6 hours of illumination, the HY5 protein accumulated to high levels at both temperatures. However, at 6 hours the levels of HY5 were ~70% higher in the cool compared to the warm, thus correlating with the phenotypic analysis of the hy5-215 mutant (Figure 5.3 A-C). In order to gain further support for increased HY5 activity at the cooler temperature we analysed the transcript levels of the well known target of HY5, CHS [71]. In agreement with reduced HY5 protein levels in the warm, CHS are only half in 27 compared to 17°C, thus lending support to our hypothesis.
Figure 5.3. HY5 activity is increased in cool temperatures

(A) Representative immunoblot of HY5 in 35S::HY5-HA seedlings grown in darkness for 3 days at 17 and 27°C before moved into red light for 1 and 6 hours. (B) Quantification of HY5-HA protein levels. Error bars represent SE of 3 biological repeats. (C) Expression levels of CHS in wt seedlings grown for 7 days in indicated fluence rate of light and temperature. IPP2 was used as control and error bars represent SE of 3 biological repeats.
5.2.4. Genetic relationship between *hy5* and *pif4*

Our genetic analysis of *hy5* has revealed that HY5 is more important in the high fluence rates of light in cooler conditions that in warmer. Furthermore, we observe a faster accumulation of overexpressed HY5-HA at 17 than 27°C suggesting that post-translational regulation of HY5 might account for a part of the temperature dependent phenotype of *hy5-215*. Interestingly, while the *pif4-101pif5-3* double mutant restored a classical fluence response curve in the warm (Figure 4.7 B), the *hy5-215* mutant displayed more of a biphasic response in the cool (Figure 5.2 A). Therefore, it appears that PIF4/5 and HY5 plays opposite roles in the temperature regulation of hypocotyl length at high fluence rates of light. Although no direct link between these proteins previously have been described, both HY5 and PIF4/5 are transcription factors able to bind the G-box sequence on DNA to regulate transcription [96, 201, 202]. Furthermore, it was noted [187] that when comparing HY5/HYH regulated genes with PIF4/5 regulated genes, as examined by microarrays [75] there is a small 20 gene overlap between PIF4/5 upregulated and HY5/HYH down regulated transcripts. Interestingly, this overlap was significantly enriched in transcript related to auxin signalling containing *IAA19*, *SAUR23* and *YUC8* but also *XTR7*. Thus, it is possible that HY5 and PIF4/5 are acting in opposite to regulate temperature dependent hypocotyl elongation by acting on the same promoters.

To investigate the genetic relationship between PIF4 and HY5 we generated the *pif4-101hy5-215* double mutant and analysed their hypocotyl length. At both temperatures we observe an intermediate phenotype between *hy5-215* and *pif4-101* (Figure 5.4 A-B), indicating that HY5 and PIF4 are acting in separate pathways. The expected redundancy in these networks (HYH, PIF5) complicates analysis and multiple
mutants should be created to fully determine their genetic relationships. However, intermediate phenotype of the *pif4-101hy5-215* double mutant is consistent with idea of HY5 and PIF4 acting on the same gene targets to regulate hypocotyl elongation.

**Figure 5.4. Analysis of the *pif4hy5* mutant**

(A-B) Hypocotyl measurements of Col-0, *hy5*, *pif4* and *pif4hy5* seedlings grown in constant red light for 7 days at 17°C (A) and 27°C (B) at indicated fluence rate. Error bars represent SE, n ≥ 18.
5.3 Discussion

5.3.1. Low GA levels in the cool promotes DELLA suppression of PIFs

Post translational regulation of the PIF proteins appears to be complex. In addition to phytochrome mediated degradation, there are several examples of interacting proteins that bind to and sequester the transcriptional activity of the PIFs, including HFR1, PAR1, phyB and the DELLA proteins [89, 121, 123, 173, 185]. As GA regulation of the DELLAs previously have been implicated in temperature regulation [135], we investigated the role of GA and \textit{della4} in different fluence rates of light and at two temperatures. Interestingly, while GA treatment had small effects in the warm, GA treatment in the cool promoted elongation preferentially at the high fluence rates of light suggesting that the DELLAs are suppressing light activation of PIF4/5 at the lower temperatures (Figure 5.1 A). This is consistent with the reported light dependent accumulation of RGA and its degradation in the warm [135, 153]. The fact that the \textit{della4} mutant or WT seedlings grown on GA still elongated in response to increased fluence rate of red light in the warm suggests that the light dependent activation of PIF4/5 is independent of GA signalling (Figure 5.1 A-D).

5.3.2. HY5 acts in the cool to promote photomorphogenesis

In our initial screen for mutants with altered temperature and light responses we identified \textit{hy5} and its negative regulator \textit{cop1} as mutants of interest (Figure 4.6 B). Consistent with HY5 action being increased in cool (4°C) conditions, we observe a relatively stronger phenotype of \textit{hy5} in 17 than 27°C at the high fluence rates of light.
Remarkably, over a fluence rate in the cool, the hy5 mutant has a tendency towards a biphasic response, similar to the wt in the warm (Figure 5.2 A) implying that HY5 acts to suppress this response at cooler temperatures. Thus, in relation to the PIFs, not only does HY5 act opposite in light signalling, but the preferred temperature for their action appears opposite. Interestingly, there are reports of HY5 and PIF3 acting on the same promoters to regulate anthocyanine accumulation [201] and it was noted that there is a overlap of genes positively regulated by PIF4 and PIF5 that are negatively regulated by HY5 and HYH [75, 187]. Moreover, this overlap was highly enriched in auxin related targets, including YUC8 as we looked at in Chapter 4. Thus, it is possible that HY5 are acting mainly in the cool, at high fluence rates of light to directly, or indirectly, suppress the expression of YUC8 while PIF4/5 are acting as promoters. This is consistent with the intermediate phenotypes observed in the pif4hy5 double mutant, considering the fact that HYH and PIF5 still are present in the plant. Interestingly, the HY5 dependent increase in anthocyanin accumulation in response to cool conditions have been shown to be regulated by GA [203], suggesting that increased DELLA levels promotes HY5 protein levels and transcriptional activity [203-205]. Thus, it is possible that biphasic response seen in the cool after application of exogenous GA is partly due to decreased HY5 activity, explaining why both GA and hy5 promotes elongation specifically in the cool.

5.3.3. Conclusions

In Chapter 4 we provided evidence that light can act to promote the activity of PIF4 and PIF5 in the warm which ultimately results in a biphasic hypocotyl fluence response curve. Interestingly, both the application of GA and in the hy5 mutant, a pif
dependent biphasic response was seen in the cooler temperatures as well (Figure 5.1 A and 5.2 A). This suggests that HY5 and the DELLAs are highly important in the cool at high fluence rates of light to promote de-etiolation and to negate the effects of PIF mediated promotion of elongation. Thus, the biphasic FRC observed in the warm, which we have shown to be a result of light dependent activation of the PIFs (Chapter 4) appears to be strongly inhibited at cooler temperatures by both the DELLAs and HY5.
Chapter 6 - Discussion

Although plant responses to heat stress and freezing conditions are relatively well characterised [206, 207], when the work for this thesis was initiated, little was known about how plants are able to sense and respond to a small change in the ambient temperature. However, a few publications had indicated that the loss of photoreceptor activity could result in dramatic temperature sensitivity in responses as flowering time and internode elongation [133, 134]. Furthermore, one study had shown that warm temperatures promote elongation of the hypocotyl and that this response is dependent on the phytohormone auxin [128].

However, over the last few years, major advances have been made in the field to which the work in this thesis has contributed [148]. Most notably, Phytochrome Interacting Factor 4 (PIF4) has recently been extensively characterised as a major regulator of responses to warm temperatures [99, 129, 135, 136, 144]. Previously characterised as negative regulator of photomorphogenesis [104], the fact that PIF4 now takes a central role in temperature signalling supports the earlier proposition that light and temperature networks are interconnected [133].

Based on the apparent importance of photoreceptor activity in temperature signalling, the work in this thesis aimed to investigate the relationship between light and temperature. In doing so, we established that the bHLH transcription factor HFR1 plays a vital role in controlling heat effects on growth by suppressing PIF4 action. We also demonstrated that the reported temperature-regulated PIF4 activity is not observed in the dark. In fact, red light over a threshold of value of 1µmol/m²/s is required to elicit this response that is strictly fluence rate dependent.
6.1. HFR1 acts to prevent excessive elongation in the warm

In the search of key regulator factors of temperature responses, through a microarray approach, we identified the transcript levels of \textit{HFR1} to be highly temperature regulated as well as dependent on \textit{phyB}. HFR1 had previously been shown to act in light signalling pathways to promote photomorphogenesis in far-red and blue light as well as acting as a negative regulator of the shade avoidance syndrome [81, 84, 87]. Given the microarray result we investigated a possible role of HFR1 in temperature signalling by analysing the hypocotyl response of the \textit{hfr1} mutant in a set of different light conditions at two temperatures. Interestingly, we found that the mutant exhibited strongly increased elongation in warm temperatures specifically in blue light (Figure 3.2). This result suggested that HFR1 action is not only light dependent as previously thought but also regulated by the ambient temperature. Our genetic analysis showed that HFR1 acts mainly downstream of the blue light receptor \textit{cry1} in these conditions, and we also provided evidence to suggest that HFR1 is stabilised and accumulates to higher levels in the warm (Figure 3.3 and 3.4). As PIF4 had recently been identified as a major positive regulator of warm growth in Arabidopsis [129], and our collaborators had shown HFR1 functions by inhibiting PIF4 activity [89], following work focused on the interplay of HFR1 and PIF4 in regulating growth at warm temperatures. Importantly, we show that the phenotype of \textit{hfr1} in the warm is largely dependent on the action of PIF4 and PIF5 suggesting that similar to in the SAS, HFR1 acts to suppress these proteins in the warm (Figure 3.5). Although PIF4 had just been described as a major regulator of the temperature induced hypocotyl elongation, we did not observe a phenotype of the single \textit{pif4} mutant in
blue light. This fact encouraged us to further characterise the \textit{pif4} mutant and we found that the temperature dependent phenotype of the mutant was most evident in red light, where increased temperatures largely failed to promote elongation (Figure 3.6). In addition, we showed that in a \textit{phyB} mutant background, the \textit{pif4} phenotype was not temperature specific, but observed across a temperate range, suggesting that phyB is required for the \textit{pif4} temperature phenotype (Figure 3.10).

Thus, by working in monochromatic light conditions, we identified two separate light signals that appear to be modulated by the ambient temperature and we propose a molecular network where red light and blue light signals together with temperature merge on the regulation of PIF4 (Figure 3.15). Under white light conditions (that include red and blue wavelengths) warm temperatures increase PIF4 activity. However, its action is moderated through a red light (phyB) and a blue light (cry1 via HFR1) arm of the network. In support of this view, we showed that removal of the red and blue light regulation of PIF4 (\textit{phybhfr1} double mutant) resulted in extremely elongated growth, specifically in the warm, due to unregulated PIF4 activity (Figure 3.12, 3.13 and 3.14).

\textbf{6.2. Identification and characterisation of a biphasic FRC in the warm}

Our hypocotyl measurements from high fluence rates of blue, far-red and red light indicated that the temperature response is most pronounced in red light while nonexistent in the dark (Figure 3.2). This suggested that the phytochromes are likely to play a major role in temperature responses and furthermore that there must be a relationship between the fluence rate of red light and the temperature response. To investigate this, we grew wild type seedlings at cool and warm temperatures in
different fluencies of red light, ranging from very low to high fluence. Consistent with previous observations, a fluence rate dependent inhibition of the hypocotyl length was observed in the lower temperatures. At 27°C, light increasingly inhibited the hypocotyl elongation in the low fluence rates similarly in 17 and 27°C, however after reaching 1µmol/m²/s, further increasing the light levels resulted in elongation of the hypocotyl. These new observations showed that in warm temperatures, the classical hypocotyl response to an increase in fluence rates is disrupted, resulting in a biphasic response (Figure 4.1 A). More specifically, in contrast to the current view of de-etiolation, increasing fluence rates of red light can promote hypocotyl elongation at warm temperatures, a finding that challenges the existing view of light signalling.

To elucidate the molecular basis of this behavioural change we examined mutants of the red light receptors and other factors known to be involved in both light and temperature signalling. As the major photoreceptor in these conditions, the phyB mutant failed to respond to increased light levels at both temperatures, suggesting that phyB acts over a broad temperature range to suppress hypocotyl elongation (Figure 4.8). The double mutant of the Phytochrome Interacting Factors 4 and 5 (pif4pif5) did not exhibit the biphasic response, implicating these PIFs in the light dependent thermal response (Figure 4.7). This suggested that at warm temperatures, red light promotes the activity of PIF4 and PIF5, a proposition that appears to conflict with the earlier observation that red light depletes PIF protein levels [103]. As we previously observed higher PIF4 levels in the warm, a simple explanation would be that high fluence rates of light promotes PIF4 accumulation in the warm. However, analysis of the transcript abundance of PIF4 and PIF5 as well as protein stability of PIF4 indicated that the increased action of the PIFs in high fluence rates
at warm temperature is not due to increase in PIF4/5 abundance (Figure 4.9 and 4.10) although PIF4 protein levels are elevated in the warm (Figure 6.1 A). Instead we looked at the transcriptional activity of PIF4 and PIF5 on a number of their confirmed targets and found that several targets appears to be upregulated by these PIFs specifically in the warm at high fluence rates of red light (Figure 4.13, 4.14 and 4.15). Interestingly, these targets included several auxin related genes, which is consistent with our data showing that NPA treatment largely suppresses the biphasic FRC (Figure 4.12). In agreement with increased transcriptional activity on targets as YUC8 at high fluence rates of light in the warm, we provide preliminary data suggesting that also PIF4 binding to the promoter is increased (Appendix B).

Thus, we have shown that warm temperatures disrupt the classically described fluence rate dependent inhibition of the hypocotyl elongation, resulting in a biphasic response. We further showed that this is due to a previously unidentified light dependent promotion of PIF4 (and likely PIF5) transcriptional activity through increased binding to their target promoters (Figure 6.1 B).

### 6.3. The effects of GA on temperature dependent hypocotyl elongation

Our data suggests that high light conditions in the warm promote the promoter binding of PIF4 to regulate Auxin signalling. Furthermore, the genetic analysis of the pif4pif5 mutant suggests that red light is required for the light dependent promotion of their activity (Figure 4.7). However, we do not know what could account for the difference in PIF behaviour at 17 and 27°C. As our data suggests that PIF4 protein levels are reduced with increasing fluence of light, thus not correlating with their transcriptional activity, a sequestering mechanism regulating the PIFs presents an
attractive idea. Indeed, HFR1, PAR1, phyB and the DELLA proteins have been shown to inhibit PIF activity by sequestering them [89, 121, 123, 173, 185]. As PAC treatment previously have been shown to inhibit the temperature response and RGA accumulates in response to light but is also temperature sensitive, we hypothesised that DELLA regulation of PIFs could contribute to the temperature control of PIF activity [129, 135, 144, 153]. Growing seedlings on GA supplemented media, which has been shown to deplete the DELLAs [121], illustrated that DELLA suppression of the PIFs is more important in cooler temperatures than in the warm as expected (Figure 5.1). Interestingly, GA treatment in the cool resulted in a biphasic behaviour in the wt but not in the pif4pif5 mutant. Thus, these results suggest that the DELLAs are important for imposing control on PIF4 and PIF5 in the cool and that the impact of DELLAs on PIF activity increases with fluence rate (Figure 6.1). This finding also suggests that perhaps the light-dependent PIF promotion of hypocotyl elongation that we observed at high temperatures could be the PIF signalling “default” state, but in the cool DELLAs suppress this activity. The light dependent activation of the PIFs however is not likely to be due to a fluence rate dependent release of PIF4 and PIF5.

6.4. HY5 acts to antagonise PIF4

By growing a number of different known light signalling mutants in low and high fluence rates of light at two temperatures we identified a possible role for the bZIP transcription factor HY5 in temperature dependent regulation of hypocotyl growth (Figure 4.6 B). A detailed analysis of the hy5 mutant revealed a large phenotype in cool conditions increasing with the fluence rate of light, while a relatively minor phenotype was observed in the warm (Figure 5.2). To our knowledge, this is the first
time a temperature dependent role for HY5 has been reported in regards to hypocotyl elongation. However, consistently with the regulation of anthocyanin accumulation, HY5 appears more active in cooler conditions [147]. It was proposed that cool temperatures inhibit the nuclear accumulation of COP1 and in agreement, we provide evidence suggesting that HY5 protein stability is increased at lower temperatures (Figure 5.3 A-B and Figure 6.1) [147]. It is therefore tempting to speculate that COP1 activity is generally decreased in the cool resulting in increased inhibition of hypocotyl elongation and that the opposite is true in the warm. However, as we also have shown that another target of COP1, HFR1 protein levels increases in the warm, the system is likely highly regulated (Figure 3.4 A). Interestingly, both HY5 and PIF4/5 have been shown to regulate auxin signalling, and comparing microarray data there is a small overlap between HY5/HYH and PIF4/PIF5 regulated genes significantly enriched in auxin related genes [75, 187]. These genes include YUC8, IAA19 and SAUR23 whose transcripts we have shown to correlate with the PIF4/5 dependent biphasic FRC in the warm (Figure 4.13 and 4.14). It is therefore possible that HY5 and PIF signalling merge on the regulation of auxin signalling in which HY5 would inhibit auxin levels in the cool while PIF4 has a positive function in the warm. As both the PIFs and HY5 has been shown to bind to G-boxes, this also grants the possibility of them competing for the same binding sites [201] [104, 208]. We further analysed their genetic relationship by measuring the hypocotyls of the pif4hy5 mutant and found intermediate phenotypes at both temperatures. This is consistent with the idea of the PIF4 and HY5 pathways are merging on the transcriptional regulation of auxin signalling and suggests that HY5 acts to repress the PIF response in the cool, like the DELLAs.
6.5. Interactions of light and temperature

HY5 and the PIFs are well known to be regulated by light. Activated phytochrome photoreceptors promote the degradation of the PIFs while promoting the stability of HY5 by inhibiting the ubiquitin E3 ligase COP1 [58, 63] (Figure 6.1 A). Furthermore, the DELLA protein RGA has been shown to accumulate to higher levels in seedlings when exposed to light [153]. Thus, light inhibits the PIFs that drive elongated growth while it promotes the accumulation of HY5 and the DELLA to further restrict elongation (Figure 6.1 A, B). In 17°C the combined action of these factors is consistent with a fluence rate dependent shortening of the hypocotyl elongation (Figure 6.1 B). Interestingly, results from mutant analysis (Figure 4.7, 5.1 and 5.2) and protein measurements (Figure 4.10 and 5.3) at 17 and 27°C suggests that these three factors are all temperature regulated. Intriguingly, while light inhibits the PIFs and promotes DELLA and HY5, warm temperatures appears to act in the direct opposite, promoting PIF activity and reducing the importance of HY5 and the DELLAs (Figure 6.1 A). As warm temperatures and light clearly have opposite effects on the phenotypic level of the young seedling, the fact that this appears true for several molecular components is compelling.
Figure 6.1 Schematic model of light and temperature crosstalk.

(A) Red light activates the photoreceptor which results in the inhibition of COP1 activity and PIF protein levels as well as the promotion of DELLAAs and PIF transcript levels. Warm temperatures act opposite to red light on COP1 activity, PIF protein levels and DELLA activity resulting in the promotion of elongated growth. (B) Representation of fluence rate dependent effects on HY5, PIF and DELLA action/activity at 17 and 27°C. The increased “activity” of PIF in 27°C at the high fluence rate compared to the middle does not correspond to increased protein levels but transcriptional activity or increased promoter binding.

However, the opposing roles of light and warm temperatures can not easily explain the biphasic fluence response curve observed in the warm (Figure 4.1). In particular, although both the HY5 and DELLA mediated suppression of hypocotyl elongation is
reduced in the warm they are still likely to be most active at the highest fluence rate of light (Figure 6.1 B). Moreover, we have shown that although PIF4 levels are promoted by warm temperatures (Figure 3.7 and 4.10), PIF4-HA is still degraded by increasing the fluence rate of light. Thus, in addition elevating the PIF protein levels, warm temperatures must also increase the activity of the PIFs specifically at higher fluence rates of light so that the combined action of HY5, DELLAs and PIFs results in elongated growth (Figure 6.1 B). We postulate that this is accomplished by increased PIF binding of promoters in the warm which was supported by preliminary ChIP data (Appendix B).

6.6. Future Perspectives

In order to conclude the work in presented in Chapter 4 the preliminary ChIP data needs to be repeated. Furthermore, performing the ChIP experiment in the phyB mutant background would reveal a possible role of phyB regulation of PIF4 promoter binding. In Chapter 5 it was suggested that PIF4/5 acts antagonistically to HY5 possibly by regulating the same transcripts. Further experiments to support this is needed, in particular qPCR data of possible common targets using single pif4 and hy5 mutants as well as the pif4hy5 double mutant. Additionally ChIP can be used to investigate the temperature and fluence rate dependent binding of both HY5 and PIF4 to promoters of interest.

The interaction of light and temperature on the molecular level has proven highly complex. In addition, we have observed unintuitive physiological responses. Hence,
follow on work would likely be aided by taking a computational modelling approach. In fact, this is already ongoing in the lab where this work was conducted.

Perhaps one of the more intriguing discoveries in this thesis is the fact that PIF4 appears to become phosphorylated in response to increasing temperatures (Figure 3.9). As these phosphorylation events also occurred in darkness the phosphorylation can not by itself explain the increased activity of PIF4 in the warm at high fluence rates of light. In any case, it would be interesting to identify these temperature dependent phosphorylation sites in order to create Ala or Asp substitutions at these sites. Introduced into the \( pif4 \) mutant could reveal the function of these phosphorylations.

Lastly, in the view of the recently published finding that PIF4 is a major regulator of thermal induction of flowering [144] it would be interesting to see what effect HFR1 has in similar conditions as HFR1 directly inhibits PIF4 by forming heterodimers [89].

In conclusions, some additional work could strengthen the conclusions of Chapter 4 and 5 and for increasing the understanding of the system, a modelling approach would likely be helpful.

6.7. Conclusion

Work conducted in this lab and in others has shown that light and temperature as external signals converges on the same molecular components in plants. This thesis represents an effort to decipher how this information is integrated. We have shown that light can buffer temperature effects through HFR1 mediated suppression of PIF4 and PIF5 activity, dependent on cry1 (Chapter 3) [148]. Conversely we have shown
that red light can boost PIF activity in the warm (Chapter 4) while suppressing its activity through a DELL A and a HY5 pathway in the cool (Chapter 5).

In conclusion, this work highlights the importance of PIFs in temperature responses as well as the importance of its regulation. In the cool, PIFs are strongly inhibited by several separate mechanisms particularly in high fluence rate of light while in the warm this restraint is alleviated leaving the PIFs free to act on plant development.
Chapter 7 – References


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Appendix A
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Table 1. Micro-array analysis showing genes expressed in a phyB and temperature dependent manner.

All genes have ≥1.5 fold-change in expression in phyB relative to Ler (t-test P-value cut-off 0.05) and show ≥1.5 fold-change in response to temperature in both Ler and phyB (t-test P-value cut-off 0.05). P-values relate to changes in transcript due to temperature.

Data acquired by Julia Foreman.
Figure 1. In blue photoperiods HFR1 protein abundance and dynamics are altered by temperature.

Immunoblot of HFR1 protein levels in 6 day old 35S::HFR1-HA hfrl seedlings grown in diurnal 12:12 blue light photo-cycles at 15°C or 25°C. Anti-HA antibodies were used to visualise HFR1. DET3 is shown as loading control. EON= End of night, 15h = 3h into following night.

Data acquired by Patricia Hornitschek.
Figure 2. PIF4 protein levels in diurnal blue light.

Immunoblot of PIF4 protein levels in 6-day-old 35S::PIF4-HA seedlings grown at 15 or 25°C in 12-h light:12-h dark diurnal blue photocycles. Protein levels are significantly different between the two temperatures ($P \leq 0.005$, paired Student’s $t$-test).

Data acquired by Patricia Hornitschek.
Figure 3. Red light dependent *HFR1* transcription is affected by temperature and accumulates during the night.

Diurnal expression of *HFR1* in WT seedlings grown in diurnal 12:12 red light at 12°C, 17°C, 22°C and 27°C. Samples were taken every 4 hours throughout day 9.

Data acquired by Julia Foreman.
Methods

Micro-array analysis
Ler and phyB-1 seedlings were grown in short-day conditions (8L:16D) at 16°C for 11 days then kept at 16°C or transferred to 22°C for 21 days. Aerial parts of the plants were collected at 4 hours post-dawn. RNA was extracted with TRIZOL (Invitrogen) then further purified with a RNeasy kit (QIAGEN) with on-column DNase digestion. Two independent experiments were carried out to attain biological replicates for each sample. RNA was sent to the NASC Affymetrix Gene Chip service where they performed ATH1 Genome Arrays. The full dataset is available to download from the NASCarrays database (ref: NASCARRAYS-394). Background correction, normalization and gene expression analysis of the array data were performed using the GC-RMA routine in GeneSpring version 7.2 (Silicon Genetics).

Q-PCR analysis
For diurnal transcript analysis, seeds were stratified for 3 days at 4°C, give a 6h white light pulse to induce germination, then grown in darkness for 18 hours at 22°C. The plates were moved to diurnal, 12:12 red light (20μmol/m²/s, florescent tubes with Rosco #19 red filter) for 24h before the temperature was changed to the indicated temperature. The samples were harvested in RNAlater (SIGMA) and cDNA synthesis was performed using ReverseAid™ FirstStrand cDNA synthesis kit (Fermentas). Real-time PCR was performed using a Rotor Gene 3000 (Corbett research). Primers used have been described in the main text.
Appendix B
PRR7 acts in the cool to suppress hypocotyl elongation at high fluencies.

Hypocotyl measurements of Col-0, *prr7*, *prr9* and *prr7prr9* grown constant red light (1.4 and 40 µmol/m²/s) for 7 days at 17 and 27°C. Error bars represent SE, n ≥ 20.
PIF4 binding to G-box containing promoter regions is promoted by light in the warm.

(A-F) Chromatin of 6 day old seedlings grown in Darkness, 1.4 and 40 µmol/m2/s of red light in 17 or 27°C was immunoprecipitated using either no antibody (-Ab) or anti-HA antibody (+Ab). Resultant DNA extracted from 35::PIF4-HA (A, C, E) or the 35S::HFR1-HA negative control (B, D, F) was analysed by qPCR using primers specific for G-Box containing promoter regions of \textit{YUC8} (A-B), \textit{XTR7} (C-D) and \textit{IAA19} (E-F). Each signal is expressed as a percentage of the signal in nonimmunoprecipitated DNA (input) extracted from the same sample and data represents the average of two technical replicates.

This data was acquired and analysed by Kristen Knox.
**METHOD**

Chromatin Immunoprecipitation was carried out essentially as previously described [209] with the exception that 35S::PIF4-HA and 35S::HFR1-HA seedlings were grown for 6 days in darkness, 1.4 or 40 µmol/m²/s of red light in 17 or 27°C. Chromatin was immunoprecipitated using anti-HA (ab9110 Abcam) and target binding was analysed using the G-box spanning primers listed below.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
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<tbody>
<tr>
<td>IAA19-F</td>
<td>ACCACCGCATCCTCAGTTG</td>
</tr>
<tr>
<td>IAA19-R</td>
<td>CGTTGGTCCACACGATAC</td>
</tr>
<tr>
<td>XTR7-R</td>
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</tr>
<tr>
<td>YUC8-R</td>
<td>AGTGATAATGCTGCGGCGGCA</td>
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