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par

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Genome-wide transcriptional responses to shade
Linking shade avoidance and auxin

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Prof. Andreas Mayer

Abstract

Plants have the ability to use the composition of incident light as a cue to adapt development and growth to their environment. *Arabidopsis thaliana* as well as many crops are best adapted to sunny habitats. When subjected to shade, these plants exhibit a variety of physiological responses collectively called shade avoidance syndrome (SAS). It includes increased growth of hypocotyl and petioles, decreased growth rate of cotyledons and reduced branching and crop yield.

These responses are mainly mediated by phytochrome photoreceptors, which exist either in an active, far-red light (FR) absorbing or an inactive, red light (R) absorbing isoform. In direct sunlight, the R to FR light (R/FR) ratio is high and converts the phytochromes into their physiologically active state. The phytochromes interact with downstream transcription factors such as PHYTOCHROME INTERACTING FACTOR (PIF), which are subsequently degraded. Light filtered through a canopy is strongly depleted in R, which result in a low R/FR ratio and renders the phytochromes inactive. Protein levels of downstream transcription factors are stabilized, which initiates the expression of shade-induced genes such as *HFR1*, *PIL1* or *ATHB-2*.

In my thesis, I investigated transcriptional responses mediated by the SAS in whole *Arabidopsis* seedlings. Using microarray and chromatin immunoprecipitation data, we identified genome-wide PIF4 and PIF5 dependent shade regulated gene as well as putative direct target genes of PIF5. This revealed evidence for a direct regulatory link between phytochrome signaling and the growth promoting phytohormone auxin (IAA) at the level of biosynthesis, transport and signaling.

Subsequently, it was shown, that free-IAA levels are upregulated in response to shade. It is assumed that shade-induced auxin production takes predominantly place in cotyledons of seedlings. This implies, that IAA is subsequently transported basipetally to the hypocotyl and enhances elongation growth. The importance of auxin transport for growth responses has been established by chemical and genetic approaches.

To gain a better understanding of spatio-temporal transcriptional regulation of shade-induce auxin, I generated in a second project, an organ specific high throughput data focusing on cotyle-

don and hypocotyl of young *Arabidopsis* seedlings. Interestingly, both organs show an opposite growth regulation by shade. I first investigated the spatio-transcriptional regulation of auxin responsive gene, in order to determine how broad gene expression pattern can be explained by the hypothesized movement of auxin from cotyledons to hypocotyls in shade. The analysis suggests, that several genes are indeed regulated according to our prediction and others are regulated in a more complex manner. In addition, analysis of gene families of auxin biosynthetic and transport components, lead to the identification of essential family members for shade-induced growth responses, which were subsequently experimentally confirmed. Finally, the analysis of expression pattern identified several candidate genes, which possibly explain aspects of the opposite growth response of the different organs.

Résumé

Les plantes ont la capacité dutiliser la composition de la lumière incidente comme signal en vue d'adapter leur développement et leur croissance à leur environnement. *Arabidopsis thaliana*, tout comme beaucoup d'autres espèces cultivées, est mieux adaptée à des habitats ensoleillés. Lorsque soumises à l'ombre, ces plantes montrent une variété de réponses physiologiques appelées collectivement 'syndrome dévitement de l'ombre'. Ces réponses incluent une augmentation de la croissance de l'hypocotyle et des pétioles, une diminution de la croissance des cotylédons ainsi qu'une réduction de la ramification et du rendement agricole.

Ces réponses sont principalement régulées par les photorécepteurs phytochromes, lesquels existent soit dans leur isoforme active, absorbant dans le rouge lointain (FR), soit dans leur isoforme inactive, absorbant dans le rouge (R). Dans la lumière directe du soleil, le rapport du rouge sur rouge lointain (R/FR) est haut, ce qui convertit les phytochromes à leur état physiologiquement actif. Les phytochromes interagissent avec des facteurs de transcriptions situés en aval de la réponse, tels que les PHYTOCHROME INTERACTING FACTOR (PIF), qui sont ensuite dégradés. Lorsque filtrée à travers une canopée, la lumière du soleil s'appauvrit fortement en rouge et ceci résulte en un faible rapport rouge sur rouge lointain, lequel rend les phytochromes inactifs. Les quantités protéiques des facteurs de transcriptions en aval de la réponse sont stabilisées, ce qui déclenche l'expression de gènes induits par l'ombre, comme *HFR1*, *PIL1* ou *ATHB-2*.

Durant ma thèse, j'ai examiné les réponses transcriptionnelles régulées par le syndrome d'évitement de l'ombre dans les plantules entières d'*Arabidopsis*. Par le biais de données provenant de microarrays et d'immunoprécipitation de chromatine, nous avons identifié, sur l'intégrité du génome, des gènes régulés par la réponse à l'ombre et dépendants de PIF4 et PIF5. Nous avons également identifié certains gènes comme cibles supposées de PIF5. Ces données ont révélé des preuves d'un lien de régulation directe entre la signalisation des phytochromes et la phytohormone auxine (IAA), qui promeut la croissance, au niveau de la biosynthèse, du transport et de la signalisation.

Par la suite, il a été montré que les niveaux de IAA libre augmentent en réponse à l'ombre. Il est

communément admis que la production d'auxine induite par l'ombre se passe principalement dans les cotylédons des plantules. Cela implique que l'auxine soit ensuite transportée vers l'hypocotyle et augmente ainsi la croissance par élongation. L'importance du transport d'auxine pour des réponses de croissance a été établie par des approches chimiques et génétiques.

Afin de mieux comprendre la régulation spatio-temporelle de la transcription par l'auxine induite par l'ombre, j'ai également généré une base de données à haut débit qui permet de focaliser spécifiquement sur l'hypocotyle et les cotylédons des jeunes plantules d'*Arabidopsis*. Lorsqu'à l'ombre, ces deux organes montrent de façon intéressante une régulation opposée de la croissance. J'ai commencé par étudier la régulation spatio-transcriptionnelle des gènes répondant à l'auxine. Ceci afin de déterminer à quel point les motifs d'expression des gènes peuvent être expliqués par l'hypothétique transport de l'auxine depuis les cotylédons vers l'hypocotyle en conditions ombragées. Les analyses suggèrent que plusieurs gènes sont en effet régulés selon nos prédictions, mais que d'autres sont régulés de manière plus complexe. De plus, l'analyse de familles de gènes, impliquées dans la biosynthèse de l'auxine et son transport, a mené à l'identification de membres essentiels pour la régulation de la croissance induite par l'ombre. Ces derniers ont par la suite été confirmés de façons expérimentale. Finalement, l'analyse de motifs d'expressions a permis d'identifier plusieurs gènes candidats qui pourraient expliquer certains aspects de la régulation opposée de la roissance dans les deux organes.

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1 Introduction

Environmental conditions are subject to constant changes to various extends. For sessile organisms, such as plants, it is imperative to constantly monitor and respond to various factors such as resource availability, temperature, photoperiod or herbivore pressure effect or guides growth and development. Plants have a high phenotypic plasticity and the final shape, size and internal morphology of organs or whole plants depend on the interplay of genetic and environmental factors. An important external cue, which affects the entire life cycle of plants, is the availability of light. Sunlight consists of various wavelengths and its composition may differ between conditions. Plants particularly use wavelengths of the blue and red spectrum as a key energy source through photosynthesis. In addition, light function as a crucial signal, which provides spatial and temporal information about the surrounding environment. Consequently, many phenotypic adaptations aim to optimized light capture by inducing growth and developmental responses through complex molecular signaling networks (Cline, 1997; Kim *et al.*, 2005b; Franklin, 2008; Casal, 2013).

1.1 Direct sunlight and shade have different spectral distributions

Direct sunlight and vegetational shade differ not only in light intensity but also in the composition of the light spectrum (Casal, 2013). Plant leaves absorb a large proportion of red light (R) and blue light, which are best suited to fuel photosynthetic processes. Longer wavelength including far-fed light (FR) penetrate leaves to a higher extent leading to relative enrichment of FR in shade (Franklin, 2008). As a consequence, the photosynthetic active radiation (PAR) below leaf canopies is reduced, whereas the R/FR ratio is increased. However, green leaves also reflect FR causing a relatively low R/FR ratio in direct proximity of neighboring plants without necessarily reducing the amount of PAR (Ballaré, 1999; Keller *et al.*, 2011; de Wit *et al.*, 2012; Pierik and de Wit, 2014).

1.2 Light-mediated growth modulation

The model organism *Arabidopsis thaliana* as well as many crop species are best adapted to sunny habitats (Kebrom and Brutnell, 2007). With increasing growth density plants need to compete for light. Too little light results in a negative energy balance and cannot sustain the organism. To avoid being shaded by nearby growing competitors, plants have evolved various strategies to detect neighboring plants including monitoring light quality, quantity and sensing volatile or soil-delivered chemicals as well as mechanical cues (de Wit *et al.*, 2012; Pierik and de Wit, 2014).

Shade intolerant plants respond to a low R/FR ratio as perceived in shaded environments with a suit of growth- and development-adapting responses collectively called shade avoidance syndrome (SAS) (Casal, 2013). Many responses to vegetational shade are already triggered by a relative reduction of R/FR under constant PAR as observed in proximity to neighboring plants. Those shade avoidance responses are more precisely referred to as neighbor detection responses (figure 1.1) and are commonly interpreted as an early warning mechanism to avoid future shading (Pierik and de Wit, 2014).

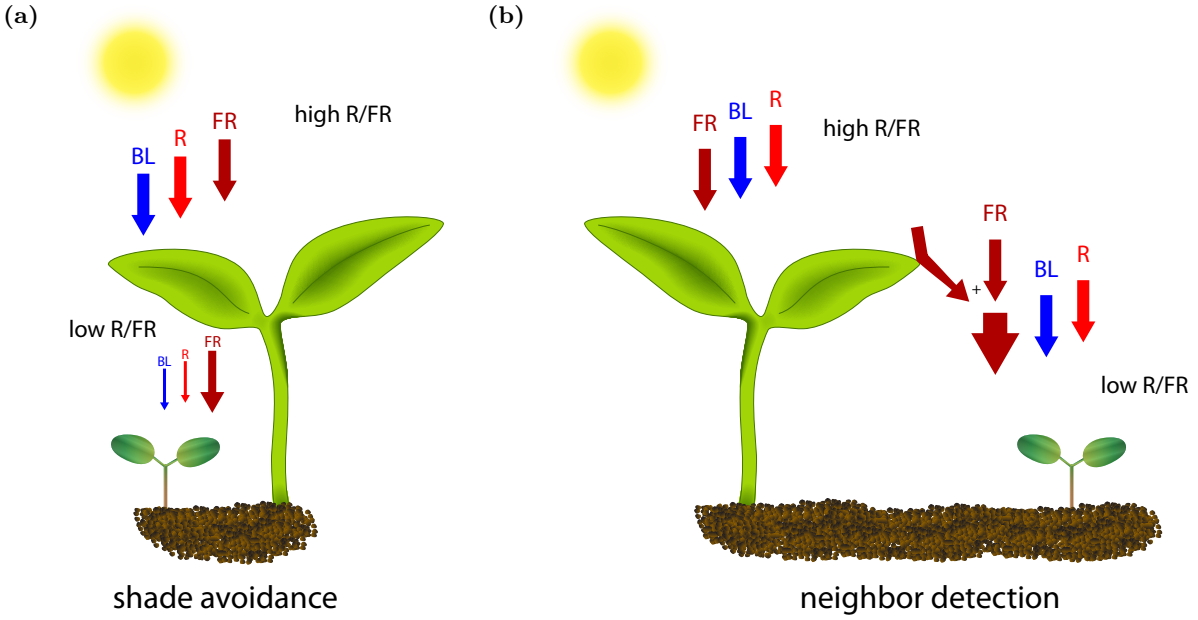

Figure 1.1: Schematic representation of shade avoidance and neighbor detection. Sunlight contains a high ratio of R to FR wavelength. (a) Light filtered through a leaf canopy gets strongly depleted in photosynthetically active wavelength such as R and B, which lead to a relative enrichment of FR. (b) FR wavelength are reflected by neighboring plant and enriches direct sunlight. In close proximity this FR enrichment can serve as early signal of competitive plants.

Low R/FR triggers different physiological responses at various developmental stages. One of the earliest responses during development is the regulation of germination by light. Red light induces germination and FR light can repress this response (Borthwick *et al.*, 1952). Thereby,

germination is prevented under unfavorable light conditions, e.g. until sufficient light can penetrate the leaf canopy. However, when seeds are exposed to continuous darkness, e.g. buried in the soil, also FR light is capable of inducing germination (Shinomura *et al.*, 1996; Botto *et al.*, 1996). Young *Arabidopsis* seedlings exposed to low R/FR conditions respond with increased growth rates of hypocotyls (embryonic stem) and petioles, which favors a higher positioning of cotyledons (embryonic leaves) to access direct sunlight (Franklin, 2008). At the same time the growth rate of cotyledons is reduced in shade (Sessa *et al.*, 2005; Roig-Villanova *et al.*, 2007; Li *et al.*, 2012; Casal, 2012).At the rosette stage, leaves show a similar response to low R/FR as described for cotyledons. The length of leaf petioles is enhanced and the ratio of leaf lamina to petiole length is reduced, allowing the leaves to spread further away from the central axis (Franklin, 2008; Moreno *et al.*, 2009; Keuskamp *et al.*, 2010). Furthermore, low R/FR induces hyponastic responses of cotyledons and leaves, which increase the elevation angle and favor their positioning above competing vegetation (Franklin, 2008; Keuskamp *et al.*, 2010; Dornbusch *et al.*, 2014). Reduced accumulation of chlorophyll in mutants that show impaired shade avoidance responses or in simulated shade was shown for *Arabidopsis*, tobacco and *Rumex* (McLaren and Smith, 1978; Casal *et al.*, 1990; Reed *et al.*, 1993). In lasting shade conditions, *Arabidopsis* adapts its leaf morphology and develops leaves with reduced stomata index (Boccalandro *et al.*, 2009; Casson *et al.*, 2009). The transition to flowering is also promoted by lasting low R/FR conditions. At the inflorescence stage in low R/FR, *Arabidopsis* shows increased apical dominance and correlative inhibition, which result in reduced outgrowth of auxiliary buds and reduced elongation growth of competing rosette branches (Reed *et al.*, 1993; Finlayson *et al.*, 2010). Lasting low R/FR promotes early flowering leading to reduced yield in crop plants (Ballaré *et al.*, 1997; Franklin, 2008). Commonly, early flowering is interpreted as an escape mechanism to the next generation: seed dispersal may allow the next generation to reach more favorable light conditions (Casal, 1993; Ugarte *et al.*, 2010). In addition low R/FR reduces the seed yield per plant (Ugarte *et al.*, 2010; Procko *et al.*, 2014). Finally, low R/FR conditions reduce defense responses against insects and herbivores (Moreno *et al.*, 2009; Ballaré, 2011).

1.3 The phytochrome family of red/far-red photoreceptors

Plants have evolved a multitude of photoreceptors that are sensitive in the R, FR, blue, UV-A or UV-B range of the light spectrum (Casal, 2013). Phototropins and cryptochromes are UV-A/blue light receptors (Christie, 2007). Blue light is also perceived by the ZEITLUPE/FLAVIN-

BINDING, KELCH REPEAT, F-BOX/LOV DOMAIN KELCH PROTEIN 2 (ZTL/FKF1/LKP2) family (Nelson *et al.*, 2000). UV RESISTANCE LOCUS8 (UVR8) responds to irradiation in the UV-B range (Rizzini *et al.*, 2011; Heijde and Ulm, 2012). R and FR light, as well as to a lower extent blue light, are perceived by the phytochrome family (Franklin, 2008). In *Arabidopsis thaliana* the phytochrome family consists of five members (phyA - phyE).

Phytochromes fall into two functional groups based on their stability in light. Type I phytochromes comprise light-labile receptors and are represented in *Arabidopsis* by a single member, phyA. Type II phytochromes are light-stable and are comprised of phyB-phyE in *Arabidopsis thaliana*. Based on size-exclusion chromatography analysis, oat phyA was reported to homodimerize (Lagarias and Mercurio, 1985; Jones and Quail, 1986). PhyB forms homo- as well as heterodimers (Sharrock and Clack, 2004) while phyC and phyE were described to form obligate heterodimers with phyB and phyD (Clack *et al.*, 2009).

Phytochromes exist in two interconvertible states, the P_r isoform with an absorption peak in R ($\lambda_{max} = 660\,nm$) and the P_{fr} isoform with an absorption peak in FR ($\lambda_{max} = 730\,nm$) (Quail, 1997). Both isoforms have partially overlapping absorption spectra and, under physiological conditions, exist in a photoequilibrium reflecting the R/FR ratio of the environment. In the absence of light, the P_{fr} form slowly converts back into P_r in a process generally referred to as dark reversion (Casal, 2013).

The intracellular localization of phytochromes is regulated by light. Phytochromes are synthesized in the cytoplasm in the physiologically inactive P_r state (Casal, 2013). Upon light activation, they translocate to the nucleus and interact with target proteins such as phytochrome interacting factors (PIFs, see next chapter; Ni *et al.*, 1999; Huq and Quail, 2002; Huq *et al.*, 2004; Khanna *et al.*, 2004; Oh *et al.*, 2004; Leivar *et al.*, 2008; Quail unpublished). In case of phyB, nuclear translocation is R mediated while phyA enter the nucleus upon R or FR perception (Nagatani, 2004; Kevei *et al.*, 2007). phyB contains a nuclear localization signal (NLS) and its nuclear import is facilitated by interaction with PIF3 (Pfeiffer *et al.*, 2012). The nuclear import of phyA depends on the two shuttle proteins *FAR-RED ELONGATED HYPOCOTYL1* (*FHY1*) and *FHY1-LIKE* (*FHL*) (Hiltbrunner *et al.*, 2005, 2006).

Degradation of phyB is mediated by Light-Response Bric-a-Brack/Tramtrack/Broad (LRB) E3 ubiquitin ligases, which bind to phyB-PIF3 complexes (Ni *et al.*, 2013, 2014).

Phytochrome responses can be classified into three groups depending on their photon flux requirements. Very Low Fluence Responses (VLFR) promote seed germination and de-etiolation responses (Botto *et al.*, 1996). They are mediated by phyA whose abundance in seeds and

etiolated seedlings is more than 100 times higher than that of other phytochromes. VLFR can be triggered by perception of low numbers of photons (10^{-4} to 10^{-1} μmol m^{-2} s^{-1}) of various wavelengths.

Low Fluence Responses (LFR) comprise all R/FR reversible responses similar to the classic germination experiment with *Lactuca sativa* seeds which established that induction of germination by red light pulses can be reversed by subsequent FR pulses (Borthwick *et al.*, 1952). Furthermore LFR follow the Bunsen-Roscoe reciprocity law, which describes the relationship between response intensity and total exposure.

High Irradiance Responses (HIR) are elicited in response to long-term FR, R, blue or UV light. The FR-HIR are specifically mediated by phyA and promote seed germination while inhibiting elongation growth in seedlings in shade conditions (Salter *et al.*, 2003; Li *et al.*, 2011).

In *Arabidopsis* different phytochromes mediate unique and redundant responses: phyA and phyB redundantly promote germination in R light (Botto *et al.*, 1996; Shinomura *et al.*, 1996).

Also synergistic, additive or antagonistic effects were reported for different phytochrome members. The regulation of flowering is antagonistically regulated by phyA and phyB. While phyA accelerates flowering (Johnson *et al.*, 1994) it is repressed by phyB (Goto *et al.*, 1991). Flowering time seems to be synergistically regulated by phyD and phyE since *phyDphyE* double mutants show earlier flowering time than the respective single mutants (Clack *et al.*, 2009).

phyB is the main photoreceptor mediating shade avoidance responses with phyD and phyE playing a minor role. *phyB* mutants have a shade-like phenotype with increased internode growth and small leaf size (Nagatani *et al.*, 1991; Reed *et al.*, 1993; Devlin *et al.*, 1998, 1999).

1.4 PIF mediates shade signals

In the past, more than 20 genes with various functions have been reported to interact with phytochromes, including several bHLH transcription factors (TF) (Bae and Choi, 2008). All phytochrome interacting basic Helix-Loop-Helix (bHLH) TFs belong to the same subgroup based on structural similarity (Heim *et al.*, 2003). This subgroup comprises 15 members of which seven physically interact with the P_{fr} confomer in low R/FR (Ni *et al.*, 1999; Huq and Quail, 2002; Huq *et al.*, 2004; Khanna *et al.*, 2004; Oh *et al.*, 2004; Leivar *et al.*, 2008; Quail unpublished) and were subsequently named PHYTOCHROME INTERACTING FACTOR (PIF; PIF1, PIF3 - PIF8). Interaction with phyB requires the Active Phytochrome B-binding motif, which is present in the protein sequence of eleven family members (Leivar and Quail, 2011). PIF1 and PIF3 contain an

additional Active Phytochrome A-binding motif and bind phyA with different affinity (Leivar and Quail, 2011). Phytochrome-PIF interaction leads to the phosphorylation of PIF1, PIF3 - PIF5 and PIF7, which eventually results in their degradation via the 26S ubiquitin-proteasome system (Bauer *et al.*, 2004; Park *et al.*, 2004; Al-Sady *et al.*, 2006; Oh *et al.*, 2006; Shen *et al.*, 2007, 2008; Lorrain *et al.*, 2008; Leivar *et al.*, 2008; Li *et al.*, 2012). Mutant combinations of *phyB* and *pif4*, *pif5* and *pif7* partially rescue the elongation phenotype of *phyB* and lead to reduced transcription levels of shade marker genes, demonstrating that PIF4, PIF5 and PIF7 act downstream of phyB during shade avoidance (figure: 1.2; Lorrain *et al.,* 2008; Li *et al.,* 2012). PIFs bind sequence-specific to G-boxes (-cacgtg-) or PIF binding E-boxes (PBE; -catgtg-) in promoter sequences. The binding strength of PIF5, but not PIF4, has been shown to be affected by the directly flanking nucleotide *in vitro* indicating that PIF4 might regulate a larger set of target genes (Martínez-García *et al.*, 2000; Huq and Quail, 2002; Moon *et al.*, 2008; Leivar *et al.*, 2008; Hornitschek *et al.*, 2009, 2012)

Not all members of the PIF subfamily of bHLH TF directly interact with phytochromes. For ALCATRAZ, BHLH23, BHLH56, BHLH119 and BHLH127 no role in photomorphogenic processes has been reported (Leivar *et al.*, 2012). Nevertheless, several further characterized members have photomorphogenesis-related functions. SPATULA (SPT) can repress germination in light by regulation of biosynthetic genes of the phytohormone gibberellic acid (Penfield *et al.*, 2005). PIF3-like 1 (PIL1) and LONG HYPOCOTYL IN FAR- RED 1 (HFR1) are rapidly and robustly upregulated in response to low R/FR (Salter *et al.*, 2003; Sessa *et al.*, 2005; Hornitschek *et al.*, 2009). Both genes contain G-box motifs in their upstream promoter region, show chromatin binding of PIF1, PIF3, PIF4 and PIF5 in the promoter and reduced transcriptional induction to low R/FR in *pif4*, *pif5* and *pif4pif5* (Lorrain *et al.*, 2008; Oh *et al.*, 2009, 2012; Hornitschek *et al.*, 2012; Zhang *et al.*, 2013). HFR1 and PIL1 are both described as negative regulators of shade avoidance responses, since *hfr1* and *pil1* mutants show enhanced hypocotyl elongation in response to low R/FR (Salter *et al.*, 2003; Roig-Villanova *et al.*, 2006; Sessa *et al.*, 2005). HFR1 can interact with both, PIF4 and PIF5 and prevent their binding to DNA, thus forming a negative feedback loop, which dynamically prevents an exaggerated response to low R/FR (Hornitschek *et al.*, 2009).

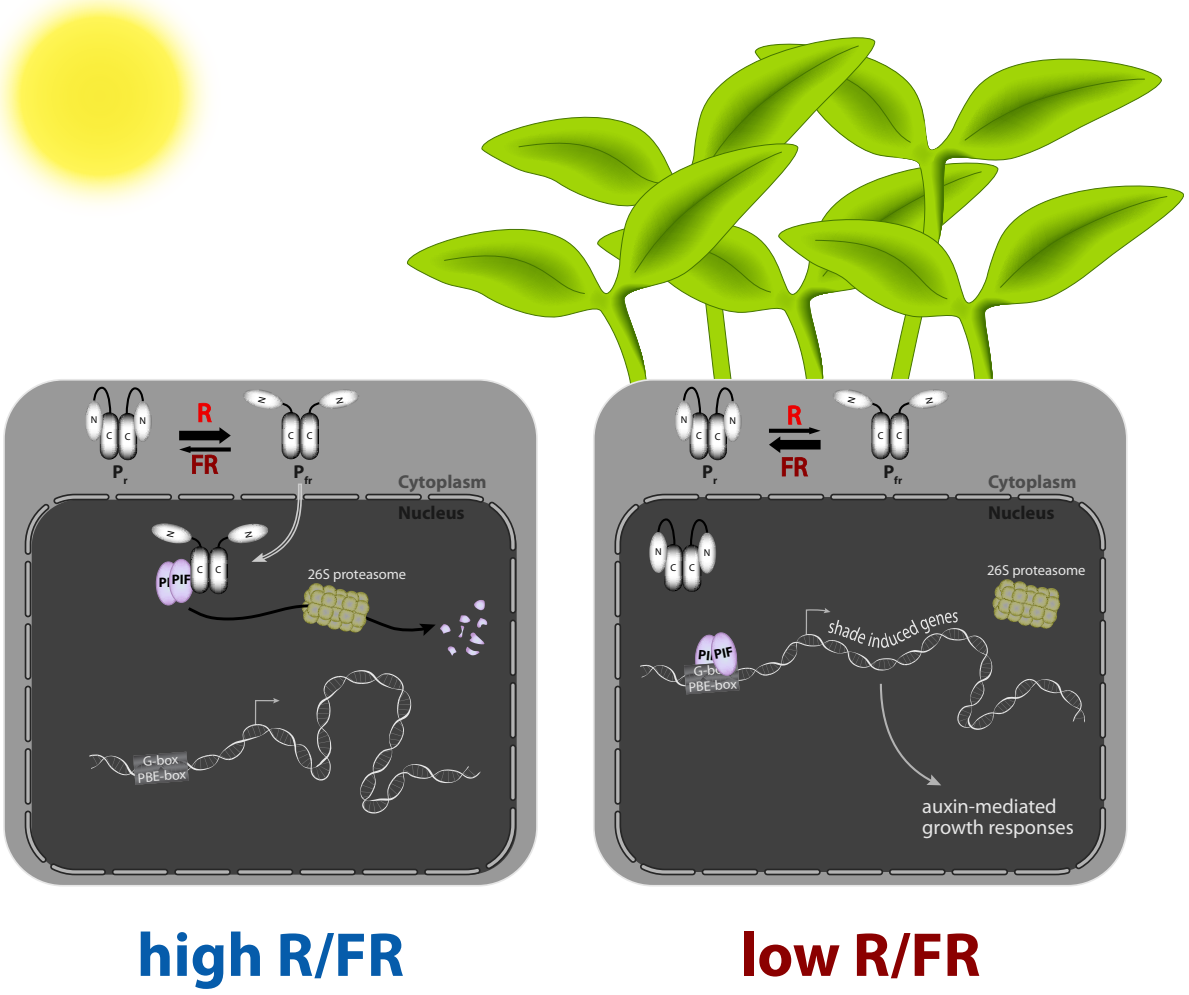

Figure 1.2: Model of the transcriptional regulation during shade avoidance
In high R/FR active phytochrome interacts with PIF proteins, leading to their phosphorylation and subsequent degradation via the 26S proteasome (left). In low R/FR conditions phytochromes are inactive. PIF protein levels are stabilized and bind to G-box and PBE DNA motifs in upstream regulatory sequences of shade regulated gene. (Adapted from (Lorrain *et al.*, 2008))

1.5 PIF abundance is subject to various internal and external cues

PIF proteins are highly expressed in etiolated seedlings. In white light, PIFs have a half-life of around 5 to 20 min and are maintained at low concentrations in high R/FR. When plants are shifted from white light back to dark PIF levels re-accumulate (Leivar and Quail, 2011). Protein levels of a hemagglutinin (HA)-tagged PIF4 were shown to peak at the end of the dark period (Nozue *et al.*, 2007; Kumar *et al.*, 2012). PIF7 shows lower rates of degradation as compared to the other PIFs (Leivar *et al.*, 2008; Leivar and Quail, 2011; Li *et al.*, 2012). Red light promotes PIF degradation while PIF levels rise in far-red light (Bauer *et al.*, 2004; Shen *et al.*, 2008; Lorrain *et al.*, 2009). In low R/FR the photoequilibrium of phyB is shifted towards

P_r and PIF3, PIF4 and PIF5 protein levels accumulate and induce transcriptional responses (Lorrain *et al.*, 2008; Leivar *et al.*, 2012). PIF1 and PIF3 are also degraded in blue light, which depends on functional phytochrome interaction domains (Bu *et al.*, 2011; Castillon *et al.*, 2009). In addition to this directly light-dependent regulation of PIF protein levels, transcript levels are under circadian control. in SD, *PIF4* and *PIF5* are transcriptionally expressed during day and night while in LD, the expression is absent during the night (Niwa *et al.*, 2009; Nozue *et al.*, 2007). The circadian clock further represses *PIF4* and *PIF5* transcript levels through the evening complex (EC) in the early evening. The EC is composed of EARLY FLOWERING (ELF) 4, ELF3 and LUX ARRHYTHMO (LUX), while LUX mediates directly binding to promoter sequences of *PIF4* and *PIF5* (Nusinow *et al.*, 2011). The evening complex was also described as in integration point of temperature and light signals. At warm temperatures, the EC is less active and promotes expression of downstream targets such as *PIF4* (Mizuno *et al.*, 2014). Roles for *PIF4* and *PIF5* in temperature dependent transcriptional regulation have been reported (Koini *et al.*, 2009; Foreman *et al.*, 2011; Toledo-Ortiz *et al.*, 2014). In response to high temperature, *PIF4* transcript levels are induced (Koini *et al.*, 2009; Franklin *et al.*, 2011; Kumar *et al.*, 2012; de Wit *et al.*, 2014). Phenotypically, high temperature induces elongation of the hypocotyl in a PIF4-dependent manner. The response to high temperature can be interpreted as a escape response to reduce exposure of cotyledons to reflected heat from the surface. Finally, *PIF5-HA* is induced by sucrose treatment in light and darkness when expressed under the control of the Cauliflower Mosaic Virus 35S promoter (35S promoter). *PIF5ox* have longer hypocotyls in the presence of sucrose, while *pif1*, *pif3*, *pif4*, *pif5*, *pif4pif5* and *pif1pif3pif4pif5 (pifq)* show a reduce response to sucrose, demonstrating a possible integration point of carbohydrate metabolism and light-regulated hypocotyl elongation (Stewart *et al.*, 2011; Liu *et al.*, 2011a).

1.6 Additional low R/FR-induced molecular responses

A similar regulatory interaction as described for PIFs and HFR1 was described for PAR1 and PAR2 on the one hand and PIF4, but also HFR1 on the other hand (Hao *et al.*, 2012; Cifuentes-Esquivel *et al.*, 2013). PAR1 and PAR2 are bHLH TF and belong to a different subfamily than the PIFs (Heim *et al.*, 2003). Similar to HFR1 and PIL1, the PARs contain an atypical bHLH domain that does not support direct interaction with DNA (Roig-Villanova *et al.*, 2007). Reduced or absent expression of *PAR1* and *PAR2* promote elongation growth while overexpression leads to a reduced overall size of mutant plants (Roig-Villanova *et al.*, 2007). PAR1

is slightly downregulated in *pif4pif5* and had a *PIF5-HA* chromatin binding site upstream of its coding sequence, suggesting a direct regulation of *PAR1* by PIF5 (Hornitschek *et al.*, 2012). PAR1 transcriptionally represses *SMALL AUXIN UPREGULATED 15* (*SAUR15*) and *SAUR68* thereby providing a link between shade avoidance and the auxin-signaling network (to be discussed further in section1.7.1) (Roig-Villanova *et al.*, 2007). In addition, PARs have been shown to interact with signaling components of the brassinosteroid pathway, BRASSINOSTEROID INSENSITIVE (BRI) 1 Enhanced Expression (BEE) 1, BEE2 and BES1-Interacting Myc-like 1(BIM1) and reduce DNA binding of BEE2 and BIM1. Functional redundancy between different BEEs and between different BIMs was suggested, based on hypocotyl elongation responses of single and higher order mutants. Nevertheless, hypocotyl elongation measurements over time under low R/FR indicate a synergistic relationship of BEE and BIM proteins (Cifuentes-Esquivel *et al.*, 2013). *bee1bee2bee3* and *bim1bim2bim3* triple mutants show reduced growth and exhibit altered growth kinetics in response to low R/FR compared to the wild-type, suggesting a regulatory mechanism through which PAR1 and PAR2 regulate growth (Cifuentes-Esquivel *et al.*, 2013).

Shade also induces the homeodomain-Leucin Zipper (HD-Zip) II transcription factors *(ARABIDOPSIS THALIANA HOMEOBOX PROTEIN (ATHB) 2 / HOMEOBOX FROM ARABIDOPSIS THALIANA (HAT) 4*, *HAT1*, *HAT2*, *ATHB4* and *HAT3*, which act as positive regulators of responses to shade (Roig-Villanova *et al.*, 2006; Carabelli *et al.*, 2013). *ATHB-2* mediates growth responses, such as cell elongation in cotyledons, hypocotyl and roots or the proliferation of the vascular system (Steindler *et al.*, 1999). Different studies suggest that in response to shade, *ATHB-2* is at least partially upregulated through a mechanism involving direct binding of PIFs to G-box sequences upstream of the *ATHB-2* coding sequence(Lorrain *et al.*, 2008; Zhang *et al.*, 2013; Hornitschek *et al.*, 2012). Treatment of *athb-2* mutants with the growth-stimulating phytohormone auxin rescues the short root phenotype (Steindler *et al.*, 1999).

XYLOGLUCAN ENDO/TRNASGLYCOSIDASE HYDROLASES (XTH) 15 / XYLOGLUCAN ENDOTRANSGLYCOSYLASE (XTR) 7 is one of the classical shade marker genes. It encodes a xyloglucan:xyloglucosyl transferase. Members of this protein family are involved in cell wall remodeling and can promote cell stiffness as well as cell wall loosening and were also linked to cortical microtubules organization under low R/FR condition (Rose *et al.*, 2002; Sasidharan *et al.*, 2014).

XTH15/XTR7 is a direct target gene of *PIF4* and *PIF5* (de Lucas *et al.*, 2008; Hornitschek *et al.*, 2012; Oh *et al.*, 2012) and upon low R/FR perception, it is quickly upregulated in a PIF4-

and PIF5-dependent manner (Lorrain *et al.*, 2008). *xth15xth17* is impaired in petiole elongation in low R/FR conditions (Sasidharan *et al.*, 2010). Several additional members of the *XTH* family respond transcriptionally to low R/FR (Sasidharan *et al.*, 2014). It is worth mentioning that more than ten *XTHs* are light-induced or -repressed in an organ-specific manner (Ma *et al.*, 2005).

1.7 Intersection of phytohormone signaling pathways with shade mediated growth regulation

Phytohormones are small signaling molecules, which may be transported over long distances. Phytohormons can act directly in the tissue of their synthesis (Santner *et al.*, 2009). Signaling pathways of several phytohormones are affected by low R/FR treatment.

Gibberellin (GA) biosynthesis is regulated by light. Upon light perception, phytochromes regulate GA biosynthesis and promote GA degradation (Alabadí *et al.*, 2004, 2008). Low R/FR induces the transcription of GA biosynthetic genes as well as GA responsiveness (Hisamatsu *et al.*, 2005; Reed *et al.*, 1996). Furthermore, several aspects of the *phyB* mutant phenotype are rescued in GA-deficient or GA-response mutants (Peng and Harberd, 1997). DELLAs are regulatory proteins of the GRAS family of putative transcription factors (Tian *et al.*, 2004; Thomas and Sun, 2004) . The gene family name was derived from the first characterized members: GAI, RGA and SCR (Pysh *et al.*, 1999). DELLAs act downstream in the GA signaling pathway and are degraded in the presence of GA (Tian *et al.*, 2004; Thomas and Sun, 2004) . Their protein levels are low in etiolated seedlings but quickly induced upon light perception. In low R/FR, the DELLA fusion protein REPRESSOR OF GA-green fluorescent protein (RGA-GFP) showed reduced expression (Djakovic-Petrovic *et al.*, 2007). DELLAs interact with PIF3 and PIF4 and likely other PIFs and thereby prevent their binding to DNA (de Lucas *et al.*, 2008; Feng *et al.*, 2008; Casal, 2013). DELLAs strongly repress PIF activity in white light . PIF5 binds to regulatory sequences of several DELLA proteins and DELLA transcript levels are induced in low R/FR presenting a possible negative feedback loop (Rieu *et al.*, 2008; Leivar *et al.*, 2012; Hornitschek *et al.*, 2012).

DELLAs and PIF4 both interact also with BRASSINAZOLE-RESISTANT 1 (BZR1) and link Gibberellin and shade signaling with the brassinosteroid pathway. Brassinosteroids are important growth factors involved in phytochrome-dependent stem and petiole growth (Luccioni *et al.*, 2002; Kozuka *et al.*, 2010). Brassinosteroids are extracellular perceived by the receptor

kinase BRI1 and co-receptor BRI1-ASSOCIATED RECEPTOR KINASE (BAK) 1, which in turn activates intracellular kinases. This results in the activation of the transcription factors BZR1 and BZR2. BZR1 shares direct target genes with PIF4 and recently, an interdependent relationship of transcriptional regulation between BZR1, PIF4 and AUXIN RESPONSE FACTOR (ARF) 6 was reported (Oh *et al.*, 2012, 2014).

Different abscisic acid (ABA) mediated responses related to drought stress or tolerance are modulated by phyB and low R/FR in several species (Dubois *et al.*, 2010; González *et al.*, 2012; Cagnola *et al.*, 2012; González-Grandío *et al.*, 2013). This can happen on a morphological level by regulating stomata density, stomata index (ratio of stomata to epidermal cells) and amphistomy, as well as by impacting on the responsiveness, e.g. the regulation of stomata conductance (Boccalandro *et al.*, 2009; Wang *et al.*, 2010; González *et al.*, 2012).

1.7.1 auxin

Auxin is an essential regulator of growth processes

The phytohormone auxin plays a major role in almost every aspect of plant growth and development (Zhao, 2014). Auxins are low molecular weight organic acids with the capacity to mediate growth responses in various plant species. The most abundant endogenous auxin found in higher plants, including Arabidopsis, is indole-3-acetic acid (IAA) (Went, 1934; Sauer *et al.*, 2013).

Several auxin overproduction mutants have been identified such as *superroot1* and *superroot2* (Boerjan *et al.*, 1995; Delarue *et al.*, 1998), and the dominant *yucca1 (yuc1)* mutant (Zhao *et al.*, 2001). Overexpression of *YUCCA* gene family members, as week as transgenic plant lines expressing the bacterial auxin biosynthesis gene, *iaaM*, lead to a more detailed description of the auxin overproduction phenotype (Romano *et al.*, 1995). When grown in light, auxin overproduction mutants have long hypocotyls and petioles, epinastic cotyledons, long and curled rosette leaves, and strong apical dominance. (Lincoln *et al.*, 1990; Boerjan *et al.*, 1995; Delarue *et al.*, 1998; King *et al.*, 1995; Barlier *et al.*, 2000; Zhao *et al.*, 2001, 2002; Marsch-Martinez *et al.*, 2002; Tobeña-Santamaria *et al.*, 2002; Cheng *et al.*, 2006; Kim *et al.*, 2007; Chen *et al.*, 2014). Several transgenic lines also display longer roots and development of adventitious roots from the hypocotyl (Boerjan *et al.*, 1995; Mikkelsen *et al.*, 2004; Gutierrez *et al.*, 2012).

YUCCAs are key enzymes of auxin biosynthesis (see below; Mashiguchi *et al.*, 2011). No obvious phenotype was reported when single *YUCCA* genes were disrupted (Cheng *et al.*, 2006). Nevertheless, the genome of *Arabidopsis thaliana* encodes 11 *YUCCA* genes and combinations of

mutants show severe developmental defects. *yuc1yuc4* double mutants have disrupted vascular patterns in leaves and flowers and are completely sterile. Reduced rosette size, impaired leaf flattening and reduced branching are more apparent in triple mutant combinations with *yuc2* or *yuc6* (Cheng *et al.*, 2006). The quintuple mutant *yuc3yuc5yuc7yuc8yuc9*, which lacks expression of all major root-expressed *YUCCA* genes, has very short and agravitropic primary roots (Chen *et al.*, 2014).

Chemical treatment of seedlings with IAA or synthetic analogs, such as picloram, also induces hypocotyl elongation of *Arabidopsis* seedlings (Sauer *et al.*, 2013; Zhao, 2014). Responses to auxin follow an optimum curve: while moderately increased auxin concentrations have a stimulating growth effect e. g. on hypocotyls, higher concentrations can rather have toxic effects (Chapman *et al.*, 2012; Grossmann, 2010). The hypocotyl elongation response can be prevented by application of auxin transport inhibitors such as 1-naphtylphtalamic acid (NPA), which interferes with the distribution of auxin within seedlings. Similar phenotypes can be observed using auxin antagonists such as α-(phenyl ethyl-2-one)-IAA (PEO-IAA) or auxinol (Hayashi *et al.*, 2008, 2012).

Shade avoidance induce auxin levels

Growth processes in response to shade are auxin dependent as shown in hypocotyl growth assays under low R/FR using various chemicals such as NPA and PEO-IAA (Hayashi *et al.*, 2008, 2012). Direct measurements of free auxin levels in five or seven-days-old seedlings revealed enhanced auxin concentrations when treated with low R/FR for one or two hours (Tao *et al.*, 2008; Li *et al.*, 2012; Hornitschek *et al.*, 2012; Hersch *et al.*, 2014). In young seedlings, increased free IAA levels were measured after two days of low R/FR treatment (Keuskamp *et al.*, 2010). This increase might be a transient response and last for a different period of time dependent on the age of the seedling, since measurements of auxin levels in entire seven-day-old seedlings showed initial increases within a few hours but no detectable differences after 24 hours (Bou-Torrent *et al.*, 2014). Similar results were obtained for *Arabidopsis* leaves and petioles (*de Wit, in preparation*). In addition, in a long-term experiment, no differences in auxin accumulation between high and low R/FR were observed in stems and leaves of tomato after four days of treatment (Cagnola *et al.*, 2012).

Shade-induced increases in free IAA levels depend on PIF4, PIF5 and PIF7, since *pif4pif5* double mutants and *pif7* single mutants do not accumulate auxin in low R/FR (Hornitschek *et al.*, 2012; Li *et al.*, 2012). Furthermore, for *PIF4-HA* over expressing plants, elevated free IAA

levels in white light were shown, further supporting the regulation of auxin levels by PIFs (Sun *et al.*, 2012). Surprisingly, *PIF5-HA* over expressing plants have lower fee IAA levels than the wild type and do not increase in low R/FR (Hornitschek *et al.*, 2012), which might be the result of the ectopic expression.

Shade avoidance promotes auxin biosynthesis in higher plants

Several naturally occurring as well as synthetic compounds display auxin activity. In higher plants auxin synthesis can occur either tryptophan dependent or independent (Bartel, 1997; Normanly *et al.*, 1993; Korasick *et al.*, 2013). A tryptophan independent pathway was proposed based on early studies showing constant or enhanced free-auxin levels in tryptophan auxotroph mutants (Normanly *et al.*, 1993). Nevertheless, the underlying molecular events are still poorly understood and the biological relevance remains elusive.

Tryptophan dependent IAA biosynthesis can be divided into four parallel pathways. The IPA pathway is considered as the primary auxin biosynthetic pathway during shade avoidance (Mashiguchi *et al.*, 2011). It is named after the intermediate compound indole-3-pyruvic acid (IPA). IPA is synthesized from tryptophan by members of the L-tryptophan-pyruvate amino-transferase family (Stepanova *et al.*, 2008; Tao *et al.*, 2008; Yamada *et al.*, 2009) and further converted into IAA by the YUCCAs. YUCCAs are flavin-containing monooxygenases and importantly mediate the rate-limiting step of auxin biosynthesis (Zhao *et al.*, 2001; Mashiguchi *et al.*, 2011; Kim *et al.*, 2007).

During shade avoidance, *YUC2*,*YUC3*, *YUC5*, *YUC8* and *YUC9* are transcriptionally induced (Tao *et al.*, 2008; Won *et al.*, 2011; Brandt *et al.*, 2012; Li *et al.*, 2012; González-Grandío *et al.*, 2013). PIF4, PIF5 and PIF7 bind to the promoter of *YUC8* and *YUC9*, suggesting that both PIFs affect auxin levels by directly regulating genes expression of auxin biosynthesis components (Hornitschek *et al.*, 2012; Li *et al.*, 2012). However, the quintuple mutant *yuc3yuc5yuc7yuc8yuc9* exhibits only a mild reduction of hypocotyl elongation in response to low R/FR (Li *et al.*, 2012), whereas the double mutant of the weak *yuc1-163* allele and *yuc4* shows strongly reduced hypocotyl elongation (Won *et al.*, 2011), indicating that various *YUCCAs* have different importance for physiological responses.

A mutant screen for plants failing to elongate specifically in response to shade identified a mutation in the *L-TRYPTOPHAN AMINOTRANSFERASE (TAA) 1* acting upstream of the YUCCAs in the auxin biosynthetic pathway (Tao *et al.*, 2008). In white light, *taa1 / shade avoidance (sav) 3* single mutants (hereafter *sav3*) display a wild-type-like phenotype whereas *taa1*

tryptophan aminotransferase related 2 (*tar2*) double mutants have strong defects in vasculature, reduced apical dominance and sterile flowers (Stepanova *et al.*, 2008). Shade-induced hypocotyl elongation is strongly impaired in *sav3* mutants, similar to the *yuc1yuc4* double mutant (Won *et al.*, 2011). Furthermore, the hypocotyl elongation defect of *sav3* mutants in low R/FR is rescued in the *vas1* (identified in a suppressor screen of *sav3*) background. *VAS1* encodes an aminotransferase described to promote the reverse reaction of TAA1 by catalyzing the conversion of IPA to L-Tryptophan (Zheng *et al.*, 2013).

Spatial aspects of auxin biosynthesis

Auxin is mainly produced in the shoot apex and subsequently transported basipetally (Sauer *et al.*, 2013). Nevertheless, different organs and tissues have been reported to locally produce auxin: In flowers, stamens depend on local auxin biosynthesis (Cheng *et al.*, 2006) and evidence for root-derived auxin produced in root tips and root hairs has been reported in several studies (Cheng *et al.*, 2006; Stepanova *et al.*, 2008; Ikeda *et al.*, 2009; Chen *et al.*, 2014). Auxin biosynthesis in response to shade depends on *TAA1*. Histochemical staining of transgenic seedlings expressing a pTAA1::TAA1-GUS reporter construct, suggests that the margin of cotyledons is the predominant site of TAA1 expression in high R/FR. Low R/FR had no detectable effect on expression levels and the expression domain (Tao *et al.*, 2008).

Auxin homeostasis

In higher plants, auxin either exists in its free form or conjugated as either low molecular weight ester conjugates with sugar moieties, low molecular weight amide conjugates with amino acids or high molecular weight conjugates with peptides and proteins. Free auxin accounts for up to 25 % of total auxin, dependent on tissue and species (Ludwig-Müller, 2011).

In *Arabidopsis thaliana*, members of the Gretchen Hagen 3 (GH3) family synthesize auxin conjugates with amino acids. Auxin conjugation activity was shown for seven members, all belonging to the phylogenetic clade II (Staswick *et al.*, 2005; Park *et al.*, 2007). Among those, transcriptional regulation of *GH3.5* is phyB dependent (Tanaka *et al.*, 2002a). Auxin amino acid conjugates are considered to be synthesized in the cytoplasm and to render auxin inactive (Ludwig-Müller, 2011). IAA-Trp is the only described amino acid conjugate with biological activity. IAA-Trp counteracts auxin root growth inhibition in a TIR1 dependent fashion. This effect seems to be different from competitive binding to the receptor (Staswick, 2009). The amino acid conjugate IAA-Asp and IAA-Glu are considered as non-reversible conjugates which

label auxin for degradation. This based on the observation that at high auxin concentrations IAA-Asp is oxidized and subsequently degraded (Tuominen *et al.*, 1994; Barratt *et al.*, 1999). The two amino acid conjugates IAA-Ala and IAA-Leu are reversible storage forms as determined by feeding experiments (Ludwig-Müller, 2011).

Auxin perception

In *Arabidopsis thaliana*, two types of auxin receptors have been described: AUXIN BINDING PROTEIN 1 (ABP1), as well as members of the TRANSPORT INHIBITOR RESISTANT 1/ AUXIN SIGNALING F-BOX PROTEIN (TIR1/AFB) family of F-box proteins (Sauer *et al.*, 2013).

TIR1/AFB auxin receptor The best characterized auxin receptors to date are the TIR1/AFB proteins. They belong to the F-box proteins family and provide specificity to the SKP1-Cullin-F-box (SCF) class of E3 ubiquitin ligases, which label target proteins for degradation via the 26S proteasome pathway (Sauer *et al.*, 2013). TIR1 forms complexes with CULLIN1, RING BOX1 (RBX1) and *Arabidopsis* Skp1-like protein (ASK) 1 or ASK2. In *Arabidopsis* the TIR/AFB family comprises six proteins. TIR1, the founding member, shares about 70 % amino acid sequence similarity with AFB1 and around 60 % with AFB2 and AFB3. AFB4 and AFB5 form an extra subgroup partially due to an amino-terminal sequence extension (Dharmasiri *et al.*, 2005b; Parry *et al.*, 2009).

TIR1 and AFB1 through AFB3 have similar transcriptional patterns in eight-day-old seedlings (Parry *et al.*, 2009). However, the translational expression domain varies strongly between single members. AFB1 has the highest abundance of TIR/AFB auxin receptor in cotyledons, based on histochemical staining of GUS reporter lines (Parry *et al.*, 2009). Individual family members also differ on the functional level. AFB1 and AFB2 cannot rescue the *tir1* auxin hypersensitive root phenotype when expressed under the control of the TIR1 promoter. However, the *tir1* root phenotype is enhanced in higher order mutants demonstrating partial functional redundancy between the TIR1/AFBs (Parry *et al.*, 2009).

TIR1/AFB signaling pathway Auxin can directly interact with the auxin receptor TIR1 (Dharmasiri *et al.*, 2005a; Kepinski and Leyser, 2005). As revealed by crystallography, it binds to the bottom of a surface pocket thereby promoting the interaction between TIR1 and Aux/IAA (AUXIN/INDOLE-3-ACETIC ACID) co-receptors. The genome of *Arabidopsis thaliana* encodes

29 Aux/IAAs, which have various affinities to TIR1/AFB auxin receptors and different degradation kinetics in vitro (Calderón Villalobos *et al.*, 2012; Havens *et al.*, 2012). In the absence of auxin, Aux/IAA proteins interact with transcriptions factors of the AUXIN RESPONSE FACTOR (ARF) family thereby preventing ARF mediated transcription (Guilfoyle and Hagen, 2007). The complex formation of TIR1, auxin and Aux/IAA proteins results in the ubiquitination and subsequent degradation of the co-receptor (Tan *et al.*, 2007; Maraschin *et al.*, 2009).

Aux/IAAs have been identified as immediate early auxin response genes. Transcriptional induction of several family members can be observed as quick as five minutes following IAA treatment (Abel *et al.*, 1995). Most Aux/IAA proteins contain four domains. Domain I confers repressor activity of ARF mediated reporter gene expression, as demonstrated by protoplast co-transfection(Ulmasov *et al.*, 1997; Tiwari *et al.*, 2001). It is also required to recruit co-repressors including TOPLESS (Szemenyei *et al.*, 2008). Aux/IAA domain II contains the hydrophobic motif GWPPV, which is required for the interaction with TIR1/AFB receptor proteins leading to Aux/IAA instaility (Ramos *et al.*, 2001). Domains III and IV mediate interaction with ARF transcription factors (Guilfoyle and Hagen, 2007; Nanao *et al.*, 2014).

In *Arabidopsis thaliana* Aux/IAA genes build a regulatory network with 23 ARFs (Guilfoyle and Hagen, 2007, 2012; Korasick *et al.*, 2014). ARF transcription factors bind sequence specifically to auxin response elements in promoter of target genes (Ulmasov *et al.*, 1999; Boer *et al.*, 2014). They are composed of an amino-terminal DNA binding domain (DBD), a central domain and except of three ARFs a carboxy-terminal domain III and IV required for Aux/IAA interaction (Nanao *et al.*, 2014; Korasick *et al.*, 2014). ARF23 contains only a DBD and could be either a pseudogene or a negative regulator of other ARF proteins. The middle domain confers based on carrot protoplast experiments either active or repressive transcriptional activity dependent on the amino acid composition (Ulmasov *et al.*, 1999). A mechanism by which ARFs repress transcription has not been described yet. Protein interactions of ARFs were traditionally described as dimerization between ARFs or with Aux/IAA proteins. Recent crystallography studies suggest that domain III and domain IV of Aux/IAA and ARFs form a type I/II PB1 domain suited for oligomerization. In planta analysis of ARF7 demonstrated that highest reporter gene activity only when both interaction surfaces were mutated. This indicates that ARF7 function can be repressed by Aux/IAAs through both domains (Guilfoyle and Hagen, 2012; Korasick *et al.*, 2014; Nanao *et al.*, 2014). It should also be mentioned that different signaling pathways might modify auxin signaling at the level of ARFs. Beside low R/FR, which promote the transcription of several *ARF* genes, BIN2, a kinase acting in the brassinosteroid pathway, modifies the activity

of ARF7 and ARF19 in the context of lateral root formation (Cho *et al.*, 2014).

ABP1 function Auxin binding protein 1 (ABP1) is the first identified auxin receptor in *Arabidopsis thaliana* (Sauer and Kleine-Vehn, 2011). The *abp1* loss of function mutant is embryo lethal (Chen *et al.*, 2001). Therefore, early research focused on biochemical approaches in heterologous system. ABP1 mediates fast responses at the plasma membrane, which lead to hyperpolarization, K^+ fluxes, cytosolic changes of pH and cell expansion (Barbier-Brygoo *et al.*, 1992; Venis *et al.*, 1992; Thiel *et al.*, 1993; Steffens *et al.*, 2001; Yamagami *et al.*, 2004). ABP1 also mediate Ca^{2+} fluxes (Shishova and Lindberg, 2010). Furthermore, ABP1 plays a role in ROP mediated cytoskeleton modification as well as clathrin-dependent endocytosis (Xu *et al.*, 2010; Robert *et al.*, 2010). Herterozygous $abp^{-/+}$ mutants have altered root development and gravitropic response and subsequent analysis revealed defects in the internalization of plasma membrane located auxin transporters of the PIN family (Robert *et al.*, 2010; Effendi *et al.*, 2011). Nevertheless, the upstream auxin receptor mediating internalization of PIN proteins is still debated since Pan *et al.* (2009) reported TIR1/AFB mediated PIN internalization independent of ABP1.

The conditional repression of *abp1* by antisense constructs or cellular immunization finally paved the way for more detailed physiological studies at various postembryonic developmental stages (Braun *et al.*, 2008). The conditional repression lines display general reduced growth responses. Leafs have reduced cell size and cell number. Furthermore, D-type cyclins, which are important for G1/S phase transition, are lower expressed in *abp1 AS* induced plants and cyclinB reporter lines of G2/M marker show reduced signal intensity (Braun *et al.*, 2008).

Studies in tobacco protoplast showed that following ABP1 inactivation the cell cycle was arrest(David *et al.*, 2007). Tobacco BY-2 cells also fail to elongated when treated with auxin (Chen *et al.*, 2001). Taken together this suggests that *ABP1* plays a role in the regulation of cell division and cell elongation.

ABP1 signaling pathway ABP1 is a small glucoprotein. Its structure is composed of a conserved auxin binding site, a central domain and a C-terminal KDEL sequence , which serves as a ER-retention signal. In solution ABP1 forms homodimers (Löbler and Klämbt, 1985; Shimomura *et al.*, 1986; Woo *et al.*, 2002) and localizes primarily in the ER lumen, but can be found as well in the extracellular space associated with the plasma membrane (Diekmann *et al.*, 1995; Xu *et al.*, 2014). Nevertheless, the highest affinity of purified ABP1 to auxin *in vitro* is about

pH 5.5 (Löbler and Klämbt, 1985; Shimomura *et al.*, 1986; Tian *et al.*, 1995), which correlates with the extracellular environment. At neutral environment as reported for the ER ABP1 has a poor affinity to auxin (Tian *et al.*, 1995). Therefore, ABP1 activity was predicted to be localized at the plasma membrane. While ABP1 auxin binding has been demonstrated by photoaffinity labeling method (Jones and Venis, 1989) and confirmed by crystallography (Woo *et al.*, 2002) the signaling mechanism has not been solved yet. Initially a conformational change in the ABP1 structure upon auxin binding was hypothesized, but the solved crystal structure of the complete protein suggests that stable disulfide bridges link both sides of the peptide and prevent movement (Woo *et al.*, 2002). ABP1 can interact with the E3 ubiquitin ligase RMA2 in vitro and in vivo, nevertheless the biological relevance needs to be further investigated (Son *et al.*, 2010).

ABP1 activity is extracellular localized and until recently the transmission of signals through the plasma membrane remained elusive. Using immunodetection experiments Xu *et al.* (2014) showed the interaction of ABP1 with the extracellular domain of TMK1. TMKs are a small subfamily of receptor-like kinases. They are composed of an intracellular serine/threonine kinase domain, a single transmembrane domain and extracellular leucine-rich repeat domain (Dai *et al.*, 2013). In the genome of *Arabidopsis thaliana* four *TMK* genes were identified (*TMK1* to *TMK4*). While single *TMK* loss-of-function mutants have no reported phenotype, *tmk1tmk4* double mutants display a strongly reduced growth in roots, hypocotyls, rosette size and siliques (Dai *et al.*, 2013). The *tmk* quadruple loss of function mutant has in addition a single cotyledon, reduced auxin-mediated pavement cell interdigitation and displays embryo lethality with slightly reduced penetrance compared to *abp1* (Xu *et al.*, 2014).

Recently the ABP1 signal integration with the TIR1/AFB pathway was discovered. ABP1 stabilizes Aux/IAA levels by a combination of altered biosynthesis and degradation rates and act therefore negatively on the TIR/AFB signaling pathway (Tromas *et al.*, 2013). This regulation is independent of TIR1/AFB (Tromas *et al.*, 2013) and take place within 8 min following ABP1 inactivation (Braun *et al.*, 2008). The interaction of both signaling pathway is further supported by the epistatic phenotype of higher order *tir1/afb* mutants over *abp1* conditional immunization lines (Tromas *et al.*, 2013). ABP1 is required for the transcriptional expression of cell wall remodeling enzymes such as expansins and XTHs. While some members of those genes highly depend on ABP1, e.g. *EXPA5* and *XTH33*, other members are such as *XTH19* are transcriptionally regulated through APB1 and TIR1/AFB (Paque *et al.*, 2014).

ABP1 also positively affects the localization of PINFORMED (PIN) auxin efflux carrier at the plasma membrane and polar auxin transport (PAT) (Robert *et al.*, 2010; Xu *et al.*, 2010; Effendi

et al., 2011). Recently, *ABP1* was linked to low R/FR responses. Heterozygote *abp1/ABP1* or weak *abp1-5* mutants show increased hypocotyl elongation in low R/FR condition and show altered transcriptional responses of shade-induced genes (Effendi *et al.*, 2013, 2014).

auxin transport Auxin is predominantly produced in cotyledon and leafs in the shoot and can also be locally synthesized in additional organs like roots (Ljung *et al.*, 2001; Chen *et al.*, 2014). Shoot derived auxin is subsequently transported basipetally towards the roots. This establishes a differential auxin concentrations forming a gradient between shoot and root as well as local maxima and minima. While generally phytohormones are distributed by diffusion, auxin is also directionally transported through tissues. The chemiosmotic model of auxin transport includes chemical properties of IAA as well as auxin influx and efflux carrier. At a low pH such as pH 5.5 in the extracellular space IAA exists mainly in anionic and only around 15 % in protonated form. The protonated form can passively diffuse through the plasma membrane. Auxin influx carrier binding anionic IAA facilitate cellular auxin uptake. At a neutral pH such as around pH 7.0 in the cytoplasm IAA exist almost completely in anionic form, which need to be actively transported across the plasma membrane, and the export depends on auxin efflux carrier (Friml and Jones, 2010; Swarup and Péret, 2012). In *Arabidopsis thaliana* several classes of auxin transporter were identified. AUXIN RESISTANT 1/LIKE AUX1 (AUX/LAX) are described as auxin influx carrier (Bennett *et al.*, 1996; Carrier *et al.*, 2008) and PINFORMED (PIN), PIN-LIKES (PILS), ATP-binding cassette transporters/multi-drug resistance/P-glycoprotein (ABCB/PGP) and WALLS ARE THIN 1 (WAT1) have efflux activity (Noh *et al.*, 2001; Santelia *et al.*, 2005; Luschnig and Vert, 2014; Barbez *et al.*, 2012; Ranocha *et al.*, 2013). Importantly, they are polar localize in some tissues such as the endodermis and can therefore establish an auxin polar transport (PAT) stream.

The PINFORMED family is named after the pin-like inflorescence phenotype of its founding members PIN1 (Gälweiler *et al.*, 1998). In *Arabidopsis thaliana* eight members PIN1 to PIN8 were identified and functionally characterized to different extends (Gälweiler *et al.*, 1998; Müller *et al.*, 1998; Utsuno *et al.*, 1998; Friml *et al.*, 2002b,a; Mravec *et al.*, 2009; Friml *et al.*, 2003; Dal Bosco *et al.*, 2012; Ding *et al.*, 2012; Nisar *et al.*, 2014). PINs fall into two subgroups with PIN1 to PIN4, PIN6 and PIN7 forming clade I and PIN5 and PIN8 clade II. All PINs have ten conserved transmembrane domains and vary in their central domain, which forms a hydrophilic loop. PIN proteins of clade I contain a long hydrophilic loop and localize with the exception of PIN6 predominantly to the plasma membrane, whereas the clade II PINs contain a shorter

loop and are either predominantly found at intracellular compartments such as the ER or in case of PIN8 at the ER or the plasma membrane (Ganguly *et al.*, 2010; Mravec *et al.*, 2009; Ganguly *et al.*, 2014). Loop swap experiments indicate that the central domain can contribute to the different localization in a tissue specific context (Ganguly *et al.*, 2014). Different clade I PINs have different expression pattern but also exhibit redundancies based on analysis of higher order mutants (Vieten *et al.*, 2005; Friml *et al.*, 2003; Grunewald and Friml, 2010). During shade avoidance PIN3 is required for a full hypocotyl elongation response (Keuskamp *et al.*, 2010).

Taken together, full shade avoidance responses depend on multiple levels of auxin metabolism and signaling. This includes auxin biosynthesis through *TAA1* and several *YUCCAs*, auxin perception by *TIR1/AFB* and *ABP1* auxin receptors, auxin signaling through multiple *IAAs* and *ARFs* as well as auxin polar transport by *PIN* proteins.

1.8 Aims

The aim of my thesis was to investigate transcriptional responses during shade avoidance on a genome wide level and thereby increase our understanding of the underlying regulatory network.

Shade avoidance responses are mediated by phytochrome B. Two important downstream transcription factors, PIF4 and PIF5, were previously identified and investigated in single case studies. In the project described in chapter II two large-scale experiments were analyzed aiming to identify at the genome level PIF4 and PIF5 regulated genes. Enrichment studies of those genes revealed a strong upregulation of genes involved in the auxin biosynthesis and signaling pathway. Furthermore, PIF5 direct target genes were identified and DNA sequences in the proximity of PIF5 chromatin binding sites revealed an enrichment of TCP transcription factor binding sites.

The project in chapter III aims to investigate the opposite growth response of cotyledon and hypocotyls to shade. Since previous analyses focused mainly on shade mediated transcript regulation identified in whole seedlings only little is known on an organ level. The current model of shade avoidance includes the upregulation of auxin biosynthesis in cotyledons followed by basipetal transport to the hypocotyl and subsequent induced hypocotyl elongation. On a transcriptional level we evaluated how general the transcriptional responses to shade can be explained by our current model. Furthermore this data set allowed us to define organ-specific transcriptional regulation to shade.

1.8.1 Research objectives

- Genome wide identification of PIF4 and PIF5 dependent transcriptionally regulated genes
- Identification of PIF5 direct target genes
- Analysis of proximal DNA sequences of PIF5 binding sites in order to identify additional DNA binding proteins involved in PIF mediated transcriptional regulation
- Identification of general trends of PIF4 and PIF5 mediated transcriptional regulation in shade
- Test and refine the shade avoidance model in respect to shade induced auxin levels and subsequent downwards transport to the hypocotyl
- Investigate the opposite growth response of cotyledon and hypocotyls
- Identification of organ-specific and opposite regulated genes in cotyledon and hypocotyl in order to increase our understanding of the growth response of both organs

2 Genome wide transcriptional regulation of shade responsive genes by PIF4 and PIF5

As discussed in the publication, we intended to study transcriptional shade regulation mediated by the close homologs PIF4 and PIF5 in a genome wide scale. To this end we compared transcriptional responses in Col-0, *pif5*, *pif4pif5* and, *PIF5ox* lines to low R/FR using an Affymetrix¨ ATH1 GeneChips assays (microarray). To gain further insight into the regulatory mechanisms, we extended the analysis with co-immunoprecipitation followed by high throughput sequencing (ChIP-seq) with the PIF5-HA overexpressing line, in order to identify PIF5 chromatin binding sites (peaks). By combining PIF5 peaks in proximity of coding sequences (CDS) with coincidental shade regulated transcriptions level we defined a list of PIF5 direct target genes. Those PIF5 direct targets included gene related to auxin biosynthesis (*YUCCA8*), auxin transport (*PIN3*) and auxin signaling (several *IAAs*).

My contribution to this publication comprises the analyses of both high throughput data sets and their comparison to a published data set. cDNA samples were prepared by Patricia Hornitschek. The statistical analysis of our microarray data was done by Sylvain Pradervand and initial ChIP-Seq analysis including read mapping and peak calling was done under supervision of Jacques Rougemont.

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FEATURED ARTICLE

Phytochrome interacting factors 4 and 5 control seedling growth in changing light conditions by directly controlling auxin signaling

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SUMMARY

Plant growth is strongly influenced by the presence of neighbors that compete for light resources. In response to vegetational shading shade-intolerant plants such as Arabidopsis display a suite of developmental responses known as the shade-avoidance syndrome (SAS). The phytochrome B (phyB) photoreceptor is the major light sensor to mediate this adaptive response. Control of the SAS occurs in part with phyB, which controls protein abundance of phytochrome-interacting factors 4 and 5 (PIF4 and PIF5) directly. The shade-avoidance response also requires rapid biosynthesis of auxin and its transport to promote elongation growth. The identification of genome-wide PIF5-binding sites during shade avoidance revealed that this bHLH transcription factor regulates the expression of a subset of previously identified SAS genes. Moreover our study suggests that PIF4 and PIF5 regulate elongation growth by controlling directly the expression of genes that code for auxin biosynthesis and auxin signaling components.

Keywords: shade avoidance, phytochrome, phytochrome-interacting factor, auxin, ChIP sequencing, *Arabidopsis thaliana*.

INTRODUCTION

Many plants are sensitive to shade from the neighboring vegetation and display a developmental response known as the shade avoidance syndrome (SAS) to adapt to this potentially threatening situation. These responses include elongation of hypocotyls, stems and petioles, elevated leaf angles (hyponasty), reduced leaf blade development and early flowering (Morelli and Ruberti, 2000; Vandenbussche *et al.*, 2005; Franklin, 2008; Ballare, 2009; Franklin and Quail, 2010). Light filtered through vegetation has a specific spectral signature with a reduction of the red to far-red ratio (R/FR) due to selective absorption of red and blue light, but not far-red, by photosynthetic pigments. In direct sunlight the R/FR ratio is above 1, while under deep shade it can drop below 0.1 (Franklin, 2008; Ballare, 2009). Under shading by

vegetation, plants can experience reduced photosynthetically active radiation (PAR) and R/FR ratio. Given that about 50% of far-red light is reflected from leaves, plants growing in the proximity of neighbors will also experience a reduction of the R/FR ratio but maintain access to normal PAR (Ballare, 1999). Many plants respond to such 'neighbor threat' by displaying responses similar to the SAS (Ballare, 1999; Keller *et al.*, 2011).

The red and far-red sensing phytochromes play a predominant role in the control of the SAS particularly under 'neighbor threat' conditions when the low R/FR ratio occurs without PAR reduction. (Ballare, 2009; Franklin and Quail, 2010; Kami *et al.*, 2010). In Arabidopsis, phyB is the major sensor of low R/FR although phyD and phyE contribute to the response (Franklin and Quail, 2010). Phytochromes are synthesized in the inactive red-light-absorbing Pr conformer that is primarily cytosolic. Upon light absorption it converts to the active Pfr form (far-red absorption maximum) that accumulates in the nucleus where it leads to rapid changes in gene expression (Nagatani, 2004; Fankhauser and Chen, 2008; Franklin and Quail, 2010; Kami *et al.*, 2010). Transfer of plants from sun to shade alters the Pfr/Ptot ratio and leads to rapid phytochrome-mediated modifications in gene expression (Devlin *et al.*, 2003; Salter *et al.*, 2003; Sessa *et al.*, 2005; Tao *et al.*, 2008). Under direct shading, which includes a reduction in blue light additional photoreceptors, the cryptochromes contribute most notably to the SAS (Sellaro *et al.*, 2010; Keller *et al.*, 2011; Keuskamp *et al.*, 2011; Zhang *et al.*, 2011).

Multiple hormones are involved in the establishment of the SAS (Morelli and Ruberti, 2000; Vandenbussche *et al.*, 2005; Franklin, 2008; Martinez-Garcia *et al.*, 2010). Both TAA1-dependent auxin biosynthesis and auxin transport are essential to induce hypocotyl elongation by a reduction in the R/FR ratio (Steindler *et al.*, 1999; Tao *et al.*, 2008; Keuskamp *et al.*, 2010). Moreover, gibberellins (GA), brassinosteroids (BR), cytokinins and ethylene also contribute to a normal SAS (Pierik *et al.*, 2004, 2004; Carabelli *et al.*, 2007; Djakovic-Petrovic *et al.*, 2007; Kozuka *et al.*, 2010; Keuskamp *et al.*, 2011).

PIF4 and PIF5, two members of the phytochrome-interacting factor (PIF) family of bHLH proteins are good candidates for a direct link between phytochrome regulation by shade and gene expression because their protein stability is controlled directly by the R/FR ratio (Lorrain *et al.*, 2008; Keller *et al.*, 2011). However the SAS is only partially affected in *pif4pif5* double mutants, a finding that indicates that additional factors mediate the SAS (Lorrain *et al.*, 2008; Cole *et al.*, 2011; Keller *et al.*, 2011). Additional transcription factors, which include several target genes of PIF5, have been implicated in the control of SAS (Steindler *et al.*, 1999; Salter *et al.*, 2003; Sessa *et al.*, 2005; Roig-Villanova *et al.*, 2007; Hornitschek *et al.*, 2009; Sorin *et al.*, 2009; Crocco *et al.*, 2010; Kunihiro *et al.*, 2011). Among them HFR1, PIL1 and PAR1 act as negative regulators of the SAS (Salter *et al.*, 2003; Sessa *et al.*, 2005; Roig-Villanova *et al.*, 2007). This negative regulation occurs, at least in part, via the inhibition of PIF4 and PIF5, which suggests the existence of complex regulatory networks controlling SAS including circadian regulation of the process (Salter *et al.*, 2003; Sessa *et al.*, 2005; Hornitschek *et al.*, 2009; Hao *et al.*, 2012; Sellaro *et al.*, 2012).

The link between auxin that is essential for the response to low R/FR and the transcriptional network described above remains poorly understood. PAR1 over-expression inhibits shade-induced expression of auxin response genes (Roig-Villanova *et al.*, 2007), while the positive regulator of SAS, ATHB2, controls auxin sensitivity through unknown mechanisms (Steindler *et al.*, 1999; Kunihiro *et al.*, 2011). Interestingly, PIF4 controls hypocotyl elongation in response to elevated temperature by direct regulation of the *TAA1* auxin biosynthesis gene (Franklin *et al.*, 2011). Moreover it has been shown that *pif4pif5* mutants display an altered sensitivity to auxin and altered expression of numerous 'auxin genes', however whether this situation is due to direct regulation of auxin signaling genes by those PIFs remains unknown (Nozue *et al.*, 2011).

In order to better understand the mechanisms underlying PIF4 and PIF5-mediated growth responses, we combined chromatin immunoprecipitation (ChIP) followed by sequencing (ChIP-seq) to identify chromatin-binding sites of PIF5 with gene expression studies. We identify a small set of shade-induced genes whose regulation depends on PIF4 and PIF5. Both transcription factors bind to promoter sequences of most of these genes, which indicates that they may be direct targets of these PIFs. Our study reveals that PIF4 and PIF5 also influence gene expression in high R/FR, particularly in low PAR. Finally, our work suggests that PIF4 and PIF5 affect auxin-mediated growth by directly controlling the expression of *YUC* genes that code for enzymes that control a rate-limiting step in auxin biosynthesis and of *IAA/AUX* auxin signaling genes.

RESULTS

Identifying genome-wide PIF5 binding sites

PIF4 and PIF5 control the SAS and directly regulate the expression of several shade marker genes (Lorrain *et al.*, 2008; Hornitschek *et al.*, 2009). In order to obtain a global view of the importance of PIF5 during shade avoidance we performed a ChIP experiment followed by ultrahigh throughput sequencing (ChIP-seq) using a PIF5-HA line that was subjected to a 2 h low R/FR treatment (Lorrain *et al.*, 2008). We generated DNA libraries, one for the chromatin (input) and one for the enriched chromatin fragments following immunoprecipitation (IP). In total, 1103 PIF5 binding sites were detected using Model-based Analysis of ChIP Sequ (MACS) (Zhang *et al.*, 2008). For further analysis we

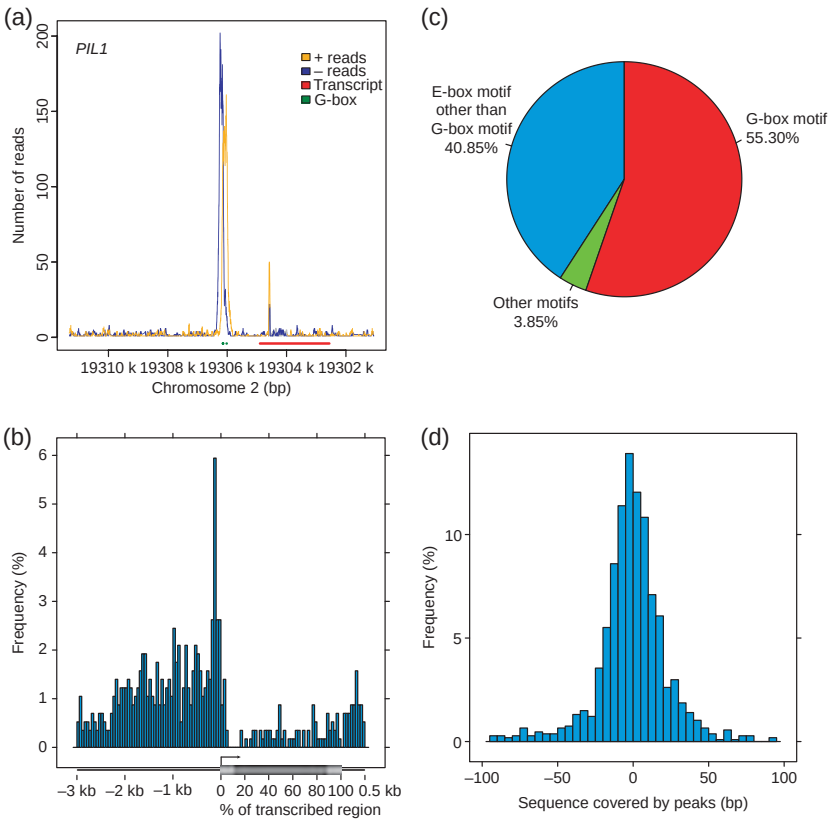

Figure 1. PIF5-HA preferentially binds to promoters containing E- and G-boxes.
(a) Sequence read distribution in the genomic region that contains *PIL1*. Reads are enriched on top of G-boxes (green dot) located in the *PIL1* promoter. Reads mapping to the + and –strands are labeled in yellow or blue respectively. The *PIL1* coding sequence (CDS) is marked with a red bar.
(b) Distribution of PIF5-HA binding loci relative to the transcriptional start site. PIF5-HA binding sites, which map within CDSs, were plotted relative to 2.5 kb (horizontal bar). Only PIF5-HA binding sites assigned to one gene were considered.
(c) Percentage of PIF5-HA binding loci containing at least one G- or E-box. Note that loci that contain a G-box may also contain additional E-boxes.
(d) Distribution of G-boxes within the 200 bp sequence covered by peaks. The *x*-axis represents the relative distance in bp to the center of peaks.

considered peaks located in the proximity of genes defined as follows: from –3000 bp of the transcript to 500 bp downstream of the transcript. This list comprises 962 peaks and identifies 1218 Arabidopsis Genome Initiative loci (Table S1). As an example the reads located on three closely spaced G-boxes present 5′ of the *PIL1* gene are presented (Figure 1a). We previously showed that these G-boxes are required for PIF5-mediated expression of a *PIL1* reporter in cell cultures (Hornitschek *et al.*, 2009). In Arabidopsis seedlings, these three G-boxes were required for shade-induced expression of the *PIL1* reporter (Figure 2). We conclude that PIF5 binding to the G-boxes of the *PIL1* promoter is important for shade-regulated expression of this gene. Moreover, the results of this experiment suggest that genes that require PIFs for normal expression and possess a nearby PIF5 binding site are likely to be direct targets of this transcription factor.

Most genes in our list contained a binding site in the promoter regions with a higher frequency towards the transcriptional start site (TSS) and fewer peaks within the transcript or immediately 3′ of it (Figure 1b). PIF5 has been shown previously to bind directly to the G-box DNA motif (5′-CACGTG-3′) (Hornitschek *et al.*, 2009). We therefore analyzed PIF5 binding peaks, defined as 200 bp centered to the peak summit, for the presence of this sequence and of the E-box (5′-CANNTG-3′), a degenerated G-box that is also bound by bHLH transcription factors. Almost all PIF5 peaks contained an E-box (96%), the majority of which was a G-box (55%) (Figure 1c). Using motif-based sequence analysis tools (http://meme.sdsc.edu/meme/intro.html) we confirmed that the G-box is highly over-represented in PIF5 peaks. G-boxes were enriched in the center of PIF5 peaks, a finding that suggested that they mediate DNA binding (Figure 1d).

Protein-binding microarrays (PBM) were used to compare the *in vivo* binding sites of PIF5 with its DNA-binding specificity (Godoy *et al.*, 2011). We included PIF4, the closest homologue of PIF5 and HFR1, in our analysis. PIF5 and PIF4 showed a strong preference for the G-box, which is the sequence that was most enriched in PIF5 peaks determined by ChIP (Figures 1, 3 and S1). In addition, binding of PIF5 to the G-boxes *in vitro* was influenced by the nucleotides immediately 5′ and 3′ of the G-box, while this situation was not the case for PIF4 (Figure 3). Moreover, this experiment demonstrated that HFR1 did not possess any sequence-specific DNA-binding capacity (Figure 3). Our data do not exclude that HFR1 heterodimers with other bHLH factors could bind to DNA. However, taken together with findings in recent publications, it is most likely that HFR1 works by preventing other bHLH factors from binding to DNA (Figure 3) (Hornitschek *et al.*, 2009; Galstyan *et al.*, 2011).

Gene Ontology (GO) enrichment analysis was performed on genes that were close to PIF5 peaks in order to identify biological processes possibly regulated by PIF5 (Table S2). The terms ‘response to light stimulus’, ‘response to red or

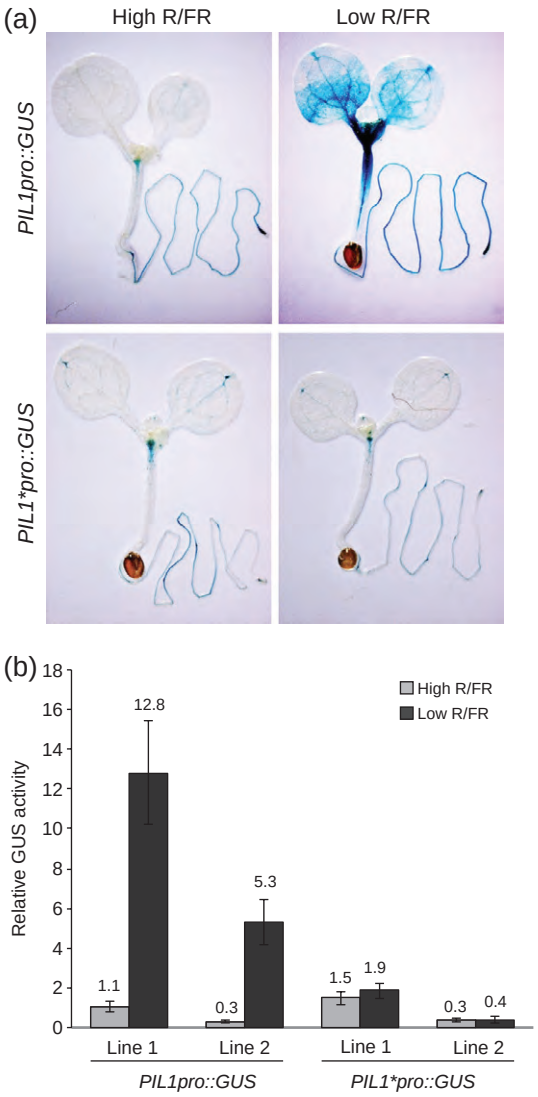

Figure 2. The shade-induced expression of *PIL1* is dependent on G-boxes. Seedlings were grown for 7 days in constant high R/FR before being transferred 5 h to low R/FR or kept in high R/FR (control). Transgenic lines carrying *PILpro1::GUS* or *PIL1*pro::GUS* (*PIL1* promoter that contains point mutations in all three G-boxes) were used.
(a) GUS staining of *PILpro1::GUS* and *PIL1*pro::GUS* lines.
(b) Quantification of *PILpro1::GUS* and *PIL1*pro::GUS* reporter gene activity using MUG assay. Results of two independent transgenic lines are presented. Data are means ± 2 standard errors (SE) of three biological replicas.

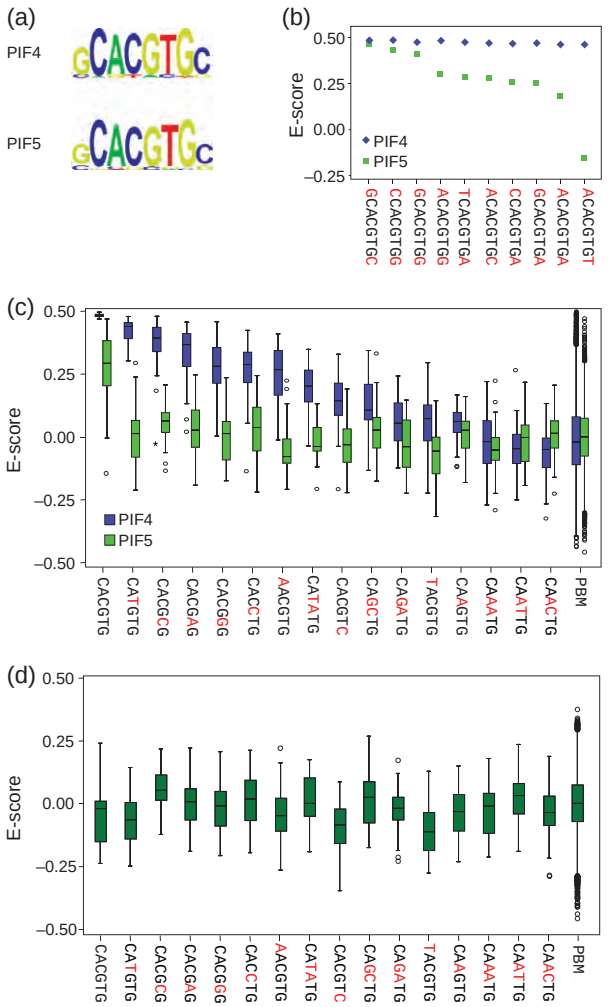

Figure 3. Identification of PIF4 and PIF5 binding sites *in vitro*.
(a) Position weight matrix representation of the first scoring 8-mers corresponding to PIF4 and PIF5.
(b) Enrichment scores (E-scores) of all the possible G-box-containing 8-mers for the two proteins tested.
(c) Box-plot of E-scores of G-box-related variants including both single-site mutations and E-boxes for PIF4 (blue) and PIF5 (green). Boxes represent quartiles 25–75%, and black line represents the median of the distribution (quartile 50%). Bars indicate quartiles 1–25% (above) and 75–100% (below), and dots denote outliers of the distribution.
(d) Box-plot of E-scores of G-box-related variants that include both single-site mutations and E-boxes corresponding to HFR1, as in (c). HFR1 did not show significant binding to any of the elements represented in the PBM.

far-red light', 'response to radiation' and 'shade avoidance' were enriched. 'Response to hormone stimulus' and especially 'response to auxin stimulus' were also strongly enriched. 'Transcription factor activity' and, interestingly, the auxin responsive 'SAUR' (small auxin-up RNA), basic helix–loop–helix and IAA/AUX proteins were also over-represented. This first analysis suggested that PIF5 might regulate light responses by controlling the expression of hormonal pathways directly, in particular auxin.

Shade-regulated gene expression in *pif4pif5*

The wild type, *pif5*, *pif4pif5* and the *PIF5-HA* line used for ChIP-seq were subjected to a 2 h low R/FR treatment or maintained in the high R/FR light to determine the importance of PIF4 and PIF5 in shade-regulated gene expression using an Affymetrix® Arabidopsis ATH1 chip. A linear model was used to compute the interaction between the genotype (*pif4pif5* versus wild type) and treat-

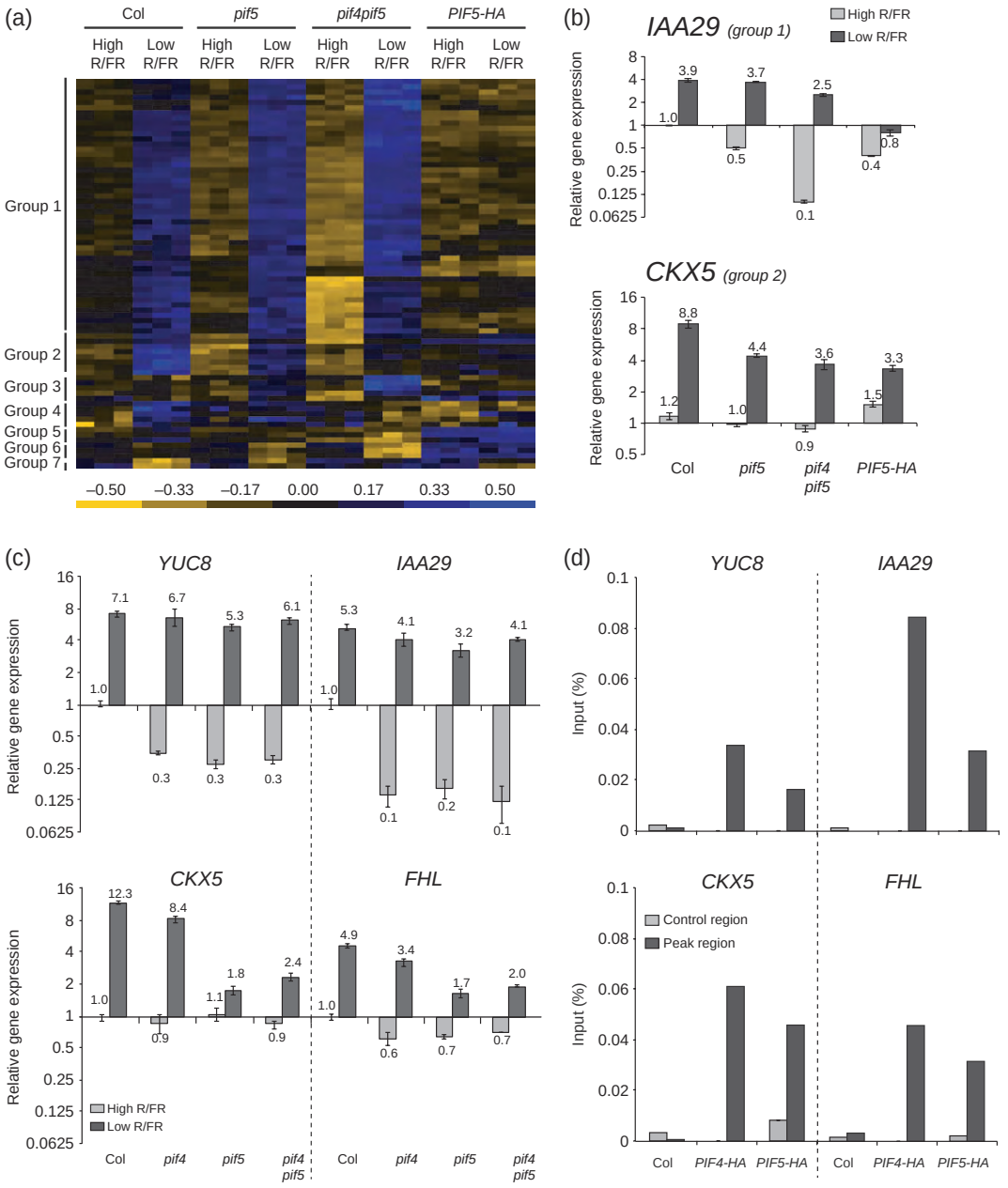

Figure 4. Genes displaying an altered regulation by a low R/FR treatment in *pif4pif5*.
(a) Hierarchical clustering of relative expression levels across all samples for 77 genes significantly (adj. *P*-value < 0.05) dependent on an interactive effect of the genotype (*pif4pif5* versus wild type) and the condition (low versus high R/FR ratio).
(b) Gene expression from the microarray experiment for a representative gene of groups 1 and 2.
(c) Gene expression determined by reverse transcription quantitative polymerase chain reaction (RT-QPCR) in response to 2 h of low R/FR. Col, *pif4*, *pif5* and *pif4pif5* seedlings were grown 7 days in constant light conditions before being moved for 2 h to low R/FR or kept in high R/FR. Expression levels were normalized to *YLS8* and *UBC* and expressed relative to the Col value in high R/FR. Error bars represent standard error of the mean of three biological replicates.
(d) Chromatin immunoprecipitation (ChIP) of PIF4-HA or PIF5-HA grown for 7 days in constant light followed by a 2 h low R/FR treatment. Immunoprecipitated DNA was quantified by QPCR using primers in the promoter region containing a G-box or control region. Data are average of technical triplicates of the QPCR. Data from one representative ChIP experiment are shown.

ment (low R/FR versus high R/FR) to identify genes misregulated by the treatment in *pif4pif5*. We identified 77 genes with significant interaction with a false discovery rate (FDR) < 0.05 (Figure 4a), meaning that genes in this list showed a significantly different fold-change following treatment in *pif4pif5* compared with the wild type (Table S3). The expression of these genes is presented as a heatmap that also includes their expression in *pif5* and

PIF5-HA (Figure 4a). Hierarchical clustering of the expression of these 77 genes identified two major groups and five smaller ones (3–7) that will not be discussed further here. The expression of the majority of these genes was regulated robustly by shade in *pif4pif5.* This situation was particularly obvious for group 1, which represented the largest set of genes. Genes belonging to group 1 presented similar expression levels in low R/FR in the wild type and in *pif4pif5.* These genes are present in this list because their expression was reduced in high R/FR-grown *pif4pif5*, leading to greater shade induction. In contrast, genes belonging to group 2 showed reduced induction by low R/FR in *pif4pif5.* This small group contains genes identified previously as dependent on PIF4 and PIF5 for regulation, such as *HFR1* and *ATHB2* (Figure 4) (Lorrain *et al.*, 2008). Considering previously published data, *PIL1*, which is not included in the ATH1 chip, would also be part of this group (Lorrain *et al.*, 2008; Hornitschek *et al.*, 2009). Genes identified in this category include *PIL2*, *CKX5* and *FHL.* GO enrichment analysis was performed in order to identify biological processes that may be misregulated in *pif4pif5* during the response to low R/FR (Table S2). Interestingly, several GO terms identified among genes close to PIF5 peaks were also found in this list. In particular, the most over-represented term was 'response to auxin stimulus' and 'AUX/IAA proteins' was also over-represented in the list of genes with a misregulated expression by shade in *pif4pif5.* This analysis provided further support for a role of these PIFs in auxin-mediated growth responses.

Direct target genes probably showed altered expression in the mutant and in binding of the transcription factor to their promoter (e.g. *PIL1*) (Figures 1 and 2) (Hornitschek *et al.*, 2009). We thus compared the list of genes in the interaction list with genes that have a PIF5 binding site in their promoter (Tables S1 and S3). We found that 39% of the genes of the interaction present a PIF5 binding peak in their vicinity. Interestingly these putative direct target genes are not distributed evenly in the different groups. Especially, eight out of the nine genes of group 2 (including *PIL1*) show a PIF5 binding site in their promoter. These data suggest that most genes that are not properly upregulated by shade in *pif4pif5* (group 2) are direct targets of these transcription factors (Figure 4).

To confirm these genome-wide data, we conducted additional gene expression and ChIP analysis on selected genes (Figure 4c,d). We present data for representatives of groups 1 and 2 of the interaction list, which contained a PIF5 binding peak determined by ChIP-seq. This experiment confirmed that genes listed in group 2 (*CKX5* and *FHL*) were primarily misexpressed in response to a low R/FR treatment in *pif4pif5.* In contrast, genes belonging to group 1 (*YUC8* and *IAA29*) showed a slightly reduced expression in *pif4pif5* exposed to low R/FR but had strongly reduced expression in high R/FR (Figure 4c). Moreover, by analyzing the expression of these genes in *pif4* and *pif5* single mutants we noticed that PIF5 played a predominant function in the expression of group 2 genes in low R/FR, while the expression of group 1 genes was reduced both in *pif4* and *pif5* grown in high R/FR (Figure 4c). ChIP experiments were conducted with the *PIF5-HA* line and seedlings expressing *PIF4-citrine-HA* under the control of the *PIF4* promoter (hereafter referred to as *PIF4-HA*). Using chromatin from seedlings exposed to a 2 h low R/FR treatment, we confirmed binding of PIF5 to 10 (out of 10) genes selected based on the presence of a PIF5 binding site and misexpression in response to shade (interaction list) (Figures 1, 4, and S2). Moreover, PIF4-HA also bound to the promoter of all tested genes (*FHL*, *CKX5*, *IAA29* and *YUC8*; Figure 4d). This finding suggests that both PIF4 and PIF5 control the expression of shade-regulated genes, including genes coding for auxin biosynthesis and signaling (Figure 4d), directly.

PIF4 and PIF5 regulate gene expression in low PAR

Our gene expression analysis identified numerous genes misexpressed in *pif4pif5* in our high R/FR conditions (Figure 4a). These conditions correspond to relatively low PAR, which prompted us to analyze more carefully the implication of PIF4 and PIF5 in low PAR. Using a FDR < 0.05 we found 521 genes whose expression differed between *pif4pif5* and the wild type (Figure 5) (Table S4). Close to 80% of these genes showed reduced expression in *pif4pif5*, suggesting that PIF4 and PIF5 promote the expression of most of these genes. Hierarchical cluster analysis identified four main expression classes (Figure 5a). Among the genes that were downregulated in *pif4pif5* only a subset was expressed at a higher level in *PIF5-HA* than in the wild type (compare groups I and II). A third cluster contained genes that were downregulated in *pif4pif5* and even further downregulated in *PIF5-HA.* Finally, the last group contained genes with a higher expression in *pif4pif5* than in the wild type. Generally speaking, the *pif5* expression phenotype was intermediate between the one of the wild type and *pif4pif5* (Figure 5a). GO terms enriched among these 521 genes include 'response to hormone stimulus', 'response to auxin', 'response to light stimulus' and 'response to radiation'; all terms that were identified in our previous GO analysis of genes nearby PIF5 peaks in low R/FR-grown seedlings.

We hypothesized that PIF4 and PIF5 might play a particularly important role in low light intensity, which prompted us to analyze seedling growth of the wild type, *pif4*, *pif5*, *pif4pif5* and the *pif1pif3pif4pif5* (*pifq*) mutants under several intensities of PAR (Figure 5b). Interestingly, while the *pif* mutants showed no significant defect in hypocotyl elongation under high PAR, phenotype strength increased with decreasing PAR (Figure 5b). Similar to the gene expression phenotype, *pif5* showed a phenotype intermediate between the wild type and *pif4pif5.* Finally, the *pifq* hypocotyl

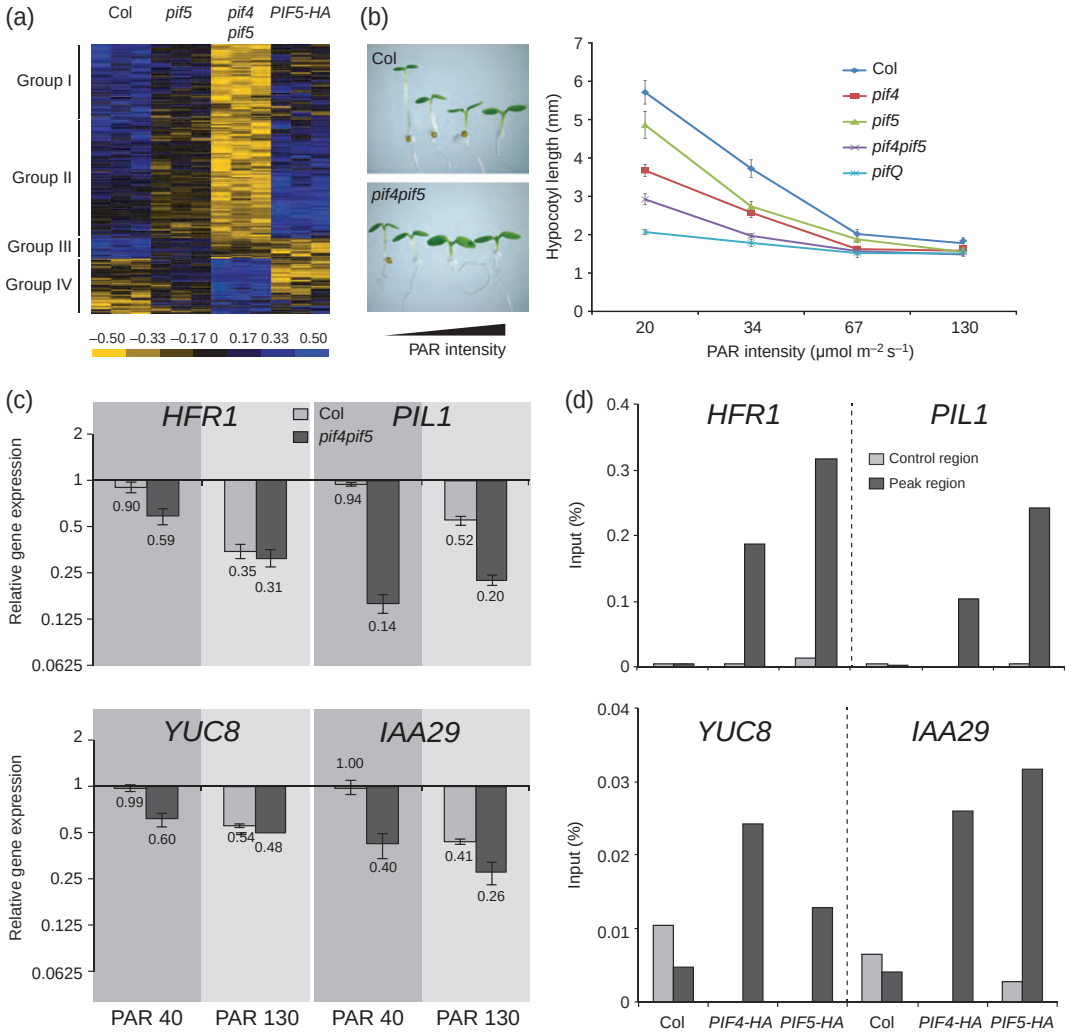

Figure 5. PIF4 and PIF5 are involved in responses to low light intensities.
(a) Hierarchical clustering of genes differently expressed between *pif4pif5* and Col-0 grown in high R/FR light.
(b) Hypocotyl length in constant white light. Seedlings were grown 4 days under different constant white light conditions before hypocotyls were measured. Representative seedlings are shown for Col and *pif4pif5* in the left panel. Data are means ± 2SE (n = 23–30).
(c) Gene expression determined by reverse transcription quantitative polymerase chain reaction (RT-QPCR) after 7 days growth in constant low or high light intensity (40 or 130 µmoles m^{-2} sec^{-1}). Expression levels were normalized to *YLS8* and *UBC* and expressed relative to the Col value in PAR 40. Error bars represent standard error to the mean of three biological replicates.
(d) Chromatin immunoprecipitation (ChIP) of PIF4 or PIF5 in high R/FR. Col, *PIF4-HA* and *PIF5-HA* lines were grown for 7 days in constant light (40 µmoles m^{-2} sec^{-1}) immunoprecipitated DNA was quantified by QPCR using primers in the promoter region containing a G-box (black bar) or control region (gray bar). Data are average of technical triplicates of the QPCR. Data from one representative ChIP experiment are shown.

elongation phenotype was stronger than that of *pif4pif5* only at the lowest fluence rate tested (Figure 5b).

In order to determine whether the hypocotyl elongation phenotype correlated with gene expression, we analyzed the expression of several genes under high and low PAR conditions by reverse transcription quantitative polymerase chain reaction (RT-QPCR). We concentrated on genes with the GO term 'auxin' as this term was strongly over-represented and auxin has been implicated in growth. The expression of these genes was lower in high than low PAR correlating with the shorter hypocotyls of seedlings grown in high PAR conditions (Figure 5c). Moreover, we found a good correlation between gene expression and hypocotyl length, as differences in gene expression between Col and *pif4pif5* are smaller in high compared to low PAR (Figure 5c). We conducted ChIP experiments in order to determine whether these genes were bound by PIF4 and PIF5 in low PAR. PIF4-HA and PIF5-HA are bound to the promoters of genes involved in auxin biosynthesis and signaling (*IAA29*, *YUC8*), which suggests that they also control growth by directly regulating auxin synthesis and signaling in low PAR but high R/FR (Figure 5d). Binding of both transcription

factors was also observed in the promoter of the shade marker genes *PIL1* and *HFR1*, which also show higher expression in low compared with high PAR (Figure 5d).

PIF4 and PIF5 control growth by directly regulating auxin signaling

Our gene expression, ChIP and physiological experiments suggested that PIF4 and PIF5 control hypocotyl elongation by controlling auxin biosynthesis and/or signaling (Figures 4 and 5). We thus determined auxin levels in the aerial parts of young seedlings maintained in high R/FR or transferred for 1 h into low R/FR because it was shown previously that such a treatment leads to an increase in auxin concentration (Tao *et al.*, 2008). We confirmed that a low R/FR treatment increased auxin concentration in the wild type. Interestingly, the shade-mediated increase was much reduced both in *pif4pif5* and in *PIF5-HA* (Figure 6). The auxin concentration in high R/FR was normal in *pif4pif5* while in *PIF5-HA* it was reduced (Figure 6).

In order to analyze auxin sensitivity of *pif4pif5*, we compared hypocotyl elongation of the mutant and the wild type grown in presence of different concentrations of picloram. This experiment showed that the auxin sensitivity of *pif4pif5* was altered particularly under low PAR (Figure 7a,b). In addition we tested the effect of picloram on gene expression and compared it with the effect of shade. *HFR1* was upregulated by shade but not picloram while *IAA29* expression was induced by both treatments in *pif4pif5* and the wild type (Figure 7c). However, the expression of *IAA29* in *pif4pif5* never reached wild type levels when seedlings were treated by picloram or shade (Figure 7c). Collectively our data suggest that PIF4 and PIF5 control hypocotyl growth, at least partially, by directly controlling the expression of genes involved in auxin biosynthesis and signaling.

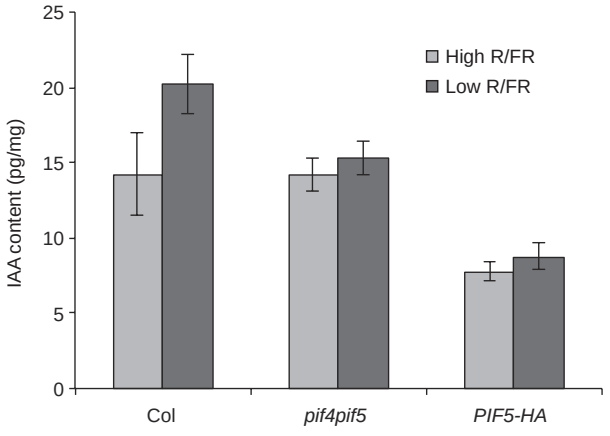

Figure 6. *pif4pif5* is affected in auxin accumulation in response to shade. WT, *pif4pif5* and *PIF5-HA* seedlings were grown 7 days in constant high R/FR light. Free IAA was measured after 1 h of high or low R/FR treatment. Data are means ± 2 standard error (SE) (n = 5).

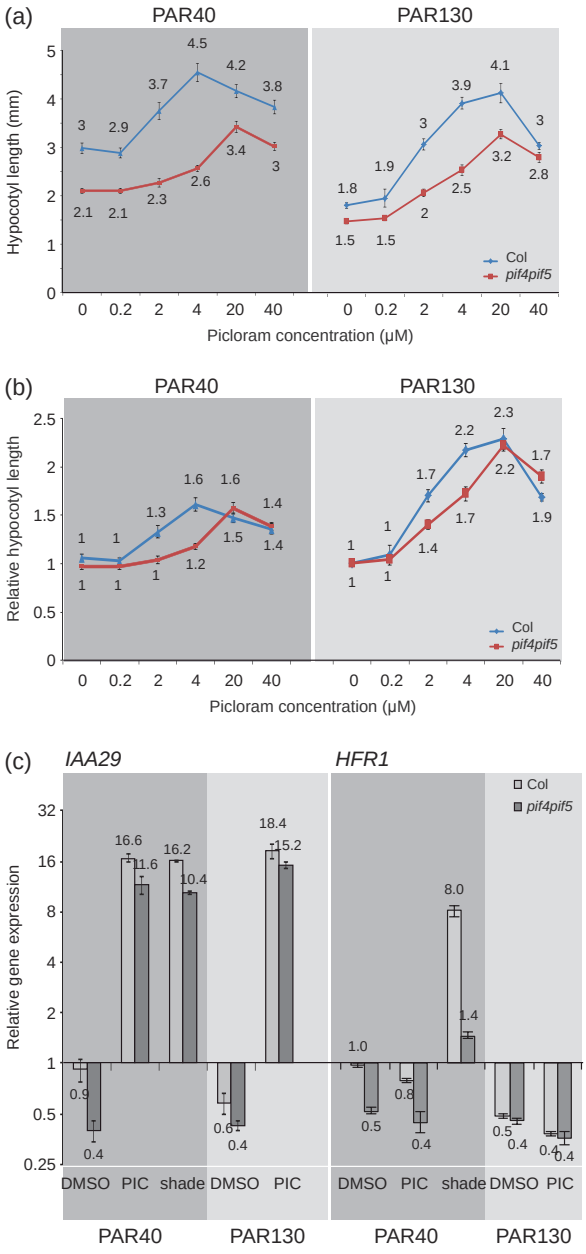

Figure 7. *pif4pif5* is affected for responses to the auxin analog, picloram.
(a) Hypocotyl length in response to picloram of seedlings grown under PAR 40 μmoles m^{-2} sec^{-1} or PAR 130 mmoles m^{-2} sec^{-1}. Seedlings were grown 4 days in constant white light conditions (40 or 130 μmoles m^{-2} sec^{-1}) before being transferred on plates containing different concentrations of picloram (PIC). They were grown for 4 more days in constant white light (40 or 130 μmoles m^{-2} sec^{-1}). Data are means ± 2 standard error (SE) (n = 36–43).
(b) Relative hypocotyl length of the data presented in panel (a), defined as the hypocotyl length relative to growth in the absence of picloram for each genotype.
(c) Gene expression in response to picloram. Col and *pif4pif5* seedlings were grown 7 days in constant white light (PAR = 40 or 130 μmoles m^{-2} sec^{-1}) before being treated for 2 h with 5 μM picloram (PIC) or moved under low R/FR conditions (PAR = 40 μmoles m^{-2} sec^{-1}) for 2 h. Expression levels determined by reverse transcription quantitative polymerase chain reaction (RT-QPCR) were normalized to *YLS8* and *UBC* and expressed relative to the Col control grown in PAR 40 Imoles m^{-2} sec^{-1} without picloram treatment (dimethyl sulfoxide). Error bars represent standard error of the mean of three biological replicates.

DISCUSSION

To get a broader view of the role of PIF5 and PIF4 during the SAS, we analyzed their contribution to gene expression in seedlings treated with low R/FR and identified PIF5 binding sites genome-wide. ChIP-seq revealed a large number of genes in the proximity of which we found PIF5 binding sites, a number that is comparable with those identified in genome-wide ChIP experiments for other transcription factors involved in light signaling (Lee *et al.*, 2007; Oh *et al.*, 2009; Ouyang *et al.*, 2011). Binding sites were abundant 5′ of the TSS with a further enrichment within the first 500 nt directly upstream of the TSS. A similar binding pattern was reported for other members of the bHLH family (Morohashi and Grotewold, 2009; Oh *et al.*, 2009). PIF5 peaks were strongly enriched in E- and G-boxes (96/55% of peaks), another feature shared with PIF1 (Oh *et al.*, 2009). By comparing the sequences bound by PIF5 *in vitro* with our ChIP-seq data, we conclude that most PIF5 binding on chromatin reflects direct binding to DNA (Figures 1, 3 and S1). Although *in vitro* PIF5 exclusively binds to G-boxes with high affinity our ChIP data shows that a sizable fraction of PIF5 ChIP peaks do not contain a G-box (Figures 1 and 3). Several hypotheses can explain this apparent paradox and future experiments are needed to understand this difference.

Interestingly, *in vitro* binding experiments show that although PIF4 and PIF5 have a preference for G-boxes, as was reported previously for several members of this family, PIF4 robustly binds to a wider range of sequences than PIF5 (Figure 3) (Martinez-Garcia *et al.*, 2000; Huq and Quail, 2002; Huq *et al.*, 2004). All the genes we tested for PIF5 binding *in vivo* were also bound by PIF4, which indicated that *in vivo* PIF4 and PIF5 share an overlapping set of binding sites (Figures 4 and 5). This finding is consistent with the additive phenotype of *pif4* and *pif5* that was reported in several situations, which included during shade avoidance (Nozue *et al.*, 2007; Lorrain *et al.*, 2008, 2009) (Figures 4 and 5). However, hypocotyl elongation in response to temperature involves PIF4 and not PIF5, which is difficult to explain based on the similar expression patterns of those genes (Nozue *et al.*, 2007; Koini *et al.*, 2009; Stavang *et al.*, 2009; Foreman *et al.*, 2011). The greater number of E-box variants efficiently bound by PIF4 may provide an explanation for the specific functions of PIF4 (Figure 3). The fact that over-expression of PIF4 leads to a stronger and more pleiotropic phenotype than over-expression of PIF5 is consistent with this hypothesis (Lorrain *et al.*, 2008).

Gene expression analyses were conducted to identify those requiring PIF4/PIF5 for normal regulation by a low R/FR treatment (Figure 4a). Among these genes a small group required PIF4/PIF5 for robust low R/FR-induced expression (Figure 4a). With the exception of one gene, all members of this group also show PIF5 binding 5′ of their TSS (Figure 4). Given that our subsequent ChIP analysis also showed binding of PIF4 to the promoters of all tested group 2 genes, they represent likely direct targets of both PIF4 and PIF5 (Figure 4). This group includes previously identified PIF5 targets *PIL1*, *HFR1* and *ATHB2* and we show that PIF4 also binds to promoter regions of these genes (Figure 4) (Hornitschek *et al.*, 2009; Kunihiro *et al.*, 2011). This situation was confirmed for *PIL1* where the 3 G-boxes present in the PIF5 binding peak are essential for shade-induced expression in seedlings (Figure 2). These data show that *PIL1* is a direct target of PIF4 and PIF5 because the transcription factors and the sequence to which they bind are both needed for robust shade-induced expression.

While some group 2 genes promote the SAS (*ATHB2*), others (*HFR1*, *PIL1*) play a negative role in shade avoidance (Salter *et al.*, 2003; Sessa *et al.*, 2005; Hornitschek *et al.*, 2009; Sorin *et al.*, 2009). The other members of this group are *PIL2*, *FHL*, *CKX5*, *ATMGL*, a B-box type zinc finger protein (At5g54470 or BBX29) and an unknown protein in the promoter of which we found no PIF5 peak (Figure 4 and Table S1). FHL mediates import of phytochrome A (phyA) into the nucleus (Hiltbrunner *et al.*, 2006; Rosler *et al.*, 2007). phyA plays a negative role in the SAS (Salter *et al.*, 2003), moreover the levels of FHY1 and FHL are limiting thus controlling the extent of phyA import into the nucleus (Rausenberger *et al.*, 2011). The shade-induced up-regulation of *FHL* may thus contribute to phyA-mediated inhibition of the SAS by promoting its import into the nucleus. BBX29 belongs to the Arabidopsis B-box family, which includes members with a role in light signaling, in particular BBX21 that negatively regulates shade-avoidance (Khanna *et al.*, 2009; Crocco *et al.*, 2010). CKX5 is involved in cytokinin catabolism and CKX6, a close homologue of CKX5, regulates the SAS (Carabelli *et al.*, 2007). CKX6 does not control hypocotyl elongation but limits leaf primordia growth in plants subjected to a shade treatment (Carabelli *et al.*, 2007). *CKX6* expression is upregulated by shade and auxin linking cytokinin-mediated responses to shade and auxin (Carabelli *et al.*, 2007). By analogy with the role of CKX6, it is conceivable that CKX5 also acts as a negative regulator of the SAS (Figure 4). *PIL2* is a member of the PIF family that also shows shade-induced gene expression, however its function is poorly understood (Salter *et al.*, 2003; Yamashino *et al.*, 2003). Finally, ATMGL is involved in methionine catabolism and its role in shade avoidance is currently unknown (Rebeille *et al.*, 2006). Collectively these data indicate that PIF4 and PIF5 directly control the expression of several genes acting as negative regulators of the shade-avoidance response (Figures 4 and 5). The relatively normal expression of many shade-regulated genes in *pif4pif5* and the reduced induction of several negative regulators of the SAS in *pif4pif5* may explain why a low R/FR signal still induces hypocotyl growth in *pif4pif5* (Figure 4) (Lorrain *et al.*, 2008; Hornitschek *et al.*, 2009; Cole *et al.*, 2011).

Our gene expression analysis showed that numerous genes are misexpressed in *pif4pif5* grown in high R/FR conditions (Figures 4 and 5). Combined with our ChIP analysis, we conclude that PIF4 and PIF5 are likely direct regulators of the expression of a number of these genes in high R/FR conditions (Figure 5). Importantly, our high R/FR conditions correspond to relatively low PAR a condition in which PIF4/PIF5 were previously shown to control growth (Keller *et al.*, 2011). Interestingly, we show that by increasing PAR we can correct both hypocotyl length and gene expression in *pif4pif5* (Figure 5). We made similar observation during de-etiolation in far-red light (Lorrain *et al.*, 2009). Many of the genes showing reduced expression in *pif4pif5* in high R/FR are strongly induced by shade in the mutant, which suggests that another transcriptional regulator controls their expression in response to low R/FR (Figure 4). Other members of the PIF family are candidates for such a function given that they bind to similar DNA sequences and can act additively (Figure 3) (Martinez-Garcia *et al.*, 2000; Huq and Quail, 2002; Huq *et al.*, 2004; Leivar *et al.*, 2008; Shin *et al.*, 2009).

Both the analysis of genes bound by PIF5 and genes misregulated in the *pif4pif5* mutant show a strong overrepresentation of the GO terms 'response to auxin stimulus' and 'response to hormone stimulus' (Table S2). This situation is remarkable in view of the strong links between auxin and shade avoidance (Morelli and Ruberti, 2000; Roig-Villanova *et al.*, 2007; Tao *et al.*, 2008; Keuskamp *et al.*, 2010; Kozuka *et al.*, 2010; Keller *et al.*, 2011). These results are also in agreement with the recent findings linking PIF4 and PIF5 to auxin-mediated growth responses (Franklin *et al.*, 2011; Nozue *et al.*, 2011). We found a large overlap when comparing the genes misregulated in our high R/FR conditions with the genes whose expression correlates with growth and requires PIF4 and PIF5 for normal expression (Nozue *et al.*, 2011) (Figure 5 and Table S5). Taken together with previous studies our data suggest that PIF4 and PIF5 modulate elongation growth responses by directly regulating auxin-controlled responses at multiple levels.

In warm conditions, PIF4 binds to the promoter and controls the expression of *TAA1* and *CYP79B2*, two genes that code for auxin biosynthetic enzymes (Franklin *et al.*, 2011). Although TAA1/SAV3 is essential for the SAS, its expression is not induced by shade and therefore rendering it unlikely that PIF4 and/or PIF5 control shade-induced growth by regulating *TAA1* expression (Tao *et al.*, 2008). However, we found that members of the YUCCA family that act downstream of TAA1 in auxin biosynthesis have PIF5 binding sites in their promoter (*YUC5*, *YUC8* and *YUC9*) (Table S1) (Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011; Won *et al.*, 2011). YUCCA proteins are rate limiting for auxin biosynthesis and increasing their expression leads to hypocotyl elongation (Zhao *et al.*, 2001; Mashiguchi *et al.*, 2011; Won *et al.*, 2011). The increased expression of several *YUCCA* genes in response to low R/FR may thus contribute to shade-induced hypocotyl elongation. We show that both PIF4 and PIF5 bind to the promoter of *YUC8* and that the gene displays reduced expression in *pif4* and *pif5* mutants, which suggests that PIF4 and PIF5 might directly control auxin biosynthesis. We thus determined auxin content in *pif4pif5* seedlings grown in high R/FR with or without a 1-h low R/FR shade treatment. Despite the reduced *YUC8* expression in *pif4pif5* grown in high R/FR we found a wild-type auxin content in aerial parts of these seedlings (Figures 4, 5 and 6). More local auxin content measurements may reveal differences between *pif4pif5* and the wild type and thus explain the shorter hypocotyl of these seedlings grown in high R/FR but low PAR. Of note, the *PIF5-HA* line that was used for ChIP-seq had the lowest auxin content of all lines despite having the longest hypocotyls (Figure 6) (Lorrain *et al.*, 2008). This finding indicates that despite a promoting effect of auxin on hypocotyl growth the auxin content in aerial parts does not simply correlate with hypocotyl length. Another unanticipated finding was that in *pif4pif5* the low R/FR-induced increase in auxin was strongly reduced although this mutant shows hypocotyl elongation in response to low R/FR both in long-term and short-term measurements (Lorrain *et al.*, 2008; Cole *et al.*, 2011). Again, more localized auxin measurements may help with the interpretation of these results.

We provide evidence for a direct link between PIF4 and PIF5 and auxin signaling by showing that PIF4 and PIF5 bind to the promoter region of *IAA29*, a gene that shows reduced levels in *pif4*, *pif5* and *pif4pif5* (Figures 4 and 5). *IAA29* expression can be induced by the addition of picloram to *pif4pif5*, however both in response to shade and in response to picloram *IAA29* expression does not reach wild-type levels in the mutant (Figure 7). In addition we analyzed hypocotyl elongation in response to picloram and consistent with a previous study found that auxin sensitivity in *pif4pif5* was altered (Figure 7) (Nozue *et al.*, 2011). Importantly auxin sensitivity was most altered in low PAR conditions, where we also found greater gene expression defects in *pif4pif5* (Figures 5 and 7). We thus suggest that PIFs modulate plant growth by directly controlling the expression of auxin signaling genes. Moreover, we propose that PIF-mediated control of auxin-driven growth might involve different mechanisms (transport, signaling, synthesis) in different situations (this work) (Franklin *et al.*, 2011).

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Seedlings were grown as described in Hornitschek *et al.* (2009) except that PAR intensity was 40 μmol m^{-2} sec^{-1}. The *pif4*, *pif5* and *pif4pif5* mutants as well as the transgenic lines were on the Columbia background (Col-0) and were described in Lorrain *et al.* (2008).

Cloning procedure and generation of transgenic lines

The generation of new transgenic lines is described in detail in the Supplementary Materials and Methods.

GUS staining and MUG assay

GUS staining and quantitative determination of GUS activity (4-methylumbelliferyl-beta-D-glucuronide, MUG assays) were performed according to standard procedures and described in detail in the Supplementary Materials and Methods.

Picloram treatment and quantification of IAA

Picloram (SIGMA-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) at 400mM. Seeds were sown on a nylon mesh on ½ strength MS (Murashige and Skoog) plates that were kept vertical during the experiment. On day 4, nylon meshes were transferred to new ½ strength MS plates containing different concentrations of picloram. Seedlings were grown for 4 more days on those plates before being photographed and measured using the ImageJ software. For gene expression analysis in response to picloram, 50 seeds were sown on a nylon mesh on ½ strength MS Petri dishes and grown for 7 days in constant light conditions. At day 7, seedlings were transferred in 1 ml of liquid ½ strength MS with 5 μM picloram or 0.01% DMSO as a control for additional 2 h.

Seedlings were pooled, weighted and frozen in liquid nitrogen for quantification of free IAA according to Andersen *et al.* (2008).

Identification of PIF4, PIF5 and HFR1 binding sites *in vitro*

Given the expected size of the DNA motif recognized by PIF4, PIF5 and HFR1 a 10 nucleotides design in PBM was chosen. In this case, we used the same PBM design as in Berger and Bulyk (2009). Protein incubation was as in Godoy *et al.* (2011) but in these cases we employed soluble protein extracts from recombinant *E. coli* cultures expressing MBP-PIF4, MBP-PIF5 and MBP-HFR1 recombinant proteins. Synthesis of double-stranded microarray and immunological detection of DNA-protein complexes were as in Godoy *et al.* (2011).

Analysis of gene expression

RNA extraction and RT-QPCR experiment were performed as described in (Lorrain *et al.*, 2009) except that results were analyzed using the qbase^{PLUS} software (http://www.biogazelle.com/products). Primer sequence is given in Table S6.

For microarray analysis samples were amplified, labeled and hybridized on Affymetrix® Arabidopsis ATH1 Genome arrays as described previously (Lorrain *et al.*, 2009). Subsequent data analysis was performed using the statistical language R (http://www.R-project.org) and various Bioconductor packages (http://www.Bioconductor.org). Normalized expression signals were calculated using RMA, and differential hybridized features were identified using LIMMA, as before (Lorrain *et al.*, 2009). We used a statistical model in which the four conditions were included as factors and then extracted the comparisons of interest as contrasts: (i) *pif4pif5* double mutant versus the wild type in high R/F; (ii) *pif4pif5* double mutant versus the wild type in low R/FR; and (iii) interaction between high and low R/FR factor and mutant/wild-type factor. *P*-values from each comparison were adjusted separately for multiple testing with the Benjamini–Hochberg method to control the FDR. Genes depicted as a heat map were mean centered and analyzed by average linkage hierarchical clustering (Cluster 3.0) and subsequently visualized using Java TreeView. GO terms belonging to the GO Biological Process or Interpro database were tested for enrichment using the DAVID knowledge resource. Microarray and ChIP-seq data can be obtained from the Gene Expression Omnibus (GEO) database (GSE35062).

ChIP sequencing

The ChIP experiment was performed as described in (Hornitschek *et al.*, 2009). A detailed description of the ChIP-seq procedure can be found in the supplementary materials and methods.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the on-line version of this article:

Figure S1. PIF5 binds with different frequency to various E-box sequences *in vivo*.

Figure S2. Chromatin Immunoprecipitation (ChIP) of PIF5-HA after 2 h in low R/FR.

Table S1. List of genes with a PIF5 peak identified by ChIP sequ in the *PIF5-HA* line transfered for 2 h into low R/FR.

Table S2. Lists of GO analysis.

Table S3. List of genes showing an altered regulation of gene expression in *pif4pif5* when comparing seedlings grown in high versus low R/FR (interaction between the genetic backgrounds and environmental conditions).

Table S4. List of genes with altered expression in *pif4pif5* compared to wild-type seedlings grown in high R/FR.

Table S5. Comparison between the genes we identified as requiring PIF4 and PIF5 for normal expression in high R/FR (Figure 5) and the genes whose expression correlates with growth and requires PIF4 and PIF5 identified by (Nozue *et al.*, 2011).

Table S6. List of primers used in this study.

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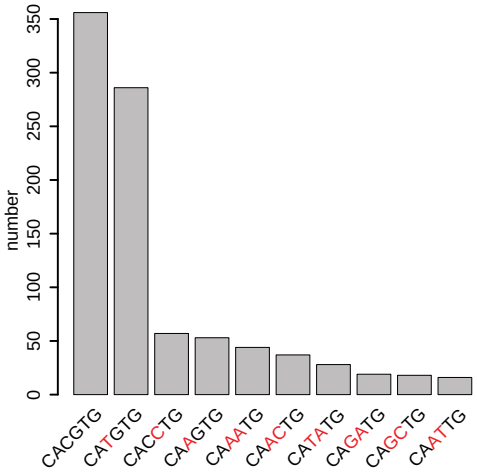
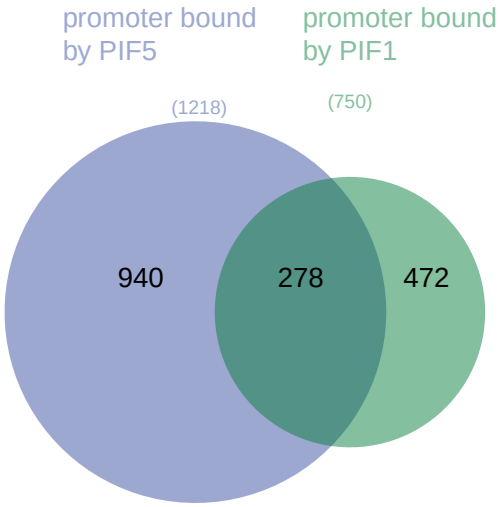


group name	nr.	complementary sequences
CACGTG	356	cacgtg; gtgcac
CATGTG	286	catgtg; gtacac; cacatg; gtgtac
CACCTG	57	cacctg; gtggac; caggtg; gtccac
CAAGTG	53	caagtg; gttcac; cacttg; gtgaac
CAAATG	44	caaatg; gtttac; catttg; gtaaac
CAACTG	37	caactg; gttgac; cagttg; gtcaac
CATATG	28	catatg; gtatac
CAGATG	19	cagatg; gtctac; catctg; gtagac
CAGCTG	18	cagctg; gtcgac
CAATTG	16	caattg; gttaac

Supplementary Figure S1: PIF5 binds with different frequency to various E-box sequences in vivo. PIF5 binding sites that were assigned to a gene locus were chosen. For each binding site the most central E-box was detected. Sequences with two E-boxes with the same distance to the peak summit were discarded from the analysis. E-box sequences were then counted and sense and antisense sequences as well as their reverse complements were grouped.



Supplementary Figure S2: Chromatin Immunoprecipitation (ChIP) of PIF5-HA after 2 hours in low R/FR. Immunoprecipitated DNA was quantified by Q-PCR using primers in the promoter region containing a G-box or control region (minimum 1 kb 3 or 5 from the peak region). Data are average of technical triplicates of the Q-PCR. (A / B) Col and/or PIF5-HA lines were grown for 7 days in constant light conditions before being shifted for 2 hours in low R/FR conditions. (C) PIF5-HA lines were grown for 14 days in constant light conditions before being shifted for 2 hours in low R/FR conditions.

Supplementary material and methods

Cloning procedure and generation of transgenic lines

Primers used in this study are listed in supplementary table 6. Fusions to the maltose-binding proteins (MBP) were generated by PCR. *HFR1, PIF4* and *PIF5* CDS were amplified respectively from the plasmids PH49, pCF402 and pCF404 using the primer pairs pPH153/154 (*HFR1*), pPH149/150 (*PIF4*) pPH151/152 (*PIF5*). Fragments were cloned *NotI/XhoI* into pMAL-c2 TEV V5. The promoter region from *PIL1* and *PIL1** were described previously in (Hornitschek et al., 2009). They were inserted into the pCB308 binary vector using *EcoRI* and *BamHI* sites. To generate the *PIF4pro:PIF4-citrine-3HA* (*PIF4-HA*) line, the *PIF4-3HA* CDS was amplified from the plasmid pCF402 (Lorrain *et al.* 2008) with the primers SL131 and SL135 and digested by *NheI* and *XhoI.* The digestion product was introduced into the pCF300 binary vector with the *BamHI-NheI* digested *PIF4pro* previously described to generate pAM02 (*PIF4pro:PIF4-3HA*). The citrine coding region was amplified by PCR using the primers SL136 and SL137, digested with *SalI* and *XhoI* and introduced into the *SalI*-digested pAM02 vector to generate the pSL90 vector (*PIF4pro:PIF4-citrine-3HA*). This construct was introduced in the *pif4-101* mutant background plants by the *Agrobacterium tumefaciens* dipping procedure.

GUS staining and MUG assay

Seedlings were grown for 7 days in constant light (high R/FR) and then either kept in high R/FR or shifted to low R/FR for the indicated times. For the GUS staining, seedlings were incubated at 37°C for 6 hours within a buffer containing

2mM 5-bromo-4-chloro-3-indolyl β-d-glucuronide (X-Gluc, Duchefa Biochimie BV), 2mM ferrocyanide, 2mM potassium ferricyanide and 50mM sodium phosphate. Stained seedlings were washed with 100% ethanol over night and then rinsed with 70% ethanol. Seedlings were observed and photographed with the stereomicroscope Nikon SMZ 1500. Biological triplicates were performed for each treatment of the MUG assay (4-methylumbelliferyl-beta-D-glucuronide). Seedlings were ground in liquid nitrogen, homogenized on ice in a buffer containing 25mM Tris (pH 7.8), 2mM EDTA, 2mM DTT, 10% glycerol, and 1% Triton X-100, and cleared by centrifugation at 12,000g for 5 min. The extract (25µl) was incubated with 500 µl MUG assay buffer (50mM $NaPO_4$ pH7, 1mM MUG, 10mM EDTA, 10mM β-mercaptoethanol, 0.1% sarkosyl, 0.1% Triton X-100) at 37°C for 2 hours. The reaction was stopped by adding 450µl of 0.2M Na_2CO_3.

ChIP sequencing

The ChIP experiment was performed as described in (Hornitschek *et al.* 2009)). The forward and the reverse primer pairs to amplify the peak and the control region are provided in supplemental S6. For the ChIP-Seq experiment 300 mg of seeds were plated on ½ strength MS. UTH-sequencing of the ChIP samples were performed at the Lausanne Genomics Technologies Facility (GTF) (http://www.unil.ch/cig/page7861_en.html). For ChIP-Seq analysis 145 bp (PIF5-HA ChIP sample) and 166 bp (input DNA control) fragments were used to generate 37 bp or 40 bp reads, respectively. The software Bowtie version 0.12.7 (bowtie -S -n 3 --best --strata --solexa1.3-quals -a -m 1) (Langmead *et al.* 2009) was used to map sequence reads to the Arabidopsis genome (TAIR8;

www.arabidopsis.org). 8.6 million (for the IP sample) and 26.4 million (for the input sample) uniquely mapping reads were selected and sequence read enrichments were identified with Model-based Analysis of ChIP-Seq (MACS) version 1.4.0alpha2 (-p 1e-7 -slocal 500) (Zhang *et al.* 2008). Genome regions identified by MACS were analyzed with Mali Salmon's PeakSplitter software (version 0.1; -v 0.21; -c 20) to determine several peaks per sequence. Sequences covered by peaks were defined as 200 bp centered to the summit positions reported by MACS or PeakSplitter. Putative direct target genes were identified using a perl script from Vivian Praz (University of Lausanne), which compares the center of peaks with gene annotations. Peaks were assigned to genes if they located 3000 bp upstream, 500 bp downstream or within an annotated region. If several genes per peak fulfill the criteria, only the immediate neighboring genes up and downstream were assigned. Subsequent analyses were performed with R (version 2.12.2).

Reference

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Complementary results

One aim of this project was to identify direct target genes of PIF5 on a genome wide scale. To this end we combined PIF5 chromatin binding sites (peaks) in low R/FR condition with microarray derived expression data of Col-0 and *pif4pif5* double mutants. In total I identified 1103 PIF5-HA chromatin binding sites. While translational start sites are defined with high confidence, regulatory upstream DNA sequences of genes (promoter) are still poorly defined on a genome wide scale. Therefore all distances between peaks and genes were expressed relative to the translational start site. As expected, a large majority was located within non-coding sequences with a clear bias towards translational start sites (publ. figure 1b, figure 2.1a). Almost half of all identified peaks (43.79 %) locate within the proximal 1000 bp upstream of CDS and only 14.36 % are more than 3000 bp apart from any annotation. Nevertheless, no common distance could be observed between PIF5 peaks and proximal genes.

PIF5 binds to G-box motifs, which occur relatively even distributed over the different chromosomes of *Arabidopsis thaliana* taken the overall chromosome length into account (1x per 4400 - 4800 bp; figure 2.1b). Peaks in proximity to CDS were similar distributed over chromosomes (figure 2.1c).

Sharp read enrichments at different genome locations detected in the PIF5-HA ChIPseq data set are interpreted at PIF5 chromatin binding sites. In the easiest case those signals originate from direct binding to DNA given that PIF5 is a bHLH transcription factor. Nevertheless, false positive signals might occur due to overexpression or indirect DNA binding through additional proteins. Under the assumption that PIF5 binds directly to DNA sequences, we expected an enrichment of G-box motifs in proximity to PIF5 chromatin binding sites. Indeed, more than 50 % of the peaks cover a G-box (publ. figure 1c) which is enriched towards the peak summit (publ. figure 1d). Other hexameric sequences such as poly(A) or poly(T) are depleted towards the peak summits. This was not observed for poly(C) or poly(G) most likely due to the base pair composition of promoter sequences, which have a reduced GC content (figure 2.2b). Among the remaining peak sequences, which do not contain a G-box, almost all contain at least one E-box variant (-CAnnTG-; publ. figure 1c). Also E-boxes are enriched toward PIF5 binding sites (figure 2.2a) providing the possibility that PIF5 might also bind to E-boxes. When E-box motifs were summarized irrespective to orientation and strand the E-box motif (-CATGTG-) had a strong enrichment (publ. Supplementary Figure S1). This motif was also enriched in several PIF high throughput datasets and later renamed in PIF binding E-box (PBE; Zhang

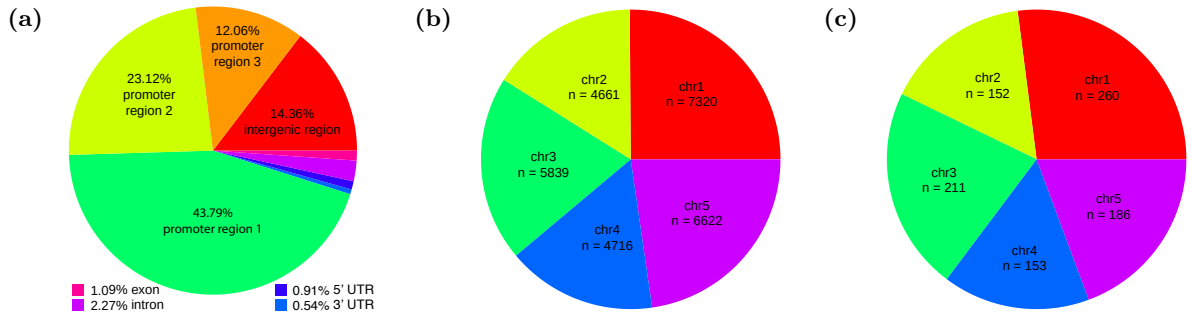

Figure 2.1: Distribution of PIF5 bindings sites and G-boxes in the genome
(a) Relative distribution of PIF5-HA chromatin binding sites over annotated regions. The immediate 3000 bp upstream of transcriptional start sites were divided into region 1 - 3 with region 1 presenting the proximal 1000 bp and region 3 the most distant 1000 bp. (b) Distribution of G-box motifs (-cacgtg-) within whole chromosomes. (c) Distribution of PIF5 chromatin binding sites proximal to CDS over chromosomes.

et al., 2013). A PIF5 DNA binding preference for the PBE-box was not detected in our in-vitro protein-binding microarray (PBM) data (publ. figure 3c). A possible explanation is the requirement of additional proteins not present in the in-vitro experiment, which bind to PIF5 and promote PIF5 DNA binding. This unknown protein might be incorporated into the same transcriptional complex, but could also be a different PIF protein, which forms heterodimers with PIF5 and thereby modify binding preferences. A PIF candidate for forming heterodimers with PIF5 is PIF4, since both PIFs regulated similar responses and PIF4 had in addition to the G-box motif strong binding preferences for several E-box motifs including the PBE-box in vitro (publ. figure 3c).

The PBM data also revealed that flanking base pairs influence PIF5 DNA binding. PIF5 has a higher DNA binding activity, when G-box motifs are flanked by S nucleotide (S = strand; G or C) of both sides (publ. figure 3b). This in-vitro binding preference was not observed for the most proximal G-box motifs to PIF5 binding sites (figure 2.2c). This discrepancy might be due to differences in the chemical environment or availability of additional proteins.

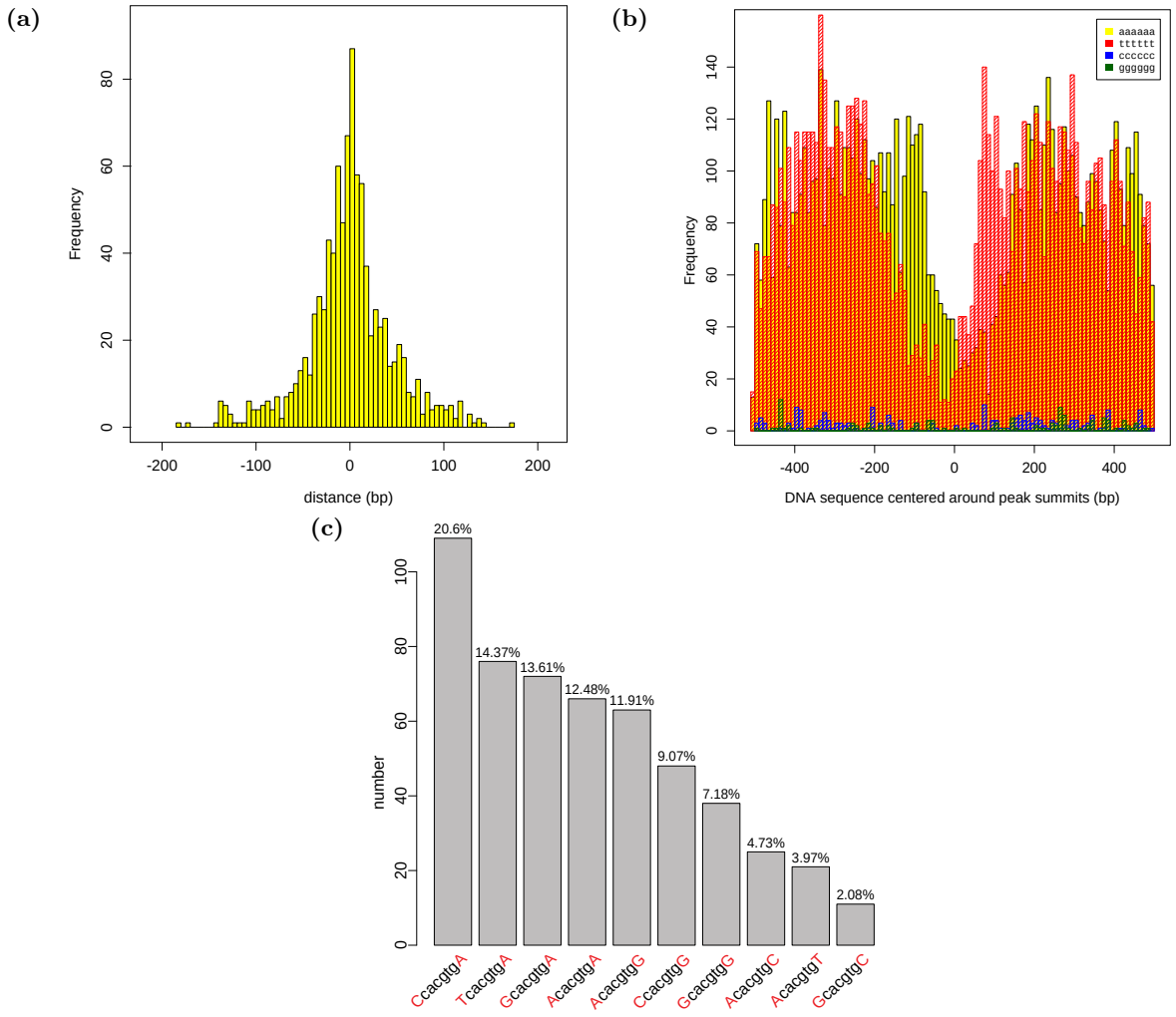

Figure 2.2: Motif distributions in proximity to PIF5 chromatin binding sites.
(a) Distribution of the most proximal E-box motif (-CAnnTG-) to the PIF5 peak summit. (b) Distribution of hexameric homo-nucleotides around the PIF5 binding site. (c) Number of flanking nucleotides of G-boxes located most proximal to PIF5 binding sites.

2.1 Characterization of CKX5 during shade avoidance

2.1.1 Introduction

Shoot branching is mediated by important processes through which plants adapt their morphology to environmental conditions. They depend on various external factors such as light conditions, and directly affect fitness and reproducibility. A plastic branching control allows plants to channel recourses into the main axis or recover after damage. A reduction in the R/FR ratio as experienced by plants in dense populations promote the outgrowth of the main stem and repress the development of lateral branches, while plant of the same species may produce a more bushy phenotype in direct sunlight (Cline, 1997; Lortie and Aarssen, 2000; Bonser and Aarssen, 2003; Finlayson *et al.*, 2010; Su *et al.*, 2011).

The development of new branches is also affected by the plant morphology and depends for instance on the number of already existing branches. Reduction in growth of new branches by signals from remote part of the plant is called correlative inhibition. This includes effects of additional lateral branches and the main shoot. Developmental control of the main shoot over lateral buds is more specifically referred to as apical dominance. The strength of the apical dominance varies between species or different branches of the same organism and can be developmentally dependent or last throughout the entire life cycle (Cline, 1997; Leyser, 2009; Thomas and Hay, 2011).

In *Arabidopsis thaliana* shoot branching occurs at the level of inflorescences and can be divided into two types. Lateral branching describes the outgrowth of rosette buds located in the axil between rosette leaves and the main shoot and secondary or auxiliary branching refers to the development of higher order branches on top of lateral branches or the main shoot. Following the outgrowth of the main shoot, bud activation occurs first at the highest cauline positions and proceeds in a basipetal wave until also lateral buds get activated. While in Col-0 all cauline buds produce branches only a fraction of lateral buds are activated (Hempel and Feldman, 1994; Finlayson *et al.*, 2010; Leyser, 2009; Reddy *et al.*, 2013).

Branching is controlled by a not well-understood regulatory network, which includes the interplay between three phytohormones auxin, cytokinin and strigolactone. Classical decapitation or chemical approaches demonstrated the importance of shoot apex derived auxin for the imposition of auxiliary bud dormancy (stage II). At this stage high cytokinin levels promote the outgrowth of buds as shown by chemical treatments (Wickson and Thimann, 1958; Faiss *et al.*, 1997; Chatfield *et al.*, 2000) and decapitation and thereby removal of shoot apex derived auxin which promote

bud outgrowth lead to upregulation of cytokinin biosynthetic genes (Tanaka *et al.*, 2006). Branch elongation (stage IV) depends on lateral branch derived auxin (Cline, 1997). Cytokinins are adenine derivatives, which are mainly synthesized in the root, but also evidence for local production in the L1 layer of the shoot apical meristem (SAM) has been reported (Kurakawa *et al.*, 2007; Chickarmane *et al.*, 2012). Analysis of mutants which alter endogenous cytokinin levels or cytokinin perception demonstrated a positive role of cytokinin on cell proliferation in the SAM (Werner *et al.*, 2003; Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Miyawaki *et al.*, 2006).

Under shade conditions *Arabidopsis thaliana* exhibits altered branching responses. This includes a reduced diameter of the main shoot, a reduced number of primary rosette branches, enhanced length of the main shoot, faster development of the highest rosette branch (branch n; branch developed from the morphologically highest bud at the main axis) and reduced length of the third highest rosette branch (branch n-2) (Finlayson *et al.*, 2010; Su *et al.*, 2011; Reddy *et al.*, 2013).

In low R/FR conditions bud outgrowth and elongation response of the main shoot and branch n and n-2 are phytochrome dependent (Finlayson *et al.*, 2010; González-Grandío *et al.*, 2013). Low R/FR regulates transcription levels of two member of the cytokinin oxidases/dehydrogenases (CKX) family, which catalyze cytokinin breakdown. CKX6 is involved in leaf development through modulation of cell division under low R/FR conditions (Carabelli *et al.*, 2007). *CKX5* transcription levels are unregulated under low R/FR in a *PIF4 PIF5* dependent manner (Sessa *et al.*, 2005; Hornitschek *et al.*, 2012; Leivar *et al.*, 2012; Nomoto *et al.*, 2012).

ckx single mutants have no striking phenotype under white light conditions (Bartrina *et al.*, 2011). Interestingly, several double mutants show increased inflorescence meristem activity, which is strongest in *ckx3ckx5* mutant combination. The corresponding phenotype under white light resembles traits of an opposite phenotype of low R/FR treated wild-type plants. *ckx3ckx5* mutants develop a main inflorescence with a larger diameter and promote seed production whereas wild-type inflorescence of shade grown plants are thinner in diameter (Bartrina *et al.*, 2011). Histochemical staining of pCKX5::GUS lines as well as in situ hybridization revealed tissue specific promoter activity including in auxiliary buds in shoots, after bolting in the rip zone of the axillary meristem and pro cambium of inflorescence stems (Werner *et al.*, 2003; Bartrina *et al.*, 2011). *CKX3* is expressed in the center of inflorescence meristems (Bartrina *et al.*, 2011) and has no reported transcriptional response to low R/FR. Despite the presence of two G-boxes within 3000 bp upstream of the transcriptional start site, no chromatin binding of PIF transcription factors has been identified.

2.1.2 Results

CKX5 was identified in several microarray analyses as shade induced gene downstream of *PIF4* and *PIF5* (Sessa *et al.*, 2005; Hornitschek *et al.*, 2012). It encodes for an oxidase/dehydrogenase involved in cytokinin breakdown and promoter activity has been reported for procambium in inflorescence stems. It is therefore tempting to speculate that shade negatively regulates cambium differentiation and thereby lateral branch elongation by lowering cytokinin levels through a signaling cascade including phyB, PIF TFs and *CKX5*. *CKX3* has no reported transcriptional response to low R/FR.

ckx3ckx5 is impaired in lateral branch elongation under low R/FR conditions

In the framework of a collaboration with Jorge Casals group I generated a *ckx3ckx5* double mutant and Santiago Trupkin and Mercedes Keller subsequently investigated *ckx3ckx5* as well as *pif4pif5pif7* for shade induced shoot branching phenotypes.

To this end they grew Col-0, *ckx3ckx5* and *pif4pif5pif7* under high R/FR conditions and kept or transferred plants to low R/FR conditions at anthesis. With such timing of low R/FR treatment Col-0 is expected to show no lateral branch elongation phenotype in response to the experimental light conditions (Casal, unpublished), whereas low R/FR treatment from earlier developmental stages on result in shade regulated lateral branch elongation (Finlayson *et al.*, 2010). Indeed, *ckx3ckx5* had a shade induced lateral branch elongation phenotype of all measured shoot branches (branch n to n-2; figure 2.3). Col-0 as well as *pif4pif5pif7* showed no alteration in branch elongation. Also the main inflorescence length of all three genotypes were unaffected by low R/FR. The rosette leaf numbers were similar between genotypes. This indicates that the different genotypes had on a morphological level a comparable branching potential, since lateral bud are located in the axil between rosette leaves and stem and their development in *Arabidopsis thaliana* is not affected by shade (Finlayson *et al.*, 2010).

In two subsequent measurements with application of low R/FR from developmentally earlier stages on, Col-0 rosette branches did not have a clear altered elongation response in low R/FR as it was expected and no difference was observed for *pif4pif5pif7* (figure 2.4). The absence of a robust low R/FR induced phenotype in Col-0 makes the interpretation of these results difficult, and is likely due to the interplay of multiple factors on the branching response such as light and nutritional state. Nevertheless, *ckx3ckx5* had a clear reduced growth response of branch n-2.

These branch elongation measurements suggest a negative regulatory function of elongation

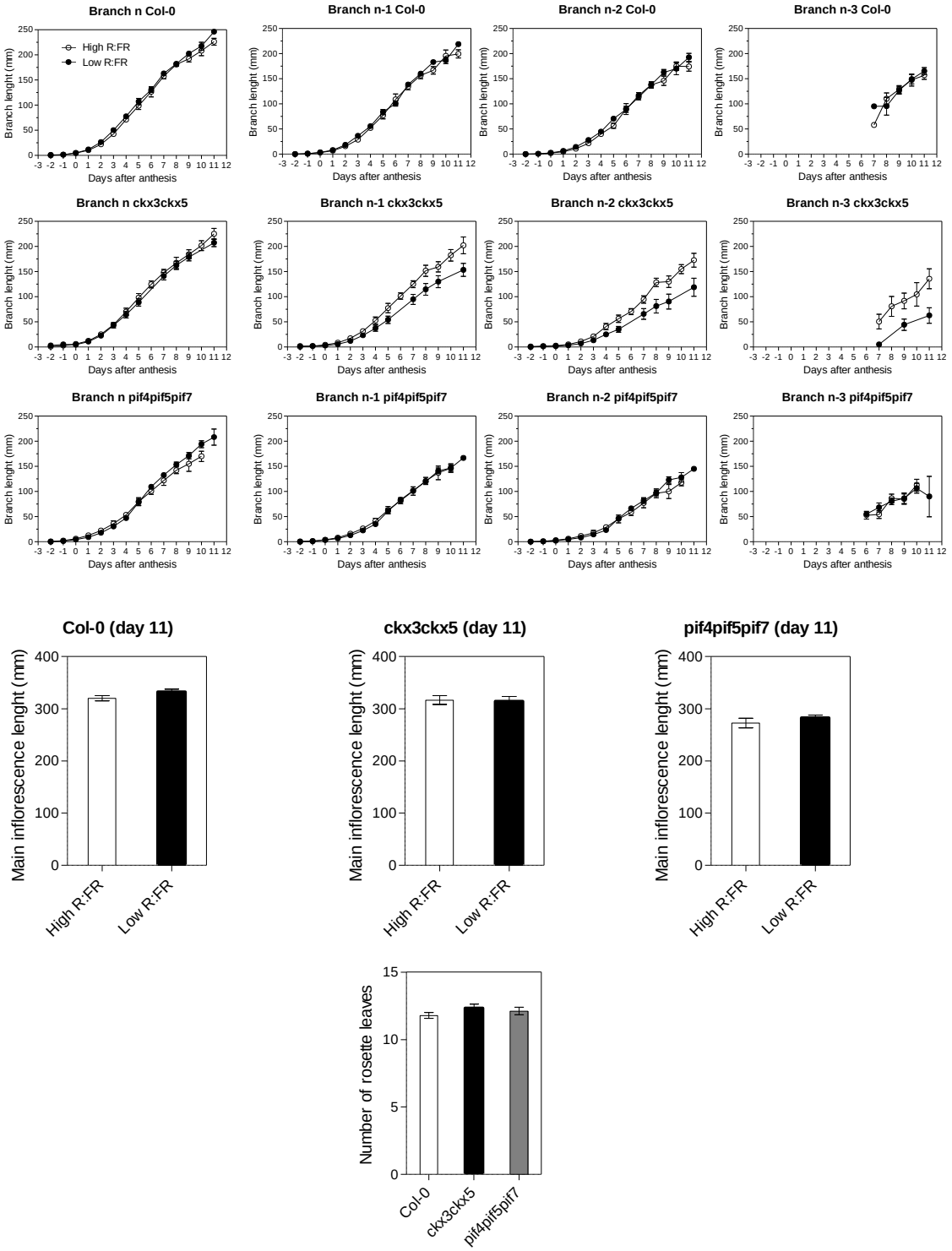

Figure 2.3: Lateral branch length of *ckx3ckx5* and *pif4pif5pif7* and Col-0.
Measurement of lateral branch length under high and low R/FR. Supplemental FR was applied at the onset of anthesis.

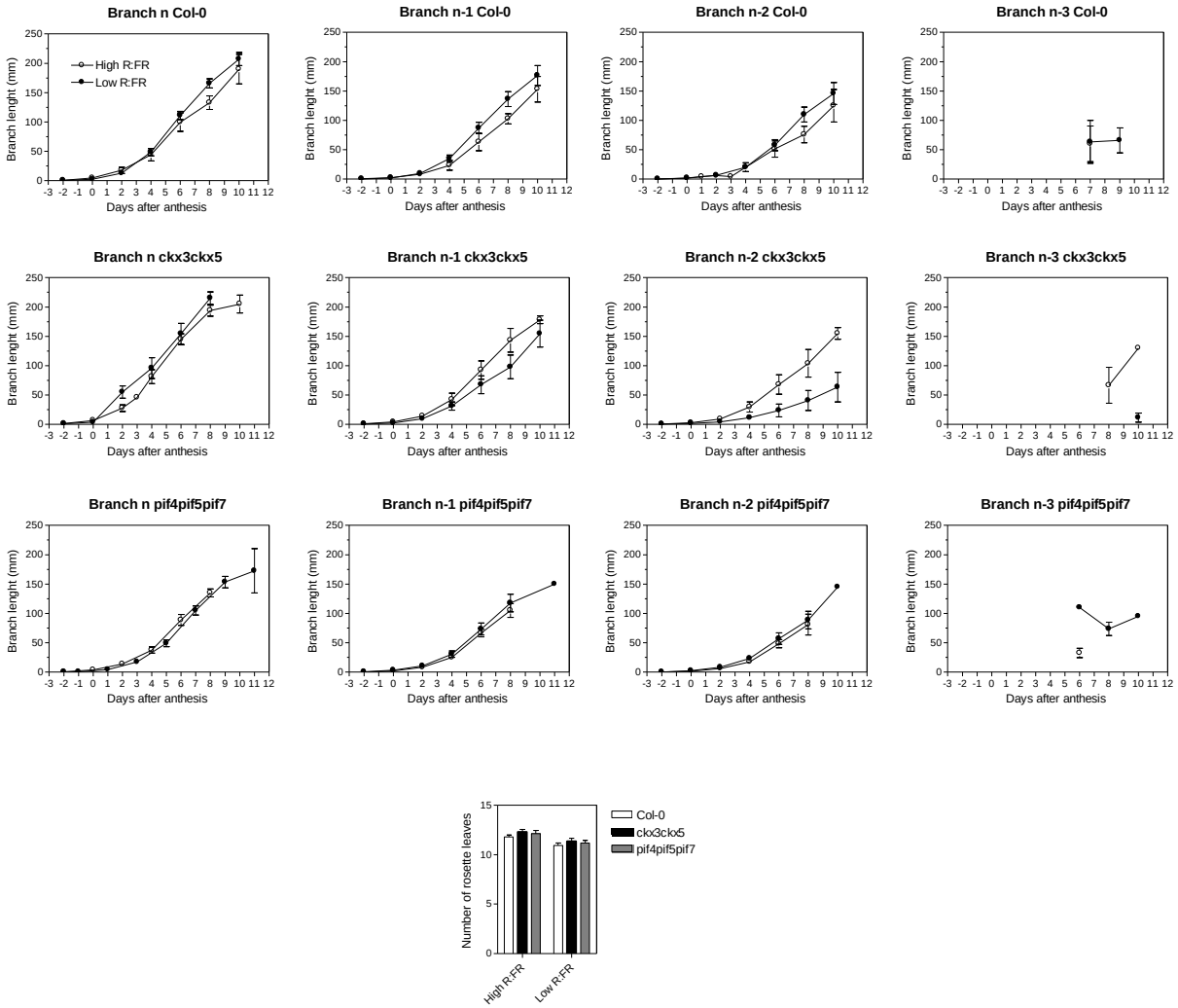

Figure 2.4: Lateral branch length of *ckx3ckx5* and *pif4pif5pif7* and Col-0.
Measurement of lateral branch length under high and low R/FR. Supplemental FR was applied two weeks after germination before the onset of anthesis.

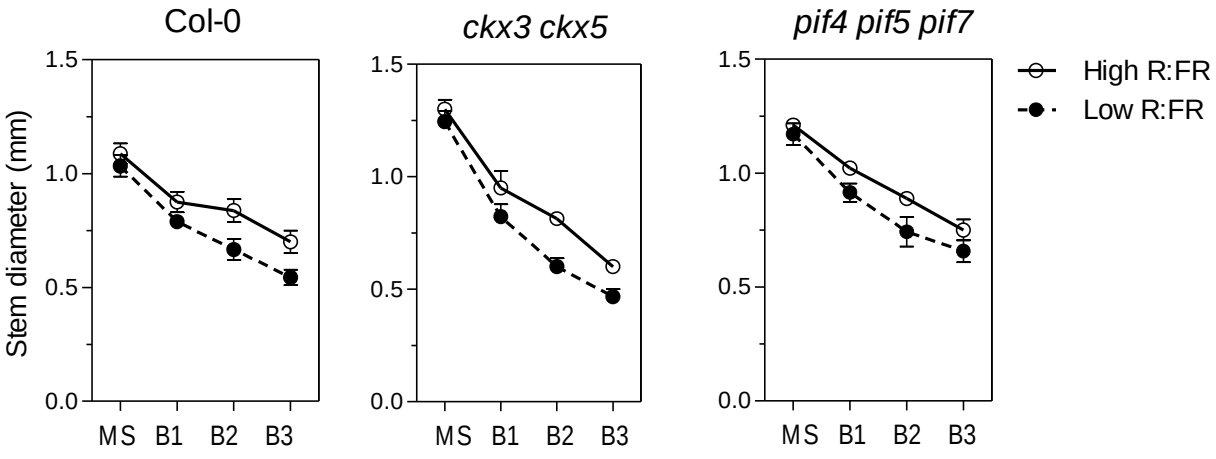

Figure 2.5: Diameter of main shoot and lateral branches
Diameter of the main inflorescence (MS) branch n to n-2 (B1 to B3) of Col-0, *ckx3ckx5* and *pif4pif5* in low and high R/FR.

growth under low R/FR condition for *CKX5* and/or *CKX3* in particular for lateral branches but not the main inflorescence. Furthermore, the observed mutant phenotype seems to be PIF independent.

Col-0 and *ckx3ckx5* show reduced lateral branch diameters Under white light conditions *ckx3ckx5* produces inflorescences with increased diameter than wild-type indicating increased meristematic activity (Bartrina *et al.*, 2011). In order to check if low R/FR also affects the diameter of lateral branches in a *ckx3ckx5* dependent fashion, Mercedes Keller measured cross-sections of main shoot and lateral branches under similar experimental condition than the previously described secondary branch elongation experiments (figure 2.5). Low R/FR had no affect on the diameter of the main inflorescence, but reduced the lateral branch diameters in all genotype. This suggests that CKX3 and CKX5 have no obvious role in regulating the diameter of inflorescences in response to low R/FR signals.

Hypocotyl elongation of *ckx3*, *cxk5* and *ckx3cxk5* in low R/FR *CKX5* was described as transcriptionally induced gene in response to low R/FR treated in seedlings (Sessa *et al.*, 2005; Hornitschek *et al.*, 2012; Leivar *et al.*, 2012; Nomoto *et al.*, 2012). To analyzed if *ckx5* or *ckx3ckx5* T-DNA insertion lines exhibit a low R/FR induced mutant phenotype at a similar developmental state, I measured hypocotyl elongation and cotyledon size of seedlings treated for three days with high R/FR followed by additional four days of high or low R/FR. The hypocotyl elongation response of *ckx* single mutants were similar to wild-type plants. Nevertheless the *ckx3ckx5* double mutant showed a slight but significant longer hypocotyl in low R/FR compared to Col-0 ($p < 8.5 * 10^{-12}$). The long hypocotyl of *ckx3ckx5* could be observed in several but not all repetitions and need therefore further investigation.

Similar to the branching phenotype a longer hypocotyl phenotype in low R/FR would suggest that *ckx3* and/or *ckx5* act as negative regulator of shade avoidance responses.

2.1.3 Discussion

The *ckx3ckx5* double mutant showed altered growth responses in lateral branches as well as hypocotyls in response to shade. Of both genes only *CKX5* has been shown to be regulated by shade. The combination of proximal chromatin binding sites of *PIF4* and *PIF5* upstream of the CDS and transcriptional regulation pattern in Col-0, *pif4pif5* and *pif1pif3pif4pif5* suggest a direct regulation in seedlings (Hornitschek *et al.*, 2012; Leivar *et al.*, 2012; Oh *et al.*, 2012).

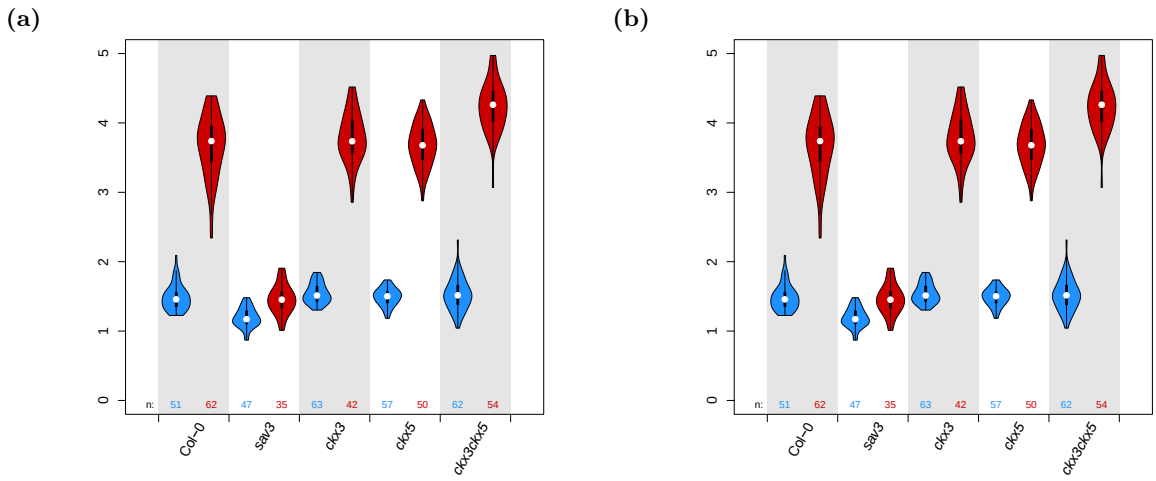

Figure 2.6: Hypocotyl length and cotyledon area measurement in high and low R/FR
Violin plot showing hypocotyl length (a) or cotyledon size (b) of Col-0, *ckx5*, *ckx3*, *ckx3ckx5* and *sav3* seedings grown for 3 days in high R/FR and subsequent 4 days in high or low R/FR.

The wild-type-like growth response of lateral branches of pif4pif5pif7 however, suggests a PIF independent effect. *CKX3* is transcriptionally expressed in the center of floral meristems and *CKX5* transcripts can be found in procambial tissue. *ckx3ckx5* has elevated cytokinin levels (Bartrina *et al.*, 2011). Auxin, which is induced during low R/FR treatment and essential for elongation growth, mutually interact with cytokinin and thereby present a possible link between cytokinin metabolism and elongation growth. Auxin negatively regulates cytokinin levels through repression of the cytokinin biosynthesis genes *IPT* or *CYP735A*. Nordström *et al.* (2004) report no detectable effect on auxin concentration in inducible *IPT* lines, whereas Jones *et al.* (2010) detected higher auxin levels in the shoot apex, young leaves and roots upon cytokinin application. Furthermore, in different studies auxin has a positive effect on *CKX1* and *CKX6* transcription (El-Showk *et al.*, 2013) and *CKX5* can be transcriptionally induced by picloram in hypocotyls within 120 min (Chapman *et al.*, 2012). In turn cytokinin regulates auxin activity at the biosynthesis level through up- or downregulation of different *YUCCA* genes, auxin conjugation through regulation of *GH3.9* and *GH3.17* and auxin signaling regulation various *Aux/IAAs* (Jones *et al.*, 2010; Brenner *et al.*, 2012).

Cytokinin also affects auxin transport in the shoot. Cytokinin reduces transcription levels of several *PIN* auxin efflux carrier including *PIN3*, *PIN4* and *PIN7* (Laplaze *et al.*, 2007), which are the central family members during shade avoidance for hypocotyl elongation (figure 3.26a). Furthermore, *PIN3* and *PIN4* GFP fusion reporter are posttranscriptionally downregulated by cytokinin in cytokinin hypersensitive *arr* octuple mutants (Zhang *et al.*, 2011). Bud outgrowth and corresponding transcription pattern of shade acclimated Col-0 change in response to high

R/FR in a positional fashion (Reddy *et al.*, 2013). It is therefore tempting to speculate that *CKX* expression affects auxin transport by regulating *PINs*. This regulation might occur in a lateral branch dependent manner due to effects of correlative inhibition. Correlative inhibition might also be affected by auxin level in the main shoot. Further insight of auxin concentration within main shoot and different lateral branches are needed to understand to what extend *ckx3ckx5* mutants affects auxin levels. Initial insight could be gained by means of auxin signaling reporter analysis in wild-type and *ckx3ckx5* background.

Cytokinin is an important regulator of vascular development, promoting procambial formation and differentiation (Jouannet *et al.*, 2015). The cytokinin biosynthesis quadruple mutant *ipt1357* fails to develop vascular cambium and can be rescued by cytokinin application (Matsumoto-Kitano *et al.*, 2008). In provascular tissue in roots, auxin and cytokinin have distinct domains of high or low signaling activity, which is required for normal vascular development (Bishopp *et al.*, 2011). Growth rates of *ckx3ckx5* might be altered due to different domain sizes of high auxin or cytokinin signaling and subsequent vascular differentiation. Low R/FR lead to elevated auxin level and thereby potentially alters the auxin to cytokinin ratio in shoots. This ratio might change to different extends dependent on tissue types and subsequently induce different physiological responses such as induced growth of lateral branches and reduced growth of hypocotyls in wild type seedling.

Local exogenous cytokinin application to buds is sufficient to break dormancy (Cline, 1997). Therefore it would be interesting to determine if the *ckx3ckx5* mutant has an altered number of outgrowing branches in response to low R/FR.

2.2 Analysis of DNA motif elements proximal to PIF5 chromatin bindings sites

2.2.1 Introduction

Phytochrome interaction factors (PIFs) form together with 8 additional PIF-like (PIL) proteins a distinct subgroup within the phylogenetic tree of are basic helix-loop-helix transcription factors (Heim *et al.*, 2003). All PIFs are involved in light regulated biological processes (Jeong and Choi, 2013) and bind to phytochromes (Ni *et al.,* 1999; Huq *et al.,* 2002; Huq *et al.,* 2004; Khanna *et al.,* 2004; Oh *et al.,* 2004; Leivar *et al.,* 2008; Quail unpublished). Where examined PIFs show DNA binding affinity to G-boxes in vitro (Martínez-García *et al.*, 2000; Huq and Quail, 2002; Shin *et al.*, 2007; Leivar *et al.*, 2008; de Lucas *et al.*, 2008; Moon *et al.*, 2008; Oh *et al.*, 2007, 2009; Kidokoro *et al.*, 2009; Hornitschek *et al.*, 2009, 2012) and have strong enrichments of G-boxes and PBE-boxes in close proximity of genome wide chromatin binding sites (Oh *et al.*, 2009, 2012; Hornitschek *et al.*, 2012; Zhang *et al.*, 2013).

Despite these common attributes, several features are shared by only a subset of PIFs, others are predominantly linked or are unique to a single PIFs. For example, only PIF3 has been shown to regulate hypocotyl elongation in response to ethylene perception (Zhong *et al.*, 2012) and PIF4 is the major regulator of hypocotyl elongation and early flowering in response to high temperatures (Franklin *et al.*, 2011; Koini *et al.*, 2009; Kumar *et al.*, 2012). PIF1 and PIF6 are positive regulators of seed dormancy (Oh *et al.*, 2004; Penfield *et al.*, 2010). PIF1 and PIF3 promote negative hypocotyl gravitropism (Shin *et al.*, 2009) and repress chloroplast development in dark (Stephenson *et al.*, 2009). PIF4 and PIF7 promote cold acclimation in short day condition (Lee and Thomashow, 2012) and PIF4, PIF5 and PIF7 are the major regulators of shade avoidance responses (Lorrain *et al.*, 2008; Li *et al.*, 2012).

Various *PIF* are expressed at different levels in different organs dependent on light availability. Based on microarray data of six days old seedlings, *PIF3* is in cotyledons the dominantly transcribed PIF in darkness, whereas *PIF5* shows the highest *PIF* expression levels in white light. In hypocotyls *PIF1*, *PIF3* and *PIF6* have more or less similar transcript level, whereas in white light *PIF3* and *PIF5* are higher expressed compared to *PIF1*, *PIF4* and *PIF6* (Ma *et al.*, 2005). Furthermore, the transcript levels of *PIF4* and *PIF7* depend on the photoperiod and are higher during the morning in long-day compared to short-day (Lee and Thomashow, 2012).

PIFs have different intrinsic properties. Only PIF1 and PIF3 bind with different strength to

phyA *in vitro* (Al-Sady *et al.*, 2006; Shen *et al.*, 2008) and phyB-PIF affinity also varies between PIF members (Huq and Quail, 2002; Huq *et al.*, 2004). Also, the DNA binding affinity varies for different motifs to different extends between PIFs. PIF4 show higher binding activity to the G-box sequence *in vitro* compared to PIF5 and show mildly reduced affinities to several hexametric sequences, which differ only in one position to the G-box motif. In contrast PIF5 binds selectively to the G-box sequence. *In vitro* binding capacity of PIF5 is furthermore strongly affected by the presence of at least one weak base directly flanking the G-box while PIF4 DNA binding capacity is unaffected (Hornitschek *et al.*, 2012).

Genome wide PIF binding to chromatin has been investigated by means of ChIP-chip or ChIPseq for PIF1, PIF3, PIF4 and PIF5 and genes in proximity to chromatin binding sites were isolated (Oh *et al.*, 2009, 2012; Hornitschek *et al.*, 2012; Zhang *et al.*, 2013). The putative direct target gene sets largely overlap, demonstrating that a large amount of targets are presumably regulated by several PIFs in similar or different biological contexts. Furthermore, these data sets also present candidate genes, which are transcriptionally regulated by single PIFs. It has to be pointed out, that those differences between the genes list reflect a combination of several factor such as growth conditions, age of seedlings, composition and duration of different light quality as well as different applied analytical methods and stringency criteria. However, more than 450 genes are putative direct target genes of at least 3 PIFs (Oh *et al.*, 2009, 2012; Hornitschek *et al.*, 2012; Zhang *et al.*, 2013; Jeong and Choi, 2013). When entire lists were compare with expression levels, not all genes showed altered relative transcript level between light treatment and control conditions. While false positive chromatin binding cannot be excluded, it is likely that several of those genes are only regulated in certain conditions or in a developmental or tissue dependent fashion. Such conditional, developmental and spatio-temporal regulation might be mediated by the availability of combinations of several transcriptional regulators. The variety of recruited DNA-binding proteins is determined by a suit of regulatory DNA sequences upstream of transcriptional start sites. The identification of conserved DNA sequences within promoter regions of coregulated transcripts allows the identification of regulatory motifs without prior knowledge. Such approaches has been successfully used in the past (Harmer, 2000; Michael and McClung, 2003) and led for instance to the identification of Sequences Over-Represented in Light-Induced Promoter (SORLIP) motifs upstream of phyA regulated genes during far-red induce de-etiolation (Hudson and Quail, 2003).

While PIF proteins mainly bind to G-boxes the composition of additional DNA motifs in proximity to PIF DNA binding sites might differ between direct target genes of various PIFs.

Those differences would contribute to different transcriptional profiles of various PIFs, which eventually result in different magnitudes or distinct physiological responses.

In this project, promoter sequences in proximity to PIF5 chromatin binding site upstream of PIF4 PIF5 dependent low R/FR regulated genes were analyzed in order to identify enriched DNA motifs. Identified enriched elements can either help to identify shared target genes of the shade signaling network with different regulatory pathway, which may or may not are functionally related or identify possible DNA binding proteins, which are part of the *PIF5* transcriptional complex. This will broaden our understanding of biologically relevant *PIF5* binding sites, which mediated transcriptional responses to low R/FR.

2.2.2 Results

Previously we generated a stringent list of *PIF5* direct target genes by extracting genes with altered transcriptional responses dependent on both, low R/FR and mutation of *pif4pif5*, which had a proximal *PIF5* chromatin binding site in the vicinity of their CDS (Hornitschek *et al.,* 2012: STab. 1). In total 29 genes passed all selection criteria and therefore share features of a regulon (common regulated response) as well as a stimulon (response to common environmental stimulus) and are suited for a motif analysis.

In order to extract a conservative set of DNA sequences, which coincides with *PIF5* binding sites, I restricted the analysis to 50 bp up- or downstream of peak summits (total length: 101 bp), which were located in non-coding sequences within 3000 bp upstream of selected genes. I also included intergenic sequences of similar size further upstream of *ATHB-2*, which contain additional *PIF5* binding sites (Kunihiro *et al.*, 2011). In total 37 DNA non-overlapping sequences were subsequently analyzed located upstream of 29 CDS genes.

I performed a hexanucleotide analysis as described in van Helden *et al.* (1998). All possible hexanucleotides were extracted with a sliding window approach from the 37 DNA sequences (sample) as well as from genome wide intergenic regions (background). Using simple binomial formulas the oligonucleotide frequencies were compared between the sample DNA set and the background. The probability to observe a given oligonucleotide n or more time, was finally used to derive a oligonucleotide length and input sequence number independent coefficient of significance (sig). This coefficient allows selecting for unexpectedly over-represented oligonucleotides. Following the assumption that DNA motifs of protein binding sites have a similar distribution within the input sequences with the G-box motif, which is bound by *PIF5*, false positively enriched oligo sequences can be detected based on a deviating relative cumulative probability distribution (Oh

et al., 2009; Hornitschek *et al.*, 2012). To this end a Kolmogorov-Smirnov (KS) statistic was applied and oligo sequences with significantly different relative distribution compared to G-boxes were excluded from subsequent analysis (figure 2.7).

In total 51 enriched hexametric sequences with sig > 0 were identified. Of those 24 share at least 4 bps with either G-box or PBE motif and were clustered accordingly (table 2.1). The remaining oligonucleotide sequences were used to query motif database Jaspar (Mathelier *et al.*, 2014). Similar sequences to TCP transcription factor binding sites of class I and class II (-GTGGGnCC- and -GTGGnCCC- respectively; Viola *et al.*, 2011; Viola *et al.*, 2012) were detected. TCP transcription factors are important development and growth regulators. *BRC1* and *BRC2*, two major regulator of shoot branching, are TCP transcription factors and are low R/FR dependent regulated (Finlayson *et al.*, 2010; González-Grandío *et al.*, 2013). Additional enriched hexameric sequences were detected, which share sequence similarity with the FBS motif. This motif is bound by PIF4 (Hornitschek *et al.*, 2012) as well as by FHY3 a zinc-finger transcription factor shown to transcriptionally regulate phyA nuclear importer *FHY1* and *FHL* as well as circadian clock genes (Lin *et al.*, 2008; Ouyang *et al.*, 2011). Also the poly GA promoter core element were slightly enriched which serves as binding site for Basic Pentacysteins (BPC).

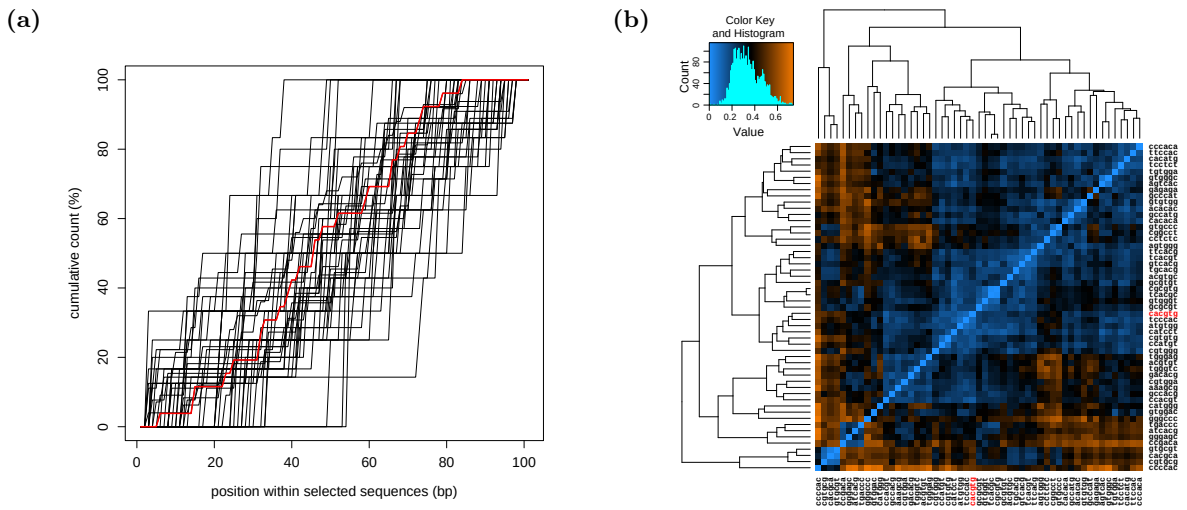

Figure 2.7: Hexanucleotide distribution within sample sequences.
Relative cumulative sum of d various enriched hexanucleotide sequences (a). Values of the G-box motif is highlighted in red. (b) Heat map representation of distribution distance to the G-box distribution derived from two-sample Kolmogorov-Smirnov tests. G-box motif is highlighted in red.

Table 2.1: Computational detection of enriched hexameric sequences. Hexamers were clustered based on sequence similarity. The first row per table section shows the consensus sequence of known promoter motifs with similar sequences to enriched hexanucleotides. Only oligo nucleotides with positive significance coefficient (sig) were cluster. Single oligonucleotides with no related additional sequence with a significance coefficient > 1 are shown in the last section of the table. Underlined bases indicate divination from the related consensus sequence. Sequences similar to TCP binding sites of clade I and II are grouped together. n (s): number of observations in sample sequence set; n (bg): number of observations in background sequence set; s freq: sample frequency; bg freq: background frequency; P(occ{b}≥n): probability to observe oligonucleotide b n or more times

sequence	n (s)	n (bg)	E(occ{b})	s freq	bg freq	P(occ{b}≥n)	sig
CACGTG	**G-box**						
tcacgt	14	14395	0.9393	0.00221	0.000148	0	inf
cgtgtg	13	8792	0.5737	0.002052	0.000091	0	inf
cgtggg	11	4859	0.3171	0.001736	0.00005	0	inf
ccacgt	25	11110	0.7250	0.003946	0.000114	0	inf
cacgtg	26	7800	0.5090	0.004104	0.00008	0	inf
acgtgt	14	17269	1.1269	0.00221	0.000178	0	7.4295
acgtgc	9	5573	0.3637	0.00142	0.000057	0	6.3431
cgtgga	8	8846	0.5772	0.001263	0.000091	0	3.4204
gtcacg	7	6990	0.4561	0.001105	0.000072	0.000001	2.9448
cgtgcg	5	2312	0.1509	0.000789	0.000024	0.000001	2.9233
gccacg	6	5339	0.3484	0.000947	0.000055	0.000002	2.4171
ttcacg	8	13427	0.8761	0.001263	0.000138	0.000004	2.0848
gacacg	6	8167	0.5329	0.000947	0.000084	0.000020	1.3775
gcacgt	5	4973	0.3245	0.000789	0.000051	0.000023	1.3226
atcacg	6	11639	0.7595	0.000947	0.00012	0.000139	0.5377
CATGTG	**PBE**						
catgtg	16	28341	1.8494	0.002525	0.000292	0	6.5035
tgtggg	11	11583	0.7558	0.001736	0.000119	0	5.9258
ccatgt	13	18795	1.2264	0.002052	0.000194	0	5.8237
gccatg	7	8393	0.5477	0.001105	0.000086	0.000002	2.4232
tgtgcg	6	5659	0.3693	0.000947	0.000058	0.000003	2.2731
tgtgtg	11	28027	1.8289	0.001736	0.000289	0.000004	2.1252
atgtgg	9	21707	1.4165	0.00142	0.000224	0.000018	1.4325
tgtgga	8	22054	1.4391	0.001263	0.000227	0.000128	0.5748
tgtgtg	9	31580	2.0607	0.00142	0.000325	0.000294	0.2142
CACGCGC	**FBS motif**						
cacgcg	5	3363	0.2194	0.000789	0.000035	0.000004	2.1343
acgcgc	4	2288	0.1493	0.000631	0.000024	0.000018	1.4180
tcacgc	5	5248	0.3425	0.000789	0.000054	0.000029	1.2121
acgcac	5	4335	0.2829	0.000789	0.000045	0.000012	1.6058
cacgca	5	5860	0.3824	0.000789	0.00006	0.00005	0.9869
GTGG-nCCC	**TCP-like**						
gtgggc	9	9675	0.6313	0.00142	0.0001	0	4.2876
tgggag	9	10821	0.7061	0.00142	0.000111	0	3.8790
agtggg	8	10061	0.6565	0.001263	0.000104	0	3.0036
gtgg-ac	8	11626	0.7586	0.001263	0.00012	0.000001	2.5403
gtgtgg	8	12310	0.8033	0.001263	0.000127	0.000002	2.3588
gggccc	6	6021	0.3929	0.000947	0.000062	0.000004	2.1203

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sequence	n (s)	n (bg)	E(occ{b})	s freq	bg freq	P(occ{b}≥n)	sig
gtgggt	7	10743	0.701019	0.001105	0.000111	0.000009	1.7305
tgggtc	3	1503	0.0981	0.000473	0.000015	0.000146	0.5175
gagag	**BPC**						
gagagg	7	14676	0.957662	0.001105	0.000151	0.000064	0.878328
agagga	8	23849	1.556233	0.001263	0.000246	0.000216	0.347269
gagaga	12	54665	3.567087	0.001894	0.000563	0.00034	0.150591
	unrelated sequences						
ttccac	9	20828	1.359102	0.00142	0.000215	0.000013	1.571942
cggcct	4	2658	0.173444	0.000631	0.000027	0.000033	1.165969
gtgccc	4	2673	0.174423	0.000631	0.000028	0.000034	1.156531

Full length FHY3 and TCP binding elements are composed of 7 or 8 nucleotides, respectively. If those binding motifs coincides with *PIF5* bound G-boxes, both elements are expected to lead to enrichment of heptameric or octameric oligo sequences in a similar analysis for 7mer or 8mer. Enriched TCP and FBS like heptameric sequences are listed in table 2.2. Those enriched sequences can be aligned along the whole sequence of the FBS motif supporting the enrichment. Using the distance of the relative cumulative sum, hexameric sequences aligning with the FBS motif cluster separately the G-box or PEB-box motif suggesting that more gene are shared by *PIF5* and *FHY3* as direct targets than expected by chance but are not necessarily interdependently regulated (figure 2.7b).

In case of the dyad-symmetric TCP motif, heptameric sequences can be preferentially aligned with only one half-site. When clustered by the distance of the relative cumulative distribution hexametric TCP binding site like sequences does not form a cluster (figure 2.7b). This also indicates that enriched sequences are not present for the entire length of the TCP consensus sequence.

2.2.3 Discussion

The motif analysis of immediate proximal DNA sequences of *PIF5* binding sites revealed two clusters of enriched hexamers with similar sequences to the FBS motif bound by FHY3 and PIF4 or TCP transcription factors binding sites.

The distribution of FBS boxes in upstream sequences of the selected genes differs from the G-box distribution and does therefore provide no strong evidence for *FHY3* and *PIF5* function

Table 2.2: Computational detection of enriched heptameric sequences. Heptamers similar to TCP or FHY3 binding sites were clustered based on sequence similarity. The first row per table section shows the consensus sequence of known DNA binding sites with similar sequences to enriched heptanucleotides. Only oligo nucleotides with positive significance coefficient (sig) were cluster. n (s): number of observations in sample sequence set; n (bg): number of observations in background sequence set; s freq: sample frequency; bg freq: background frequency; P(occ{b}≥n): probability to observe oligonucleotide b n or more times

sequence	n (s)	n (bg)	E(occ{b})	s freq	bg freq	P(occ{b}≥n)	sig
GTGGGnCCC	**TCP class I**						
cgtggga	6	1485	0.0960	0.000957	0.000015	-20.740859	5.0882
tgtggac	5	3442	0.2224	0.000797	0.000035	-12.527589	1.5110
tgggccc	4	1720	0.1111	0.000638	0.000018	0.000006	1.3223
tgtgggc	4	1934	0.1250	0.000638	0.00002	-11.622764	1.1234
aagtggg	5	3955	0.2556	0.000797	0.000041	-11.866072	1.2212
agtgggg	4	1847	0.1193	0.000638	0.000019	0.000008	1.2014
ttgtggg	5	4490	0.2901	0.000797	0.000046	-11.266255	0.9581
CACGCGC	**FBS motif**						
cacgcgc	4	1059	0.0684	0.000638	0.000011	0.000001	2.1500
gtcacgc	3	1000	0.0646	0.000478	0.00001	0.000043	0.4550
cgcgccg	2	228	0.0147	0.000319	0.000002	0.000107	0.0554

in the same transcription complex. FHY3 direct taget genes in white light and FR (Ouyang *et al.*, 2011) and PIF5 direct target genes (Hornitschek *et al.*, 2012) have only a poor overlap and do not provide further evidence for combined *FHY3* and *PIF5* target gene regulation via binding to promoter sequences. With the exception of two gene, the gene, which was initially used to select PIF5 binding sites, were also identified as PIF4 direct target genes. The identification of the FBS motif could therefore reflect the transcriptional regulation by PIF4.

Two TCP transcription factors, *BRC1* and *BRC2*, have known roles during shade avoidance. The genome of *Arabidopsis thaliana* encode for 24 TCP genes which fall into three subgroups based on sequence similarity in their DNA binding domains (Martín-Trillo and Cubas, 2010). Clade I or PCF type TCPs bind to -GTGGGnCC- consensus sequence, while CIN and CYC/TB1 type TCPs, which are summarized as TCP-Ps, bind to -GTGGnCCC- sequences (Viola *et al.*, 2012). Among class I TCPs this general rule has been confirmed by Binding Site Selection (SELEX) experiments for *TCP15* and *TCP20*, while different DNA binding preference of *TCP11* and class II DNA binding preference for *TCP16* have been reported (Viola *et al.*, 2011, 2012). Furthermore enriched motifs in proximity to *TCP4* (PCF) chromatin binding sites show variation of the established consensus sequences (Schommer *et al.*, 2008). Rice (Oryza saliva) TCPs also form heterodimers in yeast-2-hybrid assays (Kosugi and Ohashi, 2002). It is possible that heterodimerization in-planta affects DNA binding preferences. Out of 253 genes with a proximal *PIF5* chromatin bindings site which are transcriptionally regulated by low R/FR in cotyledon

or hypocotyl (see chapter II), 22 (8.7 % contain a perfect match of the class 1 TCP consensus sequence and 11 4.35 % a class 2 binding site within 1000 bp upstream of the translational start site. Furthermore, more than 90 promoter sequences contain a class 1 like sequence when with a single base mismatch in position 2-7. Therefore it is tempting to speculate members of the TCP family with that yet uncharacterized DNA binding preferences contain binding affinities with enriched hexameric oligo sequences in this analysis. A divination of octamers from the consensus sequence in one position might also reduce the strength of TCP DNA interaction. Further analysis are needed to assess the importance of TCP transcription factors during shade avoidance.

2.2.4 Materials and Method

DNA sequence selection

In *Arabidopsis thaliana* all *PIF* transcription factors bind to G-box motifs in DNA sequences and *PIF4*, *PIF5* and *PIF7* regulate gene expression in response to low R/FR perception (Leivar *et al.*, 2012; Li *et al.*, 2012). In order to select only promoter sequences form PIF5 regulated genes under low R/FR conditions, genes which depend in their transcriptional response on the R/FR ratio and *PIF4* and/or *PIF5* were matched against *PIF5* chromatin binding sites (Hornitschek *et al.*, 2012). From the retained 29 genes, 101 bp around the reported peak summit (Hornitschek *et al.*, 2012) within 3000 bp upstream of translational start sites were extracted for previously reported *PIF5* chromatin binding sites. In cade of *ATHB-2* additional reported *PIF5* chromatin binding sites further upstream were added. (Hornitschek *et al.*, 2012; Kunihiro *et al.*, 2011). Non-coding sequences have a different base pair composition than coding sequences (van Helden *et al.*, 1998). To take the base pair composition into account all peaks which felt into annotated CDS were excluded from the analysis. In total, 33 non-overlapping sequences were selected.

Enrichment analysis

Statistical analysis was performed as described in van Helden *et al.* (1998). Binomial formulas were implemented in python and motif enrichment analyses were performed between selected sequences centered around *PIF5* chromatin binding sites and genome wide non-coding sequences for oligo sequences of various length.

In the process of the analysis tables were generated which contain for all observed oligo sequences various parameters. First the number of observation of the oligonucleotide sequence b in sample and background were determined and subsequently the background frequency were

used to estimate the expected frequency ($F_e\{b\}$) of randomly selected DNA promoter sequences. The expected number of occurrences depend on the expected frequency, the motif length and the length and number of the investigated sequence and can be computed with the formula

$$E(occ\{b\}) = F_e\{b\} * 2 * \sum_{i=1}^{S}(L_i - w + 1) = F_e\{b\} * T \tag{2.1}$$

when S is the number of sample sequences, L_i the nucleotide length of sequence i and w the motif length. Consequently, the maximum number of possible observations can be summarized as T. The orientation of motifs is not taken into account and can occur on both strands, which is represented by the factor 2.

The probability to observe oligonucleotide exactly n times in the sample sequence set can be estimated with the binomial equation:

$$P(occ\{b\} = n) = \frac{T!}{(T-n)! * n!} * (F_e\{b\})^n * (1 - F_e\{b\})^{(T-n)} \tag{2.2}$$

Finally enrichment oligonucleotide sequences should have a low probability of n as well as more than n observations. Therefore the sum of all probabilities between n and the maximal possible number of observation T need to be assessed.

$$P(occ\{b\} \geq n) = \sum_{j=n}^{T} P(occ\{b\} = j) \tag{2.3}$$

To evaluate enrichment independently of motif length and the number of input sequences van Helden *et al.* (1998) suggest a significance coefficient which is the negative decadic logarithm of $P(occ\{b\} \geq n)$ and the number of possible base permutations within the motif. The number of possible permutations are

$$D = 4^w - {}^{(4^w - n_{pal})}/_2 \tag{2.4}$$

with $n_{pal} = 4^{w/2}$ or $n_{pal} = 0$ for even or odd numbers of w, respectively.

The significance coefficient is than computed with

$$sig = -log_{10}(P(occ\{b\} \geq n) * D) \tag{2.5}$$

Finally, to increase the chance of biologically meaningful enrichment a non-parametric two-sided Kolmogorov-Smirnov test was performed using R. Under the assumption that random sequences follow a different distribution than the *PIF5* bound G-box, all hexamers with a sig-

nificantly different relative cumulative distribution within all sequences of the sample set were excluded ($p < 0.05$).

3 Spatio-temporal transcriptional responses during shade avoidance

Plants have the ability to use the composition of incident light as a cue to adapt development and growth to their environment (Casal, 2013). *Arabidopsis thaliana* as well as many crops are shade intolerant plants. (Kebrom and Brutnell, 2007). When subjected to shade, these plants exhibit a variety of physiological responses collectively called shade avoidance syndrome. Such adaptive responses include increased growth of hypocotyl and petioles and decreased growth of cotyledons (Li *et al.*, 2012; Pierik and de Wit, 2014). Therefore, the single cue, low R/FR, regulates opposite growth responses in different organs.

Shade is perceived by plants as a reduction in the ratio between R and FR by the phytochrome family of photoreceptors. Shade avoidance responses are predominantly mediated by phyB. *PhyB* mutants exhibit a constitutive shade avoidance phenotype including long internodes, small leaves and early flowering (Halliday *et al.*, 1994; Devlin *et al.*, 1996). Phytochrome B is expressed in all organs of young, de-etiolated seedlings. However, histochemical staining of promotor-GUS lines indicate, that phyB has different transcriptional activities in various tissues of cotyledons, varying from a strong expression in vascular tissue to the absences in epidermal cells (Somers and Quail, 1995). Interestingly, phyB mediates several light responses in a tissue-specific manner. In *Arabidopsis thaliana* this includes the repression of seed germination (Lee *et al.*, 2012) and the control of flowering time by mesophyll-localized phyB in leaves (Endo *et al.*, 2005). In addition cotyledon/leaf-localized phyB plays an important role for growth responses to R and FR. Shielding experiments of either cotyledons or hypocotyl in *Cucumis sativus* demonstrated that R perception by cotyledons is required and sufficient to mediated hypocotyl growth inhibition in this species (Black and Shuttleworth, 1974). However, in *Helianthus annuus* no organ-specific FR perception was reported (Garrison and Briggs, 1975) and *Vigna sinensis* epicotyls respond to simulated shade (Garcia-Martinez *et al.*, 1987) showing that the site of R and FR perception vary between species. In *Brassica rapa*, which is a closely related species to *Arabidopsis* (Yang *et al.*,

1999), the major site of shade perception are cotyledons. This was determined by split light chambers and dissected seedling lacking cotyledons (Procko *et al.*, 2014). Also in *Arabidopsis thaliana* cotyledons seems to be the major site of R and FR perception as indicated by different experiments. Enhancer-trap lines expressing *phyB-GFP* in cotyledons of *phyB*-deficient seedlings rescue the elongated hypocotyl phenotype (Endo *et al.*, 2005). Furthermore, spotlight irradiation of cotyledons with supplemental FR induced GUS reporter expression in hypocotyls, which was either dependent or independent of auxin. Furthermore, local FR irradiation of hypocotyls failed to induce reporter gene expression (Tanaka *et al.*, 2002a). This indicates that low R/FR can trigger inter-organ signaling in *Arabidopsis thaliana*. This also raises the possibility that several long distance signal between cotyledons an hypocotyls exist during shade avoidance and that auxin is one of them. Nevertheless, the nature of such signals remains elusive. Consistent with important role of cotyledons in low R/FR, *pinoid (pid) wag1wag2*, which fail to produce cotyledons, does not show hypocotyl elongation in low R/FR (Procko *et al.*, 2014). *PID*, *WAG1* and *WAG2* code for three closely related serine/threonine-protein kinase, which are positive regulator of cellular auxin efflux (Cheng *et al.*, 2008). Therefore, it is difficult to distinguish between defects of low R/FR perception and general growth defects, such as auxin movement or auxin levels (Friml *et al.*, 2004).

Auxin is essential for shade-induced hypocotyl elongation. Increased auxin levels were measured in whole seedlings (Tao *et al.*, 2008; Keuskamp *et al.*, 2010; Li *et al.*, 2012; Hornitschek *et al.*, 2012; Hersch *et al.*, 2014; Bou-Torrent *et al.*, 2014). Several members of the *YUCCA* family, which encode auxin biosynthetic enzymes, are transcriptionally induced by shade. Furthermore, chromatin binding of PIF4 or PIF5 to upstream regulatory sequences of *YUC8*, *YUC3*, *YUC5* and *YUC6*, suggesting a direct link between phyB signaling and auxin biosynthesis (Oh *et al.*, 2009, 2012; Hornitschek *et al.*, 2012; Zhang *et al.*, 2013). Mutants, impaired in auxin biosynthesis, such as *yuc1yuc4*, *yuc3yuc5yuc7yuc8yuc9* and *sav3* (encoded by the *TAA1* gene) show reduced hypocotyl elongation responses in low R/FR. Cotyledons are through to be a main sites of auxin production in young seedlings in white light (Ljung *et al.*, 2001; Casal, 2013). Histochemical staining of transgenic seedling, expressing the translational fusion construct TAA1-GUS under the control of the TAA1 promoter, exhibit strong signals at the shoot apex and the margin of cotyledons (Tao *et al.*, 2008). Therefore, newly synthesized auxin in shade is assumed to be localized mainly in cotyledons and the shoot apex of seedlings. This also implies that auxin need to be downwards transported, out of the cotyledons (figure 3.1). Chemical treatment of seedlings with the auxin transport inhibitor NPA prevent shade induced hypocotyl elongation (Steindler

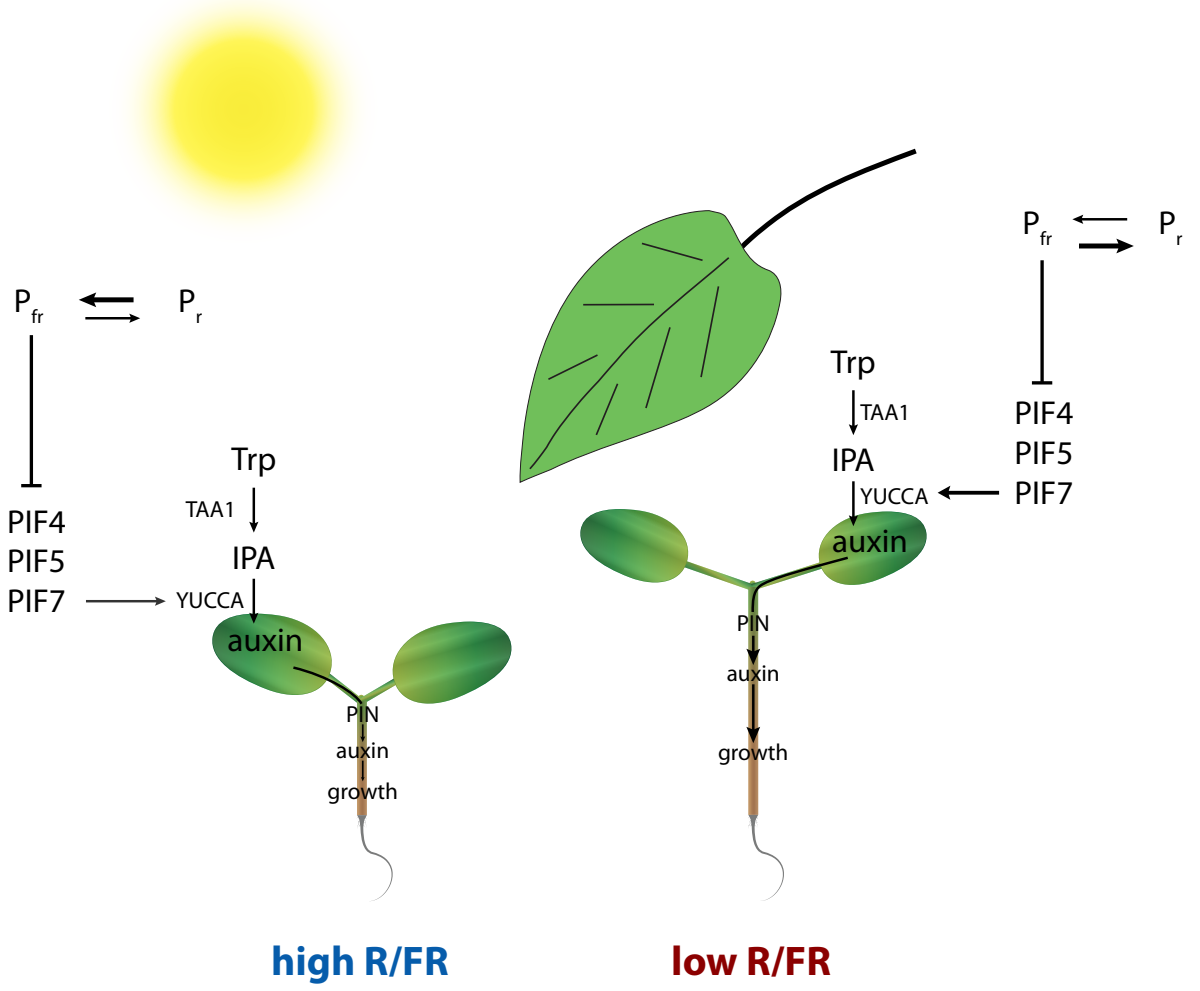

Figure 3.1: Model of shade induced signaling in seedlings
In low R/FR phytochromes induce the expression of *YUCCA* genes by repression of PIF proteins. Increased auxin biosynthesis lead to shade induced auxin production in cotyledons and subsequent basipetal transport to the hypocotyl in a PIN dependent manner.

et al., 1999; Tao *et al.*, 2008; Keuskamp *et al.*, 2010). Furthermore, NPA inhibits shade mediated induction of the auxin signaling marker DR5::GUS in hypocotyls but not in cotyledons, suggesting that auxin transport from cotyledons to hypocotyls is required wild-type gene expression in hypocotyls during shade avoidance (Tao *et al.*, 2008). Mutants of the auxin efflux carrier *pin3* show reduced hypocotyl elongation in response to shade demonstrating the importance of auxin transport (Keuskamp *et al.*, 2010). In a very recent study, free auxin levels were measured in hypocotyl sections in *B. rapa*. These measurements revealed a gradient formation from the top to the base of hypocotyls after 6 h of low R/FR treatment, supporting the notion of shade-induced auxin production in cotyledons followed by basipetal transport through petioles and hypocotyl. Furthermore, auxin responsive genes were identified, which showed correlating transcript and auxin levels (Procko *et al.*, 2014).

The project described in this chapter aims to investigate early shade-induced spatio-temporal

transcriptional responses of auxin biosynthetic and signaling component in cotyledon and hypocotyls on a genome-wide scale. It should be investigated how broad transcription responses occur, which correlate with the predicted increases of auxin through shade-induced de-novo synthesis in cotyledons and subsequent transport to hypocotyls.

Furthermore, different transcriptional responses to low R/FR in cotyledon and hypocotyls should be identified, which contribute to our understanding of the opposite growth response to shade of both organs.

3.1 Results

3.1.1 Phenotypic characterization of Arabidopsis seedlings during the first hours of supplemental far-red light treatment

Altered growth rates of cotyledon and hypocotyl upon low R/FR conditions are well-studied responses in *Arabidopsis thaliana* (Pierik and de Wit, 2014). The extent of changes in growth rate depends on several factors such as photoperiod, light intensity, R/FR ratio and genetic background (Zeevaart, 1971; Wiese *et al.*, 2007; Cole *et al.*, 2011).

In order to define how fast changes in hypocotyl growth rates in response to low R/FR treatment can be phenotypically detected in our experimental setup, wild type seedlings were grown in high light intensity white light and long day (LD) photoperiod (16 h light/ 8 h dark) for five days. On day six, 2 h after dawn, seedlings were either kept in white light or treated with white plus supplemental far-red light to create a low R/FR regime simulating FR reflection of neighboring plants. Images were taken in intervals of 30 min for a total of 16 h and the difference in hypocotyl length between time points was determined (figure 3.2a). Significantly longer hypocotyl length in low R/FR could be observed as early as two h after supplemental far-red light treatment.

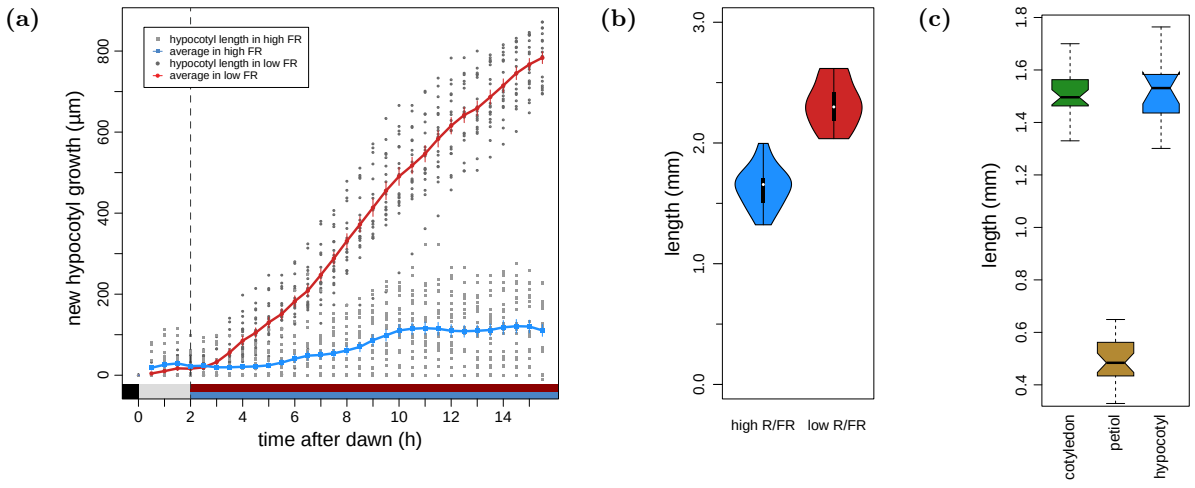

Figure 3.2: Growth measurements of five-days-old seedlings grown in LD.
Seedlings where treated with two hours white light after dawn and subsequently either kept in white light or transferred to supplemental far-red light. (a) New hypocotyl growth between measurements in high (squares) or low (dots) R/FR conditions. Solid lines show the average length per time point smoothed with a sliding window approach. The color bar at the bottom indicate the light treatment: black = night; gray = white light before begin of experiment; red = far-red light treatment; blue = white light treatment; dashed line = begin of different light treatments. (b) Hypocotyl length at ZT15.5 on day six. (c) Organ length measured after 90 min exposure to supplemental far-red light.

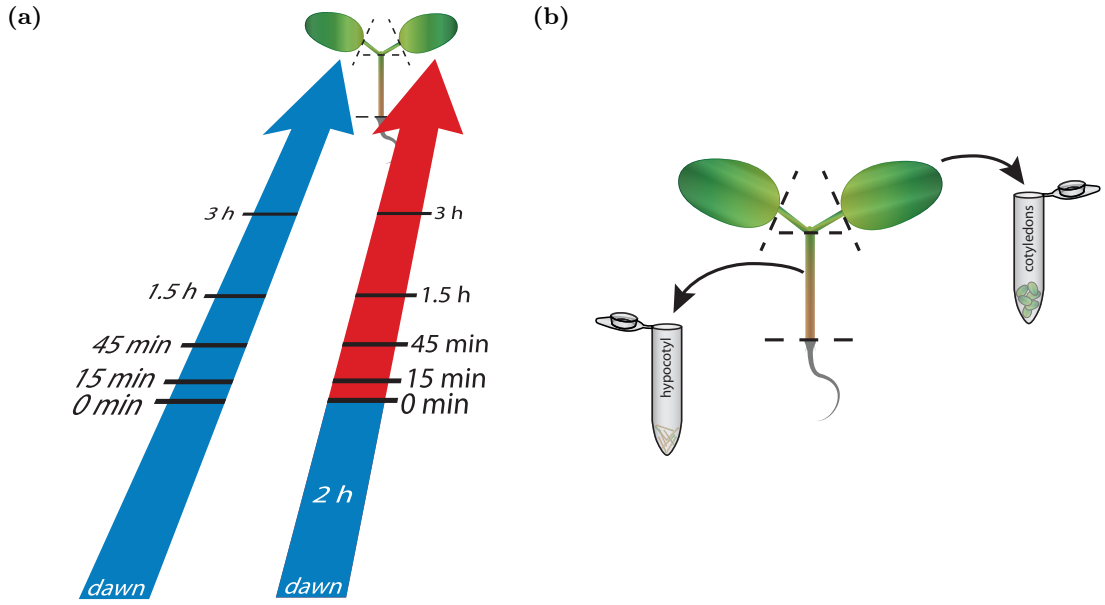

Figure 3.3: Scheme of the experimental setup
(a) After dawn of day six, seedlings were grown for two h in high R/FR (blue) and either kept in white light (blue) or transferred to low R/FR (red) condition. Samples were taken at all indicated time points in both light conditions; (b) Seedlings were dissected as indicated by the dashed lines in order to separately collect cotyledon and hypocotyl material.

3.1.2 Organ specific transcriptional profiling in response to shade

To date a lot of high-throughput experiments investigating shade avoidance responses were performed with whole seedlings at few time points (Oh *et al.*, 2009; Hornitschek *et al.*, 2012; Li *et al.*, 2012; Leivar *et al.*, 2012). However, reporter gene analyses as well as the different growth responses of cotyledons and hypocotyl suggest spatial differences of the transcriptional network in response to shade (Tanaka *et al.*, 2002b; Tao *et al.*, 2008). Size differences of various organs of seedlings suggest that extracted mRNA from whole seedlings is highly enriched for transcripts expressed in cotyledons. Transcriptional responses to low R/FR, which are hypocotyl-specific, might therefore be missing in whole seedling approaches. Furthermore, according to our current model shade-induced auxin production primarily takes place in cotyledons followed by subsequent basipetal transport to hypocotyls (de Wit *et al.*, 2014). Therefore, auxin-dependent transcriptional responses to shade might take place later in hypocotyls than in cotyledons.

To investigate early transcriptional regulation in response to low R/FR in cotyledons and/or hypocotyls I performed a time course experiment with 5-day-old seedlings in high and low R/FR condition starting at zeitgeber 2. I chose five time points (TP) over the course of three hours: 0 minutes (min), 15 min, 45 min, 90 min and 180 min. This design allows monitoring early transcriptional regulation such as *PIL1* transcript levels, which were reported to change within 8 min upon low R/FR treatment (Salter *et al.*, 2003) as well as slower changing transcript levels that

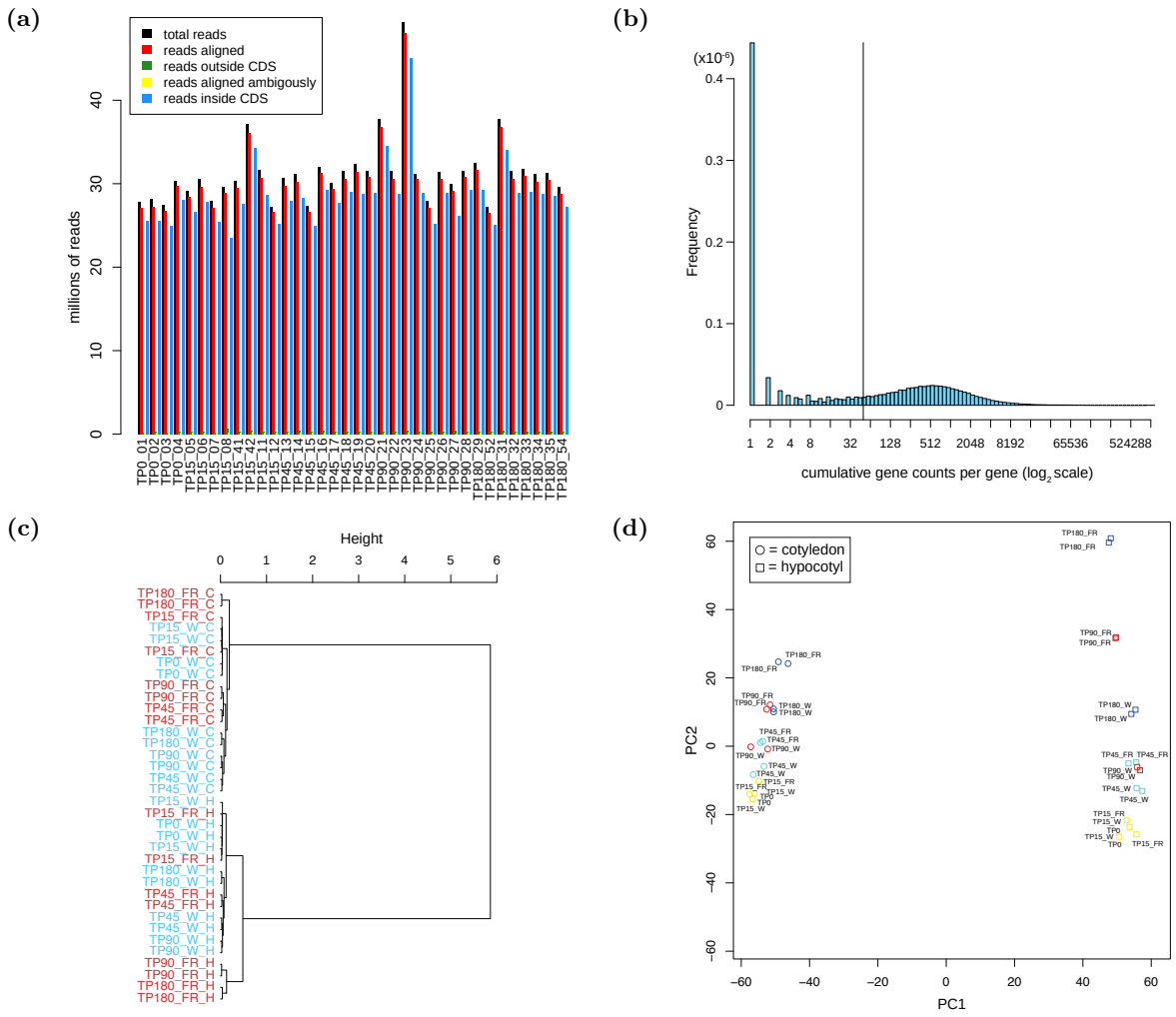

Figure 3.4: Comparison and basic evaluation of RNAseq libraries.
(a) Number of reads mapped to different genomic regions. (b) Histogram of sum of reads in all libraries mapped per gene. The vertical black line represents the cutoff at 50 counts per gene. (c) Dendrogram of clustered libraries and (d) scatterplot of the PCA analysis showing the variation between libraries summarized in the first two components of the top 5000 most variant genes. W = high R/FR; FR = low R/FR; C = cotyledon; H = hypocotyls

are detectable when changes in growth rate can be phenotypically observed in hypocotyls (figure 3.2a). In addition TP45 and TP90 flank the 1 h time point for which the earliest changes in free IAA levels have been measured up to now (Oh *et al.*, 2009; Hornitschek *et al.*, 2012; Li *et al.*, 2012). To ensure the extraction of high quality RNA, which is representative at each time point, seedlings were fixed in 100 % acetone. This method allows subsequent careful dissection of seedlings into single organ without contamination of cotyledon samples with petioles or hypocotyl samples with shoot apical meristems or roots which potentially showing opposite responses. This is important since most available phenotypic data on shade avoidance responses are either from hypocotyls or the blade of cotyledons. Having RNA precisely from those organs is desirable to try to correlate gene expression changes with phenotypic changes in the same organ.

I generated stranded libraries using the Illumina TruSeq protocol, which were subsequently

sequenced on a HighSeq 2500 sequencer by the Genomic Technologies Facility. Sandra Calderon and Sylvain Pradervand performed initial computational and statistical analysis including read mapping against the reference genome. For each library more then $25 * 10^6$ reads were mapped to coding sequences (figure 3.4a). Further analyses were restricted to coding regions with more than 50 counts summed up over all libraries (figure 3.4b). Counts for higher expressed genes approximately follow a normal distribution and can therefore be analyzed with methods well established for microarray analysis. For this data set 20957 coding sequences were selected. Counts per coding region were TMM normalized to improve across sample compatibility and $\log_2$ transformed. Comparisons of expression levels between different conditions were statistically analyzed using the moderated t-statistics implemented in the LIMMA package.

3.1.3 Organ-specific transcription is more variable than shade-specific transcription

Sandra Calderon also performed a principle component analysis of libraries in order to evaluate the variation contained in the data set (figure 3.4c and 3.4d). Figure 3.4d shows principal component 1 and 2 (PC1 and PC2) of the 5'000 genes with the most variable transcript levels. All biological replicates are highly similar. PC1, which summarized the largest variation in the data set, divides the set of libraries into two groups separating cotyledon-derived libraries from hypocotyl-derived libraries. PC2 separates libraries predominantly according to light treatment and along the time of harvesting. The separation between libraries by the light treatments is more dominant for later time points and more pronounced in hypocotyls than cotyledons. The early time points TP0 and TP15 cluster relatively closely together. This temporal component, which can be observed between libraries of high R/FR treated samples, as well as between libraries of low R/FR treated samples, suggests a circadian effect on transcriptional expression patterns in the data set. The ratio by which the temporal and the light factor contribute to PC2 depends on the number of top variable genes that were included in the analysis. For the top 5'000 genes, the light component has a slightly stronger effect on the separation of libraries through PC2 than the temporal component. When analyzing complete libraries the temporal factor contributes much stronger than the light factor to PC2 (not shown). The observation of the temporal component in PC2 demonstrates the importance of white light control samples at each time point to determine proper regulation levels between both light conditions at a given time point. Taken together, the highest variation in this data set is introduced by the different organ types while additional important factors that generated variation between libraries, are

the light treatment and a temporal component.

3.1.4 Organ marker genes show cotyledon- and hypocotyl-dependent expression patterns

The design of the sample preparation from cotyledons and hypocotyls aimed to generate organ specific transcription data without contamination from other parts of the plant. Organ marker genes can therefore provide a readout to evaluate organ identity or possible contamination of the used plant material. Those marker genes need transcriptionally be absent or present in different organs. In the past, different organ marker gene classes were defined for the AtGenExpress data set (Schmid *et al.*, 2005). To see if those organ marker genes have similar absence/presence pattern between cotyledon and hypocotyl libraries, I classified the genes based on summed read counts (figure 3.5a). The two closest related organs to cotyledon and hypocotyl investigated in the AtGenExpress data set are leaves and stems, respectively. A large fraction of leaf and stem marker genes were detected in the RNAseq data set. Except for root marker genes all other classes of organ marker genes showed low expression in cotyledon or hypocotyl sample. ⅔ of the root marker genes could be detected predominantly only in hypocotyls. Many of the root marker genes have additional organ classifications on the TAIR website indicating a possible broader expression pattern. Such types of organ marker genes may thus depend on additional factors, such as developmental stage or various external factors. Genes with experimentally well-defined organ specific expression domains such as the meristem marker genes *WUSCHEL* and *CLAVATA3* have less than five mapped reads when summed over all libraries and are consistently defined as not expressed in the RNAseq data set, demonstrating that the corresponding tissues were excluded during sample preparation.

Marker genes for leaves were all expressed in cotyledon samples. Less than half of them were also detected in hypocotyl libraries. Marker genes for stems of the rosette stage were to similar amounts detected in cotyledons and hypocotyls, respectively. Organ marker gene sets, as well as summed read counts for selected genes, showed expected tendencies in their expression patterns, which suggests the absence of contamination during sample preparation.

A key difference between cotyledons and hypocotyls is the capacity for photosynthetic processes. While cotyledons are the major photosyntheticly active parts of *Arabidopsis thaliana* seedlings, hypocotyls have only a limited number of chloroplasts even after de-etiolation. Therefore, libraries generated from cotyledon material are expected to have enriched read abundance for genes related to photosynthetic processes as compared to libraries prepared from hypocotyl

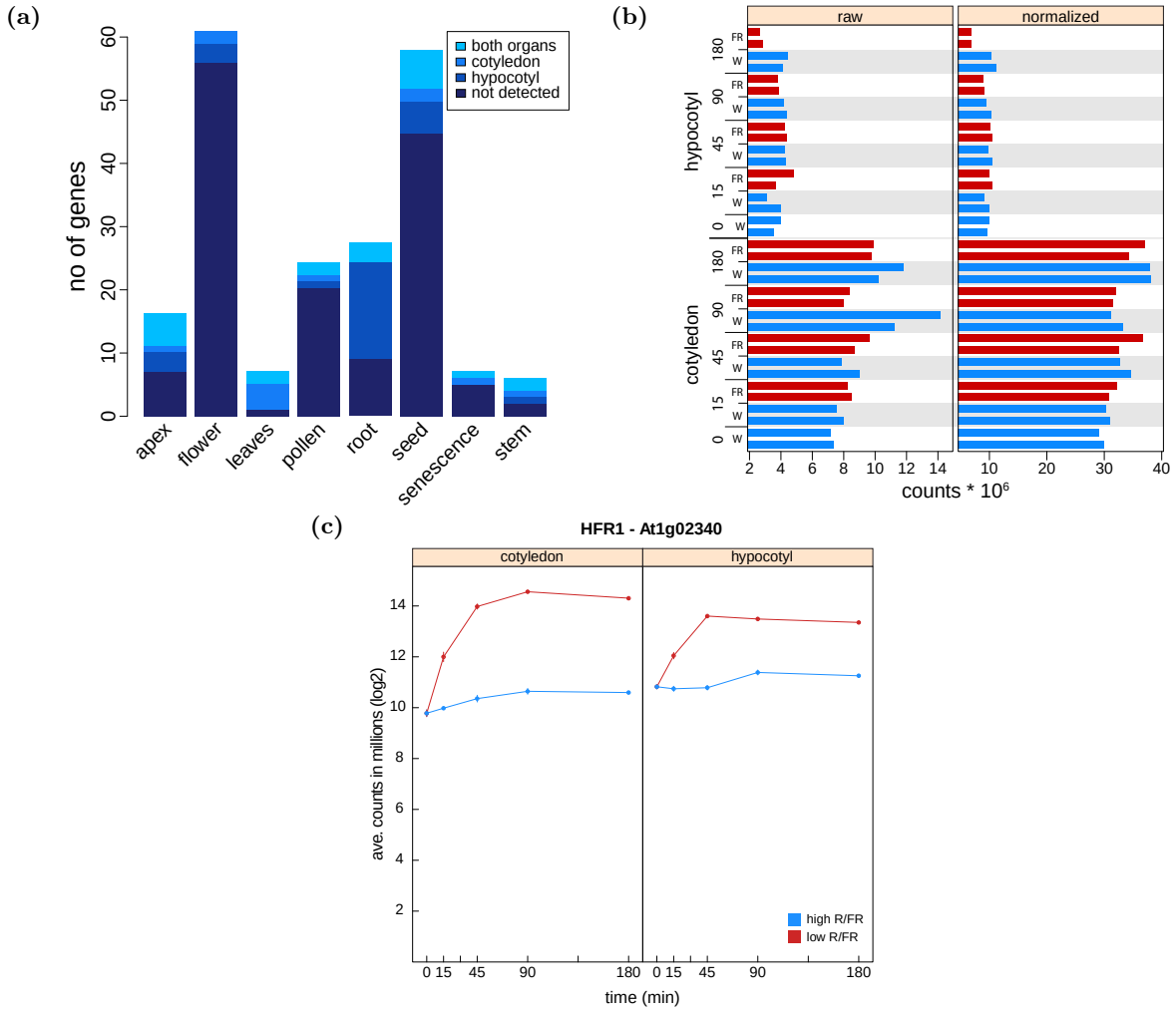

Figure 3.5: Read distribution of photosynthetic active and organ marker genes.
(a) Detected and non-detected organ marker genes as defined in Schmid *et al.* (2005). (b) Reads for all gene assigned to the photosynthesis category in the MapMan annotation file were summed over all libraries before (raw) or after (normalized) read normalization.(c) Expression pattern of the shade marker gene *HFR1*.

material. Figure 3.5b shows the sum of reads before and after normalization for genes related to photosynthesis as defined in the annotation file from MapMan (Thimm *et al.*, 2004). Average read abundance in libraries derived from hypocotyls is clearly lower than for cotyledon derived libraries (29 %) and reflects lower transcript levels in hypocotyls than in cotyledons in all libraries, as expected.

3.1.5 Low R/FR induces stronger transcriptional changes in hypocotyl than cotyledons

Using the moderated t-statistics from the LIMMA package, significantly differentially expressed genes between high and low R/FR conditions were identified for single time points per organ. Shade marker gene, such as *HRF1* (figure 3.5c), and *XTH15/XTR7* (figure 3.10e) exhibited a clear induction of transcript levels in low R/FR. In a first step, only genes with an adjusted p value lower than 0.05 in at least one organ were considered. To graphically display the distribution of fold changes (FC) between high and low R/FR within one organ, logFC values of hypocotyl were plotted over logFC values of cotyledon for separate time points (figure: 3.6).

With increasing time more differentially expressed genes were detected for both organs. The magnitude of transcriptional regulation in cotyledons and hypocotyls was comparable for TP15 and TP45, whereas at the two latest time points higher differences between transcript levels could be observed for hypocotyls. Immediate early transcriptional responses at TP15 occurred predominantly in only one organ. Only few genes responded simultaneously in both organs and transcription of almost all of those genes was induced (figure: 3.6). The number of simultaneously responding genes increased at TP45 and comprised almost solely genes with similar direction of regulation in both organs. Most of those genes were induced, but a smaller fraction had reduced transcriptions levels. Simultaneously responding genes with opposite regulation in cotyledons and hypocotyls were first observed at TP90. At the latest time point, more genes with similar regulation direction between organs were identified. At each time point, except TP45, more genes responded to supplemental far-red in hypocotyls than in cotyledons. At TP15, this was due to many genes with a subtle FC.

To define significantly differentially expressed genes sets base on p value and FC a combination of $p < 0.05$ and $|FC| > 1.5$ was chosen. These thresholds were used for all subsequent analysis if not further specified. The numbers of genes identified as significantly differentially expressed are listed in table 3.1.

In cotyledon, the overall number of significantly differentially expressed genes increased by

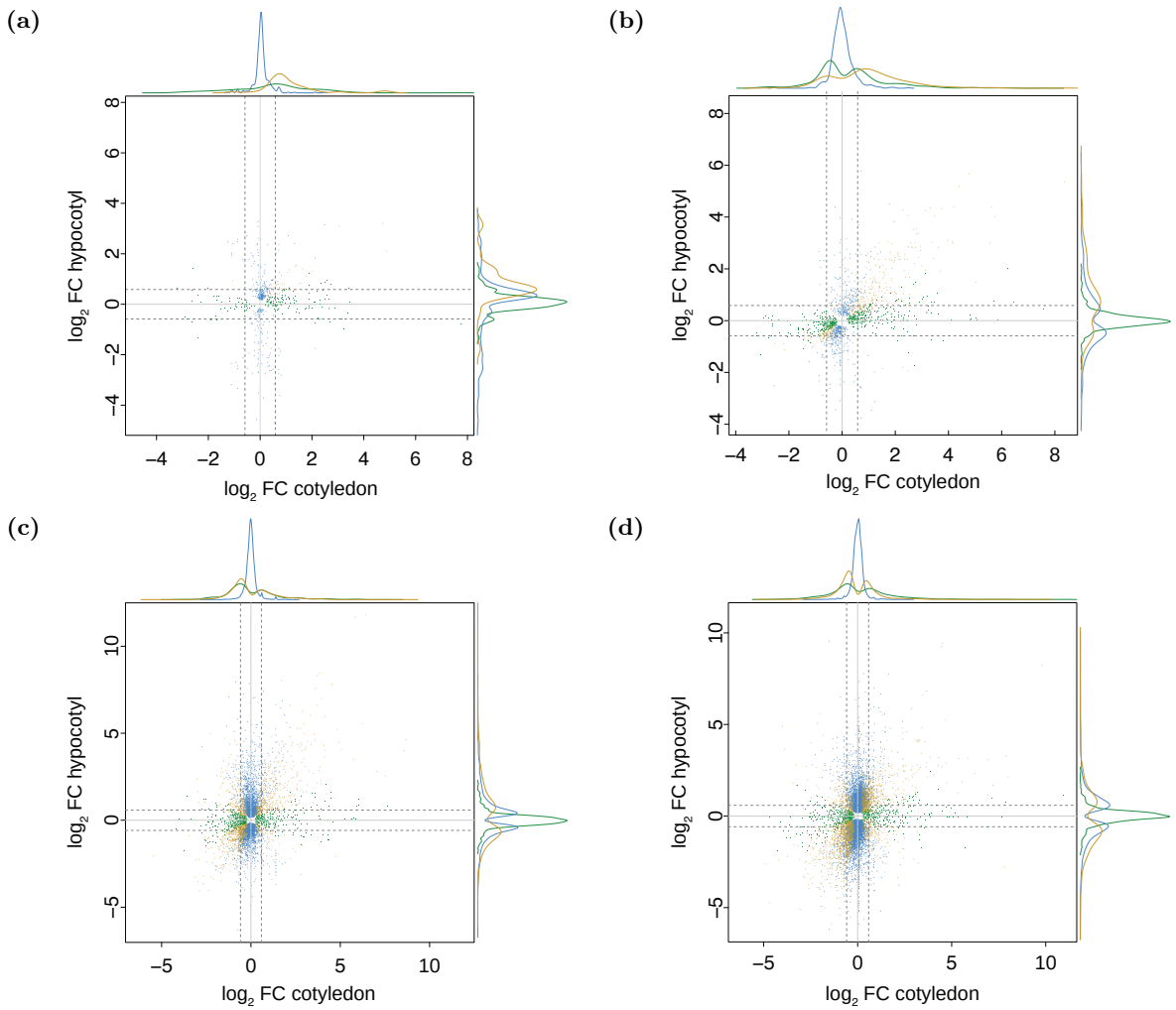

Figure 3.6: Distribution of transcriptional regulation in cotyledon and hypocotyl at different time points.
Genes with significant adjusted p values ($p < 0.05$) in at least one organ were plotted according to their logFC values for time point 15 (a), time point 45 (b), time point 90 (c) and time point 180 (d). Squares indicate a significant p value for cotyledon, circles for hypocotyls and triangle for both organs. The dashed line indicates a fold change of +/-1.5.

Table 3.1: Number of differentially expressed genes in cotyledon or hypocotyl at various time points.
Genes with a adj. p values < 0.05 and a $|FC| > 1.5$ were selected at the corresponding time point.

	cotyledon				hypocotyl			
time points	TP15	TP45	TP90	TP180	TP15	TP45	TP90	TP180
upregulated	113	422	559	708	118	354	1932	3838
downregulated	40	163	648	927	77	190	1497	4003
total	153	585	1207	1635	195	544	3429	7841

roughly 500 per time point. At the two early time points, about ¾ of the genes had upregulated transcript levels while at later time points downregulated genes increased to more than 50 %.

In hypocotyls, the increase of significantly differentially expressed gene over time is less continuous than in cotyledons. While the overall numbers of identified genes at the two first time points behaved similar to cotyledons, significantly differentially expressed genes increased about six fold and additional two fold between TP45 and TP90 and TP90 and TP180, respectively. This shows that the transcriptional landscape in hypocotyls changed to a larger extend towards the end of the time course compared to cotyledons. The relative proportion between up and downregulation is comparable for TP15 and TP45 for both organs. In contrast to cotyledons, similar numbers of genes show up and downregulated transcript levels in hypocotyls at TP90 and TP180.

3.1.6 comparison to published data

Analysis of transcriptional shade responses of organ identifies more genes compared to whole seedling approaches

In the RNAseq experiment significantly differentially regulated genes in response to low R/FR were identified for single organs. Beside the additional local information, separate sequencing of cotyledon and hypocotyl samples has the advantage that transcriptional responses which occur in only one organ are not artificially attenuated by combining those plant material with transcriptionally not responding part of plants. In this particularly experiment cotyledon samples produced roughly 10 times more total RNA than hypocotyl samples. This data set potentially provide more sensitive data especially for hypocotyls than whole seedling approaches. In this data set large gene list of significantly different regulated genes in response to low R/FR were identified. Larger numbers of identified genes compared to published data sets such as Hornitschek *et al.* (2012) or Li *et al.* (2012) may be due to various factors including different age of seedling, duration of low R/FR treatment, different analytic methods or applied stringency of criteria

used for gene identification.

The data set published in Hornitschek *et al.* (2012) was prepared from 12-day-old seedlings after 2 h of low R/FR treatment and analyzed by means of microarrays. To compare the present data set to the previous identified shade responsive gene in Col-0 form Hornitschek *et al.* (2012), gene sets were overlapped using TP90. This time point was chosen because duration of treatment is most similar and the shorter time of treatment belongs to the RNAseq analysis, which is the more sensitive method.

Most of the genes identified in Hornitschek *et al.* (2012) were also differentially expressed in cotyledon and hypocotyl at the earlier TP90 (figure 3.7a). Interestingly, a large number (150 genes, 43.23 % of all overlapping genes) of hypocotyl specific genes in our data set were previously detected with the whole seedling approach reported in Hornitschek *et al.* (2012). Additional low R/FR regulated genes were identified for both organs with many more genes for hypocotyl samples than cotyledons. A large fraction of hypocotyl specific genes at TP90 in our data are also classified as hypocotyl specific during the whole time course (67.33 % of previously identified genes (150), and 85.66 % of new identified genes (2588)). Genes of both subgroups have the tendency to be upregulated. A large fraction of cotyledon specific genes at TP90 have also cotyledon specific relative fold change level between high and low R/FR during the whole time course in our experiment. Nevertheless, those fractions are with 39 % and 50 % for previously identified (41) and previously not identified (475) genes, respectively, smaller than for hypocotyl specific genes. These ratios for cotyledon or hypocotyl specific low R/FR responsive genes show, that also a considerable number of hypocotyl specific genes can be identified with whole seedling approaches, additional genes can be identified by organ specific transcriptional analysis.

Through organ specific transcriptional analysis, further insight are gained for gene which oppositely respond in different part of the plant since whole seedling approaches potentially underestimate the significance of those responses based on pooled transcript levels. Among the previously identified genes, almost no showed an opposite regulation between cotyledons and hypocotyls in our RNAseq experiment. Nevertheless the union of additionally identified gene contains 70 % (group ch3) and 82 % (group ch4) of oppositely regulated genes.

Finally, only a small number of genes, which were previously identified, were not transcriptionally regulated at TP90. Among those 85 genes only 30 were not differentially expressed during the whole time course in both organs.

To determine if organ specific genes, which were previously not identified in (Hornitschek *et al.*, 2012) are enriched in biological processes, GO analysis was applied. For genes, which

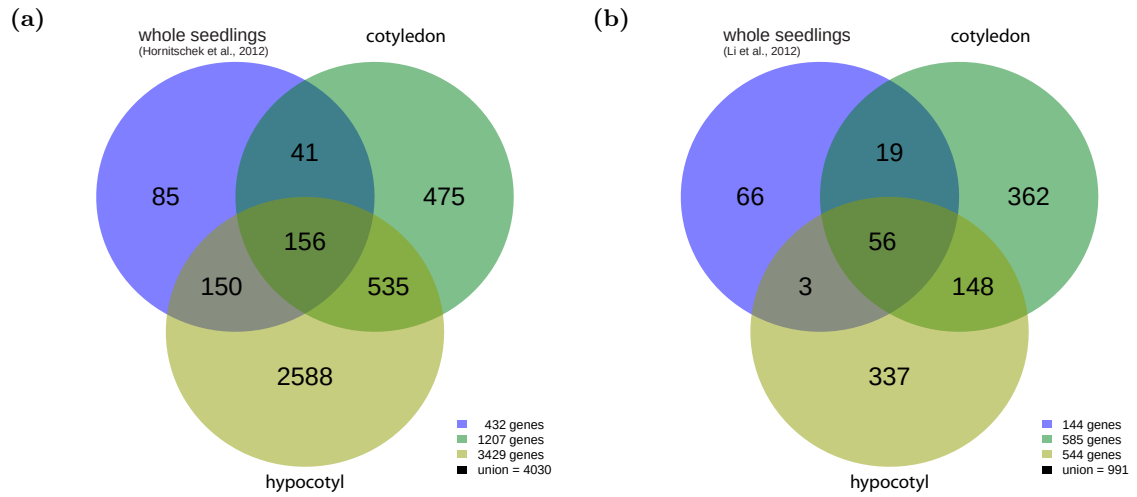

Figure 3.7: Comparison of shade responsive genes of whole and dissected seedlings
(a) Whole seedlings treated for 2 h with low R/FR and analyzed by microarrays are compared with gene sets of cotyledon and hypocotyl material at TP90. (b) Whole seedlings treated for 1 h with low R/FR and analyzed by RNAseq are compared with gene sets of cotyledon and hypocotyl material at TP45.

were uniquely identified in cotyledon at TP90 several GO categories were detected. The best-ranked enriched term was 'response to abscisic acid stimulus' (GO:0009737). Additional terms are 'regulation of abscisic acid mediated signaling' (GO:0009787) and 'negative regulation of abscisic acid mediated signaling' (GO:0009788).

Enriched GO terms for genes specifically identified in hypocotyls include the phytohormone related terms 'response to auxin stimulus' (GO:0009733), 'response to gibberellin stimulus' (GO:0009739), 'response to ethylene stimulus' (GO:0009723) and 'response to abscisic acid stimulus' (GO:0009737) as well as 'response to carbohydrate stimulus' (GO:0009743) and 'response to light stimulus' (GO:0009416).

To further compare our RNAseq data set with published low R/FR responsive genes identified with a similar method from entire Col-0 seedlings, significantly different regulate genes in cotyledon and hypocotyl at TP45 were compare with the gene set identified by Li *et al.* (2012) after 1 h of low R/FR treatment (figure 3.7b). About 55 % of genes identified by Li *et al.* (2012) were also detected in our time course experiment predominantly in both organs. Additional shade responsive genes were identified in both organs with more organ specific expressed genes at TP45.

PIF5 bound G-boxes have similar distributions of flanking bases in cotyledon and hypocotyl

In our previous publication (Hornitschek *et al.*, 2012) we identified PIF5 chromatin binding sites and analyzed the composition of the flanking bases of the most central G-box motifs to PIF5 binding peak summits (figure 2.2c). Our current RNAseq experiment allows distinguishing between PIF5 binding sites proximal to organ specific regulated genes. About 62 % of the putative direct target genes are regulated in the whole time course experiment with a clear trend toward upregulated genes. Additionally, a large number of genes were classified as hypocotyl specific regulated. While PIF5 chromatin-binding does not necessarily correlate with transcriptional activity, different groups of PIF5 direct target genes might share different trends in PIF5 DNA binding affinities. Those differences might manifest in a different composition of flanking base pairs. To check if differentially regulated genes in low R/FR, which were cotyledon-specific, hypocotyl-specific or in both organs identified, have different flanking base pairs proximal to central G-boxes of PIF5 binding sites, selected PIF5 chromatin binding sites were reanalyzed. Only few cotyledon specific regulated genes with proximal PIF5 chromatin binding site were found. Therefore the count for the absolute cotyledon specific class is considerably lower than for the two remaining classes, which makes the interpretation of the data more difficult (37 sequences for cotyledons class vs. >150 sequences for hypocotyl or both organ classes). Different analyzed classes of octameric sequences had a similar distribution to the previous analysis and no clear organ specific trend could be observed (figure 3.8).

3.1.7 Gene sets of different TPs

To investigate the transcriptional response over time in more details gene sets of different time points were compared within organs (figure 3.9). Visualization by Venn diagram revealed several global expression trends for cotyledon and hypocotyl. The expression of most genes changed in response to the stimulus at one or during subsequent time points, while very few, showed an absence/presence patter of regulation. The largest subgroups of single time points were time point specifically responding genes and their size increased with time.

Additional subgroups follow three different expression types: constantly regulated genes throughout the whole analysis (class I), transiently regulated genes at TP15 and TP45 (class IIa) or TP15 to TP90 (class IIb) and delayed responding genes either at TP45 and later (class IIIa) or at TP90 and later (class IIIb).

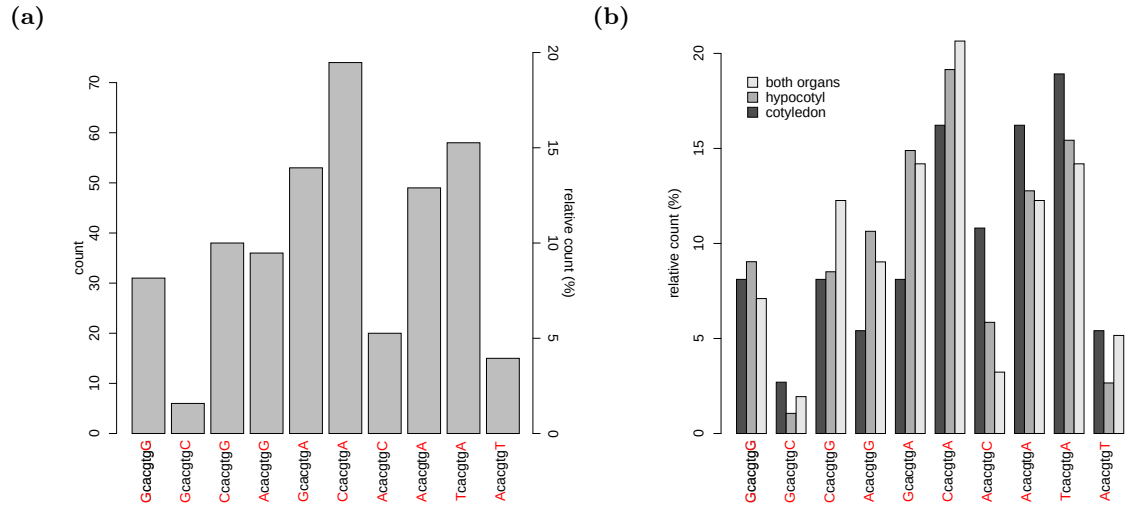

Figure 3.8: PIF5 binding site analysis proximal to shade-regulated genes in cotyledon and hypocotyl.
DNA strand independent absolute number or relative count of G-box containing octamers proximal to PIF5 chromatin binding sites with non organ (a) or organ specific (b) resolution. Values in (b) are relative to the different group sizes, which is 37 for cotyledon specific, 188 for hypocotyl specific and 155 for both organs.

Upregulated genes of class II were fewer in number than TP15 specifically regulated genes, but increase with longer response times. Almost no genes were transiently downregulated at several time points including TP15.

Class III gene numbers also increased the later the initial response was observed. This was due for both, up- and downregulated gene. Delayed responding gene lists were also larger compared to transient regulated genes.

Constantly transcriptional responses throughout the entire time course (class I) were mainly found among upregulated genes. Their numbers were similar for both organs and 11 upregulated genes were shared. Those common genes include the shade marker genes *ATHB-2*, *HFR1* and *PIL1*. Furthermore, both gene lists share the auxin signaling components *IAA2* and five members of the SMALL AUXIN UPREGULATED RNA (SAUR) gene family. In hypocotyl additional auxin signaling components such as *IAA29* and several *SAUR* genes were upregulated.

Interestingly, in cotyledons the three auxin biosynthetic genes *YUC2*, *YUC8* and *YUC9* are upregulated throughout the whole time course, while in hypocotyls none of the *YUCCA* genes respond at all time points.

Additional class I genes, which respond either in cotyledon or hypocotyls, comprise further well-known shade responsive gene. Several are PHYTOCHROME RAPIDLY REGULATED (PAR) genes, which are known to quickly respond to changes in the R/FR ratio (Roig-Villanova *et al.*, 2007). This heterogeneous group consists of basic helixloophelix (bHLH) transcription

factors, B-BOX DOMAIN PROTEIN (BBX) genes and the class 2 subfamilies γ and δ of the homeodomain-leucine zipper (HD-ZIP) family (Ciarbelli *et al.*, 2008; Crocco *et al.*, 2010). In our experiment *PAR1*, *HAT2* and *BIM1* were identified at all time points for cotyledons and *HAT1*, *ATHB-4* and *BBX29* were found in the gene list for hypocotyls.

Finally, in cotyledon *GATA2* was upregulated, which is an integrator of light and brassinosteroid signaling (Luo *et al.*, 2010).

The list of class I downregulated genes holds one gene within each organ. In case of hypocotyls, this gene has not been characterized to date. The downregulated gene in cotyledons is *TINY*. This gene belongs to the group III of the AP2/ERF superfamily of transcription factors (Nakano *et al.*, 2006). Knockout mutants exhibit overall reduced growth including reduced cell size and disorganized epidermal cell files in etiolated hypocotyls (Wilson *et al.*, 1996).

Early transient responses (class II) were observed for only few genes. Interestingly, the lists of upregulated class IIa genes include the three transcription factor *HAT1*, *ATHB-4* and *GATA2*, which respond transiently in one organs and were found in class I in the second organ as described before. More precisely, *GATA2* responded transiently in hypocotyl during the first 45 min. In contrast, *HAT1* and *ATHB-4* were transiently responding in cotyledons during the first 45 min and 90 min, respectively.

Cotyledon specific upregulated class IIa genes comprise in addition the two genes *HAT3*, which is closely related to *HAT1* (Ciarbelli *et al.*, 2008), and *ATBS1-interacting factors* (*AIF/bHLH149*), a atypical bHLH transcription factor. *AIF* was identified in a yeast-to-hybrid screen with *ACTIVATION-TAGGED BRASSINOSTEROID-INSENSITIVE 1-SUPPRESSOR 1/BASIC HELIX-LOOP-HELIX PROTEIN 135* and formally described as a negative regulator of brassinosteroid signaling (Wang *et al.*, 2009).

Finally, upregulated class IIb genes identified in both organs hold *CKX6*. *CKX6* is involved in mediating reduced cell proliferation rates in leaf primordia under low R/FR (Carabelli *et al.*, 2007).

Delayed responding genes (class IIIa), which were upregulated in both organs, included several signaling components of various phytohormones. Additional auxin signaling components were *IAA19*, *IAA4*, *SAUR9* and *SAUR10*. Of the brassinosteroid pathway *BEE1* and *BIM2* were identified (Friedrichsen *et al.*, 2002; Yin *et al.*, 2005). This list contained also the bHLH TF *JASMONATE ASSOCIATED MYC2 LIKE 2* (*JAM2*), which was placed as negative regulator

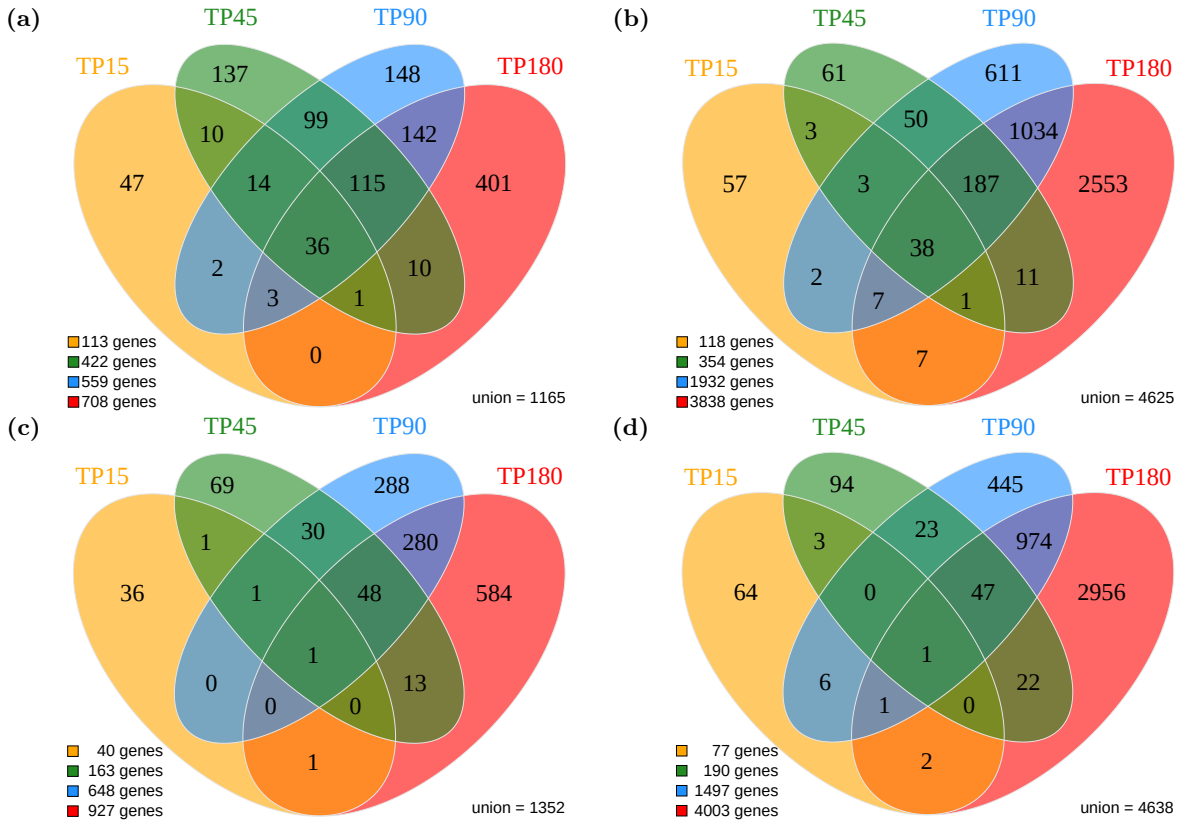

Figure 3.9: Differentially regulated genes in cotyledon and hypocotyl at different time points Venn diagram depicting upregulated (a, b) or downregulated (c, d) gene in response to low R/FR in cotyledon (a, c) or hypocotyl (b, d).

in the jasmonate signaling pathway. It therefore potentially affects various responses including wound and defense responses as well as anthocyanin biosynthesis (Sasaki-Sekimoto *et al.*, 2013).

The list of upregulated class IIIa genes of cotyledon but not hypocotyls, holds additional well known shade-induced genes such as *XTH15/XTR7*, which is involved in cell wall modification (Rose *et al.*, 2002; Lorrain *et al.*, 2008), and *PIF6*. Additional genes, encoding for auxin signaling components were present in this list such as *IAA29*, *ARF18* and the auxin efflux carrier *PIN3*. This list also contains three members of the BBX zinc-finger transcription factor family: *BBX6/COL5* was identified as a positive regulator of flowering in SD (Hassidim *et al.*, 2009). *BBX21* acts positively during photomorphogenesis and was reported as a negative regulator of shade-regulated gene expression (Datta *et al.*, 2007; Khanna *et al.*, 2009; Crocco *et al.*, 2010) and *BBX29*, which was previously shown to be PIF4 and/or PIF5 dependent upregulated in response to shade (Hornitschek *et al.*, 2012). Another gene of this list is *CKX5*, which belong to the family of cytokinin oxidase/dehydrogenase (CKX) genes mediating cytokinin breakdown (Schmülling *et al.*, 2003; Bartrina *et al.*, 2011). *CKX5* has previously been described as shade-induced gene by means of microarray analysis (Sessa *et al.*, 2005; Hornitschek *et al.*, 2012).

In hypocotyl specific, upregulated class IIIa genes include further members of auxin signaling

components. Interestingly, this list also contained the auxin biosynthetic gene *YUC3* and the auxin receptor-encoding gene *AFB1*. Finally, also genes involved in cell wall modification were identified. Those genes include *XTH22* and *XTH9*, six members of the expansine family as well as *CELLULOSE-SYNTHASE LIKE C4* (Micheli, 2001; Rose *et al.*, 2002; Hamant and Traas, 2010).

Downregulated class III genes were a heterogeneous group. Downregulated class IIIa genes in cotyledons comprise several transcription factors family members of various types such as *TCP8*, *NAI1/bHLH20*, *bHLH125* or *MYB30*. In addition *CKX7* is downregulated in cotyledon. In hypocotyls *KNAT4* and *SUC1* are downregulated at the latest three time points.

Delayed responding genes (class IIIb), which were upregulated, contained additional members of gene families listed above such as three *IAA* genes in both organs. The gene set for cotyledon sample also contains*BBX23*, a negative regulator of skotomorphogenesis (Sentandreu *et al.*, 2011). Also, transcript level of *IAA carboxylmethyltransferase 1* (*IAMT1*), *GH3.2* and *GH3.5*, which belong to two different classes of auxin modifying enzymes (Staswick *et al.*, 2005; Qin *et al.*, 2005; Park *et al.*, 2007), were upregulated. In hypocotyl *YUC8*, *TMK1*, a auxin signaling component (see below), the auxin transporters *PIN7*, *PILS3* and *PILS5* as well as several *XTH* members were upregulated.

Finally, downregulated class IIIb genes specifically in cotyledon include *YUC6*, *TAA1/SAV3*, *SAUR6* and *SAUR14* as well as the *LAX3* and *PILS5* encoding for two auxin transport proteins. In addition the Cytochrome *P450 90C1/ROT3* was found in this list. This enzyme is involved in the brassinosteroid biosynthesis. *rot3* mutants have reduced hypocotyl length predominantly under red light (Kim *et al.*, 2005a) and altered leaf shape in white light (Kim *et al.*, 1998).

3.1.8 Enriched biological processed among upregulated gene

In order to investigate general biological trends at different time points, gene ontology (GO) enrichment analysis was performed. Organs were investigated independently for each single time point discriminating between up and downregulated genes. Lists of enriched GO categories of different time points were finally compared within organs for up- or downregulated genes and visualized as Venn diagrams (figure 3.11).

The four Venn diagrams show, that all enriched term at TP15 were shared with at least one later time point (table 3.2). All GO terms can be either described as either transiently identified at one or several time points, or continuously enriched once detected. No term with complex

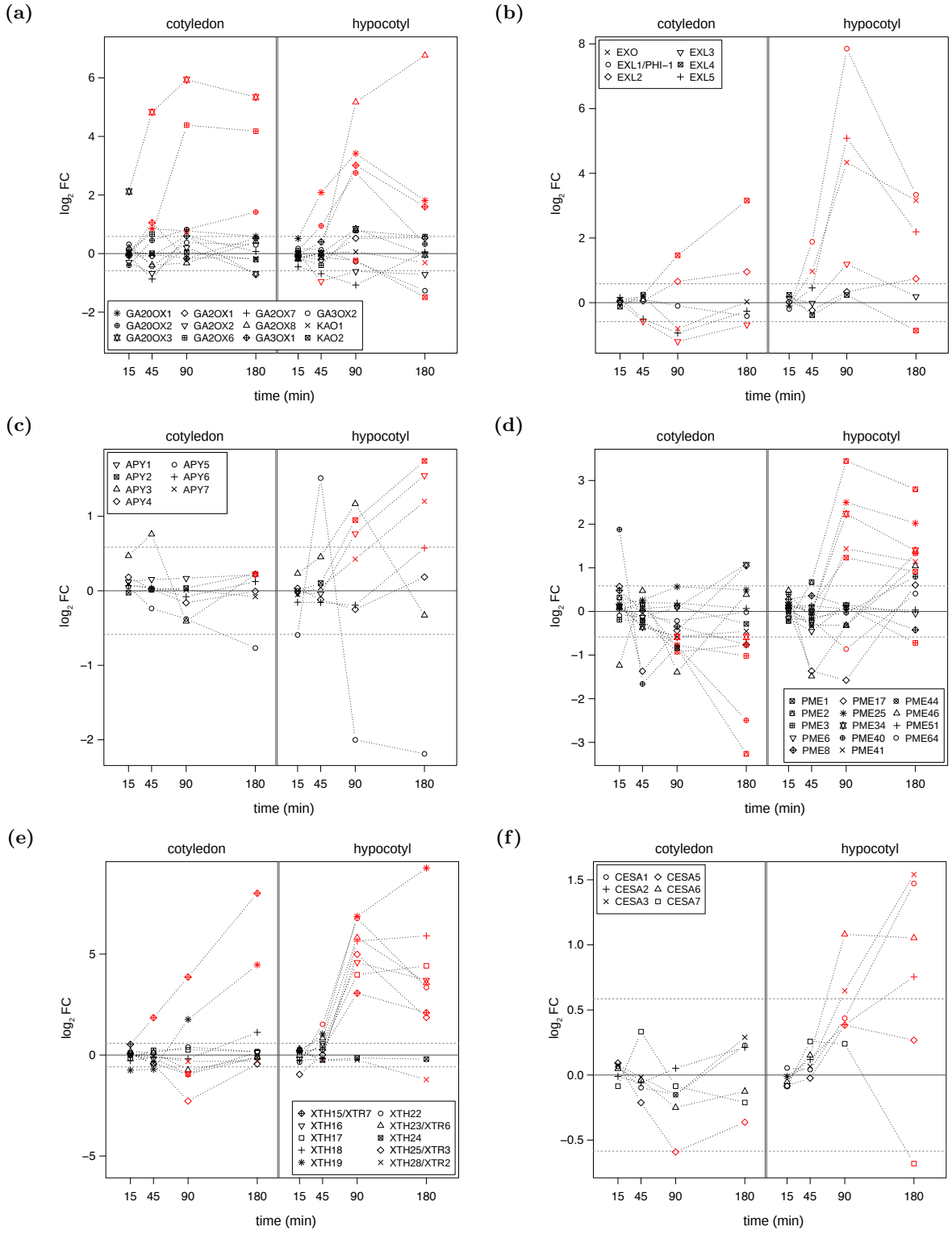

Figure 3.10: Differential regulated of hormone or cell wass related gene families in cotyledon and hypocotyl
(a) Gibberellin metabolic enzymes, (b) *EXORDIUM* gene family members expressed in cotyledon and/or hypocotyl. (c) Expression pattern of the apyrase gene family. (d,e) Relative expression pattern of cell wall modifying gene families: (d) pectin methyl transferases, (e) XTH/XTR family (f) Relative expression pattern of cellulose syntheses. Relative expression levels with a p values < 0.05 are shown in red.

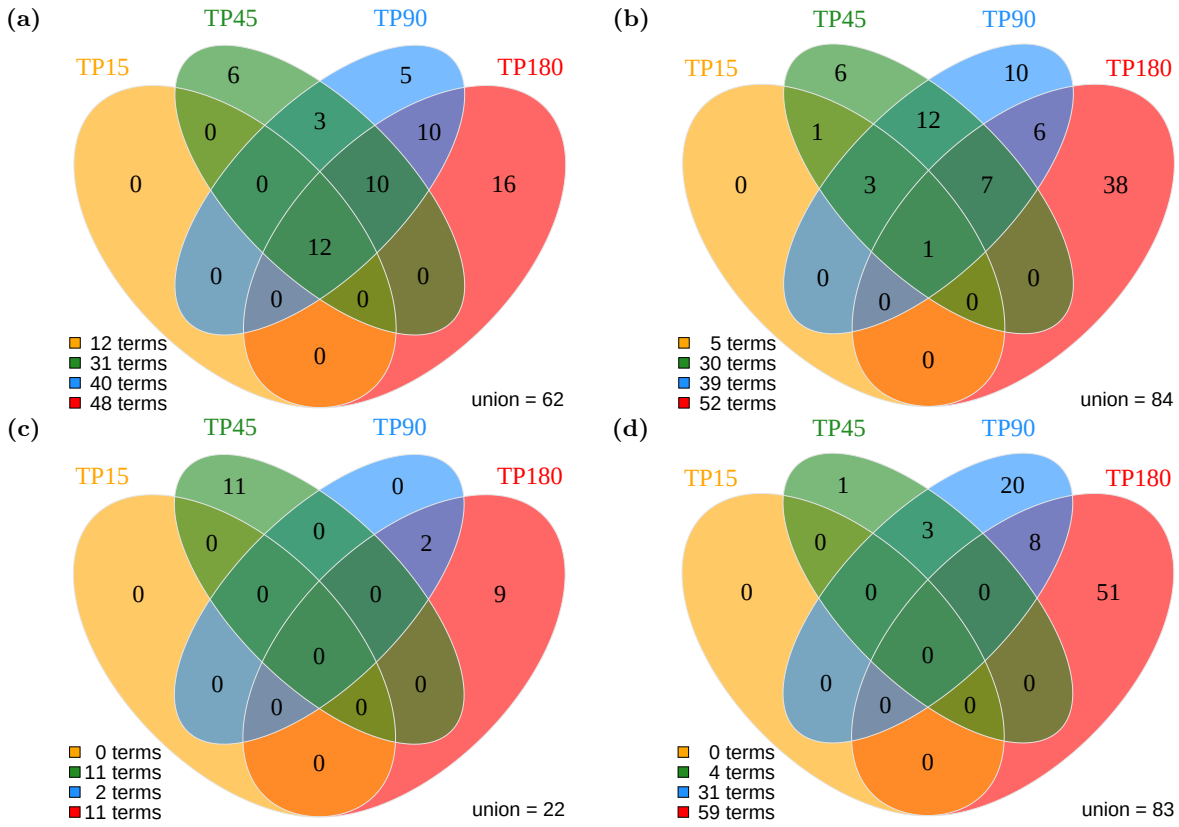

Figure 3.11: Comparison of gene ontology terms identified at different time points in cotyledon or hypocotyl.
Venn diagram of gene ontology terms (Benjamini Hochberg corrected p value < 0.05) identified per time point in cotyledon (a, c) or hypocotyl (b, d) for up (a, b) or down (c, d) regulated gene.

Table 3.2: GO terms in cotyledon enriched at all time points. Terms are sorted by the Benjamini Hochberg corrected p value from TP15.

term	description
GO:0009733	response to auxin stimulus
GO:0006350	transcription
GO:0010556	regulation of macromolecule biosynthetic process
GO:0009889	regulation of biosynthetic process
GO:0031326	regulation of cellular biosynthetic process
GO:0051171	regulation of nitrogen compound metabolic process
GO:0019219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
GO:0010468	regulation of gene expression
GO:0031323	regulation of cellular metabolic process
GO:0080090	regulation of primary metabolic process
GO:0060255	regulation of macromolecule metabolic process
GO:0009416	response to light stimulus

absence/presence pattern was detected.

Phytohormone-related enriched biological processes

auxin

Gene ontology categories describing auxin-regulated responses and regulation were detected at all time points for upregulated genes in cotyledons and hypocotyls. Interestingly, the term 'response to auxin stimulus' was already detected at TP15 as the most significant category in both organs (adj. $p < 1.4^{-4}$, cotyledons; adj. $p < 3.0^{-11}$, hypocotyls). This term remained among the best ranked categories at all later time points in both organs. Genes described by the category 'response to auxin stimulus' and found in gene lists of different time points comprise predominantly *SAUR* genes, *Aux/IAAs* some *GH3* genes and *PINs* (table 3.4).

The term 'auxin polar transport' was enriched at TP45 to TP180 in cotyledons, suggesting a increased activities of PAT at later time points. In hypocotyls this term was detected at TP90.

brassinosteroid

Brassinosteroid related GO terms were detected as early as TP45 in hypocotyls. The term 'response to brassinosteroid stimulus' was enriched at the three latest time points. At TP45 several signaling components, e.g. *EXORDIUM* (*EXO*) and *XTH22* (figure 3.10b and 3.10e), were described by this term. Several genes of the initial brassinosteriod signaling pathway are upregulated at the two latest time points. This includes the receptor *BRI1*, which were previously descriptive as low R/FR induced in whole seedlings (Devlin *et al.*, 2003), the signaling kinases *BRASSINOSTEROID-SIGNALING KINASE 1* (*BSK1*) and *BRASSINOSTEROID-*

INSENSITIVE 2 (*BIN2*) as well as the transcription factor *BZR1*. Also further downstream signaling components were differentially regulated such as *DWARF 4* (*DWF4*), *ARGOS-LIKE* (*ARL*), *EXO* and *XTH22* .

In gene list of cotyledons, the term 'response to brassinosteroid stimulus' was detected at a single time point, TP90. This suggests that the brassinosteroid pathway is predominantly transcriptionally regulated in hypocotyls and may result in stronger brassinosteroid-mediated growth responses in this organ during the first hours after initial shade perception.

gibberellin

The GO term 'response to gibberellin stimulus' was enriched in gene lists of TP45 to TP180 in cotyledons and at TP90 in hypocotyls. Several GA metabolic genes, which are part of this term, were transcriptionally regulated in both organs. In cotyledon *GIBBERELLIN 20 OXIDASE (GA20OX) 2*, *GA20OX3* and *GA2OX6* were upregulated within the initial 3 h of low R/FR treatment, while different gene family members were induced in hypocotyls: *GA20OX1*, *GA20OX2*, *GA2OX8*, *GA3OX1* and *GA20OX2* (figure 3.10a).

abscisic acid

Enrichment for abscisic acid related terms were detected only for cotyledon gene lists. From TP45 on, 'response to abscisic acid stimulus' was enriched and in additions at TP90 the term 'regulation of abscisic acid mediated signaling' and 'negative regulation of abscisic acid mediated signaling' was detected. Therefore, the abscisic acid pathway may play a cotyledon specific role during shade avoidance.

ethylene

Gene ontology terms related to ethylene were predominantly found at single time points. In hypocotyls the two terms 'ethylene biosynthetic process' and 'ethylene metabolic process' were enriched at TP45. At TP90 and TP180 'response to ethylene stimulus' was detected in both organs. Finally, at TP180 'regulation of ethylene mediated signaling pathway' and 'negative regulation of ethylene mediated signaling pathway' was identified only for cotyledons. There term suggest a transient increase of ethylene biosynthesis in hypocotyls and subsequent transcriptional induction of ethylene signaling components within the first hours in shade.

Table 3.3: Jaccard index comparing shade regulated genes in cotyledon and hypocotyl at different time points.

	TP15	TP45	TP90	TP180	union
upregulated	0.111	0.302	0.133	0.073	0.116
downregulated	0	0.063	0.131	0.114	0.148

Additionally enriched biological processes

Additional GO terms which do not directly describe biological processes of phytohormones, were enriched in both organs at different time points. The term 'response to light stimulus' was detected at all time points for cotyledons and from TP45 on in hypocotyls.

Several GO categories, which describe different growth processes, were enriched throughout the time course. For example, in cotyledon-enriched term were at TP45 'shoot system development', at TP45 and TP90 'organ development', and at TP90 'photomorphogenesis'. In hypocotyls the terms 'shoot development', 'organ development' and 'cell growth' were detected at TP45 and TP90. At TP45 to Tp180 'developmental growth involved in morphogenesis' and at TP180 'cell maturation', 'epidermis development' and 'epidermal cell differentiation' were enriched.

In cotyledons at TP90 and TP180, 'response to water deprivation' were detected, and may describe increased water demands during growth processes (Guerriero *et al.*, 2014).

For cotyledons, flavonoid related terms were enriched at TP180. These include 'flavonoid metabolic process', 'flavone biosynthetic process' and 'flavonol biosynthetic process'.

In hypocotyls at TP90 or TP180, sugar related terms were enriched. At TP90 the term 'response to carbohydrate stimulus' and at TP180 the categories 'carbohydrate biosynthetic process', 'polysaccharide biosynthetic process', 'cellular polysaccharide metabolic process', 'nucleotide-sugar biosynthetic process', and 'nucleotide-sugar transport' were detected.

Finally, also the term 'fatty acid metabolic process' was enriched for hypocotyl at TP180.

3.1.9 Enriched biological processed of downregulated gene

For downregulated genes few enriched GO categories were identified primarily single time points.

In cotyledons at TP90 and TP180 the term 'oligopeptide transport' was enriched and at TP180 the terms 'carbohydrate biosynthetic process' and 'cell wall modification' were detected.

In hypocotyl most identified GO categories for downregulated genes were enriched at TP90 and TP180. At both time points 'response to ethylene stimulus' and 'response to abscisic acid stimulus' was identified. At the last time point in hypocotyls 'salicylic acid metabolic process',

Table 3.4: Induced genes of the gene ontology term 'response to auxin stimulus' in cotyledon or hypocotyl across all time points.

time point	cotyledon	hypocotyl
TP15	-	-
TP45	AUX1, IAA3, IAA11, IAA14, IAA16, PLS, SAUR7, SAUR15, SAUR16, SAUR46, SAUR50, SAUR61, SAUR62, SAUR66, SAUR74	AUX1, PLS
TP90	GH3.1, GH3.17, GH3.18, IAA13, IAA32, LAX2, MYB61, PIN1	ARF2, ARF10, ARF19, CCD8, FQR1, GH3.2, GH3.6, HSFA4C, IAA13, IAA18, IAA20, LAX2, MYB6, NLM9, PBP1, PIN1, PIN4, SAUR4, SAUR49, SAUR76, SAUR77
TP180	ARF11, ATSEH, BT4, EIN2, IBR1, NAC1, PAP1, PIN4, PIN7, RHM1, SAUR41, SAUR71, SAUR72	ACT7, ARAC1, BT4, CAX4, IBR5, MYB16, PGP4, PHB3, PIN2, RCE1, RCN1, RHM1, RPL40A, RPN12a, SAR1, SAUR52, SAUR53, SAUR55, SAUR57, STV1, UBQ1, UBQ5, UBQ6, UBQ11, XTH4
TP15,TP45	-	-
TP15,TP90	-	-
TP15,TP180	-	-
TP45,TP90	ACS4, GH3.6, IAA1, IAA5, IAA6, MYB109, PGP19, SAUR26, SAUR27, SAUR28, SAUR29, SAUR63, SAUR64, SAUR67, SAUR68,IAA11, IAA30, MYB73, PIN3, SAUR73	IAA11, IAA30, MYB73, PIN3, SAUR73
TP45,TP180	-	-
TP90,TP180	ARGOS, GH3.2, GH3.5, IAA7, IAA34, IAA26, NLM9, PGP4, SAUR53	ACS6, AT5PTASE11, ATHB20, GH3.1, GH3.3, GH3.10, IAA7, IAA9, IAA14, IAA17, MYB30, MYB109, MYBR1, PGP19, PID, PIN7, RPS27AA, SAUR34, SAUR35, SAUR46, SAUR78, TCH2, TTL3
TP15,TP90,TP180	-	-
TP15,TP45,TP180	-	-
TP15,TP45,TP90	SAUR19, SAUR65	SAUR14, SAUR67

Continued on next page

Continued from previous page

time point	cotyledon	hypocotyl
TP45,TP90,TP180	ARF18, IAA4, IAA19, IAA29, IAA30, IAA20, MAX2, PIN3, SAUR9, SAUR10	ACS4, AFB1, ARGOS, GH3.5, HAT2, IAA1, IAA3, IAA4, IAA5, IAA6, IAA19, SAUR1, SAUR6, SAUR7, SAUR9, SAUR10, SAUR12, SAUR15, SAUR25, SAUR26, SAUR27, SAUR61, SAUR64, SAUR66, SAUR68, XTH22
TP15,TP45,TP90,TP180	ATHB-2, GH3.3, IAA2, HAT2, SAUR22, SAUR20, SAUR24, SAUR23, SAUR-like	ATHB-2, IAA2, IAA29, SAUR16, SAUR19, SAUR20, SAUR22, SAUR23, SAUR24, SAUR28, SAUR29, SAUR50, SAUR62, SAUR63, SAUR65, SAUR-like

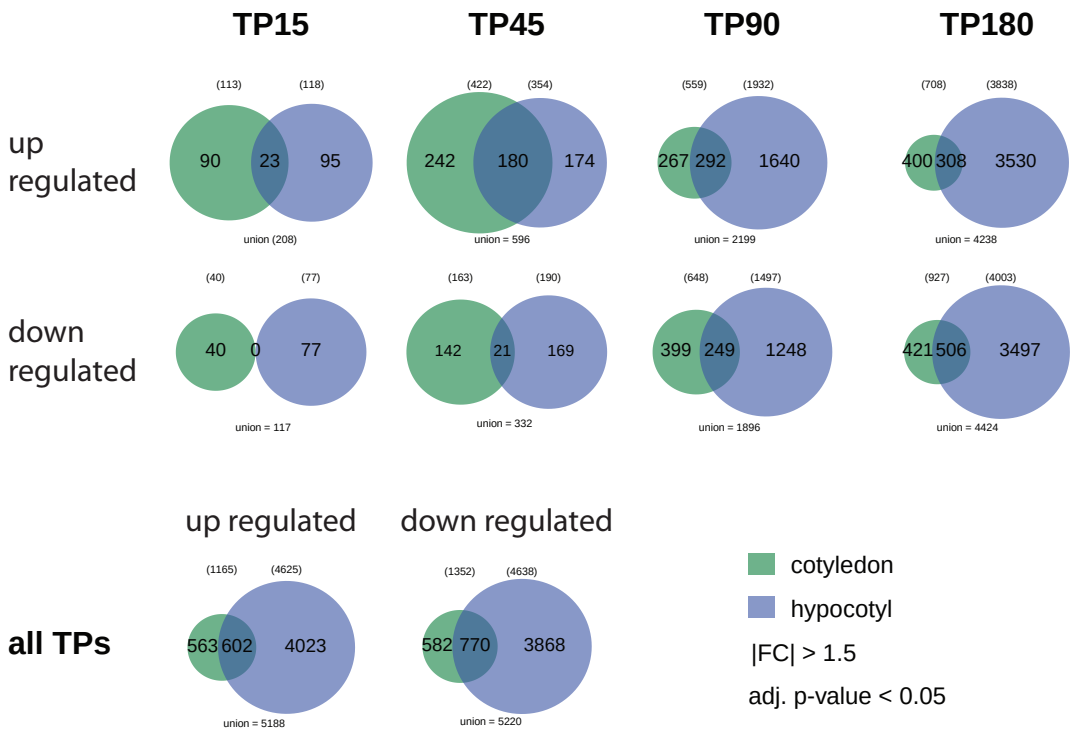

Figure 3.12: Differentially low R/FR regulated genes in cotyledon and hypocotyl compared at different time points.

as well as several defense related terms such as 'defense response to bacterium' and 'response to nematode' were enriched.

General trends comparing cotyledon and hypocotyl gene lists

To directly compare similarities and differences between cotyledon and hypocotyl at different time points, gene list of up or downregulated genes of both organs were overlapped at single time points (figure 3.12). At TP15 both list overlap with about 20 % of their genes. This ratio gets larger with increasing time before it finally decreases at TP180. Also the Jaccard index, which measures the similarity of lists by dividing the number of elements found in the intersection by the number of elements of the union, follows this trend (table 3.3). In case of downregulated genes the genes of the intersection increase over time up to 54.6 % and 12.6 % relatively to the complete lists of cotyledon and hypocotyl, respectively.

Gene ontology analysis identified more shared GO categories between cotyledons and hypocotyls for upregulated genes than downregulated genes. Enriched GO terms for downregulated gene are few in number and overlap only at the last two time points. For the upregulated gene set all enriched GO terms at TP15 of hypocotyls are also contained in the list for cotyledon. While GO categories of the intersection increase in number at TP45 the lists of enriched GO categories at the two last time points are more diverse and share less common terms (figure 3.13).

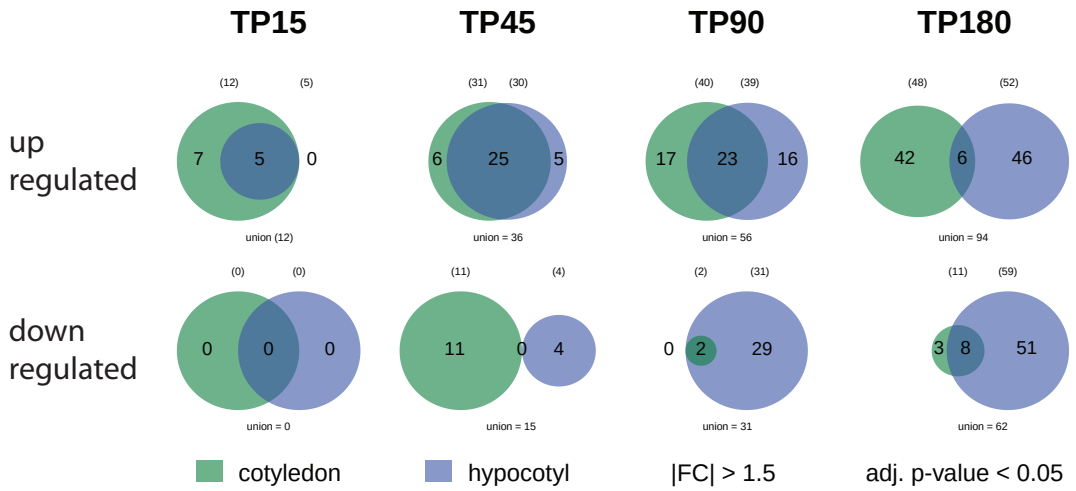

Figure 3.13: Comparison of gene ontology terms identified in cotyledon or hypocotyl at different time points.
Venn diagram showing the overlap of gene ontology terms with a Benjamini Hochberg corrected p value < 0.05.

3.1.10 Low R/FR induced genes follow few global expression pattern over time

So far, differentially expressed genes were identified at single time points and compared to each other. In order to put differentially expressed genes into a temporal context allowing the identification of regulation pattern over the first 3 h in low R/FR, relative expression levels of TP15 to TP180 were hierarchically clustered. Expected general pattern include a similar regulation direction over all time points, transient transcriptional responses in a single direction and various pattern of up- and downregulation between time points. In case of genes expressed in both organs, those patterns could occur in various combinations between cotyledons and hypocotyls.

Table 3.5: Number and organ specificity of identified genes. The first row contains absolute numbers of organ specific groups. The second section list the gene set sizes of corresponding subgroups.

cotyledon		both organs		hypocotyl		total
689		1782		7314		9785
group c1	352	group ch1	591	group h1	3698	
group c2	337	group ch2	729	group h2	3616	
		group ch3	173			
		group ch4	210			
		group ch5	23			
		group ch6	53			
		group ch7	3			

In order to consider only relative expression pattern between high and low R/FR with at least one significant time point in a given organ, all 9785 previously identified genes were divided into three classes according to their organ specificity (table 3.5). The smallest group was cotyledon

specific genes with slightly less than 700 members, followed by roughly 1800 gene differentially expressed in both organs and the largest group of 7314 hypocotyl specific genes. All three lists were hierarchically clustered using average linkage and the Euclidean distance.

The 1782 genes, expressed in both organs, split into 7 general groups based on all 8 FC values between high and low R/FR for cotyledon and hypocotyl (table 3.5). Group ch1 comprises genes, which respond during the time course with increased transcript levels of various magnitudes and kinetics (figure 3.14a). In fact, several sub clusters can be observed on the corresponding heat map, which differ in time of the first response or the time point of the strongest response. In group ch2 genes are summarized, which show opposite response pattern to group1 and are predominantly downregulated in both organs (figure 3.14b). Overall, downregulation is more pronounced at the two latest time points with a broader response at TP180 in hypocotyls. Opposite regulated genes between organs are clustered in group ch3 and group ch4 (figure 3.14c and 3.14d, respectively). The opposite regulation is most obvious when comparing TP90 and TP180. Most genes in group3 and group4 have different response kinetics in both organs. Furthermore, some genes have already altered relative expression level at earlier time points predominantly in one organ. Group ch5 to group ch7 have more complex relative expression pattern (figure 3.14e to 3.14g). Genes of group ch5 tend to be transiently upregulated in cotyledons at TP45 and TP90, and have in hypocotyls increasing FC values during the first 90 min of supplemental far-red light treatment and are downregulated at TP180. Group ch6 comprises genes, which tend to be downregulated mainly at TP45 and TP90 in both organs. Relative expression levels at TP180 are less homogeneous and only few genes are regulated in similar direction between cotyledon and hypocotyl. Group ch7 consists of only three genes. These genes have a clear transient upregulation at TP45 in cotyledon and are repressed at later time points, whereas in hypocotyls the expression is moderately induced compared to cotyledon.

Cluster analysis of organ specific regulated genes suggests for both organs a subdivision into two major groups of predominantly up or downregulated genes (figure 3.15). Many cotyledon specific shade responsive genes display the strongest response at late time points. In addition groups with clear transient response pattern at one of the three early time points can be identified. In contrast, hypocotyl specific shade responsive genes are primarily responding at TP90 and TP180.

Similar and different response to low R/FR of cotyledon and hypocotyl are potentially reflected in gene sets of either organ specific responding genes or gene sets with similar or different trends of transcriptional regulation. Gene ontology enrichment of different regulation pattern identified primarily categories unique to subgroups of organ specifically or in both organs responding genes.

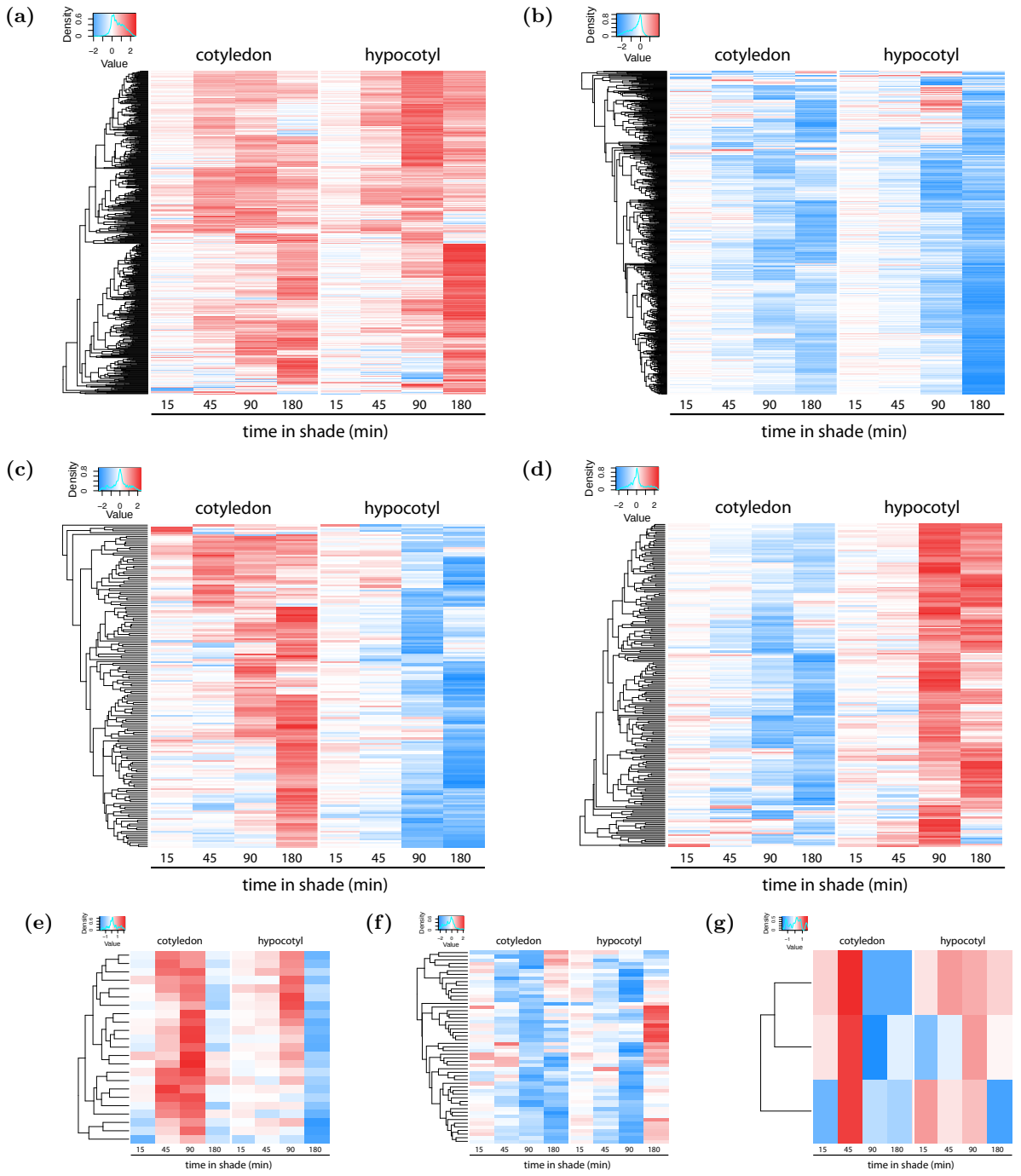

Figure 3.14: Heat map representation of hierarchically clustered and color-coded relative expression levels across all time points and organs.
Genes expressed in both tissues were subdivided into seven major groups by their general mode of regulation in response to low R/FR. (a-g) group ch1 - group ch7

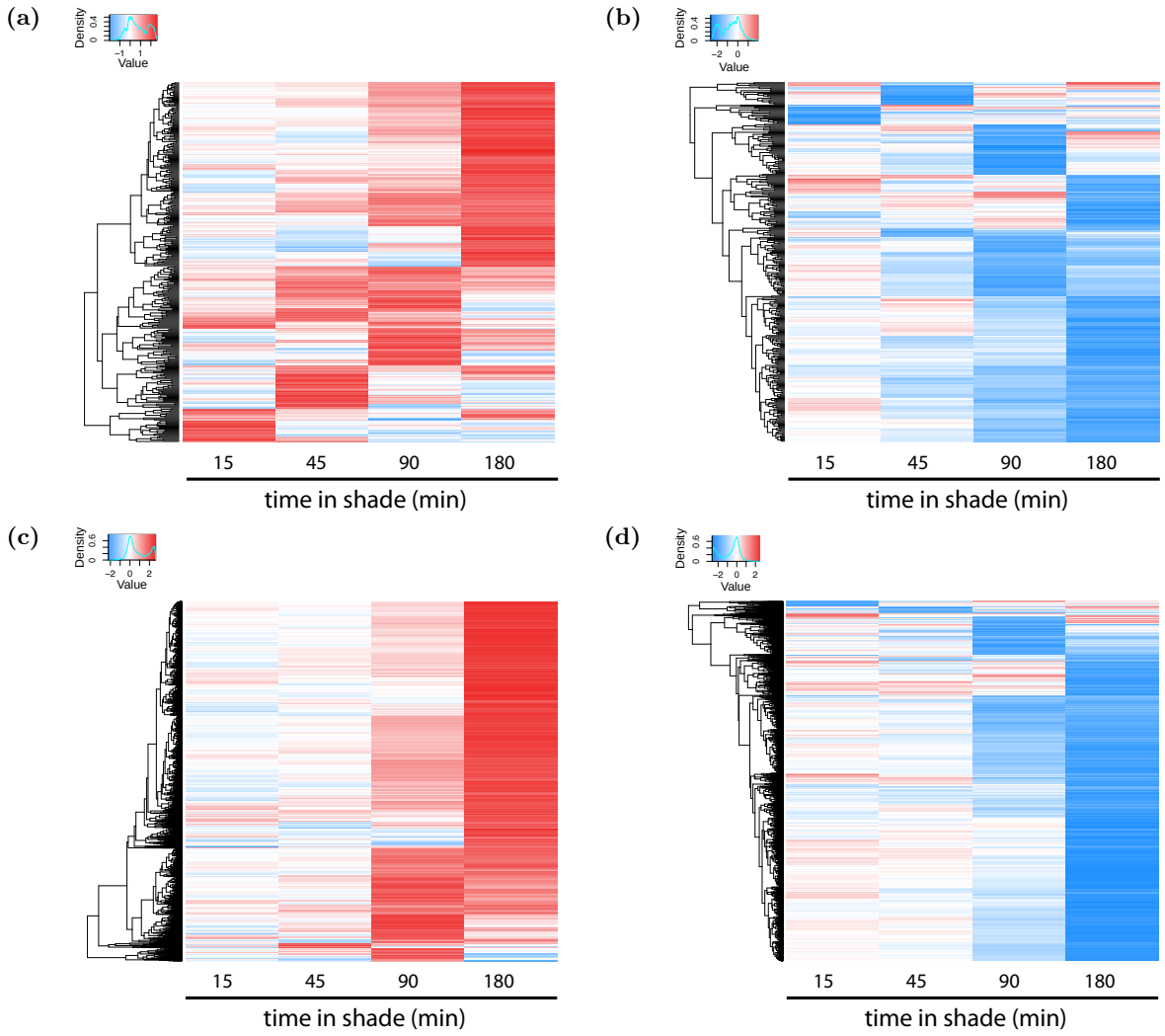

Figure 3.15: Heat map representation of hierarchically clustered and color-coded relative expression levels in low R/FR of organ specifically expressed genes.
Genes expressed either in cotyledon (a, b) or hypocotyl (c, d) were classified into two major groups.

Those few terms shared by several subgroups are rather general terms such as 'response to light stimulus' (GO:0009416). The few more specific terms are mentioned below.

Enriched terms describing responses to classical phytohormones were identified for auxin, gibberellin, abscisic acid and ethylene among upregulated gene responding in both organs (group ch1). Furthermore, this list holds the two terms 'regulation of ethylene mediated signaling pathway' and 'negative regulation of ethylene mediated signaling pathway'. The term 'auxin polar transport' is shared between upregulated genes in both organs (group ch1) and upregulated gene in cotyledons (group c1). Upregulated gene in both organs (group ch1) were further enriched for the categories 'cold acclimation' and 'response to cold'.

Genes, which were downregulated in both organs (group ch2), are enriched for the sugar-related terms glycoside, glucosinolate and S-glycoside metabolic process (GO:0016137, GO:0019760 and GO:0016143, respectively) as well as 'glycosinolate biosynthetic process' (GO:0019758). Additional sugar-related GO categories were identified for hypocotyl specific upregulated genes (group h1). Several of those terms describe modification processes of carbohydrate-sugar or nucleotide monosaccharide or monosaccharide derivatives ('glycosylation' (GO:0070085), 'protein amino acid glycosylation' (GO:0006486), glycoprotein metabolic process (GO:0009100), nucleotide-sugar metabolic process (GO:0009225), 'nucleotide-sugar biosynthetic process' (GO:0009226)). Additional sugar related categories for upregulated genes are 'polysaccharide biosynthetic process' (GO:0000271), 'carbohydrate biosynthetic process' (GO:0016051) and 'cellular carbohydrate metabolic process' (GO:0044262), while some sugar-related catabolic processes were downregulated specifically in hypocotyls (group h2; 'cellular carbohydrate catabolic process' (GO:0044275), 'carbohydrate catabolic process' (GO:0016052)). Further sugar-related terms enriched among downregulated hypocotyl specific genes (group h2) were 'monosaccharide metabolic process' (GO:0005996) and 'hexose metabolic process' (GO:0019318).

For opposite regulated gene between organs only one category, 'fatty acid metabolic process' (GO:0006631), in group ch4 was detected (downregulated in cotyledon while upregulated in hypocotyl).

The GO list for upregulated hypocotyl specific genes (group h1) include the general terms 'cell growth', 'epidermis development', 'epidermal cell differentiation' and 'shoot development' (GO:0016049, GO:0008544, GO:0009913, GO:0048367, respectively) as well as the transport related terms 'establishment of protein localization', 'vesicle-mediated transport', 'Golgi vesicle transport', 'nuclear transport' and 'intracellular protein transport' (GO:0048193, GO:0016192, GO:0006888, GO:0051169, GO:0006886, respectively). Enriched term for downregulated hypo-

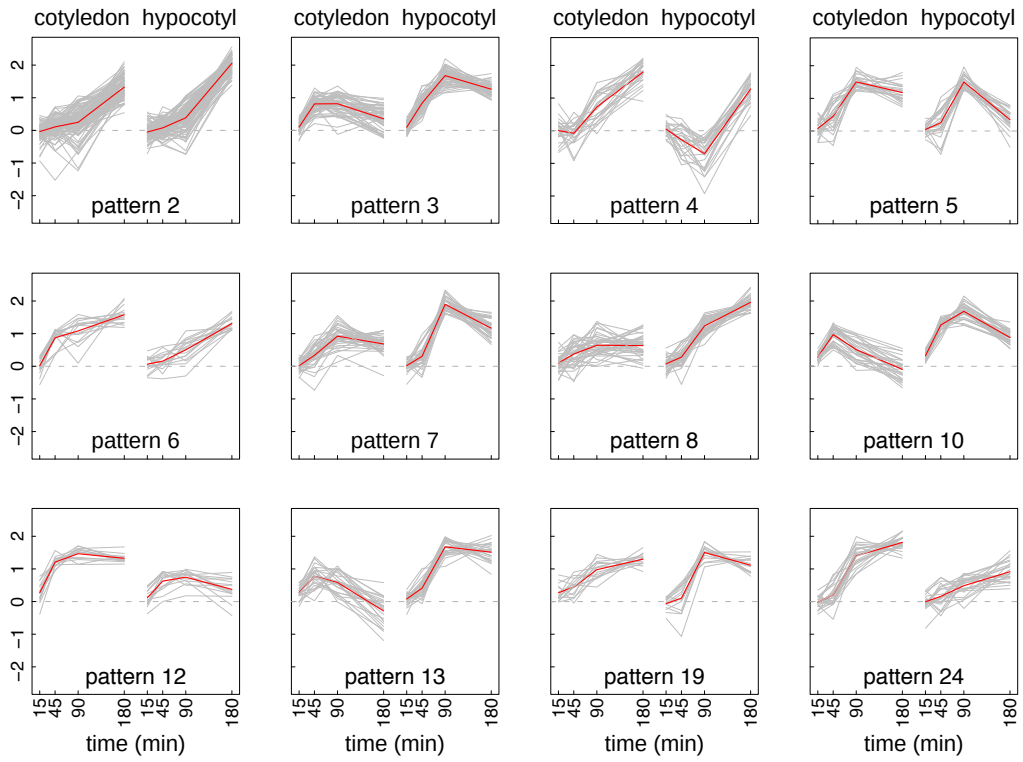

Figure 3.16: Relative expression pattern between low and high R/FR of upregulated genes in both organs.
Shown are the 12 most abundant relative expression pattern summarized in group ch1 of genes transcriptionally responding in cotyledon and hypocotyl to low R/FR. gray: relative expression pattern on single genes; red: average relative expression pattern.

cotyl specific genes (group h2) include several term related to photosynthetic processes such as 'photosynthesis, light reaction', photosynthesis, light harvesting', 'tetrapyrrole metabolic process', 'porphyrin metabolic process' and 'chloroplast organization' (GO:0019684, GO:0009765, GO:0033013, GO:0006778, GO:0009658 respectively). Also the plant defense-related terms 'defense response to bacterium' (GO:0042742) and 'innate immune response' (GO:0045087) were enriched for downregulated genes (group h2).

The list of enriched GO term of cotyledon specifically upregulated genes includes in addition to the above mentioned 'auxin polar transport term', 'flavoroid metabolic process' (GO:0009812), 'phenylpropanoid biosynthetic process' (GO:0009699) and 'phenylpropanoid metabolic process' (GO:0009698). No GO enrichment was found for cotyledon specific downregulated genes.

3.1.11 Genes with similar regulation directions are composed of various expression pattern

More precise regulation pattern within the previously described groups of genes responding in both organs as well as organ specifically responding genes (table 3.5) were defined. Each list was further subdivided by means of hierarchical clustering using average linkage and Pearson's

Table 3.6: Enriched GO categories for expression pattern ch3.

ID	name	Benjamini
GO:0009755	hormone-mediated signaling	6.07E-07
GO:0032870	cellular response to hormone stimulus	6.07E-07
GO:0009725	response to hormone stimulus	6.86E-07
GO:0009719	response to endogenous stimulus	8.92E-07
GO:0010033	response to organic substance	5.51E-06
GO:0009733	response to auxin stimulus	6.91E-06
GO:0009734	auxin mediated signaling pathway	1.94E-05
GO:0009416	response to light stimulus	7.42E-05
GO:0009314	response to radiation	8.56E-05
GO:0007242	intracellular signaling cascade	8.73E-05
GO:0009639	response to red or far red light	0.001632
GO:0009835	ripening	0.008232
GO:0009693	ethylene biosynthetic process	0.021302
GO:0009692	ethylene metabolic process	0.021302
GO:0043449	cellular alkene metabolic process	0.021694
GO:0043450	alkene biosynthetic process	0.021694
GO:0032535	regulation of cellular component size	0.041187
GO:0009628	response to abiotic stimulus	0.041435
GO:0008361	regulation of cell size	0.041515
GO:0009740	gibberellic acid mediated signaling	0.043164
GO:0010476	gibberellin-mediated signaling	0.043164

correlation as distance metric. The precise number of pattern was manually selected by two criteria. First, genes within the same group should homogeneously respond and therefore have no opposite regulation direction between time points compared to the average group pattern and second homogeneous pattern must not be further subdivides when the number of extracted pattern were increased. In group ch1 to group ch4 of low R/FR responding genes in cotyledon and hypocotyl, 55, 77, 11 and 37 pattern were extracted, respectively. No further pattern were extracted from group ch5 to group ch7.

In case of cotyledon specific shade-regulated genes 17 pattern were identified in group c1 and 13 in group c2, whereas for genes specifically responding in hypocotyls 30 and 56 pattern were defined for group h1 and h2, respectively.

Gene in group ch3 are enriched for several GO term related to phytohormones or light conditions such as 'response to auxin stimulus' or 'response to red or far red light', respectively (table 3.6). The relative expression pattern ch3 describes a fast upregulation during the first 45 min or 90 min in cotyledons or hypocotyls, respectively, followed by somewhat stably induced levels in both organs during the remaining time course. Furthermore, relative transcript levels increase with similar speed in both organ leading to higher FC values in hypocotyls at the later time points (figure 3.16).

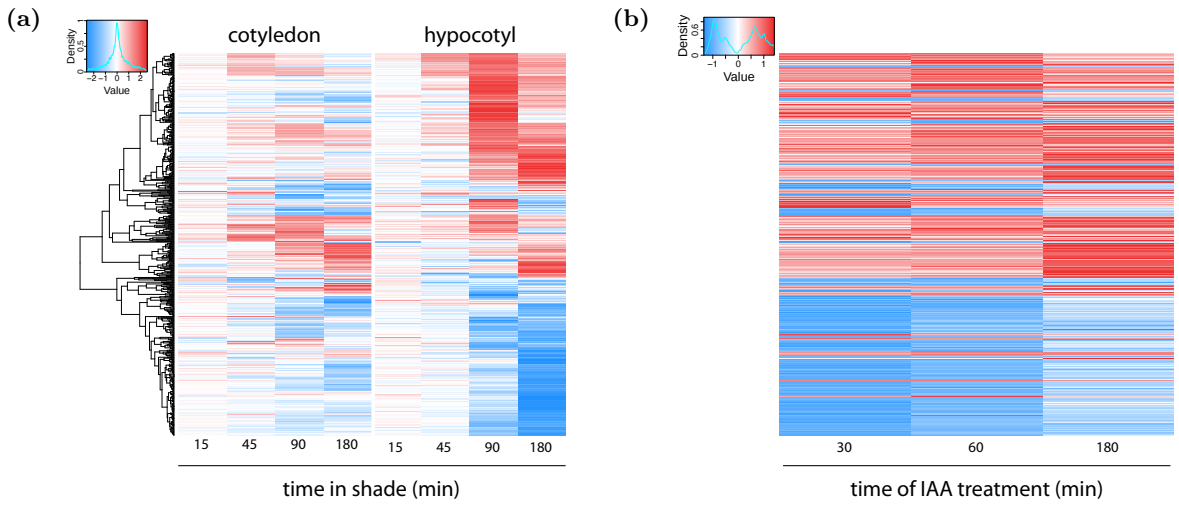

Figure 3.17: Transcriptional regulation of IAA responsive genes during shade avoidance. Heat map representation of hierarchical clustered low R/FR regulated relative transcript levels of IAA response genes reported by Nemhauser *et al.* (2006) (a) and published fold change values upon IAA treatment in similar order (b).

3.1.12 comparison to published data

Auxin responsive genes respond to low R/FR

Lists of upregulated gene for all time points in both organs were enriched for auxin responsive genes determined by gene ontology analysis (subsection 3.1.8). It is therefore interesting to analyze low R/FR induced transcriptional responses of auxin inducible gene of publicly available data. One prominent data set, which defined transcriptionally responding genes to treatment with IAA, was published by Nemhauser *et al.* (2006). In this publication, transcript levels of 30 min, 60 min and 180 min IAA treated seedlings were analyzed by means of microarrays and 791 genes were classified as up, down or complex regulated. From this list 94.3 % of genes were expressed in our time course data set and 76.6 % transcriptionally respond to low R/FR (figure 3.17). Using the classification of the hierarchical clustering analysis summarized in table 3.5 a large number of up or downregulated genes of the IAA list (81.02 %) respond in similar direction in both experiments. Furthermore, half of those genes had a hypocotyl specific response in our data set and about 6 % showed a cotyledon specific response, both, in cotyledon or hypocotyl.

Responses to auxin are dose-dependent (Tiwari *et al.*, 2001; Gray *et al.*, 2001). Different organs contain various auxin concentrations (Ljung *et al.*, 2001) and might therefore exhibit different transcriptional responses when treated with applied auxin. Recently, Chapman *et al.* (2012) identified auxin responsive genes in hypocotyls after dissection and picloram treatment for 30 min or 120 min. This data set provide an excellent opportunity to determine the similarity of transcriptionally responding genes to the two growth promoting factors, auxin and low R/FR,

focusing on hypocotyls. In our data set 70.77 % and 90.78 % of picloram responsive gene at time point 30 and 120, respectively, were expressed and further analyzed.

Shade-regulated relative transcript levels of picloram responsive genes were hierarchically cluster and visualized as heat map. Corresponding fold-change values published by Chapman *et al.* (2012) were visualized in a similar order and color code but independently from shade-regulated expression values (figure 3.18).

Most strikingly, all detected genes, which respond within 30 min to picloram, have a clear response to low R/FR in hypocotyls at TP45 and/or TP90. Furthermore, they are regulated in similar direction by picloram and low R/FR. Most of those genes do also respond in cotyledon but less pronounced than in hypocotyls. Interestingly, the majority of genes (71.74 %) showed the strongest response at TP45 in cotyledons, whereas in hypocotyls the highest differential regulation occurred later, at TP90 (figure 3.18a).

Also a large number of all genes, which transcriptionally respond to picloram treatment after 120 min, are differentially expressed between high and low R/FR (77.95 %). The strongest responses in hypocotyls to low R/FR were detected at TP90 and TP180 with roughly correspond with the length of picloram treatment. In hypocotyls, almost all (96.34 %) relative transcript levels are regulated in similar direction in response to picloram low R/FR. Transcriptional regulation in cotyledons is less similar between low R/FR and picloram treatment. Finally, the transcriptional response in cotyledons is milder than in hypocotyls (figure 3.18b).

Taken together, this analysis suggests, that genes responding to auxin respond similarly to low R/FR in hypocotyls. Furthermore, under low R/FR condition auxin responsive genes show different transcriptional regulation pattern in cotyledon and hypocotyl, which vary more with increasing time. Finally, the transcriptional response was stronger in hypocotyls and the highest amplitude for different organs occurred for several genes earlier in cotyledons than hypocotyls.

3.1.13 Auxin levels show no major changes during the first 45 min of low R/FR

Auxin can be synthesized in different organs of seedlings. The highest biosynthetic capacity and concentration have cotyledons or after emerging young leaves (Ljung *et al.*, 2001; Chen *et al.*, 2014). Auxin is required for a full shade avoidance response and free auxin levels increase in shoots or whole seedlings within 1 h of low R/FR treatment (Tao *et al.*, 2008; Hornitschek *et al.*, 2012; Li *et al.*, 2012). TAA1 is required for shade-induced increase of auxin concentration. The DR5-GUS auxin signaling marker shows induced response to shade in Col-0 but not in *taa1/sav3*

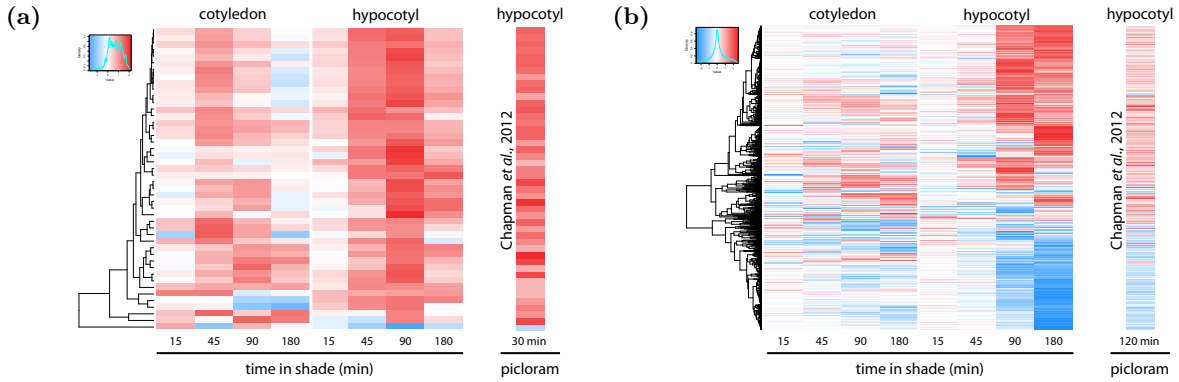

Figure 3.18: Transcriptional regulation of picloram responsive genes during shade avoidance. Heat maps representing hierarchically clustered relative transcript levels in low R/FR of gene responsive to 30 min (a) or 120 min (b) treatment with picloram in hypocotyls. The single column on the right hand side of each subfigure represents FC values as measured in Chapman *et al.* (2012). Experiments are color coded independently.

mutants. Furthermore, *sav3* does not show increased hypocotyl elongation in low R/FR conditions (Tao *et al.*, 2008). In addition, transport of auxin is essential for hypocotyl elongation in response to a low R/FR stimulus. Seedlings treated with auxin-transport inhibitor such as NPA exhibit no increased hypocotyl elongation or DR5-GUS signals in hypocotyls under low R/FR conditions (Steindler *et al.*, 1999; Tao *et al.*, 2008; Keuskamp *et al.*, 2010). As demonstrated by GUS-reporter constructs, *TAA1* is predominantly expressed at the margin of cotyledon of young seedlings, suggesting a low R/FR induced increase in auxin biosynthesis in the distal area of cotyledons. The speed by which auxin is transported has been reported for various species and organs. In *Arabidopsis thaliana* inflorescence stems auxin transport speed was reported with 7 mm h^{-1} (Kramer *et al.*, 2011). Measurements for cotyledon or leaf material of *Arabidopsis thaliana* are not available to date.

To first theoretically estimate in which time frame auxin can be transported from the outermost cotyledon margin to and through the hypocotyl, the length of cotyledon, petiole and hypocotyl was measured along the mid vein on day 6, 3.5 hours after dawn including 90 min of low R/FR treatment (figure 3.2c). On average cotyledon, hypocotyl and petioles were 1.508 mm, 1.507 mm and 0.495 mm long, respectively. Under the assumption that auxin travels in seedlings with a similar speed as reported for inflorescent stems, auxin transport over similar distances would roughly take 12.92 min in case of cotyledon or hypocotyl or 4.24 min in case of petioles. Therefore the transport of auxin from the tip of cotyledons to the top of hypocotyls can be expected to take about 17 min and the transport to the base of hypocotyls takes around 30 min.

In seedlings, shade-induced changes of auxin concentration depend on several processes including perception of a low R/FR signal, alteration of auxin biosynthesis rates and possibly auxin

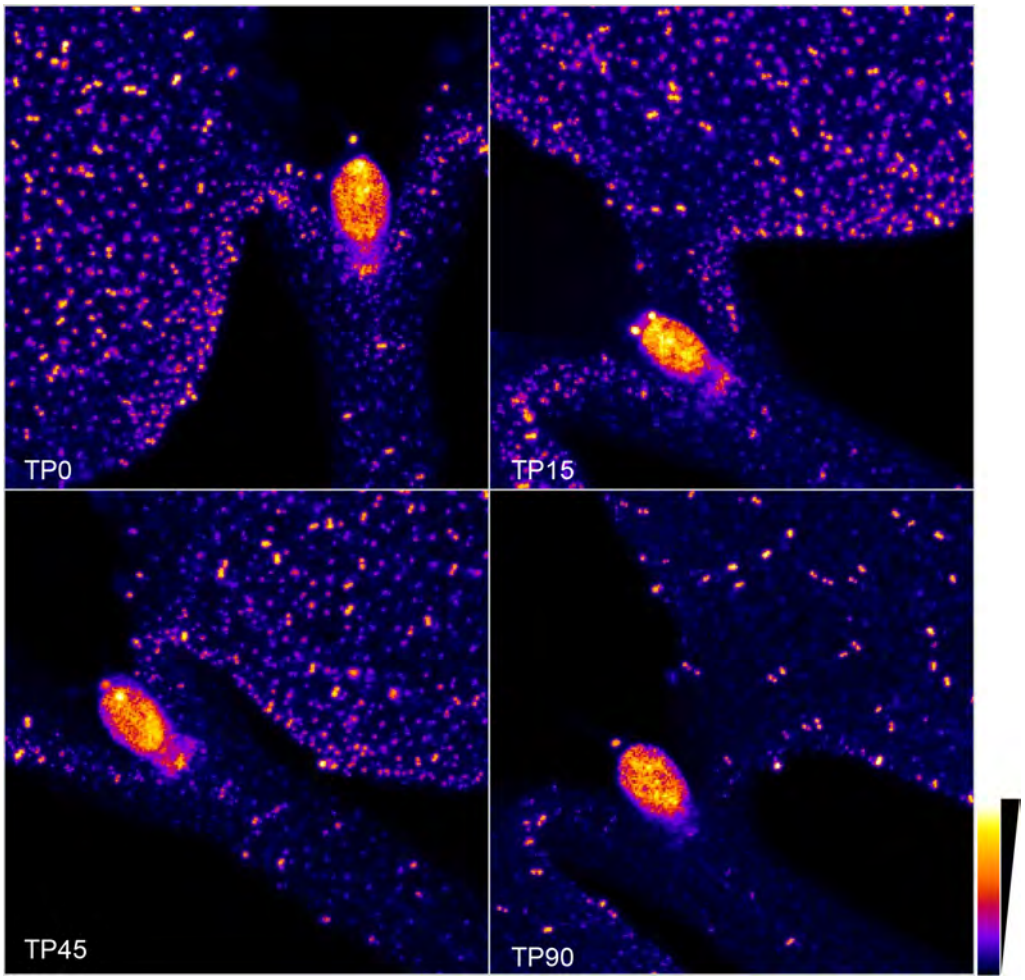

Figure 3.19: Fluorescence of the DII-VENUS-NLS auxin signaling sensor
DII-VENUS-NLS is degraded in the presence of auxin. False color image displaying DII-VENUS fluorescent signal intensities at different time points of low R/FR treatment at the base of cotyledons and the top of hypocotyls. The signal intensity in guard cells in not affected by low R/FR treatment and can be observed at all time points.

transport (Tao *et al.*, 2008; Keuskamp *et al.*, 2010). Auxin concentration were further investigated at similar time points of our RNAseq data set in order to analyzed at which time points changes in auxin level can be detected in which organ. Auxin concentration were indirectly monitored by confocal laser scanning microscopy using Col-0 plants expressing the 35S::DII-VENUS-NLS reporter gene construct. The DII-VENUS-NLS construct is composed of the auxin interacting DII domain of *IAA28*, the VENUS fluorescent protein and a nuclear localization signal (NLS) (Brunoud *et al.*, 2012). In the presence of auxin the DII domain interacts with the SCF-TIR1/AFB complex which leads to the degradation of the reporter construct hence reduced fluorescent signals indicate increased auxin levels (Brunoud *et al.*, 2012).

At TP0 and TP45 DII-VENUS-NLS signals were detected throughout the whole seeding. The emerging first leafs maintained a strong signal throughout the whole time course suggesting constant low auxin level in those organs. The same is true for guard cell, which were not affected by low R/FR. Also after 45 min of supplemental FR light treatment, no major changes in signal intensity were observed.

At TP90 DII-VENUS-NLS signals were strongly depleted in cotyledon, petiole and hypocotyl. This suggest that in those organs during the first 45 min auxin level do not significantly rise in response to low R/FR. Furthermore, after 90 min auxin level are increased throughout the whole shoot.

3.1.14 Transcriptional responses to low R/FR of auxin biosynthetic and signaling gene families

Auxin biosynthetic gene

The main auxin biosynthetic pathway in *Arabidopsis thaliana* is the TAA/YUCCA pathway (Mashiguchi *et al.*, 2011). Members of the both gene families have been shown to respond to low R/FR treatment and *sav3* or *yuc1yuc4* mutants exhibit sever reduced hypocotyl elongation in low R/FR (Tao *et al.*, 2008; Won *et al.*, 2011; Hornitschek *et al.*, 2012; Li *et al.*, 2012). Furthermore, the *yuc3yuc5yuc7yuc8yuc9* quintuple mutant has a mild hypocotyl shade phenotype (Li *et al.*, 2012). Among the *YUCCA* genes *YUC2*, *YUC3*, *YUC5*, *YUC8* and *YUC9* are transcriptionally induced by shade (Tao *et al.*, 2008; Won *et al.*, 2011; Brandt *et al.*, 2012; Li *et al.*, 2012; González-Grandío *et al.*, 2013). In our data set, all previously reported *YUCCAs* were transcriptionally regulated. In addition, our analysis identified *YUC6* as being shade-repressed in shade (figure 3.20b).

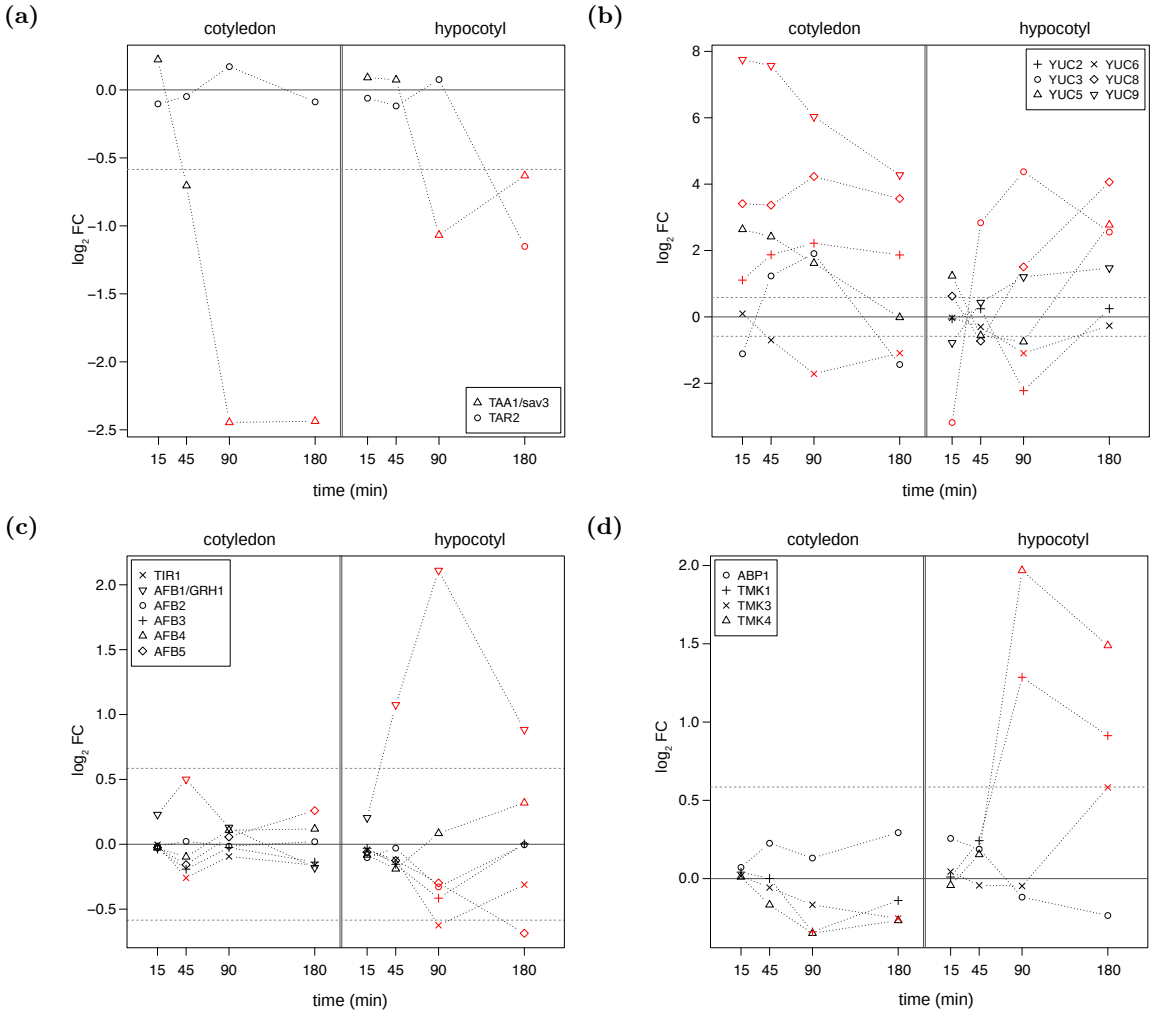

Figure 3.20: Relative transcription pattern of auxin biosynthetic and signaling genes
Relative transcription pattern of auxin related genes in cotyledon and hypocotyl between high and low R/FR. All expressed members of the auxin biosynthetic gene families TAA1/sav3 and yucca are shown in (a) and (b), respectively. (c) Relative transcript levels over time of the auxin receptor ABP1 and downstream signaling genes of the *TMK* family. Relative expression levels with a p values < 0.05 are shown in red.

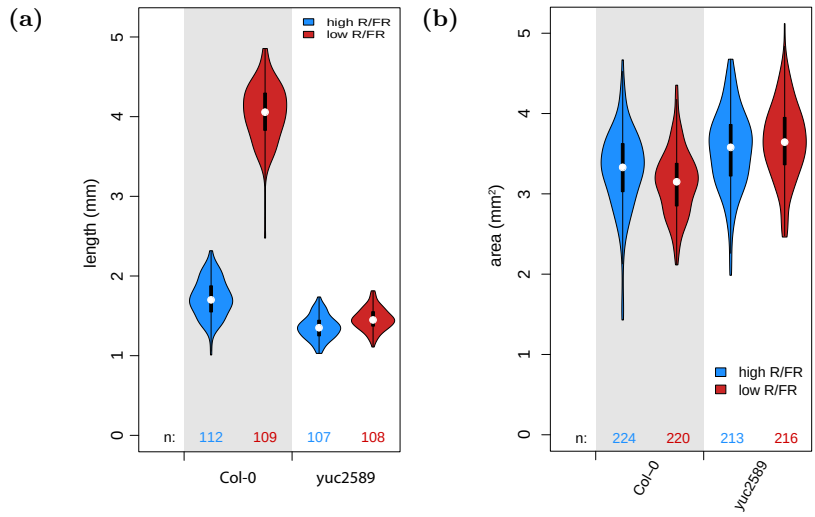

Figure 3.21: Hypocotyl length and cotyledon area measurement in high and low R/FR
Violin plot showing hypocotyl length (a) or cotyledon size (b) of *yuc2589* seedings grown for 3 days in high R/FR and subsequent 4 days in high or low R/FR.

Our data set also provide further insight into the organ specific regulation of *YUCCA* genes. *YUC2*, *YUC6* and *YUC8* were detected in both organs, while *YUC2* and *YUC8* were lowly expressed in hypocotyls. *YUC9* was cotyledon specific and *YUC3* and *YUC5* were hypocotyl specific regulated. Nevertheless, expression of *YUC3* and *YUC5* were low in hypocotyls and *YUC5* had intermediate expression levels in cotyledons.

YUCCAs had also a different temporal response to low R/FR. In cotyledon *YUC2*, *YUC8* and *YUC9* have induced transcript levels as early as 15 min of low R/FR treatment, while in hypocotyls *YUC2*, *YUC8* were detected at later time points. Also *YUC5* transcription is only induced at TP180.

As mentioned above, the *yuc3yuc5yuc7yuc8yuc9* quintuple mutant has only a mild mutant phenotype in low R/FR (Li *et al.*, 2012). This mutant has still a wild-type copy of one cotyledon-expressed *YUCCA*, *YUC2*. We hypothesized that shade-induced hypocotyl elongation depend on all three cotyledon induced *YUCCAs*. In contrast to the phenotype of previously analyzed *yuc* mutants, phenotypic analysis of the *yuc2589* quadruple mutant showed no shade-induced hypocotyl elongation in respond to low R/FR (figure 3.21).

The TIR1/AFB auxin receptor family

Auxin is perceived by two types of auxin receptors, the TIR1/AFB family of F-box proteins and the extracellular ABP1. In our experiment, all auxin receptors were strongly expressed. None of these seven receptors were transcriptionally regulated in cotyledons (figure 3.20c and 3.20d). In hypocotyls only *AFB1* was induced as early as TP45. In contrast, *AFB5* was downregulated

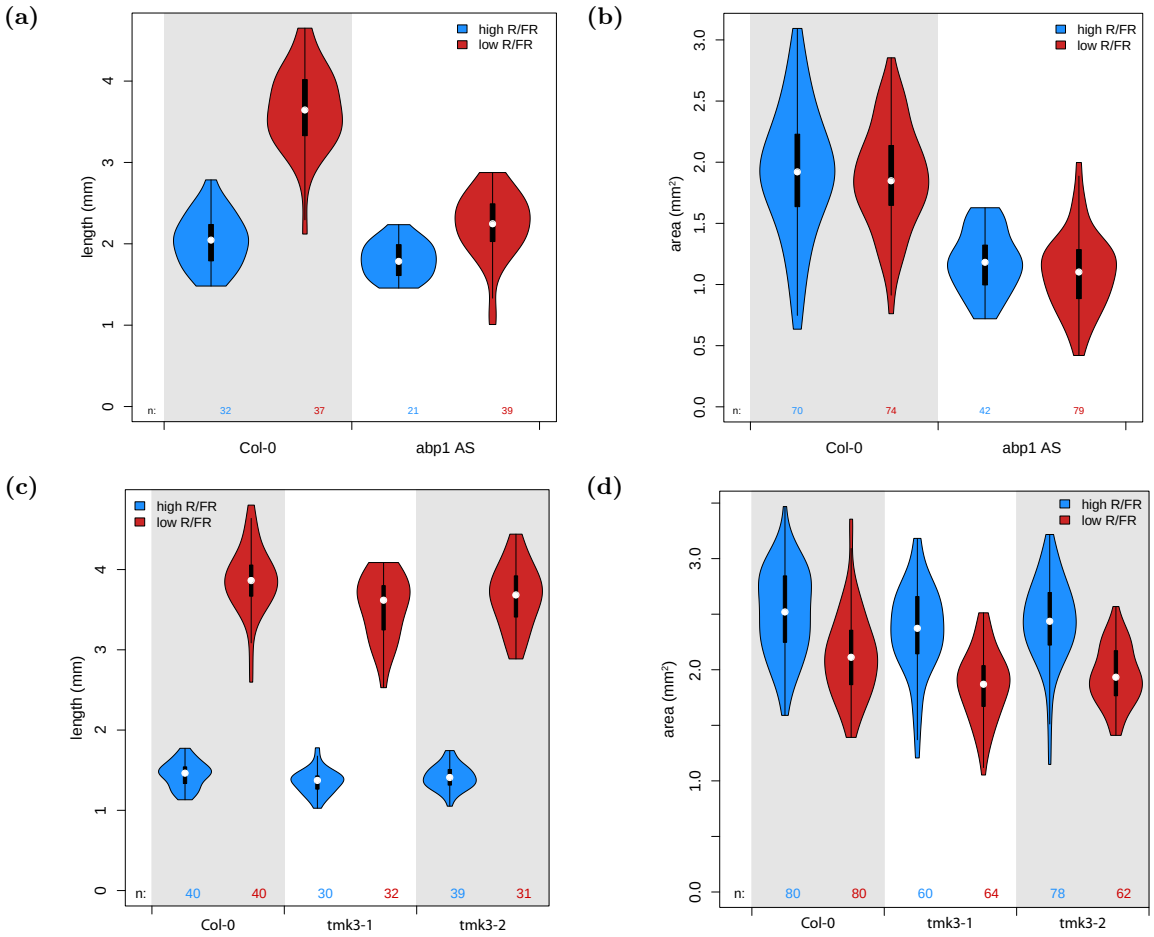

Figure 3.22: Hypocotyl length and cotyledon area measurement in high and low R/FR
Violin plot showing hypocotyl length (a, c) or cotyledon size (b, d) of *abp1* AS (a, b) or *tmk3* (c, d) seedings grown for 3 days in high R/FR and subsequent 4 days in high or low R/FR.

at TP180 and *TIR1*levels were transiently decreased at TP90.

ABP1 signaling

Early signaling components downstream of ABP1 are the receptor tyrosine kinase of the TMK family (Dai *et al.*, 2013). These integral membrane proteins were shown to interact extracellular with ABP1 and transmit signals inside the cell. Three of the four TMK genes of *Arabidopsis thaliana* were expressed in our data set. Interestingly, all three were induced specifically in hypocotyl although TMK3 had a FC of 1.497 and was therefore not identified in the global analysis (figure 3.20d). The fourth member, *TMK2*, was not included in the statistical analysis due to overall low expression levels. Therefore, two independent *tmk3* T-DNA insertion lines were tested for mutant phenotypes in low R/FR conditions. Both lines had a wild-type-like hypocotyl and cotyledon growth phenotype in low R/FR (figure 3.22c and 3.22d). Nevertheless, the hypocotyl elongation response to shade of the ethanol inducible ABP1AS transgenic line

(Braun *et al.*, 2008) was strongly impaired in simulated shade conditions (figure 3.22a and 3.22b).

Auxin-conjugating genes

The GH3 genes are early auxin induced genes and quickly respond upon low R/FR treatment (Tanaka *et al.*, 2002a; Staswick *et al.*, 2005; Park *et al.*, 2007; Nomoto *et al.*, 2012). Eight GH3s are able to catalyze the synthesis of IAA amide conjugates, which renders auxin inactive (Staswick *et al.*, 2005; Park *et al.*, 2007).

GH3.3 and *GH3.6* were earlier induced in cotyledon than hypocotyls, and *GH3.5* responded earlier in hypocotyls (figure 3.24a). *GH3.17* was classified as cotyledon specific whereas *GH3.9* was hypocotyl specifically downregulated at TP180.

The AUX/IAA family

The *AUX/IAA* genes respond quickly to change auxin levels and are involved in auxin perception by the TIR1 family (Abel *et al.*, 1995; Sauer *et al.*, 2013). As mentioned above different members respond at different time points to low R/FR (figure 3.23a to 3.23d). With few exceptions transcript levels of IAA genes were transiently or throughout the whole time course upregulated.

Moreover, several closely related IAAs had similar expression pattern such as *IAA5*, *IAA6* and *IAA19* (figure 3.23a). Finally, few IAA are organ specifically regulated in low R/FR. *IAA16*, *IAA32* and *IAA34* were cotyledon specific and *IAA9*, *IAA12*, *IAA18* and *IAA27* were hypocotyl specific regulated.

Among all *IAAs*, only in case of *IAA8*, *IAA10* and *IAA31* were no transcriptional changes detected for either cotyledon and/or hypocotyls

Auxin transcription factors

AUX/IAA genes interact with *ARF* and regulate transcription responses of downstream genes (Sauer *et al.*, 2013; Korasick *et al.*, 2014). Based on their protein sequence they are classified into six groups (Guilfoyle and Hagen, 2007). Only group 1, which is composed of five *ARFs*, showed positive transcriptional regulation of selected target genes in protoplasts experiments (Ulmasov *et al.*, 1999). Two group 1 ARFs, which are classified as activators, showed a transient response to low R/FR in our experiment. *ARF6* was repressed at TP45 in cotyledon and at the later TP90 in hypocotyls. *ARF19* responded hypocotyl specific at TP90 (figure 3.24b).

ARFs of group 2 to 5 contain a repression domain (Guilfoyle and Hagen, 2007). Interestingly, two group 2 ARFs, *ARF11* and *ARF18* and *ARF10*, a group 4 member, were oppositely regulated

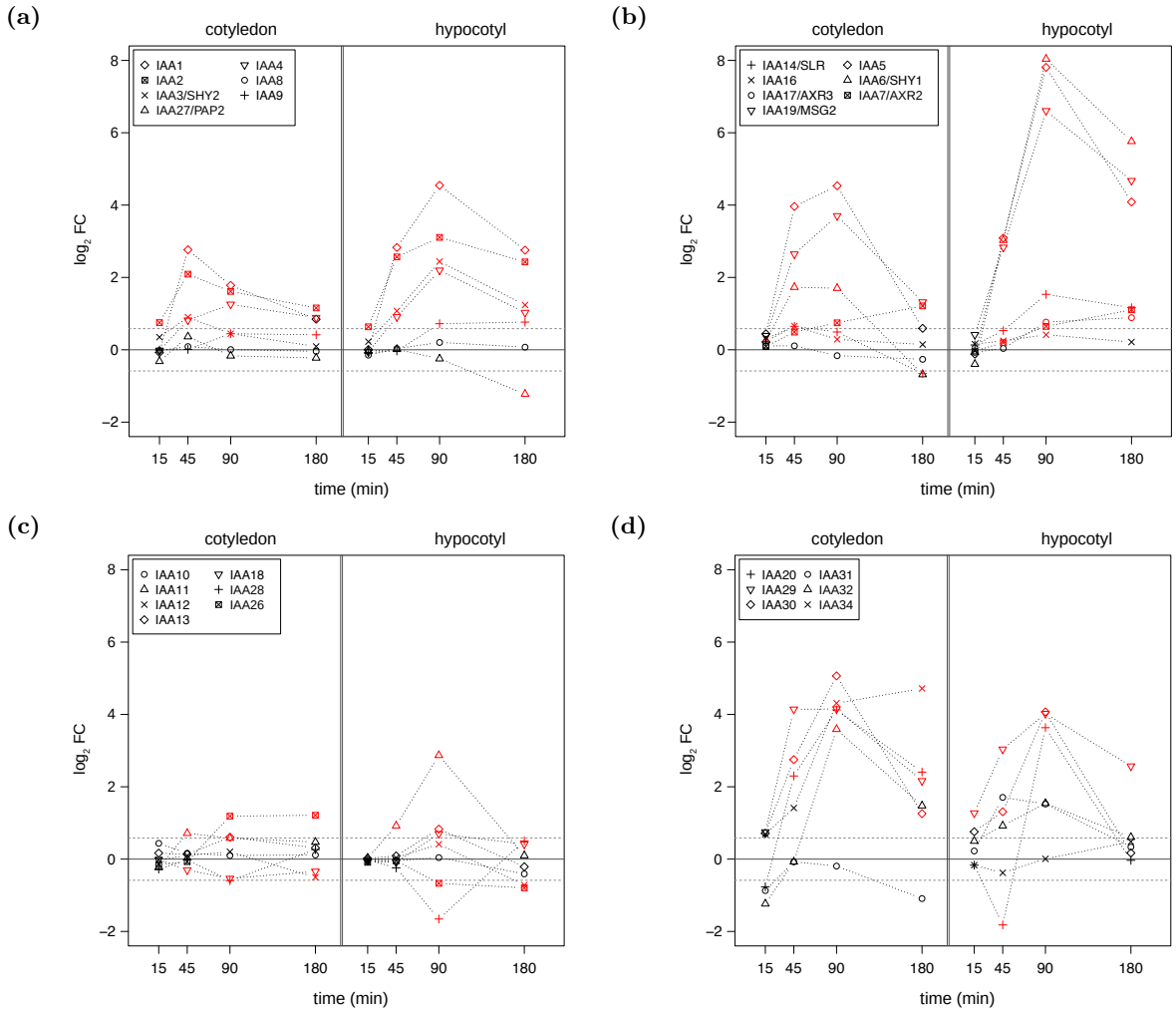

Figure 3.23: Relative transcript regulation of *Aux/IAA* genes between high and low R/FR in cotyledon and hypocotyls
(a-d) Expression patterns of *Aux/IAA* genes. *IAA* gene family was split in four groups based on the phylogenetic tree of whole amino acid sequences for visualization purpose. (e) Group II *GH3* genes. Relative expression levels with a p values < 0.05 are shown in red.

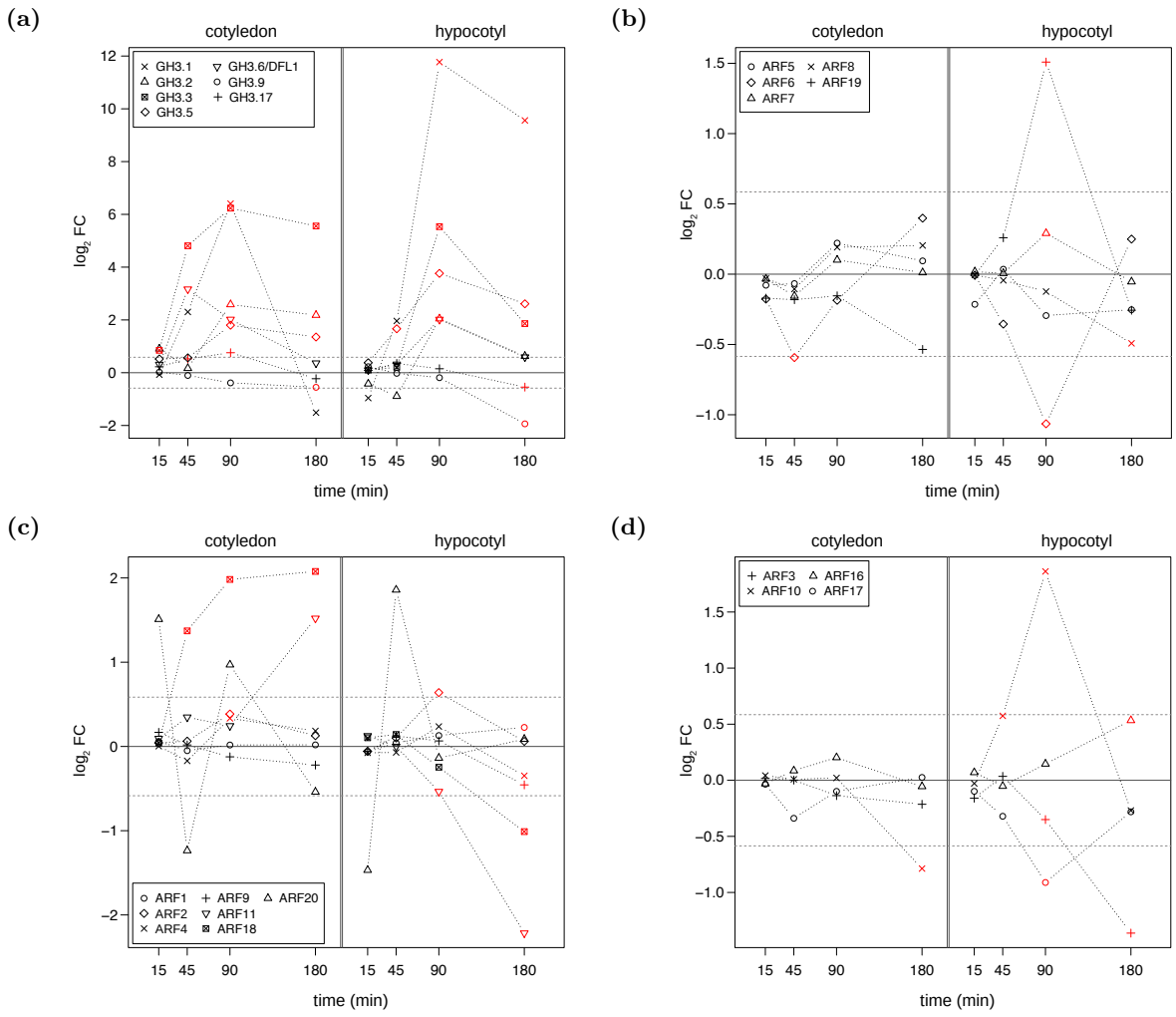

Figure 3.24: Relative transcription pattern of the *ARF* gene family
(a) Group 1 *ARFs*, (b) group 2 *ARFs* and (c) group 3, 4 and 5 *ARFs*. Relative expression levels with a p values < 0.05 are shown in red.

between cotyledon and hypocotyl, although *ARF10* was only transiently induced in hypocotyls (figure 3.24c and 3.24d).

Two additional *ARFs* responded transiently at TP90, either with induced (*ARF2*, group 2) or repressed (*ARF17*, group 5) relative transcript levels (figure 3.24c and 3.24d). Finally, *ARF3* was repressed at TP180 hypocotyl specifically (figure 3.24d).

Auxin transporters

Auxin transport is required for a full shade avoidance response. The auxin efflux carrier PIN3 has been show to play an important role during shade avoidance, since *pin3* have shorter hypocotyls than wild-type seedlings when exposed to a low R/FR regime (Keuskamp *et al.*, 2010). This suggests a role for PIN3 in the export of auxin from cotyledons and transport through petioles and hypocotyls.

In our experiment *PIN3*, *PIN4* and *PIN7* had induced relative expression levels in cotyledons. In hypocotyls all three *PINs* are induced within the first 90 min, while *PIN7* remains upregulated at TP180 (figure 3.25a). We hypothesized that *pin3pin4pin7* triple mutants are further impaired in low R/FR induced hypocotyl elongation. Therefore I measured hypocotyl elongation and cotyledon area of seedlings grown for three day in high R/FR followed by additional four days of high or low R/FR. Indeed, *pin3pin4pin7* triple mutants showed no elongation response to low R/FR. As expected *pin3* showed impaired hypocotyl elongation, but still responded to low R/FR. This demonstrates that several PINs are important for shade induced hypocotyl elongation and *PIN3*, *PIN4* and *PIN7* are required for shade-induced auxin transport (figure 3.26).

PIN2 has been previously described as root expressed PIN protein (Luschnig *et al.*, 1998). In our data set *PIN2* is hypocotyl specific upregulated in response to low R/FR. Therefore, *pin2* and *pin2pin3* were included in the hypocotyl growth assay (figure 3.26). Also *pin2* mutants were impaired in shade-induced hypocotyl elongation, and this phenotype was further enhanced in the *pin2pin3* double mutant.

Among group 2 PINs, only PIN6 has altered relative transcript levels at TP180 in hypocotyls (figure 3.25b).

A second type of transporters, which were recently identified, is the PIN-LIKES or PILS family of putative auxin carriers (Barbez *et al.*, 2012). Two of the seven *PILS* had significantly altered transcript levels. *PILS5* was downregulated in cotyledon and upregulated in hypocotyl at similar time points. Similarly, *PILS3* displayed opposite regulation pattern although FC values between high and low R/FR in cotyledon stayed below 1.5 fold (figure 3.25c).

Spatio-temporal analysis of auxin responsive genes in low R/FR

As described above, data of several research groups provide evidence that the main source of shade-induced auxin levels in seedlings are the cotyledon. Subsequently, auxin is transported downwards, and once reaching the hypocotyl, enhanced concentrations induce the expression of growth promoting genes (de Wit *et al.*, 2014). Base on this model, we hypothesized that auxin responsive genes follow in their relative transcriptional response to low R/FR the induced levels of auxin and consequently respond early in cotyledon and later in hypocotyls.

To evaluate how many auxin responsive genes are regulated in low R/FR condition accordingly to our hypothesis, a list of auxin responsive genes was defined and subsequently analyzed for the initial response to low.

The list of auxin responsive genes was defined as the union of auxin responsive gene identified

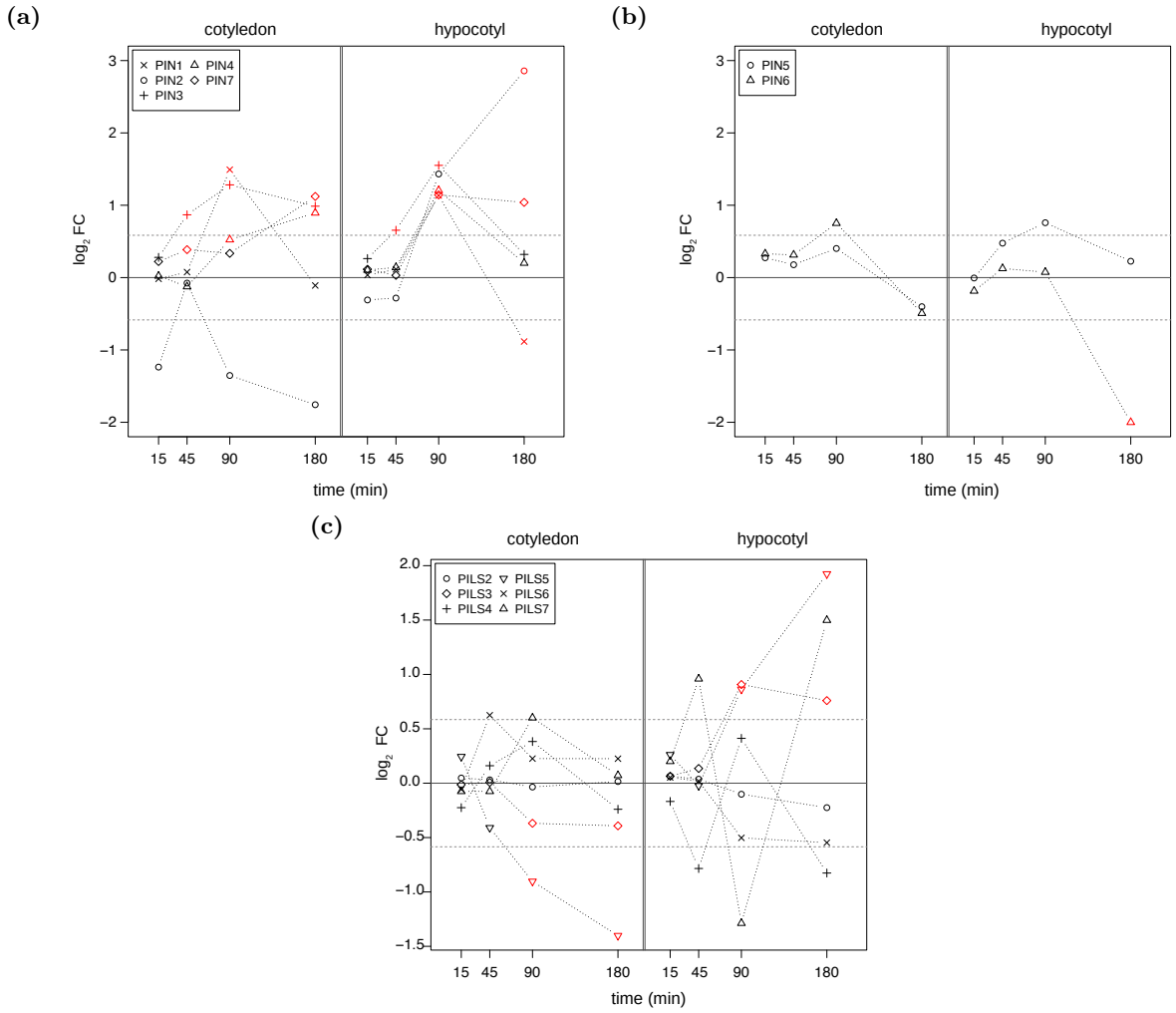

Figure 3.25: Relative transcript level of auxin transport genes
(a) group I *PINs* (b) group II *PINs* and (c) *PILS*.

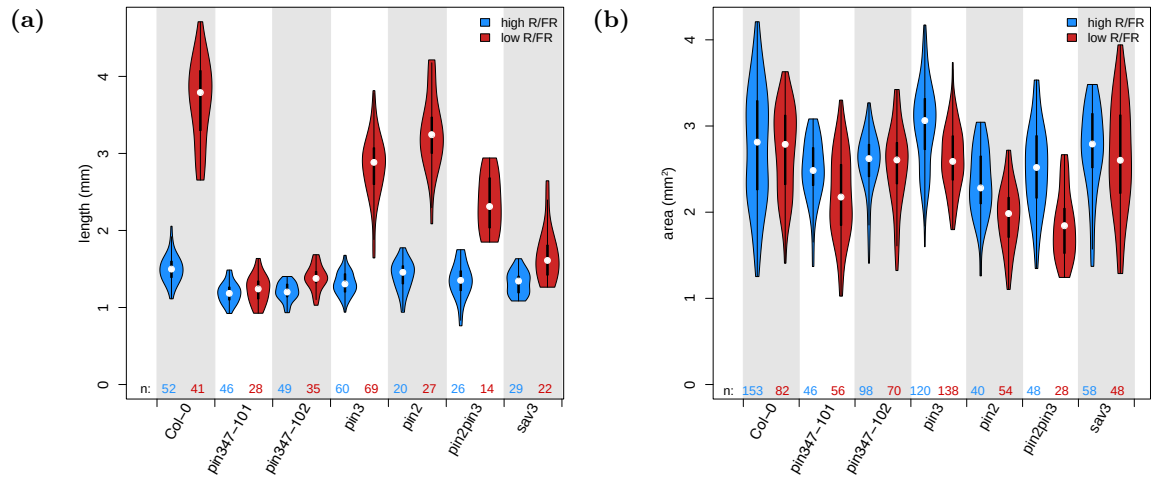

Figure 3.26: Hypocotyl length and cotyledon area measurement in high and low R/FR
Violin plot showing hypocotyl length (a) or cotyledon size (b) of *pin3pin4pin7*, *pin3*, *pin2*, *pin2pin3* and *sav3* seedlings grown for three days in high R/FR and subsequent four days in high or low R/FR.

Table 3.7: Auxin responsive genes classified by their earliest transcriptional response in cotyledon and hypocotyl to low R/FR
Auxin responsive genes were split in three groups according to the first observed response. IDs of subgroups refer to time points in cotyledons followed by hypocotyls.

first cotyledon	no	simultaneous	no	first hypocotyl	no
15-45	12	15-15	16	45-15	10
15-90	7	45-45	67	90-15	3
15-180	2	90-90	148	90-45	24
45-90	65	180-180	51	180-15	1
45-180	6			180-45	8
90-180	40			180-90	79
sum	132		282		125
	24.49 %		52.32 %		23.19 %

by Nemhauser *et al.* (2006) and Chapman *et al.* (2012) at all different time points. This list comprised 1759 of which 1595 genes are expressed in our data set. Of those genes, a total of 539 genes respond at least at one time point in both organs and are therefore suited for further analysis.

All 539 genes were classified by the time point of their first shade-regulated expression in each organ and further grouped into three categories: first cotyledon, first hypocotyl or detected at a similar time point (table 3.7). This analysis was done with adjusted p value < 0.05 and an absolute FC > 1.5. Half of the genes could be detected simultaneously and 25 % respond either earlier in cotyledon or hypocotyl. To determine it the choice of a FC > 1.5 affects the outcome of the classification the significance requirements were redefined. For different re-classifications, the adjusted p value < 0.05 was combined with either non or various FC requirements between 1.5x and 5x. As expected different FC criteria affected the overall gene number of responding genes. Nevertheless, the ratio between the categories 'first cotyledon', 'first hypocotyl' and 'simultaneous' remained constant.

It cannot be excluded, that some of the analyzed genes might be able to respond to low R/FR in an auxin independent fashion. *sav3* have a short hypocotyl in low R/FR conditions and have no significantly altered free auxin level in response to low R/FR (Tao *et al.*, 2008; Hersch *et al.*, 2014). Assuming that *sav3* mutants have no altered free auxin concentrations under low R/FR and maintain the relative auxin distribution within entire plants, shade-regulated gene of *sav3* mutants (Tao *et al.*, 2008) were excluded from the 539 auxin-responsive genes.

The remaining 62 genes were reanalyzed. The percentage of gene responding earlier in cotyledon increased to 33.87 % (21), while simultaneously responding genes remained the most dominant group with 50 % (31). 10 genes (16.13 %) responded earlier in hypocotyls compared to

Table 3.8: Auxin responsive genes grouped by regulation maxima in low R/FR condition
Auxin responsive genes of group ch1 to ch4 were classified by time points of strongest regulation. Group IDs describe corresponding time points in cotyledons followed by hypocotyl. $^{+}$ and $^{-}$ refer to all later and earlier time points, respectively.

first cotyledon	no	simultaneous	no	first hypocotyl	no
15-15^{+}	5	15-15	0	45-45^{-}	0
45-45^{+}	47	45-45	5	90-90^{-}	1
90-90^{+}	83	90-90	88	180-180^{-}	64
		180-180	164		
sum	135		257		65
	29.54 %		56.24 %		14.22 %

cotyledons.

The previous analysis of shade induced transcript levels of picloram responsive gene demonstrated, that several gene had the highest amplitude of shade-regulated transcript levels earlier in cotyledon than hypocotyl unrelated to the overall intensity of regulation (figure 3.18a). It is therefore also interesting to compare the time points of maximum response in cotyledon and hypocotyl. Due to possible different regulation kinetics in cotyledon and hypocotyl, both analysis of first response and maximum response might provide differences of shade-induced responses in both organs. The analysis of maximum responses was limited to the previously used 539 auxin-responsive genes, which belong to group ch1 to ch4 (table 3.5). About 50 % of the analyzed genes had a maximum regulation at similar time points in cotyledon and hypocotyls. More genes had earlier regulation maxima in cotyledons (29.54 %) than hypocotyls (14.22 %; table 3.8).

Induced free auxin levels in response to low R/FR were reported for the first hour of treatment in *Arabidopsis thaliana* (Tao *et al.*, 2008; Li *et al.*, 2012; Hornitschek *et al.*, 2012; Hersch *et al.*, 2014). In addition, DII-VENUS-NLS signal analysis suggests an increase of auxin levels within the first 90 min of low R/FR treatment (figure 3.19). Assuming that transcriptional responses occur quickly after auxin level changed, the first three time points might be the more sensitive time points in order to observe the dynamic regulation. The analysis of regulation maxima was therefore repeated excluding TP180. 38 genes had an earlier maximum response in cotyledon, 92 responded at similar time points and only 1 gene showed earlier response maxima in hypocotyls. Therefore, restricting the analysis to the first 90 min affected primarily the group reaching first regulation maxima in hypocotyls.

3.2 Discussion

3.2.1 Cotyledon expressed auxin biosynthetic genes are essential for shade induced hypocotyl elongation

Previous studies have established the importance of cotyledons for shade perception and induction of growth responses in hypocotyls. Shade treatment of *Brassica raps* cotyledons is sufficient to induce hypocotyl elongation (Procko *et al.*, 2014). In *Arabidopsis thaliana* phyB mutants are rescued by mesophyll-expressed phyB-GFP (Endo *et al.*, 2005). Also GUS reported gene expression in hypocotyls in response to low R/FR perception in cotyledons point towards the existence of an inter-organ signal.

Cotyledons are thought to be the major site of shade-induced auxin production. The auxin biosynthetic gene *TAA1* is predominantly expressed at the margin of cotyledons, and *sav3* hypocotyls fail to elongate in low R/FR. The *YUCCA* genes mediate a rate limiting step downstream of *TAA1* in the auxin biosynthetic pathway. Both functional *TAA1* and *YUCCAs* are required for shade avoidance, since the *sav3* phenotype can be rescued by overexpressing *YUC1* (Stepanova *et al.*, 2011; Won *et al.*, 2011). Nevertheless, hypocotyls of single *yuc* mutants as well as *yuc3yuc5yuc7yuc8cuy9* still respond to shade. In our time course experiment we show that *yuc2*, *yuc8* and *yuc9* have induced transcript levels in cotyledons and in low R/FR conditions, suggesting that *YUC2* can mediated the remaining response in the quintuple mutant. Our phenotypic analysis of *yuc2589*, which had no elongated hypocotyls in low R/FR, further support the importance of those four corresponding *YUCCA* genes. Since we did not observe a transcriptional response of *YUC5* in cotyledons, it is tempting to speculate that *YUC2*, *YUC8* and *YUC9*, but not *YUC5* play a dominant role during shade avoidance. Consequently, a *yuc2yuc8yuc9* mutant would respond similarly to shade compared to the quadruple mutant.

Interestingly, neither *YUC2* nor *YUC9* were found in ChIPseq experiments with *PIF4myc* or *PIF5-HA*. *YUC9* was described as direct target gene of PIF7 and PIF3 (Li *et al.*, 2012; Zhang *et al.*, 2013). *YUC2* has a single PBE box about 450 bp upstream of the transcriptional start site, but no PIF binding has been reported up to date. Furthermore, *YUC2* is similarly regulated in microarray experiments of *pif4pif5* and Col-0 (Hornitschek *et al.*, 2012) and showed no shade induction in *pif7* indicating that *YUC2* act downstream of *PIF7* but not PIF4 or PIF5. It is therefore likely that *YUC2* is a direct target gene of PIF7.

Almost all cells are capable of producing auxin and different members of the *YUCCA* family have different expression domain (Cheng *et al.*, 2006; Zhao, 2014). Plants mutated in all root-

expressed *YUCCAs* cannot be rescued by shoot expressed *YUC3* transgenes, demonstrating that in some contexts local auxin production is essential. Several *YUCCAs* in our experiment were expressed in hypocotyls, raising the possibility that shade-induced auxin production may also occur in hypocotyls, presumably to a lower extent than in cotyledons. Nevertheless, the phenotype of *yuc2589* makes it highly unlikely that potential hypocotyl-derived auxin in low R/FR contribute significantly to hypocotyl elongation. The impact of organ specific shade-induced auxin production could be further analyzed by cotyledon specific knockdown of various members of the *YUCCA* family.

3.2.2 Transport of shade-induced auxin requires multiple PINs

Shade induced auxin production is assumed to be predominantly located in cotyledons of young seedlings (de Wit *et al.*, 2014). This implies that auxin need to be transported downwards through the seedling in order to mediated growth responses e.g. in hypocotyls. Chemical treatments with synthetic auxin transport inhibitors demonstrated the general importance of PAT. In our experiment *PIN3*, *PIN4* and *PIN7* were upregulated in low R/FR. In addition, all three *PINs* are direct target genes of PIF4 or PIF5, demonstrating a PIF dependent regulation in shade (Hornitschek *et al.*, 2012; Oh *et al.*, 2012). Hypocotyl elongation in low R/FR of *pin3* and *pin3pin4pin7* shows that several PINs are involved in this process, and that *PIN3*, *PIN4* and *PIN7* are sufficient to prevent hypocotyls from elongating (figure 3.26 and Keuskamp *et al.,* 2010). PIN3 has been reported to relocate to the lateral side of cell upon low R/FR perception (Keuskamp *et al.*, 2010) indicating that PINs also mediate increased lateral transport in low R/FR. Therefore, it cannot be ruled out that impaired lateral transport in *pin3pin4pin7* contribute to the growth inhibition in shade. Lateral transport in hypocotyls might be important in order to ensure that auxin, transported downwards in the endodermis, reaches other cell types such as the epidermis. This might be further important, since the epidermis can be growth limiting (Savaldi-Goldstein *et al.*, 2007). As discussed in the previous section (section: 3.2.1) it is possible that auxin is to a minor extent produced also in hypocotyls during shade avoidance. Impaired lateral auxin transport in *pin* mutants most likely also interferes with locally produced auxin in hypocotyls. It would be interesting to investigate PAT in organ specific-manner. A cotyledon specific knockdown of *PIN3*, *PIN4* and *PIN7* could help to distinguish between cotyledon-derived auxin and local auxin production in hypocotyls. Our RNAseq data set could help to select organ specific promoters. Based on absolute read count number, their quotient and difference between cotyledon and hypocotyl in the RNAseq experiment as well as circadian

expression pattern extracted from genevestigator, candidate cotyledon specific promoters are regulatory upstream sequences of *CAB3*, *GLP1* and a DEFL family gene (At3g05730).

PIN2 cannot compensate for *pin3pin4pin7* triple mutations. However, *pin2* mutants display a mild hypocotyl phenotype. *PIN2* is not expressed in cotyledons, which is reflected in less than one read per library of cotyledon samples. Therefore it could be hypothesized that PIN2 is not involved in auxin export from cotyledons, but plays a minor role in transporting auxin in hypocotyls tissues. This transport could be basipetally through the hypocotyls as well as lateral between different tissue types.

3.2.3 Auxin responsive genes are enriched after 15 minutes of low R/FR treatment

Free auxin levels change within 1 h in response to shade, as demonstrated by several groups. In our experiment, significantly differentially regulated genes at TP15 were already enriched for auxin responsive genes in both organs. Changes in free auxin levels through new auxin biosynthesis in such a short time appear to rather unlikely. Our analysis of DII-VENUS signals indicates no major changes in auxin concentration within the first 45 min of low R/FR. In addition, the delay, by which changes in auxin levels are translated into changes in fluorescent signals, does not support different auxin levels within 15 min.

It is rather likely that shade induces several genes of the gene ontology term 'response to auxin stimulus ' in an auxin independent manner. In fact, several transcription factors were found at TP15, such as *ATHB-2*, which is directly regulated by PIFs (Kunihiro *et al.*, 2011). An additional gene, regulated at TP15, was *IAA2*. Several *IAA* respond as early as 5 min to applied IAA, and are therefore likely candidates of induced genes in shade by altered auxin concentrations. Nevertheless, *IAA2* is a potential target of PIF1, PIF3, PIF4 and PIF5, base on high throughput experiments. Its promoter contains a PBE box in a distance of about 20 bp to the center of the PIF5 binding peak (Hornitschek *et al.*, 2012). One of the most sensitive *Aux/IAAs* to auxin is *IAA5* (Abel *et al.*, 1995). It is no a reported direct target gene of any PIF investigated to date, and might therefore not be regulated auxin-independently. In our experiment, *IAA5* responded as early as TP45 in both organs. Transcriptional induction rates for *IAA1* to *IAA14* to auxin treatments were reported by Abel *et al.* (1995). Interestingly, the ranked list of induction rates measured after 15 min of IAA treatment, correlates well with the low R/FR induced relative transcription levels at TP45 in both organs. Taken together, the nature of the identified genes at TP15 does not provide evidence for altered auxin level at

this time point. *IAA* expression levels at TP45 rather suggest initial changes in free IAA levels around 45 min of low R/FR treatment.

3.2.4 Temporal analysis of transcriptional regulation of auxin responsive genes

Recently it has been shown, that upon low R/FR perception an auxin gradient forms in *Brassica rapa* hypocotyls. Furthermore, several genes with correlating transcript levels were identified (Procko *et al.*, 2014). Our time course analysis of auxin responsive genes, which were induced by shade, showed, that in *Arabidopsis thaliana* several genes responded later in hypocotyls than cotyledons, indicating that cotyledon derived shade-induced auxin was transported to the hypocotyl. Such genes included *GH3.3*, *GH3.6* and *IAA6* (figure 3.23). In total 25 % of auxin responsive gene exhibited an earlier response in cotyledons, showing that a considerable amount of genes support the hypothesis that genes in hypocotyls respond to cotyledon derived shade-induce auxin. Nevertheless, a large fraction (50 %) were regulated at the same time in both organs, demonstrating that the dominant fraction do not follow transcriptionally the proposed increase in auxin levels. It is likely that several of those genes can be regulated by shade in an auxin independent fashion, which could mask the time point of the initial response to auxin. A large fraction (about 20 %) of our initial gene set consists of putative direct target genes of PIF4 and/or PIF5 suggesting a possible regulation by phyB and auxin signaling pathways. Genes responding first in cotyledon or simultaneous in both organs contain similar fractions of direct target genes, suggesting a similar effect on both lists.

Nevertheless, several assumptions have to be made in order to draw direct conclusions from the above-mentioned ratios. One assumption is that gene expression is regulated with similar transcriptional rates in both organs upon signal perception. Transcription rates might also differ between cell types and in addition might depend on various regulatory factors and their intracellular concentration.

Free IAA measurements and our DII-VENUS-NLS reporter analysis suggest that increases of auxin levels can be detected between 45 min and 60 min (Tao *et al.*, 2008; Hornitschek *et al.*, 2012; Li *et al.*, 2012). In agreement with this, the most genes responding earlier in cotyledons were detected between TP45 and TP90. It is therefore possible, that a larger fraction of genes with different response times in cotyledons and hypocotyl can be identified with additional time point.

3.2.5 The ABP1 signaling genes of the TMK family are hypocotyl specifically induced to shade

Auxin binding protein 1 is the first described auxin receptor. Recently Dai *et al.* (2013) reported that APB1 interacts with integral membrane proteins of the TMK family and thereby potentially transmit signals to the interior of cell. This war further supported by various growth phenotypes to higher order *tmk* mutants. Three of the four transmembrane kinases, encoded in the genome of *Arabidopsis thaliana*, are induced in hypocotyls in response to low R/FR treatment (figure 3.20c). They were previously not identified in our microarray analysis as shade regulated gene. Single knock-out mutants of the *TMK* family have no reported phenotype suggesting an compensatory mechanism between *TMKs*. The two *tmk3* T-DNA insertion lines I phenotyped, show a wild-type-like hypocotyl elongation to low R/FR. This either suggests that low R/FR-mediated signaling responses of TMK3 can be compensated by additional members of the TMK family or that *TMK3* is not involved in this response.

All higher order mutants containing a T-DNA insertion in *tmk1* and *tmk4* are impaired in hypocotyl elongation when grown in dark, which is due to reduced cell size (Dai *et al.*, 2013). Such *tmk* mutants have also reduced cell numbers in leaf leading to smaller organs. It is therefore thought, that TMKs play different roles in different tissues. It is tempting to speculate that higher order mutants show reduced elongation growth of hypocotyls in low R/FR conditions, in particular mutant combinations with *tmk1* and *tmk4*.

As embryonic leafs, cotyledons expand mainly by cell elongation (Stoynova-Bakalova *et al.*, 2004) which is not primarily affected in leaves of *tmk* mutants. In agreement with stable transcript levels in cotyledons, these organs might be in general unaffected in *tmk* mutants by low R/FR in contrast to leaves.

In addition, *PIF5* binds to chromatin only few bp upstream of the *TMK1* 5' UTR, 117 bp upstream of the ATG. The whole intergenic region upstream of TMK1 does not contain any G-box sequence. The most likely PIF5 binding motif is therefore a PBE box located 31 bp upstream of the reported peak center (Hornitschek *et al.*, 2012). In addition, *PIF4* bind to chromatin in proximity to *TMK1* and *TMK2* (Oh *et al.*, 2012), although *TMK2* shows no transcriptional response to low R/FR in our experiment. The biological relevance of PIF binding to promoter of TMKs remains to be investigated in more details and it cannot be ruled out that also *TMK2* transcriptionally respond to shade in a tissue- or developmental state-dependent manner.

Given that *TMK1*, *TMK3* and *TMK4* have slightly higher expression levels compared to *HFR1*

in high R/FR and are not shade induce in cotyledon, it would be interesting to investigate the transcriptional response of *TMK1* in cotyledon and hypocotyl *pif* mutants. This will give further insight into the role TMKs in the phyB-mediated transcriptional network.

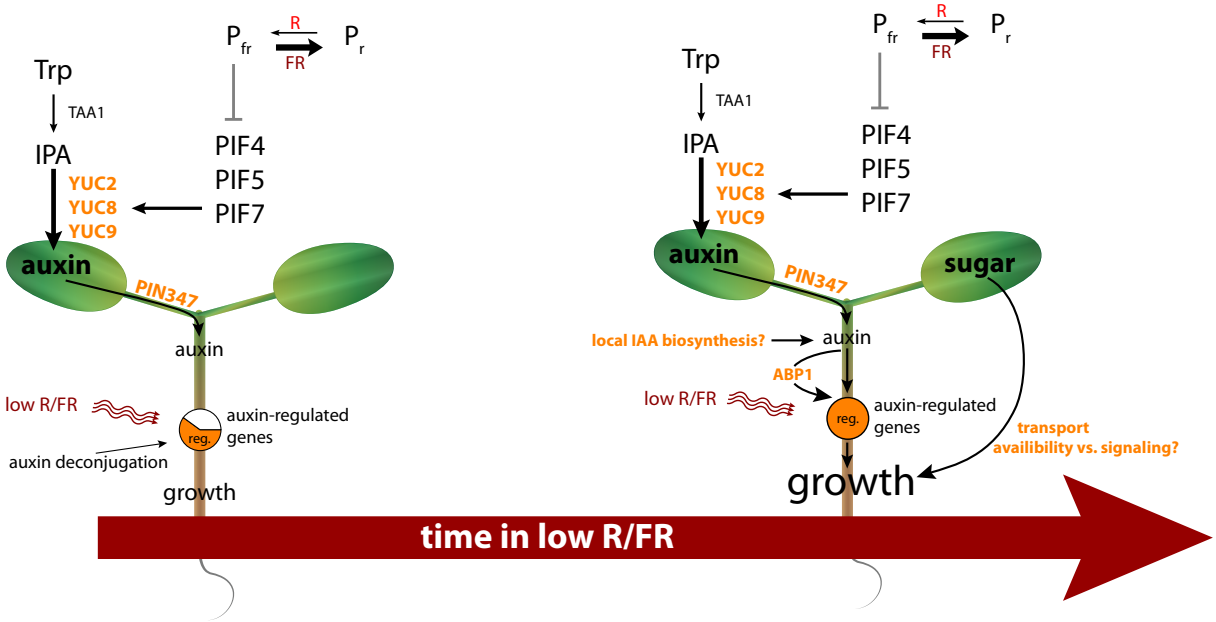

Figure 3.27: Speculative model of shade induced seedling growth.
After few minutes in low R/FR condition (left), PIF levels stabilize and induce the transcription of the auxin biosynthetic genes *YUC2*, *YUC8* and *YUC9* in cotyledons. This lead to enhanced auxin level and subsequent transport towards hypocotyls in a *PIN3*, *PIN4* and *PIN7* dependent manner. At the same time a large fraction of potentially auxin-regulated genes respond transcriptionally to additional cues, such as local low R/FR perception or relative changes between different auxin pools. These changes do not manifest on a phenotypic level at such early time.
After increased time of low R/FR perception (right) shade-induced cotyledon-derived auxin lead to enhanced levels in hypocotyls. Additional local auxin production may contribute to the overall concentration. Auxin (further) induces auxin-regulated genes, which can occur in an ABP1-dependent or ABP1-independent way. This lead to induced growth rates of the hypocotyl. Low R/FR may also affect growth through sugar transport from cotyledons to hypocotyls. Thereby, sugars may become a limiting factor for cell wall biosynthesis and/or act as a signal modulating hypocotyl growth.

3.2.6 Different transcriptional responses to low R/FR in cotyledon and hypocotyl

Transcriptionally regulated genes in opposite direction in cotyledons and hypocotyls are candidate genes which could broaden our understanding of the opposite growth response of both organs. Hierarchical clustering identified two major classes of genes, which were up- or down-regulated in different organs (figure 3.14c and 3.14d). Those groups contained genes of various functions since only one gene ontology term was detected in group ch4. This enriched category was 'fatty acid metabolic process'. Detected genes described by this term included several members of the 3-KETOACYL-COA SYNTHASE family, which are involved in the biosynthesis of

very long chain fatty acids and most likely contributed to the opposite growth responses of both organs.

Several gene families were identified, in which some members showed opposite regulation in both organs. Interestingly, *ARF11* and *ARF18* are oppositely regulated in cotyledon and hypocotyls. In a split firefly luciferase complementation assay using Arabidopsis mesophyll protoplasts *ARF18* interacts only with *IAA28* among all eleven tested IAAs (Li and Dewey, 2011). Since transcript levels of *IAA28* are relatively unaffected by low R/FR treatment, the ratio of these two proteins might change and influence downstream events. Therefore, *ARF18* might be a candidate to contribute to shade induced transcriptional changes mediating phenotypic alterations. Nevertheless, any attempt of educated guesses concerning the *ARF* gene family is highly speculative. Beside the complexity of regulatory relations within and between IAAs and ARFs, homo- and heterodimerization add another level of complexity and make predictions more challenging. Furthermore, the phosphorylation state of ARF2 has been shown to reduce the DNA binding capacity (Vert *et al.*, 2008) illustrating an additional regulatory mechanism, which might also affect transcriptional responses in low R/FR.

Additional examples of opposite regulated genes are two members of the *PILS* family of putative auxin carrier. *PILS3* and *PILS5*, showed reduced transcript levels in cotyledon and induced transcript levels in hypocotyl even tough they stayed below1.5 in case of *PILS3* in cotyledons. *PILS5* overexpression lead to reduced auxin levels and shorter hypocotyls. In contrast, *pils2pils5* double mutants have increased auxin levels and longer hypocotyls compared to wild-type plants Barbez *et al.* (2012). PILS are assumes provide similar regulatory functions as group 2 PIN proteins, since both are ER localized. The opposite regulation of *PILS5* might be related to different role of auxin in cotyledon and hypocotyl. In cotyledons reduced PILS5 transport function could shift auxin compartmentation toward the cytoplasm and contribute to basipetal auxin transport levels. In hypocotyls *PILS5* transcription levels are induced, which may be required to maintain relative auxin compartmentation compared to overall increasing levels.

Organ-specific induced or repressed genes are additional candidates, which potentially contribute to different physiological responses of cotyledons and hypocotyls. Members of several cell wall modifying enzymes respond stronger in hypocotyls than cotyledons. This includes *XTH/XTR*, *pectin methyl esterases (PME)* and cellulose syntheses.

An example of hypocotyl specific responding genes is the family of apyrases, which regulate the concentration of extracellular ATP. Suppression of *APY1* and *APY2* have been shown to limit growth and inhibit PAT (Liu *et al.*, 2012). It is therefore tempting to speculate, that hypocotyl

specific induction of apyrases in shade contribute to the opposite growth response of cotyledon and hypocotyls.

Another family, which is only in hypocotyls transcriptionally induced, is the above-discussed *TMK* family (see section: 3.2.5).

Brassinosteroid metabolism

Beside the enrichment for auxin responses, the term 'response to brassinosteroid stimulus' was detected. Brassinosteriods and auxin are well known to regulate transcription synergistically as well as interdependently. Several interaction between both pathways have been reported such as ARF2 phosphorylation by BIN2 (Vert *et al.*, 2008) or BZR1 ARF6 PIF4 cooperative binding to promoter sequences and subsequent regulation of transcription (Oh *et al.*, 2014). The identification of this term from TP45 on in hypocotyls correlates with observed induced growth phenotypes. The *BRI1* receptor is in cotyledon only transiently regulated by shade and early signaling genes such as *BIN2* and *BZR1* do only respond in hypocotyls suggesting a less important role of this pathway in cotyledons in the context of shade avoidance. Therefore the brassinosteroid pathway might contribute to opposite growth responses in seedlings during low R/FR. Possible mediators of organ specific brassinosteroid responses is the *EXORDIUM* gene family. Three family members, *EXORDIUM* (*EXO*), *EXO-like 1* (*EXL1*) and *EXL5* are strongly upregulated in hypocotyls and show no or only minor reduced relative transcription levels in cotyledons. Phenotypic analysis of a *exo* T-DNA mutant revealed over all reduced growth responses in white light. Smaller leaf cell sizes were explained with reduced cell expansion. Furthermore, in a meristematic context EXO was suggested to function as negative regulator of cell division (Farrar *et al.*, 2003; Schröder *et al.*, 2009).

Sugar related gene ontology categories are enriched in low R/FR

Sugars are important component required for various biosynthetic processes such as synthesis of cell wall components (Wolf *et al.*, 2012). They are synthesized in photosynthetic active tissue and are subsequently transported to photosynthetic inactive parts of the plant. To this end plants have evolved sugar importer and exporter, which are require for long distance transport through the phloem (Chen *et al.*, 2010, 2012; Gould *et al.*, 2012). The *SUGAR TRANSPORTER (SUT) 1* family (SUTs are named SUGAR CARRIERs (SUCs) in *Arabidopsis*) encode for sugar importer involved in phloem loading. Interestingly, in *Solanum tuberosum*, StSUT4-RNAi plants are impaired in several shade-induced growth responses including stem elongation (Chincinska

et al., 2008). In our experiment we identified several enriched sugar-related GO categories in hypocotyls at late time points. It is therefore tempting to speculate, that long-distance sugar transport is promoted by shade in *Arabidopsis thaliana*, and furthermore required for shade-induced hypocotyl elongation. The transport could be either important to satisfy the increased amounts of polysaccharide for cell wall modifications and extension in hypocotyls or serve as a signaling molecule modifying and adapting growth responses of hypocotyl to the current carbohydrate status (Lunn *et al.*, 2006; Paul, 2008). Interestingly, it has been demonstrated, that sucrose promote hypocotyl elongation in a PIF dependent manner. Single mutants of *pif1*, *pif3*, *pif4* and *pif5* had shorter hypocotyls in response to sucrose, which were further diminished in *pif4pif5* and *pif1pif3pif4pif5 (pifq)*. *PIF5ox* line showed enhanced hypocotyl elongation (Stewart *et al.*, 2011). On a molecular level, sucrose mildly represses *PIF4* and *PIF5*, but not *PIF7* transcript levels in the *circadian clock associated 1 (cca1)* background. At the same time *PIF5-HA* protein levels were increased in light and darkness (Stewart *et al.*, 2011). Similar results were reported by Liu *et al.* (2011b) and indicate that PIFs are positive growth regulators of sucrose-induced hypocotyl elongation. PIFs have also been shown to respond to glucose treatment. Sairanen *et al.* (2012) reported, that auxin biosynthesis was enhanced in *pifq* upon glucose treatment, suggesting a negative role for PIFs on auxin biosynthesis in the presence of sugar. How different growth capacities e.g. of *pifq* affect theses results and how these finding can be combined with PIF-dependent shade-induce increase of auxin levels remains elusive. It is tempting to speculate, that different sugar levels in cotyledon and hypocotyl apply various effects on growth responses. High glucose levels in cotyledon might reduce growth through the repressive effect of PIF signaling in shade. This predicts, that glucose insensitive mutants such as *glucose insensitive (gin)* (Ramon *et al.*, 2008) show increased cotyledon growth in shade. Enhance sucrose transport and subsequent higher sucrose levels in hypocotyls might promote elongation growth by stabilizing PIF protein levels.

3.3 Material and Methods

3.3.1 Material

Plant Material The time course experiment was done with *Arabidopsis thaliana* Columbia-0 (Col-0). The *yuc2589* mutant was provided by Julin N. Maloof and the *pin3pin4pin7* mutant has been generated by Martine Trevisan. Séverine Lorrain provided the *pif4pif5pif7* triple mutant.

Consumables used during the library preparation

- Agencourt AMPure XP 60 ml kit (Beckman Coulter Genomics, part # A63881)
- SuperScript II Reverse Transcriptase (Invitrogen, part # 18064)
- TruSeq Stranded mRNA LT Sample Prep Kit 48 Samples, 12 Index Set B (Illumina, part # 15032613)
- TruSeq Stranded mRNA LT Sample Prep Kit 48 Samples, 12 Index Set A (Illumina, part # 15032612)
- TruSeq Stranded mRNA LT Sample Prep Kit 48 Samples, (Box 1 of 2) (Illumina, part # 15027078)
- TruSeq Stranded mRNA LT Sample Prep Kit 48 Samples, (Box 2 of 2) (Illumina, part # 15032614)
- TruSeq Stranded mRNA LT Sample Prep Kit, 48 Samples, cDNA Synthesis PCR Box I (llumina, part # 15032611)

Used programs and versions Graphical visualizations, analysis of normalized transcription levels and gene set comparisons with published data sets were done using R 3.1.1. Used packages during the analysis process are listed in table 3.9.

3.3.2 Methods

Plant Growth Seeds of wild-type plants or transgenic lines were first size selected and than surface-sterilized for 3 min in 70 % ethanol and 0.05 % Triton X-100 followed by 10 min incubation in 100 % ethanol. Seeds were sowed on 0.8 % phytoagar containing half-strength Murashige and Skoog medium (½ MS) and subsequently stratified at 4 °C for 3 day in the darkness.

Phenotyping of growth response to shade Seedlings were grown for 4 days in high R/FR conditions and subsequently either kept or transferred for additional 3 days to low R/FR. Seedlings were dissected in order to separate cotyledons and hypocotyl and scanned. Hypocotyl length were determined using ImageJ. Cotyledon area was measured by mean of a semi-automated Matlap script provided by Dr. Tino Dornbusch.

Table 3.9: Session Into of R.
Used packages were extracted for R with the sessionInfo() command and transferred to a table format.

attached base packages	other attached packages	loaded via a namespace
splines	seqinr_3.0-7	bitops_1.0-6
grid	HH_3.0-4	caTools_1.17
stats	multcomp_1.3-4	colorspace_1.2-4
graphics	TH.data_1.0-3	gdata_2.13.3
grDevices	survival_2.37-7	gtools_3.4.1
utils	mvtnorm_1.0-0	KernSmooth_2.23-12
datasets	latticeExtra_0.6-26	leaps_2.9
methods	lattice_0.20-29	MASS_7.3-33
base	xlsx_0.5.5	plyr_1.8.1
	xlsxjars_0.6.0	Rcpp_0.11.2
	rJava_0.9-6	reshape2_1.4
	stringr_0.6.2	sandwich_2.3-0
	gplots_2.14.1	vcd_1.3-1
	RColorBrewer_1.0-5	zoo_1.7-11
	vioplot_0.2	
	sm_2.2-5.4	
	lattice_0.20-29	

Time course analysis of hypocotyl elongation *Arabidopsis thaliana* Col-0 was grown for 5 days in long-day (LD) conditions at 21 °C on inclined ½ MS plates. On day 6, seedlings were either kept or transferred to low R/FR 2 h after the light onset. Hypocotyl length were documented by time-lapse photography with intervals of 30 min and measured by mean of a semi-automated Matlab script provided by Dr. Tino Dornbusch. Significant hypocotyl elongation was determined with a two-sided t-test and a p value threshold $< 0.1 * 10^{-3}$.

Plant growth and material preparation for the time course experiment analyzed by RNAseq *Arabidopsis thaliana* Col-0 was grown for 5 days in LD at 21 °C (figure 3.28) on one horizontal plate per sample. Each plate contained 25 ml ½ MS covered with a nylon mesh. On day 6, seedlings were either kept or transferred to low R/FR 2 h after the light onset. At each time point nylon meshes with seedlings were quickly imbibed ice-cold 100 % acetone and 2x subjected to about 600 mbar below atmospheric pressure for 5 min on ice. Acetone-fixed seedlings were subsequently transferred to 70 % 4 °C cold ethanol and dissected under a binocular lens. Cotyledon and hypocotyl material of 50 seedlings per time point and light condition were collected separately in 100 % ethanol. For each time point and light condition duplicates were prepared. Plant materials were manually ground and total RNA was extracted using the RNeasy Kit (QIAGen). Finally RNA samples were precipitated using 3M NAOH (pH 5.2) and 100 % ethanol. The precipitate was visualize with glycogen and washed with 80 % ice-cold ethanol.

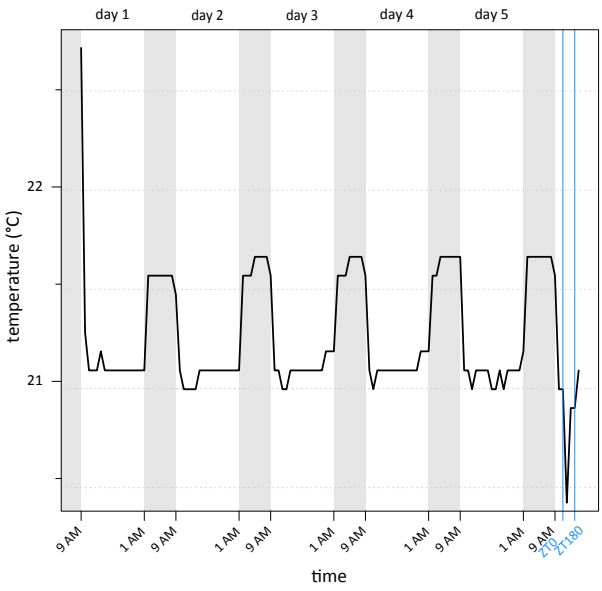

Figure 3.28: Recorded temperature during the seedling growth phase of the RNAseq experiment. Temperature was recorded with a HOBO data locker in the white light camber of a Percival AR-22L incubator while seedlings were grown for the RNAseq experiment.

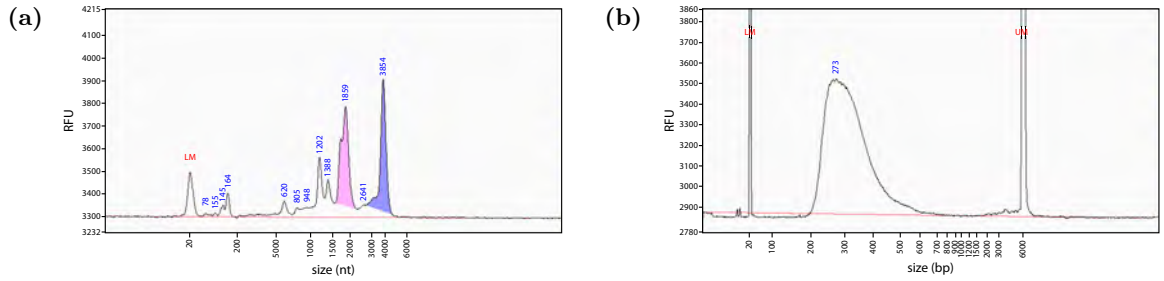

Figure 3.29: RNA sample and library quality Representative total RNA (a) or RNAseq libraries (b) quality were analyzed using a bioanalyzer Signal intensities of different nucleotide lengths were visualized as a graph. The corresponding RNA samples were extracted from cotyledons at TP0. Signal peaks were automatically detected by the bioanalyzer software and their corresponding nucleotide length are labeled in blue inside the plot area. (a) The areas underneath the 18S rRNA and 25S rRNA peaks were colored in pink and lilac, respectively. (a +b) LM = lower maker; UM = upper marker.

Library preparation Stranded libraries were prepared using 400 ng high quality RNA (figure 3.29) according to the TruSeq protocol (Illumina). This included RNA purification steps using AMPure XP beads, cDNA preparation using a mix of random and polyA primer. RNAseq libraries were subsequently sequence with a HISEQ 2500 by the Genome Technology Facility (GTF).

RNAseq analysis Read library quality control, mapping and statistical analysis was done by Sandra Calderon and Sylvain Pradervand.

Read were trimmed with Cutadapt to remove adaptor sequences and low complexity reads were removed using PrinSEQ. Reads were subsequently mapped again the TAIR9 genome using TopHat. More than $25 * 10^6$ uniquely mapping reads were identified per library. Gene count

table were generated using HTSeq and read counts were subsequently trimmed mean of m-value (TMM) and VOOM normalized and log2 transformed (Robinson and Oshlack, 2010; Law *et al.*, 2014). Statistically analyses was done using LIMMA. Subsequent analysis were done using R. Gene enrichment studies were performed using DAVID (Huang *et al.*, 2008, 2009).

4 General discussion

Several plant species favor direct sunlight and respond to shade with different adaptive responses to optimize their morphology to environmental conditions. In *Arabidopsis thaliana* various responses to shade at different developmental stages have been identified. Young seedlings show among others enhanced elongation growth of the hypocotyl and reduced growth of cotyledons (Casal, 2013). Shade is characterized by a low R/FR ratio, which plants perceive by photoreceptors of the phytochrome family (Franklin, 2008). In *Arabidopsis thaliana* shade avoidance responses are predominantly mediated by phyB (Leivar *et al.*, 2012). On a molecular level, low R/FR perception lead to a quick upregulation of several transcriptional regulators, e. g. *ATHB-2*, *HFR1* and *PIL1* (Steindler *et al.*, 1999; Salter *et al.*, 2003; Sessa *et al.*, 2005; Hornitschek *et al.*, 2009). One central class of transcriptional regulators during shade avoidance is the family of PIF transcription factors. PIFs integrate signals of various pathways such as responses to high temperature, carbohydrate availability, and various light mediated responses including shade avoidance. PIFs directly interact with phytochromes and most of them are negative regulators of the phyB pathway (Duek and Fankhauser, 2005; Casal, 2013).

For shade avoidance responses PIF4 and PIF5 are two central regulators. In the first project, we investigated PIF4- and PIF5-mediated growth responses to high and low R/FR, simulating sun and shade conditions. *pif4* and *pif5* single mutants have shorter hypocotyls in response to shade than wild-type, and the hypocotyl length is further reduced in *pif4pif5* double mutants. In the meantime PIF7 has been show to mediate similar responses to shade, and *pif4pif5pif7* might show no hypocotyl elongation in shade. Using a microarray approach we identified genome wide PIF4 and PIF5 dependent shade-regulated genes. By combining these results with chromatin immunoprecipitation (ChIP) of *PIF5-HA* we further identified direct target genes of PIF5. Similar approaches have been done for PIF1, PIF3 and PIF4, but not PIF7 (Oh *et al.*, 2009, 2012; Zhang *et al.*, 2013). The largest number of putative direct target genes was identified for PIF4, which is consistent with broad binding specificity to several E-box variants and G-box flanking bases *in vitro* and its pleiotropic phenotype when over expressed (Lorrain *et al.*, 2008; Hornitschek *et al.*,

2012; Oh *et al.*, 2012). PIF4 and PIF5 share the largest number of putative direct target genes (Jeong and Choi, 2013). To date, it is not known if common target genes of PIF4 and PIF5 are regulated by both transcription factors as the same time, if PIF4 and PIF5 competitively bind to promoter sequences or if genes are regulated by either by PIF4 or PIF5 in a tissue dependent context. Nevertheless, PIF5 has higher sequence specificity *in vitro* and binds in contrast to PIF4 only poorly to PBE boxes (Hornitschek *et al.*, 2012). Nevertheless, PIF5 binding site were enriched for this E-box motif, and might explain PIF5 chromatin binding sites without observed G-box.

Motif analysis of upstream regulatory sequences of PIF5 direct target genes, revealed an enrichment of TCP transcription factor binding sites, suggesting that similar processes are regulated by TCPs and PIFs during shade. Different TCPs regulate developmental processes e.g. leaf growth, which might be affected during shade (Palatnik *et al.*, 2003; Nath *et al.*, 2003). For example, *At*TCP4 is required for leaf development and negatively regulated cell division in yeast (Aggarwal *et al.*, 2011; Palatnik *et al.*, 2003). PhyB signaling and TCP transcription factors have been previously linked in the context on shoot branching in several species (Kebrom *et al.*, 2006; Aguilar-Martínez *et al.*, 2007; Su *et al.*, 2011). So far, it can only be speculated if in a none-branching context TCPs and PIF share common target genes and if so to what extend they regulate their expression at the same time.

Among PIF4 and PIF5 dependent shade-regulated genes, we identified a strong enrichment of the GO term 'response to auxin stimulus'. Several genes, described by this term, were in addition direct target genes of PIF4 and PIF5 such as the auxin biosynthetic gene *YUC8*. PIF4 has also been shown to bind directly to promoters of the auxin biosynthetic genes *YUC8*, *TAA1* and *CYP79B2* at high temperatures supporting the link between PIFs and the direct regulation of auxin biosynthesis (Franklin *et al.*, 2011; Sun *et al.*, 2012). Surprisingly, in low light intensities we observed reduced *YUC8* transcript levels in *pif4pif5* mutants in high R/FR, but a wild-type-like induction in low R/FR. Given that *YUC8* is also a direct target gene of PIF7, it is possible that *PIF7* is sufficient to mediate this transcriptional response to low R/FR in the absence of PIF4 and PIF5.

Free IAA levels rise quickly upon low R/FR perception. Most measurements were done after one or two hours of simulated shade treatment. Shade-induced auxin is assumed to be predominantly synthesized in cotyledons of young seedlings since *TAA1* expression was predominantly observed at their margins and is required for a full shade avoidance response. However, transcriptional regulation of auxin biosynthetic enzymes has been mainly shown for members of

the *YUCCA* family. Using RNAseq data comparing single organs at several time points in low R/FR, we were able to increase our understanding of the shade-regulated auxin metabolism. In combination with the mutant of higher order *yucca* mutants, we demonstrated that only *YUC2*, *YUC8* and *YUC9* are expressed in cotyledon and that *yuc2589* shows no hypocotyl response to shade. The mild reduction of the *yuc35789* mutants indicates that all cotyledon expressed *YUCCA* play an important role during shade avoidance.

Shade-induced auxin depends on several PIN efflux carriers. It is sufficient to knock out all PINs, which were transcriptionally induced in cotyledon. This presumably retains auxin in cotyledons and prevents the formation of an auxin gradient along the shoot as shown in *Brassica raps* (Procko *et al.*, 2014). We found that about 25 % of auxin inducible genes show regulation pattern, which correlate with the formation of a predicted auxin gradient in *Arabidopsis*. This ratio might be underestimated due to quick transcriptional response times, which take place between our time points. Furthermore, different members of gene families might have various importance in both organs. Therefore, the regulation of a molecular function might be rather reflected by the combined responses of several family members. However, not all transcriptional responses are mediated to the protein level.

Half of the auxin inducible genes respond in low R/FR at the same time point including TP15. This suggests, that low R/FR is perceived in hypocotyls and is consistent with the phyB expression domain. Nevertheless, different experiments suggests, that shade perception in hypocotyls have only minor effects on elongation responses. This might be of advantage of plants like *Arabidopsis*, which potentially shade their hypocotyl/stem with own cotyledons/leaves. Shade perception in hypocotyls and subsequent regulation of transcriptional responses, might induce the responsiveness of those organs. This could include intracellular PIN3 relocalization to the lateral side (Keuskamp *et al.*, 2010). When shade-induced auxin of cotyledons reaches the hypocotyl, enhanced lateral distribution of auxin would take place immediately. If this hypothesis is true, lateral auxin transport would be increased before overall auxin levels are enhance in hypocotyl. This could lead to transiently increased auxin level in cortex and epidermal cell leading to a transient promotion of growth. Cole *et al.* (2011) reported an multi-phasic growth pattern of hypocotyls in low R/FR with a initial phase of high growth rates, followed by a second phase of reduced growth rates and a third phase of increased growth. PIN relocalization could therefore contribute to the initial growth induction and cotyledon-derived auxin might mediate the second phase of high growth rates. Increase sensitivity of hypocotyls could also include increased transcript levels. In the context of auxin, induced transcription level of *Aux/IAAs* might be involved

in regulating auxin sensitivity.

The carbohydrate state of organs might impose an additional level of regulation of shade avoidance responses in *Arabidopsis*. In *Solanum tuberosum* sugar transport plays a important role during shade avoidance (Chincinska *et al.*, 2008). In our experiment we identified several enriched sugar related GO terms. Recently PIF have been reported to be transcriptionally induced in the presence of sucrose (Stewart *et al.*, 2011; Liu *et al.*, 2011b). Sairanen *et al.* (2012) report a repressive function of PIFs on glucose induces auxin biosynthesis rates.

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