Title: Contrasting growth responses in lamina and petiole during neighbor detection depend on differential auxin responsiveness rather than different auxin levels.

Authors: de Wit M, Ljung K, Fankhauser C

Journal: The New phytologist

Year: 2015 Oct

Volume: 208

Issue: 1

Pages: 198-209

DOI: 10.1111/nph.13449
Contrasting growth responses in lamina and petiole during neighbor detection depend on differential auxin responsiveness rather than different auxin levels

<table>
<thead>
<tr>
<th>Journal:</th>
<th>New Phytologist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>NPH-MS-2014-18667.R2</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>MS - Regular Manuscript</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>n/a</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>de Wit, Mieke; University of Lausanne, Center for Integrative Genomics Ljung, Karin; Swedish University of Agricultural Sciences, Forest Genetics and Plant Physiology; Fankhauser, Christian; University of Lausanne, Center for Integrative Genomics;</td>
</tr>
<tr>
<td>Key Words:</td>
<td>neighbor detection, shade avoidance response, auxin, PIF, leaf growth, XTH</td>
</tr>
</tbody>
</table>


Contrasting growth responses in lamina and petiole during neighbor detection depend on differential auxin responsiveness rather than different auxin levels

Mieke de Wit¹, Karin Ljung², Christian Fankhauser¹,³

1 Center for Integrative Genomics, Faculty of Biology and Medicine, University of Lausanne, CH-1015 Lausanne, Switzerland

2 Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE-901 83, Umeå, Sweden

3 Corresponding author: christian.fankhauser@unil.ch, +41.21.692.39.41

Total word count main body: 6111
Summary: 183
Introduction: 1497
Material and Methods: 567
Results: 1944
Discussion: 1762
Acknowledgements: 151

Figures: 6
Supplemental Figures: 9
Supplemental Table: 1
Contrasting growth responses in lamina and petiole during neighbor detection depend on differential auxin responsiveness rather than different auxin levels.

SUMMARY

• Foliar shade triggers rapid growth of specific structures that facilitate access of the plant to direct sunlight. In leaves of many plant species this growth response is complex because while shade triggers elongation of petioles it reduces growth of the lamina. How the same external cue leads to these contrasting growth responses in different parts of the leaf is not understood.

• Using mutant analysis, pharmacological treatment and gene expression analyses we investigated the role of PHYTOCHROME INTERACTING FACTOR (PIF)7 and the growth promoting hormone auxin in these contrasting leaf growth responses.

• Both petiole elongation and lamina growth reduction depend on PIF7. Induction of auxin production is both necessary and sufficient to induce opposite growth responses in petioles versus lamina. However, these contrasting growth responses are not due to different auxin concentrations in both leaf parts.

• Our work suggests that a transient rise in auxin levels triggers tissue-specific growth responses in different leaf parts. We provide evidence suggesting that this may be due to different sensitivity to auxin in the petiole versus the blade and to tissue-specific gene expression.

Keywords: neighbor detection, shade avoidance response, auxin, PIF, leaf growth, XTH

INTRODUCTION

The shade avoidance response is employed by plants upon perception of surrounding competitors in order to avoid future shade and thus maintain access to unfiltered sunlight. In Arabidopsis (Arabidopsis thaliana), this growth response consists of hypocotyl elongation in seedlings and of elevation (hyponasty) and
elongation of leaf petioles in older plants, which places the light capturing tissues in a higher position in anticipation of shade (Franklin, 2008; Casal, 2012). On the other hand, the growth rate of cotyledons and leaf lamina can decline upon neighbor detection (McLaren & Smith, 1978; Nagatani et al., 1991; Kozuka et al., 2010). Perception of a shade signal consequently leads to contrasting growth responses in different parts of the leaf.

Proximate neighbors are sensed through changes in light quality, mainly through a reduction in the ratio between red (R, 660-670 nm) and far-red (FR, 725-735 nm) light (Morgan & Smith, 1978; Morgan et al., 1980; Ballaré et al., 1990; Franklin, 2008; Casal, 2012). This decreased R:FR originates from absorption of R but reflection of FR by green plant tissues, and is therefore specifically signaling the presence of nearby plants. The R:FR is perceived through the phytochrome photoreceptors (phyA-E in Arabidopsis), of which phyB plays a predominant role in shade avoidance (McLaren & Smith, 1978; Nagatani et al., 1991; Franklin et al., 2003; Kozuka et al., 2010). The active, FR-absorbing conformer (Pfr) of phytochrome translocates to the nucleus (Sakamoto & Nagatani, 1996) where it interacts with a class of growth-promoting basic helix-loop-helix transcription factors called PHYTOCHROME INTERACTING FACTORs (PIFs), resulting in the phosphorylation and degradation or inactivation of these PIFs (Duek & Fankhauser, 2005; Li et al., 2012; Jeong & Choi, 2013; Leivar & Monte, 2014). Upon an increase in FR phytochrome shifts to the inactive, R-absorbing conformation state (Holmes & Smith, 1975; Smith & Holmes, 1977). The inactivation of phyB in low R:FR thus relieves the repression of the PIFs, which leads to their accumulation and subsequent binding to the G-box and PIF-binding E-box motifs of the promoters of shade-responsive genes (Hornitschek et al., 2009; 2012; Li et al., 2012; Oh et al., 2012; Zhang et al., 2013). PIF4, PIF5 and PIF7 play important roles in the shade avoidance response (Lorrain et al., 2008; Li et al., 2012), with moderate contributions of PIF1 and PIF3 (Leivar et al., 2012). The PIF-mediated transcriptional response to low R:FR leads to the induction of growth-related genes and eventually to the architectural changes that make up the shade avoidance phenotype. Transcripts encoding cell wall-modifying proteins are amongst the direct PIF targets (Hornitschek et al., 2009; Oh et al., 2009; Hornitschek et al., 2012; Oh et al., 2012). Of these, the XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASES (XTHs) show increased transcription and activity during shade (Hornitschek et al., 2009; Sasidharan et al., 2010). XTHs can cut and ligate xyloglucan chains and play a role in cell wall rigidity (Rose et al., 2002; Cosgrove, 2005). The xth15 and xth17 mutants have an inhibited petiole
elongation response in low R:FR, indicating their importance for the shade avoidance response (Sasidharan et al., 2010).

Neighbor-induced growth responses are largely mediated by a suite of hormones, of which auxin has emerged as a major player (Gommers et al., 2013; Casal, 2013; de Wit et al., 2013). Auxin is perceived in the nucleus through a set of F-box TRANSPORT INHIBITOR RESPONSE/AUXIN SIGNALING F-BOX (TIR/AFB) receptors (Dharmasiri et al., 2005). Auxin binding to a TIR/AFB receptor leads to degradation of AUX/IAA repressor proteins, which relieves their repression of AUXIN RESPONSE FACTOR (ARF) transcription factors (Guilfoyle & Hagen, 2007).

In low R:FR, auxin levels increase after 1h in wild-type Arabidopsis seedlings. This depends on de novo auxin synthesis through TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA)1, as the sav3/taa1 mutant fails to raise the auxin concentration in low R:FR (Tao et al., 2008). The rate-limiting step in this auxin biosynthesis pathway is catalyzed by the flavin-containing monoxygenases called YUCCAs (Zhao et al., 2001; Mashiguchi et al., 2011; Won et al., 2011). Interestingly, PIF4, PIF5 and PIF7 were shown to directly bind the promoters of YUCCA (YUC) 8 and YUC9 (Hornitschek et al., 2012; Li et al., 2012), which revealed a direct link between phytochrome signaling and auxin biosynthesis. Correspondingly, auxin concentrations remain at basal levels in seedlings of the pif4pif5 and pif7 mutants after exposure to low R:FR (Hornitschek et al., 2012; Li et al., 2012). The transcriptomes of the pif4pif5 and the pif7 mutants consequently show miss-regulation of many auxin-related genes in response to low R:FR (Hornitschek et al., 2012; Li et al., 2012). The sav3/taa1 mutant shows impaired hypocotyl elongation and leaf hyponasty, and altered leaf growth responses in low R:FR (Tao et al., 2008; Moreno et al., 2009), indicating that elevated auxin levels are required for the low R:FR-induced growth responses. In Arabidopsis and Brassica rapa seedlings auxin is mainly synthesized in the cotyledons and subsequently transported towards the hypocotyl during low R:FR treatment (Tao et al., 2008; Procko et al., 2014). Consistently, impaired polar auxin transport through application of the auxin transport inhibitor naphthylphtalamic acid (NPA) or mutation of the auxin export protein PINFORMED (PIN) 3 in the pin3-3 mutant abolishes hypocotyl elongation in low R:FR (Steindler et al., 1999; Tao et al., 2008; Keuskamp et al., 2010). NPA also inhibits petiole elongation in low R:FR, indicating that auxin transport is also required for shade avoidance in leaves (Pierik et al., 2009). Apart from increasing auxin levels and flow towards expanding tissues auxin signaling is directly targeted in low R:FR,
as PIF4 and PIF5 were found to bind to the promoters of *IAA19* and *IAA29* (Hornitschek *et al.*, 2012). This is however not straightforward to interpret, as there is high redundancy and negative feedback in the auxin pathway. Plants with altered PIF levels show different sensitivity to exogenous auxin and it has been predicted that auxin sensitivity may be regulated in shade to make the tissue more receptive to auxin, especially in the case of low resource availability (Nozue *et al.*, 2011; Hersch *et al.*, 2014). We currently have poor understanding how regulation of auxin sensitivity is achieved and it may be fine-tuned at various levels (Pierre-Jerome *et al.*, 2013; Bargmann & Estelle, 2014). For shade it has been suggested that the AFB1 receptor might play a role in this, as its gene expression is induced in hypocotyls of shade-treated seedlings (Hersch *et al.*, 2014).

Most shade avoidance studies have focused on hypocotyl and petiole elongation, but whether auxin also plays a role in the reduction of lamina size in low R:FR is currently not known. In leaf primordia, neighbor detection leads to rapid reduction of cell division due to auxin-dependent degradation of cytokinin (Carabelli *et al.*, 2007). However, shade also induces lamina growth reduction in older leaves when cell proliferation is largely arrested (Donnelly *et al.*, 1999; Andriankaja *et al.*, 2012), and is thus likely to affect cell expansion as well. In end-of-day far-red, a treatment that evokes a shade avoidance-like phenotype, a large amount of auxin-responsive genes are induced in both petioles and lamina (Kozuka *et al.*, 2010). Furthermore, it has been shown that an increase of auxin levels by application of auxin or NPA (thereby increasing endogenous levels) resulted in inhibited leaf growth in Arabidopsis and common bean (*Phaseolus vulgaris*) (Keller *et al.*, 2004). It therefore seems plausible that low R:FR-induced auxin biosynthesis could play a role both in growth promotion in the petiole and in growth reduction in the lamina. The effects that auxin generates in a cell are dependent on cellular context and developmental age (Kieffer *et al.*, 2010). Furthermore, active auxin transport through export carriers leads to gradient formation and different concentrations across tissues and organs (Zazimalova *et al.*, 2010). Auxin responses are known to be concentration-dependent and can follow an optimum curve as is the case for root elongation which is promoted at low auxin levels, but inhibited at higher auxin levels (e.g. Wilson & Wilson, 1991; Evans *et al.*, 1994). A similar optimum curve has been hypothesized to exist for PIF-dependent hypocotyl elongation in response to auxin (Nozue *et al.*, 2011). Organ-specific auxin responses may thus be due to different auxin levels, a difference in auxin sensitivity or a combination of both.
Here, we investigate the contrasting growth responses of the shade avoidance leaf phenotype in Arabidopsis. Our data suggests that both petiole elongation and lamina size reduction in low R:FR are an effect of PIF7-dependent auxin production in the lamina. However, we find that overall auxin levels are not significantly different between petiole and lamina, neither in control light nor after low R:FR induction. The contrasting growth responses of petiole and lamina thus rather appear to be due to different auxin responsiveness. Although abundance of the AFB1 receptor is specifically upregulated in petioles in low R:FR, a functional role for this only became apparent in absence of auxin biosynthesis. Enhanced auxin sensitivity through receptor regulation may therefore be mainly important in limiting conditions. We hypothesize that PIF7 regulates tissue-specific growth-related genes both dependent and independent of auxin.

**MATERIAL AND METHODS**

Plant growth, treatments and measurements

All mutant lines are in the Col-0 background: *hfr1-101* (Fankhauser & Chory, 2000), *sav3-2* (Tao et al., 2008), *tir1afb* mutants and 35S::*AFB1-Myc* (Dharmasiri et al., 2005), *msg2-1* (Tatematsu et al., 2004), *iaa5iaa6iaa19* (Overvoorde et al., 2005). The *pif4pif5pif7* mutant was obtained by crossing *pif7-1* (Leivar et al., 2008) with *pif4-101pif5* (Lorrain et al., 2008). Seeds were sown on soil and stratified for three days at 4°C in the dark. Plants were grown in a 16h light / 8h dark photoperiod of 220 µM m⁻² s⁻¹ at 20°C and 70% RH. After 14d plants were divided over two Percival Scientific Model I-66L incubators and acclimatized to 130 µmol m⁻² s⁻¹ for 24h. The next morning at ZT3 one incubator was supplemented with 45 µM m⁻² s⁻¹ of FR light (739 nm LEDs, Quantum Device, USA), lowering the R(640-700 nm):FR (700-760 nm) from 1.4 to 0.2, as measured by Ocean Optics USB2000+ spectrometer. 10 µM IAA (SIGMA-Aldrich), 25 µM NPA (Duchefa Biochemie), 500 µM L-Kynurenine (SIGMA-Aldrich) and 200 µM α-(phenylethyl-2-oxo)-indole-3-acetic acid (PEO-IAA, provided by H. Nozaki) solutions were freshly prepared from concentrated DMSO stocks before each application. Mock solution was similarly prepared to contain 0.1% DMSO and 0.15% Tween-20. Solutions were applied adaxially with a paintbrush, prior to the start of light treatment. After three days of treatment the third leaf was removed from ten plants per treatment and incisions were made in the lamina to allow leaf flattening. Leaves were scanned on a flatbed scanner (600 dpi) using a uniform blue background. This
allowed automated separation from the background in Matlab (Methods S1) using
the Green/Blue pixel value. Petiole base and petiole-lamina junction were selected
manually. Pixels located below the petiole-lamina junction were labeled as petiole
and above as lamina. Transformation of pixel coordinates to petiole length and
lamina area were done using the image resolution given by the scanner.

RNA extraction and RT-qPCR

Petioles and lamina of leaf 3 were separately pooled into three biological replicates
and frozen in liquid nitrogen. RNA was extracted using the Qiagen Plant RNeasy kit
with on-column DNA digestion, according to the manufacturer's instructions. For
each experiment, equal amounts of RNA were reverse transcribed into cDNA with
Superscript II Reverse Transcriptase (Invitrogen). RT-qPCR was performed in three
technical replicates for each sample (7900HT Applied Biosystems). Data was
normalized against two reference genes (YLS8, UBC) using the Biogazelle qbase
software.

IAA content and MUG assays

For IAA measurements, five biological replicates per timepoint containing 12 mg
fresh weight of petioles or lamina were harvested and frozen in liquid nitrogen. 500
pg $^{13}$C$_6$-IAA internal standard was added to each sample before extraction and
purification. Free IAA was quantified using gas chromatography – tandem mass
spectrometry as described in (Andersen et al., 2008) with minor modifications.
For AFB1-GUS quantification, three biological replicates consisting of petioles or
lamina from ten pAFB1::AFB1-GUS plants were frozen in liquid nitrogen. Proteins
were extracted from ground material with GUS extraction buffer (50mM NaPO$_4$,
10mM 2-ME, 10mM EDTA, 5% glycerol, 0.1% Triton-X). 10 µL of protein extract was
incubated in 140 µL of MUG assay buffer (1mM 4-Methylumbelliferyl-B-D-
glucuronide hydrate (SIGMA-Aldrich) in GUS extraction buffer) for 55 minutes at 37
°C. The enzyme reaction was stopped in 2M NaCO$_3$ and fluorescence
measurements were done in duplicate in a Tecan Saphire$^2$ platereader. Protein
concentrations were determined in duplicate at OD$_{595}$ using the Biorad Protein
Assay.

RESULTS

Low R:FR induces contrasting leaf responses dependent on PIF7
To study responses of lamina and petiole during neighbor detection we subjected two-week-old plants to several days of low R:FR. In agreement with previous studies (McLaren & Smith, 1978; Nagatani et al., 1991; Reed et al., 1993; Kozuka et al., 2010), leaves of low R:FR-treated plants showed a reduced lamina size and elongated petioles as compared to leaves of plants in control white light (high R:FR), which was also reflected in their biomass allocation (Fig. S1a-d). For further experiments we measured leaf 3, which reliably shows both leaf responses after three days of low R:FR treatment (Fig. 1a,b). In this leaf, the gene expression kinetics of typical shade avoidance markers (as shown for PIL1 in Fig. 1c) were largely similar for lamina and petiole. Low R:FR-induced expression of the negative shade avoidance regulator HFR1 was higher in lamina than in petioles (Fig. S2a). The lamina response in low R:FR was however not affected in the hfr1 mutant suggesting that in our growth conditions HFR1 does not play a limiting role in the reduced lamina growth during shade avoidance (Fig. S2b,c).

In seedlings, PIF4, PIF5 and PIF7 are important regulators of the shade avoidance response (Lorrain et al., 2008; Li et al., 2012) and we therefore tested petiole length and lamina size in pif mutants after three days of low R:FR. pif7 had a reduced petiole response in low R:FR (138% (1.8 mm) length increase vs. 153% (3.0 mm) in the wild-type), while the smaller pif4pif5 petioles showed a strong elongation response (166% (2.3 mm) length increase) (Fig. 1d, S3a). Similarly, lamina size was not reduced in pif7 after low R:FR treatment, while pif4pif5 still showed a tendency towards reduced lamina area (p=0.05, Fig. 1e, S3b). Although pif4pif5pif7 plants were smaller than pif7 plants, they only showed a slightly greater inhibition in petiole elongation as pif7 (130% (1.0 mm)) and no lamina size reduction in response to low R:FR (Fig. 1e,d, S3). This indicates that among the tested PIFs, PIF7 plays the predominant role in regulating these leaf growth traits in response to low R:FR conditions.

**Auxin biosynthesis is required and sufficient to induce both leaf responses**

The pif7 mutant has impaired YUCCA activation in low R:FR-treated seedlings (Li et al., 2012) and induced auxin production through the TAA1-YUCCA pathway is an important step during shade avoidance in seedlings (Tao et al., 2008). We therefore tested whether this also plays a role in the lamina and petiole responses of juvenile leaves. All four YUCCA genes that were reported to be shade-induced in seedlings showed increased expression in the lamina after 2 hours of low R:FR (Fig. 2a-c). In contrast, only YUC9, which showed the highest shade-induced expression in the lamina, also showed increased expression in the petiole. Moreover, the magnitude of
induced YUC9 expression by low R:FR was six times lower in the petiole than in the lamina (Fig. 2d). YUC8 expression was dramatically reduced in pif7 lamina compared to the wild type (Fig. 2e). Shade-regulated YUCCA expression correlated with an increase in auxin levels in wild-type lamina after 2h of low R:FR, while the auxin concentration in pif7 lamina remained similar to control light conditions (Fig. 2f), indicating that low R:FR-induced auxin biosynthesis in leaves is PIF7-dependent. Interestingly, the pif7 shade avoidance phenotype resembles that of the sav3/taa1 mutant (Fig. 2e, S4a,b), which lacks an induced auxin burst in low R:FR (Tao et al., 2008). The impaired petiole and lamina responses of pif7 could thus be due to failure to induce auxin biosynthesis upon neighbor detection.

The YUCCA expression pattern suggests that low R:FR-induced auxin production mainly takes place in the lamina (Fig. 2). Interestingly, the auxin signaling marker DR5::GUS is induced at the leaf margins in low R:FR (Fig. S4c), which in cotyledons coincides with the site of TAA1 expression (Tao et al., 2008). We therefore hypothesized that by analogy with seedlings most auxin would be produced in the leaf lamina and would subsequently be transported into the petiole. It has been shown previously that the auxin transport inhibitor NPA can inhibit low R:FR-induced petiole elongation (Pierik et al., 2009a). Application of NPA to the lamina-petiole junction was sufficient to completely inhibit the petiole response to low R:FR (Fig. S4d), suggesting that auxin flow from lamina to petiole is required. Expression of the gene coding for the auxin efflux carrier PIN3 was upregulated both in lamina and petioles in the first few hours of low R:FR treatment (Fig. S4e), which may facilitate enhanced basipetal auxin transport upon neighbor detection.

To test whether increased auxin biosynthesis in the lamina could account for the leaf responses induced by low R:FR, we applied IAA to the adaxial side of the leaf lamina and especially at the leaf margins. In comparison to mock-treated plants, application of various concentrations of IAA to the lamina induced petiole elongation and reduced growth of the lamina (Fig. 3a,b, Fig. S5a,b). This shows that increased auxin levels in the lamina can lead to both leaf phenotypes as observed in low R:FR. Moreover, the pif7 and pif4pif5pif7 mutants also responded to IAA application, suggesting that their reduced leaf phenotypes in low R:FR is mainly due to impaired auxin biosynthesis (Fig. S5c,d). Correspondingly, application of NPA to the lamina-petiole junction, which should increase endogenous auxin levels in the lamina and inhibit auxin transport to the petiole, reduced both petiole and lamina growth (Fig. 3c,d). Reduction of basal auxin levels through application of the biosynthesis inhibitor L-Kynurenine led to increased lamina size but had no effect on petiole growth (Fig. 3e,f), suggesting that basal auxin levels in control conditions are indeed sub-optimal
for lamina growth. Together, these results correspond to a model in which PIF7-dependent auxin production takes place mainly in the lamina leading to lamina growth reduction and in which auxin is subsequently transported to the petiole leading to enhanced petiole growth.

**Contrasting leaf responses are not due to different auxin concentrations**

Auxin responses can be concentration-dependent (Wilson & Wilson, 1991; Evans et al., 1994) and we therefore asked whether the contrasting growth responses of lamina and petiole to low R:FR could be due to a different auxin concentration in both leaf parts. If the lamina is indeed the site of auxin production then auxin levels might be relatively high in lamina compared to petioles. In agreement with this basal expression levels (plants grown in high R:FR) of both an auxin biosynthesis (YUC8) and an auxin responsive (IAA29) gene were higher in lamina compared to petioles (Fig. 4a,b). Thus, a further increase in auxin production upon low R:FR perception may shift the auxin optimum curve further towards growth reduction in the lamina, while auxin transported to the petiole may increase the auxin concentration further within the lower growth-stimulating range. To test this, we measured overall auxin levels in entire lamina and petioles after 0.5h, 1h, 2h and 24.5h of low R:FR treatment. Based on previously published seedling data and on the expression of the two highest induced YUCCAs in leaves (Fig. 4c,S6) we expected auxin levels to rise within this timeframe in lamina. As shown in Fig. 4d and e, auxin concentration indeed increased within 2h of low R:FR treatment and were back to basal levels after 24h. The kinetics were similar for petioles and lamina, and despite the early timepoints no indication that auxin levels first increase in the lamina could be observed. Interestingly, after 2h of low R:FR the auxin concentration reaches very similar levels in both leaf parts. These concentration data indicate that the contrasting growth responses of lamina and petiole to auxin are not due to a global concentration difference.

**Auxin responsiveness in low R:FR-treated petioles**

The different responses of lamina and petiole to increased auxin levels may alternatively be due to a difference in sensitivity to auxin, which could be under regulation of light signals. One way in which auxin sensitivity could be regulated is at the level of receptor abundance. In seedlings gene expression of the AFB1 receptor was shown to be hypocotyl-specific, which may suggest that auxin sensitivity is locally enhanced and could contribute to the shade-induced elongation response (Hersch et al., 2014). Of the four TIR/AFBs tested, only AFB1 was upregulated in low
R:FR, both in petioles and lamina (Fig. 5a,b, S7). Interestingly, AFB1 was also upregulated in petioles of pif7 (Fig. 5b). If induced AFB1 expression indeed leads to enhanced sensitivity, this might explain why the petiole response in low R:FR is not completely abolished in this mutant despite the lack of induced auxin levels. Overall, AFB1 protein levels in control light conditions were higher in petioles than in lamina (Fig. 5c), as measured by GUS activity of AFB1-GUS protein under the expression of the AFB1 promoter (Parry et al., 2009). Furthermore, although AFB1 expression levels were induced in both leaf parts in low R:FR, AFB1-GUS levels were increased only in petioles upon low R:FR treatment (Fig. 5c). Such a difference in receptor levels may play a role in the different responsiveness of the two leaf parts to auxin. Nevertheless, a role for AFB1 in shade-induced petiole elongation could not be deduced from higher-order receptor mutants lacking AFB1 or a 35S::AFB1 overexpression line (Fig. S8), which all showed a normal elongation response in low R:FR (Fig. 5d). As Aux/IAAs can act as co-receptors (Calderon-Villalobos et al., 2012; Havens et al., 2012) and IAA6 and IAA19 is induced in low R:FR (Kozuka et al., 2010; Hornitschek et al., 2012), we also tested the iaa5iaa6iaa19 triple mutant and the dominant IAA19 mutant msg2 (Fig. S8). Neither of these lines was affected in low R:FR-induced petiole elongation. The lack of a phenotype in the receptor- and iaa mutants could however be due to redundancy with the other TIR/AFBs or Aux/IAAs and/or to the fact that auxin production should still be induced in these mutants upon low R:FR perception, which could compensate a reduced sensitivity. Indeed, application of the auxin antagonist PEO-IAA that binds to the TIR/AFBs only reduced low R:FR-induced petiole elongation in the wild type at a high concentration (Fig. 5e), but led to a significantly decreased petiole response at a lower concentration in mutants with impaired induction of auxin biosynthesis (Fig. 5f,g). It is thus possible that regulation of auxin sensitivity through the TIR/AFBs may play a role in low R:FR-induced petiole elongation mainly when auxin levels are low. A similar role for auxin sensitivity was recently predicted for low R:FR-induced hypocotyl elongation in low light conditions, in which seedlings have low auxin levels (Hersch et al., 2014).

Leaf part-specific PIF7 targets

Ultimately, PIF7 should confer tissue-specific responses by regulating specific gene targets, either directly through binding to their promoters, or indirectly through auxin-mediated changes in gene expression. The cell wall-modifying proteins of the XTH family have been implicated in shade avoidance previously (Hornitschek et al., 2009; Kozuka et al., 2010; Sasidharan et al., 2010). Moreover, the expression of some
members of the XTH gene family is regulated by auxin while this is not the case for others (Yokoyama & Nishitani, 2001; Nemhauser et al., 2006; Chapman et al., 2012).

We therefore decided to analyze the expression of members of the XTH family in the petiole and the lamina of shade treated seedlings. Interestingly, XTH15/XTR7 and XTH19 showed a leaf part-specific expression pattern, with XTH15 being predominantly upregulated in the lamina and XTH19 being mainly induced in the petiole (Fig. 6a,b). This leaf part-specific induction of the XTHs in low R:FR was strongly reduced in the pif7 mutant (Fig. 6c,d) while PIF7 levels were high in both petioles and lamina (Fig. S9), which may be due to a different auxin-mediated transcriptional readout in the lamina versus the petiole.

DISCUSSION

Specificity in auxin responses depends both on auxin concentration and auxin responsiveness (Del Bianco & Kepinski, 2011). In this work, we showed that both contrasting growth responses of petiole and lamina in low R:FR are auxin-mediated (Figs. 1-3), but that auxin levels are very similar in the two leaf parts both in control light and in low R:FR (Fig. 4). This suggests that the opposite responses of petioles and lamina are not due to a difference in auxin concentration, although as we have analyzed entire petioles and lamina it remains possible that there is a concentration difference in specific cells that mediate the growth responses. Another interesting feature of the concentration measurements is that after 24h of low R:FR auxin levels were back to the base values despite elevated levels of YUC8 and YUC9 at this timepoint (Fig. 4, S3), which was shown previously in seedlings (Bou-Torrent et al., 2014). This implies that shade-induced auxin biosynthesis is transient or alternatively the transient nature of increased auxin levels may be regulated through irreversible degradation of auxin to inactive catabolites (Pencik et al., 2013). This is somewhat surprising considering the importance of auxin biosynthesis for the shade avoidance response (Tao et al., 2008) and the fact that low R:FR-mediated growth of petiole and lamina continues over multiple days (Fig. S1). The concentration kinetics may point towards a role for auxin biosynthesis especially during the first hours of shade avoidance signaling, in which auxin is required for reprogramming of developmental processes until a new growth homeostasis is reached. However, our gene expression analysis suggests that there may be additional smaller peaks of auxin production in low R:FR-grown plants as both YUC8 and YUC9 expression levels
show small rises in expression levels on days two and three of the shade treatment (Fig. 4, S6).

Our data suggests that in juvenile leaves low R:FR-induced auxin synthesis mainly takes place in the lamina (Fig. 2, S4d), although this was not apparent in our concentration measurements (Fig. 4). If the lamina is indeed the source of rising auxin levels in the petiole in low R:FR, the newly synthesized auxin would therefore have to be immediately transported away to the petiole by means of polar transport or the phloem. Speed of rootward auxin transport has been determined to be 7-8 mm h\(^{-1}\) for the Arabidopsis inflorescence, but may vary between different organs (Kramer et al., 2011). Such a transport rate could be sufficient to transport auxin from the leaf margins to the petiole in 15-day-old plants within the measured timepoints and may even be increased in shade. The PIN3 export carrier was shown to adopt a more lateral position in low R:FR-treated hypocotyls (Keuskamp et al., 2010) and PIN3 was upregulated in both petioles and lamina in low R:FR (Fig. S4e), which may result in increased protein abundance and enhanced auxin export. How far auxin can subsequently travel after excretion into the apoplast depends on the auxin influx carriers, fraction of molecules that becomes protonated and thus becomes membrane permeable, permeability of the cell membranes and cell wall thickness (Kramer, 2006; Swarup & Péret, 2012). Apoplastic acidification happens within minutes of the onset of a shade signal in Arabidopsis petioles (Sasidharan et al., 2010), which will increase the protonated fraction of auxin molecules and consequently diffusion into cells. It may thus be possible that the increased auxin concentration in petioles is due to transport from the lamina. Alternatively, low R:FR also induces auxin production in petioles. Although the \textit{YUCCA}s were predominantly upregulated in the lamina, \textit{YUC9} was also induced in the petioles after 2h of low R:FR (Fig. 2). It has been shown in ten-day-old Arabidopsis seedlings that all plant parts including hypocotyls, cotyledons, roots and leaves have the capacity to synthesize auxin, but this has not been specified for lamina and petioles separately (Ljung et al., 2001). It was shown recently that cotyledon-specific overexpression of \textit{YUC3} in a quintuple \textit{yuc} mutant background leads to an auxin overexpression phenotype in both cotyledons and hypocotyls but not in roots (Chen et al., 2014), indicating that local auxin production can be required for certain responses.

As overall auxin concentration was similar between petioles and lamina while their growth response to IAA application is opposite, it is likely that their contrasting growth in response to low R:FR-induced auxin production are due to a difference in auxin
sensitivity. Different responsiveness to auxin could be brought about by a context-specific difference in abundance of auxin signaling components, such as receptors, Aux/IAAs and/or ARFs. Regulation of environmental responses through altered expression levels of TIR/AFB receptors has been reported previously for pathogen defense and root responses to nutrient availability (Navarro et al., 2006; Perez-Torres et al., 2008; Vidal et al., 2013), and AFB1 expression shows hypocotyl-specific induction in low R:FR (Hersch et al., 2014). In juvenile leaves, AFB1 expression was upregulated both in petioles and lamina (Fig. 5a,b), but AFB1 protein levels were increased by low R:FR specifically in petioles (Fig. 5c). This however seems to play a minor role in our experimental conditions as tir/afb mutants showed a normal petiole response in low R:FR and PEODIAA treatment only affected the petiole response in Col-0 at high concentration (Fig. 5d,e). It was recently predicted by a computational model of low R:FR-dependent hypocotyl elongation that enhanced auxin sensitivity may be especially important when there is a low auxin signal (Hersch et al., 2014). Correspondingly, we found that a PEO-IAA concentration that had no effect on the wild type did inhibit petiole elongation in the sav3 and pif7 mutants, of which the latter also shows increased AFB1 expression in low R:FR (Fig. 5). Regulation of the AFB1 auxin receptor may thus be an important mechanism to ensure elongation responses in shade particularly when overall IAA levels are low. Upregulation of AFB1 receptor during neighbor detection, such as in our experimental conditions, may be important to anticipate future shading events. The Aux/IAAs and ARFs are other components of the auxin pathway that may confer specificity. End-of-day-FR was previously reported to lead to higher induction of IAA19 and IAA6 in petioles than in lamina (Kozuka et al., 2010). The Aux/IAAs act as co-receptors and different combinations of TIR/AFB – Aux/IAA have different auxin-binding affinities (Calderon-Villalobos et al., 2012; Havens et al., 2012). Abundance of different IAAs could thus determine the sensitivity of a tissue. Although we found that low R:FR-induced petiole elongation was not affected in the iaa5iaa6iaa19 and msg2 mutants (Fig. S8), it would be informative to study different combinations of higher order tir/afb – aux/iaa mutants to unravel a putative role of auxin receptor complexes in the shade avoidance response. Furthermore, different IAAs may interact with different ARFs (Vernoux et al., 2011) and thus affect transcription of different targets. Furthermore, ARFs are known to show distinct spatial and developmental expression patterns (Rademacher et al., 2011) and may be leaf part-specific. Finally, a specific auxin response may depend on tissue-specific chromatin structure, which may make certain auxin-responsive genes more or less accessible for the transcriptional machinery (Widman et al., 2014).
Here, we showed that two different genes of the XTH family, XTH15 and XTH19, show a leaf part-specific expression pattern in low R:FR which was reduced in the pif7 mutant (Fig. 6). XTH15 was previously shown to be upregulated in petioles after 24h of low R:FR treatment (Sasidharan et al., 2010). We found a similarly small upregulation in petioles after 24h (1.8 fold), but a much more significant upregulation in lamina at earlier timepoints (23 fold after 4h of low R:FR). XTHs are cell wall-modifying enzymes that can play a role in both cell wall loosening and cell wall strengthening (Takeda et al., 2002; Cosgrove, 2005; Mellerowicz et al., 2008).

Whether the induction of XTH15 and XTH19 depends on transcriptional activity of PIF7 itself or on PIF7-dependent auxin biosynthesis cannot be distinguished from our data. Previously published data shows that XTH15/XTR7 is a target of PIF1, PIF3, PIF4 and PIF5 and that its expression is reduced in the pif4pif5 mutant, while XTH19 does not appear in ChIP-seq data of PIF targets (Hornitschek et al., 2009; Oh et al., 2009; Hornitschek et al., 2012; Oh et al., 2012; Zhang et al., 2013). On the other hand, expression of XTH19 is auxin responsive (Yokoyama & Nishitani, 2001; Vissenberg, 2005; Nemhauser et al., 2006; Chapman et al., 2012; Pitaksaringkarn et al., 2014), while XTH15 does not appear to be auxin inducible (Yokoyama & Nishitani, 2001; Nemhauser et al., 2006; Chapman et al., 2012). Hence, while shade-induced XTH15 expression in the lamina may be directly mediated by the PIFs, the expression of XTH19 in the petiole may rather be due to the PIF7-mediated increase in auxin levels. Indirect evidence for this hypothesis comes from studies investigating shade avoidance with other light treatments (low blue or green shade). In response to attenuated blue light, a treatment that also leads to PIF-mediated shade responses (Keller et al., 2011), induction of XTH15 was not inhibited by PEO-IAA (Keuskamp et al., 2011) and neither was its green shade induction inhibited by NPA (Sasidharan et al., 2014). XTH19 induction however was reduced in green shade after NPA treatment and in the TAA1-mutant weiz (Sasidharan et al., 2014), showing that XTH19 expression is auxin-dependent in a shade context. As it was recently shown that PIFs and ARFs may interact to jointly regulate target genes (Oh et al., 2014), the expression of some shade-induced genes might also depend both on PIFs and auxin signaling components. Hence, different combinations of PIF and auxin-mediated transcriptional readouts may underlie the tissue-specific growth responses in the leaf.

Besides tissue-specific regulation of growth regulators such as the XTHs, there may be tissue-specific hormonal interactions that determine different organ responses. In
young leaf primordia shade-induced auxin mediates a cytokinin-mediated reduction
in cell division (Carabelli et al., 2007) and other hormones are known to be involved
in shade avoidance responses (Gommers et al., 2013). Currently we have poor
understanding of the localization and developmental windows of these hormonal
(inter)actions, although it is known that some hormones can have very localized
effect (e.g. Savaldi-Goldstein et al., 2007; Bargmann et al., 2013). Furthermore,
known negative regulators of shade avoidance may similarly play a tissue-specific
and developmental age-dependent role. We showed that HFR1 expression is
induced higher in lamina than in petioles, but that the hfr1 mutant displays wild-type
leaf responses (Fig. S2). This in contrast to hfr1 seedlings, which are known to show
enhanced hypocotyl elongation in low R:FR (Sessa et al., 2005). These findings
advocate further unraveling of the shade avoidance signaling network taking into
account tissue-specific and developmental-determined signals.

ACKNOWLEDGEMENTS

Funding in the Fankhauser lab comes from the University of Lausanne and the Swiss
National Science foundation (FNS 310030B_141181/1 to C.F.). This work was
further supported by Kempestiftelserna, the Swedish Governmental Agency for
Innovation Systems and the Swedish Research Council to K.L. We are grateful to
Tino Dornbush for providing the Matlab script to take leaf measurements and to
Séverine Lorrain for the pif4pif5pif7 mutant. We thank Hiroshi Nozaki for kindly
providing PEO-IAA, Peter Quail for the pif7-1 mutant, Joanne Chory for the sav3-2
mutant and Miguel Blazquez for the iaa5iaa6iaa19 and msg2-1 mutants. The
pAFB1::AFB1-GUS, 35S::AFB1-myc and tir/afb lines were generously provided by
Mark Estelle. We thank Hannes Richter from the Lausanne Genome Technology
Platform (LGTF) for advice on the QPCR analyses, Roger Granbom for excellent
technical assistance, Markus Kohnen for browsing previously published ChIP-seq
and transcriptomic datasets and three independent reviewers for their useful
comments on the manuscript.

REFERENCES

Andersen SU, Buechel S, Zhao Z, Ljung K, Novák O, Busch W, Schuster C,
Lohmann JU. 2008. Requirement of B2-Type Cyclin-Dependent Kinases for


Pencik A, Simonovik B, Petersson SV, Henykova E, Simon S, Greenham K,
homeostasis and gradients in Arabidopsis roots through the formation of the indole-3-acetic acid catabolite 2-oxindole-3-acetic acid. The Plant Cell 25: 3858–3870.

Perez-Torres C-A, Lopez-Bucio J, Cruz-Ramirez A, Ibarra-Laclette E,
lateral root development in Arabidopsis by modulating auxin sensitivity via a
mechanism involving the TIR1 auxin receptor. The Plant Cell 20: 3258–3272.

Auxin and Ethylene Regulate Elongation Responses to Neighbor Proximity Signals
Independent of Gibberellin and DELLA Proteins in Arabidopsis. Plant Physiology
149: 1701–1712.

Pierre-Jerome E, Moss BL, Nemhauser JL. 2013. Tuning the auxin transcriptional

Pitaksaringkarn W, Matsuoka K, Asahina M, Miura K, Sage-Ono K, Ono M,
by ANAC071 under auxin flow are involved in cell proliferation in incised Arabidopsis
inflorescence stems. The Plant Journal: n/a–n/a.

Auxin Is Required for Shade-Induced Hypocotyl Growth in Brassica rapa. Plant
Physiology 165: 1285–1301.

Rademacher EH, Moller B, Lokerse AS, Llavata-Peris Cl, van den Berg W,
Weijers D. 2011. A cellular expression map of the Arabidopsis AUXIN RESPONSE

the red/far-red light receptor phytochrome B alter cell elongation and physiological

involved in xyloglucan endotransglucosylation and endohydrolysis: current
perspectives and a new unifying nomenclature. Plant & Cell Physiology 43: 1421–
1435.


Sasidharan R, Chinnappa CC, Staal M, Elzenga JTM, Yokoyama R, Nishitani K,
Voesenek LACJ, Pierik R. 2010. Light quality-mediated petiole elongation in
Arabidopsis during shade avoidance involves cell wall modification by xyloglucan

Interactions between auxin, microtubules and XTHs mediate green shade-induced

Savaldi-Goldstein S, Peto C, Chory J. 2007. The epidermis both drives and
Morelli G, Ruberti I. 2005. A dynamic balance between gene activation and 
repression regulates the shade avoidance response in Arabidopsis. Genes & 

Smith H, Holmes MG. 1977. THE FUNCTION OF PHYTOCHROME IN THE 
NATURAL ENVIRONMENT?III. MEASUREMENT AND CALCULATION OF 
PHYTOCHROME PHOTOEQUILIBRIA. Photochemistry and Photobiology 25: 547–
550.

Steindler C, Matteucci A, Sessa G, Weimar T, Ohgishi M, Aoyama T, Morelli G, 
Ruberti I. 1999. Shade avoidance responses are mediated by the ATHBD2 HD-Zip 


Suppression and acceleration of cell elongation by integration of xyloglucans in pea 
stem segments. Proceedings of the National Academy of Sciences of the United 
States of America 99: 9055–9060.

Tao Y, Ferrer JL, Ljung K, Pojer F, Hong F, Long JA, Li L, Moreno JE, Bowman 
pathway is required for shade avoidance in plants. Cell 133: 164–176.

Yamamoto KT. 2004. MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein 
that functions together with the transcriptional activator NPH4/ARF7 to regulate 
differential growth responses of hypocotyl and formation of lateral roots in 

Vernoux T, Brunoud G, Farcot E, Morin V, Van den Daele H, Legrand J, Oliva M, 
dynamic input into robust patterning at the shoot apex. Molecular Systems Biology 7: 
508.

Vidal EA, Moyano TC, Riveras E, Contreras-Lopez O, Gutierrez RA. 2013. 
Systems approaches map regulatory networks downstream of the auxin receptor 
AFB3 in the nitrate response of Arabidopsis thaliana roots. Proceedings of the 

Vissenberg K. 2005. Differential expression of AtXTH17, AtXTH18, AtXTH19 and 
AtXTH20 genes in Arabidopsis roots. Physiological roles in specification in cell wall 

Widman N, Feng S, Jacobsen SE, Pellegrini M. 2014. Epigenetic differences 
between shoots and roots in Arabidopsis reveals tissue-specific regulation. 

and patterns of tracheary elements differentiating in pith explants. Annals of Botany 
68: 463–467.


**FIGURE LEGENDS**

**Figure 1.** Petiole and lamina responses of leaf 3 in low R:FR. Petiole length (a), lamina size (b) and relative expression of PIL1 (c) from leaf 3 (Col-0) over time. Gene expression values were calculated as fold induction relative to petiole sample at t=0. (d,e) Petiole length and lamina size of wild-type (Col-0) and *pif* mutants in high and low R:FR after 3d of treatment. Plants were 15d old at t=0. Error bars represent 2SE, *= p<0.05, Students *t*-test low R:FR vs. high R:FR within genotype. Black bar represents 8h dark period.

**Figure 2.** Auxin biosynthesis in low R:FR is PIF7-dependent. (a-d) Expression of shade-inducible *YUCCA* genes in petiole and lamina after 2h of control light (high R:FR) or low R:FR. Gene expression values were calculated as fold induction relative to lamina sample at t=0. (e) *YUC8* expression in lamina of wild-type (Col-0) and *pif7* plants over time. Expression values were calculated relative to Col-0 at t=0. (f) Auxin concentration in lamina after 2h of high or low R:FR. Error bars represent 2SE, *= p<0.05, Students *t*-test low R:FR vs. high R:FR within organ (a-d) or genotype (f).

**Figure 3.** Manipulation of auxin levels mimics low R:FR leaf responses. Petiole length (a,c,e) and lamina size (b,d,f,) of Col-0 plants after 3d of application of 10 µM
IAA or 500 µM L-Kynurenine (Kyn) to the lamina, or 25 µM NPA to the lamina-petiole junction. Error bars represent 2SE, *= p<0.05, Students t-test chemical treatment vs. mock application.

**Figure 4. Contrasting petiole and lamina responses are not due to different auxin concentration.** Basal levels of YUC8 (a) and IAA29 (b) in petioles and lamina of 15-day-old plants. Expression values were calculated as fold induction relative to petiole sample. (c) Relative expression of YUCCA8 in lamina of plants in control light (high R:FR) or low R:FR over time. Values were calculated as fold induction relative to t=0 sample in high R:FR. (d,e) Auxin concentration in petioles and lamina of plants in high or low R:FR over time. Auxin concentration data for Col-0 petioles at t=2h are the same as presented in Figure 2. Error bars represent 2SE, *= p<0.05, Students t-test. FW= fresh weight. Black bars represent 8h dark period.

**Figure 5. TIR/AFB-mediated auxin perception in low R:FR.** AFB1 expression in petioles (a) and lamina (b) of wild-type (Col-0) and pif7 after 2h of control light (high R:FR) or low R:FR. Expression values were calculated as fold induction relative to Col-0 sample in high R:FR. (c) Enzyme activity of AFB1-GUS in petioles and lamina of pAFB1::AFB1-GUS plants after 24h and 72h of light treatment. (d) Petiole length of tir1/afb mutants after 3d of light treatment. (e) Petiole elongation response of Col-0 to low R:FR after application of different concentrations of PEO-IAA. Response was measured as the difference in petiole length between plants in control light and plants in low R:FR after 3d. (f,g) Petiole elongation response to low R:FR in Col-0, pif7 and sav3, after application of 200µM PEO-IAA. Error bars represent 2SE, *= p<0.05, Students t-test low R:FR vs. high R:FR within genotype (a,b,d) or organ (c), PEO-IAA vs. mock treatment within genotype in e-g.

**Figure 6. Lamina and petiole-specific XTH expression.** Relative expression of XTH15 (a) and XTH19 (b) in petioles and lamina over time in control light (high R:FR) or low R:FR. Expression values were calculated as fold induction relative to petiole sample at t=0. (c,d) Relative expression of XTH15 in lamina (c) and of XTH19 in petioles (d) in Col-0 and pif7. Expression calculated as fold induction relative to Col-0 sample in high R:FR after 4h of light treatment. Error bars represent 2SE, *= p<0.05, Students t-test low R:FR vs. high R:FR within genotype. Black bar represents 8h dark period.
Supporting Information

Figure S1. Petiole and lamina responses of all leaves in low R:FR.

Figure S2. Role of HFR1 in shade avoidance phenotype of juvenile leaves.

Figure S3. Boxplot representation of Figure 1d,e.

Figure S4. Auxin production in the blade leads to growth responses in lamina and petiole.

Figure S5. Petiole and lamina response to IAA application.

Figure S6. Expression of YUC9 in lamina.

Figure S7. Expression of auxin receptors in leaves.

Figure S8. Low R:FR-induced petiole elongation in (co)receptor mutants.

Figure S9. PIF7 expression in petioles and lamina.

Table S1. Primer sequences used for Real Time RT-PCR.

Method S1. Matlab script for petiole length and lamina area analysis.