Mechanisms and consequences of neighbour detection in *Arabidopsis thaliana*

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Mechanisms and consequences of neighbour detection in *Arabidopsis thaliana*

De mechanismen en gevolgen
van buurplantdetectie
in *Arabidopsis thaliana*

(met een samenvatting in het Nederlands)

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Promotoren: Prof. dr. R. Pierik
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Contents

Chapter 1

General introduction

Agricultural studies in the past decades have tried to deal with an important issue: ensuring food production for the increasing human population (Schmidhuber and Tubiello, 2007). It has been estimated that in 2050, the human population on earth will rise up to 9 billion people (Tilman et al., 2011), leading to an urgent need for global food security. In order to cover these demands for food, crop productivity needs to be improved (Ballaré et al., 2012), requiring further intensification of agriculture. This intensification for higher crop productivity requires also higher plant densities, which results in higher competition between the individuals in a plant community for water, nutrients and light. This competition becomes more intense in the presence of the weeds, which take up resources but do not deliver yield.

Crop and weed competition

Crop-weed competition is a worldwide agricultural challenge since all cropping systems face this problem. Weed plants can usually produce a large amount of seeds, which can lay dormant in soil, creating a vast seed bank. Some of them can stay dormant up to 20 years (Gurney et al., 2006). Because of this, weed control is not an easy task. Weeds germinate under suitable conditions (light, nutrients, water) and grow between the crop plants (see Figure 1.1). The competition between crop and weed plants can cause 34% of the yield loss, even more than animal pests and pathogens, 18% and 16% respectively (Oerke,

Figure 1.1 | Weeds growing between the crop rows in a pomegranate (*Punica granatum*) cultivation system. Photograph was taken at Magoula, Pyrgos, Ilias, Greece, by Despoina Pantazopoulou.

2006). Multiple studies have shown that yield losses can be avoided, if weeds are removed from the field before crop emergence (Nieto et al., 1968; O'Donovan et al., 1985; Kropff and Spitters, 1991). Also studies have determined the critical period of weed control as well as the weed density threshold in order to understand the crop-weed competition (Van Acker et al., 1993; Swanton et al., 1999; Evans et al., 2003; Page et al., 2009). The use of herbicides is the most common way to suppress weeds but not a sustainable solution. Despite the herbicide use, yield loss still occurs due to weeds (Gallandt and Weiner, 2007). For that reason a more sustainable solution needs to be found.

The importance of planting pattern and the application of Evolutionary Agroecology for weed suppression and the crop performance

As mentioned in the previous paragraph herbicides are the most common way to suppress weeds but it also raises the expenses to produce crop yield (Gallandt and Weiner, 2007). Evolutionary Agroecology (Darwinian Agriculture) could be an approach towards more sustainable weed suppression, it essentially uses evolutionary theory to modify crops and crop traits to improve crop yield (Denison et al., 2003; Weiner, 2003). The general idea behind this theory is to rely on Darwinian theory regarding the enhancement of individual plant fitness, but to consider the whole group of plants (for example: a whole agricultural field) to select traits that enhance fitness of the full crop community (Weiner et al., 2017). In crop-weed competition, increasing competitive performance of the entire crop community might sustainably suppress weeds and subsequently increase crop yield (Weiner et al., 2010). Indeed, in cereals an increase in crop density combined with increased spatial uniformity in the planting patterns can lead to an increase in crop yield up to 30% (Weiner et al., 2001) while weeds are suppressed more effectively (Olsen et al., 2005b; Kristensen et al., 2008). The individual plants may not produce more, perhaps even less, but the group as a whole does. The possible explanation for these interesting results is that cereal plants growing at a more uniform pattern reduce the intraspecific competition within the cereal community and with a size advantage of the crop, this can create a better shade canopy above the weeds compare to the row planting pattern (Weiner et al., 2010). Therefore, if the competitive potential of crop community can be improved, we may create more efficient competitors (Cahill et al., 2005) against weeds. This hypothesis developed the idea that a cooperative shading strategy from the whole crop community can cause better weed suppression (Weiner et al., 2010). However this idea has not been tested yet.

Competition for light and the Shade Avoidance Syndrome (SAS) in dense canopy

High planting densities in cereal crops have positive effects on weed suppression (Weiner et al., 2010). However, the competition for light between high density-grown plants also becomes more intense (Schmitt and Wulff, 1993; Schmitt et al., 2003; Franklin and Whitelam, 2005). In addition to light, plants also compete for water and mineral nutrients (Harper, 1977). In such a crowded environment plants enhance their individual plant fitness via the elongation of stem, petiole or hypocotyl and upward movement of the leaf (Figure 1.2) in order to escape from a shading canopy. These traits characterize a very well-known syndrome called Shade Avoidance Syndrome (SAS) (Schmitt et al., 1995; Schmitt et al., 2003; Franklin, 2008). These responses facilitate photosynthesis and growth by repositioning the photosynthetic organs directly towards the sunlight (Pierik et al., 2013). But how do plants detect their neighbours in order to induce SAS? In the proximity of a neighbour plant, the horizontal reflection of Far-red (FR) light increases, while the red (R) and blue (B) are absorbed, leading to a strong decrease of the R:FR ratio in high-density growing plants (Morgan and Smith, 1976; Pierik and de Wit, 2013). The reduction of the R:FR is an early signal for upcoming competition for light, even before true shade occurs (Ballaré et al., 1990). In the presence of weeds this phenomenon becomes more intense, since weeds induce extra FR-enrichment towards the crop plants (Pierik and Testerink, 2014). During SAS, plants allocate carbon towards the elongation of stems and petioles instead of the harvestable and reproductive organs, causing a loss of yield potential (Robson et al., 1996; Boccalandro et al., 2003; Page et al., 2010). SAS-expressing plants are relatively tall and thin and have a rather weak root development, causing high susceptibility to lodging (Robson et al., 1996). Also the defence mechanism against herbivores and diseases is weakened during SAS (Izaguirre et al., 2006; Cerrudo et al., 2012). All these implications of SAS help plants reach the light, but also lead to a more open canopy, where sunlight can pass through, which facilitates weed growth. Therefore, from a weed-suppression perspective, the crop canopy should close rapidly to maximize shading weeds, and allocation of resources to the unnecessary organs needs to be minimized. Optimizing planting pattern [(Weiner et al., 2010); mentioned in pervious paragraph] as well as crop plants architecture (Robson et al., 1996; Boccalandro et al., 2003) could be a way to achieve such crop canopies. While SAS has been observed in a broad variety of crop species (Libenson et al., 2002; Chincinska et al., 2008; Green-Tracewicz et al., 2011; Whipple et al., 2011; Cagnola et al., 2012; Gong et al., 2015), it has been particularly well-studied

Figure 1.2 | Shade avoidance in *Arabidopsis thaliana* rosette plant. *Arabidopsis thaliana* grew under control (left) or low R:FR conditions (right).

in *Arabidopsis thaliana* (Moreno et al., 2009; Casal, 2013a; Gommers et al., 2013; Bongers et al., 2014; Franklin et al., 2014; Zheng et al., 2016; Ballaré and Pierik, 2017; Fiorucci and Fankhauser, 2017).

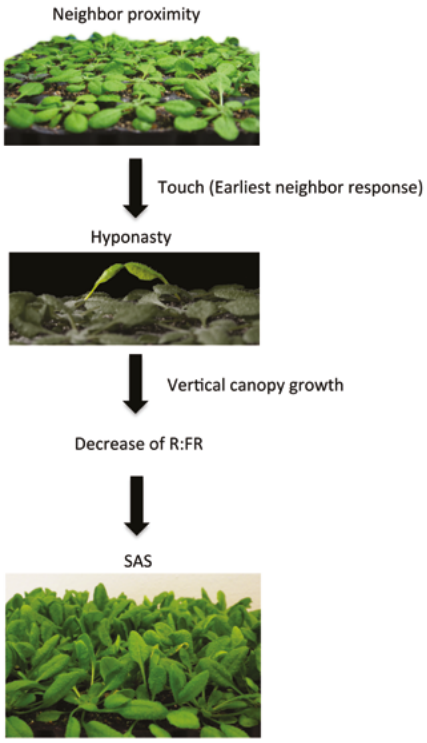

Figure 1.3 | Schematic overview of neighbour proximity sensing in the *Arabidopsis* canopy. Neighbours are first detected through touch, which changes the vertical architecture and triggers FR reflection, which then induces photoreceptor-driven shade avoidance.

Acclimations of canopy plants leading to the Shade Avoidance Syndrome

It is very important for plants growing in natural dense communities, to perceive and respond to upcoming shade signals as fast as possible. Some years ago it was found that the earliest neighbour detection mechanism in *Arabidopsis* canopies is through leaf touching. It was observed that the leaves responded to neighbour proximity with a hyponastic response (or also called upward movement of the leaf) due to leaf tip touching. This hyponastic response changed the canopy architecture from a horizontal structure to a more vertical one (de Wit et al., 2012). This vertical structure generated Far-red (FR) light reflection, leading to a low R:FR signal which resulted in the typical shade avoidance responses such as petiole elongation and further upward movement of the leaves (Figure 1.3). Two key steps characterize neighbour proximity sensing in *Arabidopsis* canopies. The initial step is the touching of leaves, which subsequently can lead to FR enrichment and full SAS expression. These steps will be explained below in detail.

Touch

Touch (among other factors such as herbivory attack, wind, rain) is one of the exogenous environmental mechanical stimuli (Toyota and Gilroy, 2013). Plants respond to mechanical stimuli by changing their growth, which was already described by Darwin centuries ago (1881). Interestingly, different plant species can respond to mechanical stimulation in very

different ways. For instance, *Mimosa pudica* is able to fold its leaves within seconds after being touched and a rapid closing trap response can also be observed in the *Dionaea muscipula* (Venus' Flytrap) after touching trichomes (Fagerberg and Allain, 1991; Malone, 1994; Braam, 2005). However most other species respond slowly over time in touch-induced response. This slow mechanical-induced response is called thigmomorphogenesis (Jaffe, 1973). Thigmomorhogenesis (derived from the Greek word *thigma* "θίγμα" which means *to touch*) describes plant morphological responses to mechanical stimulation. Some of the *Arabidopsis* thigmomophogenetic changes are overall growth reduction and delay in flowering time (Chehab et al., 2012; Lange and Lange, 2015). Several phytohormones play a role in the touch-induced morphological changes, with a strong emphasis on jasmonic acid (JA), but also ethylene, auxin, abscisic acid (ABA), brassinosteroids, and gibberellin.

Jasmonic acid

JA has a very important role in wounding and in the defence against necrotrophic pathogens and herbivorous insects (reviewed in Howe and Jander, 2008). Lipoxygenases metabolize linoleic acid (one of the fatty acids of membrane lipids) towards JA formation. These steps take place in the chloroplasts and generate the oxylipins such as the JA precursor 12-oxo-phytodienoic acid (OPDA) and methyl ester (MeJA) (reviewed in Wasternack and Hause, 2013), which have been reported to have a role in thigmomorphogenesis. The *Arabidopsis cev1* mutant and *Medicago truncatula* produce high level of MeJA and 12-OPDA after a touch treatment (Ellis et al., 2002; Trenter et al., 2008). Other touch responses related to JA, MeJA and 12-OPDA was found in *Phaseolus vulgaris* and *Bryonia dioica* (Weiler et al., 1993; Stelmach et al., 1998). JA is conjugated to L-isoleucine, yielding JA-Ile (Wasternack and Kombrink, 2009; Suza et al., 2010) that binds to the COI1-JAZ receptor complex. This binding leads to ubiquitination and degradation of JAZ proteins, that are repressors of JA-signaling (Pieterse et al., 2012). JAZ degradation subsequently allows activation of the MYC2, MYC3 and MYC4 transcription factors initiating JA response (Fernández-Calvo et al., 2011). JA signaling in *Arabidopsis* has two branches, of which the MYC branch is activated upon wounding with a synergistic action of ABA (Anderson et al., 2004; Niu et al., 2011).

Abscisic acid

ABA is a phytohormone that is involved in plant development and plasticity under different environmental conditions such as water deficiency, salinity and light (Cutler and Krochko, 1999; Verslues et al., 2006). In *Arabidopsis,* it has been shown that the PYR/PYL/RCAR family of ABA receptors have a very important role in the perception of ABA (Yin et al., 2009). The *abaQ* mutant (*pyr1/pyl1/pyl2/pyl4*) showed hyposenstitivity in root growth and germination in response to ABA (Gonzalez-Guzman et al., 2012). Under abiotic stresses, ABA levels are increased and the ABA receptors inhibit activity of a

negative regulator Phosphatase 2Cs protein (PP2Cs) (Schweighofer et al., 2004), which in turn allows activation of the positive regulator SNF1-related protein Kinase 2 s (SNRKs) (Umezawa et al., 2009). SNRKs induce ABA-responsive genes after targeting the AREB/ABF transcription factors, membrane proteins and ion channels (Sheard and Zheng, 2009; Soon et al., 2012). ABA has not only been associated with the abiotic stress but also with the thigmomorphogenic responses. Bean and rice plants showed increased ABA levels after mechanical stimulation (reviewed in Chehab et al. 2008). However the ABA function has not been unraveled in touch-induced hyponasty in *Arabidopsis* dense stands.

Long-distance leaf-wounding signaling

Long-distance leaf-wound signaling has been associated with the rapid accumulation (even in a minute) of JA and JA-Ile in the wounding areas and in distal tissues (Glauser et al., 2009; Koo et al., 2009), detection of the electrical activity (Stahlberg and Cosgrove, 1992) and expression of defence-related gene and xylem water tension relief (Stahlberg and Cosgrove, 1992; Stahlberg and Cosgrove, 1997; Farmer et al., 2014). Mousavi et al. 2013 showed that the clade 3 GLUTAMATE RECEPTOR-LIKE (GLR) proteins are associated with JA responses in distal tissues. More specifically the *glr3.3glr3.6* double mutant after wounding one leaf showed increase in *JAZ10* expression similar to wild-type in wound-treated leaf but a reduction was observed in the distal leaf. These findings indicate that propagation of electrical activity relies on GLRs, and this induces jasmonate biosynthesis in distal leaves in adult plants (Mousavi et al., 2013).

Light quality-controlled shade avoidance

When the earliest neighbour response, touch-induced hyponasty, has occurred in the *Arabidopsis* canopy, subsequent low R:FR signals are generated in the now vertically structured canopy (de Wit et al., 2012). Also, a vertical gradient in light intensity occurs in dense canopies (Monsi et al., 2005). Leaves in the upper part of a canopy are exposed to the much higher R:FR ratio and light intensity (PAR: Photosynthetically Active Radiation) compared to the lower, shaded ones. Since not all leaves in a canopy are exposed to shade or direct sunlight, horizontal and vertical variations exist within plants or even individual leaves for the light quality and quantity they are exposed to (Monsi et al., 2005; Chelle et al., 2007; Boonman et al., 2009; Crepy and Casal, 2015).

Upon low R:FR, phytochrome B (PhyB) is mostly inactivated, which prevents it from degrading PHYTOCHROME INTERACTING FACTORS (PIFs) in the nucleus (Sakamoto and Nagatani, 1996; Yamaguchi et al., 1999; Duek and Fankhauser, 2005). PIFs belong to the basic HELIX-LOOP-HELIX family of transcription factors and contain 7 members, which can interact with the phyrochromes. Among the 7 members, PIF4, PIF5 and PIF7 have a

predominant important role in shade avoidance (Lorrain et al., 2008; Koini et al., 2009; Li et al., 2012). These positive regulators induce expression of the growth promoting-genes involved in shade avoidance (Leivar and Quail, 2011; Leivar and Monte, 2014). Many hormones has been recorded to be involved in SAS, including giberrellin (GA), abscisic-acid (ABA), jasmonic acid (JA), but auxin is now accepted as dominant regulator of shade avoidance (Nozue et al., 2015; revewed in de Wit et al., 2016a; Ballaré and Pierik, 2017).

The involvement of auxin in shade avoidance

It has been shown that there is direct link between the phyrochrome signaling pathway and auxin biosynthesis (Hornitschek et al., 2012; Li et al., 2012; Bou-Torrent et al., 2014), since PIF4, PIF5 and PIF7 bind the promoter region of the auxin biosynthesis genes (*YUCCAs*) (Stepanova et al., 2008; Tao et al., 2008) which encode auxin biosynthetic enzymes required for low R:FR-induced elongation (Won et al., 2011; Nozue et al., 2015; de Wit et al., 2015). Furthermore, PIFs can also modulate auxin responsiveness by regulating genes such as *INDOLE-3-ACETIC ACID INDUCIBLE* (*IAA)29* and *IAA19* (Hornitschek et al., 2012; Li et al., 2012). It was shown recently in *Brassica rapa* seedlings that auxin biosynthesis occurs in the cotyledons, and transport to the hypocotyl subsequently induces cell elongation there under low R:FR conditions (Procko et al., 2014). In *Arabidopsis*, low R:FR-induced hypocotyl elongation also relies on auxin transport (Pierik et al., 2009), mostly through the PIN3 auxin efflux-associated protein (Keuskamp et al., 2010a). In analogy to the cotyledon-hypocotyl division of auxin dynamics, (Procko et al., 2014), de Wit et al. (2015) recently proposed a similar division within a single leaf between the lamina and petiole. All these findings show how auxin is a major regulator of shade avoidance responses.

Scope of the thesis

In this thesis, I study different aspects of shade avoidance focusing on both the regulatory molecular mechanisms as well as the functional implications. *Arabidopsis thaliana* has proven to be a successful plant model to unravel the molecular mechanisms of responses to different abiotic and biotic stresses, including shade avoidance. Therefore, I use this plant species here to study mechanisms and consequences of shade avoidance.

In **chapter 2** I describe how planting patterns and plant densities affect light quality distribution and productivity in a dense *Arabidopsis* canopy. Including a second wild type accession, the effects of planting density and pattern on competition of the community against and invading competitor is studied. The highest density at a uniform planting pattern of the focal community was highly effective at suppressing competing plants. In **chapter 3** it is studied if reduction of shade avoidance responses (such as hyponasty and petiole elongation) affects yield and if the resulting altered canopy architectures

affect canopy closure and suppression of competitors. Complete suppression of the shade avoidance responses was found to reduce performance in dense stands, and increased proliferation of invading competitors. The SAS mutants used were disturbed in all shade avoidance aspects, and it is possible that a more targeted modification of specific SAS components would be more effective in boosting, rather than reducing, performance at high density. To this end, more knowledge is needed on the mechanisms controlling different SAS responses at different stages of canopy development. The earliest neighbour response in *Arabidopsis* dense stands is through touching of the leaves causing hyponasty (upward movement of the leaf) and not through R:FR signalling (de Wit et al., 2012). In **chapter 4** the molecular mechanisms underpinning touch-induced hyponasty are studied. A combined transcriptome, mutant and physiology approach revealed that touch is a mechanical stress that relies on mechanosensing in the trichomes and involves Receptor-Like Kinases (RLKs) that have been previously proposed to act in mechanosensing. In addition, clade 3 GLRs that are associated with ion transport and electrical signal transmission are also key to this touch response. Upon touch-induced hyponasty, the canopy becomes more vertically structured, leading to induction of low R:FR signals, triggering subsequent shade avoidance responses. **Chapter 5** investigates how perception of low R:FR from different parts of the leaf lead to shade avoidance responses and how this affects plant performace. Trascriptome data elucidated that upward movent of the leaf is associated with spatial auxin dynamics, and regulation by auxin was subsequently elucidated in molecular-physiological experiments. Using 3D computational modeling, it was clarified why plants use the lamina tip to control hyponasty. Finally, **chapter 6** summarizes and briefly discusses the main conclusions from this thesis.

Chapter 2

Optimization of *Arabidopsis* planting patterns to suppress competing neighbours

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Abstract

A key issue in agriculture is the loss of crop yield due to weeds, a problem becoming more urgent with the high demands for food due to the expanding human population. Studies in cereal crop plants have shown that spatial crop uniformity at high crop plant density can suppress weeds more effectively and sustainable. High crop and weed competition means higher competition between plants for light and other resources that are required for growth. In response to this competition for light, plants induce the shade avoidance syndrome (SAS). SAS helps plants to position their leaves away from the shade through accelerated stem elongation and upward leaf movement. This channels resources towards elongation growth at the expense of storage and investment in reproductive organs. Furthermore the upward movement of the leaf increases light penetration in the canopy, thus facilitating weed growth. To study the importance of both planting patterns and shade avoidance for suppression of competitors and performance of target plants, shade avoidance mutants are needed, in addition to manipulation of planting patterns. To date, very few shade avoidance mutants in crop plants are available to study this. For that reason, we decided to use the model plant *Arabidopsis thaliana* wildtype accession Col-0 as a canopy plant and the wildtype accession L*er* as the competitor. In this chapter, we study the effect of sowing pattern and density in *Arabidopsis* canopies with or without competition with L*er*. We found that uniform-high density was the most effective one, since Col-0 yield was increased whereas L*er* was suppressed. These results indicate that uniform planting patterns, rather than conventional row planting patterns, increase plant performance and suppression of competitors.

Introduction

In the next three decades, the human population on Earth will increase from the current 7 billion to 9.3 billion people (FAO, 2017). This will result in higher demands for food that should be covered by using the already limited cultivable land of our planet. A way to achieve enhanced food production is by using land in continuous and dense cultivation systems where crop-crop as well as crop-weed competition can occur (Carriedo et al., 2016). Studies have shown that crop-weed competition leads to serious economic losses and could be the most important cause of yield loss globally (Bridges, 1994; Liebman et al., 2001), causing even more damage than pathogens do (Oerke, 2006). Weeds are defined as plants that are unusually persistent and pernicious, that significantly interfere with the growth of crops, and are optimally adapted to agroecosystems (Tull et al., 1733; Ross and Lembi, 1999; Gallandt and Weiner, 2007). Weed control is usually accomplished with the extensive use of herbicides, and although these can be effective they also have negative side effects on people's health and the environment (Buhler, 2002; Chauhan and Johnson, 2010). Worldwide, the money spent to control weeds represents the biggest percentage among all the expenses required in crop production. Therefore, there is an urgent need for novel ideas and methods in order to suppress weeds and at the same time maintain the environmental balance and sustainability.

Light is one of the main resources that plants compete for in high densities (de Wit et al., 2016b) and neighbours are detected through reflection of far-red light relative to red light by neighbour plants (Ballaré and Casal, 2000; Pierik and Testerink, 2014).

One approach to improve crop yield and suppress weeds at the same time is to change the cropping system and plant behavior through Evolutionary Agroecology approaches (Darwinian Agriculture). Studies performed in cereal crops for a period of nine years have shown the influence of the cropping pattern and density on crop and weed production. It was demonstrated that a combination of increased crop density and increased crop spatial uniformity can effectively suppress weeds, leading to enhanced crop yield (Weiner et al., 2001; Olsen et al., 2005a; Olsen et al., 2006; Kristensen et al., 2008). This has for example been shown to increase wheat yield by up to 30% (Weiner et al., 2001). It is expected that this huge improvement occurs because crop plants growing in uniform patterns can collectively create a stronger shade over the weeds than those sown in row planting patterns. In addition, there may also be less crop-crop competition within the rows. These findings led to the hypothesis that an offensive, cooperative shading strategy can be much more effective in competition of crops with weeds than the defensive, individualistic shade-avoidance strategy (Weiner et al., 2010; Weiner et al., 2017). In other words, crop plants can collectively suppress weeds much better than individual plants would be capable of. However, it remains unknown how the light quality inside these crop stands and plant architecture are affected by the density-pattern matrix.

In principal, a plant is a good competitor when it can reduce resources available to neighbours and/or be prospering even if there is low resource availability caused by neighbours (Cahill et al., 2005). The aim of this chapter is to identify how the light climate and productivity in dense stands can be affected by the crop sowing pattern.

The *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used as a model for crop and the easily recognizable Landsberg *erecta* (L*er*) was used as a competitor (weed). The major advantages of using this system, rather than true crops, is that experiments can be very rapid (because of the short life cycles), use relatively little space and importantly, we can use shade avoidance mutants as presented in chapter 3. Here we show that in a relatively uniform planting patterns Col-0 more rapidly creates a closed canopy than in a row planting pattern, leading to the suppression of the competitor (L*er*) and increase the yield simultaneously.

Results

The effect of the density and pattern on Col-0 performance

To investigate the effect of sowing pattern and density on the biomass of *Arabidopsis thaliana* (hereafter Col-0), we grew canopy plots in three different densities (high, medium and low) and two different patterns (uniform and row) (Figure 2.1A). There was a strong and significant effect from the density and planting pattern on Col-0 biomass. The row pattern produced Col-0 plants with the smallest dry weight, indicating that the intraspecific competition was higher in rows compared to the uniform pattern. In terms of planting density, Col-0 canopy plants reached lower individual plant dry weights when grown in high-density stands compared to lower densities. In contrast, the total biomass of the plot in high density and uniform pattern was higher than the rest of densities (medium, low) and than the row pattern (Figure 2.1A & 2.1B).

To establish the exact impact of the planting density and pattern of the Col-0 canopy on light quality distribution inside the canopy and the shade avoidance response, we measured the R:FR light ratios. Measurements were performed in all three densities (high, medium and low) and patterns (uniform and row) through time. The R:FR showed a reduction in all densities and patterns through time, reflecting the growing canopy (Figure 2.2A). However, the strongest and quickest decline of R:FR was observed in high density/uniform pattern, where the R:FR was decreased from approximately 2.0 to 1.1 after eight days of measurements hinting at a faster closing canopy (Figure 2.2B). This was not the case for the row pattern in high density, where the R:FR was still high, presumably because the inter row distance was bigger than in the uniform pattern. Low and medium density showed reduction of the R:FR (less than 1.5) at day 36 (Figure 2.2A), indicating that the canopy remained more open for a longer period of time.

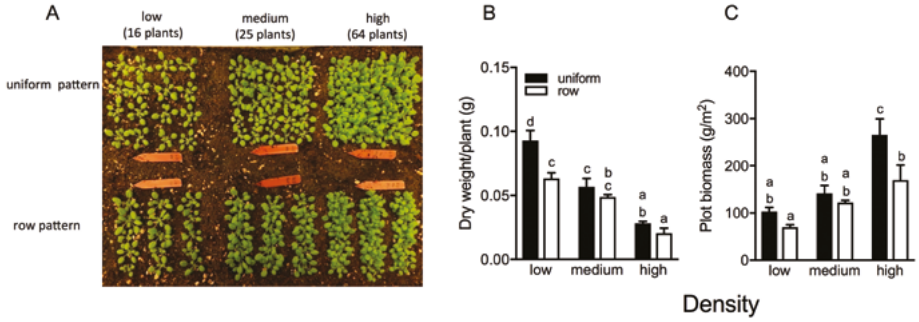


Figure 2.1 | Col-0 in high density, uniform pattern produces more biomass than at the other densities and pattern. (A) Overview of the planting system. In the upper part, Col-0 plants grow in a uniform pattern (uniform), while in the lower part in a row pattern (row) in three different densities (low, medium, high). (B) The shoot dry weight of Col-0 per plant and (C) the plot shoot biomass of Col-0 per m^2 in three different densities (low, medium, high) and two different patterns (uniform, row). Data represent mean ± SE, n = 5. Different letters indicate statistically significant differences (two-way ANOVA with Tukey's Post-hoc test, $p < 0.05$).

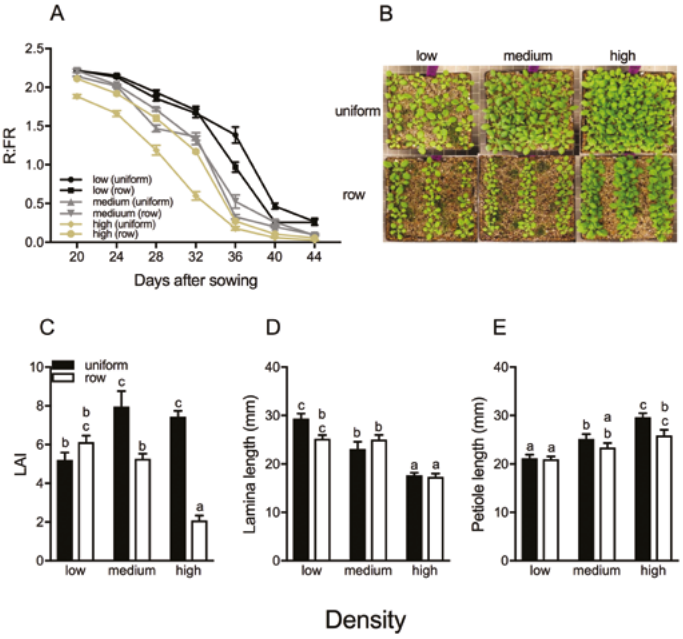


Figure 2.2 | Col-0 can induce a more efficient canopy closure at high planting density than at low density. (A) The R:FR output of Col-0, during the days of growth, in low (black lines), medium (grey lines) and high (yellow lines) densities and two patterns (uniform and row). (B) Pictures illustrate the canopy of Col-0 in the uniform (upper part) and row (down part) patterns after 28 days of growth in low, medium and high density. (C) The LAI, (D) lamina and (E) petiole length upon the three different densities (low, medium, high) and two different patterns (uniform and row). Data represent mean ± SE, n = 5. Different letters indicate statistically significant differences (two-way ANOVA with Tukey's Post-hoc test, $p < 0.05$).

The leaf area index (LAI) expresses the amount of leaf area per unit soil area and reflects the closure status of the canopy. LAI increased more strongly in the uniform pattern than in the row and mostly in the medium and high density (Figure 2.2C). Interestingly, leaf lamina length decreased with increasing plant density, irrespective of the planting pattern (Figure 2.2D). The opposite was observed for petiole length, where the high density induced the strongest elongation (Figure 2.2E). Enhanced petiole elongation, combined with reduced lamina size, are classic aspects of shade avoidance.

The effect of sowing pattern and density on competitor L*er* performance

In order to simulate the crop and weed competition we developed a system using the Col-0 accession as a canopy plant and the visually recognizable Landsberg *erecta* (L*er*) accession as a competitor. This allowed us to study the effect of sowing pattern and density on a good light competitor plant such as L*er* (Hayes et al., 2014; Nozue et al., 2015) versus a canopy plant (Col-0). There was a significant density effect on the dry weight of Col-0, with the uniform pattern at low density performing the best (Figure 2.3A). However, there was a strong competitor effect L*er* on the dry weight of Col-0, when compared to Figure 1A, especially in the row pattern (Figure 2.3A). On the other hand, the biomass of Col-0 showed a reduction in all densities and row pattern by the presence of the competitor L*er* compared to the canopies without it. Still, Col-0 performed better in the high-density uniform pattern than the other densities (Figure 2.1C & 2.3C). Interestingly, the high-density uniform pattern of Col-0 strongly affected L*er* biomass, more so than when planted in row pattern or at lower densities (Figure 2.3D). Summarizing, canopy uniformity together with a high density can enhance productivity and reduce performance of the competitor.

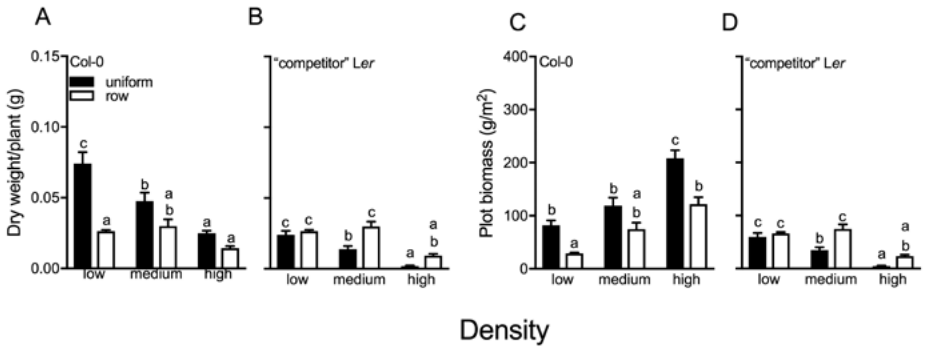


Figure 2.3 | Col-0 can reduce the biomass of competitor (Ler) in high density, uniform pattern. The dry weight per plant of (A) Col-0 and the (B) competitor (L*er*) per plant and the plot biomass per m^2 of (C) Col-0 and the (D) competitor (L*er*) in three different densities (low, medium, high) and two different patterns (uniform, row). Data represents mean ± SE, n = 5. Different letters indicate statistically significant differences (two-way ANOVA with Tukey's Post-hoc test, $p < 0.05$).

Rapid canopy closure can have a negative effect on competitor L*er* performance

We measured the light quality (R:FR) changes at the level of competitor L*er* inside Col-0 canopy, under three different densities and two patterns as shown in Figure 2.4A. Similar to Figure 2.2A, the R:FR decreased more rapidly when the canopy plants are in high-density uniform pattern (Figure 2.4B) compared to the other scenarios. After 28 days L*er* was fully covered under the Col-0 canopy at uniform-high density (Figure 2.4B). Indeed, Col-0 generated the highest LAI at that specific density, suppressing L*er,* which itself formed only a very low LAI under these conditions (Figure 2.4C & 2.4D). Under low and medium Col-0 density L*er* could reach an LAI equal to Col-0 (Figure 2.4D).

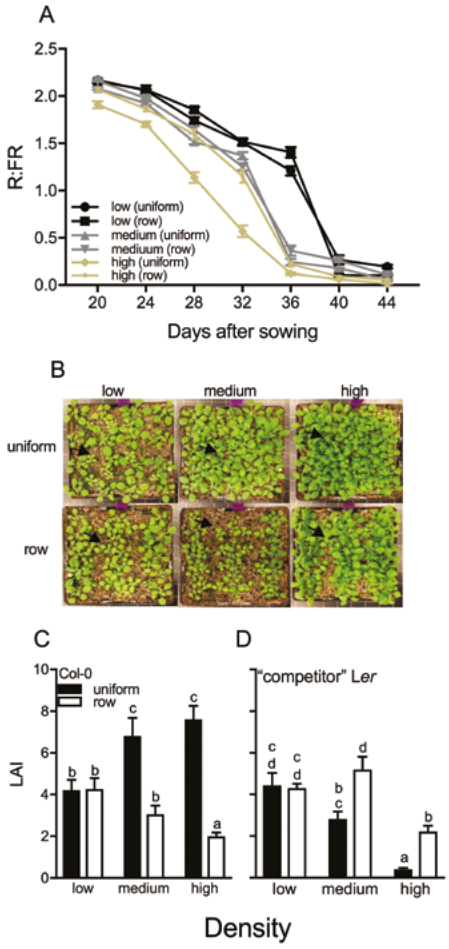


Figure 2.4 | (A) The R:FR ratio in Col-0 canopies, during the days of growth, in low (black lines), medium (grey lines) and high (yellow lines) densities and two patterns (uniform and row) during competition with L*er*. (B) Pictures illustrate the canopy of Col-0 during competition with L*er* in the uniform (upper part) and row (lower part) patterns after 28 days of growth in low, medium and high density. Black arrows illustrate the competitor L*er*. The LAI of (C) Col-0 and (D) L*er* upon three different densities (low, medium, high) and two different patterns (uniform and row). Data represents mean ± SE, n = 5. Different letters indicate statistically significant differences (two-way ANOVA with Tukey's

Shade avoidance traits of Col-0 and competitor L*er* under different densities and patterns

We measured the petiole and lamina length of L*er* and Col-0 when grown together to better understand the effect of different densities and patterns on the shade avoidance response of both. The petiole length of Col-0 in low density was significantly lower than in medium (uniform) and high density, whereas L*er* did not show a strong elongation at the high density in the uniform pattern (Figure 2.5A & 2.5B). Lamina lengths showed the reverse patterns of those observed for petiole length of the same leaves (Figure 2.5A).

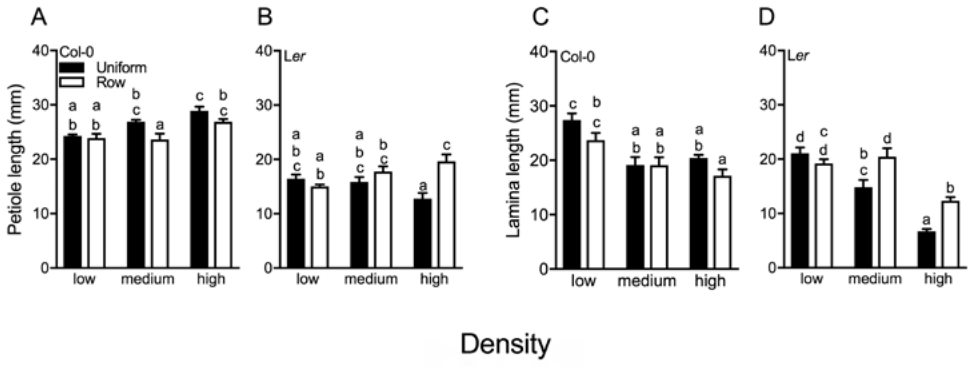


Figure 2.5 | Petiole (A and B) and lamina (C and D) length of Col-0 and the competitor L*er* under three different densities (low, medium, high) and two different patterns (uniform and row). Data represent mean ± SE, n = 5. Different letters indicate statistically significant differences (two-way ANOVA with Tukey's Post-hoc test, $p<0.05$).

Col-0 yield with or without the competitor L*er*

Crop yield is a key agronomic parameter that is reduced when competing with weeds. We tested whether L*er* could affect silique production in Col-0 when grown together. There was indeed a strong effect of the presence/absence of L*er* as a competitor on Col-0 yield (Figure 2.6A & 2.6B). The number of siliques was reduced by presence of L*er* especially in the high density, indicating that L*er* had a significant competitive interaction with Col-0 plants. Moreover there was a strong and significant effect of the density and planting pattern on Col-0 yield, in the presence but also in the absence of L*er*. The higher the density, the higher the yield of Col-0. Between the row and uniform pattern, the latter produced higher yield, and in the uniform pattern most siliques were produced per unit soil area at high-density (Figure 2.6A & 2.6B). It is known that high biomass can lead to high yield (Weiner et al., 2010; Marín and Weiner, 2014). We examined the statistical correlation between yield and the biomass in the uniform pattern with and without competition. Indeed, the correlation was positive under these conditions (Figure S2.1). Our findings suggest that if the canopy plant grows in a more uniform pattern and a relatively high-density, yield can be optimized.

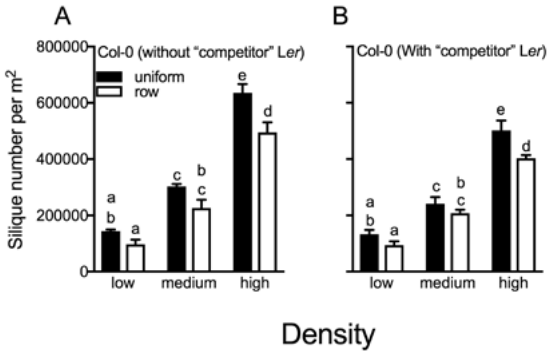


Figure 2.6 | Seed output (silique number per m^2 plot) of Col-0 without (A) or with (B) the competitor L*er* under three different densities (low, medium, high) and two different patterns (uniform and row). Data represent mean ± SE, n=5. Different letters indicate statistically significant differences (two-way ANOVA with Tukey's Post-hoc test, $p<0.05$).

Discussion

The ability of weeds to be adaptable in changing environments and their competitive potential makes them a major threat for agricultural yields when they interact with crop plants (Oerke, 2006). Studies in the past decades found that crop sown uniformity in high density can positively affect the yield and suppress weeds (Weiner et al., 2001; Olsen et al., 2005b; Olsen et al., 2006; Kristensen et al., 2008). However, plants growing at high densities experience light competition and often respond through shade avoidance responses that are associated with reduced allocation of resources towards harvestable organs (Robson et al., 1996; Boccalandro et al., 2003). It is pertinent to resolve how crop yield can be increased at high densities and how weeds can be suppressed at the same time to address the increasing global demands for food. Crop plants in high density enhance their individual plant performance through SAS (Franklin, 2008) but not the community-level performance. Inspired by Evolutionary Agroecology insights (Weiner et al., 2010) we hypothesize that reduction of SAS might enhance crop yield and increase competitiveness over the weeds. Although this will be put to the test in chapter 3, here we first developed the basic set-up for these studies in *Arabidopsis*. Consistent with studies in cereals, in *Arabidopsis* canopies planting density and uniformity also have great potential to improve yield. Indeed, at high density, uniform-planting patterns, the *Arabidopsis* Col-0 accession is very productive and can suppress growth of invading L*er* accession plants.

Uniform-high density is key to suppress competitor L*er* and increase Col-0 yield

The canopy structure leading to the fastest drop in R:FR, thus likely indicating the most rapid canopy closure and inhibition of light penetration, was the high density uniform pattern (Figure 2.4A). These conditions had a strong negative impact on the performance of competitor L*er*. Under these conditions, leaf length (sum of lamina and petiole) of L*er* is thirty times smaller than Col-0 leaf length, whereas these two genotypes reached nearly similar leaf lengths in low density (Figure 2.5). We expect that in this high density scenario, L*er* plants could not access direct light anymore. The reduced growth of L*er*, also indicated by its low LAI (Figure 2.4D), likely results from lack of light interception needed to drive photosynthesis (Norris et al., 2001) further reducing growth (Thornley and Johnson, 1990; Bullock et al., 1998; Olsen et al., 2006). The suppression of competitors on one hand, and the optimal access to light of Col-0 at uniform planting pattern on the other, probably explain the high plant mass and yield at high density uniform Col-0 planting.

It is striking to see how effective a uniform planting pattern is for yield improvement, relative to the conventional row patterns. This is not just a specific feature of *Arabidopsis*, but has also been found for field-grown wheat (Olsen et al., 2005a; Olsen et al., 2006; Marín and Weiner, 2014). It would be worthwhile investigating if the yield gain from uniform planting would outweigh the costs of changing agricultural practices to accommodate such planting patterns. These costs would involve changing sowing/planting machines and machines to maintain crop fields during the growing season. Since a uniform planting pattern would also reduce weed proliferation, less intensive maintenance of the crops during the season would be expected in uniform planting patterns.

Shade avoidance

As seen from Figures 2.3-2.5, both accessions showed pronounced shade avoidance responses. Although petiole elongation, combined with upward leaf movement (hyponasty), will increase access to light at the individual plant level, the reduced leaf lamina growth [Figure 2.4 (C&D) & 2.5 (C&D)] may counterbalance the predicted gain in photosynthesis. At least part of the shade avoidance responses will have been triggered through the drop in R:FR inside the canopies (Figure 2.4A). However, It has been shown that shade avoidance responses, particularly hyponasty, can on their turn also affect the R:FR inside the canopy by affecting the extent to which a vertical canopy structure is formed in this otherwise horizontal growing rosette species (de Wit et al., 2012). Modulating shade avoidance traits in the different canopy structures may thus also affect the light quality distributions inside these canopies.

Conclusion

Changing from row to uniform planting patterns is a promising way forward towards increased crop yield in field-grown crops such as cereals. The next challenge is to optimize the crops themselves to adopt an architecture under high densities that allows them to grow optimally, allocate strongly towards the yielded organs, and suppress weeds more efficiently. This would require plants with suppressed shade avoidance, and the impact of such adjustments are studied in chapter 3.

Materials and methods

Plant growth conditions and measurements

We used the *Arabidopsis thaliana* Col-0 wild type as a canopy plant and L*er* as the competitor. Col-0 seeds were sown in a pot with a surface area 10.5x10.5 cm filled with mix soil with substrate containing soil:perlite (2:1), with additional nutrients [6 g of slow release fertilizer (Osmocote 'plus mini' Ammonium Nitrate Based Fertilizer; UN2071; Scotts Europe BV, Heerlen, The Netherlands) and 6 g MgOCaO (17%; Vitasol BV, Stolwijk, The Netherlands]. The number of plants and the sowing distance between the plants are based on the density and pattern scheme in Figure 2.8. To simulate crop-weed competition where weeds emerge after planting of the crop plant, L*er* plants were sown in a different pot three days after Col-0. Sowing was followed by stratification for 4 days (dark, 4°C). After stratification plants were moved in a short day growth chamber (9 h/16 h of light/dark period respectively; R:FR was 2.3 and PAR = 150 µmol m^{-2} s^{-1}). When Col-0 plots were 15 days old (Col-0 seeds were sown directly in plots), the competitor L*er* (12 days old) was transplanted (see Figure 2.8) in the plot (Col-0 plots were with or without the competitor L*er*). Light spectra were checked with Ocean optics JAZ spectroradiometer (Figure S2.2). Col-0 plants grew for 44 days while L*er* grew for 41 days. After 44 days Col-0 and L*er* were harvested. Morphological and physiological measurements were performed in four plants for each plot. Petiole and lamina length of the three longest leaves from each plant were measured with a digital caliper. Leaf area was scanned and determined with image-J software. Shoot dry weight was recorded with a digital scale, after drying the tissue at 70°C oven for three days. Plot biomass and LAI were calculated from the four individuals by extrapolating to the full plot and density. Seed output was recorded in separate experiments, 3 months after sowing. Every 10 days (starting form the sowing day) plants were watered with nutrients, on all other days they were watered with tap water. When the first silique from each pot turned brown, watering was stopped. The number of siliques was measured, after two weeks of ripening.

R:FR measurements

The R:FR measurements started at day 20 [before the competition starts, (de Wit et al., 2012)] by using the Spectrosense2-Skye light sensor with a glass fiber extension with 0.6 cm light collection area. The sensor was placed inside of the canopy plot (see Figure 2.7) and measured the R:FR from four different directions and on four different positions, resulting in 16 measurements per time. When canopy closure occurred, the sensor was placed under the canopy, without causing any damage to the plants or interfering with the canopy shade. The measurements were always taken from the same position in all densities and patterns.

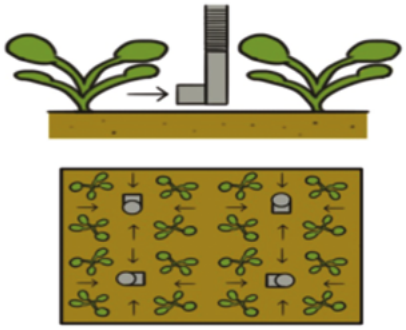


Figure 2.7 | Schematic overview of the R:FR measurements. The grey cartoon represents the R:FR sensor and the black arrows represent the direction of the measurements in each position. The sensor was placed in the 4 positions indicated in the second picture.

Experimental design of the densities and patterns with or with the competitor L*er*

For the Col-0 canopy plants three different densities were used (16 plants per pot (1111 plants m^{-2}), 25 plants per pot (2500 plants m^{-2}), 64 plants per pot (8264 plants m^{-2}); hereafter low, medium and high density respectively) and two spatial patterns [uniform (equal distance between the plants) and row (bigger distance between the rows of the plants but smaller distance between the plants within the rows)] (See Figure 2.8). In uniform pattern, the distance between the plants was 3 cm, 2 cm and 1 cm in high, medium and low density respectively. In row pattern, the distance between the row was always 5 cm while within the rows the distance between the plants were 0.6 cm, 1.25 cm and 2 cm in high medium and low density respectively. The number of competitor L*er* plants was the same in every density and pattern [16 plants per pot (1111 plants m^{-2})]. L*er* plants were transplanted in the same position in each pattern (inner part of the plot) and density and the distance between each other was 5 cm (Figure 2.8).

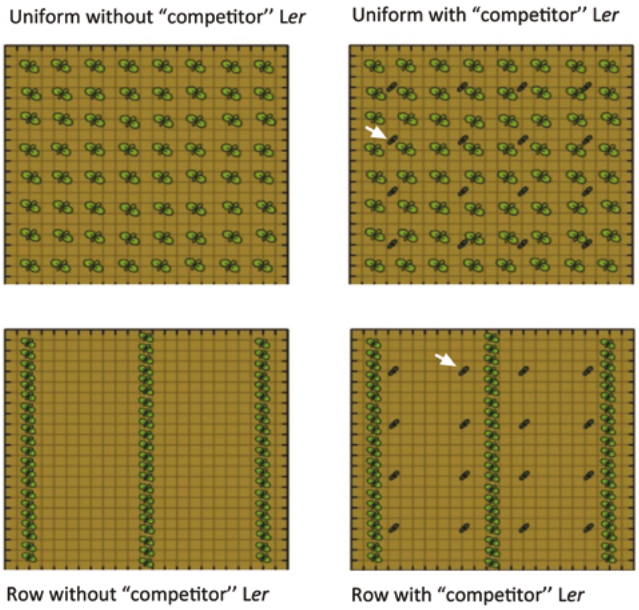


Figure 2.8 | Schematic illustrating the high density in uniform (upper part) and row pattern (lower part). The two patterns are without or with the competitor Ler (white arrows shows the position of Ler in uniform and row canopy).

Statistics

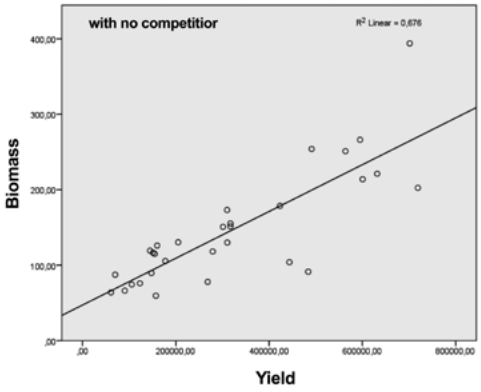
Data were analyzed by one or two-way ANOVA followed by post-hoc Tukey test. Analyses were performed with SPSS or GraphPad.

Acknowledgments

We thank Maxime Brugman for making the diagrams in Figure 2.7 and 2.8, which illustrate the experimental set up, as well as all the Plant Ecophysiology group for their help during the harvest.

Supplemental data

A

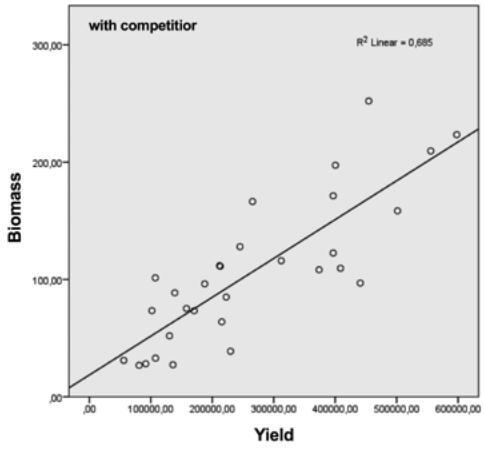


Correlations

		biomass	yield
biomass	Pearson Correlation	1	,822**
	Sig. (2-tailed)		,000
	N	30	30
yield	Pearson Correlation	,822**	1
	Sig. (2-tailed)	,000	
	N	30	30

**. Correlation is significant at the 0.01 level (2-tailed).

B



Correlations

		Biomass	Yield
Biomass	Pearson Correlation	1	,828**
	Sig. (2-tailed)		,000
	N	30	30
Yield	Pearson Correlation	,828**	1
	Sig. (2-tailed)	,000	
	N	30	30

**. Correlation is significant at the 0.01 level (2-tailed).

Figure S2.1 | The correlation of biomass and yield in Col-0 uniform pattern canopies with the absence or presence of the competitor L*er*.

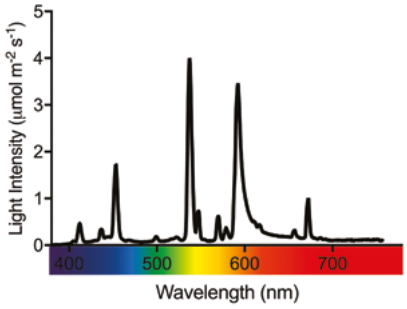


Figure S2.2 | The spectral composition of light in the growth room

Chapter 3

Changes in the canopy architecture by modifying the shade avoidance responses

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Abstract

One of the main problems in agriculture is the loss of crop yield due to weeds, a problem that becomes more urgent now that the population grows and the demand for food is even greater. The use of herbicides is the most common way to control weeds, but extensive use can negatively affect both human life and the environment. A more sustainable solution is, therefore, needed to improve crop yield and at the same time reduce harmful and expensive inputs. One way to achieve this is the application of evolutionary theory to improve agricultural yield and/or sustainability, known as Evolutionary Agroecology or Darwinian Agriculture. In this study we studied if inhibition of shade avoidance syndrome (SAS) responses to neighbours in dense vegetation can enhance the competitive power of the crop community against competitors. We approached this by growing mutants of the model plant *Arabidopsis thaliana* with mild or severe reduction of SAS in canopies together with a competitor, in this case the *Arabidopsis* accession L*er*. We observed that severe reduction of SAS in the canopy phenotype against a potent competitor (L*er*) did not enhance the canopy performance and did not contribute to suppressing the competitor. Comparison of the wild-type (Col-0) and SAS mutant canopies, revealed that complete inhibition of SAS is not beneficial for plants during competition, both in terms of canopy growth and competitive advantage against other competitors. Future studies should elucidate the optimal combination of SAS traits to increase yield and suppresses competitors.

Introduction

The rapidly increasing human population has led to a need for intensification of agriculture, in order to meet the demands for food. Intensive cultivation of crops requires higher plant density, which in turn results in increased crop-weed competition. Weeds account for massive yield losses globally (Bridges, 1994; Liebman et al., 2001) and require severe financial investments in herbicides (discussed in more detail in chapter 2).

Evolutionary Agroecology theory (also called Darwinian Agriculture) is one approach (Denison et al., 2003; Weiner, 2003; Denison, 2007) that aims at using crop performance at a community level to suppress weeds. In chapter 2 we showed that planting patterns indeed can be an instrument to suppress growth of competitors. Here we focus on the question if modifying shade avoidance responses of the canopy plants can help suppress competing plants even further.

One well-established set of responses that enhances the individual plant fitness in dense stands is the well-characterized shade avoidance syndrome, (SAS) which is observed when plants alter their morphological characteristics (Schmitt et al., 1999), in order to avoid shading by their close neighbours (Ballaré et al., 1990). The light environment in dense canopies is already changing before the start of competition for light, due to neighbouring plants reflecting far-red (FR) light (Ballaré et al., 1990). The reduction in the R:FR ratio is a reliable signal of competition, because chlorophyll-containing tissues (leaves) have the unique attribute to absorb R (and blue) light for photosynthesis and to reflect FR light (Smith, 2000; Ballare, 2009). When the ratio of R:FR decreases, SAS is induced (Franklin, 2008; Pierik and de Wit, 2013; de Wit et al., 2016a), consisting of elongation of petioles, internodes and hypocotyls, upward bending of leaves, apical dominance and early flowering (Pierik et al., 2013; Roig-Villanova and Martínez-García, 2016; de Wit et al., 2016b; Ballaré and Pierik, 2017). In this way, SAS helps plants reposition their leaves into direct sunlight, facilitating photosynthesis and growth (Pierik and de Wit, 2013). In various crops, shade avoidance responses have been observed and also in a variety of wild species, including the genetic model plant *Arabidopsis thaliana* (Aphalo et al., 1999; Cober and Voldeng, 2001; Pierik et al., 2004; Gommers et al., 2013; Casal, 2013b; Bush et al., 2015; Carriedo et al., 2016). Phytochromes are a family of R:FR reversible photoreceptors. *Arabidopsis* has 5 phytochromes, PHYA-PHYE, and PHYB is the major phytochrome in light-grown plants that controls SAS (Ballaré, 1999; Franklin et al., 2003; Franklin and Whitelam, 2005). Some decades ago, it was proposed that R light activates the phytochromes, while FR light inactivates them, and the R:FR thus determines the balance between the active (Pfr) and the inactive (Pr) form (Smth and Holmes, 1977; Bae and Choi, 2008). Under FR-enriched conditions, i.e. (future) shade, PhyB is inactivated, which prevents it from degrading the basic helix-loop-helix transcription factors, PHYTOCROME INTERACTING FACTORs (PIFs) in the nucleus (Li et al., 2012; Jeong and Choi, 2013; Leivar and Monte,

2014). The resulting PIF accumulation induces the expression of shade-responsive genes (Oh et al., 2012; Zhang et al., 2013). PIF4, PIF5 and PIF7 have the most important role in the shade avoidance pathway (Lorrain et al., 2008; Li et al., 2012), since they control the shade growth-related genes (Leivar and Quail, 2011; Leivar and Monte, 2014) that trigger changes in the phenotype of plants growing in dense stands.

Shade avoidance responses can reduce crop yield, because instead of storing resources and investing in reproduction, plants use energy and resources to elongate their stems and petioles (Robson et al., 1996; Boccalandro et al., 2003; Carriedo et al., 2016). Furthermore, shade avoidance leads to a more open crop canopy that allows more light penetration that in turn facilitates weeds growth.

In this chapter we will test the hypothesis that suppression of shade avoidance responses of a monoculture community of plants will lead to a canopy that can effectively and jointly suppress invading weeds (Weiner et al., 2010). To this end we used a high plant density at a uniform planting pattern (chapter 2) to study the impact of shade avoidance responses on collective competitor suppression. Recently it was found that very strong shade signals that normally induce strong shade avoidance in *Arabidopsis*, failed to do so in the *pif4pif5pif7* triple mutant and induced a partially reduced elongation response in the *pif4pif5* double mutant (de Wit et al., 2016b). These two shade avoidance mutants were used in this study to investigate the impact of different degrees of shade avoidance inhibition on competitive interactions.

Results

The effect of low R:FR and green shade on Col-0, *pif4pif5* and *pif4pif5pif7* performance

Although the *pif4pif5* double and *pif4pif5pif7* triple knockout mutants have reduced petiole elongation responses to shade cues in short-term experiments, we wanted to verify their responses to prolonged shade cue conditions. We studied two of the most well-known traits of SAS, leaf hyponasty and elongation (Franklin, 2008; de Wit et al., 2012; Hersch et al., 2014; de Wit et al., 2015; Ballaré and Pierik, 2017) under 13 days of shade (cue) treatment. Reduction in the R:FR resulted in the elevation of Col-0 petiole angle (hyponasty) during the first two days (day 29 and 30), while petioles elongated from day 28 until 34 (Figure 3.1B & 3.1C). *pif4pif5* showed a phenotype initially similar to wild-type both in terms of petiole angle and elongation, but the petiole elongated slightly less through time in low R:FR. On the other hand, *pif4pif5pif7* was unresponsive to low R:FR for both traits (Figure 3.1B & 3.1C). Green filter triggered a continuous shade avoidance phenotype in Col-0 (hyponasty and petiole elongation) from day 28 up to 36 (8 days) (Figure 3.1B & 3.1C). Hyponastic responses were reduced in *pif4pif5* and not observed at all in *pif4pif5pif7* under these severe shade conditions (Figure 3.1B). In general, Col-0 shade avoidance responses (hyponasty & petiole elongation) were stronger in green shade than

in low R:FR alone. Overall, *pif4pif5* was less responsive than Col-0, while *pif4pif5pif7* was fully insensitive to the different light conditions.

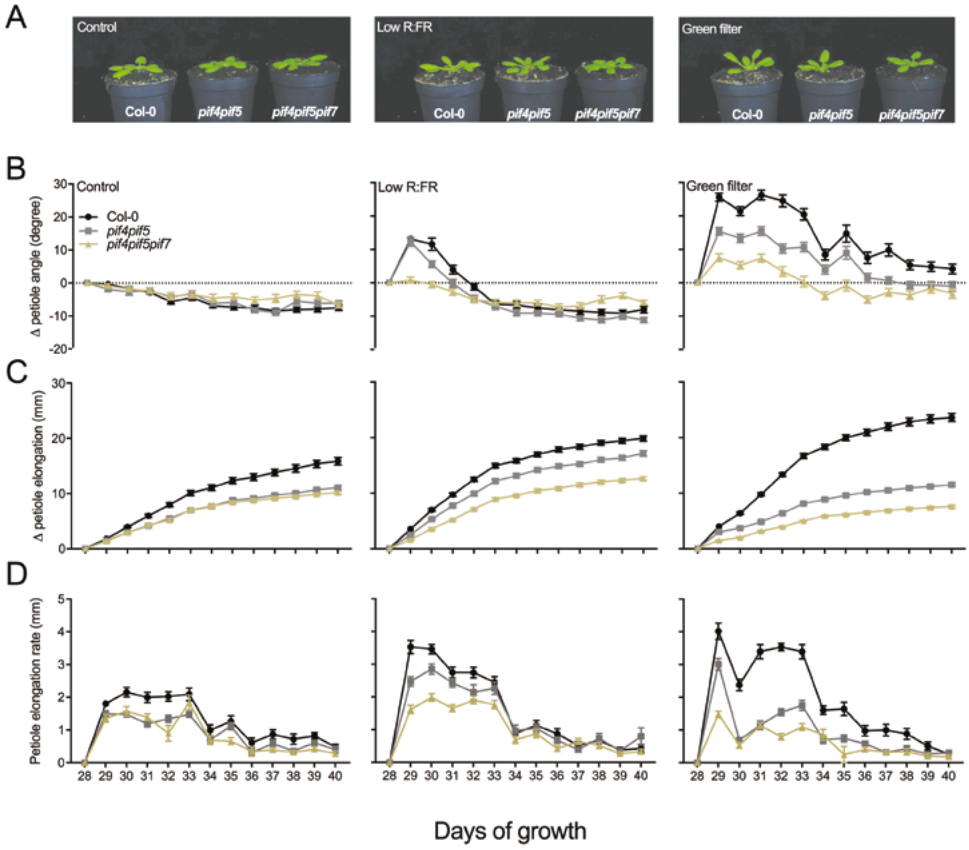

Figure 3.1 | Shade avoidance responses in Col-0, *pif4pif5* and *pif4pif5pif7* upon white light (Control), low R:FR (Low R:FR) and green filter (Green filter). (A) Pictures taken after 24 h treatment. The time lapse of (B) differential petiole angle and (C) length, as well as the (D) petiole elongation rate of Col-0 (black line), *pif4pif5* (grey line) and *pif4pif5pif7* (yellow line) upon Control (first column of the graphs), Low R:FR (second column of the graphs) and Green filter (third column of the graphs). The plants were under the light treatments for 13 days (plants were 28 days old when the light treatments start). Data represent mean ± SE, n = 15.

Shade avoidance responses in relation to competitor L*er* supression

The impact of different magnitudes of shade avoidance on competitor L*er* suppression was tested using canopies of Col-0, *pif4pif5* and *pif4pif5pif7*. As mentioned in chapter 2, Col-0 canopies growing in high density and uniform planting pattern can effectively create a closed canopy. Here, we monitored the canopy closure state through the R:FR ratio, determined by abundance of green tissues. Indeed, *pif4pif5* and *pif4pif5pif7* canopies developed R:FR decreases similar to Col-0 in the absence of competitor L*er* although starting with higher R:FR (≈2) compared to Col-0 canopy (R:FR ≈1.7) in day 20 (Figure 3.2A).

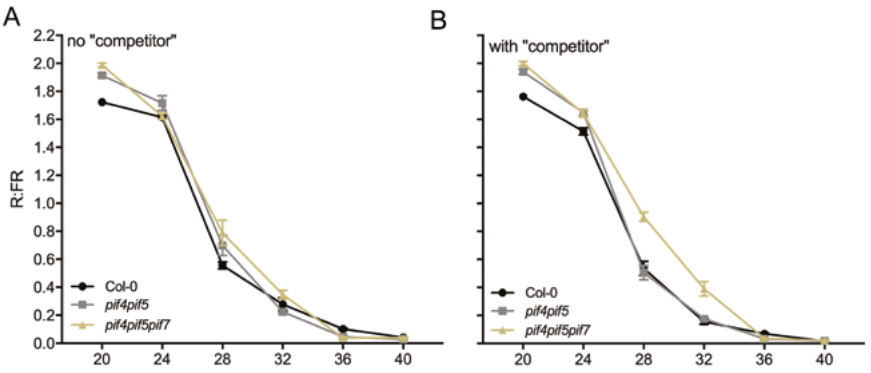

FIGURE 3.2 | The R:FR ratio inside the canopy of Col-0 (black lines), *pif4pif5* (grey lines) and *pif4pif5pif7* (yellow lines) (A) with or (B) without the competitor L*er* during the days of growth in a high density (8264 plants m^{-2}) uniform planting pattern.

The latter probably results from a reduced growth rate in the mutants compared to Col-0. In the presence of the competitor L*er*, the *pif4pif5pif7* canopy was less able to create a dense foliar shade, as indicated by a relatively high R:FR compared to Col-0 until 32 days. The *pif4pif5* and Col-0 canopies developed more rapidly, leading to rapid decline of R:FR (Figure 3.2B). Nevertheless, the LAI was not significantly different between the genotypes in the presence or absence of "competitor", but the more open canopy of *pif4pif5pif7* enchanced the LAI of the competitor L*er* (Figure 3.3A & 3.3B). Nevertheless, the biomass of *pif4pif5* was similar to Col-0 and the L*er* biomass was suppressed under these competitive conditions (Figure 3.3C & 3.3D).

SAS in both canopy and competitor growth during competition

To follow shade avoidance of the different genotypes and competitor during light competition, we measured the petiole and lamina length. The *pif4pif5pif7* canopy was open for 36 days (Figure 3.2A) and this was associated with significantly enchanced petiole and lamina length of competitor L*er*. In *pif4pif5* canopy, L*er* showed a significant increase in petiole elongation compared to L*er* plants growing under Col-0 canopy, while lamina length of L*er* was the same in both cases (Figure 3.4A & 3.4B). Col-0 canopy plants displayed the strongest petiole elongation with or without the competitor, while the petioles of *pif4pif5* and *pif4pif5pif7* were significantly less elongated. Between the mutants, in the presence of L*er*, *pif4pif5pif7* had the shortest petioles (Figure 3.5A), confirming its lack of shade avoidance. Contrary to petiole length, lamina length of the three genotypes was rather similar during competion (Figure 3.5B).

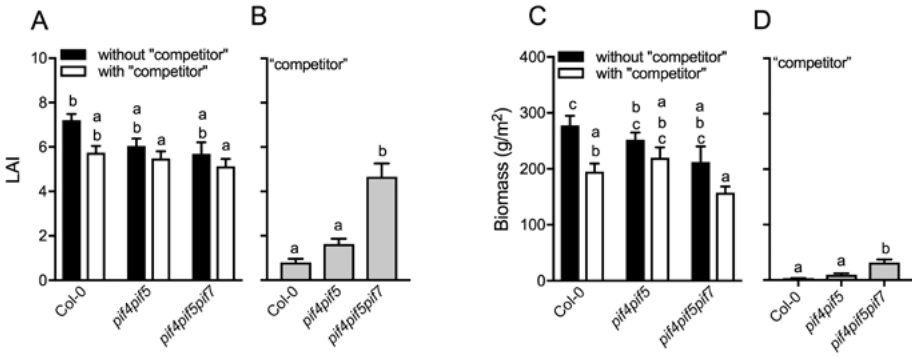

Figure 3.3 | The LAI (A & B) and the plot biomass (C & D) of Col-0, *pif4pif5*, *pif4pif5pif7* and competitor L*er* (grey bars) upon high density, uniform pattern after 44 days of growth. Canopy plots (Col-0, *pif4pif5*, *pif4pif5pif7*) grew with (black bars) or without the competitor L*er* (white bars). Data represent mean ± SE, n = 5. Different letters indicate statistically significant differences (two-way ANOVA, with Tukey's Post-hoc test, $p < 0.05$).

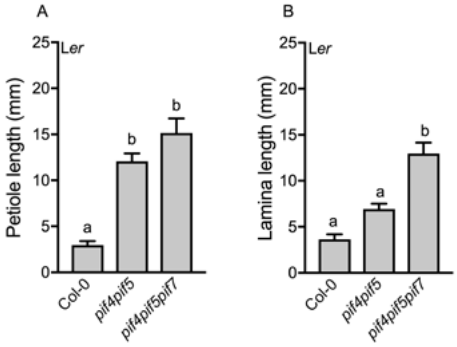

Figure 3.4 | The (A) petiole and (B) lamina length per plant of competitor L*er* under the high density, uniform canopies of Col-0, *pif4pif5*, *pif4pif5pif7*. Data represents mean ± SE, n = 5. Different letters indicate statistically significant differences (one-way ANOVA with Tukey's Post-hoc test, $p<0.05$).

Discussion

We studied competitive interactions between shade avoiding and less-shade avoiding plants (Figure 3.3 & 3.4). A signal that indicates the light competition is the change in R:FR light ratio, mostly because FR is reflected from the neighbour plant, while R is absorbed (Smith, 2000), resulting in a gradual decrease of R:FR though time (Liu et al., 2012; Yang et al., 2014) (Figure 3.2A & 3.2B).

In this chapter we tested the Evolutionary Agroecology hypothesis that collective reduction of SAS may suppress invading competitors and increase canopy productivity through collective shading. Although *pif4pif5* and *pif4pif5pif7* showed mild and severe reduction of SAS during light competition (Figure 3.1), we did not observe faster canopy closure in the two shade avoidance mutants that could lead to faster reduction of the R:FR inside the canopy compared to Col-0. A possible explanation for the observation that non-shade avoiding canopies did not close more rapidly may be that their growth rates happened to reduced relative to Col-0 (Figure 3.1D-Control conditions).

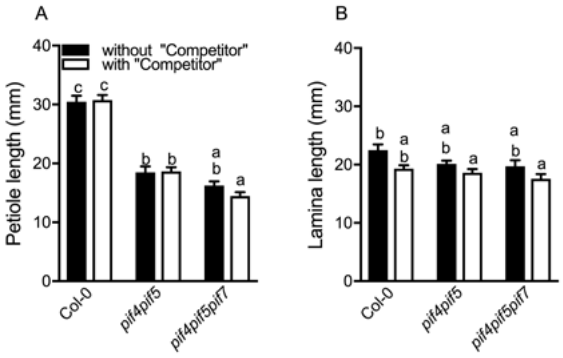

Figure 3.5 | The petiole (A) and lamina length (B) per plant of Col-0, *pif4pif5, pif4pif5pif7* canopies plants. Canopy plots (Col-0, *pif4pif5, pif4pif5pif7*) grew with (black bars) or without the competitor L*er* (white bars) in high density, uniform pattern. Data represents mean ± SE, n = 5. Different letters indicate statistically significant differences (two-way ANOVA with Tukey's Post-hoc test, $p<0.05$).

A previous study (Keuskamp et al., 2010a) showed that when Col-0 wild-type was grown in a 1:1 high density mixture with the weaker shade avoider *pin3-3,* biomass and reproductive output of *pin3-3* was reduced compared to Col-0. Indeed, the biomass and LAI of mutants *pif4pif5* and *pif4pif5pif7* with reduced petiole elongation (Figure 3.1C & 3.1D; Figure 3.4A & 3.4B) and petiole angle (Figure 3.1B) showed a trend towards reduced biomass accumulation compared to Col-0 (Figure 3.3C). However, this was in monocultures of these genotypes and might simply reflect overall reduced growth rates, especially of the *pif4pif5pif7* triple mutant. On the other hand competitor L*er* showed increased biomass, LAI and petiole length under the *pif4pif5pif7* canopy (Figure 3.3B, 3.3D & 3.4A), rather than the reduced growth predicted by Evolutionary Agroecology theory. This could be due to the strong shade avoidance responses present in L*er* (Djakovic-Petrovic et al., 2007; Hayes et al., 2014; Nozue et al., 2015). In dense stands or conditions with strong light competition (green shade) plants with the characteristics of *pif4pif5pif7* such as 1) reduced overall growth and 2) lack of upward leaf movement (Figure 3.1B-D) cannot compete against shade avoiding competitors like L*er.* Also mild reduction of SAS, such as in *pif4pif5* did not show any further suppression of L*er*. Indeed, we could observe during the experiments that L*er* could lift and elongate its leaves sufficiently to outgrow the *pif* mutant canopies and have undisturbed access to light. In a canopy system with very marginal height development, such as in *Arabidopsis*, only a very modest shade avoidance responses is sufficient for a competitor to outgrow non-shade avoiding surrounding plants. A follow-up study should include a competitor other than Ler, that lacks the ability to respond to canopy closure by shade avoidance.

To conclude, shade avoidance is an adaptive response which helps the plant to excel against their neighbours/competitors, and completely knocking out SAS does not improve plant performance. In *Arabidopsis*, the earliest means of plant neighbour detection in dense

stands is not through R:FR signalling, unlike most other species. Rather, early neighbour detection occurs through touching of the leaf tips of other plants (de Wit et al., 2012). It may be even more promising to manipulate this very early neighbour response to touch, and have a less severe intervention in plant growth, whilst still ensuring a more rapid closure of the canopy that might be more helpful in suppressing invading competitors in the canopy.

Materials and methods

Plant growth conditions and measurements

Genotypes used in this study, as a canopy plants were wild-type Col-0, *pif4-101*, *pif4-101 pif5-1* (Lorrain et al., 2008), *pif4-101 pif5-1 pif7-1* (de Wit et al., 2015) and L*er* as the competitor. The mutants were all in Col-0 background. Petiole angles of the fifth-youngest leaf were measured digitally with image J to determine hyponasty. Pictures were taken every day for 13 days, starting at day 28 (t=0). For more information for the growth conditions and the measurements see chapter 2.

Experimental design of the densities and patterns with or with the ''competitor'' L*er*

We used the uniform pattern high density based on the data of chapter 2. For more details see chapter 2.

R:FR measurements

The R:FR measurements were as described in chapter 2 using Ocean optics JAZ spectroradiometer.

Light experiments

To reduce the R:FR light ratios in the control white (W) light conditions from Philips HPI lamps (R:FR = 2.3, 180 µmol m^{-2} s^{-1} PAR), supplemental far-red LEDs (Philips Green Power FR 730 nm) were used. FR supplementation resulted in R:FR = 0.2 (180 µmol m^{-2} s^{-1} PAR). To mimic the true canopy shade, green filter (Lee filters Fern Green) was used (R:FR = 0.35 and ± 35 µmol m^{-2} s^{-1} PAR). The light spectra of the treatments were measured with an Ocean optics JAZ spectroradiometer (Figure S3.1).

Statistics

Data were analyzed by one or two-way ANOVA followed by post-hoc Tukey test. All the analyses were performed with SPSS or GraphPad.

Acknowledgements

We thank the entire Plant Ecophysiology group for their help during the harvests.

Supplemental data

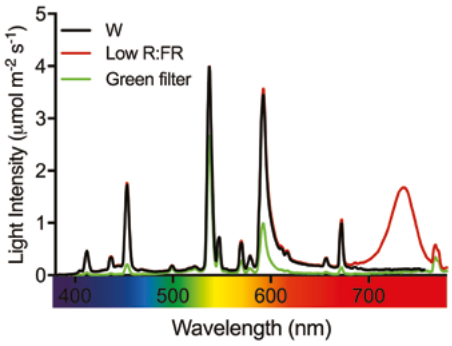

Figure S3.1 | The spectral composition of the three different light treatments: white light control (W), FR-enriched light (Low R:FR) and green shade (Green filter) in the growth room.

Chapter 4

Plant neighbour detection through touching of leaves

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Abstract

Plants growing at high densities compete for resources, including light. Light quality changes are generally known to exist as a neighbour detection cue before true shading occurs. However, previous studies have shown that the earliest neighbour responses in dense stands of *Arabidopsis* are induced through touching of leaves at their very tips. It is currently unknown how touch is sensed and how the signal is transferred from the leaf tip to the base of the petiole where local cell expansion regulates hyponastic leaf moment. Here we are studying how and where touch is sensed to trigger leaf movement and which signal transduction components are required. Although a transcriptome survey suggested regulation of jasmonate and defence genes, we found no causal evidence for involvement of the canonical JA-controlled defence pathways. We do show that trichomes are essential for touch-induced hyponasty. Several molecular components in mechanosensing and signal transduction are shown to control this response.

Introduction

Competition for light in dense stands of plants occurs frequently (Ballaré et al., 1988; Schmitt et al., 1995), and it is essential for plants to adjust to this. The best-established above-ground neighbour detection signal is the reduction of the red (R) to far-red (FR) light ratio (R:FR) (Vandenbussche et al., 2005; Franklin, 2008; Ballaré and Pierik, 2017). This reduction is caused by FR reflection and R depletion by chlorophyll in neighbouring plants (Smith, 2000). Plants respond to reduced R:FR through shade avoidance responses (SAS) (Oh et al., 2012; Zhang et al., 2013), with a central role for PHYTOCHROME INTERACTING FACTORS (PIFs) (Franklin and Quail, 2009; Leivar and Monte, 2014). SAS includes petiole, stem and hypocotyl elongation, apical dominance, early flowering and hyponasty (upward movement of the leaf) (Franklin, 2008; Keuskamp et al., 2010b; Pierik and de Wit, 2013). These physiological and morphological changes help plants reposition their leaves towards sunlight, facilitating photosynthesis and subsequent growth. In dense stands of *Arabidopsis*, the earliest neighbour response to induce SAS leading to the changes in the canopy architecture is hyponasty, but in *Arabidopsis* stands this is primarily initiated upon touching of neighbouring leaves (de Wit et al., 2012). Continuous touching of leaves can cause modest mechanical stimuli (reviewed in Chehab et al., 2008; Toyota and Gilroy, 2013), which might be different from the established light-signaling pathways. Touch is a mechanical perturbation that alters morphology and cause growth inhibition in plants (Braam and Davis, 1990; Braam, 2005; Lange and Lange, 2015); known as thigmomorphogenesis (derived from the Greek word θίγμα "*thigma*" which means *to touch*). Thigmomorphogenesis was described as mechanically-induced responses years ago (Jaffe, 1973). Jasmonic acid (JA) is involved in responses to mechanostimulations in plants, as found in *Phaseolus vulgaris* and in *Medicago trancatula* (Ellis et al., 2002; Tretner et al., 2008). Important factors in the mechanism of responses to touch (as well as in osmotic pressure and gravity responses) are mechanosensitive (MS) ion channels. The MS ion channels consist of three families; Two-pore potassium (TPK), Mscs-like (MSL) and Mid1-complementing activity (MCA) families, which are able to transduce the cellular membrane tension into ion influx (reviewed in Hamilton et al., 2015). TPKs are vacuole-localized and participate in ion homestasis. The MSL family protects cells from the osmotic stress in different organisms (bacteria, plants, fungi) while the MCA is a plasma membrane protein involved in calcium homeostasis and osmotic stress in plants (Haswell et al., 2008; Yamanaka et al., 2010; reviewed in Hamilton et al., 2015). Also the *Catharanthus roseus* Receptor-Like kinases (CrRLK1L) family, including members such as THESEUS1 (THE1), THESEUS2 (THE2) and FERONIA4 (FER4), were identified to contribute to the mechanoperception at the cellular level (Denness et al., 2011; Monshausen and Haswell, 2013). In this chapter, we tried to identify the mechanisms regulating touch-induced hyponasty. We hypothesized that this response would involve signaling in the lamina tip and signal transmission to the petiole base where differential growth between

the abaxial and adaxial side of the petiole would be required for upward movement of the leaf (Polko et al., 2012). Our transcriptome data suggested involvement of the JA and ABA (abscisic acid) hormonal pathways. These two hormones have been associated with leaf wounding responses (Lorenzo et al., 2004; Glauser et al., 2008; Glauser et al., 2009; Koo et al., 2009; Niu et al., 2011). We show, however, that JA-regulated defence pathways are not involved in hyponasty control upon touch. Here, we studied the involvement of a variety of mechanical stress-associated components, including those involved in signal detection as well as in signal transduction. We show that trichomes are essential for touch-induced hyponasty. We studied a broad range of mechanostimulation-associated components, including RLKs and mechanosensitive ion channels, that may be involved in relaying the mechanosignal from the trichomes to the petiole and provide a draft conceptual model for touch-induced hyponasty.

Results

Touch induces upward leaf movement in both *Arabidopsis thaliana* and *Nicotiana benthamiana*

It has been shown that the touching of the leaves in dense stands of *Arabidopsis* is the earliest mode of above-ground neighbour detection (de Wit et al., 2012). When a lamina tip of an *Arabidopsis* rosette plant touches a lamina tip of a neighbour plant or a transparent plastic tag, it responds with hyponastic leaf growth of approximately 20 degrees after 24 h (Figure 4.1A). This hyponastic response was increased even more after 48 h (Figure 4.1B). Similar responses were observed in an unrelated species, *N. benthamiana,* (Figure 4.1C & D), indicating that this response is not restricted to *Arabidopsis*.

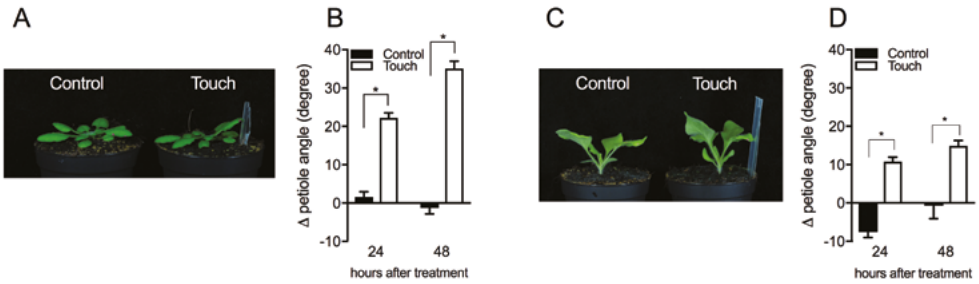

Figure 4.1 | Touch-induced hyponasty in *N. benthamiana* leaves. Representative photo of (A) *Arabidopsis thaliana* Col-0 and (C) *N. benthamiana* leaves touching transparent tags after 24 h. The differential petiole angle of touch-induced hyponasty in (B) Col-0 and (D) *N. benthamiana* after 24 h and 48 h. Data represent mean ± SE; n = 10. Statistically significant differences are indicated with asterisk, paired Student's t test ($P < 0.05$).

PIFs partially mediate touch-induced hyponasty

PIFs control shade avoidance responses, including upward movement of leaves in response to shade cues. To test the role of PIFs in touch-induced leaf movement we used *pif4, pif5, pif7* and *pif4pif5pif7* mutants and studied their response to touching. The *pif4* mutant showed almost no hyponastic response compared to wild-type (hereafter Col-0) (Figure 4.2A). In contrast *pif5* and *pif7* remained responsive similar to Col-0 (Figure 4.2B and 4.2D). Interestingly, hyponasty was still induced in the triple *pif* knockout mutant *pif4pif5pif7*, but the magnitude of the response was strongly suppressed as compared to Col-0 and similar to *pif4*. These data suggested that PIF4 is important for touch-induced hyponasty, whereas PIF5 and PIF7 are not.

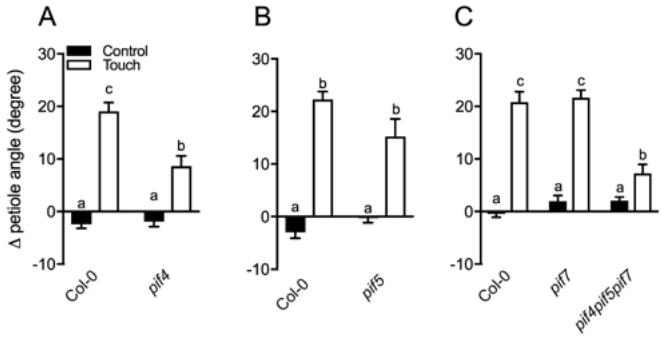

Figure 4.2 | Touch-induced hyponasty in different *pif* mutants. Differential petiole angle of Col-0 compared to (A) *pif4*, (B) *pif5*, (C) *pif7* and *pif4pif5pif7* after 24 h of touch treatment. Data represent mean ± SE; n = 8-10. Different letters indicate significant differences (two-way ANOVA with Tukey's post hoc test; $P < 0.05$).

Auxin does not have a direct role in touch-induced hyponasty

PIFs are key regulators of auxin biosynthesis, signaling and transport (de Wit et al., 2015). We therefore investigated if auxin regulates touch-induced hyponasty. The *DR5:LUC* auxin response reporter, however, did not show any differences between control and touch-treated leaves (Figure S4.1A). This suggests that auxin response is not clearly affected by touch. To test if auxin biosynthesis and transport contributed to touch-induced hyponasty, we studied two strong auxin biosynthesis mutants, *wei8* and *yuc2yuc5yuc8yuc9*, and a strong auxin transport mutant, *pin3pin4pin7*. Interestingly, all these severe auxin-associated mutants showed a Col-0-like hyponastic response to touch (Figure S4.1B & S4.1C), indicating that auxin is probably not a major regulator of this response.

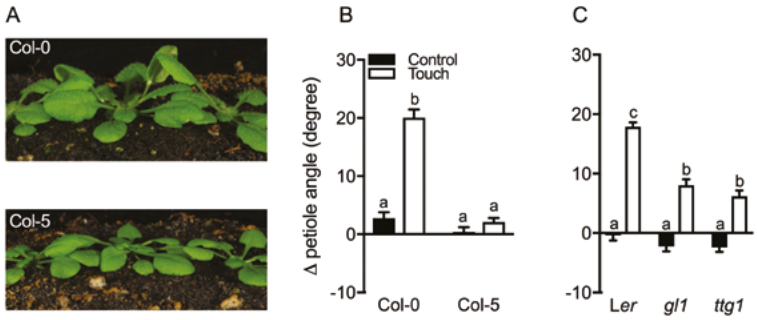

Figure 4.3 | Trichomes are required for touch-induced neighbour responses. Differential petiole angle of (A, B) Col-0, Col-5, and (C) L*er*, *gl1* and *ttg1*, 24 h after the touch treatment. Data represent mean ± SE; n = 10. Different letters indicate significant differences (two-way ANOVA with Tukey's post hoc test; $P < 0.05$).

Mechanosensing in early neighbour response

Leaf-leaf touching is a mechanical stimulation and the hyponastic response to this mechanosensing slowly increase through time, likely because of the elongation growth of the leaf. *Arabidopsis* trichomes have been recently described as an active mechanosensory switch (Zhou et al., 2017) that regulates plant responses to herbivore attack. To investigate the role of trichomes in the response to leaf-leaf touching, we compared Col-0 with Col-5, which is an accession without trichomes. We found that Col-5 showed a strongly reduced hyponastic response to touch, as compared to Col-0 (Figure 4.3A & 4.3B). The *TTG1* (Koornneef, 1981) and *GL1* (Herman and Marks, 1989) genes are positive regulators of *Arabidopsis* trichomes, and their respective mutants, *ttg1* and *gl1*, do not form trichomes. These two mutants displayed severely reduced touch-induced hyponasty as well (Figure 4.3C). We therefore concluded that trichomes are required for the hyponastic responses to touching of leaves.

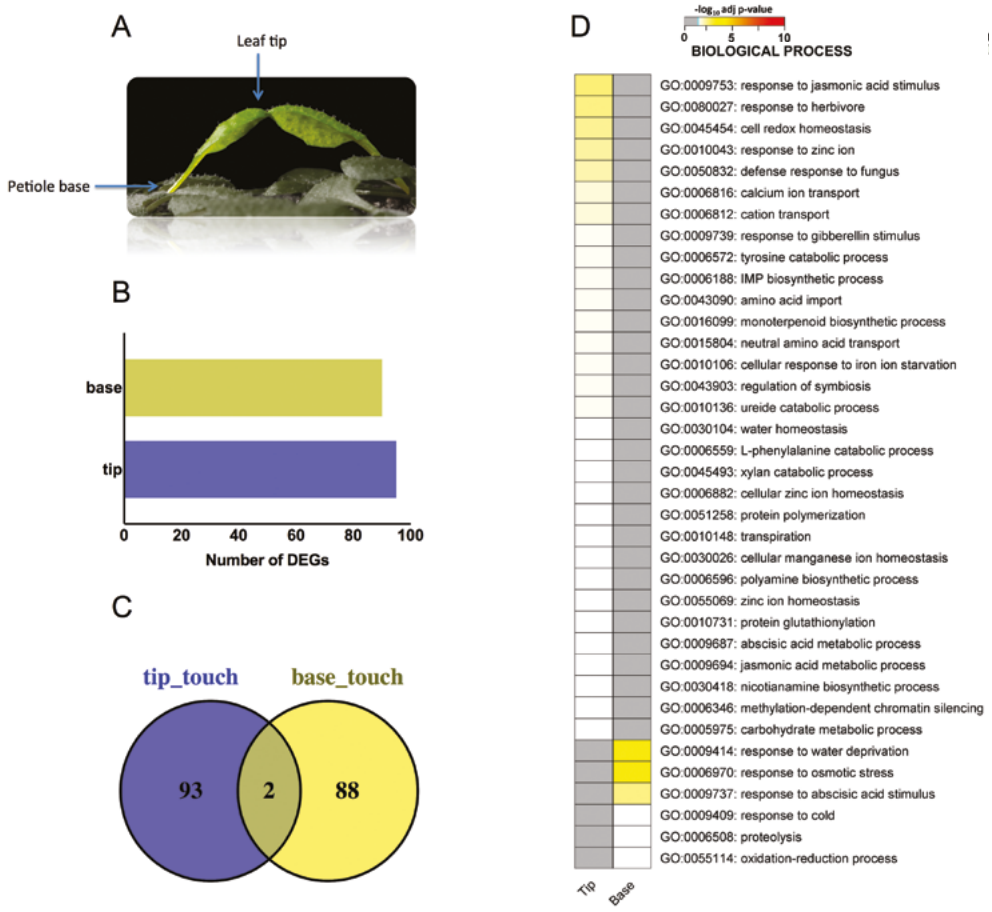

Figure 4.4 | Comparative analysis of touch-induced hyponasty in the lamina tip and the petiole base. (A) Leaf tip and petiole base tissues were harvested. (B) Number of DEGs in the lamina tip and the petiole base. (C) Comparison of differentially expressed genes (DEGs) in leaf-tip and petiole base in response to touch (adj. P-value < 0.05). (D) GO enrichment analysis of the DEGs in the lamina and the petiole base. Gray indicates the GO term is not significantly enriched in that specific tissue.

Tissue-specific transcriptomic analysis in response to touch

To investigate the mechanism of touch-induced hyponasty in depth, we performed a transcriptome analysis (using Affymetrix *Arabidopsis* Gene 1.1 ST arrays), comparing the site of perception (lamina tip) and the site of action (petiole base) (Figure 4.4A) under control (no touch) and touch conditions, using transparent tags. We found similar numbers of differential expressed genes (DEGs, adj. P-value < 0,05, relative to control plants) between the two tissues (Figure 4.4B), with almost no overlap (Figure 4.4C). Gene ontology analysis (GO) revealed a high representation of JA-related genes in the lamina tip and ABA-related genes in the petiole base (Figure 4.4D). These data suggest that different parts of the leaf have different transcriptional profiles in response to touch.

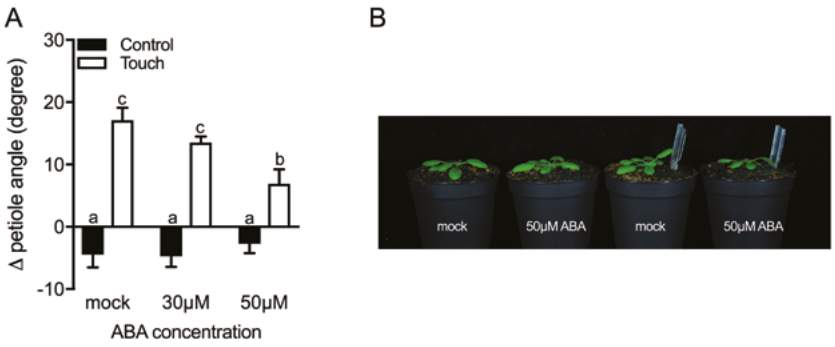

Figure 4.5 | The effect of ABA on touch-induced hyponasty. (A) Differential petiole angle response after exogenous application of different ABA concentrations (30μM and 50μM) after 24h of touch treatment. (B) illustrates the touch response of Col-0 after exogenous application of ABA (mock, 50μM) with or without the transparent tag. Data represent mean ± SE; n = 6-8. Different letters indicate significant differences (two-way ANOVA with Tukey's post hoc test; $P < 0.05$).

The Involvement of ABA and JA in the touch-induced leaf movement

To investigate the role of ABA in touch-induced leaf movement, we applied ABA in the adaxial site of the petiole and found a reduction in the hyponastic response due to touch (Figure 4.5). Furthermore, we performed physiological experiments by using mutants deficient in ABA perception (*pyr1pyl1pyl2pyl4*, referred to as *abaQ*), biosynthesis (*aba2-1*, *aba3-1*) and signaling (*areb1areb2abf3abf1*, referred to as *arebQ,* and *abi4*). None of these mutants showed a significantly altered hyponastic response to touch relative to Col-0 wild-type (Figure 4.6A, 4.6B & 4.6C) except *abi4*, which had reduced petiole angles compared to wild-type plants (Figure 4.6D). Importantly, the *abi4* mutant has no trichomes.

Since the transcriptome analysis also identified regulation of JA-associated genes (Figure 4.4C), we studied this in more detail as well. Exogenous application of OPDA (12-oxo-phytodienoic acid), which is the precursor of JA-biosynthesis and MeJA (methyljasmonate), which is the inducer of JA-biosynthesis pathway, resulted in the reduction of hyponastic response after touch treatment (Figure 4.7A-D).

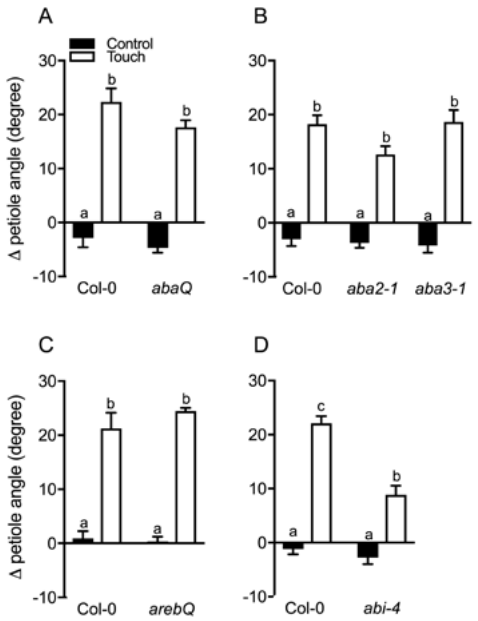

Figure 4.6 | Touch-induced hyponasty in different ABA mutants. Differential petiole angle of Col-0 compared to, (A) *abaQ*, (B) *aba2-1* and *aba3-1*, (C) *arebQ* and (D) *abi-4*, after 24 h of touch treatment. Data represent mean ± SE; n = 7 (A,C) and n = 14 (B,D). Different letters indicate significant differences (two-way ANOVA with Tukey's post hoc test; P<0.05).

The JA pathway is activated upon wounding (or herbivore attack) and regulates MYC transcription factors MYC2, MYC3, and MYC4 (Lorenzo et al., 2004). To test their role in touch responses, we used the *myc2*, *myc3*, *myc4*, *myc2myc3*, *myc2myc4*, *myc3myc4* and *myc2myc3myc4* mutants. The single knockout mutants (*myc2*, *myc3*, *myc4)* showed similar phenotypes as Col-0 (Figure 4.8A). In the higher order mutants, especially *myc2myc3* and to a lesser extent *myc2myc3myc4,* we observed reduction in hyponastic response due to touch (Figure 4.8B & 4.8C).

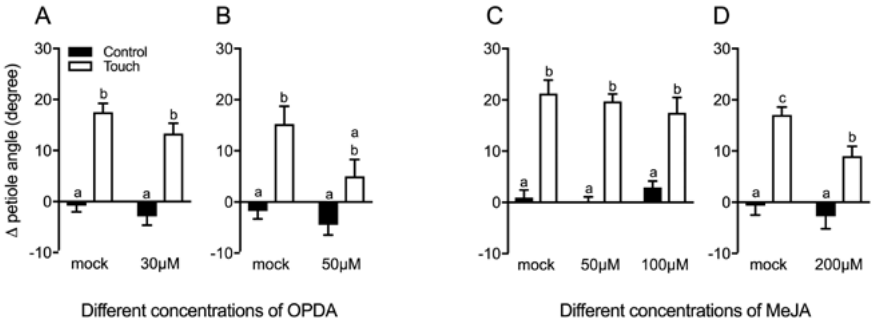

Figure 4.7 | The effect of MeJA and OPDA in touch-induced hyponasty. Differential petiole angle response after exogenous application of different (A) OPDA (30 μM and 50 μM) and (B) MeJA (50 μM, 100 μM and 200 μM) concentrations after 24 h of touch treatment. Data represent mean ± SE; n = 6-8. Different letters indicate significant differences (two-way ANOVA with Tukey's post hoc test; P < 0.05).

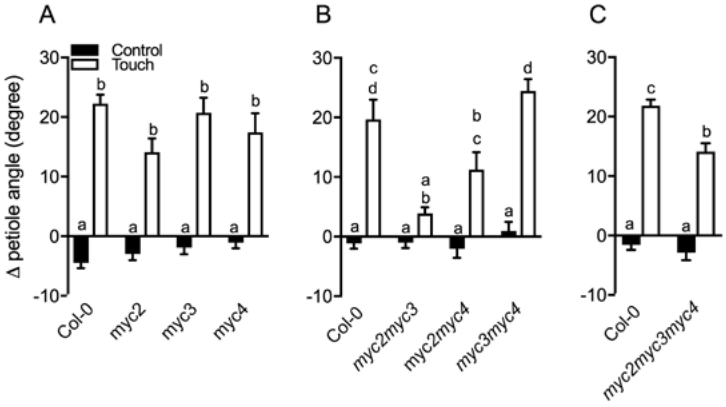

Figure 4.8 | Touch-induced hyponasty in different *myc* mutants. Differential petiole angle of Col-0 compared to, (A) *myc2*, *myc3*, *myc4* (B) *myc2myc3*, *myc2myc4*, *myc3myc4* and (C) *myc2myc3myc4*, after 24 h of touch treatment. Data represent mean ± SE; n = 16 (A,C) and n = 8 (B). Different letters indicate significant differences (two-way ANOVA with Tukey's post hoc test; $P < 0.05$).

Mechanoresponse mutants in touch-induced hyponasty

JA is associated mechanical stresses such as wounding (Farmer et al., 2014) and thigmomorphogenesis (Chehab et al., 2012). The family members of the *Catharanthus roseus* receptor-like kinases (CrRLK1L), such as THE1, THE2, HERK1, FER have been proposed as mechanosensors (Monshausen and Haswell, 2013). The *the1*, *the2*, *herk1* knockout mutants and the knockdown mutant *fer5* showed a wild-type phenotype 24h after touch treatment (Figure 4.9). Interestingly, the double knockout mutant *herk1the1* and the single knockout mutant *fer4* had clearly reduced touch-induced hyponasty after 24 h (Figure 4.9A-C) and 48 h (Figure S4.3B & S4.3C), suggesting that these could be involved in touch detection. Interestingly, a pentuple mechanosensitive ion channel mutant (*msl4msl5msl6msl9msl10*) was still able to respond to touch (Figure S4.3A).

Jasmonate biosynthesis is activated through electrical signals after wounding, with *GLUTAMATE RECEPTOR-LIKE* genes (*GLRs* 3.2, 3.3 and 3.6) having a very important role in the long-distance electrical signaling upon wounding (Mousavi et al., 2013). After testing the knockout mutants *glr3.3aglr3.6a*, *glr3.1glr3.3glr3.6* and *glr3.2glr3.3glr3.6* in the touch-induced hyponasty, we found that *glr3.1glr3.3gl3.6* had a strongly defective touch-phenotype (Figure 4.10A & 4.10B), indicating that clade 3 GLRs may redundantly regulate touch-induced leaf movement.

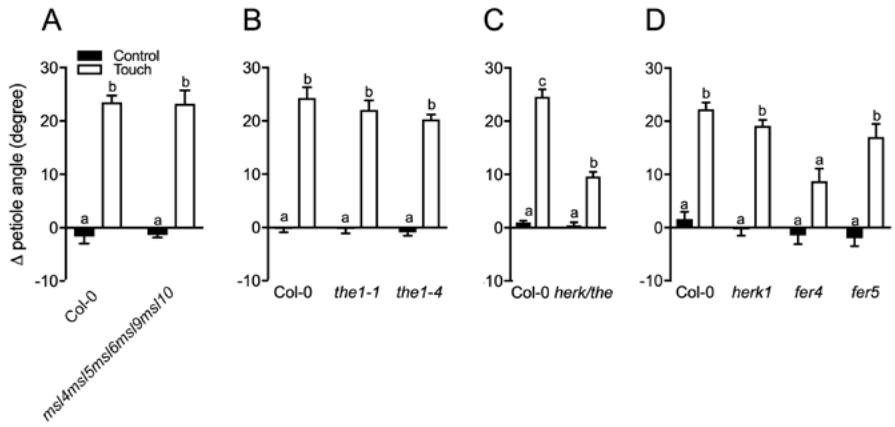

Figure 4.9 | CrRLKs are involved in touch-induced hyponasty. Petiole angle of Col-0 plants compared to several receptor-like kinase mutants (A) *the1-1*, *the1-4* (B) *herk1the1*, (C) *herk1, fer4*, *fer5* after 24 h of touch treatment. Data represent means ± SE. Different letters indicate significant difference (one way ANOVA, $P < 0.05$).

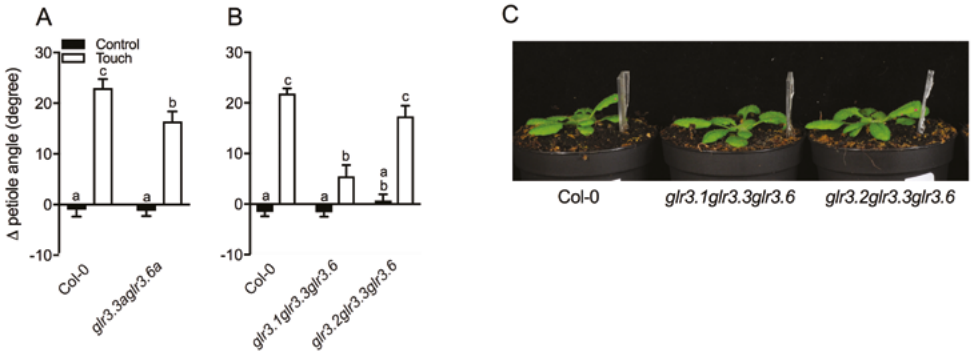

Figure 4.10 | The involvement of GLUTAMATE RECEPTOR-LIKE (GLR) proteins in touch-induced hyponasty. Differential petiole angle of Col-0 compared to, (A) *glr3.3aglr3.6a* (B) *glr3.1glr3.3glr3.6, glr3.2glr3.3glr3.6* after 24 h of touching the transparent tag. The picture (C) illustrates the touch response of Col-0, *glr3.1glr3.3glr3.6* and *glr3.2glr3.3glr3.6.* Data represent mean ± SE; n = 8-10. Different letters indicate significant differences (two-way ANOVA with Tukey's post hoc test; $P < 0.05$).

Discussion

de Wit et al., (2012) proposed that in horizontally growing rosette plants such as *Arabidopsis*, hyponasty through touching of the leaves is the earliest neighbour response at high densities. Here, we describe a similar response in *Nicotiana bethamiana*, indicating that it is not specific to *Arabidopsis* (Figure 4.1). The de Wit et al., (2012) did not elucidate the signal transduction steps involved in this response, whereas here we try to elucidate this in part.

Transcriptome data revealed signatures of the phytohormones JA and ABA in the lamina tip and the petiole base respectively (Figure 4.4D), indicating a mechanical perturbation transcriptome profile, since previous studies have shown that these two pathways are activated simultaneously after wounding effect from herbivore attack (Howe and Jander, 2008; Vos et al., 2013). MYC2, MYC3, MYC4 transcription factors with a synergetic action of ABA activate the JA pathway to wounding by herbivores (Niu et al., 2011). Using mutants of the JA pathway, we found that *myc2myc3* had a weakened hyponastic response compared to Col-0, with MYCs acting redundantly (Figure 4.6D and 4.8B,D). Mutants of the ABA pathway did not show any distinct phenotype, except the *abi4* mutant, which is known to be negative regulator of ABA signaling (Shang et al., 2010) and showed severe reduction of hyponasty (Figure 4.6D). Also exogenous application of lipid-derived metabolites MeJA and OPDA (Fonseca et al., 2009) which have been involved in thigmomorphogenetic responses due to mechanostimulations (Chehab et al., 2008) and ABA resulted in reduced hyponasty upon touch (Figure 4.5 & 4.7). Collectively, these data suggest that JA and ABA can inhibit touch-induced hyponasty.

Mechano-sensing and signal transduction in touch-induced hyponasty

Data from this chapter suggest that the JA signature may not necessarily represent defence-related processes, but rather denotes triggering of a mechanical stress response. Plant responses to touch are called thigmomorphogenesis (Jaffe, 1973) and JA is induced upon mechanostimulation in different plant species (Ellis et al., 2002; Tretner et al., 2008; Chehab et al., 2012). During insect-induced wounding, JA biosynthesis is activated through electrical signals that are propagated through the leaf. GLRs have an important role in propagating this electrical signal (reviewed in Farmer et al., 2014). Interestingly, we found that the *glr3.1glr3.3glr3.6* mutant had a severely reduced touch response (Figure 4.10B & 4.10C). This could imply that membrane depolarizations are transmitted over long-distance from the lamina tip to the petiole base. Since JA itself does not seem to be a major regulator, we propose that local induction of JA synthesis, as in herbivory (Glauser et al., 2008; Glauser et al., 2009; Koo et al., 2009; Mousavi et al., 2013), does not regulate the touch response.

Mechanical stresses may be sensed via CrRLK1L such as THE1, THE2, HERK1, FER4 (Monshausen and Haswell, 2013). Indeed, *fer4* and *herk1the1* showed a severe reduction in hyponastic response after touch (Figure 4.9B & 4.9C), suggesting that these RLKs may function in mechanosensing, or mechano-signal transmission during touch-induced leaf movement. Importantly, it appeared that trichomes are key to touch-induced hyponasty (Figure 4.3), and indeed the physical interaction between two leaf tips takes place at the trichomes. Thus, we propose that mechanosensing occurs at the level of trichomes or in the epidermal pavement cells directly surrounding the trichomes. Trichomes are indeed considered active mechanosensory switches (Zhou et al., 2017). It would be interesting

to study if the mentioned RLKs or clade 3 GLRs are indeed active in the trichomes or not. Since the *abi4* mutant lacks trichomes (Figure 4.6D), we postulate that its aberrant touch response unlikely indicates a role for ABA, but rather corroborates the need for trichomes in plant-plant signaling.

In summary, we propose that mechanosensing in or around the trichomes for example through CrRLKs may trigger a signal whose transmission to the petiole base is clade 3 GLR-dependent. Locally, in the petiole base, this then initiates differential growth between the abaxial and adaxial side, resulting in upward bending of the petiole.

A key mobile candidate regulator that could depend on ion channels, which has been associated with thigmomorphogenesis, is calcium (Ca^{2+}). Indeed, calcium has been associated with trichomes (Zhou et al., 2017), can be controlled through GLRs (kwaaitaal et al., 2011; Mousavi et al., 2013), and has been associated with touch responses through the Ca^{2+} transporters MCA1 and MCA2 located in the plasma membrane (Nakagawa et al., 2007; Van Aken et al., 2016). In the transcriptome data we indeed found a GO enrichment of calcium ion transport.

Future studies could therefore be directed at identifying if and how mobile calcium regulates touch-induced hyponasty.

Material and methods

Plant growth and measurements

Genotypes used in this study that are in the Col-0 background are: *pif4-101, pif5-1* (Lorrain et al., 2008), *pif7-1* (Leivar et al., 2008), *pif4-101pif5-1pif7-1* (de Wit et al., 2015), *abaQ, aba2-1, aba3-1* (Léon-Kloosterziel et al., 1996), *arebQ* (Yoshida et al., 2015), *abi-4, myc2* (Lorenzo et al., 2004), *myc3, myc4, myc2myc3, myc2myc4, myc3myc4, myc2myc3myc4* (Fernández-Calvo et al., 2011), *the1-1, the1-4, herk1, herk1the1* (Guo et al., 2009b), *fer4, fer5* (Guo et al., 2009a), *glr3.3aglr3.6a* (Mousavi et al., 2013), *glr3.1glr3.3glr3.6, glr3.2glr3.3glr3.6* (Nguyen et al., unpublished), *wei8* (Stepanova et al., 2008), *pin3-3pin4pin7* (Willige et al., 2013), *yuc2yuc5yuc8yuc9* (Nozue et al., 2015), *DR5:LUC* (Moreno-Risueno et al., 2010) and *msl4msl5msl6msl8msl10* (Haswell et al., 2008). Genotypes used in this study, in the L*er* background are: *gl1* and *ttg1* (Koornneeff et al., 1982). We also used Col-5 and *N. benthamiana*. Seeds were sown on Primasta® soil and stratified for 3 days (dark, 4°C), before transfering to short day (9 h light / 15 h dark) growth rooms (130-135 µmol m^{-2} s^{-1} PAR, R:FR 2.3, 20°C, 70% RH). After 11 d, seedlings were transplanted to 70 ml pots. The fifth-youngest leaf of 28 d plants was used for measuring petiole angles. Pictures were taken before (t = 0 h) and after treatment (t = 24 h) and angles were measured using image-J software. All experiments started at 10:00 in the morning (ZT = 2 h). The touch treatment was performed as described in de Wit et al., 2012.

Light experiments

$W+FR_{whole}$ (low R:FR in all the rosette plant) performed after adding supplemented FR Philips LEDs (0,05 R:FR, 125-135 µmol m^{-2} s^{-1} PAR) in the W (white light, 2,3 R:FR, 130-135 µmol m^{-2} s^{-1} PAR). For the $W+FR_{tip}$ (supplemented FR light only in the lamina tip). More details can be found in the respective paragraph in chapter 5.

Pharmacological experiments

Plants were treated with different concentrations of OPDA (Cayman Chemical, USA), MeJA (Van Meeuwen Chemicals BV, NL) on the whole lamina and ABA (Sigma-Aldrich, USA) on the petiole. All solutions contained 0.1% DMSO and 0.1 % Tween. The solutions were freshly made and they were applied right before and 5 h after the touch treatment.

Microarray data analysis

The lamina tip and petiole base from wild-type plants (Col-0) were harvested after 5 h of touch treatment. 15 petiole bases and 15 lamina tips were pooled for each sample for RNA extraction (three biological replicates per tissue / treatment, collected from three independent experiments). Affymetrix 1.1 ST *Arabidopsis* arrays were used to hybridize the samples via a commercial provider (Aros, Aarhus, Denmark). The raw data were normalized for signal intensity to remove background noise. The quality check of the data was performed using Bioconductor (packages "oligo" and "pd.aragene.1.1.st") in R software. Differential expression analysis was carried out using the Bioconductor "Limma" package in R software. Genes with adjusted p-value < 0,05 were considered as differentially expressed. Gene ontology (GO) analysis was done with GeneCodis (http://genecodis.cnb.csic.es). Clustering was based on the positive and negative logFC for each set.

Luciferase assay

DR5:LUC plants were exposed to the light or hormone treatments for 24 h. Whole shoots or single leaves were then cut and evenly sprayed with 2 mM D-Luciferin Potassium Salt (BioVision Inc.) in 0.1% (v/v) Triton X-100. Luciferase luminescence was imaged in a ChemiDoc imager (Bio-Rad) with a 40 min exposure time. The Fiji lookup table "Fire" was used to convert black and white images into color scales based on pixel intensity. Relative luciferase intensity in the petiole was analyzed by measuring mean pixel intensity of the petiole in Icy software (v1.8.6.0; http://icy.bioimageanalysis.org; De Chaumont et al., 2012).

Statistical analysis

Data were analyzed with one or two-way ANOVA followed by Tukey's HSD test using GraphPad.

Acknowledgements

We thank the entire Plant Ecophysiology group (UU) for help with tissue harvests for the transcriptome experiments and Debatosh Das for help with bioinformatics. We thank Elizabeth S. Haswell and Christian Fankhauser for sharing mutant seeds.

Supplemental data

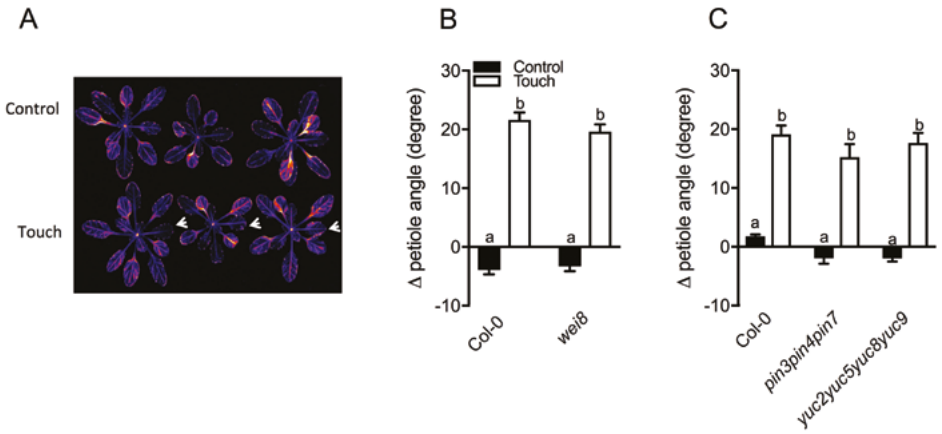

Figure S4.1 | Auxin control in touch-induced hyponasty. (A) Luciferase luminescence of auxin reporter line *DR5:LUC* in the abaxial side after 24 h of Control (upper part) and the touch treatment (lower part). White arrows indicate the touch-exposed leaf. Differential petiole angle of Col-0 compared to (B) *wei8* (auxin biosynthesis mutant) (C) *pin3pin4pin7* (polar auxin transport mutant) and *yuc2yuc5yuc8yuc9* (auxin biosynthesis mutant) after 24 h of touching the transparent tag. Data represent mean ± SE; n = 8. Different letters indicate significant differences (two-way ANOVA with Tukey's post hoc test; $P < 0.05$).

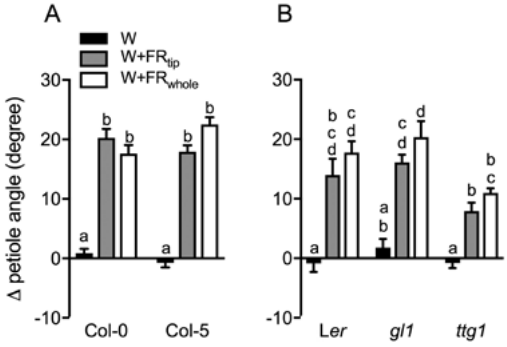

Figure S4.2 | The effect of local FR in mutants lacking trichomes. Differential petiole angle of (A) Col-0 and Col-5 and (B) Ler, *gl1* and *ttg1* under W (white light), $W+FR_{tip}$ (supplemental FR in the lamina tip) and $W+FR_{whole}$ (supplemental FR in the whole plant). Data represent mean ± SE; n = 7-9. Different letters indicate significant differences (two-way ANOVA with Tukey's post hoc test; $P < 0.05$).

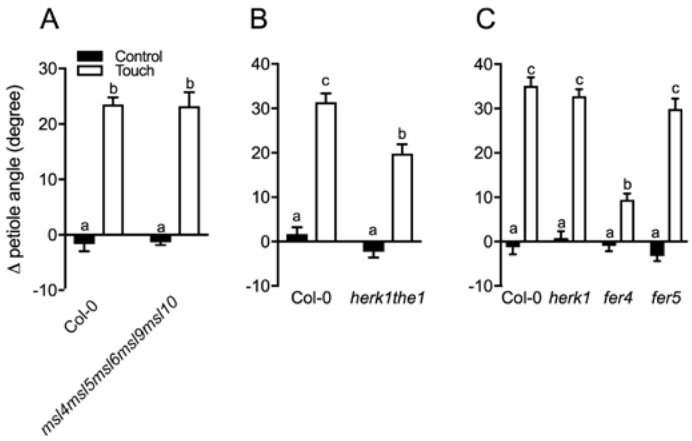

Figure S4.3 | Differential petiole angle of Col-0 compared to (A) *msl4msl5msl6msl9msl10* (B) *herk1the1* and (C) *herk1*, *fer4* and *fer5* after 24 h (A) or 48 h (B, C) of touching the transparent tag. Data represent mean ± SE; n = 7-10. Different letters indicate significant differences (two-way ANOVA with Tukey's post hoc test; $P < 0.05$).

Chapter 5

Neighbour detection at the leaf tip adaptively regulates upward leaf movement through spatial auxin dynamics

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Abstract

Vegetation stands have a heterogeneous light quality distribution, including the red:far-red light ratio (R:FR) that informs plants about proximity of neighbours. Adequate responses to changes in R:FR are important for competitive success. How the detection and response to R:FR are spatially linked and how this spatial coordination between the two affects plant performance remains unresolved. We show in *Arabidopsis thaliana* and *Brassica nigra* that localized FR-enrichment at the lamina tip induces upward leaf movement (hyponasty) from the petiole base. Using a combination of organ-level transcriptome analysis, molecular reporters and physiology we show that PIF-dependent, spatial auxin dynamics are key to this remote response to localized FR-enrichment. Using computational 3D modeling we show that remote signaling of R:FR for hyponasty has an adaptive advantage over local signaling in the petiole, since it optimizes timing of leaf movement in response to neighbours and prevents hyponasty caused by self-shading.

Introduction

Plant canopies have pronounced gradients of light intensity between the top and bottom because leaves shade one another (Monsi et al., 2005). Due to clustering of leaves, light intensities also vary horizontally. As light drives photosynthesis this creates selection pressure for plants to position their leaves for optimal light capture. Leaves do not absorb all wavelengths of the incoming light equally and light quality and therefore also differs vertically and horizontally in canopies (Monsi et al., 2005; Boonman et al., 2009; Crepy and Casal, 2015) even across the surface of single leaves (Chelle et al., 2007). Leaves preferentially absorb red (R, λ=600-700 nm) and blue (B, λ=400-500 nm) light for photosynthesis, whilst reflecting most of the far-red (FR, λ=700-800 nm) light. This leads to a relative enrichment of FR light (low R:FR ratio) in the local vicinity of leaves, a signal of neighbour proximity (Ballaré et al., 1987).

Low R:FR is sensed by phytochrome photoreceptors, mainly phytochrome B (phyB) and induces upward leaf movement (hyponasty) though differential petiole growth and elongation of stems and petioles, thus bringing the leaves higher towards the more illuminated parts of the canopy (Casal, 2013a; Pierik and de Wit, 2013; Fraser et al., 2016). Plants are modular organisms and such shade avoidance responses could thus be restricted to the specific modules that sense shade cues (De Kroon et al., 2005; Izaguirre et al., 2013; Ballaré and Pierik, 2017). Although spatial separation was shown recently for hypocotyl elongation in small *Brassica rapa* seedlings (Procko et al., 2014), only more established plants are large enough to experience light quality heterogeneity over the plant body. It is unknown if low R:FR responses in relatively mature *Arabidopsis* plants act locally or if these integrate detection from different plant parts.

Low R:FR inactivates phytochromes, leading to accumulation of active PHYTOCHROME INTERACTING FACTOR (PIF) transcription factors, notably PIF4, PIF5 and PIF7, that trigger expression of growth-promoting genes (Leivar and Monte, 2014). These include auxin signaling and biosynthesis genes (Lorrain et al., 2008; Li et al., 2012; Bou-Torrent et al., 2014; de Wit et al., 2016b), such as *YUCCAs* that encode enzymes in tryptophan-dependent auxin synthesis downstream of TAA1 (TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1) (Won et al., 2011; Nozue et al., 2015; de Wit et al., 2015). Auxin biosynthesis in seedlings occurs mostly in the cotyledons, and polar auxin transport (PAT) to the hypocotyl subsequently induces elongation in low R:FR (Procko et al., 2014). Within a leaf PAT also spatially relays signals (Sequeira and Steeves, 1954) and is required for low R:FR-induced petiole elongation (de Wit et al., 2015) and thus shade avoidance (Pierik et al., 2009; Keuskamp et al., 2010a).

We hypothesize that a spatial separation of R:FR detection and auxin-dependent growth response enables plants to deal with the spatially heterogenic light climate in dense

stands where R:FR responses are functional. Here, we show that leaf growth responses to FR-enrichment occur exclusively within the FR-exposed leaf, but within the leaf there is spatial separation: hyponasty occurs exclusively in response to remote FR-enrichment at the leaf tip, whereas petiole length responds to FR-enrichment only when sensed at the petiole itself. Integrating transcriptomics, physiology and genetics we show FR-enrichment at leaf tip induces hyponasty through auxin synthesis and transport in a PIF-dependent manner. Using a computational 3D plant model (Vos et al., 2009; Bongers et al., 2014; Evers and Bastiaans, 2016), we demonstrate why sensing R:FR at the lamina tip to control leaf angles is functionally superior to sensing it in the petiole.

Results

Site of R:FR perception determines hyponasty versus petiole elongation

We exposed different leaf regions of *Arabidopsis thaliana* Col-0 to FR irradiation spotlight of 3.5 mm diameter (Figure 5.1) and measured the effect on petiole angle and elongation. Supplemental FR light to the lamina tip ($W+FR_{tip}$) and lamina middle ($W+FR_{middle}$) induced hyponasty, whereas treatment of the petiole itself ($W+FR_{petiole}$) did not elicit any hyponasty (Figure 5.2A & 5.2B). $W+FR_{tip}$ triggered the strongest hyponastic response of all local supplemental FR treatments, similar to hyponasty in whole-plant low R:FR ($W+FR_{whole}$). Only the supplemental FR-treated leaf would respond; no systemic response was observed in the local treatments (Figure 5.2C-F). In *Brassica nigra* we found similar responses; petiole hyponasty was induced in $W+FR_{tip}$, and petiole elongation was mainly

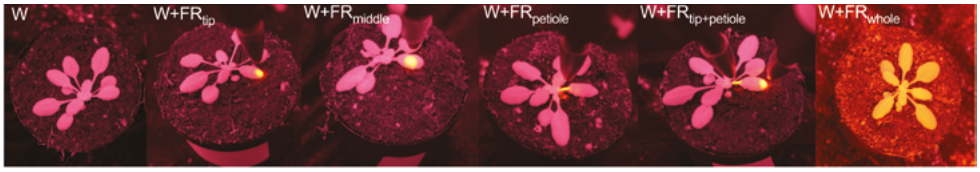

Figure 5.1 | FR irradiation spotlight in different parts of the leaf. Infra-red photographs illustrating plants in the different light treatments; white light (W), white light with supplemental FR through a 3.5mm spot (observed as bright yellow spot) on the lamina tip ($W+FR_{tip}$), the middle of the lamina ($W+FR_{middle}$), the petiole ($W+FR_{petiole}$), and lamina tip and petiole ($W+FR_{tip+petiole}$), and whole-plant FR ($W+FR_{whole}$).

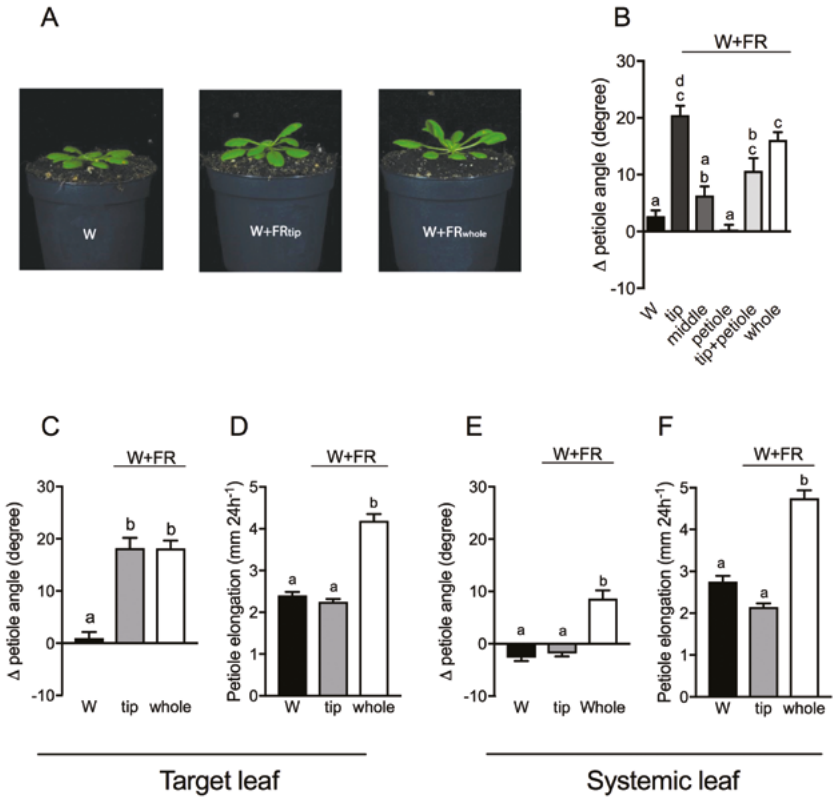

Figure 5.2 | Local FR in the lamina tip can induce hyponasty in the target leaf but not a systemic leaf. (A) Representative photographs of Col-0 plants treated with white light (W), white light with supplemented FR light to the lamina tip ($W+FR_{tip}$) and white light with supplemented FR to the whole plant ($W+FR_{whole}$). (B) Differential petiole angle for plants exposed to 24 h white light control conditions (W) and supplemented FR light to the lamina tip (tip), lamina middle (middle), the entire petiole (petiole), lamina tip plus the petiole (tip+petiole) and the whole plant (whole). The differential petiole angle (C) and petiole elongation (D) of the target leaf in W, $W+FR_{tip}$, and $W+FR_{whole}$ condition. The differential petiole angle (E) and petiole elongation (F) of the systemic leaf under the same light conditions. Data represent mean ± SE (n = 10). Different letters indicate statistically significant differences (one-way ANOVA with Tukey's Post-hoc test, $P < 0.05$).

induced by $W+FR_{petiole}$ and only marginally by $W+FR_{tip}$ (Figure 5.3). Reciprocally, R spotlight on one leaf of *Arabidopsis* plants under $W+FR_{whole}$ conditions, reduced leaf angles in the R-treated leaf only (Figure 5.4A), corroborating that the response is local to the treated leaf. Although $W+FR_{petiole}$ induced no hyponasty, it induced maximal petiole elongation (similar to $W+FR_{whole}$ and $W+FR_{petiole+tip}$), whereas $W+FR_{tip}$ did not elicit any petiole elongation (Figure 5.4B). Plants treated with $W+FR_{tip}$ showed supplemental FR-induced growth only in the abaxial side of the most basal petiole section. The area contributing to hyponasty extends slightly beyond the 3.5 mm spotlight area on the tip, as was evidenced by moving the spotlight on the tip-most half of the lamina (Figure 5.5A & 5.5B). $W+FR_{whole}$ treatment induced elongation throughout the petiole on both the abaxial and adaxial sides (Figure 5.5C).

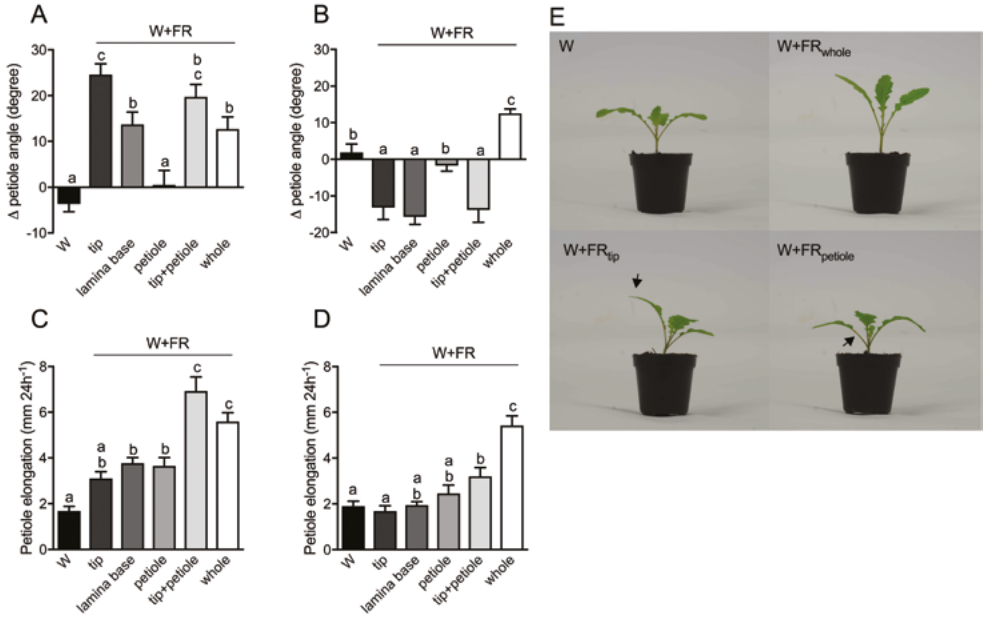

Figure 5.3 | Tissue-specific FR perception in *Brassica nigra* induces hyponastic and elongation responses. *Brassica nigra* seedlings were grown in 16 h light - 8 h dark cycle with 110-150 µmol m^{-2} s^{-1} PAR, R:FR 2.3, 20°C and 70% RH. The two first leaves of fourteen days old Brassica seedlings were measured for petiole angle and petiole elongation over 24 hours of exposure to different light treatments (cotyledons were removed two days before the treatment). Plants were grown in white light (W) or one leaf (referred to as target leaf) was treated with supplemental FR light applied to the lamina tip (tip), lamina base, petiole, lamina tip plus petiole (tip+petiole) or whole-plant (whole). Petiole angle change of the target leaf (A) and systemic leaf (B), and petiole elongation of the target leaf (C) and systemic leaf (D). Data represent mean ± SE (n = 10). Different letters represent statistically significant difference (one-way ANOVA with Tukey's Post-hoc test, $P < 0.05$). (*E*) Representative photographs of the *Brassica nigra* plants in white light (W), white light with supplemental FR for the whole-plant ($W+FR_{whole}$), and in white-light with supplemental FR light at the lamina tip ($W+FR_{tip}$) or at the petiole ($W+FR_{petiole}$) of the left leaf. Black arrows indicate the treated area with supplemental FR (lamina tip and petiole).

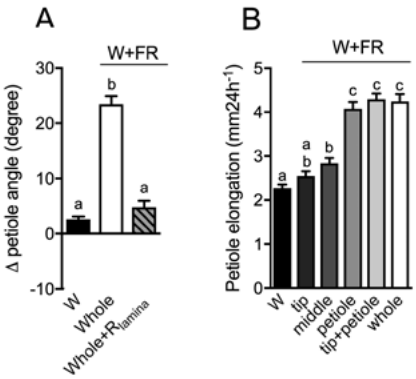

Figure 5.4 | FR detection of the petiole results in maximal petiole elongation response. (A) Differential petiole angle after 24 h exposure to W, $W+FR_{whole}$ and $W+FR_{whole}$ with supplemented R light at the lamina ($W+FR_{whole}+R_{lamina}$). (B) Petiole elongation response to 24 h exposure of similar light treatments as figure 5.2B. Data represent mean ± SE [n = 10 (A), or n = 18 (B)]. Different letters indicate statistically significant differences (one-way with Tukey's Post-hoc test, $P < 0.05$).

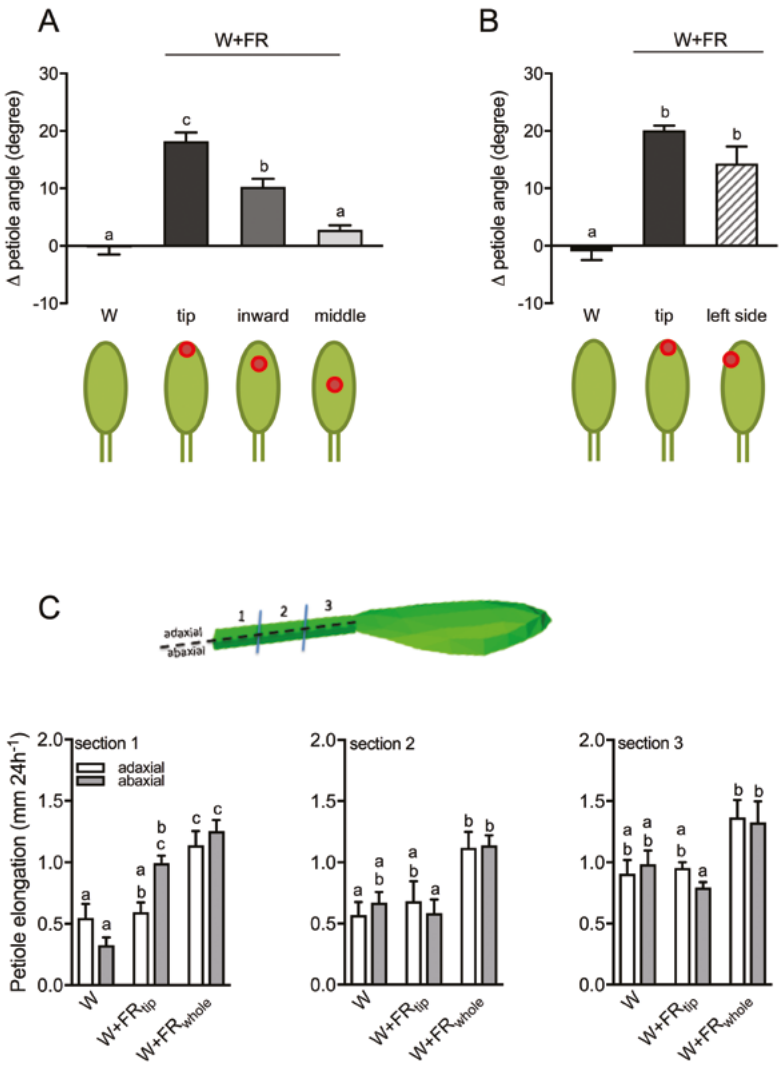

Figure 5.5 | FR-perception in the lamina tip induces the maximal hyponastic response through differential cell growth in the abaxial site of the petiole base. (A and B) The differential petiole angle of Col-0 WT in white light (W) and white light with supplemental FR at different spots on the lamina tip, including the very tip (tip), (A and B), between the lamina tip and the middle of the lamina (inward) (A), in the middle of the lamina (middle) (A), and on the left side of the lamina tip (B). Cartoons below the graphs illustrate the areas spotted with supplemental FR light (indicated as a red spot). (C) Elongation of the adaxial (white) and abaxial (grey) sides for three sections of the petiole (see leaf drawing) in W, W+FR_{tip} and W+FR_{whole} conditions. Data represent mean ± SE [n = 14 (A) and n = 7 (B, C)]. Different letters represent statistically significant difference (one-way ANOVA (A, B) or two-way ANOVA (C) with Tukey's Post-hoc test, $P < 0.05$).

Hyponastic response to FR-enrichment of the lamina tip relies on PIFs

The hyponastic response to W+FR$_{tip}$ was absent in the *phyBphyDphyE* triple mutant, confirming that it is a phytochrome response (Figure 5.6A). We therefore studied the involvement of the key PHYTOCHROME INTERACTING FACTORs (PIFs) that regulate established shade avoidance responses. Although the *pif4* mutant showed only a very mild reduction of the response to W+FR$_{tip}$, the *pif4pif5* double knockout showed no response at all. Interestingly, both mutants responded normally to W+FR$_{whole}$ (Figure 5.6B), whereas *pif7* and *pif4pif5pif7* showed a severe reduction of hyponasty under both W+FR$_{tip}$ and W+FR$_{whole}$ conditions (Figure 5.6C). These data indicate that PIF4, PIF5 and PIF7 contribute to supplemental FR-induced hyponasty.

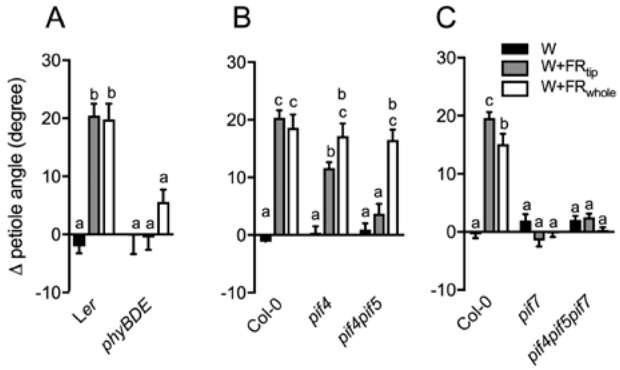

Figure 5.6 | Local FR treatment can induce hyponastic responses in a PIF-dependent manner. (A) L*er*, *phyBphyDphyE* (*phyBDE*) (B) Col-0, *pif4*, *pif4pif5* and (C) Col-0, *pif7* and *pif4pif5pif7* after 24 h of growth in white light (W) or white light with supplemented FR to the lamina tip (W+FR$_{tip}$) or to the whole plant (W+FR$_{whole}$). Data represent mean ± SE [n = 18 (A) or n = 10 (B, C)]. Different letters indicate statistically significant differences (two-way ANOVA with Tukey's Post-hoc test, $P < 0.05$).

Transcriptome analysis at sub-organ level identifies auxin signatures

A transcriptome analysis on the lamina tip and petiole base revealed more differentially expressed genes (DEGs) in W+FR$_{whole}$ than in W+FR$_{tip}$ treatment in both tissues, and more DEGs in the lamina tip compared to petiole base under W+FR$_{tip}$ (Figure 5.7A). Most DEGs in W+FR$_{tip}$ were also expressed in the respective tissues under W+FR$_{whole}$ (Figure 5.7B & Figure S5.1A), but with higher $\log_2$Fold Changes ($\log_2$FCs). Gene Ontology (GO) analysis for the common genes of each tissue revealed high representation of auxin-related processes (Figure 5.7C & S5.1B), which was confirmed by a hormonometer (Volodarsky et al., 2009) analysis (Figure S5.2A). Indeed, many DEGs in our transcriptome data were previously shown to be auxin-regulated (Nemhauser et al., 2006) as listed in Figure S5.2B-D for common and treatment-specific genes. *PIF7* transcript levels were higher in the lamina tip than in the petiole base, whereas *PIF4* and *PIF5* are equally expressed (Figure S5.2E).

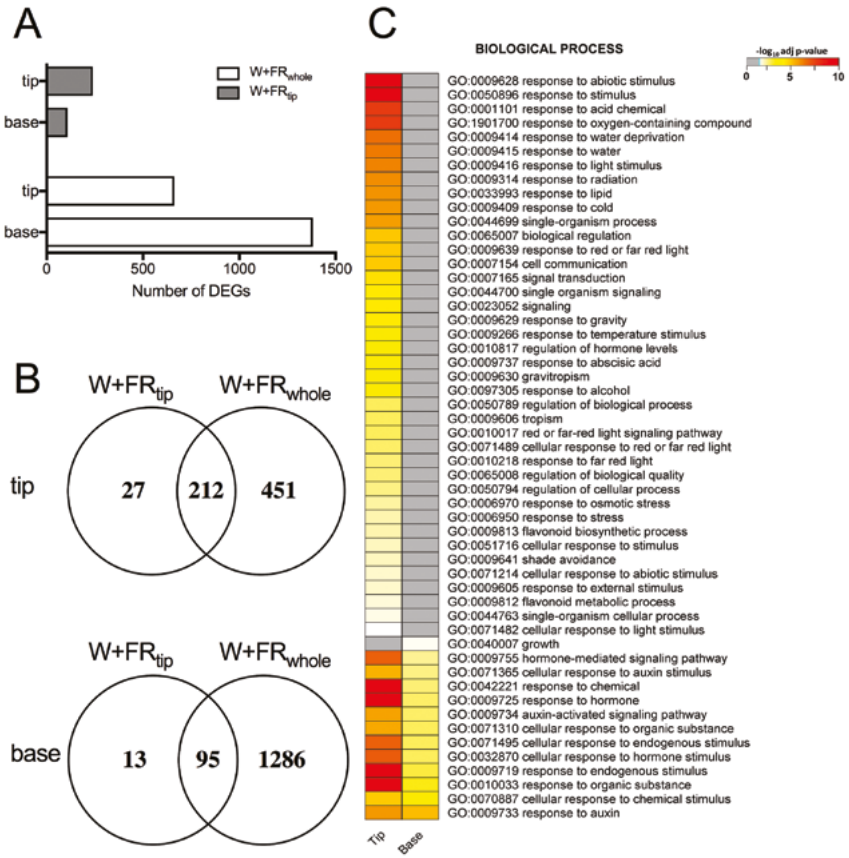

FIgure 5.7 | Comparative analysis of W+FR$_{tip}$- and W+FR$_{whole}$-induced transcriptome responses in lamina tip and petiole base. (A) Differentially expressed genes (DEGs) in lamina tip and petiole base. (B) Venn diagrams illustrate the DEGs common to the two FR treatments in the lamina tip and petiole base. (C) Gene ontology (GO) enrichment analysis for the genes common to the W+FR$_{tip}$ and W+FR$_{whole}$ conditions for each of the two tissues. Yellow-Red color scale denotes significance of the GO terms, grey indicates the GO term is not significantly enriched in that specific treatment x tissue combination.

5

Auxin regulates PIF-dependent, supplemental FR-induced hyponasty

Given the clear transcriptome auxin signatures, we studied if auxin regulates FR-induced hyponasty. Localized application of the auxin transport inhibitor NPA (1-naphtylphthalamic acid) to the lamina tip fully abolished hyponasty in both supplemental FR treatments, confirming that auxin from the lamina tip is required for hyponasty (Figure 5.8A & S5.3A). Consistently, the auxin efflux mutant *pin3* showed a reduced response and the *pin3pin4pin7* completely lost responsiveness to FR-enrichment (Figure 5.8B). The auxin biosynthesis mutant *wei8* (deficient in *WEI8/ TAA1/ SAV3*) had no response to W+FR$_{tip}$ but retained responsiveness to W+FR$_{whole}$ (Figure 5.8C). The *yuc8* and *yuc9* auxin biosynthesis mutants had marginally or not reduced hyponastic responses to supplemental FR (Fig.

S5.3B), but the *yuc8yuc9* double knockout lacked any hyponastic response to either of the supplemental FR treatments (Figure 5.8D). Importantly, expression of *YUC8* and *YUC9* in Col-0 plants was induced in the lamina tip, but not in the petiole base upon W+FR_{tip} and this was PIF7-dependent. These *YUC* genes were also induced in the petiole base under W+FR_{whole} exposure, which was PIF7-dependent for *YUC9* but not *YUC8* (Figure 5.9A & B). *PIN3* transcripts were also induced by both W+FR_{tip} and W+FR_{whole} in the tip, and by W+FR_{whole} in the base in a mostly PIF7-dependent manner (Figure 5.9C), whereas an independent shade-marker, PIL1, was less strongly PIF7-dependent (Figure 5.9D). Collectively, these data suggest that the function of PIF7 may be to induce auxin biosynthesis and transport in the lamina tip upon W+FR_{tip}.

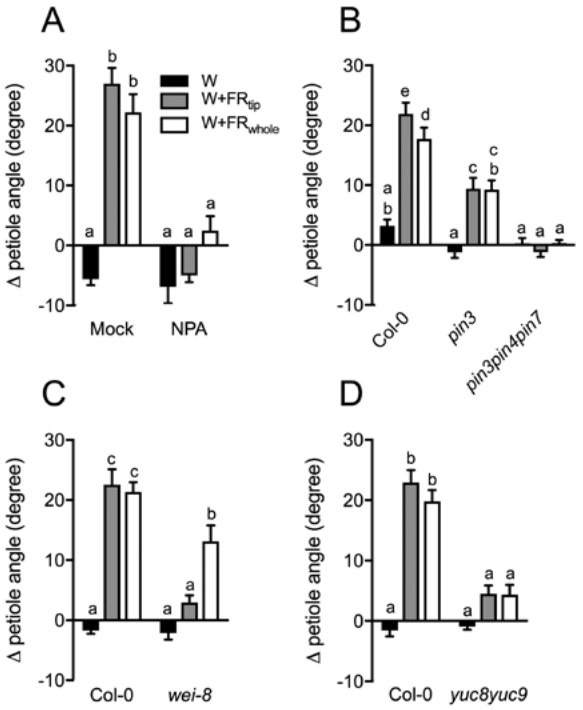

Figure 5.8 | Auxin biosynthesis and transport are required for hyponasty. (A) Differential petiole angle in response to different light conditions with exogenous application of one droplet of 50 µM NPA or mock solution to the lamina tip. (B-D) Differential petiole angle of mutants, for auxin transport [*pin3* and *pin3pin4pin7* (B)] and biosynthesis [*wei-8* (C), *yuc8yuc9* (*yuc89*) (D)] in three different light conditions. Data were collected after 24h of exposure to W and W+FR_{tip} or W+FR_{whole} conditions. Data represent mean ± SE (n = 7-10). Different letters indicate statistically significant differences (two-way ANOVA with Tukey's Post-hoc test, $P < 0.05$).

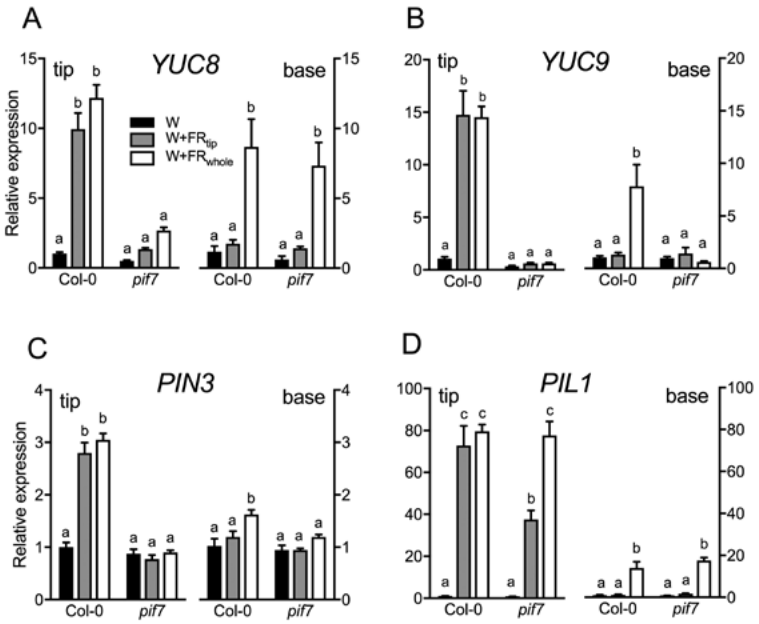

Figure 5.9 | Relative expression of four auxin related genes and shade-avoidance marker in two tissues**.** Relative expression of the auxin biosynthesis *YUC8* (A) and *YUC9* (B) genes, auxin efflux carrier *PIN3* (C) gene and shade-avoidance marker *PIL1* (D) gene for lamina tip and petiole base tissue of Col-0 and *pif7* after 5 h in W, W+FR$_{tip}$ or W+FR$_{whole}$ conditions. Data represent mean ± SE (n = 4). Different letters indicate organ-specific statistically significant differences (two-way ANOVA with Tukey's Post-hoc test, $P < 0.05$).

We visualized auxin activity with the *DR5:LUC* auxin reporter and observed clear luciferase induction in the abaxial, but not adaxial side of petiole after 7h in W+FR$_{tip}$ (Figure 5.10A & 5.10B). Luciferase activity was increased almost everywhere in W+FR$_{whole}$-exposed rosettes and was much stronger on the abaxial side compared to the adaxial side (Figure 5.10A & S5.3C). Exogenous application of a small (5 µL) droplet of 30 µM IAA at the lamina tip resulted in massive LUC reporter induction (Figure 5.10A, 5.10B & S5.3C), and also induced pronounced hyponasty (Figure 5.10C, 5.11A & 5.11B). IAA-induced hyponasty was abolished when applying NPA to the lamina-petiole junction (Figure 5.11A), confirming that IAA must be transported from the lamina to the petiole base in order to induce hyponasty there. Consistently, applying IAA directly to the abaxial side of the petiole base to bypass this pathway readily induces hyponasty (Figure 5.11C), whereas applying IAA on both sides of the petiole did not induce hyponasty (Figure 5.11D). If the *pif7* mutant lacks a W+FR$_{tip}$-induced hyponasty because of its failure to induce *YUCCAs*, it should be able to respond to exogenous IAA treatment of the lamina tip. Indeed, *pif7* responds to IAA, although slightly less than WT at low concentrations (Figure 5.10C; maximal at 30 µM IAA). Interestingly, *pif4pif5* displayed considerably reduced responsiveness to exogenous IAA, requiring at least 100 µM exogenous IAA on the leaf tip for maximal hyponasty (Figure

5.10C). In W+FR$_{whole}$ a larger section of leaf tip tissue than in W+FR$_{tip}$ is FR-triggered to produce IAA, likely increasing auxin levels. Indeed, when we illuminated a slightly larger leaf tip area than the 3.5 mm spotlight, this triggered hyponasty in *pif4pif5* (Figure S5.4A) as did very high IAA concentrations (Figure 5.10C). Illuminating a slightly larger leaf tip area with supplemental FR also restored some hyponasty in the auxin under-producing *wei8-1* mutant, similar to W+FR$_{whole}$ (Figure S5.4B).

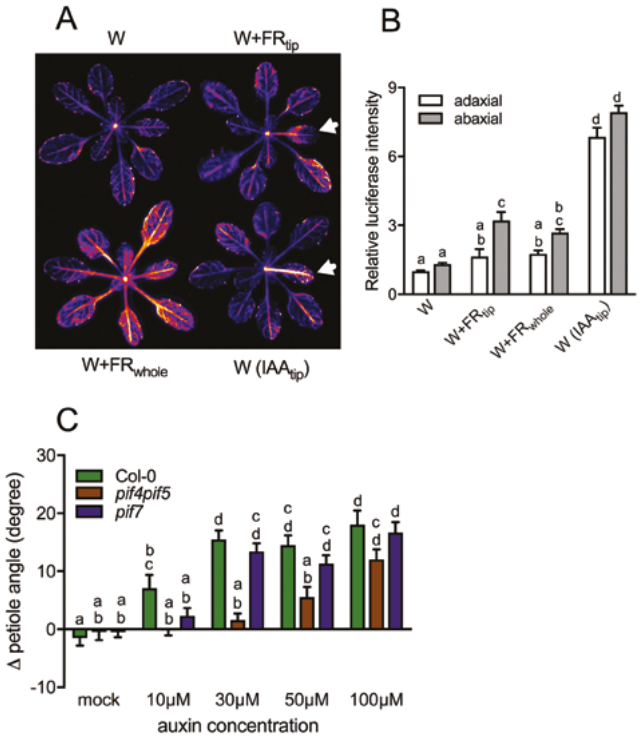

Figure 5.10 | Supplemental FR and localized IAA control auxin dynamics of leaf hyponasty**.** (A) Luciferase luminescence of the *DR5:LUC* auxin reporter on the abaxial side in W (upper left), W+FR$_{tip}$ (upper right), W+FR$_{whole}$ (lower left) and W with exogenous application of one droplet of 30 μM IAA in the lamina tip (W(IAA$_{tip}$), lower right). White arrows indicate the treated leaf in localized treatments. (B) Quantification of relative luciferase intensity on adaxial (white) and abaxial (grey) side of the petiole after exposure to W, W+FR$_{tip}$, W+FR$_{whole}$ and W(IAA$_{tip}$). Intensity values were expressed relative to those measured for adaxial sides of control W petioles. (C) Differential petiole angle of Col-0, *pif4pif5* and *pif7* after 23 h of exogenous application of different IAA concentrations in the lamina tip. Data represent mean ± SE [n = 5 (B) or n = 14 (C)]. Different letters indicate significant differences (two-way ANOVA with Tukey's Post-hoc test, $P < 0.05$).

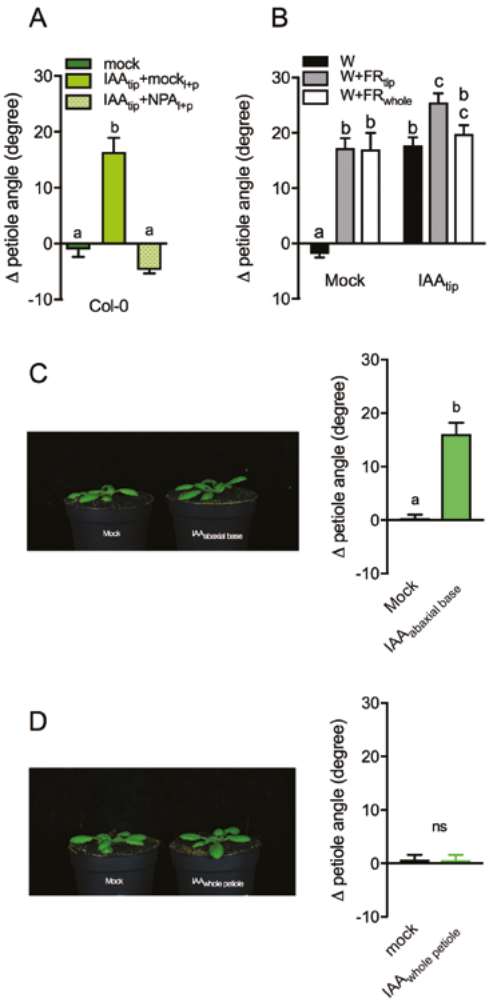

Figure 5.11 | Auxin has to be transported from the lamina tip to the petiole base to induce hyponasty. (A) Differential petiole angle of Col-0 upon IAA (30 µM) application at the lamina tip in the presence (IAA$_{tip}$ + NPA$_{l+p}$) or absence of 50 µM of NPA (IAA$_{tip}$ + mock$_{l+p}$) in the petiole-lamina junction in W light. (B) Differential petiole angle of Col-0 under W, W+FR$_{tip}$, W+FR$_{whole}$ after application of 30 µM IAA to the lamina tip. (C) Col-0 hyponastic response to exogenous auxin application to the abaxial side of the petiole base in W light (IAA$_{abaxial\ base}$) versus mock control. (D) Differential petiole angle of Col-0 upon IAA (30 µM) application at both the abaxial and adaxial side of the petiole (IAA$_{whole\ petiole}$). Data represent mean ± SE [n = 10 (A, B), n = 18 (C,D)]. Different letters indicate statistically significant differences (one-way (A) or two-way ANOVA (B) with Tukey's Post-hoc test, or paired Student's t-test (C,D), P < 0.05. ns = not significant).

Using R:FR sensing in the lamina tip for hyponasty is adaptive

Using a recently validated FSP model of *Arabidopsis* rosettes (Bongers et al., 2017) we determined the dynamics and consequences of incident R:FR on the petiole and lamina tip (Figure S5.5A) when growing in low and high density. At low density without competition, our simulations show a decreasing R:FR around the petioles, due to self-shading by newly developed leaves, whereas R:FR at the tip remained constant (Figure S5.5B). Simulations at high density showed that neighbouring plants caused a strong decrease of R:FR at the lamina tip (Figure S5.5C). Next, we simulated plant growth in 50-50% checkerboard designs of mixtures of plants sensing R:FR at the lamina tip ('Tip') and plants sensing R:FR at the petiole ('Petiole') at different canopy densities. At low density, plants approach

their neighbours later than at high density and consequently hyponasty was induced later at low density than at high density when R:FR is sensed at the tip (Figure 5.12 and Figure S5.5D). However, when sensing R:FR at the petiole, plants showed hyponasty even without interaction with neighbours (low density of 100 plants m^{-2}) and had a delayed response to neighbours at high density (2500 and 10000 plants m^{-2}) (Figure 5.12B). Raising leaves too early reduces light interception and raising them too late results in neighbour shade, which directly determines light absorption that drives carbon accumulation through photosynthesis. Accordingly, virtual 'Petiole' plants accumulate less biomass than 'Tip' plants when competing with each other (Figure 5.12), thus identifying the adaptive significance of using R:FR input from the lamina tip for hyponasty.

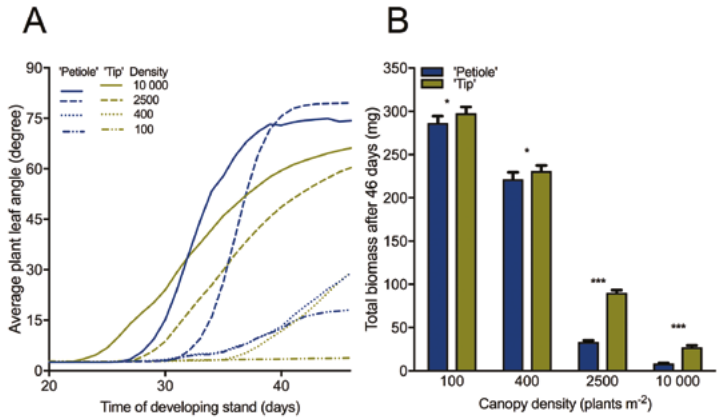

Figure 5.12 | R:FR tissue-specific perception affects plant performance growing in stands with different densities through FSP modeling. (A) Mean plant leaf angle for plant types using petiole ('Petiole' - blue) or lamina tip ('Tip' - yellow) as R:FR-detecting organ for inducing hyponasty, in four mixture stands with different densities. (B) Simulated total accumulated biomass after 46 days of growth for both plant types ('Petiole' & 'Tip') in mixture stands with four different densities. Data represent mean ± SD (n = 10). Statistically significant difference indicated by * for $P < 0.05$ and *** for $P < 0.001$, Paired Student's t-test.

Discussion

An early plant response to FR light reflected by neighbours is upward leaf movement, called hyponasty, followed by petiole elongation (Pierik and de Wit, 2013). Since light quality distribution in dense stands is heterogeneous even at the level of individual plants, we studied how localized FR exposures can elicit pronounced different shade avoidance responses. Here we show how the site of FR light detection influences the adaptive value of the elicited responses, and provide evidence that auxin spatially translates the neighbour detection cue to a growth response.

Functionality of localized R:FR detection for different responses

We found that petiole elongation is induced only when low R:FR exposure occurs on the target petiole itself, whereas hyponasty is induced only if low R:FR is sensed at the lamina tip of the same leaf (Figure 5.2 & 5.4). FSP modeling of *Arabidopsis* rosettes showed that sensing R:FR at the lamina tip indeed is the most effective way to induce hyponasty in response to neighbour proximity (Figure 5.12). The lamina tip is the first tissue to approach a neighbour and is thus the first to be exposed to neighbour-induced reductions in R:FR in stands of rosette-forming plants such as *Arabidopsis*. This is a rather robust system found in short day-grown, relatively mature *Arabidopsis* plants (this study), in much younger and long day-grown *Arabidopsis* (Michaud et al., 2017), and in another species, *Brassica nigra* (this study). The R:FR at the level of the petiole will also be reduced by self-shading and using this to induce hyponasty would be maladaptive. Since low R:FR at the petiole level does induce petiole elongation, this may be an intrinsic aspect of regular petiole length growth in *Arabidopsis*, irrespective of neighbour proximity. A previous study (Kozuka et al., 2010) showed that FR treatment of the lamina can also induce petiole elongation by giving supplemental FR at the end of the photoperiod (EODFR) to the full lamina, rather than to only the very tip of the lamina. Indeed, when we moved the FR spotlight on the lamina closer to the petiole, we observed modest stimulation of petiole elongation (Figure 5.4B).

Auxin and PIFs control hyponastic response to localized FR detection

Low R:FR light inactivates phyB, allowing PIFs to accumulate and become active to control transcription of target genes including YUCs (Leivar and Quail, 2011; Li et al., 2012; Leivar and Monte, 2014). Indeed, *phyBphyDphyE*, *pif4pif5* and *pif7* displayed a severe reduction of hyponasty in W+FR_{tip} (Figure 5.6). Interestingly, the *pif4pif5* double mutant was fully responsive to W+FR_{whole}, whereas *pif7* remained unresponsive, indicating a particularly important function for PIF7. Since hyponasty in the *pif4pif5* mutant could be rescued only by very high exogenous IAA concentrations relative to Col-0 wildtype and *pif7* (Figure 5.10C), we conclude that PIF4 and PIF5 control auxin responsiveness (Hornitschek et al., 2012; Hersch et al., 2014). The observations that *pif7* i) lacks any hyponastic response to W+FR_{whole} and W+FR_{tip} (Figure 5.6C), ii) lacks *YUC8* and *YUC9* expression in the lamina tip (Figure 5.9A and 5.9B) and iii) responds like wild-type to exogenous IAA (Figure 5.10C), suggest that PIF7 controls auxin production in the lamina tip (de Wit et al., 2015) to regulate hyponasty. Indeed, *PIF7* is also expressed more in the lamina tip than in the petiole base (Figure S5.2E). In the absence of PIF7 there will be insufficient lamina-derived IAA to drive auxin-mediated differential growth. Since we observed pronounced auxin gene expression signatures (Figure 5.8 & S5.2A-D) and luminescence of the auxin-responsive *DR5:LUC* reporter (Figure 5.10A & 5.10B) in the petiole upon FR-enrichment of the lamina tip, newly synthesized IAA from the lamina tip is probably transported to the petiole base. Indeed, polar auxin transport (PAT) mutants showed a disturbed response,

and local application of the PAT inhibitor NPA to the lamina tip marginalized the W+FR_{tip} response (Figure 5.8A & 5.8B). Following this route of auxin transport from the leaf tip, ultimately into the roots (Bhalerao et al., 2002), also explains why the response is restricted to the supplemental FR-exposed leaf and does not occur in systemic leaves.

The question then remains how newly synthesized auxin from the leaf tip remotely induces a differential growth response between the abaxial and adaxial side of the petiole base (Figure 5.5C). Since the *DR5:LUC* data indicate a differential auxin response between these two sides (Figure 5.10 versus Figure S5.3C), this could indicate an auxin concentration gradient that could drive the differential elongation growth between the abaxial and adaxial side of the petiole base (Figure 5.11C & 5.11D). We can, however, not exclude the possibility that differences in auxin sensitivity would underlie the differential *DR5:LUC* signal and growth response.

We conclude that auxin relays photoreceptor information to growth regulation over the distance of the two extreme ends of the leaf. This allows plants to sense neighbour plants as early as possible by using their most remote parts that are the first to interact with neighbours and react with an adaptive response: upward leaf movement.

Materials and methods

Plant growth and measurements

Genotypes used in this study: *pif4-101* and *pif4-101pif5-1* (Lorrain et al., 2008), *pif7-1* (Leivar et al., 2008), *pif4-101pif5-1pif7-1* (de Wit et al., 2015), *wei8* (Stepanova et al., 2008), *pin3-3* (Friml et al., 2002), *pin3-3pin4pin7* (Willige et al., 2013), *yuc8*, *yuc9*, *yuc8yuc9* (Chen et al., 2014), *DR5:LUC* (Moreno-Risueno et al., 2010) (all Col-0 background) and *phyBphyDphyE* (Lorrain et al., 2008) (L*er* background). Seeds were sown on soil, stratified (3 d, dark, 4°C), and then transferred to growth rooms (9 h light, 130-135 µmol m^{-2} s^{-1} PAR, R:FR 2.3, 20°C, 70% RH).The growth conditions of the plants were similar as in chapter 4 ("plant growth and measurements").

Light treatments

In addition to control white (W) light from Philips HPI lamps (R:FR = 2.3), far-red supplementation treatments were used: whole-plant FR-enrichment (W+FR_{whole}) through FR LED (Philips Green-Power FR) supplementation of W background (R:FR = 0.05), and localized FR-enrichment by directing FR spotlights (Ø 3.5 mm) to selective parts of the leaf, using custom FR LEDs (724-732 nm; local R:FR = 0.05). PAR was constant at 125-135 µmol m^{-2} s^{-1} for all treatments. Light spectra were recorded with OceanOptics JAZ equipment (Figure S5.6).

Pharmacological treatments

Localized auxin was applied as 5 µl droplets of 30 µM indole 3-acetic acid (IAA, Duchefa Biochemie, Haarlem, The Netherlands) and polar auxin transport was inhibited with 4 or 5 µl of 50 µM 1-N-naphthylphthalamic acid (NPA, Duchefa Biochemie, Haarlem, The Netherlands). Solutions, including mocks, contained 0.1% Tween and 0.03 (IAA) or 0.05 (NPA) % DMSO. When NPA was applied to the lamina-petiole junction, the solution was supplemented with 0.05% agar to stabilize the droplet. Solutions were applied right before commencing light treatments. IAA was applied to the abaxial side of the petiole via a small piece of filter paper sticking to the petiole base containing auxin or mock solution and rewetted with the solutions frequently.

RNA isolation and gene expression quantification

The lamina tip (top 2.5 mm) and petiole base (basal 1.5 mm) of the fifth-youngest leaf plants were harvested for RNA isolation after 5 h (Figure S5.7) of light treatments (W (control), W+FR_{tip}, W+FR_{whole}). Ten (qRT-PCR) or fifteen (microarrays) lamina tips or petiole bases were pooled as one biological replicate. RNA was isolated from three independent biological replicates per treatment and tissue (RNeasy mini kit (Qiagen), Hilden, Germany). For qRT-PCR, cDNA was synthesized using random hexamer primers (Invitrogen, Carisbad, USA). qRT-PCR was performed using Applied Biosystems ViiA 7 (Thermo Scientific, Waltham, USA) with SYBR Green MasterMix (Bio-Rad, Hercules, USA) with gene-specific primers (Table S5.1). Relative transcript abundance was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) normalized to *PEX4* (AT5G25760) and *RHIP1* (AT4G26410). For transcriptomics, samples were hybridized to Affymetrix 1.1 ST *Arabidopsis* arrays by AROS Applied Biotechnology (Aarhus, Denmark) and are deposited at GEO-NCBI (accession number GSE98643). Data were processed using Bioconductor, packages "oligo" and "pd. aragene.1.1.st" for quality check and normalization, and "limma" for differential expression analysis, in R software. Genes with adjusted P-value ≤ 0.01 and $\log_2$FC > 1 or $\log_2$FC < -1 were considered differentially expressed. Gene ontology (GO) analysis was done with AmiGO (http://amigo.geneontology.org/cgi- bin/amigo/term_enrichment; (Boyle et al., 2004). Hormonometer (http://genome.weizmann.ac.il/hormonometer; (Volodarsky et al., 2009) was used to identify hormonal signatures in our transcriptomes.

Luciferase assay

DR5:LUC plants were exposed to the light or hormone treatments for 7 h. The whole process has been described in chapter 4 in the respective paragraph.

Functional-structural plant (FSP) model

A recently developed functional-structural plant model of *Arabidopsis* rosettes (Bongers et al., 2017) was used to study the relative functional impact of R:FR-detection for hyponasty occurring at the lamina tip or petiole. Principles for plant structure, growth and light interactions are largely based on models used in previous work (Bongers et al., 2014; Evers and Bastiaans, 2016). Details of this model are described in the Supplemental Material and Methods.

Acknowledgements

We thank Jojanneke Voorhoeve for help with the *Brassica nigra* experiments and members of the Plant Ecophysiology group (UU) for help with tissue harvests for the gene expression experiments. We thank Christian Fankhauser, Ikram Blilou and Stephan Pollmann for distributing seeds. We thank Scott Hayes for excellent feedback.

Supplemental data

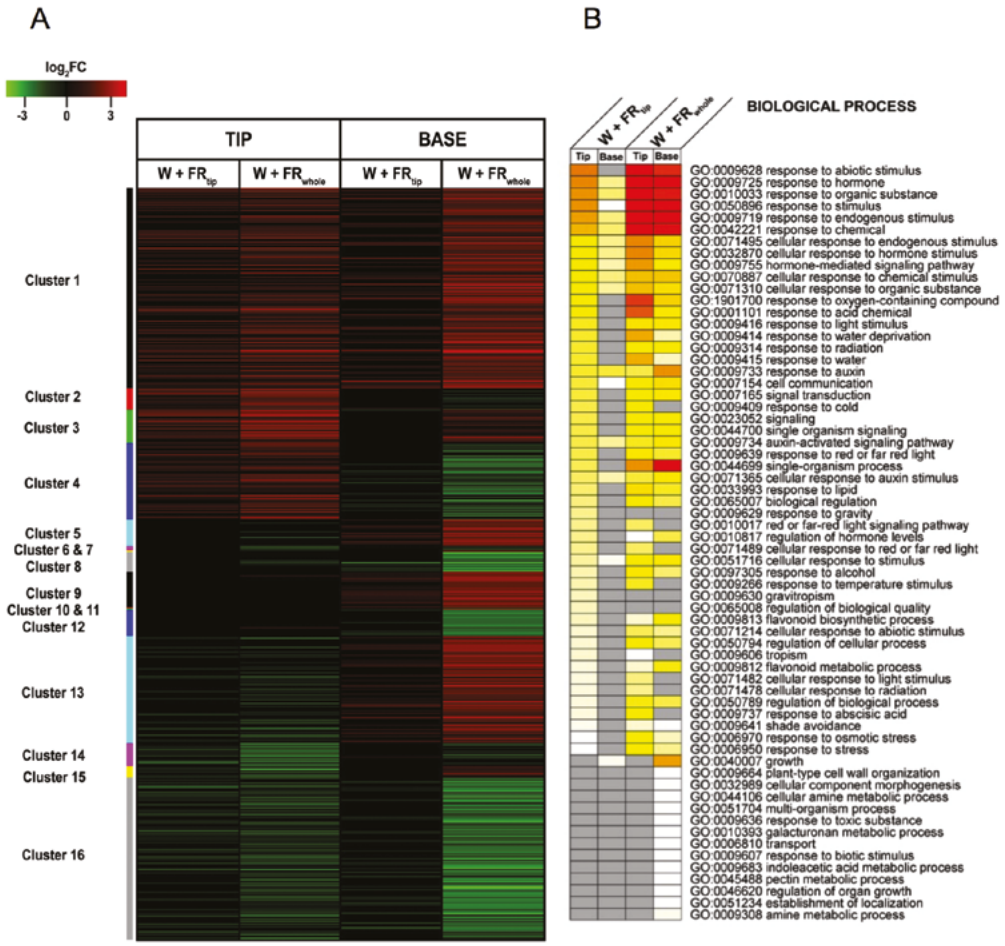

Figure S5.1 | Transcriptome analysis of lamina tip and petiole petiole base upon W+FR_{tip} and W+FR_{whole} conditions. (A) Differentially expressed genes in the lamina tip and petiole base in response to W+FR_{tip} and W+FR_{whole} light treatment (adjusted p-value ≤ 0.01 and $log_2FC > 1$ or <1), clustered based on fold change and direction (up/down) of regulation. (B) Gene ontology (GO) enrichment analysis for the lamina tip and petiole base upon W+FR_{tip} and W+FR_{whole} conditions. Yellow-Red color scale denotes significance of the GO terms, grey indicates the GO term is not significantly enriched in that specific treatment x tissue combination.

B

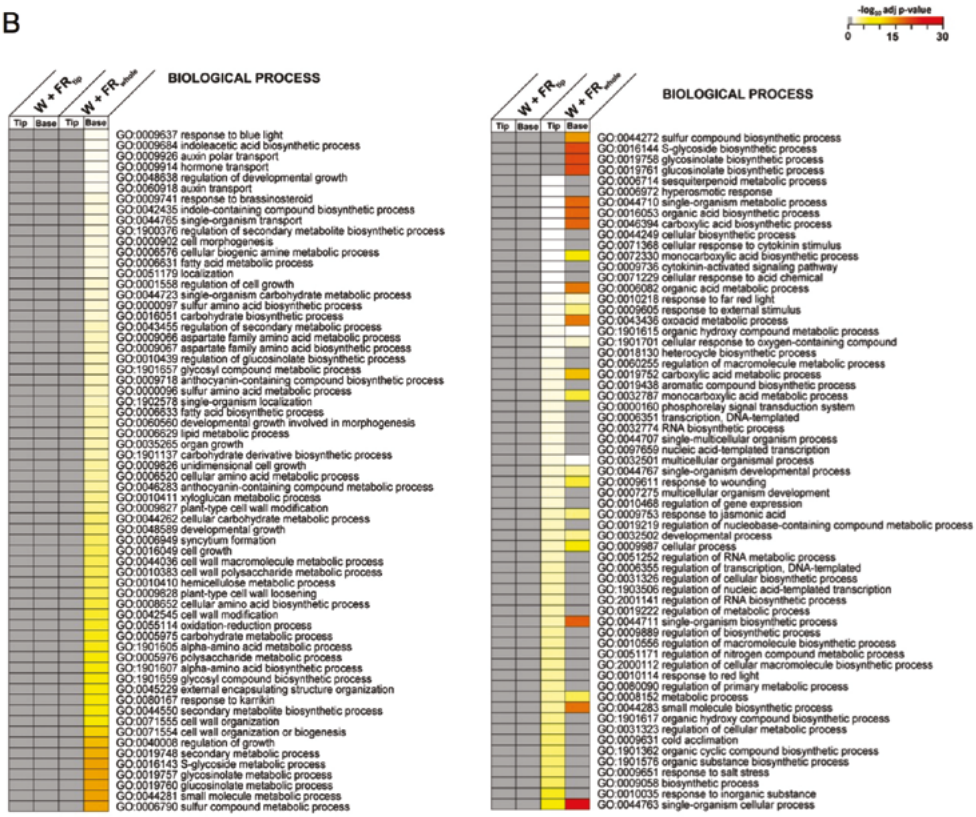

Figure S5.1 | Continued.

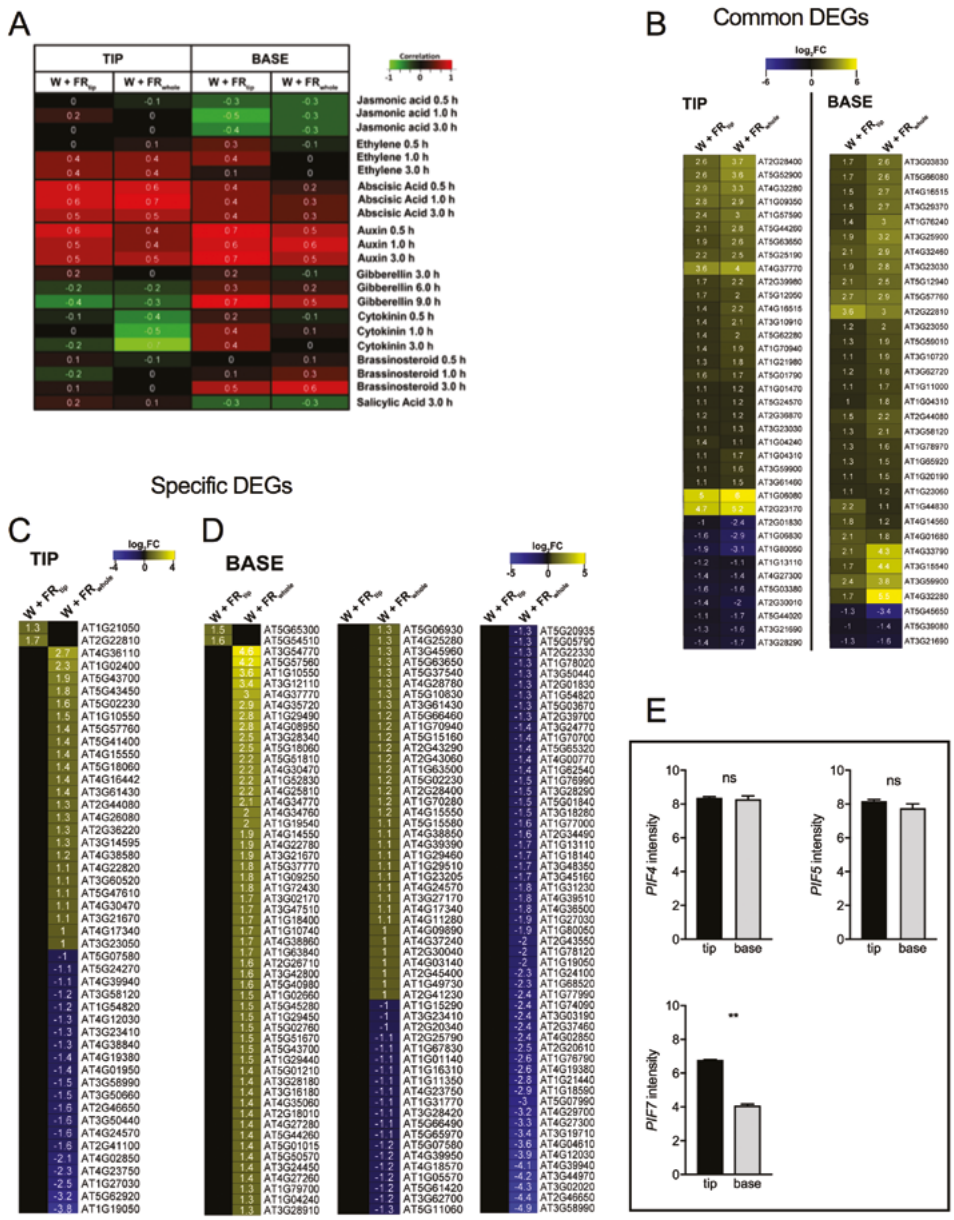

Figure S5.2 | Hormone-associated transcript patterns in the microarray data of petiole base and lamina tip under W+FR$_{tip}$ and W+FR$_{whole}$ treatments. (A) Hormonometer analysis of the lamina tip and petiole base transcriptome (Volodarsky et al., 2009). (B) Heatmap representation of the expression levels of DEGs that are associated with auxin and are shared (common) in the two tissue types between W+FR$_{tip}$ and W+FR$_{whole}$. (C, D) Heatmaps showing differentially expressed auxin-related genes in the (C) lamina tip (TIP) and (D) petiole base (BASE) that are exclusively to either W+FR$_{tip}$ or W+FR$_{whole}$. All heatmaps (B, C, D) are based on IAA-induced transcripts in ref. (Nemhauser et al., 2006). (E) Intensity of *PIF4*, *PIF5* and *PIF7* in the microarray data of the lamina tip and petiole base in W light. Data represent mean ± SE, n=3. ns, no significant difference. ** for P<0.01, Paired Student's t-test.

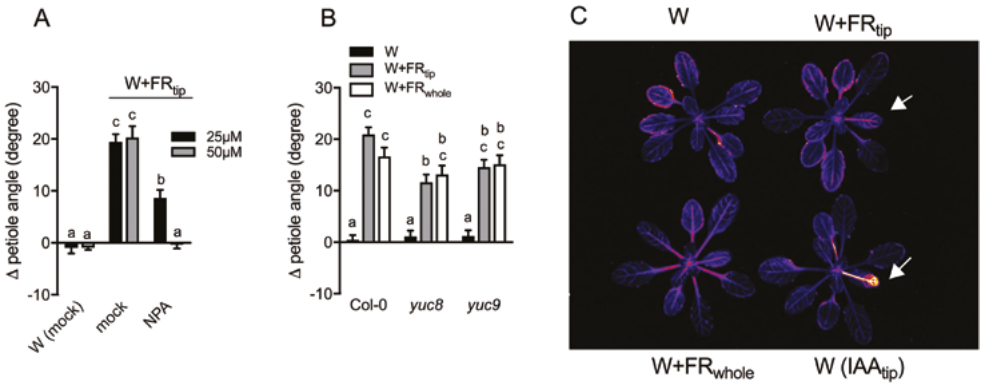

Figure S5.3 | Auxin transport and production have a key role in FR-induced hyponasty. (A) Differential petiole angle response after exogenous application of different mock or NPA concentrations to the lamina tip under W and W+FR$_{tip}$ conditions. (B) Differential petiole angle of auxin biosynthesis gene mutants (*yuc8, yuc9*) under W, W+FR$_{tip}$ and W+FR$_{whole}$ conditions. (C) Luciferase luminescence of the auxin reporter line *DR5:LUC* on the adaxial side under W (upper left), W+FR$_{tip}$ (upper right), W+FR$_{whole}$ (lower left) and W with exogenous application of 30 μM IAA to the lamina tip (W (IAA$_{tip}$); lower right). White arrows indicate the treated leaf. Data represent mean ± SE [n=6 (A), n=10 (B,C)]. Different letters indicate statistically significant differences (two-way ANOVA with Tukey's Post-hoc test, or paired Student's t-test, $P < 0.05$).

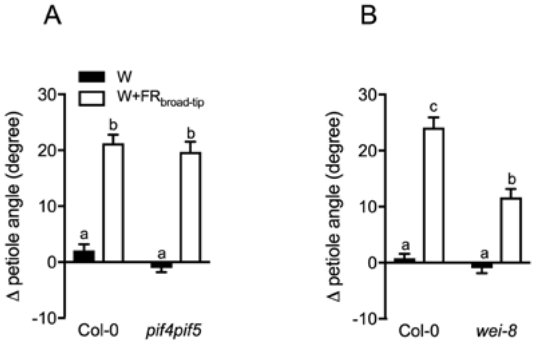

Figure S5.4 | Supplemental FR treatment of a relatively larger leaf tip area can restore the hyponastic phenotype in *pif4pif5* and *wei-8* mutant. (A, B) Differential petiole angle of (A) *pif4pif5* and (B) *wei8-1* under W light and supplemental FR light in a broad spot (7.5mm, rather than the regular 3.5 mm) on the lamina tip (W+FR$_{broad\ tip}$). Data represent mean ± SE (n = 14). Different letters indicate statistically significant differences (two-way ANOVA with Tukey's Post-hoc test, $P < 0.05$).

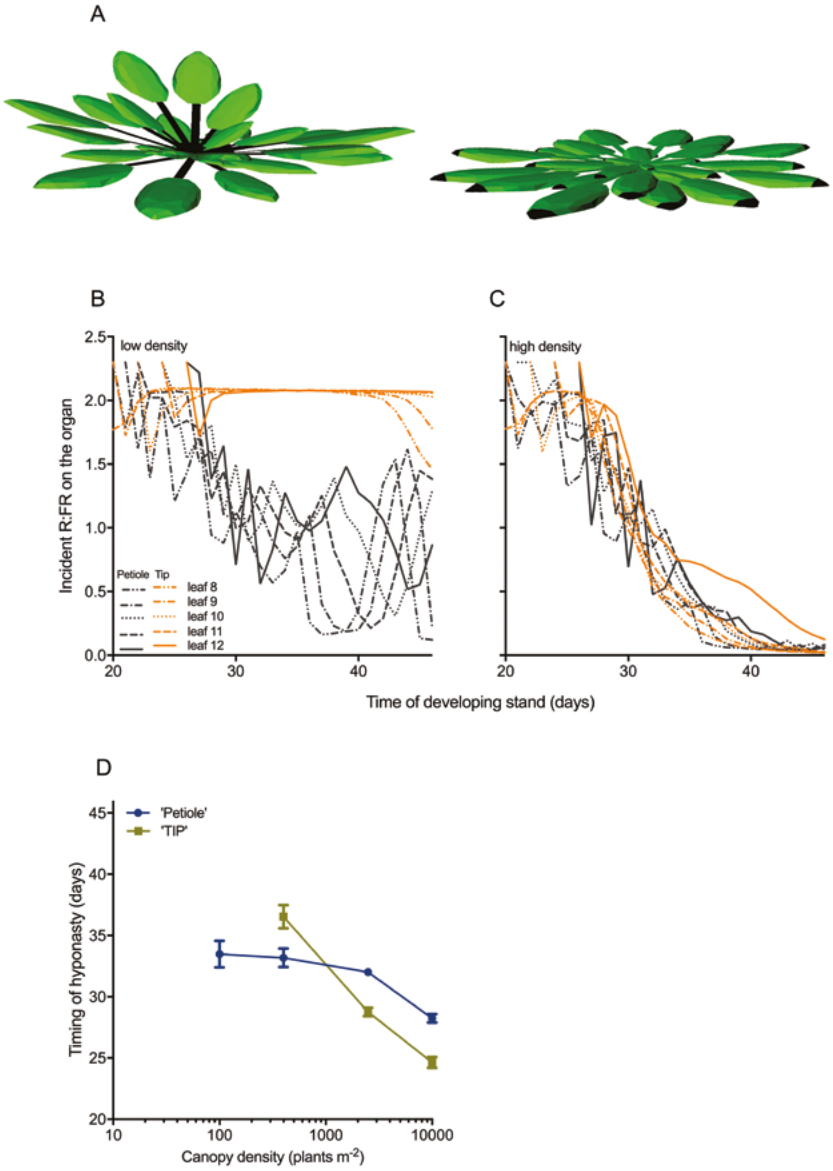

Figure S5.5 | Simulated tissue-specific incident R:FR and timing of hyponasty in different densities through FSP modeling. (A) Virtual representation of two simulated Arabidopsis plants growing at low density (100 plants m^{-2}). Two plant types were simulated that used the R:FR at the petiole (left) or at the lamina tip (right) to induce hyponasty, as identified by black coloring of the organ part. The simulated plant that used the petiole R:FR as input for hyponasty shows that leaves become hyponastic through self-shading by younger leaves. (B-C) Incident R:FR at petiole (grey) and tip (orange) of leaf rank 8 up to 12, during plant development at low density (B) or at high density (2500 plants m^{-2}; C). In these simulations, plants did not become hyponastic**.** (D) Timing of hyponasty in relation to canopy density for two plant types that use the petiole ('Petiole' - blue) or the lamina tip ('Tip' – yellow) as R:FR-detection organ to induce hyponasty. Data represent mean ± SD (n = 10).

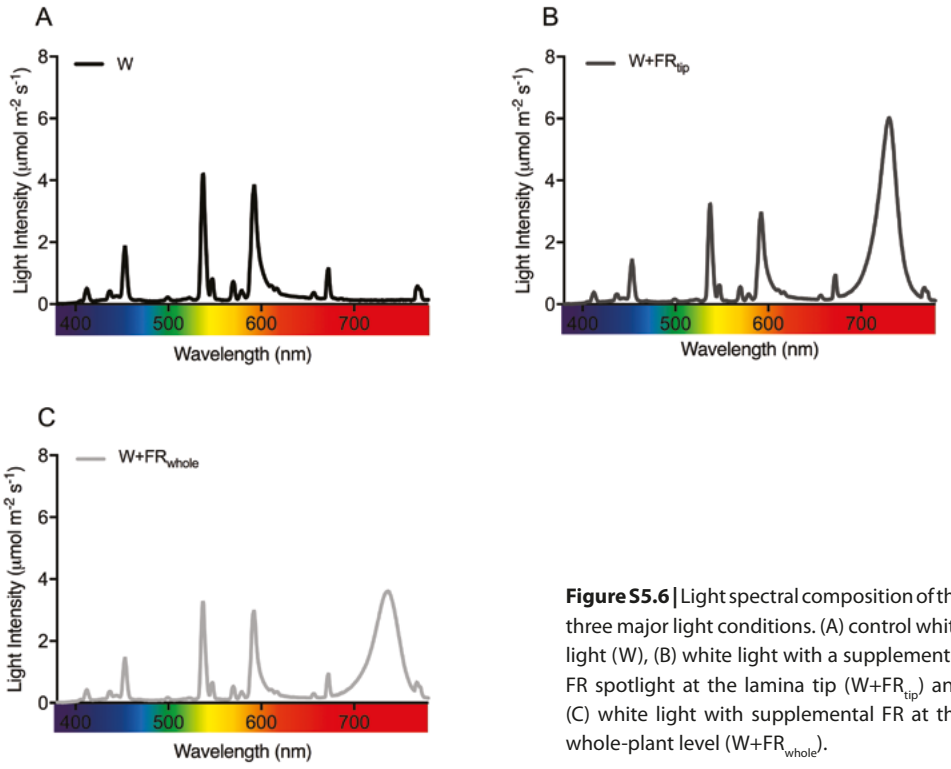

Figure S5.6 | Light spectral composition of the three major light conditions. (A) control white light (W), (B) white light with a supplemental FR spotlight at the lamina tip ($W+FR_{tip}$) and (C) white light with supplemental FR at the whole-plant level ($W+FR_{whole}$).

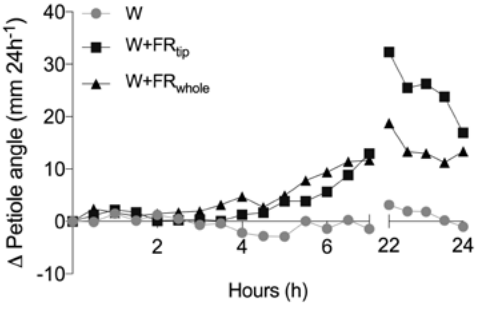

Figure S5.7 | Time lapse of differential petiole angle of Col-0 in different light treatments [control white light (W), white light with a supplemental FR spotlight at the lamina tip ($W+FR_{tip}$) and white light with supplemental FR at the whole-plant level ($W+FR_{whole}$).

SI Material and Methods

Functional-structural plant model (FSP): A recently developed functional-structural plant (FSP) model of *Arabidopsis* rosettes (Bongers et al., 2017) was used to simulate artificial *Arabidopsis* plant types, using the simulation platform GroIMP and its radiation model (https://**sourceforge**.net/projects/**groimp**/). The model is available upon request. *Arabidopsis* rosettes are represented by a collection of leaves (represented by a petiole and a lamina). Their appearance rate and shape were based on empirical data. We defined the lamina tip to represent 7% of total lamina area at the most distal part of the lamina (Figure S5.5A). In our simulations, the light source emitted Photosynthetically Active Radiation (PAR; 400 – 700 nm) at an intensity of 220 µmol m^{-2} s^{-1}, with a R:FR ratio of 2.3. In each model time step, these light rays were reflected, transmitted and absorbed by the petioles and laminas according to their wavelength-specific spectral properties. The PAR absorbed at each time step determined the amount of carbon fixation for growth, which consequently determined biomass accumulation (in mg) for each organ. The amount of biomass determined organ size, which, in turn determined the reflection, transmission and absorption of light by the simulated plants. Thus, the leaves individually grew in time in three dimensions based on leaf-level PAR absorption, photosynthesis and between-organ carbon allocation principles (explained in more detail in (Evers and Bastiaans, 2016). Simulated plant growth depended on the level of competition for light that individual plants experienced with neighbouring plants. Plant size and biomass were therefore an emergent property of the model. Hyponasty was calculated each time step by a unit-step response curve: when R:FR perception at the specific petiole or lamina tip was below a threshold of 0.5, the leaf angle increased with a fixed amount of 20 degrees, otherwise leaf angle was maintained. The angle of the leaf over time was therefore a function of the number of time steps in which this low R:FR perception occurred, with a maximum leaf angle of 80 degrees. In our model hyponastic responses influenced light competition between individual plants based on two principles. First of all, elevated leaves (i.e. leaves that are not horizontal) intercept less light than horizontally positioned leaves when plants grow solitarily. Secondly, in the presence of neighbouring plants horizontal leaves will intercept less light because of overlap with neighbour leaves. This means that positioning leaves in a horizontal or more vertical position determined total light interception that drives biomass accumulation, and this depended on the presence of neighbour plants.

Model scenario: All *Arabidopsis* stand types were constructed by placing 4 × 4 plants at different inter-plant distances (1, 2, 5 and 10 cm) resulting in four canopy densities; 10000, 2500, 400 and 100 plants m^{-2}. In stands with a low density (100 plants m^{-2}), neighbouring plants did not interact with each other, and no additional rows were simulated. Stands with a density of 400 or 2500 plants m^{-2} were simulated with two extra plant rows and stands with an extreme high density of 10000 plants m^{-2} with 4 extra plant rows to correct

for possible border effects. Plant orientations (determined by the direction of the leaves) at their positions in the field were random, just as would be the case in experimental plots. In the first simulation scenario, two stands were simulated with a density of 100 and 2500 plants m^{-2}, representing low and high canopy density, with plants that did not use the incident R:FR for signaling and therefore did not become hyponastic. In the second simulation scenario, four mixture competition stands were simulated at four densities. In these mixtures, two different plant types were placed in a 50-50% checkerboard design; the 'Petiole' plant type used the R:FR signal at the petiole and the 'Tip' plant type used the R:FR signal of the lamina tip to induce hyponasty. Because PAR absorption and R:FR perception were the driving factors of plant growth and development, plant leaf angle and biomass accumulation in different canopy densities were the emergent properties of the model. Each replicate stand consisted of 8 plants of each plant type ('Petiole' and 'Tip') that were averaged per plant type per stand. Model output was subsequently calculated as the mean of 10 replicated stands.

Table S5.1 | Primer sequences (5'→3') used for qRT-PCR were as follows.

Gene name	Primer	5' to 3' sequence
AT1G04180 (*YUC9*)	FW	AGTCCGGCGAGAAATTCAGA
AT1G04180 (*YUC9*)	REV	AACCGAGCTTCTAACGACCA
AT4G28720 (*YUC8*)	FW	TGCGGTTGGGTTTACGAGGAAAG
AT4G28720 (*YUC8*)	REV	GCGTTTCGTGGGTTGTTTTG
AT1G70940 (*PIN3*)	FW	CTTATTTGGGCTCTCGTCGC
AT1G70940 (*PIN3*)	REV	AACGTTGCCACTGAATTCCC
AT2G46970 (*PIL1*)	FW	AGACCACCTACGATGTTGCC
AT2G46970 (*PIL1*)	REV	TAGCATTTGTGGTGGTGCAT
AT5G25760 (housekeeping gene *PEX4*)	FW	TGCAACCTCCTCAAGTTCGA
AT5G25760 (housekeeping gene *PEX4*)	REV	TGAGTCGCAGTTAAGAGGACT
AT4G26410 (housekeeping gene *RHIP1*)	FW	ATTGGTGTCGCTGCTAGTCT
AT4G26410 (housekeeping gene *RHIP1*)	REV	TAAAGCCGTCCTCTCAAGCA

Chapter 6

General discussion

Improvement of crop production is essential to cover the growing demands for food (Schmidhuber and Tubiello, 2007). In order to achieve this, crop production needs to be increased at least 2% per year (Ray et al., 2013), for example by reducing the plausible causes of decreased production output. The increase of biomass, density and species of weeds in an agricultural field is one of the main causes of yield losses (Blackshaw et al., 2002; Oerke, 2006). Rice, corn and wheat are some of the most consumed crops in our daily life, and have been recorded to suffer from serious weed infestation (Khan and Haq, 2002; Yeganehpoor et al., 2015; Dass et al., 2017). Plants in agricultural fields grow at high densities in which they compete for the available resources, including light. In dense canopies, the Red (R) : Far-red (FR) light ratio is strongly reduced since the R and blue (B) light are absorbed where the FR is reflected (Morgan and Smith, 1976; Franklin, 2008). The weeds in a dense canopy intensify the R:FR reduction, because FR reflection is more enriched through them (Green-Tracewicz et al., 2011; reviewed in Pierik and Testerink, 2014). This R:FR reduction inside the vegetation triggers the shade avoidance syndrome (SAS), which maximizes the individual plant fitness by allocating the carbon resources towards petiole or stem elongation and upward movement of the leaf (Franklin, 2008; Pierik et al., 2013; Pierik and de Wit, 2013; Ballaré and Pierik, 2017) instead of the harvestable organs (Robson et al., 1996; Boccalandro et al., 2003; Page et al., 2010). Also, the reduction in root investment due to SAS (Morelli and Ruberti, 2002) stimulates unavoidable lodging, leading to a more open canopy for light penetration, thus facilitating weed growth. These SAS implications cause reduction in crop yield. The maximization of the canopy closure by reducing SAS, in order to suppress the weeds and minimize the allocation of the resources to non-edible organs could be a possible solution. In this thesis I tested two scenarios to achieve that by: 1) changing the planting patterns and 2) modifying the shade avoidance responses.

The effect of planting patterns on canopy closure and performance

A decade of experiments in cereal crops revealed that planting patterns have an effect on weed suppression (Weiner et al., 2001). More specifically, if wheat grows in high density, in relatively uniform patterns instead of rows, the weeds were suppressed up to 30% more, while the crop yield was increased (Weiner et al., 2001). This is likely due to the more efficient interception of above and below ground resources, as well as the faster and better formation of shade canopy above the weeds than in the row pattern (Olsen et al., 2005b; Olsen et al., 2006; Kristensen et al., 2008). Indeed, using a similar set-up, with *Arabidopsis* Col-0 as a canopy plant and *Arabidopsis* L*er* as a competitor, we observed comparable effects during competition (chapter 2). The canopy structure of dense, uniformly planted *Arabidopsis* induces a fast reduction of R:FR inside the canopy through time, presumably limiting access of competing L*er* to light. This limitation of light reduced

the Leaf Area Index (LAI) and biomass of L*er*, making it difficult to reach out of the Col-0 canopy to intercept light (Thornley and Johnson, 1990; Bullock et al., 1998; Norris et al., 2001). Accordingly, Col-0 canopy plants showed a relatively high plant biomass and yield under these conditions. These findings indicate that a relatively uniform planting pattern at high density can enhance canopy plant performance, whilst repressing competing plants (L*er*), a finding similar to those observed for field-grown cereal crops and weeds.

Evolutionary Agroecology: suppress SAS to suppress competitors?

Although the findings from chapter 2 are very promising, these plants do still invest in shade avoidance responses, which are investments that may go at the expense of yield and that may open the canopy for light penetration. We therefore continued with the *Arabidopsis* canopy system to test the Evolutionary Agroecology hypothesis (Weiner et al., 2017) that suppressed shade avoidance may enhance whole community fitness and competitive performance against invading competitors (chapter 3). Using mutants with mild (*pif4pif5*) or severe reduction of shade avoidance (*pif4pif5pif7*) as canopy plants and the *Arabidopsis* accession L*er* as a competitor, we found that L*er* performed better both in terms of biomass and LAI under the *pif4pif5* and *pif4pif5pif7* canopy compared to Col-0. The opposite results were true for the *pif4pif5* and *pif4pif5pif7* mutant canopy plants. The reduced growth rate of the *pif4pif5* and *pif4pif5pif7* (Figure 3.1D) and the delay in canopy closure due to the reduced petiole angle (Figure 3.1B, Figure 3.2), combined with the strong shade avoiding phenotype of L*er* (Figure 3.4) might explain why these mutants performed less than Col-0. However, chapter 2 and chapter 3 have only focused on above ground competition whereas below ground interactions can also have an effect in our system. Under shade avoidance conditions, the size of the main root is reduced (Salisbury et al., 2007; Gundel et al., 2014) and it has also been described that roots are able to detect their neighbours in shared competition zones (Caffaro et al., 2013). Follow up research studying the impact of shade avoidance on weed suppression, could use 3D computational modeling (Bongers et al., 2014) to test the impact of separate shade avoidance traits on competitor suppression. Disturbed leaf angle responses but normal elongation responses or normal leaf angle response with a reduced elongation would typically be testable scenarios. Also canopy experiments to test the Evolutionary Agroecology hypothesis, with genotypes less responsive to light competition as a competitor and *pif4pif5pif7* as a canopy, would be very helpful.

Modifying canopy architecture: early plant neighbour detection

As discussed above, and shown in chapters 2 and 3, canopy architecture influences plant growth and competitor suppression. Upward leaf movement, known as hyponasty, is a response that changes the canopy architecture from a horizontal to a vertical structure. Hyponasty in Arabidopsis stands is initially induced by touching leaf tips of neighbouring plants, even before light quality changes occur (de Wit et al., 2012). An important consequence of the change from a horizontal to a vertical canopy structure is FR light is now horizontally reflected, resulting a decrease of the R:FR, leading to SAS (de Wit et al., 2012). Thus, early responses to mechano-stimulation set in motion a chain of shade avoidance events. Understanding how to modify this in plants might help change the canopy structure for competitor suppression. We therefore set out to elucidate the physiological mechanisms regulating touch-induced hyponasty. In chapter 4, we studied touch-induced gene expression profiles and observed enrichment of biological processes related to Jasmonic acid (JA) in the lamina tip and abscisic acid (ABA) in the petiole base, hinting at an involvement of these two defence-associated hormones. However, most mutants related to JA or ABA defence pathways did not show any distinct phenotype, except *myc2myc3* and *abi4*. Possibly, the transcriptome profile of the two ends of the leaf during touch treatment could indicate a mechano-stimulation profile rather than a biotic defence-related one. This is due to the activation of JA and ABA response pathways in a synergistic manner upon wounding (which is considered as a mechanical stress; Farmer et al., 2014) after herbivore attack (Anderson et al., 2004; Fernández-Calvo et al., 2011). Moreover, JA is also tangled to mechano-stimulation due to thigmomorphogenetic responses (plant response to touch; Ellis et al., 2002; Tretner et al., 2008). In thigmonastic movement of *Dionaea muscipula* (Venus' Flytrap), the small hairs in the leaf surface are very important, since they generate an electrical signal after the insect touches (Simons, 1981). This signal triggers the differential enlargement of cells in the lobes, causing fast closure of the trap (Braam, 2005; Escalante-Pérez et al., 2011). Arabidopsis mutants, in clade 3 *GLUTAMATE RECEPTORS-LIKE* (*GLRs*) genes cannot generate long-distance electrical signalling upon wounding, (Mousavi et al., 2013). We tested some of these *glr3* mutant combinations and found strong reduction of hyponasty. Comparable to *Dionaea muscipula*, *Arabidopsis* trichomes have a key role in touch-induced hyponasty, as they are the first tissues to interact between the two-leaf tips and mutants without trichomes showed no touch-induced hyponasty (chapter 4). Since trichomes are considered to be mechanosensory switches (Zhou et al., 2017), we propose that the mechanosensing element of the touch response could be the trichomes or the epidermal pavement cells surrounding the trichomes. In addition, we found a severe reduction of touch-induced hyponasty in the mechanosensing-associated *Catharanthus roseus* receptor-like kinases (CrRLK1L) (Monshausen and Haswell, 2013) mutants *fer4* and *herk1the1*. We speculate that mechanosensing of neighbour leaves occurs in the trichomes though CrRLKs and the

signal upon trichome-trichome interaction of two leaves is possibly transmitted via clade 3 GLRs to the petiole base. In the petiole base, this leads to differential growth of adaxial and abaxial sides of the petiole, resulting in the observed hyponastic leaf movement.

Clearly, this pathway of touch-induced hyponasty is not completely resolved yet. It remains to be studied if CrRLKs and clade 3 GLRs are connected and how the spatial separation between trichomes, lamina and petiole is bridged. Using GFP-protein fusion lines of RLKs and/or GLRs could illustrate the localization of the proteins after the touch response. Studying the expression of RLKs or clade 3 GLRs related genes in the trichomes versus other tissues would further help establish if they are predominantly located in the cells that experience touch. Furthermore, our transcriptome data revealed enriched gene ontology of calcium ion transport and we also know that thigmomorhogenic responses are associated with calcium (Ca^{2+}) signalling (Knight et al., 1992; Chiasson et al., 2005; Hayashi et al., 2006). The involvement of Ca^{2+} in touch-induced hyponasty remains to be investigated.

Hyponasty: the importance of upward movement of the leaf during competition for light

In chapter 2 it is shown how an Arabidopsis canopy progresses and closes. In early stages of development where neighbour interactions are starting the canopy is not fully closed (Figure 2.1) leading to light gaps between the leaves, and heterogeneity at the level even of individual leaves. However rather little was known about how local shading in the leaf is leading to the SAS. Using supplemental FR spotlights in different regions of Arabidopsis leaf, we studied how local FR enrichment triggers shade avoidance in chapter 5. Strong hyponastic response was observed when FR light detected from the lamina tip, while the FR directly to petiole resulted to the strongest petiole elongation response (Figure 6.1). Similar findings were recorded for much younger *Arabidopsis* plants (Michaud et al., 2017) and another species, *Brassica nigra* (chapter 5). We subsequently used functional-structural plant (FSP) models (Bongers et al., 2017) to study how these perception localizations affect plant performance. We observed that FR at the leaf tip changes reliably in a neighbor-dependent manner, whereas FR at the petiole base is also lowered by self-shading by younger leaves. Responding to petiole-detected

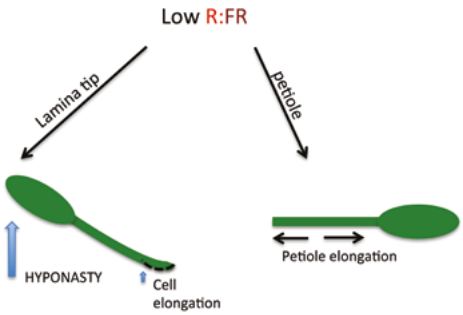

Figure 6.1 | Illumination with FR light in specific regions of the leaf induces different shade avoidance responses. Low R:FR signal in the lamina tip induces hyponasty, while low R:FR perceived in the petiole triggers petiole elongation.

FR would trigger hyponasty in response to self-shading and this inhibits photosynthesis, whereas neighbour-shade is difficult to distinguish from self-shade. The impact of hyponastic responses to FR detection at either the leaf tip or petiole base on biomass accumulation is visualized in Figure 6.2.

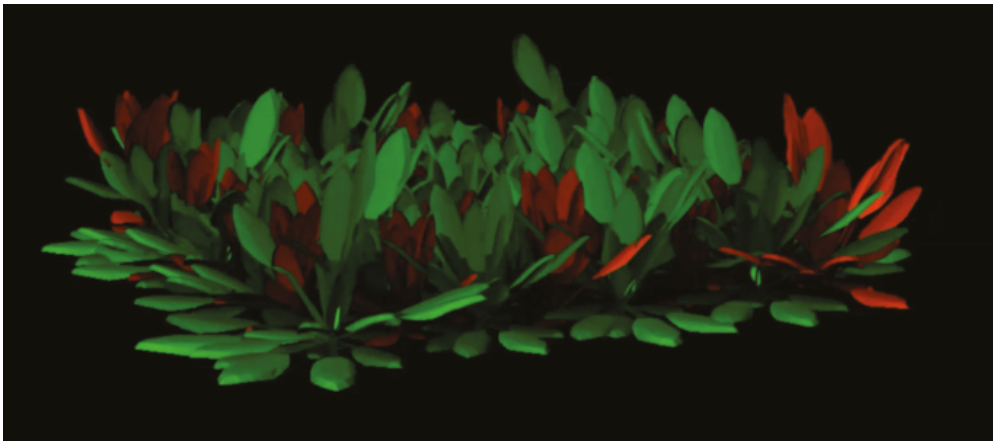

Figure 6.2 | FSP model output of simulation of *Arabidopsis* plant growth at high density, with hyponastic responses to FR detected at the petiole (red) or lamina tip (green). Video still taken from Franca Bongers' video simulation (https://www.youtube.com/watch?v =Zr5Rknu6cQc&feature=youtu.be) (Bongers et al., 2017).

The mechanism of local FR-induced hyponasty

The PHYTOCROME INTERACTING FACTORs (PIFs), especially PIF4, PIF5, PIF7 that are released after inactivation of the phytochromes (PHYB-PHYE) due to FR enrichment (Nagatani et al., 1991; Franklin et al., 2003; Kozuka et al., 2010; Roig-Villanova and Martínez-García, 2016), are key regulators of SAS (Leivar et al., 2012; Li et al., 2012; Leivar and Monte, 2014). PIFs induce expression of auxin biosynthesis genes (Hornitschek et al., 2012; Li et al., 2012; Bou-Torrent et al., 2014), such as YUCCAs upon shade (Stepanova et al., 2008; Tao et al., 2008) and auxin response genes (Hornitschek et al., 2012; Li et al., 2012). These studies on young seedlings established auxin as an important PIF-induced regulator of hypocotyl elongation in response to signals for upcoming shade (reviewed in Iglesias et al., 2017). However,

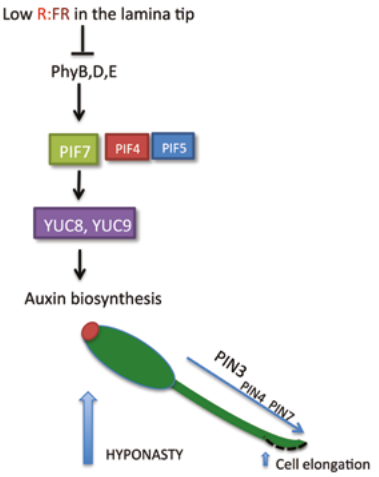

Figure 6.3 | A schematic overview of the signaling pathway of local FR-induced hyponasty. Red dot on the lamina tip represents the FR light, while arrows illustrate the directions of the pathway.

the hyponastic to shade has been poorly investigated (but see Dornbusch et al., 2014). In chapter 5, we tried to unravel the mechanism of local FR-induced hyponasty, which occurs in the petiole base. Transcriptome data from the lamina tip and petiole base showed high representation of auxin-responsive genes, suggesting that auxin could have a role in local-FR induced hyponasty. Using physiological, pharmacological and molecular approaches, we are able to provide a convincing spatially explicit, mechanistic explanation for the lamina tip-triggered hyponasty upon local FR. Local inactivation of phytochromes acts through PIF4, PIF5 and PIF7 which then induce local-synthesized auxin through YUC8 and YUC9 in the lamina tip. The newly synthesized auxin needs to be transported mostly via PIN3 (Keuskamp et al., 2010, chapter 5), but probably also via PIN4 and PIN7 (Kohnen et al., 2016, chapter 5) to the abaxial side of the petiole, causing differential cell elongation and leading to hyponasty (Figure 6.3).

There are still unanswered questions, such as: How is the newly synthesized auxin in the lamina tip able to induce differential growth between the two sides of the petiole? What is the mechanism of petiole elongation induced by FR application to the petiole itself? Transcriptome analysis at a tissue-specific level, for example separating the abaxial and adaxial side of the petiole or single cell sequencing (Rinke et al., 2013) from the abaxial versus the adaxial side could be potential approaches to answer these questions. Furthermore, our transcriptome survey also identified strong ABA gene expression signatures (Figure S5.2A) both in leaf tip (which was FR-enriched) and in the petiole base of the same leaf (which didn't receive any supplemental FR). This implies that ABA, or another messenger regulating the ABA pathway, possibly auxin, is transported to the petiole base in response to local FR-enrichment. Future studies will need to resolve if ABA is indeed regulated, and if this represents a functional component of leaf movement in response to highly localized FR-enrichment.

Conclusion and future perspectives

In this thesis, I tried to unravel the mechanisms behind different aspects of SAS. The ultimate future goal would be to use the obtained knowledge in enhancing canopy performance and yield. Hyponasty appears to be an important factor for canopy performance, since it is considered as the earliest (touch) as well as a later stage response to neighbour signals. It also determines the light interception. Darwin already discussed upward movement of the leaf (hyponasty) in plants (Whippo and Hangarter, 2009). However, the mechanism of hyponastic responses derived from SAS or touch has been poorly studied in the 21st century. After using molecular and physiological approaches, we found that touch- and local FR-induced hyponasty are determined from tissue-dependent distinct pathways. This indicates that a canopy develops progressively through different hormone-related or other signalling (mechanical stress, electrical signalling) pathways for the final shade

avoidance canopy phenotype (Figure 1.3). But there is an open question: Which hyponastic response should be manipulated for a better light interception and better closed canopy leading to a significant effect on yield? Testing the most promising mutants from the chapters 4 and 5 in high-density growing conditions (like in chapters 2 and 3) or testing different scenarios with the FSP model could possibly answer this question. Another approach could be by learning from nature. We could test in a tissue-specific manner the expression of the genes we identified (chapters 4 and 5), in *Arabidopsis* accessions with antithetical responses to shade such as shade avoidance and shade tolerance (Gommers et al., 2017). Results from these experiments will help develop strategies to inhibit SAS and increase the amount of plant resources invested in the enhancement of yield and the competitive ability of the plant (Weiner et al., 2010; Campos et al., 2016). Overall, these findings will be a step closer to identify target genes to develop crops with favourable yield characteristics.

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Summary

The global human population is rising rapidly every year, and that in turn increases the demands for food production. For that reason, crop production must be intensified. Intensification of agriculture requires crop plants to grow in high densities, creating a very competitive environment especially for space and light between the plants. This competition becomes more intense in the presence of weeds. Under these conditions, the light quality is changed inside the dense canopy due to the absorption of Red (R) and Blue (B) light and the reflection of Far-red (FR) light from the neighbour plants. A reduced R:FR ratio triggers the Shade Avoidance Syndrome (SAS). SAS is enhancing the individual plant fitness in dense stands by allocating precious carbon to petiole or stem elongation and upward movement of the leaf. This goes at the expense of carbon allocation to leaves and to harvestable organs such as seeds. These phenotypic characteristics in combination with sub-optimal carbon allocation and competition with weeds cause serious reductions of crop yield. To combat these yield reductions, sustainable ways to suppress weeds and to minimize the allocation of the carbon resources due to SAS in the unwanted organs should be developed. In this thesis, I tried to study the basic principles needed to achieve this by studying two different scenarios. The first one was to change the planting patterns and the second to modify the shade avoidance responses in dense stands.

Ten years of field experiments in wheat-weed competition showed that the spatial planting patterns and planting density have a key role in weed suppression and wheat yield. More specifically, when wheat plants are grown in relatively uniform patterns and at high density, weeds are strongly suppressed whereas the wheat production is high. Taking advantage of this knowledge and using the model plant *Arabidopsis thaliana* (especially for its variety of available mutants and the short life cycle) I tested the first scenario in **chapter 2** and **chapter 3**. *Arabidopsis thaliana* (Col-0) was used as canopy plant and another wild type *Arabidopsis* accession [Landsberg *erecta* (L*er*)] as a competitor. In **chapter 2**, I used a similar set-up as the field-grown wheat crop and I found comparable effects during competition. Col-0 canopy plants showed the highest biomass and yield in the high-density, uniform pattern while competitor L*er* showed completely the opposite. The latter could be explained by the more rapid closure of the Col-0 canopy at high versus low density, and the uniform shading as compared to when grown in rows, both limiting the access of competitor L*er* to light. Based on these findings I continued with the high-density uniform pattern in **chapter 3** where I studied the impact of shade avoidance responses. Evolutionary Agroecology hypothesis is a sustainable approach towards weed suppression where the performance of the whole canopy community could benefit from the suppression of the SAS and their competitive ability against weeds can be enhanced. I explored this concept in *Arabidopsis*, using genotypes with mild (*pif4pif5*) or severe reduction (*pif4pif5pif7*) of shade avoidance as canopy plants and the wild-type accession L*er* as an invading competitor. Performing these

experiments, I observed that the reduced growth rate of *pif4pif5* and *pif4pif5pif7* delayed the canopy closure, and this likely confounded studying the impact of shade avoidance responses. On the other hand, the competitor L*er* with the strong shade avoiding phenotype was able to escape from the poorly shade avoiding canopies and was able to increase its biomass under the reduced SAS canopy compared to the normally shade avoiding Col-0 canopy. Follow up research by using a less strongly shade avoiding invading competitor, or genotypes with uncoupled variation in hyponasty and elongation responses to shade cues could elucidate the optimal SAS traits to test the Evolutionary Agroecology hypothesis.

In order to do so, more detailed understanding of very early neighbor detection and shade avoidance induction is needed. Manipulating the earliest neighbor responses might be a promising way to maximize the canopy closure and performance in order to suppress the weeds more effectively. This is the second scenario of the thesis that was studied in **chapter 4**. Previous research in *Arabidopsis* showed that the earliest neighbor response is through touching of the leaves of neighboring leaves. This touching of the leaves induces the upward movement of the leaf (also called hyponasty), which leads to the change of canopy architecture from a horizontal to a more vertical position. Using molecular and physiological approaches I tried to elucidate the mechanism of touch-induced hyponasty. Transcriptome data upon touching revealed a mechano-stimulation profile. Moreover we observed that trichomes (hairs on the surface of the leaf) have an essential role in touch-induced upward movement of the leaf. We further found that several molecular components of mechanosensing such as the CrRLKs and signal transduction such as the clade 3 GLRs have a role in the regulation of touch-induced leaf movement.

The touch-induced hyponastic response changes the canopy architecture and this results in more vertically structured vegetation that allows for FR reflection and the resulting decrease of the R:FR light ratio. However, not all the leaves have direct access to the sunlight and they distribute asymmetrically in the canopy. This erratic distribution leads to light gaps and local differences both in terms of light quality and quantity between the leaves or even within the same leaf. In **chapter 5,** I studied how FR perception from a specific part of the leaf leads to shade avoidance responses and how this does affect plant performance. Data of this chapter elucidate that when low R:FR is sensed in the tip of the leaf, the leaf is able to induce hyponasty driven from the abaxial side of the petiole. Auxin was found to have a key role in this differential growth response. Using 3D computational modeling we showed that the plants, which responded with hyponasty upon monitoring the R:FR from the leaf tip, can accumulate more biomass than plants that would use R:FR information from the petiole itself to induce this response. Most probably, this is because the leaf tip is the first to experience a decrease of R:FR, due to neighbour reflection, and thus using this information triggers the most rapid response to neighbours. Using R:FR on the petiole to drive hyponasty would also trigger the response upon self-shading by younger leaves, which is maladaptive.

Collectively, the data show that a developing canopy activates different signaling pathways to change the canopy architecture in response to density, thus canopy architecture appears to be an important factor for canopy performance. These insights help to understand how plants sense and respond to canopy density and how canopy architecture influences canopy performance and weed suppression. Nevertheless, it remains to be proven if shade avoidance responses can be effectively tweaked to further improve canopy structure and weed suppression without impairing overall growth.

Samenvatting

De snelgroeiende wereldbevolking leidt tot een groeiende behoefte aan voedsel. Om aan die vraag te voldoen zal de voedselproductie dus intensiever moeten worden. Bij de intensivering van landbouw zullen de gewassen bij een hogere dichtheid moeten groeien wat resulteert in zeer competitieve omgeving met betrekking tot zowel de beschikbare ruimte als het beschikbare licht voor de plant. Deze competitie zal nog sterker worden wanneer er ook onkruiden tussen de gewassen groeien. In dichte vegetatie zal de hoeveelheid licht verlaagd zijn en de licht samenstelling veranderen. Dit als gevolg van het feit dat planten rood (R) en blauw (B) licht absorberen maar verrood (FR) licht juist reflecteren. Als gevolg van de aanwezigheid van buurplanten zal hiermee dus ook de verhouding tussen rood en verrood licht (R:FR) dalen in het licht wat de plant bereikt. Dit wordt door planten gebruikt als signaal voor de aanwezigheid van buurplanten. Deze verandering in de R:FR ratio initieert vervolgens schaduw vermijdende reacties bij de planten. Deze reacties verhogen de fitness van individuele planten in een dichte vegetatie, maar gaan juist ten koste van de totale opbrengst per eenheid landbouwgrond. De plant zal de beperkte hoeveelheid energie die beschikbaar is investeren in de strekking van de bladsteel (petiool) of stengel en in de opwaartse beweging van de bladeren (hyponastie). Deze verandering in investeringspatroon zal leiden tot minder bladoppervlakte en minder oogstbare plantendelen zoals vruchten en zaden. Opbrengstverlies zou geminimaliseerd kunnen worden via een duurzame manier om onkruid te onderdrukken en/of de schaduw vermijdende reactie van het gewas te onderdrukken. In mijn proefschrift bestudeer ik twee verschillende scenario's en hun invloed op de opbrengst per oppervlakte-eenheid. Ten eerste de invloed van het beplantingspatroon en ten tweede de onderdrukking van de schaduw vermijdende reactie in dichte vegetatie.

10 jaar aan veldstudies in tarwe heeft laten zien dat het ruimtelijke beplantingspatroon en de beplantingsdichtheid van dit gewas een grote rol spelen in het onderdrukken van onkruid en uiteindelijk in de opbrengst per oppervlakte-eenheid. Wanneer tarwe in een uniform patroon bij grote dichtheid wordt verbouwd, wordt het onkruid sterk onderdrukt en is de tarweproductie hoog. Bij het testen van de invloed van beplantingspatroon in **hoofdstuk 2** en **hoofdstuk 3** maak ik gebruik van deze kennis in combinatie met de modelsoort *Arabidopsis thaliana*. De *A. thaliana* accessie Col-0 werd gebruikt als plant van interesse terwijl de Landsberg *erecta* (L*er*) accessie als concurrent werd geïntroduceerd. **Hoofdstuk 2** laat zien dat het gebruik van *A. thaliana* tot dezelfde uitkomsten leidt als de veldstudies die gebruik maken van tarwe. Col-0 had de hoogste biomassa en opbrengst bij een uniforme en hoge dichtheid terwijl de concurrent L*er* juist het tegenovergestelde liet zien. Dit kan verklaard worden doordat bij een hoge en uniforme dichtheid, Col-0 de vegetatie eerder en gelijkmatiger doet sluiten in vergelijking met een lagere en ongelijke dichtheid, waarbij L*er* dus minder licht kan onderscheppen. Op basis van de deze

bevindingen heb ik in **hoofdstuk 3** de hoge en uniforme dichtheid gebruikt om het effect van de schaduw vermijdende reactie te onderzoeken. Concepten uit de zogenaamde evolutionaire agro-ecologie voorspellen dat een gewas-vegetatie voordeel heeft bij het onderdrukken van hun schaduw vermijdende reactie omdat deze als collectief dan een beter gesloten vegetatie zou vormen die onkruiden effectiever onderdrukt. Om dit concept verder te onderzoeken heb ik gebruik gemaakt van plots van *A. thaliana* genotypes die minder (*pif4pif5*) of geen (*pif4pif5pif7*) schaduw vermijdende reactie hebben met L*er* als invasief onkruid. De algeheel lagere groeisnelheid van de mutanten *pif4pif5 en pif4pif5pif7* ten opzichte van wild-type leidde ertoe dat de vegetatie zich later sloot, wat het onderzoeken van de impact van schaduw vermijdende reactie bemoeilijkte.

L*er* heeft namelijk wel een sterke schaduw vermijdende reactie en was in staat om aan de competitie van de mutanten te ontsnappen. Daarmee had L*er* een hoge biomassa terwijl dit in competitie met Col-0 duidelijk niet het geval was. Om de impact van schaduw ontwijking verder te onderzoek in de collectieve onderdrukking van concurrenten binnen een monocultuur zal er gebruik gemaakt moeten worden van een minder schaduw vermijdende concurrent in plaats van L*er*. Een andere optie is het gebruik van andere mutanten die verstoord zijn in specifieke onderdelen van de schaduw ontwijkende reactie zoals bijvoorbeeld petioolstrekking of hyponastie.

Om het bovenstaande verder te kunnen onderzoeken is er meer kennis nodig over de vroege buurplant signalen die de schaduw vermijdende reactie initieren. Eerder onderzoek heeft uitgewezen dat in *A. thaliana* het fysieke contact van bladeren het eerste buurplant detectiemechanisme is. Dit eerste fysieke contact met een blad van de buurplant resulteert in een opwaartse blad beweging (hyponastie), waarmee de architectuur van de vegetatie verschuift naar een meer verticale structuur. Om in **hoofdstuk 4** van dit proefschrift de achterliggende mechanismen van deze contact-geïnduceerde hyponastie te onderzoeken heb ik gebruik gemaakt van een moleculaire en fysiologische benadering. Genexpressie data lieten een profiel zien van mechanische stimulatie waarbij trichomen (haren op het bladoppervlak) een essentiële rol spelen. Ook bleken verschillende componenten die betrokken zijn bij de waarneming van mechanische stress zoals CrRLKs en Clade 3 GLRs nodig te zijn voor de contact-geïnduceerde hyponastie.

Zoals eerder genoemd zorgt de hyponastische response als gevolg van fysiek contact tussen bladeren voor een verticale vegetatiestructuur. De meer verticale positie van de bladeren zorgt vervolgens weer voor relatief meer horizontale FR reflectie, wat door buurplanten wordt waargenomen. In een vegetatie groeien bladeren in verschillende richtingen, waardoor de licht verdeling onregelmatig is. In **hoofdstuk 5** beschrijf ik mijn onderzoek over waar in het blad de FR waarneming plaatsvindt die leidt tot de schaduw vermijdende reactie. De data laten zien dat wanneer specifiek de bladpunt extra FR wordt gegeven, dit leidt tot hyponastische groei in de petiool. Hierbij speelt het plantenhormoon

auxine een belangrijke rol in het doorgeven van de informatie uit de bladpunt naar de petiool, alwaar de daadwerkelijke groeireactie plaatsvindt. Vervolgens hebben we met behulp van 3D computermodellen van plantengroei in hoge dichtheid laten zien dat planten die in staat waren om het R:FR signaal in de bladpunt om te zetten in een hyponastische reactie grotere biomassa hadden dan de planten die alleen reageerden wanneer het R:FR lichtsignaal de petiool zelf bereikte. Dit is te verklaren doordat in een zich ontwikkelende vegetatie de bladpunt het eerste orgaan is dat extra FR waarneemt. Daarnaast kan ook beter worden vermeden dat een blad hyponastisch wordt als de petiool door een jonger blad van dezelfde plant wordt beschaduwd.

Kortom, dit onderzoek laat zien dat in een groeiende vegetatie verschillende signalen worden waargenomen door een plant die vervolgens leiden tot een verandering in de vegetatie architectuur, waarbij deze structuur een belangrijke rol speelt in de prestatie van de desbetreffende vegetatie. Deze kennis kan worden gebruikt in onkruidbestrijding. Er moet echter nog wel onderzocht worden hoe de schaduw vermijdende reactie zodanig gestuurd kan worden dat de vegetatie structuur kan worden geoptimaliseerd zonder de algehele groei sterk te remmen.

Σύνοψη

Ο πληθυσμός της γης αυξάνεται ραγδαία και ως εκ τούτου δημιουργούνται αυξημένες ανάγκες για παραγωγή τροφής. Ένας τρόπος για να καλυφθούν οι ανάγκες αυτές είναι η εντατικοποίηση της καλλιέργειας των φυτών. Ωστόσο στην εντατική καλλιέργεια απαιτείται τα φυτά να μεγαλώνουν σε μεγαλύτερη πυκνότητα, με αποτέλεσμα να παρατηρείται αυξημένος ανταγωνισμός μεταξύ των καλλιεργούμενων φυτών για φως αλλά και για το διαθέσιμο χώρο. Η παρουσία φυτών-ζιζανίων καθιστά τον ανταγωνισμό αυτό ακόμα πιο έντονο. Σε συνθήκες πυκνής φύτευσης, οι ιστοί των φυτών που περιέχουν χλωροφύλλη (φύλλα) έχουν τη μοναδική ιδιότητα να απορροφούν το κόκκινο (R) και το μπλε (B) φως, ενώ αντανακλούν το υπεριώδες (FR) φως με αποτέλεσμα να αλλάζει η ποιότητα του φωτός μέσα στην φυτεία. Η μείωση της αναλογίας R:FR αποτελεί ένδειξη ύπαρξης ανταγωνισμού για φως και ενεργοποιεί στα φυτά το «Σύνδρομο Αποφυγής της Σκίασης» (Shade Avoidance Syndrome; SAS), ώστε να επιτύχουν μεγαλύτερη έκθεση τους στον ήλιο. Χαρακτηριστικές φαινοτυπικές αποκρίσεις των φυτών κατά τη SAS είναι η επιμήκυνση των μίσχων, των μεσογονατίων διαστημάτων και των στελεχών, η ανοδική κάμψη των φύλλων και η κυριαρχία της κορυφής. Υπό αυτές τις συνθήκες ωστόσο, τα φυτά επενδύουν στην ανάπτυξή τους και όχι στην παραγωγή σπόρων και καρπών. Η προσπάθεια αποφυγής της σκίασης σε συνδυασμό με τον ανταγωνισμό με τα ζιζάνια έχει σαν αποτέλεσμα οι καλλιεργητικές αποδόσεις να μειώνονται. Για το λόγο αυτό, κρίνεται επιτακτική η ανάγκη ανάπτυξης αειφόρων μεθόδων καταστολής των ζιζανίων και περιορισμού της σπατάλης θρεπτικών συστατικών σε μορφολογικές τροποποιήσεις λόγω της SAS. Στην παρούσα διδακτορική διατριβή ακολουθήσαμε δύο διαφορετικές προσεγγίσεις ώστε να διερευνήσουμε τις απαραίτητες βασικές αρχές προς αυτή την κατεύθυνση: Η πρώτη προσέγγιση αφορούσε την αλλαγή των προτύπων φύτευσης και η δεύτερη την τροποποίηση των αποκρίσεων του συνδρόμου SAS, σε συνθήκες πυκνής φύτευσης.

Προηγούμενες μελέτες που εστίασαν στον ανταγωνισμό μεταξύ του σιταριού και των ζιζανίων, έδειξαν με πειράματα αγρού ότι ο συνδυασμός της αυξημένης πυκνότητας φύτευσης και της χωρικής ομοιομορφίας μπορεί να οδηγήσει σε αποτελεσματική καταστολή των ζιζανίων και αύξηση της παραγωγής του σιταριού. Με βάση αυτό, στα **κεφάλαια 2** και **3** μελετήσαμε διαφορετικά πρότυπα φύτευσης χρησιμοποιώντας για τα πειράματά μας το φυτό *Arabidopsis thaliana* (με πληθώρα μεταλλαγμένων φυτών και μικρό κύκλο ζωής). Σαν καλλιεργούμενο φυτό χρησιμοποιήσαμε τον οικότυπο Col-0 του *A. thaliana* και σαν ανταγωνιστικό φυτό τον οικότυπο Landsberg *erecta* (L*er*). Στο κ**εφάλαιο 2**, χρησιμοποιώντας παρόμοιες συνθήκες φύτευσης με τα πειράματα του σιταριού στον αγρό, διαπιστώσαμε παρόμοιες επιδράσεις κατά τις συνθήκες ανταγωνισμού. Η βιομάζα αλλά και απόδοση του καλλιεργούμενου φυτού Col-0, ήταν μεγαλύτερη όταν τα φυτά φυτεύτηκαν σε υψηλή πυκνότητα και με ομοιόμορφη διάταξη σε σχέση με τα φυτά του ανταγωνιστή L*er* που βρισκόταν εντός της φυτείας του Col-0. Η περιορισμένη πρόσβαση

του ανταγωνιστή L*er* στο φως, εξαιτίας του γρήγορου "κλεισίματος" της φυτείας των φυτών Col-0 στην πυκνή φύτευση επηρέασε αρνητικά απόδοση του.

Επειδή η πυκνή και ομοιόμορφη φύτευση μείωσε σημαντικά την απόδοση του ανταγωνιστικού φυτού, στο **κεφάλαιο 3** μελετήσαμε το φαινόμενο SAS κάτω από αυτές τις συνθήκες φύτευσης. Η υπόθεση της Εξελικτικής Αγροοικολογίας αποτελεί μια αειφόρο προσέγγιση καταστολής των ζιζανίων σύμφωνα με την οποία η συνολική απόδοση της φυτείας του καλλιεργούμενου πληθυσμού, και όχι των φυτών σαν μονάδες, θα μπορούσε να αυξηθεί λόγω της καταστολής του SAS ενώ ταυτόχρονα ενισχύεται η ανταγωνιστική ικανότητα της ομάδας των καλλιεργούμενων φυτών έναντι των ζιζανίων. Στο **κεφάλαιο 3** μελετήσαμε αυτή τη θεωρία, χρησιμοποιώντας σαν καλλιεργούμενα φυτά τις μεταλλαγμένες σειρές *Arabidopsis* με ήπια (*pif4pif5*) ή έντονη (*pif4pif5pif7*) μείωση του συνδρόμου αποφυγής της σκίασης και τον οικότυπο L*er* σαν φυτό-ανταγωνιστή.

Τα αποτελέσματα των πειραμάτων αυτών έδειξαν ότι υπήρξε καθυστέρηση του "κλεισίματος" της φυτείας εξαιτίας του μειωμένου ρυθμού ανάπτυξης των μεταλλαγμένων σειρών *pif4pif5* και *pif4pif5pif7*, καθιστώντας δύσκολη τη μελέτη του ρόλου των αποκρίσεων αποφυγής της σκίασης, καθώς το φυτό-ανταγωνιστής μπορούσε να αποφύγει τη σκίαση και να αυξήσει τη βιομάζα του, σε μεγαλύτερο βαθμό σε σχέση με τα πειράματα φυτείας με τον οικότυπο Col-0. Μελλοντικές μελέτες με φυτά-ανταγωνιστές που να εκφράζουν πιο ήπιες αποκρίσεις αποφυγής της σκίασης ή μεταλλαγμένες σειρές φυτών που να παρουσιάζουν μερικές (και όχι όλες) τις μορφολογικές αποκρίσεις της αποφυγής της σκίασης, θα μπορούσαν να οδηγήσουν σε καλύτερη κατανόηση των χαρακτηριστικών του SAS που χρησιμεύουν για την αξιολόγηση της υπόθεσης της Εξελικτικής Αγροοικολογίας.

Ωστόσο για να γίνει αυτό δυνατό, χρειάζεται λεπτομερής κατανόηση των μηχανισμών που καθορίζουν την πολύ πρώιμη αντίληψη της γειτνίασης από κάποιο φυτό καθώς και αυτών που επάγουν την αποφυγή της σκίασης. Η χειραγώγηση των πρώιμων αποκρίσεων στη γειτνίαση είναι ένας υποσχόμενος τρόπος για να βελτιστοποιηθεί το κλείσιμο και η απόδοση της φυτείας, ώστε να κατασταλούν πιο αποτελεσματικά τα ζιζάνια. Αυτό αποτελεί το δεύτερο πειραματικό σενάριο που μελετήσαμε στο **κεφάλαιο 4** αυτής τη διατριβής. Προηγούμενα πειράματα με το φυτό *Arabidopsis* έδειξαν ότι η επαφή γειτονικών φύλλων αποτελεί την πιο πρώιμη αντίληψη γειτνίασης. Επακόλουθο αυτής της επαφής είναι η ανοδική κίνηση των φύλλων (ονομάζεται υπονάστια), με αποτέλεσμα την αλλαγή της αρχιτεκτονικής της φυτείας από οριζόντια σε πιο κάθετη διάταξη. Χρησιμοποιώντας μοριακές και φυσιολογικές μεθοδολογίες, προσπαθήσαμε να κατανοήσουμε την υπονάστια σαν απόκριση στην επαφή. Η ανάλυση δεδομένων μεταγραφικού RNA των φύλλων μετά από επαφή, αποκάλυψε ένα προφίλ που προσομοιάζει την απόκριση σε μηχανική διέγερση. Επιπλέον δείξαμε ότι τα τριχώματα (τρίχες στην επιφάνεια των φύλλων) καθώς και αρκετά μοριακά συστατικά της αντίληψης του μηχανικού στρες, όπως οι CrRLKs και συστατικά του clade 3 GLRs, παίζουν ρόλο στην ανοδική κίνηση των φύλλων σαν απόκριση στην επαφή με τα γειτονικά φύλλα.

Η καθετοποιημένη διάταξη της φυτείας, ως αποτέλεσμα της υποναστικής απόκρισης, επιτρέπει την αύξηση της αντανάκλασης του FR και συνεπώς τη μείωση της αναλογίας R:FR φωτός. Παρ'όλα αυτά, εξαιτίας της ασύμμετρης κατανομής των φύλλων στην φυτεία, η απευθείας πρόσβαση τους στο ηλιακό φώς δεν είναι εξασφαλισμένη για όλα τα φύλλα. Αυτή η άναρχη κατανομή των φύλλων δημιουργεί κενά διέλευσης του φωτός καθώς και διαφορές ανά σημεία στην ποιότητα και την ποσότητα του φωτός που διέρχεται τόσο μεταξύ των διαφορετικών φύλλων όσο και πάνω στο ίδιο φύλλο. Για το λόγο αυτό, στο **κεφάλαιο 5** μελετήσαμε πως η πρόσληψη του FR από συγκεκριμένα σημεία του φύλλου μπορεί να προκαλέσει αποκρίσεις σχετικές με την αποφυγή της σκίασης και πως αυτές οι αποκρίσεις μπορούν να επηρεάσουν την απόδοση του φυτού. Τα αποτελέσματα αυτού του κεφαλαίου καταδεικνύουν ότι η αντίληψη χαμηλής αναλογίας R:FR από την άκρη του φύλλου, επάγει την υποναστία του φύλλου λόγω κυτταρικών τροποποιήσεων στην "κάτω" επιφάνεια του μίσχου του φύλλου. Βρήκαμε ότι η ορμόνη αυξίνη έχει σημαντικό ρόλο σε αυτή την τροποποιημένη ανάπτυξη των φυτών. Χρησιμοποιωντας τρισδιάστατη υπολογιστική προσομοίωση, δείξαμε ότι το φυτό που ανταποκρίνεται με υποναστία, λόγω της αντίληψης του R:FR από την κορυφή του φύλλου, μπορεί να αποκτήσει μεγαλύτερη βιομάζα σε σχέση με το φυτό που αντιλαμβάνεται το R:FR στο μίσχο του φύλλου. Πιθανότατα, αυτό συμβαίνει επειδή η άκρη του φύλλου αποτελεί το πρώτο σημείο του φύλλου που «βιώνει» τη μείωση της αναλογίας R:FR, εξαιτίας της αντανάκλασης του FR από τα γειτονικά φύλλα, και κατ' αυτό τον τρόπο επάγει μια ταχύτατη απόκριση στην επερχόμενη γειτνίαση από άλλα φυτά.

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Curriculum vitae

Chrysoula Pantazopoulou was born the 23rd of August 1986 in Athens, Greece. She received her primary, secondary and higher education in Athens. In September 2004, she started her bachelor studies in Agricultural University of Athens, in the Department of Crop Science with specialization in Agronomy and Plant breeding. Her bachelor diploma thesis project was performed in the Laboratory of Agronomy under the supervision of Prof. Garyfalia Oikonomou where she studied the growth and productivity of forage plant mixture in dryland conditions. In October 2010, she joined the Master Program "Crop Protection and Environment" at the Agricultural University of Athens. During her studies, she received a "Panagiotis Triantafilidis**"** scholarship, which supported her research for two years. Her master internship was performed in the Laboratory of Agronomy and Plant breeding and Biometry under the supervision of Prof. Petros Tarantilis and Prof. Garyfalia Oikonomou on a project entitled "The adaptability of wild biotypes of aromatic and medicinal plants with carvacrol chemotype under extensive cultivation". She graduated on May 2012 with cum laude. In October 2013, she started as a PhD student in the Plant Ecophysiology group at Utrecht University. Her research was focused on the early neighbor detection during competition for light in high densities of *Arabidopsis thaliana*. This research was performed under the supervision of Prof. Ronald Pierik. During her PhD studies Chrysoula was also an active member of the PhD councils of the Graduate School of Life Science (GSLS), the Institute of Environmental Biology (IEB), both at Utrecht University, and of the national graduate school Experimental Plant Science (EPS). The results of her PhD studies are presented in this thesis.

Publications

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