DELLA protein regulation during shade avoidance

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Regulatie van DELLA eiwitten tijdens shade avoidance

(met een samenvatting in het Nederlands)

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CHAPTER 1

General Introduction

Plants are threatened by a variety of adversities in the local environment during their life cycle. Besides many abiotic stresses, such as drought, flooding, salt stress and high or low temperature, also various biotic stress factors such as pests and diseases can constrain plant growth. Furthermore, life of individual plants can be highly influenced by the proximity of other plants with which they have to compete for resources such as light, water and nutrients. This is very obvious in vegetations with high plant densities where the microclimate drastically differs from the open field. Sun irradiance decreases exponentially through the plant canopy. This affects the photosynthetic rate which can become insufficient to compensate for the carbon loss during respiration (Lambers et al., 1998). Next to shortage of light, belowground resources such as water and nutrients can also become limiting in dense vegetations. It is, therefore, no surprise that plant growth and development in dense vegetations, including crop growth and yield in agricultural fields, can be considerably constrained.

Plants can reduce the impact of these resource limitations through various morphological, anatomical and physiological traits that enable them to maximize capture of resources even when these are available in limited quantities. More specifically, in order to deal with the low light environment of a dense stand, plants can either acclimate to these conditions, or avoid their young leaves to grow in the shade altogether.

Shade acclimation helps plants to harvest sufficient light for maintaining photosynthetic capacity, despite the low light environment. To this end plants increase photosynthetic area through investment into leaf area which results in large, but thin leaf blades. Furthermore, the ratio between chlorophyll a:b can decline to maximize light harvesting (Lambers et al., 1998).

Instead of acclimating to shade, plants can also increase their competitiveness for light by avoiding shade. Plants can detect shade already in a very early stage of

canopy development and respond by a suite of responses collectively called shade avoidance. Shade avoidance responses enable plants to maximize light capture by positioning their leaves away from the shade, into the light.

Plant neighbour detection and shade avoidance responses

The most dramatic shade avoidance response is stimulation of shoot elongation, which can be initiated very quickly upon sensing shade cues (Smith and Whitelam, 1997). This allows plant to overtop neighbours and position their leaves in unattenuated daylight. Light capture can be further optimized by enhanced petiole elongation and upward movement of leaves (hyponasty). Plants that in the end fail to reach the light can accelerate the entrance into the reproductive stage leading to early flowering and seed development.

Shade avoidance responses are induced upon sensing neighbours through light signals. Once neighbours are detected, plants can adjust their morphology and physiology already before the light intensity is noticeably reduced. The light signal that leads to this early neighbour detection is the reduced ratio between red (660-670 nm) and far-red (725-735 nm) light (R:FR ratio) in light reflected by nearby neighbours. The R:FR ratio typically is lowered due to absorption of red light by chlorophyll of neighbouring plants, while far-red light is mainly scattered or reflected from surrounding vegetation. The daylight spectrum contains almost equal proportions of red and far-red light, leading to a R:FR ratio of approximately 1.2. In dense canopies, however, the R:FR ratio can be reduced to 0.2 or even less (Smith and Whitelam, 1997; Ballare, 1999; Smith, 2000). The R:FR ratio is perceived by phytochrome photoreceptors which can absorb both R and FR wavelengths. The Arabidopsis phytochrome family consists of five members (PHYA-PHYE). These photoreceptors exist in two photoconvertible forms whose photoequlibrium is primarily dependent on the R:FR ratio of the incident light (Smith, 2000). The inactive Pr form of phytochrome can absorb red light upon which it is converted into the active Pfr form. This active Pfr can be reverted back to Pr by absorption of far-red light or passively in darkness. In red-enriched daylight, the equilibrium between these two forms of phytochromes is thus

shifted towards the active Pfr form. Active (Pfr) phytochrome stimulates photomorphogenic development, consisting of such traits as inhibition of hypocotyl and stem elongation, stimulation of leaf and chloroplast development and expression of photosynthetic genes (Reed et al., 1993). In FR-enriched light, typical for dense canopies, phytochrome exists predominantly in the Pr form which is inactive and this releases the Pfr-mediated suppression of all aspects of shade avoidance responses.

The light spectral composition in canopies is not only depleted in red light, but also in blue light wavelengths, again as a consequence of chlorophyll absorption. Reduction of blue light has been shown to initiate shade avoidance responses in cucumber hypocotyls (Ballare et al., 1991b) and tobacco stems (Casal and Sanchez, 1994; Pierik et al., 2004b) and petioles (Pierik et al., 2004b). Blue light can be perceived by two families of photoreceptors: cryptochromes and phototropins. The Arabidopsis, cryptochrome photoreceptor family contains three members, CRY1, CRY2 and CRY3, although only CRY1 and CRY2 have been functionally characterized so far. The phototropin family in Arabidopsis consists of two members, PHOT1 and PHOT2. Cryptochrome signaling is related mainly to photomorphogenic responses of de-etiolating plants (inhibition of stem elongation, stimulation of leaf expansion, control of photoperiodic flowering and entrainment of the circadian clock), while phototropins mainly mediate phototropism, chloroplast relocation and stomatal opening (Christie et al., 2001; Lin and Shalitin, 2003). Studies on the importance of the low blue light signal during shade avoidance responses are still limited, and the relative importance of this signal as compared to the conventional low R:FR signal, therefore, remains elusive.

Agronomical importance of shade avoidance responses

Enhanced shoot elongation in canopies enhances light capture of individual plants in dense stands, thus enhancing their competitive performance (Schmitt, 1997). On the other hand, strong resource investment in elongation growth has its disadvantages from an agronomical point of view (Robson et al., 1996). For example, strong stem growth tends to go at the expense of investment in seeds, which are often the agronomically important organs, especially in cereals. Furthermore, shade avoiding plants develop

small root systems, leading to elongated, poorly anchored plants that have an enhanced risk of lodging. Thus, shade avoidance responses tend to reduce crop yield, especially given the fact that plant growers prefer to grow crops in very high densities, where shade avoidance cues are abundant.

A step forward towards addressing this paradox was the introduction of a dwarfed and lodging-resistant variety of wheat, called Norin 10 during the 1950s. This variety did not show excessive growth of straw, but it did show an increased grain yield. It was found later that Norin 10 carries two mutated loci *Rht-B1* and *Rht-D1* (*REDUCED HEIGHT-1*) which caused dwarfing (Borner et al., 1996). These mutations in part triggered the so-called Green Revolution which changed the methods of crop cultivation and increased yields of commercially important species. Similar mutations to *Rht-B1/Rht-D1* were also found in other crops such as maize at the *d8* and *d9 (DWARF-8*) loci (Winkler et al., 1994), rice *slr1* (*SLENDER RICE 1*- Ikeda et al., 2001) and barley *sln* (*SLENDER*, Fu et al., 2004). Importantly, these mutations all led to semi-dwarfed varieties that displayed a much reduced stem elongation response to high densities and increased yield. Although the above-mentioned genetic mutations have drastically contributed to modern agriculture in the past 50 years, the molecular identity and function of these genes was uncovered only relatively recently. This identification became possible because strong progress was made in unraveling the signal transduction pathway of the important plant hormone gibberellin (GA), in which the so-called DELLA proteins play an essential role.

Gibberellin-mediated regulation of DELLA proteins

Peng et al. (1999) showed that the *Rht1* genes are orthologues of the *GAI* (*GIBBERELLIN INSENSITIVE*) gene in Arabidopsis. *GAI* encodes a so-called DELLA protein. DELLA proteins belong to the GRAS family of proteins (named after its members GAI, RGA and SCARECROW; Pysh, et al., (1999)), which are transcriptional regulators involved in divergent processes, such as root development, phytochrome signaling and GA signaling (reviewed by Bolle et al., 2004). The Arabidopsis genome contains 33 GRAS members with highly homologous C-domains and great variation of

their N-termini. DELLA proteins are named after a 5-amino acid sequence (Aspartic acid - Glutamic acid – Leucine (2x) - Alanine) in the N-terminal domain. The DELLA protein family in Arabidopsis consists of five members: GAI (GA-INSENSITIVE), RGA (REPRESSOR OF *ga1*), RGL1, RGL2 and RGL3 (RGA-LIKE 1, 2 and 3). These proteins are located in the nucleus where they suppress the expression of genes that are responsive to GA (Peng et al., 1997; Silverstone et al., 1998; Lee et al., 2002; Cheng et al., 2004). DELLA proteins are involved in all aspects of GA-mediated development. GAI and RGA are considered mainly as repressors of vegetative growth and floral initiation. RGL2 is involved in regulating seed germination and, together with RGA and RGL1, in controlling floral development. So far, no function has been assigned to RGL3.

The precise molecular mechanism of DELLA suppression has not been elucidated yet, although it was suggested by Achard et al. (2004) that these proteins, located in the nucleus, may suppress transcript levels of GA-responsive *GAMYB* genes by regulating levels of the micro RNA mR159, which cleaves the *GAMYB* mRNA.

DELLA proteins suppress GA-mediated growth and development in the absence of GA. When present in a cell, GA binds to its receptor GID1 (GIBBERELLIN INSENSITIVE DWARF 1). GA binding to GID1 stimulates interaction between GID1 and the N-terminal DELLA domain of DELLA proteins. This promotes ubiquitination of DELLA proteins by the $SCF^{SLY1/GID2}$ ubiquitination complex and further degradation by the 26 proteasome (Dill et al., 2004; Fu et al., 2004; Ueguchi-Tanaka et al., 2005; Griffiths et al., 2007; Fig. 1).

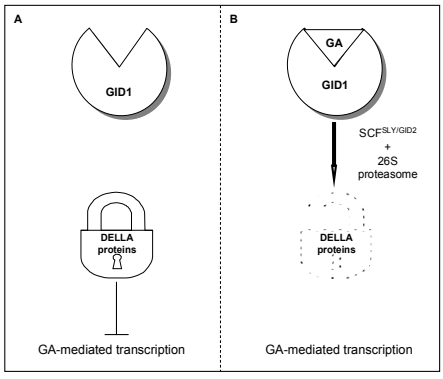

Figure1. DELLA-mediated GA signaling: A) in the absence of GA in cells DELLA proteins are suppressing expression of GA-mediated genes; B) GA presence in a cell stimulates interaction of GA-GID1 complex with DELLA proteins what promotes polyubiquitination of DELLAs by the $SCF^{SLY1/GID2}$ complex and further degradation by the 26s proteasome. This relieves transcription of GA-inducible genes.

Muangprom et al. (2005) showed that the C-terminal domain of the DELLA proteins is responsible for interaction with the F-box of the $SCF^{SLY1/GID2}$ ubiquitination complex. This domain is also functional in suppressing the expression of GA-responsive genes. Mutations in the sequence encoding this motif, as well as in the DELLA-encoding motif, confer GA-insensitive plants. Peng et al. (1997) revealed that in Arabidopsis the gain-of-function *gai* mutation in the *GAI* gene results from deletion of a 51 bp region in the open-reading frame encoding the DELLA domain of the GAI protein. The protein product of this mutated gene is a gai protein lacking a 17 amino-acid segment in the DELLA motif, rendering this protein unrecognizable to the GA-GID1 complex. This confers a GA-insensitive phenotype (dwarf, dark-green), due to enhanced DELLA levels that cannot be abolished by GA addition.

Contrary to this gain of function *gai* mutant, loss-of-function mutations in DELLA genes can partly or completely rescue GA-deficient phenotypes (Peng et al., 1997; Dill and Sun, 2001; King et al., 2001; Lee et al., 2002; Wen and Chang, 2002; Achard et al., 2006). These findings were summarized in the 'relief of restraint' model by Harberd (2003). According to this model, GA is required to oppose the repression of its negative signaling components, DELLA proteins, by stimulating their interaction with $SCF^{SLY1/GID2}$ and subsequent degradation in the 26 proteasome. This degradation relieves the growth restraint and all other aspects of GA-regulated development. The absence of DELLA restraint, as in DELLA knock-outs, counteracts many GA deficiency symptoms such as non-germinating seeds and dwarfism. On the other hand, DELLA null mutations in plants with normal GA content have only mild phenotypic effects (Peng et al.,1997; Dill and Sun, 2001).

GA and DELLA involvement in shade avoidance

The regulatory role for GA in shade avoidance has been confirmed by many studies. The first indication came from Downs et al. (1957) who noticed a similarity between phenotypes of Pinto beans (*Phaseolus vulgaris* L., cultivar 'Pinto') induced by far-red enrichment and those sprayed with gibberellic acid (GA). In general, photoconversion of phytochromes affects either GA biosynthesis or GA responsiveness depending on the

plant species and the stage of development (reviewed by Halliday and Fankhauser, 2003). For example, Nick and Furuya (1993) found that phytochrome activation reduced sensitivity of rice mesocotyls to exogenous GA. Accordingly, a study by Reed et al. (1996) on Arabidopsis hypocotyls showed that phytochrome inactivation increases responsiveness to GA. Importantly, elongation responses induced by a reduced R:FR ratio are prevented by GA-deficiency, as shown in cucumber hypocotyls (Lopez-Juez et al., 1995) and tobacco stems (Pierik et al. 2004a). Additionally, Peng and Harberd (1997) showed that both GA-deficiency and insensitivity can suppress the constitutively elongated phenotypes of Arabidopsis phytochrome deficient *phyB* and *hy1* mutants. Finally, Hisamatsu et al. (2005) have recently shown that light with a reduced R:FR ratio positively regulates the activity of the GA-biosynthesis genes *GA20ox1* and *GA20ox2* in Arabidopsis petioles.

In contrast to the well-studied role for GA in low R:FR-induced elongation, little is known about GA involvement in elongation induced by reduced blue light photon fluence rates, another neighbour detection signal. The relationship between blue light signaling and GA was recently studied by Zhao et al., (2007) in de-etiolating Arabidopsis hypocotyls. These authors showed that the activity of GA catabolic genes was transiently upregulated by blue light in a cryptochrome-dependent manner what resulted in transient decrease of the level of bioactive GA_4.

The role for GA in shade avoidance is well established, but little is known about the operating GA-signaling mechanism involved. It has been shown that the GA-resistant gai form of the DELLA protein GAI can counteract the constitutively elongated hypocotyls of *phyB* seedlings in the double *gai phyB* mutant (Peng and Harberd, 1997). A micro array study by Devlin et al. (2003) showed that prolonged low R:FR treatment reduced expression of the DELLA gene *GAI* in Arabidopsis hypocotyls. Achard et al. (2007) provided the first direct evidence on the effect of phytochrome signaling on DELLA protein abundance. These authors showed that the constitutively elongated *phyB* phenotype of the *phyB pRGA*::*GFP-RGA* line was accompanied by decreased abundance of the GFP-RGA fusion protein. Furthermore, Oh et al. (2007) found that phytochromes can also regulate the expression of DELLA genes in seeds.

DELLA proteins as crosstalk factors for other shade avoidance-associated hormones

A considerable number of studies have shown that GA signaling may interact with other plant hormones, such as auxin, ethylene and abscisic acid, at the level of the DELLA proteins (Fu and Harberd, 2003; Achard et al., 2003; Vriezen et al., 2004; Achard et al., 2006). In general, it has been noticed that manipulation of these hormones affects GA-mediated DELLA degradation. For example, application of the gaseous hormone ethylene to Arabidopsis seedlings can cause a delay in GA-mediated degradation of the DELLA protein RGA in roots, resulting in lower growth rates (Achard et al., 2003). The molecular mechanism for this interaction remains unknown as no effect of ethylene on the expression of the *RGA* gene or protein abundance was found. Next to ethylene, also auxin was found to interact with DELLA abundance. It was found that plants with impaired polar auxin transport showed delayed degradation of the DELLA protein RGA upon GA application. Likewise, the auxin resistant mutant *axr1-12* also displayed enhanced stability of this DELLA protein to GA in the roots (Fu and Harberd, 2003).

Ethylene is generally considered as a growth inhibitor and is best known for the induction of the so-called triple response in dark-grown Arabidopsis seedlings, which consists of an exaggerated apical hook, shortening and thickening of the hypocotyls and inhibition of root growth (Abeles et al., 1992). Ethylene can, however, also act as a growth stimulator, as shown by Voesenek et al. (2004) for several herbaceous species or by Smalle et al. (1997) for light-grown Arabidopsis hypocotyls. In general, effects of different ethylene concentrations on growth differ between plant species and growth conditions (light, nutrient availability) and is hypothesized to follow the pattern of a bell-curve (Pierik et al., 2006). According to this model, low concentrations of ethylene may act stimulatory on growth of plant organs, whereas higher concentrations can often become saturating or even inhibitory (e.g. Konings and Jackson, 1979; Lee and Reid, 1997). Ethylene can also act as a neighbour detection signal in canopies where it may accumulate in the shielded canopy atmosphere (Heilman et al, 1971; Pierik et al, 2004b). Furthermore, the low R:FR ratio in canopies stimulates ethylene production. For example, ethylene production was found to be increased in phytochrome mutants of different species such as sorghum (Finlayson et

al., 1998), Arabidopsis (Vandenbussche et al., 2003a) and pea (Foo et al., 2006). Moreover, Pierik et al. (2004a) showed that low R:FR can induce a strong increase of ethylene production in tobacco plants. Similar effects were found for low light-exposed Arabidopsis leaf rosettes (Vandenbussche et al, 2003a). The functional importance of ethylene regulation was shown by the fact that ethylene-insensitive transgenic tobacco plants showed reduced shade avoidance responses and were out competed by wild type neighbours (Pierik et al., 2003, 2004b).

Next to ethylene, also auxin is considered as a regulatory factor during shade avoidance responses. A study by Steindler et al. (1997) showed that R:FR-mediated changes in hypocotyl elongation of Arabidopsis seedlings can not proceed in the auxin-resistant *axr1-12* mutant or in seedlings with inhibited polar auxin transport. Furthermore, FR-enriched light can affect the expression of various auxin transport and signaling genes (Devlin et al., 2003). It is hypothesized that low R:FR light affects auxin distribution such that more auxin accumulates in the epidermal cells of the hypocotyls, thus regulating hypocotyl elongation (Morelli and Ruberti, 2002). Auxin most likely is also involved in blue light signaling, as suggested by Folta et al. (2003) who detected increased activity of auxin-mediated genes in blue light of the blue light photoreceptor mutant *cry1* as compared to wild type.

Shade avoidance is thus likely to result from the action of various (interacting) hormones that are regulated by photoreceptors sensing neighbour detection signals. The aim of this study is to elucidate the role of GA in elongation responses of Arabidopsis petioles and hypocotyls to two neighbour detection light signals, reduced R:FR ratio and reduced blue light photon fluence rates. It will be investigated if GA regulates elongation responses by mediating DELLA protein abundance and this study will try to elucidate if the GA-DELLA interaction integrates signaling of other hormones in regulating shade avoidance.

Outline thesis

The aim of this thesis is to elucidate the hormonal regulation of Arabidopsis shade avoidance responses induced by light spectral cues. To this end, elongation of petioles and hypocotyls induced by a low R:FR ratio and low blue light photon fluence rates will be studied. The focus of this research is on the functional involvement of GA and DELLA proteins in these elongation responses. To explore this, a range of DELLA mutants with normal or reduced GA content will be subjected to low R:FR and low blue light treatments and elongation of their petioles and hypocotyls will be recorded. DELLA protein abundance will be studied by using the reporter line *pRGA*::*GFP-RGA* expressing the protein fusion of the DELLA protein RGA with GFP (green fluorescent protein). Furthermore, the importance of two other plant hormones, ethylene and auxin will be investigated, with special emphasis on their interactions with DELLA proteins.
Chapter 2 will investigate the involvement of GA and DELLA proteins in low R:FR-induced elongation of petioles and hypocotyls. Hypocotyl elongation responses to low blue light photon fluence rates will be studied in Chapter 3. In this chapter it will be explored which photoreceptors trigger the hypocotyl elongation induced by low blue light and if GA and DELLAs mediate regulation of this response. As shade avoidance regulation appears to involve more hormones than just GA, Chapter 4 will explore the importance of ethylene and auxin in regulating petiole and hypocotyl elongation response to low R:FR and low blue light. These studies will focus on petiole and hypocotyl elongation induced by low R:FR and low blue light, respectively. The functionality of interactions of ethylene and auxin with DELLA proteins in regulating shade avoidance will be studied. Furthermore, DELLA-independent modes of action of these two hormones will be explored and a working mechanism for these hormonal interactions will be constructed. Chapter 5 will summarize the results from the previous chapters and aims to create a synthesis from these data.

CHAPTER 2

GA is involved in low R:FR light-induced elongation of petioles and hypocotyls through its relief of DELLA restraint

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Abstract

Plants detect neighbours in dense canopies through changes in the light intensity and light spectral composition, with the reduced Red:Far-red (R:FR) light ratio being the best known signal. This triggers a suite of plastic responses collectively called the shade avoidance syndrome. These responses allow plants to maximize light capture by enhancing shoot elongation growth towards the light. Although this process is well described, the mechanistic basis of these growth responses is still poorly understood. In this study, we aim to investigate how the plant hormone gibberellin (GA) is involved in the regulation of petiole and hypocotyl elongation responses to reduced R:FR in Arabidopsis. More specifically, it will be investigated if the growth repressing family of DELLA proteins, components of GA signal transduction, regulate shade avoidance.

Our results show that GA is required for low R:FR-induced degradation of the DELLA protein RGA. Increased abundance of DELLA proteins in GA-deficient petioles and hypocotyls prevents elongation responses to low R:FR, which could be de-repressed by GA addition or genetical DELLA knockout. Degradation of these proteins alone, however, proved not to be sufficient to induce shade avoidance, suggesting regulation of low R:FR-induced responses by other, DELLA-independent signalling pathways.

The data from this chapter have been published in The Plant Journal (2007) **51**, 117-126.

In conclusion, the DELLA proteins can be considered as a restraint on low R:FR-mediated elongation that is degraded in a GA-dependent manner. Removing DELLA restraint, although not sufficient to induce growth responses, is required to permit shade avoidance.

Introduction

Growth of individual plants in dense canopies is strongly affected by surrounding vegetation. As a result of selective wavelength absorbance by neighbouring plants, light intensity is highly reduced and the spectral composition deviates from undisturbed sunlight. To maximize light capture or to finish the life cycle before the environmental conditions become life-limiting, plants evolved a suite of traits called shade avoidance responses. It consists of enhanced petiole and stem elongation, upward movement of leaves (hyponasty) and accelerated reproduction (Smith and Whitelam, 1997). The main trigger for these responses is the reduced ratio of red to far-red radiation (R:FR) resulting from selective absorbance, reflection and transmittance of the incident light (Smith, 2000). The R:FR ratio declines from 1.2 in sun light to 0.20 or less in dense canopies (Franklin and Whitelam, 2005). This R:FR signal is perceived by the phytochrome family of photoreceptors consisting of five members (PHYA – PHYE) in Arabidopsis (Smith, 2000; Franklin and Whitelam, 2005). These photoreceptors exist in two photoconvertible forms, Pr and Pfr, and the equilibrium between them depends on light conditions. The Pfr form is the active form and suppresses all aspects of the shade avoidance syndrome. In a far red-enriched environment, i.e. dense vegetation, the prevailing form is the inactive Pr form, which relieves this Pfr-mediated suppression, and thus allows for shade avoidance responses (Smith, 2000). In Arabidopsis, FR-enriched irradiation enhances elongation of hypocotyls, petioles and flower stems and accelerates flowering (Robson et al., 1993). The cellular basis of these elongation responses relies on cell expansion (Reed et al., 1993; Kozuka et al., 2005).

Many plant hormones are involved in the regulation of shade avoidance responses (reviewed by Halliday and Fankhauser, 2003, Vandenbussche et al., 2005).

An essential role has been ascribed to gibberellin (GA) as a key regulator of cell elongation. GA-involvement in low R:FR-induced elongation was reported for many plant species, such as cucumber hypocotyls (Lopez-Juez et al., 1995), tobacco stems and petioles (Pierik et al., 2004a) and Arabidopsis hypocotyls (Reed et al., 1996). Furthermore, Peng and Harberd (1997) showed that GA-deficiency and GA-insensitivity suppressed the constitutively elongated phenotypes of the Arabidopsis phytochrome-related mutants *phyB* and *hy1*. The precise mechanism through which GA signalling regulates shade avoidance, however, remains largely unknown.

Photoconversion of phytochromes can affect either GA biosynthesis or responsiveness (reviewed by Halliday and Fankhauser, 2003). A study by Reed et al. (1996) on the growth of Arabidopsis *ga1-3 phyB* double mutant hypocotyls showed that phytochrome inactivation increased responsiveness to GA rather than its biosynthesis. On the other hand, Devlin et al. (2003) showed that exposure to FR-enriched light of Arabidopsis seedlings resulted in up-regulation of the GA-biosynthesis gene *GA20ox3* (*GA20oxidase-3*). This was confirmed for petioles of more mature Arabidopsis plants for several *GA20ox* genes (Hisamatsu et al., 2005).

Although GA involvement in shade avoidance responses has been shown in many studies, little is known how exactly the signalling pathway of this hormone is involved in regulating shade avoidance. Recent work on GA signalling has proposed the DELLA family of growth repressing proteins as crucial downstream elements in the GA-signalling pathway (Peng et al., 1997; Dill and Sun, 2001; Silverstone et al., 1998; Sun and Gubler, 2004). The DELLA protein family in Arabidopsis consists of five members: GAI (GA-INSENSITIVE) and RGA (REPRESSOR OF *ga1*) suppressing mainly vegetative growth and induction of flowering, while RGL1, RGL2 and RGL3 (RGA-LIKE 1, 2 and 3) act as repressors of seed germination and flower development (Fleet and Sun, 2005). DELLA proteins are located in the nucleus where they suppress the expression of GA-inducible genes in the absence of GA (Alvey and Harberd, 2005). DELLAs are named after the first five amino acids in the N-terminal domain which is the recognition site for the GA signal. The C-terminal GRAS domain is the functional region of these proteins. Binding of GA to its recently identified receptor GID1

(GIBBERELLIN INSENSITIVE DWARF1) targets DELLA proteins for polyubiquitination by the $SCF^{SLY1/GID2}$ E3 ligase complex and subsequent degradation in the 26S proteasome (Ueguchi-Tanaka et al., 2005, Nakajima et al., 2006).

Gain-of-function mutations in the DELLA-encoding region prevent recognition of the DELLA domain by GA and, thus, the degradation of the protein. This results in constitutively dwarfed phenotypes of *gai* and *rgaΔ17* mutants, irrespective of their elevated GA content (King et al., 2001) due to the still functional, growth-suppressing C-domain. On the other hand, loss of function mutations of DELLA-encoding genes result in constitutive GA-responsiveness which counteracts many aspects of GA-deficiency such as dwarfism and arrested development (Dill and Sun, 2001, Alabadi et al., 2004). The first indication for effects of light quality on DELLAs came from a micro-array study by Devlin et al. (2003) who detected a down-regulation of the DELLA encoding gene *GAI* induced by prolonged low R:FR treatment in Arabidopsis hypocotyls. In addition, Achard et al (2007) have recently shown lower RGA protein level in Arabidopsis seedlings lacking phytochromes A and B. Furthermore, Oh et al. (2007) found that the phytochrome signalling component PIL5 (PHYTOCHROME-INTERACTING FACTOR-LIKE 5) can bind to promoters of the DELLA *GAI* and *RGA* genes and, by stimulating expression of those genes, prevent germination of Arabidopsis seeds in darkness.

The aim of this study was to investigate if GA operates through DELLA proteins in regulating low R:FR-induced elongation in two different plant organs representing two developmental phases, petioles and hypocotyls. The importance of GA was confirmed by using GA-deficient and GA-insensitive mutants. Using DELLA gain-of-function and loss-of-function mutants, the involvement of the various DELLA proteins in low R:FR-mediated shade avoidance was studied. This was further elaborated with DELLA protein dynamics, studied with a *pRGA*::*GFP-RGA* reporter. Our data show that GA-mediated elongation responses to low R:FR act through the abundance of DELLA proteins. Removing DELLA restraint is a pre-requisite for the shade avoidance response to occur, but removal alone is not sufficient to induce low R:FR-induced elongation. This unveils the existence of (a) DELLA-independent

pathway(s) that can act in concert with degradation of the DELLA growth repressors to regulate shade avoidance responses.

Material and Methods

Plant material and growth conditions

All mutants and the transgenic DELLA-GFP reporter are in the *Arabidopsis thaliana* (L.) Landsberg *erecta* (L*er*) background (NASC accession number NW20). The *GA20ox1*::*GUS* reporter is in the Col-0 background. GA-involvement in shade avoidance was investigated in the GA-deficient *ga1-3* (Sun *et al*., 1992) and GA-insensitive *gai* (Koornneef *et al*., 1985) mutant lines. The effect of GA-deficiency on the shade avoidance phenotype was tested with the *phyB* mutant (NASC accession number N6211, Reed et al., 1993). Roles of four of the five Arabidopsis DELLA proteins were studied in the single *gai-t6* (Peng and Harberd, 1993), *rga-24* (Dill and Sun, 2001), *rgl1-1* (Wen and Chang, 2002) and *rgl2-1* (Lee *et al.*, 2002), double *gai-t6 rga-24* (Dill and Sun, 2001) and quadruple *gai-t6 rga-24 rgl1-1 rgl2-1* (Achard *et al.*, 2006) knock-out lines in a L*er* background. These DELLA knockouts were also studied in the GA-deficient (*ga1-3* background) mutants *rga-24 ga1-3* (Silverstone et al., 1998), *gai-t6 ga1-3, gai-t6 rga-24 ga1-3* (Dill and Sun, 2001), *rgl1-1 ga1-3* and *rgl2-1 ga1-3* (Lee *et al.*, 2002). Abundance of the DELLA protein RGA was examined in transgenic plants (*pRGA*::*GFP-RGA*) expressing the GFP-RGA fusion protein (Silverstone et al., 2001). The effect of low R:FR light on a GA-biosynthetic gene was studied in GUS reporter line *GA20ox1*::*GUS* in which *GA20ox1* promoter sequences drive the expression of β-glucuronidase (GUS). Seeds were generously donated by Dr. N. P. Harberd (*gai*, *gai-t6*, *rga-24, gai-t6 rga-24, gai-t6 rga-24 rgl1-1, rgl2-1* and *pRGA*::*GFP-RGA*), Dr. J. Peng (*rgl1-1*, *rgl2-1*, *rgl1-1 ga1-3* and *rgl2-1 ga1-3*), Dr. T. P. Sun (*gai-t6 ga1-3*, *rga-24 ga1-3*, *gai-t6 rga-24 ga1-3*) and Dr. P. Hedden (*GA20ox1*::*GUS*).

For petiole elongation experiments, seeds were sown in Petri dishes on filter paper soaked with water or 100 μM GA_3 (Duchefa, The Netherlands) for GA-deficient

lines. Seeds were stratified 4 d. at 4°C in the dark, and germinated for 4 d. in a growth chamber (20^{o} C, 9 h. light (200 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR); Philips Master HPI 400 W) - 15 h. dark). Seedlings were then transferred to 70 ml pots filled with a 1:2 potting soil:perlite substrate mixture and additional nutrients (Millenaar *et al.*, 2005). Plants were used for experiments 35 d. after sowing.

For hypocotyl experiments, surface-sterilized seeds were sown on agar (5.5 g l^{-1}) with low nutrients (0.22 g l^{-1} MS, Duchefa Biochemie B.V, The Netherlands), stratified for 4 d. at 4°C in the dark, and then transferred to a growth chamber. Germination of GA-deficient mutants was promoted by placing seeds in 100 µM GA_3 solution for 4 days at low temperature (4°C) prior to placing on agar. After 3 d., seedlings were used for experiments.

Treatments and growth measurements

To study the importance of GA during shade avoidance, endogenous GA levels were manipulated. GA-biosynthesis was inhibited by adding paclobutrazol which blocks the early step in GA-biosynthesis, oxidation of ent-kaurene to ent-kaurenoic acid (Rademacher, 2000). Paclobutrazol was applied by adding 20 ml of 50 µM solution to each pot 7 days prior to the light treatments or by adding the substance from a stock solution to the agar plates (1 µM final concentration). Paclobutrazol gave typical GA-deficiency symptoms, such as dwarfing and dark green leaves. This could be rescued by GA treatments which took place by daily spraying with 50 µM GA_3 solution (controls were sprayed with water) or by adding GA_3 to the plates (10 µM final GA concentration). Manipulations of the light quality took place in a white light background (Philips Master HPI-T Plus 400 W and Philips Plusline Pro 150 W). PAR in all light treatments was maintained at 140 µmol m^{-2} s^{-1}. The R:FR ratio was lowered from 1.2 to 0.25 by supplemental far-red light (730 nm LED; Shinkoh Electronics Co. Ltd., Tokyo, Japan). One day before the experiment, plants were transferred to the relevant growth cabinet for acclimation. Light treatments started on the subsequent day at 10 a.m. and lasted 24 h. Petiole lengths were measured with a digital calliper at t=0 and t=24 h. to calculate growth as the length increment over this period. Petiole elongation rates were

also determined by linear displacement transducers to follow the kinetics of petiole elongation in normal and low R:FR (Benschop *et al.*, 2005).

Hypocotyl lengths were measured after 5 d. of light treatment with a custom-built image analysis system (Sony CCD camera (type XC-77CE) combined with KS400 software (Carl Zeiss Vision, Oberkochen, Germany)).

Two-way Analysis of Variance (ANOVA), with Tukey-B post-hoc comparisons (SPSS V12.0.1), revealed if there were significant differences between genotypes and treatments. Experiments were independently repeated at least three times.

GUS assay

In order to study the effect of low R:FR treatment on the activity of a gene encoding a GA-biosynthetic enzyme, we used a GUS-reporter line *GA20ox1*::*GUS*. Leaf rosettes of the *GA20ox1*::*GUS* were detached from the roots and submerged in cold acetone (90% (v/v) for 20 seconds to enhance penetration of the staining solution. Tissue was fixed in 0.3% formaldehyde in 10 mM MES (pH 5.6), 0.3 M mannitol for 45 minutes at 4°C and washed with 100 mM Na-phosphate buffer, pH 7.2. The histochemical reaction was performed by incubating leaf material for 24 h. with 1mM XGlucA (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Duchefa, The Netherlands)) in 100 mM Na-phosphate buffer, pH 7.2, 1 mM EDTA. After staining, leaf rosettes and hypocotyls were rinsed and bleached in an ethanol series from 50 to 90% and then photographed.

DELLA abundance

The abundance of the DELLA protein RGA was studied with the *pRGA*::*GFP-RGA* reporter line (Silverstone et al., 2001; Achard et al, 2003, 2006). Fluorescence was detected with an inverted confocal laser scanning microscope (Zeiss LSM Pascal, 40x C-apochromat objective). The excitation wavelength was 488 nm, a 505-530 nm bandpath filter was used for GFP emission and a 560 nm long pass filter was used to visualize red fluorescence by chloroplasts. Images depict 149.5 µm Z-stacks of petioles and hypocotyls. GFP-RGA fluorescence of petioles and hypocotyls was determined at the same time-points as the length measurements. Although the basal, middle and

uppermost regions of hypocotyls and petioles were studied, only images from the basal region (abaxial in petioles with the mid-rib horizontally approx. half-way the image) are shown as this was the most responsive region to the light treatments. Confocal images are representative selections from at least nine replicates of at least three independent experiments. It has been shown by Western blotting with an anti-GFP antibody, that the RGA-GFP signal visualized through confocal imaging gives a reliable estimation of the abundance of this fusion protein (Achard *et al.*, 2006).

Results

Low R:FR-induced elongation of petioles and hypocotyls is GA-dependent

Petioles and hypocotyls responded to a 24 h. low R:FR treatment by increased elongation in the wild type accession L*er* (Fig. 1A and B). This response and the control length petioles and hypocotyls were reduced in GA-deficient plants, i.e. L*er* treated with an inhibitor of GA-biosynthesis, paclobutrazol, and in the *ga1-3* mutant.

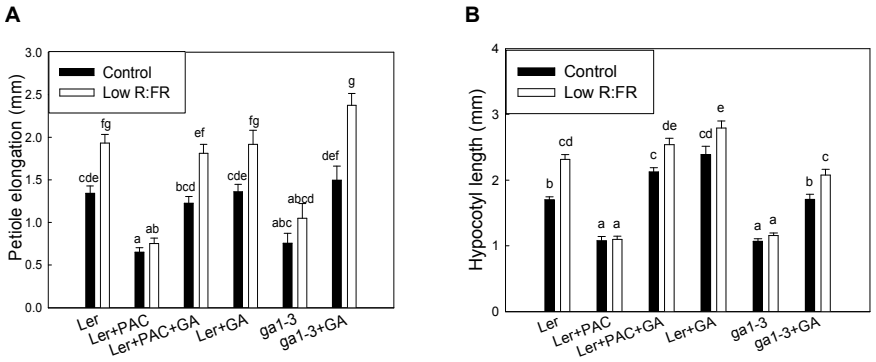

Figure 1. The effect of GA-level on low R:FR-induced elongation of A) petioles and B) hypocotyls. Data are means of 10 petioles and 30±5 hypocotyls. No overlapping letters indicate significant difference ($p<0.05$). PAC=paclobutrazol.

Furthermore, GA-deficiency abolished the constitutively enhanced elongation of *phyB* petioles (Fig. 2). Adding back GA to GA-deficient plants completely restored the elongation response to low R:FR of petioles (Fig. 1A) and promoted hypocotyl elongation in both control and low R:FR light conditions (Fig. 1B).

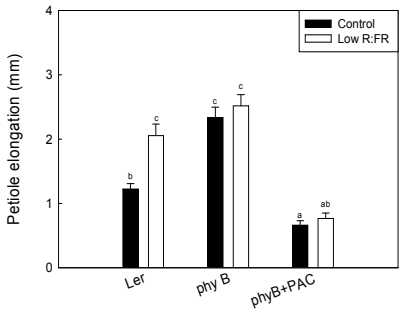

Figure 2. The effect of GA-deficiency induced by paclobutrazol (PAC) on the petiole elongation response to low R:FR of the *phyB* mutant. Data are means ± SE (n=10). No overlapping letters indicates significant difference ($p<0.05$).

GA application to L*er* petioles with a normal GA-content did not affect the growth response to low R:FR (Fig. 1A), indicating saturation by the endogenous level of this hormone. Contrary to petioles, GA addition to hypocotyls partly induced a shade avoidance phenotype in control light which partly saturated the elongation response to low R:FR treatment (Fig. 1B). However, GA application could not induce the complete low R:FR-elongation response in control light conditions.

According to a GUS assay, low R:FR treatment did increase the activity of the GA-biosynthesis gene *GA20ox1* (Fig. 3) in petioles. This activity was the highest in younger petioles and showed a basipetal gradient. *GA20ox1*::*GUS* activity in seedlings was localized in the root tips and apical meristem, rather than the hypocotyl, and was not affected by the low R:FR treatment (data not shown).

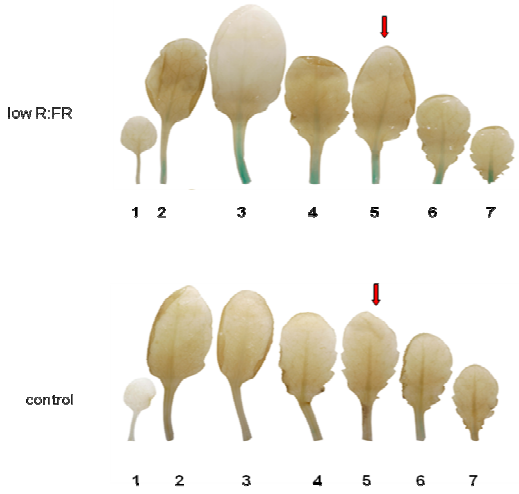

Figure 3. The effect of low R:FR on the activity of the GA-biosynthesis gene *GA20ox1* in leaves of the *GA20ox*::*GUS* reporter line. Images depict leaves of one selected rosette out of five per light treatment involved in the GUS assay. Arrows indicate the age of leaves which were selected for petiole length measurement.

The petiole and hypocotyl elongation responses to low R:FR of the GA-insensitive, DELLA gain-of-function mutant line *gai* were severely reduced (Figs. 4A and 5A). This confirms the regulatory role of GA and more specifically indicates the involvement of the DELLA protein GAI in shade avoidance. In control light conditions *gai* petioles tend to elongate somewhat slower than WT, but this effect is not significant.

DELLA removal rescues low R:FR-induced response of GA-deficient plants

Removing DELLA proteins by knocking-out DELLA-encoding genes counteracts some aspects of GA-deficiency (Dill and Sun, 2001; King et al., 2001). In plants with normal GA content, however, loss-of-function DELLA mutations have only mild phenotypic effects. As expected, single and double DELLA knock-outs hardly affected the response of petioles and hypocotyls to low R:FR treatment (Figs. 4A and 5A). However, control hypocotyls showed a constitutively elongated phenotype when four DELLAs were knocked out in *gai-t6 rga-24 rgl1-1 rgl2-1* (Fig. 5A). This indicates that removing DELLA restraint can partly induce a shade-avoidance phenotype in hypocotyls (Fig. 5A), which is clearly not the case in petioles (Fig. 4A).

The inhibited petiole elongation response to low R:FR in GA-deficient L*er* treated with paclobutrazol and in *ga1-3* was partly restored in the single DELLA knock-outs *gai-t6* and *rga-24* (Fig. 4B and C). The complete rescue of low R:FR-induced elongation in a GA-deficient *gai-t6 rga-24* (Fig. 4B and C), suggests that particularly the combined absence of the GAI and RGA proteins can completely counteract the inhibition of shade avoidance induced by GA-deficiency. Additional silencing of *RGL1* and *RGL2* genes in the quadruple DELLA mutant did not further rescue the elongation response to low R:FR in a GA deficient background, confirming that GAI and RGA are the main repressors of the low R:FR-induced elongation response in GA-deficient petioles.

In contrast to petioles, DELLA knock-out in GA-deficient hypocotyls could not completely rescue the elongation response to low R:FR (Fig. 5B and C). Although very limited, some rescue was seen in the double *gai-t6 rga-24* in the *ga1-3* background and the quadruple *gai-t6 rga-24 rgl1-1 rgl2-1* mutants (Fig. 5B and C). As in petioles,

this suggests that the main growth repressors in GA-deficient hypocotyls are the GAI and RGA proteins. However, the lack of complete rescue of low R:FR-mediated elongation could imply DELLA-unrelated repressors which remain active in GA-deficient seedlings.

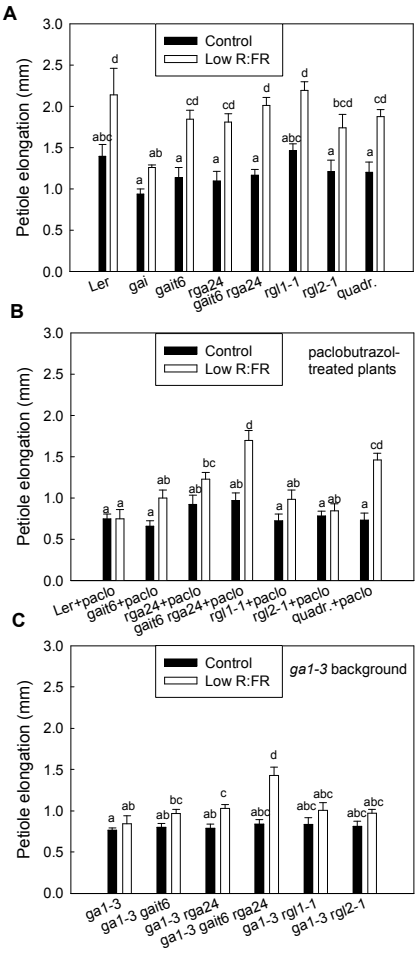

Figure 4. Low R:FR-induced petiole elongation in DELLA mutants with A) normal GA-content and reduced GA content obtained by B) paclobutrazol and C) in the *ga1-3* background. Data are means ± SE (n=10). No overlapping letters indicates significant difference ($p<0.05$).

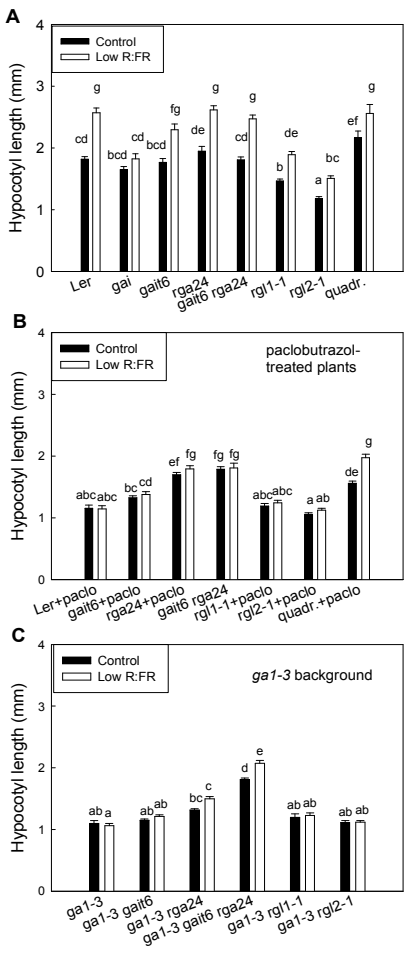

Figure 5. Low R:FR-induced hypocotyl extension in DELLA mutants with A) normal GA-content and reduced GA content obtained by B) paclobutrazol application and C) in the *ga1-3* background. Data are means ± SE (n=35). No overlapping letters indicate significant difference ($p<0.05$).

Low R:FR treatment affects the abundance of the DELLA protein RGA

The abundance of the DELLA protein RGA, visualized by the GFP-RGA fusion, was severely reduced by low R:FR treatment in petioles and hypocotyls with normal content of GA (Fig. 6A and B). This was prevented by an inhibitor of GA biosynthesis, paclobutrazol, which increased RGA-GFP abundance in both light treatments (Fig. 6A and B).

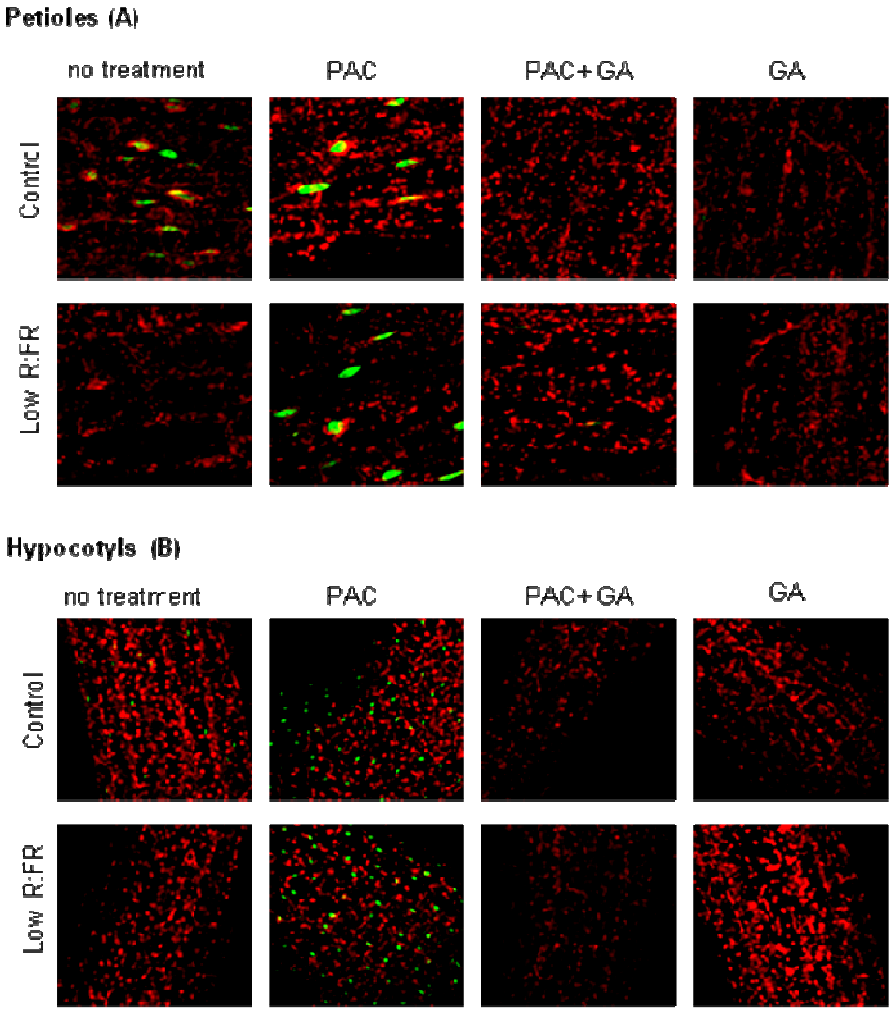

Figure 6. RGA-GFP signal of *pRGA*::*GFP-RGA* in A) petioles and B) hypocotyls in control and low R:FR treatment. Degradation of RGA induced by low R:FR was prevented by GA-deficiency in paclobutrazol-treated plants and restored by adding GA.

Adding back GA led to disappearance of the RGA-GFP signal and concomitantly the elongation response to low R:FR of petioles and hypocotyls was restored (Fig. 1A and B). These data thus indicate that low R:FR-induced DELLA degradation acts in a GA-dependent manner.

Growth kinetics showed that low R:FR treatment induced acceleration of petiole elongation starting approximately 2 h. after start of the treatment. The maximum growth rate was reached 2 h. later, i.e. 4 h. after start of the treatment and this was maintained until 6 h. after start of treatment (Fig. 7A). In parallel with these growth kinetics, the GFP-RGA signal became weaker after 2 h. of low R:FR treatment and disappeared within the next 2 h., at the time-point of the maximal elongation rate (Fig. 7B).

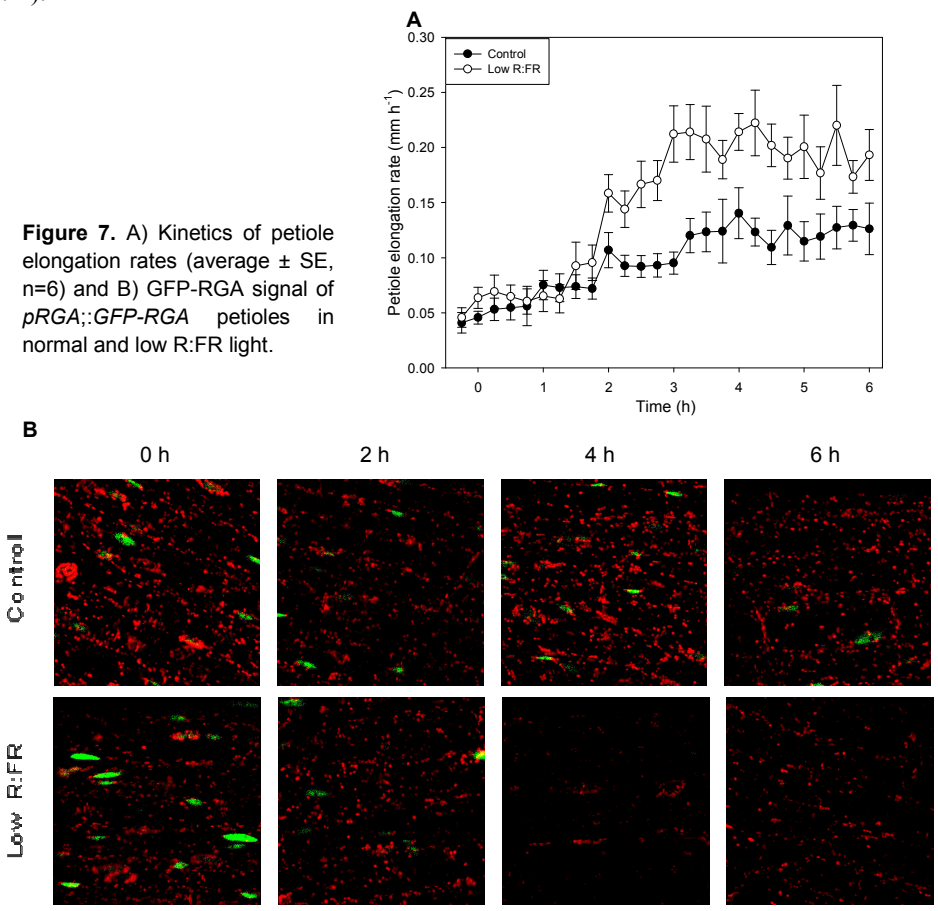

Figure 7. A) Kinetics of petiole elongation rates (average ± SE, n=6) and B) GFP-RGA signal of *pRGA*;:*GFP-RGA* petioles in normal and low R:FR light.

This indicates that the low R:FR-induced reduction in DELLA abundance coincides with the growth acceleration of petiole elongation in this light treatment.

Discussion

Elongation responses to reduced R:FR in plants result from a multitude of physiological reactions including those regulated by the plant hormone GA. Here we studied the importance of GA for low R:FR-induced shade avoidance. Furthermore, it was studied if GA involvement in shade avoidance is through low R:FR-induced degradation of DELLA proteins.

Elongation response to low R:FR is GA and DELLA-dependent

Low R:FR-induced petiole and hypocotyl elongation is highly regulated by GA. When the biosynthesis of this hormone is inhibited this leads to a severely reduced elongation response of petioles and hypocotyls to low R:FR (Fig. 1A and B). GA deficiency also suppressed the constitutive shade avoidance phenotype of the *phyB* mutant (Fig. 2). This is in agreement with data found for other species, such as cucumber (Lopez-Juez et al., 1995) and tobacco (Pierik et al., 2004). Not only GA deficiency but also reduced sensitivity to this hormone, as in the *gai* mutant, reduced shade avoidance responses of petioles and hypocotyls (Figs. 4A and 5A). This confirms findings of Peng and Harberd (1997) on *ga*i-mediated suppression of the constitutively elongated phenotype of the *phyB* mutant in the *gai phyB* double mutant. The gain-of-function *gai* mutation was generated by deletion of 51bp in the DELLA encoding region of GAI conferring a non-functional form of the binding site for the GA-GID1 complex. The functional C-domain remains active in this mutant gai protein, which can thus still suppress growth. Due to a lack of feedback regulation through GA signaling, the *gai* mutant has elevated GA levels (Peng et al., 1997). However, irrespective of these enhanced levels, *gai* plants could not fully respond to low R:FR light due to constitutive repression by the stabilized gai protein. We conclude that inducible elongation as part of the shade avoidance syndrome in Arabidopsis hypocotyls and petioles can be constrained by the DELLA protein GAI.

More evidence for DELLA involvement in low R:FR responses came from the rescue of low R:FR-induced elongation by DELLA knock-outs in a GA-deficient background. In both petioles and hypocotyls this was most pronounced in the double *gai-t6 rga-24* and the quadruple *gai-t6 rga-24 rgl1-1 rgl2-1* mutants confirming that the GAI and RGA proteins are the most important growth repressors in GA-deficient plants. This is in agreement with findings of King et al. (2001) who showed that these two proteins have distinct but also overlapping activity in plant growth regulation. Interestingly, suppressed low R:FR-induced elongation by GA-deficiency was not rescued to the same extent in petioles and hypocotyls by knocking-out DELLAs. The weaker rescue in GA-deficient hypocotyls may indicate that the regulation of low R:FR-induced hypocotyl elongation includes additional GA-dependent regulators, other than DELLAs. Another discrepancy between petioles and hypocotyls is the fact that hypocotyls of quadruple DELLA knockouts with non-reduced GA content are constitutively elongated, whereas petioles are not (Figs 4A and 5A). This constitutive shade avoidance phenotype could indicate that DELLA breakdown switches on part of the shade-avoidance machinery in hypocotyls. However, this constitutive elongation is not as strong as the full low R:FR responses indicating the existence of alternative route(s).

In summary, DELLA proteins suppress elongation responses to low R:FR in GA-deficient petioles and hypocotyls. This can be rescued completely by adding back GA to petioles and hypocotyls. Knocking-out DELLAs also results in rescued low R:FR-induced elongation of GA-deficient petioles whereas the hypocotyl elongation response is only partly rescued. The absence of constitutively enhanced petiole elongation in any of the investigated DELLA knock-outs indicates that low DELLA abundance alone does not lead to shade avoidance, whereas in hypocotyls this does induce part of the responses.

DELLA abundance is functionally regulated by the R:FR ratio

To further investigate if DELLA proteins are also functionally involved in the regulation of shade avoidance, we studied the abundance of the RGA protein in the transgenic *pRGA*::*GFP-RGA* reporter line in which the behavior of GFP-RGA

accurately reflects that of the RGA protein (Silverstone et al., 2001, Achard et al., 2006, 2007).

Enhanced elongation of petioles and hypocotyls induced by the low R:FR signal was accompanied by the disappearance of GFP-RGA signal (Fig. 6A and B). Furthermore, gradual disappearance of the GFP-RGA signal (Fig. 7B) in time coincided with increased petiole elongation rate induced by low R:FR (Fig. 7A). In petioles, this might result partly from increased GA-biosynthesis, as suggested by the higher expression of *GA20ox1*::*GUS* in low R:FR (Fig. 3). This tentative up regulation of GA biosynthesis is consistent with findings by Hisamatsu et al. (2005) who detected up regulation of both *GA20ox1* and *GA20ox2* in Arabidopsis petioles upon end-of-day far-red treatment. In agreement with this, external GA application also led to enhanced DELLA breakdown. However, GA application did not enhance petiole elongation (Fig. 1A). We, therefore, conclude that increased GA-biosynthesis and concomitant DELLA breakdown are not sufficient to induce petiole elongation. Alternatively, low R:FR may also enhance GA responsiveness, as has been shown for Arabidopsis hypocotyls (Reed et al. 1996).

The abundance of the RGA-GFP fusion protein was affected most strongly in the basal region of the petiole, which corresponds with the highest low R:FR-induced activity of *GA20ox1*::*GUS* (Fig. 3B). In this region Kozuka et al. (2005) have also found the highest increase in far-red induced cell elongation in petioles.

Although the activity of *GA20ox1*::*GUS* was undetectable in hypocotyls, enhanced GA-content in low R:FR treatment is still possible since Devlin et al. (2003) found FR-induced expression of *GA20ox3*, another member of the *GA20ox* family in Arabidopsis. If low R:FR would stimulate endogenous bioactive GA concentrations, this would likely lead to degradation of DELLA proteins, similar to petioles. This is supported by the promotive effect of GA addition on degradation of the GFP-RGA signal in hypocotyls (Fig. 6B). In contrast to petioles, attenuation of the GFP-RGA signal in hypocotyls, induced by GA addition, was accompanied by enhanced elongation (Fig.1B) suggesting that DELLA degradation itself could already induce partial shade avoidance in hypocotyls. On the other hand, hypocotyls with

experimentally fixed GA levels (plants grown on paclobutrazol and GA) still show low R:FR-induced hypocotyl elongation, suggesting that also additional regulatory mechanisms could be involved.

We have indicated that low R:FR signaling controls DELLA abundance in a GA-dependent manner. Furthermore, it was shown that a lack of DELLA degradation, as in the *gai* mutant or in GA-deficient plants, prevents the enhanced petiole or hypocotyl elongation response to low R:FR. However, artificially obtained low DELLA abundance, e.g. by GA addition or in genetic DELLA knockouts, does not necessarily lead to enhanced elongation growth. These findings together indicate that DELLA degradation is essential to low R:FR-mediated shoot elongation, but not sufficient to fully induce this response. Therefore, these data unveil the existence of alternative growth regulating signal transduction pathways that act independently of GA/DELLA to stimulate elongation growth upon perception of a reduced R:FR ratio. It will be an interesting challenge to investigate if other hormones that have been implied in the regulation of shade avoidance, such as auxin and ethylene, would have DELLA-independent modes of action to regulate shade avoidance responses to low R:FR ratio's.

Acknowledgements
We thank Ankie Ammerlaan for preparing the assembled images of *GA20ox1*::*GUS* plants.

CHAPTER 3

Hypocotyl elongation in light-grown Arabidopsis is stimulated by reduced blue light photon fluence rates in a cryptochrome and DELLA-dependent manner

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Abstract

Shade avoidance responses enable plants to compete for light in dense canopies and encompass traits such as enhanced shoot elongation and upward leaf movement. Although these responses are mostly associated with perception of a reduced Red:Far-red light ratio, reduced low blue light photon fluence rates, occurring in canopy shade, have also been shown to trigger these responses. However, relatively little is known about how this blue light signal is perceived and which hormonal processes act downstream of it to regulate these growth responses. Here we show that reduced blue light photon fluence rates induce pronounced elongation of hypocotyls in a predominantly cryptochrome-dependent manner. Furthermore, this elongation response requires the presence of the growth-promoting hormone gibberellin (GA). The GA signaling pathway includes DELLA proteins which suppress low blue-induced elongation when stabilized by GA-deficiency. The elongation response to low blue light of GA-deficient hypocotyls was, therefore, genetically rescued by DELLA knock outs. In seedlings with normal GA content, however, DELLA removal gave only a partial constitutive increase in hypocotyl length suggesting that also alternative low blue-induced processes are involved in the elongation response. We conclude that DELLA degradation is essential to allow low blue-induced hypocotyl elongation, but that this

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process alone is not sufficient to induce the full growth response, indicating the existence of alternative pathways.

Introduction

Plants in dense canopies compete for light, as this is severely depleted by the presence of surrounding vegetation. Light conditions in canopies differ in quality and intensity from the open field as a result of absorption, transmittance and reflection of incoming sunlight. Selective absorption of blue and red photons by chlorophyll of neighboring plants results in altered light quality characterized by a reduced Red:Far-red (R:FR) light ratio and reduced blue light photon fluence rates (Smith and Whitelam, 1997; Ballaré, 1999; Franklin and Whitelam, 2005; Vandenbussche et al., 2005). To increase light capture, plants evolved a suite of adaptive reactions called shade avoidance responses which enable them to increase light harvest by enhancing shoot elongation and upward leaf movement (Smith and Whitelam, 1997; Ballare, 1999; Franklin et al., 2005). It is known that the reduced R:FR ratio is an early neighbour detection signal which triggers shade avoidance responses (Aphalo et al., 1999; Ballare, 1999). In addition to this signal, low blue light photon fluence rates may also induce certain aspects of shade avoidance responses such as enhanced elongation of cucumber hypocotyls (Ballare et al., 1991b) and tobacco stems (Casal and Sanches, 1994; Pierik et al., 2004b). However, relatively little is known about how this signal is perceived in canopy-grown plants and how it is regulating growth.

Blue light in general is perceived by two groups of photoreceptors, cryptochromes and phototropins that have distinct but also overlapping functions. Cryptochromes are mainly mediating photomorphogenetic responses such as inhibition of stem elongation, stimulation of leaf expansion, control of photoperiodic flowering and entrainment of the circadian clock. Phototropins predominantly regulate phototropism, chloroplast relocation and stomatal opening (Christie et al., 2001; Lin and Shalitin, 2003). The cryptochrome photoreceptor family in Arabidopsis consists of two well-characterized members, CRY1 (Ahmad and Cashmore, 1993) and CRY2 (Lin et

al., 1998). A third family member is CRY3 (Huang et al., 2006), but the function of this cryptochrome has not been elucidated yet. CRY1 is light-stable and acts at high blue-light intensities, whereas CRY2 is light-labile and is considered to be more important at low-blue light intensities (Parks et al., 2001). Although these photoreceptors can interact with phytochromes, cryptochromes themselves are responsible for blue-light mediated inhibition of hypocotyl elongation in dark-grown Arabidopsis seedlings (Ahmad et al., 2002). It is yet unknown if elongation growth stimulated by reduced blue light in light-grown plants is regulated in an analogous manner.

Upon perception, light signals are further transduced to regulate growth by a hormone-based signaling network (reviewed by Halliday and Fankhauser, 2003; Vandenbussche et al., 2005). Several studies confirmed an important role for the plant hormone gibberellin (GA) in regulating elongation responses to low R:FR (Lopez-Juez et al., 1995; Reed et al., 1996; Peng and Harberd, 1997; Pierik et al., 2004a). It was also shown that GA-signaling components, DELLA proteins, are involved in the regulation of elongation responses to this light signal (Chapter 2). DELLA proteins are known as repressors of GA-inducible growth in the absence of this hormone. The DELLA protein family in Arabidopsis consists of GAI (GA-INSENSITIVE), RGA (REPRESSOR OF *ga1*) and RGL1, RGL2, RGL3 (RGA-LIKE 1, 2 and 3) (Fleet and Sun, 2005). Petiole and hypocotyl elongation in Arabidopsis, induced by FR-enriched light, were accompanied by enhanced degradation of the DELLA proteins in Arabidopsis hypocotyls and petioles (Chapter 2). Although the degradation of these proteins alone was not sufficient to induce shade avoidance responses in petioles and hypocotyls, it was a pre-requisite for the functionality of other, DELLA-independent, signaling pathways. It is yet unknown if a similar regulation is involved in responses to another trigger for shade avoidance, low blue light photon fluence rates.

The aim of this study was to explore how the low blue light signal is perceived and to give more insight into the involvement of GA and DELLA proteins in elongation responses in Arabidopsis. Our data indicate that low blue light photon fluence rates induce considerable elongation of hypocotyls in a cryptochrome-dependent manner. GA is essential for this elongation response, as it removes DELLA proteins which

accumulate in GA-deficient plants and prevent growth. Although DELLA degradation alone could not induce the full elongation response in hypocotyls, it was required to allow low blue-induced hypocotyl elongation.

Material and methods

Plant material and growth conditions

Photoreceptors that perceive low blue light signals were studied by using the single, double and quadruple photoreceptor null mutants *cry1* (Ahmad and Cashmore, 1993), *cry2* (Lin et al., 1998), *cry1 cry2* (Ahmad et al., 1998), *phot1-101* (Sakai et al., 2001), *phot2-5* (Sakai et al., 2001), *phot1 phot2* (Sakai et al., 2001), *cry1 cry2 phot1 phot2* (Ohgishi et al., 2004), *phyABDE* (Franklin et al., 2003) and *hy2* (Parks and Quail, 1991). GA-involvement in shade avoidance was investigated with the GA-deficient *ga1-3* (Sun *et al*., 1992) and the GA-insensitive *gai* (Koornneef *et al*., 1985) mutants. The roles of four of the five Arabidopsis DELLA proteins were studied with the single *gai-t6* (Peng and Harberd, 1993), *rga-24* (Dill and Sun, 2001), *rgl1-1* (Wen and Chang, 2002) and *rgl2-1* (Lee *et al.*, 2002), the double *gai-t6 rga-24* (Dill and Sun, 2001) double and *gai-t6 rga-24 rgl1-1 rgl2-1* (Achard *et al.*, 2006) quadruple knock-out lines in a L*er* background. These DELLA knockouts were also studied in the GA-deficient (*ga1-3*) background mutants *rga-24 ga1-3* (Silverstone et al., 1998), *gai-t6 ga1-3, gai-t6 rga-24 ga1-3* (Dill and Sun, 2001), *rgl1-1 ga1-3* and *rgl2-1 ga1-3* (Lee *et al.*, 2002). Abundance of the DELLA protein RGA was studied in transgenic plants (*pRGA*::*GFP-RGA*) expressing the GFP-RGA fusion protein. Seeds were generously donated by Dr. M. Koornneef (*cry1*), Dr. G. C. Whitelam (*cry2* and *phyABDE*), Dr. M. Ahmad (*cry1 cry2*), Dr. T. Sakai (*phot1*, *phot2*, *phot1 phot2* and *cry1 cry2 phot1 phot2*), Dr. N. P. Harberd (*gai*, *gai-t6*, *rga-24, gai-t6 rga-24, gai-t6 rga-24 rgl1-1 rgl2-1* and *pRGA*::*GFP-RGA*), Dr. J. R. Peng (*rgl1-1*, *rgl2-1*, *rgl1-1 ga1-3* and *rgl2-1 ga1-3*), and Dr. T. P. Sun (*gai-t6 ga1-3*, *rga-24 ga1-3*, *gai-t6 rga-24 ga1-3*). Most mutant and transgenic lines used in this study are in the Landsberg *erecta*-L*er* background. Exceptions are the quadruple *cry1 cry2 phot1 phot2* and the double *phot1 phot2*

photoreceptor mutant in mixed L*er*/Ws background, the single *cry2* in Col-0 background and *phot2* in Ws background. Seeds were surface-sterilized by sodium hypochlorite solution in water (20% v/v) and sown on agar plates (5,5 g l^{-1}) with low nutrients (0.22 g l^{-1} MS, Duchefa Biochemie B.V, The Netherlands). Seeds were then stratified at 4°C for 4 d. To induce germination of GA-deficient mutants, seeds were stratified in 100 µM GA_3 solution. GA-biosynthesis was inhibited by adding paclobutrazol to the agar plates (1 µM final concentration) while GA-content was rescued by adding GA_3 to the plates (10 µM final GA concentration). Germination was induced by exposing plates with seeds for 2 hours to white light one day prior to light treatment. For a subsequent 22 h., plates were kept in darkness and then transferred to the growth chamber with light treatment compartments (20^o C, 15 h. light (200 µmol m^{-2} s^{-1} photosyntheticaly active radiation (PAR); Philips Master HPI 400 W) - 9 h. dark). Blue light photon fluence rates (400-500 nm) were reduced from 26 to < 1 µmol m^{-2} s^{-1} in the low blue treatment with two layers of Lee010 Medium Yellow filter (Lee Filters, Andover, UK). PAR in all light treatments was maintained at 140 µmol m^{-2} s^{-1}. Hypocotyl lengths were measured after 7 d. of treatment with a custom-built image analysis system (Sony CCD camera (type XC-77CE) combined with KS400 software (Carl Zeiss Vision, Oberkochen, Germany)).

Two-way Analysis of variance (ANOVA), with Tukey-B post-hoc comparisons (SPSS V12.0.1) revealed if there were significant differences between genotypes and treatments. Experiments were independently repeated at least three times.

DELLA abundance

The abundance of the DELLA protein RGA was studied with the *pRGA*::*GFP-RGA* reporter line. Various recent papers have shown an excellent match between GFP-RGA data based on GFP fluorescence and Western blotting (Silverstone *et al.*, 2001, Achard et al., 2003, Achard et al., 2007), indicating the reliability of the method used here to study protein abundance. Fluorescence was detected with an inverted confocal laser scanning microscope (Zeiss LSM Pascal, 40x C-apochromat objective). Excitation wavelength was 488 nm, a 505-530 nm bandpath filter was used for GFP emission and

a 560 nm long pass filter was used to visualize red fluorescence by chloroplasts. Images depict 149.5 µm Z-stacks of hypocotyls. GFP-RGA fluorescence of hypocotyls was determined at the same time-points as the length measurements. The basal, middle and uppermost regions of hypocotyls were studied, but only images from the basal region are shown. Confocal images are representative selections from at least nine replicates of at least three independent experiments.

Results

Low blue light photon fluence rates induce hypocotyl elongation

Low blue light photon fluence rates seemed to induce elongation of petioles, detected from 2 d. of treatment onwards (Fig.1A). The promotive effect of this neighbour detection signal on petiole elongation was, however, not significant. Hypocotyls, on the other hand, showed a very strong response to low blue light evidenced by the strong (approximately threefold) increase in length (Fig. 1B), which is already visible from the first day of low blue exposure onwards (D.K. Keuskamp and R. Pierik, unpublished data).

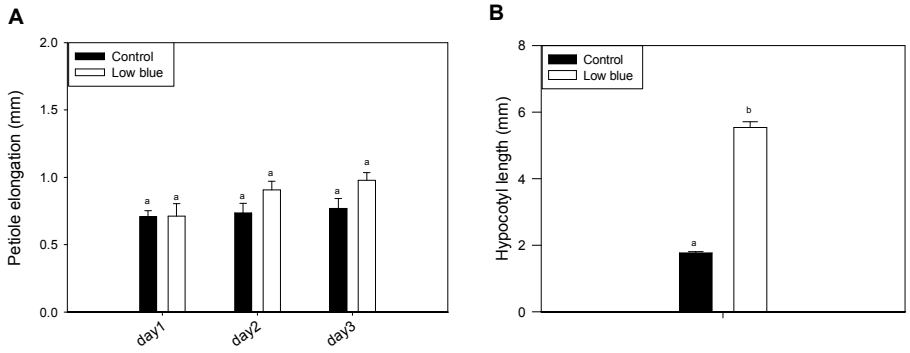

Figure 1. A) Elongation response of Ler petioles to 3d. low blue treatment; B) Elongation response of L*er* hypocotyls to 7d. low blue treatment. Data are means of 10 (petioles) and 30± 5 (hypocotyls) ± SE. No overlapping letters indicate significant difference ($p<0.05$).

Low blue, therefore, likely is more important as an early signal triggering shade avoidance in hypocotyls than in petioles, although it probably does contribute to long

term petiole elongation. For this reason, in the remainder of this study we focused on low blue-mediated elongation of hypocotyls.

Low blue light is perceived by cryptochromes

To study which photoreceptors are responsible for low blue-induced hypocotyl elongation, we used a range of single, double and quadruple photoreceptor mutants. From the data in Fig. 2 it is obvious that, among single photoreceptor mutants, only *cry1* displayed constitutively elongated hypocotyls suggesting that the CRY1 photoreceptor regulates hypocotyl elongation.

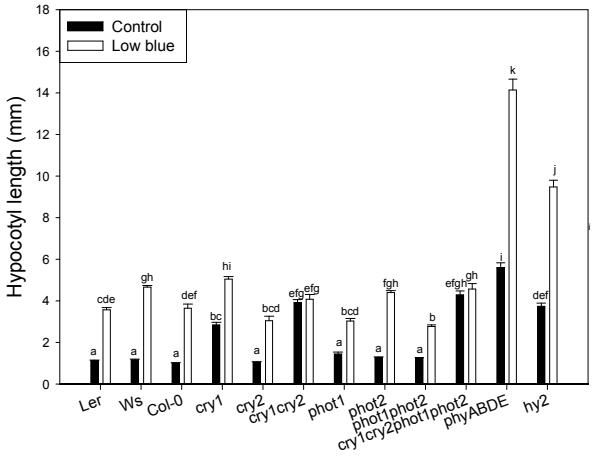

Figure 2. Hypocotyl elongation responses to 7d. low blue treatment of photoreceptor mutants. Data are means ± SE (n=30-50). No overlapping letters indicate significant difference ($p<0.05$).

However, *cry1* hypocotyls still showed a response to low blue, albeit reduced as compared to WT and this response was entirely absent in the double mutant *cry1 cry2*. This indicates that CRY1 and CRY2 act redundantly to regulate the hypocotyl elongation response to low blue. Phototropin mutants were not constitutively elongated what would suggest that these photoreceptors, unlike the cryptochromes, do not mediate hypocotyl elongation. However, the *phot1* and the double *phot1 phot2* mutants did show a reduced hypocotyl elongation response to low blue light in comparison to wild type,

although a clear and significant response remained in these phototropin mutants (Fig. 2). These data would indicate that phototropins are playing a subtle role in low blue-induced hypocotyl elongation. Since phytochromes are also sensitive to blue light, we tested the quadruple phytochrome *phyABDE* and chromophore *hy2* mutants for low blue-induced hypocotyl elongation. Despite their constitutively elongated phenotype, both mutants still responded to low blue suggesting that phytochromes are not key regulators of low blue-induced hypocotyl elongation.

GA is essential for the elongation response to low blue

The strong elongation response to low blue was severely reduced in GA-deficient hypocotyls, as shown in the wild type L*er* treated with the GA-biosynthesis inhibitor, paclobutrazol, and in the *ga1-3* mutant (Fig. 3).

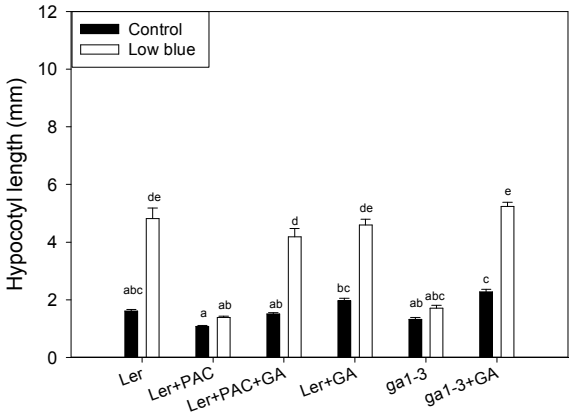

Figure 3. Hypocotyl responses to 7 d. low blue treatment. GA deficiency inhibits hypocotyl elongation response to low blue and can be rescued by GA. Data are means ± SE (n=30-50). No overlapping letters indicate significant difference ($p<0.05$).

Adding GA to GA-deficient seedlings completely rescued the elongation response to low blue (Fig. 3), thus indicating GA-specific involvement in the regulation of this response. GA importance in low blue-induced elongation was further confirmed by the reduced low blue response of the gain-of-function DELLA mutant *gai* which confers reduced responsiveness to GA (Fig. 4A). This finding is the first indication for involvement of DELLA proteins in low blue-induced elongation of hypocotyls.

DELLA knockouts partly rescue low blue-induced elongation in GA deficient seedlings

Loss-of-function DELLA mutations in plants with normal GA content did not affect the elongation response to low blue light (Fig. 4A). The effect of DELLA removal became apparent through increased absolute hypocotyl length in both light conditions of several DELLA knock-outs (*rga-24*, *gai-t6 rga-24*, quadruple; Fig.4A), indicating that DELLAs in general constrain hypocotyl growth.

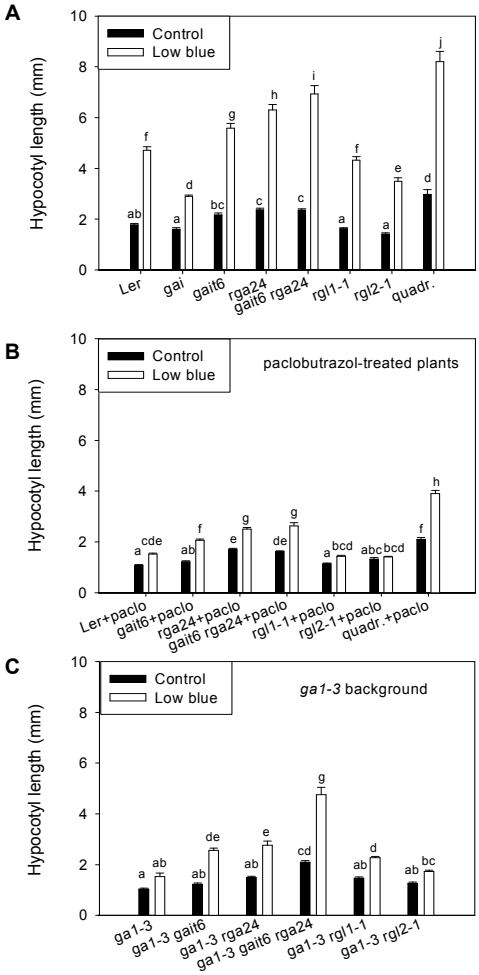

Figure 4. Hypocotyl responses to 7 d. low blue treatment. A) The DELLA gain of function *gai* mutant has reduced response to low blue, whereas the response in various DELLA knockouts is undisturbed. (B-C) DELLA knockouts can counteract and overcome the stunted hypocotyl phenotype of GA-deficient seedlings and rescue the response to low blue. Data are means ± SE (n=30-50). No overlapping letters indicate significant difference ($p<0.05$).

The elongation response to low blue light in GA deficient seedlings was partly rescued by knocking out DELLAs (Figs. 4B and C), as is obvious in the paclobutrazol-treated

quadruple DELLA knock-out *gai-t6 rga-24 rgl1-1 rgl2-1* and in *gai-t6 rga-24 ga1-3* (Fig. 4C). With respect to single DELLA knock-outs, the strongest, yet not complete, rescue of low blue-induced elongation in GA-deficient plants was found for *gai-t6* and *rga-24* (Figs. 4B and C). This suggests that the GAI and RGA proteins are the main repressors of this response in GA-deficient seedlings. These two proteins act redundantly to suppress the elongation response to low blue of GA-deficient hypocotyls.

Low blue light induced reduction of the DELLA protein RGA

Abundance of the DELLA protein RGA was visualized through GFP (Green Fluorescent Protein) fluorescence from the transgenic *pRGA*::*GFP-RGA* line. This fluorescence was detectable and strong in control light conditions, while low blue treatment induced complete disappearance of the GFP-RGA signal (Fig. 5).

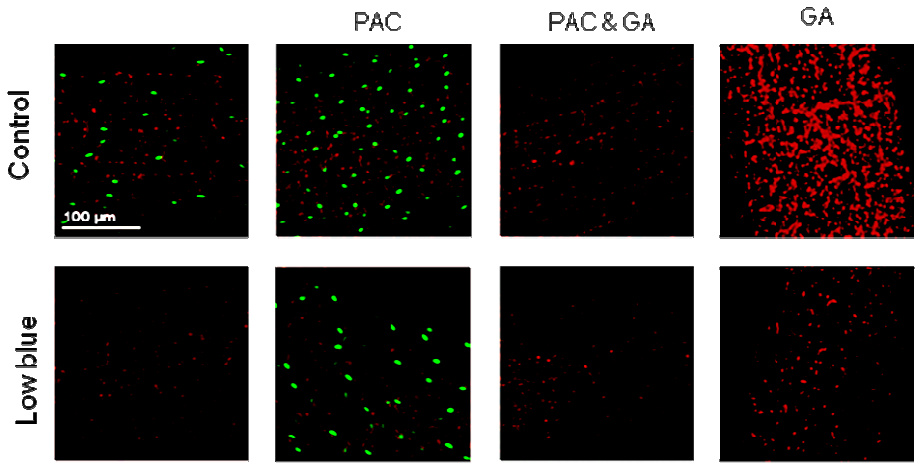

Figure 5. RGA-GFP signal of *pRGA*::*GFP-RGA* hypocotyls is reduced in low blue, this is prevented by GA deficiency (PAC = paclobutrazol), and rescued by additional GA_3.

This low blue-induced RGA degradation was prevented by paclobutrazol treatment which even increased the abundance of GFP-RGA in both light conditions (Fig. 5). Addition of GA to these GA-deficient plants induced complete disappearance of the RGA-GFP signal irrespective of the light treatment (Fig. 5). The same effect on GFP-RGA abundance was obtained in hypocotyls grown on GA only (Fig. 5). These data

indicate that at least one of the main DELLA proteins, RGA, is degraded by low blue light photon fluence rates in a GA-dependent manner.

Discussion

The focus of this study was on hypocotyl elongation of Arabidopsis seedlings as induced by reduced blue light photon fluence rates. First, it was studied which photoreceptors sense the reduction in blue light intensity to regulate hypocotyl elongation. Second, we investigated if low blue-mediated hypocotyl elongation is regulated through the gibberellin-DELLA pathway. Low blue light induces a strong elongation response in Arabidopsis hypocotyls. The effect of low blue on petiole elongation was considerably weaker and delayed compared to that of the low R:FR light signal (Chapter 2). This would suggest that low blue light is not likely to be a main trigger for early induction of shade avoidance in petioles. However, in the long run low blue signaling might still enhance shade avoidance of petioles.

Hypocotyl elongation induced by low blue light results mainly from cryptochrome signaling

Blue light in plants is mainly perceived by two photoreceptor families, cryptochromes and phototropins (Lin and Shalitin, 2003). It has been shown that blue light arrests elongation of etiolated Arabidopsis seedlings, mainly through cryptochrome-specific activity which can be preceded by transient phototropin-mediated regulation (Folta and Spalding, 2001). The response to low blue, although reduced, was still present in the constitutively elongated *cry1* mutant suggesting involvement of additional photoreceptors. Complete absence of low blue-induced elongation in the c*ry1 cry2* double mutant, which also displays constitutively elongated hypocotyls, suggests that increased hypocotyl elongation in low blue light results from the combined CRY1 and CRY2 action. Phototropin mutants did not display constitutively elongated phenotypes and did respond to low blue. This would suggest that these photoreceptors do not play a role in low blue-induced elongation. However, the *phot1* and *phot1 phot2* mutants

displayed a reduced hypocotyl elongation response to low blue, suggesting that they are in some way involved. This subtle role for PHOT1 may be explained by a transient involvement of this receptor, for example only in the early induction of the response, as has also been suggested for phototropin involvement in hypocotyl de-etiolation (Folta and Spalding, 2001). As such transient effects were not studied in our low blue experiments, this remains speculative. Finally, phytochromes can also absorb blue light but the quadruple *phyABDE* mutant and the chromophore mutant *hy2*, lacking all functional phytochrome activity (Parks and Quail, 1991), both showed normal responsiveness to low blue (Fig. 2). This suggests that phytochromes are not essential for low blue-induced stimulation of hypocotyl elongation.

GA involvement in blue light response is through degradation of DELLA proteins

Once perceived, the low blue signal is, among others, transduced into the hypocotyl elongation response. Our study shows that the presence of GA is essential for a normal reaction of hypocotyl elongation to low blue photon fluence rates, following from the seriously reduced response of GA-deficient seedlings (Fig. 3). GA-application to these plants rescued the response to control levels (Fig. 3) supporting the notion that these phenotypes result from GA deficiency and not from hypothetical unknown side-effects., GA applied to plants with a normal GA content did not induce further elongation (Fig. 3), which may indicate that normal low blue-induced elongation can not result from a putative low blue-induced increase in GA-biosynthesis only. This is in agreement with Zhao et al. (2007) who did not detect significant differences in the GA content between dark or light grown shoots of wt and *cry1 cry2* Arabidopsis seedlings. Apparently, GA regulation of low blue-mediated elongation may also involve modification at the GA response level.

GA-signaling involves regulation of the DELLA proteins that suppress GA-inducible growth (Peng et al., 1997; Silverstone et al., 1998; Dill et al., 2001). DELLA proteins are degraded in a GA-dependent manner, and the proteins are, therefore, most abundant upon GA deficiency or reduced responsiveness to this hormone. GA can form a complex with its receptor GID1 (GIBBERELLIN INSENSITIVE DWARF 1), by

GID1 binding to the N-terminal DELLA-motif. This stimulates DELLA breakdown in an ubiquitination-dependent, proteasomal pathway (Ueguchi-Tanaka et al., 2005; Nakajima et al., 2006). Gain-of-function mutations in the DELLA-encoding sequence result in a form of the DELLA-motif that is unrecognizable to the GA-GID1 complex, thus leading to increased stability of DELLA proteins (*gai* mutant). The functional, C-domain, in these proteins remains intact and active leading to constitutively dwarfed and GA-insensitive plants, irrespective of the increased GA-content in these mutants (Peng et al., 1997). In our study, the open-frame deletion of 51-base pairs in the DELLA encoding-region of *GAI* resulted in the considerably reduced elongation response to low blue of the *gai* hypocotyls (Fig. 4A). This suggests that the DELLA protein GAI can actively suppress low blue-mediated hypocotyl elongation in Arabidopsis seedlings.

Knocking-out DELLA genes can counteract some aspects of GA-deficiency (Dill and Sun, 2001; King et al., 2001). Our study showed that such DELLA null mutations can partly rescue low blue-induced elongation of GA-deficient hypocotyls (Figs 4B and C). This was most pronounced in the quadruple DELLA knock out *gai-t6 rga-24 rgl1-1 rgl2-1* grown on paclobutrazol (Fig. 4B) and the double *gai-t6 rga-24* in the *ga1-3* background (Fig. 4C). It followed that GAI and RGA are the most effective DELLA family members with respect to suppression of the elongation response to low blue. Moreover, these two proteins seem to constrain hypocotyl growth in general, since GAI and RGA knock-outs with normal GA content displayed slightly elongated phenotypes irrespective of the light environment (Fig. 4A). This agrees with studies by King et al., (2001) and Dill and Sun (2001) which showed that these two DELLA proteins are the most effective regulators of vegetative growth as compared to the RGL proteins.

The hypocotyl elongation response to low blue light photon fluence rates of GA-deficient seedlings thus is constrained by the presence of the growth-inhibiting DELLA proteins. It is, therefore, not surprising that the abundance of the DELLA protein RGA was down regulated during seedling exposure to low blue light. This was shown using the GFP reporter line *pRGA*::*GFP-RGA* whose promoter for RGA governs expression of the protein fusion GFP-RGA (Silverstone et al., 2001). The degradation of

GFP-RGA was shown to occur in a GA-dependent manner, as it was prevented by the GA biosynthesis inhibitor paclobutrazol and rescued by GA add-back (Fig. 5). However, it still remains unknown how low blue light photon fluence rates regulate DELLA abundance. Achard et al. (2007) have found that dark-induced disappearance of RGA protein can be related to a putative increased GA content. This could result from up-regulation of GA-biosynthesis genes and down regulation of those encoding GA-degradation enzymes. Our study showed that GA biosynthesis is not likely to be the sole mechanism to regulate DELLA levels in blue-deficient light. This could imply a more direct connection between cryptochrome signaling and DELLA stability. For example, this could involve an effect of cryptochromes on the interaction between DELLA proteins and the $SCF^{SLY1/GID2}$ complex that regulates poly-ubiquitination of the DELLAs in a GA-dependent manner. One possible option might be a hypothetical regulation of gibberellin receptor (GID1) levels, which is supported by the notion that *GID1* over-expression in rice leads to elongated internodes (Ueguchi-Tanaka et al, 2005). GID1 interacts directly with the $SCF^{SLY1/GID2}$ complex, and upon binding GA, promotes the DELLA-SLY1 interaction which culminates in DELLA degradation (Griffiths et al., 2007; Ueguchi-Tanaka et al., 2007). Another possibility is that cryptochrome signaling reduces DELLA transcript level through an unknown interaction with DELLA-encoding genes.

In conclusion, increased abundance of the DELLA protein RGA in our study was accompanied by suppression of hypocotyl elongation. This was counteracted by GA addition which reduced RGA abundance and rescued hypocotyl elongation. This highlights the importance of DELLA degradation to allow the growth response. However, enhanced DELLA degradation, as in GA-treated transgenic seedlings, did not increase the elongation response to low blue beyond the control level, which was also found for the multiple DELLA knock-outs. Apparently, DELLA proteins are suppressors of low blue-induced hypocotyl elongation when accumulating, as in GA-deficient plants, but their degradation alone cannot induce the complete elongation response of hypocotyls.

These data together indicate that regulation of shade avoidance in low blue-exposed Arabidopsis seedlings, next to DELLA regulation, also involves other (hormonal) signaling routes.

CHAPTER 4

Ethylene and auxin regulate shade avoidance responses mainly through DELLA-independent modes of action in Arabidopsis

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Abstract

Sensing neighbours in dense vegetations through light spectral changes triggers elongation of stems, petioles and hypocotyls thus allowing individual plants to outgrow surrounding neighbours. Previous research showed an important role for the plant hormone GA in regulating growth responses of petioles and hypocotyls induced by the canopy light signals, low R:FR ratio and low blue light photon fluence rates. GA is required in these responses to induce degradation of the growth suppressing DELLA proteins. However, DELLA degradation appeared not to be sufficient to induce these growth responses, indicating the existence of alternative regulatory pathways. We, therefore, study here two other hormones that have been implied in shade avoidance responses: auxin and ethylene. We address the question if these hormones are part of the alternative regulatory routes mediating shade avoidance in Arabidopsis, next to GA-mediated DELLA degradation. To explore this, we studied interactions between auxin, ethylene and GA during low R:FR and low blue light-induced shade avoidance of Arabidopsis petioles and hypocotyls.

We show here that auxin and ethylene are regulators of these elongation responses. Interactions of these two hormones with DELLA proteins appear to exist, but cannot completely account for the observed petiole and hypocotyl elongation responses to neighbour detection signals. This indicates that regulations through ethylene and auxin are at least two of the alternatives that operate next to GA, to regulate shade avoidance responses to canopy light signals.

Introduction

Plants perceive the presence of competing neighbours by sensing the light quality and quantity in dense stands. A reduced ratio of Red:Far-red (R:FR) light is the best known neighbour detection signal, but also blue light photon fluence rates are reduced in dense stands and perceived by plants (Ballaré, 1999; Franklin and Whitelam, 2005; Vandenbussche et al, 2005). Upon sensing these signals, plants can respond with so-called shade avoidance responses that include enhanced elongation growth of hypocotyls, stems and petioles (Smith and Whitelam, 1997; Aphalo et al, 1999). These responses enable plants to outgrow shade imposed by neighbours.

Upon perception of these light signals by photoreceptor proteins, i.e. R:FR-perceiving phytochromes and blue light-perceiving cryptochromes and phototropins, these light cues affect hormonal pathways to ultimately control phenotypic responses (Halliday and Fankhauser, 2003; Vandenbussche et al., 2005). Various studies have highlighted the importance of the growth-promoting hormone gibberellin (GA) in shade avoidance and reduced GA levels or responsiveness abolish many aspects of shade avoidance responses (Weller et al., 1994; Lopez-Juez et al., 1995; Peng and Harberd, 1997; Pierik et al., 2004a). We showed recently that in Arabidopsis GA involvement during shade avoidance acts through degradation of DELLA proteins, which are negative regulators of GA-mediated growth responses (Djakovic-Petrovic et al., 2007). Upon binding of GA to its receptor GID1, DELLA proteins are poly-ubiquitinated by the $SCF^{SLY1/GID2}$ E3 ubiquitin ligase complex and thereby targeted for degradation in the 26S proteasome (Ueguchi-Tanaka et al., 2005; Griffiths et al., 2007; Nakajima et al.,

2006; Ueguchi-Tanaka et al., 2007). We found, however, that the route of GA-mediated DELLA degradation cannot explain the full Arabidopsis elongation response to either low blue or low R:FR as the quadruple DELLA knockout mutant is not constitutively shade avoiding, and still displays pronounced shade avoidance responses to light signals (Djakovic-Petrovic et al., 2007). This implies that alternative (hormonal) routes must operate.

The hormones ethylene and auxin have previously been associated with shade avoidance responses (Steindler et al., 1999; Morelli and Ruberti, 2002; Vandenbussche et al., 2003a; Pierik et al., 2004b), but these two hormones have also been suggested to affect the stability of DELLAs in roots and the hypocotyl hook, thereby interfering with their degradation and abundance (Achard et al., 2003; Fu and Harberd, 2003; Vriezen et al., 2004). The molecular mechanism of these putative interactions is, however, unknown. It also remains elusive to what extent these putative interactions with DELLA stability are general for the various growth regulatory effects of ethylene and auxin.

Ethylene production is enhanced in constitutively shade avoiding phytochrome mutants of different species such as sorghum (*Sorghum bicolore*), Arabidopsis (*A. thaliana*) and pea (*Pisum sativum*) (Finlayson et al., 1998; Vandenbussche et al., 2003a; Foo et al., 2006). Moreover, low R:FR can stimulate ethylene production in for example tobacco (*Nicotiana tabacum*) (Pierik et al., 2004a) and *Brassica napus* (Kurepin et al., 2007). In tobacco, a regulatory role for ethylene in shade avoidance responses was shown in two steps. First, ethylene-insensitive plants showed reduced shade avoidance responses to neighbours, leading to inferior competitive ability with wild type neighbours (Pierik et al., 2003). Second, it was shown that stem elongation and leaf movement responses to reduced total light intensity and to reduced photon fluence rates of blue light, both occurring in dense stands, required intact ethylene signaling (Pierik et al., 2004b).

Ethylene is known to interact with auxin (Lehman et al., 1996; Smalle et al., 1997; Harper et al., 2000; Vandenbussche et al., 2003a; Stepanova et al., 2007; Ružička et al., 2007; Swarup et al., 2007) and auxin has also been implied in shade avoidance (Steindler et al., 1999; Morelli and Ruberti, 2002; Vandenbussche et al., 2003b).

A micro-array study by Devlin et al. (2003) showed that FR-enriched light affects the expression of several auxin transport and signaling genes in Arabidopsis. Previously, Steindler et al (1999) recorded an abolished hypocotyl elongation response to reduced R:FR ratio in the Arabidopsis auxin-resistant *axr1-12* mutant. Furthermore, the same study showed that Arabidopsis seedlings treated with an auxin transport inhibitor also showed a reduced response to this R:FR ratio. A hypothetical model for auxin action in shade avoidance was proposed by Morelli and Ruberti (2002), in which low R:FR light affects auxin distribution by stimulating lateral transport of this hormone towards the outer, epidermal, cell files of the hypocotyl.

The above-mentioned hormones do probably not act independently to regulate shade avoidance responses, but rather may act through an interacting network (Vandenbussche et al., 2005). For example, ethylene-mediated shade avoidance in tobacco acts through GA (Pierik et al., 2004a). Folta et al. (2003) showed that GA and auxin treatment could phenocopy the constitutively elongated *cry1* seedlings only when applied together, indicating that these two hormones may regulate cryptochrome-mediated hypocotyl elongation.

Here, we will study if auxin and ethylene are part of the regulatory network of light-mediated shade avoidance responses in Arabidopsis and if this occurs in a DELLA-dependent or DELLA-independent manner. Our findings indicate a regulatory role for auxin and ethylene in low R:FR-induced petiole elongation and low blue-induced hypocotyl elongation. The precise importance of their activities and interactions with DELLA proteins in regulating shade avoidance will be discussed.

Material and methods

Plant material and growth conditions

The role of ethylene in shade avoidance responses was studied by using the ethylene receptor mutant *ein4* (NASC accession number N8053, Hua et al., 1998) and ethylene signal transduction mutants *ein2-1* (NASC accession number N3071, Alonso et al., 1999), *ein-3-1* (NASC accession number N8052, Chao et al., 1997) and *ein3-1 eil1*

(Chao et al., 1997), all in the Col-0 background. Auxin involvement in shade avoidance responses was studied in petioles and hypocotyls of the auxin-resistant gain-of-function mutant *axr2-1* (NASC accession number N3077, Timpte et al., 1994) in comparison with its wild type Col-0. To study the effect of GA on auxin activity, we used the GUS reporter line *pIAA19*::*GUS* expressing β-glucuronidase (GUS) driven by the *IAA19* promoter sequence (Tatematsu et al., 2004) in the Col-0 background. Interactions between ethylene, auxin and the DELLA proteins were studied in the DELLA quadruple knock-out *gai-t6 rga-24 rgl1-1 rgl2-1* (Achard et al., 2006) and in the transgenic reporter line *pRGA*::*GFP-RGA* (Silverstone et al., 2001), both in L*er* background. To explore the interaction between ethylene and the DELLA protein RGA, we used the GFP reporter line *ctr1-1 pRGA*::*GFP-RGA* in the mixed Col-0/L*er* background. Seeds were generously donated by Dr. J. Ecker (*ein3-1 eil1*), Dr. K. Yamamoto ($pIAA_{19}$::*GUS*) and Dr. N. P. Harberd (*gai-t6 rga-24 rgl1-1 rgl2-1*, *pRGA*::*GFP-RGA* and *ctr1-1RGA*::*GFP-RGA*).

For petiole elongation studies, seeds were sown on filter paper soaked with water, stratified for 4 d. at 4°C and then germinated for 4 d. in a growth chamber with standard growth conditions (20°C, 9 h. light (200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR); Philips Master HPI 400 W) - 15 h. dark). Seedlings were then transferred to 70 ml pots with a 1:2 potting soil:perlite substrate mixture and additional nutrients (Millenaar et al., 2005). At the age of 35 days after sowing, plants were transferred to light treatment boxes for subsequent experiments.

For hypocotyl experiments, seeds were surface-sterilized by 20% (v/v) sodium hypochlorite, rinsed with water and sown on agar (5.5 g l^{-1}) with low nutrients (0.22 g l^{-1} MS, Duchefa Biochemie B.V, The Netherlands). To provoke germination of the GA-deficient mutant *ga1-3*, seeds were kept in 100 μM GA_3 solution (Duchefa Biochemie B.V, The Netherlands) for 4 d. at low temperature (4°C) prior to placing on agar. After 4 d. stratification at 4°C, plates were placed in a growth chamber. One day before the onset of low blue light treatment, plates with seedlings were transferred to a growth chamber and exposed for 2 h. to 200 μmol m^{-2} s^{-1} PAR to provoke germination.

Afterwards, plates were wrapped in aluminium foil and kept in darkness for the subsequent 22 h.

Light treatments

Light quality manipulations took place in a white light background (Philips Master HPI-T Plus 400 W and Philips Plusline Pro 150 W; 15 h. light, 9 h. dark). The R:FR ratio was lowered from 1.2 to 0.25 by supplemental far-red light (730 nm LED; Shinkoh Electronics Co. Ltd., Tokyo, Japan). Blue light photon fluence rates (400-500 nm) were reduced from 26 to < 1 µmol m^{-2} s^{-1} with two layers of Lee010 Medium Yellow filter (Lee Filters, Andover, UK). PAR in all light treatments was maintained at 140 µmol m^{-2} s^{-1}.

Treatments with hormones and their inhibitors

1-MCP and ethylene treatments

To prevent ethylene perception we used the gas 1-methylcyclopropane (1-MCP). This gas interacts with ethylene receptors thus competitively blocking ethylene binding (Sisler and Serek, 2003). Ethylene perception in petioles was blocked by 3 h. pre-treatment with 1-MCP prior to the light treatment. This gas at a final concentration of 10 µl l^{-1} was obtained by incubating EthylBloc powder (Floralife Walterboro, SC) containing 0.14 % 1-MCP with 1.5 ml water in sealed flasks for 15 minutes. The gas 1-MCP was collected from the headspace with a syringe and injected into desiccators with plants. Seedlings were pretreated with 1-MCP by injecting the same concentration of this gas (10 µl l^{-1}) into glass jars with plates. 1-MCP in jars was refreshed every day during the light treatment.

To explore the effect of ethylene on petiole elongation, plants in glass cuvettes (18 L. volume) were treated with air (21 % (v/v) O_2; 0.035 % (v/v) CO_2) supplemented with a range of ethylene concentrations (10^{-2} µl l^{-1}; 0.15 µl l^{-1} and 2 µl l^{-1}) for 24 h. in a growth cabinet (18 °C; 15 h. light (200 µmol $m^{-2}s^{-1}$ PAR; Osram 55W/840 Lumilux and Sylvania F58WT8/2023) - 9 h. dark). The effect of ethylene on seedling growth and

elongation responses was studied by applying ethylene gas (3 µl l^{-1}) or by adding the ethylene biosynthetic precursor ACC (1-aminocyclopropane-1-carboxylic acid, Sigma-Aldrich Co., USA) dissolved in water to the agar plates. Dose response curves of hypocotyl growth to different ACC concentrations (10^{-2} µM; 10^{-1} µM; 1 µm; 10 µM and 20 µM) showed that 10 µM ACC was a saturating concentration which phenocopied the effect of 3 ml l^{-1} ethylene on hypocotyl growth (data not shown).

NPA and IAA treatments

The role of auxin in shade avoidance responses of petioles and hypocotyls was studied by applying an inhibitor of auxin transport 1-naphthylphthalamic acid, (NPA, Duchefa Biochemie B.V., The Netherlands). Leaf rosettes were daily sprayed by 25 µM NPA in water starting from 72 h. before the light treatment. Seedlings were grown on MS media supplemented with 25 µM NPA.

Indol-3-acetic acid (IAA, Duchefa Biochemie B.V., The Netherlands) dissolved in ethanol to a 10 mM stock was added to the plates at a final concentration of 10 µM to investigate the interaction between auxin and ethylene signalling.

GA and paclobutrazol application

To investigate auxin-GA interactions, seedlings of the auxin resistant mutant *axr2-1* were grown on 10 µM GA (diluted from an aqueous 10 mM stock solution) added to the plates. Furthermore, to explore the effect of GA on auxin activity, the GUS reporter line *pIAA19*::*GUS* was grown on the GA biosynthesis inhibitor paclobutrazol (Duchefa Biochemie B.V, The Netherlands) dissolved in water and applied at a final concentration of 1 µM to the plates. Auxin-GA-DELLA interactions were studied in seedlings of the transgenic line *pRGA*::*GFP-RGA* grown on media containing both NPA (25 µM) and GA (10 µM).

Growth measurements

For petiole elongation studies, one day before the experiment plants were transferred to the relevant growth cabinet for acclimation. Light treatments started on the subsequent

day at 10 a.m. and lasted 24 h. Petiole lengths were measured with a digital calliper at t = 0 and t = 24 h. to calculate growth as the length increment over 24 h.

Hypocotyl length measurements were performed on seedlings that had been exposed to control light or low blue light treatment for 7 d. Hypocotyls were measured then with a custom-built image analysis system (Sony CCD camera (type XC-77CE) combined with KS400 software (Carl Zeiss Vision, Oberkochen, Germany)).

Two-way Analysis of variance (ANOVA), with Tukey-B post-hoc comparisons (SPSS V12.0.1), revealed if there were significant differences between genotypes and treatments. Experiments were independently repeated at least three times.

Ethylene production measurement

Ethylene production was measured in young, growing leaves of the Col-0 accession. Approximately 0.3 g of the fresh weight was taken by excising both blades and petioles after 3h. and 27 h. of the light treatment. Leaf material was collected in a 2.5 ml syringe with a volume of 1.5 ml. Material was incubated for 15 minutes after which the gas volume was transferred to a 1 ml syringe and injected into a gas chromatograph (GC955; Synspec, Groningen, The Netherlands). Care was taken to sample ethylene from the leaf tissue within 20 minutes to prevent wound-induced ethylene which is accumulating after this period, as verified previously.

Ethylene production in seedlings was measured in the last 24 h. of light treatment. 35 seedlings were grown in 10 ml glass flasks on 5 ml agar (5.5 g l^{-1}) with low nutrients (0.22 g l^{-1} MS, Duchefa Biochemie B.V, The Netherlands). After 6 days in low blue treatment, flasks were flushed with air and closed for a subsequent 24 h. to allow accumulation of ethylene in low blue light. A sample from the headspace was taken by a 1ml syringe and injected into a gas chromatograph (GC955; Synspec, Groningen, The Netherlands).

The ethylene concentration was calculated in moles produced per gram fresh weight per hour.

DELLA abundance

The abundance of the DELLA protein RGA was studied with the *pRGA*::*GFP-RGA* reporter line (Achard et al., 2003; Achard et al., 2006; Silverstone et al., 2001). Fluorescence was detected with an inverted confocal laser scanning microscope (Zeiss LSM Pascal, 40x C-apochromat objective). Excitation wavelength was 488 nm, a 505-530 nm bandpath filter was used for GFP emission and a 560 nm long pass filter was used to visualize red fluorescence by chloroplasts. Images depict 149.5 µm Z-stacks of petioles and hypocotyls. GFP-RGA fluorescence of petioles and hypocotyls was determined at the same time-points as the length measurements. The basal, middle and uppermost regions of hypocotyls and petioles were studied, but only images from the basal region (abaxial in petioles with the mid-rib horizontally approx. half-way the image) are shown. Confocal images are representative selections from at least nine replicates of at least three independent experiments.

GUS assay

GUS assays were performed on *pIAA19*::*GUS* rosettes and seedlings to study auxin action in low R:FR and low blue light, respectively. The expression of β-glucuronidase in this line is driven by the auxin-inducible *IAA19* promoter (Tatematsu et al., 2004). The GUS assay in leaf rosettes was performed after submerging in cold acetone (90 % (v/v) for 20 seconds and fixing in 0.3% formaldehyde in 10 mM MES (pH 5.6), 0.3 M mannitol for 45 minutes at 4°C. Tissue was washed then with 100 mM Na-phosphate buffer, pH 7.2. The histochemical reaction was performed by incubating leaf material for 24 h. with 1 mM XGlucA (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Duchefa, The Netherlands)) in 100 mM Na-phosphate buffer, pH 7.2, 1mM EDTA. After staining, leaf rosettes were rinsed by phosphate buffer and bleached in an ethanol series from 50 to 90% and then photographed.

GUS staining in seedlings was obtained after 24 h. incubation in the GUS-staining solution (1 mM X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) in 100 mM sodium phosphate, pH 7.0, with 10 mM EDTA, 1 mM $K_4Fe(CN)_6$, 1 mM

$K_3Fe(CN)_6$, and 0.1 % Triton X-100) at 37°C. After incubation, seedlings were placed in 70 % ethanol for destaining of green tissues and conservation.

Results

Low R:FR-induced petiole elongation is regulated by ethylene

A first indication that ethylene may play a role in shade avoidance responses induced by low R:FR in Arabidopsis comes from the observation that ethylene production is stimulated by this treatment (Fig. 1A). Low R:FR induced a 1.5-fold increase of ethylene emission from leaves after 3 h. of treatment. To study if ethylene is functionally involved in regulating low R:FR-induced elongation, we tested the effect of the ethylene perception inhibitor 1-MCP on this response in petioles. Fig. 1B shows that petiole elongation in response to low R:FR was inhibited by 1-MCP, indicating the involvement of ethylene in this growth response. The importance of ethylene was further established by the absence of low R:FR-induced petiole elongation in the ethylene insensitive mutants *ein4*, *ein2-1* and *ein3-1 eil1* (Fig. 1C). However, the application of a range of ethylene concentrations to leaf rosettes did not result in a significant stimulation of petiole elongation (data not shown) in the absence of a low R:FR signal. This together suggests that ethylene is required for low R:FR-induced petiole elongation, but that ethylene alone can not induce this response.

Next, we checked if ethylene signalling interacts with the DELLA proteins in regulating low R:FR-induced petiole elongation. This was done by comparing the effect of 1-MCP on the petiole elongation response of the DELLA quadruple knock-out *gai-t6 rga-24 rgl1-1 rgl2-1* and its corresponding wild type L*er*. If ethylene would functionally interact with DELLA proteins (e.g. a destabilizing effect) we would expect a normal quadruple mutant response to low R:FR in the presence of 1-MCP. However, blocking ethylene perception by 1-MCP reduced the low R:FR-induced elongation in petioles of L*er* and the elongation response of the quadruple DELLA knock-out was even completely inhibited (Fig. 1D).

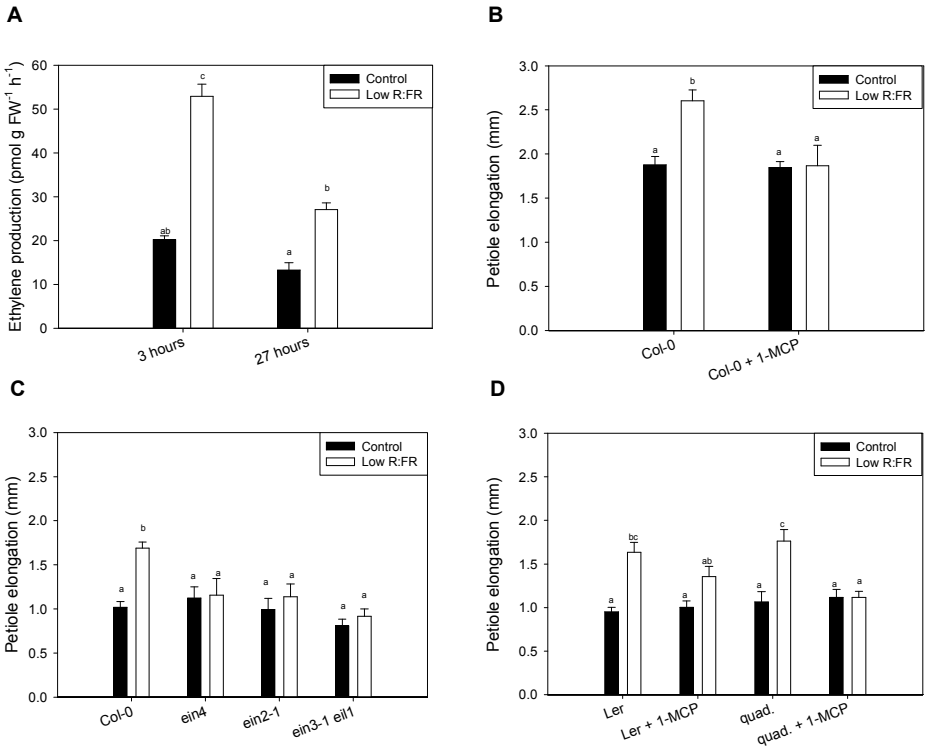

Figure 1. The involvement of ethylene in low R:FR-induced petiole elongation. A) Ethylene production in Col-0 leaves after 3 and 27 h. of the low R:FR treatment. The effect of ethylene insensitivity on petiole elongation response to 24 h low R:FR treatment in B) 1-MCP treated plants and C) ethylene mutants; D) 1-MCP effect on petiole elongation of the quadruple DELLA knock-out. Except for ethylene measurements (n=3), all data are means and SE (n=10). No overlapping letters indicate a significant difference (P<0.05).

This suggests that ethylene does not primarily act through a hypothesized interaction with DELLAs to regulate low R:FR-induced petiole elongation.

Auxin is required for low R:FR-induced increase in petiole elongation

The effect of low R:FR treatment on auxin action was studied by using the *pIAA19*::*GUS* reporter line. Low R:FR increased auxin action, detected by stronger GUS-staining in petioles (Fig. 2A). Functional involvement of auxin in regulating low R:FR-induced petiole elongation was explored by studying petiole elongation in the auxin-resistant mutant *axr2-1*, and by monitoring petiole elongation in plants that were

treated with the auxin transport inhibitor NPA. Petioles of the *axr2-1* mutant did not respond to low R:FR by elongation and also displayed constitutively low elongation rates (Fig. 2B). Blocking auxin transport by NPA in L*er* also resulted in the absence of a low R:FR-induced elongation response of petioles (Fig. 2C). These findings together indicate a regulatory role for auxin in low R:FR-induced petiole elongation.

To explore if auxin interacts with DELLA proteins while mediating shade avoidance in petioles, we monitored the effect of NPA application on the petiole elongation response of the quadruple DELLA knock-out. Results in figure 2D do not suggest a functional interaction between auxin and DELLAs since the absence of DELLAs in the quadruple knock-out did not rescue the NPA-inhibited petiole elongation response to low R:FR.

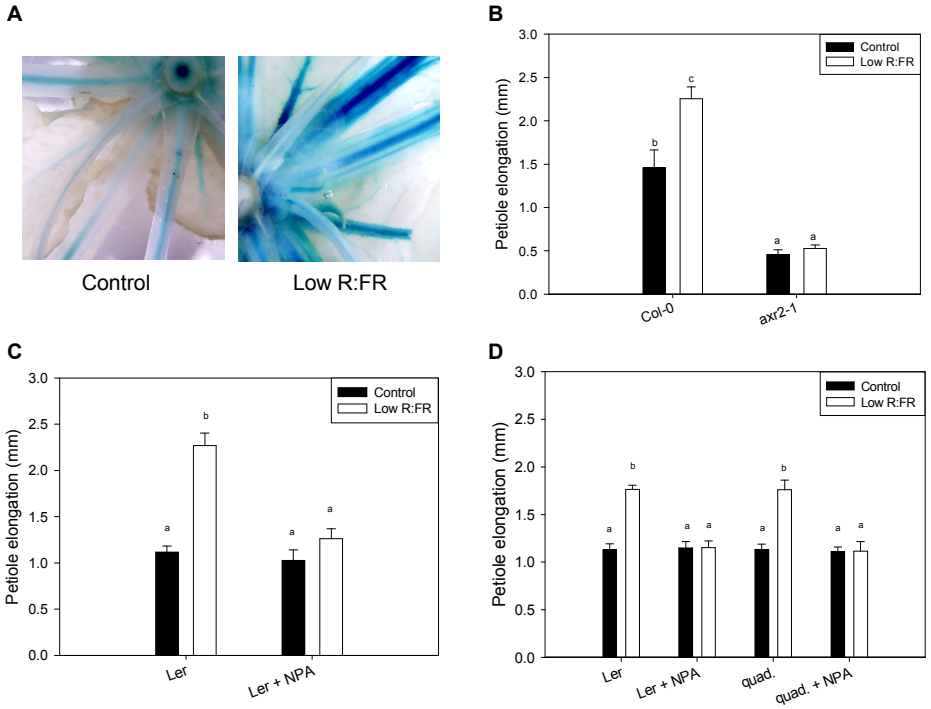

Figure 2. The role of auxin in petiole elongation after 24 h of low R:FR treatment. A) The effect of low R:FR treatment on auxin activity visualized in petioles of *pIAA19*::*GUS*. B) Petiole elongation response to low R:FR of the auxin-resistant *axr2-1* mutant. C) The effect of 25 µM NPA on low R:FR-induced petiole elongation. D) NPA effect on petiole elongation of the quadruple DELLA knock-out. Data are means (n=10) ± S.E. No overlapping letters indicate a significant difference (P<0.05).

Ethylene involvement in low blue-induced hypocotyl elongation

We have shown before that low blue light photon fluence rates induced strong elongation of hypocotyls (Chapter 3; Djakovic-Petrovic et al. (2007)). This elongation response appears to be only partly ethylene-dependent since some of the ethylene-insensitive mutants and 1-MCP-treated seedlings showed a reduced but not absent elongation response to low blue (Fig. 3B and C). Furthermore, ethylene production of Arabidopsis seedlings appeared not to be affected by low blue light photon fluence rates (Fig. 3A).

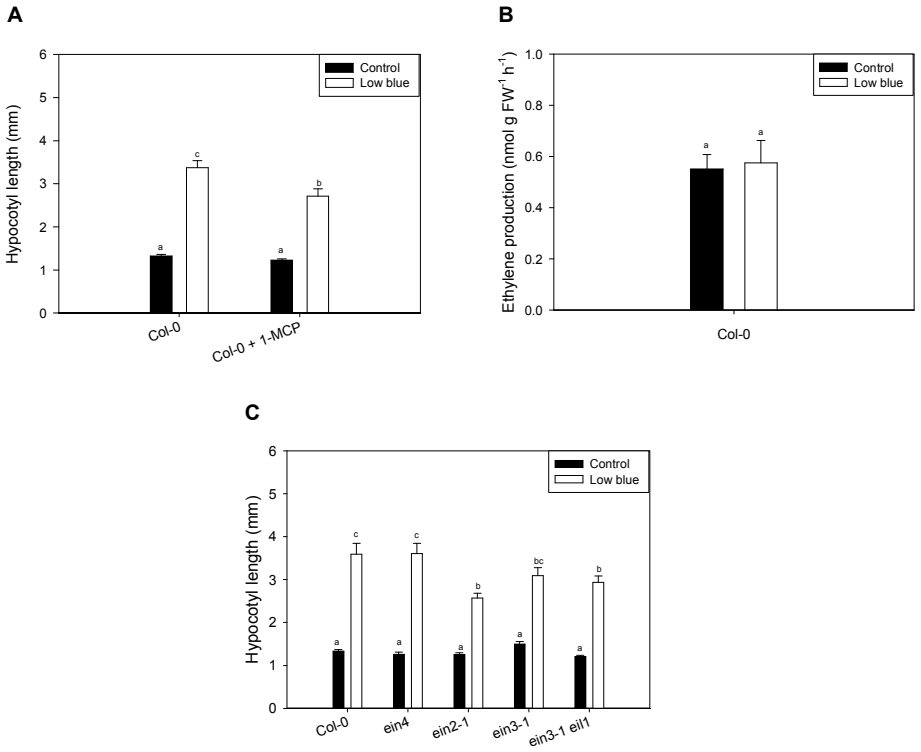

Figure 3. Ethylene involvement in low blue light-induced elongation of hypocotyls. A) Ethylene production in seedlings exposed to 24 h. low blue light treatment. The effect of ethylene insensitivity on 7 d. low blue-induced elongation response in B) 1-MCP treated seedlings and C) ethylene mutants (B). Data are means and SE (n=35). No overlapping letters indicate a significant difference (P<0.05).

However, in contrast to petioles, enhanced ethylene levels, reached through application of the biosynthetic ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid), significantly stimulated hypocotyl elongation of the wild type L*er* accession in control light (Fig. 4A). This is similar to what has been described in literature for the Col-0 accession (Smalle et al., 1997). ACC did not further stimulate hypocotyl lengths of already elongated low blue-treated seedlings, indicating that the effect of ethylene on hypocotyl length can be light-dependent or that the elongation response cascade is already saturated.

Ethylene-DELLA interactions during low blue-induced hypocotyl elongation

The reducing effect of 1-MCP on low blue-induced hypocotyl elongation was not alleviated by knocking out four out of five DELLA genes in the quadruple DELLA knock-out (Fig. 4B), suggesting that ethylene does not act through DELLA proteins. Although this quadruple DELLA knockout seems to show slightly higher growth stimulation by ACC concentration series in control light than does WT (Fig. 4A), there was no significant genotype x ACC interaction. In low blue, both WT and the DELLA quadruple knockout showed no significant response to any of the ACC concentrations tested. To further analyse if there is regulation of DELLA protein abundance by ethylene, we studied the effect of ACC on abundance of the DELLA protein RGA in the *pRGA*::*GFP-RGA* reporter. An ACC concentration of 10 µM increased GFP-RGA abundance in low blue treatment, but did not give an obvious effect in control light (Fig. 4C). Consistently, the *constitutive triple response* mutant *ctr1-1* crossed with the *pRGA*::*GFP-RGA* reporter also displayed enhanced RGA abundance in low blue (Fig. 4C). Interestingly, during ACC–induced stimulation of hypocotyl elongation in control light, RGA abundance was not detectably affected, while in low blue the lack of ACC-induced elongation was paralleled by enhanced abundance of the RGA protein. This would suggest that a positive growth response to ACC in low blue is prevented by an ACC-mediated increase of RGA abundance. However, this is unlikely as ACC could not further stimulate elongation of low blue-exposed quadruple DELLA knockouts where DELLA stabilization cannot occur.

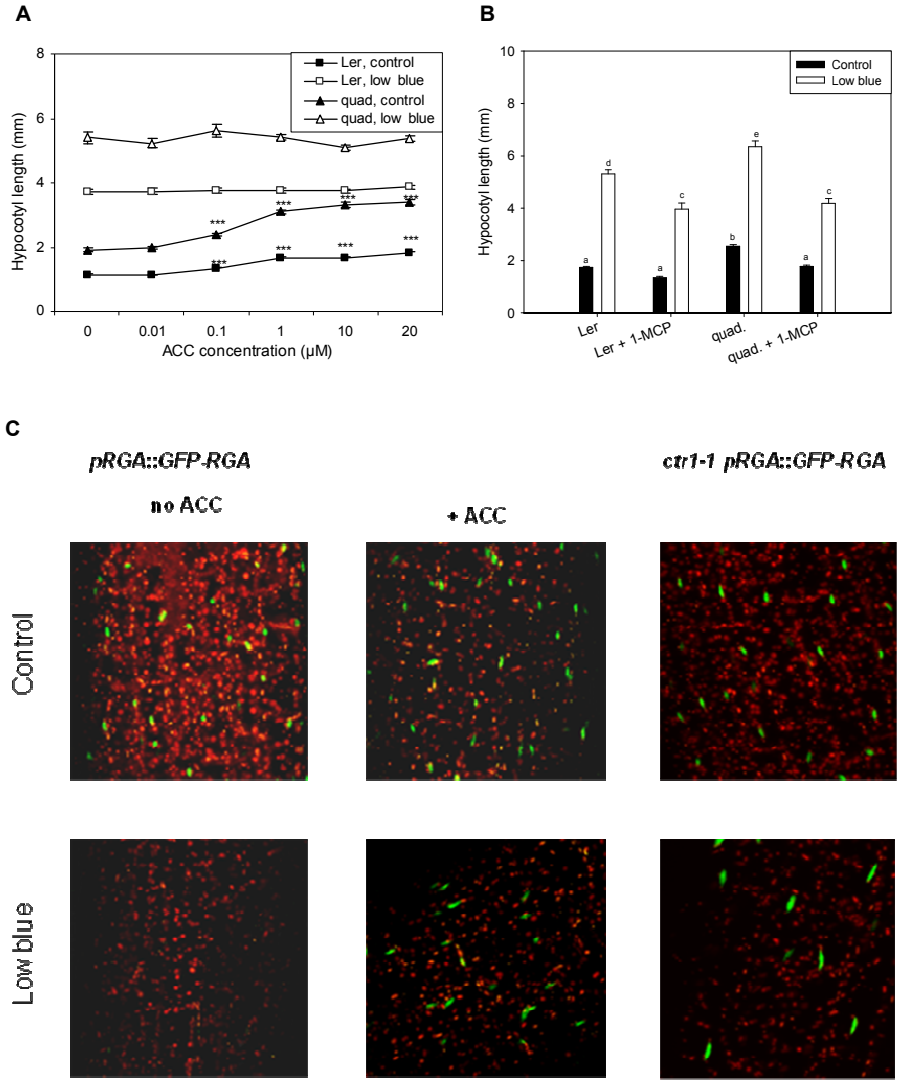

Figure 4. Interaction between ethylene signaling and the DELLA proteins in 7 d. low blue-treated seedlings. A) Dose–response curve of L*er* and the quadruple DELLA hypocotyl elongation to different ACC concentrations. B) The effect of 1-MCP on the hypocotyl elongation response of the quadruple DELLA knock-out. C) The abundance of GFP-RGA signal in ACC-treated *pRGA::GFP-RGA* and constitutive responsive *ctr1-1 pRGA*::*GFP-RGA* seedlings. Data are means and SE (n=25-35). No overlapping letters indicate a significant difference (P<0.05).

We, therefore, checked if the ACC-induced hypocotyl elongation involves GA and DELLA at all, by testing the GA-deficient *ga1-3* and GA-insensitive *gai* mutants, both having enhanced DELLA abundance. Data in Fig. 5 indicate that ACC can induce hypocotyl elongation in these mutants (in controls and in low blue treated hypocotyls) suggesting that ACC can stimulate hypocotyl elongation independently of GA and DELLA proteins.

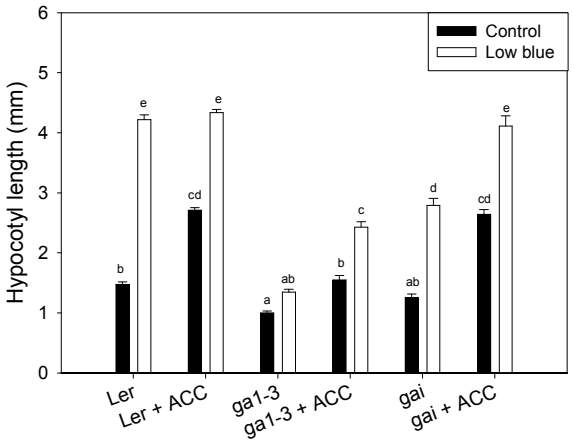

Figure 5. The effect of 10μM ACC application on hypocotyl growth and elongation response to 7 d. low blue treatment of GA-deficient (*ga1-3)* and GA-insensitive (*gai*) mutants. Data are means and SE (n=25-35). No overlapping letters indicate a significant difference (P<0.05).

Auxin is important for low blue-induced hypocotyl elongation

As ethylene appears to regulate hypocotyl elongation independently of GA, the next step was to investigate if ethylene acts alone or through interaction with auxin. Therefore, it was studied if stimulation of hypocotyl elongation by ACC can still commence in the auxin-resistant mutant *axr2-1*. Fig. 6 shows that ACC stimulates elongation of *axr2-1* hypocotyls in low blue light suggesting that ethylene can partly regulate hypocotyl elongation independently of auxin. This growth stimulation by ACC was not recorded in hypocotyls grown in control light (Fig. 6) which suggests that the interaction between ethylene and auxin depends on the light environment. Auxin-induced hypocotyl elongation did not depend on intact ethylene signalling as the auxin IAA could stimulate hypocotyl elongation in both WT and the ethylene insensitive *ein3-1 eil1* mutant (Fig. 6). In fact, IAA addition to *ein3-1 eil1* seedlings completely rescued hypocotyl elongation of this mutant to the WT level in both light conditions.

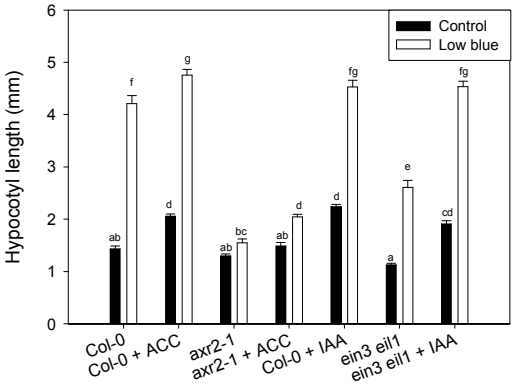

Figure 6. The effect of 10 µM ACC and 10 µM IAA on hypocotyl elongation in control light and in low blue of auxin- and ethylene-insensitive mutants. Data are means and SE (n=25-35). No overlapping letters indicate a significant difference (*P*<0.05).

The fact that the low blue response was severely inhibited in *axr2-1* indicates that auxin could be an essential component in regulating low blue-induced hypocotyl elongation. Accordingly, auxin action was enhanced in low blue-exposed hypocotyls, particularly in the more lateral hypocotyl regions, as can be derived from the enhanced *pIAA19*::*GUS* activity (Fig. 7).

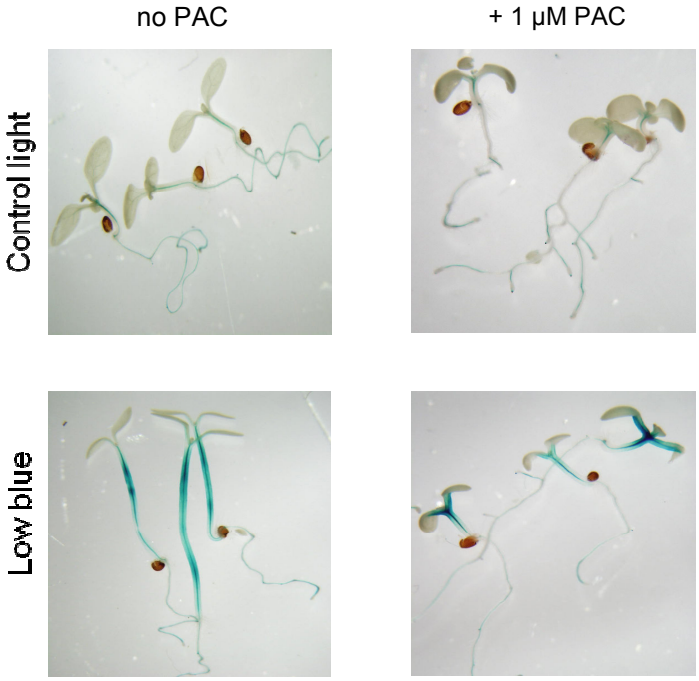

Figure 7. The effect of low blue light and paclobutrazol (PAC) application on auxin activity, as visualized with *pIAA19*::*GUS* reporter line.

This pattern of *pIAA19*::*GUS* distribution was abolished by NPA treatment (D. Keuskamp & R. Pierik, unpublished results), confirming that *IAA19* is a relevant indicator for auxin action. The pattern of *pIAA19*::*GUS* activity was not affected by GA-deficiency suggesting that low blue light affects auxin action independently of GA.

Auxin regulates low blue-induced hypocotyl elongation mainly independently of DELLA

We further tested if auxin interacts with the DELLA proteins by applying the auxin transport inhibitor NPA to DELLA knock-outs. Data in Fig. 8B indicate that NPA-induced reduction of low blue-induced hypocotyl elongation cannot be rescued by removing DELLAs.

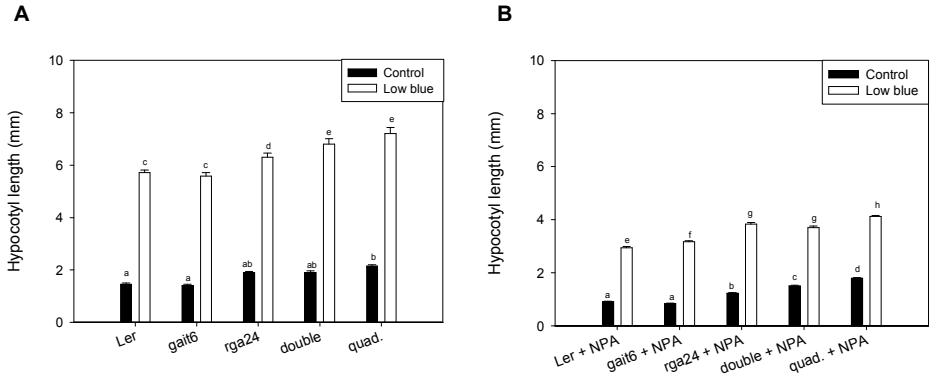

Figure 8. The effect of NPA on hypocotyl elongation response to 7 d. low blue treatment of DELLA knock-outs. A) DELLA knock outs grown in the absence of NPA. B) The effect of 25 µM NPA on hypocotyl elongation in control light and low blue. Data are means and SE (n=25-35). No overlapping letters indicate a significant difference (P<0.05).

This suggests that a putative auxin-DELLA interaction cannot explain the regulatory role of auxin in this growth response. However, NPA constitutively reduced hypocotyl length irrespective of light conditions (Fig. 8B), and this was counteracted by DELLA knockouts in control light. Therefore, we studied the effect of NPA on DELLA abundance in the *pRGA*::*GFP-RGA* reporter line. Our findings showed that NPA enhanced the GFP-RGA signal irrespective of the light (Fig. 9A) which corresponds with its general growth inhibiting effect (Fig. 8B and 9B). GA addition to NPA-treated seedlings induced complete disappearance of the GFP-RGA signal in both light

conditions (Fig. 9A) which was accompanied by fully rescued elongation in control light (Fig. 9B). The low blue response, however, was not restored completely by GA addition to NPA-treated seedlings despite the GA-induced degradation of RGA (Fig. 9A). This suggests that undisturbed auxin transport is essential for the elongation response to low blue in a DELLA-independent manner.

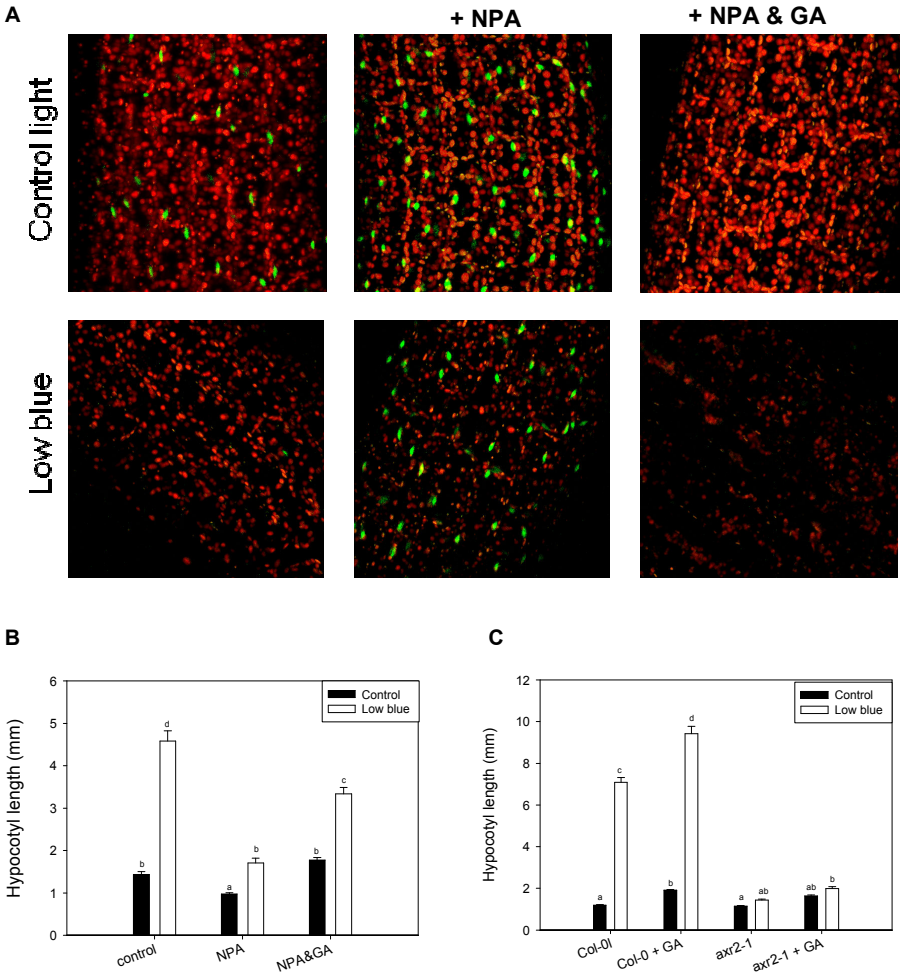

Figure 9. The effect of auxin interaction with GA-signaling on DELLA protein abundance and hypocotyl elongation. A) The effect of NPA and GA application on the abundance of the DELLA protein RGA in hypocotyls of *pRGA*::*GFP-RGA* reporter and B) corresponding hypocotyl elongation. C) Hypocotyl elongation of the auxin-resistant *axr2-1* mutant after GA application. No overlapping letters indicate a significant difference (P<0.05).

Consistent with this conclusion, it was shown that inhibited low blue-induced hypocotyl elongation in the auxin resistant *axr2-1* mutant cannot be rescued by adding GA (Fig. 9C).

Discussion

Previous studies (Chapters 2 and 3; Djakovic-Petrovic et al., 2007) showed that GA is important for light quality-induced stimulation of elongation in Arabidopsis petioles and hypocotyls to induce degradation of the growth suppressing DELLA proteins. This relief of DELLA restraint alone, however, appeared insufficient to induce elongation of petioles and hypocotyls indicating involvement of additional (hormonal) route(s) in stimulating elongation, provided that DELLA proteins are degraded. Therefore, we extended the focus in this study to two other hormonal signals, ethylene and auxin. These hormones may regulate elongation growth, induced by neighbour detection signals, but may also interact with DELLA proteins.

Ethylene involvement in the regulation of petiole and hypocotyl elongation responses to canopy light cues

Photocontrol of ethylene production has been indicated by several studies which showed that phytochrome inactivation, e.g. by low R:FR, can induce an increase in ethylene production (Finlayson et al., 1998; Vandenbussche et al., 2003a; Pierik et al., 2004a; Foo et al., 2006). Our research confirmed that low R:FR light stimulates ethylene production in Arabidopsis leaves (Fig. 1A). It was also shown that ethylene-insensitive petioles can not respond to low R:FR by enhanced elongation (Fig. 1B and C), which is consistent with the reduced responsiveness to neighbours of ethylene-insensitive tobacco (Pierik et al., 2004b). Although important for these light-mediated growth responses, ethylene application itself did not induce elongation of Arabidopsis petioles. This suggests that ethylene signaling is required for a normal growth response to low R:FR, but that an elevated ethylene concentration alone is not sufficient to induce petiole elongation.

Interestingly, hypocotyl data for low blue-induced elongation gave a somewhat different view on the role of ethylene. First, we did not detect an increase in ethylene production upon low blue light treatment (Fig. 3A) and second, ethylene insensitivity only mildly (e.g. 20% reduction in 1-MCP) affected the hypocotyl elongation response to low blue light (Fig. 3B and C). However, externally applied ACC, the biosynthetic precursor from which ethylene is produced by the non-rate-limiting ACC-oxidase enzyme family, induced clear hypocotyl elongation in control light (Fig. 4A).

Ethylene, thus, is important for low R:FR-induced petiole elongation and to a lesser extent for low blue-induced hypocotyl elongation. Furthermore, elevated ethylene levels which have also been suggested to serve as a primary neighbour detection signal in canopies (Pierik et al., 2004b), can stimulate hypocotyl, but not petiole elongation in Arabidopsis.

Do auxin and ethylene interact to regulate shade avoidance?

A considerable number of studies have shown that ethylene and auxin signaling can be intertwined at different regulatory levels to control growth of plant organs (Visser et al., 1996; Lehman et al., 1996; Smalle et al., 1997; Harper et al., 2000; Vandenbussche et al., 2003b; Stepanova et al., 2007, Ružička et al., 2007; Swarup et al., 2007). Our data on hypocotyls of Arabidopsis seedlings show that the requirement of auxin for ethylene-induced growth effects depends strongly on the light conditions. In low blue light ethylene may regulate hypocotyl elongation independently of auxin as the auxin resistant *axr2-1* mutant showed ACC-induced stimulation of hypocotyl elongation (Fig. 6). However, ACC could not significantly stimulate hypocotyl elongation in this mutant when grown in control light conditions. Blue light, thus, seems to affect functional interactions between ethylene and auxin.

Interestingly, hypocotyl length of the ethylene-insensitive *ein3-1 eil1* mutant was reduced in control and low blue light conditions, but this was rescued by application of the naturally occurring auxin IAA in both light conditions (Fig. 6). This suggests that auxin acts downstream rather than upstream of ethylene in one signal transduction cascade. Alternatively, auxin may act independently of ethylene to regulate

hypocotyl elongation irrespective of the light conditions. The observed reduced or even absent shade avoidance responses to low R:FR and low blue of the auxin resistant mutant *axr2-1* and of NPA-treated wild type plants are in agreement with findings by Steindler et al. (1999). They showed that the auxin resistant *axr1-12* mutant has a reduced R:FR response. In agreement with this regulatory role of auxin is the observed putative increase of auxin action, visualized through *pIAA19*::*GUS* staining, in petioles and hypocotyls upon low R:FR and low blue exposure, respectively (Fig. 2A and 7). The enhanced auxin action during shade avoidance seems to be particularly located in the more lateral regions of the petiole or hypocotyl, which is consistent with the model by Morelli and Ruberti (2002) on low R:FR-stimulated lateral transport of this hormone. It remains to be elucidated if the activity is enhanced really in the epidermal cells, which are thought to drive shoot elongation growth (Savaldi-Goldstein et al., 2007).

Auxin and ethylene regulate shade avoidance mainly independently of interactions with DELLA proteins

Auxin can affect GA signaling by modulating GA-inducible degradation of DELLA proteins, as shown by Fu and Harberd (2003). Our study confirms that auxin can affect DELLA abundance as the auxin transport inhibitor NPA increased abundance of the DELLA protein RGA in hypocotyls (Fig. 9A). This presumably explains the general reduction of hypocotyl growth irrespective of light conditions by NPA as this effect was strongly counteracted by multiple DELLA knock-outs (Fig. 8B). However, this interaction can not account for the reduced low blue-induced hypocotyl elongation since DELLA knockout did not rescue the elongation response to low blue in NPA-treated seedlings. Accordingly, GA application could also not rescue the elongation response in NPA treated wild type or in the *axr2-1* mutant (Fig. 9). The same can be concluded for low R:FR-induced petiole elongation which was inhibited by NPA and not rescued by multiple DELLA knock-out (Fig. 2D). Apparently, the auxin interaction with DELLA proteins, although clearly visible at the level of protein abundance, is not functional in determining the shade avoidance elongation response.

It has been suggested that ethylene, applied as ACC, can stabilize the DELLA protein RGA and thereby inhibit root elongation (Achard et al., 2003). Our data confirm that ACC can prevent degradation of the DELLA protein RGA, but only detectably so in the low blue light treatment (Fig. 4C). This blue light-dependent effect of ACC on RGA protein abundance co-occurs with a lack of ACC-induced stimulation of hypocotyl elongation in low blue light, as compared to a clear elongation response in control light (Fig. 5). The lack of an ACC effect on hypocotyl length in low blue can, however, not be related to the ACC-induced increase of DELLA abundance in this light condition since ACC did also not affect elongation in low blue-exposed quadruple DELLA knock-out plants (Fig. 4A). It even seems that ACC-mediated hypocotyl elongation is independent of GA and DELLA proteins altogether. This is because the GA-deficient *ga1-3* mutant and the DELLA gain-of-function *gai* mutant both show clear responses to ACC application in control light as well as in low blue light conditions (Fig. 5). As ethylene also seems to act independently of auxin in low blue light conditions, this might suggest interactions of ethylene with other growth regulators that were not studied here. Alternatively, it is also possible that ethylene would regulate elongation growth in a more direct manner. It is, for example, known from studies on the semi-aquatic species *Rumex palustris* that ethylene can directly affect processes such as apoplastic acidification, expansin abundance and expansin-mediated cell wall extensibility (Vreeburg et al., 2005).

In Fig. 10 the observed hormonal interactions are summarized into a schematic working model for shade avoidance in Arabidopsis. In short, our previous studies showed that low R:FR and low blue light positively regulate GA-induced DELLA degradation in petioles and hypocotyls (Djakovic-Petrovic et al., 2007). These two light signals also act through ethylene, with low R:FR stimulating ethylene production of petioles. Furthermore, ethylene itself might also act as a neighbour detection signal in canopies (Pierik et al., 2004b) that can induce elongation of Arabidopsis hypocotyls. Although ethylene can interact with DELLA proteins, ethylene most likely regulates hypocotyl elongation growth during shade avoidance in a DELLA-independent manner. In low blue light, ethylene, exogenously applied as ACC, appears to stimulate hypocotyl

elongation independently of auxin. It, however, remains to be studied if non-manipulated, endogenous ethylene levels act through auxin or not. In control light conditions, ACC-induced elongation does depend strongly on an intact auxin pathway. Auxin action itself also seems to be stimulated during shade avoidance and auxin is essential to the growth responses observed. It is possible that auxin action is regulated in an ethylene-dependent manner but this has not been fully resolved yet. Auxin on its turn, seems to stimulate DELLA degradation in hypocotyls, but likely acts independently of DELLAs to stimulate shade avoidance responses of low blue-exposed hypocotyls and low R:FR-exposed petioles.

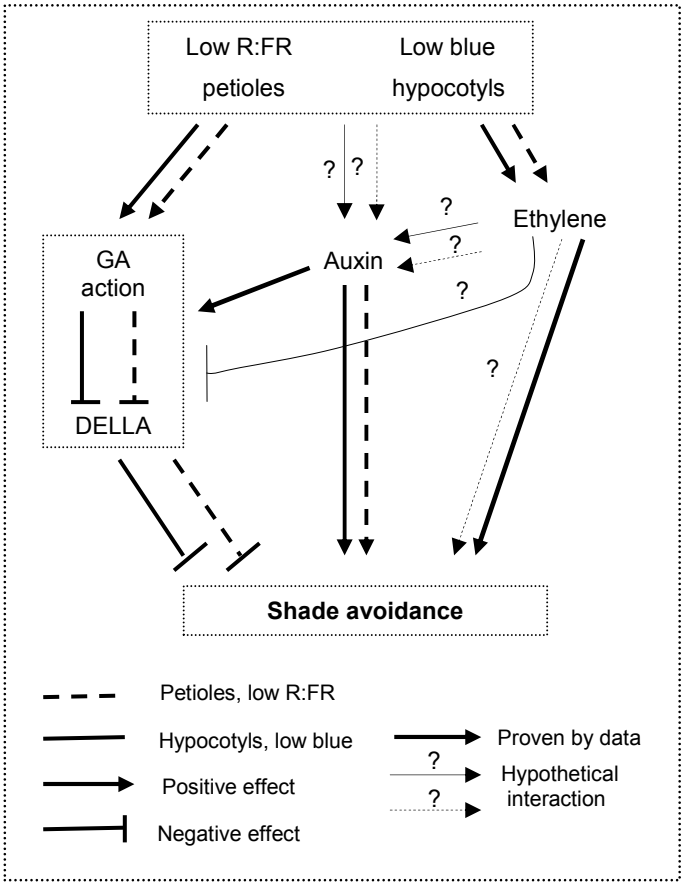

Figure 10. Schematic summary of the effect of ethylene and auxin action and their interaction with GA-DELLA signaling in regulating shade avoidance responses in petioles and hypocotyls.

In summary, this research shows that ethylene and auxin signaling can be considered as alternative pathways to GA-DELLA in regulating light quality-induced petiole and hypocotyl elongation. These two hormones can interact with DELLA proteins but the growth responses result mainly from DELLA-independent signaling route(s). It will be an interesting challenge for future research to study whether these interacting hormonal routes explain the complete regulation of shade avoidance elongation responses, or if still more regulatory routes operate in parallel to the ones elucidated here.

CHAPTER 5

Summarising Discussion

The aim of this study was to investigate the regulatory role of the plant hormone GA and its interaction with DELLA proteins in shade avoidance responses in Arabidopsis. Shade avoidance responses help plants to grow away from neighbour-imposed shade in dense vegetations and consist of traits such as petiole and hypocotyl elongation. These traits are induced upon perception of neighbour detection light signals: reduced ratio between red and far-red (R:FR ratio) and reduced blue light photon fluence rates. We studied elongation growth of Arabidopsis petioles and hypocotyls induced by low R:FR and low blue light. Ethylene and auxin were also included in this research as additional hormonal signals, which may regulate shade avoidance and interact with DELLA proteins. The importance of interactions between the above mentioned hormones and DELLA proteins in determining hypocotyl and petiole elongation induced by neighbour detection signals were investigated. This study reveals the importance of DELLA proteins in GA-signaling that regulates shade avoidance responses in Arabidopsis petioles and hypocotyls. However, it also identifies that DELLA-mediated regulation alone is not sufficient, and that DELLA-independent modes of action mediated by auxin and ethylene are also important signaling pathways.

Shade avoidance in Arabidopsis

Arabidopsis thaliana (L.) displays all aspects of shade avoidance when exposed to canopy light signals and responds with enhanced petiole and hypocotyl elongation (Robson et al., 1993). Red and far-red light in Arabidopsis are perceived by five phytochrome photoreceptors (PHYA-PHYE) that are involved in the regulation of all aspects of light-mediated development (Chory et al., 1996). Blue light in Arabidopsis is perceived by the cryptochrome family of photoreceptors which contains three members (CRY1, CRY2 and CRY3) and by phototropins (PHOT 1 and PHOT 2). The importance of both low R:FR and low blue signals in triggering elongation was shown by Ballare et

al. (1991) who performed a study on photocontrol of stem elongation of *Datura ferox* (L.). This study showed that both FR-enrichment and low blue light photon fluence rates can induce stem elongation and that these two signals act additively. Further study on cucumber hypocotyls (Ballare et al., 1991b) confirmed the promotional effect of low blue light on elongation.

In this thesis was investigated the effect of these two different neighbour detection light signals on the elongation growth of hypocotyls and petioles in Arabidopsis. We found that the effect on elongation can depend on the developmental stage of treated plants. For example, while low R:FR induced substantial elongation of petioles detected already after 2 hours of treatment (Chapter 2), low blue photon fluence rate had a delayed and relatively weak effect on petiole elongation (Chapter 3). On the other hand, low blue light induced vigorous hypocotyl elongation (Chapter 3) as compared to low R:FR, which in turn induced a much milder hypocotyl elongation response (Chapter 2). The latest finding is consistent with PHY A action which is abundant in early developmental stages and suppresses elongation of seedlings in a FR-enriched environment (Parks and Spalding, 1999). According to our study, the low blue light-induced hypocotyl elongation response results mostly from cryptochrome perception. The role of phototropins in regulating hypocotyl elongation growth induced by low blue light seems to be less pronounced.

Involvement of GA and DELLA proteins in regulation of petioles and hypocotyl elongation responses to neighbour detection signals

Perception of light signals is translated into growth responses through the action of a hormonal network (reviewed by Halliday et al., 2003; Vandenbussche et al., 2005). The role of GA in promoting shade avoidance traits has been established in several studies (reviewed by Garcia-Martinez et al., 2001 and Halliday et al., 2003). Pierik et al. (2004a) showed that exogenous GA application can induce shade-avoidance traits in tobacco such as stem and petiole elongation and leaf hyponasty. In our study, however, GA application induced additional elongation only in hypocotyls and not in petioles (Chapter 2). This may suggest that enhanced elongation induced by shade light cues can

only in hypocotyls result from increased GA biosynthesis. An interesting observation is that GA application to seedlings of the L*er* accession induced hypocotyl elongation only in control light (Chapters 2 and 3), whereas growth enhancement by exogenous GA of hypocotyls of the Col-0 accession was recorded in both control and low blue light (Chapter 4). This indicates differences in saturation by the endogenous GA level for hypocotyl elongation in low blue light between different Arabidopsis accessions and points out the importance of choosing particular accession. Differences between L*er* and Col-0 accessions for responses to external signals were already noticed in a study by Millenaar et al. (2005) that showed different responsiveness of hyponastic growth of petioles to ethylene application.

The importance of GA in shade avoidance has been demonstrated in many studies by observing the effect of reduced GA levels on shade avoidance traits. For example, Folta et al. (2003) found that GA-biosynthesis is regulated in a cryptochrome-dependent manner and that GA deficiency suppresses the elongated phenotype of the *cry1* mutant. Pierik et al. (2004a) showed abolished shade avoidance traits in GA-deficient tobacco. GA deficiency and reduced GA responsiveness may also suppress the constitutively elongated phenotype of *phyB* hypocotyls, as shown by Reed et al. (1996) and Peng and Harberd (1997), respectively. We used a similar approach to confirm the importance for GA in both low R:FR and low blue-induced elongation of petioles and hypocotyls (Chapters 2 and 3). Our study showed that GA deficient petioles and hypocotyls cannot respond to low R:FR and low blue by elongation growth (Chapters 2 and 3). Observed shade-avoidance traits were inhibited or severely reduced also in the GA-insensitive *gai* mutant (Chapters 2 and 3). This mutant carries an open-frame deletion of 51bp in the sequence encoding the DELLA motif which leads to a stabilized form of the gai protein. The gai protein belongs to the DELLA family of growth suppressors that are regulated by GA. The DELLA family in Arabidopsis contains five members (GAI, RGA, RGL1, RGL2 and RGL3) that suppress different aspects of GA-regulated growth and development (reviewed by Alvey and Harberd, 2005). A study by Dill et al. (2001) showed that the main suppressors of GA-mediated growth are the GAI and RGA proteins. Accordingly, our study showed that these two proteins are also the

main regulators of low R:FR and low blue-induced petiole and hypocotyl elongation (Chapter 2 and Chapter 3). This was concluded after performing growth studies on DELLA knock-outs in which GA biosynthesis was inhibited either by the *ga1-3* mutation or paclobutrazol application, a chemical inhibitor of GA biosynthesis. The inhibiting effect of GA-deficiency on shade avoidance in petioles and hypocotyls was partly rescued in the *gai- t6* and *rga-24* single knock-outs and this effect was enhanced in their double and quadruple knock-outs. Interestingly, DELLA knock-outs in plants with normal GA content gave only mild phenotypic effects, obvious only in mildly elongated hypocotyls of the quadruple DELLA knock-out seedlings (Chapters 2 and 3). This is different from DELLA knock-outs in some other species, such as rice (Ikeda et al., 2001) or barley (Fu et al., 2002). In these two monocots, that both express one DELLA protein rather than five in Arabidopsis, DELLA knock-outs conferred constitutively elongated shoots. Obviously, the impact of DELLA proteins on growth depends on the plant species and, in our study, on the light condition. On the other hand, we studied four out of five DELLA proteins in Arabidopsis, which leaves the theoretical possibility that the putative activity of the RGL3 member of the DELLA family would suppress an elongated phenotype. There are, however, no indications that RGL3 would regulate vegetative growth.

We observed that low R:FR and low blue detection led to DELLA protein degradation in a GA-dependent manner. Although in low R:FR-exposed petioles upregulation of the *GA20ox1* gene was observed, it remains to be elucidated if this light signal enhances endogenous GA levels. If it does, this would suffice to degrade DELLAs as GA application led to complete loss of GFP-RGA signal in the *pRGA*::*GFP-RGA* reporter line. However, this does not automatically lead to enhanced elongation growth in petioles (Chapter 2). In contrast to petioles, attenuation of the GFP-RGA signal, induced by GA addition in hypocotyls, was accompanied by enhanced elongation (Chapter 2). This suggests that DELLA degradation alone already partly induced shade avoidance in hypocotyls. According to Achard et al. (2007) increased GA content can account for promotion of the DELLA degradation in hypocotyls of dark-grown seedlings. These authors suggested that light-induced

inhibition of hypocotyl elongation, paralleled by DELLA accumulation, resulted from a decrease in GA content. Although endogenous GA levels were not measured in this study, this putative decrease of GA was ascribed to light-induced reduction of the activity of GA biosynthetic genes and upregulation of GA-catabolic ones. Furthermore, these authors showed that the DELLA accumulation results from phytochrome signaling as they detected lower RGA-GFP protein level in the *phyA* and *phyB* phytochrome mutants. In conclusion, the active form of phytochrome in light may promote accumulation of the DELLA proteins by decreasing GA-biosynthesis. Furthermore, Zhao et al. (2007) showed the importance of cryptochromes to mediate blue light-induced upregulation of the GA-catabolic *GA2ox1* and suppression of the GA-biosynthetic *GA20ox1* and *GA3ox1* genes. This resulted in a transient decrease of GA_4 when etiolated seedlings were transferred to light. Interestingly, these authors did not record significant changes of GA content in dark or light grown shoots between WT and *cry1 cry2* seedlings suggesting that cryptochromes may regulate GA responsiveness rather than biosynthesis.

The impact of ethylene and auxin and their interaction with DELLA proteins in regulating shade avoidance responses

Degradation of DELLA proteins was required to allow low R:FR and low blue-induced elongation of petioles and hypocotyls (Chapters 2 and 3). However, DELLA degradation alone was not sufficient to induce elongation in petioles (Chapter 2) and only partly induced hypocotyl elongation (Chapters 2 and 3). This is consistent with the existence of other, DELLA-independent, signaling pathways that operate in regulating shade avoidance responses. Therefore, we extended the focus of our study to two other hormonal signals, ethylene and auxin, which are recorded to be involved in the regulation of shade avoidance (Steindler et al., 1999; Morelli and Ruberti, 2002; Finlayson et al., 2003; Vandenbussche et al., 2003; Pierik et al., 2004a,b) and, additionally, may interact with GA-DELLA signaling (Achard et al., 2003; Fu and Harberd, 2003; Vriezen et al., 2004).

Ethylene is considered as a canopy signal that accumulates and triggers some shade avoidance traits when applied at the appropriate concentration, such as hyponastic leaf movements and stem elongation of tobacco plants (Pierik et al., 2004a). The effect of ethylene application to petioles and hypocotyls in our study was obvious only in hypocotyls which responded by enhanced elongation in control light (Chapter 4). This effect of ethylene application to seedlings was confirmed by applying the biosynthetic precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC), which was used to further study hormone interactions. As for GA, application of ACC differently affected hypocotyl elongation of L*er* and Col-0 seedlings. ACC-induced hypocotyl elongation in L*er* was recorded only in control light whereas Col-0 displayed elongation in both control and low blue light condition (Chapter 4). This again emphasizes the importance of the accession used in the study. An interesting observation is that ethylene biosynthesis was differently affected in leaves and seedlings by low R:FR and low blue light signals. While in leaves low R:FR treatment induced a strong increase of ethylene production, in hypocotyls this increase was not recorded in low blue light (Chapter 4). This may point to tissue specificity in ethylene production (leaves vs. seedlings) or light signal-specificity. Interestingly, the impact of ethylene on petiole elongation responses to low R:FR and hypocotyl elongation in low blue was also different. Whereas ethylene insensitivity clearly prevented low R:FR-induced elongation of petioles, ethylene insensitive hypocotyls showed reduced but not absent low blue-induced elongation (Chapter 4). As for the effect of GA application, these findings point again to subtle differences in the impact of a particular hormonal signal in the regulation of elongation responses in different plant tissues or light environments.

It has been shown by several studies that ethylene can interact with auxin signaling to control growth of plant organs (Visser et al., 1996; Lehman et al., 1996; Smalle et al., 1997; Harper et al., 2000). Our study showed that ethylene can regulate low blue-induced hypocotyl elongation independently of auxin since ACC added to the auxin-resistant *axr2-1* mutant partly rescued its elongation response in low blue (Chapter 4). On the other hand, Smalle et al. (1997) showed that auxin can induce hypocotyl elongation in ethylene-insensitive seedlings, as was also found in our study

(Chapter 4). This indicates that auxin could regulate hypocotyl elongation independently of ethylene.

Auxin involvement in shade avoidance responses of petioles and hypocotyls was confirmed by reduced or even absent elongation responses to low R:FR and low blue of the auxin resistant mutant *axr2-1* and of NPA-treated wild type plants (Chapter 4). This is in agreement with findings by Steindler et al (1999) who showed that hypocotyls of the auxin resistant *axr1-12* mutant displayed a reduced R:FR response. The regulatory role for auxin in shade avoidance was further supported by the putative increase of auxin action, visualized through *pIAA19*::*GUS* staining in petioles and hypocotyls upon low R:FR and low blue exposure, respectively (Chapter 4).

Next to interacting with auxin, ethylene can also affect the abundance of DELLA proteins. Achard et al. (2003) applied ethylene to the reporter line *pRGA*::*GFP-RGA* and noticed a delay in the GA-inducible degradation of the DELLA protein RGA in roots which resulted in reduced growth. Our research on hypocotyls, however, showed that an ACC-induced increase in RGA abundance does not necessarily lead to reduced hypocotyl length. In control light, ACC stimulates hypocotyl elongation without an obvious effect on RGA abundance. In low blue, however, ACC increased RGA abundance which, surprisingly, did not reduce hypocotyl elongation (Chapter 4). Furthermore, a comparable study on the effect of 1-MCP on wild type and the quadruple knock-out revealed that ethylene interaction with DELLA proteins is not functional in determining hypocotyl elongation induced by low blue light (Chapter 4). Moreover, ACC could even induce elongation of GA-deficient and GA-unresponsive hypocotyls strongly suggesting that GA is not involved in ethylene-induced growth.

According to a study by Fu and Harberd (2003), auxin also modulates GA-inducible degradation of DELLA proteins. These authors noticed that application of NPA, mutation in the auxin-efflux regulator *PIN1*, removal of the shoot apex or compromised auxin signaling in the *axr1-12* mutant resulted in inhibited GA-induced RGA degradation in roots. This is in agreement with our study which showed that NPA treatment to *pRGA*::*GFP-RGA* seedlings results in higher RGA abundance and concomitant growth reduction irrespective of the light condition (Chapter 4). A study by

Frigerio et al. (2006) showed that auxin stimulates GA-biosynthesis by upregulating GA biosynthesis genes and suppressing the activity of those for GA degradation. This finding may explain the NPA-induced accumulation of the RGA protein observed in our study. It is further supported by disappearance of the GFP-RGA signal after adding GA to NPA-treated plants. Although GA reduced RGA below detectable level, it rescued only growth in control light (Chapter 4). Apparently, the reducing effect of NPA-inhibited auxin transport in low blue light on hypocotyl elongation could not be rescued by removing DELLAs suggesting that auxin regulates low blue-induced elongation independently of DELLA proteins. This was confirmed by the lack of a complete rescue for hypocotyl elongation in low blue of NPA-treated DELLA knock-outs (Chapter 4), although we can not exclude suppressive activity of the remaining DELLA protein RGL3 whose function has not been elucidated yet. On the other hand, we showed that growth responses strongly depend on GA and DELLAs, since a low blue-induced increase of auxin activity, as seen in *pIAA19*::*GUS*, is not effectively stimulating hypocotyl elongation in GA-deficient plants which have high DELLA abundance.

Conclusions and future perspectives

Our study confirmed that Arabidopsis petioles and hypocotyls responded with enhanced elongation to canopy light cues. The importance of GA regulation of these elongation responses was established. GA is required to induce degradation of DELLA proteins that accumulate in GA-deficient plants and prevent elongation responses to low R:FR and low blue light. In the absence of DELLAs, as in knock-outs, elongation responses to low R:FR and low blue can proceed even in GA-deficient plants. This is in accordance with the 'relief of restraint' model of Harberd (2003). However, degradation of almost all DELLAs alone is not sufficient to induce elongation responses. Our study showed that ethylene and auxin act next to GA-DELLA interaction to induce shade avoidance traits in petioles and hypocotyls. These two hormones were found to interact with DELLA abundance, but these interactions could not account for the full expression of shade avoidance traits in Arabidopsis. Apparently, the DELLA-independent signaling

pathways of ethylene and auxin are more important in regulating petiole and hypocotyl elongation induced by low R:FR and low blue light. However, signaling pathways of auxin and ethylene cannot result in elongation growth if DELLA proteins are highly abundant as in GA-deficient plants. On the other hand, DELLA knock-outs cannot rescue elongation responses of petioles and hypocotyls induced by low R:FR and low blue abolished by disturbed ethylene and auxin actions. Apparently, shade avoidance is regulated by more than one signaling pathway which, together with others, forms a network in which the outcome of the mutual interactions is tightly regulated.

This thesis presents a novel step into studying hormonal interactions and involvement of DELLA proteins in regulating shade avoidance. Individual plants that were subjected to light conditions similar to those in canopies were studied. However, other conditions such as humidity, ethylene accumulation and nutrient deficiency can affect the observed hormonal interactions and their impact on growth responses in the realm of dense vegetations. Therefore, it will be an interesting challenge to verify the observed forms of regulation in plants grown in dense canopies.

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Samenvatting in het Nederlands

(Summary in Dutch)

Planten groeien vaak in dichte vegetaties, met veel buurplanten om zich heen waarmee ze concurreren om het beschikbare licht. Onderin het bladerdek van dichte vegetaties is namelijk bijna geen licht beschikbaar om te kunnen groeien. Planten kunnen echter reacties vertonen, zogenaamde *shade avoidance* responsen, waardoor ze in staat zijn om boven buurplanten uit te groeien en zo de lichtinvang te optimaliseren. Klassieke shade avoidance responsen zijn het opklappen van de bladeren en vervolgens versnelde strekkingsgroei van stengels en bladstelen, reacties die zeer snel optreden nadat schaduwsignalen door de plant zijn waargenomen. Mocht een plant uiteindelijk toch niet in staat blijken om het volle zonlicht te bereiken kan door middel van een andere shade avoidance respons de bloei worden versneld, zodat op z'n minst een beperkt aantal zaden kan worden geproduceerd.

Buurplant detectie door middel van lichtsignalen

Planten kunnen deze shade avoidance responsen al opstarten voordat de hoeveelheid licht in een zich ontwikkelende vegetatie beperkend is. Het is niet alleen de hoeveelheid, maar ook de kleur van het licht dat bepalend is. Het signaal dat planten gebruiken voor deze gevoelige en vroege buurplantdetectie is de verhouding tussen rood (660-670 nm) en verrood (725-735 nm) licht, de zogenaamde R:VR ratio. De R:VR ratio daalt in een vegetatie doordat rood licht wordt geabsorbeerd door planten voor fotosynthese, terwijl verrood licht wordt gereflecteerd. De R:VR ratio in vol zonlicht is ongeveer 1.2, terwijl de verhouding tot ver beneden de 0.2 kan dalen in dichte vegetaties. De R:VR verhouding wordt door planten waargenomen met specifieke fotoreceptor eiwitten, fytochroom genaamd. Fytochroom bestaat in een actieve en een inactieve vorm en de balans tussen die twee vormen wordt bepaald door de R:VR ratio. In dichte vegetaties leidt de lage R:VR tot relatief weinig activatie van fytochroom, waardoor de onderdrukking van shade avoidance wegvalt.

Het licht in dichte vegetaties is niet alleen arm aan rood licht, maar bevat ook relatief weinig blauw licht, opnieuw een gevolg van absorbtie voor fotosynthese. Blauw licht wordt gesignaleerd door andere fotoreceptoren, te weten cryptochroom en fototropine. De gerduceerde blauw lichtniveaus in het licht in een vegetatie kunnen, net als de lage R:VR ratio, shade avoidance responsen induceren.

Eenmaal waargenomen leiden de lichtsignalen tot fysiologische veranderingen op het niveau van groei-regulerende plantenhormonen. Gibberelline (GA) is zo'n hormoon en dit hormoon wordt beschouwd als de klassieke groeistimulator tijdens shade avoidance. Verhoogde GA niveaus leiden bijvoorbeeld vaak tot versnelde strekkingsgroei van stengels en bladstelen. Er is echter weinig bekend over de GA signaal transductie componenten die betrokken zijn bij shade avoidance reponsen. Dit is onderwerp van studie in dit proefschrift.

Gibberelline signalering

In de signaal transductie route van gibberelline is een belangrijke rol weggelegd voor de zgn. DELLA eiwitten. DELLA eiwitten hebben een groeiremmend effect en zijn negatieve regulatoren van alle GA-responsen die tot nu toe bekend zijn. De DELLA eiwit familie in *Arabidopsis thaliana*, zandraket, bestaat uit vijf familieleden, te weten GAI (GA-INSENSITIVE), RGA (REPRESSOR OF GA1-3), RGL1, RGL2 en RGL3 (RGA-LIKE 1, 2 en 3). DELLA eiwitten beïnvloeden de transcriptie van GA-respons genen en zijn derhalve gelokaliseerd in de celkern. GAI en RGA worden beschouwd als de belangrijkste twee DELLA eiwitten in de regulatie van vegetatieve groei, maar ze beïnvloeden ook de bloei. RGL2 is betrokken bij de regulatie van zaadkieming en reguleert samen met RGL1 en RGA de ontwikkeling van de bloeiwijze. Er is tot op heden geen functie bekend voor RGL3. Als er gibberelline aanwezig is in de cel kan dit binden aan de GA-receptor, GID1 (GIBBERELLIN INSENSITIVE DWARF 1). Binding van GA aan GID1 stimuleert de interactie van DELLA eiwitten met een zgn. ubiquitinatie complex, $SCF^{SLY1/GID2}$, en deze interactie zorgt ervoor dat het DELLA eiwit gemarkeerd wordt, met een staart van ubiquitine moleculen, voor afbraak. Deze

interacties hebben tot resultaat dat DELLA eiwitten op een GA-afhankelijke manier worden afgebroken.

Er zijn verschillende DELLA mutanten bekend in Arabidopsis, zowel *gain-of-function* als *loss-of-function* mutanten. Gain-of-function DELLA mutanten, bijv. *gai*, kunnen zodanig gemuteerd zijn dat het DELLA eiwit nog wel functioneel actief is, maar niet meer door het eerder genoemde ubiquitinatie complex kan worden herkend. Dit leidt tot een constitutieve aanwezigheid van het eiwit en daardoor tot een permanente onderdrukking van GA responsen. DELLA loss-of-function mutanten daarentegen kunnen juist de symptomen van GA-deficiëntie, zoals bijv. slechte kieming en dwerggroei, onderdrukken. In dit proefschrift werden deze typen mutanten ingezet om inzicht te verkrijgen in de regulatie van shade avoidance reacties door GA en DELLA eiwitten. Ook werd een moleculaire reporter, een fusie eiwit van RGA met GFP (Green Fluorescent Protein), gebruikt om de dynamiek van DELLA eiwitten in de plant te bestuderen.

Het belang van gibberelline en DELLA eiwitten tijdens shade avoidance

In **hoofdstuk 2** werd bestudeerd of en hoe DELLA eiwitten worden gereguleerd tijdens shade avoidance reacties van volwassen planten en zaailingen, geïnitieerd door lage R/VR ratio's. Gebleken is dat laag R/VR-geïnduceerde petiool- en hypocotyl strekking sterk afhankelijk is van de aanwezigheid van gibberelline in de plant. Remming van de endogene GA concentraties resulteerde in sterk verhoogde niveaus van het DELLA eiwit RGA, met als gevolg een nagenoeg afwezige strekkingsreactie op verlaagde R/VR ratio. Echter, wanneer er geen functionele DELLA eiwitten aanwezig waren, zoals in de DELLA loss-of-function mutanten, bleek GA-deficiëntie geen effect te hebben op deze reacties en vond er dus een ongestoorde reactie op laag R/VR plaats. Deze data laten zien dat GA noodzakelijk is voor shade avoidance reacties op laag R/VR doordat het zorg draagt voor de afbraak van de groeiremmende DELLA eiwitten.

In **hoofdstuk 3** werd een vergelijkbare studie uitgevoerd aan zaailingen die niet aan lage R/VR ratio's, maar aan verlaagde blauw licht hoeveelheden werden blootgesteld. Dit had ook een sterke strekkingsreactie van de hypocotylen tot gevolg en

verliep volgens grotendeels vergelijkbare GA-DELLA interacties als beschreven voor de R/VR reacties. Uit zowel hoofdstuk als hoofdstuk 3 bleek dus dat GA nodig voor shade avoidance omdat het de afbraak van DELLA eiwitten reguleert, maar het bleek ook dat in de afwezigheid van zowel GA als DELLA er nog steeds een volwaardige shade avoidance reactie kan optreden. Dit laatste gegeven laat zien dat er naast GA waarschijnlijk nog andere hormonen een rol spelen bij de regulatie van shade avoidance.

DELLA als mogelijk kruispunt van signaleringsroutes

Het is bekend dat, naast GA, ook andere hormonen de stabiliteit van DELLA eiwitten kunnen beïnvloeden. Zo is bijvoorbeeld aangetoond in worteltoppen dat de aanwezigheid van het plantenhormoon auxine de snelheid van GA-afhankelijke DELLA afbraak positief beïnvloedt. Naast auxine zijn ook voor een derde plantenhormoon, het gasvormige ethyleen, interacties met DELLA stabiliteit beschreven. Naast endogene hormonen lijken ook externe milieucondities, zoals zout en licht, via de DELLA eiwitten plantengroei te reguleren. Het is om deze reden dat DELLA eiwitten veelal worden beschouwd als een essentieel kruispunt voor een variatie aan signaleringsroutes die de groei van planten afstemt op interne en externe condities.

Alternatieve regulatieroutes voor shade avoidance

Ofschoon GA een belangrijke hormonale regulator van shade avoidance reacties blijkt te zijn, zijn ook de eerder genoemde hormonen auxine en ethyleen regelmatig in verband gebracht met dit proces. Zoals eerder al is aangegeven kunnen deze hormonen functioneren via regulatie van DELLA eiwit stabiliteit. De vraag in **hoofdstuk 4** was echter of deze twee hormonen, naast een interactie met GA en DELLA, ook via DELLA-onafhankelijke mechanismen strekkingsgroei kunnen reguleren. Hiertoe werd allereerst vastgesteld of deze twee hormonen inderdaad belangrijk waren voor de bestudeerde reacties en dit bleek grotendeels het geval te zijn. Het bleek dat remming van het transport van auxine de strekkingsgroei reacties blokkeerde en dit correspondeerde met verhoogde niveaus van het DELLA eiwit RGA. Echter, deze interactie tussen DELLA en auxine is waarschijnlijk niet functioneel tijdens shade

avoidance. Remming van auxine transport remde namelijk shade avoidance ook in DELLA loss-of-function mutanten waar DELLA stabilisatie niet op kan treden. Op vergelijkbare wijze werd aangetoond dat ook ethyleen een belangrijke hormonale speler is tijdens shade avoidance, maar opnieuw slechts ten dele via interacties met DELLA eiwitten.

Afsluitend kan worden gesteld dat hormonal regulatie van shade avoidance in Arabidopsis verloopt via een netwerk van interacterende hormonen. GA blijkt essentieel te zijn voor de benodigde afbraak van groeiremmende DELLA eiwitten. Auxine en ethyleen beïnvloeden ook de stabiliteit van deze eiwitten, maar reguleren de strekkingsgroei reacties waarschijnlijk ook via DELLA-onafhankelijke mechanismen.

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Curriculum vitae

Tanja Djaković-Petrović was born on the 6th of May 1971 in Belgrade, Serbia. She completed the secondary shool for biochemistry and molecular biology in Belgrade in 1990. In 1991 she started Bachelor studies at the Faculty of Agriculture, University of Belgrade, and graduated in 1996. The same year she commenced Master study at the Deparment of Plant Physiology and acquired MSc degree in Plant Physiology in 2000. From 2001 until 2003 she was employed in the department of Plant Physiology of the Maize Research Institute –'Zemun Polje' in Belgrade. In Septembar 2003 she started her PhD project at Utrecht University, Department of Biology, reearch group Plant Ecophysilogy. From December 2007 she has been working at Seminis, the Seed Technology Department, in Enkhuizen, The Netherlands.