KINETIC AND GENETIC ANALYSIS OF EARLY PHOTOMORPHOGENIC INTERACTION IN THE MODEL PLANT *ARABIDOPSIS THALIANA*

By

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To my lovely wife, Wenjing, for her sweet “push”, my parents and sister, for their undemanding love!

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LIST OF ABBREVIATIONS

µm	Micrometer
µmol	Micromolar
Arabidopsis	*Arabidopsis thaliana*
BL	Blue light
cm	Centimeter
CRY	Cryptochrome apoprotein
cry	Cryptochrome holoprotein (with chromophore covalently attached to apoprotein)
d	Days
FAD	Flavin adenine nucleotide
FMN	Flavin mononucleotide
FrL	Far-red light
g	Grams
GL	Green light
h	Hours
HRR	Hypocotyl-randomization-response
LED	Light-emitting diode
LOV	Light-oxygen-voltage
min	Minutes
NPA	1-N-Naphthylphthalamic acid
PAR	Photosynthetically active radiation
Pfr	Far-red-absorbing phytochrome
PHOT	Phototropin apoprotein
phot	Phototropin holoprotein
PHR	Photolyase-homologous region

PHY	Phytochrome apoprotein
phy	Phytochrome holoprotein
P/L	Petiole: leaf ratio
Pr	Red-absorbing phytochrome
qPCR	Quatitative polymerase chain reaction
RL	Red light
R/FR	Red to far-red ratio
s	Second
SSLP	Simple sequence length polymorphism
SAS	Shade avoidance syndrome
UV	Ultraviolet

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Work presented here seeks to add resolution to light-signaling models by describing how specific wavelengths of light are sensed and integrated. Hypotheses tested in this work concentrate on two areas: 1) how green light (GL) interacts with known photosensory systems and 2) identifying the signaling components for GL transduction. Many of the GL effects described oppose the typical light responses, but the underlying mechanisms are poorly understood. The work here investigated the interaction between GL and red light (RL) or far-red light (FrL) in the regulation of early photomorphogenesis. The results demonstrate an attenuation effect of GL on RL/FrL-induced hypocotyl-growth-inhibition. Unexpectedly, this GL effect is not observed when the blue light (BL) receptor phot1 or its interacting component, NPH3, is absent. Careful re-examination of this response under conditions of dim BL and RL/FrL regenerated the negation effects on RL/FrL, indicating that the GL negation of RL/FrL response is actually a dim BL effect from a small amount of <500 nm light present in green LED light source. Results also demonstrate that dim BL delays apical hook opening through the action of phot1. These phot1-mediated dim-BL-effects likely require coordinated action of the plant hormone, auxin. Results uncover a particular situation where BL postpones

RL/FrL-guided photomorphogenesis. Physiologically, the phot1-ajustment of RL/FrL responses may enable an emerging seedling to better utilize its limited resources in reaching a better photosynthetically favorable position. Work presented here also describes a natural accession of *Arabidopsis thaliana*, Knox-10, which may contain genetic variations in the GL signaling pathway. Knox-10 displayed a greatly reduced, GL-stimulated hypocotyl-elongation-response. The same genotype also showed atypical, directional hypocotyl growth (i.e., a lack of hypocotyl-randomization-response [HRR]) under RL. Linkage mapping results indicate that the HRR phenotype co-segregates with several molecular markers on chromosome one. In addition, Knox-10 also showed increased hyponastic growth under certain light conditions during early vegetative growth. Collectively, results presented in this work expand the knowledge of light signaling models by investigating how specific wavelengths of light are sensed, transduced, and integrated. This information expands what we know about light signaling models and may be applicable to future agricultural practices.

[1]CHAPTER 1
LITERATURE REVIEW

Plants are sessile organisms. Their physiological and morphological plasticity are greatly influenced by ambient light signals. Finely tuned light-regulated plant growth and development (photomorphogenesis) are achieved by the orchestrated actions of multiple photo-sensing systems. The genetic basis as well as the molecular mechanisms of the signaling transductions for ultraviolet B (UV-B, 280-315 nm), ultraviolet A (UV-A, 315-400 nm), blue (400-500 nm), and red/far-red light (600-800 nm) have been described in a great detail in the past decades. However, our knowledge of green light (GL) in the regulation of plant growth and development is relatively limited. The molecular basis for the sparsely documented GL responses is even less understood. The lack of understanding of this waveband prevents us utilizing or designing lighting conditions for modern agricultural practices or develops a comprehensive understanding of plant light-sensing systems.

Plant Photoreceptors and their Responses to GL

Plant photoreceptors share the common structural arrangement of a photon-absorbing chromophore covalently or non-covalently bound to an apoprotein (Möglich et al., 2010). In *Arabidopsis thaliana* (hereafter *Arabidopsis*), photoreceptor apoproteins are abbreviated in uppercase format, such as PHY (phytochrome), CRY (cryptochrome), and PHOT (phototropin). The holoproteins (apoprotein attached with chromophore) are designated with lowercase, for instance, phy, cry, and phot (Quail et

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al., 1994; Lin et al.; Briggs et al., 2001). The discovery of plant photoreceptors was boosted by the use of genetic mutants in the model plant *Arabidopsis thaliana* (hereafter *Arabidopsis*) during the 1980s to 1990s. The phytochrome (phy) family (phyA-phyE) mainly absorbs red and far-red wavebands through its chromophore phytochromobilin (Franklin and Quail, 2010). The term of phytochrome, referring to “plant” and “color” in Greek, was originally proposed by Dr. Warren Butler, according to Dr. Harry Borthwick. Although the first purification of phytochrome pigment from plant was made in the 1959 by Butler’s group, the characteristics of the photo-convertibility of this pigment in response to RL/FrL light has been described by Borthwick in 1952 (Borthwick et al., 1952; Butler et al., 1959). In the 1980s, Maarten Koornneef described several light mutants in his seminal work, ‘Genetic Control of Light-inhibited Hypocotyl Elongation in *Arabidopsis thaliana* (L.) Heynh’ (Koornneef, Rolff, and Spruit, 1980). One of those mutants, *hy3*, which is insensitive to RL, was later shown to be deficient in phyB (Somers et al., 1991). Two years later, a new class of long hypocotyl mutant *hy8*, which is insensitive to FrL, was found to be deficient in phyA (Parks and Quail, 1993).

Based on light stability, phytochromes can be divided into two groups, the ‘Type I’ light labile phytochrome and the ‘Type II’ light stable phytochromes (Chen, Chory, and Fankhauser, 2004). The phyA receptor is the only ‘Type I’ phytochrome and is most abundant in etiolated seedlings (Clough and Vierstra, 1997). By contrast, the phyB through phyE receptors are relatively light-stable (Sharrock and Clack, 2002; Franklin and Quail, 2010). Functional phytochromes require the formation of homodimers or heterodimers, however, heterodimers between phyA and phyB-E has not yet been found (Sharrock and Clack, 2004; Clack et al., 2009). Phytochromes exist in plants with

two forms, the Pr (red-light-absorbing) inactive form with a maximum absorption peak at 670 nm, and the Pfr (far-red-absorbing) active form with the absorption peak near 730 nm (Rockwell, Su, and Lagarias, 2006). These two forms are in a dynamic photo-equilibrium depending on red to far-red ratios from ambient light environment. The absorption of RL leads to the activation and translocation of Pfr form phytochromes from cytosol into the nucleus (Huq, Al-Sady, and Quail, 2003). Translocation of phyA into the nucleus requires the presence of FAR-RED-ELONGATED HYPOCOTYL 1 (FHY1) and FHY1-LIKE (FHL) (Rösler, Klein, and Zeidler, 2007). Inside the nucleus, phytochromes interact with PHYTOCHROME-INTERACTING FACTORS (PIFs, bHLH transcription factor) where they mediate the degradation of PIFs, and release the suppression of photomorphogenesis-related gene expression (Bae and Choi, 2008). Phytochromes regulate many aspects of plant growth and development, such as seed germination, hypocotyl growth rate inhibition, chloroplast development, hook opening, shade avoidance, and flowering time (Smith, 1995; Chen, Chory, and Fankhauser, 2004; Franklin and Quail, 2010). These physiological changes often are accompanied by the adjustment of gene expression (Tepperman et al., 2001; Tepperman et al., 2004; Tepperman, Hwang, and Quail, 2006).

Cryptochromes (cry), phototropins (phot), and other Light-Oxygen-Voltage (LOV) domain proteins are blue and UV-A light sensors (Kami et al., 2010). *Arabidopsis* has three cryptochromes (cry1, cry2, and cry3), two phototropins (phot1 and phot2), and three other LOV domain proteins (ZTL, FKF1, and LKP2). The identification of cryptochromes started with analysis of genetic mutants. The gene responsible for the BL insensitive phenotype in the *hy4* mutant described in Koornneef's paper was cloned

and sequenced in 1993 by Ahmad and Cashmore. The gene was re-designated *CRY1* (Koornneef, Rolff, and Spruit, 1980; Ahmad and Cashmore, 1993; Lin et al., 1995). The structure of this photoreceptor is related to that of DNA photolyases, but the DNA repair activity is not observed (Lin and Shalitin, 2003; Chen, Chory, and Fankhauser, 2004). The N-terminal of this protein contains a photolyase-homologous region (PHR) domain that binds to its chromophore flavin adenine dinucleotide (FAD) (Lin et al., 1995; Liu et al., 2011). The cry2 receptor is thought to be mostly nuclear localized, while cry1 can be found in both the cytosol and the nucleus (Lin and Shalitin, 2003; Yu et al., 2007; Yu et al., 2009). Upon BL irradiation, cryptochromes are phosphorylated to their active form and initiate photomorphogenic programs (Shalitin et al., 2002; Shalitin et al., 2003). There are two major pathways for cryptochromes to control gene expression. One is through the blue-light-dependent interactions with the transcription factor CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX (CIB), which regulates photomorphogenesis- and flowering-related gene expression. The other is through the suppression of CONSTITUTIVELY PHOTOMORPHOGENIC 1(COP1) E3 ligase activity. COP1 targets transcription factors, like LONG HYPOCOTYL 5 (HY5, basic leucine zipper transcription factor) and CONSTANS (CO), which are important for de-etiolation and flowering initiation process, respectively (Liu et al., 2011).

The plant phototropic response was described as early as the 1880s by Charles and Francis Darwin in the seminal work, ‘The Power of Movements in Plants’ (Holland, Roberts, and Liscum, 2009). However, until the1990s, the photo-sensing molecule initiating this response remained undefined. The use of an *Arabidopsis* mutant with an impaired phototropic response by Winslow Briggs’s group finally led to the molecular

identification and characterization of the *PHOT1*/ *NON-PHOTOTROPIC HYPOCOTYL* (*NPH1*) gene as a BL receptor governing phototropic response (Liscum and Briggs, 1995; Huala et al., 1997; Christie et al., 1998; Christie et al., 1999). The phototropins contain two LOV domains (LOV1 and LOV2) at the N terminus that non-covalently bind to a flavin mononucleotide (FMN) chromophore (Christie et al., 1999). Although similar in structure, it has been demonstrated that only the LOV2 domain is essential for phototropism. The LOV1 domain seems to be a suppressor for LOV2-domain-mediated kinase activity (Kimura and Kagawa, 2006). Later studies on phot1 and phot2 demonstrated their roles in regulating chloroplast movements (Kagawa et al., 2001; Sakai et al., 2001), stomatal opening (Kinoshita et al., 2001), rapid inhibition of hypocotyl growth (Folta and Spalding, 2001a; Folta et al., 2003a), and regulation of mRNA transcript stability (Folta and Kaufman, 2003). Phototropins are plasma-membrane-associated proteins but the functional nature of this association is still unclear (Christie, 2007). BL irradiation leads to the dissociation of phototropins from the plasma membrane and localization to the cytosol or Golgi apparatus (Sakamoto and Briggs, 2002; Kong et al., 2006). Two phototropin interacting components, NON PHOTOTROPIC HYPOCOTYL 3 (NPH3) and ROOT PHOTOTROPISM 2 (RPT2), have been shown to be essential for hypocotyl and root phototropism, respectively (Motchoulski and Liscum, 1999; Sakai et al., 2000). Both proteins consist of a BTB/POZ domain at the N terminus that serves as a critical motif for protein-protein interactions (Kimura and Kagawa, 2006).

Other LOV domain family proteins, like ZEITLUPE (ZTL), FLAVIN-BINDING KELCH REPEAT F-BOX (FKF), and LOV KELCH REPEAT PROTEIN 2 (LKP2), all

consist of the light-sensing LOV domain and the proteolysis-associated F-box domain (Möglich et al., 2010). Such structures enable these proteins to direct protein degradation in a light-dependent manner. The biological function of ZTL is closely related to plant circadian clock control. ZTL targets a central circadian regulation oscillator protein TIMING OF CAB EXPRESSION 1(TOC1), mediating its degradation (Mas et al., 2003). The stability of the ZTL protein is enhanced by its direct interaction with GIGANTEA (GI, a key determinant of plant flowering time). This GI-ZTL interaction can be increased by three-fold under BL (Kim et al., 2007). *GI* is also transcriptionally regulated by the clock and the interaction of ZTL and GI might indirectly lead to the dissociation of TOC1 from ZTL (Somers and Fujiwara, 2009). FKF1 is reported to be essential in generating diurnal *CO* transcript accumulation

(Imaizumi et al., 2003). The mechanism of its regulation partially relies on the degradation of CYCLING DOF FACTOR 1 (CDF1, a *CO* transcription repressor), mediated by FKF1-CDF1 interaction (Imaizumi et al., 2005). LKP2 is a nuclear-localized protein with similar function with ZTL in regulating TOC1 degradation (Yasuhara et al., 2004; Baudry et al., 2010).

UV RESISTANCE LOCUS 8 (UVR8) is the UV-B receptor that was molecularly characterized in 2011 (Rizzini et al., 2011). Interestingly, the discovery of this gene dates back to 2002, where its mutation resulted in a hypersensitive UV-B response due to the reduced expression of genes conferring UV protection (Kliebenstein et al., 2002). The absorption of UV-B is mediated by a tryptophan pyramid structure in UVR8 protein itself, rather than a bound chromophore (Rizzini et al., 2011; Christie et al., 2012). Activation by UV-B leads to the rapid monomerization of UVR8 dimers and monomeric

UVR8 accumulation in the nucleus (Kaiserli and Jenkins, 2007; Rizzini et al., 2011). In the nucleus, UVR8 binds to chromatin through histones and interacts with COP1 to initiate UV-protection-related gene expression (Cloix and Jenkins, 2008; Favory et al., 2009; Christie et al., 2012). Meanwhile, UV-B-induced UVR8 monomerization is also reversible *in vivo*, which constantly disrupts UVR8-COP1 interaction (Heijde and Ulm, 2013; Heilmann and Jenkins, 2013). It was shown that two UVR8-interacting proteins REPRESSOR OF UV-B PHOTOMORPHOGENESIS (RUP1 and RUP2) were responsible for the redimerization process, mediating the regeneration of reactivatable UVR8 homodimers post UV-B exposure (Heijde and Ulm, 2013).

The green-yellow portion of the spectrum also has been shown to contribute to plant development through established red-blue photosensing pathways, as the known photoreceptors can respond to green wavebands. For instance, phytochrome can be converted to the far-red absorbing, biologically active form by exposure to GL. GL establishes a phytochrome equilibrium favoring the active Pfr form (Hartmann, 1967), and GL is sufficient to activate phy responses like the seed germination in *Arabidopsis* (Shinomura et al., 1996). Cryptochromes exist in three interconvertable flavin redox states, flavoquinone (FAD), flavosemiquinone (FAD·), and flavohydroquinone (FAD^-). Upon excitation by BL, the flavin chromophore is reduced to a semiquinone FAD· that can absorb light green and yellow light (Banerjee et al., 2007). However, FAD· is the active form mediating cry response. Illumination with green-yellow light (563±10 nm) results in the conversion of FAD· to FAD^- and inactivates cry (Banerjee et al., 2007; Bouly et al., 2007). Similar inactivation of cry-mediated responses by GL has been observed in an additional report (Sellaro et al., 2010). However, there are green

responses that cannot be accounted for genetically (Folta, 2004; Dhingra et al., 2006; Zhang, Maruhnich, and Folta, 2011). They persist in null photoreceptor backgrounds and typically operate in the opposite direction of normal light responses. These lines of reasoning suggest that a yet-to-be-defined GL sensor that may mediate these responses.

GL in the Regulation of Plant Growth and Development

The GL-mediated plant responses have been described throughout the history of plant photobiology research. The following paragraphs are a summary of contemporary cases where GL effects have been documented. Examination of these examples may guide further inquiries into how GL may affect plant growth and develop hypothesis to test the underlining mechanisms.

Seed Dormancy and Germination

Light is critical in conditioning seed dormancy release and subsequent germination. Different light wavebands produce various effects on this process. Different plant species exhibit ranging responses to blue and GL on dormancy release (Goggin and Steadman, 2012). For some plant species, like ryegrass (*Lolium rigidum*), seed dormancy release is stimulated by dark stratification. Germination of most dormant seeds cannot be stimulated by light unless they are given a 20-d-dark stratification treatment prior to the light exposure. Seeds stratified in FrL have a germination rate close to dark-stratified seeds. However, seeds stratified in BL maintain dormancy regardless of the presence or absence of FrL light. Interestingly, GL acts similarly to blue light to inhibit dormancy release in the absence of BL (Goggin, Steadman, and Powles, 2008). An action spectrum was devised for the response and showed that BL- or GL-mediated dormancy maintenance in ryegrass has three efficient peaks: 460, 510,

and 550 nm (Goggin and Steadman, 2012). Since the inhibition of dormancy release between 510 to 550 nm wavelengths is absent, they proposed that the GL effect at 550 nm was probably mediated by a novel photoreceptor other than cryptochrome (Goggin and Steadman, 2012).

Seedlings Establishment

Plants adjust hypocotyl or stem elongation in response to ambient light conditions. Typically, as the seedling emerges from the soil the elongation growth of *Arabidopsis* seedlings slows down rapidly, accompanied with the formation of photosynthetic apparatus. BL strongly inhibits stem elongation growth under high-fluence-rate illumination (Folta and Spalding, 2001a; Ahmad et al., 2002). This effect is mainly mediated by phot and cry and maintained as long as the BL is present. RL and FrL reduce hypocotyl elongation acting principally through phyB and phyA, respectively. Computer-aided image capture and analysis has revealed the precise timing of early elongation events, showing that blue, red and far-red effects are observed within minutes of illumination (Parks, Folta, and Spalding, 2001). The general rule is that light causes the developing seedling to cease rapid elongation and adopt a strategy of vegetative aerial growth in a sufficient light environment.

However, the same imaging equipment captured an unusual trend in response to GL with comparable fluence rates used in safelights. When illuminated, the seedling growth rate would not decrease. Instead, seedlings would grow faster than the dark rate, at times approaching 150% of the etiolated pace (Folta, 2004). The response to GL was observed within minutes of illumination and reversed to the dark rate when the light was toggled off. The response persisted in all photoreceptor mutant backgrounds, suggesting that the response was either a redundant response of several photoreceptor

classes to dim GL, or perhaps mediated by a novel receptor. The second explanation appears more attractive, as it was difficult to define how sensitive light sensors could be activated by GL, yet drive responses that were opposite to normal light activation. The same study also showed that seedlings grown under dim (<4 μmol/m^2s) red and BL were shorter than those grown under the same conditions with supplemented GL. A mechanism for this later phenomenon was shown in greater detail by another study (Bouly et al., 2007). They reported that the addition of 563 nm GL led to a longer hypocotyl than white or BL basal controls, despite increasing the fluence rate of GL. Here, addition of visible light resulted in plant responses consistent with a low-light environment. Further examination of this GL counteraction effect in photoreceptor mutants suggested that the BL component was cry-dependent, consistent with other studies (Sellaro et al., 2010). The difference is that the strongest variations were observed under low light conditions, a conclusion not parallel to the results of Sellaro et al. (2010).

Vegetative Growth

In the 1960s, Klein and co-workers performed a series of studies on the effects of near ultraviolet and GL to plant growth. A common theme that emerged from these studies is that the green wavebands (510-585nm) repress the growth of a wide range of organisms, including algae, fungi, higher plants, and even plant cell cultures (Klein, 1964; Klein, Edsall, and Gentile, 1965). These findings are consistent with Went's results that showed tomato seedlings reached higher dry weight under reduced GL conditions compared with white light controls (Went, 1957; Folta and Maruhnich, 2007). More recent data from Dougher and Bugbee also indicate that yellow light (580- 600 nm) reduces the dry mass of lettuce (Dougher and Bugbee, 2001). However, other

reports also provide different views regarding to GL effect on plant growth. NASA scientists conducted a series of experiments on plant growth in order to design an appropriate lighting system for space missions. One interesting result from these studies was that, when keeping the photosynthetic photon flux stable, lettuce grown in red, blue, and green LED light condition had a larger leaf area and higher shoot fresh and dry weight than lettuce grown in red-blue conditions alone (Kim et al., 2004a, b). Their interpretation of this result is that although RL and BL are more effective for photosynthesis in several reported crops, GL might penetrate plant leaves more efficiently than RL and BL, and therefore may increase carbon fixation deep within the plant tissue (Sun, Nishio, and Vogelmann, 1998; Nishio, 2000; Kim et al., 2004b; Terashima et al., 2009).

Shade Avoidance

Phytochromes represent the principal mechanism plants use to adjust growth to a shaded environment. In such an environment, the red to far-red ratio significantly drops. The change in red to far-red ratio is readily detected by phytochromes and plants develop elongated stems and leaf petioles, as well as an increased leaf angle (relative to the horizontal orientation) accordingly. These responses are known collectively as Shade Avoidance Syndrome (SAS) (Smith and Whitelam, 1997). In addition, plants in a shade environment also experience a reduction of BL intensity, which can also induce shade-like responses (Ballaré, Casal, and Kendrick, 1991; Ballaré, Scopel, and Sánchez, 1991; Pierik et al., 2004; Keuskamp et al., 2011). Shade-like phenotypes have also been observed under conditions with sufficient BL and a high red/far-red light ratio. Under the artificial shade of an electronic LED canopy, the relative amounts of blue, red and GL were individually mixed, and induced shade responses that were GL dependent

(Zhang, Maruhnich, and Folta, 2011). Genetic tests show that the GL-mediated shade avoidance response persisted in *phyA*, *phyB*, *cry1*, and *cry2* mutant backgrounds. The phot mutants were not tested because phot photoreceptors have a limited absorption in the green portion of the spectrum (Kennis et al., 2003), and active phot receptors promote leaf expansion in low light environments (Takemiya et al., 2005). The result observed here was exactly the opposite of phot's function. The physiological and genetic evidence suggests that an alternative means of sensing GL may be responsible to this response. Another interesting finding from this report is that the induction of traditionally-induced shade avoidance maker genes (such as *HAT4* and *PIL1*) are not induced as they are in far-red-mediated shade avoidance responses in wild-type plants. The physiological response is consistent with far-red action, yet the accompanying gene expression differences are not in concordance. Further examination of the shade-related gene expression was performed in photoreceptor mutants and showed that shade-induced increases in *HAT4* and *PIL1* transcripts are normal in the *cry1*, *cry2*, or *cry1cry2* mutant backgrounds (Zhang, Maruhnich, and Folta, 2011). These results indicate that cry receptors are actively repressing the gene expression changes that are associated with shade avoidance, uncoupling the morphological and molecular changes. The results also provide data that suggest cry receptors are actively responding to GL, repressing shade response-associated gene expression.

Flowering

The transition from vegetative growth to floral development is greatly affected by light (Guo et al., 1998; Mouradov, Cremer, and Coupland, 2002). Different wavebands of light exhibit distinct roles in the regulation of floral initiation. RL slows floral induction via the phyB receptor, while BL accelerates the induction mainly through the cry2

receptor (Guo et al., 1998; Valverde et al., 2004). As mentioned previously, GL can reverse BL mediated stem growth inhibition by inactivating cry1. If a similar mechanism is at play, the cry2 receptor may also be GL sensitive, allowing it to be inactivated. The report from Banerjee et al. (2007) tested this hypothesis. They found that the time needed for BL treated *Arabidopsis* plants to flower was significantly delayed by addition of GL (563±12 nm) under a short-day photoperiod. Consistent with this outcome, the cry2-mediated induction of *FLOWERING LOCUS T* (*FT*) transcript levels were also abolished by co-irradiation with GL, and the green effects were not observed in *cry2* mutant background (Banerjee et al., 2007). These results indicate that flowering is inactivated by alteration of the cry2 signaling state.

However, in other plant species, the flowering initiation is affected differently by GL, in contrast to the aforementioned scenario. The heading time of wheat does not seem to be affected by BL (400-500 nm), but plants grown in high fluence rate green-yellow light (500-600 nm) require fewer days to reach 50% heading (Kasajima et al., 2007). Analysis of inductive wavebands showed that 540 nm gave the strongest flowering stimulation effect (Kasajima et al., 2008; Kasajima, Inoue, and Mahmud, 2009).

Anthocyanin Accumulation

Anthocyanin biosynthesis is another BL-dependent response tied to cry1 (Ahmad, Lin, and Cashmore, 1995; Lin, Ahmad, and Cashmore, 1996). It is common in plants, and is well described in *Arabidopsis*, lettuce, tomato, and rapeseed (Ahmad, Lin, and Cashmore, 1995; Giliberto et al., 2005; Chatterjee, Sharma, and Khurana, 2006; Zhang and Folta, 2012). However, when GL (582 ±10 nm) is simultaneously delivered with BL, anthocyanin accumulation is lower than with BL treatment alone (Bouly et al.,

2007; Zhang and Folta, 2012). The extent of anthocyanin reduction depends on the fluence rate of GL delivered in concert with BL (Zhang and Folta, 2012). Close examination of this response in *Arabidopsis cry1* mutant indicates that it is a cry-dependent GL response (Bouly et al., 2007). This finding also points out the GL paradox—as visible light increases with the supplementation of GL, the magnitude of light driven response decreases.

Stomatal Opening

BL stimulates the opening of stomata through the redundant actions of phot1 and phot2 BL receptors. This response has a typical action spectrum that peaks at 450 nm with two additional shoulders at 420 nm and 470 nm (Karlsson, 1986; Kinoshita et al., 2001). Several reports have indicated that GL can reverse blue-light-dependent stomatal opening or reduce stomatal conductance among several plant species, such as *Vicia faba*, *Arabidopsis thaliana*, *Nicotiana tabacum*, *Pisum sativum*, and *Lactuca sativa* (Frechilla et al., 2000; Talbott et al., 2002; Kim et al., 2004a). The action spectrum for the GL reversal effect matches well with that of BL activation but with a 90 nm shift towards red (Frechilla et al., 2000). The extent of this GL reversal effect is dose-dependent, with full negation achieved when GL fluence rate is twice that of BL (Frechilla et al., 2000).

Gene Expression

Photoreceptors initiate many processes that culminate in changes of plant form and/or function, accompanied by a supporting set of gene expression alterations (Tepperman et al., 2001; Folta et al., 2003b; Tepperman et al., 2004). During the transition from dark growth to light growth, the phyA receptor transduces environmental light information to the downstream genes, of which almost half are transcription factors

(Tepperman, 2001; Tepperman, 2004). GL also induces transcriptional profile changes. Microarray analyses based on the early stem kinetics reported in Folta (2004) show that many of the transcripts up-regulated by GL are typically induced by phytochromes. This observation is not surprising because phyA is extremely sensitive and abundant in the dark-grown seedling. The curious finding is that a set of light induced plastid genes, including *psaA*, *psbD*, and *rbcL* were down-regulated by a GL pulse relative to dark levels. The GL-mediated down-regulation of plastid gene transcripts accumulation is obvious within the fluence range of 10^0 to 10^4 µmol/m^2, and occurs within 15 min after the GL pulse. This response is GL specific since it was not observed under red, far-red, blue, or red plus BL pulses. The time course and fluence response of this GL mediated plastid response correlated well with the green-light-stimulated hypocotyl response. The down regulation of plastid transcripts is normal in *phyA*, *phyB*, *cry1*, *cry2*, *phot1*, and *phot2* mutant backgrounds, indicating that they are controlled by redundant photoreceptors function or possibly a novel receptor.

Arabidopsis Natural Variations in Light Signaling Transduction

Due to their sessile nature, plants must adapt to the local environment once established. Natural genetic variation in *Arabidopsis* provides researchers with invaluable resources for discovering functional alleles, especially those that underlie adaptive responses. *Arabidopsis* has a geographically wide distribution. Functions of nearly 100 genes have been identified from *Arabidopsis* and crop plants (rice, maize, etc.) by the use of naturally occurring variants (Alonso-Blanco et al., 2009). Pioneering studies have also discovered alternative photoreceptor alleles for *PHYA* (Maloof et al., 2001), *PHYB* (Filiault et al., 2008), *PHYC* (Balasubramanian et al., 2006), *PHYD* (Aukerman et al., 1997), and *CRY2* (El-Assal et al., 2001) from natural *Arabidopsis*

accessions. The detailed discussion about light-sensing natural variants will be further addressed in the Chapter 4. The hypothesis for using natural variations for the GL study is that GL may confer some adaptive advantages in certain environments, but not in others. It is possible to find candidate *Arabidopsis* accessions with impaired or altered GL response by monitoring adaptive responses. In addition, the genetic resources for *Arabidopsis* provide another opportunity to test the relationship to genes identified and the GL response. The increased amount of genomic information released from '*Arabidopsis* 1001 Genomes Project' empowers the capability and efficiency of photoreceptor and photoresponse research.

Light responses to the environment are well understood in Arabidopsis and other systems. Although GL responses have been historically documented, little is known about their precise effects or transduction mechanism. The goal of this work is to understand the integration of light signals during plant development and incorporate the roles of the GL portion of the spectrum into existing models.

[2]CHAPTER 2
INVESTIGATION OF THE INTERACTIONS BETWEEN BLUE-GREEN LIGHT AND RL/FrL DURING EARLY PHOTOMORPHOGENESIS

Introduction

The dark-grown seedling undergoes a dramatic developmental shift as it transitions to the light environment. The emerging seedling monitors incident irradiation for the proper adjustment of morphology, metabolism and development to best fit the current environment. The relative quanta from various regions of the spectrum inform the seedling of time, place and proximity to neighbors. Ultraviolet, blue, red and far-red light (UV, BL, RL, FrL, respectively) each drive a suite of morphological, physiological, biochemical and molecular changes that prepare the juvenile plant for optimal growth in a given environment (Chen, Chory, and Fankhauser, 2004; Kami et al., 2010).

The events of early photomorphogenic development include conspicuous alterations in plant form, particularly a suppression of stem growth elongation (Parks, Folta, and Spalding, 2001; Vandenbussche and Van Der Straeten, 2004). The rate of stem elongation is faster in darkness, yet is rapidly and robustly suppressed by UV, BL, RL or FrL. Inhibition of hypocotyl growth rate is dependent upon several separate photo-sensing systems that coordinate downstream events with great precision. The genetic control of light-mediated stem elongation has been greatly facilitated by the use of photomorphogenic mutants coupled with specialized computer-assisted imaging (Wang et al., 2009), allowing high-resolution analyses of growth kinetics as plants adapt to the light environment. For example, in RL, stem growth inhibition is mediated by phyA for

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the first 3 h, and then maintained by phyB thereafter (Parks and Spalding, 1999). BL regulates hypocotyl growth through phot1 for the first 30 min of irradiation (Folta and Spalding, 2001a), followed by inhibition dependent on the actions of cry1, cry2 and phyA (Folta and Spalding, 2001a; Folta and Spalding, 2001b). This second phase is antagonized by phyB. It is logical that the wavebands that best affect photosynthetic rate would be effective in promoting photomorphogenic development. The effects of GL on stem growth control have also been examined. In end-point analyses, GL has been shown to negate BL effects on hypocotyl elongation through cry1 by reducing the redox state of cry's flavin chromophore (Bouly et al., 2007). Examination of seedling growth during the first minutes and hours of photomorphogenic growth shows that GL induces a transient increase in stem growth rate. Illumination with constant GL in conjunction with RL and BL leads to a longer hypocotyl than that produced by RL or BL alone (Folta, 2004), yet it has not demonstrated if this effect was cry or phy dependent.

The relationship between BL and GL was tested in *Arabidopsis* seedlings, and indicated that the BL/GL ratio controls stem growth via cryptochrome receptors in light conditions that mimic natural conditions (Sellaro et al., 2010). While low GL illumination leads to an increase in stem growth rate during the initial stages of de-etiolation, after several days the stem of a green-light-grown seedling is shorter than a comparable dark-grown seedling (Young, Liscum, and Hangarter, 1992; Goto, Yamamoto, and Watanabe, 1993). This discrepancy can almost certainly be attributed to green-light activation of cry and phy during prolonged irradiation periods. However, the detailed information about how GL interacts with cryptochromes and phytochromes in the early transition from a dark-grown to light-grown environment has not been thoroughly

described. Although it has been shown that GL can negate BL-induced stem growth inhibition in the end-point assay, in the initial stages (minutes to hours), supplementation of GL to BL does not attenuate BL-induced stem inhibition, but instead enhances BL effects (Wang et al., 2013). The interactions of GL and phytochrome during the initial stages of photomorphogenesis are even less understood. Although in the dim RL background, the stimulation effect of GL to hypocotyl elongation is enhanced, the development of GL response is independent of RL (Folta, 2004). The purpose of this study is to examine how GL might interact with RL/FrL photo-sensing systems throughout various time courses by monitoring the sensitive responses of the elongating hypocotyl in wild-type and mutant seedlings. Such study might help elucidate how GL modulates phy-mediated responses under shaded environment.

Results

GL Attenuates RL-Mediated Stem Growth Inhibition

To test the interactions between GL and RL photo-sensing systems on early hypocotyl elongation, RL and GL co-irradiation experiments were performed. As shown in Fig. 2-1A, the hypocotyl elongation rate in RL started to rapidly decrease after 45 min, reaching a constant reduced growth rate after 120 min (approximately 50% of dark growth rate). The addition of dim GL significantly attenuated RL induced stem growth inhibition. The maximum opposition of RL response by GL co-irradiation during the three-hour time-course of the experiment was approximately 75% of the dark growth rate. The extent of the antagonizing effect by GL depended on the fluence rate of RL, but not GL itself under the fluence rates tested (Fig. 2-1B, C). The increase in stem growth rate was reflected in longer hypocotyls after extended treatment periods. From

48-96 h significant differences in hypocotyl length were observed between RL-treated seedlings and those co-illuminated with GL (Fig. 2-1D).

Genetic Analysis of the GL Opposition of RL Response

GL caused an increase in initial stem elongation that mirrored the time-course of RL-induced inhibition. The hypothesis was that the two actions were mediated by separate signaling systems, and not directly through GL inactivation of the phy receptor. The GL negation of RL response could also be acting act through the cry receptor in a manner that is different from its activation by BL, or BL and GL in combination, as in Zhang et al. (2011). Analysis of photosensory mutants allowed further exploration into this process. The GL opposition of RL-induced inhibition was tested in *phyA* and *phyB* single mutant backgrounds. Under monochromic RL conditions, the degree of stem-growth-rate inhibition in the steady-inhibition-stage of the *phyA* mutant was moderately reduced compared to wild-type, consistent with earlier reports (Parks and Spalding, 1999). Initial, rapid inhibition (45min~120min) was impaired in the *phyA* mutant, yet long-term inhibition (>2h) was sustained through the action of phyB. In contrast to the *phyA* mutant, *phyB* mutants exhibited normal initial-rapid inhibition (45min~120min), but showed less inhibition upon prolonged illumination. Although the RL-induced stem inhibition was partially impaired in the *phyA* or *phyB* mutants, the GL-mediated attenuation effect persisted in both mutant backgrounds (Fig. 2-2A, B). These data are consistent with other reports that early GL responses are not phytochrome driven (Folta, 2004; Dhingra et al., 2006).

GL can act directly on cryptochromes to affect plant responses (Bouly et al., 2007; Sellaro et al., 2010; Zhang, Maruhnich, and Folta, 2011). Cryptochromes have also been shown to influence mRNA and protein accumulation even in response to RL,

but the cry-driven hypocotyl growth responses have not been observed to be red-sensitive (Ahmad et al., 2002; Yang et al., 2008). Therefore, the GL opposition of RL-induced growth inhibition was tested in *cry* mutant backgrounds. The results are presented in Fig. 2-2C and indicate that GL reversal of RL response persists in *cry1cry2* mutant background.

Similarly, no role for phototropin (phot) receptors has been demonstrated in a RL response. For completeness, the GL opposition to RL was tested in *phot* mutants. While RL responses were normal in *phot* mutants, the GL reversal of RL response was not observed in the *phot1-3* mutant background (Fig. 2-2E). This result was confirmed using the *phot1-5* allele (data not shown) as well as the *phot1phot2* double mutant (Fig. 2-2F). The GL effect was absent in all three mutant backgrounds. However, the GL opposition of the RL response was normal in *phot2-1* mutant seedlings (Fig. 2-2D). Interestingly, RL-mediated stem growth inhibition was also slightly impaired in the *phot1phot2*, double-mutant background. NPH3 is a key component mediating a subset of phot-driven responses. Thus the GL opposition effect on RL was further tested in the *nph3-6* mutant background. Consistent with the *phot1* result, *nph3-6* mutant seedlings failed to reverse the RL stem-response under co-irradiation conditions (Fig. 2-2G). Together, the data demonstrate that phot1, but not any other photoreceptors tested, is required for GL opposition to the RL response, and NPH3 is also required for this interaction.

The GL Opposition to RL Stem Response does not Affect other phy Responses

Phy-mediated changes in gene expression during de-etiolation have been well described (Tepperman et al., 2001; Tepperman et al., 2004). It is therefore possible to test if the reversing effect of GL is restricted to growth responses in the hypocotyl, or perhaps affects a wider suite of phy-mediated processes. The transcript levels of phy-

induced genes were monitored in response to RL and a combination of RL and GL. Transcript levels were assessed 140 min after the onset of illumination, the time point corresponding to the most pronounced level of GL response. The results are presented in Fig. 2-3A. *HY5*, *SPA1*, *PKS1*, and *LHY* transcript levels were highly induced after 140 min of RL treatment. Comparable transcript accumulation levels were observed with GL co-irradiation. No effect was observed even after increasing GL fluence rate. Two additional time points were tested to exclude the possibility that the GL effect on gene expression may not coincide directly with the timing of the stem-growth response, yet no effect was observed (Fig. 2-3B).

GL also Reverses Early FrL-Induced Stem Growth Inhibition

Unlike RL, the FrL-induced stem growth inhibition during initial photomorphogenesis is solely mediated by the phyA receptor (Parks and Spalding, 1999). To investigate the interactions between GL and FrL in the regulation of initial stem elongation, the stem growth kinetics of 2-d-old etiolated seedlings were monitored. FrL caused rapid, stem-growth-rate inhibition after 45 min of irradiation, in agreement with the time point of RL-induced inhibition. However, the time course of maximum inhibition for FrL was found to be shortened and exhibited a greater amplitude, even at a relatively lower fluence rate (2.5 μmol/m^2s). GL (0.5 μmol/m^2s) co-irradiation with FrL reversed the FrL effect (Fig. 2-4A). A higher (50 μmol/m^2s) FrL fluence rate could only be slightly reversed by GL (Fig. 2-4B). Examination of seedlings in long-term end-point experiments showed that the relative stem length in FrL (2.5 μmol/m^2s) was not affected by addition of GL (0.5 μmol/m^2s) (Fig. 2-4C).

GL Negation of FrL Stem Response also Requires phot1 and NPH3

The photoreceptor mutants were tested for the GL reversal of the FrL response. As shown in Fig. 2-5A, FrL-induced stem growth inhibition was absent in the *phyA* mutant background, consistent with reports that phyA mediates the FrL stem response (Parks and Spalding, 1999). Addition of GL to FrL did not significantly affect the growth kinetics in the *phyA* mutant. In the *phyB* mutant, the FrL-induced stem inhibition was enhanced. GL partially reversed the FrL stem response in *phyB* mutant seedlings (Fig. 2-5B). The *cry1cry2* double mutant seedlings showed a normal FrL stem inhibition response, as well as the GL reversal effect (Fig. 2-5C). The *phot2* mutant seedlings exhibited a slightly reduced FrL stem response and normal GL reversal effect (Fig. 2-5D). When tested in *phot1*, *phot1phot2*, and *nph3-6* mutant seedlings, the FrL response was normal, but the GL effect was completely absent (Fig. 2-5E, F, G).

GL Negation of RL Stem-Response can be Phenocopied by Supplementation of Dim BL

The observation that GL acts through phot1 and its signaling component, NPH3, to negate RL/FrL stem response was at first difficult to reconcile. Early GL stem growth promotion responses (those occurring within 1h) have been shown to be phot1-independent (Folta, 2004). Also, the chromophore-bound LOV-domain has been shown to have virtually no absorption above 500 nm (Kennis et al., 2003). However it is important to test the formal hypothesis that the GL response shown here may be a response to the slight amount of BL emitted by the green LED (peak 530 nm). To test this hypothesis, seedling growth rate kinetics were monitored when co-irradiated with dim BL (0.1 µmol/m^2s) and RL. The dim BL negated the RL-induced stem inhibition in these trials, and the response was more effective in reversing RL response than GL

(Fig. 2-6A). Because the RL induced stem inhibition started as early as 45 min after light onset, it was of interest to test reciprocity, that is, if inhibition could be reversed by a brief BL pulse delivered at this time point with the same fluence as the longer, dim treatment.

Seedlings were then grown under RL conditions as above, and then were treated with a 3 μmol/m^2s BL pulse for 6 min at 45 min time point. This brief pulse was sufficient to activate phototropins, but not cry-mediated BL stem inhibition. The results in Fig. 2-6B indicate that the BL pulse only caused a slight negation of RL stem response. This BL light response was tested in *phot1* and *nph3* mutant seedlings to verify that it was a phot response. Again, *phot1* and *nph3* mutant seedlings failed to negate RL stem response under co-irradiation conditions (Fig. 2-6C, D). Parallel responses were observed in response to FrL (Fig. 2-7).

Discussion

GL and Phytochrome in the Regulation of Photomorphogenic Responses

GL responses in seedlings have been shown to oppose activities of other wavebands. Such GL responses have been hypothesized to be the basis of adaptive behaviors in developing seedlings that aid in acclimation to a specific light environment. Although much is known for blue, red, and far-RL (BL, RL, and FrL) in the control of hypocotyl elongation during photomorphogenic development, the interactions between GL and the aforementioned photosensory systems are less well understood. Using high-resolution imaging, photomorphogenic mutants, and narrow-bandwidth illumination, such interactions may be understood in greater detail.

The stem growth inhibition induced by RL or FrL was at least partially reversed by simultaneous illumination with low fluence rate GL (Figs. 2-1, 2-4). The GL effect was

obvious after 120 min and produced enough reversal of RL induced inhibition to change end point stem lengths (Fig. 2-1D). Analysis of phy-mediated gene expression shows that while stem growth is reversible, RL-driven gene expression is not, suggesting that the GL effect is happening early in signaling and at the cellular level, and is not causing a direct reversal of phy or phy signaling (Fig. 2-3).

Genetic analyses indicated that the early reversal persisted in *cry* and *phy* mutants, but did not persist in two alleles of *phot1* mutants or the *phot1phot2* double mutant (Figs. 2-2, 2-5). The *phot2* mutant exhibited a wild-type response. The response was impaired in the *nph3* mutant, a functional interacting partner of phot1 required for some aspects of phot1 signaling. The interactions between phot1 and phy systems have been described by several reports. For example, the amplitude of the phototropic response can be enhanced by pretreatment or co-irradiation of RL (Janoudi and Poff, 1992; Parks, Quail, and Hangarter, 1996; Janoudi et al., 1997). A brief RL pulse 2 h prior to BL illumination inhibited the translocation of plasma-membrane-localized PHOT1-GFP to the cytosolic compartments (Han et al., 2008).

At first there were several interpretations for a phot-mediated GL reversal of RL induced inhibition through a novel sensor, but they involved complicated models and assumptions to reconcile the unexpected result. However, one hypothesis remained to be tested—the reversal of RL-mediated inhibition might be due to phot activation from a small amount of BL produced by the GL-emitting diode. The possibility seemed unlikely because BL and phototropins inhibit stem elongation (Folta and Spalding, 2001a). However, it was important to formally test the hypothesis. In a subsequent series of experiments low fluence rate BL was substituted for GL, and was shown to reverse the

RL response, perfectly phenocopying the GL response (Fig. 2-6). These findings show that the GL reversal of the RL response was actually a low fluence rate BL response revealed by a GL treatment. A simplified model describing the interactions of different photosensory systems together with its mediating photoreceptors is proposed (Fig. 2-8).

Phototropin 1 and its Negation Effect on RL/FrL

The findings that phot1 actively responds to low fluence rates of BL to negate the RL/FrL stem response were unexpected and add seedling growth promotion to the expanding list of roles for phototropin in plant tropic responses (Christie, 2007). BL and phototropin stem growth responses have always been described as inhibitory, yet the data in this report show that under specific RL or FrL conditions the phototropins can promote elongation. Phototropins have been shown to drive expansion growth of leaves and increases in fresh weight in low-light environments (Takemiya et al., 2005). The effects observed in the current study may be related to phot activity after the developing seedling has committed to growth in a light environment and switched to optimization of organ expansion and position. This interpretation is consistent with the overarching roles for phototropins, as they tend to adjust parameters of plant form and content to optimize position of specific organs or organelles (Spalding and Folta, 2005). It also is worth noting that the *phot2* mutants respond normally, and *nph3* mutants fail to respond, showing that the phot1-driven stem promotion is phot2 independent and NPH3 dependent.

The significance of these findings lies in adding another layer of understanding to photosensor interplay in low light environments. Coaction between GL and phy, cry and phot has revealed unexpected mechanisms of action that are separate from the proposed green sensor. This contrasts against unexplained GL actions in etiolated

seedlings as well as in shade-avoidance responses (Folta, 2004; Dhingra et al., 2006; Zhang, Maruhnich, and Folta, 2011). Coactivation of multiple sensory systems in developing seedlings shows that GL reverses cry-mediated responses over the first several days and augments cry responses within minutes of illumination. Here we show that the response to GL is really just a response to BL, yet low fluence rates of BL that impart an uncharacteristic effect. Minor amounts of BL insufficient to activate cry, activate phot1 and increase seedling growth in the presence of RL, consistent with what occurs in mature plants (Takemiya et al., 2005). Here narrow bandwidth illumination, monitoring stem growth kinetics, and photomorphogenic mutants provide additional resolution into light coaction during early photomorphogenic development.

Materials and Methods

Plant Materials

The genotypes tested are identical to those previously assessed for responses to blue (Folta and Spalding, 2001a; Folta and Spalding, 2001b) and green (Folta, 2004) light: *cry1-304*, *cry2-1*, *phot1-3 (nph1-3) phot1-5 (nph1-5)*, *phot2-1*, *phyB-5,* and *nph3-6*. *Arabidopsis* accession Columbia (Col-0) was used as the wild-type.

Light Sources and Treatments

Light treatments were generated using narrow-bandwidth LED light supplied either by arrays of 3 W LEDs (American Bright LED, Chino, CA USA) or Floralamp LED arrays (Light Emitting Computers, Victoria, BC Canada). The emission spectrum of all light sources is viewable on-line at www.Arabidopsisthaliana.com/lightsources. Light composition was assessed using an Apogee spectroradiometer (Apogee Instruments, Inc., Logan, UT) and Spectra Whiz software (StellarNet, Inc., Tampa, FL).

Hypocotyl Elongation Assays

For end-point assays (24-96 h in light), *Arabidopsis* seeds were surface-sterilized briefly by immersion in 95% ethanol on blotting paper in a laminar flow hood. Upon drying, the seeds were distributed in Petri dishes on minimal media (1mM KCl and 1mM $CaCl_2$) solidified with 1% Difco agar (Beckton, Dickinson and Co, Sparks, MD). The plates were covered in foil and then stratified for 48-72 h at 4°C. The seeds were then treated with 6-8 h of fluorescent white light (16 µmol/m^2s) at 23°C to synchronize germination. Seedlings were grown in absolute darkness at 23°C for 40-48 h. Germinating seedlings were then moved under experimental light conditions without a photoperiod. At least 30 seedlings were measured per treatment in two to three independent experiments. Analysis of stem growth kinetics was performed by scanning petri dishes of seedlings on a flatbed scanner, and then measuring seedlings at high magnification using ImageJ 1.44 (http://imagej.nih.gov/ij/index.html). The mean of 30-42 seedlings is reported for each light condition.

For short-term kinetic assays, *Arabidopsis* seeds were surface-sterilized and synchronized for germination as described above. Then, different experimental light treatments were applied to 2-3 d old etiolated seedlings and images were acquired in a 5-min-interval and analyzed according to the method described (Wang et al., 2009).

Quantitative RT-PCR Analysis

The 3-d-old dark grown seedlings were treated with red or red plus GL at specified time points and harvested into liquid nitrogen. Total RNA was extracted by using the Qiagen RNeasy Mini kit (Qiagen Cat # 74904) according to the manufacturer's protocol. 1 µg RNA was reverse transcribed using the Improm-II Reverse Transcriptase (Promega Inc., Madison, WI). Quantitative RT- PCR was performed using the StepOne

Plus system (Applied Biosystems, USA) based on SYBR Green chemistry. All primers were designed by the Primer Express 2.0 software (Applied Biosystems, USA). *UBC21* (AT5G25760) was used as the reference gene (Czechowski et al., 2005). All primer sequences are listed in Table 2-1. The relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Accession Numbers

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative or GenBank/EMBL databases under the following accession numbers: *UBC21* (AT5G25760), *HY5* (AT5G11260), *PKS1* (AT2G02950), *SPA1* (AT2G46340), and *LHY* (AT1G01060).

Table 2-1. Primers used in qRT-PCR assay in Chapter 2

Primers	Sequences (5'-3')
qUBC21-5'	TTAGAGATGCAGGCATCAAGAG
qUBC21-3'	AGGTTGCAAAGGATAAGGTTCA
HY5-QF	AGAGTTTCAGCTCAGCAAGCAA
HY5-QR	TCTGTTTTCCAACTCGCTCAAG
PKS1-QF	AGAAGCAGCATGAACAAGACACA
PKS1-QR	TGATCCTTCTTCGTGTTTCGAA
SPA1-QF	TGACTTTGAAGCAAACGTTTTTG
SPA1-QR	CGAACTTTTTTTCTCTTCTGGTCAT
LHY-QF	AGCCAAGTTGGGAACATAAACAA
LHY-QR	TTCCAATCGAAGCCTTTTGC

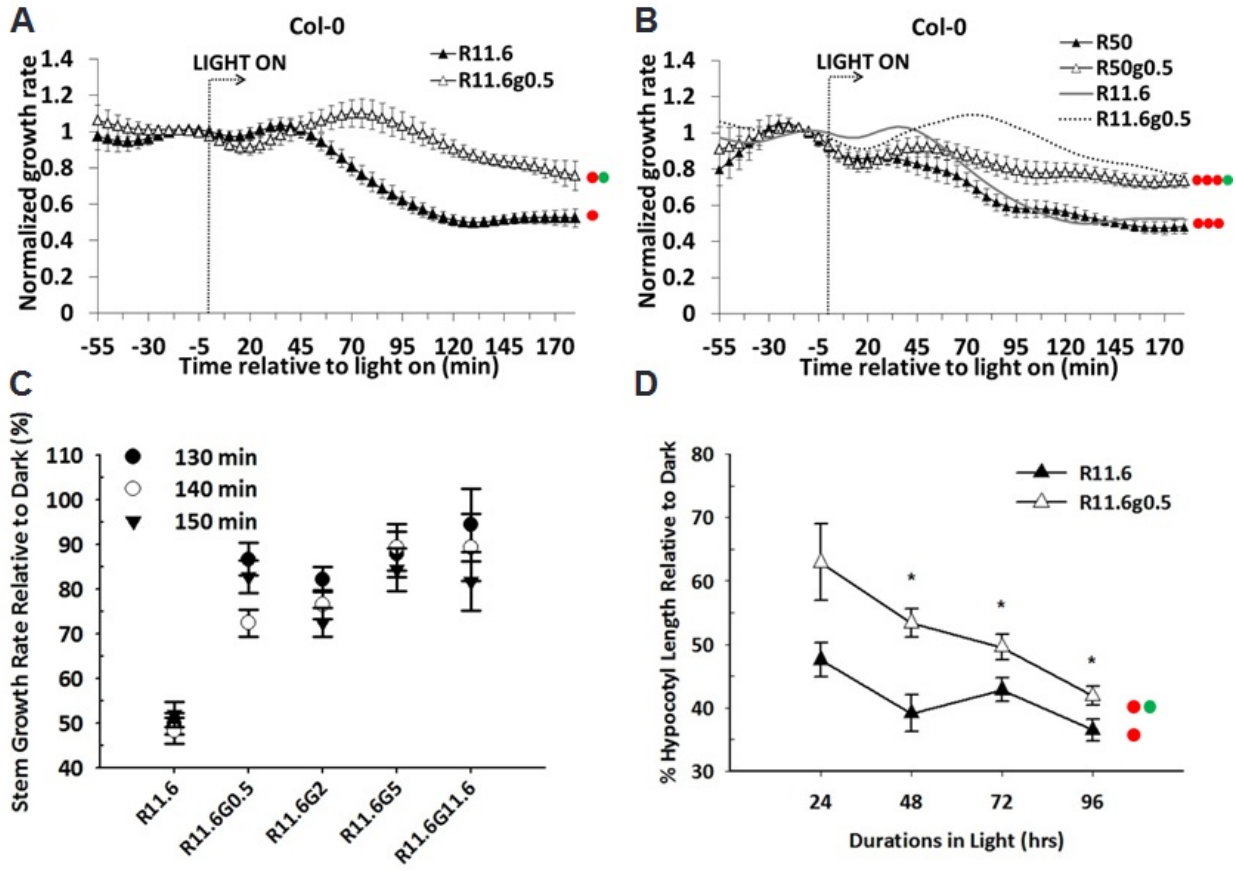

Figure 2-1. GL attenuates RL-induced stem inhibition. 2-day-old or 1-day-old etiolated *Arabidopsis* Col-0 seedlings were tested for short-term or long-term stem growth kinetics, respectively. (A) The short-term stem-growth-rate kinetics of seedlings in red (solid triangles) or red plus GL (open triangles) condition. R11.6= red, 11.6 µmol/m^{2}s, g0.5=green, 0.5 µmol/m^{2}s. Error bars represent S.E.M. (13≤n≤17). (B) Increasing RL fluence rate to 50 µmol/m^{2}s (R50) reduced the GL opposition effect. Error bars represent S.E.M. (9≤n≤14). (C) The extent of the opposition effect on RL does not depend on GL fluence rates tested. Three time points (130 min, 140 min, and 150 min) were selected for the comparison. Error bars represent S.E.M. (7≤n≤17). (D) The long-term stem growth kinetics of seedlings in red or red plus green conditions. Error bars represent S.E.M. of one experiment (17≤n≤24). Each experiment was performed at least twice with similar results. (*, p<0.05)

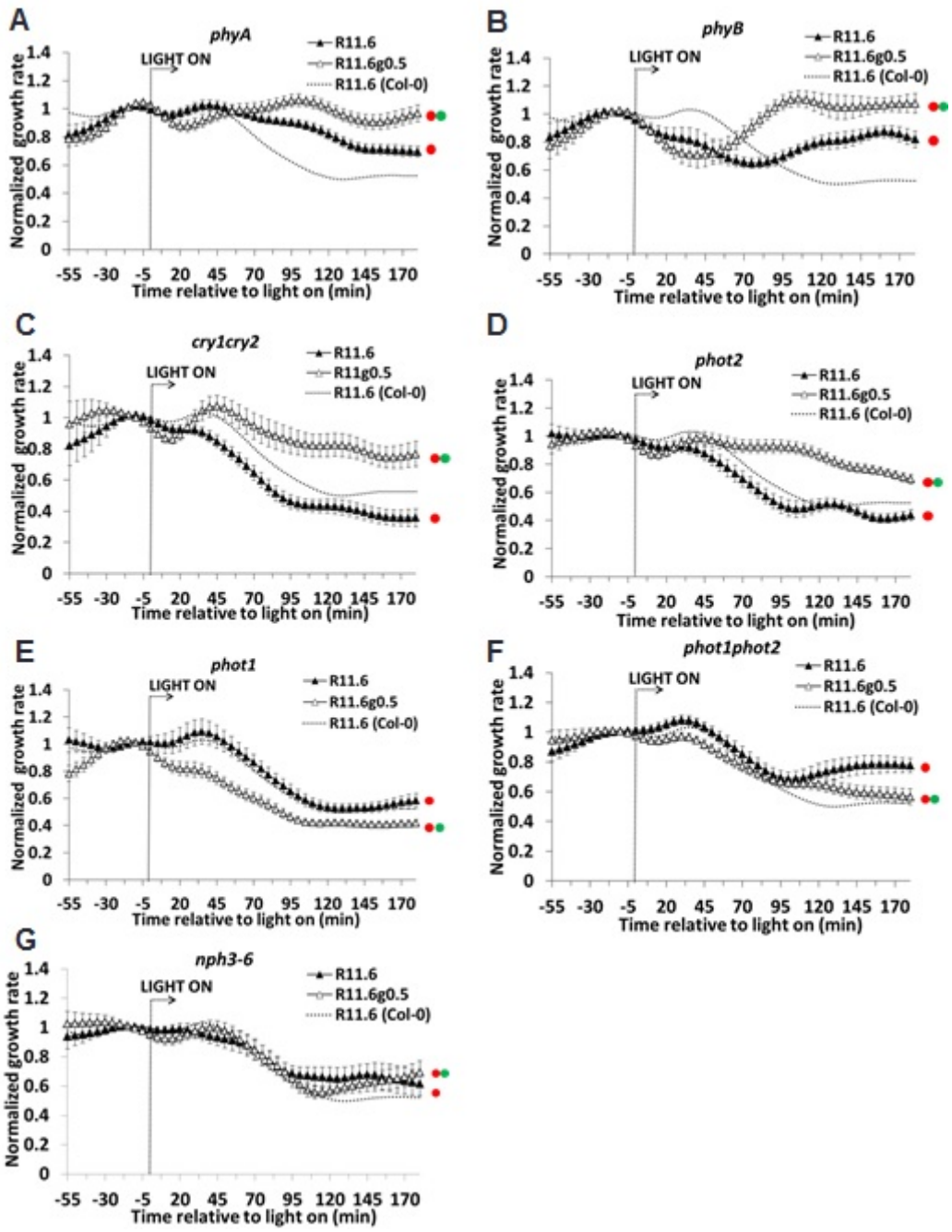

Figure 2-2. GL opposition effect on RL requires phot1 and NPH3. The GL negation effect on RL was further tested in (A)-(G) *phyA*, *phyB*, *cry1cry2*, *phot2*, *phot1*, *phot1phot2*, and *nph3* mutant backgrounds. In all panel, the line with solid triangles represents the growth-rate kinetics of mutant seedlings in RL, and the line with open triangles represents the growth-rate kinetics of mutant seedlings in red plus GL. Growth-rate kinetics of wild-type Col-0 seedlings in RL was included for comparison (dashed line). Error bars represent S.E.M. ($9 \leq n \leq 19$).

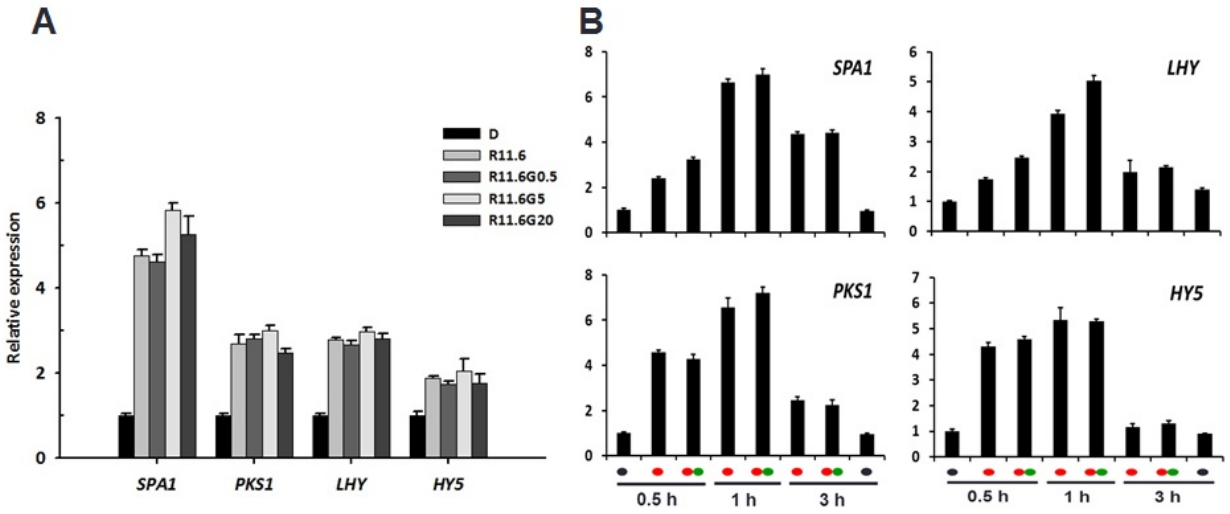

Figure 2-3. The GL opposition to RL stem responses does not affect the expression of RL-early-induced marker genes. (A) The expression of RL-early-induced marker genes were not affected by addition of three different fluence rates (0.5, 5, and 20 µmol/m^2s) of GL. Time point 140-min was chosen for this comparison. Transcript levels were normalized to dark (D) control seedlings**.** (B) Time course comparison (30-min, 1-hour, and 3-hour) for the expression of marker genes in red (11.6 µmol/m^2s) and red plus green (0.5 µmol/m^2s) conditions. Error bars represent S.E.M. of three technical replicates. Same experiment was performed twice with similar result.

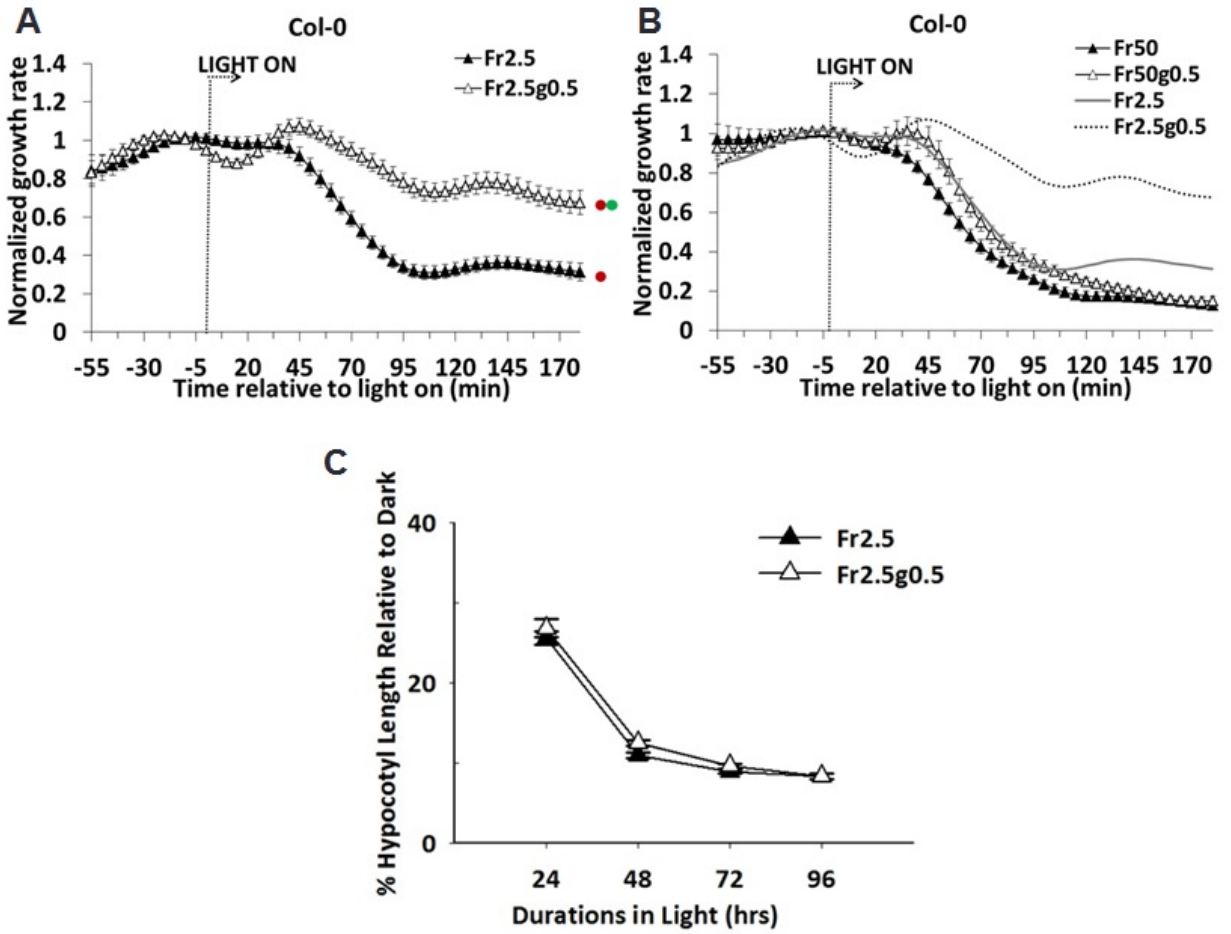

Figure 2-4. GL negates FrL-induced stem inhibition in short-term analyses. (A) The short-term, stem-growth-rate kinetics of seedlings in far-red (solid triangles) or far-red plus GL (open triangles) condition. Fr2.5=far-red, 2.5 µmol/m^2s; g0.5=green, 0.5 µmol/m^2s. Error bars represent S.E.M. (11≤n≤17). (B) Increasing FrL fluence rate to 50 µmol/m^2s (Fr50) largely reduced GL opposition effects. Error bars represent S.E.M. (10≤n≤15). (C) The long-term stem growth kinetics of seedlings in far-red or far-red plus GL condition. Hypocotyl length is displayed as percentage of that observed for the dark grown seedlings. Error bars represent S.E.M. of one experiment (16≤n≤24). Same experiment was performed at least twice with similar result.

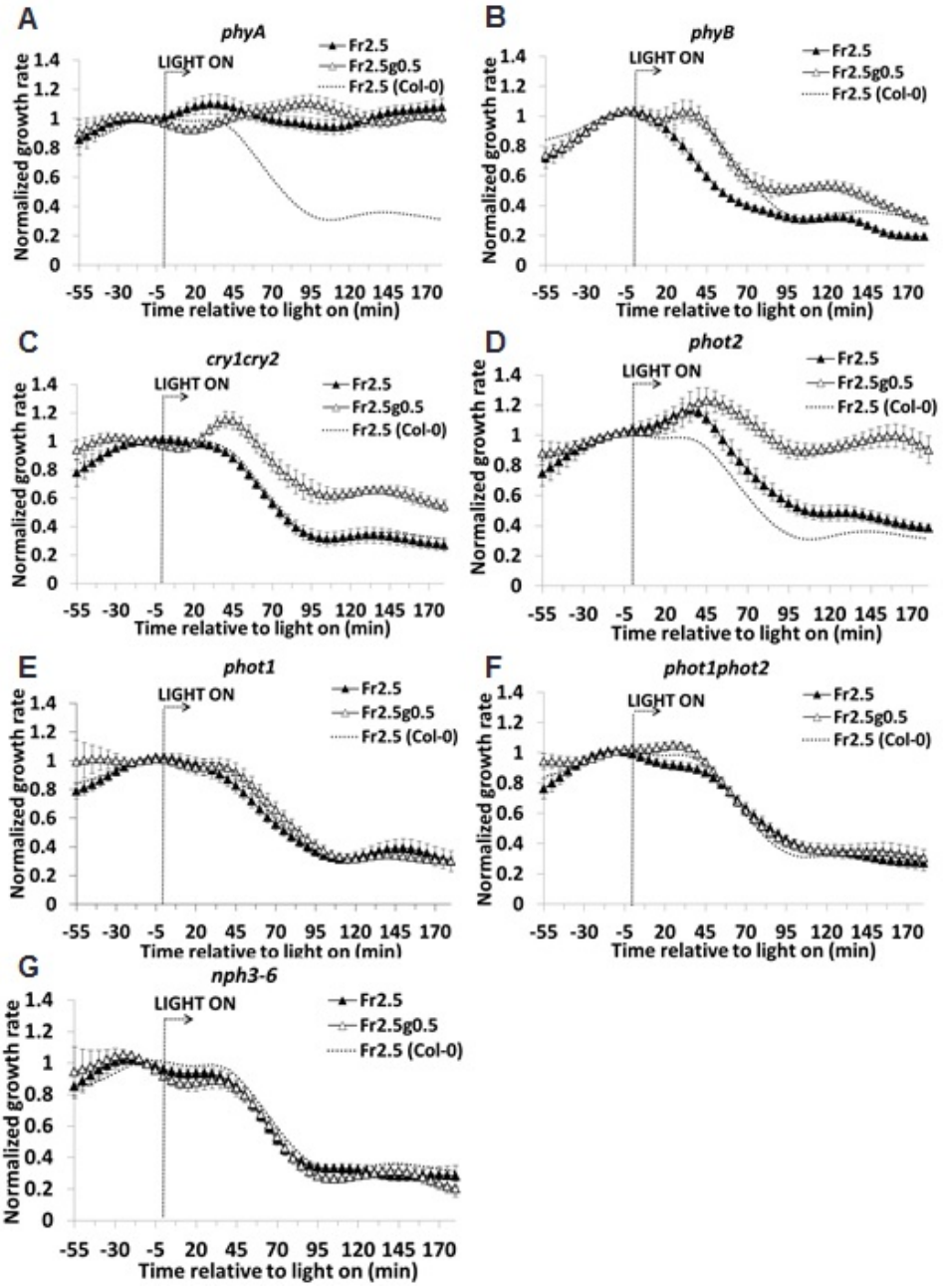

Figure 2-5. GL opposition to FrL stem response also requires phot1. The GL opposition to FrL was examined in (A)-(G) *phyA*, *phyB*, *cry1cry2*, *phot2*, *phot1*, *phot1phot2*, and *nph3* mutant backgrounds. In all panels, lines with solid triangles represent growth rate kinetics of mutant seedlings in FrL, and lines with open triangles represent growth-rate kinetics of mutant seedlings in far-red plus GL. Growth-rate kinetics of wild-type Col-0 seedlings in FrL was included for comparison (dashed line). Error bars represent S.E.M. ($10 \leq n \leq 16$).

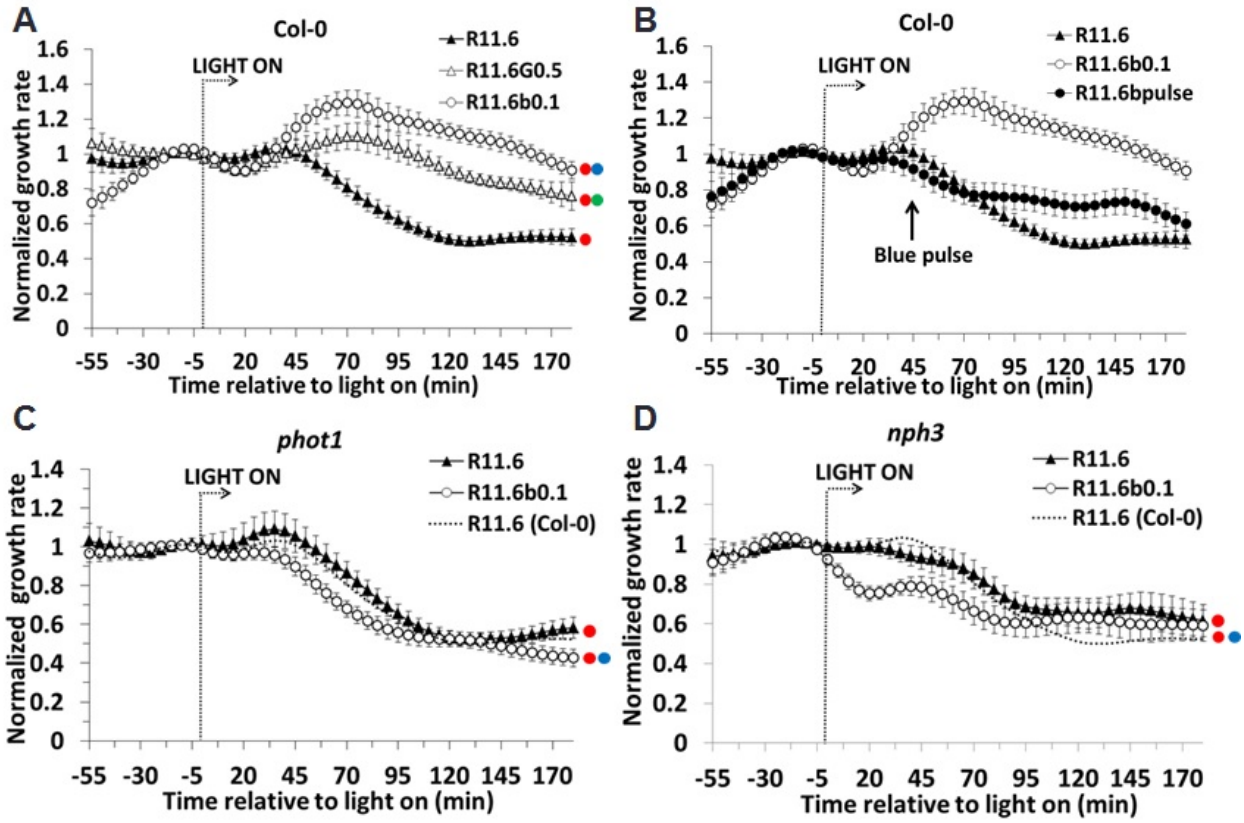

Figure 2-6. BL negates RL stem inhibition through phot1. The BL negation effect on RL was examined in (A) continuous dim BL (0.1 µmol/m^{2}s) and (B) brief blue pulse (3 µmol/m^{2}s, 6 min) conditions. Genetic tests were performed in (C) *phot1* and (D) *nph3* mutant seedlings under co-irradiation conditions. In all panels, line with solid triangles represents growth rate kinetics of seedlings in RL, and lines with open circles represent growth rate kinetics of seedlings in red plus dim BL conditions. Lines with solid circles in (B) represent the growth rate kinetics in red plus blue pulse conditions. Growth rate kinetics of wild-type Col-0 seedlings in RL was included for comparison (dashed line) in (C) & (D). Error bars represent S.E.M. (10≤n≤12).

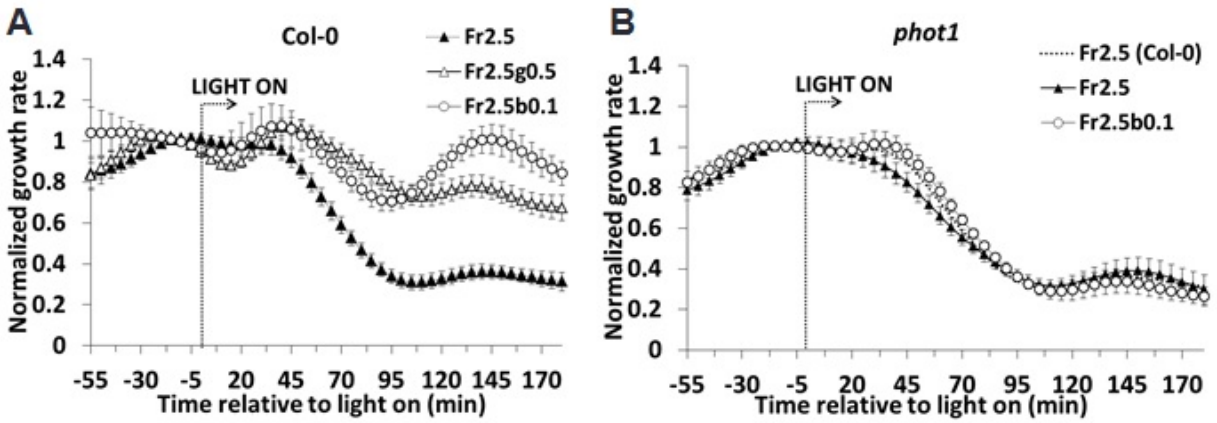

Figure 2-7. BL attenuates FrL-induced stem inhibition through phot1. (A) The BL negation effect on responses to FrL were examined in continuous dim BL (0.1 µmol/m^2s) condition. (B) Genetic tests were performed in *phot1* mutant seedlings under co-irradiation conditions. In all panels, lines with solid triangles represent growth rate kinetics of seedlings in FrL, and lines with open circles represent growth rate kinetics of seedlings in far-red plus dim BL conditions. Growth rate kinetics of wild-type Col-0 seedlings in FrL were included for comparison (dashed line) in (B). Error bars represent S.E.M. ($8 \leq n \leq 17$).

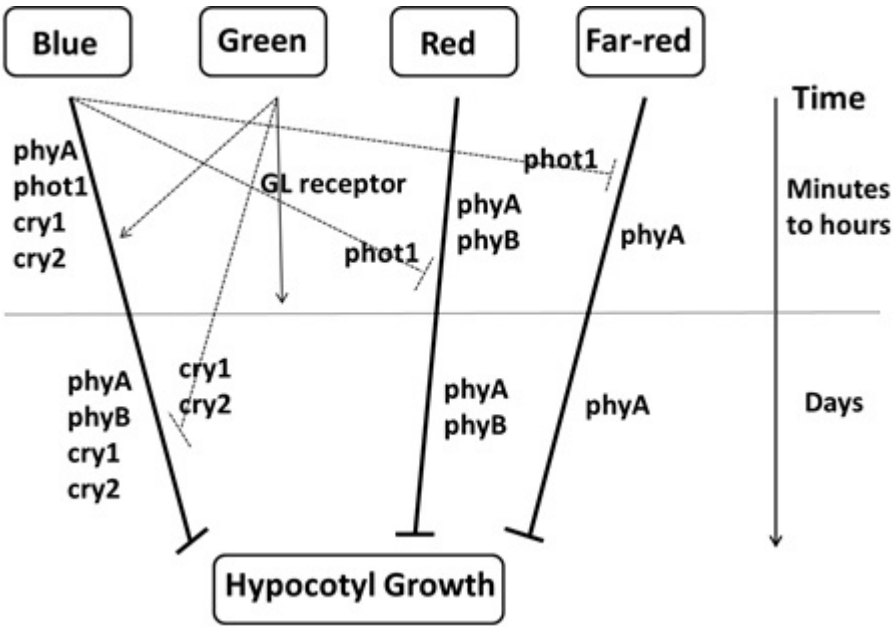

Figure 2-8. Simplified diagram of light-regulated hypocotyl growth during early photomorphogenesis. The blue-light mediated stem-growth inhibition requires the participation of multiple photoreceptor family members. The phyA, phot1, cry1, and cry2 are responsible for the early (minutes to hours) stem inhibition. However, in the long-term (days), the phyA, phyB, cry1, and cry2 are the major receptors that mediate inhibition growth. The RL-induced hypocotyl (stem) growth inhibition mainly acts through the phyA and phyB receptors. However, the phyA receptor is the major contributor mediating the far-red induced stem growth inhibition. The GL alone promotes hypocotyl growth through an unknown receptor (s) during early stem growth. The GL antagonizes the blue-light-induced stem growth inhibition by inactivating cry1 and cry2 receptors in the long-term (96 h) but enhances inhibition in the short-term (minutes to hours). Dim BL (0.1 µmol/m^2s) attenuates RL-mediated stem inhibition in both early and long-term stem growth through the phot1 receptor, but only negates FrL induced stem inhibition in early stem growth. Dashed lines with stop end represent the interactions between different monochromatic light. Horizontal dashed line crossed with the “Time” line separates early and long-term stem growth as well as the receptors involved in each light condition.

CHAPTER 3
MECHANISMS OF PHOT1-MEDIATED BL ATTENUATION OF RL EFFECTS

Introduction

When a seedling reaches light environment, the apical hook opens, the cotyledon unfolds, and the hypocotyl elongation slows down. These developmental changes are characteristics of photomorphogenesis. Frequently the light environment is not optimal, such as under the shade of leaves or coverage by plant debris or other objects. Developmental programs are adjusted when a plant moves from an optimal to suboptimal light environment. The previous chapter described how GL could modulate RL/FrL responses under unfavorable light conditions during seedling establishment. The results from that study uncovered a role for phot1 in attenuating phy-mediated responses through dim BL components of green LED light. The mechanism of phot1-reversal of RL/FrL stem-responses under such conditions remains unclear.

Interactions between photoreceptors have been described extensively (Ahmad and Cashmore, 1997; Casal and Mazzella, 1998; Parks, Cho, and Spalding, 1998; Mas et al., 2000; Folta and Spalding, 2001b; Folta and Spalding, 2001a; Folta et al., 2003a). For example, the BL-induced hypocotyl-growth inhibition consists of two stages: 1) the fast inhibition stage occurs within the first 30 min of BL onset and is mediated by phot1, and 2) the prolonged inhibition stage occurs next and is mediated by cry (Parks, Cho, and Spalding, 1998; Folta and Spalding, 2001a; Folta et al., 2003a). However, phy receptors also play an important role in the cry-mediated stem-inhibition induced by BL, since cry-function depends on phy (Ahmad and Cashmore, 1997; Casal and Mazzella, 1998; Mas et al., 2000; Folta and Spalding, 2001b). Similarly, the BL-induced phototropic response probably involves the actions of at least six photoreceptors (Casal,

2000; Whippo and Hangarter, 2003). The degree of phot-mediated phototropic bending partially depends on the phy-antagonizing effect on hypocotyl agravitropism (Lariguet and Fankhauser, 2004; Rösler, Klein, and Zeidler, 2007; Kami et al., 2012). Additionally, cry receptors also work together with phot to modulate phototropism according to the fluence-rate of BL (Whippo and Hangarter, 2003). The fact that both phot1 and NPH3 are defective in the BL-negation of phy-mediated stem-response but are normal in RL/FrL-induced stem-inhibition indicates the phototropism response is probably operating independently and overrides the phy-response at the cellular level. If this is the case, the phot1-negation of the phy-response would be affected by factors that are important for phototropism.

Auxin and its transport play an important role in hypocotyl elongation and plant tropic responses (Lehman, Black, and Ecker, 1996; Jensen, Hangarter, and Estelle, 1998; Raz and Ecker, 1999; Collett, Harberd, and Leyser, 2000; Vandenbussche et al., 2010; Žádníková et al., 2010). The transport of auxin is mediated by influx and efflux transporter family proteins, such as the PIN-FORMED (PIN) family, the ATP-binding cassette (ABC) family, and the AUXIN PERMEASE/LIKE AUX (AUX1/LAX) family (Kerr and Bennett, 2007 246; Zažímalová et al., 2010). Several members of the aforementioned transporter families have been suggested to mediate phototropic response or interact with its signaling components (Christie et al., 2011; Ding et al., 2011; Haga and Sakai, 2012; Wan et al., 2012). It is possible that some of these auxin transporters may mediate the phot1-negation of phy-responses.

There is another formal alternative explanation for the phot1-negation of phy-stem-response. A common genetic component connecting phot- and phy-signaling

pathways might mediate this response, such as the proteins in the PHYTOCHROME KINASE SUBSTRATE (PKS) family. This protein family has been shown to participate in the phy-signaling-pathway, as well as in regulating plant tropic responses in various tissues and developmental stages. Those proteins (PKS1, PKS2, PKS4) are plasma membrane associated and they physically interact with phy, phot, and NPH3 (Fankhauser et al., 1999; Lariguet et al., 2003; Lariguet et al., 2006; Boccalandro et al., 2008; Schepens et al., 2008; de Carbonnel et al., 2010; Demarsy et al., 2012). It has been speculated that PKS proteins might serve as the molecular link between phy- and phot-mediated responses (Lariguet et al., 2006; Demarsy et al., 2012).

In addition, since the phot1-attenuation of the phy-mediated stem-response described in Chapter 2 was only examined under very low BL (0.1 µmol/m^2s) conditions, it is not known how it may be affected at higher fluence-rates where phot1 has been shown to mediate stem-growth-inhibitory responses (Folta and Spalding, 2001a). Meanwhile, higher BL fluence-rates also activate cry receptors. How cry may affect phot1-mediated negation-response to phy under low- and high-BL conditions will be tested.

Results

The Roles of cry in phot1-Mediated BL Attenuation of RL-Induced Stem Inhibition

Previous data show that dim-BL attenuated RL/FrL -induced hypocotyl-growth-inhibition through phot1 and its interacting component NPH3 (Chapter 2). In the current study, the effect of different BL fluence rates in the negation of the RL stem-growth response was further examined. A fluence rate of 1.5 µmol/m^2s was still able to negate RL stem growth inhibition, but the amplitude of this attenuation was significantly reduced compared to that at 0.1 µmol/m^2s (Fig. 3-1A). Increasing the BL fluence rate to

10 μmol/m^2s enhanced the RL-induced stem-inhibition. Meanwhile, the phot1-mediated fast (within 30 min of BL onset) stem growth inhibition was greater in amplitude with the increase of BL fluence rate (Folta and Spalding, 2001a). In the long-term end-point assay, the seedlings grown in monochromic RL conditions showed less stem inhibition than those grown in RL with addition of different fluence rates of BL (Fig. 3-1B), indicating that the BL-negation of RL-induced stem-inhibition only exists for several hours. However, cry receptors are also active under BL. To define the roles of cry in the BL-negation of the RL stem-response, same short- and long-term stem growth responses were tested in *cry1*, *cry2*, and *cry1cry2* mutant backgrounds. In the short-term assay, both *cry1* and *cry2* mutant seedlings exhibited a higher hypocotyl growth rate than wild-type seedlings under RL plus high BL (10 μmol/m^2s) conditions. The *cry1cry2* mutant seedlings even showed a higher growth rate under RL plus high BL conditions than wild-type seedlings grown in RL (Fig. 3-1C). In the end-point assay, *cry1* mutant seedlings showed less hypocotyl growth inhibition than wild-type seedlings under RL plus high BL conditions. The *cry1* seedlings showed increased inhibition when BL was added to RL (Fig. 3-1D). On the contrary, *cry2* seedlings showed the same levels of inhibition as wild-type seedlings under RL plus high BL conditions. The increased inhibition induced by supplementing dim-BL to RL was absent in *cry2* seedlings (Fig. 3-1D).

BL Adjusts phy-Induced Hook Opening Process

RL not only inhibits hypocotyl elongation growth, it also stimulates apical hook opening and chlorophyll biosynthesis. To test whether BL can affect other RL-induced early photomorphogenic responses, the apical hook opening kinetics and chlorophyll content were examined under RL and RL plus dim-BL conditions. Interestingly, addition

of dim-BL to the background of RL delays the hook opening process. Seedlings treated with RL for 180 min have an average hook angle of 67.6 (±9.5) degrees. However, when dim BL (0.1 μmol/m^2s) was co-irradiated with RL, the hook angle was only 28.1 (±6.3) degrees (Fig. 3-2A). Eventually, the apical hook was fully opened with no visible differences in both experimental light conditions after 24 h irradiation (data not shown). Similarly, the FrL-induced hook-opening was also delayed by supplementation of dim-BL in the short-term but not long-term experiments (Fig. 3-2B). Increasing BL fluence rate to 1.5 μmol/m^2s did not significantly change the effect on hook opening. However, higher fluence rate of BL (≥10 μmol/m^2s) did accelerate the RL-induced apical hook opening (Fig. 3-2C), which is consistent with the report that both wavebands are inductive for the apical hook opening during the de-etiolation process (Liscum and Hangarter, 1993b). On the other hand, the chlorophyll accumulation kinetics were also measured and compared between the RL and RL plus dim-BL treated seedlings, with no significant differences observed (Fig. 3-2D).

The Roles of cry and phot1 in the BL-Mediated Adjustment of Hook Opening Response

The phot1 receptor and BL have been shown to negate RL/FrL-induced hypocotyl-inhibition (Wang et al., 2013). It is of interest to test whether this delayed hook opening response is also phot1 mediated. Fig. 3-3A and B showed that no significant differences in the hook opening kinetics were observed between RL/FrL and RL/FrL plus dim BL treated *phot1* mutant seedlings. The *nph3* mutant seedlings were also subjected to this test, but it was difficult to obtain a robust RL-induced hook opening response for this mutant (Fig. 3-3C). In addition, the enhanced RL-induced hook

opening by supplementation of high BL (≥10 μmol/m^2s) was largely impaired in *cry1* and *cry1cry2* mutant seedlings (Fig. 3-3D).

The phot1 Adjustment of RL-Induced Stem-Inhibition and Hook-Opening Requires Normal Auxin Transport

The role of auxin in the regulation of cell differentiation, cell elongation, and maintenance of the apical hook structure has been well documented (Lehman, Black, and Ecker, 1996; Jensen, Hangarter, and Estelle, 1998; Raz and Ecker, 1999; Collett, Harberd, and Leyser, 2000; Vandenbussche et al., 2010; Žádníková et al., 2010). The evidence that BL under very low fluence-rate conditions can attenuate or delay RL-induced stem-growth-inhibition and apical-hook-opening lead to the speculation that auxin might serve as the mediator of this response. To test this possibility, a synthetic auxin transport inhibitor, 1-N-Naphthylphthalamic acid (NPA), was supplemented into the minimal media and the response was measured. The basic experiment was a dose-response test under identical light treatments. The results showed that at an NPA concentration of 0.5 μM the absolute hypocotyl growth rate kinetics were similar between treated- and untreated-seedlings under RL. However, the BL-antagonism-effect on RL was largely impaired (Fig. 3-4A). When the NPA concentration was increased to 1 μM, this BL-negation-effect on RL was fully eliminated, although the RL-induced hypocotyl-growth-inhibition was slightly enhanced (Fig. 3-4B). In terms of hook opening kinetics, NPA delayed the response to RL at two different concentrations (1 and 5 μM) in our short-term assays (Fig. 3-4C, D). Nevertheless, the delayed apical-hook-opening by dim-BL to RL was completely abolished at the NPA concentration of 5 μM.

Genetic Tests of Auxin Transporter Mutants for BL-Attenuation of RL Response

The directional flow of auxin from cell to cell is partly mediated by auxin transporters. These transporters facilitate transport of auxin molecules into (influx carriers) and out of (efflux carriers) a cell in response to external signals (like light) or internal signals (such as other phytohormones). Some of these transporters, such as PINs, ABC transporters, and AUX/LAX transporters have been implicated in tropism responses (Friml et al., 2002; Stone et al., 2008; Christie et al., 2011; Ding et al., 2011; Rakusová et al., 2011; Haga and Sakai, 2012). To test the hypothesis that one or several of these transporters are involved in the BL-negation of the RL-response, several PIN transporter mutants (*pin1*, *pin3*, *pin4*, and *pin7*), one ABC transporter mutant (*abcb19/pgp19*), and one auxin influx transporter mutant (*aux1*) were examined for the BL-negation of the RL-hypocotyl-response based on their hook/hypocotyl expression patterns (Stone et al., 2008; Wu et al., 2010; Žádníková et al., 2010). Transporter mutants tested maintained a normal or near-normal ability to counteract RL inhibition with BL. Specifically, *pin1*, *pin3* and *pin7* mutant seedlings responded to RL or RL plus dim BL like wild-type seedlings (Fig. 3-5A, B, D). The *pin4* mutant seedlings showed a slight reduction in the amplitude of the BL negation of RL-induced hypocotyl-inhibition (Fig. 3-5C). The *abcb19* mutant seedlings were tested at a higher RL fluence rate (50 µmol/m^2s) in order to obtain a robust RL-stem-inhibition. Similarly, the degree of BL attenuation of RL stem-response in *abcb19* was not significantly different from wild-type seedlings (Fig. 3-5E). Interestingly, the *aux1* mutant seedlings treated with BL and RL also showed an partly impaired BL-negation-effect on RL, with a hypocotyl growth rate kinetics overlapped with that of RL-treated wild-type seedlings (Fig. 3-5F),

although the RL-induced hypocotyl-inhibition was also slightly enhanced in this background.

The phot1-Attenuation-Response to RL Functions Normally in Single *pks* Mutant Backgrounds

The PKS proteins have been reported to serve as signaling components in both the phy-signaling-pathway and phototropism. It is therefore reasonable to test the role of PKS proteins in the phot1-mediated opposition to RL-induced growth inhibition. In order to obtain a robust RL stem inhibition response, a higher fluence rate of RL (50 $\mu mol/m^2s$) was used. All *pks* single mutants (*pks1*, *pks2*, *pks4*) showed similar hypocotyl growth rate kinetics under these RL conditions. However, when dim-BL was added, they responded as wild-type in the BL suppression of RL stem inhibition response (Fig. 3-6A, B, C).

Discussion

Adjustment of phy-Mediated Photomorphogenic Programs by phot1

In the developing seedling light guides early morphological and physiological changes, e.g. hypocotyl-growth-inhibition, apical-hook-opening, chloroplast formation, and cotyledon unfolding. These developmental changes are mediated by multiple signaling systems that are initiated by specific photoreceptors. The phy receptor family (phyA-E) mainly responds to RL and FrL wavebands. In the initial stage of RL-induced hypocotyl growth inhibition, phyA mediates the fast growth inhibition response which happens within 1-3 h after RL onset (Parks and Spalding, 1999; Wang et al., 2013). The phyB receptor redundantly mediates this fast response, but contributes to a greater extent in maintaining this inhibition over subsequent days. Supplemental BL in an RL background activates two additional photosenory systems, the cry and the phot. The

cry-mediated BL response generally accelerates/enhances the process of RL-induced early photomorphogenic events (Fig. 3-1A, B; Fig. 3-2C). Such cry effects are most robust under high (>10 μmol/m^2s) BL fluence rates. Tests of *cry* mutants for the enhanced stem-inhibition and accelerated hook-opening responses suggest that cry1 and cry2 redundantly mediate the response, yet only cry1 plays the major role in stimulating the hook-opening (Fig. 3-1C, D; Fig. 3-3C). This observation is in disagreement with an earlier report that suggests cry1 does not affect hook-opening kinetics under high fluence rate BL irradiation (Wang et al., 2009). Since the experiments from this study were performed under RL and BL co-irradiation conditions, it is possible that the activation of phy receptors might modulate cry functions in response to BL. Such phy and cry interactions have been reported in several studies (Ahmad and Cashmore, 1997; Casal and Mazzella, 1998; Mas et al., 2000).

The phot mediated BL response is more intriguing. The phot receptor induces the primary hypocotyl growth inhibition response to BL irradiation, but then switches roles to do the opposite when RL is applied (Fig. 3-1A; Fig. 3-2A, B). The effect of BL to slow RL-mediated hypocotyl growth inhibition and apical hook opening likely represents an adaptive response. A young seedling actively detects the ambient light for the proper allocation of its limited energy reserve. Low BL may signal an unfavorable light environment in the early stages of photomorphogenesis, leading to more elongated growth.

BL signals do not operate strictly through phototropins. Under low flunce rate BL conditions the cry2 receptor can also be activated. The results from genetic tests of hypocotyl growth in *cry* mutants suggest that cry2 suppresses the phot1 mediated

reversal of RL response over days (Fig. 3-1D). These observations indicate the intricate interplay between photoreceptor families in adjusting plant growth and development into the new light environment.

An important aspect of these experiments is that they were conducted under narrow-bandwidth illumination conditions that were intended to isolate specific receptor contributions. Under low light environments in natural settings, GL may also be enriched, especially under the shade of leaves. GL has been shown to inactivate cry function in long-term illumination conditions (Banerjee et al., 2007; Bouly et al., 2007; Sellaro et al., 2010), thus GL might help to maintain the phot1-negation of the RL stem-response in the prolonged irradiation conditions. However, why the *cry2* or *cry1cry2* mutant seedlings did not show longer hypocotyl under dim BL plus RL conditions than that under RL alone is not clear. One possible explanation is that the phot1-negation effect is only transient (a duration of hours) and phy might dilute this effect in the long-term, or perhaps, a yet-to-be identified GL signaling pathway provides additional stem-growth-promotion under low light environments (Folta, 2004; Wang et al., 2013).

Auxin Transport and its Possible Involvement in the phot1 Action

Auxin regulates cell differentiation, growth and tropic response (Lehman, Black, and Ecker, 1996; Raz and Ecker, 1999; Vandenbussche et al., 2010; Žádníková et al., 2010). Disruption of auxin transport by the auxin transport inhibitor NPA suppresses both phototropic and gravitropic responses (Jensen, Hangarter, and Estelle, 1998; Friml et al., 2002; Nagashima, Uehara, and Sakai, 2008). Meanwhile, the maintenance of an apical hook structure requires the presence of an auxin gradient at the concave side of the hook. Application of NPA eliminates this auxin gradient and stimulates hook opening of dark grown seedlings (Wu et al., 2010). Additionally, the hypocotyl elongation of light-

grown *Arabidopsis* seedlings requires auxin transport (Jensen, Hangarter, and Estelle, 1998). The evidence that NPA can suppress both BL-mediated repression of RL-induced hypocotyl growth inhibition and hook-opening indicates a possible role for auxin transport in these responses (Fig.3-4). RL not only regulates auxin biosynthesis, but also affects the transport, content, and distribution of auxin (Nagashima et al., 2008).

The phot1-mediated adjustment of RL-induced hypocotyl and hook responses is not likely mediated by phot1-antagonizing phy-regulated transcription (Chapter 2, Fig. 2-3). Instead, this response may be a result of phot1-modulation of auxin-transporter localization and activity. Consistent with the latter scenario, phot1 and its signal transducer, NPH3, modify the cellular distribution of PIN3 and PIN2 in the hypocotyl and root, respectively (Ding et al., 2011; Wan et al., 2012). Mutation of the *PIN3* gene reduces the hypocotyl curvature towards continuous unilateral white light illumination (Friml et al., 2002; Ding et al., 2011). However, a recent study suggests that PIN3 and PIN7 mediate the pulse-induced first positive phototropic response, but not the continuous-light-induced, second positive phototropic response when the seedlings were placed vertically on the media (Haga and Sakai, 2012).

Mutations in *ABCB19* lead to abnormal localization of the PIN1 transporter, and the mislocalization of PIN1 transporter ultimately enhances hypocotyl phototropism (Noh et al., 2003). Furthermore, phot1 can inhibit ABCB19 activity by direct phosphorylation of this protein in a HeLa cell system. Reduction of ABCB19 transporter activity suppresses basipetal auxin transport, resulting in more auxin channeled in lateral directions through PIN3, depending on direction of light exposure (Christie et al., 2011). In the present study, *pin1*, *pin3*, *pin7*, and *abcb19* showed normal BL driven

opposition of RL hypocotyl growth inhibition. Only *pin4* showed slight reductions in the amplitude of this attenuation (Fig. 3-6). This result agrees with Haga's findings, but it is also possible that multiple PIN and ABCB transporters act redundantly to mediate this response. The *aux1* mutant was originally discovered as a root agravitropic mutant, and its role in unilateral BL-induced hypocotyl phototropism was subtle when NPH4/ARF7 was functional (Bennett et al., 1996; Marchant et al., 1999; Stone et al., 2008). However, AUX1 does mediate the recovered phototropism response in the conditional aphototropic mutant *nph4/arf7* background when RL is co-irradiated with BL (Stone et al., 2008). Meanwhile, AUX1 has also been suggested to facilitate auxin transport from the source (leaf) to the sink (root) in *Arabidopsis* seedlings (Marchant et al., 2002).

Another explanation is that the attenuated RL-induced hypocotyl growth inhibition was due to the altered auxin transport from shoot to downward sections through the action of AUX1. Furthermore, AUX1 only represents one possible pathway to load auxin into a cell. Passive auxin diffusion and other AUX/LAX family members may contribute additional loading capacities (Yang et al., 2006). If all of these factors are taken into account, it may explain why BL opposition of the RL-induced inhibition response was only partly abrogated in *aux1* mutant background.

An alternative explanation for this result is that the impaired BL response is a result of the slightly enhanced RL-induced hypocotyl growth inhibition in the *aux1* mutant itself. BL may not be able to fully attenuate the enhanced RL inhibition under such a scenario. It should be noted that in this study the light treatments were provided from vertical direction in parallel to seedling growth, so the seedling was growing toward the light source. Light direction guides photoreceptor activation and the cellular

distribution and activities of auxin transporters. Considering the localization of AUX1, it may be possible that AUX1 has an important role in mediating longitudinal growth, and what was witnessed in this series of experiments is essentially phototropism guiding the plant upward in the presence of BL signals.

PKS Proteins and the Oppositional BL Response

Several studies have postulated that the PKS proteins might serve as a molecular link between phy- and phot-mediated responses (Lariguet et al., 2006; Demarsy et al., 2012). Therefore, it was important to test the hypothesis that the BL-reversal of RL hypocotyl growth inhibition is mediated by one or more of the PKS proteins. The results from systematic analysis of *pks* mutants revealed no significant differences compared to wild-type seedlings. Although these data do not provide any support for the proposed hypothesis, they do not exclude the possibility that multiple PKS proteins might redundantly participate in this response. This possibility may be tested in the future using multiple *PKS* mutations in a common background.

The significance of this study lies in that it uncovers how BL may adjust RL/FrL responses, here regulating the apical hook opening, during initial photomorphogenesis when light conditions are unfavorable. This response is once again mediated by phot1, and appears to require the modulation of auxin transport and distribution in hook/hypocotyl sections of a seedling. Physiologically, this small modulation of plant developmental programs may be a meaningful part of early development, maintaining the apical hook through the soil until sufficient light is sensed.

Materials and Methods

Plant Materials

The *Arabidopsis* photoreceptor mutants tested in this study are identical to those previously assessed (Folta, 2004): *cry1-304*, *cry2-1*, *phot1-3* (*nph1-3*). The *nph3-6* mutant seeds were obtained from Dr. Mannie Liscum. The *pks1-1*, *pks2-1*, and *pks4-1* single mutant seeds were obtained from Dr. Christian Fankhauser. The *pin3-5* (CS9364), *pin4-3* (CS9368), and *pin7-2* (CS9366) were ordered from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University (Columbus, OH). The *abcb19-3* mutant seeds were provided by Dr. Edgar Spalding. The *pin1* (CS86744) and *aux1-7* (CS3074) mutant seeds are gifts from Dr. Bala Rathinasabapathi. All genotypes tested in this study are in Columbia (Col-0) background, which was used as wild-type.

Light Sources and Treatments

Light treatments were generated using narrow-bandwidth LED light supplied by custom LED arrays (Light Emitting Computers, Victoria, BC Canada). The peak wavelengths of BL, RL, and FrL are 470, 630, and 720 nm. The emission spectrum of all light sources is viewable on-line at www.Arabidopsisthaliana.com/lightsources. Light fluence rates were measured using a LI-COR LI-250 photometer with a PAR sensor (LI-COR, Lincoln, NE).

Long-term Hypocotyl Length Measurement

For end-point assays (24-96 h in light), *Arabidopsis* seeds were surface-sterilized briefly by immersion in 95% ethanol on blotting paper in a laminar flow hood. Upon drying, the seeds were distributed in Petri dishes on minimal media (1mM KCl and 1mM $CaCl_2$) solidified with 1% Difco agar (Beckton, Dickinson and Co, Sparks, MD). The plates were covered in foil and then stratified for 48-72 h at 4°C. The seeds were then

treated with 6-8 h of fluorescent white light (16 µmol/m^2s) at 23°C to synchronize germination. Seedlings were grown in absolute darkness at 23°C for 40-48 h. Germinating seedlings were then moved under experimental light conditions without a photoperiod. At least 30 seedlings were measured per treatment in two to three independent experiments. Analysis of stem growth kinetics was performed by scanning Petri dishes of seedlings on a flatbed scanner, and then measuring seedlings at high magnification using ImageJ 1.44 (http://imagej.nih.gov/ij/index.html). The mean of 30-42 seedlings is reported for each light condition.

Chlorophyll Extraction and Measurement

The Arabidopsis seedlings were grown on half-strength MS medium in a 4-cm petri dish for 3 days, and then treated with RL or RL plus BL. Seedlings were harvested at specific time points and weighted. Then, seedlings were transferred into 1 mL DMF in a 1.5 mL tube and let sit for at least 2 hours in the dark. The absorbance of each tube at 647nm and 664nm were measured using a SmartSpec™ 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, CA). The Chlorophyll concentration was calculated according to the method described (Porra, Thompson, and Kriedemann, 1989). Three biological replicates (three petri dishes for each light condition) were included for this experiment.

Time-course Hypocotyl Growth and Hook Opening Kinetics

For the time-course assays of hypocotyl growth and hook opening kinetics, *Arabidopsis* seeds were surface-sterilized and synchronized for germination as described above. Then, different experimental light treatments were applied to 2-3 day old etiolated seedlings and images were acquired in a 5-min-interval and analyzed according to the method described (Wang et al., 2009). For the auxin transport inhibition

experiments, the minimal media were supplemented with NPA (Sigma-Aldrich Cat # 33371) at different concentrations. Germinating seedlings grown on minimal media without NPA were transferred onto the NPA containing media. Then, a 40-60 min incubation time in darkness was given to seedlings to absorb NPA before light experiments. In all time course experiments, at least 8 seedlings were assayed.

Statistical Analysis

Means of replicates from long-term hypocotyl growth assays were subjected to statistical analysis of one way ANOVA by the multiple-range Tukey-Kramer test ($p \leq 0.05$) using the JMP®Pro 10.0 statistical package (SAS Institute).

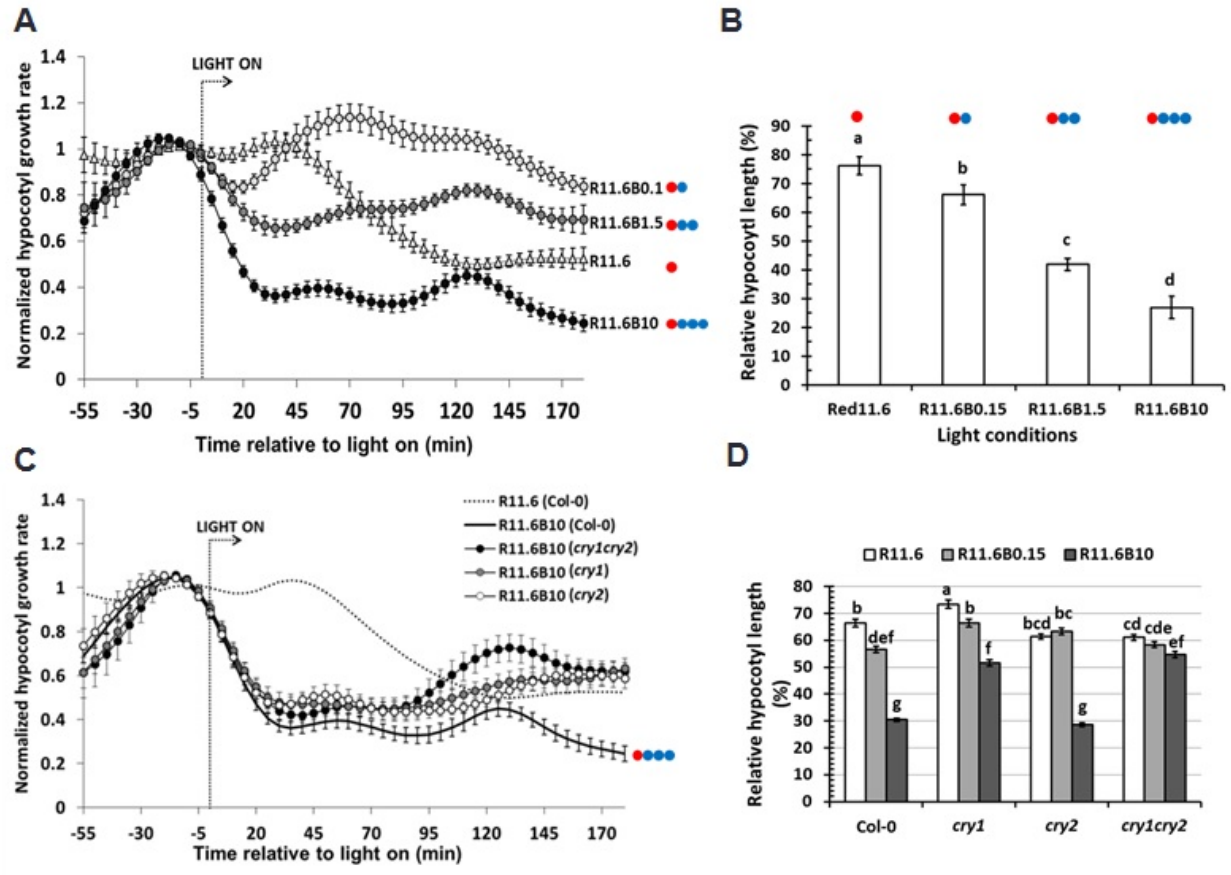

Figure 3-1. BL fluence-rate response tests of the BL-attenuation of RL-hypocotyl-response in wild-type and *cry* mutant backgrounds. (A) Increasing BL fluence-rate reduced the extent of BL-negation of RL stem-response in the time-course assay. R11.6= RL, 11.6 µmol/m^{2}s, B0.1=BL, 0.1 µmol/m^{2}s, B1.5= BL, 1.5 µmol/m^{2}s, B10=BL, 10 µmol/m^{2}s. Error bars represent S.E.M. (8≤n≤12). (B) Long-term (2 days) RL and BL co-irradiation reduced the hypocotyl length compared to the RL-treated seedlings alone. (C) The hypocotyl-growth-inhibition enhanced by adding 10 µmol/m^{2}s of BL to RL was partially mediated by cry1 and cry2. (D) The cry2 alone repressed the hypocotyl elongation under dim BL (0.15 µmol/m^{2}s) conditions in the long-term assay; cry1 and cry2 redundantly suppressed the hypocotyl elongation under higher BL (10 µmol/m^{2}s) conditions. Different letters in (B) and (D) represent statistically different means ($p < 0.05$).

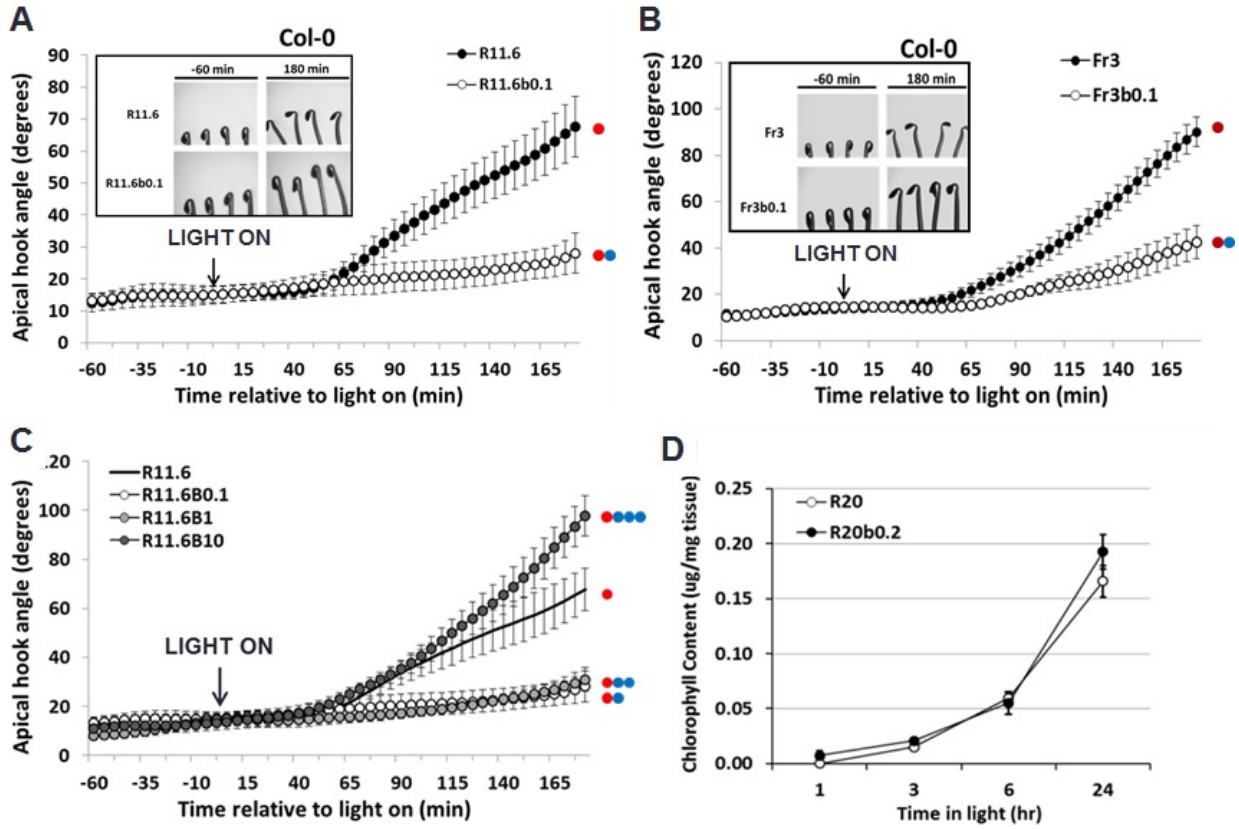

Figure 3-2. BL delays phy-induced hook-opening. (A) RL stimulates apical-hook-opening (dark circles), addition of dim BL delays RL-induced hook-opening (open circles); (B) FrL promotes apical-hook-opening (dark circles), dim-BL also delays FrL-induced hook-opening (open circles); (C) Increasing BL fluence rate to 1 µmol/m^2s still attenuates RL-induced hook-opening (light gray circles), but 10 µmol/m^2s BL enhanced RL-induced hook-opening; (D) RL- and RL plus dim BL- treated seedlings have comparable chlorophyll accumulation kinetics.

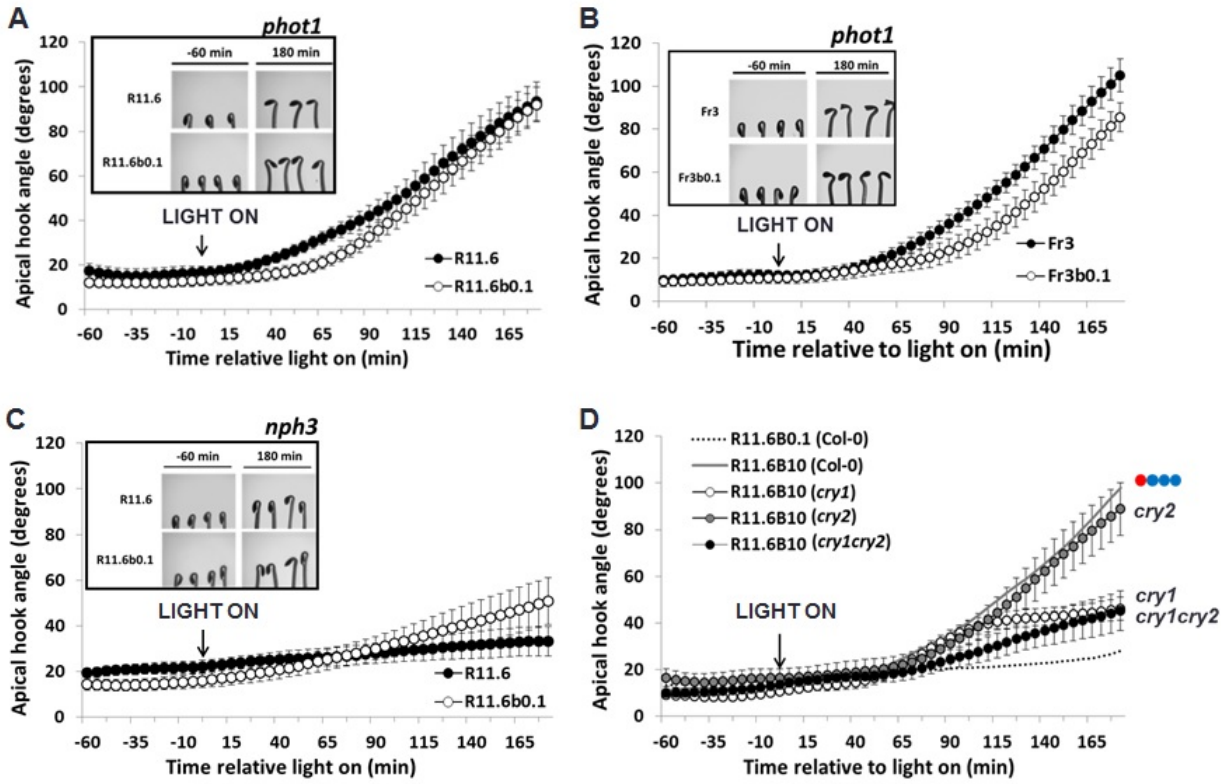

Figure 3-3. BL attenuation of RL/FrL-induced hook-opening is absent in the *phot1* mutant. (A, B) The dim-BL-attenuation of RL/FrL-induced hook-opening was absent in the *phot1* mutant background. Dark circles: RL alone; open circles: RL plus dim BL. Inset: RL or RL plus BL treated seedlings at the beginning (-60 min relative to light onset) and the end (180 min) of one representative short-term experiment; (C) BL-attenuation of RL hook-response in *nph3* mutant background; (D) The enhanced RL-induced hook-opening by higher fluence-rate BL was normal in the *cry2* (gray circles) mutant, but was partially impaired in *cry1* (open circles) and *cry1cry2* (dark circles) mutants.

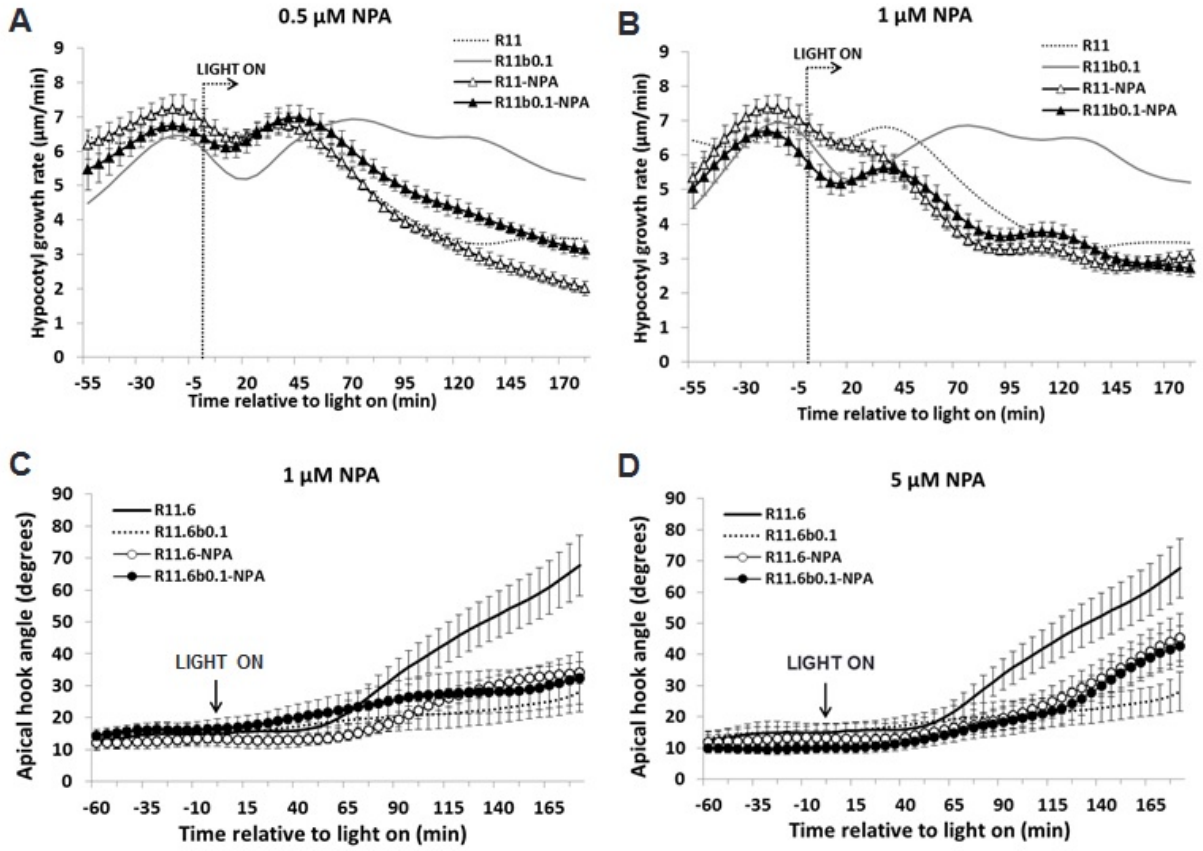

Figure 3-4. Normal auxin transport is required for the dim BL effect. The BL-negation of RL-induced hypocotyl-growth-inhibition is partially suppressed by 0.5 µM NPA (A) and fully suppressed by 1 µM NPA (B). The RL-induced hook-opening was delayed when tested on NPA containing medium at two different concentrations: 1 µM NPA (C) and 5 µM NPA (D). However, the BL-attenuation of RL-induced hook-opening was abolished when treated with 5 µM NPA. In panels (A) & (B), open triangles represent the absolute hypocotyl growth rate under RL (11.6 µmol/m^2s) conditions; dark triangles represent the hypocotyl growth rate under RL plus dim BL (0.1µmol/m^2s) conditions. In panels (C) & (D), open circles represent the hook opening kinetics under RL conditions; dark circles represent the hook opening kinetics under RL plus dim BL conditions. Wild-type Col-0 in response to RL (dotted line) and RL plus dim BL (gray line) without NPA treatment were included for comparison in all panels.

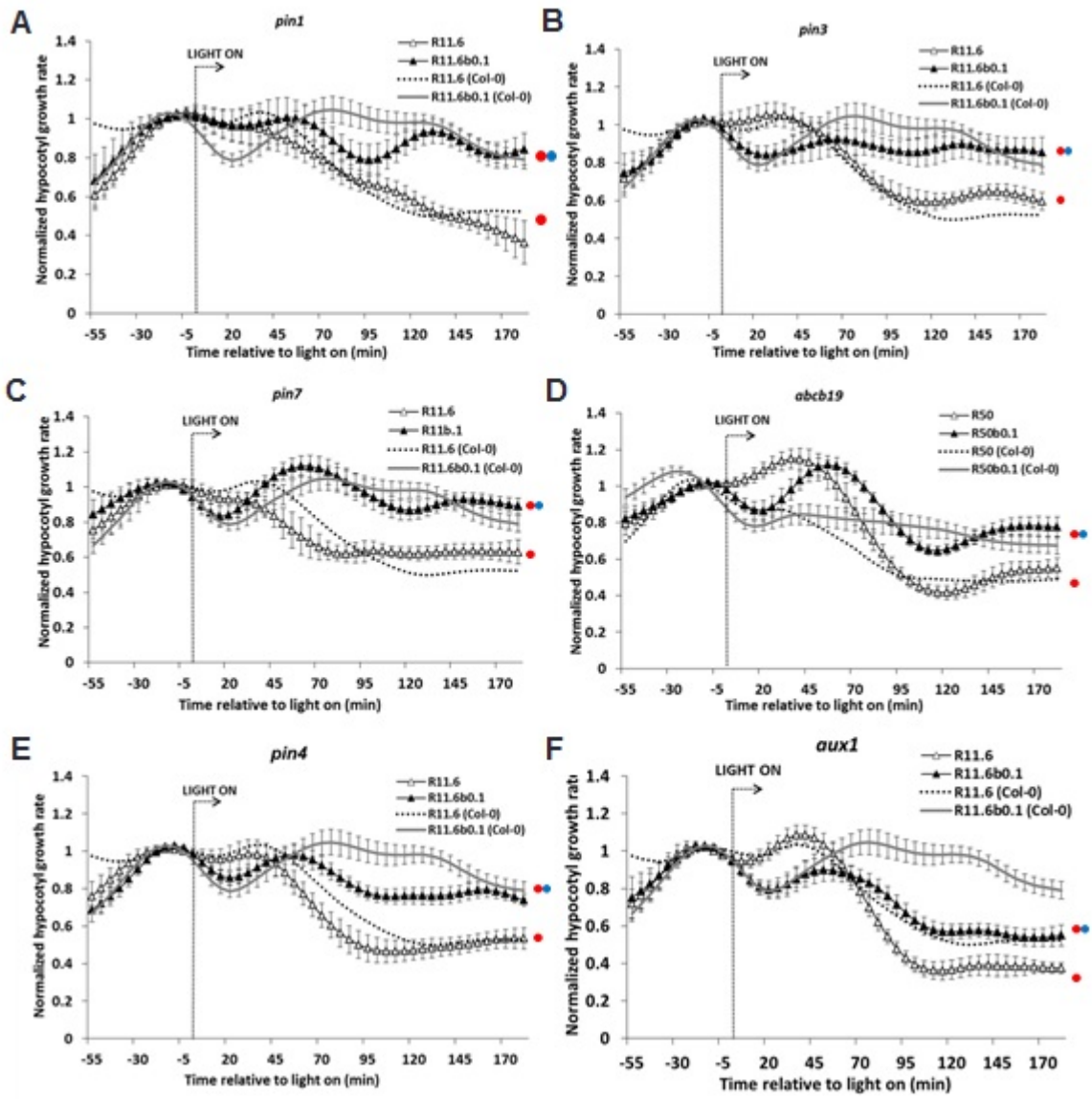

Figure 3-5. Genetic tests of the dim-blue-reversal of RL hypocotyl-response in auxin transporter mutants. The dim-BL-negation of RL-induced hypocotyl growth inhibition response was tested in *pin1* (A), *pin3* (B), *pin4* (C), *pin7* (D), *abcb19* (E), and *aux1* (F). In all panels, open triangles represent the hypocotyl growth rate under RL (11.6 or 50 µmol/m^{2}s) conditions; dark triangles represent the hypocotyl growth rate under RL plus dim BL (0.1µmol/m^{2}s) conditions. Wild-type Col-0 in response to RL (dotted line) and RL plus dim BL (gray line with error bars) were included for comparison.

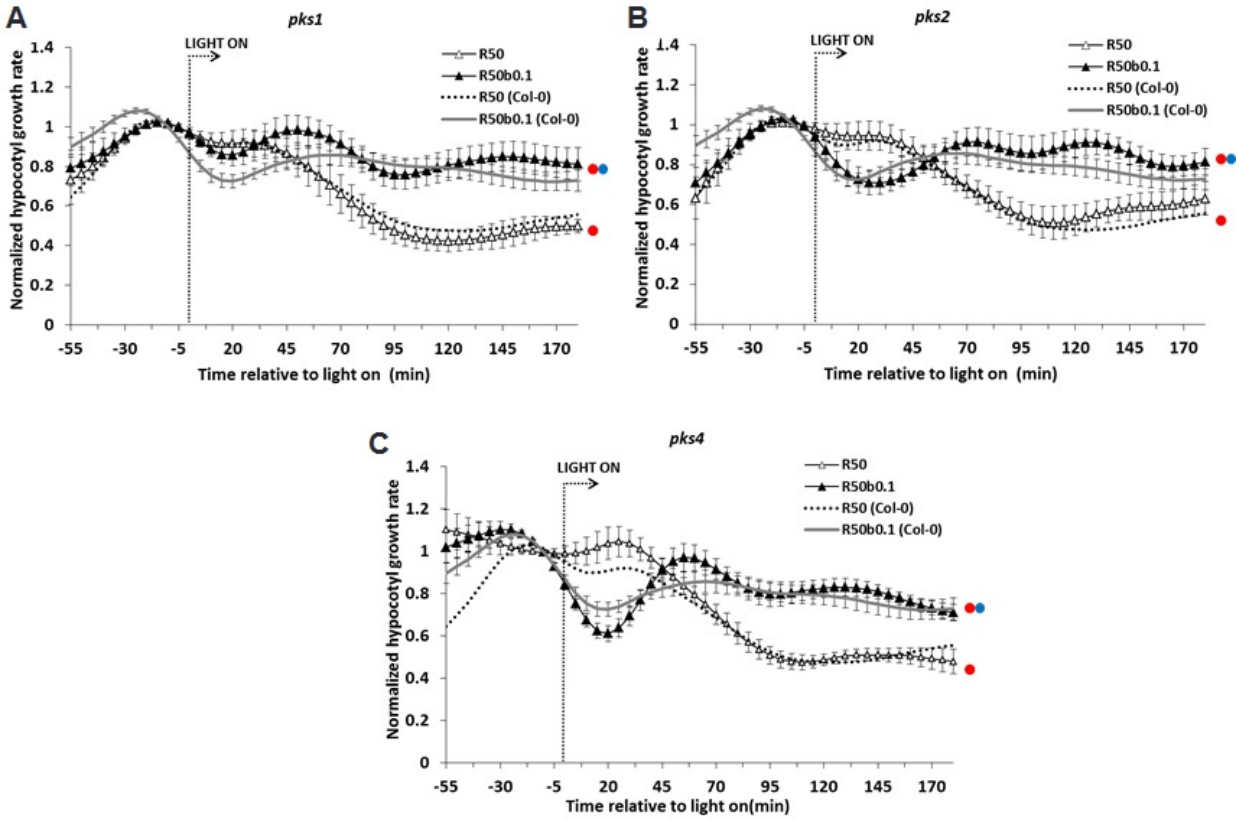

Figure 3-6. The blue-attenuation to RL hypocotyl-response functions normally in single *pks* mutant backgrounds. The dim-BL-negation of RL-induced hypocotyl growth inhibition response was normal in *pks1* (A), *pks2* (B), and *pks4* (C) mutant backgrounds. In all panels, open triangles represent the hypocotyl growth-rate under RL (50 µmol/m^2s) conditions; dark triangles represent the hypocotyl growth-rate under RL plus dim BL (0.1 µmol/m^2s) conditions. Wild-type Col-0 in response to RL (dotted line) and RL plus dim BL (gray line with error bars) were included for comparison.

CHAPTER 4
DISCOVERY OF CANDIDATE GL SIGNALING COMPONENT FROM *ARABIDOPSIS* NATURAL ACCESSIONS

Introduction

Arabidopsis has a broad geographic distribution in the world. It has been naturalized many places in the Northern hemisphere, from the Eurasian continent to North America (Alonso-Blanco and Koornneef, 2000). The adaptability to various climates is impressive. *Arabidopsis* is found from 68°N (North Scandinavia) to 0° (mountains of Tanzania and Kenya) latitudinally, which is much wider than its naturally distributed relatives in the *Brassicaceae* family (Koornneef, Alonso-Blanco, and Vreugdenhil, 2004). The wide geographic distribution of this species embraces substantial differences in growth habitats. Thus, the phenotypic variations displayed among naturally occurring accessions might be able to reflect the genetic adaptations to certain environmental conditions (Alonso-Blanco and Koornneef, 2000).

While previous chapters detailed the characterization of GL responses that ultimately led to the identification of non-GL findings, this chapter centers on a genetic approach to identify GL-signaling-components. The hypothesis for this study is that among the naturally occurring accessions, there are genotypes that are not sensitive to GL due to the genetic alternations in GL signaling genes. Such genetic variations may confer adaptive advantages in specific growth environments.

In the past decades natural genetic variation in wild accessions has led to the identification of signaling components responsible for the phenotypic variation. For instance, a single amino acid change from a conserved methionine to a threonine at position 548 in PHYA from the Lm-2 accession (collected from wheat field of France, 48°N) reduced its FrL sensitivity approximately 100-fold compared to Col-0 (Maloof et

al., 2001). Similarly, dominant gain-of-function allele of CRY2 in the Cvi background was shown to be mutation methionine to valine at position 367. The mutation led to enhanced CRY2 stability and an early flowering phenotype under short photoperiods (El-Assal et al., 2001). It is difficult to generate helpful hypotheses about the physiological and ecological relevance of these genetic variations, since they were limited to single accessions in their original habitat (Filiault et al., 2008). However, it should be noted that both variations, instead of disrupting the overall function, only affect a subset of protein functions of these two photoreceptors. In addition, the lost-of-function of *PHYD* allele is present in the Wassilewskija (Ws) natural accession, and its functional redundancy with *PHYB* indicates it is not essential in some natural environments (Aukerman et al., 1997). Thus, it seems that the pleiotropic nature of photoreceptor proteins favors changes in the photoreceptor outputs, rather than disrupting the overall function (Maloof et al., 2001).

Photoreceptors are more likely to be the common target for natural selection. Allelic variations that change photoreceptor activities are commonly found among naturally occurring variants (Balasubramanian et al., 2006; Filiault et al., 2008). Assessment of *PHYC* haplotypes among more than 220 *Arabidopsis* strains illustrated that the Col-0 *PHYC* is more active at the northern latitudes. Interaction between *FRIGIDA* (*FRI*, a vernalization control gene) and '*PHYC* X latitude' explains 10% of the variation in short-day flowering conditions. Here a *PHYC* allele contributes to the late flowering phenotype of *FRI*-positive accessions in northern latitudes (Balasubramanian et al., 2006). Another study examined *PHYB* sequences from 33 *Arabidopsis thaliana* and *Arabidopsis lyrata* individual accessions, and found 14 nonsynonymous

polymorphisms. All of these 14 polymorphism sites fall outside the functionally important GAF and PHY domains. One of them, I143L, associated with variations in RL response (Filiault et al., 2008). The aforementioned naturally occurring photoreceptor variations support the hypothesis that naturally-occurring mutations confer changes in plant behaviors that change sensitivity to the light environment. These findings indicate that variation in ecotypes could be a resource for gene discovery and structure-function analysis.

The same ecotypes may harbor alterations that affect responses to GL. GL modulates several aspects of plant growth and development. This GL signaling pathway together with its regulated responses might confer adaptive advantages under certain light environments. Because GL has been shown to alter seedling development, flowering time and shade-avoidance responses, variation in phenotypes under GL may lead to discovery of genes playing a role in GL signaling or response.

This chapter evaluates GL responses in various *Arabidopsis* ecotypes. A forward genetic approach of mutagenized seedlings is not feasible because phenotypes are subtle or could be obscured by effects of the cry-semiquinone. Scoring GL-induced shade-avoidance phenotypes is difficult because many light signals affect the response. In addition, many loci are known to contribute to leaf inclination and hyponastic responses, so screens would reveal many false positives. The same limitations apply to the natural variants, and it is impractical to use these GL-based assays to screen ecotypes. Instead, the original assay used to identify the GL response was used to monitor seedling stem growth response when adapting to the new light environments.

Results

Response of Natural *Arabidopsis* Accessions to GL

To identify potential GL-signaling components, forty-two *Arabidopsis* natural accessions were tested for the GL-stimulated hypocotyl-elongation-response. In the typical GL-response, hypocotyl-growth-rate increases after 20 min of GL and reaches its maximum between 30 min to 45 min (Folta, 2004). Among those tested accessions, 38 accessions showed this canonical GL-stimulated hypocotyl growth response (Fig. 4-1A, represented by An-1). Four accessions exhibited reduced GL-response or no response (Fig.4-1B, represented by Knox-10). A summary of the initial screening results shows the normalized growth rate were 30 min after exposure to GL. Data from various accessions were compared with the growth rate of Col-0 ecotype under both dark and GL treatment conditions (Table 4-1). These data showed that the Knox-10, Fab-4, Eden-1, and Lov-1 accessions displayed a significantly lower growth rate (*p-value* <0.05) than GL-treated Col-0 seedlings. In fact, their response was most similar to the growth rate of dark-grown Col-0 seedlings. However, only Knox-10 showed a consistent defect in growth when measured in subsequent generations.

RL-Induced Hypocotyl-Randomization Response (HRR) is Defective in Knox-10 Ecotype

Although Knox-10 showed a significant reduction in the GL-stem-response, it is possible that the observed phenotype may be due to genetic variations in known photo-sensing systems. To test if Knox-10 has a normal response to other light wavelengths, end-point hypocotyl elongation assays were conducted under BL, GL, RL, and FrL. The growth of Knox-10 under these light conditions was not distinguishable from Col-0 (Fig. 4-2A).

However, one additional phenotypic variation of this accession was observed when it was treated in continuous RL for days. Typically, *Arabidopsis* seedlings grown under RL conditions do not grow upright. Instead, they grow in a relatively randomized fashion relative to the gravitational vector (seedlings grow nearly flat or bending on horizontal plates) (Liscum and Hangarter, 1993a; Robson and Smith, 1996). This randomized growth is referred to as the hypocotyl-randomization-response (HRR). Knox-10 exhibits a defective RL-induced HRR (Fig. 4-2 B, C). When 1-d-old Col-0 and Knox-10 seedlings were grown in continuous RL for three days, the percentage of seedlings with a hypocotyl curvature (relative to vertical) between 0-40 degrees was around 28% for Col-0 and 88% for Knox-10 (Fig. 4-2D). Furthermore, when the hypocotyl curvatures of 4-d-old dark-grown seedlings were measured, Knox-10 also showed less HRR than Col-0 seedlings (Fig. 4-2E).

Hyposensitivity of Knox-10 to Changes in RL-Fluence-Rate

Since Knox-10 showed impairment HRR, the fluence-rate-dependence of this response was examined. The 2-d-old dark-grown Knox-10 and Col-0 seedlings were treated with RL at three fluence rates (1, 10, and 80 μmol/m^2s), and then hypocotyl length and curvature were measured at the end of the 24-h experiment. Increasing RL fluence rate from 1 to 80 μmol/m^2s significantly reduced the relative hypocotyl length of Col-0 seedlings from 82.7% (±1.6%) to 71.2% (±1.4%) (Fig. 4-3A). However, the relative hypocotyl length of Knox-10 seedlings did not significantly differ at these two RL fluence rates (80.0 ±1.8% and 76.5 ±2.0%, respectively). Knox-10 exhibited the HRR at lower fluence rates, yet the HRR was significantly reduced with the increase of higher fluences of RL in Col-0 seedlings. Fluence rate had no effect on hypocotyl curvature in Knox-10 seedlings (Fig. 4-3B).

Map Location of the RL-Induced HRR Gene

To identify the gene (s) responsible for the RL-induced HRR, the Col-0 ecotype was crossed into Knox-10 ecotype, and F1 seeds were harvested and tested for the RL-induced HRR. The HRR was examined in F1 seedlings. The progeny displayed an intermediate or quantitative phenotype, as the response scored was HRR or no HRR. Seedlings exhibited a range of phenotypes. The relative frequency of seedlings could be grouped into several HRR classes (0 degree to 180 degrees, at 20-degree intervals) between the angles observed in Col-0 and Knox-10 (Fig. 4-4A, B). In the F2 segregation population, hypocotyl-curvature distributions in response to RL showed the similar pattern as in the F1 seedlings (Fig. 4-4C, D), and the segregation ratio did not follow Mendalian segregation.

To locate the causal gene responsible for the defective HRR in Knox-10 ecotype, F2 seedlings that showed an upright- or near-upright-growing phenotype were selected as the mapping population. The result from rough mapping using Simple Sequence Length Polymorphism (SSLP) markers showed that several markers located on the lower arm of chromosome one displayed a biased segregation towards the Knox-10 genotype. These markers include nga280, NF5I14, nga111, and nga692, which cover about 8 Mb of genomic region (Table 4-3). The causal gene is likely to be located within this genetic interval.

Increased Hyponastic Growth in Knox-10 Ecotype

During the early stages of vegetative growth (from first to fourth pair of true leaves), Knox-10 displayed an increase in hyponastic growth (i.e. elongated stem, petiole, and reduced petiole angle) compared to the Col-0 ecotype (Fig. 4-5A). Typically, plants develop such growth behaviors in response to dense canopies/shade,

elevated temperatures, or immersion in water (Cox et al., 2003; Vandenbussche et al., 2005; Koini et al., 2009). The hyponastic growth behaviors induced by shading signals are collectively referred as shade avoidance syndrome (SAS) (Smith and Whitelam, 1997; Franklin, 2008). In shaded environments the light spectrum is FrL- and GL-enriched, as well as BL-reduced, all of which can independently induce SAS (Franklin, 2008; Keuskamp et al., 2011; Zhang, Maruhnich, and Folta, 2011).

The defect in RL-induced HRR and the hyposensitivity to RL fluence-rate changes might be connected with the increased hyponastic growth in Knox-10 ecotype. The hypothesis is that the altered function of this downstream component leads to abnormal responses to RL that ultimately affects its responsiveness to R: FR ratio changes. To further test this hypothesis, three light combinations were designed: a) B30G30 (blue and green light, 30 µmol/m^2s each); b) R10B30G30 (adding 10 µmol/m^2s red light to B30G30); c) R10B25G25 (keeping the same photosynthetically active radiation [PAR] in (a) by reducing blue and green to 25 µmol/m^2s each).

The results showed that plants grown in blue and green light conditions (B30G30) developed typical shade avoidance responses in both Col-0 and Knox-10 ecotypes. There were no significant differences in the petiole angles between these two ecotypes, but Knox-10 plants showed a significantly higher petiole: leaf ratio (P/L) than Col-0 plants (Fig. 4-5B, C, D). Additional RL (R10B30G30) significantly reduced the P/L ratio in both ecotypes. Still, Knox-10 plants showed a higher P/L ratio than Col-0 plants grown with B30G30 (Fig. 4-5C). Although the petiole angles were increased under R10B30G30 in Col-0 plants, they were not significantly different from B30G30-treated plants (Fig. 4-5B, D). When keeping the same PAR, Col-0 plants grown with

R10B25G25 exhibited attenuated shade avoidance responses, with significantly increased petiole angles and decreased P/L when compared to the B30G30-treated plants. The enhanced attenuation of SAS was not observed in Knox-10 plants treated under these same conditions (Fig. 4-5B, C, D).

Knox-10 Responds Normally to RL at the Level of Transcriptional Regulation of Shade-Induced Marker Genes

To test whether this increased hyponastic growth was a defect in the light detection or light response, the relative transcript levels of several shade-induced marker genes were examined in these two ecotypes. Marker genes include those that accompany with shade avoidance response: *PIL1*, *HFR1*, and *ATHB2* (Carabelli et al., 1996; Salter, Franklin, and Whitelam, 2003; Sessa et al., 2005). Supplementation of RL significantly reduced the transcript levels of *ATHB2* and *HFR1* genes in both Col-0 and Knox-10 plants. *PIL1* transcript levels were not affected (Fig. 4-6). While Knox-10 showed increased hyponastic growth than Col-0 plants, the transcript levels of these three marker genes did not significantly differ between these two ecotypes under the tested light combinations.

Relationships between the Defective RL-Induced HRR and the Increased Hyponastic Growth

The reduced HRR and increased hyponastic growth in Knox-10 raises a fundamental question: what is the connection between these two traits. These two traits seem to allow plants to grow more upright and taller so that they might outcompete neighbors. Does one common genetic component controls these two phenotypes? To test this hypothesis, the HRR phenotype was assessed in the F2 segregation population. Seedlings that showed upright or flat hypocotyl curvatures were selected and grouped into F2-UP (upright F2 seedlings) and F2-DOWN (flat F2 seedlings). After

8-10 d of recovery, seedlings were moved to light chambers with the same light combinations (B30G30, R10B30G30, and R10B25G25) and treated for 9 d. Petiole angles and P/L ratio were compared.

Surprisingly, the petiole angles of F2-UP- and F2-DOWN-plants were all similar to Knox-10 plants (more upward) in all three light conditions (Fig. 4-7A). Meanwhile, all F2-UP- and F2-DOWN-plants showed higher P/L (more elongated petioles) under R10B25G25 light conditions (Fig. 4-7B) than that of Col-0 plants grown under the same conditions. Furthermore, the F2-UP-plants showed increased P/L than F2-DOWN-plants only under R10B30G30 conditions (Fig. 4-7B). In summary, co-segregation was not observed between the defective HRR seedlings and increased hyponastic growth.

Examination of Amyloplasts

Conversion of etioplasts to amyloplasts is potentially behind the Col-0 HRR. This hypothesis was tested by I_2-KI staining dark-grown and RL-grown seedlings from both ecotypes. No significant differences were observed in the conversion process between Col-0 and Knox-10 seedlings (Fig. 4-8). After 24-h RL treatment, both accessions showed greatly reduced amyloplasts in the hypocotyls.

Discussion

Arabidopsis Natural Variations and GL Response

The original rationale of this study was to identify signaling components of GL using *Arabidopsis* natural variations. *Arabidopsis* grows across a wide latitudinal range in the world which makes it very suitable for adaptive traits analysis (Koornneef, Alonso-Blanco, and Vreugdenhil, 2004). GL affects many plant responses that oppose the effects of other wavelengths, especially under low-light environments. This feature may

confer adaptive advantages in a specific light environment, but not others. The hypothesis is that the genetic variations in GL-signaling proteins may lead to abnormal GL-responses in naturally occurring variants. Such variations might be neutral or positive to the overall fitness

Using hypocotyl-growth-rate imaging, 42 *Arabidopsis* accessions were tested for GL-stimulated growth (Table 4-1). Four accessions showed reduced or no GL response in the original screen. However, only Knox-10 consistently showed the reduced-GL-response that was transmitted to its progeny. Since seed size, age, maturity, and the environmental conditions (light, photoperiod, temperature, nutrient, etc.) during seed maturation can all influence the subsequent seedling development (Leishman and Westoby, 1994; Rice and Dyer, 2001; Luzuriaga, Escudero, and Pérez-García, 2006), it is likely that some of the putative candidate accessions were affected by non-genetic effects.

Impaired HRR in Knox-10 Accession

Knox-10 and Col-0 showed phenotypically similar responses in many other light conditions. Relative hypocotyl length was comparable between Col-0 and Knox-10 indicating that the overall light-signal transduction network is intact and functional for Knox-10 (Fig. 4-2A). Knox-10 displayed a defective RL-induced HRR (Fig. 4-2B, C, D) suggesting that a specific phy-mediated response is affected by the genetic variation. The Knox-10 gene controlling this response is probably positioned downstream of this particular pathway and may be essential for upright hypocotyl growth, since the 4-d dark-grown Knox-10 seedlings also displayed less HRR than Col-0 seedlings (Fig. 4-2E). One possibility is that this component is a regulator of HRR, and the phy receptors

are probably targeting this component under RL/FrL, antagonizing or enhancing its function.

Alternatively, the reduced HRR could simply result from an alternation in a structural gene that controls cell differentiation or growth in the dark-grown seedling that is revealed by RL treatment. The mechanism of phytochrome-induced HRR remains poorly understood. However, the PHYTOCHROME-INTERACTING FACTORS (PIFs) have been reported to play an essential role in the hypocotyl position relative to gravity in RL. Four loss-of-function *PIFs* (*PIF1*, *PIF3*, *PIF4*, and *PIF5*) confer HRR even in darkness (Shin et al., 2009). Sedimentable amyloplasts in the shoot/hypocotyl have been proposed to function in the gravity sensing (Morita and Tasaka, 2004), and PIFs have been demonstrated to repress conversion of amyloplast to etioplast (Stephenson, Fankhauser, and Terry, 2009). In the follow-up study, the authors hypothesized that phy might disrupt hypocotyl gravitropism by degrading PIFs. The results from their study supported this hypothesis, showing that the *pif1pif3pif4pif5* quadruple mutants did not contain amyloplasts, but still exhibit HRR even in darkness.

Endodermis-specific expression of *PIF1* in the quadruple mutant background restored both the amyloplast phenotype and hypocotyl growth against gravity (Kim et al., 2011). In the present study, the amyloplast-to-etioplast conversion was also examined in Knox-10 accession by I_2-KI staining of dark-grown and RL-grown seedlings. No significant differences were observed in the conversion process between Col-0 and Knox-10 seedlings (Fig. 4-8). After 24-h RL treatment, both accessions showed greatly reduced amyloplasts in the hypocotyls. Despite this, the gravitropic and phototropic responses are normal in the Knox-10 accession (data not shown).

These results indicate that the lack of a RL-induced HRR in Knox-10 is not likely caused by defects in the sensing or response to gravity by the hypocotyl, but instead by a component linking phy-signaling and the gravity sensing/response. Such components have been described in several studies. One such component is PHYTOCHROME KINASE SUBSTRATE4 (PKS4), which has been suggested to modulate RL-induced HRR. Mutation or overexpression of *PKS4* in *Arabidopsis* seedlings resulted in reduced or enhanced HRR (Schepens et al., 2008). Another component is GRAVITROPIC IN THE LIGHT (GIL1). Mutation of *GIL1* gene led to a reduced HRR (Allen et al., 2006). Nonetheless, how these two components mediate the RL-induced HRR remains unclear. Since both genes are located on chromosome 5 and our rough mapping position is on chromosome 1 (Table 4-3), these two genes are not likely underlying the Knox-10 phenotype. However, they could be the additional loci that contribute to the reduced HRR in Knox-10 as a quantitative trait.

Hyposensitivity to RL Fluence Rate Changes and Increased Hyponastic Growth

The Knox-10 accession was less responsive to RL fluence rate changes in terms of the hypocotyl elongation and HRR (Fig. 4-3). One possible explanation for this result is that genetic variation in this response controls cell differentiation and/or elongation growth. Candidate components include signaling or response pathways for auxin, or even modulators of auxin transport and distribution. RL might integrate with auxin signaling, response, or transport through the action of such a component. Disruption of the functions of this component abolished the connections between RL and auxin in this particular response. Alternatively, this component may contribute a physical constraint for cell differentiation or elongation, but the normal gravitropic and phototropic responses of Knox-10 seedlings do not seem to support such an explanation. The

increased hyponastic growth in Knox-10 during early vegetative development represents another potentially adaptive trait that may be physiologically relevant for this accession in its natural habitat. The elongated stems and petioles might enable juvenile plants to out-compete neighboring plants or adapt to light-poor environments. Plants in frequently flooded environments are expected to develop elongated petioles/internodes at a higher frequency than do those in rarely flooded places (Bailey-Serres and Voesenek, 2008). The same suite of morphological changes that a plant employed in response to flood stress is called the low-oxygen escape syndrome (LOES). Signals that can induce LOES include gaseous plant hormone, ethylene, as well as the signals associated with changed rate of photosynthesis and/ or reduced levels of carbohydrates (Evans et al., 1994; Lynn and Waldren, 2001, 2002; Mommer et al., 2005). Plants in supraoptimal temperature conditions also develop hyponastic growth phenotypes (van Zanten et al., 2009). *Arabidopsis* ecotypes that originated from lower latitudes have more erect leaf angles than that of ecotypes from northern latitudes (Hopkins, Schmitt, and Stinchcombe, 2008). An sudden increase in temperature from 22 °C to 28 °C induces leaf inclination and petiole elongation in *Arabidopsis* plants. This high-temperature-induced hyponastic growth is genetically controlled by *PHYTOCHROME-INTERACTING FACTOR4* (*PIF4*) (Koini et al., 2009).

The molecular mechanism for the increased hyponastic growth in Knox-10 plants is unclear. One possibility is that the hyposensitivity of Knox-10 to RL fluence-rate-changes might contribute to this trait. Since low red: far-red ratio perceived by phy is one of the mechanisms that could induce hyponastic growth, a signaling component downstream of this pathway might be altered in Knox-10, which leads to the

hyposensitivity to red: far-red ratio changes. If this is the case, the hyposensitivity to red: far-red ratio changes might explain why supplementation of RL to the background of B30G30 or B25G25 only attenuated the shade-avoidance phenotypes in Col-0, but has minimum effects in Knox-10 (Fig. 4-5). However, examination of the transcript levels of shade-induced marker genes under three light combination conditions did not reveal any significant differences between Col-0 and Knox-10 plants (Fig. 4-6). This result suggests that Knox-10 can respond to RL normally in the early steps of phy-mediated shade avoidance pathway, at least at the transcriptional level of those shade-associated genes.

In this study there is an uncharacteristic disassociation between the shade-induced genes and the morphological changes observed. GL-induced SAS produces plants with increased hyponastic growth and the transcript levels of the shade-induced marker genes are actively repressed by the cry1 and cry2 receptors (Zhang, Maruhnich, and Folta, 2011). The results observed here suggest that Knox-10 may possess variations in the response to shade with respect to coincident gene expression, consistent with the observations that a discrete set of light responses are affected in the Knox-10 background.

RL-Induced HRR and Increased Hyponastic Growth

The RL-induced HRR and the increased hyponastic growth in Knox-10 were tested for co-segregation to determine if they were governed by common genes. The results from this test were inconclusive, yet suggested that these traits were not genetically linked. All of the F2 plants from the ‘Knox-10 x Col-0 cross’ showed reduced petiole angles (more upward), as did Knox-10 plants under B30G30, R10B30G30, and R10B25G25 light conditions (Fig. 4-7A). The petiole: leaf ratio (P/L) of F2-UP and -

DOWN plants was difficult to interpret. However, the general trend is the F2-UP plants seem to have larger P/L compared to Col-0 plants at the two RL supplementation conditions. The F2-DOWN plants only showed larger P/L than Col-0 plants under R10B25G25 condition in which the increased hyponastic growth was most pronounced between Knox-10 and Col-0 plants (Fig. 4-7B). One explanation for this result is that the increased hyponastic growth is not linked with the defective RL-induced HRR. The increased P/L in F2 plants compared with that of Col-0 plants may be due to the Mendelian segregation of a second gene controlling hyponastic growth. Thus, the mean P/L from the F2 plants would be larger than Col-0 but smaller than Knox-10 plants. However, the petiole-angle result seems to conflict with such an explanation, since all F2 plants showed similar petiole angles to Knox-10 plants. The alternative explanation for this result is that the increased hyponastic growth in F2 plants might be due to the effects from the Knox-10 genetic background. Such hypotheses could be tested by reciprocal crosses in future studies.

On the other hand, the early response to GL and the HRR are highly correlated. The Knox-10 plants show a diminished response to GL and to RL, but the data indicate that these are not simply phy-responses, a result supported by gene expressions analysis. The data indicate that the defect leading to both responses is downstream, at the level of cellular response to light. Both responses are likely affected by GL or RL independently, as two signals ultimately affect an elongation response. A second interpretation is that the GL and RL responses are both mediated by the hypothetical GL receptor, which could have substantial absorbance in the red portion of the spectrum.

The phy-mediated HRR may be physiologically significant for early seedling growth and development. Seedlings emerging from soil encounter changing low-light environments, such as when covered by dense canopies, shaded by neighboring plants, buried by plant debris, or even germinated under water. In some of these light environments the reduction of BL could amplify the action of phy-mediated-HRR, thus enhancing its antagonistic effect on hypocotyl gravitropism and rendering more robust hypocotyl/stem phototropism (Lariguet and Fankhauser, 2004). In addition, the enrichment of far-red and GL promotes hypocotyl/stem elongation growth to escape from adverse light environments (Vandenbussche et al., 2005; Zhang, Maruhnich, and Folta, 2011). Impairment in the phy-mediated HRR has been proposed to confer a fitness disadvantage in seedling emergence and survival (Allen et al., 2006). Whether such a defect would hinder the early establishment of Knox-10 seedlings is an interesting question to be investigated in future studies.

However, the increased hyponastic growth in Knox-10 plants during early development may also be physiologically relevant. In the early stage of seedling growth and development, Knox-10 plants exhibit longer stems and smaller cotyledon angles compared with Col-0 plants under low-light conditions (Fig.4-2A). Such increased hyponastic growth persisted at least to the stage of fourth pair of true leaves. This phenotypic variation might compensate for the defective phy-mediated HRR if the latter do affect the fitness of Knox-10. Since the phototropic response of Knox-10 is normal, the increased hypocotyl growth might enhance the likelihood that the emerging seedlings would reach optimal light and/or oxygen as quickly as possible.

These data provide a stepping-off point to analyze additional genes in the genetic interval defined, as well as perform high-throughput sequencing to identify the causal genes in these responses. The use of a natural accession provides insight into a number of altered light responses. It will now be of interest to identify the genes affected.

Materials and Methods

Plant Materials

A set of 96 *Arabidopsis* natural accessions (Stock #: CS22660) were ordered from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University (Columbus, OH).

GL-Stimulated Hypocotyl Elongation Screening Assay

Seeds of individual accessions were germinated as described previously. 2-d-old seedlings (approximately 2–3 mm, the stage exhibiting the most robust and rapid hypocotyl elongation) were transferred to 1% Difco agar plate oriented vertically in front of a CCD camera (EDC1000N; Electrim Corp., Princeton, NJ, USA) with a close-focus lens (K52–274; Edmund Scientific, Barrington, NJ, USA). A non-photomorphogenic infrared light source was placed behind the seedlings to allow visualization of seedlings during the dark period. Digital images were taken at 5-min-interval for 1 h in the dark to establish the dark growth rate, and at 5-min-interval for 1h after GL illumination. GL was supplied by a Floralamp LED arrays (Light Emitting Computers, Victoria, BC Canada) at a fluence rate of 0.2~0.5 μmol/m^2s. A custom software application, written in the Lab View environment (National Instruments, Austin, TX, USA) were used to calculate growth rate data from the series of digital images (Folta, 2004).

End-Point Hypocotyl Length and Curvature Measurements

Seed germination and growth conditions were the same as described in previous Chapters. Analysis of hypocotyl length and curvature was performed by scanning petri dishes of seedlings on a flatbed scanner covered with black cloth, and then measured seedlings at high magnification using ImageJ 1.44 software (http://imagej.nih.gov/ij/index.html). The hypocotyl length in response to different wavebands was normalized to the mean hypocotyl length of dark-grown seedlings of each accession. The mean of 30-40 seedlings is reported for each light condition. The hypocotyl curvature was defined as the angle between hypocotyl orientation and vertical position. Same experiment was performed at least twice.

Mapping of the RL-Induced HRR Gene

Col-0 plant was crossed into Knox-10 plant under a binocular microscope. F2 segregation population was screen for the RL-induced HRR under continuous RL (8 $\mu mol/m^2s$) conditions for 4 days. Individual F2 seedlings with upright hypocotyl orientation were selected and transferred into soil for later DNA extraction. Selected F2 plants were maintained in growth chamber under white light (60-80 $\mu mol/m^2s$, 16h light/8h dark) conditions with a constant temperature at 21 °C. Genomic DNAs were extracted from the third pair of true leaves using SDS (Sodium Dodecyl Sulfate) method as described (Edwards, Johnstone, and Thompson, 1991). Briefly, leaf samples (approximately 1 cm^2) were transferred into 2.0 mL Eppendorf tubes, then each tube was added with one metal bead and 500 µL extraction buffer (200 mM Tris-HCl [pH 8.0], 25 mM EDTA [pH 8.0], 250 mM NaCl, 0.5% SDS). Leaf tissues were then homogenized on a shaker (Mini-Beadbeater, BioSpec) at maximum speed for 1 min. After centrifugation, about 400 µL supernatant was transferred into a fresh 1.5 mL

Eppendorf tube. Then, 400 µL isopropanol were added into each tube and mixed immediately. After leaving it at room temperature for 2 min, samples were centrifuged at full speed for 5 min. Then, supernatant was removed and the pellet was washed with 500 µL 70% ethanol. The pellet was dried gently and dissolved in ddH_2O. Sequencing information and chromosome locations of SSLP markers were obtained from the ABRC website (Table 4-2). PCR reactions were performed on a thermocycler (Mastercycler® pro S, Eppendorf) with the following program: 94°C 1 min, 35x (94°C 15 s; 55°C 15 s; 72°C 30 s), 72°C 5 min, hold at 10°C. 3%-3.5% agorose gel was used to obtain a good resolution of the SSLP bands.

Hyponastic Growth Measurement

9-d-old Col-0 and Knox-10 plants grown under white light (36 µmol/m^2s, 16h light/8h dark) were transferred to different light chambers and treated for another 9 days with regular watering. Then, whole plants were carefully removed from the soil, the petiole angles of the second pair of true leaves were recorded by a digital camera, and then the leaves were flattened on the adhesive side of black electrical tape and covered with plastic film. Then, leaf samples were scanned at 600-dpi resolution on a standard flatbed scanner and measured using ImageJ 1.44 software (http://imagej.nih.gov/ij/index.html) for the leaf and petiole length.

RNA Extraction and Quantitative RT-PCR Analysis

The aboveground tissues (40~60 mg) of young plant treated with different light combinations were harvested into liquid nitrogen at the same time of the day for each repeated experiment. Total RNA was extracted by using the Qiagen RNeasy Mini kit (Qiagen Cat # 74904) according to the manufacturer's protocol. Resulting RNA was treated with DNase I (Fermentas Cat # EN0521), and cleaned with Qiagen RNeasy Mini

kit. The cDNA was synthesized from 0.5 µg cleaned RNA using the Improm-II Reverse Transcriptase (Promega Inc., Madison, WI). Quantitative RT- PCR was performed using the StepOne Plus system (Applied Biosystems, USA) based on SYBR Green chemistry.

Three biological replicates (individual plants) were analyzed for each light treatment per each ecotype, and for each of these replicates, reactions were performed in duplicate. A given reaction included 10 µL EvaGreen qPCR MasterMix-ROX (ABM, CANADA), 5.0 µL of cDNA sample (diluted 10x from cDNA reaction), and 100 nM of each gene-specific primer (Table 4-4) in a final volume of 20 µL. All the primers were designed by the Primer Express 2.0 software (Applied Biosystems, USA). *YLS8* (*At5g08290*) was used as the reference gene (Czechowski et al., 2005). The relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Iodine Staining of Endodermal Amyloplasts

Endodermal amyloplasts of Knox-10 and Col-0 seedlings that treated with RL or dark were visualized by iodine staining according to method described (Kim et al., 2011). Briefly, seedlings were fixed in FAA (5% Formaldehyde, 45%Ethanol, 5% Acetic acid) solution for 24 h in dark at 4 °C. Then after fixation, seedlings were rinsed in 50% (v/v) ethanol once and stained in Lugol solution (Sigma Cat # 32922) for 2 min. Seedlings were de-stained in 1:1:1 trichloroacetic acid: phenol: lactic acid for 1 min and carefully mounted on slides with a drop of de-staining solution for the light microscopic observation (Nikon AZ100, Japan). Images were taken and analyzed by NIS Elements Imaging Software (Nikon).

Statistical Analysis

Means of replicates from long-term hypocotyl growth assays were subjected to statistical analysis of one way ANOVA by multiple-range Tukey-Kramer test ($p \leq 0.05$) using the JMP®Pro 10.0 statistical package (SAS Institute).

Table 4-1. Statistical analysis of hypocotyl growth rate between different accessions and Col-0 in response to GL treatment

Ecotypes	# of seedlings	Mean growth rate*	S.E.	Two tail *p-value***	Two tail *p-value****	Ecotypes	# of seedlings	Mean growth rate*	S.E.	Two tail *p-value***	Two tail *p-value****
Col-0 (Dark)	15	0.973	0.059	0.001		UII2-5	6	1.231	0.249	0.266	0.166
Col-0	20	1.532	0.125		0.001	Bil-7	5	1.678	0.12	0.582	0.000
Knox-10	36	1.154	0.069	0.006	0.116	Lov-1	6	0.863	0.082	0.009	0.318
Pu2-23	6	1.206	0.123	0.187	0.069	Var2-1	6	1.21	0.096	0.186	0.048
Mrk-0	6	1.714	0.184	0.475	0.000	Omo2-3	6	1.788	0.276	0.356	0.000
Mt-0	5	1.55	0.183	0.949	0.001	C24	12	1.251	0.086	0.118	0.011
Rss-10	6	1.224	0.147	0.218	0.069	Kondara	6	1.395	0.1	0.511	0.001
Gu-0	5	1.334	0.084	0.449	0.005	Lov-5	6	1.361	0.156	0.491	0.009
HR-10	6	1.369	0.164	0.514	0.010	W.s	6	1.711	0.318	0.536	0.003
An-1	10	1.513	0.082	0.917	0.000	Tul-0	12	1.257	0.202	0.228	0.151
Sq-8	5	1.344	0.081	0.472	0.005	S96	12	1.231	0.103	0.105	0.031
Mz-0	6	1.654	0.116	0.616	0.000	Col-3	5	1.324	0.106	0.429	0.009
Br-0	5	1.225	0.103	0.246	0.048	Nossen	6	1.6	0.263	0.804	0.003
Wei-0	6	1.189	0.126	0.166	0.094	Col-prl	9	1.507	0.193	0.914	0.004
Nd-0	13	1.44	0.207	0.689	0.029	Gre-0	6	1.309	0.148	0.369	0.019
Pro-0	6	1.382	0.2	0.560	0.016	Ler	6	1.957	0.371	0.171	0.001
Tamm-27	12	1.217	0.089	0.084	0.026	Kend-L	6	1.529	0.227	0.992	0.004
Fab-4	12	0.908	0.090	0.001	0.539	Vi-0	9	1.334	0.089	0.322	0.002
Kas-2	6	1.415	0.198	0.649	0.010	Be	4	1.327	0.126	0.484	0.016
Nd-1	6	1.151	0.13	0.127	0.166	Djion	6	1.8	0.197	0.300	0.000
Eden-1	6	0.825	0.098	0.006	0.202	Muhlen	6	1.686	0.187	0.546	0.000
Fab-2	6	1.411	0.172	0.631	0.006	RLD	6	2.01	0.473	0.170	0.003

*: Hypocotyl growth rate was normalized to that in dark. **: Student t-test was conducted between ecotype and GL-treated Col-0. ***: Student t-test was conducted between ecotype and Dark-treated Col-0. For simplicity, the hypocotyl growth rate at the time point 30-min after GL was onset was selected for this comparison. However, the pattern of growth rate kinetics were also taking into account when determine whether it was truly a candidate accession.

Table 4-2. SSLP markers used in Chapter 4

Chromosome	SSLP marker	Sequence	Col-0 (bp)	Knox-10 (bp)	Map position (bp)
1	nga63	AACCAAGGCACAGAAGCG ACCCAAGTGATCGCCACC	111	89	3551000
1	F21J9-83201	CAGATTTCTTGCCAAGTTTCATC GAGACGAAGAAGATGGATTCTG	189	<189	8655241
1	nga280	CTGATCTCACGGACAATAGTGC GGCTCCATAAAAAGTGCACC	105	85	20873698
1	NF5I14	GGCATCACAGTTCTGATTCC CTGCCTGAAATTGTCGAAAC	196	>196	24370345
1	nga111	TGTTTTTTAGGACAAATGGCG CTCCAGTTGGAAGCTAAAGGG	130	>130	27353212
1	nga692	AGCGTTTAGCTCAACCCTAGG TTTAGAGAGAGAGAGCGCGG	119	<119	28836552
2	nga1145	CCTTCACATCCAAAACCCAC GCACATACCCACAACCAGAA	213	<213	683626
2	CIW3	GAAACTCAATGAAATCCACTT TGAACTTGTTGTGAGCTTTGA	230	>230	6402846
2	nga361	AAAGAGATGAGAATTTGGAC ACATATCAATATATTAAAGTAGC	114	<114	13222414
2	AthBIO2b	TGACCTCCTCTTCCATGGAG TTAACAGAAACCCAAAGCTTTC	141	>141	18012804
3	nga162	CATGCAATTTGCATCTGAGG CTCTGTCACTCTTTTCCTCTGG	107	89	4608277
3	CIW11	CCCCGAGTTGAGGTATT GAAGAAATTCCTAAAGCATTC	179	>179	9774308
3	CIW20	CATCGGCCTGAGTCAACT CACCATAGCTTCTTCCTTTCTT	absent	200	20762868
3	nga6	TGGATTTCTTCCTCTCTTCAC ATGGAGAAGCTTACACTGATC	143	>143	23031050
4	CIW5	GGTTAAAAATTAGGGTTACGA AGATTTACGTGGAAGCAA	164	144	737954
4	CIW6	CTCGTAGTGCACTTTCATCA CACATGGTTAGGGAAACAATA	162	>162	7892624
4	CIW7	AATTTGGAGATTAGCTGGAAT CCATGTTGATGATAAGCACAA	130	<130	11524350

Table 4-2. Continued.

Chromosome	SSLP marker	Sequence	Col-0 (bp)	Knox-10 (bp)	Map position (bp)
4	nga1139	TAGCCGGATGAGTTGGTACC TTTTTCCTTGTGTTGCATTCC	114	<114	16444151
5	nga225	GAAATCCAAATCCCAGAGAGG TCTCCCCACTAGTTTTGTGTCC	119	absent	1507103
5	nga139	AGAGCTACCAGATCCGATGG GGTTTCGTTTCACTATCCAGG	174	<174	8428133
5	phyC	CTCAGAGAATTCCCAGAAAAATCT AAACTCGAGAGTTTTGTCTAGATC	207	>207	14007897
5	CIW9	CAGACGTATCAAATGACAAATG GACTACTGCTCAAACTATTCGG	166	<166	17044001
5	EG7F2 (CAPS marker)	GCATAGAATTTGACGATAACGAGC GATCTGTGTAGGACTACGAGAC	one band	two bands	24626811

Table 4-3. Candidate map location of the RL-induced HRR gene

SSLP marker	chromosome	AGI map (nuc_sequence)	segregation ratio
nga280	1	20873698 - 20873802 bp	5:37:18*****
NF5I14	1	24370345 - 24370540 bp	4:35:20
nga111	1	27353212 - 27353339 bp	3:33:24
nga692	1	28836552 - 28836670 bp	4:34:21

*****: Represents the ratio of $marker_{(Col\text{-}0)}$: $marker_{(Col\text{-}0\&Knox\text{-}10)}$: $marker_{(Knox\text{-}10)}$

Table 4-4. Primers used in qRT-PCR assay in Chapter 4

Primers	Sequences (5'-3')
qUBC21-5'	TTAGAGATGCAGGCATCAAGAG
qUBC21-3'	AGGTTGCAAAGGATAAGGTTCA
qYLS8-5'	ATGAGACCTGTATGCAGATGG A
qYLS8-3'	ATGACCGTAGAAGGATCGTAC A
ATHB2-QF	TGAGCCCACCCACTACTTTGA
ATHB2-QR	CGGGACCGACACGTGTTC
PIL1-QF	TGGGTGCAGCAGCAACAC
PIL1-QR	CCGGTTGCTTGAACATTCATAG
HFR1-QF	ACCACATGCTGACGGCAAT
HFR1-QR	ATCATGTGATTCGCCGGATT

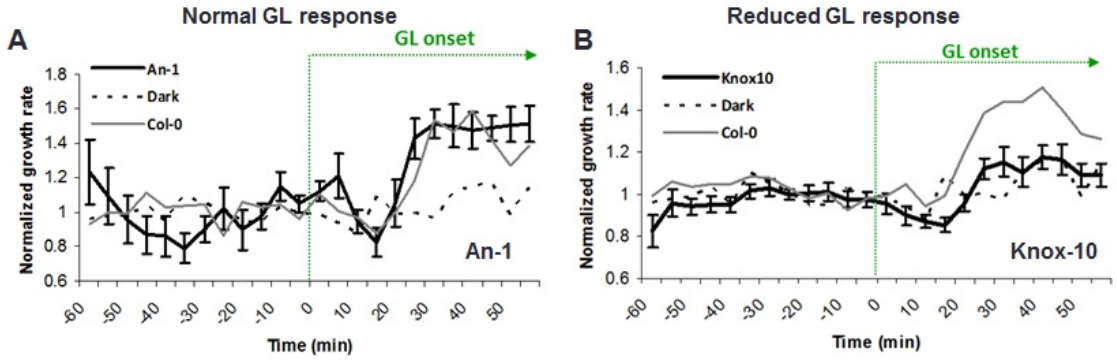

Figure 4-1. Initial response of the GL-stimulated-hypocotyl-growth in *Arabidopsis* ecotypes. (A) Normal GL-response ecotype An-1 (dark line, n=10). (B) Reduced GL-response ecotype Knox-10 (dark line, n=36). Error bars represent S.E.M. Growth rate kinetics of Col-0 seedlings in response to GL (gray line, n=20) and darkness (dash line, n=15) were included for comparison.

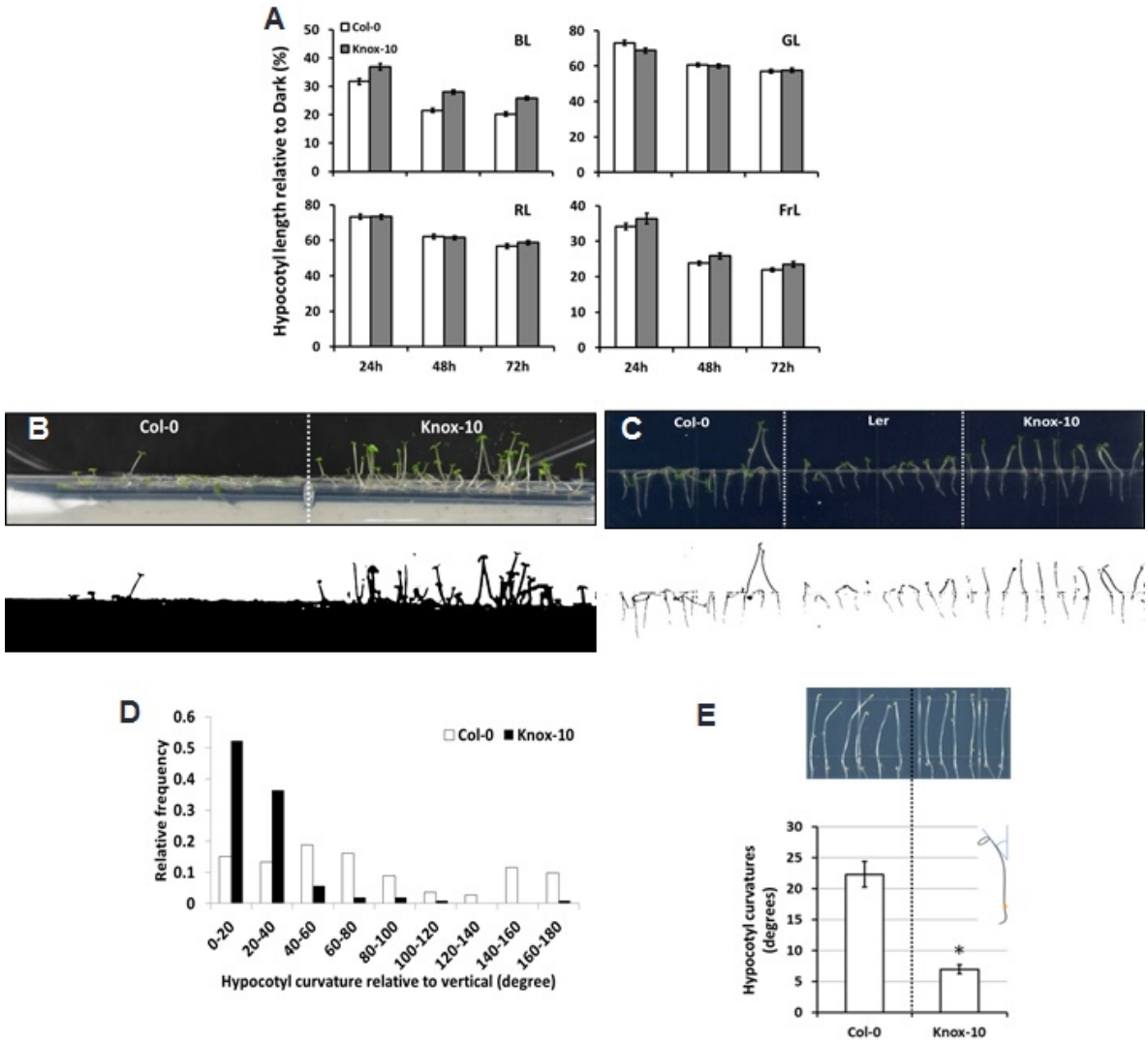

Figure 4-2. Knox-10 is defective in the RL-induced hypocotyl-randomization response. (A) Relative (to dark control) hypocotyl length of Knox-10 seedlings in response to different light wavebands in the end-point assays. BL (blue light, 10 µmol/m^2s), GL (green light, 10 µmol/m^2s), RL (red light, 10 µmol/m^2s), FrL (far-red light, 4 µmol/m^2s). (B) 4-d-old Col-0 (left) and Knox-10 (right) seedlings grown in continuous RL (10 µmol/m^2s). Petri dish was placed horizontally. (C) 4-d-old Col-0 (left), Ler (middle) and Knox-10 (right) seedlings grown in continuous RL (10 µmol/m^2s). Petri dish was placed vertically. The white line in the middle is the gridline of the petri dish. (D) Relative frequencies of hypocotyl curvature in response to continuous RL irradiation for Knox-10 and Col-0 seedlings. (E) 4-d-old dark grown Knox-10 seedlings showed less hypocotyl-randomization response compared to Col-0 (top panel). Means of at least 30 seedlings were reported. Error bars represent S.E.M. (*, p<0.05, student T-test)

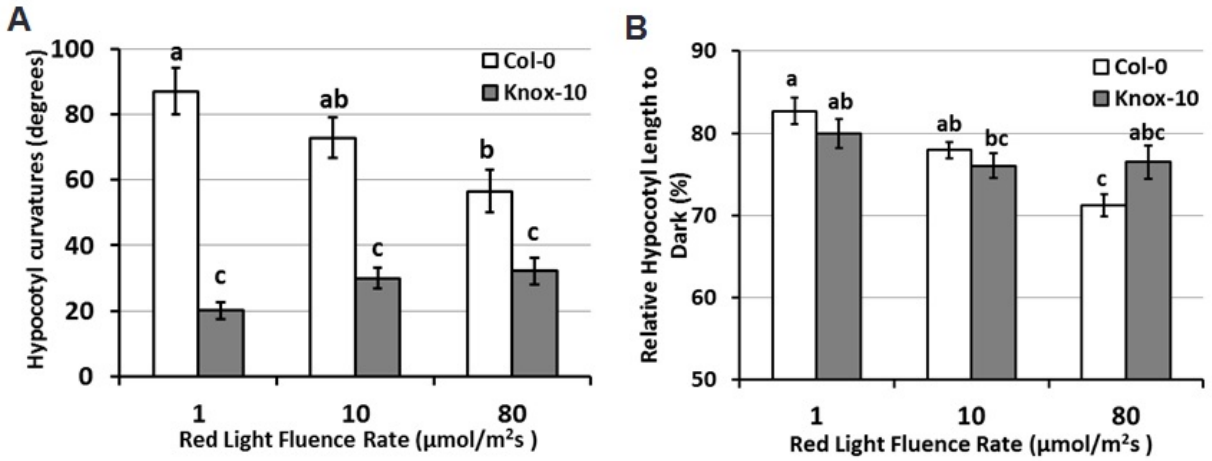

Figure 4-3. Knox-10 is hyposensitive to changes in RL fluence rate. (A) Hypocotyl curvatures of 2-d-old Col-0 and Knox-10 seedlings treated with continuous RL at different fluence rates for 24 h. (B) Relative (to dark control) hypocotyl length of 2-d-old Col-0 and Knox-10 seedlings treated with continuous RL at different fluence rates for 24 h. Different letters represent statistically different means ($p < 0.05$).

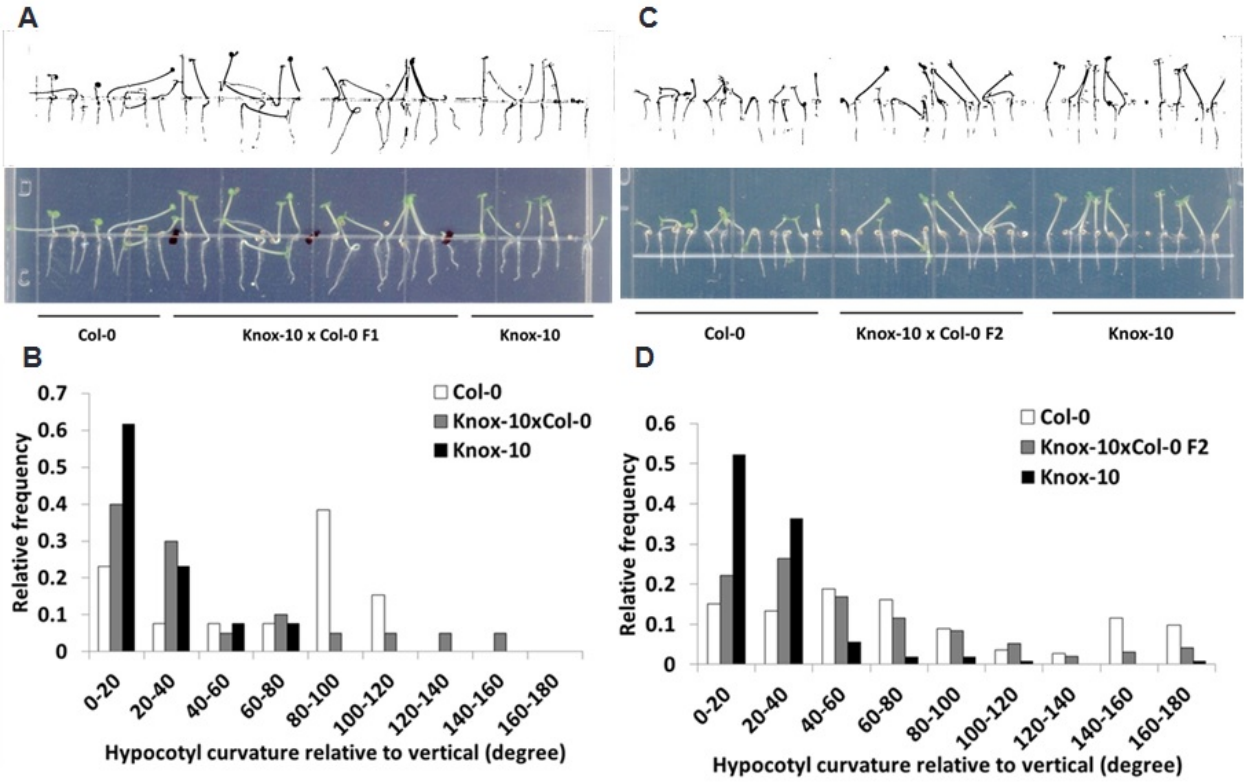

Figure 4-4. Hypocotyl-randomization-curvature distribution in 'Knox-10 x Col-0' F1 and F2 plants treated with continuous RL for 3 days. (A) Representative RL-induced hypocotyl-randomization response in F1 seedlings; (B) Hypocotyl randomization curvature distribution of F1 seedlings (n=20); (C) Representative RL-induced hypocotyl-randomization response in F2 seedlings; (D) Hypocotyl randomization curvature distribution of F2 seedlings (n=95). The white line in the middle of the bottom panel in (A, C) is the gridline of the petri dish.

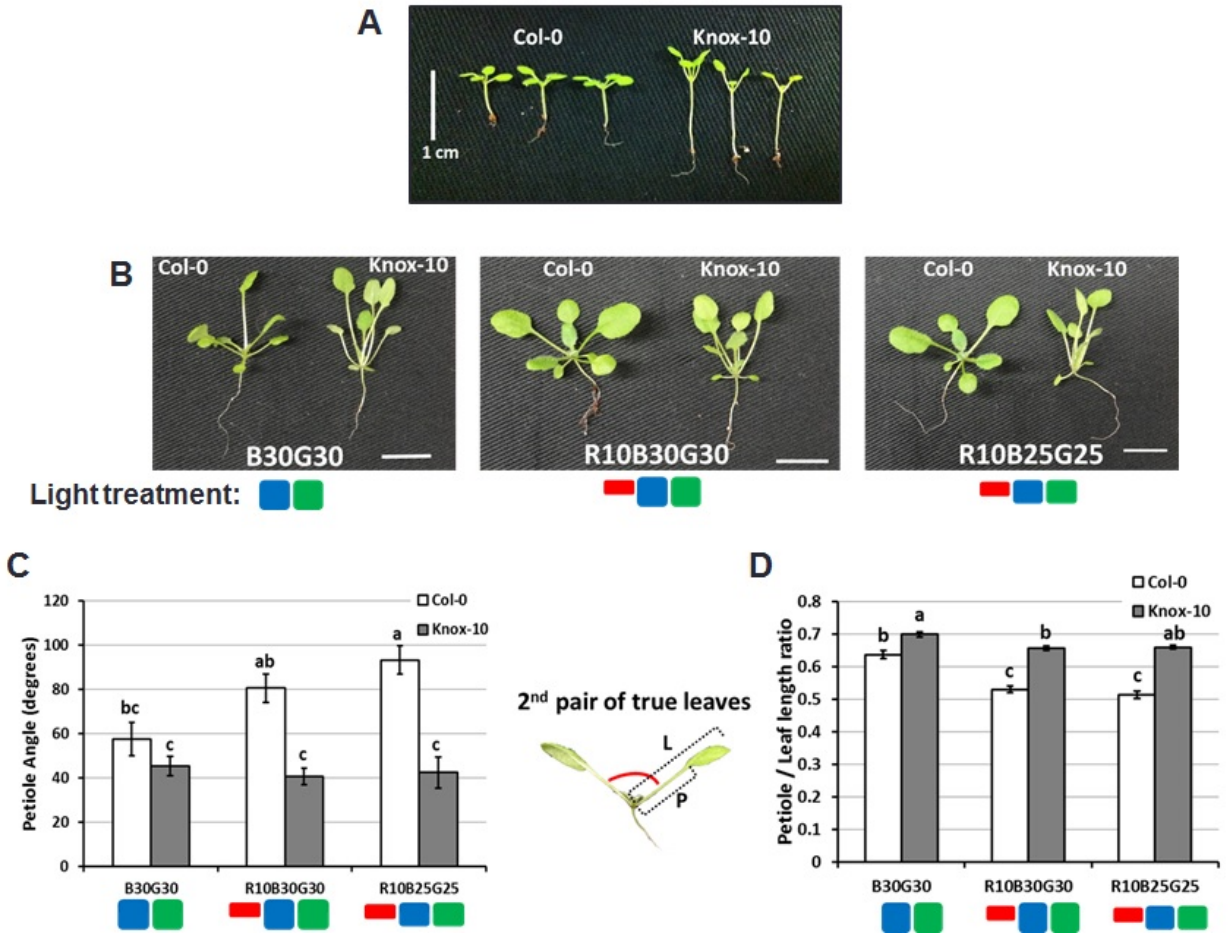

Figure 4-5. Knox-10 plants (first to third true leaf stage) showed increased hyponastic growth. (A) 9-d-old Col-0 and Knox-10 plants grown in 36 µmol/m^{2}s white light conditions with long-day photoperiod; (B) Representative Col-0 and Knox-10 plants grown at different light conditions after 9 days. B, G, and R represent blue, green, and red light, numbers represent the fluence rate used (µmol/m^{2}s). Scale bar: 1cm; (C) Petiole/leaf length ratio of Col-0 and Knox-10 plants under different light treatments (8≤n≤12); (D) Petiole angles of Col-0 and Knox-10 plants under different light treatments (4≤n≤6). Different letters in (C) and (D) represent statistically different means ($p < 0.05$). Error bars represent S.E.M. Same experiment was performed at least twice with a similar result.

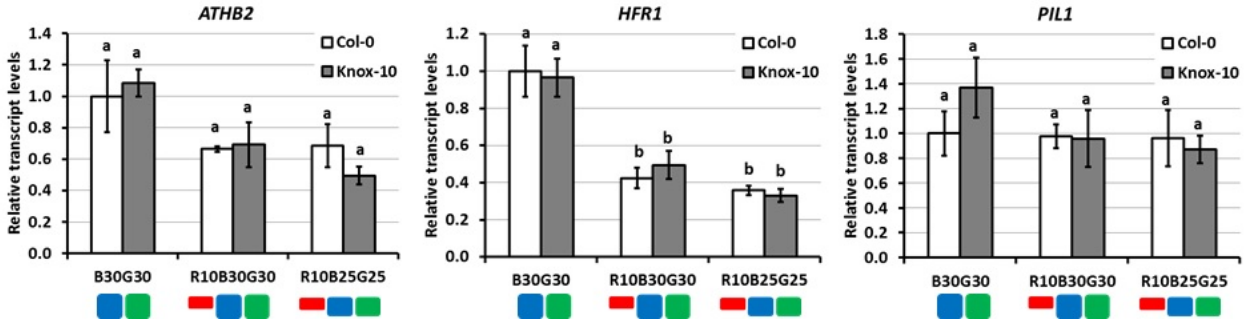

Figure 4-6. Relative transcript levels of shade-induced marker genes in Col-0 and Knox-10 plants in response to different light treatments. Error bars represent S.E.M of three individual plants. Different letters represent statistically different means ($p < 0.05$).

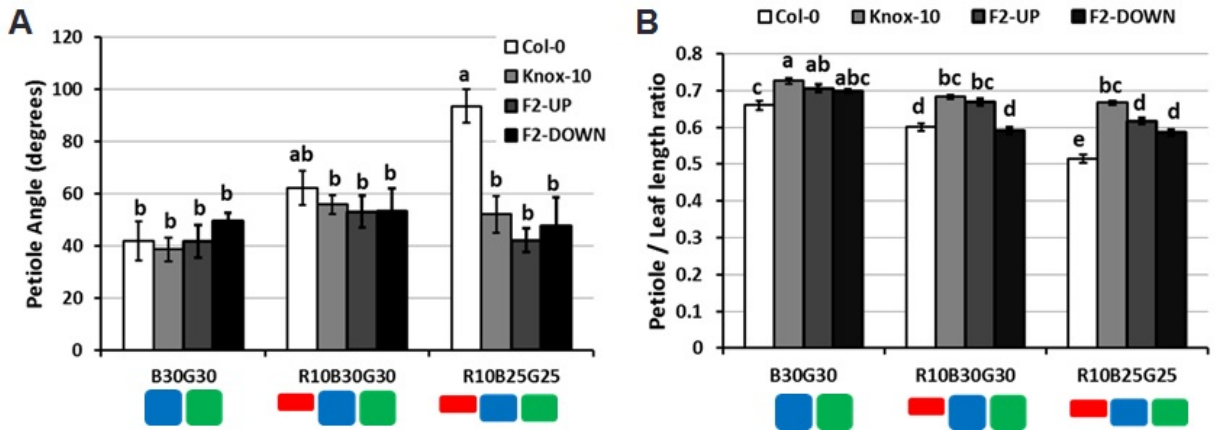

Figure 4-7. Genetic relationships between RL-induced hypocotyl-randomization-response (HRR) and increased-hyponastic-growth. (A) Petiole angles of Col-0, Knox-10, F2-UP (upright seedlings in RL-induced HRR), and F2-DOWN (flat seedlings in RL-induced HRR) plants under different light treatments, $5 \leq n \leq 9$; (B) Petiole/leaf length ratio of Col-0, Knox-10, F2-UP, and F2-DOWN plants under different light treatments, $10 \leq n \leq 18$. Different letters represent statistically different means ($p < 0.05$). Error bars represent S.E.M.

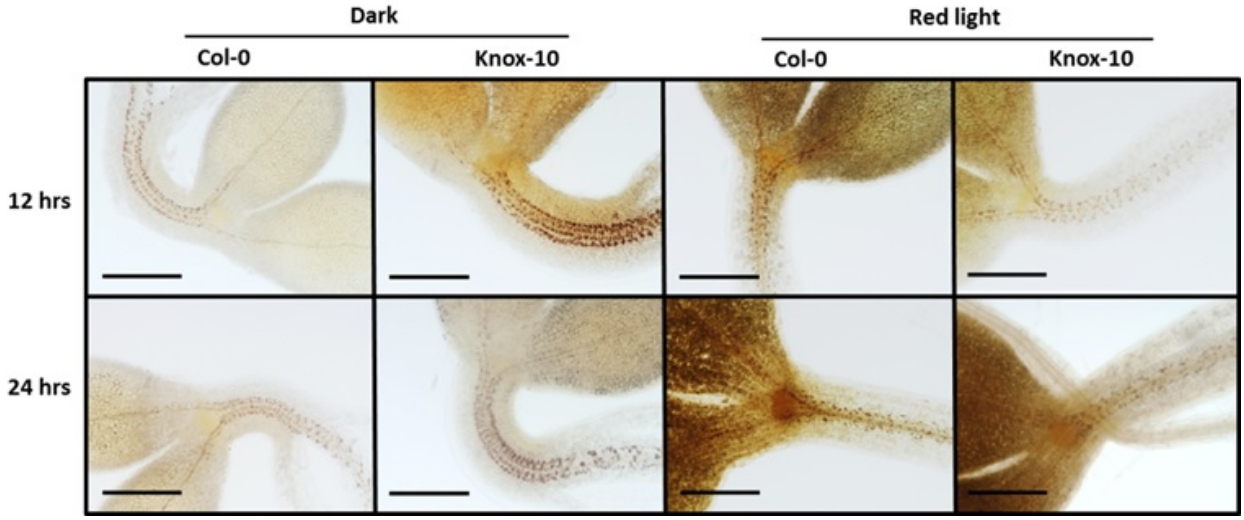

Figure 4-8. The RL-regulated amyloplast development is normal in Knox-10 ecotype. Representative I_2-KI staining patterns in the endodermis of dark-grown and RL-grown Col-0 and Knox-10 seedlings were shown. Scale bars, 200 μm.

CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

The study of GL photobiology is a challenging endeavor. One obstacle in this research is that multiple photosensory systems can be activated or inactivated by GL wavelengths. Thus, the interpretation of phenotypic responses to GL illumination becomes complicated and should be approached cautiously. Work presented in Chapter 2 demonstrated these challenges. The original rationale for that study was to examine the interactions between GL and RL/FrL in the regulation of early photomorphogenic events. By the use of high-resolution imaging system, such interactions could be illustrated with great precision for time and spatial position. At first, it was surprising to find that GL actually can suppress RL/FrL-induced hypocotyl-inhibition. Before this study, reports have shown that GL can attenuate the long-term hypocotyl-inhibition induced by low RL and BL co-irradiation (Folta, 2004).

Later studies indicate that this effect was actually mediated by GL inactivation of cry receptors (Bouly et al., 2007). The cry receptors serve as the GL: BL ratio sensors to regulate plant growth and development in natural light environments (Sellaro et al., 2010). The role of GL-negation of the RL-induced stem-inhibition has not been reported. This response is not likely mediated by inactivation of cry, since cry receptors have a limited role in the RL-induced stem-inhibition. The genetic tests of this GL-negation of RL stem-response further excluded this possibility. The finding that phot1 is mediating this GL effect is unexpected, leading to a complicated situation that was difficult to interpret. The simple explanation is that phot1 was actively responding to a small amount of BL from the lower-end spectrum of green LED light, and it was confirmed when GL was replaced by dim BL. This result finally solved the puzzle, but it also

pinpoints another obstacle in GL photobiology research: the presence of small amounts of blue and yellow light from GL spectrum distribution. Development of better light source with narrow band-width would reduce this type of contamination and misinterpretation.

BL has been always considered to inhibit hypocotyl growth and promote photomorphogenesis. Work presented in Chapters 2 and 3 demonstrated that it is not always the case. Under certain light conditions, such as RL/FrL with dim BL, BL can actually delay the photomorphogenic process induced by RL/FrL through the action of phot1 at least for hours (Fig. 2-1, 2-4, 3-2). In the long-term (days), this attenuation is antagonized by cry, and leading to a shorter hypocotyl in dim BL and RL than that in RL alone (Fig. 3-1D). One thing should be noticed is that the longer hypocotyl observed in GL and RL co-irradiation compared to RL alone (Fig. 2-1) could not be regenerated by supplementing of dim BL to RL, even in the *cry2* or *cry1cry2* mutant backgrounds. If the long-term, GL-negation of RL-induced stem inhibition was simply a result of activation of phot1 and inactivation of cry by GL, we would expect the same result when it was tested with dim BL and RL in *cry* mutant backgrounds. Thus, it seems to suggest other factors unique to GL might mediate this additional effect of hypocotyl elongation promotion. Under BL, those factors might not be activated. Such hypotheses need to be tested in future studies.

The plant hormone auxin has been shown to play important roles in plant tropism responses and stem elongation. Work presented in Chapter 3 demonstrates the possible involvement of auxin in the mediation of the BL suppression of RL-induced hypocotyl-inhibition and apical hook opening events (Fig. 3-4, 3-5F). The transport of

auxin from cell to cell is partly mediated by its transporters. Those transporters functionally may overlap with each other depending on their expression patterns and cellular localization. The functional redundancies of these transporters may explain why there was no complete abolishment of the BL-negation effect on RL in single transporter mutant backgrounds. Tests in higher order transporter mutants may help to illustrate the roles of each transporter in the mediation of this BL effect.

Utilization of genetic variation found in *Arabidopsis* ecotypes could provide an alternative path that might lead to the discovery of GL-signaling components. GL affects many plant responses in a way that seems to be diametric to other photosensory systems (Chapter 1). Such effects may confer plants with an adaptive advantage in specific light environments. Test of the GL-stimulated stem-response among naturally occurring variants may help identify candidates that are not properly responding to GL. Studies performed in Chapter 4 present one of such candidate, Knox-10. The Knox-10 accession responded to different wavebands phenotypically similar to Col-0. However, it showed defective RL-induced hypocotyl-randomization-response (HRR) besides the reduced GL-response. Even in the dark-grown conditions, Knox-10 seedlings still exhibited less HRR than Col-0. These results indicate that the reduced HRR in Knox-10 is actually not specific to RL. RL illumination seems to better present the defect in Knox-10 accession. Studies on the transcriptional regulation of shade-induced marker genes further support such reasoning, because Knox-10 responded normally to the RL signal. The genetic variation causing this altered HRR response probably results from a gene that specifically controls upright plant growth. The whole genome sequencing of the bulked-F2-segregates is currently underway. Meanwhile, the transcriptional differences

among Knox-10, Col-0, and the F3 plants (Knox-10 phenotype in the HRR test) under dark and RL conditions are being generated through an RNA-seq method. When these two methods are combined, we might be able to identify the candidate genes that are responsible for the defective GL response and reduced HRR response.

The increased-hyponastic-growth in Knox-10 plants may be eco-physiologically significant. The elongated stem and petiole might increase the possibility that the emerging seedlings or juvenile plants would reach a better photosynthetic light condition as quickly as possible. In addition, the increased-hypocotyl-growth and normal phototropic response may compensate for the defective phy-mediated HRR if the latter affects the fitness of Knox-10 plants. The connections between the reduced HRR and the increased-hyponastic-growth in Knox-10 plants are of interest to investigate. However, the preliminary result of this test was inconclusive. All F2 plants showed Knox-10-like petiole angles, which are all smaller than that of Col-0 plants. Since F2 plants are in Knox-10 genetic background, a reciprocal cross has been made to test whether this is resulted from the effects of genetic background.

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BIOGRAPHICAL SKETCH

Yihai Wang grew up in Xinglong County 90 miles away from the Capital Beijing, China. Before came to the University of Florida for his doctorate study, he earned his bachelor's degree in Plant Pathology at Agricultural University of Hebei and master's degree in Transgenic Plant Biosafety at Chinese Academy of Agricultural Sciences. He joined the Plant Molecular and Cellular Biology program in 2009. Under the guidance of Dr. Kevin Folta in the Department of Horticultural Sciences, he conducted his research on GL photobiology, focusing on the interactions between GL and other photosensory systems, and the identification of potential GL signaling component from *Arabidopsis* natural variations. He received his Ph.D. from the University of Florida in the summer of 2013.