

GREEN LIGHT IN PHOTOMORPHOGENIC DEVELOPMENT

By

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To Elie and my Mom.

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LIST OF ABBREVIATIONS

LED	Light-emitting diodes
PAR	Photosynthetically active radiation
phy	Phytochrome
cry	Cryptochrome
nm	Nanometer
PPF	Photosynthetic photon flux
phot	Phototropin
LOV	Light, oxygen, voltage domain
FMN	Flavin mononucleotide
ABA	Absciscic acid
P _{FR}	Far-red-absorbing phytochrome
P _R	Red-absorbing phytochrome
FAD	Flavin adenine dinucleotide
R	Red light
B	Blue light
G	Green light
d	Days
h	Hours
P	P-value
A	Absorbance
g	Grams
Col	Columbia
<i>Ler</i>	<i>Landsberg erectus</i>
MS	Murashige and Skoog

HCl	Hydrochloric acid
mm	Millimeter
#	Number
diff	Differentiated
lvs	Leaves
obs.	Observation
cm	Centimeter
m	Meter
s	Second
C	Celsius
PCR	Polymerase chain reaction
min	Minute
KCl	Potassium Chloride
CaCl ₂	Calcium Chloride

Abstract of Dissertation Presented to the Graduate School
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GREEN LIGHT IN PHOTOMORPHOGENIC DEVELOPMENT

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Light quality, quantity, and duration provide essential environmental cues that shape plant growth and development. Over the last century, researchers have worked to discover how plants sense, integrate, and respond to red, blue, and far-red light. Green light is often considered a “benign” wavelength with little to no effect in plant development. However, sparse experiments in the literature demonstrate that green effects are often counterintuitive to normal light responses and oppose red- and blue-light-induced responses. Green light effects on plant growth and development are described here through the use of custom, tunable LED, light-emitting diode, chambers. These light sources allow for specific light qualities and quantities to be administered. The effects of green wavebands were assessed when red and blue photomorphogenic systems were active to answer the question: Are the effects of an inhibitor (green light) more evident in the presence of inducers (red and blue light)?

In seedlings, supplemental green light increased hypocotyl elongation opposite to classical inhibition of hypocotyl elongation associated with growth in light and induced by red and blue wavebands. Results indicate that added green light induced a reversion of light-grown phenotypes. In mature plants, supplemental green light induced phenotypes typical of the shade-avoidance syndrome, including elongated petioles, smaller leaf areas, and leaf hyponasty. These

responses are typical of lower-light conditions or far-red enriched environments. Contrary to far-red-light-induced shade-avoidance, data indicate green delays flowering. In *Arabidopsis* and strawberry plants, anthocyanin levels also decreased when green light was added to red and blue light treatments, which is again opposite to normal light-induced phenotypes. Photoreceptor mutants were tested and indicate green light effects in early development are cryptochrome-dependent. However, green-light-induced shade-avoidance responses were cryptochrome-independent. A candidate gene approach was used to identify other elements required for green light sensing and/or response. Defects in some green light responses were observed for mutants in *CCD8/Max4*, a putative carotenoid cleavage enzyme with high sequence similarity to a critical enzyme in animal vision. These data support a role for green light in plant development which opposes normal light-induced responses and indicate the existence of at least two green light sensing systems.

CHAPTER 1 INTRODUCTION

Light quality, quantity, and duration provide essential environmental cues that guide plant growth and development. The effects of red, far-red, and blue light have been well-characterized. Recent work demonstrates that green light, historically considered ineffective relative to potent far-red, red, and blue wavebands, plays an important role in seedling development which opposes normal photomorphogenic growth (red- and blue-light-induced responses). Green light effects are often subtle and examined under monochromatic green light regimes. These experiments were designed to test the effects of an apparent inhibitor (green light) in the presence of inducers (red and blue light) of typical light responses. Perhaps one reason that green light effects are under-represented in the literature is due to the fact that it is difficult to observe the effects of an inhibitor when forward-acting systems are inactive. For these experiments, light emitting diodes (LED), which provide narrow-bandwidth light, were used to test the effects of green light in the presence of red and blue light on the growth of *Arabidopsis thaliana* plants. Green-light-induced effects were observed under combinatorial light regimes in newly germinated seedlings as well as mature plants.

In early development, the addition of green light resulted in longer hypocotyls. These green light effects were induced by low fluence green light but inhibited as fluence rates of green increased. The responses were also cryptochrome-dependent. Mature plants grown under red and blue light (RB) treatments displayed characteristics typical of normal photomorphogenic growth, i.e. short overall plant stature, open and expanded leaf blades, and leaves oriented relatively parallel to soil. When green light was added to the red and blue light background (RB+G), total light quantity was increased, and plants displayed characteristics associated with shade-avoidance syndrome, i.e. increased petiole elongation, increased leaf angle, and decreased leaf

area. Here increased total fluence (RB+G compared to RB) caused phenotypes associated with lower-light phenotypes, and as green light was added to the red and blue light background these lower-light associated phenotypes became more apparent. Shade-avoidance responses are also induced by far-red light enrichment (or low ratios of red to far-red), low blue light, and low photosynthetically active radiation (PAR). There was very little to no far-red light (<.0001) present under these treatments. Also, blue light quantities were the same between treatments (RB and RB+G) and plants grown under RB exhibited characteristics typical of normal photomorphogenic growth. PAR was increased in RB+G treatments compared to RB, while plants grown under RB+G displayed characteristics associated with shade-avoidance responses and plants grown under RB did not. Therefore, the shade-avoidance phenotypes seen in plants grown under RB+G can only be attributable to green wavebands.

Although most green light effects in mature plants mimicked shade-avoidance responses, preliminary data show that supplemental green light delayed flowering. This delay of flowering is opposite to the promotion of flowering demonstrated by the shade-avoidance syndrome associated with low ratios of red to far-red wavelengths. These results indicate a point of divergence between far-red- and green-light-induced shade-avoidance syndromes and suggest the existence of a novel green sensing system. Support for this novel sensing system was that these phenotypes persisted in all photosensory mutants tested, including *phyA phyB*, *cry1 cry2*, and *phot1 phot2* mutants.

In addition to this, a further candidate gene approach was taken in which a gene with high sequence similarity to a visual cycle enzyme, *Ccd8*, was found to be required for green-light-induced down-regulation of plastid transcripts early in seedling development.

The aim of this project was to characterize the role of green light in plant development and to establish tools to elucidate components of the green light sensing pathway(s). Little is known about green light's role in plant development thus far, but experiments in the literature contribute to a common theme-- green light responses seem to work against or antagonize normal light-induced phenotypes, such as red- and blue-light-induced effects. The results support this theme and increase our understanding of how plants sense and respond to their light environment. In addition, this work demonstrates the effectiveness of using LED-based arrays and provides the tools necessary to expand this work to identify other green light pathway components. These tools can also be used to assess green light effects on other plant species. If green light negatively affects a quality of interest in a given species, manipulation of the light environment through colored mulch in the field or colored filters within greenhouses may lead to increased crop production and/or commercial plant production in greenhouses.

CHAPTER 2 LITERATURE REVIEW

Introduction

Over a century of photomorphogenic research describes how light plays an important role as an environmental signal in addition to its roles in photosynthesis. Experiments have identified and described the effects of red, blue, and far-red light signaling pathways. Although, new components are discovered, the complex pathways that transduce these light signals are generally well-defined. A less-studied, but equally important waveband imparting environmental information is green light. A complete story of how plants sense and respond to green wavebands, or even a full range of green-light-inducible responses is not yet available. However, this review of the literature describes and examines experiments that indicate a role for green light in shaping plant growth and development.

One general idea presented herein is that although red and blue light promote photomorphogenesis, green wavebands tend to have opposite effects and temper responses to red and blue light. A common theme in biology is that organisms employ opposing systems to tightly monitor, adjust, and constrain developmental programs, and a negative influence of a green light sensory system would be consistent with this idea. Examination of the literature surrounding light mediated plant developmental research reveals several reports that support this view. For the purposes of this literature review, “green light” is expanded to represent the green and yellow portions of the spectrum (500-600 nm). Green wavebands have been traditionally considered as developmentally inconsequential outside of their partial forward stimulation of red and blue light responses (phytochromes [phys] and cryptochromes [crys] can absorb green light, although inefficiently). However, there are many examples in the literature where monochromatic or

broadband green light treatments elicit effects on plant growth and development that do not conveniently fit with known light sensory systems.

The accepted dogma in light biology is that all wavebands of visible light promote photomorphogenesis. However, evidence indicates that wavebands in the far-red region of the electromagnetic spectrum counter typical light-induced plant growth in some contexts. Borthwick et al. (1952) illustrated how far-red wavebands, inefficient for photosynthesis, impart important environmental information. Generally, far-red light counters the developmental processes initiated by red light, and the ratio of red to far-red wavelengths dictate the activity of molecular, biochemical, and morphological processes (Quail, 2002; Devlin et al., 2003; Chen et al., 2004; Casal and Yanovsky, 2005). This is a prime example of how wavebands with little impact on plant metabolism can function as a cue for adjustment of plant form and composition. These modifications may conserve valuable resources and provide increased fitness to plants growing in lower-light environments. Green light is typically enriched in natural environments that are also enriched for far-red light, i.e. any environment that is covered by overhanging foliage such as the understory of a plant canopy or in a densely-packed field. Therefore, a logical hypothesis is that green light may induce similar responses as far-red light.

This chapter presents evidence that green light responses oppose those of red and blue light, and are at times similar to those of far-red wavebands. Although the green signaling components are largely unknown, this work demonstrates a role for green wavebands in plant growth and development. However, interpretation of classical work presents some difficulties for the following reasons: 1. Experiments were usually performed under broadband light conditions not exclusively emitting green light, 2. Some studies measured light in foot-candles or ergs, 3. Light treatments were not equalized across the spectrum, and/or 4. Researchers used

combinations of light treatments that obscured simple interpretation. However, some of these experiments did indicate that green wavebands cause a specific set of responses not easily attributable to red, far-red, or blue light and their receptors. The elucidation and characterization of genetic elements in *Arabidopsis thaliana* that transduce red, far-red, and blue signals and their corresponding mutants now provides tools that allow green light responses to be studied in isolation from other light sensory inputs. Many of the responses induced from the green light portion of the spectrum are counterintuitive, often opposing typical light effects (Klein et al., 1965; Ahmad et al., 1998; Frechilla et al., 2000; Talbott, 2002; Eisinger et al., 2003; Folta, 2004; Dhingra et al., 2006; Bouly et al., 2007).

There are many examples in natural environments where green light is enriched, thus emphasizing the importance of exploring the way in which plants sense and respond to these wavebands. For example, under the cover of leaves, whether in the understory of a plant canopy or the shade of neighbor plants in a densely-packed field, plants are subjected to a pronounced contrast to the unfiltered solar illumination. Primarily, there is a decrease in radiant flux and a shift in the ratio of visible to far-red wavelengths (Figure 2-1). Although red and blue wavebands are absorbed by overhanging foliage, far-red wavebands are transmitted through leaves and enriched in the understory. In addition to far-red enrichment, ratios of red and blue to green light shift towards green light, since green light is readily reflected from plants as well as being transmitted through them. Green light moves efficiently through the plant body, playing more of a role in photosynthesis than red or blue light in some contexts (Sun et al., 1998), suggesting that green light may prove useful as a signal to tissues not directly exposed to the light environment. Potential green light effects may also vary with developmental context, since an etiolated

seedling emerging from the soil has negligible chlorophyll and will allow green light to penetrate as efficiently as blue, red, and far-red light.

Phytochromes and Cryptochromes are Green Light Receptors

Phytochromes and cryptochromes absorb and respond to green light. This is likely a major contributor to the lack of exhaustive studies on green light. Although phytochromes are principally regarded as red/far-red reversible pigments, they absorb well in the blue portion of the spectrum and are extremely sensitive to all light qualities, especially in dark-grown seedlings where light labile phyA is abundant (Goto et al., 1993). In addition to being sensitive to all light qualities, these receptors will initiate responses to low levels of light. In Arabidopsis, green light stimulates germination effectively through phyA and phyB systems (Shinomura et al., 1996). Green light establishes an active phy pool and even the most miniscule “safelight” green light treatments activate robust plant responses (Mandoli and Briggs, 1981; Steinitz et al., 1985; Dhingra et al., 2006).

The cryptochromes regulate plant responses to blue and UV-A light (Lin, 2002; Spalding and Folta, 2005), and recent evidence indicates biological activity of a green-light-sensing state (Banerjee et al., 2007; Bouly et al., 2007). Malhotra et al., (1995) showed that these chromoproteins contain a flavin and a pterin as the light-excitabile moieties. The presence of both the flavin and the pterin indicated that the cryptochromes rely on intramolecular electron transfer as part of their signaling mechanism (Malhotra et al., 1995). Lin et al. (1995) overexpressed Arabidopsis CRY1 in transgenic tobacco. The overexpressors exhibited hypersensitivity not only to blue, but also to broadband green light, indicating that cryptochromes could direct stem growth inhibition even when stimulatory wavelengths were red-shifted. However, recent reports by Bouly et al. (2007) and Banerjee et al. (2007) show that the green-light-absorbing state of cry1 and cry2 reverses blue-light-induced responses. These findings are discussed in detail in the

final sections of this chapter. In addition, at least one additional light sensor system, or previously undefined aspect of a known system mediates specific effects of green wavebands. Here too, green responses tend to arrest or attenuate the physiological phenotypes associated with normal photomorphogenic progression. Therefore, green light responses can be characterized as those that are *cryptochrome dependent* and those that are *cryptochrome independent*.

Clearly, the phytochromes and cryptochromes can both function as sensitive green light photoreceptors in plants, but the efficiency of these systems in processing the green light signal is poor relative to their capacity to respond to red and blue wavebands. With this in mind, green light effects could be the result of low-level coaction between red and blue sensory systems, as outputs from minimal phy and cry activation may present what mistakenly appear to be green-light specific phenotypes. This possibility has likely led to deprioritization of research on green light signaling pathways. However, current researchers possess genetic and physiological tools that allow green light effects to be dissected from those induced by developmentally dominant wavelengths, red and blue. Other tools that make elucidation of green light responses and pathways possible are the availability of high power, narrow bandwidth light sources, access to double/triple photoreceptor mutants, and growth assays with great sensitivity. Together these tools have been used to characterize the often subtle effects of green illumination.

Early Green Light Effects on Vegetative Growth

In his book “Experimental Control of Plant Growth”, Frits Went (1957) examined the effects of light quality on seedling growth by assessing tomato (*Lycopersicon esculentum*) seedling dry weight. The results of these experiments have been reproduced in Figure 2-2 and illustrate that seedlings grown under red and blue light produce more vegetative tissue than those grown under the same fluence rate of white light (consisting primarily of red, blue, and green

wavebands). This result indicates that the added green light component has a negative effect on seedling growth in terms of dry weight; however, green light in these experiments was added at the expense of red and blue light. Since red and blue are known to promote photomorphogenic development (in this case, increased seedling dry weight), lower seedling mass for plants grown under white light is expected due to decreased red and blue light. However, further experimentation led to the discovery that as fluence rates increased, plants grown under white light acquired a given mass before reaching a plateau, whereas plants grown under conditions where green light was reduced achieved a higher dry weight at saturation. In other words, plants grown under reduced green light were able to reach greater dry weights before reaching a plateau, regardless of how much fluence rates were increased. This result indicates a negative role for green light in photomorphogenic development, illustrated in this case by the maximal vegetative growth that may be achieved.

Green Light Affects Organ Growth and Stature

Similar results were found by Klein and colleagues in the 1960's when they discovered a negative effect of green wavebands on tissue culture growth. In these experiments, tissue culture growth was examined under depleted and supplemental green light conditions. Taking Went's experiments one step further, the effects of various light qualities on growth inhibition were examined and Klein found that the most deleterious wavelengths peaked at 550 nm (Klein, 1964).

Although this work demonstrated a negative role for green light in tissue growth, Klein later extended this work to mature plants and observed additional effects of green light on marigold (*Tagetes erecta L.*), tomato, and impatiens (*Impatiens balsamina L.*) (Klein et al., 1965). For reduced green light treatments, lavender filters were used together with fluorescent and incandescent bulbs to effectively reduce the level of green light. For supplemental green

treatments, these wavelengths were added to a background of white light. Results were complementary to Went's data (1957) and indicated that, when grown under green-light-depleted conditions, marigold height, fresh weight, and dry weight increased 30-50% over full-spectrum treatment. The authors interpreted these data to mean that removal of green light enhanced plant growth. However, this interpretation is complicated by the fact that lavender filters effectively reduce blue light as well as green light. Therefore, enhanced growth seen under "reduced green light" may have resulted from reduced photosynthetically active radiation (PAR) (400-700 nm). Here, lower PAR may be responsible for taller plants since red and blue wavebands inhibit stem elongation. Total photosynthetic photon flux (PPF) was only recorded for white light sources, which were 1000 and 500 footcandles (approximately 200 and 100 $\mu\text{moles m}^{-2} \text{s}^{-1}$), and not under lavender filters. The lack of recorded PPF for all treatments leads to difficulties in interpretation of these data.

More definitive conclusions can be drawn from experiments in which green light was added to a background of white light. Under conditions of enhanced green light, plants were shorter and had lower ratios of dry to fresh weight. In these experiments supplemental green light treatments apparently represent greater fluence rates when compared to white light alone, since green light was added to the white light background. Data showed that increased fluence rates under white plus green light led to less vegetative growth than white light alone. These results are consistent with those of Went (1957) and recent studies by Dougher and Bugbee (2001).

Dougher and Bugbee (2001) compared the growth of lettuce (*Lactuca sativa*) seedlings under metal halide and high pressure sodium lamps. The authors were careful to control for phytochrome equilibrium, quantities of blue, red, and far-red light, and relative ratios between them. The only difference between treatments was the green light component (580- 600 nm).

Therefore, when data indicated differences in dry mass, leaf area, and chlorophyll content, meaningful conclusions could be drawn. Trends agreed with previously documented findings-- green light has an inhibitory effect on plant growth.

Green Light and Tropism

Blue light is known to induce plant movements such as the bending of some structures towards light and the relocation of chloroplasts, and the responsible photoreceptors are the phototropins (Briggs and Christie, 2002; Spalding and Folta, 2005). In addition to blue light, green also stimulates phototropic responses (Steinitz et al., 1985). Experiments demonstrated that green light would promote bending towards light but required more time and 10-fold greater fluence rates to generate an equivalent response to that of blue light (Steinitz et al., 1985). In addition to defining a role for green light in this response, the authors argued that green wavelengths were acting through the blue light receptor, which was later identified as phot1 (Steinitz et al., 1985). The green-light role was later verified when *phot1* mutants failed to demonstrate wild-type phototropic responses to green wavelengths (Liscum and Briggs, 1996). When an action spectrum was examined, green-light-induced phototropic responses were found to be more efficient as wavelengths approached the blue portion of the spectrum, i.e. shorter wavebands of green light were more effective in stimulating this response (Steinitz et al., 1985). Whether these data are the result of phot sensitivity or blue contamination of green light sources is unknown, but results indicate that phot1s are green light receptors in addition to crys and phys. Later analysis of photocycling by the oat phot1 LOV2 domain indicated that blue light activation drives formation of a green- and red-light-absorbing flavin mononucleotide (FMN) triplet state that is extremely transient (on the order of nanoseconds) (Swartz et al., 2001; Kennis et al., 2003). Due to their short-lived nature, it is unlikely that these species would play any kind of meaningful role in phototropin activity. The work by Steinitz et al. (1985) that elucidated an

action spectrum for green-light-induced phototropic bending utilized carefully-defined illumination conditions that combined narrow-bandpass filters with sharp cut-off filters that resulted in little to no transmission below 500 nm. Therefore, when data indicated considerable differences in dry mass, leaf area, and chlorophyll content, they could draw significant conclusions. Trends agreed with previously documented findings-green light has an inhibitory effect on plant growth.

In addition to a possible role in the bending of some plant parts towards light, green wavelengths have been implicated in root tropisms (Klein, 1979). In these experiments, root growth habits of curly cress (*Lepidium sativum*) were examined. Phytochromes are known inducers of diageotropic root growth. In curly cress, however, positive gravitropic responses are not phytochrome-dependent. This allows for examination of green light effects on root growth independent of phy involvement. Data indicated that green light slows geotropic root curvature. Examination of specific green wavelengths indicated that the response was most effective when 546 nm light was administered, whereas wavelengths shorter than 520 nm or greater than 580 nm had no effect on slowing normal root responses. These experiments identified the most effective wavebands for this green light response and strengthened the link between green wavebands and root tropic responses. Interestingly, this work also demonstrated a reversal of green-light-induced inhibition of root growth by other light qualities. Here, wavebands distributed at 620 nm were most effective at reversing green light effects, whereas irradiation near 660 nm (phytochrome's peak absorption) had no effect. In addition to red light responses, blue wavelength effects were observed, and demonstrated that blue did not reverse green light responses. These results are consistent with a cry-independent mechanism.

In addition to stem and root tropic responses, green wavelengths have been associated with leaf movements. Leaf inclination has been linked to phytochrome induction in environments with low ratios of red to far-red. Studies by Mullen et al. (2006) demonstrated that changes in leaf angle required phyA, B, and E. Interestingly, the green light component of white light induced the response in *hyl* and *phyA phyB phyE* mutants, indicating that although phyA, B, and E were required for low red: far-red induction of leaf inclination, they were not required for green-light-induced changes in leaf inclination. These experiments demonstrated that although green light induced changes in leaf position that were similar to those in low red: far-red environments, the wavebands operated via two separate light sensing systems. In addition to the *phy* mutants, *npq1* mutants were examined, since the latter are required for green-light-induced reversal of blue-induced stomatal opening (Frechilla et al., 2000; detailed below). The *npq1* mutants showed green-light-induced leaf inclination similar to that of wild type. Results indicated that NPQ1 was not required for this green light response and provided a precedent for the idea that there could be multiple green sensors involved in green light responses. In addition to *npq1*, the *npq2* mutants with lesions in a gene required for ABA synthesis were unable to induce the leaf-inclination response. Application of ABA restored normal leaf angles, so a change in ABA levels may be one result of green light responses.

Heliochrome

Work described in the previous section demonstrated that green light effects on leaf inclination mimicked those of shade environments with a low red to far-red ratios. Experiments by Tanada also examined interactions between green and far-red light. The results of this and later work led to the suggestion of a far-red/green light reversible receptor. The author later coined the term “heliochrome” since this appeared to act similarly to phytochrome. Initial experiments were based on the closing of *Albizia julibrissin* pinnules (Tanada, 1982). *Albizia*

julibrissin pinnules exhibit nyctinastic closure which is delayed by far-red light 710-730 nm. This delay, which requires substantial fluence rates ($18-43 \mu\text{mol m}^{-2} \text{s}^{-1}$) of far-red light was completely negated by co-illumination with dim green light ($0.01-5 \mu\text{mol m}^{-2} \text{s}^{-1}$). The pinnules were also examined in experiments similar to those of Borthwick's early work (1952) describing phy toggling after red and far-red light application. The pinnules demonstrated a similar toggling response to green and far-red light treatments, whereas red light had no effect (Tanada, 1982). These experiments indicated the existence of a green light/far-red reversible sensor.

In addition to green light, blue was found to stimulate reversal of the far-red-induced delay in pinnule opening (Tanada, 1984). Green light could completely negate the blue light effect. Tanada concluded that the far-red absorption state of heliochrome was sensitive to blue as well as green light.

A similar connection between far-red and green light was reported in *Brassica campestris* (Tanada, 1984). In this species far-red light induces prolific flowering when given as an end-of-day pulse. As in the pinnule experiments, green light (550 nm) given at low fluence rates was able to effectively reverse far-red (710 nm) induced flowering effects, whereas red light could not. Similar to pinnule opening, this response exhibited toggling effects like those observed for red, far-red toggling by Borthwick (1952).

Together these studies describe green light responses that actively reverse far-red-light-induced responses. In 1997, Tanada proposed that heliochrome was a heme-based receptor that toggled between far-red and green light sensing states. Tanada's work illustrates another example from the literature where green light effects cannot be easily reconciled with known photoreceptors and implies the existence of a novel green light sensor.

Green Light Opposes Stomatal Opening

Zeiger and colleagues (Frechilla et al., 2000) demonstrated that a brief pulse of green light could prevent blue-light-mediated stomatal opening in *Vicia faba* epidermal peels. Closer evaluation of this phenomenon revealed a blue-green reversible dichromaticity, as the quality of the last pulse of light dictated the physiological response observed. If green light was followed by blue, then stomates opened, whereas, if the pulsed light sequence was green, blue, green, the stomates remained closed. These researchers went on to show that the unusual stomatal response was dose-dependent with the most significant effect occurring at a ratio of 2:1 for green:blue (Talbot et al., 2002). An action spectrum revealed 540 nm light to be the most effective wavelength for reversal (Frechilla et al., 2000). In addition, this response persisted in a background of red light, indicating that the blue-green pathway was separate from previously described phytochrome or photosynthesis-driven pathways that influence stomatal aperture. Later Talbot et al. (2002) observed this blue-green effect in a diverse suite of plant species, demonstrating that the effect was evident throughout the plant kingdom.

The authors suggested that the molecular entity mediating the absorption and response to blue and green light may toggle between active and inactive states similar to the phytochrome P_{FR} and P_R states (Frechilla et al., 2000). One candidate put forth was a carotenoid, zeaxanthin, which was previously described as a candidate for blue-light-induced stomatal opening (Frechilla et al., 1999). The action spectrum for this particular response (green-light-induced stomatal closure) matched the absorption spectrum for a carotenoid, but was red-shifted by 50 nm. The results were confirmed by Eisinger et al. (2003) who showed that a green-light pulse could negate the effect of ultraviolet light on stomatal opening.

Later the phototropins were implicated in the control of stomatal aperture (Kinoshita et al., 2001). In combination with the studies by Zeiger's group, this finding suggested that the

phototropins may be blue-green reversible, yet analyses of the phototropin LOV (light, oxygen, voltage) domain did not support this hypothesis. Absorbance in the green was found to be highly transient (on the order of a few nanoseconds) (Swartz et al., 2001; Kennis et al., 2003) .

This discrepancy was addressed by Talbott et al. (2003) with a genetic study in *Arabidopsis* mutants. Mutants analyzed were *phot1 phot2* double mutants and *npq1* mutants, that had a lesion in violaxanthin de-epoxidase, thus minimizing zeaxanthin accumulation (Niyogi et al., 1998). Results revealed that NPQ1, but not *phot1* or *phot2*, was required for the blue-green stomatal response pathway. In this study, the authors examined various mutants together with red- and blue-light-induction of stomatal opening, and the potential far-red/green light reversibility of the responses. The *phot1 phot2* and *npq1* mutants behaved like wild type plants in terms of the red/far-red stomatal response. Further investigation demonstrated viable red/far-red and blue-green light reversibility for stomatal responses of *phot1 phot2* double mutants, however these mutants required higher fluence rates of light for blue-induction of stomatal opening. These data confirm that *phot1* and/or *phot2* regulate the blue light stomatal response; however they do not appear to be the photoreceptors mediating the blue-green light reversible facet of stomatal opening. Interestingly, *npq1* mutants lacked the capacity to respond to blue light, but maintained red-induction and far-red reversal. These tests demonstrated the existence of independent stomatal regulatory pathways, defined discrete roles for NPQ1, *phot1*, and *phot2* in the response, and further illustrated the role of NPQ1 in blue-green reversible guard cell action (Talbott et al., 2003).

Later work by this group showed that green light effects involved a circadian component that was most prevalent in the morning (Talbott et al., 2006). This study supports the involvement of NPQ1 in the blue-green stomatal pathway, since the *npq1* mutant did not respond

to blue or green light. However, *phot1 phot2* double mutants and wild type plants showed similar responses. This work, in conjunction with other analyses, indicated that the effects of green light were conditional and that the use of mutants and specific light treatments was required to delineate specific pathways. An additional report demonstrated a role for cryptochromes in guard cells, and indicates that this system was distinct from that mediating phot effects (Mao et al., 2005). Recent research shows that cryptochromes undergo toggling from active to inactive states depending on green and blue light (Bouly et al., 2007). Therefore, it will be important to test if cryptochromes are relevant to the blue-green reversibility of stomatal opening.

Green Light Effects on Leaf Growth and Stomatal Conductance

The effects of green light on stomatal opening noted by Zeiger's group were extended to whole plants by NASA scientists. Plant growth in artificial environments remains a key provision to long-term space colonization. Therefore, NASA scientists have explored the effects of combinatorial light conditions on plants. Many of these studies simply focused on the effects of narrow-bandwidth red and blue sources compared to conventional sources (Brown et al., 1995; Goins et al., 1997; Yorio et al., 2001). One concern emerged when plants were grown under certain light conditions. Plants appeared black or purple when grown under red and blue LEDs. This rendered it difficult for the potential crew to monitor plant growth and health, and also, miscolored plants were not as visually appealing (Kim et al., 2004).

With the goal of making plants appear green, NASA scientists assessed the effects of green light supplementation to a red and blue background. The result was that addition of this allegedly benign wavelength generated conspicuous effects. The experiments differed from those performed by Went and Klein in that in these experiments PPF was kept constant and the proportion of added green light was varied. This approach had the advantage of keeping metabolism static, yet the disadvantage of skewing activation of photosensory networks

contributing to developmental responses. These studies also used different species and developmental states relative to earlier studies. For this reason the results must be considered independently of the previously described work.

In these reports, the effects of combinatorial red, blue, and green (RB+G) light treatments on leaf growth and stomatal conductance in lettuce were compared to red and blue (RB) alone (Kim et al., 2004, 2004). Green light supplied by green fluorescent lamps was added to a background of red and blue LED light. There was very little (if any) far-red light in these experiments, which is important for discounting potential phytochrome interpretations. The authors discovered that lettuce plants grown in RB+G treatments produced larger, thinner leaves that had higher specific leaf area when compared to those grown with RB alone (Kim et al., 2004). Also, plants grown under RB treatments demonstrated higher stomatal conductance when compared to those under RB+G, with the lowest stomatal conductance reported in plants grown under green fluorescent lamps alone (Kim et al., 2004). Additionally, while stomatal conductance was greater under cool white fluorescent lights than in RB+G treatments, the dry mass of the plants was greater in the latter. This result implies that weaker stomatal conductance did not negatively affect carbon assimilation (Kim et al., 2004). Plant dry mass was greatest under RB+G treatments (where 24% of the spectrum was broadband green light) when compared to RB, the opposite of the effects noted by Went (1957; Figure 2-2). However, these results do agree with previous findings that plants grown in RB+G treatments had larger specific leaf areas than those grown under RB treatments (Kim et al., 2004). These experiments demonstrate that supplemental green light affects plant physiology in conditions where red and blue systems are saturated. It remains to be seen whether these effects are cry-dependent or cry-independent, as they were performed in species where photoreceptor mutants are not yet available.

Early Stem Elongation

Once blue-green reversibility was identified in plants, namely in stomatal aperture pathways, experiments to examine green light effects in early development were initiated (Parks et al., 2001). For these studies, hypocotyl elongation rate was examined in 2-d-old dark-grown seedlings. This organ represents a highly sensitive system in terms of light response. For example, previous work using high-resolution image capture techniques were employed with *Arabidopsis* mutants to identify discrete roles of phytochromes (Parks and Spalding, 1999), cryptochromes (Folta and Spalding, 2001) and phototropins (Folta et al., 2003) in acclimation to the early light environment.

In these experiments, dark-grown seedlings were exposed to a pulse of blue light (a strong inhibitor of hypocotyl elongation) followed by a pulse of green light. A characteristic phot response was observed in which seedlings exhibited a normal inhibition in their first-phase of growth (Folta and Spalding, 2001; Folta et al., 2003). However, within minutes, and only after receiving a green light pulse, seedling growth accelerated to 150% of the rate observed in darkness. The effect of green light was unlike any previously described, and was also opposite to the accepted dogma that light inhibits hypocotyl elongation and that growth rate is maximal in darkness. This unusual green-light-induced increase in stem elongation rate was subsequently examined in greater detail. Single, etiolated seedlings were tested for the elongation response to a brief green light pulse. Within minutes of a dim-green-safelight-quality light pulse, the dark grown seedling elongated faster than it had in complete darkness (Folta, 2004). The response was dose-dependent, obeyed the Bunsen-Roscoe Law of Reciprocity, and was observed in response to a pulse barely detectable by eye. Green-light-pulse-induced growth acceleration was transient, with growth rates declining after an hour to those exhibited by dark-grown seedlings. Interestingly, green-light-mediated growth induction persisted in *cry*, *phy*, *phot* and *npq1*

mutants, indicating either that the response was mediated by redundant function between known receptor classes or that it was initiated by a novel light sensor. The response also persisted in a background of dim red light, suggesting that phytochrome was not required for this response. These data indicate that these green light responses are not initiated by the major red and blue sensors, and do not require NPQ1, a zeaxanthin synthesis enzyme that regulates stomatal aperture (Frechilla et al., 1999). Similar studies of early seedling establishment attributed the long-term blue-green reversibility to cry receptors (Bouly et al., 2007) and will be discussed further below. Together these studies delineate cry-independent and cry-dependent mechanisms associated with stem elongation and acclimation to the light environment. They also provide more evidence of green wavebands acting in opposition to red and blue wavebands.

Green Light Down Regulates Plastid Transcript Accumulation

Stem elongation experiments suggest that green light imparts developmentally-influential information to seedlings; in addition it defines a time point at which to explore green light effects on the transcriptome of a developing seedling. Since stem elongation was induced by a brief pulse of green light and was well-established, if not complete, at 60 min (Folta 2004), gene expression was analyzed at this specific time point. Microarray experiments were performed in which 2-d-old dark-grown seedlings were exposed to a brief pulse of green light, or dark.

The data presented both anticipated and unexpected results (Dhingra et al., 2006). For instance, a suite of genes known to be controlled by phyA (including *Hy5*, *Pks1*, and *ELIP*) was induced, as though the plants were illuminated with red or far-red light (as in Tepperman et al., 2001). This result was predicted and also demonstrated the phytochrome system was responding correctly. Although these phytochrome-induced transcripts increased, a number of chloroplast transcripts (including several previously shown as light-inducible) sharply decreased after a pulse of green light. Examination of candidate transcripts showed that the response was rapid

(occurring within 15-30 min), sensitive (with a threshold $<10^1 \mu\text{mol m}^{-2}$) and obeyed the Bunsen-Roscoe Law of Reciprocity. Parallel effects of green light were observed in tobacco (*Nicotiana tabbacum*), indicating that this response was not confined to Arabidopsis (Dhingra et al., 2006). Similar to early green-light-induced stem elongation, green-light-induced down-regulation of plastid transcripts persisted in all photomorphogenic mutant backgrounds tested. This suggests that the green light signal is likely dependent upon a novel sensory system. Both of these green light responses (the increased stem elongation and the down-regulation of light-induced chloroplast transcripts) are consistent with previous work. Both of these responses suggest that green wavebands provide cues that indicate a suboptimal light environment for seedlings, and thus lead to a less advanced photomorphogenic habit. Responses to green light may allow seedlings to conserve valuable resources and direct their elongation beyond competing seedlings to regions of increased photosynthetic efficiency.

A Connection to Plant Biomass

Sommer and Franke (2006) presented evidence that the treatment of seeds with green lasers could lead to enhanced fresh weight of plants at the time of harvest. Previous work by these authors examined laser light effects in speeding wound healing (Sommer et al., 2001) and increasing cell vitality in animals (Sommer et al., 2002). They subsequently expanded their examination of high-fluence-rate light effects to plants, and found that dry carrot, radish, and cress seeds treated with a green light laser or intense green light LEDs produced plants with significantly greater biomass than control plants (no light pretreatment). All plants shared equal conditions after the seeds were irradiated. All seedlings emerged at the same time, so the differences observed later could not be simply attributed to phytochrome-induced enhancement of germination in laser-treated seeds. Mature radishes and carrots from laser treated seeds were twice as large as controls (Sommer and Franke, 2006).

While interesting, statistical rigor was thin in these experiments, and the authors also did not account for the possible explanation that phytochrome would have been activated by their treatment, and this could have led to an advanced developmental state of the irradiated seedlings. With a resultant head start, seedlings might establish faster and more completely than their non-irradiated siblings. However, follow up experiments compared red to green light laser treatment of *Arabidopsis* seeds. Green light treated seeds germinated and emerged later than red treated seeds, yet still exhibited a larger end-point root phenotype, suggesting that the pre-illumination did not drive a phy-induced enhancement of early development (A. Sommer communication with K.Folta).

Cryptochrome-Dependent Green light Effects

Cryptochromes have been implicated in green-light-induced responses. To understand cry involvement in these responses, one must first understand cry structure. In 1995 two reports described association between cry1 and two potential chromophores, flavin adenine dinucleotide (FAD) and a second chromophore, 5,10-methylenyl tetrahydrofolate (Lin et al., 1995; Malhotra et al., 1995). Studies in plants and fungi since the late 1960's suggested that flavoreceptor signaling would involve changes in the redox state of the chromophore. This possibility has since been confirmed, since a significant amount of cryptochrome produced in insect cells was found to exist in the semi-reduced form (Lin et al., 1995). Biological consequences of the flavosemiquinone have been identified in *Phycomyces* (Galland and Tolle, 2003) and *Arabidopsis* (Bouly et al., 2007). Figure 2-3 depicts a model of the redox states of the flavin chromophore and how they relate to photosensor activity. Dark-grown plants contain the fully oxidized chromophore (FAD) that upon blue irradiation will convert to a semi-reduced (FADH[•]) or fully reduced (FADH⁻) chromophore. The semi-reduced form is the biologically active, yet green light absorbing. Addition of green light drives full reduction and inactivation (Bouly et al.,

2007). These chromophores likely undergo toggling similar to red/far-red toggling observed by Borthwick (1952). The degree of biological activity is determined by the relative quanta of blue and green light, and resulting pools of semi-reduced, fully-reduced, and fully-oxidized chromoproteins.

This theoretical model is supported by new biological evidence. Studies of cryptochromes in insect cells demonstrated that the initial light reactions in cryptochrome signaling depend on electron transfer from conserved tryptophan or tyrosine residues to reduce a flavin chromophore (Giovani et al., 2003). This is as predicted by models derived from photolyases (except that the latter possess the reduced flavin as a chromophore). The action spectrum for cry1-mediated inhibition of hypocotyl elongation shows a peak at 450 nm (Ahmad et al., 2002), consistent with the oxidized flavin chromophore when the receptor is catalytically active. Bouly et al. (2007) show that green light (563 nm) can reverse the effect of blue light on hypocotyl elongation in developing seedlings, consistent with previous reports of green light reversal of blue and red irradiation (Figure 8; Folta, 2004). Bouly et al. (2007) also show that this effect is cry dependent, since green-light-induced stem elongation is not present in cry double mutants, supporting the green-blue reversibility model. The cry2 receptor has been shown to be light labile (Ahmad et al., 1998). Bouly et al. (2007) tested CRY2 accumulation in response to pulses of blue light, and blue light followed by finite pulses of green light. The results show that blue light degradation of CRY2 can be reversed by a short, single pulse of green light, suggesting that the semi-reduced, active state is transient and subject to adjustment by green wavelengths. These data provide evidence of a dichromatic modulation of cryptochrome activity.

Since cry2 has a profound effect on controlling flowering time (Guo et al., 1998; Valverde et al., 2004), effects of green wavebands on flowering were observed in *Arabidopsis*

plants grown under short day (non-inductive) conditions followed by transfer to blue, green, or simultaneous green and blue light conditions (Banerjee et al., 2007). Plants transferred to blue light flowered earlier than those remaining in white light. However, when plants were moved to blue and green light conditions, the addition of green light negated the blue light effect. Green light inhibition of flowering agrees well with earlier studies where removal of green light enhanced flowering in marigold (Klein et al., 1965), and addition of green light suppressed flowering in carnation and lettuce (Vince et al., 1964). In a mechanistic context, the levels of FT transcript were shown to only accumulate in blue light conditions, again illustrating the antagonistic effects of green light (Banerjee et al., 2007). It will be interesting to see how CONSTANS localization and stability are affected by green wavebands, as this central regulator is strongly dependent upon stabilization imparted through cry2 (Mockler et al., 1999; Valverde et al., 2004).

The uncovering of a green-light-induced cry sensing state presents many interesting questions. First, is the effect of green light simply that of negating blue light responses, or is the green light cry state involved in stimulating discrete responses/ interactions with other light response pathways? Second, how do green wavebands affect other known cry responses?

Is The Photosensor Class Complete?

Clearly green wavebands play a role in regulating plant growth and development, and at least a portion of the responses are dependent on cryptochromes. The extent that blue-green cryptochrome relations affect plant growth and development are of interest. However, clearly there is evidence of green light responses that are cry-independent. There are several logical candidates for such green light responses, as well as the possibility that green wavelengths are detected by a sensor which has yet to be discovered. Zeiger and colleagues have proposed that zeaxanthin, a carotenoid required for the blue-green reversible stomatal regulation, toggles

between an “active” and “inactive” state depending on green and blue light levels (Frechilla et al., 2000).

Other intuitive candidates provide a basis for further investigation. An abundant flavoprotein was isolated from the membranes of *Cucurbita pepo* and *Phycomyces* (Hertel, 2005). The protein was later shown to have homology with type-1 aquaporins (Lorenz et al., 2003). *In vitro* analyses indicated that the protein binds flavin and that the binding can be reversed with chemical or blue light treatment. Although the *in vitro* work by Lorenz et al. (2003) presents no evidence of light effects above 500 nm, the protein remains an interesting candidate as a green light receptor *in vivo*. Such a protein would be a logical candidate for green-light-induced stem elongation (Folta, 2004), since such robust short-term growth accelerations would likely require a rapid change in turgor.

CRY3 (or CRY-DASH) is another flavoprotein with high local sequence similarity to cryptochrome photoreceptors and photolyases. Recently this protein has been shown to act as a photolyase specific for single-stranded DNA (Selby and Sancar, 2006), suggesting that it would not likely be functioning as the green light photosensor for orphan responses. However, CRY-DASH localizes to the chloroplast (Kleine et al., 2003), and the CRY1 protein from *Vibrio cholerae* binds RNA. Since at least one of the green light responses appears to require RNA metabolism (Dhingra et al., 2006), Cry3 genes are good candidates for roles in green light plastid transcript responses. In addition, cry3 is the only photoreceptor that is up-regulated in green light (Dhingra et al., 2006; supplemental data).

The existence of other sensors is implied by data in the literature. GCR1 (G-protein coupled receptor 1) in *Arabidopsis thaliana* has the strongest homology to the major photoreceptor of animal vision in terms of structural topology (Colucci et al., 2002). GCR1 has

been found to interact with the alpha subunit of the heterotrimeric G-protein in plants (Pandey and Assmann, 2004), and recently it has been shown to be required for blue-low-fluence-mediated *Lhcb* induction in etiolated seedlings (Warpeha et al., 2007). G-proteins have been pharmacologically (Neuhaus et al., 1993) and functionally (Okamoto et al., 2001) linked to light responses. Since mutants in these components are readily available they could be tested in the suite of green light assays. Studies of potential chromophores for plant light receptors identified *all-trans* retinal in tomato extracts (Lorenzi et al., 1994), the same isomer later shown to be most active in fungal and microbial opsins. Other evidence identifies retinal-binding proteins in plants and implicates retinoids in blue-light regulation of stomatal aperture (Paolicchi et al., 2005). Another potential contributor to green-light-induced responses is heliochrome, a hypothetical far-red/green light reversible heme-based receptor (Tanada, 1997), which has yet to be identified.

Since green-light associated phenotypes are subtle and often require specialized equipment to visualize, large genetic screens are not practical for identifying potential mutants. Yet, based on the literature, the loss of a non-cryptochrome green light sensor would be predicted to exhibit a light-dependent, hyper-photomorphogenic phenotype. Such mutants were isolated by Pepper et al. (2001) in mutant screens conducted under low fluence rate white and green light. Complete characterization has not been reported. It is likely that components of the green light sensing system have been isolated from the many screens performed under red, blue, or white light. In support of this notion, many photomorphogenic signaling mutants have been well studied yet do not fit conveniently into cry or phy pathways. These mutants represent an excellent starting point for further tests, now that several cry-independent green light assays have been defined.

Conclusions

Recent findings describing cry-dependent and cry-independent responses add to evidence that green light provides developmentally-influential information to plants. For the most part, recent work agrees with previous findings indicating a negative role for green light in photomorphogenesis and strengthens the argument that these wavebands impart responses atypical for red or blue light. In this way, green light may be functioning in a manner similar to far-red light, serving as a cue for suboptimal conditions for photosynthetic activity. This possibility is supported biologically since green and far-red enriched environments are similar in nature. Many examples exist in biology in which multiple systems oppose each other in order to fine tune responses and conserve valuable resources. In this sense, green wavebands may lead to conservation of valuable resources and extension into regions of more optimal light. Similar to the literature reviewed here, work in the following chapters describes roles for green light which are atypical to other light-mediated responses.

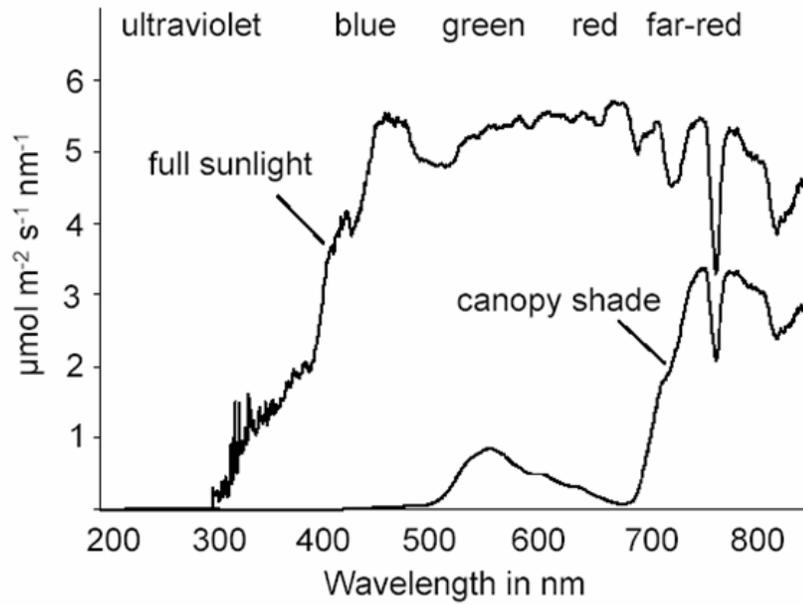


Figure 2-1. Quantum energy distribution of full sunlight and under the shade of leaves (canopy shade). Light conditions were measured at noon in mid-April in Gainesville, FL (29.67° N), using a StellarNet spectroradiometer (From Folta and Maruhnich, 2007).

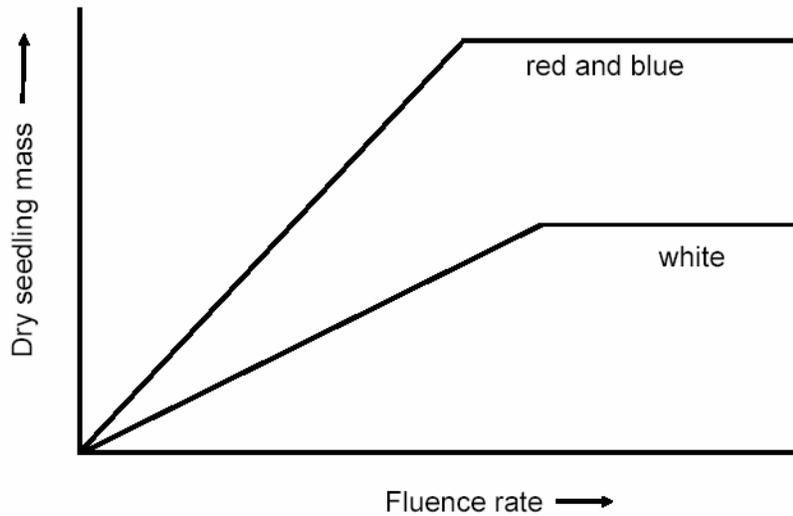


Figure 2-2. Data reproduced from Went's green-depletion experiments in 1957. Tomato seedlings were grown under white light or lavender filters (lower green light) and the dry mass of seedlings was measured after six days. The data show that at low fluence rates, red and blue light were more efficient than white light (red, blue, green) in promoting vegetative growth, as expected. However, at higher fluence rates the white light grown plants achieved a lower biomass than those grown in green-light-depleted conditions, even when light intensity was high. The author interpreted these findings as evidence for inhibition of growth by green light. (Figure adapted from F.Went, *Experimental Control of Plant Growth*, 1957).

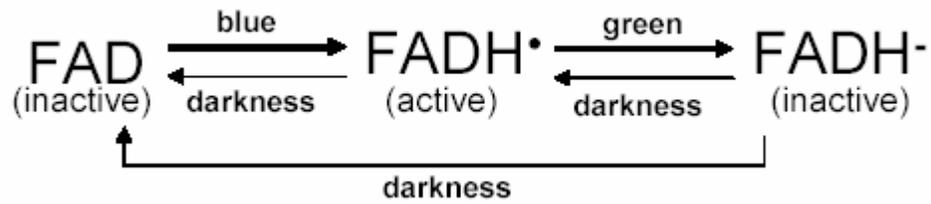


Figure 2-3. The proposed photocycle for plant cryptochromes. In darkness the cryptochrome flavin chromophore exists in the oxidized form (FAD), rendering the photoreceptor inactive, yet stable, thus facilitating its accumulation to high levels. Stimulation by blue light drives reduction of FAD to FADH[•], the active signaling state (and green light absorbing state) of the receptor. Illumination with green light further reduces the chromophore to its fully-reduced form (FADH⁻) that inactivates the receptor. Dark reversion returns the flavin from all reduced forms to the oxidized, blue-absorbing state. (Figure adapted from Bouly et al., 2007).

CHAPTER 3 GREEN LIGHT EFFECTS ON EARLY DEVELOPMENT

Introduction

The seedling undergoes a marked shift in developmental program as it transitions from growth in darkness to growth in light. Many environmental stimuli are integrated by plant systems and morphogenesis is altered to maximize survival and fitness. One of the most critical environmental cues is light, since light quality, quantity, and duration clearly affect seedling growth and development. In addition, light signals the seedling of time, place, and proximity to neighbors. Different wavebands drive suites of morphological, physiological, biochemical, and molecular changes that prepare the juvenile plant for optimal growth in its changing light environment. For young seedlings, the process of photomorphogenesis is typified by expansion of the cotyledons, opening of the apical hook, and induction of transcripts associated with light-induced development. One of the most conspicuous changes is an alteration of stem growth rate. The rate is rapid in darkness, yet is quickly and robustly repressed when exposed to specific light qualities.

Inhibition of hypocotyl growth rate depends on several separate photosensory systems that orchestrate downstream events with precision. Growth rate is strongly suppressed by exposure to continuous UV, blue, red, or far-red light (Parks and Spalding, 1999, reviewed by Chen et al. 2004; Suesslin and Frohnmeyer 2003). In red light, stem growth is inhibited by phyA for the first 3 h, followed by the influence of phyB (Parks and Spalding, 1999). Blue light controls stem growth through phot1 for the first 30 min of irradiation, followed by inhibition mediated by cry1, cry2, and phyA (Folta and Spalding, 2001a). This second phase is antagonized by phyB (Folta and Spalding, 2001b). It is not surprising that red and blue wavelengths have profound effects in stem elongation since these regulate photosynthetic rate in the developing seedling. The close

relationship between development and metabolism aids adjustment of the newly emerged seedling to ambient conditions, through changes in gene expression that ultimately optimize body plan.

While all other wavebands in the visible and flanking spectrum inhibit early stem elongation, monochromatic green light induces a robust, yet transient increase in stem growth rate (Folta, 2004). Green light induction of stem growth challenges the currently-accepted model that etiolated seedlings exhibit the most rapid rate of elongation and that added light stimulates inhibition of hypocotyl elongation. Light added as green wavebands leads to a program more reminiscent of a skotomorphogenic growth. This acute green light response is not affected by mutation of known photoreceptors or co-irradiation with dim-red light, suggesting it is mediated by a novel sensory system (Folta, 2004). Another response linking green light to programs conflicting with red and blue growth is chloroplast transcript accumulation (Dhingra et al., 2006). This work clearly demonstrates that green light induces a decrease in specific plastid transcripts which opposes their red and blue induction (Dhingra et al., 2006). In these experiments, a short, pulse of green light, like that in the above studies, was found to decrease certain chloroplast transcripts (such as *psaA* and *psbD*) that are central parts of photosystems I and II. This effect of green light agrees with periodic reports during the last 50 years that proposed a negative role for green light during de-etiolation (see Chapter 2 for references). Regulation of chloroplast transcript accumulation in this manner could have a selective advantage for resource conservation, since green light is much less effective at stimulating photosynthesis when compared to red and blue light and is often enriched in environments with low light or photosynthetically active radiation (PAR). It follows that in this and in stem

elongation, green light (or a high green to blue or red ratio) may signal a suboptimal light environment.

Monochromatic light treatments have allowed characterization of specific light sensing systems and their contributions to plant growth and development. Combinatorial light treatments are less prevalent in the literature. However, coaction between blue and red sensing systems has been examined (Fuglevand et al., 1996; Ahmad and Cashmore, 1997; Guo et al., 1998; Neff and Chory, 1998; Guo et al., 2001) and tests of red, blue, and/or green light supplementation in the growth of specific crop plants has been presented (Kim et al., 2004, 2004). Similarly, recent work by Bouly et al. examines the effects of combinatorial light conditions on *Arabidopsis* seedlings using broadband white light supplemented with green LED sources (2007). Since green wavebands influence early events in stem growth (within 15 min) and also influence long-term growth (days), it is important to test the interaction and crosstalk between light sensing systems.

Given that monochromatic green light has been shown to induce effects that oppose normal photomorphogenic growth, experiments in this chapter were devoted to assessing green light effects in the presence of red and blue light during early seedling growth. Based on influence of monochromatic green light in stem and chloroplast transcript regulation, our hypothesis was that green light would negatively affect photomorphogenic progression in early seedling development. Seedlings were grown under narrow-bandwidth, red, blue, and green light-emitting diodes (LEDs) that allowed precise control of light quality and quantity, thus decreasing the extraneous wavebands present in broadband light sources. Therefore, the phenotypes observed can be more tightly correlated with each of the wavebands.

Results

Hypocotyl Elongation

Initial experiments demonstrated that supplemental green light (G) added to a background of red and blue light (RB) increased hypocotyl elongation, increased lateral root number, and slightly delayed true leaf development relative to RB controls (hypocotyl data: Figure 3-1; $P < 0.0001$). The first experiments utilized conditions similar to those reported by Folta (2004), where administration of low-fluence rate light demonstrated a role for monochromatic green light in stem elongation. RB+G treatments had a higher fluence rate (an additional $1 \mu\text{mol m}^{-2} \text{s}^{-1}$) relative to RB, yet generated longer hypocotyls (a phenotype associated with lower-light or dark conditions). Therefore, it was unlikely that the resulting phenotype was due to increased light in RB+G treatments. To test this possibility, plants were grown under either supplemental green (G), red (R), or blue (B) light added to a background of RB (Figure 3-1). For these experiments RB was set to $R = 2 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $B = 2 \mu\text{mol m}^{-2} \text{s}^{-1}$ and +G/+B/+R added at $1 \mu\text{mol m}^{-2} \text{s}^{-1}$. Hence, the total photosynthetic photon flux (PPF) was $4 \mu\text{mol m}^{-2} \text{s}^{-1}$ for RB and $5 \mu\text{mol m}^{-2} \text{s}^{-1}$ for RB+G, RB+B, and RB+R treatments. Plants were measured after 10 d of treatment. Although hypocotyl growth is typically measured earlier in development (2-6 d) the 10-d point allowed for appraisal of the low-fluence phenotype at its most significant phase. After 10 d of treatment, plants grown under RB+B had shorter hypocotyls than those grown in RB, and hypocotyls of plants grown in RB+R conditions were the same or shorter than those from RB treatments. These results indicate that hypocotyl elongation observed in plants grown under RB+G was caused by green wavebands and not increased light when compared to those under RB. These experiments also indicated that the increased lateral root phenotype observed in RB+G was due to increased light, or photosynthetically active radiation (PAR), rather than to added green light specifically, since this phenotype was also observed under RB+B and RB+R treatments.

Green-Light-Induced Hypocotyl Elongation is Dose-Dependent

To determine the photophysiological parameters of the green light response, fluence rate/response experiments were carried out where increasing amounts of green light were added to a background of RB that equaled the $4 \mu\text{mol m}^{-2} \text{s}^{-1}$ delivered for experiments shown in Figure 3-1. Increasing amounts of green light were administered as 0, 1, 10, and $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure 3-2). Data in Figure 3-2 demonstrate that although small amounts of green light ($1 \mu\text{mol m}^{-2} \text{s}^{-1}$) added to a background of RB promote hypocotyl elongation, quantities of 10 and $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ inhibit elongation. As was the case for monochromatic green, green light added in quantities which exceeded a given fluence rate (between 1 and $10 \mu\text{mol m}^{-2} \text{s}^{-1}$) inhibited hypocotyl elongation, most likely due to green light stimulation of the phytochrome and cryptochrome photosystems.

Other Observations

In addition to effects on hypocotyl elongation, leaf development in seedlings grown under supplemental green light appeared delayed. Leaf parameters observed were: petiole length of the first leaf, and anthocyanin content and chlorophyll content. Petiole length of the first leaf was measured under all light conditions (RB, RB+G, RB+R, and RB+B) (data not shown). In RB+R and RB+B conditions, the petiole lengths of the first leaves were predictable and consistent between experiments. However, RB+G effects were variable between experimental replicates. In some instances, significant differences were observed, but in others, variability was evident that could not be attributed to the experimental treatment. Therefore, no consistent effect of green light on petiole length was apparent in the present studies. Although leaf area was not measured for these treatments, the leaf area of seedlings grown under supplemental green light had visually smaller overall leaf area than those grown under RB+B and RB+R treatments. The largest leaves

formed under RB+B conditions, followed by smaller ones from the RB+R treatment, and the smallest leaves in RB and RB+G environments.

Anthocyanin and Chlorophyll Levels

Since supplemental green light decreases anthocyanin levels in mature plants and in seedlings (mature plants: Chapter 4 of this work, seedlings: Bouly et al., 2007), anthocyanin levels were examined in these experimental conditions. Chlorophyll content was also examined since leaves under +G treatments appeared lighter green than those from +R and +B conditions. Both metrics were quantified after 10 days treatment under conditions that stimulated stem elongation. In Figure 3-3, data from these experiments is expressed as absorbance at a given wavelength (530 nm for anthocyanins and 657 nm for chlorophyll) per gram fresh weight. No discernable trends emerged from anthocyanin or chlorophyll assays at this developmental time point and at these fluence rates.

Since green light inhibits anthocyanin accumulation in mature plants (Chapter 4 this work) and seedlings (Bouly et al., 2007), and no discernable differences were detected in the data presented in Figure 3-3, it was important to examine the effect of higher fluence rates of green light. The rationale was that the quantum efficiency of cry absorption decreases as the wavelength becomes red-shifted, so higher fluence rates may be required to induce cry reversal. While anthocyanin levels were similar under RB+G ($G = 10 \mu\text{mol m}^{-2} \text{s}^{-1}$) treatments relative to RB+G ($1 \mu\text{mol m}^{-2} \text{s}^{-1}$) and RB, anthocyanin levels were greater under RB+G ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$) treatments.

Photoreceptor Mutants

Green-light-induced hypocotyl elongation was examined in known photoreceptor mutants. The *cry1 cry2* and *phyA phyB* mutants were grown under the established conditions where green light effects were conspicuous. Figure 3-5 depicts the results of these experiments.

Under all treatments, the photoreceptor mutants exhibited longer hypocotyls than did wild type seedlings. This was expected, since these mutants lack the red light and blue light photoreceptors that inhibit hypocotyl elongation. Therefore, RB treatments were set to a value of 1 so that growth patterns could be better compared to wild type trends. First, *cry1 cry2* double mutants were examined. There was no significant difference in hypocotyl length detected for *cry1 cry2* mutants after 10 d under any of the conditions tested. These data are consistent with cryptochromes mediating green-light-induced elongation, similar to experiments by Bouly et al. (2007).

In addition to testing the major sensors involved in blue-induced inhibition of hypocotyl elongation, effects of the major contributors to red-induced inhibition (phyA and phyB) were examined (Figure 3-5). These mutants responded as wild type to green-light-induced hypocotyl elongation. These data indicate that neither phyA nor phyB are required for green-light-induced stem elongation.

Discussion

Green light antagonizes normal photomorphogenic growth when delivered to an etiolated seedling in the presence of red and blue light. Similar to monochromatic green light studies, low fluence rates of green light added to a red and blue background promoted hypocotyl elongation, whereas higher fluence rates inhibited elongation. Previously identified green light effects in seedling development are subtle and detected only with minute-to-minute high-resolution imaging equipment (Folta, 2004), or by analysis of specific gene expression (Dhingra et al., 2006). However, in this report we present more prominent, visually clear evidence that green light opposes the normal progression of light-mediated development. These findings expand and further support previously published data that seedlings grow taller when supplemented with green light (Bouly et al., 2007; Folta, 2004). Collectively, these findings constitute an exception

to the accepted dogma that hypocotyl elongation rate is proportionately limited by higher fluence rates of light.

Although green light has been found to decrease anthocyanin levels in seedlings (Bouly et al., 2007) and in mature plants (Chapter 4 of this work), green-light-induced down-regulation of anthocyanins was not observed under these conditions. These differences in green light responses may be due to variations in developmental stages. However, work done by Bouly et al. (2007) demonstrated that green light down-regulated anthocyanin accumulation in early seedling development, which is consistent with mature plant studies (Chapter 4). This suggests that the lack of green-light-induced down-regulation in these experiments is more likely due to fluence rates insufficient to stimulate anthocyanin production in the first place. Experiments in Bouly et al. (2007) and Chapter 4 of this work observed these effects under greater fluence rates compared to work described within this chapter. Here, supplemental green (blue and red) was added as $1 \mu\text{mol m}^{-2} \text{s}^{-1}$ to a background of $4 \text{ total } \mu\text{mol m}^{-2} \text{s}^{-1}$ RB. These treatments represent very low fluence rates of light. Lack of green-light-induced down-regulation may be the result of insufficient up-regulation of anthocyanins.

However, green light added at a level of $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ to a background of $4 \mu\text{mol m}^{-2} \text{s}^{-1}$ RB induced an increase in anthocyanin accumulation. As in stem elongation, this quantity of green light likely stimulates crys and phys, both of which are known to stimulate anthocyanin accumulation. Whether or not this increase in anthocyanin accumulation in plants grown under $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ green light (G) is directly linked to green wavebands can be addressed by examining anthocyanin levels under similar fluence rates of supplemental red and blue. Interestingly, Bouly et al. (2007) found that green light down-regulated anthocyanin accumulation under conditions supplemented with green light (20). However, experiments by

Bouly et al. (2007) also utilized greater background fluence rates than used in the present experiments. This may explain differences in observed phenotypes. Under conditions described within this chapter, increased anthocyanin accumulation under RBG (20) may be the result of increased leaf development when comparing RBG (20) to all other treatments. RBG (20) treatments also accumulated more chlorophyll than RB, RBG (1), and RBG (10). This may also be related to increased leaf development in RBG (20) when compared to other treatments.

After examination of green light effects in early development, photoreceptor mutants were tested under the conditions where green-dependent effects were observed. Although green-light-induced elongation is opposite to red and blue inhibition, *crys* and *phys* absorb green light and *crys* have been implicated in green-light-induced stem elongation in early development (Bouly et al., 2007). Wild type promotion of hypocotyl elongation by supplemental green light was not observed in *cry1 cry2* mutants indicating that *crys* are required for green-light-mediated stem elongation. An argument can also be made that *cry* mutants are maximally elongated and cannot further increase their length under supplemental green light because mutants under all conditions, even +R (where *phy* inhibition is present), exhibited equally elongated hypocotyls. However, red-induced inhibition is not robust at these fluence rates. In addition, other mutants (such as *phyA phyB* mutants) were able to expand beyond this length (Figure 3-5), indicating that *crys* are physically able to expand and the lack of increased elongation under supplemental green light results from loss of *cry*-mediated, green-light-induced responses. To further clarify this issue, *cry* double mutants can be observed under increased RB backgrounds in order to stimulate *phy*-mediated inhibition of elongation.

In addition, phy mutants were examined. The *phyA phyB* mutants demonstrated wild type trends, i.e. supplemental green light induced hypocotyl elongation. These data imply that neither *phyA* nor *phyB* are required for this green light response.

Experiments in this chapter provide evidence of a role for green light in early stem elongation which opposes forward photomorphogenic progression, and the effects of red and blue light. These experiments reinforce previous work, which showed monochromatic green-light-induction of stem elongation (Folta, 2004). This work also supports recent findings that *crys* are required for green-light-induced stem elongation during seedling development (Bouly et al., 2007).

Materials and Methods

Plant Materials

Arabidopsis thaliana genotypes tested are identical to those previously assessed for responses to blue (Folta and Spalding, 2001, 2001) and green light (Folta, 2004) light; *cry1-304 cry2-1* mutants (in the Col-0 background), *phyA201 phyB-5* mutant (in the *Ler* background), and Col-0. Wild type references within the text refer to Col-0 ecotypes.

Light Sources and Treatments

Actinic light treatments were generated using narrow-bandwidth LED light supplied by American Bright LEDs driven by custom electronics as described (Folta et al., 2005). Fluence rate was further attenuated with neutral density filters (layers of M-209 and/or M-211, Cinemills Corporation, Burbank, CA) when needed. The emission spectrum of all light sources is viewable on-line at www.arabidopsisthaliana.com/lightsources. Light composition was assessed using an Apogee spectroradiometer and Spectra Whiz software.

Hypocotyl Elongation Assays

Arabidopsis seeds were sterilized using the following protocol: seeds were immersed in 0.01% Tween 20, shaken for 15 minutes, at which point liquid was evacuated and 50% bleach added plus shaking for 10 minutes, rinsed several times (2-3) with sterile diH₂O, quickly washed with 70% ethanol, and then rinsed 8-9 times with sterile diH₂O and dried in the laminar flow hood. Once dry, seeds were sprinkled on media containing 10 ml/ L macronutrients for MS media, 500 µl/L iron for MS media, 0.50 g/L MES, 1% sucrose, and 1% Phytoagar, with a pH of 5.8. The plates were covered in foil, stratified for 48-72 h at 4°C, and then the seeds were treated with 5 min to 1 h of fluorescent white light (~16 µmol m⁻² s⁻¹) depending on genotype requirements (i.e. Col 5 min, *cry1 cry2* mutants 40 min, *phyA phyB* mutants requires longer treatments--1 h in order to effectively stimulate germination). Seedlings were grown in absolute darkness at 23°C for approximately 40 h, and then germinated seedlings of similar size were transferred to square Petri plates containing previously described media under dim white light (<1 µmol m⁻² s⁻¹). Exposure to white light prior to transfer to experimental conditions did not affect the experimental outcome (not shown). Uniform etiolated seedlings were distributed across the plate. The seedlings were imaged with an 8.0 megapixel digital camera with an appropriate size standard (time 0), and then were moved to experimental light conditions. Seedlings were imaged at various intervals within a 10 day period.

For measurement of stem elongation, plates were scanned after 10 days of treatment and images were imported into ImageTool 3.0 software where they were measured relative to a size standard. The length of hypocotyls in pixels was determined using ImageTool software on enlarged images with 0.01 mm resolution. Pixels were converted to linear measurement using size standards. Seedlings height was graphed in Excel.

Anthocyanin and Chlorophyll Levels

Aerial portions of seedlings were harvested after 10 d of treatment, weighed and either moved to -80°C for storage or soaked directly in 100% methanol: 1% HCl, vortexed, and placed at 4°C in the dark (wrapped in foil) overnight. The next morning samples were vortexed, centrifuged quickly, and absorbance was measured at 530 nm (for anthocyanin) and 657 nm (for chlorophyll). Quantities were then expressed as the total absorbance at a given wavelength per fresh weight of the sample (in grams).

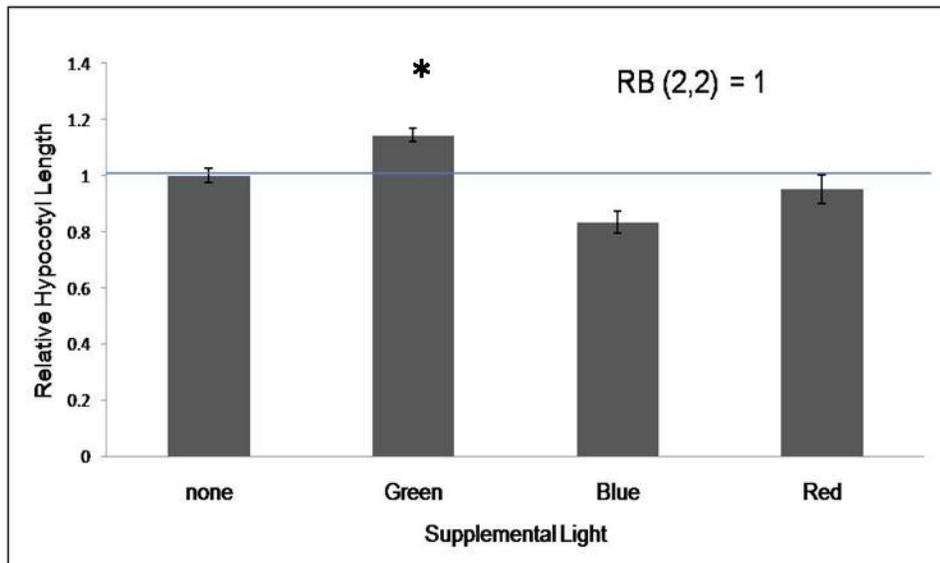


Figure 3-1. Supplemental green-light-induced hypocotyl elongation is specific to green wavebands. 10-d-old *Arabidopsis* plants were grown under RB conditions ($R=2 \mu\text{mol m}^{-2} \text{s}^{-1}$, $B=2 \mu\text{mol m}^{-2} \text{s}^{-1}$) with supplemental G, R, or B= $1 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Y-axis). Hypocotyls were measured as described in Materials and Methods. The RB treatment alone was set to 1 and the experimental data are shown as length relative to RB. Asterisk indicates statistical difference between RB and RBG treatments based on student's t-test ($P < 0.0001$). Each column represents at least 20 seedlings in at least 2 experimental replicates. Error bars show standard error of the mean.

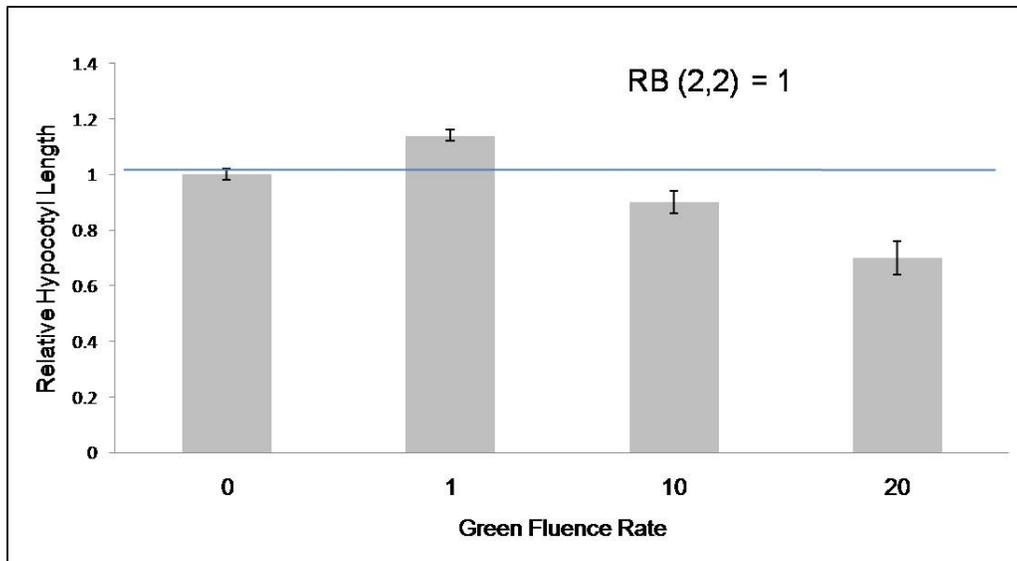


Figure 3-2. Fluence rate/response experiment. 10-d-old *Arabidopsis* plants were grown under RB conditions ($R=2 \mu\text{mol m}^{-2} \text{s}^{-1}$, $B=2 \mu\text{mol m}^{-2} \text{s}^{-1}$) and supplemented with G at 0, 1, 10, and $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Y-axis). Hypocotyls were measured and data are expressed relative to hypocotyl lengths under RB (0G). Data represent at least 20 seedlings and at least 2 experimental replicates per treatment. Error bars represent standard error of the mean.

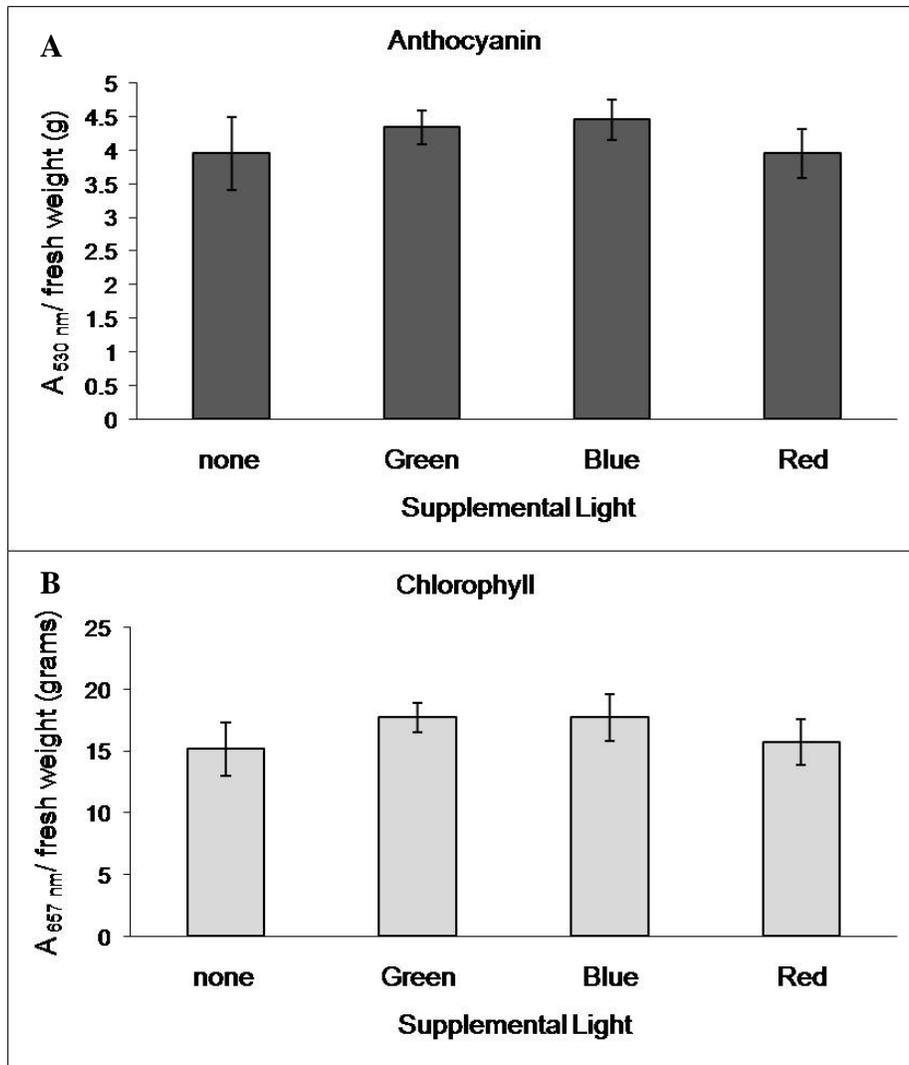


Figure 3-3. Anthocyanin and chlorophyll levels for 10-d-old Col seedlings grown under RB with +G, +B, and +R as indicated (X- axis). A) Anthocyanin levels measured as the absorbance of the sample at 530 nm per gram fresh weight (Y-axis). B) Chlorophyll levels measured as the absorbance of the sample at 657 nm per gram fresh weight (Y-axis). At least 20 seedlings and 2 experimental replicates were quantified for each treatment. Error bars represent the standard error of the mean.

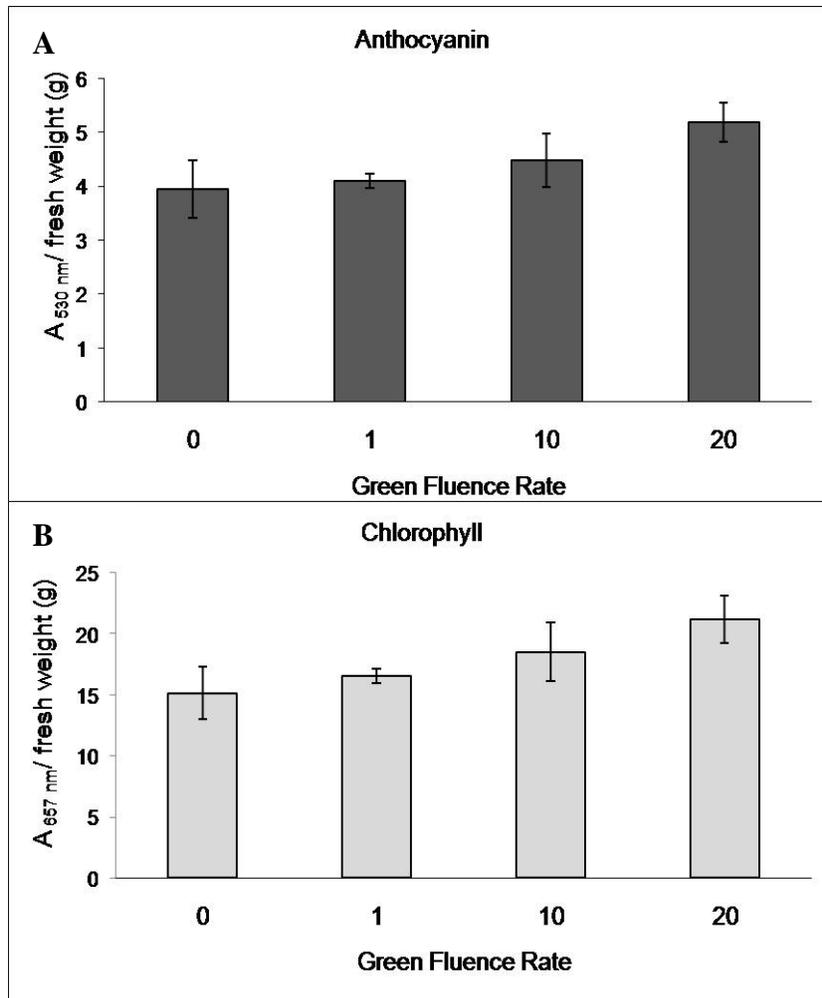


Figure 3-4. Anthocyanin and chlorophyll levels for 10-d-old Col seedlings grown under increasing fluence rates of green light. For these experiments, RB was given with $R=2 \mu\text{moles m}^{-2} \text{s}^{-1}$ $B=2 \mu\text{moles m}^{-2} \text{s}^{-1}$ with increasing amounts of green light at the following fluence rates: 0, 1, 10, and 20 $\mu\text{moles m}^{-2} \text{s}^{-1}$ (X-axis). A) Anthocyanin levels as measured by the absorbance of the sample at 530 nm/fresh weight (g) (Y-axis). B) Chlorophyll levels as measured by the absorbance at 657 nm of the sample/fresh weight (g) (Y-axis). At least 20 seedlings and 2 experimental replicates were observed for each treatment. Error bars represent the standard error of the mean.

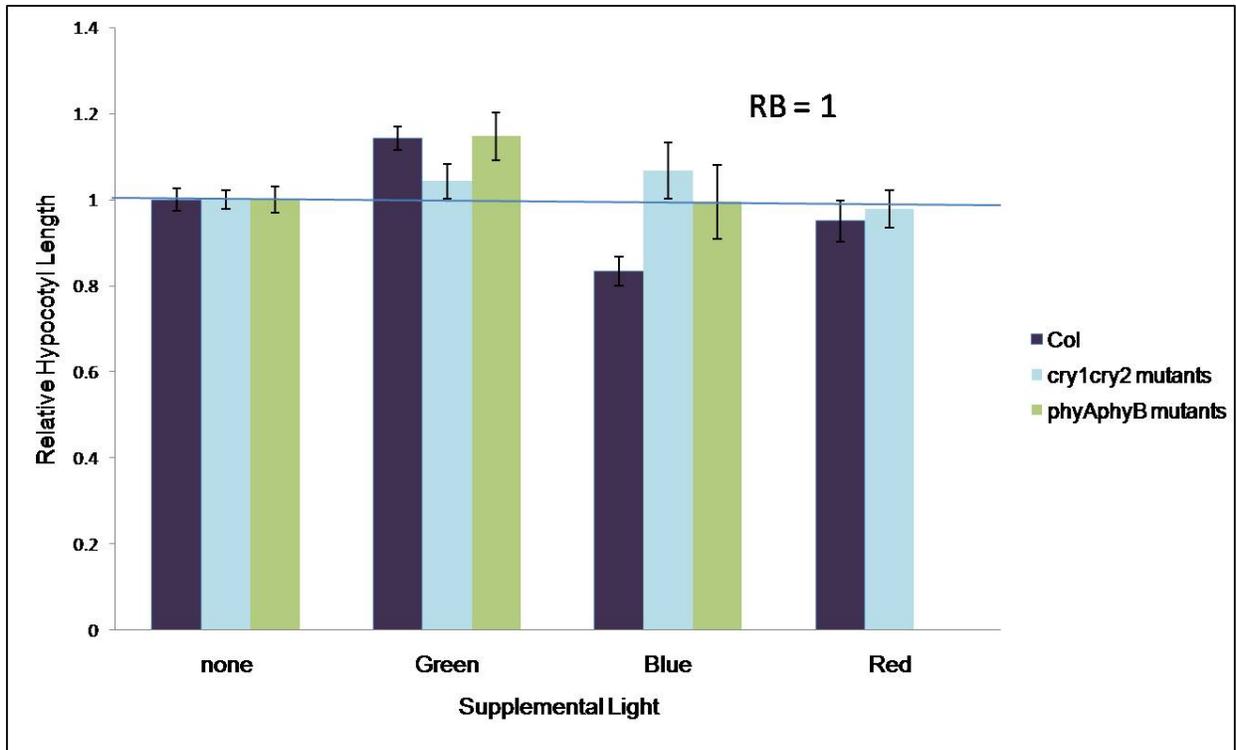


Figure 3-5. Hypocotyl elongation experiments for photoreceptor mutants (Wild type [Col], in purple, *cry1 cry2* mutants in blue, *phyA phyB* mutants in green). Average hypocotyl lengths were measured after 10-d with RB and +G, +B, +R treatments for all genotypes. Quantities are expressed relative to RB with average hypocotyl lengths under RB set to 1 for all genotypes. At least 20 seedlings and 2 experimental replicates were performed for these experiments. Error bars represent the standard error of the mean.

CHAPTER 4 GREEN LIGHT EFFECTS ON MATURE PLANTS

Introduction

Plants are anchored organisms, therefore their capacity to compete and survive relies on the extent to which they can sense and respond to even minute changes in their ambient environment. Incident irradiation provides an important package of environmental information. Light quantity, quality, and duration all have meaningful effects on plant growth and development (Chen et al., 2004; Spalding and Folta, 2005). One example of a plant response to changes in the light environment is the shade-avoidance syndrome, a genetic program that alters plant form and gene expression to acclimate to a light-limited environment. Induction of this response is regulated by red: far-red ratios. Low ratios of red to far-red light reflect shading and high plant density, as far-red light is readily transmitted through plant tissues in the canopy while red and blue wavelengths are mainly absorbed by overhanging foliage (Smith and Whitelam, 1997; Ballare, 1999; Kim et al., 2005; Vandenbussche et al., 2005). Other light environments that initiate shade-avoidance responses are low levels of blue light or photosynthetically active radiation (PAR, 400-700 nm). Like far-red light, green light can also pass through plant tissue and is present in higher amounts than red and blue in the understory of a canopy (Klein, 1992). While little is known about green light sensing and signal transduction, experiments suggest that green wavebands provide important information. Supplemental green light seems to oppose de-etiolation responses that are initiated by red and blue light (Chapter 2, Klein 1992; Folta 2005; Went 1957; Klein et al., 1965; Kim et al., 2004a; Kim et al., 2004b; Frechilla et al. 2000, Talbott et al., 2003; Folta, 2004, Brudler et al., 2003).

In early seedling development, specific examples of this apparent opposition between green light and red or blue light have been described. For example, green light promotes

hypocotyl elongation during early seedling development and this rate of elongation surpasses dark-growth rates. In contrast, growth rates are inhibited by all other light qualities tested (red, blue, far-red, UV-B) (Folta 2004, reviewed Chen et al. 2004; Suesslin and Frohnmeyer 2003). In addition, a brief pulse of dim green light causes a decrease in the accumulation of specific plastid mRNAs (Dhingra et al., 2006). These transcripts encode proteins required for photosynthesis and they typically increase in abundance after red or blue light treatments (Dhingra et al., 2006). In addition green light was found to induce hypocotyl elongation in combinatorial light treatments (Chapter 3). These are three examples in which green wavebands play an antagonistic role to normal photomorphogenic development. Green-light-enriched environments are usually also enriched for far-red light. Therefore, green light may signal a suboptimal light environment much as do far-red wavelengths, and thus induce phenotypes similar to those of the far-red-induced shade-avoidance syndrome.

Another goal of this work was to test candidate genes for roles in green light pathways. Recent work by Bouly et al. (2007) and Chapter 3 of this work indicate that cryptochromes are involved in green light sensing as it pertains to early stem elongation in combinatorial light conditions. Bouly et al. (2007) demonstrate that when the cryptochrome flavin chromophore absorbs blue light it is converted to a green-light-absorbing semiquinone. Green light then reverts the active light sensor state to an inactive state. Bouly et al. (2007) suggest that the cryptochrome receptor may toggle between an inactive, blue-light-absorbing, and active, green-light-absorbing, form much like phytochrome receptors. While Bouly et al. (2007) demonstrate cry-dependence; other studies show cry-independence (Folta 2004) of green light responses.

Experiments described in this chapter test the effects of green light in the presence of red and blue wavelengths. To isolate the roles of green light, far-red light has been excluded from

experimental conditions. Custom, adjustable LED (light-emitting diode) lighting systems were built so that specific quantities and qualities of light could be administered. Experiments were conducted to test the hypothesis that green light, represented by wavebands generally considered to be photomorphogenically inert, will induce adjustments in morphology characteristic of shade-avoidance symptoms induced by far-red enriched environments.

Results

Plants were grown for 15 d under RB conditions ($\sim 65 \mu\text{mol m}^{-2} \text{s}^{-1}$ R light, $\sim 15 \mu\text{mol m}^{-2} \text{s}^{-1}$ B light) or supplemented with green light (RGB; same as RB treatment with $\sim 15\text{-}20 \mu\text{mol m}^{-2} \text{s}^{-1}$ G light). Representative plants from each treatment are shown in Figure 4-1. Plants grown under RB conditions displayed normal photomorphogenic growth, i.e. open and expanded leaves, leaves oriented relatively parallel to soil, and shorter petioles, compared to plants grown under RGB. Phenotypes observed under RGB mirrored those of plants subjected to low red, high far-red light environments. Plants showed characteristics associated with the shade-avoidance response despite having been grown under higher fluence rates of light (Figure 4-1). Plants grown under supplemental green had smaller leaf blades, longer petioles, and displayed a hyponastic response when compared to plants grown under RB.

Rosettes from over 19 individual 15-d-old plants were dissected from two separate experiments. An entire leaf whorl from a representative wild-type plant is presented in Figure 4-2A and exhibits a conspicuous, shade-avoidance habit under RGB. A series of morphological metrics were measured including leaf length, leaf blade area, leaf blade length, leaf width, petiole length, and leaf inclination angle. All indicated symptoms consistent with the shade-avoidance syndrome. The most diagnostic measurements were petiole length as a function of total leaf length (Figure 4-2B) and leaf blade area (Figure 4-2C). These were measured for one of the leaves from the second pair of true leaves (referred to as the second leaf from here on). These

experiments demonstrate that supplemental green light induces elongation of petioles. The data are presented as petiole length relative to total leaf length (Figure 4-2B), since the latter varied slightly. The percent of the total length occupied by petiole is a much more dependable indicator of the phenomenon among all genotypes studied. The addition of green wavebands leads to increased petiole elongation at the expense of the leaf blade. The petiole represented 42% of the total leaf length under RB, yet comprised 56% of the RGB leaf (Figure 4-2A and 4-2B). Similar results were observed for Col-0 and *Ler* ecotypes although phenotypes were more prominent in Col-0 ecotypes (*Ler* not shown).

Leaf blade area clearly was affected by additional green light. The results indicate that addition of green light to RB reduced leaf area by 64% when compared to control plants grown under RB treatments alone (Figure 4-2A and 4-2B). This finding is contrary to the accepted rule that irradiation with a greater number of photosynthetically-active photons causes increased leaf expansion. Similarly, clear hyponastic leaf orientations were observed in the upright growth habit of leaves, which is consistent with lower-light-induced phenotypes. A capacity to reach over neighbors may provide selective advantage to plants and enrich fitness. Under RB the first and second pairs of rosette leaves were positioned parallel to the soil with only minor upward deviation. Under RGB the elongated petioles angle sharply upright, reaching above the rosette plane (Figure 4-1). The results indicate that the simple addition of narrow-bandwidth green light promotes the shade avoidance response.

Green-light-induced shade-avoidance responses in mature plants do not likely result from activation of known photosensors because it is observed under constant blue and red light conditions that excite, if not saturate, known photosensory pathways. However, there are several reasons to test the green light response in photomorphogenic mutants. First, blue-light sensing

cryptochromes have been shown to absorb green light and have been found to be required in stem elongation in early seedling development (Bouly et al., 2007; Chapter 3) and flowering (Banerjee et al., 2007). We also tested phytochrome mutants for their ability to respond to green wavebands. Phytochromes absorb in the green region of the spectrum and contribute to shade-avoidance responses. However, reported phytochrome effects are induced by enriched far-red, or low ratios of red to far-red, and there is little to no far-red under these conditions ($<.0001$). Also, red light was enriched under our conditions, which would have stimulated phytochromes to their active P_{FR} form. This in turn would have promoted positive photomorphogenic growth and a full-sun growth habit. Therefore, if phy are directing shade-avoidance syndrome through green light it would require participation of a novel mechanism. Another reason to test these mutants is that if the influences of red- and blue-light-sensing systems that advance photomorphogenic development are removed genetically, the genetic mechanism that underlies the inhibitory green light system may become even more apparent.

Responses to experimental conditions for Figure 4-1 and 4-2 were also tested using red and blue photoreceptor mutants: *phyA phyB*, *cry1 cry2*, and *phot1 phot2* mutants. Except for *phot1 phot2* mutants, the leaf metrics described in Figure 4-2 were measured for the second leaf of each of these mutants. Lack of *phyA* and *phyB* or *cry1* and *cry2* receptors consistently and significantly resulted in amplification of the effects of green light. The addition of green light resulted in increased petiole length in the second leaf under both RB and RGB conditions for the mutants. Therefore, differences in petiole lengths were not as prominent as in wild-type plants. This was expected, since these mutants are missing the genetic elements that inhibit elongation, therefore are much more elongated than wild type plants under RB conditions. These organs have less potential to elongate, so the differences between RB and RGB would be of a lesser

magnitude. However, general trends were consistent with responses of wild type plants, since the addition of green light enhanced growth. For each of these mutants, the addition of green light also decreased leaf blade expansion relative to the RB-grown plants (Figure 4-3A and 4-3C). With supplemental green light, leaves of *phyA phyB* mutants only expanded to 54% of those from RB-grown plants. In *cry1 cry2* mutants, this was more pronounced, since added green light limited expansion to only 38% of that observed for leaves under RB conditions. These values can be compared to those of wild-type plants, where RGB conditions limited leaf expansion to 64% of RB alone. In addition to inhibiting petiole elongation, activation of cryptochromes and phytochromes has been shown to increase leaf expansion (Cerdan and Chory, 2003; Kozuka et al., 2005). The results presented here are consistent with the hypothesis that a green light transduction system opposes mechanisms of RB-mediated leaf expansion. The differences in leaf expansion were more apparent than those in petiole elongation for these mutants (Figure 4-3A compared to 4-2A). In general, when phy and cry receptors were excluded, the effects of green light persisted but were less conspicuous. These genetic studies indicate that the major phytochromes and cryptochromes are not required for green-light-induced shade-avoidance responses.

It became immediately obvious that the *phot1 phot2* mutants were not useful to these analyses because the phot1 and phot2 photosensors are required for robust light-mediated leaf expansion (Sakamoto and Briggs, 2002). Green-light-induced opposition of expansion cannot be accurately measured in the absence of vigorous phot-mediated expansion. Leaves for these mutants were considerably reduced and shriveled so accurate measurements were unattainable. However, although not possible to quantify, shade avoidance trends were visibly evident in *phot1 phot2* plants grown under supplemental green light (data not shown).

Effects of increasing fluence rates of green light to a fixed background of red and blue light (RB conditions as in Figure 4-1) was examined. Since the response was robust with the addition of only $15 \mu\text{mol m}^{-2} \text{s}^{-1}$, the threshold for the response was evaluated for 0.5, 4.5 and $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ of supplemental green light. Results in Figure 4-4 indicate that even a small quantity of green light photons (s^{-1}) can significantly influence the shape and position of leaves. Figure 4-4A shows representative plants grown under 0.5, 4.5 and $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ of added green light. Very little difference was observed in response to addition of $0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ green light. An obvious change in leaf inclination can be induced with a threshold between 0.5 and $4.5 \mu\text{mol m}^{-2} \text{s}^{-1}$, and is more pronounced at the higher green fluence rate. Petioles of plants grown under $4.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ supplemental green light were 10% longer than those grown under $0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ added green light, and petioles from plants grown under $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ were 18% longer than those grown under $0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ green light (Figure 4-4B). Measurements taken from the second leaf of individual plants show that the addition of $4.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ green light to a background of red and blue induces a significant decrease in leaf area.

These data (Figure 4-4C) also demonstrate that higher fluence rates of green light did not lead to a significant further change in leaf area. The quantitative data presented in Figure 4-4 are slightly different from those presented in Figure 4-1, despite the common ecotype and experimental light treatment. This discrepancy can be reconciled by age and pretreatment. Plants used in later experiments were permitted a longer time to germinate and grow under white light (96 h vs. 1 week) before moved to experimental conditions. The longer germination duration was chosen because the traits of interest were more robust and consistent when plants were permitted to grow under a complete spectrum prior to transfer to experimental conditions.

The observed fluence-rate/response relationship indicates that the green light signal affects the growth of plant organs in a dose-dependent manner and these results are consistent with green-light-induced phenotypes observed in Figures 4-1 and 4-2. The low threshold is evidence that a sensitive photosensor modulates the response to supplemental green light.

These experiments were repeated and observed over a 3 week period. At the end of this period, plants grown under RGB ($G= 15 \mu\text{mol m}^{-2} \text{s}^{-1}$) were larger than plants grown under RB or RGB ($G= 4.5 \mu\text{mol m}^{-2} \text{s}^{-1}$) conditions (observation). Those receiving RB and RGB ($G= 4.5 \mu\text{mol m}^{-2} \text{s}^{-1}$) treatments were similar in size. Plants grown under RGB (4.5) showed leaf hyponasty similar to 2-week-old *Arabidopsis* plants grown with G at either $4.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ or $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure 4-1), whereas plants grown under RGB (15) did not. The size of the leaf blades of plants grown under RGB (15) treatments may have negatively impacted leaf inclination, since a substantial part of the blade is far from the stem. This increase in resistance distance could have led to lower leaf inclination. Interestingly, after three weeks of treatment, approximately 45% of plants grown under RB and RGB (4.5) treatments were flowering while this was evident for only 8% of plants grown in RGB (15) treatments. Data are consistent with previous observations (Kim, Hyeon-Hye, K. Folta; not shown).

Another interesting observation was a visible difference in anthocyanin accumulation between 3-week-old plants grown under increasing fluence rates of green wavelengths (Figure 4-5). Added green light (increasing from left to right in Figure 4-5) resulted in decreased anthocyanin accumulation. Differences were most evident at the base of petioles. This region was darkest purple in plants grown under RB treatments, with slightly less pigment detected in plants from RGB ($G= 4.5 \mu\text{mol m}^{-2} \text{s}^{-1}$) treatments, and anthocyanins were barely visible in plants grown under RGB ($G= 15 \mu\text{mol m}^{-2} \text{s}^{-1}$) conditions.

Recent work by Bouly et al. (2007) demonstrated that green-light-induced regulation of anthocyanin accumulation was mediated by cryptochromes. To test this finding with our lighting system *cry1 cry2* double mutants were grown under RB and RBG (15) conditions and anthocyanin levels were compared to those of wild type plants grown under RB and RBG (15) conditions. For these treatments RB and RBG were administered with $R=45-55 \mu\text{mol m}^{-2} \text{s}^{-1}$, $B=13-15 \mu\text{mol m}^{-2} \text{s}^{-1}$, and $G=14-15 \mu\text{mol m}^{-2} \text{s}^{-1}$ respectively. The quantities of red light are slightly less in these experiments than in previous experiments (Fig 4-1 and 4-2), because this is the maximum quantity attainable for these LED chambers. However, these quantities of red light are given at levels that fully establish a high P_{FR} to P_R equilibrium and saturate photosynthesis, as shown in comparable studies by Dr. Hyeon-Hye Kim (personal communication). Wild type *Arabidopsis* plants grown under RB and RBG (15) treatments showed similar trends as in the previous experiments (Fig 4-1 and Fig 4-2). The *cry* mutants maintained wild type responses to added green light, i.e. down-regulation of anthocyanin accumulation.

To expand this work to horticultural crops, diploid strawberry, *Fragaria vesca*, was tested under RB and RGB conditions. The disadvantage of studying strawberry is that only a small number of plants can be subjected to a given experimental condition at the same time. Nonetheless, preliminary results indicate that small amounts of supplemented green light, as in *Arabidopsis* plants, influence plant growth and development. Moreover, green light effects are consistent with low-light phenotypes.

Strawberry plants grown under RGB were compared to those under RB, with $R=45-55 \mu\text{mol m}^{-2} \text{s}^{-1}$, $B=13-15 \mu\text{mol m}^{-2} \text{s}^{-1}$, and $G=14-15 \mu\text{mol m}^{-2} \text{s}^{-1}$ respectively. Plants were monitored over a nine week period and the following parameters were observed: leaf number, number of fully-differentiated leaves (leaves with 3 leaflets/ petiole), number and length of

runners, number of plants flowering at nine weeks, color of leaves, anthocyanin accumulation, petiole length and leaf area. Data after nine weeks of treatment indicated that mean responses of RBG-treated plants (Figure 4-7) were reduced for every parameter examined.

When compared to *Arabidopsis*, *Fragaria vesca* responses showed some similar trends. In both species, plants appeared to be more vigorous under RB conditions, whereas plants receiving RBG treatment appeared less healthy, despite the increased photon flux. Plants under the latter conditions also had characteristics similar to lower-light phenotypes. Plants grown under RB conditions had more runners per plant (2 or more runners were present in 10 out of 13 RB-grown plants, 4 out of 7 RBG-grown plants) and more advanced leaf morphology (i.e. more fully differentiated leaves (3 leaflets/ petiole) (Figure 4-7). Petiole lengths were the same under both light treatments, which differs from the increase in petiole elongation seen under RBG for *Arabidopsis* plants. No clear flowering effects were observed. Differences in anthocyanin levels were visible in petioles. Petioles collected from 4 plants from each treatment demonstrated that supplemental green light decreased anthocyanin levels, much as seen in *Arabidopsis* plants. For anthocyanin levels, the absorbance of each sample was read at 530 nm ($A_{530\text{ nm}}$) and reported relative to fresh weight (grams). $A_{530\text{ nm}}/\text{g}$ levels were 106.9 (+/- 28.6) for RB treatments, and 46.1 (+/- 10.27) for RBG (15) treatments. Leaf area of the largest leaf from each plant was measured and averages were the same between treatments (data not shown). Thus far, data indicate that supplemental green light affects plant growth and development in multiple species.

Discussion

Experiments described in this chapter demonstrate that green wavebands, like far-red, induce shade-avoidance phenotypes. Here, added green light caused an increase in petiole length, decrease in leaf area, and increase in leaf hyponasty in a dose-dependent manner. In addition, supplemental green light decreased anthocyanin accumulation when compared to RB. These data

support the hypothesis that a green-light-sensing system tempers physiology, growth, and development driven by red and blue light.

Although green-light-induced stem elongation in early seedling development was found to be cry-dependent (Chapter 3; Bouly et al., 2007), all photoreceptor mutants tested in these experiments demonstrated wild-type trends. Results indicated that there may be several sensory systems which initiate green light signals.

In addition, these experiments demonstrate similarities and differences between far-red- and green-light-induced responses. Far-red- and green-light-enriched environments induce petiole elongation and leaf hyponasty. However, far-red also induces early flowering, whereas green light delays it (Kim, H-H and K. Folta; unpublished obs., observation in this chapter). Relationships between far-red and green light will be important targets for future studies. Questions that have arisen from work shown here are the following: 1. How does far-red affect green-light-induced shade-avoidance? 2. Is the response attenuated or relatively similar in the presence of both green light and far-red wavebands? 3. How will far-red light affect flowering in the presence of green light? 4. Will there be an intermediate effect when both systems are stimulated?

Another interesting extension of this work will be its potential influence on plant ecology, measured as the natural variation present in *Arabidopsis* accessions representing specific regions, climates, and culture conditions. Ecotypes may vary in their green-light-induced responses. While similar trends were seen between Columbia (Col-0) and *Landsberg erectus* (Ler) ecotypes, differences between RB and RBG treatments were much more apparent in Col-0 ecotypes. These differences may be due to naturally larger rosette diameters in Col-0 ecotypes. This may allow for more variability in elongation and leaf hyponasty whereas more compact

rosette diameters in *Ler* do not permit as much shade manipulation. Other *Arabidopsis* ecotypes may show still greater variability for their responses to supplemental green light. Previous work demonstrated that *Arabidopsis* accessions were quite varied for shade avoidance responses induced by low ratios of red to far-red (Botto and Smith, 2002).

Ethylene (Pierik et al., 2004), gibberellins (Pierik et al., 2004) and auxin (Morelli and Ruberti, 2000) have all been implicated in mechanisms underlying the shade avoidance response. Interaction between these and green-light-sensing-pathways are also points of interest. Additionally, the transcriptome associated with induction of shade avoidance by low ratios of red to far-red has been defined (Devlin et al., 2003). It describes a distinct set of transcripts affected by simulated shade (Devlin et al., 2003). Green light may affect these transcripts, as well as those involved in anthocyanin biosynthetic gene regulation. A better understanding of how, when, and where green-light-induced shade avoidance responses occur will help dissect their convergence with effects of phytochrome and blue light systems.

Results of this work indicate that the previously unrecognized effects of green light have a prominent influence on tailoring plant growth and development to conditions set by the natural environment. However, the effects are not apparent in plants grown under high fluence rates of white light. It is more likely that the green-light-sensitive system is directing plant responses under marginal light conditions, such as foliage shade or laboratory conditions containing unnatural ratios of narrow-bandwidth LED light. This finding extends to a practical level, as the results suggest the reconsideration of artificial lighting regimes for plant growth. Greenhouses and growth chambers are typically supplied with fluorescent fixtures that principally emit light equivalent to the RGB conditions used in the present experiments (although fluorescent bandwidth is greater due to accessory fluors that expand the bandwidth relative to the discrete

peaks seen in RGB LED treatments). Through addition or subtraction of the green light component of visible light, it is clearly possible to accelerate, skew, or potentially manipulate plant growth and development. In addition to greenhouse conditions, this work is relevant to densely-packed fields of crop species. Since similarities were found between green light responses of *Arabidopsis* and *Fragaria vesca*, these may extend to other plant species as well.

Materials and Methods

Plant Materials

The genotypes tested are identical to those previously assessed for responses to blue light (Folta and Spalding, 2001); *cry1-304 cry2-1*, *phot1-5 phot2 (nph1-5cav1-1)*, and *phyA201 phyB-5*. The *phyA phyB* mutant is in the *Ler* background; all others are in the Col-0 background.

Plant Growth

For *Arabidopsis thaliana*: Plants were grown in soil in flats modified to fit precisely under the LED arrays (made with American Bright LEDs) with even distribution of light.

Approximately 50 seeds from each genotype studied were applied to 16 cm² of wetted soil surface using a funnel to assure localization of each genetic line. Each flat contained a grid of 4 x 3 genotypes. The seeds were stratified at 4°C for 72 h and then transferred to white fluorescent light for 96 h (Figures 4-1 through 4-3) or 1 week (Figure 4-4) to ensure that wavelength-specific effects were attributable to green light effects on plant development and not germination timing. Seedlings were transferred to positions under LED arrays and watered approximately every third day with saturating amounts of 0.1x commercial fertilizer dissolved in distilled water. Plants were grown under constant irradiation to dampen the effects of the circadian oscillator on stem elongation. The first treatment consisted of irradiation from only red and blue LED light with a total fluence rate of ~75 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (15 $\mu\text{mol m}^{-2} \text{s}^{-1}$ blue light). The next treatment consisted of same red-blue treatment supplemented with green light (total fluence rate ~90 $\mu\text{mol m}^{-2} \text{s}^{-1}$). In

both treatments, the average fluence rate was obtained from sampling multiple positions in the plant-growth grid area.

For *Fragaria vesca*: Seeds were sterilized using the following method: Seeds were covered with 0.01% Tween 20 and shaken for 15 min, rinsed with sterile diH₂O, covered in 70% ethanol and shaken for 1 min, rinsed several times, then covered with 25% bleach and shaken for 10 min, then rinsed several times, place in sterile water and placed on a shaker until germinated. Germinated seedlings were transplanted to soil, covered with Saran Wrap and placed under treatments, acclimated, and left under treatments for nine weeks. Leaf area measurements were taken by first affixing the largest leaf per plant to a piece of tape, then taking a high-resolution picture of the leaf with a size standard, and then measuring the area with ImageTool as described below for Arabidopsis.

Light Sources

Actinic light treatments were generated using narrow-bandwidth LED light supplied by American Bright LEDs controlled by custom electronics as described (Folta et al., 2005). Light composition was assessed using an Apogee spectroradiometer and Spectra Whiz software. The emission spectra of all light sources are viewable on-line at www.arabidopsisthaliana.com/lightsources.

Measurement

Whole plants and plant organs were harvested, and then flattened and affixed in their native positions to adhesive electrical tape. Specimens were imaged with a digital camera and measurements of organ size were performed using UTHSCSA Image Tool (Version 3.0 for Windows) with comparisons to adjacent size standards.

Measurement of rosette diameter was performed using the same software indicated above.

Anthocyanin Accumulation

Tissues were either soaked in 100% Methanol: 1% HCl directly (Fluence rate/ response experiments) or first ground with liquid nitrogen (all other tissues) and then soaked overnight at 4°C (wrapped in foil). The next day tubes were centrifuged at maximum speed and absorbance at 530 nm was measured. Measurements were recorded and divided by the total amount of fresh tissue used to get the absorbance at 530 nm per weight (grams), or $A_{530 \text{ nm}} / \text{g}$.

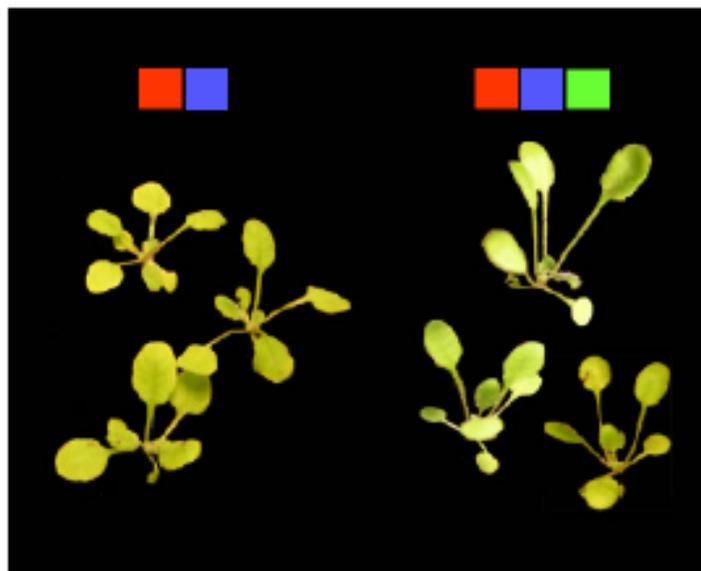


Figure 4-1. Supplemental green light induced a shade response. Wild-type (Col-0) Arabidopsis plants were grown under red and blue LED light (RB; Red light $\sim 65 \mu\text{mol m}^{-2} \text{s}^{-1}$, Blue light $\sim 15 \mu\text{mol m}^{-2} \text{s}^{-1}$) or identical RB conditions supplemented with green light (RGB; same as RB with $\sim 15\text{-}20 \mu\text{mol m}^{-2} \text{s}^{-1}$ Green light) for 15 d. Conspicuous differences in leaf area, petiole length and leaf hyponasty were observed under RGB, responses typically indicative of low-light conditions or a decreased red to far-red ratio. The ratio of red to far-red light was identical between conditions (with negligible far-red detected).

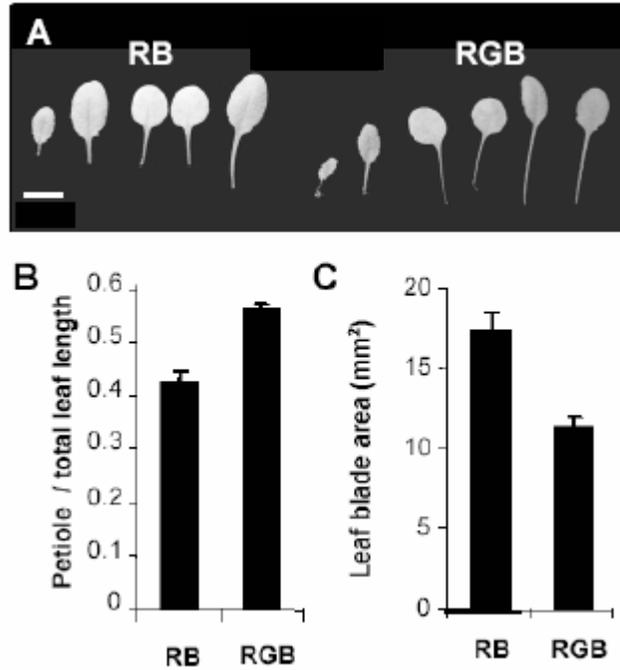


Figure 4-2. Supplemental green light induced petiole elongation and inhibited leaf expansion. A) Individual wild-type rosettes from a single representative plant grown under RB or RGB were dissected and conspicuous leaf attributes were measured. B) Petiole length divided by total leaf length. C) The mean leaf blade area from leaves. The measurements in B and C were derived from the second leaf from at least 30 individual plants. Error bars represent standard error of the mean. Bar = 5 mm.

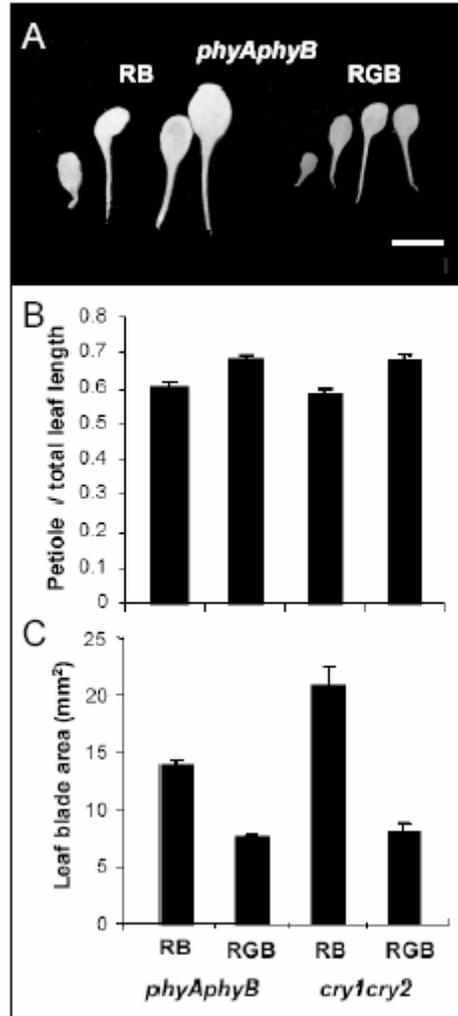


Figure 4-3. Supplemental green light effects are maintained in photoreceptor mutants. The effect of green light was tested in photoreceptor mutants, plants lacking functional genes encoding known photoreceptors for red, blue and far-red light. The *phyA phyB* and *cry1 cry2* mutants were grown under RB and RGB, their rosettes were dissected and leaf attributes quantified. A) Leaf whorls from a single representative *phyA phyB* plant grown under RB or RGB conditions. B) The average petiole length relative to total leaf length and C) Mean leaf blade areas. All measurements were obtained from the second leaf of at least 19 individual plants. Error bars represent standard error of the mean. Bar = 5 mm.

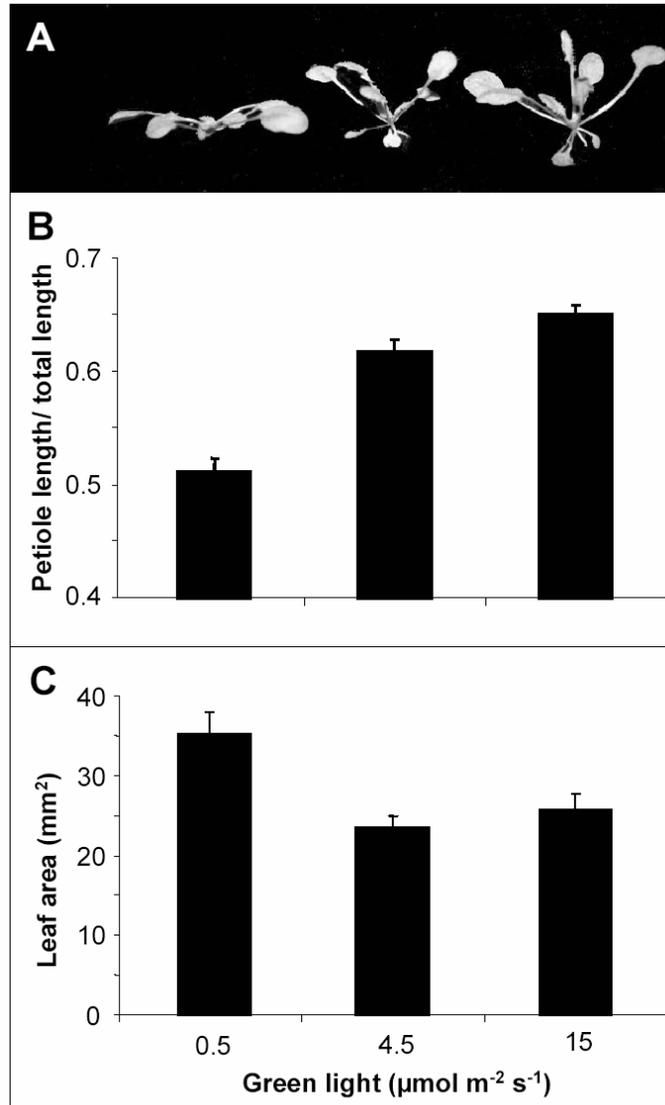


Figure 4- 4. Green light responses are fluence-rate-dependent. Green light was added at 0.5, 4.5 and 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to a constant background of RB, as in Figure 4-1 and physical attributes of plants were photographed and quantified. A) A dose-dependent change in leaf hyponasty, petiole length and leaf area is observed in representative plants. B) The mean petiole length as a function of total leaf length measured from at least 15 plants, except in the 0.5 G treatments where only 6 plants were measured. C) The average leaf blade area (derived from the second leaf).

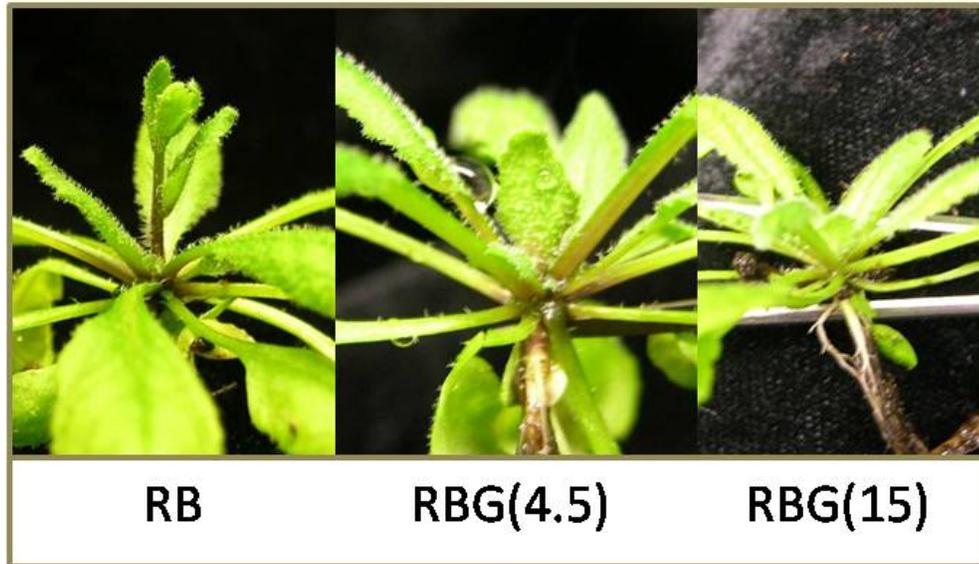


Figure 4-5. Anthocyanin accumulation under increasing amounts of supplemental green light is visible as purple color near the base of the petiole for plants grown under RB, RBG ($G=4.5 \mu\text{mol m}^{-2} \text{s}^{-1}$) and RBG ($G=15 \mu\text{mol m}^{-2} \text{s}^{-1}$).

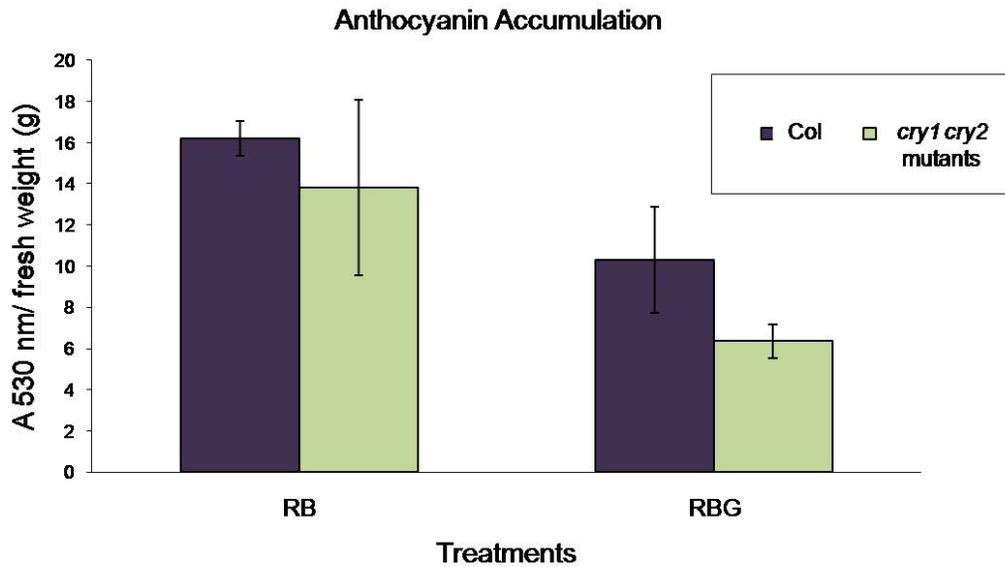


Figure 4-6. Anthocyanin accumulation decreased under supplemental green light for wild type (Col) plants (purple) and *cry1 cry2* mutants (green) when leaves (including petioles) were examined after RB or RBG treatments. Anthocyanin levels are represented as the absorbance at 530 nm/ fresh weight (in grams). Data represent at least 5 leaves plus petioles per treatment and at least 2 experimental replicates. Error bars represent the standard error of the mean.

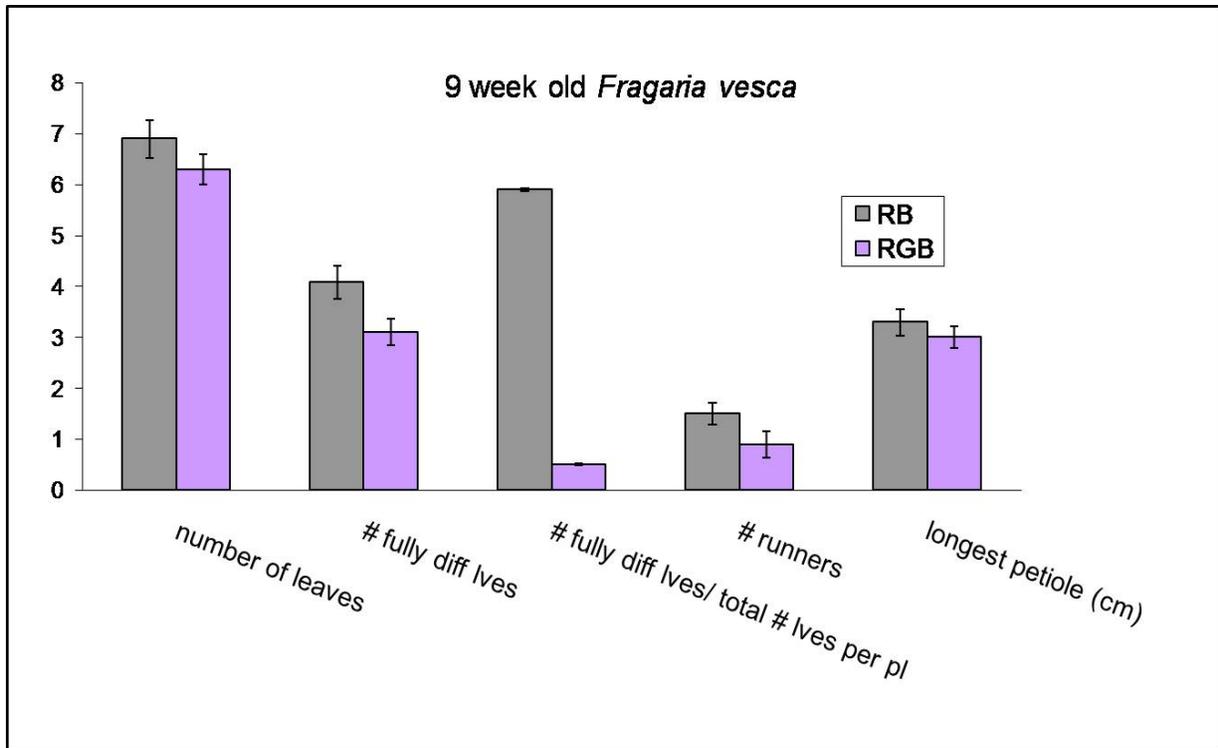


Figure 4-7. Vegetative features of *Fragaria vesca* after 9 weeks growth under RB (grey columns) and RGB (purple columns) light environments. From left to right, column 1 shows the average number of leaves per plant, column 2 shows the average number of fully differentiated leaves (3 leaflets/ petiole) per plant, column 3 shows the average number of fully-differentiated leaves per total number of leaves per plant, column 4 shows the average number of runners per plant, and column 5 shows the average length (in cm) of the longest petiole per plant. Error bars represent the standard error of the mean.

CHAPTER 5
CANDIDATE GENE APPROACH TO IDENTIFY GREEN LIGHT PATHWAY
COMPONENTS

Introduction

Light is a critical signal that guides developmental “decisions” in dark-grown seedlings. Effects of red, blue, and far-red light in regulating molecular, physiological, and biochemical events during seedling development have been well-described. When compared to red and blue light pathways, green light pathways are less well-defined.

Evidence of green light opposing normal photomorphogenic progression has been presented (See Chapter 2). However, many of these experiments were limited by light sources and light measuring equipment. Recent work examining seedling responses to green light substantiates historical findings. Folta (2004) demonstrated that a short pulse of dim green light induced an increase in hypocotyl elongation. This response followed the Bunsen-Roscoe Law of Reciprocity and was present in a background of dim-red light. In addition, green-light-induced responses were present in all photoreceptor mutants tested indicating that this green light response may be sensed through a novel photoreceptor. Also, Zeiger and colleagues (Frechilla et al., 2000) showed that green light negates blue-induction of stomatal opening and Talbott et al. (2003) indicated that this response requires NPQ1. Also, Dhingra et al. (2006) demonstrated that green light induces a decrease in plastid transcript accumulation which again opposes red and blue induction. In addition to such evidence from the literature, experiments within Chapter 3 of this work demonstrate green light opposition to red and blue inhibition of hypocotyl elongation under combinatorial light treatments. Together these experiments demonstrate a negative role for green light in normal light-induced phenotypes in early development. Although complete green-light signal transduction pathways for these responses have not been described, some green light responses have been linked to crys (Bouly et al., 2007; Chapter 3 of this work), while others are

cry-independent (Folta, 2007). These discrepancies indicate there are multiple green light photoreceptors.

To elucidate green light pathway components, a candidate gene approach was implemented and experiments are described herein. Examination of Arabidopsis genomic sequence led to identification of several genes with high sequence similarity to visual cycle components. One gene had high sequence similarity to *Rpe65*, a gene necessary for chromophore synthesis/modification in animals (*Max4/Ccd8* in plants) (Baylor, 1996; Moiseyev et al., 2005). The *max*, more axillary branching, mutants were isolated in a screen for regulators of lateral branching (Sorefan et al., 2003). Two of these mutants, *max4* and *max3* (*ccd8* and *ccd7* respectively), cleave multiple carotenoids in *E. coli* and are thought to act in the same branching pathway in plants (Booker et al., 2004; Schwartz et al., 2004). Experiments indicate that the cleavage product of CCD7 and CCD8 is likely to be a mobile regulator of auxin transport (Schwartz et al., 2004; Bainbridge et al., 2005; Bennett et al., 2006). Expression data show that CCD8 is localized to the chloroplast (Auldridge et al., 2006).

In animals, RPE65 is required for conversion of the *all-trans* retinal to 11-*cis* retinol, the preferred chromophore in animal vision (Baylor, 1996, Moiseyev et al., 2005). Although retinoids, specifically, *all-trans* retinal have been identified in plants (Lorenzi et al., 1994), a formal function has yet to be described. Roles for retinal-based receptors have been identified in other organisms such as *Pelvetia* (Robinson et al., 1998), and have light sensor roles in this species. CCD8 is thus an interesting candidate for contribution to photosensing or signaling mechanisms in plants.

Experiments described in this chapter examined green-light-induced responses in *ccd8* mutants, using previously established conditions that demonstrate green light responses (Folta

2004, Dhingra et al. 2006). Results demonstrated that CCD8, whether involved in sensing or subsequent responses, is required for green-light-induced down-regulation of chloroplast transcript levels and may be involved in long-term green-light-induced stem elongation.

Results

Isolation and Characterization of T-DNA Mutants

For this study, T-DNA insertion lines were obtained through the Salk Institute. Lines used were homozygous and verified both by PCR and by the phenotypic loss of apical dominance characteristic of homozygous *ccd8* mutants (Auldridge et al., 2006). The T-DNA insertion locations are shown in Figure 5-1.

Hypocotyl Elongation

Dark-grown 2-d-old mutant- and wild-type-seedlings, were transferred to narrow-bandwidth, monochromatic green light treatments and evaluated after 3 d (72 hours). The *ccd8* mutants exhibited light hypersensitive phenotypes such as shorter hypocotyls and more open, expanded cotyledons relative to wild type seedlings (Figure 5-2).

If this hypersensitive response is specific to green light, then one interpretation of this result is that CCD8 is required for normal, green-light-induced hypocotyl elongation. Although green light specificity was not examined in these experiments (i.e. comparing mutant and wild type under other wavelengths to account for general light sensitivity) mutant and wild type seedlings were the same length in the dark, indicating an effect on light-mediated stem elongation and not simply a defect in elongation growth.

Because these mutants displayed aberrant green light phenotypes, they were examined with finer resolution as described by Folta (2004). In this report, a brief pulse of green light induced a transient increase in hypocotyl elongation rate (Folta, 2004). In the present work, the *ccd8* mutants responded normally to green light (Figure 5-2) (Folta, 2004). The data indicate that

CCD8 is not required for stem elongation in 48-h-old seedlings exposed to monochromatic green light.

Chloroplast Transcript Regulation

In addition to effects observed on hypocotyls in early development, green light induced decreases in plastid transcripts, which is opposite to red and blue induction (Dhingra et al., 2006). Chloroplast transcript accumulation was examined for mutants and wild type seedlings after a brief pulse of green light as defined in Dhingra et al. (2006). In wild type seedlings, a brief pulse of green light down-regulated chloroplast transcript accumulation as in Dhingra et al. (2006). In the *ccd8* mutants, an increase rather than decrease was observed in plastid transcript abundance (specifically *psaA* and *psbD*) following a brief pulse of green light (*psaA*: Figure 5-4; data not shown for *psbD*).

Gene expression experiments in Figure 5-4 indicate that *ccd8* mutants do not generally show aberrant light-regulated gene responses. Levels of ELIP mRNA, a phytochrome-induced nuclear-encoded transcript, are up-regulated in both *ccd8* mutants and wild type seedlings following a brief pulse of green light. In microarrays described by Dhingra et al. (2006), ELIP and some other phy-induced transcripts were up-regulated following a brief pulse of green light. This was expected since green light stimulates phytochrome and indicates that differences between mutant and wild type in gene regulation are specific to green light (Figure 5-4).

Pharmacological Studies

Since CCD8 is required for green light affects on chloroplast transcript regulation, the next experiments were designed to test the role of CCD8 in this response. Pharmacological experiments were conducted based on the role of RPE65 in vision. Affects of exogenous *all-trans* retinal (ATR), a putative product of CCD8 activity and potential chromophore, on green light chloroplast transcript regulation were evaluated. The *ccd8* mutant seedlings were grown in

darkness in the presence of micromolar and sub-micromolar amounts of *all-trans* retinal (ATR).

Chloroplast transcript accumulation was observed after dark or green light treatments.

Micromolar and sub-micromolar amounts of *all-trans* retinal were able to partially rescue green-light-induced down-regulation of chloroplast transcripts; however, a complete dose-response was not observed over the range of concentrations tested (Figure 5-5).

Discussion

Candidate genes were identified through sequence analysis and tested for green-light-induced responses including green-light-induced stem elongation in 2-d-old seedlings, long-term stem elongation (72 h), and down-regulation of chloroplast transcript accumulation. The *ccd8* mutants were aberrant for long-term stem elongation under monochromatic green light and for green-light-mediated down-regulation of plastid transcripts. These experiments suggest that CCD8 may play a role in long-term, green-light-induced stem elongation. However, it is also possible that light hypersensitivity in general, i.e. to all wavebands, could be the culprit of the advanced light phenotypes observed in the mutant. To answer this question, affects of other wavebands on long-term stem elongation should be examined. The *ccd8* mutant growth kinetics showed wild type trends when exposed to a brief pulse of green light. CCD8 is not required for this early green light stem response. As in other light sensing and signaling regimes, CCD8 may be involved with some, but not all, green-light-induced responses.

When chloroplast transcript accumulation was examined, the *ccd8* mutants did not behave as did wild type seedlings. Rather than wild-type, green-light-mediated down-regulation of plastid transcripts, transcript levels increased in *ccd8* mutants following a brief pulse of green light. This response suggests there may be an underlying antagonism between phytochrome stimulation of *psaA* and green light repression, and that CCD8 is required for this response.

Application of micromolar and sub-micromolar amounts of *all-trans* retinal (ATR), a potential chromophore, partially rescued wild type green light responses. Partial rescue of the wild type response may be the result of the delivery method used in these experiments. Addition of ATR to media may not allow efficient uptake by seedlings. Also, ATR is hydrophobic, toxic to plants, and light-labile. Therefore, when these plates were exposed to light, for the purpose of synchronizing germination, the ATR might have started breaking down and therefore may not have been readily available to seedlings. One improvement to this procedure would be to spray plants with *all-trans* retinal after 2 d growth, and before the green light pulse. Another possible reason for partial rescue and lack of a dose-response is that ATR effects are not specific to green light and can cause a more general plant stress response. Although this is a possibility, it is unlikely, since sub-micromolar amounts induced at least partial responses.

Complementation tests using wild type dog or mouse versions of CCD8 may help resolve CCD8's role in green-light-induced down-regulation of chloroplast transcripts because the chemical activity for CCD8 has been well established in these organisms. It will also be interesting to evaluate *ccd8* mutants for other green-light-induced responses as indicated in Chapter 3 and 4 of this work. Also, CCD7 has been linked to the same branching pathway as CCD8 (Sorefan et al., 2003; Booker et al., 2004; Schwartz et al., 2004). Observation of *ccd7* mutants and *ccd7 ccd8* double mutants under these conditions is also of interest, since CCD7 and CCD8 are in the same lateral branching pathway.

Materials and Methods

Plant Lines

Two homozygous T-DNA insertion mutants were used in this study (SALK_082552 and SALK_072750). They were isolated from the Salk Institute Genomic Sequence Library and T-DNA insertion points are indicated in Figure 5-1.

Photomorphogenic Development

Seeds were surface sterilized and placed on minimal media containing 1mM KCl, 1mM CaCl₂, and 1% DIFCO agar. The plates were vernalized for at least 48 h at 4°C, and then positioned vertically under treatments. Following 48 h in a 37°C incubator, plates were placed in conditions and monitored under treatments. Light treatments were generated using fluorescent light and colored filters. For Figure 5-2, seedlings were imaged after 72 h treatment with an 8.0 megapixel digital camera.

Transcript Analysis

2-d-old dark-grown seedlings were exposed to a brief pulse of green light (100 $\mu\text{mol m}^{-2}$ total fluence), wrapped in foil, and moved to dark for one hour. At the end of 1 h, plates were dropped into liquid nitrogen. RNA extractions were done following the Qiagen RNAeasy Plant Mini Kit. Northern blots were probed with randomly primed ³²P PCR products.

Retinal Treatments

The *all-trans* retinal (from Sigma-Aldrich, St. Louis, MO) was re-suspended in ethanol and the concentration was determined spectrophotometrically. For long-term growth experiments *all-trans* retinal was added directly to cooled minimal media and dispensed into plates under dim red light.

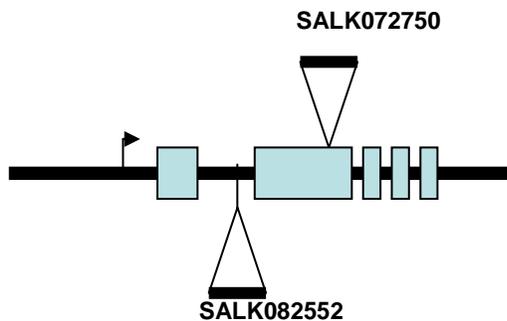


Figure 5-1. Insertion sites for the *ccd8* T-DNA lines obtained via the Salk Institute and evaluated in this study. Blue boxes represent exons.



Figure 5-2. The *ccd8* mutants are defective for green-light-induced stem elongation. Representative seedlings of *ccd8* mutants (left) and wild type (Col) (right) are shown after 72 h of growth under green light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$).

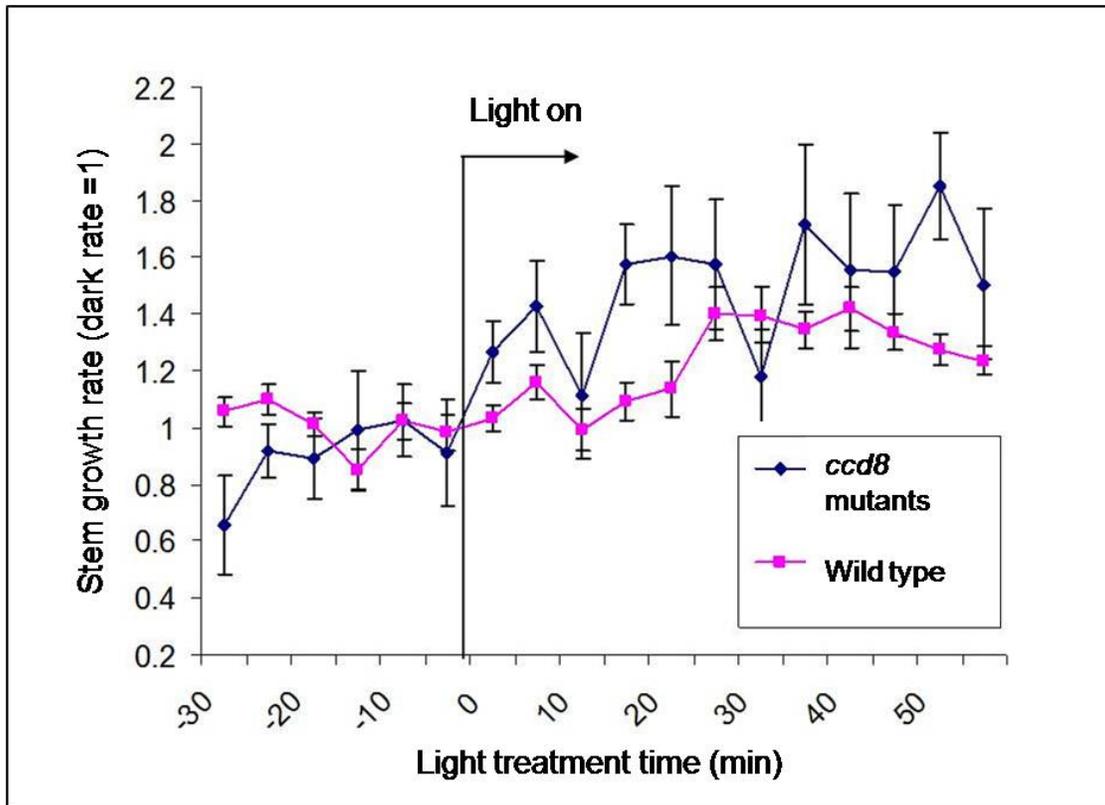


Figure 5-3. The *ccd8* mutants demonstrate green-light-induced hypocotyl elongation in 2-d-old seedlings. Stem elongation relative to dark rate (1) is shown on the Y-axis. The X-axis represents time (min) following treatment (positive values) and time before treatment (negative values) or time at treatment (time 0). Green light was given at time 0. At least 20 seedlings for each genotype were evaluated with 2 experimental replicates.

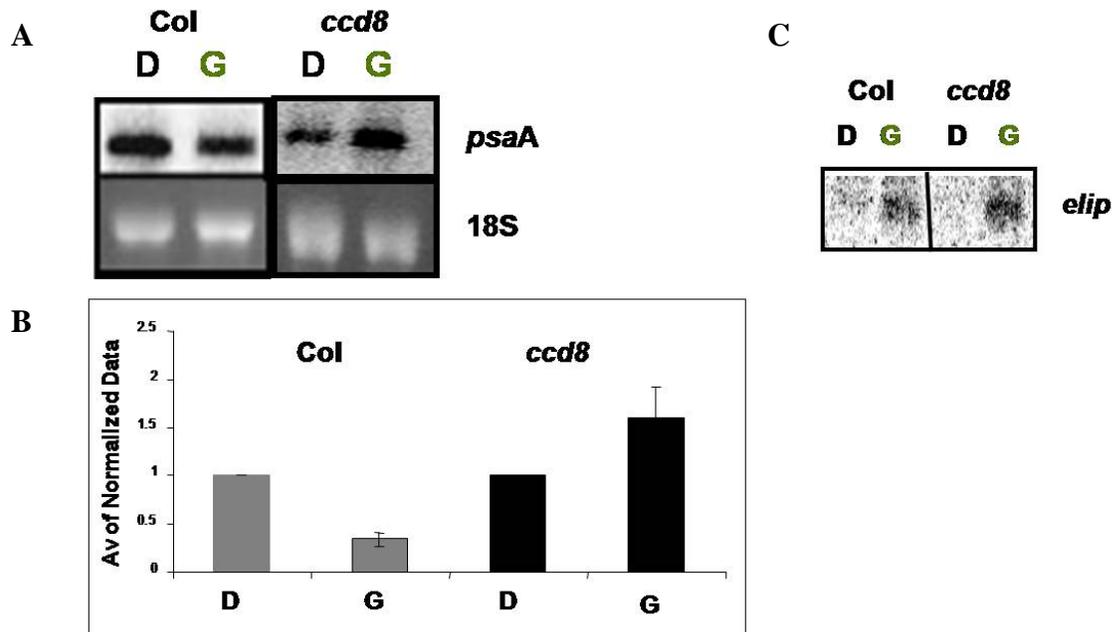


Figure 5-4. The *ccd8* mutants were aberrant for green-light-mediated down-regulation of chloroplast transcripts (*psaA*) but wild type for phy gene regulation. A) Northern blots probed with *psaA* after either dark (D) or pulsed green light (G) treatments for Col and *ccd8* mutants. Ethidium stained agarose gels show 18S levels. B) Quantified data from 5 or more biological replicates. C) Northern Blot of Col and *ccd8* mutants probed with ELIP (a phytochrome-induced, nuclear-encoded transcript) after dark (D) or green light (G) treatments.

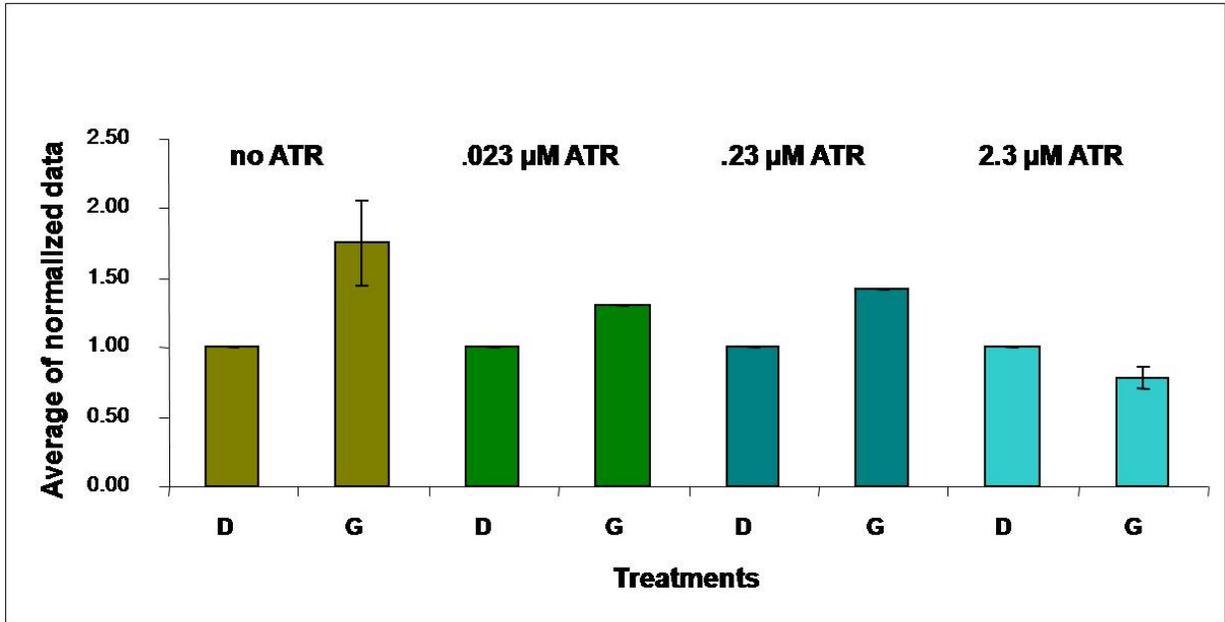


Figure 5-5. Partial rescue of green-light-mediated down-regulation of chloroplast transcripts in *ccd8* mutants with micromolar and sub-micromolar amounts of ATR. The *psaA* transcript accumulation is shown following a brief pulse of green light (G) or dark (D) treatments expressed relative to dark levels. Increasing amounts of *all-trans* retinal (ATR) are indicated. The Y-axis indicates the average of several (when error bars are present) experiments normalized to dark levels.

CHAPTER 6 FUTURE GREEN LIGHT RESEARCH

Experiments herein demonstrate specific roles for green light in shaping seedling and mature plant growth and development. All green-light-induced effects demonstrate a negative role for green light in de-etiolation. Tunable, narrow-bandwidth LEDs (light emitting diodes) were used in these experiments and provide conditions with which to test green light effects going forward. In early seedling development, green light induced an increase in stem elongation in the presence of red and blue light at low fluence rates which was dependent on cryptochromes. Preliminary data indicate that green light seems to delay true leaf development. These parameters should be addressed in future experiments.

In addition to early development, green-light-induced responses were identified in mature plants. Green light added to a background of red and blue light induced symptoms associated with shade-avoidance syndrome. In these experiments, increased light (RGB) caused lower-light phenotypes. Supplemental green light induced an increase in petiole elongation, decrease in leaf area, leaf hyponasty, and decrease in anthocyanin accumulation. Preliminary experiments indicate that green light may also delay flowering. Shade-avoidance responses occurred in a dose-dependent manner and were present in all photoreceptor mutants tested. An obvious extension of this work is in isolation of green light pathway components. Experiments described here provide the conditions with which to observe mutants. Interestingly, diploid strawberry, *Fragaria vesca*, displayed some similar trends when compared to Arabidopsis plants grown under supplemental green light. Consistent with Arabidopsis trends, strawberry plants under RB appeared healthier in general when compared to plants under RGB in preliminary experiments. Also, supplemental green light induced a down-regulation of anthocyanin accumulation as in Arabidopsis. Plants under RB had more runners per plant and more fully-differentiated leaves

than RGB treated plants. Strawberry experiments should be repeated and more species should be examined for green-light-induced responses going forward. By understanding how plants sense and respond to green wavebands, improvements in crop production and greenhouse growth can be made. Because these wavebands for the most part induce lower-light phenotypes, manipulation of light environments (via supplemental or reduced green light depending on species of interest) will allow for increased desired effects.

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BIOGRAPHICAL SKETCH

Stefanie Maruhnich was born in Durham, North Carolina and shortly after moved to Aliquippa, Pennsylvania, and later to Destin, Florida where she spent most of her formative years. Upon graduating from Fort Walton Beach High School in Fort Walton Beach, Florida, she received several scholarships to the University of Florida where she began undergraduate studies majoring in botany. She was accepted into Maria Gallo's lab as an undergraduate research assistant. The summer before receiving her B.S. in botany, she was awarded a research internship in Harry Klee's laboratory at the University of Florida. The following August she began the Plant Molecular and Cellular Biology Program in Kevin Folta's laboratory.