NOTE TO USERS

PHYTOCHROME AND SUCROSE EFFECTS ON EARLY ACCUMULATION OF ANTHOCYANINS IN *ARABIDOPSIS THALIANA*

by

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ABSTRACT

PHYTOCHROME AND SUCROSE EFFECTS ON EARLY ACCUMULATION OF ANTHOCYANINS IN *ARABIDOPSIS THALIANA*

by

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Light is one of the most important abiotic factors that affects a plant's growth and development. Characterization of genetic mutants has been a powerful method for elucidating light signaling pathways. The core of this research stems from the ability of a class of photoreceptors known as phytochromes to harvest and utilize light energy. Phytochromes are essential to the process of photomorphogenesis—how plants grow and respond to their light environment—and they act in conjunction with available carbon to promote the production of a group of pigment molecules known as anthocyanins. In an effort to ascertain how light and sugar sensory pathways affect anthocyanin accumulation, a screen was developed to isolate mutants that are defective in components that bridge these pathways. By varying the duration and intensity of far-red light, and utilizing an array of sugars and sugar analogs, conditions that yield maximum anthocyanin accumulation were determined. Using these conditions for a genetic screen, a series of *li*ght response *d*eficient (*lid*) mutants

that accumulate little or no anthocyanin in response to far-red (FR) light and sucrose during early seedling development were identified, and two of these mutants were characterized in depth. *lid3* is physiologically similar to wild type except in the early anthocyanin accumulation response, and lid3 may regulate mRNA abundance of the same flavonoid biosynthesis genes that are mediated by phytochrome A. Rough mapping places *LID3* on the long arm of chromosome 1. *lid4* is affected in numerous phytochrome A-controlled processes, but its complex phenotypic segregation pattern yielded inconclusive mapping results. The data presented in this dissertation provides insight into how anthocyanins accumulate as a function of phytochrome A activation, sucrose-sensing pathways, and the developmental stage in *Arabidopsis thaliana.*

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CHAPTER 1. INTRODUCTION

INTRODUCTION

Without the sun, life could not exist on earth. Light energy is utilized directly or indirectly by all living species. Plants detect and transduce light signals of specific wavelengths to designated cellular compartments where targeted molecules stimulate an array of responses. Through the process of photomorphogenesis, plants use this external environmental cue to adapt their growth and development to elicit these responses and for improved genetic transfer to the next generation. The most commonly studied photomorphogenic responses include seed germination, hypocotyl elongation, apical hook opening, cotyledon expansion, greening, anthocyanin accumulation, shade avoidance, phototropism, flowering time and circadian rhythms.

In the absence of light, photomorphogenesis cannot be induced. Instead, skotomorphogenesis, plant growth without light, occurs. Under these conditions plants take on an etiolated phenotype of extremely long hypocotyls, pale coloring, a closed apical hook and little cotyledon expansion. Light stimulates the de-etiolation process, which gives plants their familiar appearance of short hypocotyls, green color, and expanded cotyledons. De-etiolation is initiated and controlled by several classes of photoreceptors including phytochromes, which are the most widely studied of the photoreceptors. While each light receptor absorbs a specific wavelength and has independent functions,

photomorphogenic pathways are not completely isolated from each other. In addition, input into these pathways is not linear. The interaction between photoreceptors and/or their interaction with target molecules creates a web of connections throughout the cell and the organism.

To dissect this web of responses, the mustard-family plant *Arabidopsis thaliana* has been used as a model organism. Its easy growth, short life cycle, ability to self-pollinate and produce an abundance of seeds makes it ideal for physiological work. Its small genome of five chromosomes has also allowed it to be a molecular model for plants. In 2000 its genome was fully sequenced, making it an even more powerful tool for discovering molecular pathways in the cell (Initiative*, 2000)

A. PLANT PHOTORECEPTORS

In *Arabidopsis* there are two cryptochromes (CRY1 and 2), two phototropins (PHOT1 and 2), and five phytochromes (PHYA-E) (Quail, 2002). Cryptochromes and phototropins control blue/ultraviolet-A responses (B/UV-A). The most well characterized photoreceptors are the phytochromes, which are activated by red (R) and far-red (FR) light.

Cryptochromes are photolyase-like receptors which mediate responses such as inhibition of hypocotyl elongation, flowering and circadian clock mechanisms (Lin, 2000). CRYs do this both independently and by interacting

with different factors such as light receptors PHYA and B, and the nuclear transcription factor COP1 (Hennig et al., 1999).

Phototropins are correlated with regulation of blue-light-mediated phototropism (Short et al., 1994). *PHOT1* was initially isolated through the mutant *nph1* that is defective in phototropic responses. Soon after, *PHOT2* was identified through its sequence homology to *PHOT1* (Sullivan and Deng, 2003). Recent evidence establishes a link between phototropins and phytochromes. This was demonstrated in a lower plant, *Adiantum*, which contains a chimeric phototropin/phytochrome photoreceptor.

B. PHYTOCHROMES

Phytochromes are the most widely studied of the photoreceptors, and are the major focus of the research presented in the following chapter.

I. Phytochrome Structure

The phytochrome holoprotein may be homo- or heterodimeric (depending on the type of phytochrome), with each monomeric subunit approximately 120-130 kD (Hershey et al., 1985; Sharrock and Quail, 1989). The amino terminus consists of four domains P1-P4. P1 is serine rich and predominantly plays functional roles; P2 is related to Per-ARNT-Sim (PAS) domains which at the N-terminus only loosely resembles the consensus sequences. This domain is typically involved in protein-protein interactions (Schepens, 2004). The P3

domain is the site of a thioether attachment for a tetrapyrrole chromophore. It is the chromophore that is responsible for absorbing light. The P4 domain is designated the PHY domain. At the carboxy terminus of the holoprotein several conserved regions are present; 2 PAS domains which are involved in protein-protein interactions and which better resemble the consensus sequences; the Q (Quail) Box to which key interacting factors such as PIF3 and NDPK2 are thought to bind; the sites of dimerization for the holoprotein; and two histidine kinase-related domains (HKRD) are also present, although no His kinase activity has been observed.

In general, it has been thought that the C-terminus controls most activating functions of phytochromes, but Matsushita and colleagues (2003), by expressing transgenic phytochrome fusion constructs, showed that in the absence of the C-terminus, the phyB N-terminus can initiate somewhat normal phyB responses if fused to domains from other proteins that allow homodimerization and nuclear localization (Matsushita et al., 2003). Their results indicate that phyA and phyB homodimerization is controlled by the C-terminus.

II. Phytochrome Photoreversibility

Phytochromes consist of a five-member gene family, phyA-E. They absorb predominately in the red (R) and far-red (FR) regions of the spectra. Each phytochrome can exist as one of two alternate forms, dependent on light

absorption by the tetrapyrrole chromophore. This structural cis/trans isomerization propels the signaling induced via phytochromes. This unique mechanism is possible because phytochrome is initially created in its inactive form called the P_r, or red-absorbing form. Upon stimulation by absorption of a photon, the molecule undergoes a conformational change into the active form called the P_{fr} form or far-red absorbing form. Early experiments showed that the phytochrome molecule can be switched back and forth between the P_r and P_{fr} isoforms to reach a dynamic equilibrium dependent on the light intensity and wavelengths (Butler et al., 1959; Mackenzie et al., 1975).

III. Phytochrome Classes and Interactions

Phytochromes are divided into two classes based on their light lability. Type I phytochromes (phyA in *Arabidopsis*) are abundant in etiolated seedlings, but upon exposure to continuous red (Rc) or white light (Wc), their level drops 50- to 100-fold because of the rapid degradation of the holoprotein and repression of phy gene transcription. Active phyA ($phyA_{fr}$) is degraded quickly via ubiquitin-mediated proteolysis. Type II phytochromes (phyB through phyE in *Arabidopsis*) are the most abundant phytochromes in green plants because of their light stability in the active form (Quail et al., 1995). These two phytochrome classes are based on their expression patterns and the apparent stability of the encoded proteins after photoconversion.

As a plant develops, initial control over photomorphogenesis switches from type I to type II phytochromes. Initially, with the onset of germination, the wavelengths reaching the seed/seedling through a dense canopy are enriched in FR light. Type I phytochromes are the more abundant at this stage and in control of FR-mediated photomorphogenesis such as hypocotyl inhibition, cotyledon expansion, anthocyanin accumulation, and the phyA-preconditioned block of greening response. As the plant grows it responds differently to the ambient light wavelengths. PhyB mainly controls R-mediated responses such as hypocotyl elongation, circadian clock, and flowering. PhyC-E are less well characterized. However, in the absence of phyB, phyD has been shown to partially assume some of its roles. Based on intensive analyses with different mutants, and overexpressor lines, it is clear that phytochromes have unique, yet overlapping functions and expression patterns (Goosey et al., 1997; Sharrock et al., 2003).

In Wc a mutation in the *PHYA* gene does not cause an easily distinguishable phenotype. Such a mutation, however, is easily identified when seedlings are grown in FRc. Under strictly FRc conditions, phyA mediates the inhibition of hypocotyl elongation. Under Rc, phyB controls the inhibition of hypocotyl growth, although phyA does play a minor cooperative role (Koornneef et al., 1980). In fact, phyA and phyC seem to act redundantly with

phyB in the Rc mediation of hypocotyl elongation. Additionally, the regulation of rosette leaf morphology is also controlled via the synergistic action of these phytochromes.

R/FR light responses including control of leaf morphology and flowering time appear to be controlled by the overlapping function of PhyB, D, and E as demonstrated through mutations in these genes. In particular, the shade-avoidance response—increased stem elongation and reduced leaf production occurring under low light or FR-enriched light characteristic of dense growth habitats—seems to be particularly redundant between phyB and D. This functional overlap may be a reflection of their sequence homology (Aukerman et al., 1997).

As mentioned previously, plant photoreceptors have unique and overlapping functions. The de-etiolation process is riddled with seemingly endless combinations of interactions. An array of phytochrome downstream signaling factors control the de-etiolation process, modulated by inputs from additional light receptors and other abiotic factors.

IV. Phytochromes May Be Homo- or Heterodimeric

Recent studies indicate that the phyA (type I) protein portion can only exist as a homodimer. PhyB (type II) has also been shown preferentially to form homodimeric proteins, although it can also dimerize with other phytochromes.

The other type II phytochromes appear to function as either heterodimers or homodimers. The dimerization affinities for either form do not appear to be light-regulated. The ability of type II phytochromes to assume homodimeric or heterodimeric conformations explains the myriad negotiations phytochromes can initiate in terms of physiological responses.

V. Phytochrome Fluence and Irradiance Responses

The degree of phytochrome responsiveness is dependent in part on the quantity of light activating the phytochromes. This quantity of light is the fluence and is measured as the number of quanta impinging on a surface, regardless of direction (Papiez, 1994). The fluence rate, then, is the number of photons falling on a surface in a given amount of time, while the irradiance is a measure of the energy of unidirectional light impinging on a surface per unit time. The phytochrome fluence responses can be broken down into three categories: Very Low Fluence Responses (VLFR), Low Fluence Responses (LFR), and High Irradiance Responses (HIR).

VLFRs are driven by a range of fluences confined to a small amount of light compared to LFR and HIRs. These VLFRs are not FR-reversible because once R has caused photoconversion, a saturating flash of FR will only convert approximately 97% of the P_{fr} form back to the P_r form. That 3% that remains in the P_{fr} form is enough to continue photomorphogenesis. In fact, the FR pulse is

sufficient in itself to initiate the VLFR because the 3% P_{fr} form created by FR will initiate photomorphogenic responses. These responses are regulated by phyA.

LFRs are responses which are reversible with pulses of R and FR light due to the photoreversibility of the phytochrome molecule. Significantly more light is necessary for LFRs than for VLFRs. LFRs are defined by the number of photons absorbed, and adhere to the law of reciprocity, i.e., a brief pulse of intense light will produce the same response as exposure to dim light for a longer period so long as the total photon fluence is the same and specific photon parameters for the LFR are not exceeded. LFR responses include seed germination, most aspects of de-etiolation, R-induced anthocyanin production, and many other photomorphogenic responses.

HIRs are dependent on the fluence rate and the time of exposure. Typically, they are elicited by continuous light exposure. Therefore, HIR responses are not photoreversible. HIR responses for etiolated seedlings peak in the FR light, and have been shown to be controlled by phyA. However, in green plants the peak is in R and therefore controlled by phyB and other type II phytochromes. This phenomenon is most likely a result of the light-labile character of type I phytochromes. HIR responses include anthocyanin accumulation, inhibition of hypocotyl elongation, cotyledon growth, and many other light-mediated processes that are the focus of the work presented here.

VI. Phytochrome Cellular Activity

The phytochrome apoprotein is first synthesized in the cytosol. Once it assembles with the plastid-produced chromophore, the full phytochrome holoprotein remains in the cytoplasm in the inactive (P_r) form until the chromophore captures a photon, causing a conformational change in the phytochrome molecule to the active form (P_{fr}); this change then stimulates a cascade of events.

As mentioned previously, phytochromes have a His-kinase-related domain. Although no evidence of histidine-kinase activity exists for eukaryotic phytochromes, those in prokaryotes do show histidine-kinase activity (Vierstra and Davis, 2000; Wu and Lagarias, 2000), indicating that plant phytochromes evolved from those in common ancestral prokaryotes, but lost their histidine-kinase activity.

In 1998, Yeh *et al* used purified oat phyA expressed in yeast and revealed Ser/Thr kinase autophosphorylation activity in a light-regulated manner. Research continues to attempt to pinpoint the specific sites for *in vivo* phosphorylation of phytochromes. There are several critical S residues which, when phosphorylated, may maintain phytochromes in the P_{fr} form. These phosphorylated residues may also be important for protein-protein interactions and eventual ubiquitin-mediated degradation (Hunt and Pratt, 1981).

The conformational change upon light activation not only causes phosphorylation events to ensue, but also reveals a nuclear localization sequence causing phytochromes to translocate into the nucleus (Sakamoto and Nagatani, 1996; Kircher et al., 1999). Evidence using phy:GFP fusion proteins in transgenic plants confirm that all five *Arabidopsis* phytochromes follow this model of nuclear localization once stimulated by light. Interestingly, both phyA and phyB accumulate in the cytosol after light mediated reconversion to the P_r form. However, the phyA P_r form that has been cycled from the P_{fr} form has also been shown to localize to the nucleus in some cases (Kircher et al., 1999), suggesting that phyA and B may have both cytosolic and nuclear functions. Furthermore, while a significant proportion of active phyA and B molecules translocate to the nucleus, the majority of these proteins remain in the cytosol. Additionally, while phyC-E:GFP fusion proteins move to the nucleus upon light activation, phyC-E are also seen in the cytosol.

C. CYTOPLASMIC SIGNALING EVENTS

Recent evidence suggests that phytochromes may transduce their signals through cytosolic messengers such as calcium, CaM, cGMP, G-protein and others (Chory, 1994; Barnes et al., 1995; Quail et al., 1995). The P_{fr} form of phytochromes may activate G-proteins localized at the cell membrane. Through this interaction, numerous pathways are then stimulated. One involves Ca^{2+} and

calmodulin, stimulating chloroplast development; another requires Ca^{2+} and cGMP, which are needed for the inducing anthocyanin production (Li et al., 1991; Wu et al., 1996).

Besides G-proteins, other molecules are activated or deactivated by phytochrome photoconversion and lead to other photomorphogenic responses. Two novel cytosolic factors that interact directly with phytochromes were identified in a yeast-two-hybrid screen: phytochrome kinase substrate 1 (PKS1) (Fankhauser et al., 1999) and nucleoside diphosphate kinase 2 (NDPK2) (Choi et al., 1999). PKS1 is a negative regulator of phyB responses and is phosphorylated via serine/threonine kinase activity. NDPK2 is not exclusively localized in the cytosol; it is also found in the nucleus. NDPK2 becomes more active with increased levels of phyA P_{fr}. While *ndpk2* has normal hypocotyl elongation, the defects of a T-DNA insertion in the gene leads to its phenotypic defects which include a decreased sensitivity to both R and FR light with regard to both hook opening and cotyledon expansion (Choi et al., 1999).

PhyA also interacts with auxin stimulated transcription factors known as Aux/IAA. This was demonstrated by using recombinant Aux/IAA proteins from both *Arabidopsis* and *Pisum Sativum* (pea) which interact *in vitro* with recombinant *Avena sativa* (oat) phyA. One possible interaction suggested by

Colon-Carmona *et al* 2000 involves phytochrome-dependent phosphorylation of Aux/IAA proteins.

D. NUCLEAR PHY-INTERACTING FACTORS

Once the phytochrome molecule has entered the nucleus, it is exposed to a distinct set of potential signaling intermediates. Each phytochrome isoform will interact with a specific group of factors. PhyA and phyB share a subset of interacting factors; for instance, phytochrome interacting factor 3 (PIF3). PIF3 is a bHLH transcription factor with a PAS-related motif localized in the nucleus (Ni et al., 1998), and its abundance is light-regulated. PIF3 binds specifically to a G-box promoter *cis* element and, upon light activation, the P_{fr} form associates with PIF3. PhyB binds to PIF3 more readily than does phyA (Zhu et al., 2000). Consistent with this result is the greatly reduced responsiveness to R and somewhat lower sensitivity to FR observed in plants with low levels of PIF3 (Ni et al., 1998).

PIF4 is a phyB-specific negative regulator which preferentially binds the active form of phyB and has little affinity for phyA. PIF4 binds to the same G-box DNA sequence motif that PIF3 recognizes, although PIF4 does not seem to be involved in regulating other light-regulated promoters of circadian genes such as CAA1 and LHY that are targets of PIF3. It has been argued that there may be nucleotides outside the core G-box hexanucleotide sequence, which could

provide *cis*-element specificity whereby PIF3 and PIF4 could act preferentially toward various light-regulatory target promoters *in vivo* (Martinez-Garcia et al., 2000; Huq and Quail, 2002).

Transgenic *Arabidopsis* plants overexpressing *CRY2* were used to interpret the interactions of phyB and cry2. Both co-immunoprecipitation and physiological experiments confirm a direct interaction between these photoreceptors (Mas et al., 2000). Compared with wild-type, *cry2* mutants exhibit photomorphogenic abnormalities in their W perception, have an extended period of expression of CAB:luciferase under various W fluences (although none under R), as well as increased expression under B. This may suggest the necessity for multiple wavelengths that are necessary for full cry2 function. Phytochromes may have a synergistic role in this cry response (Mas et al., 2000).

The physiological interconnections of photoreceptors were further established for *Arabidopsis in vitro* by interactions in yeast cells expressing C-terminal fragments of cry1 and phyA. It appears that both cry1 and cry2 can be phosphorylated by a purified recombinant phyA. Additionally, certain *cry1* mutant alleles interfere with the functions of phytochrome *in vivo*, again suggesting direct interaction (Ahmad et al., 1998).

E. OTHER DOWNSTREAM SIGNALING COMPONENTS

The signaling networks activated by the selective absorption of light by photoreceptors and their direct or indirect engagement with downstream factors are intricate and complex. A number of research approaches have been used to tease out each signaling molecule in the hope of piecing together these complex pathways. The process of de-etiolation changes the expression of approximately 30% of the *Arabidopsis* genes as shown by microarray analysis (Ma et al., 2001); (Tepperman et al., 2001). This approach provides further insight into photoregulation. Although photoreceptors are activated via specific light wavelengths, the regulatory system responses are all part of a unit mechanism which allows photomorphogenesis to take place (Ma et al., 2001).

From constitutively photomorphogenic mutants in darkness to mutants insensitive, hyposensitive or hypersensitive to specific light wavelengths, genetic mutants have been a key tool in pulling apart the light signaling pathways. Below is a summary of some of the components which have been isolated and which are relevant to our study.

The COP/DET/FUS family blocks photomorphogenesis in darkness. Mutants in this pathway display a light-grown phenotype when grown in the dark. Some members of this family form the COP9 signalosome; a multiprotein complex found in the nucleus. This large molecule is suggested to play a role in

nuclear protein degradation (Quail, 2002). Constitutively photomorphogenic 1 (COP1) is another member of the COP/DET/FUS family of proteins. It contains a zinc-binding ring finger motif as well as a WD-40 repeat. In the dark COP1 resides in the nucleus. Upon activation of phytochromes by light, COP1 translocates out of the nucleus and into the cytoplasm. It functions as a ubiquitin E3 ligase (Osterlund and Deng, 1998). Specifically COP1 binds to HY5, a photomorphogenesis-inducing bZip transcription factor, and delivers HY5 to the COP9 complex for degradation. However, cry1 and cry2 disable COP1 by direct interaction (Wang, 2001; Yang et al., 2001), releasing HY5 to induce transcription of several genes including chalcone synthase and RBCS (Yang et al., 2001).

Arabidopsis de-etiolated (*det1*) mutants are phenotypically light-grown when grown in the dark. DET1 may be involved in photomorphogenic regulation through various photoreceptors (Chory et al., 1989; Pepper et al., 1994). DET1 is nuclear-localized and binds to non-acetylated amino-terminal tails in the nucleosome at the core histone H2B. It appears to regulate photomorphogenesis by remodeling chromatin(Benvenuto et al., 2002). Additional roles for DET1 include acetyl transferase complex formation and nucleosome binding to light-activated genes, regulating gene expression via acetylation of histone tails (Benvenuto et al., 2002; Schroeder et al., 2002).

DET2 encodes an enzyme in the brassinosteroid biosynthesic pathway. Its sequence homology to mammalian genes and subsequent functional analysis indicate that DET2 is a reductase in brassinosteroid biosynthesis (Li et al., 1996). *det2* mutants can be identified by their partially de-etiolated phenotype in the dark (Chory et al., 1991).

While it is clear that photoreceptors and photoreceptor substrates interact with each other in a network, specific downstream branching pathways can be further dissected when certain factors show responses to only one group of photoreceptors; for instance Fhy1, Fhy3, and Far1. These proteins appear regulated solely through phyA and appear to be nuclear localized positive regulators. Lesions in these genes yield phenotypic defects in two or more FR light (phyA) mediated responses including hypocotyl elongation, apical hook opening, cotyledon expansion, FR-preconditioned block of greening, anthocyanin accumulation, and gravitropic sensitivity.

Yeast-two-hybrid analysis and co-immunoprecipitation from live tissue show that *FHY3* and *FAR1* can form homo- or hetero-complexes. These two genes contain limited sequence homology and, based on their ability to suppress the each other's mutant phenotype, indicate that they may be part of the same branch pathway (Hudson et al., 1999; Wang and Deng, 2002). FHY1 does not appear to directly interact with FHY3 or FAR1; however FHY3 proteins do

appear to regulate the transcript levels of FHY1. Additionally *fhy3far1* double mutants have a more severe phenotype than either one by itself. It is clear that their gene products influence each other (Desnos et al., 2001; Wang and Deng, 2002).

As the branching pathways extend and become more specialized, downstream components may ultimately affect only a single light-regulated response. Inhibition of hypocotyl elongation is apparently the only response affected by mutations in HFR1, REP1, RSF1 and LAF1 (Fairchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000; Ballesteros et al., 2001). All these gene products are nuclear localized. LAF1 and HFR1 encode transcription factors that are involved in specific subsets of phyA-regulated responses (Wang and Deng, 2002). Specifically, HFR1 is expressed heavily in FR yet does not appear to bind directly to phyA or phyB. It does, however, form homodimers or heterodimers with PIF3, which can bind phyA and phyB directly (Fairchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000; Ballesteros et al., 2001). The ability of HFR1 to heterodimerize, coupled with its nuclear localization and bHLH motif, suggest a functional role in the phyA signaling pathway that mediates hypocotyl growth (Fairchild et al., 2000). It is interesting that although HFR1 may mediate phyA and even cryptochrome pathways, it does not appear to be involved with HY5, (Kim et al., 2002; Duek and Fankhauser, 2003)

delineating another branch of phyA-specific hypocotyl growth inhibition, uncoupled from that containing HY5.

There are a number of other early phyA signaling-specific positive regulators which play various functional roles. FIN219 is involved in the light inactivation of COP1 and other positive regulators via ubiquitin-mediated proteolysis. In addition to its light regulation, the *FIN219* gene product is induced by auxin and is homologous to the GH3 family of proteins.

Communication at the subcellular level is also regulated in part by light. Protoporphyrin IX is a light-specific signaling factor that aids in communication between the nucleus and plastids. The proper transport and subsequent distribution of protoporphyrin IX is mediated via the gene product of LAF6, which encodes an ATP-binding-cassette (atABC1) protein (Hare et al., 2003) involved in intracellular transport. The *laf6* mutant is hyposensitive to light.

In addition to positive regulators, there are a number of negative regulators in phyA signaling. EID1 is an F-box-containing protein with a leucine zipper required for proper functioning. EID1 may be involved in ubiquitin-dependent protein degradation. EID1 specifically controls phyA-mediated HIR responses (Büche et al., 2000). A separate, yet interacting, pathway includes SPA1, which mostly mediates VLFR based on experiments with *eid/spa1* double mutants, which show interference in a wide range of fluences (Zhou et al., 2002).

SPA1 has high sequence similarity to COP1, and is also nuclear localized. *spa1* shows hypersensitivity to FR in a phyA-dependent manner, but it does not display the typical *cop1* mutant phenotype of constitutive photomorphogenesis in the dark, nor does it translocate to the cytosol in the light. SPA1 and COP1 appear to act as suppressors at different times. Saijo, (Saijo et al.) showed that SPA1 interacts directly with COP1. Specifically, SPA1 can mediate the degradation of LAF1 via COP1 (Seo et al., 2003).

A duplication of the chromosomal section that includes *SPA1* resulted in *SPA2* which was identified by its sequence similarity to *SPA1* (Laubinger and Hoecker, 2003). Other *SPA1*-like genes including *SPA3* and *SPA4* were identified by their apparent homology. *SPA1, SPA3*, and *SPA4* show homologous sequences in their kinase-like region, putative coiled-coil domains, and the six WD-40 repeats. As anticipated, the actions of these three genes are FR-light mediated through phyA, and all act as negative regulators. However, SPA3 appears to function as a repressor regulated via other phytochrome species as well (Laubinger and Hoecker, 2003).

As mentioned, SPA1 interacts with COP1 in repressing photomorphogenesis. SPA3 and SPA4 also share these binding affinities to COP1 via their coiled-coil domain yet it is difficult to speculate exactly how all these factors interact, as neither SPA3 nor SPA4 appear to possess a nuclear

localization sequence (Osterlund and Deng, 1998; Hardtke and Deng, 2000; Laubinger and Hoecker, 2003). Where COP1-mediated HY5 degradation occurs in the nucleus, perhaps interaction between SPA3 and SPA4 occurs after COP1 shuttles out of the nucleus following light exposure. Meanwhile, nuclear-localized HY5 protein is no longer degraded via COP1; however another negative regulator, SUB1 may continue to suppress the light-dependent accumulation of HY5 transcript (Guo et al., 2001).

While we have been focusing on light signaling factors that respond to phyA only, there are other signaling components that affect phyA and phyB. One in particular, called Pseudo-Response Regulator 7 (PRR7), was originally suggested to be involved in maintaining circadian rhythms and is a member of a small gene family in *Arabidopsis* (Kaczorowski and Quail, 2003). PRR7 was isolated because *prr7* mutants are defective in de-etiolation; in particular two mutants have shown reduced sensitivity to Rc and FRc. The two mutants were found to be allelic. PRR7:β-glucuronidase fusion proteins localize to the nucleus. Various phenotypes observed in *prr7* mutants include poor regulation of CCA1, LHY, and TIMEING –OF-CAB1, suggesting a role for PRR7 in circadian rhythm regulation. PRR7 is an example of the complex talk between signaling systems in *Arabidopsis*, namely de-etiolation by both phyA and phyB and regulation of circadian rhythms (Kaczorowski and Quail, 2003).

PhyA and phyB share other common signaling intermediates. The negative regulator COG1, when overexpressed in transgenic plants, shows R and FR light hyposensitivity in responses which were inversely dependent on COG1 mRNA transcript levels. *COG1* antisense lines display hypersensitivity to both R and FR light. This gene is inducible via both phyA and phyB. In FR light phyA induces COG, and in R light, phyB can induce COG expression. This nuclear-localized protein is one of a family with a Dof (*D*NA binding with *o*ne *f*inger) domain and its pattern of expression implies that it is positively regulated by both phyA and phyB, and thus is involved in light-signaling in *Arabidopsis* (Yanagisawa, 2002; Kanyuka et al., 2003; Park et al., 2003).

Studies of phytochrome signaling in *Arabidopsis* reveal highly intricate interconnected signaling systems during the de-etiolation process. Continued examination of this process regularly yields new signaling components. Many of these signaling intermediates have been identified through genetic mutants, and the corresponding genes have yet to be cloned. Therefore, definitive roles for these factors in signal transduction remain to be deduced. Among the mutant loci deficient in FR light photomorphogenesis are: *fin2* (Soh et al., 1998), *fin4* (Fry et al., 2002), *fin5* (Cho et al., 2003), and *psi1* (Genoud et al., 1998). Mutants deficient in R responsiveness not yet cloned include *red1* (Wagner et al., 1997), *pef2*, *pef3* (Ahmad and Cashmore, 1996), and *srl1* (Huq et al., 2000). Pef1, a

seemingly positive regulator, appears to show defects in both phyA and phyB signaling (Ahmad and Cashmore, 1996). As these genes are cloned and molecularly characterized, the role of phytochrome signaling pathways in the de-etiolation process will be further elucidated.

F. PHYSIOLOGICAL RESPONSES MEDIATED THROUGH PHYA

Phytochrome molecules play intrinsic roles in the photomorphogenesis of plants. The focus of the present thesis is on the initial de-etiolation process of plants specifically mediated by FR light through phyA. $PhyA_{fr}$ inhibits hypocotyl elongation, aids in apical hook opening and cotyledon expansion, blocks chlorophyll production, and promotes anthocyanin synthesis. While all these phenotypic traits were noted if present, the main focus of this work is on anthocyanin accumulation during de-etiolation as mediated by phyA and carbon availability.

I. Anthocyanins: Plants and People

Anthocyanins are a group of glycosylated flavonoids that produce hues such as reds, pinks, purples, and blues on plants. The color of the anthocyanin is influenced by the array of side groups, hydroxyl and methoxyl groups, aromatic acids attached, pH, or their formations as supramolecular anthocyanin complexes when chelated to metal ions. The colors of anthocyanins are of utmost importance in attracting pollinators to flowers and fruits for pollination and seed

dispersal. (Li et al., 1993) showed that anthocyanins and other flavonoids protect the plant from harmful UV/B radiation and high-light conditions. More evidence of their "sunscreen" capabilities has been discerned from fall foliage studies.

Understanding how anthocyanins are produced has become especially relevant because of their apparent roles in promotion of human health. They are implicated in both protection and treatment of diseases; such as heart disease and certain cancers (Ghiselli, 1998). Kamei H., (Kamei, 1998) concluded from their studies that anthocyanins from Italian red wine were effective in absorbing free radicals and reactive oxygen species, and in inhibiting lipoprotein oxidation and platelet aggregation. Therefore, anthocyanins may serve as a major agent in preventing heart disease as they appear to act as dietary antioxidants. Kamei H., (Kamei) also showed the red wine extract to slow growth of the human colon cancer cell line HCT-15. Kong (2003) suggested that antioxidant activity affects human LDLs *in vitro*. Studies with Cabernet red wine showed that streptozotocin lowered the sugar concentration in urine and blood serum and also halted the production of free radicals in rats with diabetes. The antioxidant properties of anthocyanins are a function of their ability to donate hydrogens and electrons, chelate metals, and bind proteins. Additional benefits attributed to anthocyanins include inhibiting the effects of nitric oxide (NO) and reducing ulcers (Wang, 2002). In light of the important roles anthocyanins play across

kingdoms, the current studies may have importance beyond the role of understanding light signaling and carbohydrate regulation of gene expression.

II. Sugars and Anthocyanins

Many factors are involved in the accumulation of anthocyanins, including light quality, quantity, and duration, as well as nutrients the plant takes up from the soil or accumulates through its own metabolic processes. While nitrogen and exogenous sugars play a major role in the regulation of seedling growth, the role of nitrogen uptake in anthocyanin production remains controversial (Martin, 2002). Unpublished experiments from our lab (Estrella and Short, unpublished) indicate that available nitrogen strongly influences anthocyanin content in young seedlings, and both nitrate and ammonium forms are included in the standard Murishige and Skoog (MS) media used in all experiments.

Different sensing systems are used to detect mono and disaccharides in plants. Xiao (2000) proposed that glucose can be taken up and used independently by at least three signaling pathways . The first is an AtHXK1 (*Arabidopsis thaliana* hexokinase 1)-dependent pathway which directs gene expression via correlated AtHXK1-mediated signaling. The second is a glycolysis-dependent pathway that is facilitated by the catalytic activity of AtHXK1. The *AtHxk1* mutant can be rescued by the heterologous yeast Hxk2 protein. Finally, there is a AtHXK1-independent pathway that controls genes

such as CHS and PAL1—both enzymes that are required for anthocyanin biosynthesis. Sugar metabolites are well known to act as signaling molecules in multiple pathways that regulate patterns of gene expression in a coordinated fashion(Harrington, 2003).

Sugar enhances the phyA-mediated enhancement of anthocyanin accumulation but suppresses the phyA-controlled FR-preconditioned block of greening (Barnes et al., 1996; Dijkwel et al., 1997b). Experiments with a series of mutants known as *sucrose-uncoupled (sun)* showed that both FR light and sucrose affect anthocyanin accumulation. Sucrose appears to stimulate SUN7 as a negative regulator of cotyledon opening and inhibition of hypocotyl elongation, yet acts as a positive regulator in the block of greening. SUN6 appears to negatively regulate the block-of-greening response (Dijkwel et al., 1997a).

An additional pathway affected by sugars is the production of β-amylase in *Arabidopsis*. It is speculated that HBA1 maintains β-amylase at a low-level. Until exogenous sucrose levels rise, LBA1 and LBA2 are stimulated and increase β-amylase expression (Mita, 1997). *hba1* (higher β-amylase 1) recessive mutants show increased anthocyanins in the petioles. It is clear that sugar signaling influences a diverse array of pathways.

III. Other Sugar Sensing Mutants

The subject of the present study is the effects of light signaling and carbon source availability on anthocyanin accumulation. Various *Arabidopsis* mutants have been isolated based on defects in sensing or responding to exogenous sugars.

Glucose affects a variety of processes within a plant. While glucose signaling and glucose metabolism appear to be uncoupled in many instances (Harrington, 2003; Moore B, 2003), the regulatory mechanisms underlying monitoring of internal glucose and external sugars is poorly understood. There is evidence that glucose signaling is mediated in part through hexokinase 1 (*At*HXK1), but it is unlikely that this is the only mechanism (Harrington, 2003; Moore B, 2003).

The glucose-insensitive (*gin*) mutants have been used to elucidate sugar signaling in *Arabidopsis*. The *GIN1* gene product mediates plant development in response to the antagonistic roles of both sugar (glucose) and hormones (ethylene) (Zhou, 1998). The *gin2* mutant appears insensitive to the hormone auxin, yet is hypersensitive to the hormone cytokinin (Moore B, 2003). Both *gin5* and *gin6* show that glucose regulates ABA (absisic acid) levels through *GIN5* and also regulates ABA signaling through *GIN6/ABI4* (Arenas-Huertero, 2000).

Sugar-insensitive (*sis1*) was isolated in a screen for plants which are insensitive to the typical inhibitory affects of a high level of soluble sugars in early seedling development(Gibson, 2001). *sis4* and *sis5* were revealed to be allelic to the ABA-biosynthesis mutant *aba2* and the ABA-insensitive mutant *abi4*, respectively. All of these mutants displayed decreased inhibitory affects of mannose as well (Laby, 2000; Gibson, 2001).

G. CONCLUSION

Studies of various mutants affirm that the signaling networks for plant development are intricately interconnected and tightly regulated. While light and sugar signaling are intertwined with anthocyanin and chlorophyll accumulation, sugar and light pathways are also modulated by hormones, which may also affect anthocyanin and chlorophyll accumulation. However, in the sugar-sensing mutants isolated thus far, neither altered anthocyanin nor chlorophyll phenotypes have been reported, indicating that the mutations may be involved in pathways separate from the branches of sugar signaling.

The present discussion on some of the complex signaling involved in *Arabidopsis* de-etiolation merely brushes the surface of how these pathways merge and interact. Stemming from the known information regarding the physiology of phytochromes, anthocyanin accumulation, and carbon uptake, experiments were designed to better understand the connections among these

interconnected pathways. The study includes optimization of a set of screening conditions to isolate mutants that are defective in light-regulated anthocyanin accumulation, results of screening a large population of mutagenized seeds, and physiological and molecular characterization of two of the isolated mutants designated *li*ght response *d*eficient (*lid)* 3 and 4.

CHAPTER 2. FORMULATING A SCREEN

A. ABSTRACT

One of the primary goals of this study was to create a screen to elucidate phytochrome and sugar signaling and their interactions to regulate anthocyanin accumulation. Previous reports implicate phytochrome and sugar as key players driving increased anthocyanin accumulation in young *Arabidopsis* seedlings. The objective was to optimize conditions for maximum anthocyanin accumulation and then further test the interactions of FR light, sugar signals and developmental stage as they affect anthocyanin content. Growth for 3 days (d) in 8.6 μmol m^{-2} s^{-1} FR on a medium containing 2% sucrose yields maximum accumulation of anthocyanins. FR light activates the phyA pathway and, in the presence of sucrose during a specific developmental window, causes *Arabidopsis* seedlings to accumulate peak levels of anthocyanin. These parameters were then used to initiate a screen for mutants affected in these pathways.

B. MATERIALS AND METHODS

Fluence Rate Responsiveness

The experiments shown were done on MS (Murashige & Skoog) media solidified with 0.8% agar and supplemented with 2% sucrose. Wild type Ws seeds were sown, given standard germination initiation conditions of 3 d at 4°C in D, followed by 3 h W and 21 h of D at 23°C. Plates with seeds were then exposed to intensities of FR light ranging from 0.0001-8.6 μmol m^{-2} s^{-1} using light

emitting diode sources (Quantum Devices, Barneveld WI) with λ_{max} of 735 nm and half bandwidth of 20 nm. Fluence rates up to 178 μmol m^{-2} s^{-1} were tested subsequently to confirm saturation (see chapter 3). Light spectral output was measured with a LiCor LI-1800 spectroradiometer (Lincoln, NE). After 3 d anthocyanins were extracted and quantified. One hundred seedlings per experiment were weighed and then anthocyanin was extracted with 1 ml acidic methanol (99% methanol and 1% HCl). After 24 hours of extraction in darkness at 4°C, the tissue was centrifuged at 14,000 *g* for 5 min and the supernatant was measured spectrophotometrically at 530 nm and 657 nm using a Spectronic™ GENESYSTM 5 (Thermo Electron Corp). Calculated values based on the formula: (A_{530}-(A_{657}*0.25)) were obtained and normalized to both the seedling number and the fresh weight. Each data point represents at least three independent experiments.

Optimizing screening conditions

Initial anthocyanin determination experiments were done with seedlings on mesh which floated in Magenta boxes (GIBCO) on liquid MS media supplemented with various sugars at a range of concentrations as indicated. Seeds were surface-sterilized, sown on a mesh boat and placed on appropriate liquid media inside the Magenta boxes. Boats were placed at 4°C in darkness for 3 d, transferred to W for 3 h to induce germination, then returned to D at 23°C

for 21 h. Mesh boats with seeds were transferred in the dark with sterilized forceps to the appropriate new Magenta box with the indicated liquid media, then exposed to the appropriate light conditions as indicated. Since 2-deoxyglucose and mannose are known to inhibit germination, sugars were added in complete darkness after germination had begun, immediately prior to transfer of the plants to FR.

Final screen conditions

The *lid3* mutant, along with a series of other putative mutants, was identified under 8.6 μmol m^{-2} s^{-1} FR light for 3 d on 2% sucrose and MS medium containing 0.8% agar. The progeny were rescreened in three successive generations, and backcrossed twice. Seeds were sown and plates were put in the dark for three days at 4°C. The plates then underwent three hours of W and 21 h of D before transfer to the FR light.

Screens for Reduced Anthocyanin Content

Seedlings exhibiting visibly reduced anthocyanin coloration in the initial screen were planted individually in soil, allowed to mature and self-fertilize, and seeds were collected and sown for confirmation of a heritable phenotype. Seedlings from each line were counted and weighed and then anthocyanin was extracted with 1 ml acidic methanol as described above. After incubation at 4°C for 24 h, the supernatant was measured spectrophotometrically as described. In

the refined second screen, visual phenotypes were scored for each of three generations and anthocyanin was then quantified for *lid3* and *lid4* as mentioned previously.

C. RESULTS—DETERMINATION OF SCREEN PARAMETERS

The first goal was to establish optimum screening conditions in which maximum anthocyanin accumulation could easily be visualized. Four basic environmental variables were tested:

1.) Fluence rate of FR light

2.) Organic carbon source provided exogenously

3.) Concentration of the carbon source being provided

4.) Developmental time course for anthocyanin accumulation

I. Optimizing FR Intensity for Maximum Anthocyanin Accumulation

The intensity of light can greatly affect the physiology of a plant. Chapter 1 discussed the different types of responses that can be elicited via varying intensities of light. In the present experiments, continuous FR light was used, activating the FR-HIR. A range of intensities were tested from nearly 10^{1} μmol m^{-2} s^{-1} down to 10^{-4} μmol m^{-2} s^{-1}. 8.6 μmol m^{-2} s^{-1} nearly saturated the level of anthocyanin accumulation, and therefore this intensity was used except where noted (Figure 1 and Chapter 3).

II. Determination of Anthocyanin Content with Various Carbon Sources and Concentrations

To determine the classes of carbon sources that would cause anthocyanin to accumulate at high levels, a variety of sugars and sugar analogs were tested to narrow the mechanism of increased anthocyanin seen with increased carbon availability. Three formal possibilities were assessed: 1.) The sugar activates a specific sensor, initiating a cascade of events leading to increased anthocyanin production or maintenance; 2.) the sugar acts non-specifically as a metabolic energy source for the seedlings, causing the observed response, or. 3.) the sugars cause a non-specific osmotic effect that stimulates anthocyanin accumulation.

Before addressing the first possibility, it should be emphasized that specific sensing systems for hexose and sucrose are known to exist in *Arabidopsis* (Smeekens, 2000). Thus, both monosaccharides and disaccharides were tested. The first question was whether the known hexokinase sensor had to be activated by a specific sugar or if in fact a cascade of events would flow after stimulating the sensor. Glucose, fructose, and galactose were used to activate this sensor, as well as two non-hydrolyzable sugar receptor activators, 2-deoxyglucose and mannose which both cause the activation of the hexokinase sensor but the plant cannot utilize these sugars efficiently as a carbon source. If anthocyanin accumulation increased with 2-deoxyglucose and mannose in the media then it

would seem that the simple activation of a hexose sensor could be the cause of increased anthocyanin content. 2-deoxyglucose was added at .02% (as opposed to 2%) because it has been shown to be effective at activating *At*Hxk1 at this concentration, and it is toxic at higher levels.

To test if any energy source, regardless of the type of sugar, could cause an increase in anthocyanin, sucrose, glucose, fructose, and raffinose were examined. If these sugars increased anthocyanin levels more than without sugar or with non-metabolizable sugars or analogs, it suggests that a carbon source is responsible for the observed increase in stored anthocyanins.

Adding a carbon source to a media will increase the osmotic movement out of the tissues which can stress the seedlings and alter responses in the developing seedlings. To insure that an osmotic effect is not the underlying cause of the accumulation of anthocyanin, a range of metabolizable and non-metabolizable sugars were examined. Mannitol and sorbitol are not easily transported into the roots, but will cause an equivalent osmotic effect to occur. Neither of these sugars appeared to induce increased levels of anthocyanin as compared to the control media (MS).

The results of these treatments (see Figure 2) indicate that sucrose yields the greatest amount of anthocyanin in the *Arabidopsis* tissues. Solely activating a sensor or introducing an osmoticum does not alone cause anthocyanin

accumulation. Additional energy sources of glucose and fructose did cause more anthocyanin to accumulate, but less than sucrose. This difference may result from differential transport or activation of a specific sucrose receptor. However, the simplest explanation is that sucrose is hydrolyzed into glucose and fructose, effectively doubling the molar concentrations of available monosaccharides in the tissue compared with equimolar concentrations of glucose or fructose alone.

III. Optimization of Sucrose Concentration to Yield the Greatest Anthocyanin Accumulation

Sucrose increases the anthocyanin content in tissues in a concentration dependent manner. Above approximately 4% sucrose, plants grow poorly and above 6% the plants will die. To determine the most effective levels of exogenous sucrose for inducing anthocyanin accumulation, a range of sucrose concentrations were applied, varying between 0-4%. With no sucrose added to the standard MS nutrient medium, anthocyanins did not accumulate in seedlings to any statistically significant levels. Any trace of anthocyanins detected is most likely a result of the seed coat whose brownish color is the result of anthocyanins. Increasing levels of sucrose up to 2% resulted in concomitant increases in anthocyanin content (Figure 3). At higher sugar concentrations, anthocyanin levels did not increase, indicating that the system was saturated.

IV. Effects of Developmental Stage on FR-Induced Anthocyanin Accumulation

After one day in FR, the emerging seedlings have barely broken the seed coat. Anthocyanin accumulation can be quantified, although most of the measurable pigment is residual anthocyanins from the seed coat. By day two, nascent seedlings have visibly emerged from the seed coat. Again, while anthocyanin accumulation can be assessed at this stage, many of the seedlings have not fully emerged and the attached seed coat still contributes significantly to the total pigment content, which may account for higher anthocyanin levels at day 2 (Figure 4). By the third day, seedlings have fully emerged from the seed coat and have actively growing hypocotyls. At this stage, anthocyanin accumulation is easily assessed and thus was chosen for our screen. Thus, while it appears from the data that the second day yields more anthocyanin than the third, the high variability and the contribution of the seed coat would have decreased the reliability of the screen. Beyond 3 d, the anthocyanin level drops rapidly, returning nearly to baseline levels after 5 d.

D. THE INTERACTION OF SUCROSE, FR LIGHT, AND DEVELOPMENT

I. Phytochrome and Sugar are Required Simultaneously

After determining that maximal anthocyanin accumulation could be achieved after 3 d of 8.6-μmol m^{-2} s^{-1} of FR light using media supplemented with

2% sucrose, it remained unclear whether sucrose and FR light were needed simultaneously for maximum anthocyanin production, or if either signal could persist, making the tissue fully competent to respond to the other input. Therefore, a system was devised that allowed for rapid changes of the growth medium, the light conditions, or both during the course of early development.

Initially, seeds were sown on a floating mesh "boat" in a sterile transparent Magenta box containing a liquid medium with 2% sucrose. After the standard treatment of 3 d at 4°C in darkness, 3 h of W, and 21 h of D, the boat was transferred in complete darkness to a new box containing medium lacking sucrose. This new box was transferred to FR light for 2 d or 3 d. The data show that the sugar signal wanes by the third day, yielding the same amount of anthocyanin as if the seedlings had never been treated with sucrose (Figure 5). Therefore, the sucrose signal can not persist to the 3 d developmental time where maximum anthocyanin normally is accumulated.

Next, seeds were sown on boats floated on liquid media without sucrose, and led through the same initial light conditions: 3 d cold, 3 d W, 21 h D. Seeds were then incubated in FR light for 2 d. An aliquot of sucrose was added in the dark to bring the sucrose concentration to 2%. Seedlings were then left in the dark for 1 d after which anthocyanin accumulation was measured, corresponding to a total developmental time of 3 d. These seedlings were not

able to accumulate anthocyanin above the levels found in 3 d D-treated seedlings. Interestingly, approximately the same amount of anthocyanin accumulated whether FR-grown seedlings received sucrose only on the final day of the three day time course or throughout the entire 3 d (Figure 6). These observations indicate that not only are FR and 2% sucrose necessary, but the developmental stage of the plant also plays a determinative role in anthocyanin accumulation.

II. Age Affects PhyA- and Sucrose-Induced Anthocyanin Accumulation.

To further test the importance of the age of the plant and the timing of FR and sugar treatment with regard to anthocyanin accumulation, a complex series of experiments was performed. As seen in the time course experiment, with one day of dark (germination initiation) followed by 1-5 days of FR exposure, peak anthocyanin levels appear at the 2-3 day developmental time period (Figure 4). What was not clear was whether the time course was reflecting the absolute or developmental age of the tissue, or whether the anthocyanin accumulation was a response to the length of exposure to FR.

Therefore, the effects of extending the dark period before administering FR light were tested to separate the effect of age of the *Arabidopsis* seedlings from those of the FR exposure time on anthocyanin accumulation. After a 1, 2, 3, or 4

d dark extension, a constant 2 d of FR was administered to the seedlings. As above, at 1 d dark and 2 d FR, again, maximum anthocyanin accumulation is seen in the RLD ecotype seedlings. With the 2 d dark extension followed by 2 d FR, anthocyanin accumulation drops dramatically, suggesting that the development of the tissue regulates the effectiveness of light and sugar inputs, and that the decrease in anthocyanin observed after 4 and 5 d exposure to FR is not a direct response to the light, but rather a developmentally regulated competence to respond to the ambient signals (Figure 7). By 3 d and 4 d of dark, followed by subsequent 2 d FR exposure, no anthocyanin can be detected in the seedlings, indicating that the critical responsive age of the seedlings has passed. Thus, the developmental stage of the plant is another critical factor that affects anthocyanin accumulation, in conjunction with phytochrome and sucrose.

E. Screening for Mutants in Light-, Sugar-, and Development-Regulated Anthocyanin Accumulation

To dissect the intersecting pathways yielding the observed physiological responses, a screen was initiated to detect those *Arabidopsis* seedlings which produced no or low anthocyanin under otherwise optimal pigment accumulation conditions. The seed lines screened included 196 T-DNA-insertion lines from the Koncz N4298 (Col-O background) pool, and the 6500 pools of 100 independent

lines from the Feldmann CS6502 (Ws background) T-DNA tagged lines. Additionally, 92J (EMS mutagenized RLD ecotype, Lehle Seeds) seed lines were also screened.

In the first screen, over 50,000 potentially T-DNA tagged or EMS mutagenized M_3 seedlings from the backgrounds indicated above were screened from seeds obtained from The *Arabidopsis* Information Resource (TAIR) seed stock center. Thirty-nine putative mutants were isolated, and anthocyanin was quantified in the progeny of selfed putative mutants; three of these putative mutants bred true for the low anthocyanin phenotype after more extensive screening (Figure 8). Of the three, none cosegregated with the T-DNA insert bearing hygromycin resistance. Two of the isolated mutants, designated *lid1* and *lid2*, were examined by Fang Qi, a Ph.D. student, in his dissertation work.

In a second screen, an additional 60,000 potentially T-DNA-tagged lines from the CS6502 set were screened (Table 1). Initially, 71 putative mutants were identified, grown to maturity, and selfed for retesting. Progeny of 35 of these lines maintained low anthocyanin, but of these putative mutants, only two bred true into the third generation and were designated *lid3 and lid4*. Neither mutant had the ability to grow on hygromycin, indicating the loss of the T-DNA insertion. Characterization of the physiology and genetics of these mutants is the focus for the remainder of this dissertation.

CHAPTER 3. Lid3

V. *lid3* is Deficient in Early Anthocyanin Accumulation

VI. Semi-Quantitative RT-PCR Data Further Suggests a Role for lid3 in Anthocyanin Accumulation

VII. The Potential Role of lid3

A. ABSTRACT

Myriad responses in plants cannot function without light. Plants have evolved photoreceptors to absorb light of specific wavelengths. Phytochromes control mainly R and FR photomorphogenic responses. Over the past few decades, a plethora of mutants have been used by plant researchers to begin unraveling these photosensory pathways, but much remains unclear. To understand the intricate pathways that influence phytochrome signaling, a genetic screen was initiated to find mutants that could be used to determine how FR light and sucrose affect anthocyanin accumulation.

The anthocyanin-deficient mutant *lid3* (*li*ght response *d*eficient 3) was identified in a screen designed to uncover links between phyA signaling, sucrose signaling, and anthocyanin accumulation. This mutant displays significantly less anthocyanin than wild-type at 3 d FR on a media of 2% sucrose. Normally, these conditions yield high levels of anthocyanin accumulation in young *Arabidopsis* seedlings.

Semi-quantitative RT-PCR shows that on 2% sucrose after 3 d of FR light, levels of the first major light-regulated anthocyanin biosynthetic enzyme, chalcone synthase, are present at wild-type levels in *lid3*. However, chalcone isomerase and dihydroflavonol reductase, downstream phenylpropanoid

biosynthetic enzymes, appear to be down-regulated in *lid3* compared with wild type, indicating a potential phytochrome A regulatory role at these steps.

In the wild type, RT-PCR quantitation of transcripts from plants grown with or without sugar indicates that sucrose is a necessary ingredient for accumulating transcripts of enzymes in this pathway. Without sucrose, wild-type levels of these transcripts appear less abundant than those from plants grown with sucrose. In fact, DFR mRNA is undetectable. The difference may indicate why wild-type seedlings grown without sucrose produce little or no anthocyanin, even with a functional lid3. In the absence of sucrose, *lid3* yields reduced CHS transcript levels compared with wild type, and both CHI and DFR messages are undetectable. These results suggest that while CHI and DFR transcript accumulation requires sucrose and a functional lid3 protein, sucrose and lid3 appear to be acting in their own separate signaling pathways.

Plants bearing the *lid3* mutation appear to have normal physiological phenotypes in R, B, W and darkness as well as the typical FR-preconditioned block of greening response. This otherwise normal light responsiveness indicates that *lid3* is a mutant specific to the phyA-regulated branch of a pathway involved in anthocyanin accumulation in the early development of *Arabidopsis* seedlings.

Rough mapping data positions *lid3* on the bottom arm of chromosome 1 between SSLP markers nga111 and nga280. The present analysis indicates the involvement of *lid3* in phyA signaling in determining the amount of anthocyanin synthesis visualized in young *Arabidopsis* seedlings. Although sucrose is a necessary component of anthocyanin biosynthesis, based on low mRNA transcript levels, it is unlikely that the lid3 protein directly interacts in the sucrose regulatory pathway. *LID3* may encode a novel light-regulated protein which affects flavonoid biosynthesis through control of CHI and DFR. The transcription levels of anthocyanin biosynthetic genes appear sucrose-dependent in both wt and *lid3*. Further experiments on *lid3* will help elucidate these important developmental pathways.

B. MATERIALS AND METHODS

Plant materials

Wild-type seed lines No-O, L*er*, RLD and Ws as well as *hy5* (*hy5-1*) and *phyA (phyA-101)* mutant strains were obtained from the *Arabidopsis* Seed Stock Center at The *Arabidopsis* Information Resource (TAIR; Columbus, OH). *lid3* is from a Koorneef T-DNA tagged Ws-2 background numbered CS2623.

Screen conditions for T-DNA-tagged insert

lid3 seedlings were grown on 0.8% agar plates with hygromycin to test for growth which would imply an intact T-DNA insert. However, the hygromycin

resistance marker associated with the T-DNA was not functional; *lid3* appears to have lost the insert and does not germinate in the presence of hygromycin.

Light sources

Light exposures to initiate germination and plant growth were from cool-white fluorescent bulbs. FR was from high-output, 735-nm LED sources (Q-beam 2001, Quantum Devices, Barneveld, WI). For chlorophyll synthesis inhibition experiments, FR light was filtered through far-red plastic resin (FRF700, Westlake Plastics, Lenni Mills, PA) to eliminate minor R emission from the LED source. Except as noted, the filtered FR was given at a fluence rate of 4 μmol m^{-2} s^{-1}. R was from 660-nm Q-beam LED sources (Quantum Devices, Barneveld, WI) at a fluence rate of 15 μmol m^{-2} s^{-1}. B was from cool-white fluorescent bulbs filtered through # 824 blue Plexiglas (8.6 μmol m^{-2} s^{-1}). Light intensity and spectral output were measured with a spectroradiometer (LI-1800, LI-Cor, Lincoln, NE).

Germination initiation

Seeds were surface sterilized for 20 min in a solution of 20% commercial bleach (final sodium hypochlorite concentration 0.05%[w/v]) and 0.01% SDS, then rinsed five times in sterile deionized distilled water. Seeds were sown on 100-mm Petri plates with sterile medium containing 0.8% (w/v) agar and MS salts (Murashige and Skoog, 1962) (Invitrogen, Carlsbad, CA). Additions of

sucrose or other carbon sources were added as per experimental requirements described. Plates were kept in darkness at 4°C for three days for stratification and then induced to germinate by exposure to cool white fluorescent light (approximately 75 μmol m^{-2} s^{-1}) for 3 h, then placed in the dark at 24°C for 21 h. Plates were then transferred to appropriate light conditions as indicated for each experiment.

Screen conditions

lid3 was identified as part of a screen under 8.6 μmol m^{-2} s^{-1} FR light for 3 d on 2% sucrose and MS medium containing 0.8% agar as described in chapter 2. Plants were backcrossed twice, and three generations of progeny were retested. Mutant seedlings were scored for anthocyanin accumulation under FRc as in the original screen.

Anthocyanin extraction

One hundred seedlings per experiment were weighed and then anthocyanin was extracted with 1 ml acidic methanol (99% methanol and 1% HCl). After 24 h of extraction in darkness at 4°C, the tissue was centrifuged at 14,000 *g* for 5 min and the supernatant was measured spectrophotometrically (ABS1: 530, ABS2: 657) using The Spectronic™ GENESYSTM 5 by Thermo Electron Cooperation (Madison, WI). (Madison, WI). Values were calculated based on the formula: (Abs1-(Abs2)*0.25). The calculated values per seedling

number and fresh weight were determined and graphed on Microsoft Excel. Each data point represents at least three independent experiments.

Chlorophyll measurements

Seeds were sown onto 0.8% agar-solidified MS medium without sucrose. Germination was initiated with 3 h W followed by 21 h of D at 24°C. Then plates were set under 4.3 μmol m^{-2} s^{-1} FR for 1, 2, or 3 d, and then brought out into the W for 5 d. Seedlings were then counted, weighed, and chlorophyll was extracted for 24 h at 4°C in 1 ml of 80% acetone. Tissue was pelleted at 14,000 g for 5 min and supernatants were measured spectrophotometrically as above. Total chlorophyll concentration was calculated by chlorophyll *a* + chlorophyll *b* using the formulas:

Chl $a = (12.7*A_{663})-(2.69*A_{645})$; Chl $b = (22.9*A_{645})-(4.48*A_{663})$

Each data point in Figure 12 represents the mean of at least three independent experiments. Chlorophyll content was normalized to seedling number and fresh weight.

Hypocotyl measurements

Seeds were sown on square petri plates on 0.5x MS media solidified with 0.8% agar. Plates were oriented vertically such that seedlings would grow up along the surface of the agar. All experiments began with 3 d of D at 4°C for stratification, then 3 h of W followed by 21 h of D to initiate seed germination.

Then various light conditions were administered and the 3 day-old seedlings were photographed with slide film adjacent to a ruler for calibration. Projected images were traced on a digitizer and Sigma Scan software (SPSS, Chicago) was used to calculate the hypocotyl lengths.

RNA extractions

Total RNA was extracted after pre-treatment with 3 d FRc on agar-solidified MS media with or without supplemental 2% sucrose. Seedlings were weighed and RNA was extracted according to the protocol of the Ambion (Austin, TX) RNAqueous small-scale phenol-free total RNA isolation kit. Total RNA in a 1/20 dilution was measured spectrophotometrically for purity and quantity at 260 and 280 nm, and the RNA integrity was confirmed by gel electrophoresis.

Semi-quantitative RT-PCR

Reverse transcription PCR of the extracted RNA was performed according to the instructions provided with the Ambion (Austin, TX) RETROscript RT-PCR kit protocol. 8 μl of RNA were used in a total volume of 12 μl and heated with supplied oligo(dt) at 70°C for 5 min. Samples were immediately placed on ice for 2 min. A 20 μl total volume RT-reaction mix was made and heated at 42°C for 1 h, then heated at 85°C for 5 min followed by standard PCR conditions. Primers were designed and used according to the procedure of Qi (Qi, 2004).

Mapping

The *lid3* mutant was crossed by Dr. L. Hobbie (Adelphi University) into Col-O ecotype which was also provided courtesy of his laboratory. F_1 plants were allowed to self and F_2 seeds were screened for reduced anthocyanin after growth in 3 d FRc on 2% sucrose MS salts medium. Approximately one week later, positive segregants were transplanted into soil (a mixture of 3 parts Sunshine Mix: 1 part Perlite: 1 part Vermiculite). Three days after bolting, DNA was extracted using the Sigma REDExtract-N-Amp Kit XNA-P. F_3 seeds were grown and retested to confirm that all F_2 parents chosen were homozygous for the *lid3* mutant locus. All F_2 positive homozygous recombinants were used in PCR-based simple sequence length polymorphism (SSLP) mapping. The *lid3* locus was mapped via genetic linkage analysis to known SSLPs and linkage to the bottom arm of chromosome 1 was confirmed. Additional mapping of other potential map locations yielded no linkage to any of these areas.

C. RESULTS

I. Isolation of the *lid3* Mutant

One of the main goals of the present study was to discover novel components involved in the phytochrome regulatory pathway and their interactions with sucrose signaling networks that regulate anthocyanin accumulation. After 3 d FR on 2% sucrose, a condition that specifically induces

anthocyanin accumulation through phyA, *lid3* was identified by its reduced ability to accumulate anthocyanins around the cotyledons and upper hypocotyl. This visible phenotype is displayed in Figure 9. The putative mutant was backcrossed twice into its wild type background, Ws, and retested for heritability three times. In addition, three generations of outcrossing allowed for segregation away from most secondary mutations in the *lid3* line.

F_1 seeds from the first backcross were grown up and F_2 seeds collected to be examined for *lid3* recessiveness. Chi square values were calculated from the segregating population revealing that *lid3* displayed a 3:1 ratio consistent with classic Mendelian genetics, implicating *lid3* as a single nuclear recessive trait (see Table 2).

II. Mapping lid3

To determine the nature of the *LID3* gene, genetic characterization and mapping were initiated. The homozygous *lid3* mutant in its native Ws ecotype was crossed into the Columbia ecotype. F_1 seeds were allowed to grow and self-fertilize to obtain a segregating population. F_2 seeds were collected for screening and obtaining an F_2 mapping population. As in the backcross to Ws, these seeds maintained their mutant phenotype in a typical 3:1 Mendelian ratio after 3 d FR on 2% sucrose-supplemented MS medium (see Table 3).

Seedlings exhibiting the *lid3* low anthocyanin phenotype were chosen and transplanted to soil. DNA extracted from an excised leaf of each recombinant plant was used as the template for SSLP mapping. The recombination frequencies showed linkage of the *LID3* locus to flanking SSLP markers nga280 and nga111, located on the bottom arm of chromosome 1. Table 4 displays a summary of the *lid3* mapping data.

F_3 seeds were collected from the F_2 plants and retested for low anthocyanin in all progeny to confirm homozygosity of each recombinant line. The F_3 data confirmed the *LID3* rough map position (see Figure 10).

III. *lid3* Displays Normal Physiology in White Light and Darkness

Visual phenotypes were assessed for *lid3* in both etiolated and de-etiolated seedlings. When grown in W, *lid3* is indistinguishable from the wild type (Ws) displaying the typical de-etiolated appearance of short hypocotyls and green expanded cotyledons. In the dark, *lid3* is also indistinguishable from Ws, each having the typical etiolated phenotype of long hypocotyls, closed cotyledons and an apical hook.

IV. Hypocotyl Length Is Unaffected in FR, R, or B

Due to its ease for visual screening assessment, hypocotyl length has been a means of identifying components of a number of signaling pathways for decades, including phytochrome signaling.

To examine whether other FR-mediated responses are affected by a mutation of the lid3 protein, phyA-controlled inhibition of hypocotyl elongation was evaluated in 3-day-old FR-grown seedlings. Hypocotyl length in FR was comparable to that of wild type Ws (see Figure 11). No apparent effect of the *lid3* mutation on apical hook opening, cotyledon separation, or cotyledon expansion was observed

Next, hypocotyl lengths in R were measured to explore possible links to the phyB pathway. PhyB plays a role in hypocotyl inhibition in R. To determine whether responses regulated by phyB or other stable phytochromes may also be affected by lid3, hypocotyl elongation was tested under R for differential growth. The *lid3* mutant displayed wild-type lengths (see Figure 11).

Phytochromes have a low but significant absorbance in the B region of the spectrum. B photoreceptors cry1 and cry2 play photomorphogenic roles by inhibiting hypocotyl elongation. Quantitative measurements of hypocotyl lengths grown for 3 d in B yielded no significant difference between wild type (Ws) and the *lid3* mutant (see Figure 11).

V. Mutations in lid3 Do Not Affect the FR-Preconditioned Block of Greening Response

After 3 d FRc and subsequent exposure to W, wild-type *Arabidopsis* seedlings remain pale and soon die. This effect is caused by phyA, which blocks

transcription of NADH-protochlorophyllide oxidoreductase, (PORA, PORB), the final step in chlorophyll biosynthesis. The *lid3* mutant under these conditions also remains pale and does not green-up. A *phyA* null mutant exposed to FRc for three days and then brought into the W for 3 d will green-up because no phyA is present to inhibit POR transcription (Barnes et al., 1996). This lack of greening response, however, can be rescued if the media contains sucrose (see Figure 12).

VI. *lid3* Maintains Wild-type Levels of Chlorophyll Biosynthetic Enzyme Message Levels

To further characterize the observed phenotypic response, wild-type, *lid3* and *phyA* seedlings were grown under FRc for 3 d and subsequently exposed to W for 5 d. RNA was extracted and RT-PCR results confirmed that *lid3* lacks PORA and PORB expression under these conditions, just as wild type does, while *phyA* expresses both of these transcripts (see Figure 13). RUBISCO (RBCS) was used as the control.

VII. *lid3* Has a Developmental Deficiency in Anthocyanin Accumulation

As shown in the previous chapter, 2% sucrose is needed to obtain maximal anthocyanin accumulation. To determine the role of development in accumulation of anthocyanin in the *lid3* mutant, a time course examining Ws, *lid3* and *phyA* (*phyA-101*) seedlings was used to monitor anthocyanin accumulation

over the course of 1-5 days of growth in FRc on 2% sucrose-supplemented MS agar plates (see Figure 14).

In FRc, phyA is the exclusive photoreceptor involved in anthocyanin accumulation. Therefore, *phyA* null mutants display almost no detectable anthocyanin under these growth conditions. At days 2-4, *lid3* displays significantly less anthocyanin than its wild type, Ws, but more than *phyA*. The most obvious discrepancy between Ws and *lid3* can be seen at 3 d. This finding indicates that there may be a blockage of flavonoid production that is most pronounced at the 3 d developmental stage of *lid3*, and that the regulatory step affected by this mutation is tied into a specific part of the developmental program. These results also show that *lid3* can produce anthocyanins (also visualized in the seed coat), but that the developmentally regulated accumulation at 3 d is deficient even under normally optimal conditions. Figure 15 reveals that at 3 d FR on 2% sucrose this lower level of anthocyanins is maintained at different FR intensities.

VIII. Lid3 Affects Light-regulated Abundance of Flavonoid Pathway Structural Gene Messages

To determine how lid3 regulates anthocyanin accumulation, the relative levels of several flavonoid biosynthesis gene transcripts for enzymes involved in anthocyanin biosynthesis were investigated. Key enzyme transcript levels such

as phenylalanine ammonia lyase 1 (PAL1), chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR) were examined using semi-quantitative RT-PCR on RNA from plants grown for 3 d in FRc both with and without supplemental 2% sucrose. CHS, CHI, and DFR are known to be regulated by phyA (Kubasek et al., 1992; Shirley et al., 1995). PAL1 is an early flavonoid biosynthetic enzyme in *Arabidopsis* that has not been shown to be phyA-regulated. Both PAL1 and UBQ were used as controls (see Figure 16). These results show that at 3 d FR, *lid3* contains normal levels of *CHS* mRNA, but reduced amounts of CHI and DFR messages. This differential regulation may implicate a light-dependent regulatory role for lid3 on CHI message levels, while the expression of DFR may result from a lack of CHI or its metabolic products, or may be affected by lid3 directly as well. As expected, the *phyA* null mutant yields reduced mRNA expression of all the enzymes tested, including CHS.

Without exogenous sucrose, the transcript levels of all three enzymes are either reduced or undetectable. In the wild type, DFR transcript is not evident, which may contribute to the lack of anthocyanins accumulated under these conditions.

D. DISCUSSION

PhyA is the exclusive photoreceptor involved in early flavonoid biosynthesis in FR light. Increased carbon availability contributes greatly to

increased anthocyanin levels, but is not sufficient in the absence of a FR signal. These finding, determined from our previous data, enabled the design of a novel screen to isolate several mutants, including *lid3*. Phytochrome signal transducing elements that affect anthocyanin accumulation have begun to be identified, yet much is still unclear. Lid3 appears to be a novel light regulated factor which acts independent of sucrose signaling based on RT-PCR data which shows that with no sucrose, the level of CHS is less in both the wild type and the mutant *lid3* and that in general, *lid3* appears to have enzyme message regulation that is more similar to *phyA*. The regulated converging of phytochrome A and sucrose pathways, with the direct or indirect aid of lid3, into the flavonoid biosynthetic pathway at the appropriate time and developmental stage allows the accumulation of anthocyanin in young *Arabidopsis* seedlings.

I. *lid3* Is Not a *phyA* Allele

Once *lid3* was isolated, it was crossed into *phyA* to ensure that what was selected was not a weak *phyA* allele. F_1 seedlings of *phyA* X *lid3* display a wild-type phenotype and in the F_2, a segregating population of *phyA*, *lid3*, and wild-type are easily distinguished. Based on this complementation test, *lid3* is not an allele of *phyA*. Subsequent mapping data described below support this finding.

II. *lid3* Is Not an Allele of *transparent testa (tt)* Genes

Various previously reported mutants are also deficient in anthocyanin accumulation. The determination that *lid3* is not of the class of mutants known as *transparent testa* (*tt*)—so named because they lack anthocyanin pigmentation in their seed coat—was confirmed by several means. This group of mutants is easily identified simply by their lightly pigmented seed coats, which have a yellow to light brown color. This coloration is in contrast to that of normal seeds, which exhibit a deep brown color. This light color results from the inability of these mutants to produce anthocyanins at any time. *lid3* displays a rich brown seed coat indicating it does have the capacity for anthocyanin production in other tissues, at other developmental stages, and under different ambient growth conditions. Additionally, based on RT-PCR results and accumulation of anthocyanins in the seed coat, it is unlikely that there are mutations in loci *TT4* and *TT3* which control CHS and DFR, respectively. It is unlikely that *LID3* contains a point mutation at these loci because that would affect anthocyanin accumulation throughout the development of the plant. As shown, lack of anthocyanins in *lid3* appears only in 3 d old seedlings under the screen conditions applied. In the *lid3* mutant, both of these enzymes are expressed on 2% sucrose, although at reduced levels. Finally, as described below, *lid3* does not map to a position corresponding to any of the described *tt* loci.

III. Lid3 Maps to the Bottom Arm of Chromosome 1

Recombination frequency data link *lid3* to markers on the bottom arm of chromosome 1 between genetic SSLP markers nga280 and nga111 at a position of approximately 100cM. Further mapping to SNP markers between the Ws background ecotype of *lid3* and the outcross ecotype Col-O consistently link *LID3* to this position. Primers for SNPs located within the region of interest were designed in sets of four using the ARMS-PCR protocol of Ye *et al* 2001(Ye, 2001). They include: F15H21 (SNPs: 70303, 89249, 9766), F4N21 (SNPs:16031, 44198, 70303, 89249,90488,90792,9766), F23C21 (SNPs:, 2027, 1894, 2276, 4424, 10153, 10102), T4M14 (SNPs: 25976,26331), T27F4 (SNPs: 36683, 36723, 38959, 38067,36593,36590), F9N12 (SNPs: 45309,57301,43191,43418,43271,43159). Results of the SNP linkage analyses are consistent with *LID3* mapping to a position around 100 cM on chromosome 1. 2 SNPs (2027, 4424) located at approximately 85 cM on chromosome 1 showed a recombination frequency of 15 cM (see Table 4).

No other anthocyanin-deficient mutant has been reported to map near this region. The ICX1 gene is somewhat close to this region (map position 91cM) but loss-of-function mutations in this gene yield increased levels of CHS expression in white light (Wade et al., 2003). The *icx1* phenotype appears inconsistent with that observed in *lid3*, but it cannot yet be ruled out as a candidate gene.

IV. *lid3* Is Not Defective in Other phyA Photomorphogenic Responses

a. Hypocotyl elongation inhibition

To ensure that lid3 is truly mediating exclusively through phyA signaling and not pathways from other photoreceptors, we examined other photomorphogenic responses in the *lid3* mutants. The inhibition of hypocotyl growth is a physiological response that is controlled by several photoreceptors, and the relative elongation depends on the light conditions. To define more precisely the role of lid3 in light signaling, and to confirm that lid3 acts exclusively in phyA signaling, hypocotyl lengths in W, D, FR, R, and B were measured. In all five conditions *lid3* displays hypocotyl lengths not significantly different from those of the Ws wild type. This result indicates that *lid3* is not in a branch of the phyA pathway that regulates hypocotyl inhibition, nor is it involved in hypocotyl elongation responses originating from the other photoreceptors. Taken together, *lid3* does not appear to influence hypocotyl growth under phyA, phyB, or cryptochrome control.

b. FR-preconditioned Block of Greening

In the FR-preconditioned block of greening response, *lid3* has normal functioning of phyA which blocks PORA and PORB at this stage. This finding indicates that *lid3* is uncoupled from this branch of phytochrome signaling. After 3 d of FRc and 5 d of W, *lid3* seedlings cannot green-up. Additionally RT-

PCR results indicate that PORA and B are not expressed at this time in *lid3*, comparably to wild-type and opposite from *phyA*. Therefore the signaling pathway in which phyA mediates chlorophyll production is also unaffected in *lid3*. These data further suggest that if *lid3* is a phyA signaling component, it is exclusive to anthocyanin accumulation.

V. *lid3* Is Deficient in Early Anthocyanin Accumulation

lid3 was isolated phenotypically as an anthocyanin-deficient mutant. Of the various parameters tested, the only condition in which *lid3* could be distinguished from the wild type was at 3 d FR on 2% sucrose, where anthocyanin levels are significantly lower in the mutant compared to those in Ws. Recent studies indicate that anthocyanin accumulation in FR is regulated by the photoreceptor phyA, and our data support the hypothesis that *lid3* is in this pathway.

Additional molecular experiments using semi-quantitative RT-PCR suggest a role for *lid3* in mediating CHI and DFR in conjunction with sucrose availability. CHS levels appear to be at normal levels on 2% sucrose, implying that the production of anthocyanin is not simply a linear progression based on buildup of upstream substrate as discussed by (Dooner et al., 1991). The fact that the message levels of these genes are low in *lid3* and in *phyA* in the absence of sucrose suggests that lid3 may be involved in phyA signaling. In addition, these

data confirm that sucrose plays an integral role in anthocyanin accumulation, and that it acts at least in part by regulating the transcript levels of these enzymes. Finally, these data suggest that sucrose is necessary for the action of lid3 in promoting anthocyanin biosynthesis at the level of message abundance for key metabolic enzymes.

VI. Semi-Quantitative RT-PCR Data Further Suggests a Role for lid3 in Anthocyanin Accumulation

Our data support lid3 as a light-regulatory component, and with the availability of sucrose as a carbon source, it may aid in up-regulation of anthocyanin accumulation. W and D physiological experiments demonstrate that *lid3* is part of a specific light-dependent response. Because the *lid3* phenotype is only observable in FR light, it is likely that *lid3* is exclusively regulated through phyA signal transduction. Until the *LID3* gene is identified, it will remain uncertain whether the light-dependence is through post-translational regulation of pre-existing lid3 protein or through regulation of *LID3* gene expression.

In wild-type plants grown without exogenous sucrose, messages for CHS and CHI are detectable, albeit at much lower levels than in plants on 2% sucrose. On the other hand, DFR message is not detectable without sucrose added to the medium. The previously postulated idea that there is a coordinated regulation

of flavonoid biosynthetic enzyme abundance regulated through substrate abundance appears to be in question based on our data. Instead, it appears that individual enzymes are regulated transcriptionally by various inputs, and that these inputs (sugar, light, development) affect message levels corresponding to distinct sets of enzymes. The absence of DFR transcript—as well as the reduction in CHS and CHI transcripts—sheds light on how sucrose regulates the amount of anthocyanin in the tissues.

Qualitatively, the *lid3* mutant largely mimics the effect of a *phyA* mutation on abundance of CHS, CHI, and DFR message levels. In most cases, the quantitative reduction in message level is less severe in *lid3* than in *phyA*, however. The only exception to this trend is that on 2% sucrose, CHS mRNA levels in *lid3* more nearly match those of wild type than of the *phyA* mutant. This anomaly may indicate the existence of somewhat parallel branches linking phyA to different biosynthetic enzymes, which are affected differently by inputs such as sucrose availability. The fact that *lid3* does contain detectable messages for CHI and DFR may also be a function of the comparatively brief developmental window in which lid3 affects anthocyanin accumulation. These mRNAs may be vestiges of transcriptionally downregulated genes or newly made products of the genes that are induced by a lid3-independent mechanism. Regardless of the molecular mechanism, lid3 appears to have a regulatory function on enzymes of

the anthocyanin biosynthetic pathway by increasing CHI and DFR transcript levels. Whether lid3 is a transcription factor or acts indirectly to increase mRNA abundance remains to be determined.

VII. The Potential Role of lid3

PhyA mediates photomorphogenesis of young FR-grown seedlings which affects at least three phenotypically distinct responses: anthocyanin accumulation, FR-preconditioned block of greening, and inhibition of hypocotyl elongation. All these responses are readily observable and immediately apparent when comparing wild type with a *phyA* null mutant. Mutants that display intermediate phenotypes or are affected in specific subsets of these responses have been used for dissecting the phyA signal transduction network.

The present data show that *lid3* functions exclusively in FR light-regulated responses, and is deficient only in the pathway which involves anthocyanin accumulation at 3 d; this phenotype is mediated through the photoreceptor phyA at this stage. We hypothesize that *lid3* is active at the branch of a phyA signal transduction pathway exclusive to anthocyanin production. The *lid3* mutant phenotype can only be observed in FR on a media with 2% sucrose, and semi-quantitative RT-PCR data suggest a role for lid3 in the up-regulation of CHI and DFR transcripts. Wild type under the same conditions shows significantly higher transcript levels for CHS, CHI, and DFR and displays the resultant higher

anthocyanin accumulation. Based on the extremely low wild type anthocyanin accumulation when sucrose is omitted, it is apparent that both inputs are necessary. The possibility of a novel regulatory system emerges from the wild type RT data. Without sucrose, a block on anthocyanin accumulation apparently exists, at least at the level of DFR mRNA, specifically indicating that even with the accumulation of upstream enzyme messages—and presumably of their enzymes and enzymatic products—DFR message remains absent. Thus, if message levels roughly approximate enzyme levels, the anthocyanin biosynthetic genes are not solely controlled by substrate availability.

Without sucrose, RT-PCR products using the *lid3* mutant mRNA templates are more similar to levels of the same messages in a *phyA* mutant. These data provide further evidence that *lid3* is involved in the light regulation of anthocyanin accumulation via the phyA pathway, rather than in the sucrose-dependent up regulation of these pigments. In the presence of sucrose, CHS mRNA expression levels are equivalent in wild-type and in *lid3*. However, in the absence of sucrose, CHS transcript levels are modestly reduced in *lid3* compared with wild type. Therefore, lid3 may play an active role in regulating this early step in flavonoid synthesis, but sucrose and/or other factors may overwhelm any affect of the lid3 protein on CHS message abundance. The fact that wild-type levels of CHS message are present in *lid3* plants grown on 2% sucrose—yet little

or no anthocyanin is detectable—confirms that CHS expression is not sufficient to instigate a metabolic cascade resulting in visible accumulation of anthocyanin in *lid3*. The *lid3* deficiency can be understood by measuring CHI and DFR message levels, which are down-regulated in the mutant even in the presence of 2% sucrose. Without functional lid3, the transcript level of CHI is reduced. This reduction may then lead indirectly to down-regulation of DFR, or lid3 may directly or indirectly play a role in the regulation of both of these enzymes. These data suggest that under conditions that would normally maximize anthocyanin accumulation in early development, the absence of functional lid3 protein prevents production of anthocyanins or, alternatively, promotes their degradation. This finding further coincides with the contention that the anthocyanin enzymatic pathway is not coordinately regulated simply by accumulation of biosynthetic precursors as in other plants, such as maize and petunia (Napoli et al., 1990; Dooner et al., 1991).

Regulation of these enzymes may also involve separate individual factors. Without sucrose, anthocyanins are not readily produced even in the wild type. This fact may indicate that lid3 activity or abundance is itself affected by sucrose availability, or that there is substantial post-transcriptional regulation of the pathway by sucrose. Sucrose appears to up-regulate the transcripts of all three enzymes tested in the wild type. Together, lid3 and sucrose are integral

components in establishing the full anthocyanin biosynthetic pathway at the 3 d developmental time period of *Arabidopsis* seedlings. They do not however appear to be regulated within the same pathway. Instead, lid3, (via phyA induction) and sucrose signals converge at the anthocyanin biosynthetic pathway at either individual or multiple points to enhance the accumulation of anthocyanins. Figure 17 displays a model with a possible role for lid3.

In the absence of sucrose, the expression of CHI and DFR in *lid3* and *phyA* mutants are comparable, suggesting that lid3 is part of the same FR light-regulated pathway. Under these conditions, wild-type seedlings still transcribe CHS, but levels of CHI are lower than those in seedlings with sucrose supplementation, and DFR accumulation is undetectable. This mRNA deficiency in the wild type is reflected in the paucity of accumulated anthocyanins in the absence of sucrose. In sum, both sucrose and lid3 are necessary but neither is sufficient to drive this pathway. Further mapping of the *LID3* gene and subsequent cloning will undoubtedly elucidate further the role of lid3.

Photophysiological data, RT-PCR quantitation of key flavonoid biosynthesis enzyme messages, and mapping data suggests that lid3 is a novel signaling component of a late phyA pathway that affects anthocyanin accumulation and appears to be influenced by sucrose availability.

CHAPTER 4. Lid4

D. DISCUSSION

I. lid4 is Not an Allele of phyA

II. The lid4 Gene Product Interferes with phyA-mediated Anthocyanin Accumulation

III. lid4 Attenuates the Block of Greening in FR similarly to phyA

IV. Lid4 Functions in Hypocotyl Growth Inhibition Mediated Only Through FRc.

V. Lid4 Function or Expression May Be Under Temporal Control

VI. Possible Function of lid4 in phyA Signaling

VII. Mapping of the lid4 locus is not Practical

A. ABSTRACT

The mutant *lid4* was isolated in the screen described previously, in which seedlings were isolated based on a phenotype of no or low anthocyanin in 3 d FR on 2% sucrose. Semi-quantitative RT-PCR data of major anthocyanin biosynthetic enzyme mRNA suggest that chalcone synthase (CHS) and chalcone isomerase (CHI) message levels are slightly lower in *lid4* than those in wild-type, whereas dihydroflavonol reductase (DFR) transcript levels are absent in the mutant. Without sucrose, the mRNA levels of all of these enzymes drop dramatically, indicating the necessity for sucrose signaling for observation of the anthocyanin phenotypes. Additionally, these data imply that *lid4* is acting most strongly in a light regulatory pathway.

In addition to exhibiting reduced anthocyanin levels, *lid4* also displays an elongated hypocotyl and closed cotyledons in FRc and is deficient in the FRc-preconditioned block of greening response; *lid4* will de-etiolate similarly to a *phyA* mutant. RT-PCR shows that *lid4* is unable to block PORA transcription, also comparably to a *phyA* mutant.

Complementation analyses show that *lid4* is not an allele of phyA. Based on the physiological data, lid4 appears to play an early role in three phyA

signaling pathways involving anthocyanin and chlorophyll accumulation as well as in hypocotyl elongation inhibition.

B. MATERIAL AND METHODS

Plant Materials and Growth Conditions

lid4 was identified in the same screening conditions as described in chapter 3. Briefly, seeds were sterilized and sown on MS media with 2% sucrose, given germination initiation conditions, and exposed to FR for 3 d. Mutagenized seedlings were screened for no to low visible anthocyanin. *lid4* was isolated from *Arabidopsis* Biological Resource Center seed pool CS2637, a T-DNA mutagenized seed line generated by Dr. K. Feldmann.

Genetic Analysis

To assess the dominance of the *lid4* allele, the homozygous mutant was backcrossed twice into its parental wild type, Ws. *lid4* was backcrossed twice and χ^2 scores of the F_2 populations were calculated. *lid4* was also crossed into the *phyA*-101 null mutant to determine whether *lid4* harbored a weak *phyA* allele.

Physiological Analyses and Semi Quantitative RT-PCR

Anthocyanin was quantified spectrophotometrically as described in chapter 3. RT-PCR primers were identical to those discussed in Qi (2004). Chlorophyll was quantified spectrophotometrically as described in chapter 3. RT-PCR on transcripts involved in chlorophyll accumulation was tested by using

the primers and method of Qi (2004). Hypocotyl measurements were performed as described in chapter 3.

Mapping

Outcrosses to No-O and L*er* ecotypes were generated for genetic linkage mapping of the *lid4* locus. SSLP- and SNP-based mapping were attempted in recombinant populations to both ecotypes. The F_2 population of the *lid4* X No-O crosses yielded ambiguous results so further testing was done with *lid4* X Ler. An F_2 mapping population was tested and SSLP mapping was initiated on those mutants which resembled a "pure" (tall, low anthocyanin, and appressed cotyledons) *lid4* mutant phenotype. The SSLP markers did not show linkage to any of the tested position in the *Arabidopsis* genome for *lid*4.

C. RESULTS

I. Isolation of the Mutant *lid4*

The isolation of *lid4* occurred under the same conditions as the previously described screen. In *lid4*, anthocyanin levels were barely visible. In fact, *lid4* looks superficially like a weak *phyA* allele, displaying not only reduced amounts of anthocyanin, but also an elongated hypocotyl and closed cotyledons under FRc; however, unlike *phyA*-101, no apical hook was present under these light conditions and sucrose concentrations (see Figure 18).

II. Complementation Analysis

Since phyA controls these responses to FRc, we crossed *lid4* with *phyA* to determine whether *lid4* is a novel allele of phyA. The *lid4phyA* F_1 heterozygote yielded a wild-type phenotype, indicating that the genes belong to different complementation groups. In the F_2 population we counted the segregation of *lid4*, *phyA*, and wild-type and found that, because of the similarity in their phenotypic nature, scoring was inconclusive. However, based on the F_1 wild-type phenotype, we tested this mutant further.

III. The *lid4* Mutant Phenotype is Not Observed in Wc or Darkness

To ensure that the mutant phenotype is light-regulated, we compared *lid4* mutants and wild type grown in Wc and in darkness. In either light treatment, *lid4* grows identically to Ws in terms of hypocotyl elongation, greening, cotyledon expansion and separation, apical hook opening, and anthocyanin accumulation.

IV. Backcross Analyses

Backcrosses into its parental ecotype, Ws, were used to determine whether the isolated allele of *lid4* is recessive. F_1 plants were allowed to grow-up, self fertilize, and F_2 seeds were collected. χ^2 analysis of the F_2 progeny did not diverge significantly from the expected Mendelian ratio of 3:1 in this first

assessment. A χ^2 statistic of 0.01 was calculated when comparing the observed and expected ratios in the F_2 segregation patterns. The homozygous mutant segregants from this analysis were again backcrossed to Ws, but yielded conflicting results. Some crosses yielded an acceptable χ^2 of 0.2 for the expected 3:1 ratio, implying that *lid4* is a recessive single-locus trait. These results, however, were inconsistent with the segregation patterns of subsequent crosses which gave a segregating population inconsistent with a single-locus recessive characteristic.

Backcrosses which yielded unexpected F_2 segregation patterns were inconsistent with typical Mendelian ratios. In prior crosses, the multiple *phyA*-like phenotypes cosegregated as expected from a single locus, whereas subsequent crosses yielded combinations of phenotypes indicative of multiple loci contributing to the phenotype. In an attempt to score the phenotypes in a 3:1 ratio, "short with anthocyanin" was considered wild and "tall with no anthocyanin" was counted as the mutant (see Table 5). However, seedling elongation behaved as a continuous quantitative trait, with a large proportion of seedlings falling between the categories of "short" and "tall". Similarly, anthocyanin accumulation was difficult to score reliably, with many seedlings accumulating intermediate levels of anthocyanins, and the pigment level did not correlate with the elongation response. To test whether the F_2 progeny could be

grouped into a two-locus recessive mutant model, resulting in a 9:3:3:1 ratio, they were scored as short/anthocyanin, tall/anthocyanin, short/no anthocyanin, and tall/no anthocyanin (see Table 6). This assignment, too, proved difficult to score given the varieties of phenotypic combinations visualized. Under this assessed segregation, a χ^2 statistic of 223 was calculated, indicating that the null hypothesis could be rejected and that the observed results are significantly different from the 9:3:3:1 expected. Similarly, when the parameters were adjusted to treat these segregating populations as a single-locus segregating population with an expected 3:1 ratio, the χ^2 value was 33, again indicating a significant difference between the observed and expected values. Additionally, continued growth of the lineage of a "pure" (tall, low anthocyanin, and appressed cotyledons) *lid4* phenotype yielded a continuous segregating population of phenotypes into F_3 and F_4 generations.

V. Assessment of Anthocyanin Accumulation in *lid4*

a. Anthocyanin quantification

To examine the accumulation of anthocyanins, *lid4*, Ws, and *phyA* were sown on MS plus 2% sucrose plates and, after stratification, were grown for 3 d in FRc. Anthocyanin was quantified, and the data suggest that *lid4* produces anthocyanin levels similar to those of *phyA*, both of which contain much lower levels than does Ws (see Figure 19).

b. Accumulation of mRNA for key flavonoid biosynthesis enzymes

In an attempt to identify how lid4 increases the levels of anthocyanins, and whether the mechanism is consistent by which phyA induces anthocyanin accumulation, we examined transcript levels of genes coding for several major anthocyanin biosynthetic enzymes using semi-quantitative RT-PCR, standardized to the constitutive ubiquitin (UBQ) mRNA level (see Figure 20). With RNA isolated from seedlings grown under standard screening conditions, slightly lower CHS and CHI expression was detected in *lid4* extracts compared with those of wild-type, but the difference is not as dramatic as for the DFR message, which did not accumulate in *lid4*. These data suggest that transcript levels for DFR—a key enzyme in anthocyanin biosynthesis—require the *LID4* gene product to maintain high levels of expression at the level of transcription or transcript stability. Message levels for CHS in *lid4* appear similar to those in *phyA*. For CHI, *phyA* does not maintain any detectable transcript, whereas *lid4* retains a low but detectable level. By the DFR stage, both *lid4* and *phyA* have lost any detectable RNA message.

Message levels for the first enzyme in flavonoid biosynthesis, PAL1, were unaffected by the *lid4* mutation or the *phyA* mutation. Transcript levels for these enzymes measured under the same light conditions without sucrose yielded a severe down regulation of all the tested flavonoid pathway mRNAs from CHS to

DFR. This finding suggests *lid4* is a light-regulated player and that sucrose induces up-regulation of all these enzymes, at least at the transcript level.

c. Assessment of anthocyanin in a FR time course

To assess whether the anthocyanin accumulation pattern deviated from that of wild type at a time other than 3 d, a time course for anthocyanin content was established from 1 d to 4 d in FR light on 2% sucrose (see Figure 19). Throughout the time course, *lid4* maintained anthocyanin levels consistent with those of *phyA*. That is, at no time during early development do anthocyanin levels become high. The fact that *lid4* follows a pattern similar to that of *phyA* provides further evidence that that the *LID4* gene product may be a component of phyA signaling.

d. Fluence response

Responsiveness to various FRc fluence rates may help define the role of lid4 in light regulation of anthocyanins. Fluence rates from 5 - 178 μmol $m^{-2}sec^{-1}$ were tested for their effectiveness in maintaining increased anthocyanin levels in wild-type and *lid4* seedlings. Anthocyanin accumulation saturated near the standard fluence rate of 8.6 μmol $m^{-2}sec^{-1}$, consistent with the original screen conditions as determined in chapter 2 (see Figure 21).

VI. Does *lid4* Retain the Ability to Produce Chlorophyll After FR Exposure?

a. Chlorophyll quantification

In 3 d FRc, the action of phyA blocks transcription of NADH-protochlorophyllide oxidoreductase (POR), an enzyme necessary for the final step in chlorophyll biosynthesis. When this inhibition occurs, seedlings brought into the W will fail to become green. This process can be overridden by exogenously provided sugars such as sucrose (Barnes et al., 1996). In the absence of a signal from phyA, as in the case of the *phyA*-101 mutant, POR transcription is not blocked and the seedlings retain the ability to green up under the aforementioned light treatment. *lid4* also has this ability, suggesting that it plays a role in the same segment of phyA signaling (see Figure 22).

b. Accumulation of mRNA for POR enzymes

To further test the greening response in *lid4*, semi-quantitative RT-PCR specific to each member of the POR gene family was done. Like *phyA*, *lid4* shows an up-regulation of PORA indicating that lid4 plays an inhibitory role in FRc in a similar fashion to phyA (see Figure 23).

VII. Hypocotyl Growth of *lid4*

The inhibition of hypocotyl elongation in FRc is regulated by phyA (Boylan and Quail, 1991). In Rc, growth is regulated by phyB and in Bc, by

cryptochrome. To determine whether the *lid4* mutant is affected in this growth response, hypocotyl lengths of mutant and wild-type plants grown under each of these three light conditions were measured.

In R and B, *lid4* produced hypocotyls comparable in length to those of wild-type seedlings (see

Figure 25 and Figure 26). However, in FR *lid4* hypocotyl lengths were intermediate between those of wild type and *phyA* (see Figure 24).

VIII. Mapping

To initiate mapping, *lid4* was first crossed into the No-O ecotype. F_1 plants were allowed to self-fertilize, and the resulting F_2 seeds were collected. Screening for the full *lid4* phenotype (tall, closed cotyledons, minute anthocyanin accumulation) proved difficult. Very few of these seedlings exhibited all of the expected characteristics. Instead, a variety of combinations of these phenotypes were displayed in the recombinants. Attempts to map the locus using only those individuals exhibiting the triple phenotype yielded no clear linkage to the locations of established SSLP markers.

Because of variability of light responses among different ecotypes, the No-O background may not be competent to display the full *lid4* phenotype in the F_2, so *lid4* was crossed into wild-type background, L*er*. In theory this background should at least show more polymorphism from Ws than does No-O. F_1 plants

were grown up, selfed, and F_2 seeds collected. The F_2 population segregated in a slightly atypical Mendelian 3:1 ratio (see Table 7). The χ^2 value was 5.5 with a p-value of 0.02. Again, *lid4* F_2 seedlings only exhibiting the full set of light response phenotypes were selected and used for mapping, but no obvious genetic linkage emerged from the analyses (see Table 8). Additional screening of positive F_2 seedlings in the F_3 generation was analyzed by both standard screen conditions as well as by ability to green after FRc treatment.

D. DISCUSSION

The *lid4* mutant was isolated based on our screen to find new components in *phyA* signaling that affect the pathway branch regulating the accumulation of anthocyanin in conjunction with sucrose. RT-PCR data confirmed that *lid4*'s low accumulation of anthocyanin is likely a result of down-regulation of key anthocyanin biosynthetic enzymes. Upon further physiological investigations, it became clear that lid4 also plays a role in other phyA-controlled physiological responses including the inhibition of hypocotyl elongation and the FRc-preconditioned block of greening. Measurements of specific mRNA targets by RT-PCR suggest a role for lid4 in blocking PORA accumulation in FRc light. Hypocotyl growth phenotypes in dark, white light, red and blue light are consistent with those in wild-type plants. However, FR is not able to inhibit

hypocotyl elongation fully, giving rise to a hypocotyl length between those of wild type and *phyA*.

IV. *lid4* is Not an Allele of phyA

While *lid4* shows so many of the visual phenotypes associated with a *phyA* null mutant, complementation analyses with the *phyA-101* null allele indicate that *lid4* does not correspond to an allele of *phyA* (ecotype RLD). F_1 progeny exhibit an apparently wild-type phenotype. The segregating population of F_2 seedlings proved difficult to evaluate because of the similar phenotypes observed between *lid4* and *phyA*. However, since F_1 plants show full complementation, it seems that the mutation in *lid4* is not in the *PHYA* gene. Further evidence lies in the recombination frequency 47.9cM of SSLP marker NGA59 which shows that *Lid4* is not linked to the top of chromosome 1 where the PHYA locus is found (see Table 8).

V. The *lid4* Gene Product Interferes with phyA-Mediated Anthocyanin Accumulation

Initially, we quantified anthocyanin accumulation after 3 d FR on 2% sucrose, the original conditions used for the screening of this mutant. Spectrophotometric data confirm the visual observation that *lid4* accumulates less anthocyanin than wild type. Additional molecular examinations were performed under the same conditions using semi-quantitative RT-PCR in order

to evaluate the mechanism of anthocyanin depletion. Anthocyanin biosynthesis is accomplished via a series of enzymatic steps. One of the first enzymes in this pathway is PAL1, which, although it is not phytochrome-regulated, it is a necessary initial component for the entire flavonoid metabolic pathway (Ohl et al., 1990). Products of PAL1 are also used for other physiologically important compounds, including alkaloids, simple phenolics, complex phenolics, and other phenylpropanoids. Levels of PAL1 at 3 d FR on 2% sucrose or without were equal in Ws, *lid4* and *phyA*, confirming that this enzyme is not phytochrome-regulated under conditions in which the anthocyanin content is strongly light-controlled.

An array of enzymes and molecules take part in the steps that lead from PAL1 to CHS, the first committed enzyme in the flavonoid pathway. While phyA signaling influences CHS mRNA content, sucrose also up-regulates the amount of transcript for this enzyme. CHI and DFR are involved further downstream in this anabolic chain. Transcripts for these enzymes are strongly upregulated under the screening conditions, whereas in the absence of sucrose little or no transcript can be detected by RT-PCR. This phenomenon is most prevalent in the wild type, but it is also seen in *lid4* and *phyA* (see Figure 20).

The RT-PCR data suggest that lid4 may play a regulatory role in the phenylpropanoid pathway starting at the CHS enzyme. The pattern of lowered

expression continues with CHI message levels and is even more pronounced for DFR transcript levels. Since sucrose stimulates CHS, as does phyA, we speculate that the *lid4* transcript level may be higher at this stage because of these factors. Without sucrose, the levels of all enzyme transcripts tested are much lower or non-existent in the wild type, suggesting that sucrose plays a role vital for anthocyanin accumulation at the mRNA level. While CHS levels are faint, CHI and DFR messages are undetectable without sucrose in Ws. CHS levels are even fainter in *lid4* than in Ws, and in both CHI and DFR mRNA transcripts can not be detected in the wild type, *lid4*, or *phyA*. It appears that with sucrose stimulating anthocyanin accumulation, the *lid4* mutation specifically inhibits accumulation of these late enzyme transcripts. Because the sucrose-regulated CHS messages are unaffected by the *lid4* mutation, it is unlikely that lid4 is involved directly in sugar signaling. On the other hand, the mRNAs downregulated in *phyA* are also reduced in *lid4*, suggesting that the *lid4* mutation is affecting an upstream light regulatory step.

The role of lid4 in anthocyanin accumulation appears to be strongly light-dependent. Although the presence of sucrose induces accumulation of anthocyanin biosynthetic enzyme transcripts, *lid4* still shows a reduction of expression of a subset of major anthocyanin biosynthetic enzyme transcripts under these conditions similar to—but not identical with—the effects of a *phyA*

mutation on these messages. The fact that some transcript is present is an indication that *lid4* is probably not of the *tt* mutant class, which is consistent with apparently normal anthocyanin content of the seed coats. *lid4* also does not appear to be a *sun* mutant based on its apparent specificity for the phyA regulatory pathway.

Although *LID4* does not appear to code for a gene encoding flavonoid biosynthetic enzymes, it may encode a signaling intermediate in the branch of the phyA transduction chain regulating flavonoid biosynthesis, based on its effect on transcript levels of CHS, CHI, and DFR and the similarity to effects of *phyA* on these mRNA levels. To further examine whether the *lid4* phenotype is consistent with a role in light signaling, anthocyanin accumulation was measured over a 5 d time course. These data showed that *lid4* follows a pattern similar to that of *phyA* and consistent with semi-quantitative RT-PCR data.

VI. *lid4* Attenuates the Block of Greening in FR Similarly to *phyA*

To examine the pleiotropy of the *lid4* mutation among recognized phyA-mediated responses, the FRc-preconditioned block of greening response was tested in the mutants and in wild type. A time course in FR light was performed to establish whether *lid4* alters the preconditioned block of greening response over a 5 d period. The *lid4* mutant largely phenocopies *phyA*, although after 4 d FR *phyA* retains its ability to green up upon transfer to W, whereas *lid4* only

prevents the block of greening through the first 3 d. This reduced responsiveness to FR is consistent with *lid4* behaving like a weak *phyA*-allele. Consistent with the other phenotypes observed in *lid4*, it does not appear that *LID4* encodes a chlorophyll biosynthetic gene because PORA in FRc is unregulated, and greening occurs normally in W. Transcripts for a series of enzymes involved in greening were examined to attempt to narrow the point of *lid4* deregulation of this pathway. Expression levels of chlorophyll genes including CAB (which encodes chlorophyll a/b binding protein), PORA, B, and C all show transcript levels equivalent to those in wild-type. While CAB, PORB and PORC mRNAs from *lid4* were on par with wild-type levels, the expression of one of the three *POR* enzymes, PORA, was upregulated in *lid4*, suggesting that lid4 is specifically involved in the normal phyA-mediated inhibition of PORA in FRc.

VII. Lid4 Functions in Hypocotyl Growth Inhibition Mediated Only through FRc

Visually, the normal FRc-mediated inhibition of hypocotyl elongation is defective in the *lid4* mutant. This *lid4* effect is typical of *phyA* loss-of-function mutants (Quail et al 1995). Quantitative measurements of the hypocotyls showed that the phenotype is less severe than in a *phyA* null mutant, but that the effect is significantly different from wild-type growth inhibition (see Figure 24).

To determine whether the difference results from a general growth cessation, as well as the light conditions that reveal the growth phenotype, hypocotyl lengths after growth in various light conditions were measured. In R, B, W, and darkness, the mean hypocotyl lengths of *lid4* were not significantly different from those of the wild type. These results affirm the role of *lid4* specifically in phyA-mediated signaling, and again indicate a weak *phyA*-like phenotype.

VIII. *lid4* Function or Expression May Be Under Temporal Control

lid4 was isolated in a screen where wild-type *Arabidopsis* plants will accumulate maximal levels of anthocyanins. The developmental stage of the seedlings, as well as external abiotic conditions to which the plant is exposed, is crucial for this increase. The *lid4* mutant under these conditions fails to accumulate high levels of anthocyanins, suggesting its integral role in the process of regulating anthocyanin production and/or degradation at this stage in development. However, it seems that the role of FRc (acting through phyA and lid4) in controlling anthocyanin content is extremely specific to this early developmental stage; if viewed later, no distinction in anthocyanin accumulation will be detectable because the wild-type seedlings degrade the stored pigments.

Despite the extreme nature of the phenotype in *lid4* mutants at early stages of development, the effects of the mutation are largely imperceptible in

more mature tissues. An adult *lid4* plant is indistinguishable from wild-type, as is a *phyA* null mutant.

IX. Possible Function of *lid4* in phyA Signaling

Physiological characterization of *lid4* suggests its probable role as a phyA signaling intermediate. Light has been shown to be a regulatory stimulus in anthocyanin production via anthocyanin biosynthetic enzymes such as CHS, CHI, and DFR (Christie and Jenkins, 1996). Previous reports of other phyA pathway mutants with low anthocyanin content show a decreased expression of the corresponding transcript levels(Wang and Deng, 2002; Wade et al., 2003). *lid4* also contains lower transcript levels of these enzyme mRNAs at 3 d FR on 2% sucrose, as analyzed by semi-quantitative RT-PCR (see Figure 20). These data imply that the lack of anthocyanin accumulation in *lid4* may be a result of direct or indirect regulation of genes encoding flavonoid biosynthesis via transcription of CHS, CHI, and DFR. Alternatively, lid4 may regulate degradation of the mRNA coding for these enzymes. Precise elucidation of lid4 functional mechanisms remains to be determined following cloning of the gene or genes.

X. Mapping of the *lid4* Locus is Not Practical

Despite initial data showing *lid4* corresponding to a recessive gene at a single genetic locus, further backcrossing data and outcrosses of *lid4* to various ecotypes, including L*er* and No-O, have yielded F_2 populations that are

inconsistent with this interpretation. Instead of the expected Mendelian 3:1 ratio in the F_2 generation, mapping populations did not conform to previous segregation (see Table 7).

Original F_2 visual scoring of anthocyanin accumulation, under the standard screening conditions, yielded the expected 3:1 ratio. However, upon closer examination, it was apparent that the pleiotropic characteristics of the *lid4* phenotype did not cosegregate as expected for a single genetic locus. Therefore, only those F_2 seedlings displaying the full range of "pure" *lid4* phenotypes (tall, low anthocyanin, and appressed cotyledons) were used in the mapping studies, under the assumption that if multiple loci were involved, all of the linkage groups would be identified in this population.

The puzzling circumstances of *lid4* continued when additional characterizations of F3 lines segregated into at least eight phenotypes. This left only 43 positive lines to map with out of an original mapping population of 108. F_3 lines were then chosen to test the F_4 population. Unfortunately, this led to continued segregation of the seed lines. The nature of the mutant seemed to expand into multiple unlinked loci.

Follow-up studies on this mapping population using an alternate phenotype—the FRc-preconditioned block of greening response—also yielded inconsistent segregation data and no significant linkage to any of the markers

tested. Because the same F_3 seed populations were used for the greening response as were used in the anthocyanin screen, comparisons between *lid4* lines identified for their "pure" *lid4* phenotype and *lid4* lines pulled out for their ability to green were examined for correlation of the two phenotypes. However, the responses did not reliably cosegregate, thus dwindling the original mapping population of 108 to 21 lines. Mapping was attempted with DNA lines which appeared positive for both anthocyanin and greening, but no map position was identified. As with the previous mapping attempts, however, the full genome was not fully examined because of a dearth of polymorphic SSLPs and SNPs between Ws and L*er*. Ultimately, all mapping attempts on *lid4* were then attenuated because of:

1.) Lack of confidence in *lid4* recombinants

2.) Odd segregation populations found in all out-crosses with *lid4*

3.) Inability to gain positive F_2 recombinants that did not segregate in the F_3 and F_4 populations.

While *lid4* phenotypically is an interesting mutant (or series of mutants), the inability to locate a locus or loci corresponding to its phenotype makes the line difficult to evaluate further. Additional characterizations of the *lid4* line will have to be put on hold pending evaluation of the curious disaggregation of the phenotypes. That multiple phyA-mediated phenotypes would be generated

through several separable loci seems beyond the credibility of coincidence, but there is insufficient information to understand the mechanism that might generate this confounding result.

Despite the possible effects of more that one locus or a possible epigenetic effect occurring in *lid4*, the importance of the physiological characterizations of this mutant are still prevalent. The *lid4* mutant phenotype can be generated again and again by selfing, indicating the hereditability of its traits over generations. The nature of the hereditability of these traits, caused by either combined mutated loci, or one locus contributing to the array of varied phenotypes, is testament that possible investigations, of an unknown nature at this point, in experimental techniques could prove useful and informative in examining the phyA light regulatory pathway. Until such analytical tools can be deduced, we can use the data presented here to formulate the existence of a novel upstream regulatory element or a series of elements, which aids phyA in its three major photomorphogenic responses: anthocyanin accumulation, chlorophyll FRc-preconditioned block of greening response, and hypocotyl inhibition.

Due to the difficult nature of *lid4*, specific placement in the phyA signal transduction pathway is a challenge. Based on physiological data, including RT-

PCR, it appears that lid4 is an upstream regulatory element in phyA-regulated photomorphogenesis.

Until an explanation of the odd segregation of *lid4* can be found or the results better interpreted, mapping can not be pursued and thus, further characterizations of this intriguing mutant are impractical.

TABLES AND FIGURES

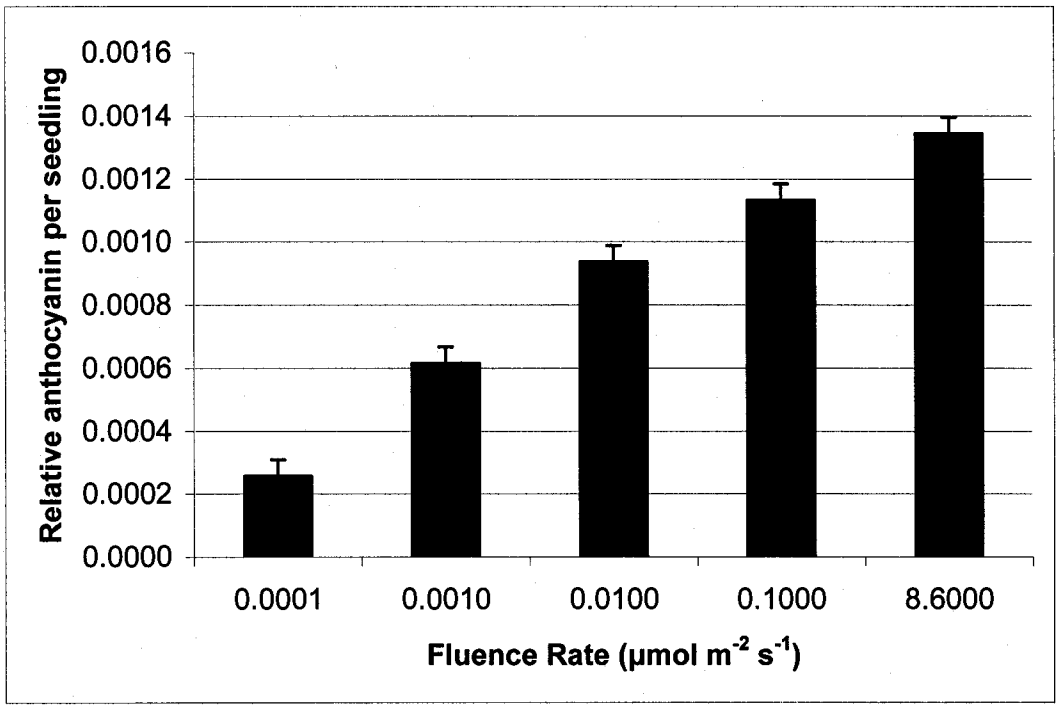


Figure 1. Maximum anthocyanin accumulation occurs at 8.6 µmol m^{-2} s^{-1}. Intensity experiments were done on MS (Murashige-Skoog) media with 0.8% agar and 2% sucrose. Wild type RLD seeds were sown, given standard germination initiation conditions of 3 d cold/D, 3 h W, followed by 21 h of D. Plates with seeds were then exposed to intensities of FR light ranging from .0001-8.6 µmol m^{-2} s^{-1}. After 3 d, anthocyanin was quantified spectrophotometrically. Error bars represent +/- 1 S.D.

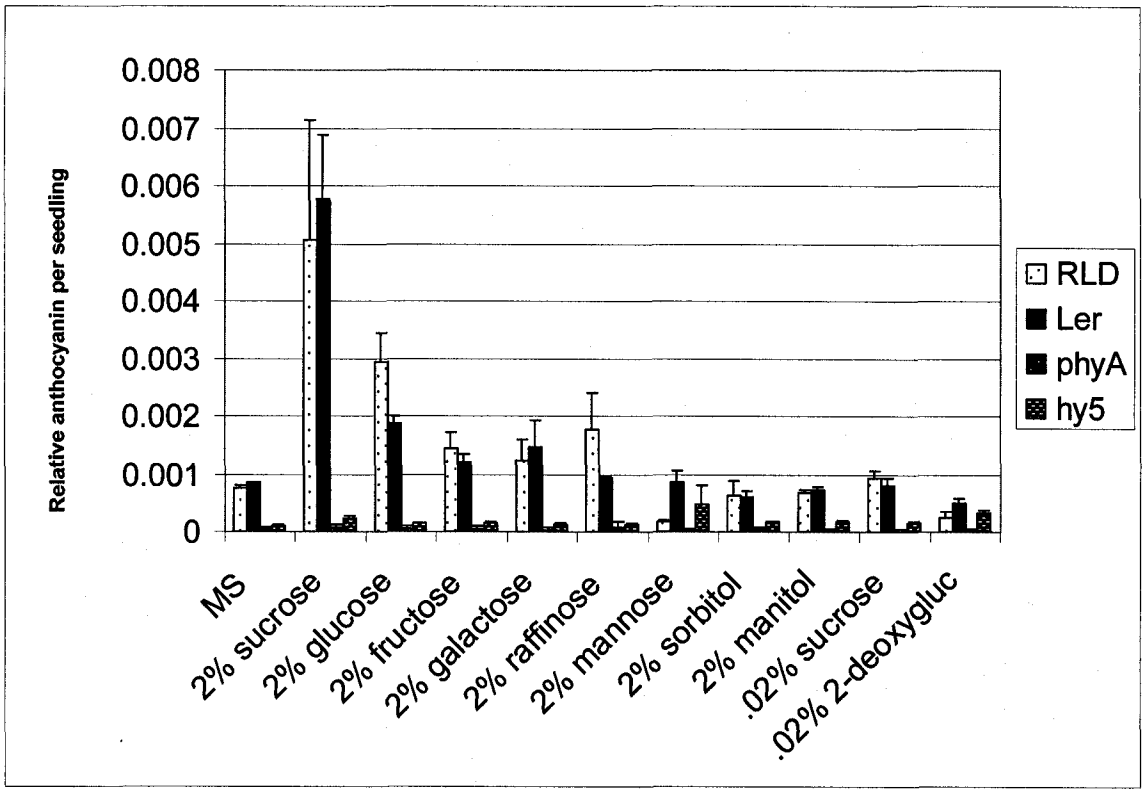


Figure 2. Maximum anthocyanin accumulation occurs on 2% sucrose.
On a liquid media of MS, seeds of wild types RLD and L*er* and mutants *phyA* and *hy5* were sown on mesh boats and placed in magenta boxes. Boxes were then incubated in the dark at 4°C for 3 d, the exposed to 3 h of W, followed by 21 h of D to induce germination, and then 3 d FR light to induce anthocyanin accumulation. Sugars were added after 1 d in the FR because 2-deoxyglucose is known to inhibit germination. After 3 d, anthocyanin was quantified and measured as mentioned in the text. Error bars represent +/- 1 S.D.

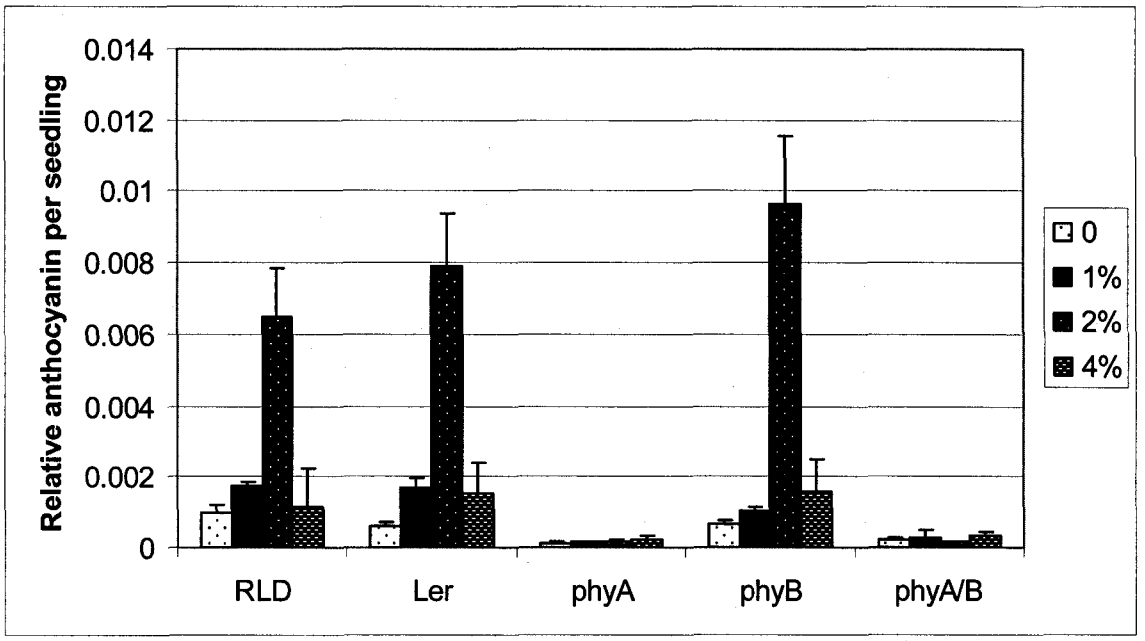


Figure 3. 2% sucrose yields maximal anthocyanin accumulation in FR. Seeds were sown on MS media with 0.8% agar and 0-4% sucrose. Wild type RLD and L*er* and mutants *phyA, phyB* and *phyA/B* seeds were sown, given standard germination initiation conditions and then exposed to FR for 3 d. Anthocyanin was quantified as stated in the text. Error bars represent +/- 1 S.D.

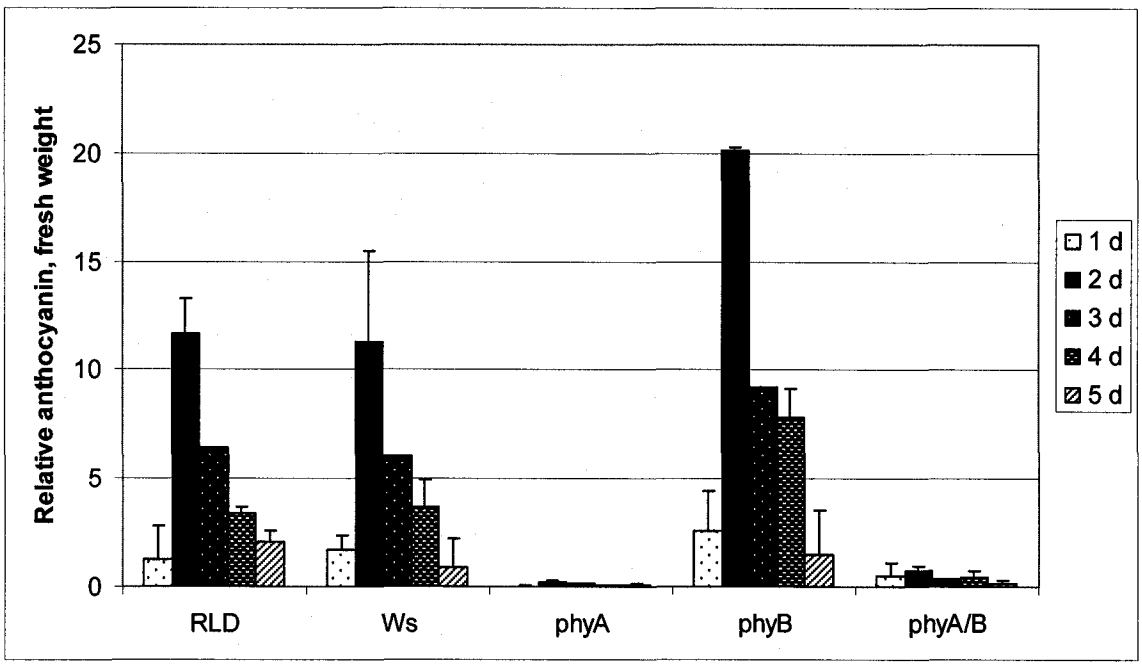


Figure 4. 3 d FR on 2% sucrose is the optimum time for assessing anthocyanin accumulation.

Wild types RLD and Ws, and mutants *phyA, phyB,* and *phyA/B* were sown on a media of MS plus 2% sucrose. After germination initiation conditions, seeds were left in the FR for 1-5 d and anthocyanin was quantified. Although it appears that 2 d FR yield the greatest anthocyanin accumulation, this may be due to the inability to separate seedlings from the seed coat at this time. By the third day, seedlings are fully out of the seed coat. At this stage anthocyanin accumulation is easily assessed and thus was chosen for our screen. Error bars represent +/- 1 S.D.

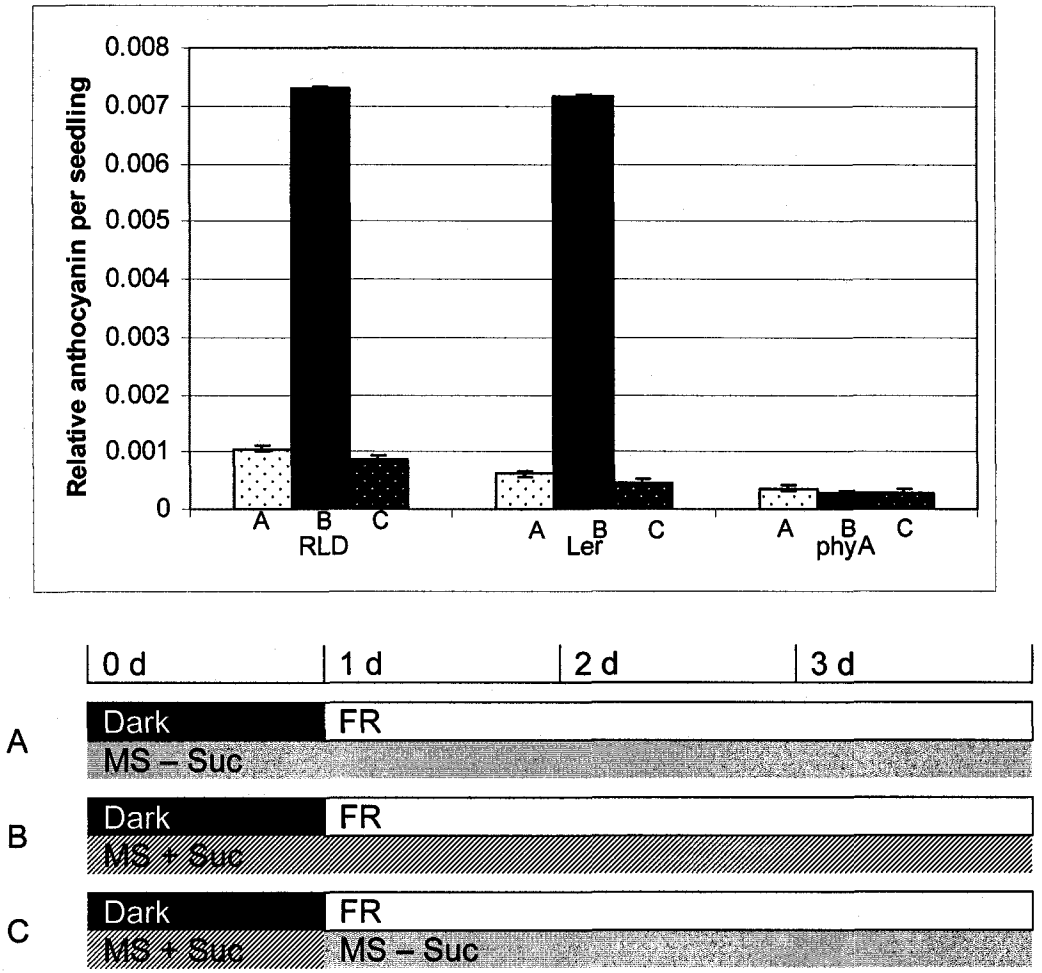


Figure 5. Sucrose is required for FR light-dependent anthocyanin accumulation at 3 d FR.

Wild types RLD and L*er*, and *phyA*-null seedlings were given three different treatments to assay whether a sucrose signal, if given during germination initiation, could persist if taken away immediately before the seedlings were exposed to FR light (treatment C). Sucrose and FR are needed simultaneously for anthocyanin accumulation to be maximized (treatment B). Treatment A represents the control where no sucrose was administered. Error bars represent +/- 1 S.D.

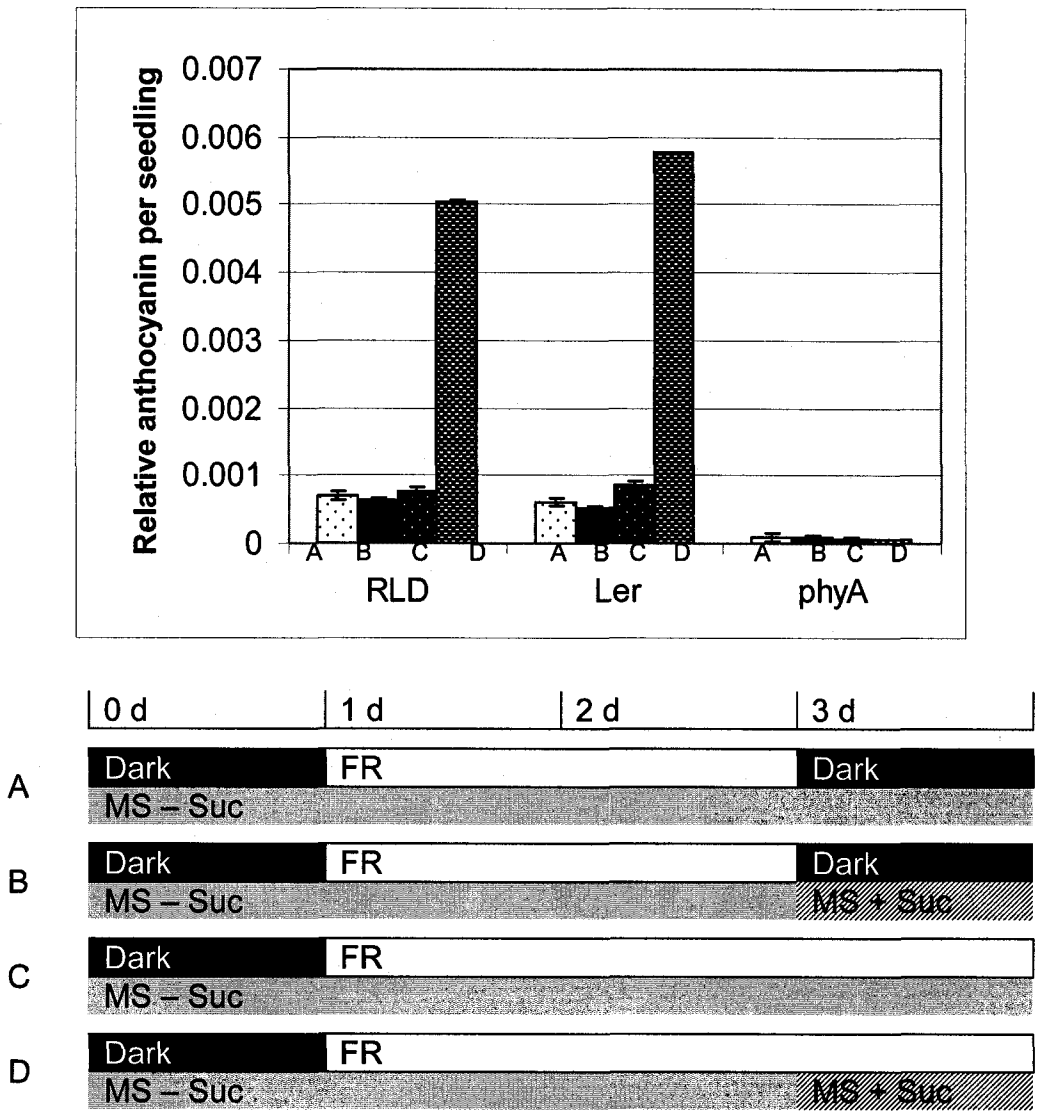


Figure 6. Simultaneous FR and sucrose are required for anthocyanin accumulation.

Wild types RLD and L*er* and the *phyA* null mutant were used to assay the necessity for simultaneous FR and sucrose on the accumulation of anthocyanins. Treatment A is a control in which no sucrose is given and the seedlings are transferred to the dark for the end of the 3 d developmental time period. In treatment B, a 2% sucrose solution is administered to media where the seedlings are growing, but the plants are put in the dark for the final 24 hours. In treatment C, no sucrose is ever in the media and the seedlings are allowed to grow in FR for the full 3 d. In treatment D, seedlings remain in the FR for the full 3 d, but sucrose is only added for the final 24 hours. Error bars represent +/- 1 S.D.

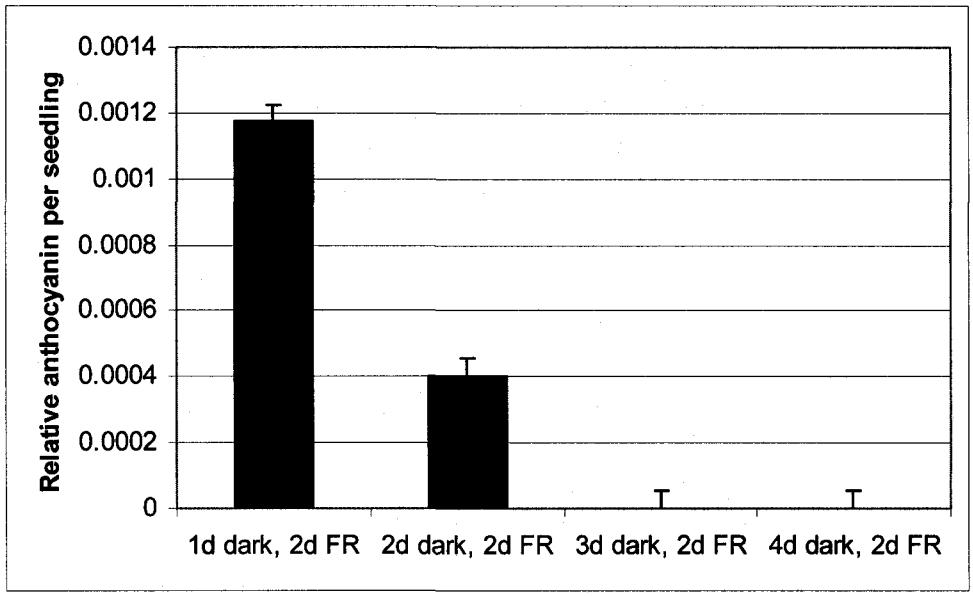


Figure 7. The developmental stage of *Arabidopsis* seedlings is crucial for anthocyanin accumulation.

These experiments were done with wild type RLD on MS medium with 2% sucrose. From the time course experiments (Figure 4) we know that as the age of the plant increases in the FR light the anthocyanin accumulation peaks at the 2-3 d developmental time period and then wanes. In this experiment we attempted to separate the length of FR exposure from the developmental age of the seedlings by leaving them in the dark from 1-4 d before giving them a constant FR exposure. Then anthocyanin accumulation was quantified as described. Error bars represent +/- 1 S.D.

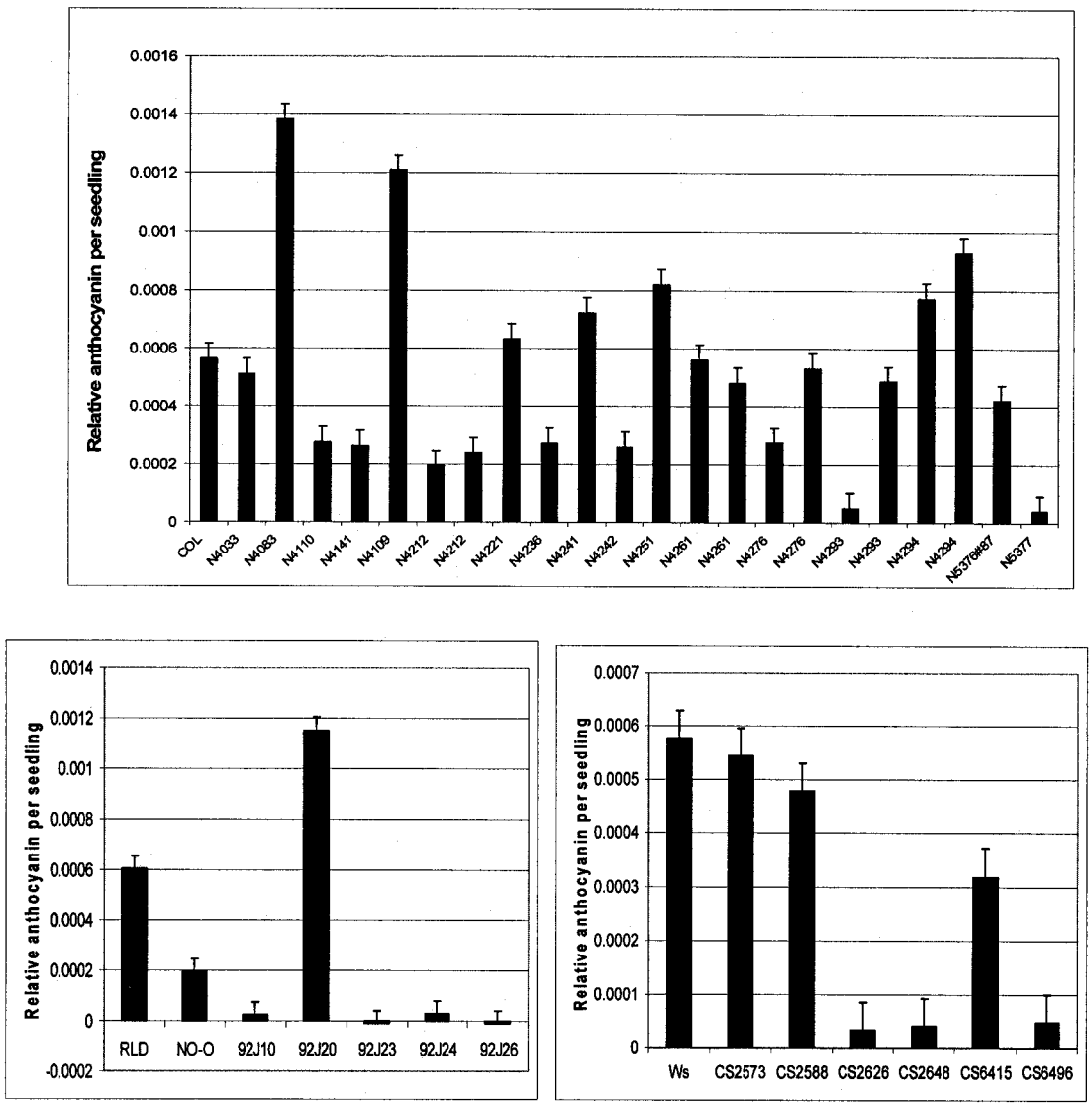


Figure 8. Screen 1 yielded a number of putative mutants.
Over 50,000 M3 seed lines were screened fromN4, 92J and CS lines which were either EMS mutagenized, or potentially T-DNA tagged. After screening in three successive generations for heritability of the visual phenotype, anthocyanin accumulation was quantified spectrophotometrically from the above putative mutants. Qi (2004) characterized these mutants further, with particular concentration on N4141 and N4242, subsequently named *lid1* and *lid2*, respectively. Error bars represent +/- 1 S.D.

Table 1. *lid3* and *lid4* were isolated from sequential genetic screens.

- 60,000 potentially T-DNA tagged seeds were screened
- 35 putative mutants were isolated
- 2 mutants bred true for a third screening, *lid3* and *lid4*

SECOND SCREEN

84 seed lines were screened. Each line contained 500-1,200 seedlings (approximately 72,000 seedlings total scored). In the table below, "+" represents "no anthocyanin" and "-"represents "anthocyanin."

	Test		
Seedline	#1	#2	#3
CS4105	1	+	-
CS4161	1	+	-
CS4015	1	+	-
CS4296	1	+	-
CS4127	1	+	-
CS4024	1	+	-
CS4226	0		
CS4185	0		
CS4114	1	+	-
CS6493	2	+	2 -
CS6489	2	-	
CS4220	0		
CS4283	1	+	-
CS4136	2	2 +	2 -
CS4075	0		
CS4112	1		
CS4159	0		
CS4092	2	+	-
CS4141	0		
CS4224	2	2 +	2 -
CS4034	1	+	-
CS6494	2	-	
CS6483	2	+	-
CS6492	0		
CS6486	0		
CS6491	3	3 +	3 -
CS4216	1	+	-

	Test		
Seedline	#1	#2	#3
CS4022	1	+	-
CS6485	1	+	-
CS4143	0		
CS2609	1	+	-
CS2631	1	+	-
CS2360	1		
CS2606	2	+	-
CS2632	4	+	-
CS4276	1	+	-
CS46483	2	+	+
CS2624	4	-	
CS2622	0		
CS2639	1		
CS2636	1	-	
CS2643	1	+	-
CS2613	0		
CS2621	0		
CS2623	**2**	**1 + 1 -**	**+**
CS2646	0		
CS2616	1		
CS2644	1	+	-
CS2652	1	-	
CS2608	0		
CS2611	0		
CS2620			
CS2642	0		
CS2648	0		

	Test		
Seedline	#1	#2	#3
CS2627	0		
CS2610	1		
CS2645	0		
CS2628	0		
CS2629	0		
CS2634	0		
CS2640	1		
CS2629	1	+	-
CS2653	0		
CS2633	0		
CS2641	3	1 + 1 -	-
CS2617	1		
CS2619	1		
CS2630	0		
CS2618	0		
CS2615	7		
CS2654	0		

	Test		
Seedline	#1	#2	#3
CS2614	0		
CS2626	1		
CS2638	0		
CS2651	0		
CS2635	0		
CS2625	0		
CS2607	0		
CS2637	**1**	**+**	**+**
CS2649	0		
CS2612	2	-	
CS4292	0		
CS4632	0		
CS4137			
CS4186			

Table 1 continued.

Wild Type: Ws **Mutant: *lid3***

Figure 9. Visible anthocyanin phenotypes of mutant *lid3* and its background wild type, Ws.
Photographs taken after 3 d of FR on a media of 2% sucrose. Ws accumulates anthocyanins in the upper region of the hypocotyl and around the edges of the cotyledon. These pigments are severely reduced in the *lid3* mutant.

Table 2. Backcrossing data reveals that *lid3* is a recessive trait.

	lid3	Ws	Total	Chi Square	p Value
Backcross #1	**18**	**55**	**73**	**.25**	**0.5-0.7**
Backcross #2	**23**	**63**	**86**	**.1**	**0.7-0.8**

lid3 was backcrossed into its native ecotype, Ws. F_1 seeds were then grown up and F_2 seeds collected and examined for *lid3* segregation. Chi Square and p values were calculated from these segregating populations, revealing that *lid3* displays a 3:1 ratio consistent with classic Mendelian genetics and implicating *lid3* as a single nuclear recessive trait. The process was repeated by crossing the *lid3* segregates from backcross #1 again into Ws.

Table 3. Outcrossing data confirm *lid3* as a recessive trait.

Outcross data	*lid3*	Col-O	Total	Chi Square	P Value
F2 population	212	698	907	1.28	0.2-0.3

The homozygous *lid3* mutant in its native Ws ecotype was crossed into the Columbia (Col-O) ecotype (courtesy of L. Hobbie laboratory at Adelphi University). F_1 seeds were allowed to grow and self-fertilize to obtain an F_2 segregating population. F_2 seeds were collected for screening as well as for obtaining an F_2 mapping population. As in the backcross to Ws, these seeds maintained their mutant phenotype in a typical 3:1 Mendelian ratio after 3 d FR on 2% sucrose.

Table 4. Markers tested with recombination frequencies for segregation with the *lid3* phenotype.

SSLP/SNP Marker	Chromosome Location	Map Position (cM)	Number Scored	Recombination Frequency (cM)
NGA63	1	11.0	43	38
NGA280	1	83.8	98	17
2027	1	85.0	43	15
4424	1	85.0	43	15
NGA111	1	115.0	98	19
NGA168	2	73.0	43	50
NGA162	3	20.0	43	43
NGA112	3	87.8	43	50
NGA8	4	25.0	43	46
NGA1139	4	83.0	43	50
NGA139	5	50.0	43	38
SO191	5	80.0	43	46

Seedlings exhibiting the *lid3* reduced-anthocyanin phenotype were chosen and transplanted to soil. DNA extracted from an excised leaf of each recombinant plant was used as the template for SSLP-based mapping. The recombination frequencies showed linkage of the *LID3* locus to flanking SSLP markers nga280 and nga111, located on the bottom arm of chromosome 1. The following chart displays a summary of the *lid3* linkage analysis data. F_3 seeds were collected from the F_2 plants and retested for low anthocyanin in all progeny to confirm homozygosity of each recombinant line. The reconfirmed lines supported the *LID3* rough map position established initially.

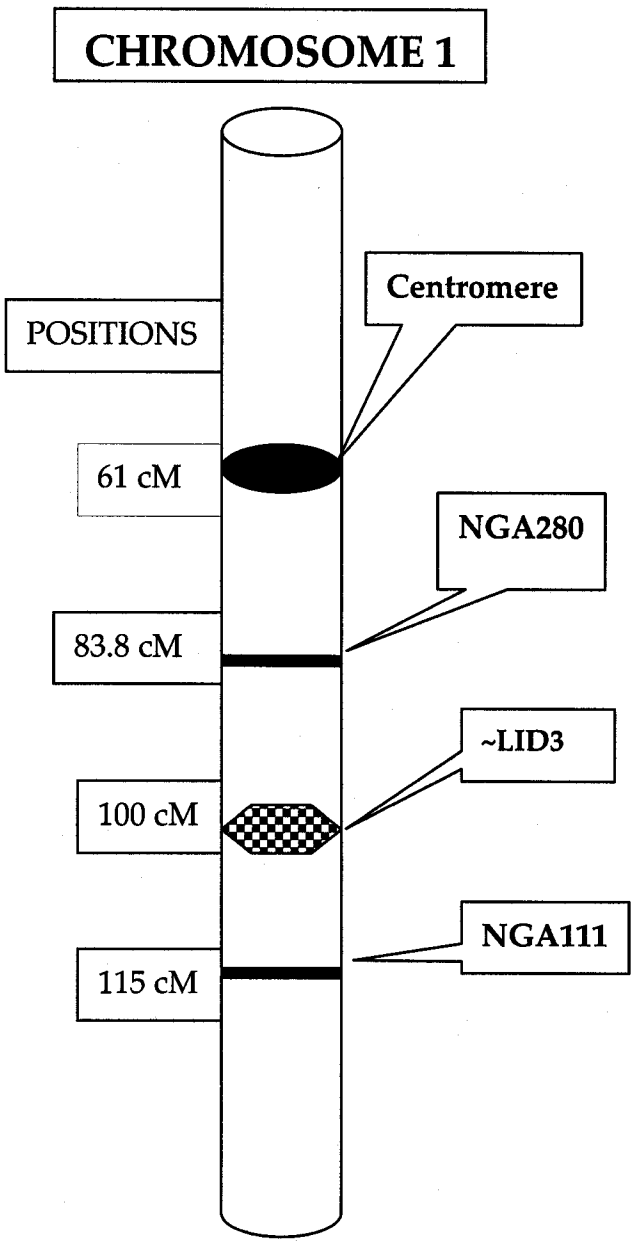


Figure 10. Rough mapping positions *lid3* on the bottom arm of chromosome 1. *LID3* maps to the bottom arm of chromosome 1 at a standard position of approximately 100 map units. Position is based on flanking SSLP markers NGA111 and NGA280. Recombination frequency places *lid3* 17cM from NGA280 and 19cM from NGA111.

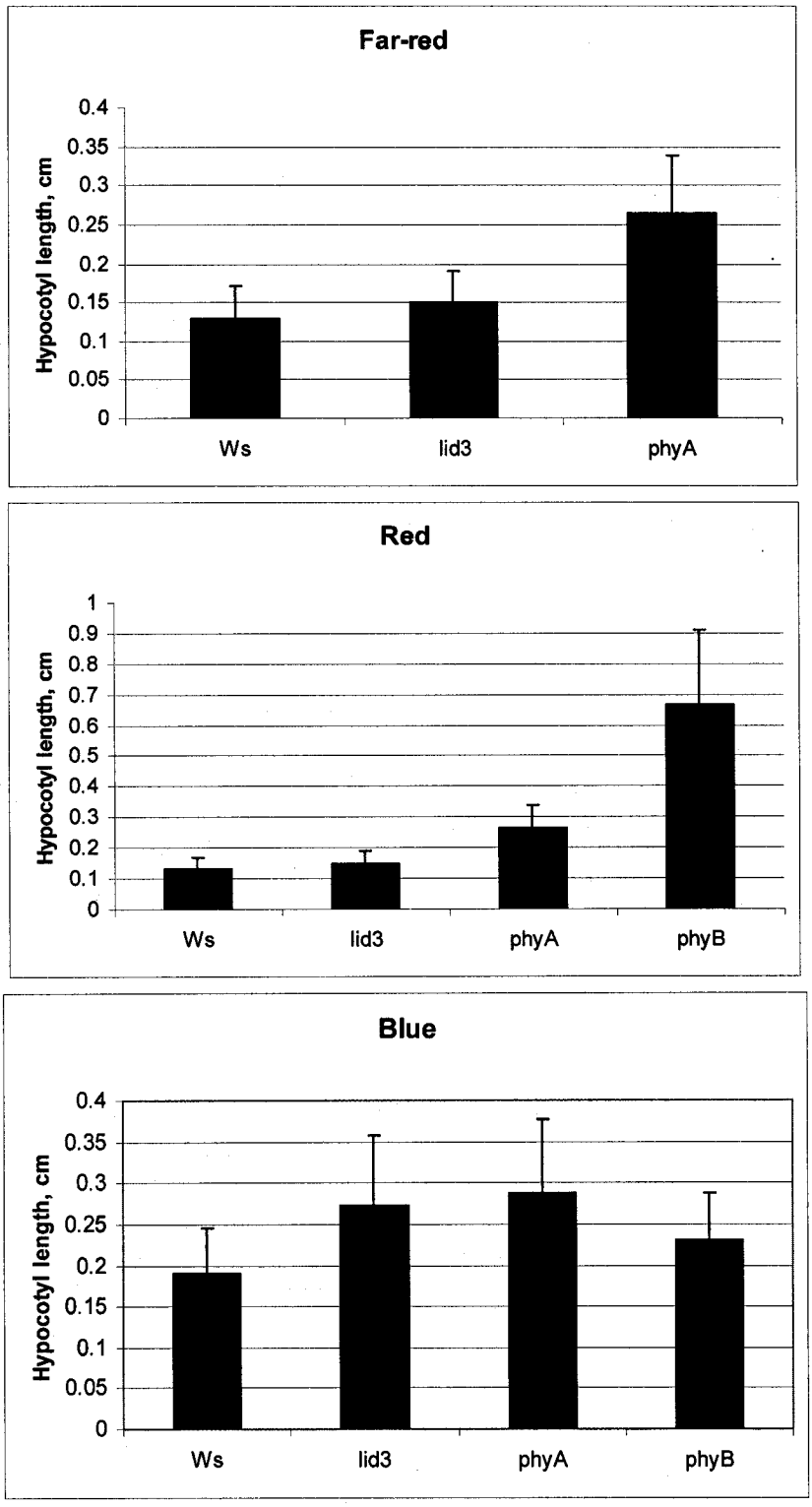
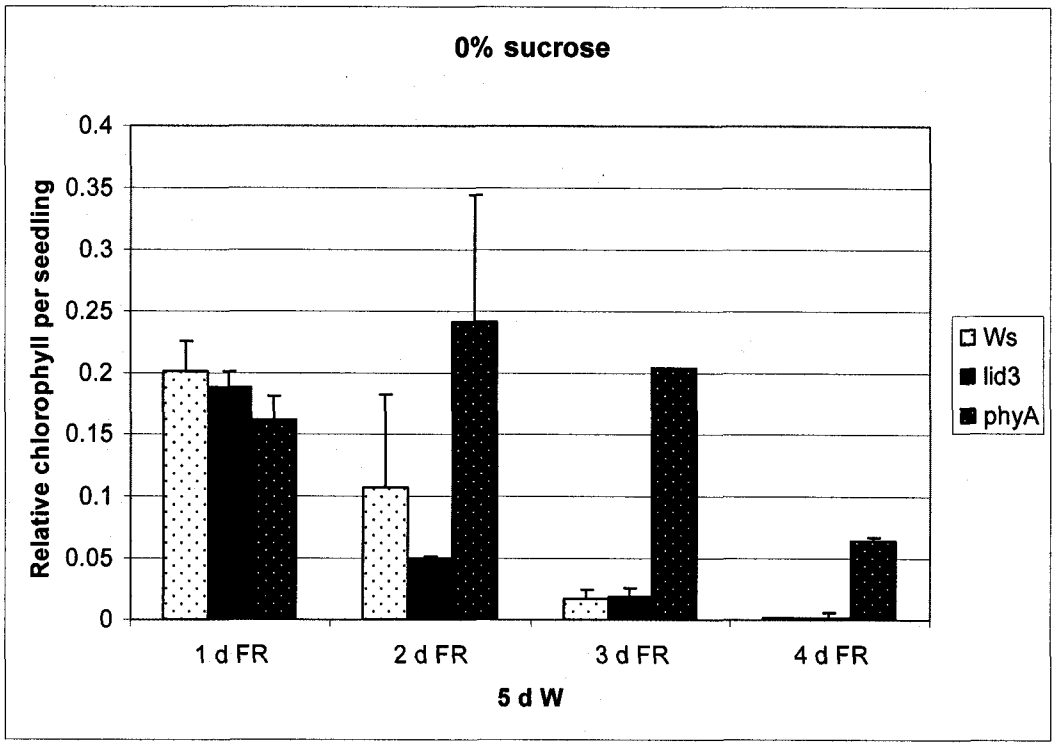


Figure 11. The *lid3* mutation does not affect inhibition of hypocotyl elongation in response to FR, R, or B light. Hypocotyls of Ws, *lid3*, *phyA*, and *phyB* sown on standard MS medium in 3 different light conditions were grown and measured as described in the text. Error bars represent +/- 1 S.D.



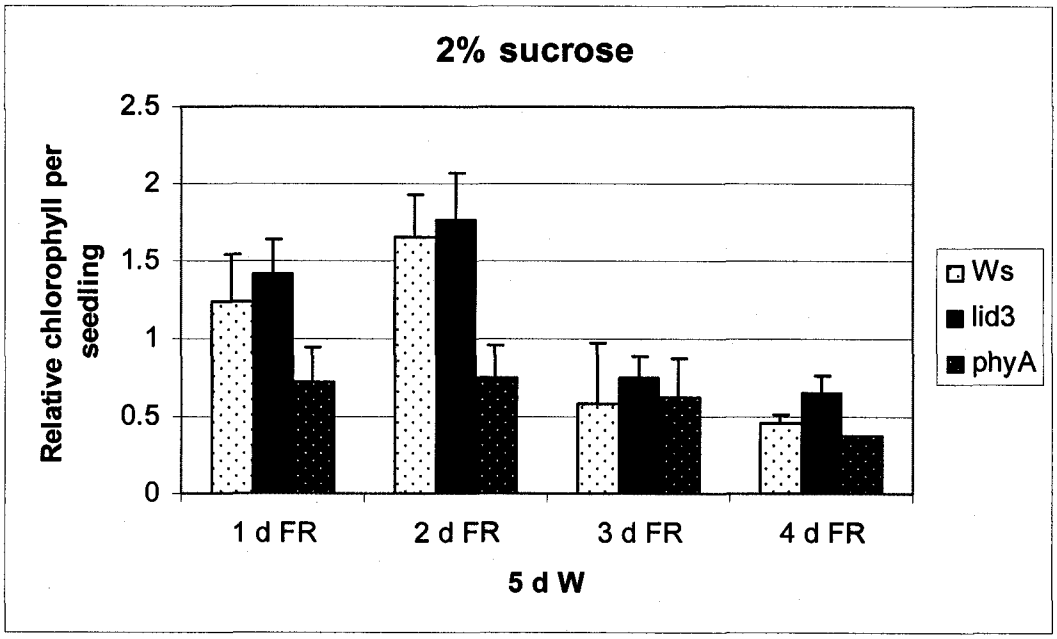


Figure 12. The *lid3* mutants do not differ significantly from Ws in chlorophyll accumulation. Seedlings were sown on a media of 2% sucrose or 0% sucrose. Chlorophyll accumulation was measured spectrophotometrically in seedlings exposed from1-4 d of FR followed by 5 d of W treatment. The preconditioned block of greening response in both Ws and *lid3* is strongly attenuated in the presence of sucrose. Error bars represent +/- 1 S.D.

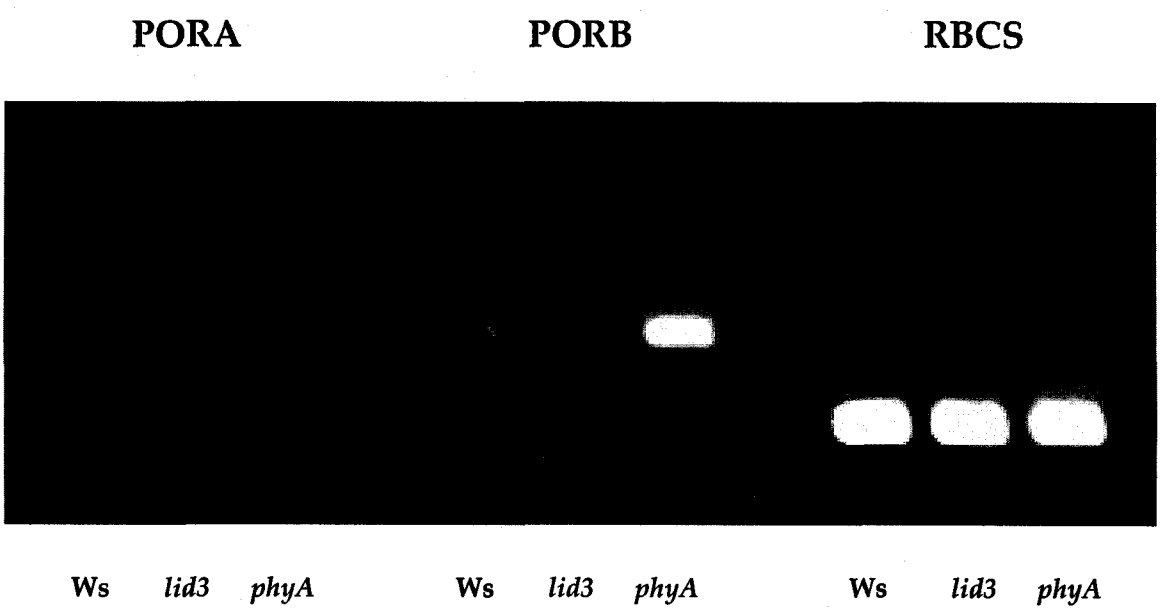
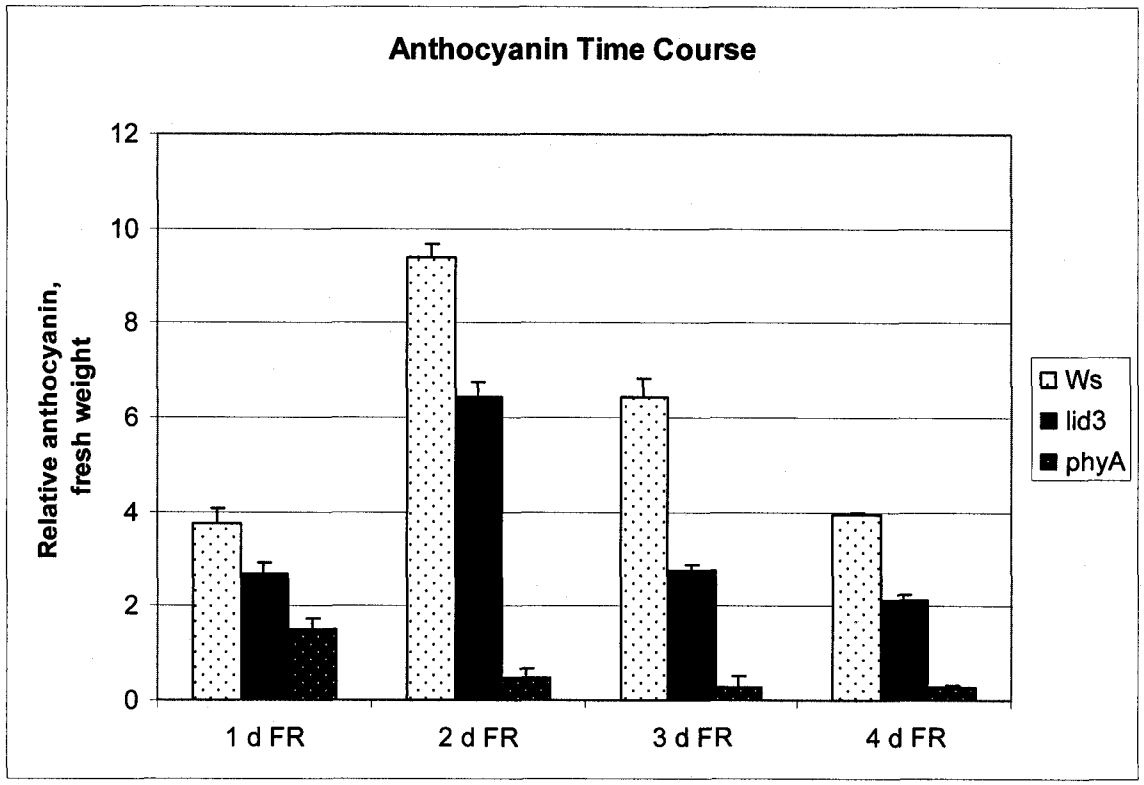


Figure 13. Message abundance of chlorophyll synthesis enzymes is largely unaffected by the *lid3* mutation.
Semi-quantitative RT-PCR of two chlorophyll biosynthetic enzymes protochlorophyllide oxidoreductase A (PORA) and PORB display *lid3* at wild type levels. Total RNA was extracted from Ws, *lid3*, and *phyA* after growth under conditions that would normally elicit a FR-preconditioned block of greening response in Ws, as described in the text.

A



B

		1 d FR	2 d FR	3 d FR	4 d FR
WS versus *lid3*	t-value	1.0034	1.4652	6.0492	2.7626
	p-score	0.99	0.99	0.01	0.99

Figure 14. *lid3* has significantly less anthocyanin after 3 d FR than does Ws.
A. Quantitative determination of anthocyanin accumulation in *Arabidopsis* seedlings grown for 3 d FR on 2% sucrose. Wild type (Ws), *lid3*, and the *phyA* null mutant were treated under conditions shown to elicit maximal anthocyanin accumulation as described in the text. Anthocyanin content was measured spectrophotometrically. Error bars correspond to +/- S. D. Each reference point is the average of at least 3 experiments.
B. Student's pairwise two-tailed t-test analyses of anthocyanin content confirm that *lid3* differs significantly from wild type Ws at 3 d FR.

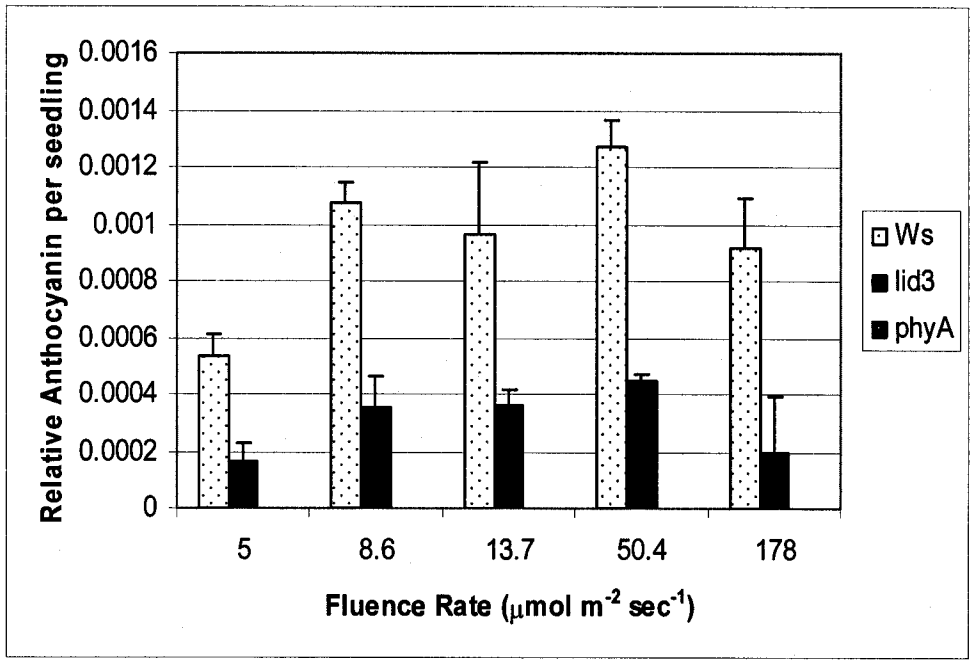


Figure 15. *lid3* contains significantly less anthocyanin than Ws over a range of photon fluence rates.

Seedlings were sown on a medium of 2% sucrose and exposed to 3 d of FR. These data confirm that saturation for anthocyanin accumulation is near 10^1 μmol m^{-2} s^{-1} in both wild type and *lid3*. Error bars represent +/- 1 S.D.

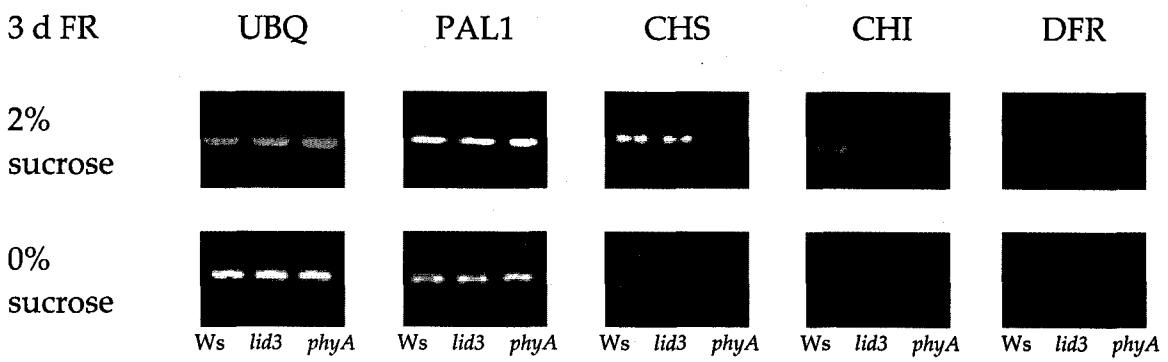


Figure 16. Semi-quantitative RT-PCR of four anthocyanin biosynthetic enzymes.

RNA was extracted from plants grown under the indicated conditions. Ubiquitin (UBQ) was used as a control. Enzyme messages tested are shown in order of their sequence in the biosynthetic pathway starting with PAL1. CHS is the first anthocyanin biosynthetic enzyme shown to be light and sucrose regulated. On 2% sucrose, Ws and *lid3* transcripts for PAL1 and CHS appear to be of nearly equal intensity, in contrast with those for CHI and DFR, which are both downregulated in *lid3*. *phyA* shows more severe downregulation of these transcripts starting from CHS, as expected. Without exogenous sucrose, CHS message of Ws is dimmer than on the sucrose media. This trend follows through to DFR where no transcript is detected regardless of background. *lid3* appears to have a lower message level for CHS than wild type, and transcripts are undetectable for CHI and DFR in the *lid3* mutant. p*hyA* does not maintain transcripts for any of these enzymes.

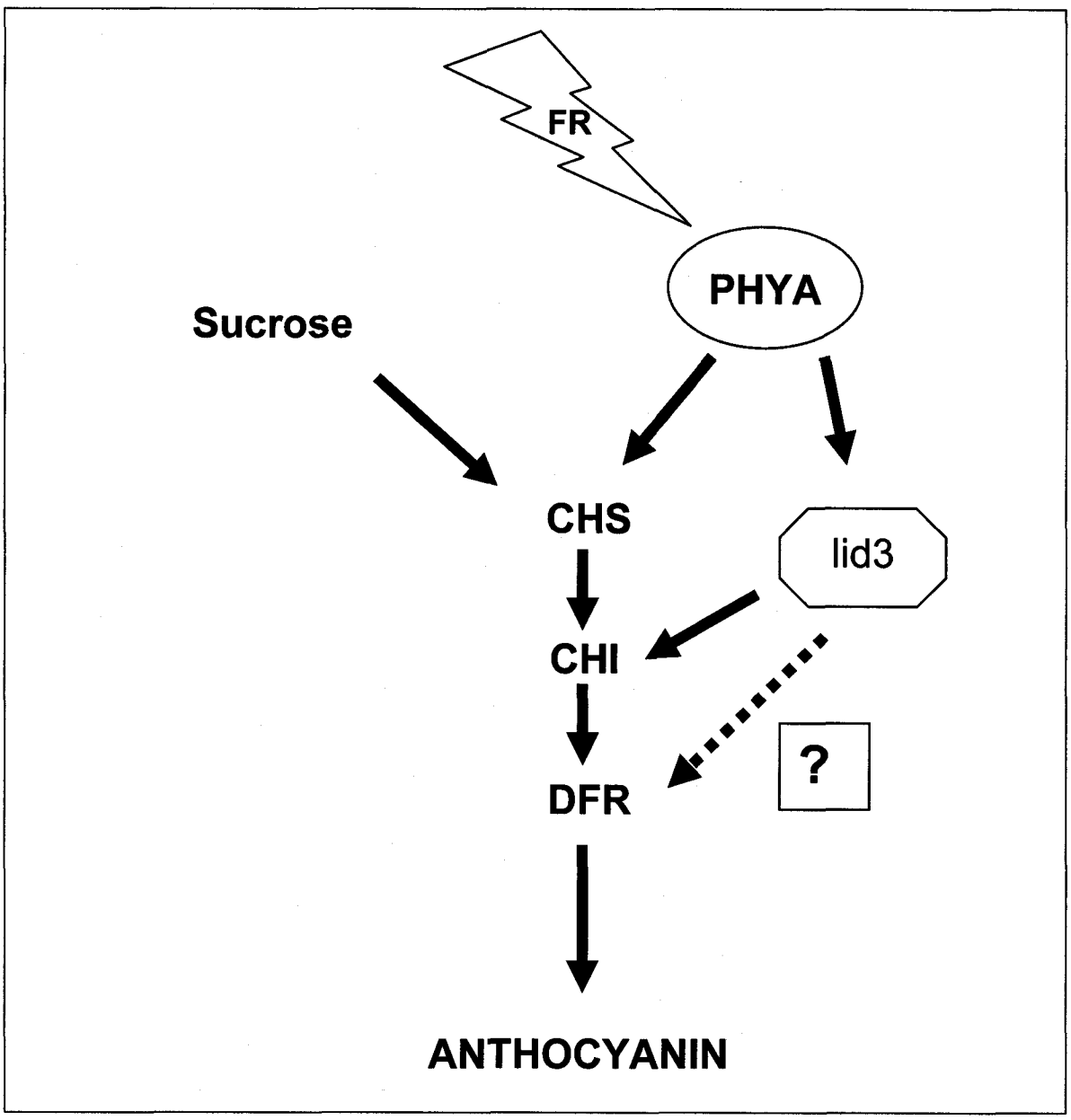
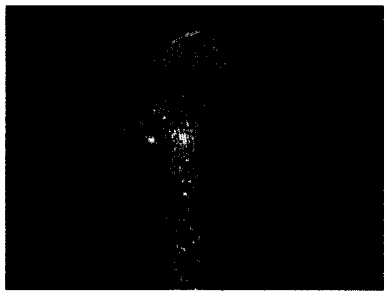
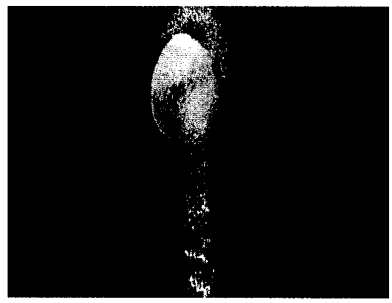


Figure 17. Working model for lid3 function.
Based on physiological and semi-quantitative RT-PCR, *lid3* appears to affect the accumulation of anthocyanins in a light dependent manner at the level of chalcone isomerase (CHI). Lid3 may affect DFR levels directly, or it may function indirectly through its regulation of CHI and the resulting metabolic products.





Wild Type: Ws **Mutant: *lid3***

Figure 18. Ws and *lid4* visible phenotypes under screen conditions.
Wild type Ws (left) at 3 d FR grown on a media of 2% sucrose shows typical expanded cotyledons and anthocyanin around the cotyledons and upper region of the hypocotyl. Cotyledons of the *lid4* mutant (right) typically remain closed and no anthocyanin is observed under these conditions.

Table 5. Backcrosses of *lid4* do not yield the expected 3:1 Mendelian Ratio in F_2 populations.

	Observed	Expected	χ^2	p value
lid4	379	327	33	9.2125e-9
Ws	57	109		

Table 6. F_2 backcrosses of *lid4* are not consistent with a two-locus 9:3:3:1 Mendelian Ratio

Height	Anthocyanin	Observed	Expected	χ^2	p value
Short	Yes	436	270	224.11	0
Tall	Yes	29	92		
Short	No	9	92		
Tall	No	19	30		

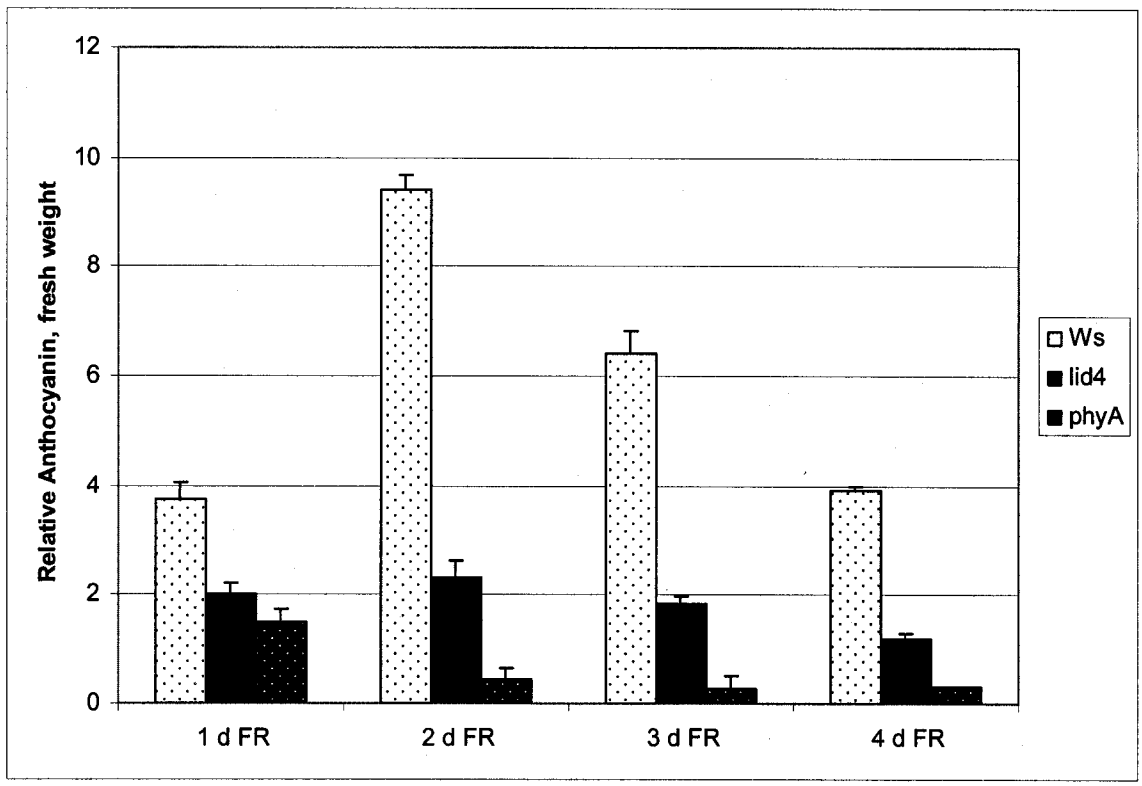


Figure 19. *lid4* displays *phyA* like anthocyanin accumulation in FR.
Wild type (Ws) and mutants *phyA* and *lid4* were plated on 2% sucrose, given germination initiation conditions, 3 d of FR, and the anthocyanin content was quantified. At each day, *lid4* maintains reduced levels of anthocyanin compared with Ws. Error bars represent +/- 1 S.D.

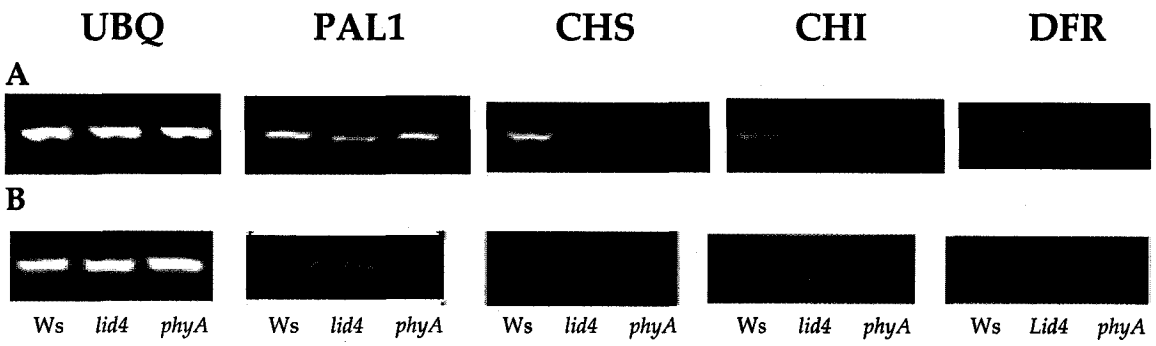


Figure 20. The *lid4* mutation affects transcripts for major anthocyanin biosynthetic enzymes in a *phyA*-like manner.

A. After seedlings were grown under standard screen conditions, RNA was extracted from Ws, *lid4* and *phyA* seedlings. The levels of control UBQ and PAL1 appear indistinguishable in all 3 seed lines. For CHS, CHI, and DFR, there is a severe downregulation of these transcripts in both *lid4* and *phyA*.

B. After seedlings were grown under standard screen conditions lacking exogenous sucrose, RNA was extracted from Ws, *lid4*, and *phyA* seedlings. While the relative transcript levels of UBQ and PAL1 appear largely unaffected, the level of CHS transcript appears downregulated, and the CHI and DFR transcripts are undetectable in the wild type. Transcript levels of CHI and DFR in *lid4* and *phyA* appear to follow parallel expression patterns, although the CHS transcript in *lid4* is slightly more abundant than it is in *phyA*.

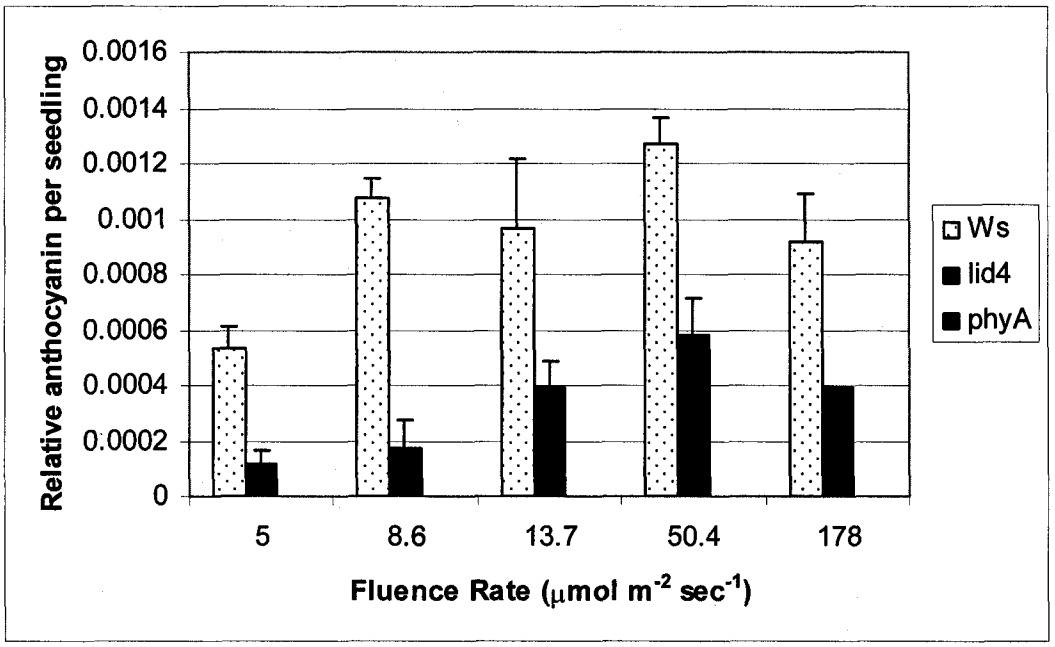


Figure 21. Over a range of fluence rates, *lid4* maintains a reduced level of anthocyanin compared to the wild type, Ws.
Under screen conditions, Ws, *lid4* and *phyA* were tested in a range of fluence rates from 5-178 μmol m^{-2} s^{-1}. Although 8.6 μmol m^{-2} s^{-1} appears to be the saturation point for anthocyanin accumulation in wild type, the saturating fluence rate is somewhat higher for *lid4*. Error bars represent +/- 1 S.D.

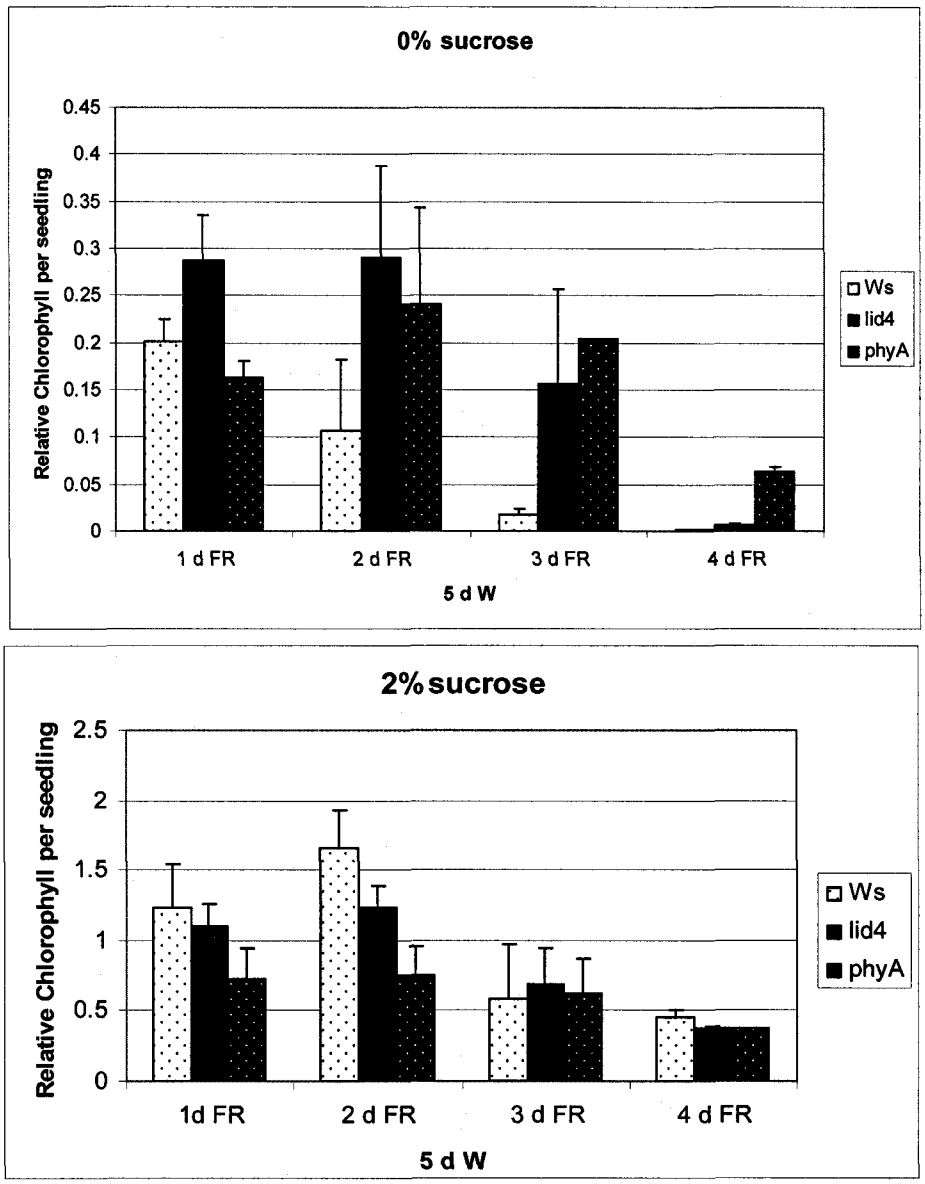


Figure 22. The *lid4* FR block of greening response is inhibited
lid4 follows a *phyA*-like lack of the preconditioned block of greening response and continues to green up without sucrose. 2% sucrose overrides the FR block of greening even in wild-type. Ws, *lid4* and *phyA* were sown on MS medium with or without supplementary 2% sucrose. A time course from 1-4 days in the FR, all followed by 5 d of W were then administered and chlorophyll content quantified. Error bars represent +/- 1 S.D.

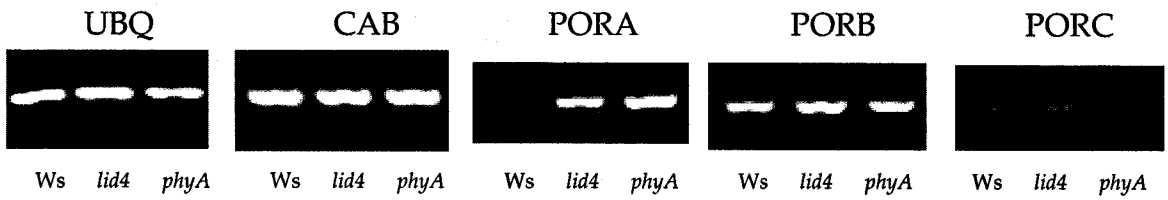


Figure 23. The *lid4* mutation prevents the FR-repression of PORA message accumulation.

Message levels for chlorophyll biosynthetic enzymes measured by semi-quantitative RT-PCR suggest that lid4 plays a role in the phyA-mediated downregulation of PORA for the FR-preconditioned block of greening response. After growth in 3 d of FR and 3 d of W as described in the text, RNA was extracted from Ws, *lid4*, and *phyA* seedlings. The results indicate that *lid4* is acting like a *phyA* mutant in terms of the block of greening response initiated by FR light, controlled by phyA. PORC mRNA is apparently expressed at more nearly wild-type levels than is seen in *phyA*.

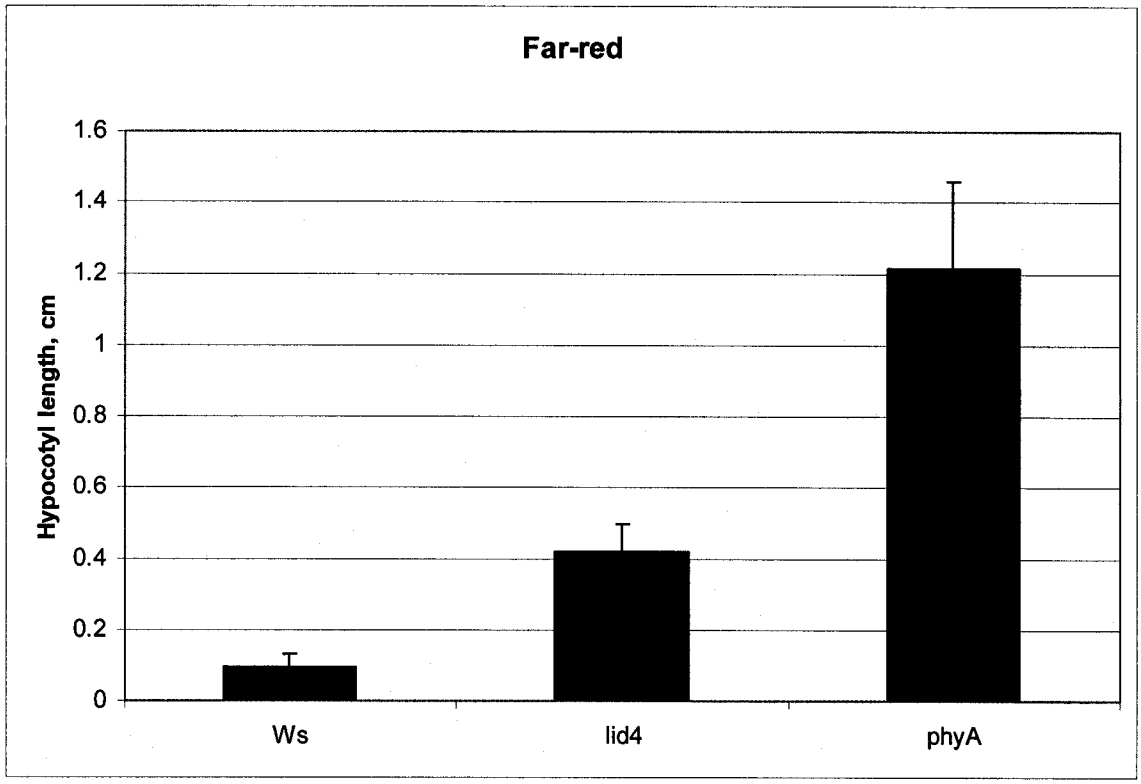


Figure 24. Hypocotyl growth in far-red light
Wild type (Ws) and *lid4* and *phyA* seeds were sown on square plates with agar-solidified MS medium. Germination was initiated as described in the text, and the plates were oriented vertically for 3 d growth in FR light. Hypocotyl lengths were measured as indicated in the text. Error bars represent +/- 1 S.D.

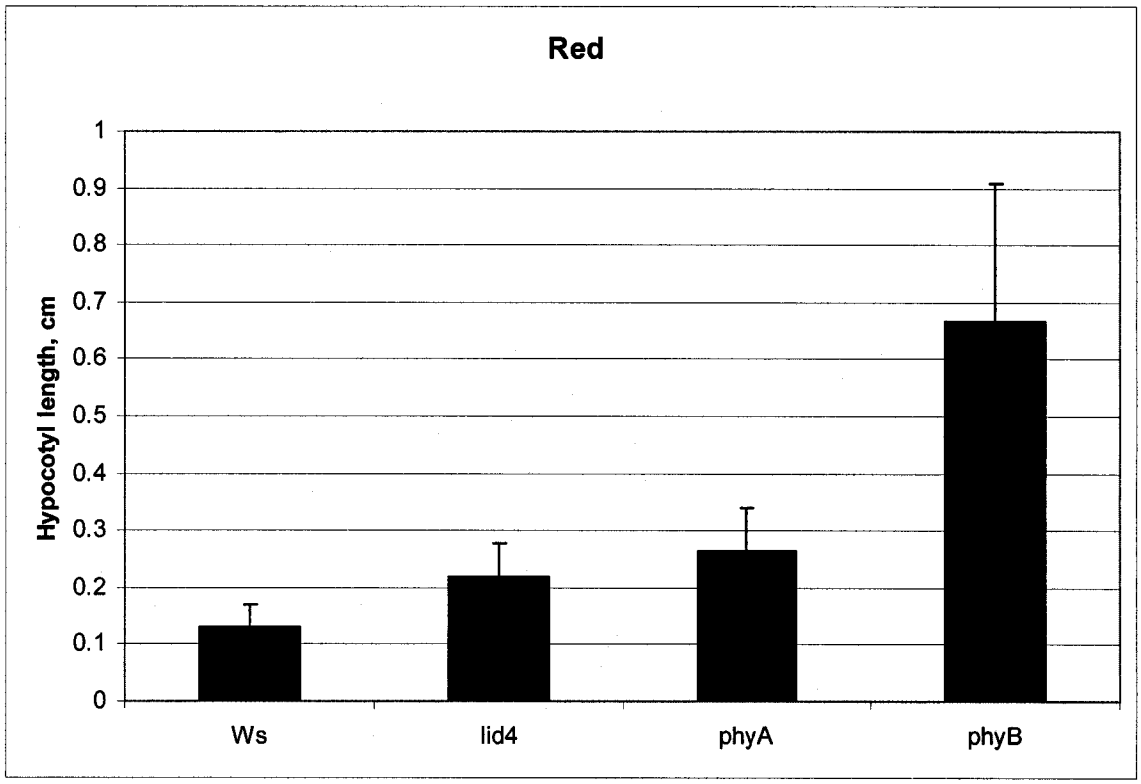


Figure 25. Hypocotyl growth in red light
Wild type (Ws) and *lid4* and *phyA* seeds were sown on square plates with agar-solidified MS medium. Germination was initiated as described in the text, and the plates were oriented vertically for 3 d growth in R light. Hypocotyl lengths were measured as indicated in the text. Error bars represent +/- 1 S.D.

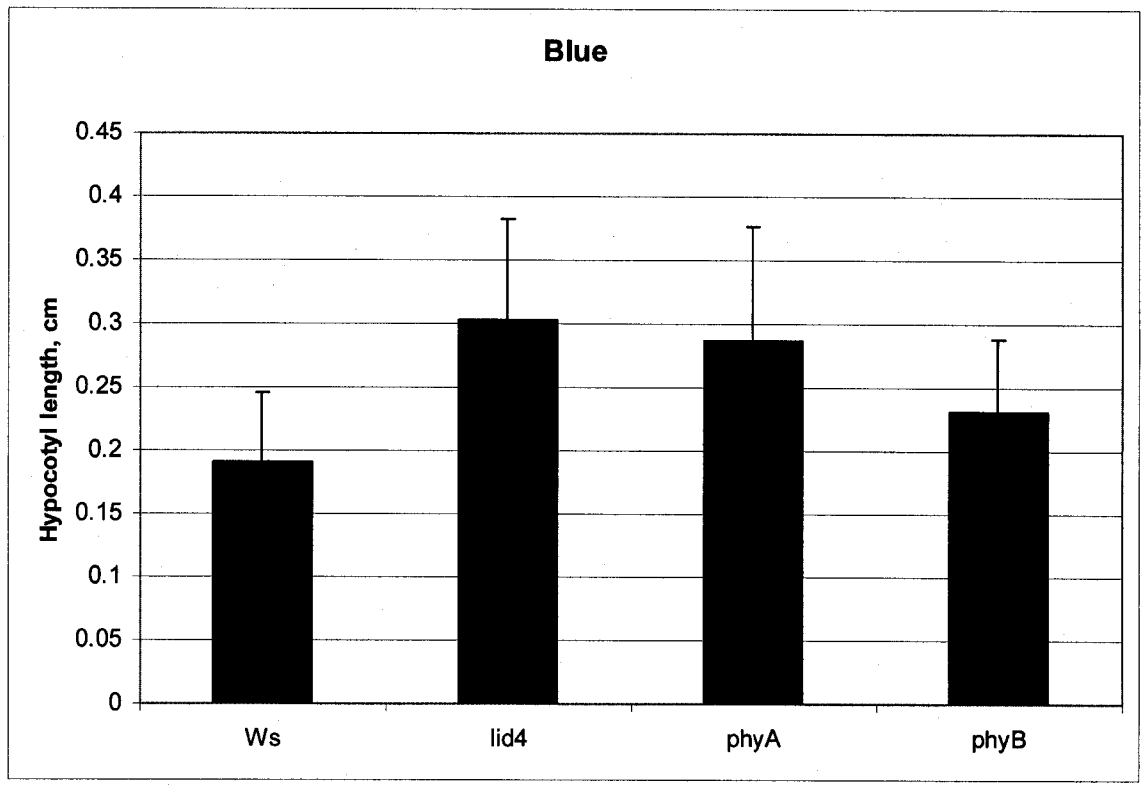


Figure 26. Hypocotyl growth in blue light
Wild type (Ws) and *lid4* and *phyA* seeds were sown on square plates with agar-solidified MS medium. Germination was initiated as described in the text, and the plates were oriented vertically for 3 d growth in B light. Hypocotyl lengths were measured as indicated in the text. Error bars represent +/- 1 S.D.

Table 7. Outcross of a *lid4* 3:1 Mendelian Ratio

	Observed	Expected	χ^2	p value
lid4	34	48	5.44	0.0196
L*er*	158	144		

Table 8. Attempted SSLP mapping of *lid4*

SSLP Marker	Chromosome Location	Map Position (cM)	Number Scored	Recombination Frequency (cM)
NGA59	1	2.9	21	47.9
NGA248	1	30.0	21	50.0
NGA111	1	115.0	21	50.0
NGA692	1	119.0	21	54.0
NGA1145	2	10.0	21	47.0
NGA6	3	86.0	21	47.0
SNPT16	4	77.0	21	49.0
NGA158	5	18.0	21	47.0
NGA151	5	30.0	21	50.0
SO191	5	65.0	21	50.0

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