ISOLATING AND CHARACTERIZING PHYTOCHROME RESPONSE MUTANTS IN *ARABIDOPSIS THALIANA*

by

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ABSTRACT

Isolating and characterizing phytochrome response mutants in *Arabidopsis thaliana*

by

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Light, acting both through photosynthesis and through a series of specific morphogenic photoreceptors, regulates nearly every stage of whole plant growth and development. Several major classes of photoreceptors have been isolated and well characterized, such as phytochromes, cryptochromes and phototropins (Cashmore *et al.*, 1999; Quail, 2002a, b). By characterizing mutants from these photoreceptors, the functions of the individual photoreceptors and of intermediate components in the signal transduction pathways initiated by these photoreceptors have been gradually clarified. (Quail, 1991; Short and Briggs, 1994; Smith, 1995; Fankhauser and Chory, 1997; Quail, 2002a).

In order to study phyA-induced pathways, we initiated a screen to look for genetic mutants with lower anthocyanin accumulation, a commonly used phenotype for studying phyA-stimulated transduction chains. As a result, four mutants were identified and designated *lid* (*li*ght-*d*eficient). Two of these mutants, *lid1* and *lid2*, are the primary subject illustrated in this dissertation. These mutants exhibit reduced some, but not all, phyA-induced mutant phenotypes. These results indicate that both *lid1* and *lid2* maybe novel genetic mutants in the phyA signaling pathway, and that both *LID1* and *LID2* may encode proteins not yet implicated in light responses. The genetic, physiological and biochemical characterizations of both *lid1* and *lid2* may help in elucidating light-mediated responses.

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CHAPTER 1 INTRODUCTION

CHAPTER 1 INTRODUCTION

I. Photomorphogenesis

Light is among the most important environmental components controlling plant development, physiology, and gene expression (Kendrick and Kronenberg, 1986). Plants not only use light energy for photosynthesis, they also use light signals to initiate numerous other effects to control plants' structural development or morphogenesis. Ecologically significant processes in which light signals are important include seed germination, etiolation and de-etiolation, root development, proximity perception and shade avoidance, floral induction and the rate of flowering, gravitropism, phototropism, circadian rhythms, and induction of bud dormancy and tuberization.

Arabidopsis thaliana is widely used as a genetic model in molecular and developmental biology, as well as in physiology and cell biology. It has a short life cycle and small genome, making it an excellent model system for genetic and molecular manipulations (Meyerowitz, 1990). For our studies of plant photoperception, seedling development of *Arabidopsis* has proven to be very useful because it is easy to follow two different strategies of development in *Arabidopsis*: skotomorphogenesis and photomorphogenesis. Skotomorphogenesis is plant growth devoid of light. Seedlings appear bleached, elongated, and cotyledons do not expand. In the light-grown plants,

(photomorphogenesis), seedlings tend to be shorter, they turn green as chloroplasts develop, and the cotyledons separate and expand (Fig 1).

To interpret the light signal, one can imagine that there is a perception-transduction response chain inside the plant. Most photomorphogenic responses in higher plants appear to be under the control of one of two classes of photoreceptors with distinct light absorption characteristics: phytochromes (Neff et al., 2000), which absorb primarily in the red (R) and far-red (FR) regions of the spectrum; and cryptochromes (Ahmad and Cashmore, 1996) and phototropins (Christie et al., 1998) which absorb in the blue (B) and UV-A regions of the spectrum. Each group of photoreceptors operates both independently and in concert with the others to regulate plant development (Fig 2).

II. Light receptors

1. Phytochrome

Of the photoreceptors in *Arabidopsis*, the best understood are the phytochromes. It is now well established that phytochromes play a critical role in almost every stage of plant development. The discovery and isolation of phytochrome and the demonstration of its importance as a pigment that controls photomorphogenic responses is one of the most brilliant and important of all physiological accomplishments. Phytochromes were discovered in 1945 by Borthwick and Hendricks during their pursuit of the mechanisms plants use to determine day length (Briggs, 1975). Although phytochromes absorb most efficiently in the R and FR wavelengths, they can also absorb B at a relatively low level.

In *Arabidopsis*, there are five different phytochromes: PHYA, which is also called type I phytochrome, and PHYB, PHYC, PHYD and PHYE, which combined make up type II phytochromes (Kendrick and Kronenberg, 1994). Protein sequence comparison points to four major gene duplication events during the evolution of *Arabidopsis* phytochromes (Mathews *et al.*, 1994). The earliest gene duplication gave rise to homologues of *Arabidopsis PHYA* and *PHYC* on one hand and *PHYB/D* and *PHYE* on the other. The later duplications resulted in the divergence of *PHYA* from *PHYC* and of *PHYE* from *PHYB/D.* The divergence of *PHYB* and *PHYD* took place much more recently (Mathews and Sharrock, 1997). The proteins encoded by these five phytochrome genes have similar size and have high sequence similarity along their

entire lengths, with locally very high regions of identity. Hence, R/FR sensing in higher plants is mediated by a diverse but structurally conserved group of soluble photoreceptors (Quail, 1991; Clack *et al.*, 1994; Mathews *et al.*, 1994).

The holoprotein of phytochrome is depicted as a Y-shaped structure which is composed of a homodimer of two 120kD subunits and attached chromophores. Each monomer is composed of two discrete structural domains: an N-terminal domain, which perceives light signals in the R/FR wavelengths, and a C-terminal domain which recognizes the downstream phytochrome interacting factors (PIFs), such as PIF3 (phytochrome interacting factor 3) (Ni *et al.*, 1998), NDPK2 (nucleoside diphosphate kinase 2) (Choi *et al.*, 1999), and PKS1 (phytochrome kinase substrate) (Fankhauser *et al.*, 1999). These two domains are connected via a flexible hinge region (Wagner *et al.*, 1996). The N-terminus is about 74kD and contains a unique tetrapyrrole chromophore covalently attached via a thioether linkage. This cysteinyl attachment is autocatalytic and indispensable for the phytochrome to perform its normal functions. Mutations in the chromophore pocket or in the binding between phytochrome apoproteins and chromophore result in plants that do not respond normally to light. Several conserved subdomains/motifs have been identified in the C-terminal domain, including a pair of the Per-Arnt-Sim (PAS) motifs which play a key role in protein-protein interaction and inter-domain communication; a Q (Quail) box through which phytochrome binds to PIF3 and NDPK2 to start the signal cascade, a dimerization motif which links two homodimers covalently, and two histidine kinase related motifs which are located in the

C-terminal 250 residue region, although they lack His kinase activity and their role is not well understood.

After the attachment of chromophore through the conserved cysteinyl residue, the phytochrome can exist in either of two forms: a red-absorbing form called P_r with an absorption maximum at 666 nm, and a far-red-absorbing form called P_{fr} with an absorption maximum at 730 nm. These two forms are photointerconvertible. Figure 3 shows the absorbance of P_r and P_{fr} form of phytochrome. The pigment is first synthesized inside the plant in the P_r form, which accumulates in the darkness. The P_r form is stable and in most cases physiologically inactive. Upon absorption of a photon of light, the P_r form is changed to the P_{fr} form. This phototransformation involves a *cis*- to *trans*-isomerization of one of the double bonds within the chromophore, a 31° reorientation of the chromophore relative to the polypeptide, phosphorylation of the Ser_{599} and possibly other Ser residues, and multiple conformational changes within the polypeptide, especially near the N terminus.

The P_{fr} conformation is physiologically active. Therefore, after phototransformation, the P_{fr} form of phytochrome will trigger signal transduction pathways that lead to induction of various responses. Type I phytochrome (represented by phyA) is very unstable in its P_{fr} form and is degraded quickly by specific ubiquitin-mediated proteolysis (half-life 1 to 1.5 hours in darkness, which is approximately 100 times greater than the rate of P_r degradation). The P_{fr} form of type II phytochromes (phyB-E) is much more stable. Upon absorption of another photon, the P_{fr} form can

convert back to the P_r form. This conversion will cancel many phytochrome-mediated responses controlled by the P_{fr} form. On the other hand, P_{fr} can also revert to P_r in darkness by a non-photochemical, temperature-dependent reaction.

2. Cryptochrome

Cryptochromes are receptors of blue light and UV-A radiation. They are flavoproteins, each carrying two chromophores, a pterin or a deazaflavin at one site and FAD at another (Briggs and Huala, 1999; Cashmore *et al.*, 1999). Two members of this family, cryptochrome 1 and 2 (cryl and cry2) have been identified in *Arabidopsis* (Cashmore *et al.*, 1999). These two genes show strong similarity to each other and to bacterial DNA photolyase genes, which encode flavoproteins that catalyze blue/UVA-dependent repair of DNA damage (Ahmad and Cashmore, 1993; Sancar, 2000). However, cryptochromes do not exhibit photolyase activity despite the apparent homology. Homologues have also been found in animals and fungi.

Mutation of the *HY4* locus in *Arabidopsis* yields seedlings that fail to show blue-light-induced inhibition of hypocotyl elongation (Koornneef *et al.*, 1980). Ahmad & Cashmore cloned and sequenced the wild-type *HY4* gene, which encodes a 681-amino acid protein, and renamed it *CRY1* (Ahmad and Cashmore, 1993; Lin *et al.*, 1995). In addition to growing long hypocotyls, *Arabidopsis* mutants at the *CRY1* locus show decreased cotyledon expansion, increased petiole and flower stem elongation, and increased leaf expansion in white light-grown seedlings (Jackson and Jenkins, 1995).

The *CRY2* gene in *Arabidopsis* encodes a protein of 619 amino acids with extensive similarity to CRY1 in the photolyase-like domain, even though there is significant divergence between the C-terminal domains of cry1 and cry2 (Lin *et al.*, 1996). Unlike cry1, cry2 is strongly down-regulated by blue light at the protein level (Ahmad et al., 1998; Lin et al., 1998). Later studies on both single and double mutants indicate that the roles of cry1 and cry2 are partially redundant (Mockler *et al.*, 1999). Cry2 also plays a major role in photoperiodic timing. Seedlings carrying *cry2* mutations exhibit greatly extended flowering times in long days or in continuous light (Guo *et al.*, 1998; Briggs and Huala, 1999).

3. Phototropin

Phototropins are the most recently characterized group of plant photoreceptors (Briggs and Christie, 2002). Phototropin 1 (Phot1) is a 996-residue protein that has 2 LOV domains (for *l*ight, *o*xygen, and *v*oltage-regulated PAS-related domains) at the amino terminus and a classic Ser/Thr-kinase domain at the carboxy terminus (Huala *et al.*, 1997). Each LOV domain also binds to a flavin mononucleotide (FMN) chromophore. A second *Arabidopsis* phototropin gene, *PHOT2*, was isolated on the basis of sequence similarity to *PHOT1* (Kagawa *et al.*, 2001). *PHOT2* encodes a slightly smaller protein than *PHOT1*, with 58% sequence identity and 67% sequence similarity (Jarillo et al., 2001). Both receptors perceive the direction and intensity of blue light. Both receptors have Ser/Thr-kinase activity in their C-terminal halves, and perform light-activated autophosphorylation and transient light-activated formation of a

flavin C (4a)-cysteinyl adduct (Briggs *et al.*, 2001; Crosson and Moffat, 2001; Salomon *et al.*, 2001; Swartz *et al.*, 2001). Like phytochromes, Phot1 and Phot2 also show light-activated conformation changes and subsequent dark recovery (Salomon *et al.*, 2000; Kasahara *et al.*, 2002). Both phot1 and phot2 mediate phototropism. However, phot1 responds to light pulses or low levels of continuous light, whereas phot2 responds only at high fluence rates of continuous light (Kanegae *et al.*, 2000). Both phot1 and phot2 also mediate light-activated chloroplast movement. Phot1 drives accumulation of chloroplasts at the cell surface over a wide fluence range, whereas phot2 directs the accumulation at a low fluence rates and has no effect at higher fluence rates (Jarillo et al., 2001; Kagawa et al., 2001; Sakai et al., 2001).

III. Initial events in phytochrome signaling

1. Nuclear translocation of phytochrome

The phytochrome apoproteins are synthesized within the cytosol, where they assemble with chromophore produced in the plastids. In darkness, both phyA and phyB holoproteins remain in the cytosol. Both phyA and phyB tagged with green fluorescent protein (GFP) or glucuronidase (GUS) in tobacco (Kircher *et al.*, 1999) and in *Arabidopsis* (Yamaguchi *et al.*, 1999) show that both phytochromes are transported into the nucleus after photoconversion to the biologically active Pfr form. Translocation of phyB only occurs in the P_{fr} form, whereas translocation of phyA can occur either in the P_{fr} or P_r form which has been cycled through P_{fr}. The import of phyB is slow, maximized within 3 hours, while the import of phyA is much more rapid (about 15 min). Translocation of phyB is activated in R and suppressed in FR. Translocation of phyA is maximized under continuous FR (Kircher *et al.*, 1999; Hisada *et al.*, 2000). Recently, using full-length phytochrome:GFP fusion proteins in transgenic plants, Nagy and Schäfer showed that all five phytochromes exhibit nuclear translocation upon light induction (Nagy and Schafer, 2000). However, in etiolated seedlings, phyC-phyE:GFP fusion protein can be detected both in the cytosol and in the nucleus even though phyC-phyE:GFP fusions accumulate in nuclei in a light-dependent manner (Kircher *et al.*, 2002). These results imply that nucleo-cytoplasmic partitioning of phyC, phyD and phyE may not to be completely light-dependent. The details of how phytochromes are transported into nucleus are not clear.

Inside nuclei, phytochromes directly interact with transcription factors, such as PIF3 which can then bind to the G box DNA sequence CACGTG (Neuhaus *et al.*, 1997; Ni *et al.*, 1999). This association activates the expression of the primary target genes [such as the Myb-class transcription factors *CCA1* (*CIRCADIAN CLOCK-ASSOCIATED PROTEIN 1*) and *LHY* (*LATE ELONGATED HYPOCOTYL*) which have G-box motifs in their promoters;(Wang and Deng, 2003). The protein products encoded by these primary target genes (many of which are transcriptional regulators), in turn, regulate the expression of the secondary target genes [e.g. the regulation of *CAB* (chlorophyll a/b binding protein) expression by *CCA1*], thus generating a transcriptional network controlling different aspects of phytochrome physiology.

2. Phytochrome as a light-regulated kinase

It was hypothesized that plant phytochromes are light-regulated enzymes more than 40 years ago (Borthwick *et al.*, 1969). However, determining the identity of this enzymatic activity has proven to be difficult. In the mid-1980s, McMichael *et al.* were able to detect Ser kinase activity from highly purified phytochrome preparations from plants. This Ser-kinase activity was believed to be intrinsic to the photoreceptor (McMichael and Lagarias, 1990). However, this proposal has remained unresolved mostly because phytochromes lack the consensus sequences that define eukaryotic protein kinases. Recent detection of kinase activity in purified phytochrome preparations from recombinant heterologous organisms (Yeh and Lagarias, 1998)

supports the idea that at least oat phyA is a functional protein kinase. Sequence comparisons show that the C-terminal half of some phytochromes is weakly similar to bacterial histidine kinases (Schneider-Poetsch, 1992). Moreover, Vierstra and Davis (2000) have demonstrated the existence of phytochromes in prokaryotes, and these photoreceptors have demonstrable *in vivo* histidine-kinase activity (Vierstra and Davis, 2000; Wu and Lagarias, 2000). These results not only outline an evolutionary history for phytochromes that was once believed to be unique to plants; they also support the hypothesis that histidine kinase activity is associated with plant phytochromes (Vierstra and Davis, 2000). Research on phytochrome kinase activity has advanced considerably with the recent development of recombinant systems to express and purify plant phytochromes in yeast (Wahleithner *et al.*, 1991; Wu and Lagarias, 1996). Using purified oat phyA produced in yeast, Yeh *et al.* was able to show that phyA autophosphorylates on Ser/Thr rather than on His/Asp (Yeh and Lagarias, 1998), and the autophosphorylation is regulated by light and requires chromophore. Therefore, phytochromes are eukaryotic Ser/Thr kinases with His kinase ancestry, although there is some doubt as to whether the His-kinase-related domain is responsible for the phosphorylation activity.

It is not yet certain where autophosphorylation occurs *in vivo* and if other specific kinases phosphorylate phytochromes. One possible target candidate is mapped to the Ser-rich N terminus, most likely Ser_7 (Lapko *et al.*, 1997). If serine residues in the N-terminus of phyA are mutated to alanines, the resulting mutant proteins exhibit

hyperactivity compared with wild-type phytochrome (Jordan *et al.*, 1997), suggesting that this site is important for the attenuation of phytochrome signaling activity.

Another site that is phosphorylated in the active P_{fr} form is in the serine residue of the hinge region (Ser_{599}). In the P_{fr} form of phytochromes, the phosphorylation of Ser_{599}, combined with the change of tertiary structure of the N-terminal region, will expose the chromophore and the hinge region (Vierstra *et al.*, 1987; Lapko *et al.*, 1998). If Ser_{599} is replaced by Lys, *in vitro* kinase assays have shown that, for this mutant form of phyA, both autophosphorylation of phyA and phosphorylation of phytochrome kinase substrate 1 (PKS1) are no longer light-regulated (Fankhauser *et al.*, 1999).

It is known that reversible protein phosphorylation is essential for controlling the flow of many signaling processes. A recent study has identified a flower-specific, phytochrome-associated protein phosphatase (FYPP), which might also participate in the regulation of phyA signaling. FYPP dephoshorylates phyA in a light-dependent manner and modulates phyA-mediated light signals in the control of flowering time (Kim et al., 2002).

3. Factors that directly interact with phytochrome

To elucidate phytochrome signal transduction pathways, considerable effort has been applied to studying downstream factors and their physiological responses. As a

result, numerous positive and negative regulators in phytochrome signaling networks have been identified (Kim et al., 2002) (Quail, 2002).

Factors that interact with both phyA and phyB

PIF3

Ni *et al.* first identified a phytochrome-interacting factor, PIF3, in a yeast two hybrid screen using the C-terminal domain of *Arabidopsis* phyB as bait (Ni *et al.*, 1998). Subsequent experiments showed that PIF3 binds to active forms of both PHYA and PHYB, but with a 10-fold preference for PHYB (Min *et al.*, 1998). These data imply that PIF3 functions in the converged pathways for both phyA and phyB signal transduction. The binding between PIF3 and PHYA/PHYB takes place only after light-induced conversion to the P_{fr} form. Upon reconversion of phyB to its P_r form, it will quickly dissociate from PIF3 (Ni *et al.*, 1999; Zhu *et al.*, 2000). In addition, *Arabidopsis* seedlings that express reduced levels of PIF3 show strongly reduced phenotypic responsiveness to R light, which is perceived by phyB, and partially reduced responsiveness to FR light, which is perceived by phyA (Ni *et al.*, 1998), supporting the importance of phytochrome-PIF3 binding to signal transduction *in vivo*. However, since genetic screens have identified multiple factors that are specific either to phyA or phyB pathways (Quail, 1998; Deng and Quail, 1999; Hudson, 2000), even though PIF3 is important in phyA and phyB signaling, it is unlikely to be the only mechanism through which phytochrome signaling passes.

PIF3 is a basic helix-loop-helix (bHLH) protein with a PAS motif. Upon its production, PIF3 localizes constitutively to the nucleus (Ni*et al.*, 1998), consistent with the light-mediated nuclear translocation of the phytochromes. PIF3 binds in a sequence-specific fashion to a G-box DNA sequence, CACGTG (Martinez-Garcia *et al.*, 2000). On one hand, PIF3 can regulate the expression of light-regulated genes through direct interaction with the G-box sequence (Martinez-Garcia *et al.*, 2000). On the other hand, other protein families, most notably bZIP proteins, can also bind to G-boxes, which may suggest that there is competition between PIF3 and other G-box-binding proteins.

PKS1

PKS1 was isolated in a yeast two-hybrid screen using the C-terminus of *Arabidopsis* PHYA as bait (Fankhauser *et al.*, 1999). Further study shows that PKS1 can interact with the C-terminal His-kinase-related region of both phyA and phyB. Kinase assays *in vitro* identified PKS1 as a substrate for light-regulated phytochrome kinase activity. *In vivo* experiments show that PKS1 is phosphorylated in a phytochrome-dependent manner, and that the active P_{fr} form of the phytochrome is much more effective than the P_r form at phosphorylating PKS1 (Fankhauser *et al.*, 1999). However, PKS1 does not appear to associate differentially with the two forms of phytochrome despite their different effects on PKS1 phosphorylation. Mutation of the phyA Ser_{599} residue, which is normally autophosphorylated in P_{fr}, disassociates PKS1 phosphorylation from light regulation (Fankhauser *et al.*, 1999), implying that phosphorylation, not binding, is the important step in its regulation (Fankhauser *et al.*, 1999). Overexpressing *PKS1* yields seedlings with elongated hypocotyls when grown in

white light, which indicates that the *PKS1* overexpressor is less sensitive to red light, and that PKS1 may function as a negative regulator in phyB signaling (Fankhauser *et al.*, 1999). Subcellular localization studies indicate that PKS1 protein locates constitutively to the cytosol (Fankhauser *et al.*, 1999), which is different from the positions of other phytochrome-interacting factors, such as PIF3. These results have led to several hypotheses on the function of PKS1 that have not yet been tested.

NDPK2

Like PKS1, *Arabidopsis* NDPK2 was isolated in a yeast two-hybrid screen using the C-terminus of *Arabidopsis* phyA as bait (Choi *et al.*, 1999). Like PIF3, NDPK2 interacts preferentially with the P_{fr} form of both PHYA and PHYB. Using an NDPK2:GFP fusion protein, Choi *et al.* has shown that NDPK2 is expressed in both the nucleus and cytoplasm. Therefore, NDPK2 and phytochromes share the same subcellular space (Choi *et al.*, 1999). Moreover, a significant increase in NDPK2 activity is observed in the presence of the P_{fr} form of phyA, but not its P_r form (Choi *et al.*, 1999).

Evidence that NDPK2 may function in a subset of phytochrome-regulated photomorphogenic responses has come from characterization of seedling deetiolation in an *Arabidopsis* mutant that contains a T-DNA insertion in the NDPK2 gene (Choi *et al.*, 1999). Study of the *ndpk2* mutant has revealed severe defects in hook opening and cotyledon separation, even though the effect on hypocotyl elongation is marginal (Choi *et al.*, 1999), indicating that NDPK2 acts as a positive factor in both phyA- and phyB-

mediated signal transduction, but is not essential for phyA- and phyB-mediated hypocotyl elongation.

Subcellular localization and yeast mutant-rescue studies suggest that NDPK2 may function as a transcriptional regulator in both phyA- and phyB-mediated pathways. Zimmerman *et al.* showed that *Arabidopsis* NDPK2 protein can bind to the yeast *HIS4* promoter *in vitro*, and, when expressed in yeast, it induces *HIS4* transcription (Zimmermann *et al.*, 1999). Moon *et al.* found that NDPK2 regulates a plants' responsiveness to oxidative stress and enhances the phosphorylation of H_2O_2-activated endogenous proteins (Moon *et al.*, 2003). However, these NDPK2-mediated responses are not believed to be under control of phytochromes.

Factors that interact specifically with phyA

CRY1 and CRY2

CRY1 and CRY2 are photoreceptors that carry out a variety of blue-light sensing functions. However, far-red/red light-sensing phytochromes have been repeatedly shown to modulate blue light responsiveness (Gaba *et al.*, 1984; Mohr, 1986, 1994). Pretreatment of plant tissue with red light can significantly enhance cryptochrome-dependent responses. Studies with phytochrome null mutants have revealed that the full function of cryptochromes requires a minimal amount of phytochrome (Mohr, 1986, 1994). Using yeast-produced recombinant oat phyA, Ahmad *et al.* showed that recombinant preparations of both CRY1 and CRY2 can be

phosphorylated *in vitro* by phyA on serine residues in the C-terminus, and no phosphorylation of CRY1 or CRY2 was observed in the absence of phytochrome (Ahmad et al., 1998). Several regions in the C-terminus of phyA are essential for this function. The degree of phosphorylation did not change dramatically under different light conditions: red, blue or far red. These data show that, in contrast to PKS1, CRYs can be phosphorylated by either the P_r or P_{fr} form of phyA (Ahmad et al., 1998), suggesting that both forms of phytochrome are effective in the phosphorylation reaction or that the small amount of P_{fr} produced by B or FR is sufficient to yield the full phosphorylation response (Wong *et al.*, 1986; Wong *et al.*, 1989).

Given that both phytochromes (Sakamoto and Nagatani, 1996; Kircher *et al.*, 1999; Yamaguchi *et al.*, 1999) and cryptochromes (Cashmore *et al.*, 1999; Guo *et al.*, 1999) localize to the nucleus, the phosphorylation of CRY1 and CRY2 by phytochrome may indicate that photoactivation of phytochrome enhances their activities in blue-light signaling.

Aux/IAA

Auxin/indole-3-acetic acid (*Aux/IAA*) genes encode short-lived transcription factors that are induced as a primary response to the plant growth hormone IAA or auxin. Gain-of-function mutations in *Arabidopsis* genes, *SHY2/IAA3*, *AXR3/IAA17*, and *AXR2/IAA7* cause pleiotropic phenotypes consistent with enhanced auxin responses, possibly by increasing Aux/IAA protein stability (Timpte *et al.*, 1994; Leyser *et al.*, 1996). Out of these three mutants, *shy2* was identified in suppressor screens of

phytochrome mutants. Colon-Carmona *et al.* have shown that there is direct interaction between Aux/IAA gene products and phyA by using recombinant Aux/IAA proteins from *Arabidopsis* and pea (*Pisum sativum*) and recombinant phytochrome A from oat (*Avena sativa*) *in vitro* (Colon-Carmona *et al.*, 2000). Phytochrome-dependent phosphorylation of Aux/IAA proteins is proposed to provide one molecular mechanism for integrating auxin and light signaling in plant development (Colon-Carmona *et al.*, 2000).

Factors that interact specifically with phyB

PIF4

Huq *et al.* used genetic screens under continuous red light (Rc) to identify mutants that are deficient in phytochrome signal transduction pathways (Huq et al., 2000; Huq et al., 2000). One of the mutants, called *srl2*, was isolated from the screen because of its hypersensitivity to Rc irradiation. The protein encoded by the *SRL2* gene has been designated PIF4 (phytochrome interacting factor 4) (Huq and Quail, 2002). Sequence analysis revealed that, like PIF3, PIF4 contains a region with strong homology to the bHLH superfamily of transcription factors, suggesting that PIF4 binds to the G-box DNA motif just as PIF3 does (Littlewood and Evan, 1998; Atchley *et al.*, 1999). Like PIF3, PIF4 also has a nuclear localization domain which indicates that it likely functions inside the nucleus (Dehesh *et al.*, 1995). Subcellular localization of GUS-PIF4 fusion protein also confirmed that PIF4 is a nuclear protein and is translocated into the nucleus (Huq and Quail, 2002). Since phyB is the major

photoreceptor for Rc irradiation and *srl2* lacks a significant FR phenotype, PIF4 is thought to be involved selectively in phyB signaling, interacting with the P_{fr} form of phyB and only very weakly with phyA. The hypersensitivity of *srl2* to Rc irradiation revealed that PIF4 is a negative regulator in the phyB signaling pathway (Huq and Quail, 2002).

Although PIF3 and PIF4 are closely related bHLH proteins, both are involved in phytochrome signaling pathways, and both are translocated into the nucleus, they have significant differences in their roles in signal transduction. For one, PIF3 interacts with both phyA and phyB and plays a role in both pathways, whereas PIF4 is specific to the phyB pathway. For another, PIF3 has been reported to control the expression of key regulatory genes, such as *CCA1* and *LHY*, and so far no target gene has been found to be regulated by PIF4.

ELF3

ELF3 (EARLY FLOWERING 3) was identified through a mutation that caused early flowering in both long and short photoperiods (Zagotta *et al.*, 1992). The *elf3* mutant exhibited pale leaves and elongated hypocotyl and petioles, phenotypes usually associated with defective light perception (Zagotta *et al.*, 1996). *ELF3* encodes a novel 695-amino acid protein that may function as a transcriptional regulator (Hicks *et al.*, 2001). Using a fusion construct of the *ELF3* promoter and the firefly luciferase coding region (Millar *et al.*, 1992), Covington *et al.* demonstrated that *ELF3* expression is regulated by the internal circadian clock. Study of both day- and night-phased circadian

markers such as chlorophyll a/b binding protein (*CAB* or *lhcb*) and *LHY* showed that ELF3 is regulating normal circadian rhythms of both these markers (Covington *et al.*, 2001), even though study on rhythmic expression of the *CCR2* gene in the *elf3* mutant revealed that ELF3 may not be required for the clock function in the absence of light (Covington *et al.*, 2001; Hicks *et al.*, 2001).

Since the synchronization of the clock to day-night cycles is mediated by phytochromes and cryptochromes in *Arabidopsis*, recent studies on ELF3 have been concentrated on its role in light signaling networks. Liu *et al.* showed that ELF3 can interact with the C-terminal domain of phyB *in vitro* (Liu *et al.*, 2001), but is unable to bind a similar region of phyA, suggesting that ELF3 is specific to the regulation of phyB responses. It has also been suggested that phyB and ELF3 can form a signaling complex that controls early events in plant development. Loss-of-function mutations at the *ELF3* locus result in deficiencies in light regulation, and ELF3 overexpression causes hypersensitivity to light, indicating that ELF3 might function as a positive regulator in phyB signaling, in a manner similar to that of both PIF3 and PIF4 (Liu *et al.*, 2001; Carre, 2002).

Genetic analyses show that ELF3 requires PHYB function in early morphogenesis, but not for the regulation of flowering time. Therefore, the process by which ELF3 controls flowering is likely independent of phyB signaling. Several other studies have also shown that ELF3 may function in association with other photoreceptors (Zagotta *et al.*, 1996; Reed *et al.*, 2000; Liu *et al.*, 2001).

ARR4

Both plants and bacteria use a "two component system", consisting of a histidine protein kinase that senses a signal input and a response regulator that mediates the output. The *Arabidopsis thaliana* response regulator 4 (ARR4) is homologous to these regulators (Brandstatter and Kieber, 1998; Imamura *et al.*, 1999). A direct interaction between ARR4 and phyB has been demonstrated as ARR4 co-immunoprecipitates with phyB, and ARR4 copurifies with both the P_r and P_{fr} forms of phyB, but not with phyA. By the yeast two-hybrid assay, ARR4 interacts with full-length phyB and with the N-terminus of phyB, but not with any other phyB domains. It does not interact with the N-terminal domain of phyA. In addition, the N-terminus of phyB does not interact with other types of the *Arabidopsis thaliana* response regulators such as ARR2 and ARR5 (Sweere *et al.*, 2001).

ARR4 is expressed in both light-grown and dark-grown seedlings. It can be found in stems, leaves, and flowers but not in roots. Thus, the expression pattern of ARR4 overlaps in great part with that of phyB. Intracellular partitioning of an ARR4:GFP fusion protein demonstrated that the chimera was detectable in both the cytoplasm and the nucleus independent of irradiation (Sweere *et al.*, 2001). Accumulation of ARR4 is induced by Wc, Rc and by a 5 min R pulse in dark-grown seedlings, but not by FR light. The inductive effect of Rlight could be reversed by a subsequent FR light pulse (Sweere *et al.*, 2001). These data suggest that the P_{fr} form of phyB dominates the photoregulation of ARR4 expression. Transgenic *Arabidopsis*

plants overexpressing ARR4 display hypersensitivity to R light, but not to light of other wavelengths. This hypersensitivity suggests that ARR4 may stabilize the Pfr form of phyB, reducing its dark reversion to P_r as is consistent with its binding characteristics, and that it specifically affects phyB-dependent photomorphogenic responses (Sweere *et al.*, 2001).

ZT1/ADO1

ZTl/ADO1 encodes a protein containing a divergent PAS domain that is similar to the LOV domains of *Arabidopsis* photoreceptors PHOT1 and PHOT2. As in the *elf3* mutant, the period of cyclical gene expression is altered in *ado1* mutants, suggesting ADO1 is a circadian clock component. Moreover, under constant W or B light, the *ado1* mutant exhibits longer hypocotyls than that of wild-type *Arabidopsis* seedlings. Both phenotypes are controlled by phytochromes and cryptochromes. Using the yeast two-hybrid system, Jarillo *et al.* showed that there is a direct interaction between ADO1 and the C-terminal domain of both cry1 and phyB, indicating that ADO1 is a positive regulator of phyB and cry1 regulated pathways (Jarillo et al., 2001).

IV. Early phytochrome signaling components

Current research on plant development in response to changing light conditions has uncovered numerous signaling components downstream of the photoreceptors, with the majority of our understanding coming from the examination of de-etiolation in *Arabidopsis* seedlings (Quail, 2002; Sullivan and Deng, 2003). Recent microarray studies have shown that, during de-etiolation, expression of approximately 30% of genes in the *Arabidopsis* genome have been altered (Ma *et al.*, 2001; Tepperman *et al.*, 2001). The identification of mutants defective in different aspects of de-etiolation and under different light conditions have identified signaling intermediates downstream of photoreceptors (Lin, 2002) (Moller et al., 2002; Quail, 2002; Wang and Deng, 2002). However, much of the work has been put into phyA- and phyB-specific signaling and little is currently known regarding phyC, phyD, or phyE signaling pathways.

Early phyA signaling components

Several phyA signaling intermediates have been characterized at the molecular level. For example, *LAF6 (LONG AFTER FR 6)* encodes an ATP-binding cassette (AtABC1) protein which may be involved in coordinating intercompartmental communication between plastids and the nucleus (Moller *et al.*, 2001). *PAT1* (phytochrome A signal transduction 1) encodes a cytoplasmic protein which is a new member of the GRAS (*GAI, RGA, SCR*) family and acts at an early step of phyA signaling (Fry *et al.*, 2002). FIN219 (far-red insensitive 219) is a GH3-like protein and

also exists in the cytosol. The expression of *FIN219* is auxin-inducible, which may be a key factor coordinating auxin responses with light regulation (Hsieh *et al.*, 2000). FHY1 is a novel light-regulated protein that accumulates in dark-grown but not in FR-grown hypocotyl cells (Desnos *et al.*, 2001). The *far1* (far-red impaired response) mutant was isolated by mutagenizing *Arabidopsis* seedlings that overexpress the *PHYA* gene and screening for mutants that display a phenotype similar that of a to *phyA* mutant (Hudson *et al.*, 1999). *FHY3* encodes a protein homologous to FAR1, and overexpression of either *FHY3* or *FAR1* suppresses the mutant phenotype of both genes (Wang and Deng, 2002). FIN2 works at an early step of phyA signaling and may function at a branch point in phyA-mediated responses (Soh *et al.*, 1998). Mutations in *FHY1, FHY3, LAF6, FAR1, FIN2, PAT1* and *FIN219* all exhibit a far-red-specific phenotype similar to that of phyA mutants such as long hypocotyl, apical hook opening, and cotyledon separation, even though there are subtle distinctions among them in phenotypes such as the FRc preconditioned block of greening.

In contrast to the many mutants with a reduced phyA response, only two mutants (*spa1* and *eid1*) have been shown to enhance phyA-specific light responses (Hoecker *et al.*, 1998; Buche *et al.*, 2000; Wang and Deng, 2003). Genetic and physiological results indicate that both SPA1 and EID1 function as negative regulators in the phyA signaling network. SPA1 is a WD (tryptophan-aspartic acid)-repeat protein that also shares sequence similarity with protein kinases (Hoecker *et al.*, 1999). EID1 is a novel F-box protein that contains a leucine zipper and acts by targeting activated components of the phyA signaling pathway for ubiquitination and degradation (Dieterle

et al., 2001). SPA1 and EID1 are involved in different but interacting phyA-mediated physiological responses. SPA1 functions as a negative regulator in both VLFR and the FR-HIR, whereas EID1 is only involved in signaling cascades regulating the FR-HIR (Zhou *et al.*, 2002). Recent reports show that both SPA1 and EID1 are involved in regulated proteolysis of various phyA signaling intermediates. Therefore, SPA1 and EID1 may negatively regulate phyA-mediated networks through controlling target protein degradation (Dieterle *et al.*, 2001; Hoecker and Quail, 2001).

Early phyB signaling components

There are fewer signal pathway mutants that have been identified for phyB than for phyA. However, those that have been found are instrumental to our understanding of phyB signaling networks. *GI* (*GIGANTEA*), identified as a circadian clock-controlled gene involved in control of flowering time, acts in phyB signal transduction (Fowler *et al.*, 1999). GI modulates phyB signaling at distinct phases of the life cycle under Rc, and GI may function as a phyB signaling intermediate in photoperiodic control of flowering (Huq et al., 2000). Several possible membrane-spanning and nuclear localization domains in the *GI* gene suggest that GI is constitutively localized in the nucleoplasm (Huq et al., 2000). Both *pef2* and *pef3* (*phytochrome-signaling early flowering 2-3*) mutants show early flowering under short days and reduced inhibition of hypocotyl elongation under R, but no impairment was observed under FR-HIR conditions (Ahmad and Cashmore, 1996). By mutagenizing an *Arabidopsis* line that overexpresses phyB, the *red*1 (*red elongated 1*) mutant was isolated by screening for

revertants of the enhanced de-etiolation response (Wagner *et al.*, 1997). *Srl1* mutants show hypersensitivity to red light, suggesting the SRL1 is a negatively acting component specific to phyB signaling (Huq et al., 2000). The molecular identities of these genes are currently unknown. Mutation of the newly described GH3-related gene DFL2, causes a short hypocotyl when overexpressed under red and blue light and a long hypocotyl when an antisense DFL2 is expressed under R. The expression of DFL2 is induced transiently by R pulses (Takase *et al.*, 2003).

PhyA and phyB joint signaling network

Mutant *pef1* (*phytochrome-signaling early flowering 1*) shows a reduced sensitivity to both Rc and FRc light in the inhibition of hypocotyl elongation response (Ahmad and Cashmore, 1996). This recently isolated EARLY-PHYTOCHROME-RESPONSIVE 1 gene (*EPR1*) encodes a novel nuclear-localized protein that is regulated by both phyA and phyB. EPR1 is a MYB-class protein with a single MYB domain highly similar to those of the circadian oscillator proteins CCA1 and LHY, which are also under the control of phytochromes. Moreover, EPR1 suppresses its own expression, suggesting that this protein is part of a regulatory feedback loop (Kuno *et al.*, 2003). *LSH1* (light-dependent short hypocotyls 1) encodes a nuclear protein of a novel gene family. The dominant *lsh1*-D allele causes hypersensitive responsiveness to Rc, FRc and Bc light (Zhao *et al.*, 2004).

V. Downstream phytochrome signaling network

One large class of *Arabidopsis* genes, designated *COP/DET/FUS*, has been shown to be important for the repression of photomorphogenesis in darkness. At least 11 mutant loci in this class have been isolated so far. Since these mutant seedlings exhibit a light-grown phenotype when grown in darkness, the wild type loci are generally considered to act negatively in darkness to suppress photomorphogenesis. These mutations are pleiotropic and the proteins defined by these loci act late in the signaling process downstream of both phytochrome and cryptochrome signaling (Chory et al., 1989; Deng et al., 1991; Hou et al., 1993; Wang and Deng, 2002). One of these genes, constitutively photomorphogenic 1 (*COP1*), functions as a light-inactivatible repressor of photomorphogenesis. COP1 is a RING-finger protein with WD-40 repeats and shows nuclear enrichment in darkness but not in light (Deng *et al.*, 1992; von Arnim and Deng, 1994). Acting as an E3 ubiquitin-protein ligase, COP1 assists the degradation of specific proteins through ubiquitination (Osterlund et al., 1999; Osterlund et al., 2000; Schwechheimer et al., 2001). One of the targets of COP1 is HY5, a constitutively nuclear bZIP transcription factor which binds to the promoters of light inducible genes, such as chalcone synthase (*CHS*). HY5 functions as a positive regulator downstream of phytochrome in the signaling network affecting plant photomorphogenic development (Oyama *et al.*, 1997; Chattopadhyay *et al.*, 1998). COP1 interacts directly with HY5 and targets its proteasome-mediated degradation in the nucleus. Hence, COP1 and HY5 act antagonistically in regulating seedling development (Ang et al., 1998; Osterlund et al., 2000; Osterlund et al., 2000).

In addition to COP1, gene products from other identified *COP/DET/FUS* loci act as negative regulators of photomorphogenesis. Among these loci, six (CSN1-CSN4, CSN7 and CSN8) encode six of the eight subunits of the COP9 signalosome (CNS) protein complex (Wei and Deng, 1999; Schwechheimer and Deng, 2000; Serino and Deng, 2003). Other than photomorphogenesis, the CNS regulates many other aspects of plant development, such as auxin responsiveness, disease resistance, and flower development (Peng et al., 2001, 2001). Like COP1, the CNS regulates plant development through the control of ubiquitin-proteasome-mediated protein degradation (Schwechheimer *et al.*, 2001; Schwechheimer *et al.*, 2002). Even though COP1, DET1, and COP10 are not required for CSN biogenesis, genetic and biochemical evidence indicates that they cooperate with the CSN to induce degradation of positive regulators of photomorphogenesis, such as HY5 (Osterlund et al., 2000; Holm et al., 2002).

Arabidopsis de-etiolated 1 (*det1*) mutants also develop like light-grown seedlings even when grown in the dark. Like COP1, DET1 is a nuclear-localized protein and it functions downstream of multiple photoreceptors to regulate morphogenesis and gene expression in response to light (Chory et al., 1989; Pepper et al., 1994). DET1 is a component of a complex that contains UV-DDB1, which is part of histone acetyltransferase complexes in animal cells. DET1 may bind to the nucleosomes of light-induced genes and is subsequently displaced by light-dependent acetylation of histone tails, thus permitting gene expression (Benvenuto *et al.*, 2002). *det2* mutants also display partially light-grown phenotypes in the dark (Chory *et al.*, 1991). The

deficiencies of *det2* can be restored by application of brassinosteroids (BR), indicating that *det2* is a BR-deficient mutant defective in the brassinosteroid synthesis pathway. The function of DET2 as a reductase in the brassinolide biosynthetic pathway gains support from sequence comparisons showing that DET2 shares significant homology with mammalian steroid 5 alpha-reductases, and by *in vitro* functional assays (Li *et al.*, 1996).

Two proteins, HFR1 and LAF1, have previously been shown to be involved in the early phyA signal pathway because gene mutants exhibit a reduction in seedling responsiveness specifically to FRc, not to Rc. HFR1 is an atypical basic helix-loop-helix (bHLH) transcription factor closely related to PIF3, and LAF1 is a Myb-type transcription activator (Fairchild *et al.*, 2000; Ballesteros *et al.*, 2001). However, recent studies show that they act downstream of COP1 in the phyA signaling pathway. HFR1 is required for *cop1*-mediated photomorphogenic seedling development (Kim et al., 2002). However, it is not clear how HFR1 maintains FR specificity given the highly pleiotropic role of COP1. LAF1 is ubiquitinated by COP1 and overexpression of COP1 leads to a decrease in the cellular LAF1 concentration (Seo *et al.*, 2003).

VI. Dose-Response Relations in Photomorphogenesis

Phytochrome responses are dependent on the fluence levels of light applied. There are three general kinds of phytochrome responses based on the fluences required to elicit them: very low fluence responses (VLFRs), low fluence responses (LFRs), and high irradiance responses (HIRs).

Very low fluence responses (VLFRs)

The photon-fluence requirements for VLFRs are in the range of 10^{-6} to 10^{-3} μmol m^{-2} s^{-1}. Dark-grown seedlings are capable of responding to very low levels of light. For example, the R level that can promote an increase in sensitivity of cereal grain seedlings to a subsequent phototropic stimulus is much lower than that required to induce the conversion of sufficient Pr to Pfr to induce classic low fluence responses. The VLFRs can not be nullified by FR because even FR light is sufficient to convert enough P_r to P_{fr} to initiate the response (Mandoli and Briggs, 1981). Because VLFRs are extremely sensitive to P_{fr}, exposure to long-wavelength FR or green "safelight" is sufficient to elicit and even saturate this type of effect. To study VLFRs, imbibed seeds must not to be exposed to any light other than the light being used to investigate the VLFR response itself.

It is important to identify which of the phytochromes is responsible for the effects, since the VLFRs have important ecological consequences for responses such as

for seed germination. Because phyA accumulates to relatively high concentrations in dark-grown seedlings, it has been suggested that phyA operates as the sensor for small amounts of light. Under FR, the amount of phyA P_{fr} is sufficient to induce the VLFRs. Under R, even though the amount of phyA P_{fr} is drastically reduced because of degradation, it can still induce VLFR since the majority of phyA is in its P_{fr} form. Studies on phytochrome mutants confirm that the VLFR is specifically controlled through phyA.

Low fluence phytochrome responses (LFRs)

The photon-fluence requirements for LFRs fall within the range of 10^{-1} to 10^{2} μmol m^{-2} of R. The LFRs are "typical" phytochrome responses in terms of photon requirements. They include most of the classic R/FR photoreversible responses such as shade avoidance and leaf movement. The LFRs can be nullified by subsequent FR because of the photoconversion of most of the P_{fr} back to the P_r form. Under R, a large proportion of phyB molecules are in the P_{fr} form. These molecules recognize downstream factors to induce the LFR. However, under pulsed or continuous FR, the proportion of phyB molecules in the P_{fr} form is below the threshold for inducing LFRs.

Both VLFRs and LFRs can be induced by brief pulses of light. The total fluence is a function of two factors: the fluence rate (μmol m^{-2} s^{-1}) and the irradiation time. Thus, a brief pulse of red light will induce a response if the light is sufficiently bright, and conversely, very dim light will be inductive if the irradiation time is long enough.

This reciprocal relationship between fluence rate and time is known as the law of reciprocity. Both VLFRs and LFRs obey the law of reciprocity.

High irradiance responses (HIRs)

The HIRs require continuous light over several hours or longer and are dependent on the actual fluence rate rather than on the total fluence. Under natural conditions, plants are exposed to long periods of light with a high fluence rate. Therefore, the HIRs are very important because they reflect conditions of the natural environment. HIRs generally share the following characteristics: full expression of the response requires prolonged exposure to high irradiance; the magnitude of the response is a function of the fluence rate and exposure duration within a certain range; and in contrast with LFRs, HIRs are not fully red, far-red photoreversible and do not obey the law of reciprocity (Shropshire, 1972; Mancinelli, 1980).

HIRs, such as the inhibition of stem or hypocotyl growth, have usually been studied in dark-grown, etiolated seedlings. PhyA is the phytochrome that controls HIRs responding to FR (FR-HIRs) and phyB is the phytochrome that controls HIRs responding to R (R-HIRs). These responses include inhibition of hypocotyl elongation, enlargement of cotyledons, regulation of numerous genes, and others.

VII. The role of phytochrome in seed germination and seedling development

The plant phenotypes discussed in this section are all phytochrome-controlled responses. They are all induced by the P_{fr} form of phytochrome and inhibited by the conversion or degradation of the active P_{fr} form. They are also the major characteristics which were studied in characterizing the novel phytochrome signal transduction pathway mutants isolated in our laboratory.

Seed germination

The germination of dormant seeds is controlled by genetic factors, as well as environmental factors such as light and temperature (Koornneef *et al.*, 2002). In unfavorable environmental conditions, the seed embryo is able to remain dormant in a dry state for extended periods until those unfavorable conditions no longer exist (Koornneef *et al.*, 2002). In *Arabidopsis*, the germination of dormant seeds is mediated entirely by phytochromes (Casal *et al.*, 1998). In the LFR-mediated germination of *Arabidopsis* seeds newly imbibed in darkness, the P_{fr} form of phyB plays the primary role, since there is very little phyA in seeds (McCormac *et al.*, 1993). Shinomura *et al.* showed that phyA can only induce germination after a prolonged imbibition of seeds (Shinomura *et al.*, 1994). Shortly after imbibition, FR is an inhibitory factor because it decreases the amount of phyB P_{fr} which is needed for germination. A low R:FR ratio of the light environment will prevent phyB-induced germination, typical of red/far-red

reversible LFRs. Several days after imbibition occurs, phyA is produced at high levels in the seeds, which allows phyA-mediated germination under VLFR and FR-HIR conditions (Shinomura *et al.*, 1996; Casal *et al.*, 1998). Recent studies on a *phyAphyB* double mutant indicate that another red/far-red reversible photoreceptor other than phyA and phyB is involved in germination regulation. Further experiments on *phyC*, *phyD* and *phyE* mutants showed that phyE also activates seed germination (Yang *et al.*, 1995; Poppe and Schafer, 1997; Hennig *et al.*, 2002). Studies on the *cop1* mutant showed that its germination rate is still fully under phytochrome control even though the mutants show totally different phenotypes than the wild type when grown in the dark. This finding suggests that germination and photomorphogenesis during seedling development involve two independent developmental commitments (Deng *et al.*, 1991) and that not all phytochrome-regulated pathways converge on COP1.

De-etiolation

After seed germination, *Arabidopsis* seedlings follow one of two developmental templates. In darkness, seedlings follow skotomorphogenic development with long hypocotyls, closed cotyledons, and very little chlorophyll or anthocyanin. In contrast, seedlings grown in the light display photomorphogenic development with short hypocotyls, anthocyanin and chlorophyll accumulation, and open, expanded cotyledons that are capable of photosynthesis (Sullivan and Deng, 2003). The regulation of de-etiolation involves a complex interplay of several photoreceptors, including phytochromes and cryptochromes (Nemhauser and Chory, 2002; Wang and Deng,

2002): phyA perceives primarily FR light, phyB perceives R light and both cry1 and cry2 function in the perception of B light during de-etiolation (Ahmad and Cashmore, 1993; Lin et al., 1998; Quail, 2002). Studies on *cop/det/fus* mutants suggest that etiolation is the growth pattern that applies when the default pathway of photomorphogenesis is suppressed (Chory et al., 1989; Deng and Quail, 1992; Smith, 1995).

In *Arabidopsis,* the opening of the apical hook and separation of cotyledons are stimulated by R, B and FR light. PhyA and phyB do not appear to be major factors for cotyledon expansion in W-grown seedlings. Full cotyledon expansion in R light is promoted by the cooperation of phyB and phyA. On the other hand, phyA is responsible alone for the separation and expansion of these "seed leaves" under FR (Liscum and Hangarter, 1993) (Reed *et al.*, 1994).

Inhibition of hypocotyl elongation is the most readily apparent and easily quantified phenotype used to study the functions of phytochromes. Multiple photoreceptors control hypocotyl elongation. In *Arabidopsis*, phyA, phyB, and cry1 all have been shown to contribute to the inhibition of hypocotyl cell elongation (Young *et al.*, 1992). *PhyA* mutants produce long hypocotyls under FRc. Under Rc, only *phyB* mutants exhibit long hypocotyls, probably because phyA is quickly degraded in these conditions. *phyB* mutants also produce long hypocotyls under Wc light, but even null mutants do not have a hypocotyl as long as that of skotomorphogenic seedlings. The double mutants between *phyB* null mutants and *phyA*, *phyD* or *cry1* display enhanced

long hypocotyl phenotypes, indicating the co-action of multiple photoreceptors that sense W light during de-etiolation (Neff and Van Volkenburgh, 1994; Chory *et al.*, 1996). The multiple photoreceptors that control inhibition of hypocotyl elongation may be partially redundant.

Most plants form anthocyanin pigments and other flavonoids in specialized cells in one or more of their organs, and this process is frequently promoted by light. Flavonoids are a group of secondary metabolites ubiquitous among higher plants. They have an enormous range of important functions within the plant, including floral coloration, protection against pathogens, and plant-microbe interactions. Flavonoids also perform an important role in pollen recognition and photoprotection from damaging ultraviolet (UV) wavelengths (Koes *et al.*, 1993) (Li *et al.*, 1993). High level production of flavonoids requires sugars as a provider of carbon atoms and light to induce the expression of the enzymes in the synthetic pathway (Short, 1999; Kong *et al.*, 2003).

The synthesis of a glycosylated group of flavonoid pigments, the anthocyanins, has been heavily studied in a wide variety of commercially important plants as well as in *Arabidopsis*. Studies show that at least several of the enzymes in the anthocyanin synthetic pathway, including chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR), are regulated by a complex combination of external and internal signals, including visible and UV light (Short and Briggs, 1994; Short *et al.*, 1994; Batschauer *et al.*, 1996), temperature (Feinbaum and Ausubel, 1988),

developmental stage (Kaiser and Batschauer, 1995), hormones such as cytokinins (Batschauer *et al.*, 1996), and carbohydrates (Tsukaya *et al.*, 1991). Therefore, the flavonoid/anthocyanin pathway has become a model system for studying interactions of photoreceptor sensory pathways with other stimulus-transduction chains. Young *phyA* null mutants growing under FRc produce very little anthocyanin, since phyA induces anthocyanin production in seedlings. Increasing the sucrose concentration within a limited range will also increase the accumulation of anthocyanin. Therefore, mutants in anthocyanin production could be involved in phyA- or sugar-mediated pathways, or in developmental stage signaling.

The consequences of de-etiolation, such as chlorophyll accumulation and subsequent transition of the proplastid or etioplast to the chloroplast, are also regulated by phytochromes. Negative regulators such as COP/DET/FUS can suppress the development of the chloroplasts. The mutation of these genes results in the absence of etioplasts and in partial chloroplast development in complete darkness, accompanied by cotyledon expansion, arrest of hypocotyl elongation, and normally light-specific cell type differentiation.

One of the phenotypes used to analyze mutants is the ability to green; that is, to accumulate chlorophyll and maintain it in chloroplasts. The rationale underlying these this experiments is that phyA is able to block subsequent greening during growth in FRc. FRc irradiation prevents the accumulation of protochlorophyllide oxidoreductase (POR) by a strong phyA-dependent inhibition (Barnes *et al.*, 1996). POR is a critical

enzyme for chlorophyll synthesis and chloroplast development. Therefore, if young seedlings are exposed to FRc for three days before transfer to Wc, all wild type seedlings will die because they lack the POR enzymes, but the *phyA* mutant will survive and turn green because POR has not been down-regulated. If wild type seedlings are exposed to FRc for less time (such as 2 days or less), some POR persists and the seedlings can recover after transfer to Wc (Barnes *et al.*, 1996). The repression of POR by phyA can be prevented by adding sucrose into the medium. However, sucrose does not suppress this effect purely by providing a nutrient source, but by controlling signal transduction (Barnes *et al.*, 1996).

Shade-avoidance responses

In the natural environment, plants are in constant competition with their neighbors for resources, including light. A plant that grows under a canopy can either adapt to reduced light (shade tolerance) or escape by changes in growth (shade avoidance) (Smith and Whitelam, 1997). The angiosperms like *Arabidopsis* have evolved impressive mechanisms to avoid shade. It is now fully accepted that shade-avoidance reactions are all initiated by a single environmental signal, the reduction in the ratio of R to FR radiation. Cumming first reported observations linking phytochromes to shade avoidance responses (Cumming, 1963). The shade avoidance responses include stimulation of elongation growth (Child *et al.*, 1981) and acceleration of flowering (Halliday *et al.*, 1994). All these shade-avoidance responses can be simulated by growing plants under low R:FR ratio conditions, mimicking growth under

a leaf canopy or in proximity to other photosynthetic competitors (Morgan and Smith, 1981; Child and Smith, 1987).

The striking similarities between the phenotypes of *Arabidopsis phyB* mutants and the phenotypes of wild-type plants displaying shade avoidance indicate that phyB is likely responsible for mediating shade avoidance responses (Nagatani *et al.*, 1991; Somers *et al.*, 1991; Devlin *et al.*, 1992). Null alleles of the *Arabidopsis phyB* mutant resemble wild-type plants that are given FR during the photoperiod or end of day (EOD) FR treatments (Goto *et al.*, 1991; Robson *et al.*, 1993; Halliday *et al.*, 1994). However, phyB does not appear to be the sole mediator of the shade avoidance syndrome in *Arabidopsis*, since *phyB* null mutants also show increased elongation growth responses to reduced R:FR ratio, even though similar experiments show that phyA is unnecessary for shade avoidance in *Arabidopsis* (Yanovsky *et al.*, 1995; Devlin *et al.*, 1996; Whitelam and Devlin, 1997). Yanovsky *et al.* showed the action of phyA in plants exposed to low R:FR ratio antagonizes that of phyB in the control of elongation growth, causing an exaggerated elongation response (Yanovsky *et al.*, 1995; Smith and Whitelam, 1997). This antagonism suggests that the role of phyA in shade avoidance responses may be to limit phyB and thus "fine tune" the light responses. A redundant role of phyD and phyE has been demonstrated by using *phyBphyD* and *phyBphyE* double mutants in the study of shade avoidance (Devlin *et al.*, 1998; Devlin *et al.*, 1999).

Phototropism

Phototropism was one of the first light-regulated plant photomorphogenic developmental responses to be studied actively (Darwin, 1881). Most studies on phototropism, or the directional curvature of organs, concentrate on the growth of seedling stems, which show positive phototropism (growth toward light) and primary roots, which show negative phototropism (growth away from light). At least two distinct photoreceptor molecules have been suggested for phototropic curvatures induced by high fluence rate blue light: phot1 and phot2 (Konjevic et al., 1989). Genetic studies in *Arabidopsis* demonstrated that phot1 is responsible for normal phototropic response to UV-A, blue, and green light, and it is presumed that the light-activation of the phot1 LOV domain is critical to its signaling properties (Khurana and Poff, 1989; Huala *et al.*, 1997; Taylor and Zhulin, 1999). The second receptor, phot2, was found somewhat later, after the discovery that a *phot1* mutant can display some phototropic responsiveness. Phot1 functions to some extent under all fluence rate conditions, while phot2 has a redundant function to phot1 specifically under high-fluence conditions (Jarillo et al., 2001) (Sakai *et al.*, 2001). Moreover, *phot1phot2* double mutants fail to exhibit seedling phototropic responses at either low or high fluence rates, confirming that phototropism in *Arabidopsis* is entirely mediated by blue light through Phot1 and Phot2 (Sakai *et al.*, 2001). Although phototropins control blue-light mediated phototropism in etiolated plants, both phyA and phyB have been shown to modulate the sensitivity to phototropic stimuli acting through phototropins (Chon and Briggs, 1966; Hangarter, 1997; Liscum, 2002).

Circadian Clock

The perception of day length, or photoperiod, allows organisms to adjust their development in anticipation of annual seasonal changes. A typical circadian system includes multiple input pathways, one or more central oscillators, and a number of output pathways. Light is the bests-characterized entraining stimulus in plants (Devlin and Kay, 2001; Yanovsky and Kay, 2002). PHYA, PHYB, PHYD, PHYE, CRY1 and CRY2 gene products have all been shown to play roles in the establishment of period length (Millar *et al.*, 1995; Somers *et al.*, 1998; Devlin and Kay, 2000). PHYA is the predominant clock photoreceptor under R or B, and PHYB and CRY1 are the main photoreceptors at high intensities of R or B (Somers *et al.*, 1998). Using *cry1cry2* double mutants, Devlin *et al.* showed that both CRY2 and CRY1 are required for the establishment of period at intermediate intensities of B (Devlin and Kay, 2000). Similar studies using a *phyAphyBcry1cry2* quadruple mutant showed that other photoreceptors also function in controlling the clock, since this quadruple mutant can still maintain circadian cycling. Devlin *et al.* demonstrated that PHYD and PHYE are among these photoreceptors controlling the clock by examining *phyAphyBphyD* and *phyAphyBphyE* triple mutants under high intensity red light (Devlin and Kay, 2000).

CHAPTER II. ISOLATION AND CHARACTERIZATION OF THE *LID1* MUTANT

A. Abstract

The first mutant we isolated from our mutant screen for putative phyA-pathway mutants is *lid1*, which is the primary subject illustrated in this chapter. *Lid1* mutants exhibit reduced phyA-induced anthocyanin accumulation in far red light, but lack the other physiological phenotypes that are typically found in early phyA pathway mutants, such as longer hypocotyls and closed cotyledons. The low-anthocyanin production by *lid1* is not affected by different durations and intensities of the FRc and different concentrations of exogenously applied sucrose in a limited range. Moreover, transcript levels of several genes in the anthocyanin biosynthesis pathway which previously have been shown to be down-regulated by phyA are considerably reduced in *lid1* compared with those in wild type. These results indicate that *lid1* maybe a novel genetic mutant in the phyA signaling pathway, and that *LID1* may encode a specific regulator of flavonoid synthesis or turnover of structural genes. Unfortunately, the finding that lid1 may have a mixed parental ecotype renders the gene cloning of lid1 impractical in the near term.

B. Materials and Methods

Plant material and growth conditions

Seeds were surface sterilized for 20 min in a solution of 20% commercial bleach (final sodium hypochlorite concentration 0.05%[w/v]) and 0.1% SDS, then rinsed in 50ml sterile deionized distilled water. Seeds were sown on 100-mm Petri plates containing 0.8% (w/v) agar and MS salts (Murashige and Skoog, 1962). Plates were kept in darkness at 4°C for three days, transferred to white light for 3h to induce germination (approximately 50 μmol m^2 s^{-1} at 24°C), and then placed in the dark at 24°C for 21h. After dark treatment, plates were transferred to appropriate light conditions as indicated for each experiment.

FRc light was obtained from high-output, 735-nm LED source (Q-beam 2001, Quantum Devices, Barneveld, WI) and filtered through far-red plastic resin (FRF700, Westlake plastics, Lenni Mills, PA) to eliminate minor red-light emission from the LED source. A fluence rate of 9.7 μmol m^2 s^{-1} was used except as indicated. Rc was from 660-nm Q-beam LED sources (Quantum Devices, Barneveld, WI) at a fluence rate of 40 μmol m^2 s^{-1}. Light intensity and spectral output were measured with a Li-Cor LI-1800 spectroradiometer (Lincoln, NE).

Columbia (Col-O) and RLD wild types and *phyA-101* mutant strain were obtained from the Arabidopsis Biological Resource Center.

Mutant isolation and genetic characterization

Approximately 10,000 M3 plants from ethylmethanesulfonate (EMS) mutagenized and from T-DNA tagged *Arabidopsis* lines were grown for 2d under FRc. Putative mutants showing low visible anthocyanin levels after 2d FRc treatment were selected, allowed to self fertilize, and the M4 seed re-evaluated for inheritance of the low anthocyanin phenotype. Selected mutants were backcrossed to the parental ecotype three times, and the progeny treated under different light and media conditions as indicated to characterize the mutants' phenotypes for other known phytochrome controlled responses, such as hypocotyl elongation and the FR-dependent block of greening.

Genetic analysis of mutations

Mutants that showed heritable mutant phenotypes from the M4 were backcrossed into their parental ecotype Col-O. F_1 progeny were allowed to self-fertilize and F_2 progeny were screened for low anthocyanin under 2d FRc. F_3 plants were re-screened to confirm the homozygosity of their F_2 parents. F_3 seedlings were then examined for the segregation of the mutant phenotype. The results were subjected to statistical analysis to determine whether the phenotype is consistent with a signal recessive mutation.

Analysis of anthocyanin contents in seedlings

After surface sterilization, seeds from each line were plated on MS medium supplemented with various concentrations of sucrose as indicated. Seeds were stratified in 4°C for 3d. After inducing seed germination by exposing plates to Wc for 3h, plates were returned to darkness for 21h, then transferred to FR (9.7 μmol m^{-2} sec^{-1}) for 2d at 24°C. The intensity of FRc and the duration of FRc treatments were adjusted in later experiments as indicated. Subsequently, the seedlings were homogenized in 1% (v/v) HCL in methanol, and anthocyanins were extracted from these seedlings by shaking overnight. The anthocyanins were quantified spectrophotometrically, as calculated by the following formula:

$$A_{530}-(0.25*A_{657})$$

At least fifty seedlings were used per extraction and each data point represents the mean of at least three independent experiments. The amount of anthocyanin per seeding and the standard deviation from these independent experiments were then calculated.

Measurement of total chlorophyll

After surface sterilization, seeds from each line were sown on 100-mm Petri plates containing 0.8% (w/v) agar and MS salts (Murashige and Skoog, 1962) without supplementary sucrose. Seeds were stratified in 4°C for 3d. After the induction of germination by 3h Wc (75 μmol m^{-2} sec^{-1}) followed by 21h dark, plants were grown in the conditions indicated for each experiment. Seedlings were collected into 1.5ml

eppendorf tubes after the time specified for each experiment. The number of seedlings was counted and combined with 80% acetone in the dark for 24h for total chlorophyll extraction. Samples were centrifuged to remove seedling debris and the absorbance of the supernatant was measured spectrophotometrically as described (Chory *et al.*, 1991). Total chlorophyll concentration was calculated as chlorophyll *a* + chlorophyll *b*, as determined with the formulas:

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

Each data point represents the mean of at least three independent experiments. The amounts of chlorophyll per seedling and the standard deviations from these independent experiments were then calculated.

For determining chlorophyll production in Wc-grown (75 μmol m^{-2} sec^{-1}) tissues, seedlings were left in Wc at 24°C for 5d before the plants were collected. For measuring the FR-dependent block of greening, seedlings were exposed to FRc (9.7 μmol m^{-2} sec^{-1}) at 24°C for 3 days followed by Wc (75 μmol m^{-2} sec^{-1}) for 3d before the seedlings were collected.

Hypocotyl length measurements

After surface sterilization, seeds from each line were sown and stratified as described previously. After the induction of germination by 3h Wc (75 μmol m^{-2} sec^{-1}) followed by 21 h dark, plants were grown in each light condition and for each time indicated. Seedlings were placed horizontally on 0.8% agar plates, photographed with

Ektachrome 200 slide film (Kodak, Rochester, NY) with a reference ruler, and the slides were projected onto a Kurta 1212 digitizing tablet for accurate measurements of the hypocotyls. Hypocotyl lengths were traced and the lengths and basic statistics were computed with SigmaScan Pro software (Jandel Scientific, San Rafael, CA). Measurement of hypocotyl lengths was done with at least 50 seedlings per treatment per experiment. Each experiment was replicated at least three times.

Extraction of total RNA

After inducing germination, seedlings of mutants and wild types grown under the indicated light conditions were collected and weighed, then frozen in liquid nitrogen. The total RNA from these seedlings was isolated by using a total RNA kit (Ambion, Austin, TX) according to the manufacturer's instructions. The total RNA concentration was measured spectrophotometrically at A_{260}, and the RNA quality was confirmed by gel electrophoresis.

Semi-quantitative RT-PCR

RT-PCR was done in two steps with the RT-PCR kit (Ambion, Austin, TX) according to the manufacturer's instructions, the first RT step with Oligo(dT) as primer and the second steps with specific primers for each gene. All the gene-specific primers were designed to span at least one intron, so as to exclude amplification of genomic DNA contamination. The amplified fragments were confirmed by checking the size of

RT-PCR product electrophoretically. The RT-PCR amplification was performed in a Mastercycler (Brinkmann) as follows: 30 s denaturation at 95 °C , 30 s annealing at 60 °C, and 1 min extension at 72 °C for 30 cycles.

Ubiquitin 1 (*UBQ1*) positive control primers used in the RT-PCR experiments were purchased from Sigma-Genosys. The *UBQ1*-forward primer 5'-TTCGTGAAAACCTTGACCGGC-3' and *UBQ1*-reverse primer 5'-TCTTCTTCTTAGGCCTCAA-3' amplify a RT-PCR product of 378 bp and would be expected to produce a contaminating genomic DNA product of 997 bp. Gene-specific primers from *PAL1, CHS, CHI* and *DFR* were also ordered from Sigma-Genosys. *PAL1*-forward primer 5'-GGTTGAATTCCTGCTAATTTGAAAGC-3', *PAL1*-reverse primer 5'-GGTTGAATTCAACTATTGGTAACAGTG-3' which amplify a RT-PCR product of 505 bp and a genomic DNA product of 972 bp, *CHS*-forward primer 5'-GGTTGGATCCCTGAGAACCATGTGCTTCAG-3', *CHS*-reverse primer 5'-GGTTGGATCCGGAGGTAGTGCAGAAGACG-3' which amplify a RT-PCR product of 324 bp and a genomic DNA product of 410 bp; *CHI*-forward primer 5'-GGTTGGATCCTCAACAGAGTGATCACTAAC-3', *CHI*-reverse primer 5'-GGTTGAATTCCAACCGACTCAACAATG-3' which amplify a RT-PCR product of 738 bp and a genomic DNA product of 1165 bp; *DFR*-forward primer 5'-GGTTGGATCCTCGAGACTATACTTGAAG-3', *DFR*-reverse primer 5'-GGTTAAGCTTTGTTCGTGCCACCGTTCG-3' which amplify a RT-PCR product of 840 bp and a genomic DNA product of 1260 bp.

Mapping

Mutants of *lid1* were mapped by genetic linkage analysis against known SSLP microsatellite gene markers. *lid1* was crossed to multiple ecotypes, including No-O and Ws. The F_2 progeny were screened for low anthocyanin under FRc. These seedlings were transferred to soil and grown for two weeks before a leaf was removed for DNA extraction. Total genomic DNA was extracted based on the method by Edwards (Edwards *et al.*, 1991). F_3 plants were re-screened to confirm the homozygosity of the F_2 parent. The DNA isolated from those lacking segregation in the F_3 generation were used for mapping of the phenotype relative to PCR-based simple sequence length polymorphism (SSLP) genomic markers (Bell and Ecker, 1994).

C. Results

I. Isolation of *lid1* mutant

In order to isolate genes encoding proteins involved in the phytochrome regulatory pathway, we initiated a screen for mutants with low anthocyanin production when seedlings were grown for 2 days in FRc, a condition in which phyA is the sole phytochrome inducing anthocyanin accumulation. M_4 seed pools from 10,000 mutagenized M_3 plants were screened. Since sucrose is required for high-level anthocyanin production, all seedlings were screened on MS medium (Murashige and Skoog, 1962) supplemented with 2% sucrose. After the primary screen, 27 putative mutants were identified that showed genetically heritable altered light response defects. Out of this group of mutants, four were identified as possible light signal transduction pathway mutants and named *lid1*, *lid2*, *lid3* and *lid4* (*li*ght *d*eficient), based initially on low anthocyanin accumulation (Fig 4). The characterization of the *lid1* mutant is the focus of this chapter.

lid1 was identified by visual comparison of anthocyanin content in young seedlings of the mutant and its background ecotype Columbia (Col-O). Seeds of *lid1*, Col-O and the *phyA*-null mutant, *phyA-101*, were sown on MS medium containing 2% sucrose. After seedlings were grown for 2 days in FRc, the *lid1* mutant was identified by comparing the color of its hypocotyl with that of wild type. This effect is shown in Fig 5.

To determine whether the *lid1* phenotype is the result of a single recessive mutation, the homozygous *lid1* mutant was backcrossed into its parental ecotype Columbia (Col-O). F_1 seeds were grown up and allowed to self-fertilize to collect F_2 seeds. In the F_2 population, a chi square value was calculated from the segregation of the seedlings between the wild type and mutant phenotypes. This chi square value (χ^2= 1.728) corresponds to $P \approx 0.189$. The segregation pattern of these seedlings did not differ significantly from the expected 3:1 ratio of a typical recessive Mendelian segregation. Therefore, the pattern is consistent with *lid1* segregating as a single nuclear recessive trait (Table 1).

II. *lid1* has abnormal anthocyanin production

Seeds of *lid1*, Col-O, *phyA-101* and RLD (the parental ecotype of *phyA-101*), were sown on medium containing 2% sucrose. Anthocyanins were extracted after all seedlings received 2d FRc. Since among all phytochromes, phyA is solely responsible for inducing anthocyanin accumulation in FRc, the *phyA* null mutant accumulates much lower levels of anthocyanins (about 10%) than does either wild type. The anthocyanin level in *lid1* is lower than that produced by Col-O (about 33% less), but significantly higher than that produced by *phyA-101* (Fig 6).

Several genes that encode anthocyanin biosynthetic pathway enzymes, such as chalcone synthase (*CHS*), chalcone isomerase (*CHI*), and dihydroflavonol-4-reductase

(*DFR*), are known to be regulated transcriptionally by phyA (Shirley *et al.*, 1995; Kong *et al.*, 2003). Since *lid1* displays lower anthocyanin accumulation when grown in FRc for 2 d, levels of message encoding these enzymes was tested by semi-quantitative RT-PCR. Total RNA from *lid1* and wild type seedlings treated with the same light conditions was extracted, and *CHS*, *CHI*, and *DFR* gene-specific primers were used to test the approximate amounts of gene transcripts using semi-quantitative RT-PCR. An early flavonoid biosynthetic pathway enzyme, *PAL1*, which is not believed to be regulated by the phytochromes, is included. The results show that *lid1* has lower transcripts of all three genes when grown in FRc (Fig 7).

The duration of FRc treatment on seedlings affects phyA-controlled anthocyanin accumulation *Arabidopsis*. Within the first few days, the longer the wild type seedlings were kept under FRc, the more anthocyanin was produced by the seedlings. In order to test the effects of the length of FRc treatment on anthocyanin production of *lid1*, seedlings of all lines were grown under FRc for 2d, 3d and 4d and the anthocyanin content was assayed. In all conditions, *lid1* accumulates about two thirds of amount of anthocyanin that is accumulated by Col-O, but much more than the amount accumulated by *phyA* (Fig 8a). Both Col-O and *lid1* produce more anthocyanin as FRc exposure is increased.

The intensity of FR light has great impact on photomorphogenic responses. PhyA both initiates FR-HIR and VLFR depending on the intensity and exposure time to FR. In order to determine whether the intensities of FR have any effect on the

abnormally low content of anthocyanin in *lid1*, the amount of anthocyanin in seedlings was measured under a series of FR fluence rates. Seeds were sown on medium containing 2% sucrose and grown for 3 d under FRc using different fluence rates. The FR fluence rates were varied over nearly 3 orders of magnitude, but the relative anthocyanin accumulations among the 4 genotypes remained essentially constant (Fig 8.b). The results show that the reduced *lid1* accumulation of anthocyanins is retained at different FR fluence rates.

Metabolizable sugars alter the responsiveness of plants to some PHYA-specific signaling pathways. Anthocyanin production is one of the photomorphogenic responses that is regulated by both phytochrome and sugar signals. It is not clear how signals from phytochrome and from sucrose are related to each other. Therefore, the reduced anthocyanin phenotype of the *lid1* mutant may be caused by a defect in the sucrose sensory pathway, in the phytochrome pathway, or in a step common to both. Seedlings growing in sucrose-containing media produce more anthocyanin than seedlings that have been grown in media without sucrose. Therefore, low anthocyanin accumulation in *lid1* may implicate a deficiency in the signal transduction pathway initiated either by sucrose or by phytochrome. Anthocyanin content in seedlings grown on media with different concentrations of sucrose was determined. Seedlings were treated with 2 d FRc before measurements were taken. When seedlings grew on media without sucrose, *lid1* still contained about 50% of the anthocyanin found in Col-O (Fig 8.c). Changes in sucrose concentration did not change the relative difference in anthocyanin production between *lid1* and Col.

III. Some photomorphogenic responses are unaffected in the *lid1* mutant

Many of the phyA signaling component mutants that have been described previously display longer hypocotyls than does wild type (Quail, 2002; Wang and Deng, 2003). To examine whether the *lid1* lesion specifically affects anthocyanin accumulation, or whether other phyA-mediated responses are also deficient, hypocotyl lengths were measured in wild type and mutant seedlings after 3d in FRc. The results indicate that *lid1* is not deficient in phyA-controlled hypocotyl elongation (Fig 9a). Similar experiments show that *lid1* also displays similar hypocotyl lengths with Col-0 when both are grown under Rc (Fig 9b) and under Bc (data not shown), light conditions in which phyA is not the primary light receptor. Therefore, lid1 may not be a factor in photoreceptor-mediated hypocotyl elongation.

Chloroplast movement and chlorophyll production are also known to be light-regulated. To assay the chlorophyll production in the mutant, *lid1* and wild type seedlings were grown under Wc for 5d and the amount of chlorophyll contained in each line was measured. The data show that there is no difference between the quantity of chlorophyll produced by *lid1* and that produced by Col (Fig 9c). Continuous white light is not perceived significantly by phyA under these conditions, presumably because the phyA concentration in Wc-grown tissues is negligible. In Wc, other phytochromes and cryptochromes are the primary light sensors (Nemhauser and Chory, 2002; Wang and

Deng, 2002). Therefore, *lid1* does not appear to be deficient in the regulatory pathway controlling chlorophyll production by these photoreceptors.

FRc pre-conditioned block of greening is another phenotype regulated exclusively by phyA. If wild-type seedlings are treated with 3d FRc immediately after germination and then exposed to 3d Wc, they are unable to produce chlorophyll because the POR enzymes are strongly down-regulated transcriptionally by phyA (Runge *et al.*, 1996; Sperling *et al.*, 1997). The amounts of chlorophyll produced in *lid1* and wild type seedlings after they were subjected to this light regime were measured (Fig 9d). Like wild type, *lid1* does not produce detectable chlorophyll after FRc treatment.

IV. Initial genetic characterization and mapping of *lid1*

In order to pinpoint the site of *LID1* in the genome, SSLP markers were used to attempt mapping of the mutated gene. The homozygous *lid1* mutant was crossed to the No-O ecotype and F_1 seeds were allowed to self-fertilize and produce the F_2 population. Phenotypic segregation was consistent with that shown in backcrosses, with the *lid1* mutants retaining low anthocyanin accumulation under FRc. Additional mapping populations were developed by crossing the *lid1* mutants to Ws and L*er* ecotypes, and the F_2 seedlings with low anthocyanin after 3d FRc selected and grown to maturity. To confirm that the selected F_2 seedlings were homozygous, each line was retested in the F_3 generation to eliminate individuals that were potentially heterozygous. Upon confirmation of homozygosity in each F_2 by the lack of F_3 segregation, the DNA that

had been extracted from F2 seedlings was used for mapping *lid1*. These DNA samples were used as templates for PCR with primers flanking SSLP microsatellite markers, such as nga8, nga111, nga151, nga172, nga249, and nga280. However, these markers gave contradictory mapping results: the sizes of amplified bands shown by gel electrophoresis imply that the original uncrossed *lid1* has No-O alleles of SSLP markers nga151, nga249, nga280, and Col alleles of SSLP markers nga8, nga111, nga172 (Fig 10), indicating that the *lid1* mutant is not in a pure Col-O ecotypic background.

The inconsistent background of *lid1* may be the result of mishandling of mutant seeds during manipulation in the laboratory. To confirm that the original *lid1* mutant was not contaminated after being obtained from the Arabidopsis Biological Resource Center, the original seed lot was re-screened, *lid1* re-isolated from the pool, and the DNA subjected to new PCR analysis. The same non-Col-O markers were observed in the independently isolated line, indicating that the error occurred previously.

D. Discussion

The phytochrome family of sensory photoreceptors transduces environmental light signals to responsive nuclear genes by complicated and poorly defined pathways. Since the discovery of the phytochrome system, people have been trying to identify the signal transduction mechanism and the signaling intermediates through which light perception by photoreceptors is coupled with changes in gene expression and plant development. For three decades, especially after recent development of molecular techniques and the sequencing of *Arabidopsis* genome, numerous downstream-signaling components of the phytochrome pathways have been identified by screening for severely altered photomorphogenic phenotypes using plants grown under controlled light conditions. Most of these components identified are part of the phyA pathway, since phyA is unique in mediating FR light responses in *Arabidopsis* (Quail, 2002; Sullivan and Deng, 2003; Wang and Deng, 2003). Among the identified positive regulators of phyA signaling, most loss-of-function mutants exhibit only partial defects in phyA-mediated responses compared with the phenotypes of *phyA* null mutants, suggesting that phyA signaling involves multiple branches or parallel pathways controlling both overlapping and distinct physiological responses (Wang and Deng, 2002). Also, loss of function of some signaling constituents may result in either less dramatic or partial phenotypic deficiencies. One of the phyA-controlled phenotypes of particular interest is the production of flavonoids in FRc-grown young seedlings. Flavonoid biosynthesis is controlled by a range of endogenous and environmental

signals, including light as detected through phytochrome. PhyA is the only phytochrome regulating flavonoid production in FRc grown seedlings. By screening for mutants producing slightly less anthocyanin than wild type, we have isolated a class of potential phyA pathway mutants that would not have been recognized using most previous approaches.

I. *LID1* is not an allele of *transparent testa* (*tt*) genes

Since *lid1* displays the phenotype of low anthocyanin production, it is necessary to compare it with previously identified mutants which are deficient in the synthesis of flavonoids. So far, 21 loci required for flavonoid biosynthesis have been identified, and the loci are collectively named *transparent testa* (*tt*) because the phenotype is due to a reduction or absence of pigments in the *testa* (seed coat) (Koornneef, 1981; Chapple *et al.*, 1992; Shirley *et al.*, 1995; Debeaujon *et al.*, 2001). Several genes have been identified that encode flavonoid metabolic enzymes, such as *TT3*, *TT4* and *TT5*. Loss-of-function mutations in any of these single-copy genes disrupt the synthesis of anthocyanin pigments in the testa layer of the seed coat, as well as in the plant organs. Each of these genes has been identified as a specific locus of anthocyanin biosynthesis pathway genes: *TT4* as chalcone synthase (CHS), *TT5* as chalcone isomerase (CHI), and *TT3* as dihydroflavonol 4-reductase (DFR). (Chang *et al.*, 1988; Feinbaum and Ausubel, 1988; Shirley *et al.*, 1992). Mutants in each of these *TT* loci display distinctive visual yellow pigmentation in the seed coat. Other *TT* genes also have been linked to the flavonoid biosynthesis pathway. For example, *TT6* encodes flavonol 3-hydroxylase

(Pelletier and Shirley, 1996; Wisman *et al.*, 1998) and *TT7* encodes flavonol 3'-hydroxylase (Koornneef *et al.*, 1982; Schoenbohm *et al.*, 2000). Both *tt2* and *tt8* are regulatory mutants (Shirley *et al.*, 1995) which encode a MYB protein (Nesi *et al.*, 2001) and a basic helix-loop-helix protein (Nesi *et al.*, 2000), respectively.

The *lidl* mutant is unlikely to represent an allele of any of these *tt* loci for several reasons. First, a trivial identification of *tt* mutants is distinguished by seed coat color, such as yellow (*tt1-5, tt8*) and pale brown (*tt6, tt7, and tt9*) (Koornneef, 1981; Koornneef *et al.*, 1982; Koornneef, 1990). However, the seed coat color of *lidl* is indistinguishable from the brown of wild type. Second, young *lidl* seedlings accumulate less anthocyanin and display less pink color than wild type, but *lidl* adult plants have similar amounts of anthocyanin to wild type in all vegetative tissues. This pattern is different from that shown by all known *tt* mutants which have visibly less anthocyanin in vegetative tissues such as leaves, stems, and flowers. Third, the reduced color shown in all the *tt* mutants is independent of the light conditions. However, the mutant phenotype of *lidl* is FR-dependent. White light-grown *lidl* does not significantly differ from wild type in anthocyanin production. Fourth, the flavonoid metabolic enzymes (*CHS, CHI and DFR*) are coded by single-copy genes and null mutations in one of these genes, such as *tt3, tt4, tt5, tt6,* or *tt7* will prevent anthocyanin production regardless of the inducer. Therefore, *lidl* is not a null allele of these genes due to its anthocyanin accumulation pattern in response to different light conditions. Fifth, pleiotropic effects have been described for mutations at several of *tt* loci, including simultaneous effects on trichome development (*ttg*), on pigmentation of

vegetative tissue, and on other characteristics of the seed (Koornneef, 1981; Koornneef *et al.*, 1982; Koornneef, 1990; Leon-Kloosterziel *et al.*, 1994). Mutation of *LID1* did not show the same pleiotropic phenotypes.

II. lid1 may be a downstream component of the phyA signaling pathway.

Based on our existing data, the most consistent hypothesis is that LID1 is a component of the phyA signaling network. Seedlings phenotypes of *lid1* are altered in FRc light but not in Wc or Rc light, and these phenotypes are not restricted by intensities and treatment duration of FRc. Reduced anthocyanin content in FRc-grown seedlings suggests that *lid1* is deficient in the pathway that regulates anthocyanin production initiated by FRc. Anthocyanin accumulation is normal in *lid1* when grown under Rc or Wc, indicating that irradiation with FRc is not only sufficient, but also critical to the low anthocyanin phenotype. Studies have shown that phyA transcriptionally induces genes that encode anthocyanin biosynthetic pathway enzymes, such as *CHS, CHI*, and *DFR* (Shirley *et al.*, 1995; Kong *et al.*, 2003) and FRc-grown *lid1* mutants display reduced transcript levels of all three genes. Moreover, changing the duration of FRc or intensity was not sufficient to mimic the wild type anthocyanin levels, indicating that it is not merely the threshold of sensitivity to FRc or the developmental competence to respond to FRc that is affected. In *Arabidopsis*, seedling responses to FRc light are primarily, if not solely, mediated by phyA through both HIRs and VLFRs. FR is perceived by phyA and passed through signaling intermediates in the phyA pathway. Therefore, low anthocyanin in *lid1* is the result of a deficiency in what

is normally a phyA-regulated pathway. The fact that *lid1* produces normal amounts of phyA protein and, more importantly, the phyA protein in *lid1* undergoes normal degradation upon illumination of Rc light, shows that *LID1* is not an allele of the *PHYA* gene and does not affect normal accumulation of PHYA apoprotein. Therefore, phyA in the *lid1* mutant is likely to be functional and the mutation in *lid1* is in the signaling intermediates or factors being regulated by or regulating these intermediates.

It should be emphasized that anthocyanin biosynthesis in *lid1* is subject to temporal control. Young FR-grown *lid1* seedlings contain significantly less anthocyanin than wild type seedlings of the same age. However, after the seedlings are transferred into pots with soil for a week, there is no longer any visible difference between the amount of anthocyanin produced by *lid1* and that produced by wild type.

III. lid1 may define part of late phyA-mediated responses

Since phyA mediates a variety of morphological, biochemical and molecular responses (Smith, 1995), it is expected that the subsets of responses are regulated by different branches of the phyA downstream signaling pathways through different signaling intermediates. Some of previously identified phyA pathway mutants resemble *phyA* null mutants, while others are only involved in subsets of phyA-regulated responses (Quail, 2002; Sullivan and Deng, 2003; Wang and Deng, 2003). The *lid1* mutation, as reported here, also affects only a subset of phyA-mediated responses. FRc-grown *lid1* seedlings display low anthocyanin content. However, neither seed

germination nor other aspects of photomorphogenic seedling development, such as inhibition of hypocotyl growth, cotyledon opening and expansion, apical hook opening, and the FRc light preconditioned block of greening, are altered in the *lid1* mutant. The simplest interpretation of these results is that anthocyanin accumulation regulation diverges from the other branches of the phyA signal transduction pathway, and that lid1 is a component in this specific regulatory branch. Alternatively, lid1 may work in these pathways in a redundant manner and the mutation in *LID1* does not alter any of these phenotypes so that they are quantitatively differentiated from those of wild type. A third formal possibility is that this allele of *LID1* is leaky, such that the more sensitive growth responses are unaffected, whereas the anthocyanin response has a higher signal threshold that is not met in *lid1*. Therefore, the lack of pleiotropic effects of mutations at the *LID1* locus suggests that *LID1* may encode a specific regulator of flavonoid synthesis or turnover structural genes.

IV. lid1 may be a novel genetic mutant in the phyA signaling network

Previous studies on phyA pathway mutants have revealed multiple positive regulators in phyA signal transduction. However, none of these mutants displays a phenotype pattern similar to that of *lid1*. For the mutants that display low anthocyanin accumulation, such as *far1* (Hudson *et al.*, 1999), *fhy3* (Wang *et al.*, 2002), and *fin2* (Soh *et al.*, 1998), all have secondary phenotypes such as elongated hypocotyls, low germination rate, or closed cotyledons, which are absent in *lid1*. By comparing mutant phenotypes between *lid1* and any previously reported phyA pathway mutants, it is

unlikely that *LID1* is an allele of any of these genes. Alternatively, if *LID1* were a weak allele of one of these genes, *lid1* may have lost a subset of its functions. Some of previously described phyA pathway mutants also have reduced anthocyanin content, but never as the exclusive phenotype.

V. Possible function of lid1 in the phyA signaling pathway

The physiological characterization of *lid1* is consistent with its possible role as a signaling intermediate in the phyA-initiated pathway regulating anthocyanin production, but separate from the pathways leading to other photomorphogenic responses. Previous research on light controlled anthocyanin production showed that CHS, CHI and DFR are three anthocyanin biosynthesis enzymes that are regulated by a variety of environmental and endogenous stimuli, including light (Mol *et al.*, 1996). Some of the previously identified phyA pathway mutants that have low anthocyanin production contain lower amounts of transcripts for anthocyanin biosynthesis genes, such as *CHS* (Wang and Deng, 2002; Wade et al., 2003). Using semi-quantitative RT-PCR, we showed that there are lower transcript levels of *CHS*, *CHI* and *DFR* genes in *lid1* than in wild type. These data suggest that the reduced anthocyanin content in *lid1* is the result of direct or indirect down-regulation of structural genes required to produce flavonoids. At least part of the function of lid1 in *Arabidopsis* is to maintain normal levels of the anthocyanin biosynthesis pathway gene transcripts. Lid1 may regulate the mRNA levels by regulating either the gene transcription or RNA degradation, but the

apparent regulation of messages for several genes in the biosynthetic pathway implies a coordinated regulatory function. The precise function of lid1 is not clear at this time.

VI. Gene cloning of lid1 is not practical

In order to clarify the role of lid1 in the phyA pathway, we attempted to identify and isolate *LID1* from the *Arabidopsis* genome. Using SSLP markers for genetic linkage analysis, we found that part of *lid1* chromosome 3 and chromosome 4 surrounding genetic marker nga172 and nga8, and part of chromosome 1 surrounding genetic marker nga111 are consistent with markers of the Col-O ecotype, but part of chromosome 1 surrounding genetic marker nga280 and part of chromosome 5 surrounding genetic markers nga151 and nga249 correspond to alleles in the No-O ecotype (Fig 10). In order to locate genes in the *Arabidopsis* genome using linkage mapping techniques, polymorphisms have to be shown between different ecotypes using selected genetic markers. These markers then are used to calculate recombination frequency with respect to the presumed mutation. Since *lid1* displays markers that are inconsistent with a single ecotype background, it is not practical to locate the position of the *LID1* gene in the *Arabidopsis* genome without extensive backcrossing.

The finding that lid1 may have a mixed parental ecotype adds another possible explanation for the observed phenotype. Different ecotypes display different levels of anthocyanin under various conditions. For example, No-O does not produce as much anthocyanin as Col-O does. Even though *lid2* accumulates less anthocyanin than either

Col-O or No-O, we can not rule out the possibility that a naturally occurring variant allele from the alternative ecotype is responsible for the phenotype.

CHAPTER III. ISOLATION AND CHARACTERIZATION OF THE *LID2* MUTANT

A. Abstract

Another mutant identified in the screen described in the previous chapter is *lid2* (*li*ght-*d*eficient 2), which is deficient in some, but not all, far-red light-induced responses. The reduced-anthocyanin phenotype displayed by far-red light-grown *lid2* seedlings during the initial screen proved not to be heritable. However, this mutant exhibits reduced chlorophyll accumulation in white light and also exhibits reduced responsiveness to both blue and red light. *Lid2* also has other mutant phenotypes that seem unrelated to the phyA pathway, such as low anthocyanin accumulation in white light-grown seedlings. However, *lid2* is also deficient in the FRc-induced block of greening which is controlled exclusively by phyA. Based on controlled light experiments, chlorophyll accumulation appears to be uncoupled from normal phytochrome A regulation. Initial mapping data suggest that the lid2 gene codes for a protein not yet implicated in light responses. The mapping experiment has narrowed the likely candidates for *LID2* to one of about two dozen genes in the telomeric end of the short arm of chromosome V.

B. Materials and Methods

Plant material and growth conditions

Plant material and growth conditions used for *lid2* characterization are similar to those described in the previous chapter for *lid1* characterization.

Mutant isolation and genetic characterization

lid2 was originally identified from the same screen as *lid1*, as a mutant with low anthocyanin accumulation in the hypocotyl and cotyledons of young *Arabidopsis* seedlings grown in FRc in the presence of sucrose. However, anthocyanin quantification of *lid2*XCol-O F_2 and F_3 seedlings showed that the decreased anthocyanin produced by *lid2* is not genetically heritable. However, further physiological characterizations of *lid2* showed that 5d Wc grown seedlings produce consistently lower amounts of chlorophyll than does wild type and that this trait is fully heritable as a single Mendelian locus. *Lid2* mutants were then exposed to different light conditions to characterize mutant responses to other phytochrome controlled responses, such as hypocotyl elongation and the FR-mediated block of greening.

Genetic analysis of mutations.

Mutants that showed heritable reduced-chlorophyll phenotypes were backcrossed into their parental ecotype Columbia (Col-O) and crossed into the null *phyA-101* as described in the previous chapter. F_2 seedlings were examined for the segregation of the mutant phenotype. The results were subjected to chi square statistical analysis.

Germination assessment

Seeds were surface-sterilized as described previously. Exactly 50 seeds were sown on 100-mm Petri plates containing 0.8% (w/v) agar and MS salts. Plates were irradiated with FR light (9.7 μmol m^{-2} s^{-1}) for 15 min and then kept in dark at 4°C for 3 d. The seeds were then irradiated with FR light for 24 h and germination frequency was measured immediately following the FR treatment. Three independent experiments were performed.

Cotyledon separation

Seeds were surface-sterilized as described previously. Seeds were sown on 100-mm Petri plates containing 0.8% (w/v) agar and MS salts. Plates were kept in darkness at 4°C for 3 d and then transferred to W for 3 h to induce germination (approximately

50 μmol m^2 s^{-1} at 24°C) and then placed in the dark at 24°C for 21h. After dark treatment, plates were transferred to FR light (9.7 μmol m^{-2} s^{-1}). The number of seedlings with opened cotyledons were counted after the plates remained in FR for 2 d. Seedlings were judged to have open cotyledons when the two cotyledons were separated by at least 30°. At least 50 seedlings from each genotype were included per experiment, and each experiment was replicated at least three times, and the proportion of seedlings displaying open cotyledons was determined.

Analysis of pigment content in seedlings

Anthocyanin was extracted in acidic methanol and quantitated spectrophotometrically using the method described in the previous chapter. Chlorophylls *a* and *b* were extracted in 80% acetone and quantitated according to the method described in the previous chapter. Each experiment was replicated at least three times.

Hypocotyl length measurements

Hypocotyls of *Arabidopsis* seedlings were measured with a digitizing tablet as described in the previous chapter. Each experiment was repeated at least three times.

Flowering time in long days

Seeds were surface-sterilized as described previously. At least 50 seeds were sown on 100-mm Petri plates containing 0.8% (w/v) agar and MS salts. Plates were kept in darkness at 4°C for 3 d and then transferred to Wc (75 μmol m^{-2} s^{-1}) for 7 d. Seedlings were then transplanted to soil and grown under Wc until the initial inflorescence was observed emerging in the center of the rosette. The time interval between the germination of the seeds and the appearance of the inflorescence was recorded. Also, the number of rosette leaves at that time was counted.

Semi-quantitative RT-PCR

Total RNA was extracted using the method described in the previous chapter. RT-PCR protocols and primers for genes encoding anthocyanin biosynthetic pathway enzymes were identical to those described in the previous chapter.

Control gene primers used in RT-PCR experiments were ordered from Sigma-Genosys. Chlorophyll a/b binding protein 2 (*CAB2*) forward primer 5'-CTTGGCTCACTTTCCGGGAAC-3' and reverse primer 5'-CAGGCAGCCCATGGTACGGATC-3' were expected to give a product of size 674 bp to both RT-PCR and genomic DNA products. Ubiquitin 1 gene (*UBQ1*) forward primer 5'-TTCGTGAAAACCTTGACCGGC-3' and *UBQ1* reverse primer 5'-

TCTTCTTCTTAGGCCTCAA-3' were expected to give a product of size 378 bp for RT-PCR products and 997 bp for genomic DNA products.

Gene-specific primers for *PORA*, *PORB*, and *PORC* were also ordered from Sigma-Genosys. The 765 bp *PORA* RT-PCR and 1,034 bp genomic DNA product were amplified from the primer pair 5'- TTCAACCCGCCTGCTAGTCCC-3' and 5'- TTCTTTGGTCTCCTCTGCTTTC -3'. The 1029 bp *PORB* RT-PCR product and 1283 bp genomic DNA product were amplified from the primer pair 5'- TTCTTCATCATCTTTCAAGGAC-3' and 5'- CAGCTCCAATAAACCCCTGATTTC -3'. The 1063 bp *PORC* RT-PCR product and 1,404 bp genomic DNA product were amplified from the primer pair 5'- TCCTTCTACCATTTCAATCC -3' and 5'- CCAATACACTCCTGACTTCCC -3'.

Gene-specific primers for the 26 genes in BAC F7A7 were ordered from Sigma-Genosys. The sequences of these primers are listed in the Table 7. Each pair of primers was designed to amplify RT-PCR products of about 500 bp and larger genomic DNA products near 1 kb, depending on the lengths of the introns being flanked.

Phytochrome Extractions and Spectrophotometric Assays

After 4d growth in darkness, fresh or frozen *Arabidopsis* seedlings were rapidly homogenized in ice-cold Extraction Buffer [100mM Tris-HCl, 140mM $(NH_4)_2SO_4$, 50% ethylene glycol, 5mM EDTA, and 0.5 mM PMSF (pH 8.3)], with 10% v/v pH7.8

polyethylenimine (PEI) at a ratio of 1 ml extraction buffer per g fresh weight tissue. The homogenate was centrifuged at 25,000g for 15 min, and the supernatants were precipitated by ice-cold saturated $(NH_4)_2SO_4$ to a final concentration of 36% saturation, left on ice for 1~2 min, and centrifuged at 25,000g for 20 min. The pellets were resuspended in Resuspension Buffer (50mM Tris, pH7.8, 5.0 mM EDTA and 10mM iodoacetamide [added immediately before use]), and were used for spectrophotometric scans using a dual beam spectrophotometer (AVIV UV-VIS-IR Model 14DS, Beckman Coulter, Fullerton, CA).

Immunoblot Analysis

Approximately 120 mg of seeds from each of the *lid2* and *phyA* mutants and the Col-O and RLD wild types were plated on two identical sets of plates and treated as described above. After inducing germination with 3 h Wc, plates were kept in darkness for 4 d. One set was exposed to Rc for 3h immediately before harvesting, while the other set remained in darkness. Seedlings from each set of plates corresponding to each line were harvested under dim-green light and flash-frozen in liquid nitrogen. Tissue was homogenized, total cellular proteins were extracted, and homogenates were subjected to PEI and ammonium sulfate precipitation to obtain a phytochrome-enriched fraction as described (Xu *et al.*, 1995). SDS-PAGE and immunoblotting on nitrocellulose were carried out according to methods described previously (Xu *et al.*, 1995; Short, 1999). The nitrocellulose membranes were stained with 1mg/ml Ponceau S in 1% acetic acid to confirm equivalent protein loading. Visualization of blots was

performed with the PHYA apoprotein-specific monoclonal antibody 073D (developed by Dr. J. Shanklin, provided courtesy of Dr. Peter Quail) and a VectaStain ABC-AP antibody-detection system (Vector Laboratories, Burlingame, CA) using a colorimetric substrate as previously described (Short *et al.*, 1992). The blot shown is representative of three independent replicates.

Mapping

Mutants of *lid2* were mapped by crossing into the No-O ecotype. The F_2 progeny were screened for low chlorophyll under Wc and the ability to produce chlorophyll after three days in FRc followed by three days in Wc. These seedlings were transferred to soil and grown for two weeks before a leaf was removed for DNA extraction as described previously. The DNA isolated from those lacking segregation in the F_3 generation were used for mapping relative to PCR-based simple sequence length polymorphism (SSLP) (Bell and Ecker, 1994), and single nucleotide polymorphism (SNP) (Ye *et al.*, 2001) markers.

Amplification of Full Length Gene Fragments

Eight sets of the forward and reverse primers were designed for each candidate gene including at least one thousand base pairs of upstream and one thousand base pairs downstream of the gene to maximize inclusion of regulatory elements. Primers were designed using a web-based program at:

http://alces.med.umn.edu/websub.html

An ApaI or a SmaI site was engineered onto the 5' end of forward and reverse primers, respectively, along with 3 to 5 additional nucleotides to improve subsequent restriction digestion and cloning of the amplified genes. Each set of primers was used to run PCR reactions with BAC-F7A7 as template to amplify the target fragments with the Takara *LA Taq*^{TM} proofreading DNA polymerase mix (PanVera, Madison, WI).

The amplified fragments from PCR reactions were ligated into the pGEM T-easy vector (pGEM-T and T-easy vector system (Promega, Madison, WI)), and the ligation products were used to transform DH5a competent cells by heat shock (Sambrook *et al.*, 1989).

DH5a *E. coli* strains were grown in sterilized LB (10g tryptone, 5g yeast extract, 5g NaCl per liter) medium containing selective antibiotic 50ug/ml kanamycin. The LB plates supplemented with 1% agar were cultured at 37°C overnight to screen for positive colonies.

Positive clones were selected on plates with X-gal (50μg/ml) and IPTG (20μg/ml) for loss of ß-galactosidase activity for further screening. Only those white colonies that were shown to contain the target insertions by PCR screening were picked for growth in liquid culture, and transferred to LB liquid medium with antibiotic grown at 37°C while shaking at 250 rpm overnight prior to plasmid extraction. Plasmids were extracted with the *Wizard plus SV miniprep DNA purification system* (Promega,

Madison, WI). The inserted fragments were excised with ApaI and SmaI for later insertion into the pGreen binary vector (Hellens *et al.*, 2000). For each gene, at least three individual clones were selected to minimize the chance of propagating a PCR amplification error. The fragments were then religated to the pGreen (www.pGreen.ac.uk) binary vector, and transformed by heat shock to DH5a competent cells generated by the $CaCl_2$ method (Sambrook *et al.*, 1989). Positive colonies were screened and plasmids were extracted as above. Those pGreen vectors with the genomic fragment insertions, along with pSoup plasmid required for pGreen maintenance and virulence of the host *Agrobacterium*, were used to co-transform *Agrobacterium tumefaciens* strain GV3101 (Koncz, 1986) by electroporation with the Bio-Rad Gene Pulser II with the Capacitance Extender Plus module (Hercules, CA). Positive clones identified from among colonies growing on kanamycin (50μg/ml) and tetracycline (5μg/ml) were used to transform *lid2* mutant *Arabidopsis* plants as described below.

Plant Transformation

Agrobacterium was cultured in sterilized LC medium (10g tryptone, 5g yeast extract, 8g NaCl per liter) containing selective antibiotics kanamycin (50ug/ml) and tetracycline (5ug/ml). Plates containing LC plus 1% agar were grown for 3d at 28°C to screen for positive colonies.

Liquid culture of *Agrobacterium* was typically started by picking a positive clone from each construct, growing in 250ml liquid LC medium with 50ug/ml

kanamycin and 5ug/ml tetracycline overnight (roughly 18 hours). Cells were harvested by centrifuge at 5000g for 20 min at room temperature, then resuspended in a solution containing of 5.0% sucrose and 0.02% Silwet L-77 (Lehle Seeds, Round Rock, TX) to a cell density of approximately 0.80 OD_{650} prior to use.

The plants selected for *Agrobacterium* transformation had their initial bolt removed to encourage multiple bolt stems. After about 3d, new bolts were dipped in the *Agrobacterium* inoculum in a 50ml falcon tube. Plants were inverted into the suspension such that all above ground tissue were submerged, and plants were removed after 3-5 sec with gentle agitation. Plants were covered with clear plastic domes, and allowed to recover overnight in low light intensity. Domes were removed approximately 24 hours after the treatment. After 7d growth, the same plants were dipped again using an identical inoculum, but with 0.04% Silwet L-77. Plants were grown for about four weeks until siliques were brown and dry, and seeds were harvested.

C. Results

I. Isolation of *lid2* mutant

A second mutant isolated from the previously described screen is *lid2*, which displays abnormal chlorophyll production. After growth under 5 d Wc, *lid2* shows a visibly paler green color than its background ecotype Columbia (Col-O), caused by the measurably decreased production of chlorophyll in *lid2* (Fig 11).

In order to test whether *LID2* is a single recessive trait in the *Arabidopsis* genome, we backcrossed homozygous *lid2* mutants into its parental ecotype Col-O. F_1 seeds were grown up and allowed to self-fertilize to collect F_2 seeds. In the F_2 population, the chi square value was calculated from the segregation of the seedlings. This chi square value (χ^2= 0.715) yields a $P \approx 0.398$. Thus, the segregation of these seedlings did not differ significantly from a 3:1 ratio (Table 2.a), consistent with its segregating in a typical Mendelian fashion as a single nuclear recessive trait.

Since phyA is the phytochrome that induces anthocyanin production when seedlings are treated with FRc, *lid2* was crossed with *phyA* to obtain the *lid2phyA* double mutant. The double mutant *lid2phyA* displays both the FRc-dependent long hypocotyl phenotype of *phyA* and reduced chlorophyll accumulation phenotype of the *lid2* mutant. In the F_2 population, the chi square was calculated from the segregation of the seedlings to determine whether the observed pattern differed significantly from the

expected 9:3:3:1 ratio. This chi square value (χ^2= 1.684) yields a $P \approx$ 0.640. These results are consistent with *LID1* and *PHYA* being independent, unlinked loci (Table 2b).

II. Chlorophyll accumulation is uncoupled from normal phyA control

The *lid2* mutant has abnormal chlorophyll accumulation.

To measure quantitatively the chlorophyll content in young seedlings, seeds from Col-O, *lid2*, *phyA*, *lid2phyA,* AOX (phyA overexpressor (Boylan and Quail, 1991)), *lid2*AOX, and RLD were sown on MS plates without supplementary sugar and grown under Wc for 5 d. The chlorophyll quantified from 5-d-old seedlings revealed that the *lid2* mutant accumulated only about one quarter the chlorophyll of the Col-O wild type, whereas *phyA* contained a chlorophyll level consistent with that of wild type. Interestingly, the *lid2phyA* double mutant accumulates much less chlorophyll than either wild type or *phyA,* comparable to the amount of chlorophyll produced by *lid2.* Chlorophyll accumulation of AOX was also tested to examine further the relationship between phyA and lid2 in controlling chlorophyll production. The chlorophyll level of AOX is similar to that of Col-O and *phyA*, whereas the chlorophyll level of *lid2*AOX is similar to that of *lid2* (Fig 12a). These results indicate that LID2 is epistatic to PHYA in the signal transduction pathway. Furthermore, after the *lid2* seedlings are transplanted into soil, they gradually produce more chlorophyll and become visibly indistinguishable from the wild type. There is no significant difference between the chlorophyll content in mutant seedlings and that in wild type seedlings two weeks after transplantation.

Since phytochromes have a smaller absorbance peak in the blue region of the light spectrum and, more importantly, blue light receptors (cry1 and cry2) also induce the production of chlorophyll, a similar experiment was done using exclusively R to exclude any influence of B on the chlorophyll production of the seedlings. When seedlings are grown under Rc, phyB plays the major role in photoreception and regulation. PHYA is also degraded rapidly under Rc after it is photoconverted to the P_{fr} form. Chlorophyll content of wild type and mutant seedlings under Rc follows similar accumulation patterns to those measured from Wc-grown plants. Double mutants *lid2phyA* and *lid2*AOX also exhibit chlorophyll levels that are lower than those of *phyA* and AOX seedlings, but similar to that of *lid2* (Fig 12b).

NADH-protochlorophyllide oxidoreductases (POR) are the enzymes that catalyze the final step in chlorophyll biosynthesis and are encoded by a three member gene family. The transcript levels of these genes are regulated by phytochromes (Barnes *et al.*, 1996). Since transcript levels of the *POR* genes show different patterns in response to illumination (Oosawa *et al.*, 2000), the mRNA levels of all three *POR* genes was examined using semi-quantitative RT-PCR. The level of PORA message is reduced in both wild type and in the *lid2* mutant after six days of illumination of Wc. However, *lid2* has significantly lower accumulations of both PORB and PORC mRNA than does wild type, which may account for the reduced production of chlorophyll in the *lid2* mutant (Fig 14). In order to study the relationship between lid2 and phyA on regulation of *POR* gene expression, we examined the transcript levels of the *POR* genes in

lid2phyA and *lid2AOX* double mutants. Both *lid2phyA* and *lid2AOX* lack any detectable PORA mRNA. Both *lid2phyA* and *lid2AOX* have detectable levels of both PORB and PORC mRNA comparable to those found in *lid2* alone and distinctly lower than those of wild type (Fig 14).

lid2 is deficient in the FR-induced suppression of greening

Active phyA strongly inhibits the expression of *POR* genes, so wild type seedlings treated with 3 d FRc can not produce chlorophyll even when transferred back to Wc (Armstrong *et al.*, 1995; Barnes *et al.*, 1996). Figure 15 shows a visual comparison of *lid2* and wild type (Col-O) seedlings after they are treated with 3 d FRc followed by 3 d Wc. Col-O was unable to produce chlorophyll and appears very pale. The phyA mutant does not down-regulate *PORs* and can produce high amounts of chlorophyll. Furthermore, *lid2* also produces chlorophyll and appears green, although it retains the paler appearance observed in Wc compared with the deep green color of the *phyA* mutant. Therefore, *lid2* is both partially deficient in the normal FRc-induced block of greening, but also lacks the full light-induced accumulation of chlorophyll.

Because the block of greening is mediated exclusively by phyA during the growth of seedlings in FRc (Runge *et al.*, 1996), young seedlings of Col-O, *lid2*, *phyA*, *lid2phyA*, *AOX*, *lid2AOX* and RLD were treated in FRc for 2, 3 or 4 d before transfer to Wc for 3 d to recuperate, and the amount of chlorophyll accumulation was measured (Fig 16). When the seedlings were treated with FRc for 4 d, only *phyA* and *lid2phyA*

double mutants fully recuperated by producing chlorophyll. Seedlings of the other genotypes died from lack of chlorophyll. Among seedlings treated to FRc for three days, Col-O was unable to produce chlorophyll, whereas *lid2* recovered and produced a moderate amount of chlorophyll, at a level approximately 50% of that produced by *phyA*. This difference is similar to the reduced chlorophyll accumulation observed in *lid2* under Wc without FRc pre-irradiation. *lid2phyA* double mutants produce similar amounts of chlorophyll to those of the *phyA* mutant, indicating that the *lid2* mutation does not actively inhibit chlorophyll accumulation in the absence of a phyA signal. However, AOX and *lid2*AOX produce almost no detectable chlorophyll after 3 d FRc treatment. Among seedlings treated with FRc for 2 d before transfer to Wc, only AOX exhibited a reduction in chlorophyll content. There is no significant difference between the amounts of chlorophyll maintained by Col-O, *lid2* and *phyA*.

There are two possible explanations for chlorophyll being detected in *lid2* mutant lines during the block of greening experiment. First, all seedlings may uniformly produce a low level of chlorophyll because of a defect in the phyA pathway, preventing full repression of the *POR* genes. Second, a few seedlings may produce high amounts of chlorophyll for trivial reasons—such as late germination that might reduce the effective exposure time to FRc—whereas most of the seedlings produce little or no chlorophyll. In order to test which of these two situations lead to increased chlorophyll measurement in mutants, the proportion of seedlings that turn green after the block of greening experiment was assayed. For Col-O, *lid2*, *phyA* and *lid2phyA*, almost all seedlings within each group exhibited a uniform phenotype. However, for 2d and 3d FRc-treated

AOX and *lid2*AOX seedlings, there was greater variation within the population, with a significant percentage of seedlings greening while others were unable to produce chlorophyll at all (Table 3).

Previous reports have shown that down-regulation of *POR* transcripts in FRc accounts for the block of greening response. Therefore, the mRNA levels of *POR* genes in seedlings of both wild type and mutants were determined after they were treated with 3 d FRc. Only *phyA* and *lid2phyA* exhibited high levels of PORA transcripts, whereas Col-O, *lid2*, AOX and *lid2*AOX all displayed undetectable levels of PORA mRNA. This result is consistent with previous reports that PORA message levels decrease upon illumination (Armstrong *et al.*, 1995). For transcripts of PORB and PORC, *phyA* and *lid2phyA* apparently contain about twice as much PORB and PORC mRNA as *lid2* does. Col-O, AOX and *lid2*AOX did not produce detectable PORB transcript and contained only a very small amount of PORC transcript (Fig 17a).

To determine whether transcriptional regulation of *POR* genes was consistent with the greening response, the levels of *POR* mRNA for seedlings that were treated for 3 d in FRc followed by 3 d in Wc were measured. Under these conditions, none of the lines displayed detectable levels of PORA transcript. However, the pattern of PORB and PORC mRNA abundance was significantly affected by genotype. Both phyA and *lid2phyA* displayed slightly higher PORB and PORC transcript levels than did *lid2*. Both Col-O and AOX have almost undetectable PORB transcript and very low PORC transcript accumulations (Fig 17b).

III. The *lid2* mutant phenotype is independent of sucrose regulation

It is long been known that sucrose can suppress the FRc-dependent block of greening in wild type *Arabidopsis* seedlings (Whitelam *et al.*, 1993; Barnes *et al.*, 1996). The results of work on *s*ucrose-*un*coupled (*sun*) mutants provide evidence for a close interaction between sucrose and light signaling pathways (Dijkwel *et al.*, 1997). Therefore, it is important to distinguish whether low chlorophyll in *lid2* is caused by a defect in a phytochrome-mediated or a sucrose-mediated pathway.

In *Arabidopsis*, sucrose levels can alter gene expression, allowing the organism to adapt to the changing photosynthetic environment. Many photomorphogenic responses are also regulated by sucrose (Koch, 1996). Previous experiments have shown that *lid2* produces less chlorophyll than wild type under the same light conditions in the absence of sucrose in the medium (Fig. 12.a). To determine the role of lid2 in this potentially sucrose-mediated pathway, *lid2* was grown for 5d under Wc on medium containing 2% sucrose and consequent chlorophyll content was assayed (Fig 13). *lid2* and wild type display the same differential pattern of chlorophyll accumulation regardless of the presence of sucrose. *Lid2* still produces approximately one quarter of the amount of chlorophyll produced by wild type and both double mutants accumulate less chlorophyll than their respective parental ecotypes.

To determine whether the *lid2* mutation affects the capacity to green in the presence of sugars, seedlings were subjected to the block of greening experiment on media supplemented with 2% sucrose. As has been shown previously, the FRc repression of greening is overcome by supplemental sugars. At the same time, *lid2* retains the pattern of reduced chlorophyll accumulation compared with that of Col-O, as seen when the two lines are grown in Wc. *lid2phyA* and *lid2*AOX also have similar chlorophyll levels to *lid2*, which are lower than those in Col-O and RLD ecotypes (Fig 18).

IV. *lid2* displays deficiencies in multiple phytochrome-controlled responses.

There are many physiological responses directly or indirectly regulated by phytochrome. Therefore, it is important to determine whether there are additional phenotypes other than chlorophyll production that are associated with the *lid2* mutant. Thus, several well characterized phytochrome-controlled photomorphogenic responses were tested.

***lid2* has abnormal anthocyanin production that is uncoupled from phyA regulation.**

Anthocyanins are plant pigments that are produced starting from very early stages of seedling development (Shirley *et al.*, 1995). Some mutations in phyA transduction components cause reductions in the FRc-induced accumulation of

anthocyanins (Quail, 2000; Wang and Deng, 2003). The amounts of anthocyanins accumulated in 2-d-old FRc-treated seedlings from Col-O, *lid2*, *phyA*, *lid2phyA*, AOX and *lid2*AOX were quantitated (Fig 19.a). The pattern of anthocyanin accumulation in seedlings with the *lid2* mutation is similar to that of the corresponding background with the wild type *LID2* allele, indicating that lid2 is probably not involved in the FRc-mediated response.

The production of anthocyanin in 5-d-old Wc-treated seedlings was also tested. Since phyA is degraded within a few hours following R or W illumination, anthocyanin accumulation in Wc grown seedlings is regulated primarily by photoreceptors other than phyA and by other factors, such as sucrose. Interestingly, *lid2* accumulated less anthocyanin (about 50%) than did wild type (Col-O) in this condition. Furthermore, *lid2phyA* produced much more anthocyanin than did *phyA* alone (Fig 19b). These results suggest that lid2 may work as a regulator in anthocyanin production under conditions where phyA is not likely to be the primary inducer. Moreover, *lid2phyA* and *lid2*AOX contain lower amounts of anthocyanin than *phyA* and AOX, respectively (Fig 19b). They both produce similar amount of anthocyanin to *lid2*. Based on these results, it appears that the control of lid2 on anthocyanin production is not phyA-dependent.

Since *lid2* displays normal anthocyanin accumulation when grown in FRc for 2 d and lower anthocyanin when grown in Wc for 5 d, the transcript levels of the genes that encode several key anthocyanin biosynthetic pathway enzymes were tested. Total RNA from *lid2* and wild type seedlings treated with the same light conditions was

extracted, and *CHS*, *CHI*, and *DFR* gene-specific primers were used to test the approximate levels of gene transcripts using semi-quantitative RT-PCR. The results show that *lid2* has normal transcript levels of all three genes when grown in FRc (data not shown) and lower transcripts of all three genes when grown in Wc (Fig 20).

lid2 seeds exhibit lower germination rates.

Since phyA induces the germination of imbibed seeds under both FR-HIR and VLFR conditions, the number of germinated seedlings was scored for seeds treated with 24 h FRc (Fig 21). The criterion for determining germination is the presence of an initial break in the seed coat and the emergence of the radicle tip. In all cases, *lid2* has a lower germination percentage than Col-O. Since phyA induces germination when seedlings are treated with FRc, *phyA* exhibits a lower germination percentage than does the RLD wild type. Moreover, both *lid2phyA* and *lid2*AOX display a lower germination proportion than even *phyA* (Fig 21). Theses results imply that lid2 is a factor that induces seed germination, and that lid2 may act downstream of phyA in the regulation of seed germination.

lid2 is normal in a subset of de-etiolation responses.

Among the changes that seedlings undergo during the transition from skotomorphogenesis to photomorphogenesis is the opening of cotyledons. FRc, low fluence Rc, and high fluence Bc are known to stimulate hook opening and cotyledon

separation (Nagatani *et al.*, 1993; Whitelam *et al.*, 1993). Under Wc and Rc, phyB plays the major role in mediating this response (Neff and Van Volkenburgh, 1994). Under FRc, phyA plays the major role. Seedlings from wild type and mutants were grown in Wc or FRc for 3d and the cotyledon separation was scored. Since phyA induces cotyledon opening, *phyA* null mutants do not display open cotyledons when treated with FRc. *Lid2* did not affect the separation of cotyledons in either 3d Wc or FRc (Fig 22a).

Since most phyA pathway signal intermediate mutants are less sensitive to FR-induce inhibition of hypocotyl elongation, the hypocotyl lengths of 3-d-old FRc-treated seedlings of Col, *lid2*, *phyA*, *lid2*phyA, AOX and *lid2*AOX were measured. No significant difference in hypocotyl elongation was observed as a result of the *lid2* mutation. Since sucrose can induce the elongation of hypocotyl-an effect opposite to that of phytochrome, the hypocotyl lengths were also quantitated when all seedlings were grown for 3d in FRc in medium supplemented with 2% sucrose. As expected, sucrose causes an incremental increase in hypocotyl length. However, these experiments did not yield any meaningful differences in hypocotyl length between *lid2* and Col-O (Fig 22b). PhyB is primarily responsible for inhibiting the hypocotyl elongation when seedlings are grown in Rc. Hypocotyl lengths of 3-d-old Rc-treated seedlings indicate that there is no effect of the *lid2* mutation on hypocotyl length in Rc-treated seedlings (Fig 22c). These results imply that lid2 is not involved in the pathway that directly targets inhibition of hypocotyl elongation initiated by either phyA or phyB.

Floral initiation is regulated by a complex network of inputs including phytochromes and cryptochromes. Delayed flowering is a phenotype observed in some light signaling network mutants. Seedlings of wild type and mutants were grown under Wc and the time interval between the induction of seed germination and observation of the first floral bud was measured. No significant difference between flowering time of *lid2* and that of wild type was observed (Table 4).

lid2 has normal PHYA production and degradation:

The absence of the PHYA apoprotein strongly affects FRc responses, while deficiencies in phytochromobilin production decrease responsiveness to both FRc and Rc by depriving all phytochromes of chromophore (Parks *et al.*, 1989; Dehesh *et al.*, 1993; Parks and Quail, 1993). The formal possibility that *lid2* leads to decreased production of phyA holoprotein was tested by immunoblotting seedling extracts and probing with phyA-specific monoclonal antibodies (Xu *et al.*, 1995; Short, 1999). Western blots with extracts of Col, *lid2*, *phyA* and RLD grown for 4d in darkness and either left in darkness or exposed to 3h Rc were tested for the dark accumulation and for the light-induced degradation of the phyA protein (Figure 23). Dark grown *lid2* maintained a wild-type level of phyA protein, and exposure to Rc for 3 h led to the light-dependent loss of immunochemically detectable PHYA protein in *lid2*, as it did in wild type.

V. Initial genetic characterization and mapping of lid2

A complement of 489 *lid2*XNo-O F_2 progeny were selected for mapping based on the reduced chlorophyll phenotype when *lid2* plants are grown under Wc. Genomic DNA was extracted from these lines for mapping purposes. Linkage with SSLP marker CTR1.2 first revealed that the *LID2* gene is on the short arm of chromosome 5, yielding 24 out of 978 possible recombination events (Fig 24) corresponding to +/-2.5 cM. However, there are no reported SSLP markers that are located nearer to the expected *LID2* locus than CTR1.2. This 5 cM region encompasses more than 200 genes. Therefore, SNP markers were used as this method is PCR-based and therefore more easily assayed than other marker types, and there are abundant single-nucleotide polymorphisms reported within the predicted region.

Recombination ratios from four SNP markers further helped to identify the location of the *LID2* gene (Fig 24). One SNP marker is located within BAC- T20L15 at position 87542, and yielded 5 recombinants out of 978 chromosomes, indicating a position within 0.5 map units from *lid2*. A second marker was within BAC-T10O8 at position of 60207 and yielded 1 out of 978 possible recombinants, indicating a position 0.1 map unit from *lid2*. Two other markers are within BAC-F7A7. One is at position of 35622, and the other at position of 78751. Neither marker yielded any recombination out of the 489 plants tested. These mapping results indicate that *LID2* is probably located within BAC F7A7, near the telomere of chromosome 5. Recombination was

calculated by using the number of Col-O alleles detected divided by the total number of alleles (No-O and Col) in F_2 progeny (Table 5).

Twenty six genes are located within BAC F7A7 (Table 6), and several of them are likely candidate genes that encode proteins functioning in light-related pathways, but most of them are putative proteins without functional attribution. Since no gene in F7A7 has been reported as taking part in phyA signaling or in reduced chlorophyll production, the *LID2* gene encodes a protein not yet implicated in light-mediated responses.

As it has proven difficult to ligate long genomic fragments into binary vectors and transfect *Agrobacterium* and *Arabidopsis* plants, the region was subdivided by designing PCR primers that can amplify 4-7 kb fragments of F7A7, including one or two genes and the flanking regions. Fifteen fragments covering the entire BAC were amplified and cloned into pGEM. These cloned fragments are at various stages of preparation for insertion into binary vectors, and a few have been used to transfect *lid2* plants in an attempt to rescue the mutant phenotype (Table 7) and identify the likely *LID2* candidate.

Since *LID2* is one of the genes in BAC F7A7, and a mutation in the promoter or in a residue contributing to instability of the message would cause a change in *LID2* gene expression, the amount of transcript for each of twenty-five out of the 26 genes in BAC F7A7 was assessed using gene-specific primers with semi-quantitative RT-PCR

(Table 8). For five of these genes, no gene transcript was detected. There are eleven genes for which equal amounts of message were detected between *lid2* and Col-O wild type seedlings (Fig 25.a). Ten genes displayed apparently different transcript levels between *lid2* and wild type (Fig 25.b). Although some gene transcript levels were invariant between mutant and wild type, they cannot be excluded as possible candidates for *LID2*, as the mutation in *LID2* may not result in an abnormal transcript level.

D. Discussion

A second mutant was isolated from putative *phyA* pathway mutants and designated *lid2*. Interestingly, *lid2* does not produce a heritably lower amount of anthocyanins, although it was identified by screening for seedlings with reduced anthocyanin levels. However, other photomorphogenic deficiencies displayed by *lid2* made it noteworthy for further study.

Preliminary observations of FRc-grown *lid2* indicated that *lid2* maintained slightly less anthocyanin than Col-O does. Then seeds of *lid2* were collected following self-fertilization and the physiological characteristics of the seedlings that developed from these seeds were studied further. Anthocyanin extraction and quantitation from FRc-grown seedlings showed that the reduced anthocyanin phenotype was not consistent, indicating the original low anthocyanin phenotype was not genetically heritable. However, simultaneous tests on chlorophyll production, germination, and other physiological responses that are observable by controlled illumination have yielded significant differences between *lid2* and wild type. These differences are not limited to FRc-mediated responses. Moreover, immunoblot analysis indicates that the amount of the phyA apoprotein in *lid2* is comparable to that in wild type. Based on these observations and other characterizations, we believe *lid2* is affected both in phyA-regulated pathways and in pathways initiated by other unidentified factors.

I. *lid2* uncouples a set of physiological responses from normal phyA regulation

5-d-old Wc-grown *lid2* seedlings appear dramatically less green than the wild type, indicating the cotyledons of *lid2* accumulate less chlorophyll than do those of the wild type (Fig. 11). Chlorophyll quantitation confirmed that *lid2* accumulates less than 25% of the wild type level of chlorophyll (Fig 12a). Chlorophyll measurement of Rc- and Bc-grown seedlings yielded similar results, even though *lid2* produced close to 30% of the chlorophyll produced by the wild type growing in Rc, a slightly higher proportion than in Wc (Fig 12b). These results may suggest that the lid2 signal may not depend on one specific R or B photoreceptor, and that it generates a similar pattern of low chlorophyll content in all light conditions tested.

Even though the chlorophyll accumulation and subsequent transition of the proplastid or etioplast to the chloroplast are under the control of phytochromes, it is not certain that lid2 is part of the phytochrome pathway or pathways initiated by other receptors in regulating chlorophyll accumulation under Wc. The mutation could also be in a structural gene of chlorophyll biosynthesis, causing a deficiency in chlorophyll accumulation. However, just like wild type, dark grown *lid2* seedlings do not accumulate chlorophyll, showing that the lid2 signal is directly or indirectly initiated by light receptors. Furthermore, if *LID2* encoded a chlorophyll biosynthetic gene, the down- regulation of *POR* in FRc should still occur. Alternatively, lid2 may still work in the phyA pathway, assuming that the reduced amount of photoactive phyA under Wc can induce chlorophyll accumulation and the lack of phyA signal because of the *lid2*

mutation results in reduced chlorophyll. These results also indicate that lid2 is not among the negative regulators such as COP/DET/FUS that can suppress the development of the chloroplasts, since the mutation of these negative regulators results in photomorphogenic development of dark grown seedlings. Even though *lid2* produces chlorophyll in an amount that is more than 70% lower than in wild type, mutation of *LID2* does not completely block the production of chlorophyll, suggesting that either *lid2* is not a null loss-of-function mutant, that lid2 works in a redundant manner such that null mutation in *LID2* can not completely eliminate the production of chlorophyll, or that it is involved in a regulatory step that acts in concert with an unrelated, intact regulatory mechanism. There is not enough evidence to favor one hypothesis over another at this point. Even though less chlorophyll is produced in *lid2*, the chlorophyll a/b ratios in *lid2* mutants were not significantly different from those in wild type (data not shown), indicating that *lid2* mutants do not lack completely the light-harvesting complex, since chlorophyll b is found exclusively bound to the light-harvesting chlorophyll a/b binding proteins (Kaplan and Arntzen, 1982).

Further characterizations corroborate the idea that some of the physiological responses affected by lid2 are not under control of phyA. For example, 5d Wc-grown *lid2* seedlings produce less anthocyanin than wild type (Fig 19b), and 3d FRc-grown *lid2* and wild type contain similar levels of anthocyanin (Fig 19a). Anthocyanin production in Wc-grown seedlings is regulated in plant development by a variety of stimuli, including pathogens, metabolites, and light (Dixon and Paiva, 1995; Mol *et al.*, 1996), but not by R or FR light (Fuglevand *et al.*, 1996; Wade *et al.*, 2001). However,

phyA induces anthocyanin biosynthesis in FRc-grown young seedlings. The fact that FRc grown *lid2* seedlings have similar anthocyanin accumulation to wild type indicates that phyA-mediated anthocyanin production is normal in *lid2*. However, even though Wc-grown *lid2* accumulates less anthocyanin, the phenotype is not consistent with *LID2* being an allele of any of the *TT* genes (Feinbaum and Ausubel, 1988; Shirley *et al.*, 1992; Nesi *et al.*, 2000, 2001), which are genes required for flavonoid biosynthesis or regulation and result in pale seed coat color not seen in the *lid2* mutant.

Even though *LID2* does not appear to be a gene encoding a flavonoid biosynthetic enzyme or a signaling intermediate in phyA-controlled flavonoid biosynthesis, it does affect the transcript levels of all three flavonoid metabolism genes tested. Semi-quantitative RT-PCR shows that 5 d Wc-grown *lid2* seedlings have very low *DFR* and *CHI* transcript levels and about 50% of the *CHS* transcript level that wild type accumulates (Fig 20). These results indicate that both phyA and lid2 mediate transcript levels of all three metabolic genes, but they probably work through separate mechanisms.

II. lid2 function or expression may be under temporal control

As stated before, based on the characterization of mutant phenotypes, *lid2* seedlings that grow for more than two weeks do not show a significant difference in chlorophyll production compared with wild type, indicating that the mutation of *LID2* primarily affects early response regulation. Therefore, the expression or activity of *LID2* may be under the temporal control such that the mutation of *LID2* is only conspicuous in young seedlings.

The hypothesis of temporal control of *LID2* expression is also supported by the study of anthocyanin accumulation. Previous experiments show that 5d Wc-grown *lid2* seedlings display lower anthocyanin levels, but this mutant phenotype no longer exists in seedlings that grow longer than two weeks after germination. Anthocyanin measurements using seedlings older than two weeks reveal no difference in anthocyanin accumulation between *lid2* and wild type.

Mature *lid2* plants do not manifest other light-receptor-mediated phenotypes, such as altered flowering time (Table 4). These results suggest that lid2 works as a positive regulator in both chlorophyll and anthocyanin accumulation only in young seedlings, and its effects diminish with the development of the seedlings. The diminishment may be caused by the regulation of *LID2* expression or activity, increased function of a redundant factor, or the antagonistic effects of another temporally regulated factor.

III. lid2 acts negatively to regulate parts of the phyA signaling network

Previous studies on the FR-induced block of greening showed that both PORA and PORB are depleted in wild type seedlings and that the depletion is exclusively controlled by phyA (Barnes *et al.*, 1996; Runge *et al.*, 1996). Even though low chlorophyll production in Wc-grown *lid2* seedlings does not appear to be phyA-related, *lid2* is also deficient in the phyA-induced block of greening. Nearly all 3d FRc-treated *lid2* seedlings recuperate and produce chlorophyll, whereas all wild type seedlings die from the lack of chlorophyll, indicating that lid2 is indispensable for phyA-mediated inhibition of chlorophyll production (Fig 16). Moreover, *lid2* can only accumulate one third the chlorophyll of a *phyA* null mutant, indicating either that lid2 works in a redundant manner to another component in the phyA pathway, that the mutation of *LID2* only affects part of lid2 function, or that the absence of functional lid2 only partially contributes to loss of POR suppression. Several lines of evidence are consistent with lid2 acting in the phyA signaling pathway. There is no additive affect of the *phyAlid2* double mutation on chlorophyll accumulation after FRc exposure, whereas *lid2* in a phyA overexpressor AOX background partially relieves the hypersensitivity exhibited in AOX alone. The amount of chlorophyll the double mutant produced is less than that produced by *phyA*, suggesting that lid2 may not be completely epistatic to phyA in the signal transduction chain and that lid2 may be able to mediate chlorophyll production regardless of the existence of a phyA signal. However, the double mutant *lid2phyA* still produces twice as much chlorophyll as *lid2*, indicating that phyA exerts

some effects in FRc-induced inhibition of chlorophyll production in the *lid2* mutant. Therefore, lid2 may work both as a negative regulator in the phyA pathway and have an antagonistic effect on phyA signaling, perhaps integrating the phytochrome signal with other inputs to regulate the ultimate response.

Even though lid2 and phyA may work partially independently in controlling chlorophyll production, phyA still plays a dominant role in this regulation since there is very little difference in chlorophyll accumulation between *phyA* and *lid2phyA* and there is a dramatic difference between amount of chlorophyll produced by *lid2* and by *phyA* mutants.

It has been shown that Wc-grown AOX seedlings display a short-hypocotyl phenotype and that the amount of PHYA does not decrease in abundance as much as it does in wild type when seedlings were exposed to light (Boylan and Quail, 1991), suggesting that phyA-induced signal transduction may be at least partially active under Wc in AOX seedlings. In order to understand the relationship between phyA and lid2 pathways, AOX and *lid2*AOX were included in the physiological analyses. In the block-of-greening experiments, 3d FRc-treated *lid2*AOX showed much lower chlorophyll production than *lid2*—despite its being higher than in wild type—indicating that the effect of overexpressed phyA can still partially overcome the mutation of *LID2* (Fig 16). Again, this could mean that the mutation is leaky, such that the high signal input from phytochrome in AOX exceeds the threshold for limited suppression of *POR* genes,

or that an alternate signaling mechanism partially circumvents the *lid2* block between phyA and *POR* repression.

Consistent with the phenotypic and genetic data, immunoblotting using a phyA-specific antibody demonstrates that *lid2* produces a normal amount of PHYA apoprotein and that the phyA in *lid2* undergoes normal degradation upon illumination with Rc light (Fig 23). These data indicate that *LID2* is not an allele of the *PHYA* gene (as confirmed by its map position) and that lid2 does not affect normal accumulation of PHYA apoprotein or its attachment to chromophore. Therefore, *lid2* has apparently functional phyA, and lid2 instead acts downstream as a signaling intermediate in a branch of the phyA pathway.

IV. The FRc-induced lid2-mediated block of greening is duration-dependent.

The block of greening responses are highly dependent on the duration of exposure. *lid2* seedlings treated with 4 d FRc behave like wild type. Among the lines tested, only *phyA* and *lid2phyA* are capable of producing chlorophyll. Therefore, the lack of a phyA input makes irrelevant the function of *lid2* in blocking suppression of POR, and nearly all seedlings produce chlorophyll regardless of the state of lid2. On the other hand, in the presence of a normal phyA suppressive signal, wild type lid2 allows the suppressive signal to propagate and few or no seedlings produce chlorophyll, while in the *lid2* mutant the suppression is attenuated but not lost.

If all lines receive only 2 d FRc before transfer to 3 d Wc, there is very little suppression of *POR* even in wild type, and the absence of lid2 does not dramatically alter the chlorophyll accumulation even though 2 d FRc-treated *lid2* and double mutants accumulate slightly less chlorophyll than wild type, possibly because of the normally slightly lower chlorophyll content of *lid2* in Wc. Since AOX contains increased phyA signal input, the phyA-mediated suppression in AOX is also increased and less chlorophyll is produced. However, *lid2* can attenuate the increased phyA signal in AOX, preventing the block in chlorophyll production and returning *lid2*AOX to a more nearly wild type phenotype.

If all lines receive 3 d FRc before transfer to 3 d Wc, wild type greening is severely inhibited. The absence of lid2 blocks the suppression and most seedlings can produce chlorophyll (94%, Table 3) but the amount produced in each seedling is still reduced compared with that produced by the *phyA* null mutant. The suppression in AOX also results in very little chlorophyll accumulation in this condition. However, *lid2* can no longer as successfully block the increased phyA signal in AOX as it does in 2 d FR-grown seedlings, and as a result, *lid2*AOX double mutant produces less chlorophyll than does the *lid2* single mutant. Comparisons between individual seedlings within each group also show increased variability in the pattern of greening, possibly suggesting that these growth conditions are near a threshold for the block of greening in this genotype such that some plants can produce a limited amount of chlorophyll, while others are unable to accumulate any significant amount. The decrease of chlorophyll accumulation in *lid2*AOX is from the inability to produce any chlorophyll by 70% of

seedlings and the reduced chlorophyll production by the other 30% (Table 3). The decrease of chlorophyll accumulation in *lid2* may arise from its general inability to produce as much chlorophyll as wild type in Wc, but the percentage of individuals that turn green is still 100% (Table 3).

V. lid2 may regulate chlorophyll production by regulating PORB and PORC transcript levels.

In *Arabidopsis*, the biosynthesis of chlorophyll is light-dependent in the step of enzymatic reduction of Pchlide to Chlide, independent of photomorphogenic photoreceptors (Bauer *et al.*, 1993), but under normal light conditions the reduction of Pchlide to Chlide accompanies the simultaneous photoconversion of the P_r form of phytochrome to the P_{fr} form, which triggers plant photomorphogenesis (Chory, 1991; Thompson and White, 1991). Since Wc-grown *lid2* produces less chlorophyll than wild type and FRc grown *lid2* is deficient in the bock of greening, a possible candidate in chlorophyll biosynthesis that may be mediated by lid2 is one or more of the POR enzymes that, under light regulation, catalyze the photoreduction of Pchlide in plastids using Pchlide itself as the photoreceptor (Koski *et al.*, 1951; Griffiths, 1978; Apel *et al.*, 1980). Semi-quantitative RT-PCR using total RNA as template and gene-specific nucleotide sequences as primers revealed that *lid2* accumulates significantly less PORB transcript and slightly less PORC transcript than does wild type under 5d Wc, and no detectable PORA transcript was found in either wild type or *lid2* under the same conditions (Fig 14). Therefore, one function of lid2 may be in controlling the levels of

PORB and PORC mRNAs, and the down-regulation of PORB and PORC by *lid2* is phyA-independent in Wc grown seedlings. These conclusions are supported by similar experiment using *lid2phyA* and *lid2AOX* double mutants, which yield similar PORB and PORC message levels with *lid2* alone, indicating that in Wc, lid2 is not affected by the level of phyA in conditions where phyA is missing or rapidly degraded. However, since AOX displays very low PORB transcript, it appears that enough phyA persists in AOX to inhibit some accumulation of PORB message, also consistent with the reduced degradation of phyA in AOX under Wc.

Semi-quantitative RT-PCR on RNA from plants exposed to 3d FRc or to 3d FRc followed by 3 days Wc also show the regulation of PORB and PORC message levels by lid2. In both cases, *lid2* accumulates more PORB and PORC transcripts than does wild type, consistent with the higher chlorophyll accumulation in *lid2*. Since the FR-HIR-induced block of greening is regulated primarily by phyA, it is reasonable to believe that the phyA signal which inhibits accumulation of PORB and PORC transcripts passes through lid2.

VI. The *lid2* phenotype is not modified by sucrose

Compared with other phyA pathway mutants, such as *fin219*, whose phenotype exhibits sucrose-dependence, the *lid2* phenotypes are not altered by the addition of sucrose to the medium. Unlike *fin219*, *lid2* does not respond to exogenously applied sucrose any differently from wild type in terms of hypocotyl elongation or anthocyanin

production (Hsieh *et al.*, 2000). Previous studies have shown that sugar availability can alter many photomorphogenic responses through regulating sugar-responsive gene expression (Koch, 1996), and that many phytochrome-mediated phenotypes, such as anthocyanin production, seed germination, and chlorophyll accumulation, are also regulated in part by sugars. However, adding sucrose to the growth medium does not increase chlorophyll production in Wc-grown *lid2* seedlings any more than in wild type.

Previous studies showed that exogenously supplied sucrose suppresses the FR block of greening (Barnes *et al.*, 1996). Mutants and wild type seedlings grown under 3 days FRc followed by 3 days Wc on medium with 2% sucrose were measured and their respective chlorophyll content assayed. Even though all lines, including wild type, recuperated and accumulated chlorophyll, *lid2* still produced less chlorophyll than did the wild type (Fig 18). This result implies either that lid2 works downstream of phyA and that the part of the pathway encompassing lid2 is not under the direct control of sucrose, or that lid2 may work in a pathway separate from phyA and the lid2 pathway is not under the control of sucrose.

The chlorophyll accumulation results suggest that lid2 is not part of the signal transduction chain initiated by sucrose in controlling chlorophyll production. Since other FR irradiation effects, such as the inhibition of hypocotyl elongation, is also suppressed by sucrose (Whitelam *et al.*, 1993), the hypocotyl length of *lid2* and wild type with or without sucrose in the medium was measured. No difference was observed in responsiveness to sucrose between *lid2* and wild type (Fig 22b, Fig 22c). Therefore,

exogenously supplied sucrose does not negate the effect of *LID2* mutation, further supporting the contention that lid2 is not part of the sucrose signaling pathway, and that addition of moderate sucrose concentrations (up to 4%) does not differentially affect these responses.

VII. lid2 is a novel genetic mutant that defines a branch of phyA-mediated responses.

As stated previously, the downstream signaling pathway of phyA includes branches responsible for subsets of FRc-controlled responses, and many intermediates take part in passing the signal. The *lid2* mutation, as reported here, also controls a subset of phyA-mediated responses, primarily affecting the phyA-mediated block of greening. However, other photomorphogenic responses of seedlings, such as inhibition of hypocotyl growth, cotyledon opening and expansion, apical hook opening, and anthocyanin accumulation are apparently not under the control of *LID2*. The data imply either that these physiological responses diverge early from the predominant phyA signal transduction pathway and lid2 is only in effect after the point of divergence, or that lid2 may work in these pathways in a redundant manner. The lack of pleiotropic effects of mutations at the *LID2* locus suggests that *LID2* may encode a specific regulator of chlorophyll production, and it functions late in the phyA signaling network.

The comparison between the amount of chlorophyll produced by *lid2* and that produced by wild type and other mutants in FRc-induced block-of-greening

experiments indicates that *lid2* mutant allele is either leaky or redundant in controlling chlorophyll accumulation. After 3d FRc treatment, *lid2* accumulates more chlorophyll than does wild type. When there is no phyA signal, lid2 is largely irrelevant, and *phyA* and *lid2phyA* produce similar amounts of chlorophyll. When there is a strong phyA signal present, lid2 attenuates the hypersensitivity, and thus helps attenuate the block of greening. Therefore, *lid2*AOX accumulates more chlorophyll than does AOX.

Genetic mapping of lid2 reveals that *LID2* is located very close to the top of chromosome 5, an area in which no genes reported to play a role in phytochrome signal transduction have been identified (Table 6). Sequence comparisons of potential candidate genes in the region encompassing *LID2* have not yielded similarities between these genes and light-regulatory pathway genes. Therefore, *lid2* appears to be a novel mutant with multiple roles, part of which is in a phyA-controlled signal transduction pathway.

In order to identify the *LID2* gene from the area delineated by linkage analysis, 12 of the 26 putative genes identified from the *Arabidopsis* genome sequence were cloned into the pGEM vector, of which 8 have been transferred to the pGreen binary vector (Hellens *et al.*, 2000) and transformed into *Agrobacterium tumefaciens* strain GV3101 (Koncz, 1986). Four of these have been used to transform *lid2* mutants, but none of the four genes tested has been able to rescue of the mutant phenotype.

Because of the labor involved in attempting to rescue with each gene, the possibility that transcript levels of lid2 are severely diminished because of a mutation in the promoter or in a residue necessary for maintaining RNA stability was tested. Using gene specific primers (Table 8), we compared the transcript levels of these genes from wild type to those in *lid2*. Five genes display an undetectable level of transcript both in wild type and in the *lid2* mutant. Ten genes display similar levels of message in *lid2* compared with the respective RNA levels in wild type, and the other ten genes display different level of message (Fig 25.a, Fig 25.b). One of the genes that shows altered transcript levels may correspond to the *LID2* gene, since mutation of *LID2* may cause a change in the amount of transcripts through changing gene transcription or RNA degradation. However, it is also possible that mutation of *LID2* does not alter the steady state level of *LID2* transcript, but simply the function of the lid2 protein. Furthermore, the change of these transcripts may be the indirect consequences of *LID2* mutation, since lowering chlorophyll would be expected to lead to numerous genetic and physiological changes, and reduced phyA signal has been shown by microarray analysis to affect expression of a large proportion of the genes in *Arabidopsis*. Therefore, although these transcript abundance changes may be important for understanding the function of lid2, the identification of the *LID2* gene will require completion of the rescue experiments and confirmation of the lesion by sequencing.

VIII. Working model for lid2 function

Based on the evidence presented above, lid2 is likely a phyA signaling intermediate. On the other hand, Wc-grown *lid2* lacks the full complement of chlorophyll; therefore lid2 may also be a signaling intermediate in a pathway initiated by a different factor. Fig 26 illustrates a simplified lid2 working model in which lid2 works to integrate both pathways to induce chlorophyll accumulation through maintaining normal levels of PORB and PORC. In the phyA-mediated pathway, lid2 works as a negative regulator and phyA inhibits chlorophyll production by passing an inhibitory signal through lid2. In the other unidentified pathway, lid2 is a positive regulator and induces chlorophyll accumulation. Light conditions determine how each pathway is balanced to determine whether the output inhibits (as with a strong phyA signal) or enhances (as with a strong alternate signal) PORB and PORC expression. Under Wc, the phyA pathway has little effect since most phyA is degraded, so lid2 predominately works as a positive regulator to induce the production of chlorophyll. As a result, the mutation of *LID2* causes a reduced level of chlorophyll accumulation. The mutation of phyA prevents any inhibitory signal from entering the pathway, so wild type and *lid2* mutants both produce chlorophyll, but the maximum induction is inherently reduced by the *lid2* mutation. Under FRc, the phyA pathway has a dominant role in controlling subsequent chlorophyll production and lid2 works as a negative regulator in this pathway. Mutation of *LID2* largely blocks the normal phyA suppression of greening, leading to increased chlorophyll accumulation. In the

meantime, examination of the *lid2*AOX double mutant reveals that either *lid2* is a leaky mutant or lid2 works redundantly in the phyA pathway since the mutation of *LID2* causes significant changes in chlorophyll accumulation in the AOX background.

It is expected that future identification of the *LID2* gene will help clarify its function in regulating photomorphogenesis, and that determination of factors that interact with the lid2 protein will define its place in cellular pathways.

Segregation of *lid1* Mutations (892 F_2 seedlings analyzed)		
Phenotype	**Col-O**	***lid1***
No. of seedlings	**652**	**240**
$\chi^2 = 1.728$ $P \approx 0.189$		

Table 1. Genetic analysis of *lid1* mutation. χ^2 was based on the expected segregation ratio of 3:1. Seedlings grew under FRc for 2 days. The phenotypic criterion used for *lid1* was low observable anthocyanin. Based on the χ^2 value, it is unlikely that these values differ significantly from the expected.

Segregation of *lid2* Mutations (986 F_2 seedlings analyzed)		
Phenotype	**Col**	***lid2***
No. of seedlings	**728**	**258**
$\chi^2 = 0.715$ $P \approx 0.398$		

(a)

Segregation of *lid2phyA* Double Mutations (608 F_2 seedlings analyzed)				
phenotype	**wild type**	***phyA-101***	***lid2***	***lid2phyA***
No. of seedlings	**336**	**108**	**126**	**38**
$\chi^2 = 1.684$ $P \approx 0.640$				

(b)

Table 2. Genetic analysis of the *lid2* mutant F_2 populations. χ^2 was based on expected segregation ratios of 3:1 (a) and 9:3:3:1 (b). Seedlings grew under Wc for 5d. The phenotypic criterion used for *lid2* was low visible chlorophyll content (a) and the criterion used for *phyA-101* was long hypocotyls (b). Based on the χ^2 value, it is unlikely that these values differ significantly from the expected.

	Col	*lid2*	*phyA-101*	*lid2phyA*	AOX	*lid2*AOX
2dFR	**86%**	**98%**	**100%**	**100%**	**24%**	**80%**
3dFR	**4%**	**94%**	**100%**	**100%**	**0%**	**30%**
4dFR	**0**	**4%**	**100%**	**94%**	**0%**	**2%**

Table 3. Percentage of seedlings able to green in Wc following 2, 3, or 4d FRc. Seedlings from Col, *lid2, phyA-101, lid2phyA*, AOX and *lid2*AOX were treated with 2d, 3d or 4d of FRc, then transferred to Wc. The percentage of seedlings that appeared green after 3d Wc treatment was then calculated.

Col-O	*lid2*	*phyA-101*	*lid2phyA*	AOX	*lid2*AOX
21.9±0.9 days	22.3±0.8 days	23.3±1.0 days	23.4±0.8 days	22.8±1.3 days	22.8±1.2 days

Table 4. Flowering time of mutants in long days. Seeds of wild types Col-O and RLD, mutants *lid2* and *phyA*-null mutant *phyA-101*, and the phyA overexpressor AOX, as well as double mutants *lid2phyA* and *lid2*AOX were sown on MS medium. 7-d-old seedlings were then transferred to pots with soil. The number of days between germination induction and appearance of the first inflorescence was recorded. Values represent means +/- S.D.

marker	T10O8_60270 (SNP)	F7A7_35622 (SNP)	F7A7_78751 (SNP)	T20L15_87452 (SNP)	CTR1.2 (SSLP)
# of heterozygous	1	0	0	5	20
# of homozygous (No-O)	0	0	0	0	2
# of homozygous (Col)	488	489	489	483	467
Recombination ratio	0.1%	—	—	0.5%	2.5%

Table 5. The recombination ratio for each SSLP or SNP marker. The number of seedlings that have only Col allele, only No-O allele, or both alleles were counted. The recombination ratio was calculated.

Sequence ID	5' coordinate	3' coordinate	Gene product name
At5g01480	649	1890	CHP-rich zinc finger protein, putative
At5g01490	3248	5790	cation exchanger, putative (CAX4)
At5g01500	6676	8988	expressed protein
At5g01510	9415	12810	putative protein
At5g01520	14456	16058	expressed protein
At5g01530	16743	17902	light-harvesting chlorophyll a/b binding protein
At5g01540	20992	18944	receptor lectin kinase, putative
At5g01550	24242	22176	receptor lectin kinase, putative
At5g01560	27886	25829	receptor lectin kinase, putative
At5g01570	29026	29817	putative protein
At5g01580	31511	30419	putative protein
At5g01590	31908	33854	expressed protein
At5g01600	37251	35806	ferritin 1 precursor
At5g01610	38732	39651	expressed protein
At5g01620	40539	42478	expressed protein
At5g01630	48568	42774	putative protein
At5g01640	49770	49099	putative protein
At5g01650	51690	50391	Macrophage migration inhibitory factor (MIF) family
At5g01660	55802	52161	putative protein
At5g01670	59657	61513	aldose reductase, putative
At5g01680	64297	61653	cation/hydrogen exchanger, putative (CHX26)
At5g01690	65067	68002	cation/hydrogen exchanger, putative (CHX27)
At5g01700	70149	68505	protein phosphatase 2C (PP2C)
At5g01710	72907	71366	expressed protein
At5g01720	78048	74775	F-box protein family, AtFBL3
At5g01730	85218	80676	putative protein

Table 6. The genes on BAC F7A7. There are 26 genes (some are putative) in BAC F7A7. Their length and position on the BAC are shown. Annotations are based on information from the Arabidopsis Information Management System (AIMS).

Sequence ID	PCR amplified	cloned into pGEM	cloned into pGreen	transformed into plants
At5g01480				
At5g01490	X	X		
At5g01500	X	X		
At5g01510	X	X		
At5g01520	X	X	X	X
At5g01530	X	X	X	X
At5g01540	X			
At5g01550	X	X		
At5g01560	X	X	X	X
At5g01570	X	X	X	X
At5g01580	X	X	X	
At5g01590	X	X	X	
At5g01600	X	X	X	
At5g01610	X	X	X	
At5g01620	X			
At5g01630	X			
At5g01640	X			
At5g01650	X			
At5g01660	X			
At5g01670	X			
At5g01680	X			
At5g01690	X			
At5g01700	X			
At5g01710	X			
At5g01720				
At5g01730				

Table 7. list of clones that are in different stages of plant transformation.

Sequence ID	Forward primer	Reverse primer
At5g01480	5'-CGCTGAAGACGAAAAGCCTG-3'	5'-CTCAATTGGTGACGTGGATG-3'
At5g01490	5'-CCTCTTCCCGGCCGTTCCTC-3'	5'-CATCACGAAGCTGCTTGCTC-3'
At5g01500	5'-CGGTCGTAATGGCGGCGGAG-3'	5'-GACGTCATGCCAGCACAAGC-3'
At5g01510	5'-CCCCAAACTCGAACAATGAC-3'	5'-CTCCAATTCCATCCTTGGAC-3'
At5g01520	5'-GTGGACTGATTGTCATTTCG-3'	5'-GGGACAAGATTTGGATCAG-3'
At5g01530	5'-CCGGCCTCTTTGGTACCCAG-3'	5'-CCTGTTGCAGCCGCTTGAAC-3'
At5g01540	5'-CTCCTCTCCGTTTAGATACC-3'	5'-GAATTCGGATCCCATCGCAG-3'
At5g01550	5'-CTCCTCTCGCGTGCAATTCC-3'	5'-CTCTGATCGTAGCTCTATCG-3'
At5g01560	5'-CCTTCGTCGTAACCAGATCC-3'	5'-GGTGTTGCTGTTCCTCTTC-3'
At5g01570	5'-GATCAGTCGAGGAATTTCG-3'	5'-GGATACAGCCTGAGGTCTTC-3'
At5g01580	5'-GCTTAGTGTCTCCACCGAAC-3'	5'-CGAAGCTCTATGCCCCTTC-3'
At5g01590	5'-CAACGAAGATCCAGTTTTCG-3'	5'-CAAGTCGGTCTTTATACCACC-3'
At5g01600	5'-GCATCTGGTCGAAATGCCAAAC-3'	5'-CAATGTACGCATACTTTGAC-3'
At5g01610	5'-GGTTGGATCCTACTGGTTAGG-3'	5'-CGGAGACCATTTCTTTGAACC-3'
At5g01620	5'-CTCTTCAACGAGCGTAGCATTC-3'	5'-GCCCTGAAAATGGTGAGGTG-3'
At5g01630	5'-GGACTCTTTGCCGAAGTTCATG-3'	5'-CTTTACTTACATGCCAGAGGG-3'
At5g01640	5'-GCCGCTGAGGAGGCAGAGGG-3'	5'-CCACCACCGCAACAGACACC-3'
At5g01650	5'-GAGTCGCCCCGTTCCAACC-3'	5'-CCATTCTCTCGGAAGCTTCC-3'
At5g01660	5'-CTGCCACTGCTGAATATCCTC-3'	5'-CCAGACTGATGGCCAGTGGAGC-3'
At5g01670	5'-GCCGCCCACGCCGTTGTCAC-3'	5'-CGTTTCTCCAACCAGGATGC-3'
At5g01680	5'-CGTGTCTGATTCCAACCATG-3'	5'-CAAGGGATCTACGATGTCC-3'
At5g01690	5'-GGGAGGTTAGCACTTGCATCC-3'	5'-GTCGAGTTTACCGTTGCTGAC-3'
At5g01700	5'-GTCCGAGCGGCTCGCTGAACC-3'	5'-CCGTGAATTCGAGGACATTC-3'
At5g01710	5'-GCACCCCTCTTTGTCTGAGCG-3'	5'-GTGGAAAGTGCTGGATTCCGG-3'
At5g01720	5'-CCTGCCCCGCTTAAAAGTGAGG-3'	5'-GTTGGCGTCGGAGATTTAGGGG-3'
At5g01730	5'-CCATGTAACTTGCCCCAGAGC-3'	5'-CCCATTCTCTCTCAAACCGATC-3'

Table 8. Gene specific primers for genes in BAC F7A7.

Sequence ID	no detectable transcript	similar amounts of transcripts	more transcript in Col-O	more transcript in *lid2*
At5g01480	X			
At5g01490	X			
At5g01500		X		
At5g01510			X	
At5g01520			X	
At5g01530		X		
At5g01540				X
At5g01550	X			
At5g01560				X
At5g01570			X	
At5g01580			X	
At5g01590		X		
At5g01600		X		
At5g01610		X		
At5g01620		X		
At5g01630				X
At5g01640				X
At5g01650			X	
At5g01660		X		
At5g01670		X		
At5g01680	X			
At5g01690	X			
At5g01700		X		
At5g01710				X
At5g01720		X		
At5g01730		X		

Table 9. Genes in the BAC F7A7 are grouped based on the comparison on the amounts of gene transcript between in 5 d Wc-grown *lid2* seedlings and in 5 d Wc-grown Col-O seedlings. Comparisons are based on semi-quantitative RT-PCR as described in the text.

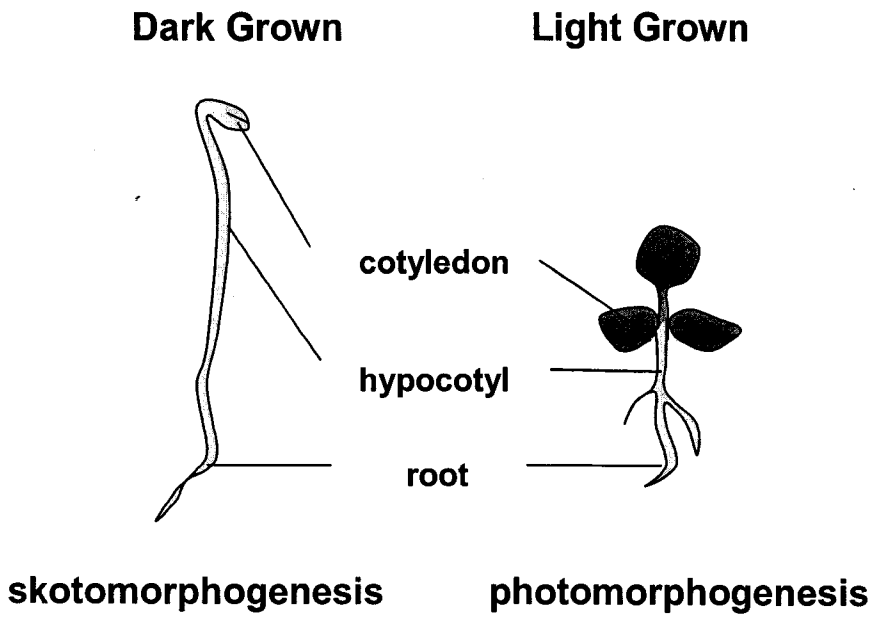


Fig 1. Skotomorphogenic and photomorphogenic growth in *Arabidopsis* seedlings.

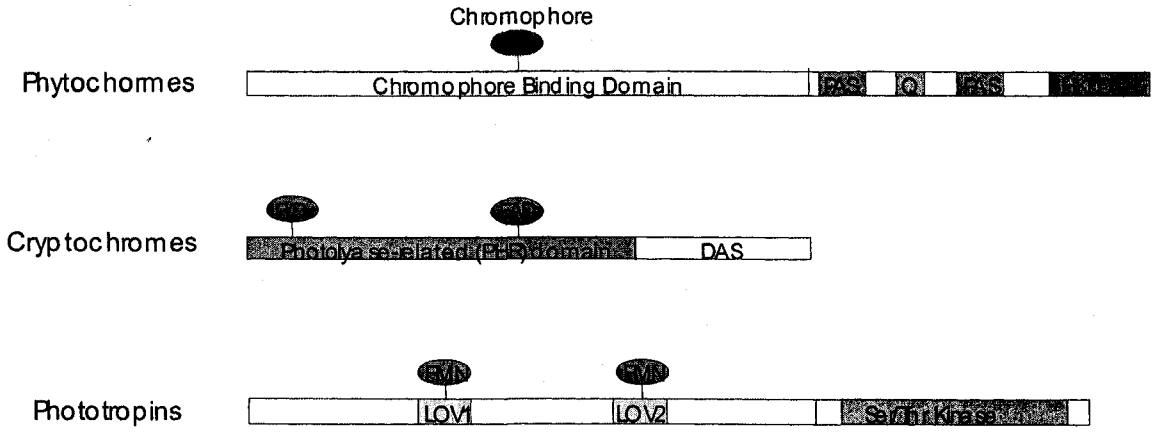


Fig 2. Domain structures of three classes of photoreceptors.

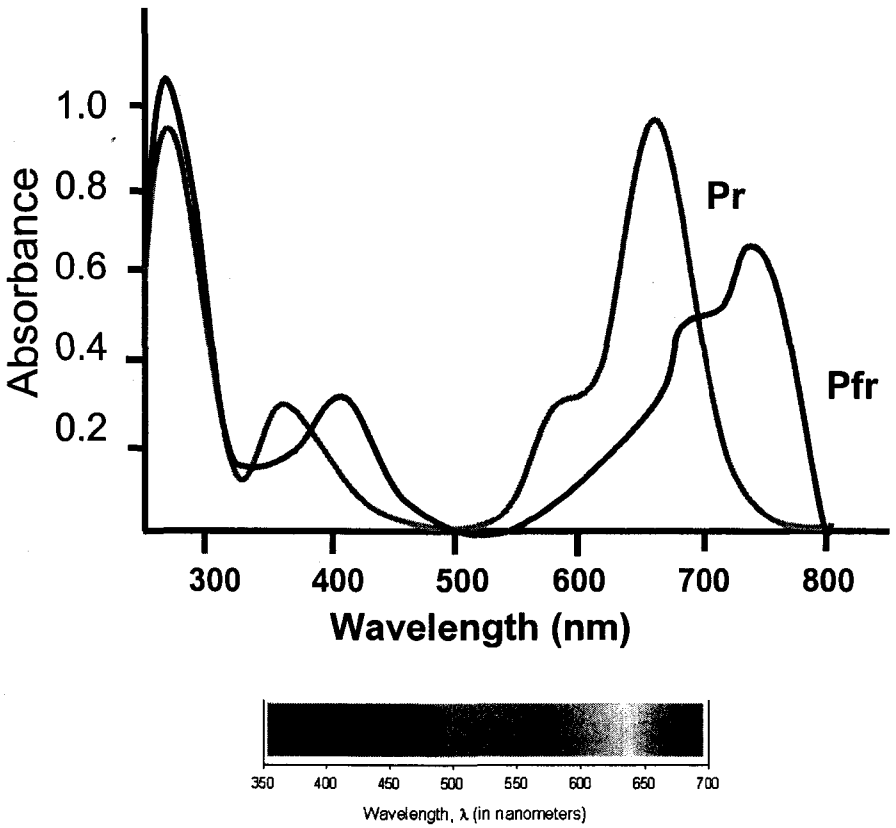


Fig 3. Absorbance spectra of Pr and Pfr forms of phytochromes.

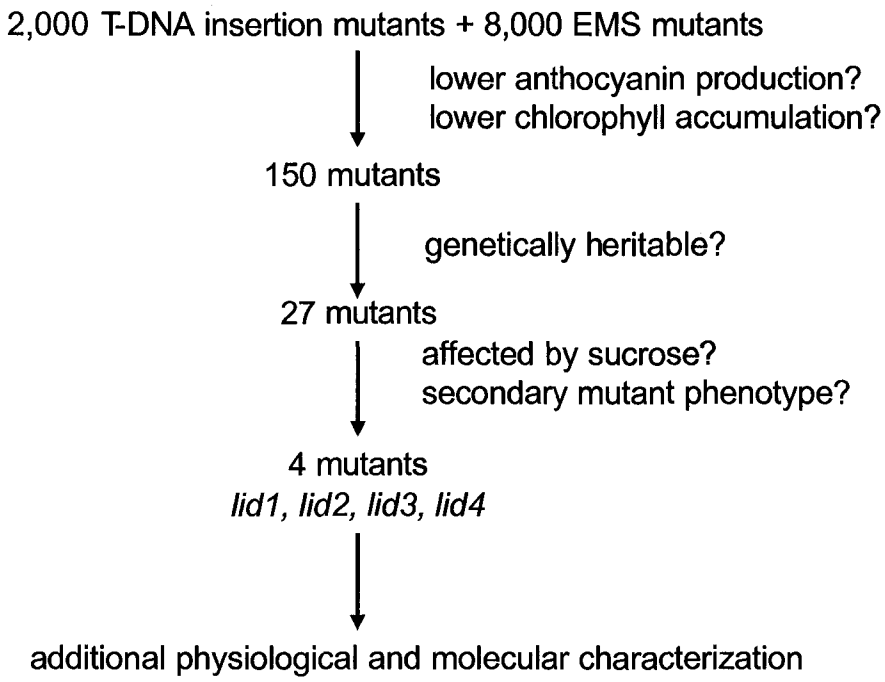


Fig 4. Flow diagram for the *lid* mutant screen.

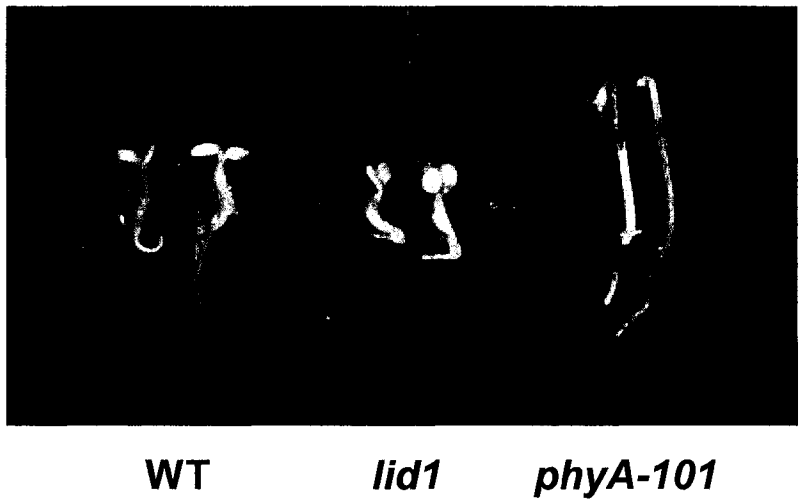


Fig 5. Visible photomorphogenic phenotypes of *lid1* seedlings. Col-O wild type (WT), mutant *lid1*, and *phyA*-null mutant *phyA-101* seedlings were grown for 3 days in continuous far-red light (FRc).

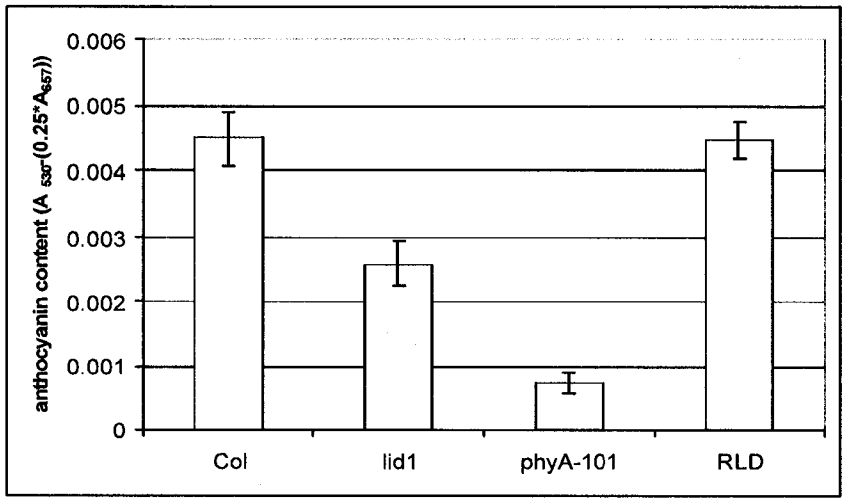


Fig 6. Response of anthocyanin levels to FRc in wild type and in mutant seedlings. The amount of anthocyanin accumulated in seedlings of the *lid1* mutant, wild type Col-O, the *phyA*-null mutant *phyA-101*, and wild type RLD were measured. Seeds were sown on MS medium containing 2% sucrose and grown for 2 days under FRc. Error bars represent +/- S.D. Experiments were repeated at least three times.

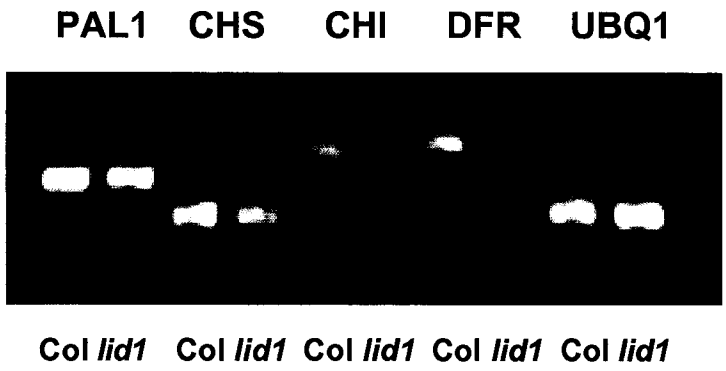


Fig 7. Transcript accumulation of anthocyanin biosynthetic pathway genes. The levels of transcripts for *PAL1*, *CHS*, *CHI*, and *DFR* were measured in seedlings of wild type Col-O and the *lid1* mutant. *UBQ1* was included as a general transcription-level control. Total RNA was isolated from 2-d-old FRc-grown seedlings.

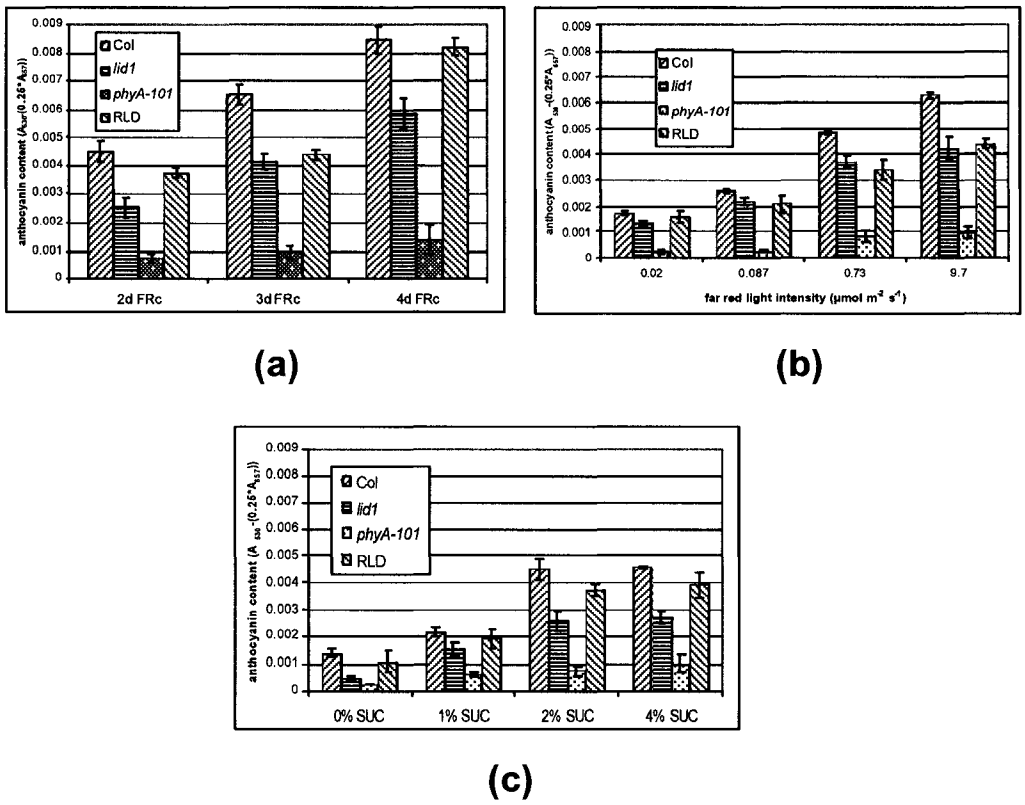


Fig 8. Anthocyanin levels unde r different durations or intensities of FRc and different sucrose concentrations in wild type and mutant seedlings. The amount of anthocyanin accumulated in FRc grown seedlings of the *lid1* mutant, wild type Col-O, the *phyA*-null mutant *phyA-101*, and wild type RLD was measured. Seeds were either sown on medium containing 2% sucrose while receiving different periods of exposure to FRc of 9.7 µmol m^{-2} sec^{-1} (a) or on medium containing 2% sucrose while receiving FRc of different intensities (b), or sown on medium containing different concentrations of sucrose while receiving FRc of 9.7 µmol m^{-2} sec^{-1} (c). Error bars represent +/- S.D. Experiments were repeated at least three times.

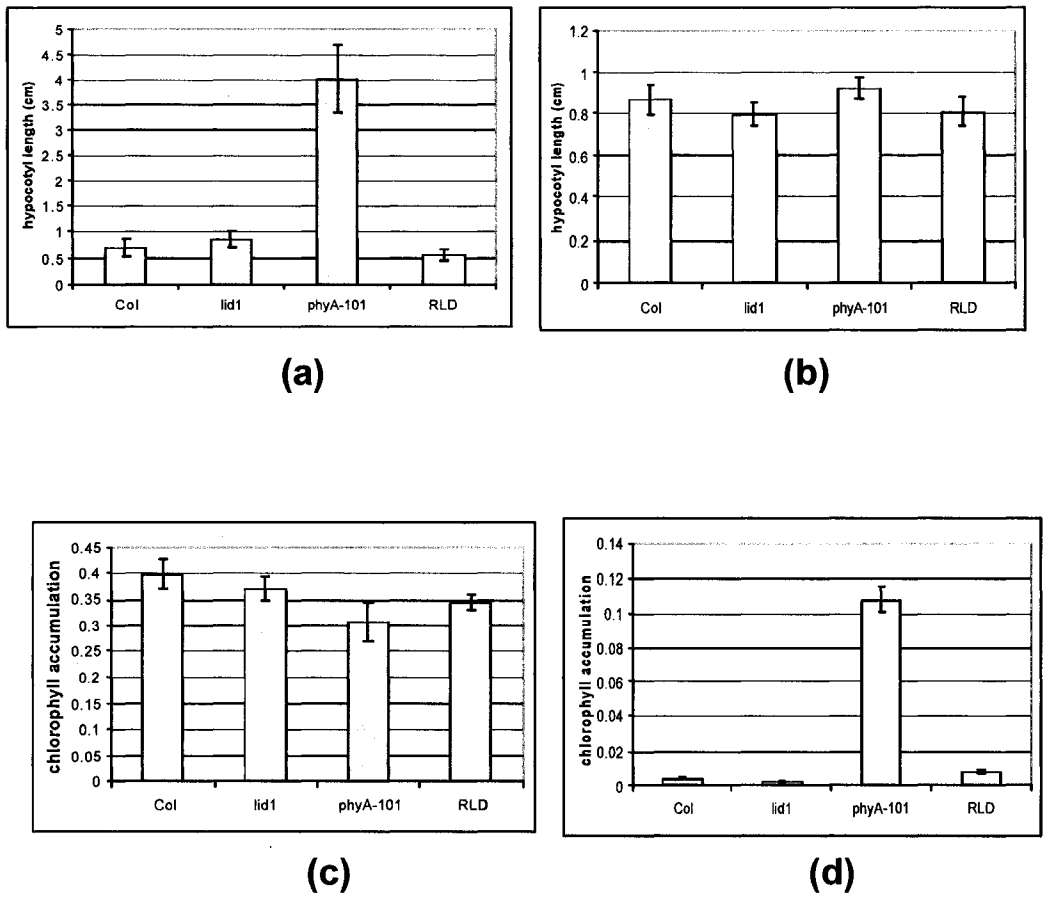
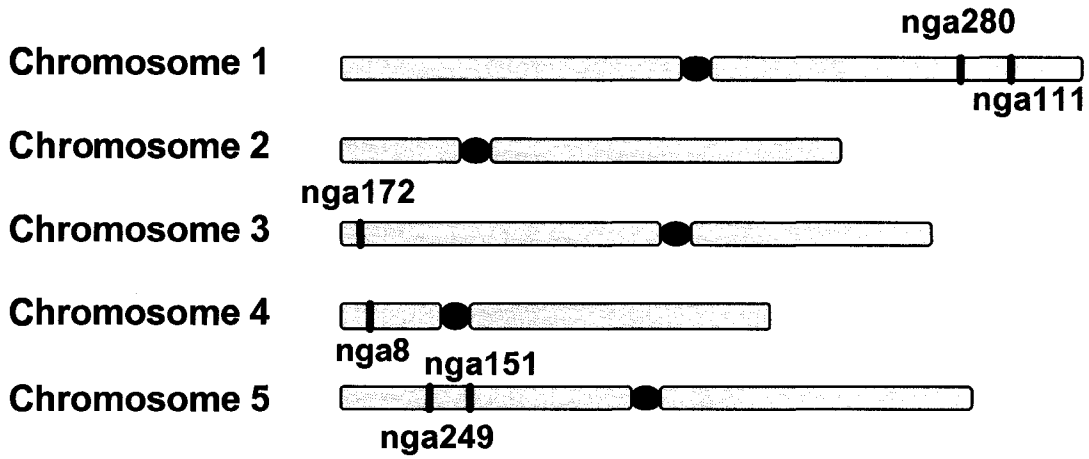


Fig 9. Some typical photomorphogenic responses are not affected in *lid1*, including the elongation of hypocotyls in FRc (a) or in darkness (b), chlorophyll production in Wc (c) and in the FR-mediated block of greening (d). Seedlings were grown in medium without sucrose and treated either with FRc for 2 days (a) or with Rc for 2 days (b) or with Wc for 5 days (c) or with FRc for 3 days followed by Wc for 3 days (d). Error bars represent +/- S.D. Experiments were repeated at least three times.



	Col allele	No-O allele
SSLP Markers	nga8	nga151
	nga111	nga249
	nga172	nga280

Fig 10. The SSLP markers are inconsistent with Col-O as the genetic background of *lid1*.

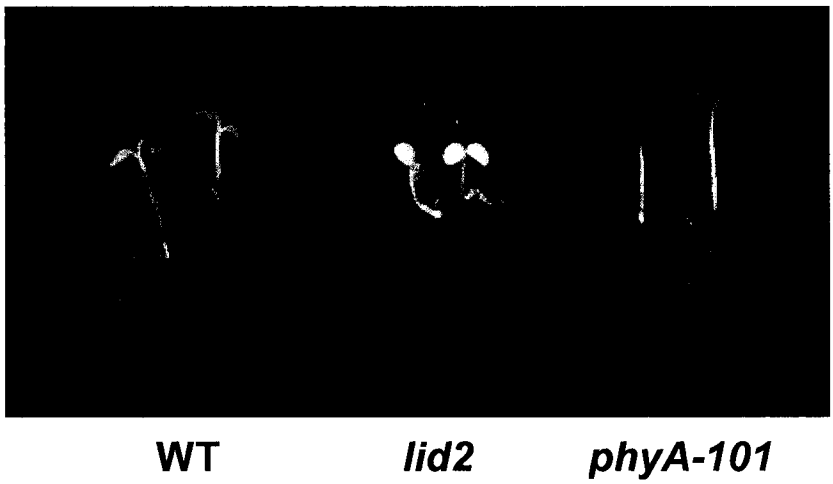


Fig 11: Visible photomorphogenic phenotypes of *lid2* seedlings. Col-O wild type (WT) and *lid2*, *phyA*-null mutant *phyA-101* seedlings were grown for 5 days in continuous white light (Wc).

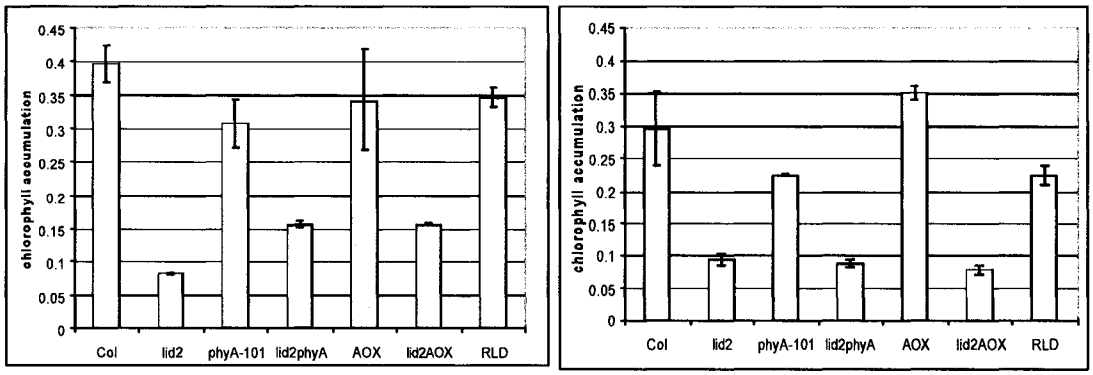


Fig 12. Quantitative determination of chlorophyll accumulation in seedlings grown for 5 days in Wc (a) or 5 days in Rc (b). Total chlorophyll was extracted from seedlings of wild types Col-O and RLD, mutants *lid2* and *phyA*-null mutant *phyA-101*, and the phyA overexpressor AOX, as well as double mutants *lid2phyA* and *lid2*AOX. The amount of chlorophyll was measured spectrophotometrically. Error bar represent +/- S.D. Experiments were repeated at least three times.

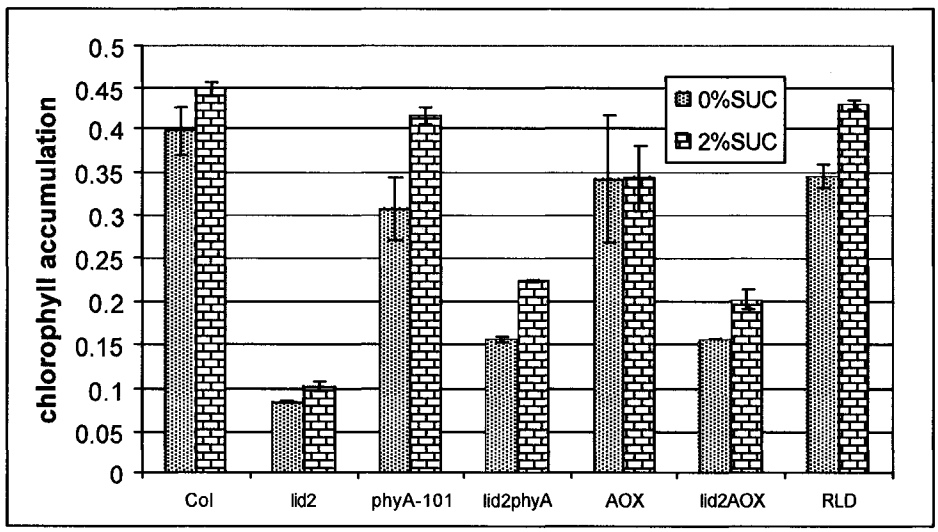


Fig 13. Effect of 2% sucrose on chlorophyll accumulation after 5 d growth in Wc. Total chlorophyll was extracted from seedlings of wild types Col-O and RLD, mutants *lid2* and *phyA*-null mutant *phyA-101*, and the phyA overexpressor AOX, as well as double mutants *lid2phyA* and *lid2*AOX. The amount of chlorophyll was measured spectrophotometrically. Error bars correspond to +/- S.D. Experiments were repeated at least three times.

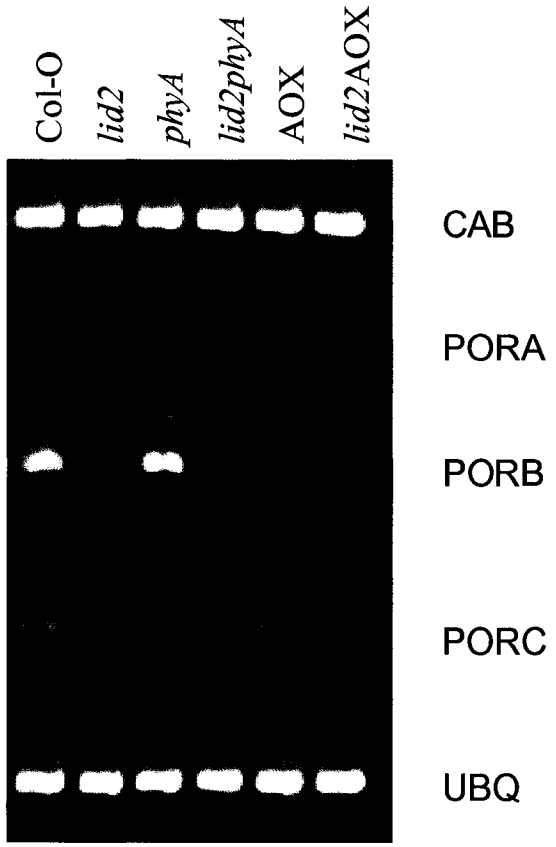


Fig 14. The *lid2* mutation causes a reduction in PORB mRNA accumulation. Levels of POR transcripts in seedlings of wild type Col-O, mutants *lid2* and *phyA*-null mutant *phyA-101,* and the phyA overexpressor AOX, as well as double mutants *lid2phyA* and *lid2*AOX were measured by gene-specific semi-quantitative RT-PCR. Total RNA was isolated from 5-d-old Wc-grown seedlings.

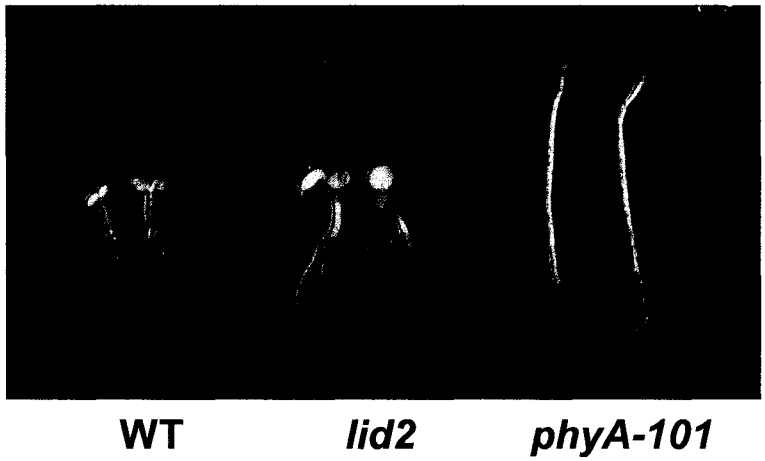


Fig 15. Visible effects on the FRc-mediated block of greening of *lid2* seedlings. Col-O wild type (WT), and mutant *lid2* and *phyA*-null mutant *phyA-101* seedlings, were grown for 3d in continuous far-red light (FRc) followed by 3 days in continuous white light (Wc).

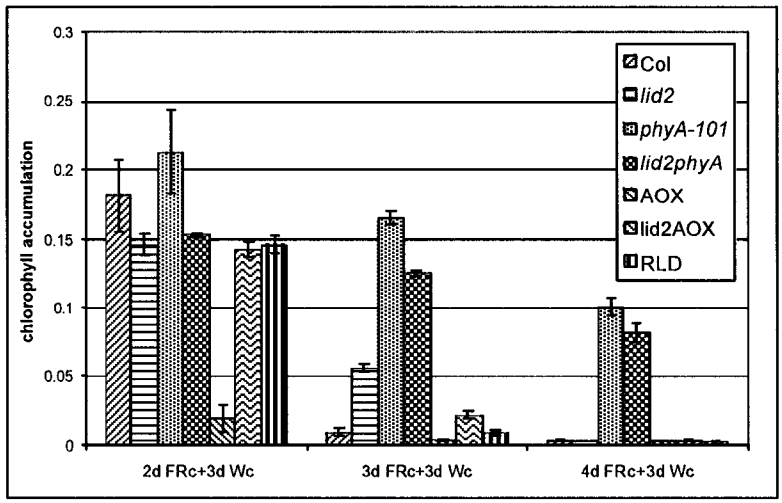


Fig 16. Quantitative determination of chlorophyll accumulation in seedlings after exposure to FRc followed by Wc. Seedlings were grown under the light conditions shown in the graph. Total chlorophyll was extracted from seedlings of wild types Col-O and RLD, mutants *lid2* and *phyA*-null mutant *phyA-101*, and the phyA overexpressor AOX, as well as double mutants *lid2phyA* and *lid2*AOX. The amount of chlorophyll was measured spectrophotometrically. Error bars represent +/- S.D. Experiments were repeated at least three times.

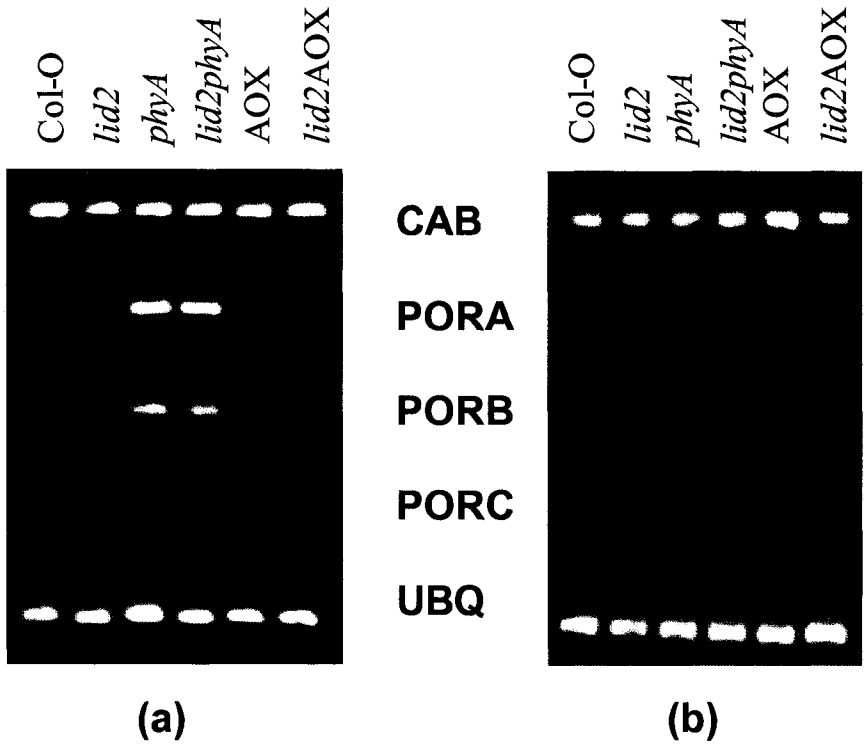


Fig 17. The *lid2* mutation results in increased levels of PORB and PORC under FRc. The relative levels of POR transcripts in seedlings of wild type, mutants *lid2* and *phyA-101,* and phyA overexpressor AOX, as well as double mutants *lid2phyA* and *lid2*AOX were determined by semi-quantitative RT-PCR. Total RNA was isolated from seedlings grown for 3d in FRc (a) or 3d in FRc followed by 3d in Wc (b).

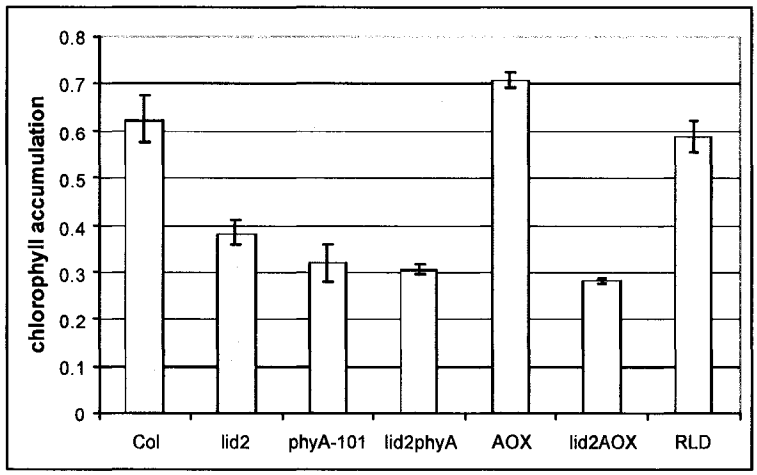
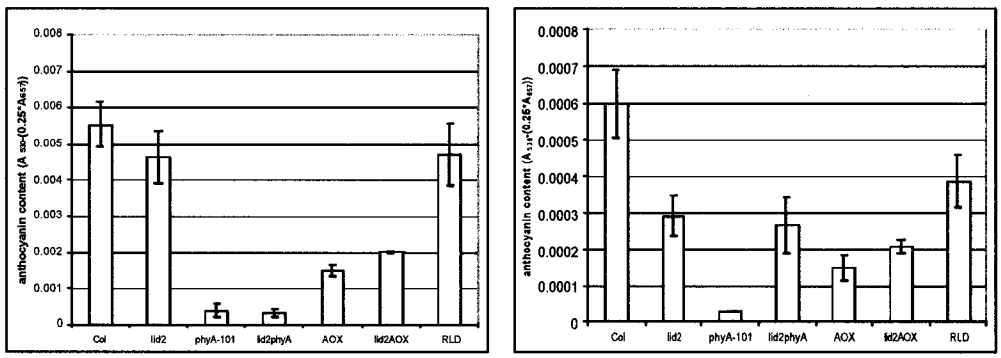


Fig 18. Quantitative determination of chlorophyll accumulation in seedlings grown 3d in FRc followed by 3 days in Wc.
Seedlings were sown in MS medium containing 2% sucrose. Total chlorophyll was extracted from seedlings of wild types Col-O and RLD, mutants *lid2* and *phyA*-null mutant *phyA-101*, and the phyA overexpressor AOX, as well as double mutants *lid2phyA* and *lid2*AOX. The amount of chlorophyll was measured spectrophotometrically. Error bars correspond to +/- S.D. Experiments were repeated at least three times.



(a) (b)

Fig 19. Quantitative determination of anthocyanin content in seedlings grown 2d in FRc (a) and 5d in Wc (b). Seedlings were sown on MS medium containing 2% sucrose. The amount of anthocyanin accumulated in seedlings of the *lid1* mutant, wild type Col-O, the *phyA*-null mutant *phyA-101*, and wild type RLD were measured. Anthocyanins were extracted from seedlings and measured spectrophotometrically. Error bars represent +/- S.D. Experiments were repeated at least three times.

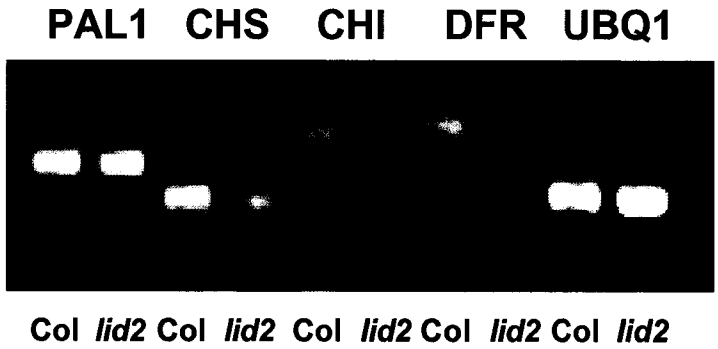


Fig 20. Transcript levels of anthocyanin biosynthetic pathway genes. The levels of transcripts for PAL1, CHS, CHI, DFR and UBQ1 were measured in seedlings of wild type Col-O and *lid2* mutant. Total RNA was isolated from 5-day-old Wc grown seedlings.

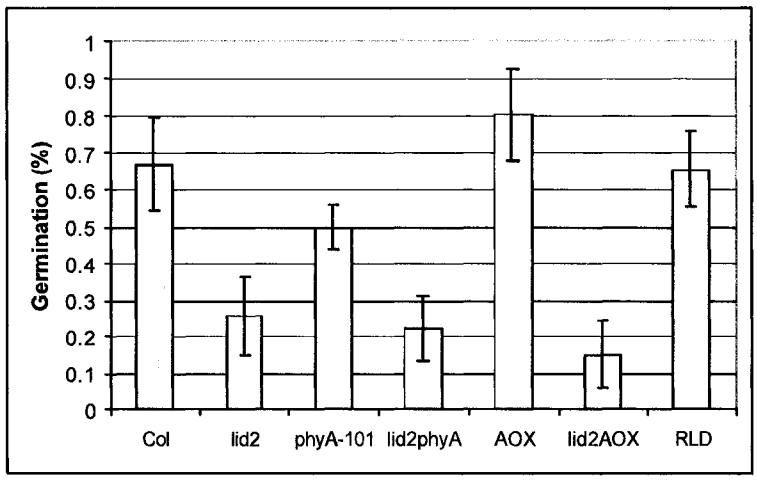


Fig 21. Germination frequencies of *lid2* exposed to 24 hours of FRc light. 50 seeds of WTs (Col and RLD), the *lid2* mutant, *phyA* null mutant *phyA-101*, phyA overexpressor AOX, and double mutants *lid2phyA* and *lid2*AOX were tested as indicated in the text. Error bars correspond +/- S.D. Experiments were repeated at least three times.

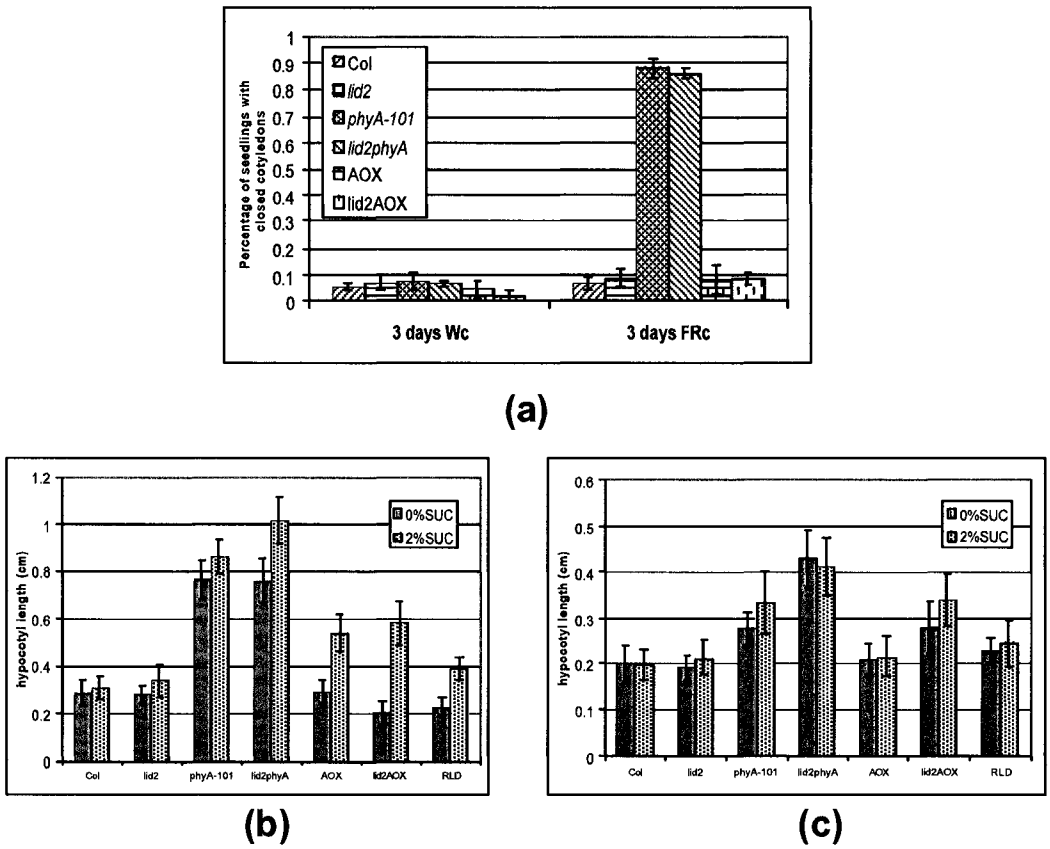


Fig 22. Some photomorphogenic responses are not altered in *lid2*. (a) Cotyledon separation. 50 seeds were sown on MS medium and exposed to 3 d Wc or 3 d FRc. The percentage of seedlings that displayed closed cotyledons was assessed as described in the text. Analysis of hypocotyl elongation after treatment with 3d FRc (b) and Rc (c). Seedlings were grown in medium with or without 2% sucrose for hypocotyl measurements, as described in the text. Error bars represent +/- S.D. Experiments were repeated at least three times.

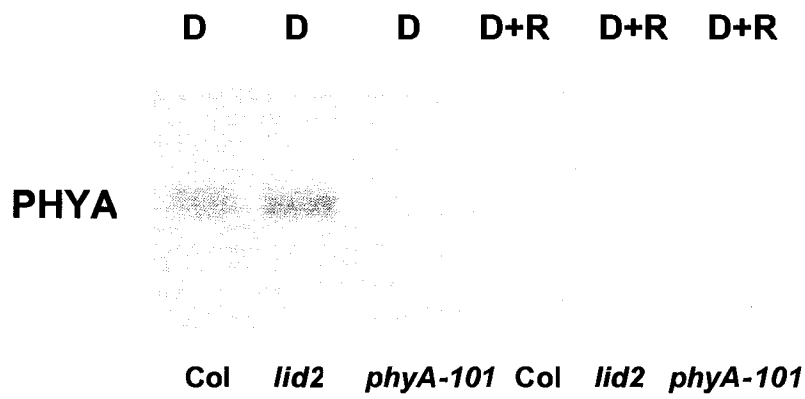


Fig 23. *lid2* has normal PHYA production and degradation.
A phyA-enriched protein fraction was extracted from 5-d-old dark (D) or 5-d-old dark followed by 3h R (D+R) grown Col-O, *lid2,* and *phyA* null mutant *phyA-101* seedlings. The immunoblots were probed with a phyA-specific antibody. An equal amount of total protein was loaded in each lane.

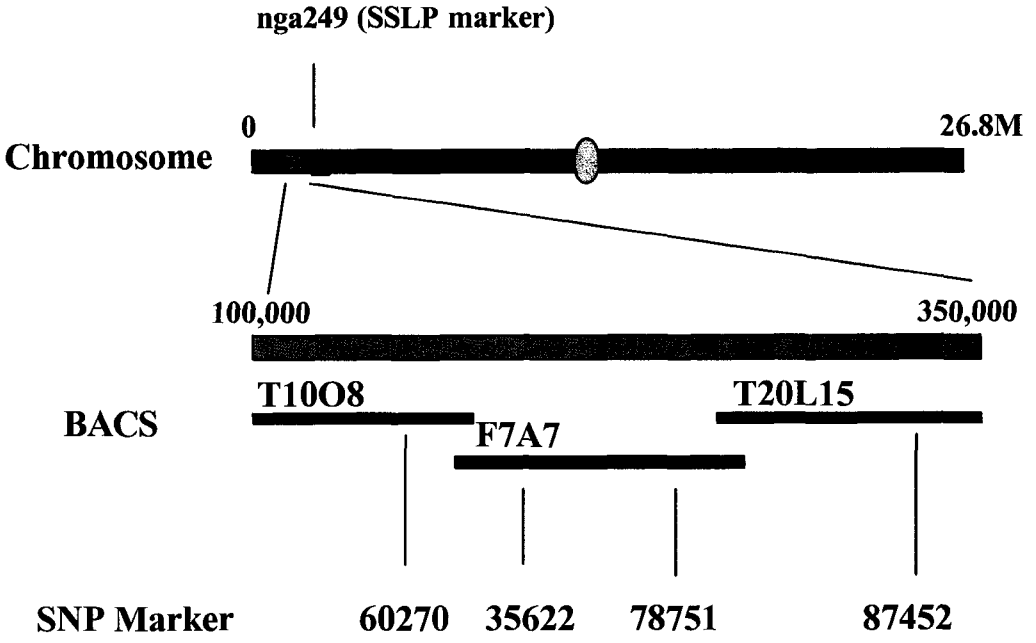
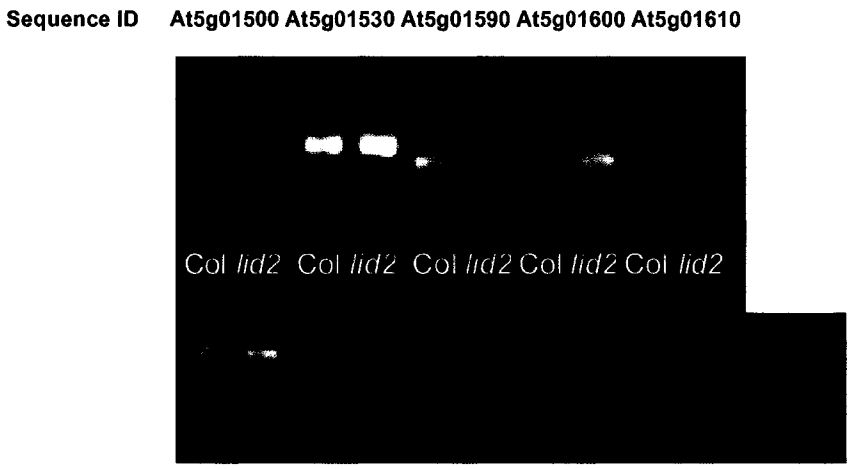
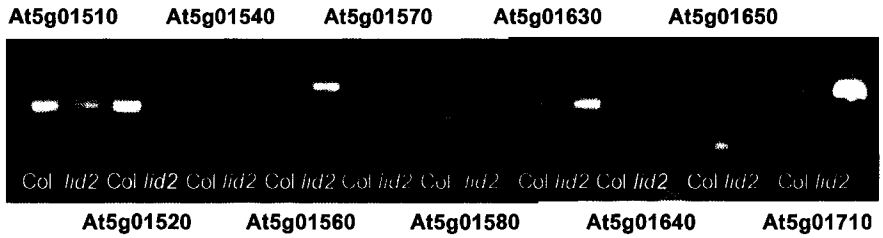


Fig 24. SSLP marker and SNP markers used in the mapping of *lid2*. The relative positions of BACs in the chromosome, the length of each BAC, and the approximate positions of each SNP marker are labeled.



(a)



(b)

Fig 25. F7A7 gene transcripts in Col-O and in *lid2*. Some genes display similar levels of transcript in Col-O and in *lid2* (a) and some genes display different levels of transcript (b). The levels of transcript were determined by semi-quantitative RT-PCR. Total RNA was isolated from 5-d-old Wc-grown seedlings.

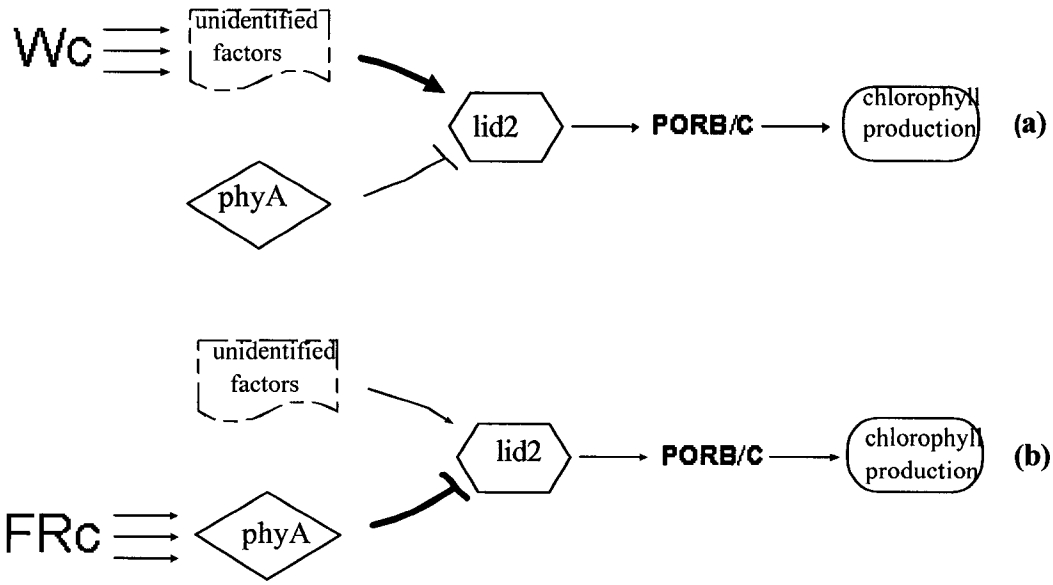


Fig 26. Working model for lid2 function. lid2 works both in the phyA pathway and in a pathway initiated by an as yet unidentified receptor. Under FRc, lid2 works predominately in the phyA pathway and under Wc, lid2 works predominately in the pathway that is initiated by the unidentified receptor.

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