1996

Molecular Genetic Analysis of Cyclic GMP Signaling in Higher Plants

Robert B. McGrath

Molecular Genetic Analysis of Cyclic GMP Signaling in Higher Plants

A thesis presented to the faculty of
The Rockefeller University
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

By
Robert B. McGrath

27 March 1996
The Rockefeller University
New York

Proverb

"Black and white are densities of more complicated grays."

-William Trevor, Excursions in the Real World

"If you get confused, listen to the music play."

-Robert Hunter and Jerry Garcia, "Franklin's Tower"

Acknowledgments

I first became interested in plants during my sophomore year in college when I read an article in Scientific American about the way light regulates gene expression in plants. Coincidentally, the article was written by Nam-Hai Chua and Phyllis Moses, a postdoctoral fellow in his lab. Given my interest in plants, it is no surprise that I chose The Rockefeller University for graduate study, because Nam's lab has been and continues to be a leader in plant research. The notion that there are two sides to every issue was shattered when I began working with Nam. It immediately became clear that there are many different ways to investigate a question, and that more incisive experiments can be designed based on the way the question is asked. I feel very fortunate to have had Nam as my advisor for the last five years because he has taught me the most important thing a teacher can impart to his or her student, the ability to think critically. This training above all others has been the most important aspect of my development as a scientist and will serve me well for the rest of my life, and I cannot thank Nam enough for that.

I also thank the members of the Chua Laboratory, past and present, for their guidance and willingness to help me. In particular, I would like to acknowledge Tim Brears, Chris Bowler, Andrew Millar, Sharla Short, Stewart Gillmor, Ueli Klahre, Raphael Mayer, and Ronaldo Quaggio for their friendship and help. By the same token, I thank Dr. Michael Young and Dr. Nat Heintz for serving on my advisory committee and guiding me during my tenure at Rockefeller. Dr. Gloria Coruzzi and Dr. Steve Kay also deserve my thanks for all of the help I received from them during my first years here.

I would like to thank Helen Shio of the Rockefeller Electron Microscopy facility for the excellent work she contributed to this project. I particularly enjoyed collaborating with Dr. Yan Wu on our investigations of sucrose signaling, and thank her for sharing her insights on such a complex question. The majority of this project would not have been possible without the generous gift of zaprinast provided by Dr. Les Bell of the Monsanto Company. Dr. Naoko Nishizawa was particularly helpful in interpreting the results of

electron microscopy experiments. I would like to thank Dr. Curt Horvath and Dr. Claude Desplan for their help in the production of figures for my thesis. I would also like to thank Dr. Joanne Chory, Dr. Joe Ecker, Dr. Garry Whitelam, Dr. Roger Hangarter, and Dr. Jeff Dangl for their very useful discussions of my data, and for communicating unpublished results from their laboratories.

I have been fortunate to have worked with Diane Shevell and Diana Horvath for the last five years, and Simon Barnes for the last two. These three people have had a significant impact on my development as a scientist and as a person. I have come to greatly value and respect their scientific opinions, and would not hesitate to ask them for help about any problem. More important, though, I have come to value their friendship, which has helped me over numerous bumps in the road, and I only hope that I have returned that friendship in kind.

No project of this undertaking can be done alone. I am lucky to have had the great support of my family during my stay here. I particularly thank them for asking me about my project. The details may have been Greek to them, but they cared enough to ask because they knew how important this was to me. My father did not live long enough to see me achieve my goal, and I dedicate this thesis to him. Finally, I could not have completed this project without the love and support of Margret Einarson. Her encouragement helped me through many difficult times, and she has helped me to see things in a whole new way.

Table of Contents

List of Illustrations

List of Tables

Abbreviations

au	Symbol for the type I phytochrome-deficient *aurea* mutant of tomato
B	Blue light
FR	Far-red light
fus/det/cop	Class of mutations termed *fusca* (also called *de-et*iolated and *co*nstitutively*p*hotomorphogenic) that is characterized by the production of large amounts of anthocyanin in the seed, and a light-grown appearance when grown in the dark.
HIR	High irradiance response
hy	Long *hy*pocotyl; a class of mutants characterized by elongated hypocotyls when grown the light. This appearance is normally characteristic of dark-grown plants.
M1	The first generation of a mutagenesis. These plants are actually treated with mutagen
M2	The second generation of a mutagenesis. These plants are the progeny of M1 plants, and are generally used for genetic screens.
M3	The third generation of a mutagenesis. These plants are the progeny of M2 plants, and may be rescreened for additional phenotypes of mutants isolated during the primary genetic screen.
P_r	The abbreviation designating the red absorbing form of phytochrome
P_{fr}	The abbreviation designating the far-red absorbing form of phytochrome
Phy	Phytochrome apoprotein
phyA or phyB	Holoprotein of phytochrome A or phytochrome B
phyA or *phyB*	The symbol of mutant plants deficient in phytochrome A or phytochrome B, or the abbreviation for the mutant phytochrome A or phytochrome B gene.
PHYA or *PHYB*	The abbreviation for the wild type phytochrome A or phytochrome B gene
$phyA_r$ or $phyB_r$	The abbreviation for the red light-absorbing form of phytochrome A or phytochrome B
$phyA_{fr}$ or $phyB_{fr}$	The abbreviation for the far-red light-absorbing form of phytochrome A or phytochrome B

R	Red light
VLFR	Very low fluence response
W	White light
WT	Wild type

Abstract

Understanding the mechanisms by which light signals are transmitted within a plant to elicit changes in physiology and gene expression is a primary goal of current research. Recently, significant insights have been gained regarding the signal transduction pathway that is regulated by the red/far-red light receptor phytochrome. Moreover, it has become clear that the phytochrome signaling pathway functions within the context of a global regulatory network that exists in plants and permits fine control of a multitude of responses to various environmental conditions. In this study, investigation of the basic mechanisms of phytochrome signal transduction, and the way this signaling pathway interacts with sugar signals was undertaken. Pharmacological studies of sucrose signal transduction in a soybean cell culture demonstrated that sucrose is a potent inducer of anthocyanin biosynthetic gene expression, and that its inductive effects are mediated by the small signaling molecule cyclic GMP (cGMP). In addition, sucrose is an inhibitor of phytochrome-regulated gene expression, but the manner in which it achieves these effects remains undefined. It was also found that the signaling pathways of sucrose and phytochrome synergistically interact to induce the expression of chalcone synthase. A novel genetic screen was developed based on a phytochrome-dependent phenotype induced in constant red and far-red light by the cGMP-dependent phosphodiesterase inhibitor zaprinast in *Arabidopsis thaliana*. Mutations at a locus designated *ZAP1* were found to confer resistance to zaprinast in both red and far-red light, and resulted in plants with elongated hypocotyls relative to wild type plants. Additional phytochrome-regulated responses were also found to be altered in *zap1* mutants. The late flowering phenotype of *zap1* mutants led to the discovery that the previously-identified *ft* mutant was resistant to zaprinast in red light. Studies of gene expression in this mutant led to the determination that mutations of the *FT* locus disrupt the synergistic integration of phytochrome and sucrose signals, causing reduced expression of anthocyanin biosynthetic genes. The results described here have strong implications for the nature of

phytochrome signaling and the way it interacts with other pathways in a global regulatory network.

Chapter 1
Introduction

The environmental stimulus that produces the most profound effects on plants is light. Light induces radical developmental changes in young plants, is instrumental in establishing the period of biological rhythms, has profound influence on developmental processes in older plants, and is the primary source of energy for the production of glucose via photosynthesis (Kendrick and Kronenberg, 1994). Plants perceive light by a group of regulatory molecules termed photoreceptors, different classes of which are responsible for detecting light of different wavelengths. Light in the UV-B range is detected by a specific receptor, whereas UV-A and blue light (B) are perceived by at least one type of receptor, termed cryptochrome (Kendrick and Kronenberg, 1994). Red (R) and far-red (FR) light are perceived by a class of photoreceptors termed the phytochromes (Quail, 1991; Furuya, 1993). Studies of all of these photoreceptors has been very intense over the last fifty years. The UV-B receptor is very poorly characterized, as was the case with cryptochrome until recently, when a mutation in the photoreceptor was identified, and it was subsequently cloned (Ahmad and Cashmore, 1993). The best characterized photoreceptors by far, though, are the phytochromes.

Phytochrome: Molecular Biology and Biochemistry

Phytochrome is comprised of a family of large proteins of approximately 120 kDa to which are attached a linear tetrapyrrole chromophore (Furuya, 1993). They have been demonstrated to be phosphoproteins, and exist as dimers *in vivo* (Hunt and Pratt, 1980). Phytochrome is synthesized as an apoprotein that attaches its chromophore, phycocyanobilin, by an autocatalytic mechanism (Furuya and Song, 1994). This physiologically-inactive form of the photoreceptor is capable of absorbing light with a peak in the R range of the visible light spectrum of 666 nm, and is termed P_r. Absorbance of R converts the protein to its physiologically-active conformation, termed

P_{fr}, which is able to absorb light with a peak absorbance in the FR range of the spectrum at 730 nm. Absorbance of FR converts the active form, P_{fr}, back to its inactive form, P_r (Furuya, 1993). Thus, phytochrome is a photoreversible molecule with an active and inactive state that is dictated by the wavelength of light that it absorbs.

Phytochrome has been determined to mediate a number of physiological responses that can be classified into three distinct groups based on the amount of light required for induction. Very low fluence responses (VLFRs) can be stimulated by light fluences totaling a mere 10^{-4}-10^{-2} μmol m^{-2}, and may be induced by both R and FR (Smith et al., 1991). Because FR can induce the response as well as R, these responses are not photoreversible. Low fluence responses (LFRs) can be induced by total light fluences of 1-1000 μmol m^{-2}, and the inductive effects of R are photoreversible by subsequent FR treatment (Smith et al., 1991). High irradiance responses (HIRs) are primarily induced in dark grown, etiolated plants by treatment with high fluence rate light for extended periods of time (Smith et al., 1991). FR is a far more potent inducer of HIRs than R, but R appears to be able to cause them under limited circumstances (Leu et al., 1995). Because of the long duration of light treatment that is required to induce them, HIRs are not photoreversible. All three types of response may be manifested at both the physiological level and at the level of gene expression.

Biochemical characterization of phytochrome indicated that a large pool of spectrophotometrically-detectable photoreceptor was present in etiolated plants that rapidly disappeared shortly after light irradiation (Furuya, 1989). Subsequently, it was found that phytochrome was present in light-grown plants, but at much lower levels than those seen in etiolated plants (Pratt et al., 1991). It was proposed and later confirmed, that two pools of phytochrome were present in plants, a light-labile pool termed type I, and a light-stable pool termed type II (Quail, 1991). Cloning of phytochrome genes from a number of species, including oat (*Avena sativa*) (Hershey et al., 1985), rice (*Oryza sativa*) (Kay et al., 1989a), and *Arabidopsis thaliana* (Sharrock and Quail, 1989; Clack et

al., 1994), indeed demonstrated that phytochrome was encoded by a multigene family. In *Arabidopsis*, five genes have been identified and termed *PHYA*, *PHYB*, *PHYC*, *PHYD*, and *PHYE* (Sharrock and Quail, 1989; Clack et al., 1994).

PHYA cDNAs were isolated from libraries constructed from RNA isolated from etiolated seedlings that contain large amounts of spectrophotometrically-detectable phytochrome. Thus, it was thought that *PHYA* would encode type I phytochrome. RNA gel blot analysis demonstrated that the *PHYA* gene was expressed at very high levels in the dark, and that expression was rapidly repressed after the onset of light treatment (Kay et al., 1989b). Nuclear run-off transcription assays demonstrated that repression of rice *PHYA* occurred directly at the level of transcription (Kay et al., 1989b), and studies of oat *PHYA* expression indicated that repression of transcription occurred within 5 minutes after light illumination commenced (Lissemore and Quail, 1988). It thus appeared that *PHYA* was an excellent candidate for type I phytochrome.

In order to understand how *PHYA* transcription is regulated, deletion analysis of the oat *PHYA* promoter was undertaken. It was found that three regions of the promoter within 1 kilobase (kb) of the transcriptional start site were required for activation of the gene (Bruce and Quail, 1990; Bruce et al., 1991). Two functionally redundant elements termed PE1 and PE2 are located distal to the transcriptional start site, whereas the third element, termed PE3, is located within 100 bp of the transcriptional start site, and is absolutely required for efficient *PHYA* transcription (Bruce et al., 1991). In addition, linker scanning mutagenesis identified a region immediately downstream of PE3 that mediates the light-dependent repression of *PHYA* transcription, and has been designated RE1. Moreover, it was found that the PE1, PE3, and RE1 regions of the promoter are protected from digestion in *in vitro* DNase I protection assays (Bruce et al., 1991). Through comparison of the rice and oat *PHYA* promoters, it was found that whereas PE3 and RE1 are common to both promoters, PE1 is not. A different sequence element that was important for transcriptional activity, termed GT-2, was identified in the rice

promoter (Dehesh et al., 1990). The DNA binding protein that recognizes this element and a similar element located in close proximity termed GT-3, was cloned and called GT-2 (Dehesh et al., 1992). It appears as though both oat and rice utilize similar promoter elements proximal to the TATA box to regulate transcription, but diverge distally. Nevertheless, both species require the presence of two positively-acting elements for efficient *PHYA* transcription (Dehesh et al., 1992).

Immunological detection of phyA in plant extracts indicated that the protein was rapidly lost following light treatment (Pratt et al., 1974). Studies of protein stability indicated that the P_r form of phyA ($phyA_r$) was stable for approximately 100 hours (Quail et al., 1973). Photoconversion of $phyA_r$ to $phyA_{fr}$ dramatically reduced the stability of the protein, as the half life of $phyA_{fr}$ was determined to be 60 minutes (Quail et al., 1973). phyA degradation is energy dependent, and $phyA_{fr}$ becomes conjugated to ubiquitin, a protein that tags proteins for rapid degradation (Pratt et al., 1974; Shanklin et al., 1987; Finley and Chau, 1991). Searches for possible ubiquitination sites on phyA identified the region of phyA between amino acids 742 and 790, that contains three lysines that could serve as ubiquitin conjugation sites (Shanklin et al., 1989). Interestingly, this region of phyA appears to become exposed on the surface of the molecule following conversion to $phyA_{fr}$ (Grimm et al., 1988). In addition, a protein motif termed the PEST sequence that has been found in numerous highly unstable proteins, has been identified in phyA in a region adjacent to the chromophore (Vierstra and Quail, 1985). At present, the precise phyA degradation mechanism has not been conclusively determined.

A third mechanism controlling phyA abundance appears to function as well. Specifically, the presence of a series of degradation products on RNA gel blots measuring *PHYA* mRNA abundance indicates that the mRNA is very unstable. It was demonstrated that these degradation products did not result from the RNA isolation procedure, and reflected *in vivo* degradation (Seeley et al., 1992). Unlike repression of *PHYA*

transcription and P_{fr} degradation, though, mRNA degradation is not enhanced by light treatment, as it is rapidly degraded both in the absence and presence of light. From this work, it was determined that the half-life of *PHYA* mRNA is approximately 60 minutes, a time substantially shorter than the observed 30 hour half lives of many soybean RNAs (Silflow and Key, 1979; Seeley et al., 1992). Clearly, a complex set of mechanisms have evolved to ensure that phyA levels can be very tightly regulated.

Genes encoding *PHYB*, *PHYC*, *PHYD*, and *PHYE* were cloned from *Arabidopsis*, and it was found that their sequences are considerably divergent from *PHYA* (Sharrock and Quail, 1989; Clack et al., 1994). Moreover, the sequences of *PHYB-E*, are divergent as well, with *PHYB* and *PHYD* being the most homologous at 80% (Clack et al., 1994). The expression patterns of *PHYB-E* were very similar, in that expression of all four genes appeared to be constitutive (Sharrock and Quail, 1989; Clack et al., 1994), and expression of *PHYB* was the highest of the four (Sharrock and Quail, 1989). There was no evidence of light repression of any of these genes. In addition, immunological detection of phyB and phyC demonstrated that the proteins are stable in the light, and that phyB is twenty fold more abundant than phyC (Somers et al., 1991). Antibodies that specifically recognize phyD and phyE have not been reported, so it is not possible to assess their relative abundances. Thus, *PHYB-E* are considered type II phytochromes (Sharrock and Quail, 1989; Clack et al., 1994). Identification of regulatory elements and factors that bind them has not been performed on any of these promoters.

With the existence of multiple forms of phytochrome within the same plant and within the same cell, it is difficult to ascribe particular physiological and molecular functions to individual species. Progress has been made, however, because of the use of transgenic plants that overexpress different phytochrome isoforms, and because of the isolation of mutants that are deficient in different phytochrome species. The effects of phytochrome mutation and overexpression on molecular and physiological aspects of plant development will be considered.

PHYA *and* PHYB *overexpression*

Cloned *PHYA* and *PHYB* genes have been overexpressed from the strong constitutive 35S promoter of cauliflower mosaic virus (CaMV) in a number of different species, including tobacco (Nagatani et al., 1991b), tomato (Boylan and Quail, 1989), and *Arabidopsis* (Boylan and Quail, 1991; Wagner et al., 1991). Overexpression of either isoform results in a dwarf phenotype of mature plants, and dramatically-shortened hypocotyls of seedlings when grown in white light (W), indicating that the plants are hypersensitive to light (Boylan and Quail, 1989; Boylan and Quail, 1991; Nagatani et al., 1991b; Wagner et al., 1991). Growth in R produces similar inhibition of hypocotyl elongation in both the *PHYA* and *PHYB* overexpressers, whereas growth in FR evokes a hypersensitive response in *PHYA* overexpressers only (Boylan and Quail, 1991; McCormac et al., 1993; Boylan et al., 1994). This indicates that phyA and not phyB can be activated by FR. Overexpression of these peptides routinely increases the amount of phytochrome by 5-15 fold (Kay et al., 1989c; Wagner et al., 1991). A study examining the effect of *PHYB* copy number demonstrates that two additional copies of the *PHYB* gene are sufficient to induce the light hypersensitivity seen in overexpressing lines (Wester et al., 1994). Clearly, the cells are profoundly sensitive to the amount of active phytochrome they contain, and respond accordingly.

Mutational analysis of phytochrome

Mutations affecting all phytochromes

Genetic screens were undertaken to isolate mutants of *Arabidopsis* that were deficient in their response to light. In these screens, mutagenized seedlings were grown in W, and plants that retained the morphology of dark-grown, etiolated seedlings were selected as mutants (Koornneef et al., 1980). Normally, light inhibits hypocotyl elongation, induces the opening of the apical hook, and stimulates cotyledon expansion and chloroplast development. The plants selected as mutants displayed the features of etiolated seedlings, possessing long hypocotyls, closed apical hooks, and unexpanded

cotyledons (Koornneef et al., 1980; Chory et al., 1989a). Three of these mutants, *hy1*, *hy2*, and *hy6* were demonstrated to have long hypocotyls in W, R, and FR. Resistance to the latter two light treatments indicate defects in phytochrome, as they are unresponsive to R growth inhibition, and have lost the FR-HIR. Moreover, spectrophotometric analysis of these mutants demonstrated that they lacked detectable phytochrome (Koornneef et al., 1980; Chory et al., 1989a). Immunochemical detection indicated that *hy1* and *hy2* contained detectable Phy apoprotein, but proteolysis assays indicated that it was not photoreversible (Parks et al., 1989). Subsequently, it was found that the mutations in *hy1*, *hy2*, and *hy6* could be rescued by phytochrome chromophore precursors, demonstrating that the mutations affected chromophore biosynthesis (Parks and Quail, 1991; Nagatani et al., 1993). Given the likelihood that all phytochromes use the same chromophore, these mutants are likely to be deficient in all phytochromes.

Mutations affecting PHYB

The initial screen for *Arabidopsis* light response mutants yielded one mutant, termed *hy3*, which possessed a long hypocotyl in white and R light, yet had a normal response to FR (Koornneef et al., 1980). Subsequently, it was found that *hy3* mutants lacked detectable phyB polypeptide, whereas they possessed normal phyA (Nagatani et al., 1991a; Somers et al., 1991). Sequencing the *PHYB* gene in these mutants demonstrated that it contained numerous lesions, confirming the identity of the *HY3* locus as *PHYB* (Reed et al., 1993). Similar mutations that affect the *l*ong *h*ypocotyl (*lh*) mutant of cucumber (*Cucumis sativa*) and the *e*longated *in*ternode (*ein*) mutant of *Brassica* also appear to affect *PHYB* (Lopez-Juez et al., 1990; Devlin et al., 1992). The *t*emporarily *r*ed light *i*nsensitive (*tri*) mutant of tomato (*Lycopersicon esculentum*) also lacks a phyB-like polypeptide, but the effects of the deficiency are confined to the first two days following the transition from dark growth to growth in R (van Tuinen et al., 1995b).

Mutations affecting PHYA

The initial screens to isolate light response mutants in *Arabidopsis* did not yield mutants in the *PHYA* gene. The fact that *phyB* mutants retained their FR HIR despite being deficient in other phytochrome-mediated responses suggested that phyA mediated the FR-HIR. Mutants that displayed the phenotype of etiolated seedlings when grown in constant FR (FR_C) were selected as potential mutants (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). Since mutations in *hy1*, *hy2*, and *hy6* also conferred FR insensitivity, secondary screens to eliminate them from the pool were devised (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). This resulted in the isolation of several *Arabidopsis phyA* mutants whose identities were confirmed by sequencing the *PHYA* gene and finding lesions, or demonstrating chromosomal rearrangements of the *PHYA* gene in radiation-induced mutants (Dehesh et al., 1993; Whitelam et al., 1993; Reed et al., 1994). In addition to the *Arabidopsis* mutants, tomato mutants that possessed elongated hypocotyls in B and low fluence rate R were isolated. Subsequently, it was found that these mutants were not responsive to FR, and were named *f*ar-*r*ed *i*nsensitive (*fri*). The *fri* mutation mapped very close to the known location of the *PHYA* gene on chromosome 10, and the mutants were shown to lack immunochemically-detectable phyA polypeptide and spectrophotometrically-detectable type I phytochrome (van Tuinen et al., 1995a). Thus, like the *Arabidopsis* mutants, tomato *fri* mutants are considered *PHYA* deficient.

A second tomato mutant termed *aurea* (*au*), possesses elongated hypocotyls in W, much like the *phyB* and *hy1* mutants of *Arabidopsis* (Koornneef et al., 1985). Unlike these mutants, though, *au* mutants were found to lack immunochemically- and spectrophotometrically-detectable phyA, while retaining detectable phyB (Parks et al., 1987; Sharma et al., 1994). The difference in light sensitivity relative to other *phyA* mutants is difficult to explain, although it has been proposed that *au* affects chromophore

biosynthesis. If this were the case, one would not expect that phyB would still be functional. Identification of the nature of the *au* mutation clearly requires further study.

Involvement of phytochrome in physiological responses

The production of plants that overexpress phyA or phyB and the identification of mutations that specifically affect each of these genes has allowed the investigation of the different physiological roles each form of phytochrome plays. As mentioned earlier, phytochromes play a major role in a number of physiological processes, including seed germination, end-of-day light responses, and flowering, as well as regulating gene expression. In addition to R and FR perception and response, these phenomena and the prospective roles of different phytochrome isoforms in them will be considered below.

Perception of red and far-red light

The initial screens for *Arabidopsis* light response mutants utilized W for selection, and the chromophore biosynthetic mutants *hy1, hy2, and hy6*, as well as the *phyB* mutant were selected . All four mutants were found to be insensitive to W and R, and the chromophore mutants were also demonstrated to be insensitive to FR (Koornneef et al., 1980; Chory et al., 1989a). As *hy1, hy2, and hy6* are deficient in all phytochromes, it was likely that a phytochrome other than phyB conferred FR light responsiveness. Isolation of *phyA* mutants based on FR sensitivity bore out this prediction (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). Measurements of sensitivity to different wavelengths of light based on the degree to which hypocotyl elongation is inhibited has proven to be a fairly accurate indicator of photoreceptor function. *phyA* mutants display very long hypocotyls when grown in FR, and have slightly longer hypocotyls than WT in R (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). Similar results have been obtained with the phyA-deficient *fri* mutant of tomato, which is insensitive to FR, and is also partially insensitive to R (van Tuinen et al., 1995a). By contrast, *phyB* mutants possess very long hypocotyls in R, yet retain WT responsiveness to FR (Koornneef et al., 1980). Thus it is possible to ascribe different light sensing functions to

phyA and phyB. By virtue of the responsiveness of *phyA* mutants to R and FR, one can conclude that phyA is the sole mediator of responses to FR, and contributes to R responsiveness to a lesser degree. That phyA is rapidly degraded and its production is inhibited in R may explain why phyA does not contribute more to R responses. Conversely, phyB appears to contribute only to R responsiveness, as it responds to FR like WT plants. Work with *phyAphyB* double mutants bears out these assertions. The double mutant shows equal insensitivity to FR as the *phyA* mutant alone, demonstrating that additional loss of phyB does not further reduce FR-responsiveness (Reed et al., 1994). Moreover, the double mutant is approximately 1.5 times longer than phyB mutants alone when grown in R, demonstrating that removal of phyA eliminates additional R-responsiveness (Reed et al., 1994).

Studies in plants that overexpress phyA or phyB support the results obtained from the mutant studies. *PHYA* overexpression yields plants that are hypersensitive to W, R, and FR (Boylan and Quail, 1991; Nagatani et al., 1991b; McCormac et al., 1993). This clearly demonstrates that the presence of more phyA in cells than normal under light-grown conditions confers additional R-responsiveness. Similarly, overexpression of *PHYB* causes plants to display hypersensitivity to W and R, yet there is no effect on FR-responsiveness (Wagner et al., 1991; McCormac et al., 1993). If *PHYB* copy number is increased from 2-4 copies per diploid genome and expression is regulated by the *PHYB* promoter, plants display similar hypersensitivity to light as overexpressing plants which increase the amount of phyB 15-fold (Wester et al., 1994). Thus, it is clear that cells are extremely sensitive to the amount of active phytochrome they contain.

The differential sensitivity of phyA and phyB to FR indicates that intrinsic differences between the molecules endow phyA with the FR-sensory capacity that phyB lacks. Domain swapping experiments in which the chromophore-bearing amino terminus of phyA was fused to the carboxy terminus of phyB demonstrate that plants that overexpress this fusion possess the same FR hypersensitivity as plants that overexpress

native phyA (unpublished data cited in Quail et al., 1995). In the reciprocal experiment, overexpression of a fusion of the chromophore-bearing amino terminus of phyB to the carboxy terminus of phyA did not confer FR hypersensitivity (unpublished data cited in Quail et al., 1995). Thus, the ability to sense FR resides in the amino terminus of phyA. This photosensory specificity may reside in the first 52 amino acids at the amino terminus, as deletion of these residues induces a dominant negative phenotype with regard to FR sensitivity when expressed *in vivo* (Boylan et al., 1994).

Germination

Numerous environmental factors have been demonstrated to affect the germination of seeds, including light and temperature. The stimulatory effect of R on the germination of *Lactuca sativa* seeds, and the reversibility of this effect by subsequent treatment with FR was one of the first phytochrome-regulated responses to be discovered (Borthwick et al., 1952). Studies of germination with *Arabidopsis* plants containing null mutations in *PHYA* and/or *PHYB* have greatly aided the identification of which phytochromes are involved in germination. It was found that the germination of wild type (WT) seeds in the dark was reduced relative to those germinated in constant R or W, where germination rates approached 100% (Johnson et al., 1994; Reed et al., 1994; Shinomura et al., 1994). In addition, FR was found to reduce the level of germination compared to seedlings grown in darkness (Reed et al., 1994; Shinomura et al., 1994). *phyA* mutants showed similar germination responses as WT when grown in darkness, W, and R, but germination in FR was totally absent (Johnson et al., 1994; Reed et al., 1994; Shinomura et al., 1994). A similar reduction in germination response to FR was seen in putative *phyA* mutants of tomato (van Tuinen et al., 1995a). Germination of *phyB* mutants was virtually abolished in darkness, yet showed normal responses to constant R and W (Reed et al., 1994; Shinomura et al., 1994). Pulses of R were ineffective in stimulating *phyB* germination (Cone and Kendrick, 1985). Interestingly, germination of

phyB mutants in FR was enhanced relative to WT (Reed et al., 1994; Shinomura et al., 1994).

The following conclusions can be drawn from these data. First, $phyB_{fr}$ is responsible for dark germination. Loss of this photoreceptor completely abolished germination in darkness. Since phyB is detectable in WT dry seed, whereas phyA is not, it is likely that conversion of $phyB_r$ to $phyB_{fr}$ in dry seed stimulates germination (Shinomura et al., 1994). Indeed, briefly treating seeds with FR reconverts all $phyB_{fr}$ back to $phyB_r$, and results in the loss of dark germination (Shinomura et al., 1994). Second, phyA is required for germination in FR. Loss of phyA by mutation completely eliminates this response. It is interesting to note that germination of *phyB* seeds in FR increases with longer periods of time in which the seeds are imbibed prior to FR treatment (Shinomura et al., 1994). This may be explained by the observation that whereas dry seeds lack phyA and its promoter is inactive, imbibition results in a very strong activation of *PHYA* transcription (Somers and Quail, 1995b). Thus, as phyA accumulates over time, the ability to germinate in FR is acquired. This is supported by the observation that tobacco seeds that overexpress phyA have the ability to germinate in FR, unlike untransformed controls (McCormac et al., 1991). Because phyA in imbibed seeds is not immunochemically-detectable though, the amount of phyA required for this response is exceedingly small (Shinomura et al., 1994). Third, in constant W or R, germination can be mediated by other photoreceptors, possible phyC, phyD, phyE, or the blue light receptor cryptochrome. This conclusion may be drawn from the observation that *phyAphyB* double mutants germinate like wild type seeds under these conditions (Reed et al., 1994). Finally, $phyB_r$ appears to antagonize phyA-mediated germination in FR. In WT, germination in FR is low compared to dark-grown or W controls. Removal of phyB by mutation results in a substantial increase in the ability to germinate in FR (Reed et al., 1994; Shinomura et al., 1994). This indicates that the $phyB_r$ produced by FR treatment represses the positive effects produced by FR activation of phyA.

End-of-day FR and shade avoidance responses

It has been proposed that the primary function of phytochrome as a growth regulator is to sense the ratio of R:FR, and induce responses in the plant that allow it to thrive under these conditions (Smith, 1994). For instance, plants growing under dense canopies frequently receive light that has been reflected from other plants which has been depleted of R wavelengths via chlorophyll absorption, rendering it rich in FR. Thus when R:FR is low, the cue transmitted via phytochrome stimulates growth so that the plant can grow taller than its competitors and obtain R-rich light (Smith, 1994). This is termed the shade avoidance response. Similarly, plants that receive high doses of FR prior to darkness tend to elongate more during the ensuing dark cycle. This phenomenon is termed the end-of-day FR response (EOD FR) (Smith, 1994). Because the effects of these light treatments are observed in light-grown plants, type II phytochrome is thought to mediate the responses. Indeed, mutations in *PHYB* block the EOD FR in *Arabidopsis* and the *ein* mutant of *Brassica* (Nagatani et al., 1991a; Devlin et al., 1992), and block the shade avoidance response of *Arabidopsis*, as assessed by hypocotyl length (Robson et al., 1993). Other markers for the shade avoidance response can be scored as well, including petiole growth and early flowering. These phenotypes are not perturbed in *Arabidopsis phyB* mutants, implicating another type II phytochrome in the response (Robson et al., 1993). *phyA* mutants display normal shade avoidance and EOD FR responses, indicating that this isoform plays no role in the response (Nagatani et al., 1993).

Flowering

The effect of phytochrome action on flowering was first observed in studies of short day plants that require long periods of darkness every day to induce flowering. Treating these plants with light in the middle of their dark cycle is equivalent to extending the period of light growth, with both treatments delaying the onset of flowering (Borthwick and Hendricks, 1960). Plants possessing phyB deficiencies like the *phyB* mutant of *Arabidopsis* have been shown to flower significantly earlier than WT (Goto et

al., 1991). Plants grown in light with a low R:FR also flower significantly earlier than those grown under a high R:FR. *phyB* mutants flower earlier under these conditions as well, demonstrating that another light-stable phytochrome is involved in this response (Robson et al., 1993). In general, *phyA* mutants flower at essentially the same time as WT, but the promotive effect of flowering by day length extensions in *Arabidopsis* is significantly reduced in *phyA* mutants (Bagnall et al., 1995). This is expected because FR is most effective at promoting flowering in this manner and a FR-HIR appears to be involved, the induction of which must be mediated by phyA.

It may be predicted that if the loss of phyB results in early flowering, then overexpression of *PHYB* should result in late flowering. This is not the case, however. Like *phyB* mutants, plants that overexpress *PHYB* flower significantly earlier than the untransformed controls. Similarly, whereas *phyA* mutations have little effect on flowering under normal conditions, plants that overexpress *PHYA* flower early as well (Bagnall et al., 1995). It is clear that regulation of flowering time is mediated by a number of factors, including light, but this process is much too complex to allow accurate prediction of the effects on flowering caused by major perturbations of plant physiology.

Regulation of Gene Expression

Phytochrome has been shown to regulate the expression of a large number of genes, in both a positive and negative manner. Genes that are positively-regulated by light include those encoding the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (*RBCS*), chlorophyll a/b binding proteins (*CAB*), and chalcone synthase (*CHS*), the enzyme that catalyzes the first committed step in anthocyanin biosynthesis (Batschauer et al., 1994). Genes that are negatively-regulated by light include asparagine synthetase (*AS1*), *PHYA*, and *PHYB* (Lam et al., 1994; Somers and Quail, 1995a). Ascribing regulatory functions to particular forms of phytochrome has again been addressed using phytochrome-deficient mutants. It has been found that the induction of *CAB* expression by red light pulses is slightly reduced in the chromophore

deficient mutant *hy1* (Chory et al., 1989a), and is normal in *phyA* and *phyB* mutants (Reed et al., 1994), demonstrating that both forms are able to mediate induction. In *phyAphyB* double mutants, however, normal induction of *CAB* is not observed. Expression is usually detectable within 4 hours, but instead, expression is not detectable until 12 hours after treatment in the double mutant, indicating that another phytochrome has taken over the function (Reed et al., 1994). In the case of *CHS*, it has been found that induction by both R and FR is absolutely dependent on the presence of phyA (Barnes et al., 1996b). *phyA* mutants display no detectable *CHS* expression in FR and considerably reduced expression in R, although B-mediated induction is normal (Barnes et al., 1996b).

Repression of *PHYA* and *PHYB* expression is also mediated by phytochrome. Use of promoter::β-glucuronidase (*GUS*) fusions in mutant backgrounds has demonstrated that repression of *PHYA* expression can be mediated by either phyA or phyB when treated with W (Somers and Quail, 1995a). This is important because it allows proper repression of *PHYA* expression under all conditions in which phytochrome is active. *PHYB* repression, however, is mediated almost solely by phyB itself under the conditions tested (Somers and Quail, 1995a). It remains to be determined whether phyA can mediate repression under growth in FR. Because the intricacies of gene regulation clearly extend to the level of individual photoreceptors, no blanket statements can be made with regard to which isoform modulates gene expression. Rather, differential regulation will have to be assessed on a case by case basis.

Genetic Analysis of Phytochrome Signaling

The earliest genetic screens have proven fruitful in producing mutations that directly affect the photoreceptors themselves (Koornneef et al., 1980). Despite cloning the genes encoding the photoreceptors, little insight has been gained regarding the steps through which a phytochrome-mediated signal is transduced. The phytochrome-mediated effects of light on plant development, morphology, physiology, and gene expression have been used as the basis for a large number of genetic screens designed to identify

components of the phytochrome signaling pathway. Recent efforts to identify signaling components by genetic means have focused on the isolation of gain-of-function and loss-of-function mutants.

Gain-of-function mutants

Isolation of loss-of-function phytochrome mutants was achieved by selecting mutant plants that retained an etiolated, dark-grown phenotype despite being grown in the light (Koornneef et al., 1980). It was therefore reasoned that gain-of-function mutants could be isolated by selecting mutants possessing a light-grown morphology despite growth in the dark. A large number of mutations isolated in this manner have been found to be of the *fusca* (*fus*) class of mutants, which were originally isolated based on accumulation of abnormally high amounts of anthocyanin in the seed coat (Castle and Meinke, 1994; Misera et al., 1994). These mutations have also been designated *de-et*iolated (*det*) and *co*nstitutively *p*hotomorphogenic (*cop*) (Chory et al., 1989b; Deng et al., 1991). Thus, *cop1* is allelic to *fus1*, *det1* to *fus2*, *cop8* to *fus8*, *cop9* to *fus7*, *cop10* to *fus9*, and *cop11* to *fus6* (Misera et al., 1994). All of these mutants possess short, thick hypocotyls, open, expanded cotyledons, and no apical hook when grown in the dark (Chory et al., 1989b; Deng et al., 1991; Wei and Deng, 1992; Castle and Meinke, 1994; Wei et al., 1994). This morphology is very similar to that of light-grown plants. In addition, the plants produce large amounts of anthocyanin in the dark, a phenomenon that usually requires light stimulation. Examination of gene expression demonstrated that *fus* plants express the light-inducible genes *CAB*, *RBCS*, and a number of chloroplast-encoded genes at very high levels in the dark (Chory et al., 1989b; Deng et al., 1991; Wei and Deng, 1992; Wei et al., 1994). The *CHS* gene was expressed in the wrong cell types at exceedingly high levels, reflecting the large amount of anthocyanin that accumulates in mutant plants (Chory and Peto, 1990). In *det1* plants, despite aberrant gene expression in the dark, *CAB* and *RBCS* are shut off in a normal manner when light-grown plants are returned to darkness (Chory et al., 1989b). In these *fus* mutants, chloroplast maturation

begins in the dark (Chory et al., 1989b; Deng et al., 1991; Wei and Deng, 1992; Wei et al., 1994), and plastids abnormally develop into chloroplasts in the roots of *det1* and *cop1* plants, rather than starch-containing amyloplasts (Chory et al., 1989b; Deng and Quail, 1992a). Germination is light-independent in the *det1* mutant, whereas it is regulated normally by phytochrome in the other *fus* mutants tested (Chory et al., 1989b; Deng et al., 1991; Wei and Deng, 1992; Wei et al., 1994). Unlike WT plants, *fus* mutants develop true leaves during extended growth in the dark, and produce pale green, dwarf plants when grown in the light (Chory et al., 1989b; Deng et al., 1991; Wei and Deng, 1992; Wei et al., 1994). Weak alleles of *det1* and *cop1* have been isolated and are fertile, but all *cop8*, *cop9*, *cop10* and *fus6* alleles are lethal (Misera et al., 1994). Clearly, these mutants display very pleiotropic phenotypes, seemingly causing the constitutive activation of a number of light-dependent processes.

The wild type DET1 protein was proposed to act as a negative regulator of photomorphogenesis, as the recessive nature of the mutation indicates that photomorphogenesis can proceed in the dark only in the absence of DET1 (Chory et al., 1989b). Cloning the *DET1* gene did not reveal significant insights into the mechanism by which DET1 functions. *DET1* is expressed at very low levels, and is not regulated by light. In addition, no significant homology to other proteins was found, although DET1 appears to contain nuclear localization signals (Pepper et al., 1994). DET1-GUS fusion proteins expressed in transfected *Arabidopsis* protoplasts did, indeed, localize to the nucleus, supporting the proposition that DET1 is a nuclear protein (Pepper et al., 1994). Nevertheless, how DET1 functions to repress photomorphogenesis is not known.

The recessive nature of the *cop1* mutation also placed it in the same proposed negative regulator class as *DET1* (Deng et al., 1991). Cloning of the *COP1* gene yielded some possible insights into how the WT protein functions. WT COP1 contains a proposed Zn^{2+} -binding domain of the ring finger class, putative coiled-coil forming domains, and WD-40 repeats reminiscent of the β-subunit of the heterotrimeric GTP-

binding protein (G protein) transducin (Deng et al., 1992b). It has been proposed that the WD-40 repeats of COP1 allow it to interact with signal transduction pathway components and migrate to the nucleus to interact with DNA (Deng et al., 1992b). It has been found that COP1 bears significant homology to $TAF_{II}80$, a *Drosophila* protein that is a component of the general transcriptional activator TFIID (Dynlacht et al., 1993). In addition, the WD-40 repeats of such global transcriptional repressors as SSN6 and TUP1 have been implicated in the formation of repressive chromatin structures (Cooper et al., 1994). It is possible that the negative regulatory function of COP1 may be achieved in a similar manner. It has recently been shown that COP1-GUS fusion proteins are present in the nucleus in the dark, and cytoplasm in the light, consistent with the notion that COP1 regulates photomorphogenesis by repressing transcription in the dark. Similarly, it has been shown that light can stimulate the relocation of COP1 from the nucleus to the cytoplasm (von Arnim and Deng, 1994). It should be noted, however, that dark-grown plants of the same age as those that are assayed for COP1 localization after being shifted to the light, were not tested as a control. It is therefore possible that COP1-GUS migrates to the cytoplasm independently of light. Nevertheless, like DET1, COP1 likely plays a role in regulating a number of light-dependent processes.

Cloning of *COP9* and *FUS6* gave little indications of their mode of action *in vivo*. FUS6, though, displays homology to the human GPS1 protein, which is able to suppress a yeast *gpa* mutation that affects an α subunit of a heterotrimeric G protein involved in mating pheromone signaling (Castle and Meinke, 1994). Antibodies produced against COP9 have been used to immunoprecipitate a large protein complex that is greater than 500 kDa (Wei et al., 1994). This complex elutes as a larger complex with a broad elution profile from dark-grown plants, and elutes at a smaller size with a narrow elution profile from light-grown plants. Moreover, studies of *cop8* and *fus6* demonstrate that the complex is not detectable in these mutants, indicating that in WT plants, COP8 and FUS6 are part of this complex or are required for its formation or stability (Wei et al., 1994). It

is quite intriguing to propose that this large complex is an important light-dependent regulator of photomorphogenesis.

The genes defined by these *fus/det/cop* mutations have been proposed to be negative regulators of light responses. The major weakness of the studies of these mutants is that specificity for phytochrome and light signaling has not been demonstrated, as the possible activation of other pathways has not been investigated. Recent results demonstrate that a number of pathways are derepressed in some of these mutants (Dr. Raphael Mayer, The Rockefeller University, personal communication). It has been found that in *cop1* and *cop9* mutants, defense-related genes and genes regulated by hypoxia are aberrantly induced, with higher expression observed in *cop9* plants. By contrast, only defense-related genes are overexpressed in *det1* plants. It is clear that the effect of mutations in *fus/det/cop* genes is not confined to phytochrome signaling. Given the fact that high levels of hormones like cytokinin can induce de-etiolation like *fus/det/cop* mutations, it is possible that the phenotypes seen in these mutants are not specific to light (Chory et al., 1994). At this point, it is impossible to distinguish the effects of derepressing one pathway from another.

Another gain-of-function mutation that is not of the *fus/det/cop* class is the *Arabidopsis* mutant *det2*. This mutation is very similar to *det1* in that it possesses a short hypocotyl, no apical hook, and open, expanded cotyledons when grown in the dark (Chory et al., 1991). Like *det1*, it accumulates anthocyanin in the dark, but *CHS* expression is not as high. In addition, the derepression of light-inducible genes in the dark is significantly less than that of *det1*. There is no chloroplast development in the dark in *det2* plants, and although leaves are initiated during dark growth, they arrest, and no more are produced (Chory et al., 1991). Light-grown plants are dwarves compared to WT, but in *det2* plants, leaf senescence is delayed and repression of light-inducible genes is delayed upon dark adaptation (Chory et al., 1991). Cloning of *DET2* provided significant insights into the function of the WT protein. Homology to animal steroid

biosynthetic enzymes indicated that DET2 is necessary for the synthesis of plant steroid hormones termed brassinosteroids. Indeed, growth of *det2* mutants on brassinosteroids completely rescued the mutant phenotype (Li et al., 1996). Thus, whereas *det2* does not define a light signaling intermediate, it clearly demonstrates that brassinosteroids are an important part of the regulatory network governing light-regulated development (see below).

The *cop4* mutant of *Arabidopsis* was isolated based on its lack of an apical hook and open, expanded cotyledons as a dark-grown plant, while retaining an elongated hypocotyl (Hou et al., 1993). *cop4* mutants display cellular differentiation in their cotyledons that is more like light-grown plants than those grown in the dark, yet plastids remain undifferentiated. In addition, a number of light-inducible genes are expressed in the dark in *cop4* plants, and promoter::GUS fusion experiments indicate that the misregulation of *CAB* expression occurs between -1281 and -250 of the promoter. *cop4* mutants also display aberrant shoot gravitropic responses, a known phytochrome-regulated response (Hou et al., 1993). It is possible that the *cop4* mutation may identify a component in the light signaling pathway.

Recently, transgenic screening approaches to identify mutations that primarily affect gene expression have been undertaken. Using plants that contain the *CAB3* promoter fused to a hygromycin resistance gene has permitted the isolation of mutants that survive when grown on hygromycin in the dark. Under normal conditions, the *CAB3* promoter is inactive in the dark and the seedlings are sensitive to hygromycin. In the *d*ark *o*verexpression of *c*ab 1,2, and 3 (*doc1, doc2, doc3*) mutants of *Arabidopsis*, however, expression of the resistance gene from the *CAB3* promoter in the dark allows the plants to survive (Li et al., 1994). Overactivity of the transgene reflects higher levels of expression of endogenous *CAB* in the dark, with higher levels seen in *doc1* than *doc2* and *doc3*. The levels of *CAB* expression in the light are equivalent to WT, so the effect of the mutation is confined to the dark. The *doc* mutants look like WT, except *doc1* is

shorter in stature (Li et al., 1994). These mutations may affect the repression mechanism that shuts off *CAB* expression in the dark, whereas the *CAB* induction mechanism functions normally. Thus, it is possible to genetically-separate the mechanism of light dependent gene induction and repression.

The *l*ight *i*ndependent *p*hotomorphogenesis (*lip*) mutation of pea (*Pisum sativum*) appears to confer a *det*-like phenotype. This mutant possesses a short epictoyl, an open apical hook, and enlarged apical bud when grown in the dark (Frances et al., 1992). Dark-grown *lip* plants undergo some cellular differentiation, and chloroplasts partially develop. In addition, light-inducible genes are aberrantly expressed in the dark, and induction of these genes by light is greater in *lip* mutants, though the kinetics of gene expression are like WT. Dark-grown plants contain less spectrally and immunochemically detectable phytochrome, indicating that phytochrome expression may be repressed in the dark. Finally, adult light grown plants develop as dwarves (Frances et al., 1992). These mutants are similar to *fus/det/cop* mutants, yet lack noticeable anthocyanin accumulation and are not lethal. This may reflect a different type of mutation, or *lip* may simply be leaky.

The *h*igh *p*igment (*hp*) mutant of tomato is notable for its dark green plants and fruits, and its shorter stem heights when grown in monochromatic light (von Wettstein-Knowles, 1968). It gets its name from the fact that it produces copious amounts of anthocyanin in red light. Double mutant studies with the phyA-deficient *aurea* mutant demonstrate that penetrance of *hp* absolutely requires type I phytochrome (Adamse et al., 1989). Experiments have demonstrated that *hp* mutants contain normal amounts of phytochrome and are not hypersensitive to P_{fr} levels (Peters et al., 1992). Thus, it appears that the *hp* mutation affects the amplification of phytochrome signals. As it is recessive, it is likely that HP acts to antagonize the step at which the phytochrome signal is amplified to produce its response. For example, one photon of light activates one phytochrome molecule which interacts with multiple secondary signaling intermediates.

These intermediates interact in turn with multiple downstream effectors. This effectively amplifies the initial light signal. HP may act to rein in this step to maintain a measured light response.

Like the *doc* mutants, the *i*ncreased *c*halcone synthase e*x*pression 1 (*icx1*) mutant of *Arabidopsis* was isolated based on a transgenic screening approach. This mutant was selected based on higher levels of *CHS*::*GUS* expression in dim light than WT (Jackson et al., 1995). The mutant displays similar increased expression of the endogenous *CHS* gene, and other anthocyanin biosynthetic genes are overexpressed as well. In addition, *icx1* mutants accumulate twice as much anthocyanin as WT, and display an aberrant distribution of trichomes on the surface of leaves (Jackson et al., 1995). The similarity of *icx1* to *hp* with regard to anthocyanin accumulation suggests that the *Arabidopsis* mutant may be *hp*-like. More work needs to be done with *icx1* to determine which photoreceptor system is affected in this mutant.

The mutants described above all produce a marked effect on the expression of light-inducible genes. Other putative light mutants have been isolated whose primary effect is on morphology. Two of these *Arabidopsis* mutants have been termed *det3* and *diminuto* (*dim*). Both mutations cause plants to adopt a de-etiolated phenotype when grown in the dark (Cabrera y Poch et al., 1993; Takahashi et al., 1995). Moreover, *det3* mutants develop like light-grown plants in the dark, producing rosette leaves and eventually flowering (Cabrera y Poch et al., 1993). Neither *dim* nor *det3* exhibit any chloroplast development in the dark, light-inducible genes are not expressed, and light-grown plants of both mutants are extremely reduced in stature (Cabrera y Poch et al., 1993; Takahashi et al., 1995). Cloning of *DIM* did not give any indication of its mode of action, as it bears no significant homology to other known proteins, although it is predicted to possess an FAD-binding domain (Takahashi et al., 1995). Recently, it has been shown that like *det2*, *dim* can be complemented by brassinosteroids (Dr. Nam-Hai Chua, The Rockefeller University, personal communication). It is clear, though, that it is

possible for a plant to develop with light-grown morphology without altering the normal regulation of light-inducible gene expression.

The *cop2*, *cop3*, and *a*ltered *m*eristematic *p*rogram 1 (*amp1*) mutants of *Arabidopsis* are similar to *det3* and *dim* in that they partially develop as light-grown plants in the dark, without affecting light-inducible gene expression (Chaudhury et al., 1993; Hou et al., 1993). These three mutants have no apical hooks in the dark, and develop open, expanded cotyledons, yet retain elongated hypocotyls. Both *amp1* and *cop2* also develop true leaves in the dark (Chaudhury et al., 1993; Hou et al., 1993). *amp1* mutants flower significantly earlier than WT, and have accelerated vegetative growth, producing more leaves in a shorter period of time than WT. Because *amp1* mutants contain six-fold higher levels of cytokinin than WT, the effects of this mutation may not be restricted to light signaling (Chaudhury et al., 1993). The *cop3* mutant has been found to express ethylene-inducible genes at a lower level then WT (R. Mayer, personal communication). This is consistent with the fact that it is allelic to *hookless* (*hls*), a mutation identified in a screen for ethylene mutants that do not produce apical hooks in the dark. Cloning of *hls* indicates that the protein bears homology to acetylases, and recent data have indicated that HLS is necessary for restricting the spatial distribution of the auxin indole-3 acetic acid (IAA) in the region of the apical hook (Dr. Joseph Ecker, University of Pennsylvania, personal communication). Thus, *hls/cop3* mutants may have little direct involvement in light-dependent signaling.

Loss-of-function mutants

Compared to the wealth of putative gain-of-function mutants that have been isolated, relatively few loss-of-function mutants aside from the photoreceptors have been identified. In the initial screen for *Arabidopsis* light response mutants, only one mutation, *hy5*, did not define a photoreceptor. The long hypocotyl phenotype in W, R, FR, and B indicates that *hy5* mutants are insensitive to these wavelengths of light, while retaining sensitivity to UV-B (Koornneef et al., 1980). *hy5* mutants behave like WT with

regard to germination, chlorophyll production, and flowering time, but produce less anthocyanin in the light than WT (Chory, 1992). Some genetic studies have placed *hy5* in pathways parallel to light signaling, and others indicate functional interactions with *cop1* (Chory, 1992; Ang and Deng, 1994). To date, however, no definitive role in any signal transduction pathway has been confirmed for HY5.

The *f*ar-red elongated *hy*pocotyl 1 and 3 (*fhy1* and *fhy3*) mutants of *Arabidopsis* have been isolated based on their etiolated appearance when grown in constant FR (Whitelam et al., 1993). Both of these mutants develop normally under other light conditions, and they have been determined to be non-allelic to *phyA* mutants. Thus, they appear to define phyA- and FR-specific signaling intermediates. *fhy1* plants have been shown to be deficient in FR-mediated induction of *CHS* and other anthocyanin biosynthetic genes. As these genes are regulated by a cGMP-dependent branch of the phytochrome signaling pathway (see below), *fhy1* may specifically disrupt this branch (Barnes et al., 1996b). *phyA* mutants are defective in FR-mediated germination, but *fhy1* mutants behave like WT (Johnson et al., 1994). Thus, *fhy1* appears to be involved in phyA signaling, but is not involved in all phyA-dependent responses. *fhy3* has not been as well-characterized, but its responses to growth in monochromatic light indicates that it is hypersensitive to R (Whitelam et al., 1993). In addition, *fhy3* flowers earlier than WT (Bagnall et al., 1995). More work with this mutant is required to assign it a particular role in phyA signaling.

The last group of loss-of-function mutants were isolated based on a transgenic phenotype in *Arabidopsis*. Expression of *CAB3*::alcohol dehydrogenase (*ADH*) fusions in the light results in the death of the plant when grown on allyl alcohol. *CAB* *u*ndere*x*pressed 1 (*cue1*) mutants survive this treatment because *CAB* expression is significantly reduced under all light wavelengths tested, including R, FR, and B (Li et al., 1995). The mutant does not green in the interveinal regions, as *CAB* and *RBCS* expression is reduced in mesophyll cells only. This results in a plant with a reticulated

appearance. *CAB* is expressed normally in other regions like paraveinal tissue. *cue1*, therefore, represents a mutation that affects light-dependent gene expression in mesophyll cells only (Li et al., 1995). It is reported that many other *cue* mutants have been isolated, and it is possible that some of them may define specific steps of the phytochrome signaling pathway (Li et al., 1995; Dr. Joanne Chory, Salk Institute, personal communication).

The purpose of isolating both gain-of-function and loss-of-function mutants in the same signaling pathway is to be able to utilize epistasis testing to order the activity of each gene product in a linear pathway. Epistasis testing was used with the mutants described above, and pathways were proposed based on their epistatic relationships (Chory, 1992). It should be noted, however, that the epistatic relationships are not necessarily valid. For instance, it was found that all of the *fus/det/cop* mutants were epistatic to mutations in phytochrome and the blue light receptor when grown in both the dark and the light (Chory, 1992). To demonstrate epistasis, the phenotype of one mutant must mask that of the other. In dark-grown plants, photoreceptor mutants do not possess a phenotype different from wild type, whereas *fus/det/cop* mutants do. *fus/det/cop* mutants cannot be considered epistatic to the photoreceptors in dark-grown plants, because there is no specific photoreceptor-deficient phenotype in the dark to mask. In light-grown plants, the *fus/det/cop* mutant phenotype is clearly epistatic to the phenotype of photoreceptor mutations. Nevertheless, the demonstration that *fus/det/cop* mutants display derepression of a number of different signaling pathways (R. Mayer, personal communication), indicates that activation of a parallel pathway may be able to bypass photoreceptor deficiencies, and makes the claim of epistasis less valid. It must be demonstrated that the phenotypes of *fus/det/cop* mutants result solely from derepression of light signaling before epistasis can be claimed. From the studies described above, a role in the phytochrome signaling pathway that has been partially defined by biochemical analysis (see below) can only be tentatively ascribed to FHY1.

Biochemical Analysis of Phytochrome Signaling

Like all receptors, phytochrome initiates a signal transduction pathway that ultimately results in physiological processes like seed and spore germination, leaf unrolling, protoplast swelling, and the activation and repression of specific R-responsive genes. Until recently, identification of signaling molecules that convey phytochrome signals has proven difficult, with only a few specific molecules being implicated to function in this pathway. In the last few years, a number of putative signaling molecules have been identified by biochemical means. In this section, the known components of the phytochrome signaling pathway will be described.

GTP binding proteins

Pharmacological studies of the protoplast swelling response to R first implicated G proteins as a component of the phytochrome signaling pathway. Protoplasts that had been electroporated to induce uptake of GTP-γ-S, a non-hydrolyzable analogue of GTP and an activator of G proteins, swelled in darkness. Conversely, cells electroporated with GDP-β-S, a GDP analogue that inhibits G proteins, were incapable of swelling in response to R (Bossen et al., 1990). Subsequently, it was found that binding of GTP-γ-S in crude extracts of etiolated oat seedlings was enhanced by R (Romero et al., 1991). In addition, treating etiolated seedlings with cholera toxin, a compound that ADP-ribosylates heterotrimeric G proteins and activates them, induced *CAB* gene expression, and inhibited *PHYA* gene expression (Romero et al., 1991). These data indicate that by activating G proteins, it is possible to stimulate a red light response in the absence of light. Further, these data indicated that the G proteins involved were heterotrimeric G proteins, and not small G proteins such as RAS.

These observations were confirmed and extended in microinjection experiments in the phyA-deficient *aurea* mutant of tomato. Microinjection of purified oat phyA into individual *au* hypocotyl cells, followed by R treatment, stimulated three light-regulated processes, chloroplast development, anthocyanin production, and activation of the

expression of a *GUS* reporter gene fused to a wheat *CAB* promoter (Neuhaus et al., 1993). These phenomena were seen in the injected cells only, indicating that the phytochrome response is a cell-autonomous event. Subsequently, cells injected with GTP-γ-S in the absence of phyA were able to respond in an identical manner as those injected with purified phyA and treated with red light. Similarly, cells injected with cholera toxin alone were capable of inducing all three responses (Neuhaus et al., 1993). This effect was enhanced by co-injection with low concentrations of GTP-γ-S. In a related experiment, cells into which purified phyA and GDP-β-S were injected and subsequently treated with R were unable to respond like those cells injected with phyA alone (e.g. no chloroplasts developed, anthocyanin was not produced, and the *CAB*::*GUS* fusion was not induced; Neuhaus et al., 1993). Subsequent experiments in a photomixotrophic cell culture supported these observations (Romero and Lam, 1993). All of these data strongly indicate that heterotrimeric G proteins are involved in the phytochrome-regulated signal transduction cascade.

Heterotrimeric G proteins are known to act very early in mammalian signal transduction pathways, stimulating other molecules that cause the production of new secondary signaling molecules (Simon et al., 1991). It is therefore of great interest to identify signaling molecules that act downstream of phytochrome and its associated G proteins.

Calcium

The possibility that calcium ions (Ca^{2+}) played a role in the phytochrome signal transduction pathway was first postulated based on observations that calcium played important roles in mammalian signal transduction, and that phytochrome appeared to control ion fluxes across the plasma membrane (Roux and Yguerabide, 1973; Haupt and Weisenseel, 1976). This proposal was expanded by the suggestion that the P_r to P_{fr} conversion induced by R caused an increase in cytosolic levels of calcium which, in turn, had direct effects, or acted through calcium binding proteins to activate enzymes and alter

cellular metabolism (Roux, 1984). A large number of examples in the years that followed appear to support this model, at least in general terms.

Fern spore germination, leaf unrolling, and protoplast swelling

It has been shown that fern spore germination is controlled by phytochrome (Wayne and Hepler, 1984). It was found subsequently that spores did not germinate if Ca^{2+} was not supplied in the medium. Moreover, inhibitors of Ca^{2+} uptake like La^{3+} and Co^{2+} also blocked germination, but were only effective if they were present in the medium before R treatment (Wayne and Hepler, 1984). Addition after R treatment had no effect, indicating that the Ca^{2+} requirement is transient. Treatment of spores with a Ca^{2+} ionophore in the dark also stimulated germination, demonstrating that the response pathway is poised to respond, and that control of Ca^{2+} uptake is the key regulatory step in the germination response (Wayne and Hepler, 1984). Atomic absorption studies have demonstrated that spores contain more Ca^{2+} after R treatment, consistent with the model that phytochrome regulates Ca^{2+} uptake (Wayne and Hepler, 1985). Inhibition of the Ca^{2+}-binding protein calmodulin (CaM) also blocked germination, indicating that CaM may transmit Ca^{2+} signals to downstream targets (Wayne and Hepler, 1984).

Two other phenomena have also been shown to be regulated by phytochrome in a Ca^{2+}-dependent manner, leaf unrolling in monocots and protoplast swelling (Bossen et al., 1988; Viner et al., 1988). It is thought that the two phenomena are related to the same process, as a correlation between the two has been established (Zhou et al., 1990). Both leaf unrolling and protoplast swelling are induced by R, but Ca^{2+} must be supplied in the medium for the response to occur (Bossen et al., 1988; Viner et al., 1988). Incubation with inhibitors of slow, "L-type" voltage gated Ca^{2+} channels like verapamil and the dihydropyridine nifedipine specifically inhibited the R-mediated responses (Tretyn et al., 1990). As with the case of spore germination, leaf unrolling and protoplast swelling could both be induced by treatment with Ca^{2+} ionophores in the absence of R (Viner et al., 1988; Tretyn et al., 1990). Fluorescence imaging of wheat leaf protoplasts

demonstrated that Ca^{2+} levels indeed rise after R treatment, but that the increase is transient, returning to normal levels within one minute after irradiation (Shacklock et al., 1992). In addition, the transient Ca^{2+} increase could be inhibited by subsequent FR treatment. In the wheat experiments, protoplasts swelled soon after the Ca^{2+} transient was observed.

Ultimately, it was shown that release of Ca^{2+} from caged molecules in protoplasts caused an increase in cytoplasmic Ca^{2+} levels followed shortly thereafter by protoplast swelling (Shacklock et al., 1992). These data conclusively demonstrate that Ca^{2+} is a mediator of red light-induced protoplast swelling. Protoplasts, however, are not capable of activating light-regulated genes in response to R. One cannot conclude, therefore, that the swelling effects seen in protoplasts are induced by the same signal transduction pathway that is responsible for stimulating the transcription of genes in response to light.

Gene expression

Studies performed in a photomixotrophic soybean cell line and microinjection in intact plants have allowed examination of the role played by Ca^{2+} in the induction of R-responsive genes. Experiments performed with inhibitors of CaM action, have demonstrated that CaM is involved in the induction of *CAB* gene expression in soybean cells in response to light (Lam et al., 1989; Bowler et al., 1994b). Treating dark-adapted cells with the Ca^{2+} ionophore ionomycin slightly induced *CAB* expression, indicating that Ca^{2+} is capable of inducing gene expression in the absence of light (Lam et al., 1989).

Microinjection experiments have conclusively demonstrated that Ca^{2+} is capable of inducing the expression of some R-inducible genes (Neuhaus et al., 1993). Microinjection of Ca^{2+} into *au* cells to a concentration of 500 nM is sufficient to induce expression of a *CAB*::*GUS* reporter gene construct and stimulate partial chloroplast maturation independently of phyA and R. This concentration of Ca^{2+} did not induce anthocyanin production, however (Neuhaus et al., 1993). The observed minimum concentration of Ca^{2+} required to induce these processes is in close agreement with the

levels of Ca^{2+} observed in wheat leaf protoplasts following R irradiation (Shacklock et al., 1992). It was also found that inhibitors of Ca^{2+} and CaM action could block the induction of *CAB*::*GUS* and endogenous *CAB* expression, and chloroplast maturation that was stimulated by G protein agonists in both cell culture and microinjection experiments (Neuhaus et al., 1993; Bowler et al., 1994b). Injection of activated CaM with a threshold concentration of 5000 molecules was also sufficient to induce expression of *CAB*::*GUS*, and to promote partial chloroplast maturation (Neuhaus et al., 1993). From these data, it is clear that Ca^{2+}/CaM plays a very important role in the phytochrome signal transduction pathway, but some other signaling molecule must be required to stimulate anthocyanin accumulation.

Cyclic GMP

In microinjection experiments in mutant *au* cells, it was found that whereas injecting Ca^{2+}/CaM stimulated partial chloroplast differentiation and *CAB*::*GUS* expression, it did not induce anthocyanin biosynthesis (Neuhaus et al., 1993). Subsequent experiments demonstrated that injection of cyclic GMP (cGMP) induced anthocyanin biosynthesis (Bowler et al., 1994a). It was also found that co-injection of cGMP with *CHS*::*GUS* reporter fusions, resulted in *GUS* expression. Moreover, cGMP did not stimulate *CAB*::*GUS* expression (Bowler et al., 1994a). Similar results were obtained in a light-responsive soybean cell culture. Incubation of cells with a membrane-permeable cGMP analogue, 8-Br-cGMP, in the dark, resulted in induction of endogenous *CHS* expression, whereas the endogenous *CAB* genes remained uninduced (Bowler et al., 1994a). Co-injection of Rp-cGMPS, a specific cGMP inhibitor, into *au* along with purified phyA selectively blocked R-induced anthocyanin production and *CHS*::*GUS* induction, whereas induction of *CAB*::*GUS* was unaffected, and chloroplasts partially developed (Bowler et al., 1994a). Production of anthocyanin stimulated by GTP-γ-S was also inhibited by Rp-cGMPS, indicating that cGMP acts downstream of the heterotrimeric G protein(s) (Bowler et al., 1994a). These experiments demonstrated that

cGMP functions as a component of the phytochrome signaling pathway, and that cGMP and Ca^{2+} regulate separate responses.

Immunofluorescence studies of the immature chloroplasts whose development was induced by injection of Ca^{2+}/CaM indicated that they only contained the components of photosystem II (PS II), light harvesting complexes (LHC) I and II, and ATP synthase (Neuhaus et al., 1993). They completely lacked the components of photosystem I (PS I), and cytochrome b_6f. Microinjection of cGMP could not stimulate any chloroplast maturation, yet when cGMP and Ca^{2+}/CaM were co-injected, the chloroplasts developed fully (Bowler et al., 1994a). Moreover, the amount of cGMP required to activate PS I and cytochrome b_6f genes was ten-fold lower than that required to activate the anthocyanin biosynthetic genes (Bowler et al., 1994a). These data indicate that whereas the Ca^{2+}/CaM and cGMP branches of the pathway regulate separate responses, they also function together to control the production of PS I and cytochrome b_6f. These results were supported by the observation that a promoter::*GUS* fusion of the ferredoxin NADP oxidoreductase (*FNR*) gene, whose product is a component of PS I, was only induced when both Ca^{2+}/CaM and cGMP were co-injected (Bowler et al., 1994a).

Phosphorylation

The discovery that purified oat phytochrome contained an associated protein kinase activity raised the possibility that phytochrome initiated its signal transduction chain by phosphorylation (Wong et al., 1986). Other studies demonstrated, however, that the associated kinase activity could be separated from phytochrome upon further purification (Grimm et al., 1989; Kim et al., 1989). The associated kinase activity was possessed by a 60 kDa protein that existed in aggregates of approximately 450 kDa in native polyacrylamide gels (Grimm et al., 1989). Immunoblotting experiments revealed that phytochrome was not a component of the 450 kDa complex. Attempts to stimulate by phosphorylation an intrinsic kinase activity of phytochrome were unsuccessful (Grimm et al., 1989). These data clearly indicated that oat phytochrome was not a protein

kinase. This is in contrast to phytochrome from the moss *Ceratodon purpureus*, which contains a protein kinase domain that is catalytically active *in vitro* (Thummler et al., 1992; Algarra et al., 1993), although the functional relevance of this kinase domain remains to be determined.

Nevertheless, it has been shown that phytochrome is phosphorylated *in vivo* (Hunt and Pratt, 1980). It is possible that the associated kinase may help regulate phytochrome activity, as it has been demonstrated that the associated kinase phosphorylates phytochrome in its serine-rich amino terminus (McMichael and Lagarias, 1990). One or more serines in this region appear to be necessary for regulation of phytochrome activity (Stockhaus et al., 1992), as mutation of the first ten serines at the amino terminus to alanine resulted in phytochrome molecules that possessed greater biological activity *in vivo* than the wild type molecule. Alteration of these serines removes potential phosphorylation sites, and, apparently, attenuation of phytochrome activity by negative regulators. The activity of the purified phytochrome-associated kinase has been shown to be stimulated by both Ca^{2+} and cGMP (Grimm et al., 1989). This observation raises the possibility of a negative feedback regulatory mechanism in which phytochrome activity is inhibited by its second messengers.

Whereas phosphorylation of phytochrome itself may be important, phosphorylation of molecules downstream is likely to be necessary to transduce and amplify the initial light signal. Indeed, it has been shown that light stimulates the reversible phosphorylation of a number of proteins. In the case of pea nuclear proteins, light-stimulated phosphorylation was shown to be Ca^{2+}-dependent (Datta et al., 1985). Similarly, phosphorylation by a casein kinase of a DNA binding activity termed AT-1, which binds to AT-rich regions of some light-regulated promoters, was found to alter the binding activity (Datta and Cashmore, 1989). G box binding factors (see below) of parsley (*Petroselinum crispum*) were found to be phosphorylated in response to light treatment, and subsequently translocate to the nucleus (Harter et al., 1994). In addition,

other proteins have been identified in oat coleoptile tips and wheat leaf protoplasts that are phosphorylated upon light treatment (Otto and Schafer, 1988; Fallon et al., 1993).

Transcription Factors

The changes in gene expression regulated by the phytochrome signaling pathway are ultimately mediated by transcription factors. Over the past ten to fifteen years, numerous groups have identified elements in the promoters of light-regulated genes that are required for proper transcriptional regulation. The elements identified include palindromic G-boxes (CACGTG) (Izawa et al., 1993), GT boxes (GGTTAA) (Green et al., 1987), AT-rich sequences (Datta and Cashmore, 1989), and CA-rich sequences (Sun et al., 1993). Factors that bind these elements have been identified in electrophoretic mobility shift assays, and in many cases, have been cloned.

As described earlier, GT-2 is a factor that binds to the GT-2 and GT-3 boxes of the rice *PHYA* promoter (Dehesh et al., 1990). The protein contains a putative DNA binding domain termed the trihelix motif, and contains acidic domains that are reminiscent of transcriptional activators (Dehesh et al., 1992). A similar DNA binding activity termed GT-1 was identified by its ability to bind the GGTTAA-containing box II element of the pea *RBCS-3A* promoter (Green et al., 1987). The observation that removal of box II from the promoter reduces transcriptional activity supports the notion that the factor that binds it is a transcriptional activator (Green et al., 1987). Cloning of this factor revealed that like GT-2, GT-1 contains a trihelix motif and acidic domains (Gilmartin et al., 1992; Perisic and Lam, 1992). In addition, GT-1 has been shown to bind its target sequence as a dimer (Hiratsuka et al., 1994).

The promoters of numerous light-inducible genes also contain elements termed G-boxes that contain an ACGT core that are flanked by additional nucleotides that determine DNA binding specificity. The factors that bind these elements generally belong to the basic-leucine zipper (b-Zip) class of proteins that bind their target sequences as dimers (Izawa et al., 1993). Mutation of these elements from a number of

promoters, including parsley (*Petroselinum crispum*) *CHS*, dramatically reduces transcriptional activity (Block et al., 1990). The G-box element within the parsley *CHS* promoter is located within a 56 base region designated Unit I. The Unit I element has been shown to be light responsive (Weisshaar et al., 1991), but the specific factors that function as activators via Unit I binding are not known. Three b-Zip proteins designated CPRF-1, CPRF-2, and CPRF-3 were cloned based on their abilities to bind the Unit I G-box (Weisshaar et al., 1991). The G-box sequence (CACGTG) is also the consensus binding site for myc transcription factors (CANNTG; Roth et al., 1991). Ten bases downstream lies a putative binding site for myb transcription factors (C/TAACG/TG) which has also been demonstrated to be required for proper transcriptional activity (Block et al., 1990; Roth et al., 1991). The proximity of myc and myb binding sites is potentially important because the maize genes *R* and *B*, which regulate anthocyanin biosynthesis, are of the *myc* and *myb* classes of transcription factors, respectively (Dooner et al., 1991). It is not known which factors, b-Zip or *myc/myb* confer light responsiveness.

Other sequence elements have been identified in light-responsive promoters that are important for proper gene activation. These elements include CA-rich and AT-rich sequences of the *Arabidopsis CAB140* promoter and pea *CAB* and *RBCS* promoters, respectively (Datta and Cashmore, 1989; Sun et al., 1993). Deletion or mutation of the CA-1 and AT-1 elements is correlated with reduced promoter activity. DNA binding activities, named, not surprisingly, CA-1 and AT-1, have been identified, and it has been determined that the binding activities of both are altered by phosphorylation (Datta and Cashmore, 1989; Sun et al., 1993). The kinase activity that phosphorylates AT-1 was deduced to be an NII-type casein kinase based on phosphate donor specificity and divalent cation requirements (Datta and Cashmore, 1989). Phosphorylation is likely to play an important role in regulating the terminal elements of the signaling pathway, and identification of phosphorylation requirements for factor binding activity supports this. Nevertheless, it has not been definitively established that any of the cloned transcription

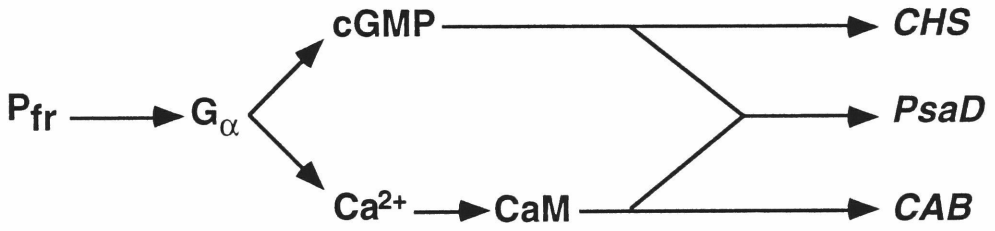
factors or identified binding activities are specific targets of the phytochrome signaling system. Moreover, demonstration that specific promoter elements are targets of one signaling pathway or another remains to be achieved.

A Model of Phytochrome Signaling

The information obtained from the biochemical analyses described above allows the construction of a basic model of the phytochrome signaling pathway (Figure 1). It is proposed that photoconversion of P_r to P_{fr} causes a structural change in the phytochrome molecule that allows it to interact with at least one heterotrimeric G protein and activate the signaling pathway. The mechanism by which phytochrome, a cytosolic molecule, interacts with membrane-localized G proteins is unclear, but there is some evidence that phytochrome can interact with membranes (Sineshchekov et al., 1994). The activated G proteins stimulate three distinct signaling pathways. The first pathway requires Ca^{2+}/CaM, and activates the expression of the genes encoding PS II, the two light harvesting complexes, *RBCS* and ATP synthase. The second pathway requires high levels of cGMP, and stimulates the expression of anthocyanin biosynthetic genes. The third pathway requires both Ca^{2+}/CaM and low levels of cGMP, and stimulates the expression of the genes encoding PS I and the cytochrome b_6f complex.

The different levels of cGMP required to activate the cGMP and Ca^{2+}/cGMP pathways allows flexibility in which genes are activated depending on the light conditions the plant encounters. In low levels of light, the Ca^{2+}/cGMP pathway would be activated despite low levels of phytochrome pathway activity, and PS I components would be synthesized, allowing photosynthesis to proceed. In high levels of light, phytochrome signaling would be activated to maximal levels, stimulating production of anthocyanin biosynthetic genes. In high levels of light, anthocyanins would be necessary because they would protect the plant from UV damage. In low levels of light, the danger of UV damage is minimal, and anthocyanins are not required. Thus the plant does not have to expend energy on unnecessary protein synthesis.

Figure 1: Model of the phytochrome signal transduction pathway (adapted from Bowler et al., 1994b).


cGMP
CHS
Pfr
Gα
PsaD
Ca2+
CaM
CAB

A second level of regulation was observed in microinjection and cell culture experiments. It was found that if the Ca^{2+}/CaM pathway were inhibited, genes regulated by the cGMP pathway were superinduced (Bowler et al., 1994b). This indicated that the Ca^{2+}/CaM pathway negatively-regulated the cGMP pathway *in vivo*. Indeed, it was found that increased levels of calcium that would hyperactivate the Ca^{2+}/CaM pathway tended to repress the cGMP pathway. Conversely, it was found that high levels of cGMP could negatively regulate the Ca^{2+}/CaM and Ca^{2+}/cGMP pathways as well (Bowler et al., 1994b). This phenomenon of cross-regulation has been termed reciprocal control. Thus, it appears that the activity of the phytochrome signaling pathways is controlled by very precise mechanisms that confer a high degree of flexibility in responding to various light conditions.

A Global Regulatory Network

Considerable evidence has been accumulated that demonstrates that physiological and molecular responses to phytochrome may be modified by a variety of other factors. In particular, phytochrome-regulated processes have been shown to be enhanced or repressed by hormones, sugars (Tsukaya et al., 1991), organellar signals (Taylor, 1989), biological rhythms (Nagy et al., 1986), developmental programs (Kubasek et al., 1992), cell type-specific signals (Li et al., 1995), temperature (Leyva et al., 1995), and other photoreceptor systems (Mohr, 1994). Moreover, phytochrome modulates responses mediated by these other pathways as well. All of this evidence demonstrates that the phytochrome signaling pathway is part of a much larger, global regulatory web that governs the responses of a plant to the vast majority of environmental conditions it encounters in its life cycle.

Among the hormones that interact with phytochrome signaling, abscisic acid (ABA), cytokinins, and brassinosteroids achieve notable effects. ABA has been found to effectively inhibit the expression of a number of phytochrome-inducible genes, including *CAB* and *RBCS* (Bartholomew et al., 1991). Moreover, it has been found to be a potent

inducer of genes that are negatively-regulated by light (Williams et al., 1994), and ABA degradation may also be enhanced by phytochrome activity (Kraepiel et al., 1994). All of these observations underscore the antagonistic relationship between phytochrome and ABA signaling.

It has been found that mutations of the *DET1* locus can be phenocopied by the addition of exogenous cytokinin. The effects of cytokinin treatment include the induction of light-regulated genes in the absence light (Chory et al., 1994). Moreover, the *amp1* mutant, which displays partial photomorphogenesis in the dark, possesses six-fold higher levels of cytokinin than WT (Chaudhury et al., 1993). Similarly, *det2* mutations also cause a light-grown appearance in the dark (Chory et al., 1991). Demonstration that *det2* and *dim* plants can be rescued by brassinosteroids indicates that they play an important role in the maintenance of an etiolated morphology (Li et al., 1996; N.-H. Chua, personal communication). One can predict that phytochrome activity represses brassinosteroid effects and enhances cytokinin activity to induce photomorphogenesis.

Similarly, sugars, temperature, and blue light photoreceptor systems have all been shown to enhance phytochrome responses (Tsukaya et al., 1991; Mohr, 1994; Leyva et al., 1995). In addition, sugars have also been observed to have antagonistic effects on photosynthetic gene expression (Sheen, 1990). The interplay of phytochrome signaling with circadian rhythms is equally complex, because light signals establish the period of biological clocks, and the clocks modulate the levels of gene expression in both the dark and the light (Millar and Kay, 1991). Understanding the mechanisms by which each signal transduction pathway interacts with another is crucial to understanding global mechanisms regulating a plant's response to its environment.

In the studies described here, new approaches are taken to investigate phytochrome signal transduction and the interactions of this pathway with others in the global regulatory web. A novel genetic screen has been developed to isolate new mutants that specifically affect phytochrome signaling. The mutants obtained from this screen are

analyzed to identify the effects their mutations have on phytochrome signaling, and what role the WT gene products play in the transmission of phytochrome signals. An examination is made of the signaling pathway regulated by sucrose, the predominant form in which carbohydrate is stored and transported in higher plants (Salisbury and Ross, 1985). The effects of sucrose on stimulating and repressing the expression of phytochrome-regulated genes is examined in order to understand the nature of the interaction between the two pathways. Ultimately, these studies provide a clearer insight into the mechanisms of phytochrome signal transduction, and the manner in which it functions in the context of a global regulatory network.

Chapter 2

Characterization of Sucrose-Dependent Signaling

Aside from the profound effects light has on plant development, it is essential for the most important set of chemical reactions on earth, photosynthesis. The radiant energy possessed by photons of light is harvested by chlorophyll and converted into usable chemical energy in the form of ATP and NADPH. This energy is then used in the dark reactions of the Calvin Cycle for the fixation of atmospheric carbon and production of glucose, which is subsequently converted into sucrose, the principal form in which carbon fixed during photosynthesis is stored and transported (Salisbury and Ross, 1985). Clearly, regulation of photosynthesis and carbohydrate metabolism is of critical importance to plants.

As described previously, light induces the expression of genes encoding components of the photosynthetic apparatus, and phytochrome transduces a significant portion of these light signals via the tripartite pathway described above (Figure 1). At a certain point, though, significant amounts of sugars have been produced, and signals from within photosynthetic cells (source tissues), as well as from organs to which sugars are transported (sinks), stimulate and repress the expression of genes required for sugar metabolism and photosynthesis itself (Neales and Incoll, 1968). It has been demonstrated that sugars stimulate the expression of metabolic genes like sucrose synthase and ADP-glucose pyrophosphorylase (Muller-Rober et al., 1990; Karrer and Rodriguez, 1992). Moreover, sugars have been demonstrated to repress the transcription of the starch hydrolytic enzymes α- and β-amylase as well as inhibit photosynthetic rate, chlorophyll biosynthesis, and genes encoding components of the sugar-recycling glyoxylate cycle (Edelman and Hanson, 1971; Yu et al., 1991; Goldschmidt and Huber, 1992; Graham et al., 1994; Mita et al., 1995).

Similarly, sugars have been shown to have profound effects on the expression of phytochrome-inducible photosynthetic genes. Sheen (1990) first demonstrated that high levels of glucose, fructose or sucrose repressed the expression of light-inducible promoter::reporter gene fusions in maize mesophyll protoplasts. The response of these reporter constructs demonstrated that regulation occurred at the transcriptional level. Subsequently, these observations were confirmed with sugars at concentrations that are much closer to physiological levels, although glucose and fructose were more than twice as effective at inhibiting gene expression than sucrose (Jang and Sheen, 1994). Similar experiments performed in light-responsive cell suspension cultures of *Chenopodium rubrum* and *Brassica napus* have demonstrated that sugars could repress the expression of endogenous *RBCS* and *CAB* genes, respectively (Harter et al., 1993; Krapp et al., 1993).

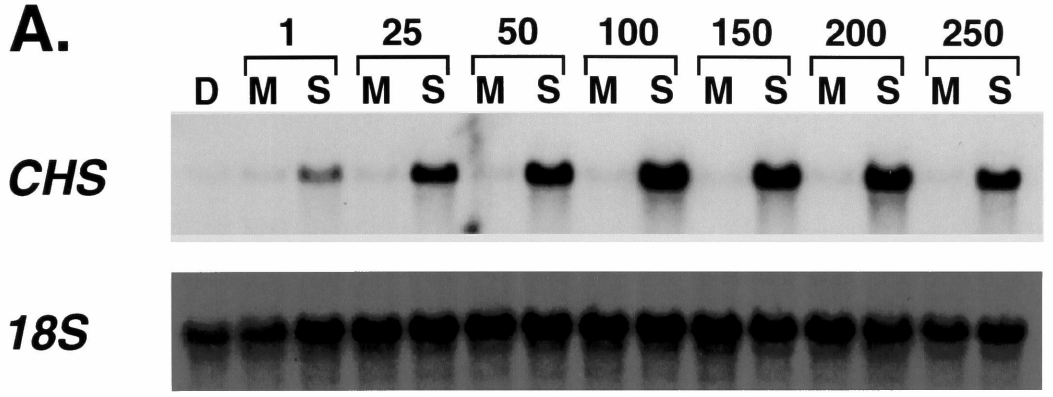
Conversely, sugars have also been shown to enhance the expression of phytochrome-inducible genes and physiological processes. It has previously been shown that sucrose stimulated the accumulation of anthocyanin in flowers, a phenomenon that was enhanced when the plant hormone gibberellin was added as well (Weiss and Halevy, 1989). Similarly, accumulation of anthocyanins in the *fus6* mutant of *Arabidopsis thaliana* was found to require the presence of sugars in the medium (Castle and Meinke, 1994). Osmotic substitutes like sorbitol had no stimulatory effect, demonstrating that the sugar itself was required. Moreover, expression of *CHS::GUS* fusions in *Arabidopsis* was found to be activated by sugars, with sucrose being the most potent inducer (Tsukaya et al., 1991). The induction of this anthocyanin biosynthetic gene correlates well with the observed sugar-dependent increases in anthocyanin production seen previously. The light- and nitrate-inducible nitrate reductase (*NR*) gene was similarly found to be activated by sucrose (Cheng et al., 1992). The induction of *CHS* and *NR* was observed using promoter::reporter gene fusions, demonstrating that control occurred at the transcriptional level.

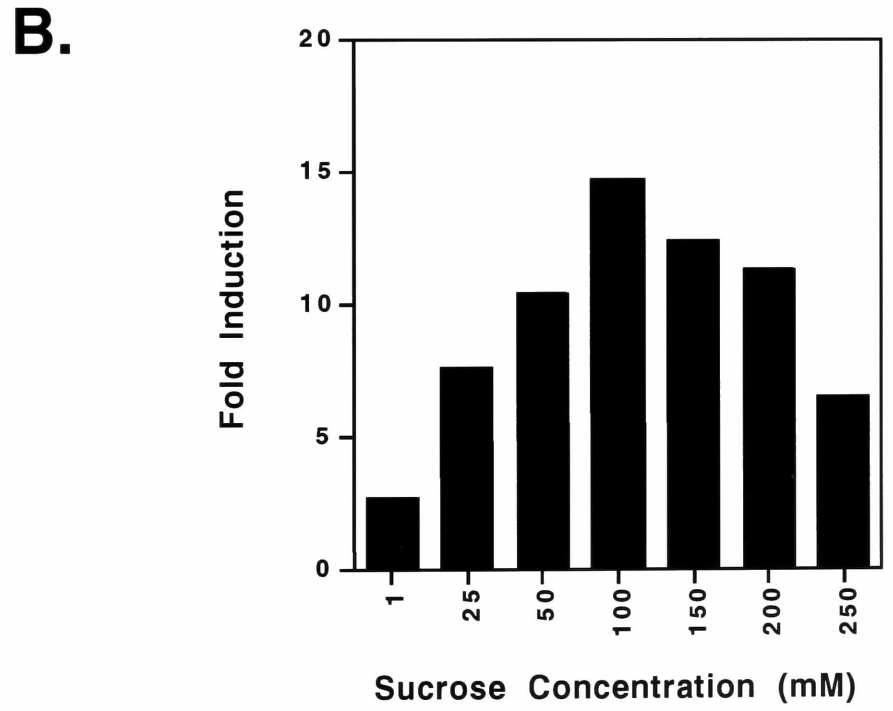
The positive and negative effects sucrose has on phytochrome-inducible genes indicates that the signal transduction pathways regulated by each may functionally interact *in vivo*. To better understand the nature of these interactions, the regulation of phytochrome-responsive genes by sucrose was examined in a light-inducible soybean (*Glycine max*) cell suspension culture. Such analysis has proven to be very effective in the past, as it played a significant role in the elucidation of the phytochrome signal transduction pathway (Neuhaus et al., 1993; Bowler et al., 1994a). The experiments described below investigate the sucrose-mediated induction of *CHS*, and repression of the phytochrome-mediated induction of *CAB* as well as *PSAD*, a gene that encodes a protein that is an integral component of photosystem I. The results indicate that sucrose induces *CHS* expression via a cGMP-dependent pathway, and that repression of *CAB* and *PSAD* occurs through a heretofore undescribed mechanism.

Sucrose-mediated induction of CHS *expression*

Preliminary experiments indicated that sucrose stimulated *CHS* expression in both the dark and the light. These data are consistent with the observation that sucrose stimulated expression of a *CHS::GUS* transgene in *Arabidopsis* plants (Tsukaya et al., 1991). To measure the responsiveness of soybean suspension cultures to sucrose, a dose response test was performed. Cells that had been adapted to darkness for three days were treated with different concentrations of sucrose, ranging from 1-250 mM. As a control for osmotic effects that may arise from sucrose treatment, equimolar concentrations of the non-metabolizable monosaccharide mannitol were added to separate cultures in parallel. Following three hours of sugar treatment in continuous darkness, cells were harvested, RNA was isolated, and *CHS* mRNA levels were assessed by gel blot analysis. It was found that *CHS* expression was induced to increasing levels in a dose dependent manner, such that a maximal induction of 15-fold over dark controls was observed at extracellular sucrose concentrations of 100 mM (Figure 2A and 2B). At sucrose concentrations of 1 mM, a level that reflects those observed under physiological conditions, a 2-fold increase

Figure 2: RNA gel blot analysis of dose responsive induction of *CHS* in response to sucrose. (A) 10 day old light-grown SB-P soybean cell cultures were adapted for 3 days in darkness. An aliquot of cells was harvested as a dark control (D), and cells were split into 30 ml cultures. Mannitol (M) or sucrose (S) was added to each culture to the final concentrations listed above the lanes (mM), and cells were further incubated in the dark for 3 hours prior to harvest. RNA gel blots were sequentially hybridized with a *CHS* cDNA and an *18S* rDNA. (B) Quantification of the induction of *CHS* expression by sucrose. mRNA levels were quantified by phosphorimage analysis, and *CHS* mRNA levels were normalized to *18S* rRNA levels to correct for differences in loading.


A.
1
25
50
100
150
200
250
D
M
S
M
S
M
S
M
S
M
S
M
S
M
S
CHS
18S


B.
Fold Induction
20
15
10
5
0
1
25
50
100
150
200
250
Sucrose Concentration (mM)

of *CHS* expression was observed. Similar concentrations of mannitol had no effect on *CHS* expression, demonstrating the requirement for sucrose in this response. At sucrose concentrations above 100 mM, *CHS* mRNA levels declined. At these concentrations, strong osmotic effects may occur, and other pathways that repress *CHS* expression may be induced.

To further investigate the effects of sucrose on *CHS* expression, an extended time course of sucrose treatment was performed. In this experiment, dark-adapted cultures were treated with 10 mM sucrose in the dark for 18 hours. Gel blot analysis of RNA harvested over the duration of the time course demonstrated that *CHS* was induced to appreciable levels (Figure 3). *CHS* mRNA was clearly detectable within one hour of sucrose addition, and reached maximal levels three hours after treatment began. Subsequently, *CHS* expression declined, such that by twelve hours, levels were similar to those of dark controls. Parallel treatments with mannitol produced little measurable effect on *CHS* expression.

It is striking that the time course of *CHS* expression in response to sucrose is remarkably similar to that observed in cultures induced with light (Bowler et al., 1994a). In the case of light, a similar rapid induction and equally rapid decline of *CHS* mRNA levels was observed. It was also found that the phytochrome-mediated induction of *CHS* was insensitive to the protein synthesis inhibitor cycloheximide (CHX), demonstrating that the light response pathway does not require *de novo* protein synthesis (Dr. Chris Bowler, UNINA, Italy, personal communication). To address the same question with regard to sucrose, dark adapted cells were treated with sucrose in the absence or presence of 30 μg/ml CHX. It was found that sucrose-mediated induction of *CHS* is also insensitive to CHX, displaying identical levels of *CHS* expression as well as identical kinetics (Figure 4). This demonstrates that a sucrose response pathway pre-exists in cells, allowing a rapid response to changing sugar levels.

Figure 3: RNA gel blot analysis of the time course of induction of *CHS* expression by sucrose. 10 day old light-grown cultures were adapted to darkness for 3 days, after which an aliquot of cells was collected as a dark control (D). The large culture was split into 2x210 ml cultures to which mannitol or sucrose was added to a final concentration of 10 mM. Cells were further incubated in darkness, and aliquots were harvested at the times indicated (hours). RNA gel blots were hybridized with a *CHS* cDNA and an *18S* rDNA.

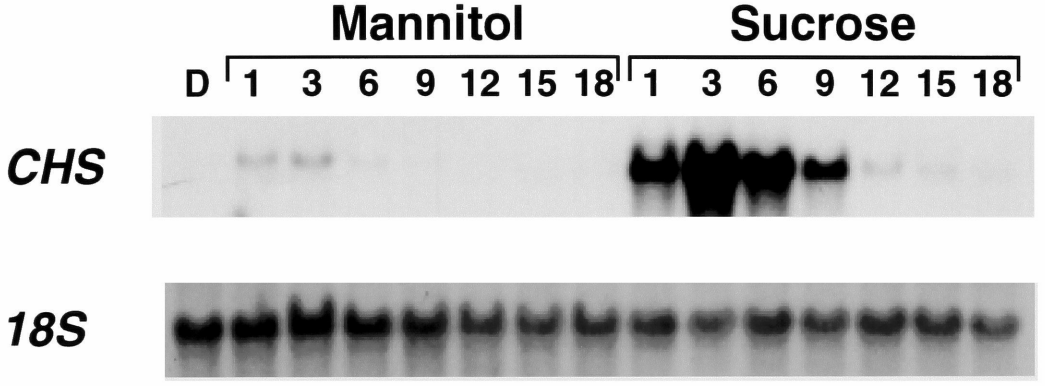
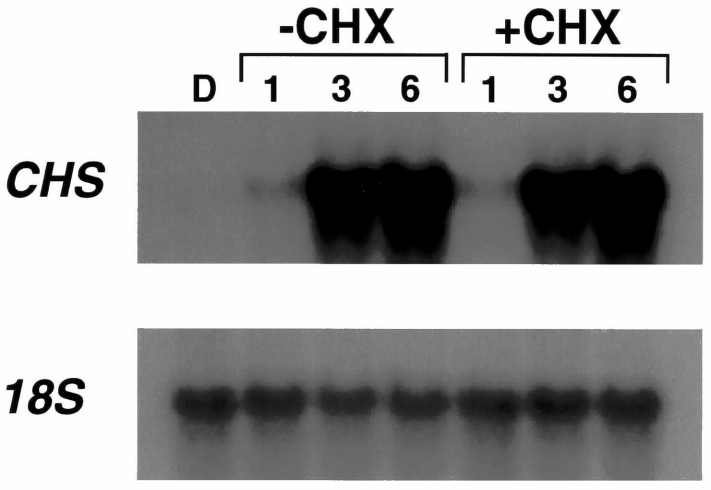

Mannitol
Sucrose
D 1 3 6 9 12 15 18 1 3 6 9 12 15 18
CHS
18S

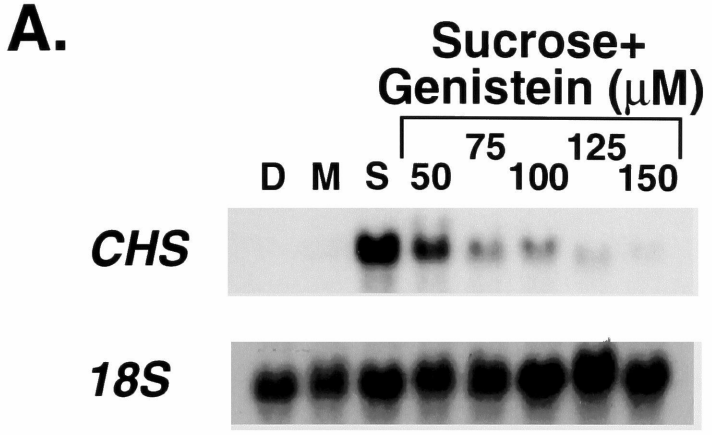
Figure 4: RNA gel blot analysis of the effect of cycloheximide on sucrose-induced *CHS* expression. 10 day old light-grown cultures were adapted to darkness for 3 days, after which an aliquot was collected as a dark control (D). Cells were split into 2x90 ml cultures to which sucrose was added to a final concentration of 100 mM. Cycloheximide was added to a final concentration of 30 μg/ml (+CHX) or water was added as a control (-CHX). Cells were further incubated in the dark, and aliquots were harvested at the times indicated (hours). RNA gel blots were hybridized with a *CHS* cDNA and an *18S* rDNA.

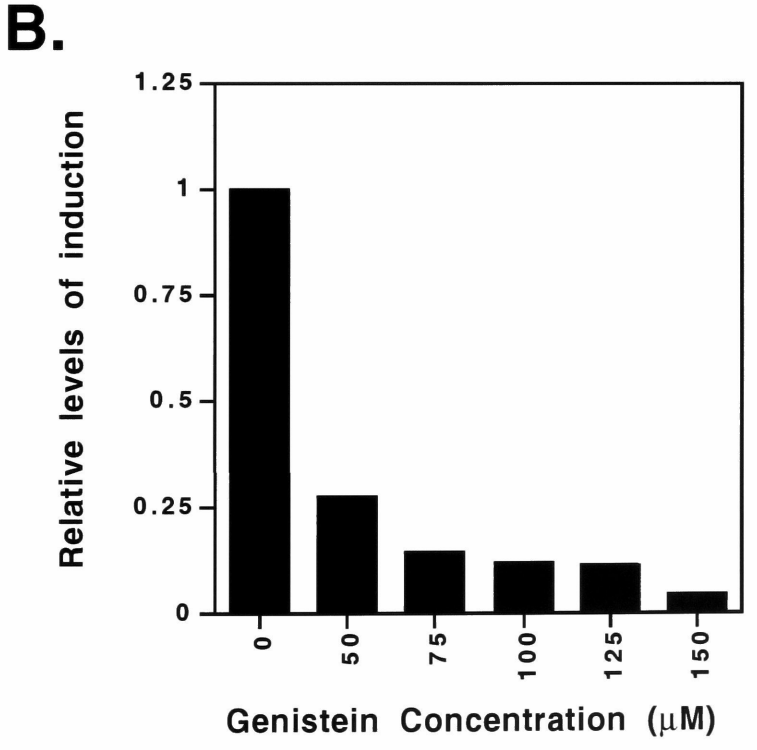

-CHX
+CHX
D
1
3
6
1
3
6
CHS
18S

The similarity of light and sucrose induction of *CHS* raised the possibility that the two stimuli utilized similar signaling pathways to induce *CHS* expression. An additional characteristic of phytochrome induction of *CHS* is that it is sensitive to the general tyrosine kinase inhibitor genistein (Bowler et al., 1994b). Figure 5A shows the effect of genistein on the sucrose-induced expression of *CHS*. It was found that at increasing genistein concentrations, the ability of sucrose to induce *CHS* expression was significantly inhibited. Quantification of this phenomenon determined that inhibition of *CHS* expression was 75% at the lowest concentration tested (50 μM), and increased to approximately 90% (Figure 5B). Virtually identical genistein sensitivity was also seen when the cells were induced with 100 mM sucrose as well (data not shown). These results differ from those seen in experiments investigating phytochrome-mediated induction of *CHS*, as expression was completely abolished with 100 μM genistein ((Bowler et al., 1994b); Figure 9, compare *CHS* expression in Mannitol-Gen and Mannitol+Gen samples at the 3 hour time point). This difference may reflect an intrinsic difference between the two pathways.

The decline of *CHS* expression in extended treatments with light has been attributed to rapid turnover of cGMP, the molecule responsible for phytochrome-mediated induction of *CHS* (Figure 1). It has been proposed that cGMP turnover may be mediated by a cGMP-dependent phosphodiesterase (cGMP PDE), as it was demonstrated recently that the decline of *CHS* mRNA levels during prolonged light treatments could be blocked by zaprinast, a specific inhibitor of cGMP PDEs (Bowler et al., 1994b). This observation supports the idea of cGMP turnover as a means of reducing signal flow through the cGMP pathway during prolonged light treatment. Given the strong similarity between the regulation of *CHS* expression by sucrose and the cGMP-dependent pathway of phytochrome, it was possible that sucrose may utilize cGMP as well to regulate *CHS* expression. If this were the case, the decline of *CHS* mRNA levels seen in extended sucrose treatments may result from activity of a cGMP PDE.

Figure 5: RNA gel blot analysis of dose responsive repression of sucrose-induced *CHS* expression by genistein. (A) 10 day old light-grown cultures were adapted to darkness for three days, at which time an aliquot of cells was collected as a dark control (D). Cells were split into 7x30 ml cultures to which mannitol (1 culture, M) or sucrose (6 cultures, S and those treated with genistein) was added to a final concentration of 10 mM. To five of the sucrose-treated cultures, genistein was added to the final concentrations indicated above the lanes (μM). All cultures were incubated for 3 hours in darkness following sugar addition. RNA gel blots were hybridized with a *CHS* cDNA and an *18S* rDNA. (B) Quantification of the repression of *CHS* expression by genistein. mRNA levels were quantified by phosphorimage analysis, and *CHS* mRNA levels were normalized to *18S* rRNA levels to correct for differences in loading.


A.
Sucrose+
Genistein (μM)
75
125
D M S 50 100 150
CHS
18S


B.
Relative levels of induction
1.25
1
0.75
0.5
0.25
0
0
50
75
100
125
150
Genistein Concentration (μM)

To test this hypothesis, zaprinast was used in extended sucrose treatments in an attempt to block the decline of *CHS* mRNA levels. Zaprinast has recently been found to enhance the expression of *CHS* in *Arabidopsis* plants (see below). To confine its effects to the time period during which *CHS* levels decline, zaprinast was added during the period when decline in expression had begun. In this experiment, dark adapted cells were treated with levels of sucrose that stimulate a maximal response (100 mM) for 12 hours in the dark, at which time *CHS* mRNA levels are declining (Figure 3). Zaprinast was then added, and the expression of *CHS* over the next 10 hours was observed. It was found that in untreated cells, *CHS* expression was still detectable 15 hours after sucrose treatment began, but declined thereafter (Figure 6). In zaprinast-treated cells, however, *CHS* mRNA levels persisted for an additional 7 hours, remaining detectable 22 hours after the initiation of sucrose treatment (Figure 6). These results suggest that the decline of *CHS* expression results from the activity of a zaprinast-sensitive molecule that is likely to be a cGMP PDE, and that induction of *CHS* occurs via a cGMP-dependent step. The latter assertion was borne out in a series of microinjection experiments in the *au* mutant of tomato, in which it was found that Rp-cGMPS, a specific inhibitor of cGMP-dependent processes, specifically blocked the sucrose-mediated induction of *CHS* (McGrath et al., manuscript in preparation).

Previous studies have demonstrated that the hexose sugars glucose and fructose can activate the expression of a number of genes, including *CHS* (Tsukaya et al., 1991). To examine if these hexoses had similar effects in soybean cell cultures, equimolar concentrations of sucrose, glucose, and fructose were added to dark-adapted soybean cultures. It was found that all three were able to induce *CHS* expression, but sucrose was most effective, whereas glucose was least effective (Figure 7). The effectiveness of sucrose at inducing *CHS* expression is consistent with previous results, but glucose had been found to be more effective than fructose. The differences between the two sets of results may reflect the fact that in this study, cultured cells were used, whereas in the

Figure 6: RNA gel blot analysis of the effect of zaprinast on the decline of *CHS* mRNA levels following prolonged induction by sucrose. 10 day old cultures were adapted to darkness for 3 days, at which time an aliquot of cells was collected as a dark control (D). Cells were split into 2x210 ml cultures to which mannitol or sucrose was added to a final concentration of 100 mM. Cells were incubated for a further 12 hours in darkness, at which time aliquots of cells were collected to assess *CHS* expression prior to zaprinast treatment (12). Both subcultures were split into 2x90 ml cultures to which zaprinast was added to a final concentration of 100 μM (+Zap), or dimethyl sulfoxide (DMSO) was added as a control (-Zap). Cells were further incubated in darkness, and aliquots were collected at the times indicated (hours). RNA gel blots were hybridized with a *CHS* cDNA and an *18S* rDNA.

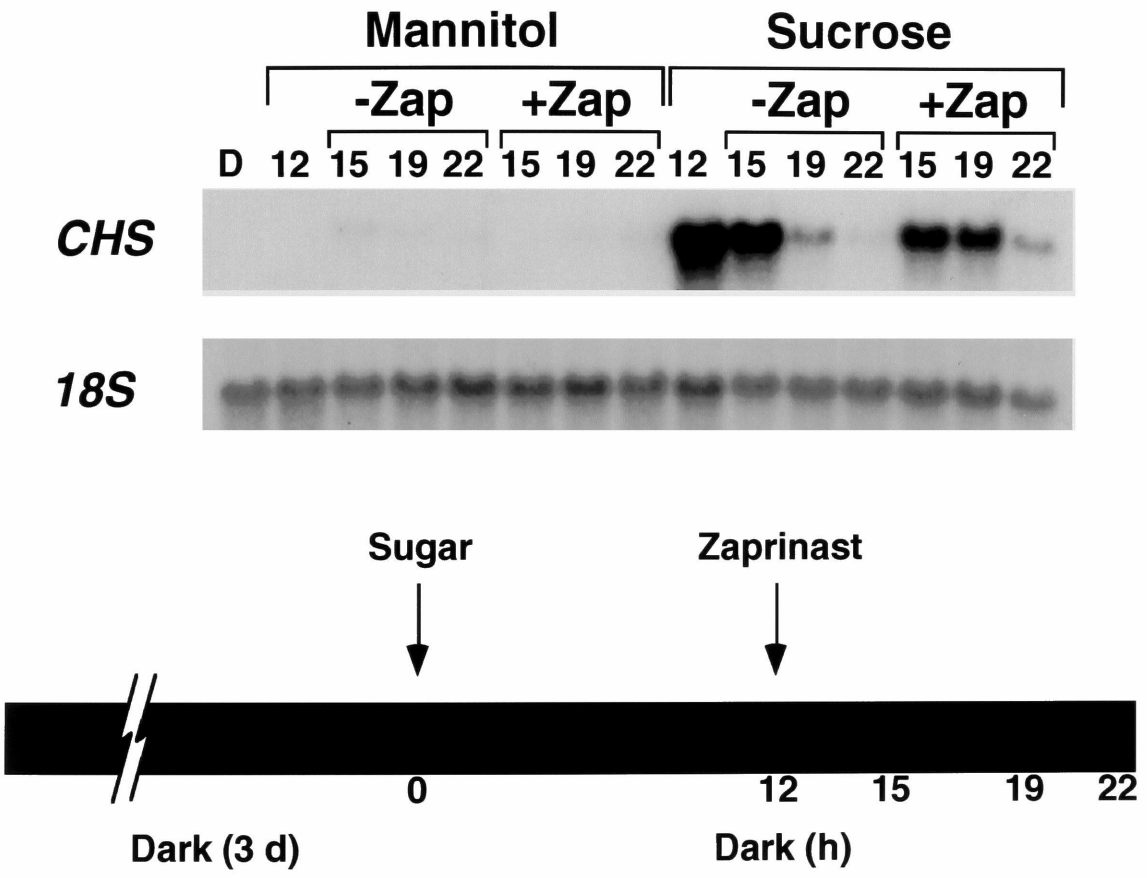
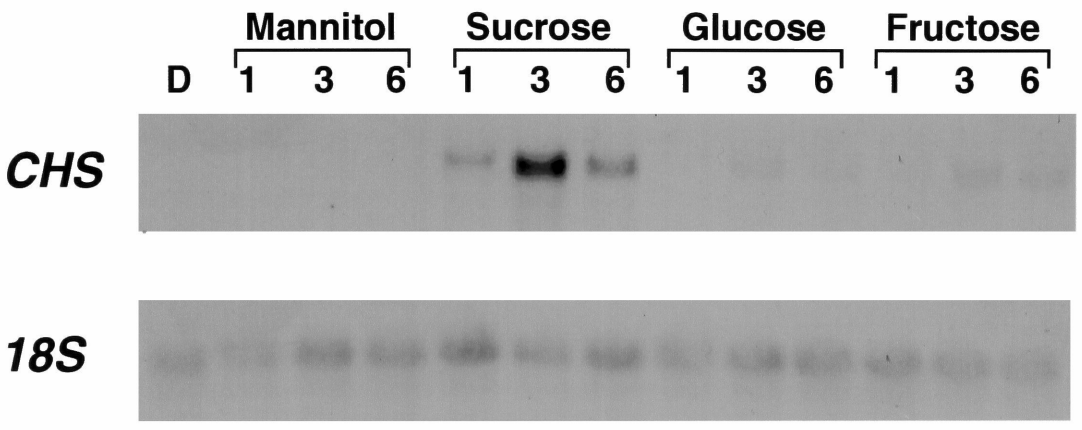

Mannitol
Sucrose
-Zap
+Zap
-Zap
+Zap
D 12 15 19 22 15 19 22 12 15 19 22 15 19 22
CHS
18S
Sugar
Zaprinast
0
12 15 19 22
Dark (3 d)
Dark (h)

Figure 7: RNA gel blot analysis of the effect of glucose and fructose on *CHS* expression. 10 day old light-grown cultures were adapted to darkness for 3 days, at which time an aliquot of cells was harvested as a dark control (D). Cells were split into 4x90 ml cultures to which mannitol, sucrose, glucose, or fructose was added to final concentrations of 10 mM. Cells were incubated further in darkness, and aliquots were harvested at the times indicated (hours). RNA gel blots were hybridized with a *CHS* cDNA and an *18S* rDNA.

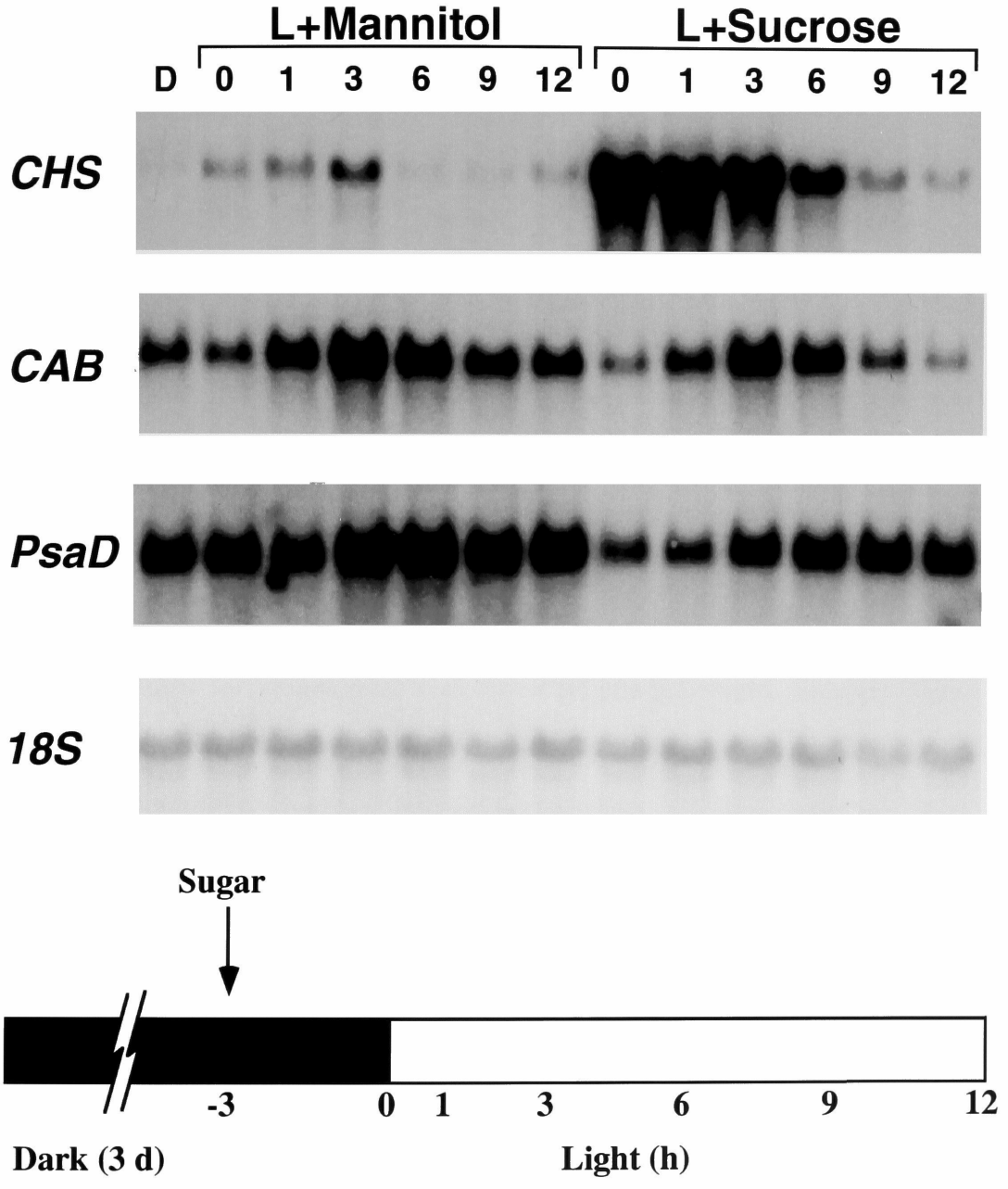

Mannitol
Sucrose
Glucose
Fructose
D
1
3
6
1
3
6
1
3
6
1
3
6
CHS
18S

previous study, experiments were performed in whole leaves or young plants. Moreover, the expression of the endogenous *CHS* gene was determined in these experiments, whereas the activity of *CHS::GUS* fusion genes were assayed in the *Arabidopsis* system (Tsukaya et al., 1991).

Effects of sucrose on phytochrome-regulated gene expression

As described earlier, sugars have been demonstrated to repress the expression of a number of photosynthetic genes, including *RBCS* and *CAB* (Harter et al., 1993; Krapp et al., 1993). Early experiments in soybean cells demonstrated that sucrose inhibited the expression of *CAB* and *PSAD*, targets of the Ca^{2+}- and Ca^{2+}/cGMP-dependent pathways of phytochrome, respectively (Neuhaus et al., 1993; Bowler et al., 1994a). The effects of sucrose on gene expression over an extended time course in the light was examined. Dark-adapted soybean cells were pre-incubated with 10 mM sucrose or mannitol for 3 hours prior to a shift to constant R (50 μmol m^{-2} s^{-1}). Samples taken immediately prior to the shift to R demonstrate that sucrose induced *CHS* expression (Figure 8). It was found that whereas light induced *CHS* mRNA to modest levels in mannitol-treated cultures, the combination of sucrose and R elevated *CHS* expression to much higher levels. Moreover, the kinetics of *CHS* expression in R+sucrose more closely resembled those seen in light alone, declining by 6 hours in light-treated cells, and by 9 hours in dark-adapted cells (compare Figure 3 and Figure 8). Expression of both *CAB* and *PSAD* was found to increase rapidly in mannitol-treated cells, such that within 6 hours of light treatment, maximal levels of expression were reached. The expression levels of *PSAD* remained high during the remainder of the time course, whereas *CAB* mRNA levels declined, perhaps reflecting the circadian regulation of this gene (Nagy et al., 1986; Millar and Kay, 1991). In the presence of sucrose, however, the expression of both genes was reduced (Figure 8). A higher degree of repression was observed at sucrose concentrations of 100 mM (data not shown). These data demonstrate that sucrose is capable of

Figure 8: RNA gel blot analysis of the effect of sucrose on phytochrome-inducible gene expression. 10 day old light-grown cultures were adapted to darkness for 3 days, at which time an aliquot of cells was harvested as a dark control (D). Cells were split into 2x180 ml cultures to which mannitol or sucrose was added to a final concentration of 10 mM. Cells were incubated for a further three hours in the dark, at which time aliquots of cells were harvested (0). Cultures were shifted to constant red light (50 μmol m^{-2} s^{-1}), and aliquots were harvested at the times indicated (hours). The times reflect the onset of light treatment. RNA gel blots were hybridized with a *CHS* cDNA, *CAB* cDNA, *PSAD* cDNA, or *18S* rDNA. A schematic representation of the experiment is indicated below.

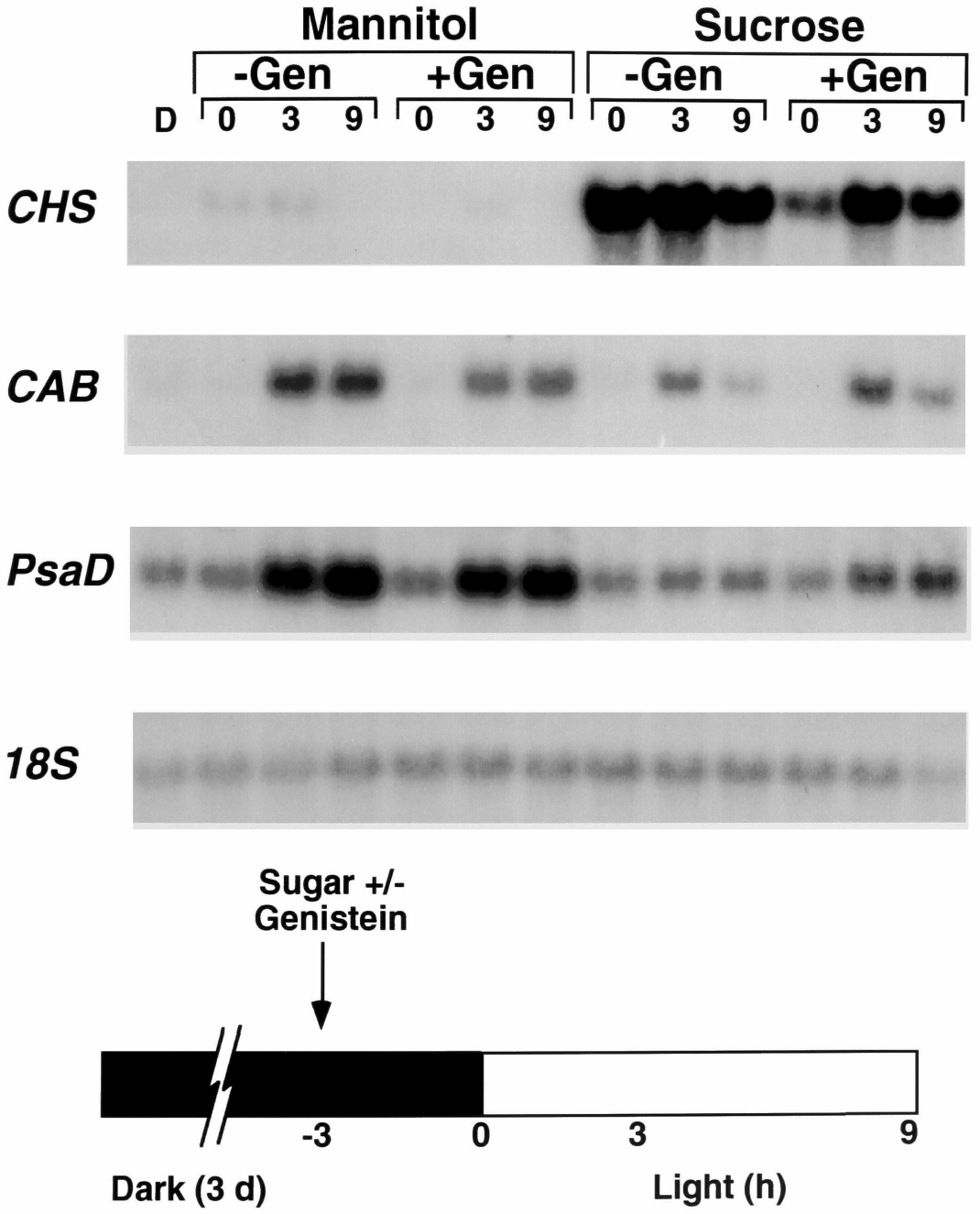

L+Mannitol
L+Sucrose
D
0
1
3
6
9
12
0
1
3
6
9
12
CHS
CAB
PsaD
18S
Sugar
-3
0
1
3
6
9
12
Dark (3 d)
Light (h)

repressing the expression of targets of both the Ca^{2+}- and Ca^{2+}/cGMP-dependent pathways of phytochrome in soybean cells.

The high levels of *CHS* expression induced by sucrose and the dependence of this pathway on cGMP suggest that sucrose stimulates the production of large amounts of cGMP to achieve its inductive effects. It has previously been demonstrated that the expression of Ca^{2+}- and Ca^{2+}/cGMP-dependent pathway target genes may be repressed by high levels of cGMP in both soybean cells and microinjection experiments in *au* hypocotyls (Bowler et al., 1994b). This repression mechanism has been termed reciprocal control. It is possible that the high levels of cGMP produced by sucrose signaling functions in a similar manner, resulting in the repression of genes induced by the Ca^{2+}- and Ca^{2+}/cGMP-dependent pathways. It has been shown that cGMP-dependent reciprocal control is inhibited by genistein (Bowler et al., 1994b). Thus, if sucrose utilizes reciprocal control mechanisms to repress gene expression in soybean cells, it may be possible to relieve that repression with genistein.

In this experiment, dark-adapted cells were treated with mannitol or sucrose, with or without genistein for 3 hours prior to shift to R (Figure 9, bottom). Samples taken prior to the shift to R demonstrate that genistein inhibits the induction of *CHS* by sucrose (Figure 9). Upon shift to R, light stimulated the induction of *CHS* in mannitol-treated cells to a lesser degree, but this induction was inhibited by genistein, an observation that is consistent with previous results. Whereas genistein inhibited the expression of *CHS* in response to either R or sucrose alone, it did not effectively inhibit *CHS* expression when the two acted in concert (Figure 9, compare Sucrose-Gen and Sucrose+Gen). Both *CAB* and *PSAD* are induced in mannitol-treated cells, and unlike *CHS*, are not inhibited by genistein. Sucrose inhibited the expression of both genes, however, but genistein was unable to significantly relieve that repression (Figure 9). These data suggest that sucrose does not utilize reciprocal control to inhibit gene expression. This conclusion is supported by microinjection experiments in *au* hypocotyls, in which it was determined

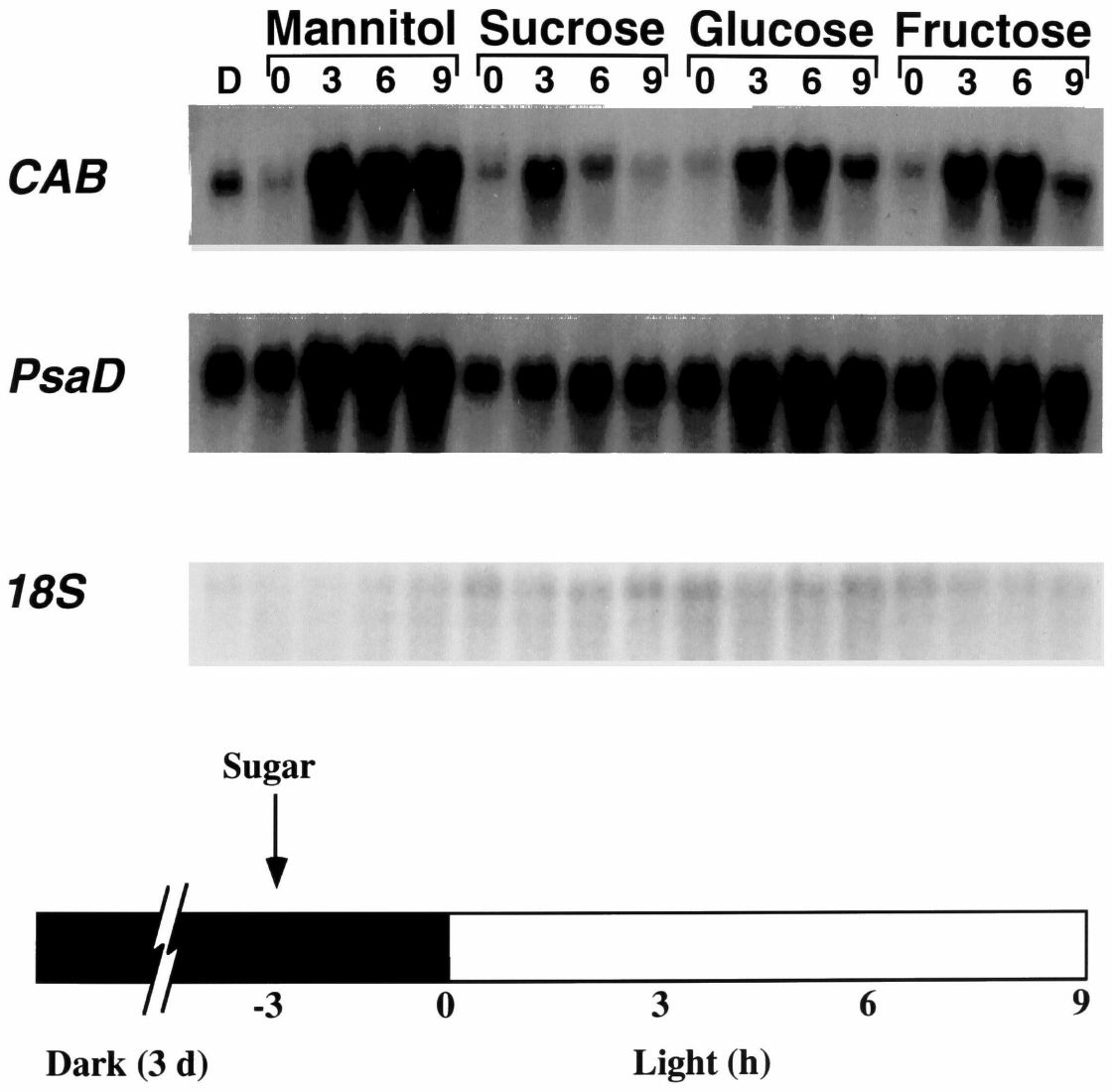
Figure 9: RNA gel blot analysis of the effect of genistein on the sucrose-mediated repression of phytochrome-inducible gene expression. 10 day old light-grown cultures were adapted to darkness for 3 days, at which time an aliquot of cells was harvested as a dark control (D). Cells were split into 4x90 ml cultures, of which, 2 received mannitol and 2 received sucrose to final concentrations of 100 mM. 1 mannitol-treated and 1 sucrose-treated culture were treated with 125 μM genistein (+Gen), and the remaining 2 cultures were treated with dimethylsulfoxide (DMSO) as a control (-Gen). The cultures were incubated in the dark for a further 3 hours, at which time aliquots were harvested (0). Cells were then shifted to constant red light (50 μmol m^{-2} s^{-1}), and aliquots were harvested at the times indicated (hours). The times refer to the onset of light treatment. RNA gel blots were hybridized with a *CHS* cDNA, *CAB* cDNA, *PSAD* cDNA, or *18S* rDNA. A schematic representation of the experiment is indicated below.


Mannitol
Sucrose
-Gen
+Gen
-Gen
+Gen
D
0 3 9
0 3 9
0 3 9
0 3 9
CHS
CAB
PsaD
18S
Sugar +/-
Genistein
-3
0
3
9
Dark (3 d)
Light (h)

that repression of *CAB* by sucrose did not require cGMP (McGrath et al., manuscript in preparation). Thus sucrose induces and represses gene expression by two separate mechanisms that utilize different downstream signaling components.

To examine whether sucrose and fructose were effective inhibitors of gene expression, dark-adapted cells were treated with either sucrose, glucose, or fructose for 3 hours prior to a shift to R. It was found that whereas sucrose effectively inhibited the expression of both *CAB* and *PSAD*, neither glucose nor fructose had much effect (Figure 10). These results are in contrast to those obtained in maize mesophyll protoplasts and autotrophic *Chenopodium* cultures (Sheen, 1990; Krapp et al., 1993; Jang and Sheen, 1994). In these systems, glucose and fructose were both potent inhibitors of photosynthetic gene expression. The discrepancies between these results may be reconciled by the manner in which the experiments were conducted. In the soybean experiments, sugars are added to dark-adapted cultures, and the ability to prevent the induction of genes is assayed. In the maize and *Chenopodium* systems, sugars are added to light-grown cultures in which active transcription of photosynthetic genes is occurring (Sheen, 1990; Krapp et al., 1993; Jang and Sheen, 1994). In this case, the ability to shut off the active transcription of photosynthetic genes is assayed. The inhibition of quiescent genes and repression of actively-transcribed genes may occur by fundamentally different mechanisms. Physiologically, this may reflect the metabolic status of cells *in planta*. Photosynthetically-active cells produce large amounts of hexose sugars (Salisbury and Ross, 1985). When a threshold concentration of glucose or fructose is achieved, a signal could be transmitted to repress the transcription of photosynthetic genes. Conversely, cells that have taken up sucrose during dark periods may contain high amounts of sugars during the first few hours after light illumination, and not require the production of more. Photosynthetic genes could then be actively repressed.

Figure 10: RNA gel blot analysis of the effect of glucose and fructose on phytochrome-inducible gene expression. 10 day old light-grown cultures were adapted to darkness for 3 days, at which time an aliquot of cells was harvested as a dark control (D). Cells were split into 4x120 ml cultures to which mannitol, sucrose, glucose, or fructose were added to final concentrations of 10 mM. Cells were incubated for a further 3 hours in darkness, at which time aliquots of cells were harvested (0). Cultures were shifted to constant red light (50 μmol m^{-2} s^{-1}), and aliquots were harvested at the times indicated (hours). The time refers to the onset of light treatment. RNA gel blots were hybridized with a *CAB* cDNA, *PSAD* cDNA, or *18S* rDNA. A schematic representation of the experiment is indicated below.


Mannitol
Sucrose
Glucose
Fructose
D
0 3 6 9
0 3 6 9
0 3 6 9
0 3 6 9
CAB
PsaD
18S
Sugar
-3
0
3
6
9
Dark (3 d)
Light (h)

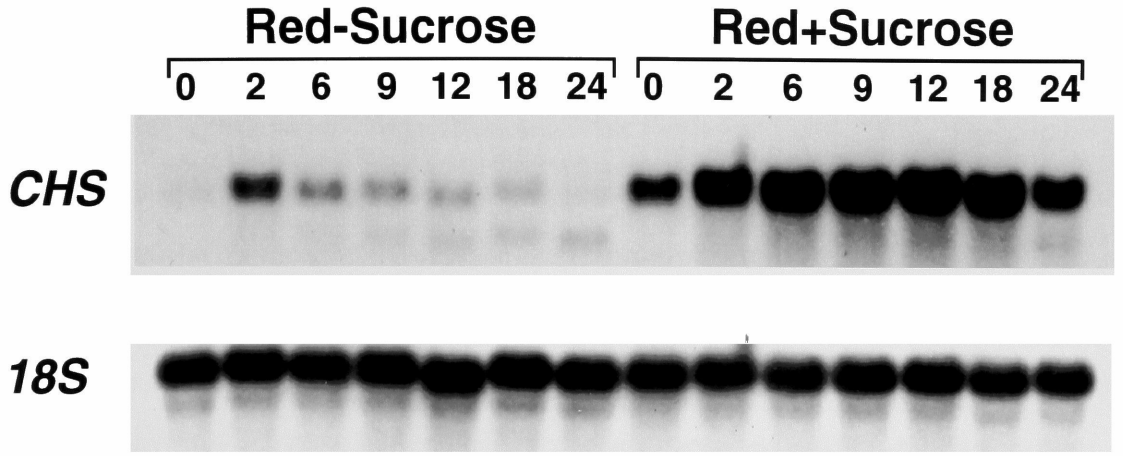
Synergistic interactions between sucrose and phytochrome

The observation that genistein does not effectively inhibit *CHS* expression when phytochrome and sucrose act in concert (Figure 9), suggests that a new, genistein-insensitive induction mechanism has been established. This may reflect a synergistic relationship between sucrose and phytochrome in which both act in concert to promote a beneficial physiological response under certain conditions. For example, under high light conditions, harmful ultraviolet rays may cause severe damage in plant cells. Under these conditions, though, large amounts of sugars would be produced by photosynthesis, and the sucrose produced would act in concert with phytochrome, both acting through cGMP, to produce protective anthocyanins.

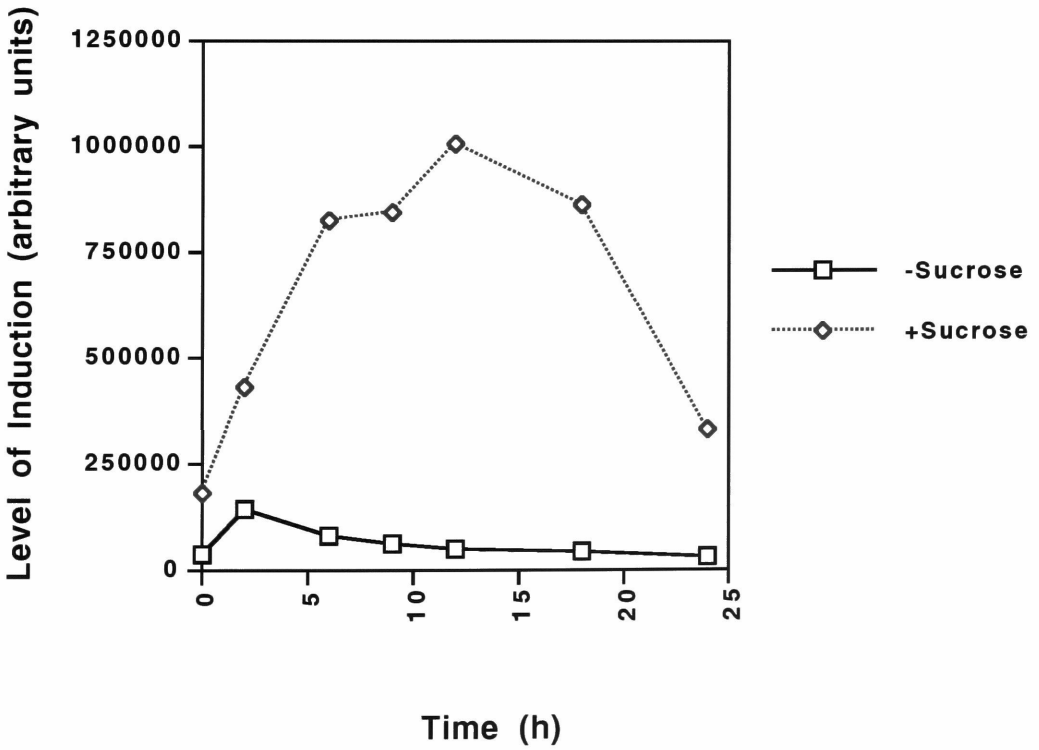
The synergistic relationship between sucrose and phytochrome is easily seen in the induction of *CHS* by R in *Arabidopsis*. Without sucrose present, R induces *CHS* expression transiently, reaching a peak at 2 hours, and declining rapidly thereafter (Figures 11A and 11B). Addition of sucrose to the medium stimulates *CHS* expression to significantly higher levels. In addition, expression persists for 18 hours, declining rapidly by 24 hours (Figures 11A and 11B). The level of induction and dramatic alteration of kinetics is too strong to be explained by a simple additive interaction. Clearly, the two pathways act together in a manner that renders *CHS* expression subject to a different method of regulation.

Figure 11: RNA gel blot analysis of the synergistic interactions of sucrose and phytochrome that regulate *CHS* expression in *Arabidopsis thaliana*. A. Wild type *Arabidopsis* seedlings were grown for 4 days in constant darkness on 1X MS salts with or without 90 mM sucrose. Seedlings were subsequently shifted to constant red light (35 μmol m^{-2} s^{-1}), and samples were collected over the next 24 hours at the time points indicated (hours). The time refers to the onset of light treatment. RNA gel blots were hybridized with a *CHS* cDNA and an *18S* rDNA. B. mRNA levels were quantified by phosphorimage analysis, and *CHS* mRNA levels were normalized to *18S* rRNA levels to correct for differences in loading.

A.


Red-Sucrose
Red+Sucrose
0 2 6 9 12 18 24 0 2 6 9 12 18 24
CHS
18S

B.


Level of Induction (arbitrary units)
1250000
1000000
750000
500000
250000
0
0 5 10 15 20 25
Time (h)
-Sucrose
+Sucrose

Chapter 3

Characterization of a Novel Phytochrome-Dependent Phenotype in *Arabidopsis thaliana*

Genetic analysis of light signal transduction pathways has resulted in the isolation of the photoreceptors phyA (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993), phyB, and cryptochrome, as well as the identification of genetic loci required for phytochrome chromophore biosynthesis (Koornneef et al., 1980; Chory et al., 1989a). None of the putative gain-of-function mutants isolated so far has been demonstrated to specifically affect light signaling, and of the few loss-of-function mutants that have been isolated, only *fhy1* has been shown to disrupt phytochrome-dependent signaling at the morphological level and at the level of gene expression (Whitelam et al., 1993; Barnes et al., 1996b). It is therefore of great interest to isolate new mutants that specifically disrupt phytochrome-dependent signaling.

The genetic screens for mutants that retain elongated hypocotyls in W have likely been saturated (Millar et al., 1994), and none of the screens for gain-of-function mutants that possess light-grown features in the dark has yielded a phytochrome pathway-specific mutant. New transgenic screening approaches have yielded new mutants that may specifically affect phytochrome signaling (Jackson et al., 1995; Li et al., 1995), and physiological analysis of photoreceptor mutants has indicated that it may be possible to screen for new mutants based on well-characterized, phytochrome-mediated responses (Johnson et al., 1994; Bagnall et al., 1995). A third method exploits the pharmacological sensitivity of the phytochrome signaling pathway to various chemicals that disrupt or enhance pathway function. It has been found that the Ca^{2+}- and Ca^{2+}/cGMP-dependent branches of the phytochrome pathway may be specifically disrupted by agents that interfere with Ca^{2+} and CaM activity, like nifedipine and trifluoperazine, respectively (Bowler et al., 1994b). Moreover, the same pathways may be blocked by the general

kinase inhibitor staurosporine. Similarly, the cGMP- and Ca^{2+}/cGMP-dependent branches may be disrupted with cGMP antagonists like Rp-cGMPS, whereas only the cGMP-dependent pathway may be inhibited by the general tyrosine kinase inhibitor genistein (Bowler et al., 1994b). In addition, the cGMP-dependent phosphodiesterase (cGMP PDE) inhibitor zaprinast inhibits the desensitization of the signaling pathway to constant light (Beavo and Reifsnyder, 1990; Bowler et al., 1994b). All of these compounds have been found to have effects at the level of single cells (Neuhaus et al., 1993).

In this section, the effects of zaprinast on phytochrome-mediated processes in *Arabidopsis thaliana* are investigated. Zaprinast was found to induce a novel, phytochrome-dependent phenotype in whole seedlings, alter normal physiological responses to FR, alter cellular ultrastructure, and cause profound effects on phytochrome-regulated gene expression. The effects of zaprinast are investigated under different light regimes and in the context of different mutants that are defective in light signaling. The results suggest a role for a target of zaprinast, likely to be a cGMP PDE, in phytochrome signaling, and a model of the effects of zaprinast on the primary phytochrome signal transduction pathway is proposed.

It was found that when WT seedlings were grown on sucrose in the presence of zaprinast under constant high fluence rate R (35 μmol m^{-2} s^{-1}), they developed a striking phenotype. Under normal conditions, seedlings grown under R possess short hypocotyls and open, expanded green cotyledons. Under these conditions, phytochrome regulates the inhibition of hypocotyl elongation, induction of the genes necessary for proper chloroplast development, and induction of agravitropic growth (Chory et al., 1989a; Robson and Smith, 1996). When grown in the presence of zaprinast, however, hypocotyls are markedly shorter, cotyledons are bleached and relatively unexpanded, and they exhibit an exaggerated agravitropic response (Figure 12). The exaggerated agravitropic response seen in zaprinast-treated seedlings is very interesting because when

Figure 12: The effect of zaprinast on the development of wild type *Arabidopsis thaliana*. Wild type seedlings were grown for 5 days in constant red light (35 μmol m^{-2} s^{-1}) on media containing 1X MS salts with 90 mM sucrose. The two seedlings on the left are untreated controls, and the two seedlings on the right were treated with 175 μM zaprinast for the duration of the experiment. Scale bar = 2 mm.

sucrose is present in the medium, the hypocotyls of light-grown seedlings normally display strong negative gravitropism, growing vertically (0°) (Caspar and Pickard, 1989). These observations suggest that zaprinast causes the seedlings to over-respond to light. Namely, hypocotyl elongation is inhibited to a greater degree, and a stronger phytochrome signal overcomes the gravitropic response stimulated by sucrose. Nevertheless, the phenotype caused by zaprinast may be explained by three different possibilities. First, zaprinast may be generally toxic to young seedlings. Second, the toxicity may be light-dependent, resulting from a breakdown of the drug over extended light treatments, producing toxic degradation products. Third, the toxicity may be dependent on the activity of the phytochrome signaling pathway. It is proposed that a cGMP PDE may mediate cGMP turnover during prolonged light treatment, resulting in the down-regulation of the cGMP pathway (Bowler et al., 1994b). If this PDE were inhibited by zaprinast, cGMP may accumulate to very high levels and cause the dramatic effects seen in zaprinast-treated plants.

Subjective assessment of dose response experiments indicated that 175 μM zaprinast produced the most reproducible response of the concentrations tested. Moreover, it was found that sucrose was absolutely required for the inhibition of hypocotyl elongation and cotyledon bleaching phenotypes (data not shown), but was dispensable for induction of the agravitropic phenotype. It has been demonstrated recently that phytochrome-dependent agravitropic responses are mediated by a VLFR, a response that would be predicted to activate the pathway to very low levels (Robson and Smith, 1996). Thus, the very low levels of cGMP that may accumulate in the presence of zaprinast when no stimulus such as light is applied may be sufficient to stimulate agravitropism. In addition, it was found that effectiveness of the drug decreased at lower fluence rates of R (see below), and constant light treatment was required, as dark intervals during treatment appeared to allow seedlings to recover from the effects of zaprinast (data not shown). To investigate the mechanism by which zaprinast achieves its effects, WT

and mutant *Arabidopsis* seedlings of different genotypes were grown on zaprinast in darkness or under various light conditions.

Effects of zaprinast on hypocotyl elongation

Dark-grown WT seedlings display a typical etiolated appearance, possessing elongated hypocotyls, closed apical hooks, and unexpanded cotyledons (Figure 13). Identical dark-grown phenotypes are possessed by the phytochrome chromophore-deficient *hy1* mutant (Parks and Quail, 1991), a *phyB* null mutant (Reed et al., 1993), the blue light receptor-deficient *hy4* mutant (Ahmad and Cashmore, 1993), a *phyA* null mutant (Reed et al., 1994), a *phyAphyB* double mutant, and the putative phyA signal transduction mutant *fhy1* (Whitelam et al., 1993) (Figure 13). Growth on zaprinast in the dark does not induce dramatic morphological changes in seedlings of any genotype (Figure 13). Seedlings still possess long hypocotyls, closed apical hooks, and unexpanded cotyledons. Despite the fact that the hypocotyls remain long, zaprinast-treated seedlings are measurably shorter by 25-30% (Figure 17A; Table 1). This result may reflect the fact that seeds are treated with high fluence rate white light (40 μmol m^{-2} s^{-1}) to induce germination. $phyB_{fr}$ that is produced by this treatment may be present during early seedling development and stimulate the production of cGMP which causes the observed hypocotyl shortening.

When seedlings are grown in constant R, WT, *hy4*, *phyA*, and *fhy1* seedlings possess short hypocotyls, and open, expanded green cotyledons (Figure 14). By contrast *hy1*, *phyB*, and *phyAphyB* mutants retain elongated hypocotyls, relatively unopened apical hooks, and small, green cotyledons (Figure 14). This disparity results from the fact that phyB is the primary perceiver of R, and because the latter three mutants do not possess normal phyB, they cannot undergo normal photomorphogenesis. Growth of seedlings on zaprinast induces a dramatic phenotype in WT, *hy4*, *phyA*, and *fhy1* seedlings, as they possess extremely short hypocotyls, small, bleached cotyledons, and display exaggerated agravitropism (Figure 14). Measurement of hypocotyl lengths

Figure 13: The effect of zaprinast on the development of dark-grown *Arabidopsis* seedlings. Seedlings of the genotypes indicated to the left of each panel were grown for 5 days in constant darkness on media containing 1X MS salts with 90 mM sucrose, with or without 175 μM zaprinast present in the medium for the duration of the experiment. The two seedlings on the left of each panel are untreated controls, and the two seedlings on the right were treated with zaprinast. Scale bar = 2 mm.

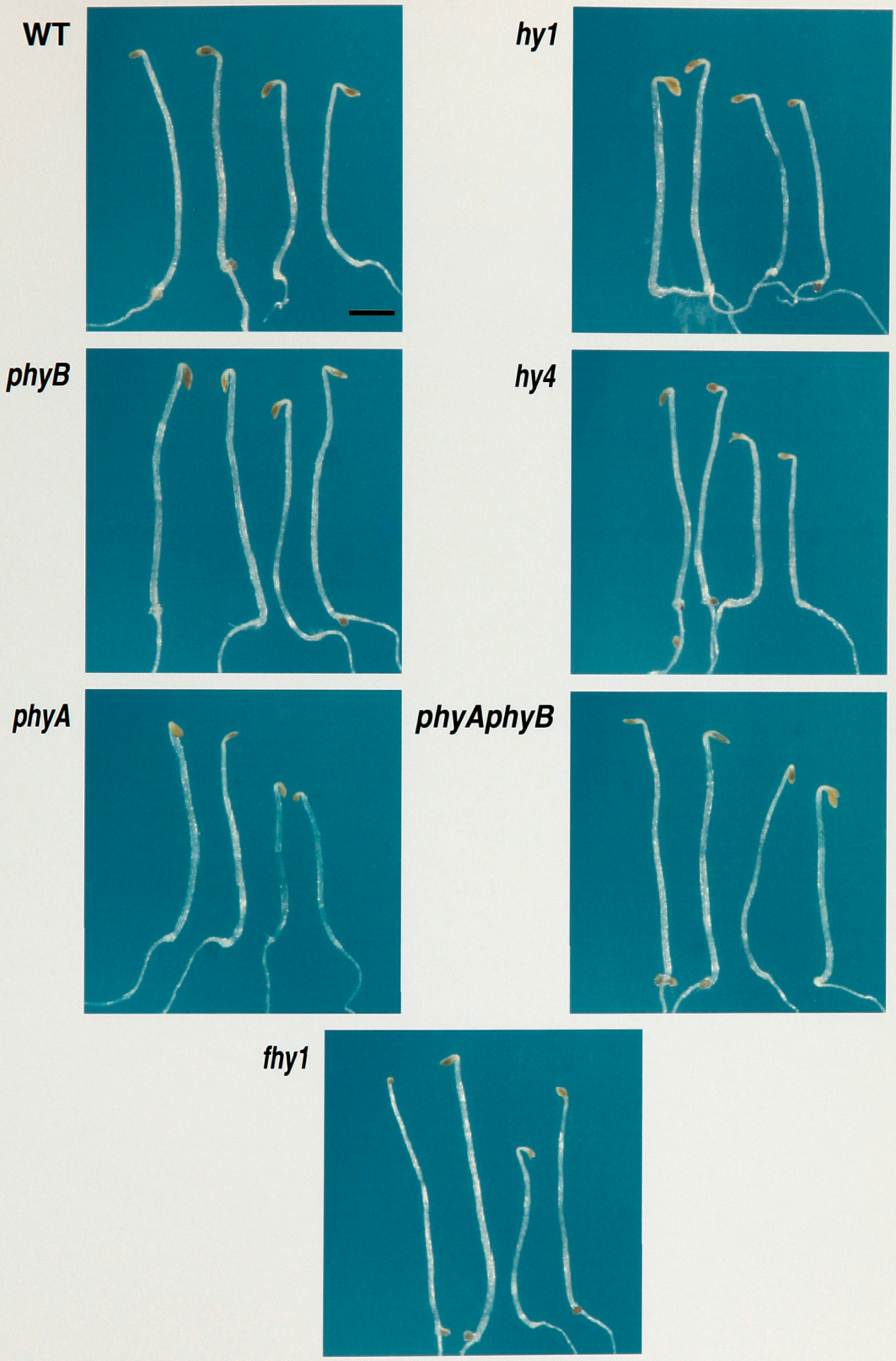
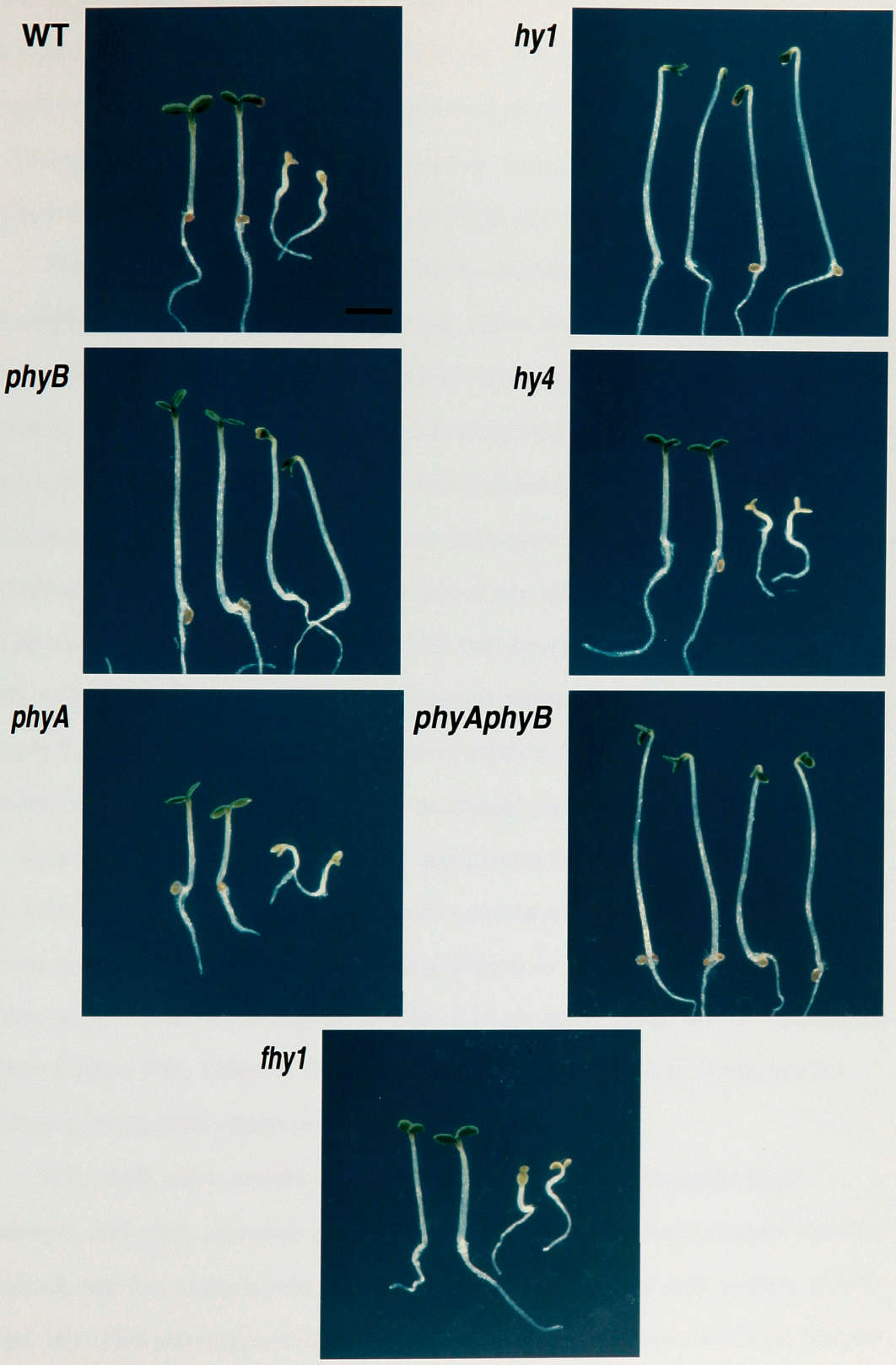

WT
hy1
phyB
hy4
phyA
phyAphyB
fhy1

Figure 14: The effect of zaprinast on the development of red light-grown *Arabidopsis* seedlings. Seedlings of the genotypes indicated to the left of each panel were grown for 5 days in constant red light (35 μmol m^{-2} s^{-1}) on media containing 1X MS salts with 90 mM sucrose, with or without 175 μM zaprinast present in the medium for the duration of the experiment. The two seedlings on the left of each panel are untreated controls, and the two seedlings on the right were treated with zaprinast. Scale bar = 2 mm.


WT
hy1
phyB
hy4
phyA
phyAphyB
fhy1

indicates that these mutants are inhibited approximately 75% by zaprinast (Figure 17B; Table 1). The phyB-deficient mutants, however, still possess longer hypocotyls, green cotyledons, and relatively unopened apical hooks (Figure 14). Moreover, hypocotyl elongation in these mutants is only inhibited by approximately 25% (Figure 17B; Table 1). This value is very close to that observed in dark-grown seedlings (Table 1). These data indicate that zaprinast requires intact phyB to achieve its effects in R.

When grown in constant FR, WT, *phyB*, and *hy4* seedlings possess short hypocotyls, open apical hooks, and expanded, yellow cotyledons (Figure 15). Greening does not occur in FR because the formation of chlorophyll requires red light (Salisbury and Ross, 1985). Seedlings of the *hy1*, *phyA*, and *phyAphyB* genotypes, however, possess long hypocotyls, closed apical hooks, and unexpanded cotyledons (Figure 15). *fhy1* plants also possess long hypocotyls, but they have open apical hooks, and small expanded cotyledons (Figure 15). These four mutants look like etiolated seedlings because they lack phyA, the photoreceptor activated by FR that regulates a FR high irradiance response (HIR), or lack a component of the phyA signaling pathway. When grown on zaprinast, WT, *phyB*, and *hy4* seedlings are dramatically inhibited, possessing extremely short hypocotyls, small cotyledons, and display an exaggerated agravitropic response (Figure 15). Hypocotyl elongation is inhibited by approximately 80% in these plants (Figure 17C; Table 1). By contrast, the FR-insensitive phenotype is not altered by zaprinast in mutants deficient in phyA or phyA signaling (Figure 4). Moreover, hypocotyl elongation in these mutants is inhibited only by 15-20%, a level similar to that seen in dark-grown controls (Figure 17C; Table 1). Thus, it is clear that phyA signaling is required for zaprinast to produce its effects in FR.

WT, *phyB*, *phyA*, and *fhy1* seedlings grown in constant B possess short hypocotyls, and open, expanded green cotyledons (Figure 16). This contrasts with *hy4*, *phyAphyB*, and *hy1* mutants, which possess elongated hypocotyls in B, relative to WT (Figure 16). This phenotype is expected of *hy4*, because it lacks the blue light receptor

Figure 15: The effect of zaprinast on the development of far-red light-grown *Arabidopsis* seedlings. Seedlings of the genotypes indicated to the left of each panel were grown for 5 days in constant far-red light (5 μmol m^{-2} s^{-1}) on media containing 1X MS salts with 90 mM sucrose, with or without 175 μM zaprinast present in the medium for the duration of the experiment. The two seedlings on the left of each panel are untreated controls, and the two seedlings on the right were treated with zaprinast. Scale bar = 2 mm.

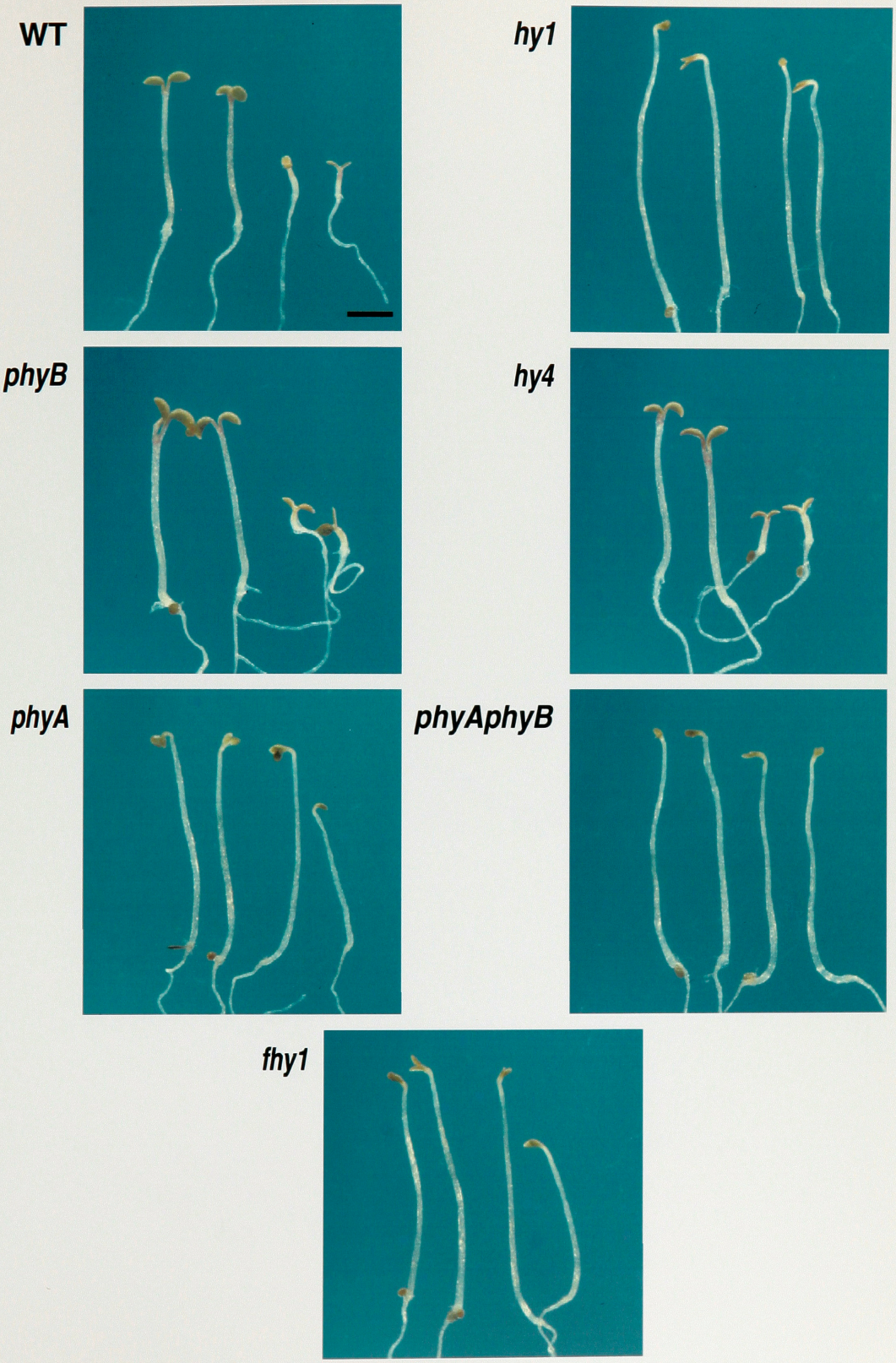
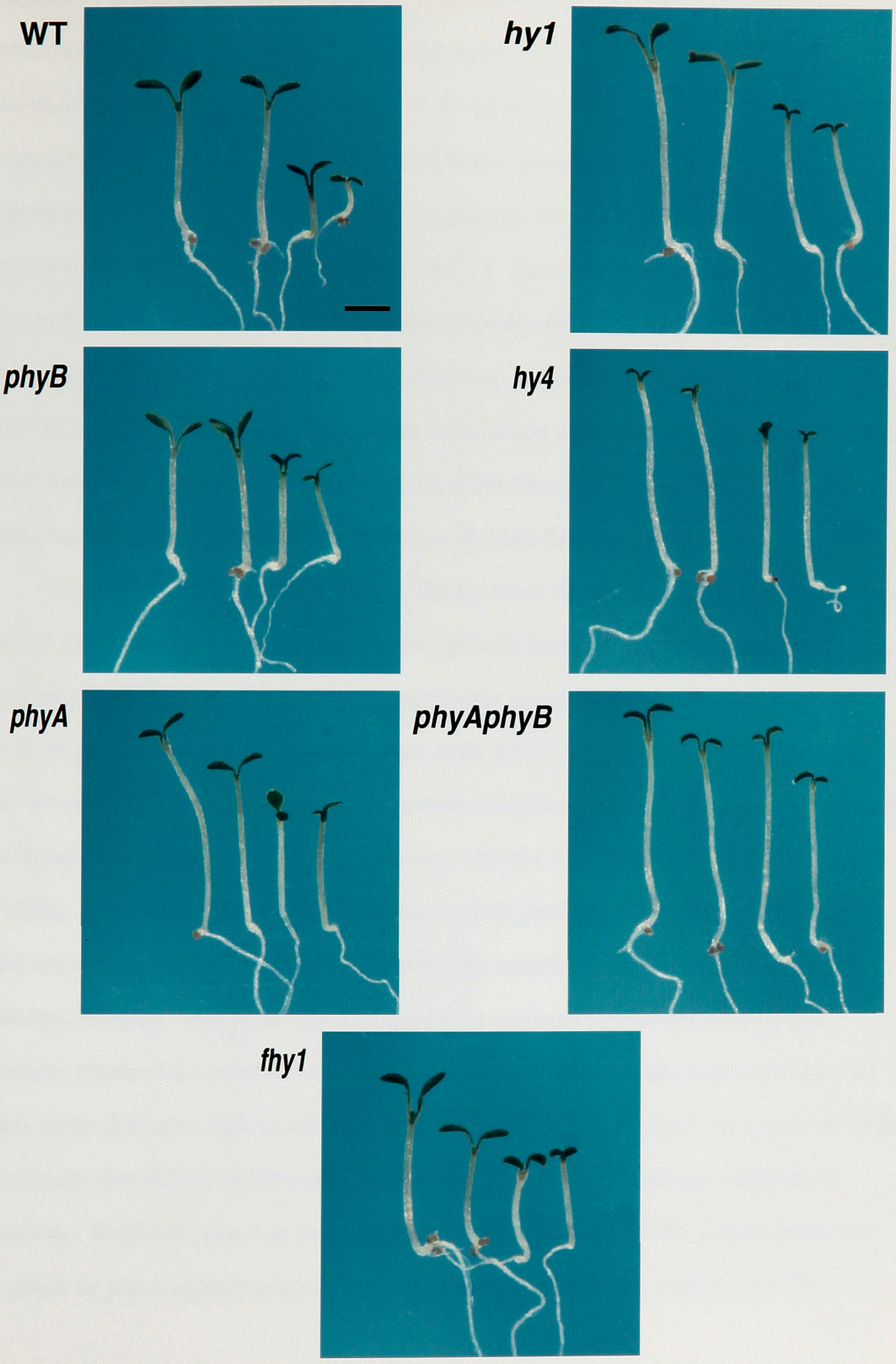

WT
hy1
phyB
hy4
phyA
phyAphyB
fhy1

Figure 16: The effect of zaprinast on the development of blue light-grown *Arabidopsis* seedlings. Seedlings of the genotypes indicated to the left of each panel were grown for 5 days in constant blue light (20 μmol m^{-2} s^{-1}) on media containing 1X MS salts with 90 mM sucrose, with or without 175 μM zaprinast present in the medium for the duration of the experiment. The two seedlings on the left of each panel are untreated controls, and the two seedlings on the right were treated with zaprinast. Scale bar = 2 mm.


WT
hy1
phyB
hy4
phyA
phyAphyB
fhy1

cryptochrome, and is unable to respond to blue light signals. The long hypocotyls possessed by *phyAphyB* and *hy1* mutants is unexpected because they contain intact cryptochrome signaling systems, and should respond normally to B. It is known however, that phytochrome absorbs light in the B region of the spectrum, and may be activated by it (Mancinelli, 1994). When all seven genotypes are grown on zaprinast in the presence of B (20 μmol m^{-2} s^{-1}), their hypocotyls are all significantly shortened by approximately 60% (Figures 16 & 17D; Table 1). The notable exception to this is *phyAphyB*, which is only shortened by approximately 50% (Table 1). These data imply that the hypocotyl shortening induced by zaprinast in constant B requires either phyA or phyB. *hy1* mutants, which are presumably deficient in all phytochromes, are affected in a similar manner to WT, perhaps reflecting the leakiness of this mutation, the penetrance of which may be masked under these conditions by high fluence rate B.

The following conclusions may be drawn from these data. First, it is clear that the effect of zaprinast is not merely the result of general toxicity. Uniform hypocotyl shortening is observed in the dark in all genotypes tested (Table 1), but the dramatic effects on growth that are observed in some light-grown plants are not manifest in the dark. Second, the effect of zaprinast on growth is light-dependent, but it is not a general light-dependent toxicity such as that observed with the herbicide norflurazon (Susek et al., 1993). Rather, the observation that only certain genotypes are affected whereas others are normal under different light conditions rejects the notion of general light-dependent toxicity. Third, the effect of zaprinast is phytochrome-dependent, and moreover, requires the presence of the appropriate type of phytochrome to produce its effects under different light conditions. phyB is the primary mediator of responses to R, and mutants that lacked phyB were found to be insensitive to zaprinast under these conditions. Similarly, phyA is the sole mediator of responses to FR, and mutants that lack phyA or phyA signaling were insensitive to zaprinast when treated with FR.

Figure 17: The effect of zaprinast on the hypocotyl elongation of *Arabidopsis* seedlings grown in darkness, red light, far-red light, and blue light. Seedlings were grown under each light condition as described in the legends to Figures 13-16. A representative sample of these seedlings was plated out on agar plates that were subsequently photographed with a scale bar included. The images were projected, the hypocotyl lengths were measured, and the actual hypocotyl lengths were calculated based on the scale bar contained in the projected image. A. Dark; B. Red light; C. Far-red light; D. Blue light. The hypocotyl lengths represent the mean of 30 seedlings. Standard errors are indicated by the error bars. In cases where no error bar is present, the standard error was too small to be visible in the figure.

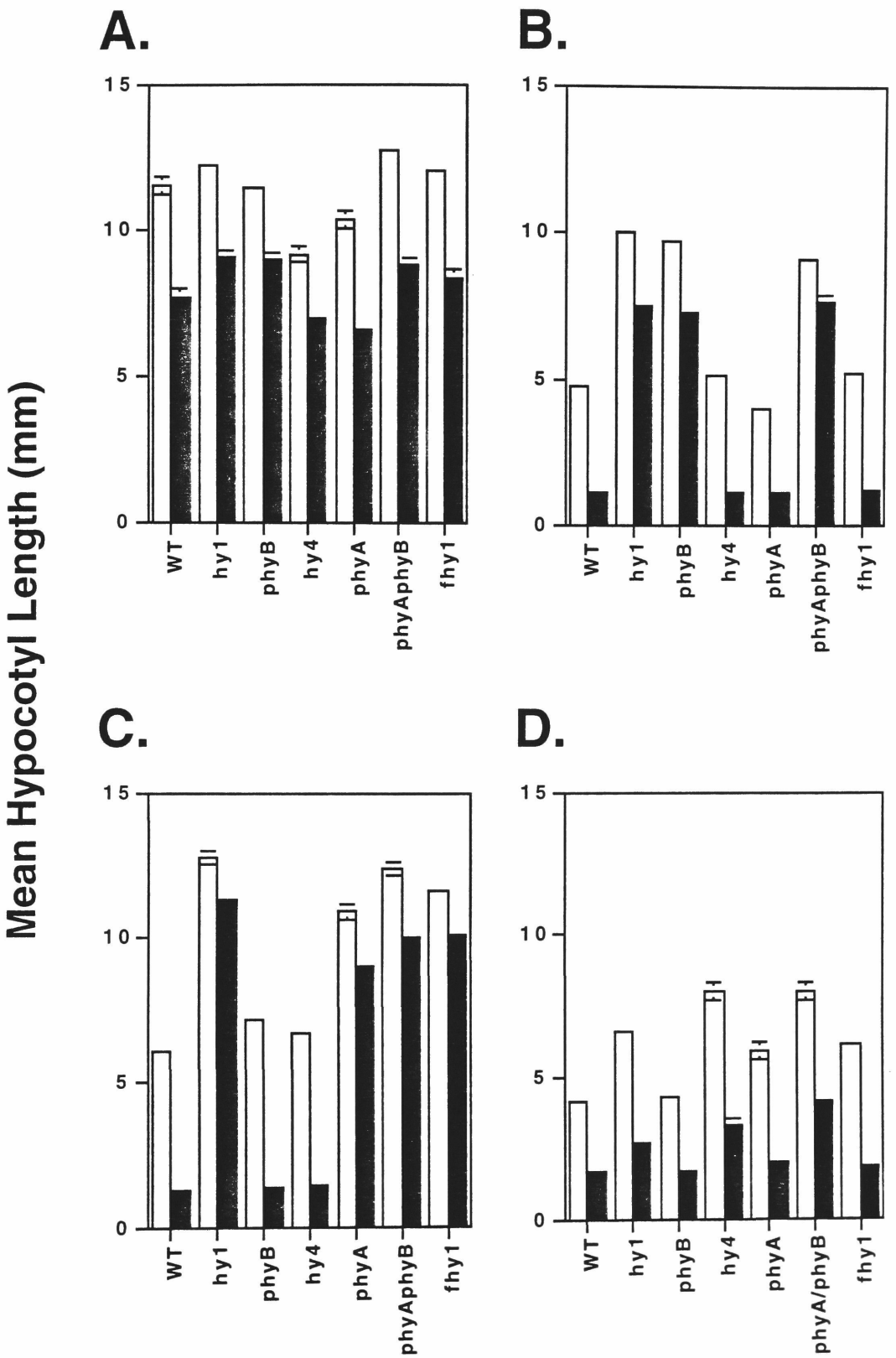

A.
B.
C.
D.
Mean Hypocotyl Length (mm)
15
10
5
0
WT
hy1
phyB
hy4
phyA
phyAphyB
fhy1
phyA/phyB

Table 1: Effect of zaprinast on hypocotyl elongation in the genotypes listed. Seedlings were grown for 5 days in darkness or constant red (35 μmol m^{-2} sec^{-1}), far-red (5 μmol m^{-2} sec^{-1}), or blue (20 μmol m^{-2} sec^{-1}) light. The hypocotyls of zaprinast-treated and untreated seedlings were measured, and the numbers listed in the table represent the percent inhibition by zaprinast relative to untreated controls.

Genotype	Dark	Red	Far-red	Blue
Wild type	33	76	79	60
hy1	26	25	11	60
phyB	21	25	81	61
hy4	24	77	79	60
phyA	36	70	17	66
phyAphyB	30	16	19	48
fhy1	30	77	13	71

It may be argued that mutants with long hypocotyls simply may not be inhibited enough under different light conditions to behave like WT in the presence of zaprinast. The hypocotyls of insensitive mutants, however, were found to be inhibited to the same degree under light-grown conditions as those of all genotypes when treated with zaprinast in the dark. As many of the mutations tested are known null mutations in the photoreceptors, this suggests that any increased sensitivity to zaprinast requires intact photoreceptors or an intact signaling pathway. Second, in the one experiment where they were tested, long hypocotyl *hy5* (Koornneef et al.,1980) mutants were found to possess hypocotyls of equal length to WT when both were grown on zaprinast in R (data not shown). Therefore, even mutants with elongated hypocotyls may be inhibited to the same short length as WT if their phytochrome signaling systems are intact. From these data, it is clear that zaprinast may be used to gauge the activity of the phytochrome signaling pathway under all conditions in which it is active.

The requirement for the inclusion of sucrose in the medium to induce the phenotype observed in zaprinast-treated seedlings raises the question of why phytochrome activation alone is not sufficient to induce the phenotype. Studies of gene expression have demonstrated that phytochrome stimulates its cGMP-dependent pathway transiently and to low levels (Barnes et al., 1996b). The dramatic effects on hypocotyl elongation and cotyledon bleaching produced by zaprinast appear to require prolonged, high level activity of the pathway. It has been found that the ability of phytochrome to stimulate anthocyanin accumulation, and most likely the expression of anthocyanin biosynthetic genes as well, decreases over time under conditions of continuous illumination by light, as the primary wavelengths of light that promote the expression of anthocyanin biosynthetic genes shift from red to blue (Mancinelli, 1983).

The light-dependent induction of anthocyanin biosynthetic genes, which are targets of the cGMP-dependent pathway of phytochrome, is regulated by a developmental program as well, and expression of these genes is inhibited by 4 days after germination

(Kubasek et al., 1992), an event that occurs during the time frame of this experiment. The presence of sucrose in the medium may help overcome the insufficient levels of expression produced by phytochrome alone, and may bypass some of the developmental regulatory mechanisms that appear to repress the activity of the pathway. This may be accomplished by the synergistic interactions of phytochrome and sucrose that result in hyperactivation of this pathway that is observed at the level of gene expression (Figure 11).

Effects of zaprinast on chloroplast development

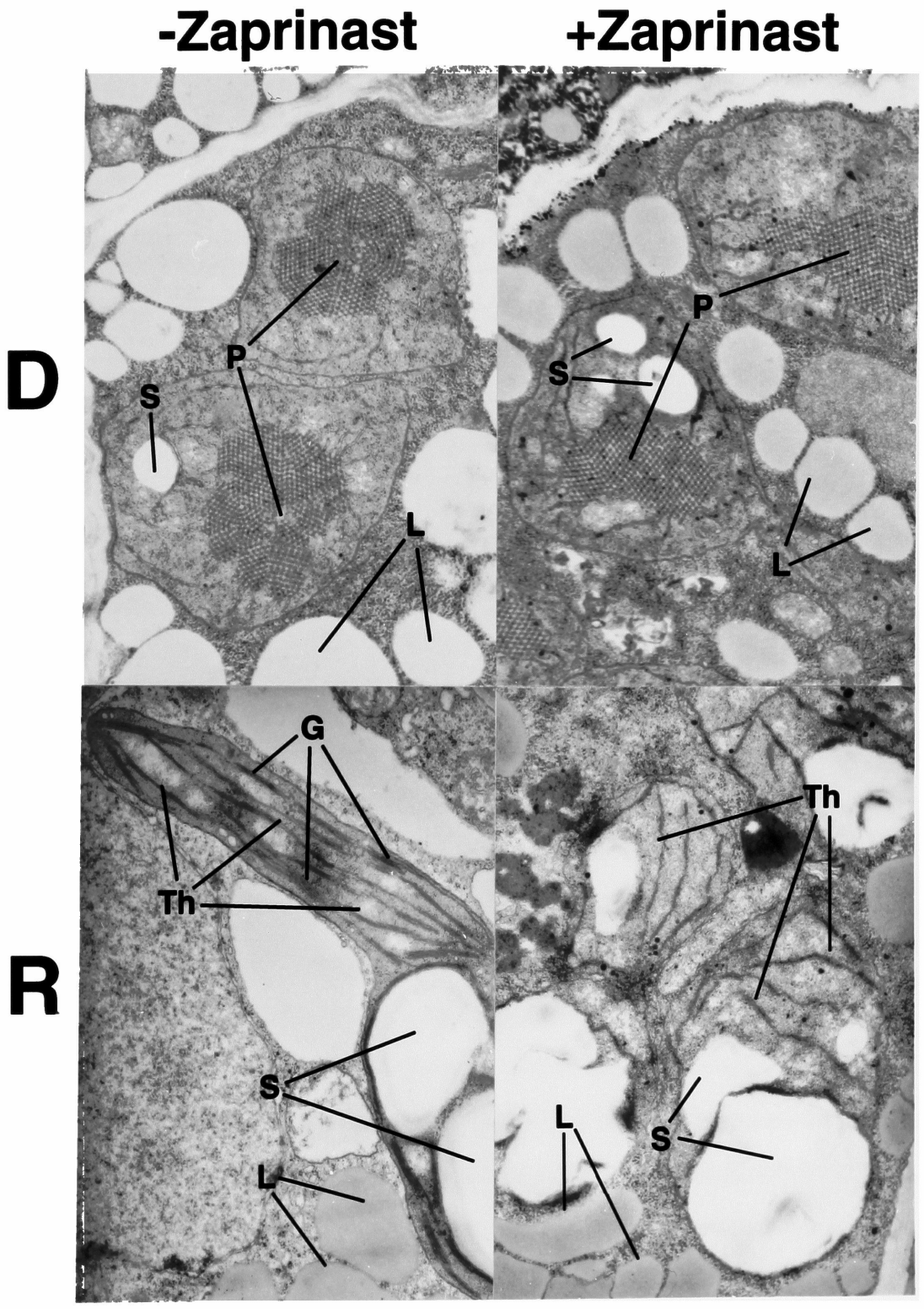
As described above, the effects of zaprinast were not confined to the inhibition of hypocotyl length. One of the most striking phenotypes was the bleached cotyledons possessed by sensitive plants when grown in R (Figure 12) and constant W (data not shown) in the presence of zaprinast. To investigate the defect that causes the cotyledons to appear bleached, cotyledons were examined by transmission electron microscopy. It was found that when WT seedlings were grown in the dark, zaprinast had little effect on the cellular structure (data not shown). This is in stark contrast to the effect of zaprinast when seedlings were grown in constant R (data not shown). The most obvious difference is the presence of large amounts of nutrient storage bodies in the forms of starch granules, lipid droplets, and protein bodies in the cells of light-grown, zaprinast-treated plants. In addition, cells from zaprinast-treated seedlings appear to lack a large central vacuole. In contrast, cells from untreated plants possess very little stored nutrients compared to zaprinast-treated plants, and contain large central vacuoles.

These differences may be reconciled by the observation that zaprinast-treated seedlings do not exhibit any marked growth in the light compared to control seedlings. Because growth does not proceed, there is no need to break down stored nutrients into usable forms. Thus, storage bodies would not disappear over the course of zaprinast treatment. Other experiments have determined that storage bodies do not disappear and subsequently reaccumulate over the 5 day time course, supporting this argument (data not

shown). Similarly, dark-grown zaprinast-treated seedlings do not possess the large number of storage bodies possessed by their light-grown counterparts. This may be explained by the fact that a large amount of cell elongation and division occurs during dark growth. Since zaprinast has little effect on dark growth, breakdown of stored nutrients would occur at a similar pace as that seen in control seedlings, resulting in no observable ultrastructural differences in the dark. The lack of an observed central vacuole in light-grown zaprinast-treated plants may be explained by the fact that the vacuole does not tend to form in plants until most protein storage bodies are broken down (Matile, 1975; Hara and Matsubara, 1980). Therefore, the continued presence of storage bodies in zaprinast-treated plants prevents the normal formation of the vacuole.

Closer analysis of the plastids has suggested the reason why light-grown zaprinast-treated seedlings possess bleached cotyledons. Plastids from dark-grown control and zaprinast-treated seedlings were morphologically indistinguishable from each other (Figure 18). Both plants possessed etioplasts that contained large prolamellar bodies and little thylakoid membrane development. The prolamellar body contains large amounts of protochlorophyllide (PC) and the enzyme protochlorophyllide oxidoreductase, which catalyzes the conversion of PC to chlorophyllide upon light illumination, permitting chlorophyll biosynthesis to proceed (Barnes et al., 1996a). The prolamellar body disappears upon light treatment, and a normal chloroplast containing significant thylakoid membrane development and a large number of grana stacks is formed (Figure 18). This does not occur in light-grown zaprinast-treated seedlings. Plastids of these seedlings do not contain prolamellar bodies, but thylakoid development is significantly retarded, and no grana stacks are formed (Figure 18). Zaprinast does not induce chloroplast development in the dark (Figure 18), consistent with the observation that it does not induce photomorphogenesis in dark-grown seedlings (Figure 13). Moreover, the light-dependent dispersal of the prolamellar body is unaffected by zaprinast (Figure 18). Thus, the primary effect of zaprinast on plastid development is the

Figure 18: Transmission electron microscopic analysis of the effects of zaprinast on plastid development in wild type *Arabidopsis*. Wild type seedlings were grown in darkness or constant red light (35 μmol m^{-2} s^{-1}) for 5 days on media containing 1X MS salts and 90 mM sucrose, with or without 175 μM zaprinast. Representative seedlings were selected from each growth condition, fixed and examined by electron microscopy as described in Materials and Methods. P: prolamellar body; S: starch granule; L: lipid droplet; Th: thylakoid membrane; G: grana stack. Magnification: Dark: 20,000X; Red: 13,850X


-Zaprinast
+Zaprinast
D
R
P
S
L
G
Th

inhibition of thylakoid membrane development, and subsequent grana stack formation. Such poorly developed plastids would produce a bleached cotyledon phenotype.

Effects of zaprinast on phytochrome-dependent CAB *gene expression*

The bleached cotyledons cannot result from a light-dependent modification of zaprinast that renders it toxic to plastids, because earlier experiments demonstrated that zaprinast does not cause this phenotype in the cotyledons of all light-grown plants. Rather, the effect of zaprinast is correlated with phytochrome signaling. In order to determine if zaprinast altered the expression of phytochrome-inducible genes that may be required for proper chloroplast formation, gel blot analysis of RNA isolated from plants grown in constant R or FR in the presence of zaprinast was performed. It was found that in untreated control plants, both R and FR induced the expression of *CAB* to very high levels over a 48 hour time course (Figures 19 and 20B, and 21 and 22B, respectively) . In both cases, expression of *CAB* displayed the circadian oscillations that have been previously observed (Millar and Kay, 1991). In the presence of zaprinast, however, *CAB* expression was markedly reduced in both R and FR (Figures 19, 20B, 21, and 22B, respectively). Zaprinast did not appear to dramatically affect circadian cycling, as peaks and troughs of expression were still apparent. *CAB* genes encode the chlorophyll A/B binding proteins that reside in the thylakoid membranes in the chloroplasts. Therefore, by inhibiting the expression of genes that are required for normal chloroplast assembly and function, zaprinast may cause the bleached cotyledon phenotype that is observed.

The repression of *CAB* expression by zaprinast is not unexpected. It was found previously that zaprinast inhibited the expression of *CAB* genes in soybean cells, and the expression of a *CAB::GUS* fusion construct in microinjection experiments in *au* cells (Bowler et al., 1994b). This inhibition was also caused by high levels of cGMP, or by compounds that stimulated cGMP accumulation like sodium nitroprusside. In each of these cases, the repressive effects of these compounds on *CAB::GUS* expression could be relieved by directly blocking cGMP-dependent processes with the inhibitor Rp-cGMPS

Figure 19: RNA gel blot analysis of the effect of zaprinast on red light-inducible gene expression. Wild type seedlings were grown for 4 days in constant darkness for 4 days on media containing 1X MS salts with 90 mM sucrose, without (-Zaprinast) or with (+Zaprinast) 175 μM zaprinast. Seedlings were then transferred to constant red light (35 μmol m^{-2} s^{-1}). Samples were harvested over the next 48 hours at the times indicated (hours). RNA gel blots were sequentially hybridized with cDNAs of *CHS* and *CAB1*, and an *18S* rDNA.

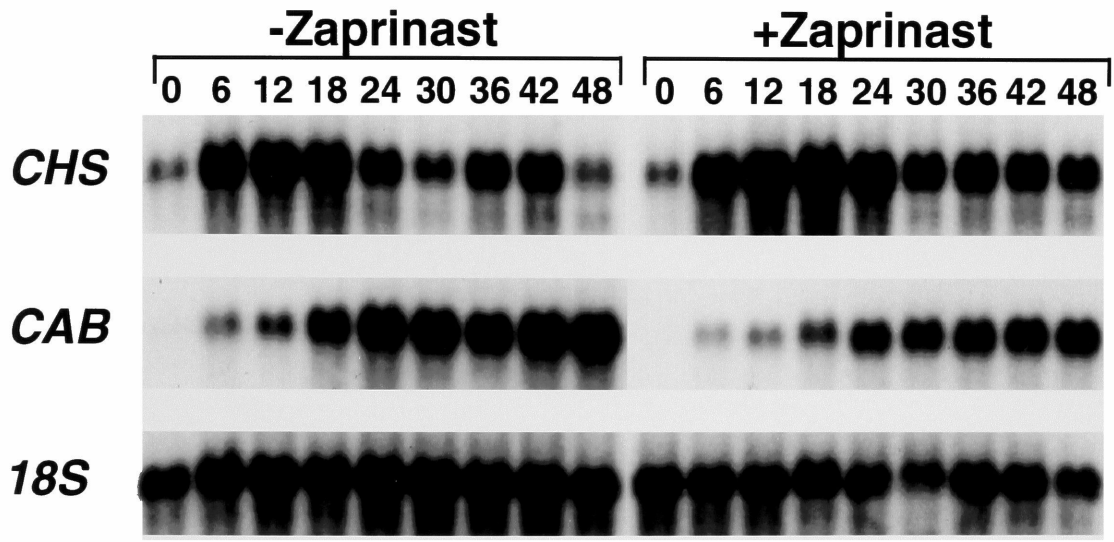
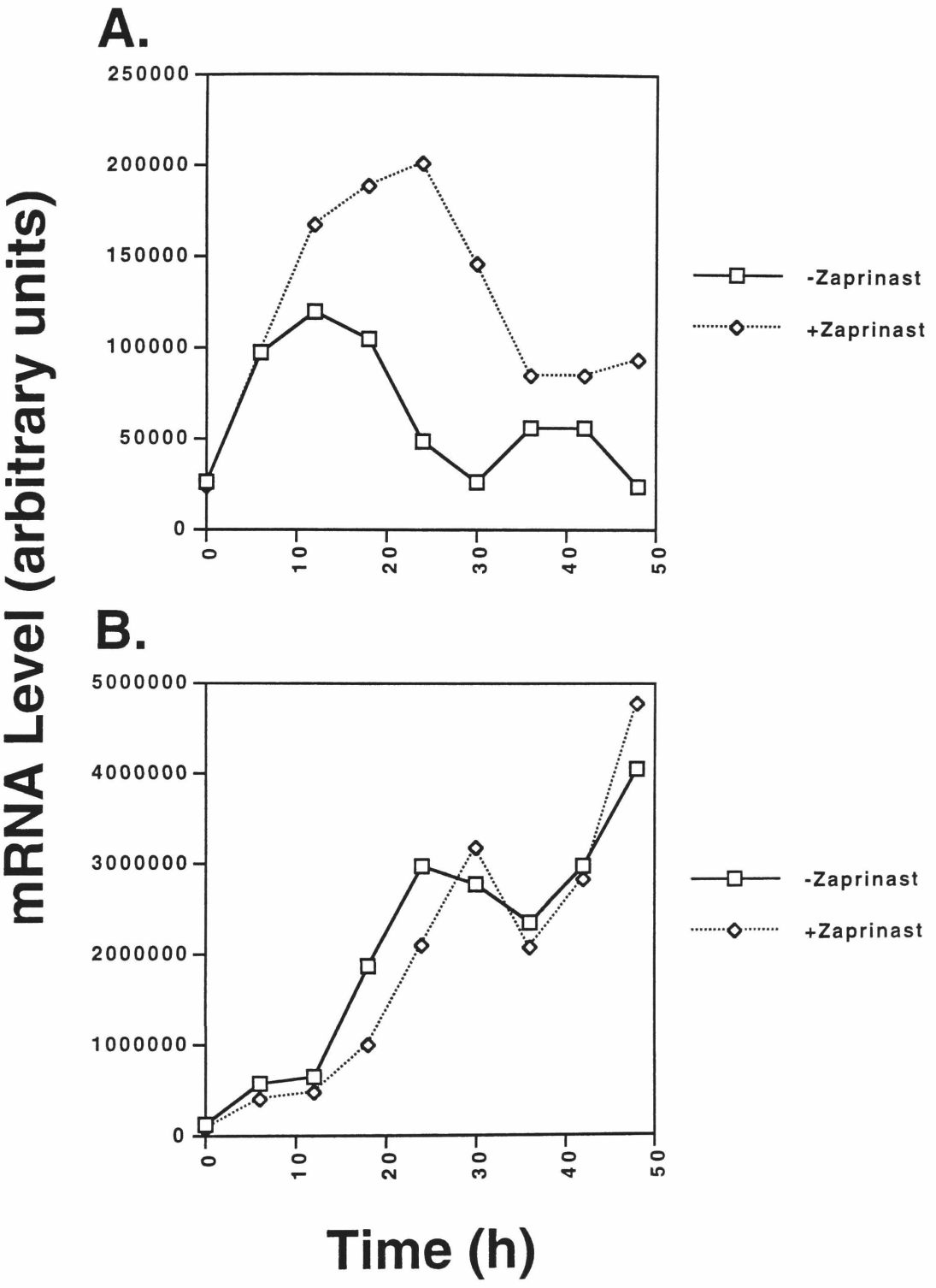

-Zaprinast
+Zaprinast
0 6 12 18 24 30 36 42 48
0 6 12 18 24 30 36 42 48
CHS
CAB
18S

Figure 20: Quantification of the effect of zaprinast on red light-induced *CHS* and *CAB* gene expression. Seedlings were grown and treated with red light as described in the legend to Figure 19. mRNA levels of *CHS* (A.) and *CAB* (B.) were determined by phosphorimage analysis, and were corrected based on *18S* rRNA levels to control for loading errors.


A.
250000
200000
150000
100000
50000
0
0
10
20
30
40
50
-Zaprinast
+Zaprinast
B.
5000000
4000000
3000000
2000000
1000000
0
0
10
20
30
40
50
-Zaprinast
+Zaprinast
mRNA Level (arbitrary units)
Time (h)

(Bowler et al., 1994b). Thus, it appears that zaprinast inhibits *CAB* expression by raising cGMP concentrations in the cell to abnormally high levels. Negative regulation of Ca^{2+}-dependent *CAB* expression by cGMP is termed reciprocal control. Zaprinast was also found to inhibit normal phytochrome-mediated chloroplast development in *au* via cGMP as well (Bowler et al., 1994b). Thus, the data obtained in *Arabidopsis* seedlings extend to the level of whole plants the observations that zaprinast inhibits the expression of genes and development of chloroplasts that is regulated by the Ca^{2+}- dependent pathway of phytochrome. These data clearly suggest that cGMP-dependent reciprocal control functions in whole plants as well.

Effects of zaprinast on phytochrome-dependent CHS *expression*

Zaprinast has been shown to inhibit the desensitization of *CHS* expression during extended light treatments in soybean cells, suggesting that a cGMP PDE regulates activity of the cGMP-dependent pathway of phytochrome (Bowler et al., 1994b). In addition, the effects of zaprinast on *CAB* gene expression suggest that cGMP accumulates to much higher levels in zaprinast-treated plants. To examine the effects of zaprinast on the expression of *CHS* in whole plants, gel blot analysis of RNA isolated from plants treated with constant R or FR was performed. In constant R, *CHS* in untreated plants was induced to high levels, peaking between 6 and 12 hours after treatment commenced (Figures 19 and 20A). Expression rapidly declined shortly thereafter, returning to basal levels by 24 hours. A second small peak of expression that is observed at 36 hours is consistent with the observation that *CHS* expression may be regulated in a circadian manner to some degree (Deikman and Hammer, 1995). Zaprinast stimulated higher levels of *CHS* expression over the entire duration of the time course (Figures 19 and 20A). It was found, however, that despite the fact that levels of *CHS* mRNA were higher overall, they still declined rapidly after 18 hours. Moreover, the kinetics of that decline were similar in both zaprinast-treated and control plants (Figure 20A), indicating that a

Figure 21: RNA gel blot analysis of the effect of zaprinast on far-red light-inducible gene expression. Wild type seedlings were grown in constant darkness for 4 days on media containing 1X MS salts with 90 mM sucrose, without (-Zaprinast) or with (+Zaprinast) 175 μM zaprinast. Seedlings were then transferred to constant far-red light (5 μmol m^{-2} s^{-1}). Samples were harvested over the next 48 hours at the times indicated (hours). RNA gel blots were sequentially hybridized with cDNAs of *CHS* and *CAB1*, and an *18S* rDNA.

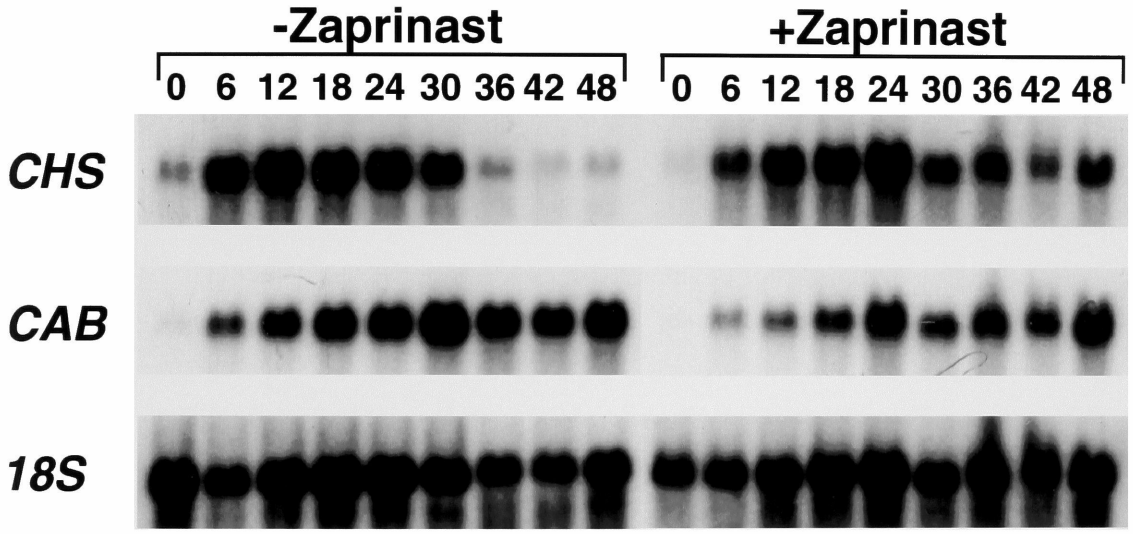
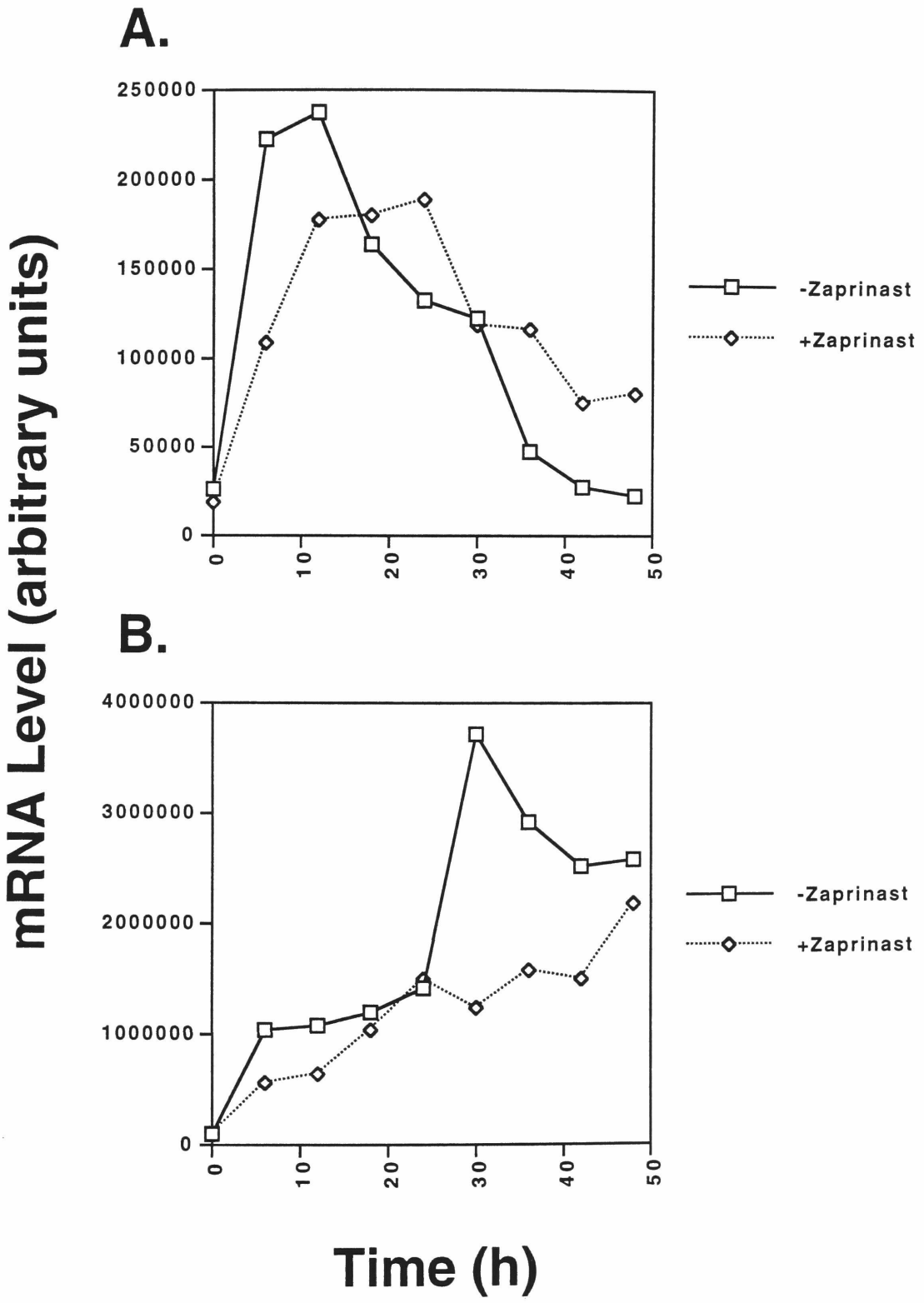

-Zaprinast
+Zaprinast
0 6 12 18 24 30 36 42 48
0 6 12 18 24 30 36 42 48
CHS
CAB
18S

Figure 22: Quantification of the effect of zaprinast on far-red light-induced *CHS* and *CAB* gene expression. Seedlings were grown and treated with red light as described in the legend to Figure 21. mRNA levels of *CHS* (A.) and *CAB* (B.) were determined by phosphorimage analysis, and were corrected based on *18S* rRNA levels to control for loading errors.


A.
250000
200000
150000
100000
50000
0
0
10
20
30
40
50
-Zaprinast
+Zaprinast
B.
4000000
3000000
2000000
1000000
0
0
10
20
30
40
50
-Zaprinast
+Zaprinast
mRNA Level (arbitrary units)
Time (h)

desensitization mechanism operates in R independently of the zaprinast-sensitive component.

In constant FR, *CHS* expression is induced to high levels in untreated controls, reaching maximal expression by 24 hours and declining thereafter, such that expression returns to basal levels by 48 hours (Figures 21 and 22A). Treatment with zaprinast results in higher levels of *CHS* expression with similar amplitude and kinetics over the first half of the time course (Figures 21 and 22A). Whereas *CHS* mRNA levels decline over the second half of the time course in untreated controls, *CHS* expression is maintained at higher levels for an additional 12-18 hours in zaprinast-treated plants before declining. In this case, because zaprinast can delay desensitization for a significant period of time, it is likely that a zaprinast-sensitive target such as a cGMP PDE is at least partly responsible for the declining *CHS* levels observed in control plants.

The normal desensitization kinetics observed in the presence of zaprinast in R as well as the eventual decline of *CHS* expression in FR indicate that another mechanism to negatively regulate this branch of the phytochrome signaling pathway may exist. One obvious possibility is the destruction of phyA. It has been shown that phyA is required for *CHS* induction in R as well as FR (Barnes et al., 1996b), although sucrose can bypass this requirement in *phyA* mutants (Figure 23). In constant R, if phyA is required for induction, sucrose may be able to stabilize $phyA_{fr}$ for an extended period of time, allowing strong induction of *CHS*, before being degraded after 18 hours. Experiments with *phyA* mutants, however, indicate that this is not the case. Figure 23 shows that *CHS* expression is induced in *phyA* mutants in the presence of sucrose, and zaprinast enhances this induction. Nonetheless, just as in the WT, *CHS* levels rapidly decline in *phyA* mutants regardless of the presence of zaprinast (Figures 23 and 24A). Moreover, the kinetics of the decrease of *CHS* expression in *phyA* mutants with and without zaprinast are comparable to those seen in WT (compare Figures 20A and 24A). These data suggest that a third mechanism that negatively regulates the cGMP-dependent branch that is

Figure 23: RNA gel blot analysis of the effect of zaprinast on red light-induced gene expression in *phyA* mutants. *phyA* seedlings were grown in constant darkness for 4 days on media containing 1X MS salts with 90 mM sucrose, without (-Zaprinast) or with (+Zaprinast) 175 μM zaprinast. Seedlings were then transferred to constant red light (35 μmol m^{-2} s^{-1}). Samples were harvested over the next 48 hours at the times indicated (hours). RNA gel blots were sequentially hybridized with cDNAs of *CHS* and *CAB1*, and an *18S* rDNA.

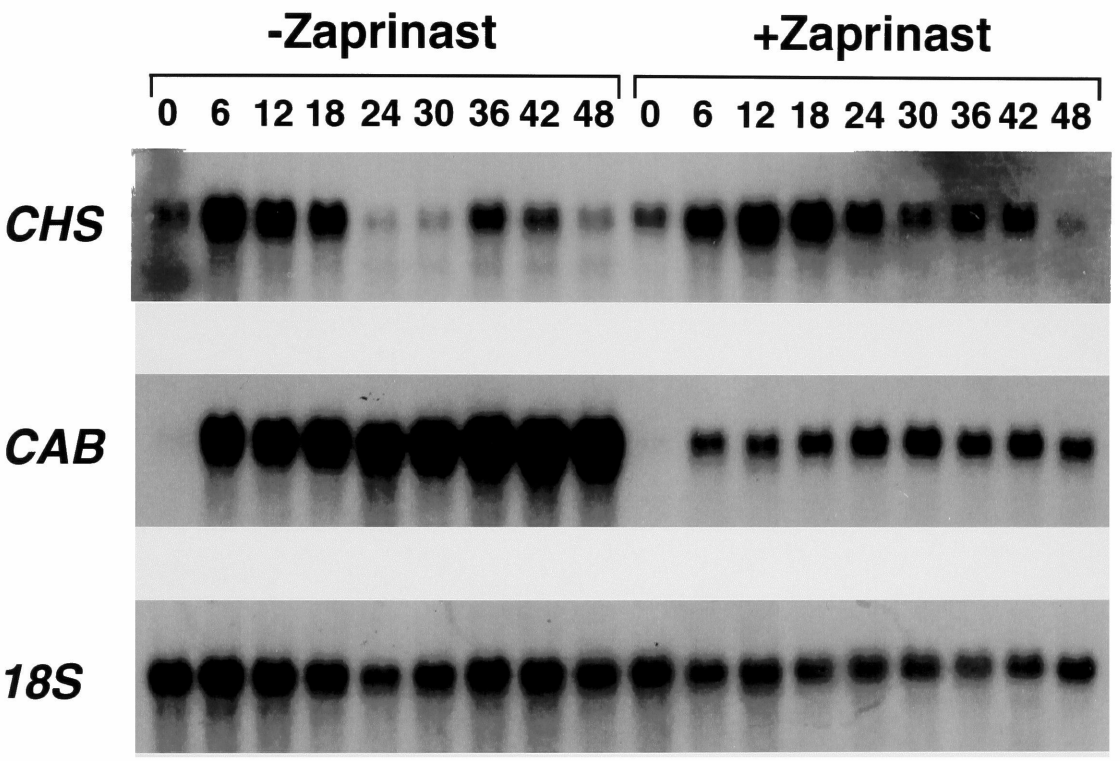
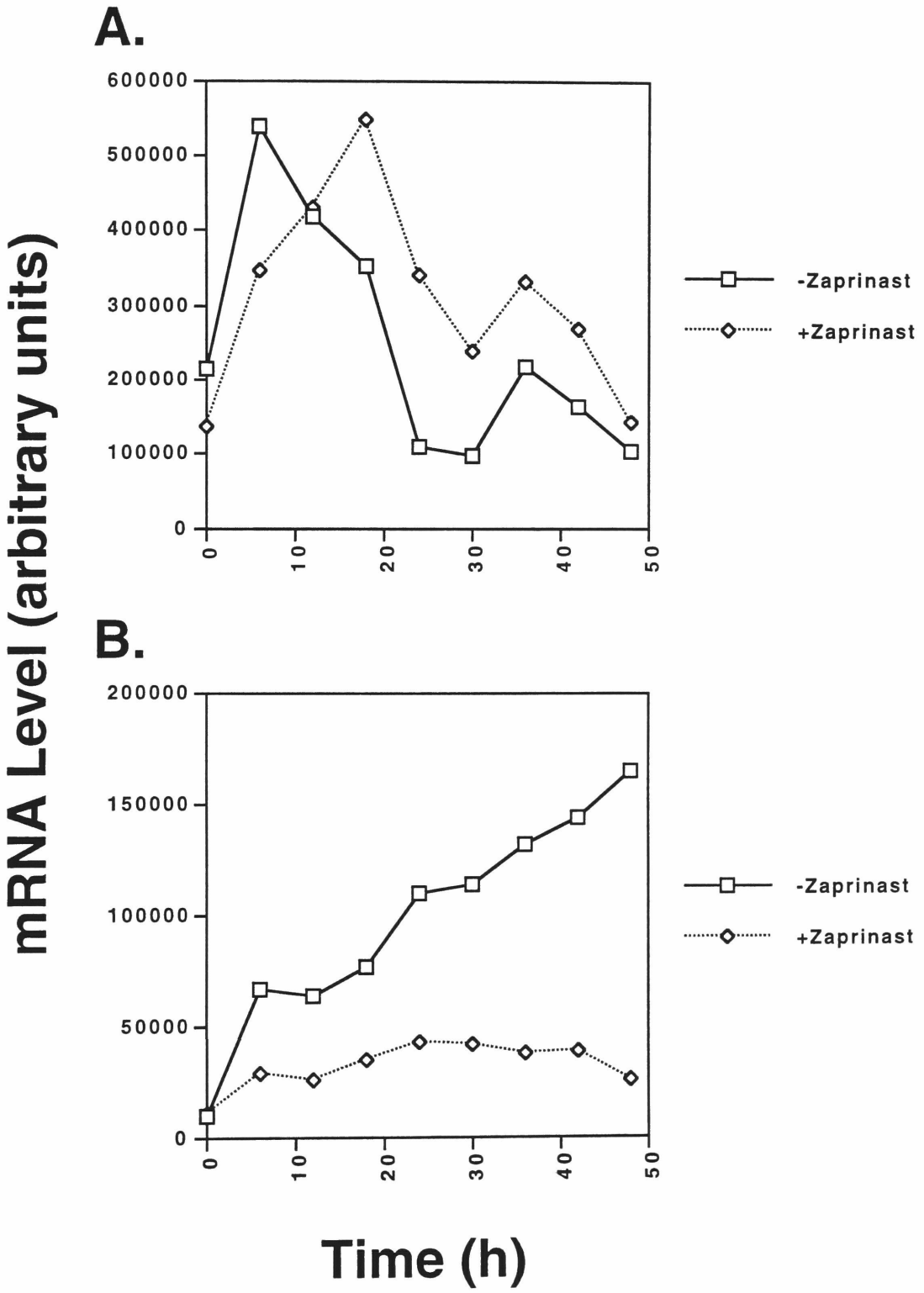

-Zaprinast
+Zaprinast
0 6 12 18 24 30 36 42 48 0 6 12 18 24 30 36 42 48
CHS
CAB
18S

Figure 24: Quantification of the effect of zaprinast on red light-induced *CHS* and *CAB* gene expression in *phyA* mutants. Seedlings were grown and treated with red light as described in the legend to Figure 23. mRNA levels of *CHS* (A.) and *CAB* (B.) were determined by phosphorimage analysis, and were corrected based on *18S* rRNA levels to control for loading errors.


A.
600000
500000
400000
300000
200000
100000
0
0
10
20
30
40
50
-Zaprinast
+Zaprinast
B.
200000
150000
100000
50000
0
0
10
20
30
40
50
-Zaprinast
+Zaprinast
mRNA Level (arbitrary units)
Time (h)

independent of both phyA degradation and cGMP PDE activity is present in plants, functions primarily in R and perhaps to some degree in FR. It is also interesting to note that the expression of *CAB* is still significantly repressed in zaprinast-treated *phyA* mutants, demonstrating that under R conditions, phyA is not a primary mediator of reciprocal control (Figures 23 and 24B).

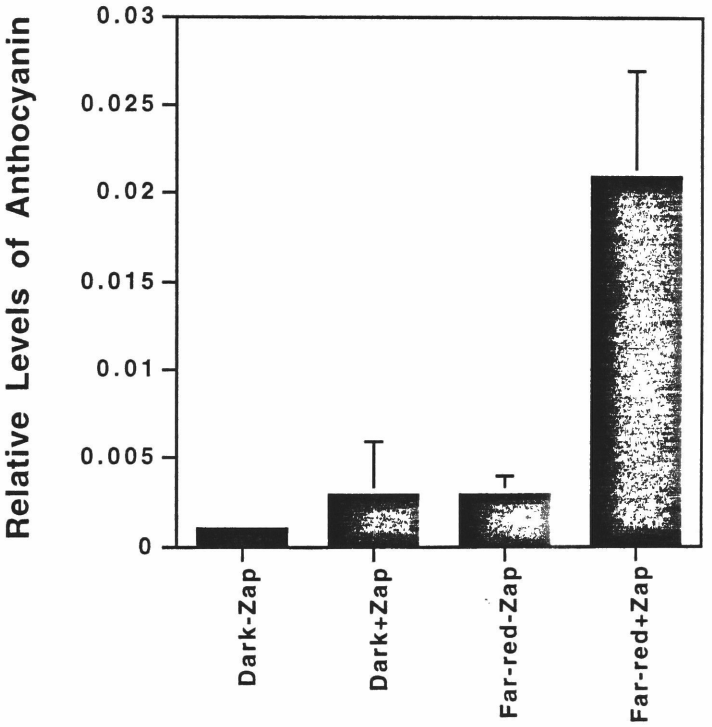
Effects of zaprinast on anthocyanin accumulation

A prediction that may be made based on the expression of *CHS* in the presence of zaprinast is that CHS enzyme levels will be increased. Because CHS performs the first committed step in anthocyanin biosynthesis (Shirley et al., 1995), it is possible that FR-stimulated anthocyanin accumulation may be enhanced. To test this, WT seedlings were grown in the dark on sucrose with or without zaprinast for 4 days, and were subsequently maintained in darkness or shifted to constant FR for an additional 3 days. Anthocyanins were extracted, and it was found that zaprinast had no effect on the accumulation of anthocyanins in the dark (Figure 25). By contrast, zaprinast increased the level of anthocyanin produced in FR by approximately 5 fold (Figure 25). These data confirm that zaprinast is able to enhance phytochrome-mediated responses at both the molecular and physiological levels.

Effects of zaprinast on gravitropic responses

An additional phenotype of zaprinast treatment was the enhanced agravitropic response observed in plants grown on zaprinast in both R and FR. This phenotype was striking because sucrose normally promotes negative gravitropism in the hypocotyls of light-grown plants through the action of statoliths, gravity sensors contained in plastids that are composed entirely of starch (Audus, 1962). Since zaprinast-treated seedlings contain significantly more starch than untreated controls (data not shown), they should be even more likely to exhibit negative gravitropism. This is not the case, however. It has been demonstrated that the agravitropic response of the hypocotyls of *Arabidopsis* seedlings is regulated by phytochrome (Robson and Smith, 1996). Hypocotyls of

Figure 25: Quantification of anthocyanin levels in zaprinast-treated seedlings. Wild type seedlings were grown in constant darkness for 4 days on media containing 1X MS salts and 90 mM sucrose, with or without 175 μM zaprinast. Seedlings were subsequently maintained in darkness or transferred to constant far-red light (5 μmol m^{-2} s^{-1}) for an additional 3 days prior to extraction and quantification of anthocyanins. Relative levels of induction express the difference of the absorbances at 530 nm and 657 nm per 50 seedlings. The levels represent the average of three experiments. Error bars indicate the standard error. Where error bars are not present, the standard error was smaller than can be represented at this scale.


Relative Levels of Anthocyanin
0.03
0.025
0.02
0.015
0.01
0.005
0
Dark-Zap
Dark+Zap
Far-red-Zap
Far-red+Zap

etiolated seedlings grown without sucrose are vertical, displaying negative gravitropism, whereas pulses of R or W can induce hypocotyls to grow at random angles instead of vertical, displaying agravitropism. Recent work has demonstrated that agravitropism can be stimulated by either phyA or phyB (Robson and Smith, 1996). For that reason, it is of interest to characterize the gravitropic phenotype induced by growth on zaprinast.

WT seedlings were grown vertically on plates without sucrose, with or without zaprinast, in constant darkness for 5 days. Subsequently, the plates were photographed, and the angles of the hypocotyls were measured relative to vertical. It was found that without zaprinast, hypocotyls of WT seedlings displayed strong negative gravitropism. The frequency distribution of seedlings growing at various angles displays strong clustering around vertical (0°) (Figure 26A). An accurate measure of the degree of agravitropism is expressed by the standard deviation of the distribution of the angles of the hypocotyls of seedlings relative to vertical (Liscum and Hangarter, 1993; Robson and Smith, 1996). In the case of the untreated seedlings, the standard deviation of 15° is very similar to that observed in other studies (Liscum and Hangarter, 1993; Robson and Smith, 1996; Table 2). In contrast, zaprinast induced the hypocotyls of seedlings to grow at a wide range of angles in the absence of light. The frequency histogram of these seedlings demonstrates that there is a random distribution of angles relative to vertical (Figure 26B). The calculated standard deviation of 70° is very similar to that observed for light-grown seedlings (Liscum and Hangarter, 1993; Robson and Smith, 1996; Table 2). Therefore, it appears that zaprinast is able to induce this phytochrome-dependent phenomenon in the absence of light.

It has been found that the incidence of increased randomization of hypocotyl angles in the dark results from a VLFR produced by the photoconversion of P_r to P_{fr} in seeds prior to germination (Robson and Smith, 1996). Thus, it is possible that zaprinast prevents the degradation of cGMP that may be produced by this VLFR, and an agravitropic response results. To test this, phytochrome-deficient mutants were examined

Figure 26: The effect of zaprinast on gravitropic growth in wild type. Wild type seedlings were grown in constant darkness on vertical plates for 5 days on media containing 1X MS salts, without (A.) or with (B.) 175 μM zaprinast. The plates were photographed, the images were projected, and the orientations of hypocotyls relative to vertical (0 °) were determined. Hypocotyls growing to the left of vertical are designated negative (-), and those growing to the right of vertical are designated positive (+). The results are expressed as a histogram of the number of seedlings growing at each angle relative to vertical.

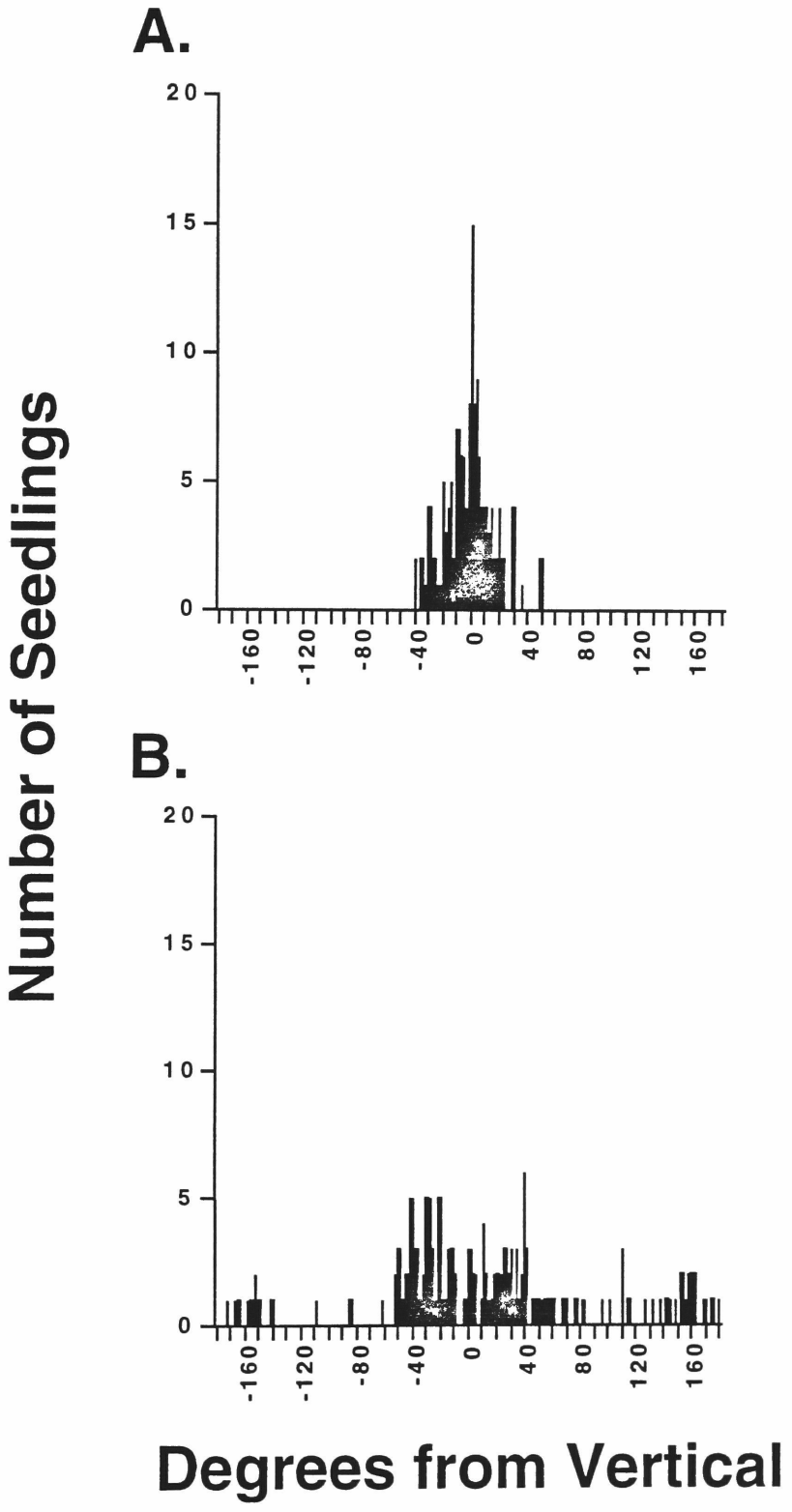

A.
B.
Number of Seedlings
Degrees from Vertical
20
15
10
5
0
-160
-120
-80
-40
0
40
80
120
160

Table 2: Effect of zaprinast on gravitropism in etiolated *Arabidopsis* mutants deficient in phytochrome or phytochrome signaling. Seedlings were grown for 5 days in constant darkness in the absence or presence of 175 μM zaprinast. The angles of the hypocotyls relative to vertical were subsequently measured. SD refers to the standard deviation of the frequency distribution of the angles of plants relative to vertical (0°). The angles of at least 175 seedlings in each treatment were measured in each experiment. Low SD values reflect strong negative gravitropism whereas high SD values reflect more random distributions.

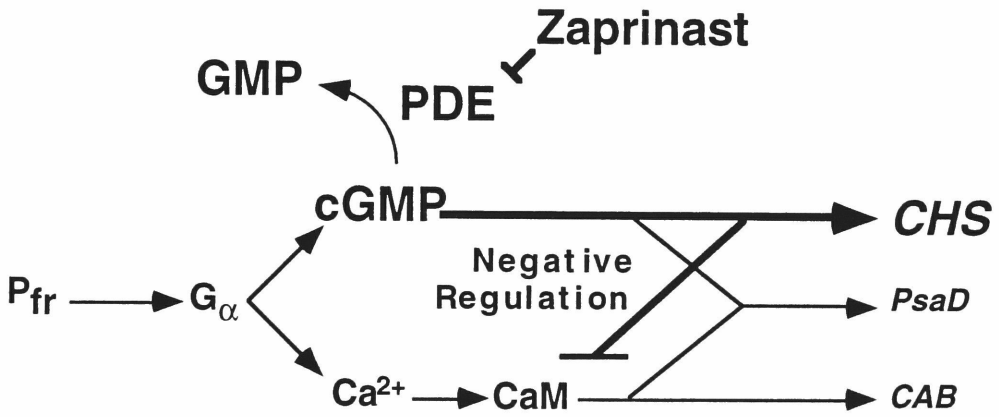
Genotype	SD
Wild type-zaprinast	15°
Wild type+zaprinast	70°
phyA-zaprinast	14°
phyA+zaprinast	71°
phyB-zaprinast	15°
phyB+zaprinast	76°
phyAphyB-zaprinast	16°
phyAphyB+zaprinast	72°
fhy1-zaprinast	13°
fhy1+zaprinast	85°

for their agravitropic responses to zaprinast. *phyA*, *phyB*, and *phyAphyB* mutants all displayed strong negative gravitropism in the dark in the absence of zaprinast (Table 2). By contrast, zaprinast induced the randomization of hypocotyl growth in each of these mutants in the absence of light (Table 2). These results are particularly striking in the case of the *phyAphyB* double mutant because it has been found that light cannot induce randomization in these plants (Robson and Smith, 1996). The phyA signal transduction mutant *fhy1* was also tested, and it was found that it displayed normal negative gravitropism in the absence of zaprinast (Table 2). Like the other mutants, the hypocotyls of *fhy1* plants were similarly randomized by zaprinast (Table 2).

These data demonstrate that the stimulus for the agravitropism observed in zaprinast-treated seedlings does not originate from phyA or phyB. Because *phyAphyB* double mutants do not display randomization even in the light, it is thought that phyC, phyD, and phyE do not contribute to gravitropic responses. Moreover, the sensitivity of *fhy1* to zaprinast demonstrates that the signaling pathway that regulates gravitropism does not require FHY1 for normal activity. It is interesting to note that the *cop4* mutant of *Arabidopsis* which possesses an open apical hook and expanded cotyledons in the dark, as well as expression of some light-regulated genes, also displays aberrant shoot gravitropism (Hou et al., 1993). The reason for the agravitropic phenotype that is induced by zaprinast is not clear. Since there is some inhibition of hypocotyl elongation in the dark in addition to the induction of agravitropism, the two phenomena may be related. Both may result from cGMP that accumulates during development independently of light, although the signaling pathways that may produce cGMP are unknown. Conversely, either or both responses may result from non-specific effects of zaprinast that occur by a mechanism that is currently undefined.

The phenotypes displayed by zaprinast-treated plants may all be explained by the hypothesis that zaprinast induces overactivation of the cGMP-dependent branch of the phytochrome signal transduction pathway (Figure 27). In this model, cGMP levels would

Figure 27: Model of the effects of zaprinast on the phytochrome signal transduction pathway in *Arabidopsis thaliana*.


Zaprinast
GMP
PDE
cGMP
CHS
Negative
Regulation
Pfr
Gα
PsaD
Ca2+
CaM
CAB

accumulate to very high levels, and the plant would respond to them in a manner proportional to those levels. It has been found that plants grown under higher fluence rates of light are shorter than those grown under low fluence rates (Mancinelli, 1994)(see below). In this situation, increased flow through the signaling pathway causes the plant to inhibit hypocotyl elongation to a greater degree. Similarly, in plants that contain zero, one, two, or four copies of the *PHYB* gene, those that contain four copies are shorter than those with no *PHYB*, and those with one or two copies possess intermediate lengths (Wester et al., 1994). Clearly, plants are exquisitely sensitive to the amount of signal produced by phytochrome. The high levels of cGMP caused by zaprinast also inhibit proper chloroplast development and *CAB* gene expression through a cGMP-dependent reciprocal control mechanism, as has been seen in soybean cells and microinjection experiments in *au* hypocotyls (Bowler et al., 1994b). Consistent with its proposed role as an inhibitor of cGMP PDEs, zaprinast inhibits the desensitization of *CHS* to FR, and increases the expression level of *CHS*. This results in 5-fold greater levels of anthocyanin produced in FR. Finally, zaprinast induces agravitropic responses in dark-grown plants, possibly through a cGMP-dependent mechanism. These results demonstrate that zaprinast may be a powerful tool for assaying the function of the cGMP-dependent branch of the phytochrome signal transduction pathway, and may be useful for the isolation of mutants that specifically affect this branch.

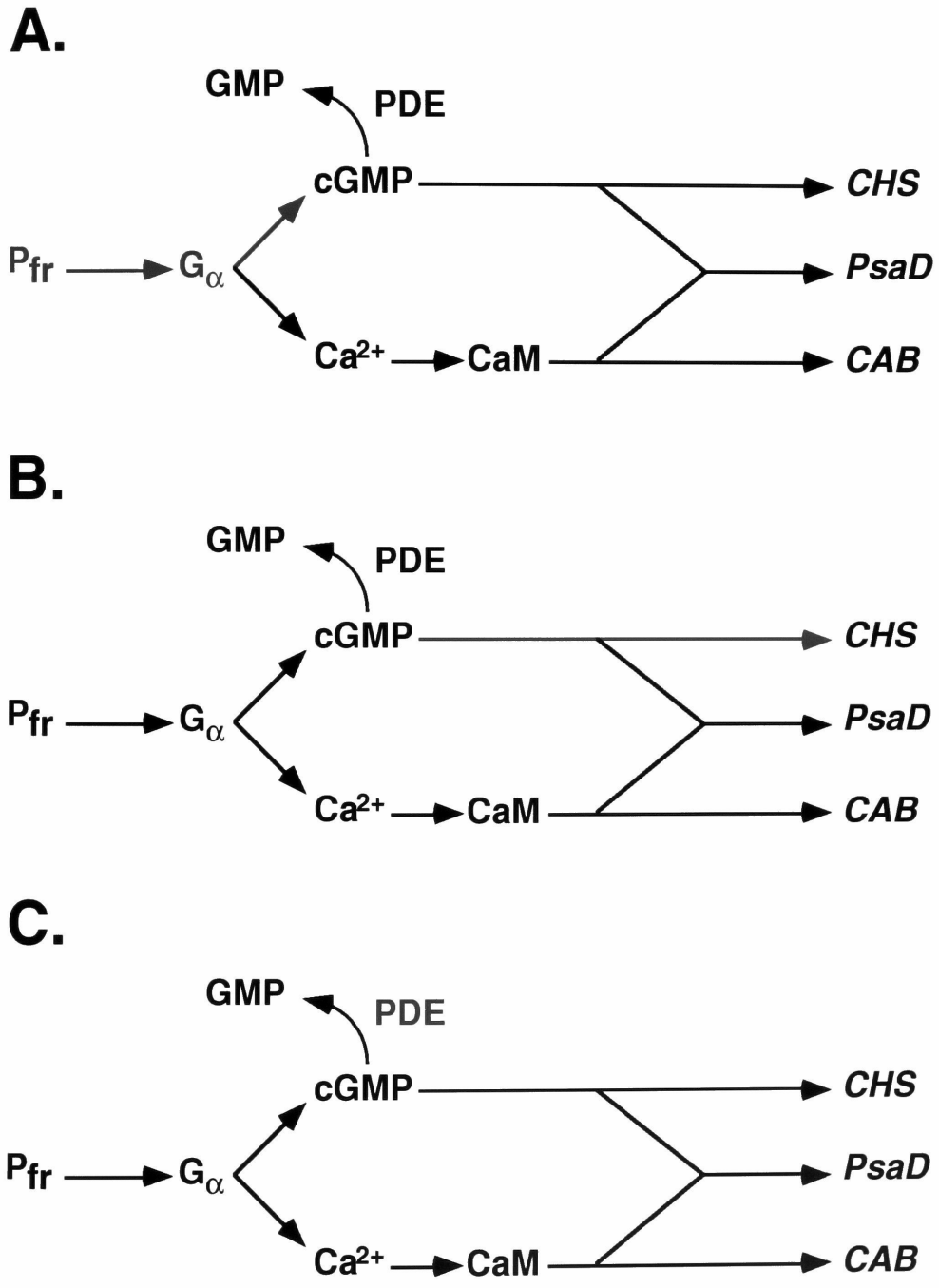
Chapter 4

Isolation and Characterization of Zaprinast-Resistant Mutants of *Arabidopsis thaliana*

The phenotype induced by the cGMP-dependent phosphodiesterase inhibitor zaprinast in WT seedlings of *Arabidopsis thaliana* clearly requires the activity of phytochrome. The fact that mutants that are deficient in phytochrome or phytochrome signaling are resistant to the effects of zaprinast under different light conditions suggests that phytochrome signaling mutants may be isolated based on resistance to zaprinast. Given the likelihood that zaprinast achieves its effects by allowing cGMP to accumulate to very high levels, it is possible that mutations that specifically affect the cGMP-dependent branch of the phytochrome signaling pathway may be isolated. Moreover, the resistance of phytochrome-deficient mutants to zaprinast under R (*phyB*) and FR (*phyA*) suggests that mutations that affect phytochrome-dependent cGMP production in response to light may be recovered. Such mutations would affect components that act early in the pathway, such as phytochrome itself, a heterotrimeric GTP-binding protein (G protein), or an as yet unidentified component (Figure 28A). Additionally, components that act later in the pathway, such as guanylyl cyclase, could also be affected. One could distinguish between mutations in early acting and late acting components based on patterns of gene expression. Mutations that affect early steps would very likely affect the expression of genes that are targets of both the cGMP- and Ca^{2+}/CaM-dependent signaling branches. A mutation that affects a component that functions later in the pathway may specifically affect the expression only of genes that are the targets of the cGMP-dependent branch.

A second class of mutations would have no effect on cGMP production, but would affect downstream signaling components that are directly or indirectly regulated by cGMP, such as kinases, phosphatases, or transcription factors (Figure 28B). These

Figure 28: Regions of the phytochrome signaling pathway that may be mutated in zaprinast-resistant mutants of *Arabidopsis*. A. Early-acting components that regulate cGMP production. B. Components whose activities are regulated by cGMP. C. cGMP-dependent phosphodiesterase. Elements indicated in red are those affected by mutations.


A.
GMP
PDE
cGMP
CHS
Pfr
Gα
PsaD
Ca2+
CaM
CAB
B.
GMP
PDE
cGMP
CHS
Pfr
Gα
PsaD
Ca2+
CaM
CAB
C.
GMP
PDE
cGMP
CHS
Pfr
Gα
PsaD
Ca2+
CaM
CAB

mutations would cause reduced expression of target genes of the cGMP-dependent pathway, and would be predicted to have little effect on the activity of the Ca^{2+}/CaM-dependent pathway. A third class of mutations would directly affect the cGMP PDE itself (Figure 28C). Such a mutation would render the PDE insensitive to zaprinast, but not affect intrinsic phophodiesterase activity. This would allow normal cGMP turnover in the presence of the inhibitor. Mutations of this sort would be expected to have little effect on the expression of cGMP-dependent pathway target genes. In addition, the absolute requirement for sucrose to induce the zaprinast phenotype suggests that mutations that alter the integration of phytochrome and sucrose signals may be isolated as well. Such mutants would be distinguished by normal expression of phytochrome-regulated genes in the absence of sucrose, whereas stimulation of gene expression by sucrose may be altered. Finally, mutations that affect the uptake of zaprinast from the medium may be recovered as well.

In this study, a genetic screen was performed in order to isolate new mutations that disrupt phytochrome signaling. Four mutants were recovered from the screen, and two were characterized in detail. These two mutants were found to be allelic, and affected phytochrome-dependent processes at the physiological level. It was found that the zaprinast-resistant mutants flowered later than WT plants, so previously identified late-flowering mutants were tested for zaprinast sensitivity as well. Of those tested, two were found to be resistant to zaprinast, and one was characterized in further detail. Studies of gene expression in the mutant demonstrate that phytochrome-dependent signaling is not altered, but the integration of sucrose signals with those of phytochrome is affected.

Isolation of zaprinast-resistant mutants of Arabidopsis thaliana

The Landsberg *erecta* (L(*er*)) ecotype of *Arabidopsis* was selected for screening because the majority of photomorphogenic mutants isolated to date are of this ecotype (Koornneef et al., 1980; Whitelam et al., 1993), and problems of ecotype differences

would be avoided in any subsequent double mutant studies. Moreover, tests of other ecotypes for zaprinast sensitivity indicated that drug sensitivity is ecotype dependent. For instance, the Columbia ecotype is sensitive to zaprinast, whereas the WS ecotype is not (data not shown). In addition, polymorphic genetic markers have been identified in L(*er*), making it useful for subsequent genetic mapping (Bell and Ecker, 1994). To isolate zaprinast-resistant mutants, 150,000 EMS-mutagenized M2 seeds representing 37,500 M1 parents were screened. Seedlings were grown on media containing MS salts, 90 mM sucrose, and 175 μM zaprinast for 5 days under constant high fluence rate R (35 μmol m^{-2} s^{-1}). Mutants were selected as plants possessing elongated hypocotyls, large, expanded green cotyledons, and reduced agravitropism relative to WT plants when grown in the presence of zaprinast. By this selection scheme, 150 putative mutants were selected and transferred to soil. As predicted, several of these putative mutants possessed phenotypes similar to *hy* mutants (data not shown), retaining elongated hypocotyls and closed apical hooks, demonstrating that photoreceptor-deficient mutants may be recovered in this screen. Seeds were harvested from the 50 plants that survived, and were rescreened under identical conditions. Of these, 4 lines retained zaprinast resistance in the M3 generation, and were selected for further characterization. These 4 plants were isolated from 3 independent pools, and were designated ZE20-1, ZE20-2, ZE35-2, and ZE47-1 (for zaprinast screen *E*MS pool #-isolate).

As adult plants all 4 mutants possess very similar phenotypes that are quite different from WT, in that they are quite large, fertile plants, and have reduced apical dominance that is reflected by increased numbers of secondary inflorescences relative to WT (data not shown). The fact that the ZE20-1 and ZE20-2 mutants are so similar and originate from the same pool suggests that these two mutants may be derived from the same mutational event. ZE35-2 and ZE47-1 were selected for backcrossing and further characterization. F_1 seedlings resulting from the backcross of ZE35-2 or ZE47-1 to WT (WT plants were used as the pollen donor) were all sensitive to zaprinast in R (Table 3).

Table 3: Genetic analysis of zaprinast resistant mutants.

Cross	Generation	Zap^s	Zap^r	Ratio of WT/mutant	χ^2
L(*er*)xZE35-2	F_1	13	0		
	F_2	956	340	2.81	1.053[a]
L(*er*)xZE47-1	F_1	22	0		
	F_2	1422	482	2.95	0.101[a]
ZE35-2 xZE47-1	F_1	0	32		

[a]χ^2 values are based on the prediction of 3:1 segregation.

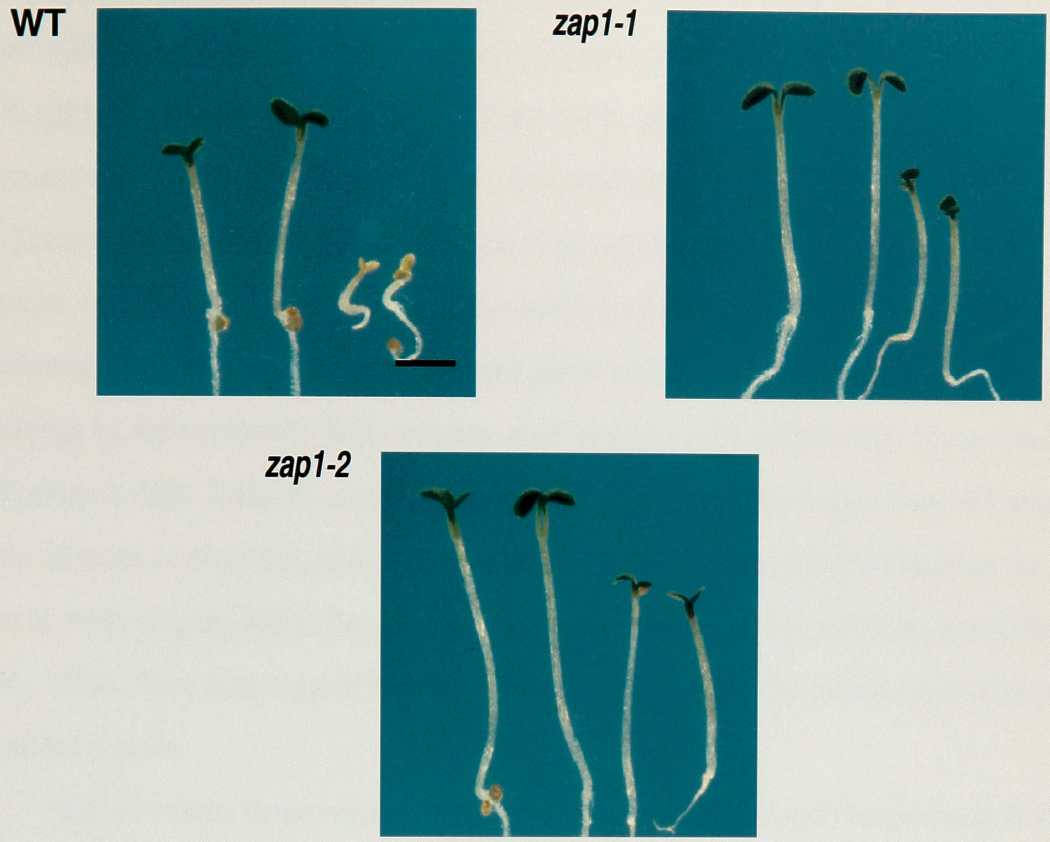
These plants were removed from R and grown in low fluence rate W to allow recovery from the effects of zaprinast. F_2 seedlings that resulted from the self-fertilization of F_1 plants displayed segregation of zaprinast resistance at a ratio of approximately 3 sensitive:1 resistant for both mutants. Chi-square analysis indicated that zaprinast resistance segregates as a single gene recessive mutation in both ZE35-2 and ZE47-1 ($p > 0.25$ and >0.50, respectively; Table 3). To test for allelism, the two mutants were crossed with ZE47-1 used as the pollen donor. In the F_1 generation, all seedlings resulting from this cross were resistant to zaprinast, demonstrating that the two mutations are allelic (Table 3). The locus affected by these mutations will hereafter be referred to as *ZAP1* (for *zap*rinast resistant), and the 35-2 allele will be referred to as *zap1-1* and 47-1 will be referred to as *zap1-2*.

Effects of zap1 *mutations on hypocotyl elongation*

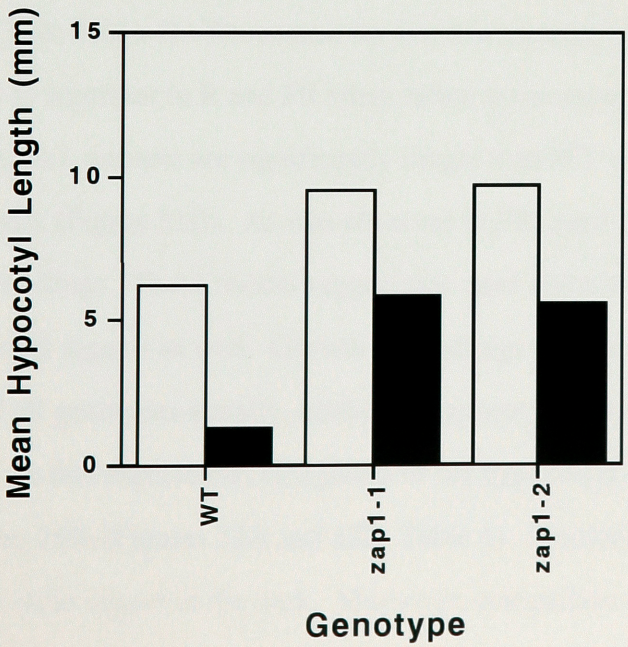
As described above, *zap1* mutants were isolated based on their resistance to zaprinast in constant high fluence rate R. Quantification of zaprinast resistance in *zap1* mutants yielded surprising results. Whereas WT seedlings possessed dramatically shortened hypocotyls and bleached cotyledons when treated with zaprinast, both *zap1-1* and *zap1-2* possessed longer hypocotyls and expanded, green cotyledons (Figure 29A). Hypocotyl elongation was inhibited approximately 80% in WT seedlings, but zaprinast inhibited hypocotyl elongation only 50% in *zap1* mutants (Figure 29B; Table 4). This result is important, but may be secondary compared to the phenotype of *zap1* mutants in the absence of zaprinast. Specifically, it was found that the hypocotyls of both mutants were approximately 50% longer than those of WT plants (Figure 29B). As described above, hypocotyl length is a fairly accurate measure of phytochrome pathway activity, as mutants deficient in phytochrome or phytochrome signaling possess longer hypocotyls than those of WT plants (Koornneef et al., 1980; Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). These results suggest that *zap1* mutants are deficient in responses to R, at least with regard to hypocotyl length.

Figure 29: The effect of *zap1* mutations on zaprinast sensitivity in red light. A. Seedlings were grown for 5 days in constant red light (35 μmol m^{-2} s^{-1}) on MS salts with 90 mM sucrose, with or without 175 μM zaprinast present in the medium for the duration of the experiment. The two seedlings on the left of each panel are untreated controls, and the two seedlings on the right were treated with 175 μM zaprinast. Scale bar = 2 mm. B. Quantification of the effect of zaprinast on hypocotyl elongation. Seedlings were grown in the conditions described above, and were transferred to an agar plate on which they were photographed. The photographs were projected, and the hypocotyl length of each seedling was determined as described in Materials and Methods. The open bars represent the mean hypocotyl length of control seedlings, and shaded bars represent those of zaprinast-treated seedlings. Standard error bars that are not visible are too small to be distinguished at this scale. Means represent the average hypocotyl length of 30 seedlings.

A.


WT
zap1-1
zap1-2

B.

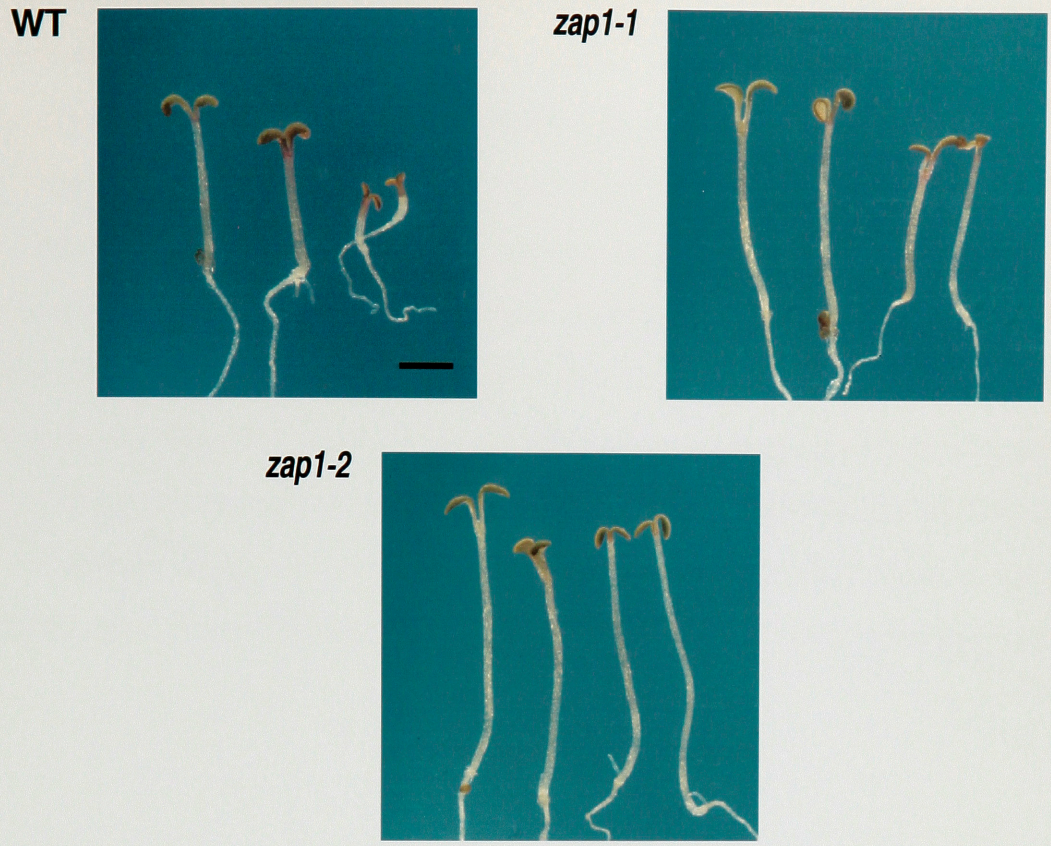

Mean Hypocotyl Length (mm)
15
10
5
0
WT
zap1-1
zap1-2
Genotype

Similar experiments were performed with *zap1* mutants in FR. As before, WT seedlings were significantly affected by zaprinast in FR, possessing extremely short hypocotyls, and small, relatively unexpanded cotyledons (Figure 30A). As was observed in R, *zap1* mutants were resistant to zaprinast in FR, possessing elongated hypocotyls and large expanded cotyledons (Figure 30A). As described above, cotyledons do not green in FR because the light-dependent conversion of protochlorophyllide to chlorophyllide requires specific wavelengths of R that the seedlings do not receive in these experimental conditions (Barnes et al., 1996a). Zaprinast inhibited hypocotyl elongation in WT seedlings by approximately 80%, whereas *zap1* mutants were inhibited by approximately 25% (Figure 30B; Table 4). *zap1* mutants were also significantly longer than WT plants in the absence of zaprinast, and *zap1-2* mutants were longer than those possessing the *zap1-1* allele (Figure 30B). Because responses to FR are mediated solely by phyA (Reed et al., 1994), these data suggest that *zap1* mutations may affect the transduction of phyA-mediated signals.

Earlier results demonstrated that zaprinast inhibits hypocotyl elongation in B by approximately 60%, regardless of genotype. In this experiment, zaprinast did not effectively inhibit hypocotyl elongation in WT or *zap1* mutants when grown in constant B (Figures 31A and 31B; Table 4). The reason for this discrepancy is not clear, as plants displayed sensitivity to zaprinast in R and FR when tested at the same time. It was also found, however, that *zap1* mutants are significantly longer than WT when grown in B in the absence of zaprinast (Figure 31B). As was observed in FR, *zap1-2* mutants were also longer than *zap1-1* seedlings. These results suggest that *zap1* mutations may affect the proper transduction of B signals as well. Growth of seedlings on zaprinast in the dark affected seedlings of all genotypes equally, inhibiting hypocotyl elongation approximately 25%. In this experiment, elongation of the hypocotyls of WT seedlings was again inhibited by 25% (Figures 32A and 32B; Table 4). Similarly, *zap1* mutants were inhibited to the same degree in the dark. Moreover, comparison of seedlings grown

Figure 30: The effect of *zap1* mutations on zaprinast sensitivity in far-red light. A. Seedlings were grown for 5 days in constant far-red light (5 μmol m^{-2} s^{-1}) on MS salts with 90 mM sucrose, with or without 175 μM zaprinast present in the medium for the duration of the experiment. The two seedlings on the left of each panel are untreated controls, and the two seedlings on the right were treated with 175 μM zaprinast. Scale bar = 2 mm. B. Quantification of the effect of zaprinast on hypocotyl elongation. Seedlings were grown in the conditions described above, and were transferred to an agar plate on which they were photographed. The photographs were projected, and the hypocotyl length of each seedling was determined as described in Materials and Methods. The open bars represent the mean hypocotyl length of control seedlings, and shaded bars represent those of zaprinast-treated seedlings. Standard error bars that are not visible are too small to be distinguished at this scale. Means represent the average hypocotyl length of 30 seedlings.

A.


WT
zap1-1
zap1-2

B.

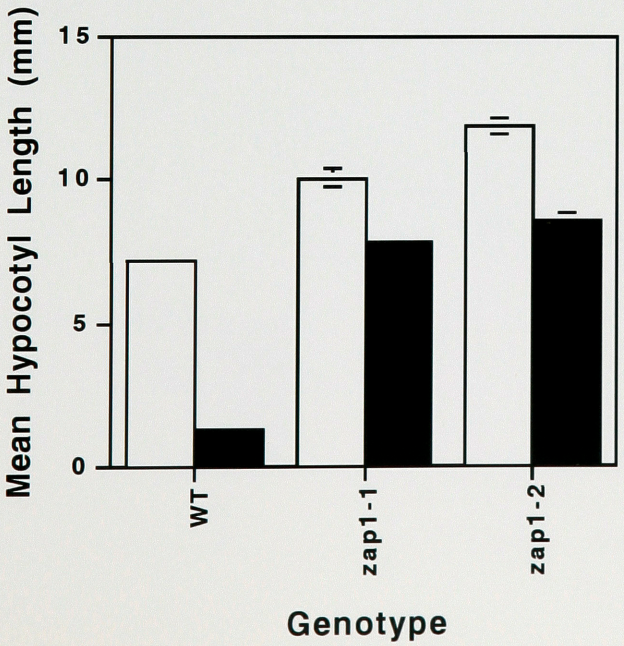

Mean Hypocotyl Length (mm)
15
10
5
0
WT
zap1-1
zap1-2
Genotype

Figure 31: The effect of *zap1* mutations on zaprinast sensitivity in blue light. A. Seedlings were grown for 5 days in constant blue light (20 μmol m^{-2} s^{-1}) on MS salts with 90 mM sucrose, with or without 175 μM zaprinast present in the medium for the duration of the experiment. The two seedlings on the left of each panel are untreated controls, and the two seedlings on the right were treated with 175 μM zaprinast. Scale bar = 2 mm. B. Quantification of the effect of zaprinast on hypocotyl elongation. Seedlings were grown in the conditions described above, and were transferred to an agar plate on which they were photographed. The photographs were projected, and the hypocotyl length of each seedling was determined as described in Materials and Methods. The open bars represent the mean hypocotyl length of control seedlings, and shaded bars represent those of zaprinast-treated seedlings. Standard error bars that are not visible are too small to be distinguished at this scale. Means represent the average hypocotyl length of 30 seedlings.

A.


WT
zap1-1
zap1-2

B.

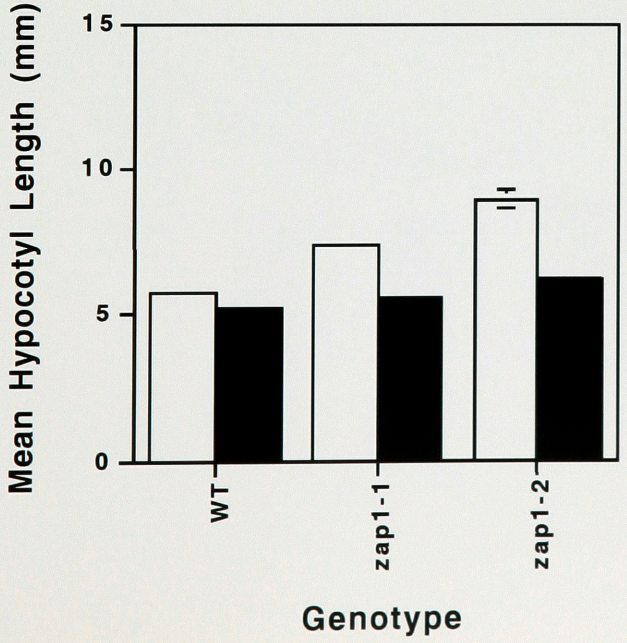
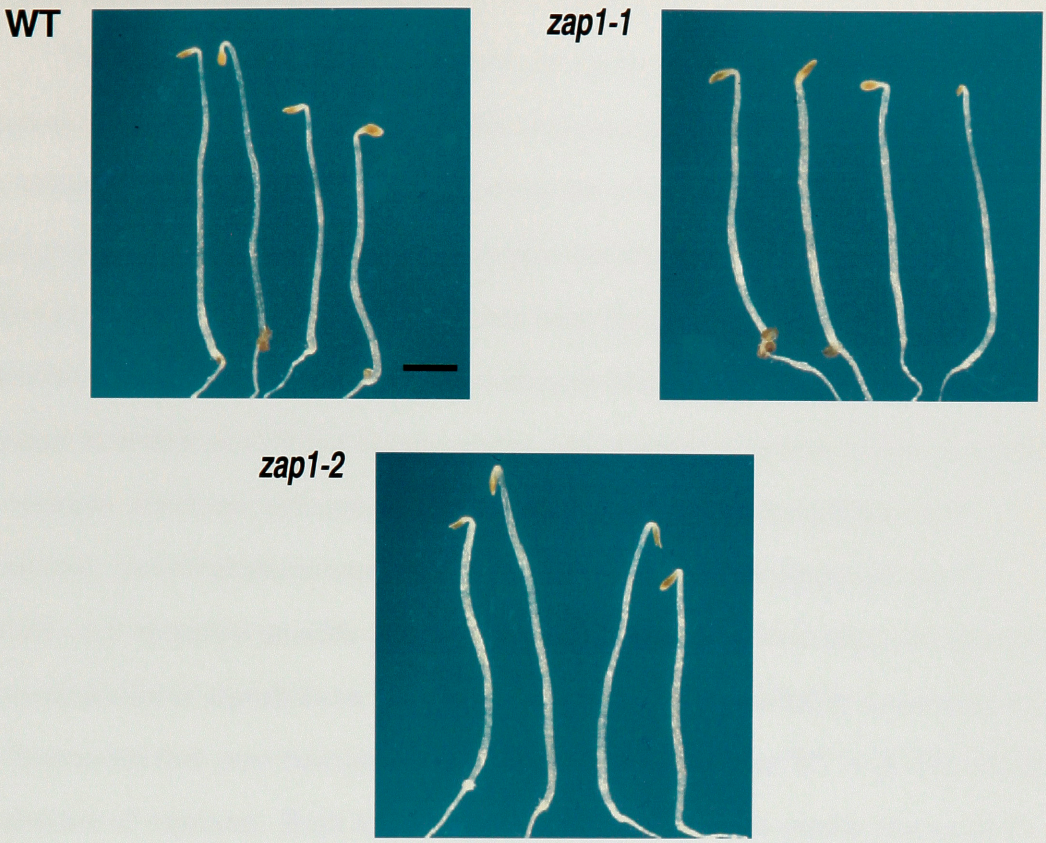
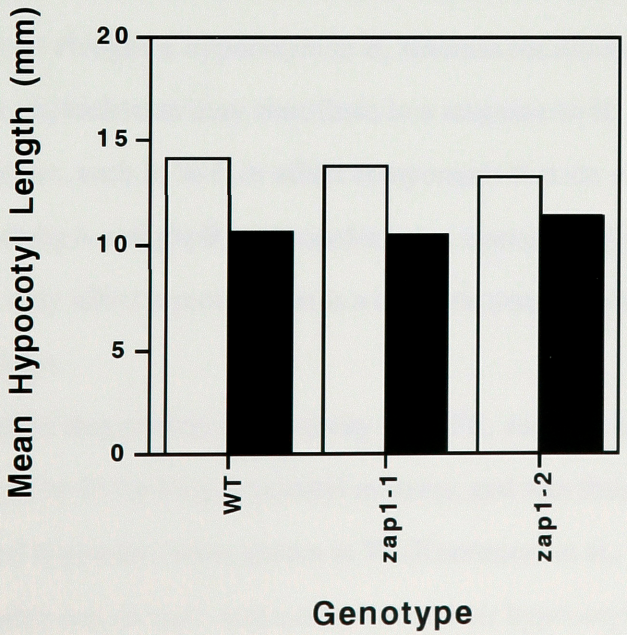

Mean Hypocotyl Length (mm)
15
10
5
0
WT
zap1-1
zap1-2
Genotype

Figure 32: The effect of *zap1* mutations on zaprinast sensitivity in darkness. A. Seedlings were grown for 5 days in constant darkness on MS salts with 90 mM sucrose, with or without 175 μM zaprinast present in the medium for the duration of the experiment. The two seedlings on the left of each panel are untreated controls, and the two seedlings on the right were treated with 175 μM zaprinast. Scale bar = 2 mm. B. Quantification of the effect of zaprinast on hypocotyl elongation. Seedlings were grown in the conditions described above, and were transferred to an agar plate on which they were photographed. The photographs were projected, and the hypocotyl length of each seedling was determined as described in Materials and Methods. The open bars represent the mean hypocotyl length of control seedlings, and shaded bars represent those of zaprinast-treated seedlings. Standard error bars that are not visible are too small to be distinguished at this scale. Means represent the average hypocotyl length of 30 seedlings.

A.


WT
zap1-1
zap1-2

B.


Mean Hypocotyl Length (mm)
20
15
10
5
0
WT
zap1-1
zap1-2
Genotype

in darkness in the absence of zaprinast indicates that the hypocotyl lengths of both WT and *zap1* mutants, regardless of the allele, are equal (Figure 32B).

The results described above are quite intriguing. Zaprinast resistance was observed in *zap1* mutants in both R and FR, whereas hypocotyl elongation in the mutants was inhibited in darkness to the same extent as that observed in WT seedlings. This distinction is important because it demonstrates that the mutation does not affect the uptake of zaprinast from the medium. If this were the case, there would be no significant inhibition of hypocotyl elongation in *zap1* mutants in darkness. The resistance to zaprinast in both R and FR that is observed in *zap1* mutants is similar to that possessed by *hy1* mutants, which are deficient in phytochrome chromophore biosynthesis and presumably lack all phytochromes (Parks and Quail, 1991), and *phyAphyB* double mutants. It is therefore possible that *zap1* mutations affect a protein that participates in the transmission of signals from both phyA and phyB. This possibility is enhanced by the observation that *zap1* mutants possess longer hypocotyls than WT in R, FR, and B in the absence of zaprinast. Both *hy1* and *phyAphyB* plants possess similar phenotypes. Because phytochrome absorbs light in the blue region of the spectrum in addition to R and FR, it may participate in some responses to B (Mancinelli, 1994). Neither *phyA* nor *phyB* mutants possessed elongated hypocotyls in B, whereas the double mutant did, suggesting that either phytochrome may contribute to a response to B. Mutations that affect both phytochromes, such as *hy1*, or affect components that are common to the signaling pathways of phyA and phyB, may confer some insensitivity to B. *zap1* mutations, therefore, may affect a protein that is a common component to the phyA and phyB signaling pathways.

Another mutation that confers insensitivity to R, FR, and B is *hy5*. This mutant was isolated in a screen in W for long hypocotyl mutants, and was found to possess a significantly elongated hypocotyl when grown in W (Koornneef et al., 1980). This phenotype contrasts with that of *zap1* mutants, because their hypocotyls were found to be

Table 4: Effect of zaprinast on hypocotyl elongation in the zaprinast resistant mutant *zap1* under the indicated light conditions. Seedlings were grown for 5 days in darkness or constant red (35 μmol m^{-2} sec^{-1}), far-red (5 μmol m^{-2} sec^{-1}), or blue (20 μmol m^{-2} sec^{-1}) light. The hypocotyls of zaprinast-treated and untreated seedlings were measured, and the numbers listed in the table represent the percent inhibition by zaprinast relative to untreated controls.

Genotype	Dark	Red	Far-red	Blue
Wild Type	25	79	81	9
zap1-1	24	38	22	24
zap1-2	14	42	28	31

only slightly elongated when grown in W (Figure 35, compare -EOD FR lanes of WT and *zap1* mutants). It may be argued that *zap1* mutants are merely weak alleles of *hy5*, but this is unlikely because unlike *zap1* plants, *hy5* seedlings are sensitive to zaprinast in R (data not shown). Nevertheless, complementation tests must be performed to demonstrate this conclusively.

Because *zap1* mutants possess long hypocotyls when grown in the light, it is possible that they simply affect some of the elements that control the rate of cell elongation at the cellular level, such as growth regulators like auxin or brassinosteroids (Wang et al., 1993; Romano et al., 1995). This is unlikely because it was found that the hypocotyls of WT and *zap1* mutants were of equal length when grown in the dark (Figure 5B), demonstrating that mutant plants elongate at the same rate as WT. It is also unlikely that *zap1* mutations affect basic elements of the cell that mediate changes in cell elongation such as cytoskeletal components (Shibaoka, 1994), because the cotyledons of *zap1* mutants green normally in the presence of zaprinast, and cytoskeletal components are not likely to be involved in processes like chloroplast development. The effects of *zap1* mutations are also not confined to conditions in which seedlings are grown on sucrose, because it was found that *zap1* mutants possess longer hypocotyls than WT in R, FR, and B when grown in the absence of sugars (data not shown). It is therefore possible that *zap1* mutations affect components of the phytochrome signaling system.

Effects of zap1 mutations on phytochrome-regulated processes

Recent work with phytochrome-deficient mutants has uncovered the roles played by individual forms of phytochrome in many physiological processes that are regulated by this photoreceptor system. Similar studies with the phyA signaling mutant *fhy1* have demonstrated that it is involved in some phyA-regulated processes, but not all (Whitelam et al., 1993; Johnson et al., 1994). To test whether mutations of the *ZAP1* locus specifically affect phytochrome-regulated responses at the physiological level,

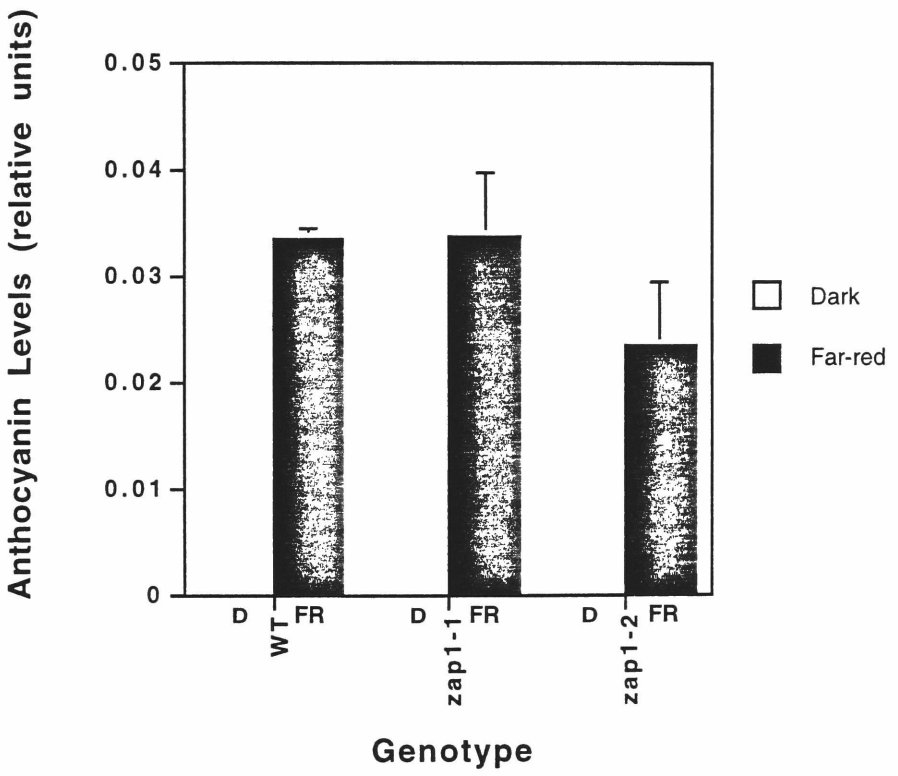
anthocyanin production in FR, flowering time, end-of-day FR responses, and germination were studied in *zap1* mutants.

Anthocyanin accumulation in far-red

Production of anthocyanin pigments is stimulated by a number of elicitors such as pathogen attack and light, including UV-B, B/UV-A, R, and FR (Chappell and Hahlbrock, 1984; Beggs and Wellmann, 1994). Anthocyanin production stimulated by R and FR is mediated by phytochrome, and the FR response is mediated solely by phyA. Moreover, the induction of anthocyanin accumulation by phytochrome requires the presence of sugars in the medium. It has recently been found that FR stimulates significant accumulation of anthocyanin in the presence of sucrose, but no anthocyanin is detectable in its absence. Similarly, induction of anthocyanin in FR requires phyA, as *phyA* null mutants do not produce anthocyanin in FR, regardless of the presence or absence of sucrose. Mutations of the *FHY1* locus, which encodes a putative component of the phyA signaling pathway, also inhibit the production of anthocyanin in FR (Dr. Simon Barnes, The Rockefeller University, personal communication). Thus, the ability to produce anthocyanin in FR appears to be an indicator of the function of the phyA photoreceptor and associated signal transduction pathway.

For that reason, the ability of *zap1* mutants to produce anthocyanin in FR was examined. Seedlings of WT and both alleles of *zap1* were grown in darkness on sucrose medium for 4 days, and subsequently shifted to constant FR (5 μmol m^{-2} s^{-1}) or maintained in darkness for an additional 3 days. Following treatment, anthocyanins were extracted and quantified by spectrophotometry. It was found that neither WT nor *zap1* plants produced detectable anthocyanin when grown solely in the dark. Both WT and *zap1-1* produced comparable levels of anthocyanin after 3 days of FR treatment (Figure 33). *zap1-2* mutants also produced anthocyanin in FR, but it was measurably lower than the levels observed in both WT and *zap1-1* plants. These data suggest that the *zap1-2* mutation is able to partially inhibit phyA-dependent anthocyanin accumulation.

Figure 33: The effect of *zap1* mutations on anthocyanin production in far-red light. Seedlings were grown for 4 days in constant darkness in 1X MS salts containing 90 mM sucrose. Seedlings were subsequently treated with constant far-red light (5 μmol m^{-2} s^{-1}) or were maintained in darkness for an additional 3 days. Anthocyanins were extracted and measured as described in Materials and Methods. Open bars represent dark grown controls, and shaded bars represent plants treated with far-red light. The mean values of detectable anthocyanin of 50 seedlings in 3 independent experiments are presented. Standard error values are indicated.


Anthocyanin Levels (relative units)
0.05
0.04
0.03
0.02
0.01
0
D
FR
WT
zap1-1
zap1-2
Genotype
Dark
Far-red

Flowering time

By a mechanism that is regulated by a large number of factors, the vegetative meristem undergoes a developmental conversion in which it ceases to produce vegetative structures like rosette leaves, and produces an inflorescence containing reproductive structures (Besnard-Wibaut, 1981). Flowering time has been shown to be regulated by day length, and interruptions of dark periods with R or FR can promote flowering, suggesting a role for phytochrome (Borthwick and Hendricks, 1960). A characteristic of mutants that are deficient in phyB is that they flower early, suggesting that the photoreceptor is required for normal flowering responses (Goto et al., 1991). It may be predicted that because the loss of phyB signals causes early flowering, increased phyB signaling should cause plants to flower late. This is not the case, however, as it has been found that transgenic plants that overexpress *PHYB* also flower early (Bagnall et al., 1995). Thus, any increased signaling that may result from phyB overproduction has the opposite effect of that predicted. It is possible that the dramatic changes in physiology that may result from the complete loss of phyB or its overproduction may be interpreted by the plant as a significant stress, and other factors that regulate flowering may act to promote early flowering. It would be interesting to determine the effect of subtly altering the amount of phyB on flowering.

Because loss of phyB causes changes in flowering time, whereas null mutations in *PHYA* have no effect, it may be concluded that flowering is a phyB-regulated response. The long hypocotyl phenotype in R possessed by *zap1* mutants suggests a defect in phyB responses. For that reason, the effect of *zap1* mutations on flowering time was determined. Seeds of WT and both alleles of *zap1* were sown in soil, and grown under light:dark cycles of 16h:8h. Plants were examined over the course of the next 35 days for the emergence of the inflorescence, and the number of days which had passed until the inflorescence was observed was considered the time to flowering (Koornneef et al., 1991). In addition, the number of rosette leaves was determined for each plant at the time

of inflorescence emergence, as there is a strong correlation between the number of rosette leaves produced and flowering time (Koornneef et al., 1991). For instance, plants that flower early possess fewer rosette leaves than WT, whereas plants that flower late possess more rosette leaves.

It was found that WT plants flowered within 29.5 days, and produced approximately 8 rosette leaves during that time (Table 5). *zap1-1* mutants flower within 32 days, and *zap1-2* mutants flower within 33. Both mutants produce approximately 10 rosette leaves during that time (Table 5). Thus, *zap1* mutants flower later than WT. The differences in flowering time are more striking when the distribution of individual plants flowering on each day is examined. Flowering of WT plants occurs primarily on days 29 and 30. *zap1-1* plants begin flowering on day 29, and continue flowering with a fairly equal distribution through day 34. By contrast, *zap1-2* mutants begin flowering on day 28, but the vast majority flower on day 34 (Figure 34). These data suggest that like their effects on anthocyanin production, the *zap1-1* and *zap1-2* mutations are different, with *zap1-2* having a more marked effect.

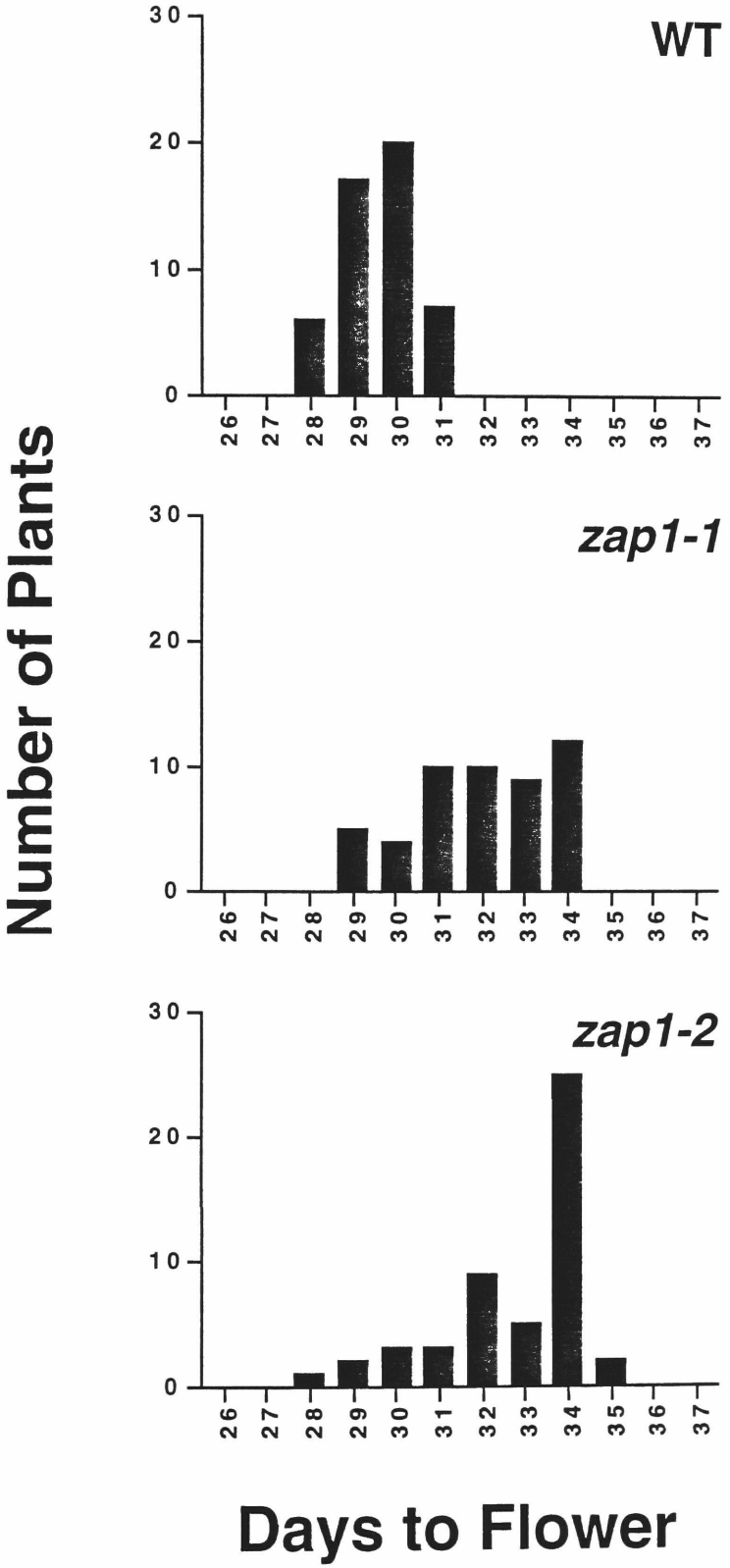
End-of-day far-red responses

Another indicator of phyB activity is measured by the end-of-day FR response (EOD FR). It has been found that if plants that are grown under a light:dark cycle are treated with FR immediately prior to shifting them to darkness, over time, they grow taller than plants that did not receive FR treatment (Smith, 1994). It is thought that EOD FR treatment converts all phytochrome back to the P_r form, and removes the inhibitory effects on stem elongation produced by P_{fr}. Recent work with *phyB* mutants has demonstrated that they are deficient in EOD FR responses, suggesting that conversion of $phyB_{fr}$ back to $phyB_r$ relieves inhibitory effects on stem elongation during the subsequent dark period (Nagatani et al., 1991a; Devlin et al., 1992). It was also found, however, that some EOD FR responses, such as petiole growth, are unaffected in *phyB* mutants, suggesting a role for another light-stable phytochrome (Robson et al., 1993).

Table 5: Flowering responses of *zap1* mutants. Plants were grown in soil under 16 hour/8 hour day/night cycles until flowering. Flowering time refers to the number of days from sowing to the appearence of the inflorescence. Number of rosette leaves refers to the number of leaves produced during the vegetative phase of growth prior to the appearance of the inflorescence. The numbers represent the means of 50 plants.

Genotype	Flowering Time (Days +/- SE)	Number of Rosette Leaves (+/- SE)
Wild type	29.56 +/- 0.13	8.44 +/- 0.11
zap1-1	32.00 +/- 0.23	10.04 +/- 0.15
zap1-2	32.84 +/- 0.24	10.12 +/- 0.23

Figure 34: The effect of *zap1* mutations on flowering time. Plants were sown in soil, and grown for 35 days in a light:dark cycle of 16 h:8 h. Plants were observed every day during that time period, until an inflorescence was detected. The day after sowing on which the inflorescence was detected is considered the number of days to flowering. Each bar represents the number of plants flowering on each day. The flowering time of 50 plants was determined for each genotype.


WT
zap1-1
zap1-2
Number of Plants
Days to Flower
0
10
20
30
26
27
28
29
30
31
32
33
34
35
36
37

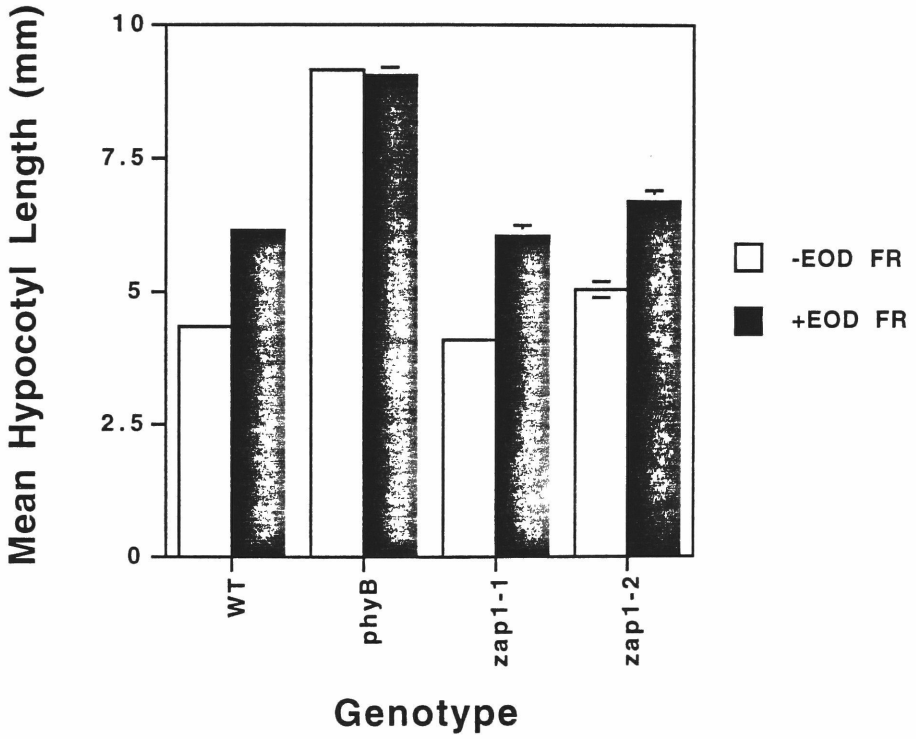
Nevertheless, some EOD FR responses appear to be an indicator of phyB pathway activity.

For that reason, the effect of *zap1* mutations on the EOD FR response was assessed. WT, *phyB*, and both *zap1* mutants were grown on minimal medium without sucrose for 3 days in white light with a light:dark cycle of 8h:16h. Subsequently, some seedlings were treated with FR for 15 minutes immediately before shifting them to darkness. It was found that EOD FR treatments stimulated stem elongation in WT plants, but had no effect on *phyB* plants (Figure 35). Both *zap1-1* and *zap1-2* plants responded like WT plants to EOD FR treatments, but the response of *zap1-2* plants was slightly reduced (an increase of 1.6 mm vs. 2.0 mm). This difference is minimal, as WT plants were 1.8 mm longer on average. Thus, *zap1* mutants appear to possess normal EOD FR responses, at least with regard to hypocotyl length.

Germination

The observation that R promoted the germination of seeds was one of the first processes demonstrated to be regulated by phytochrome (Borthwick et al., 1952). Studies of germination in mutants deficient in phytochrome or phytochrome signaling have recently provided insights into the functions played by individual phytochromes in the germination process. Specifically, it was found that germination in the dark is mediated by $phyB_{fr}$, and that $phyB_r$ may have inhibitory effects on germination in FR. Moreover, phyA is absolutely required for germination in FR, but is dispensable for germination in other conditions (Johnson et al., 1994; Reed et al., 1994; Shinomura et al., 1994). Mutations of the putative phyA signaling component FHY1 have no effect on germination in FR, suggesting that it is not involved in this phyA-mediated response (Johnson et al., 1994). The germination of *zap1* mutants was examined in preliminary experiments under different light conditions to determine if the WT protein is involved in phytochrome-mediated germination. It was found that all genotypes tested germinated robustly in W and R (data not shown). In the dark, *phyB* mutations resulted in poor

Figure 35: The effect of *zap1* mutations on end-of-day far-red responses. Seedlings were grown on medium containing 1X MS salts for 3 days in white light following a light:dark cycle of 8 h:16 h. Subsequently, half of the plants of each genotype were treated with far-red light (5 μmol m^{-2} s^{-1}) for 15 minutes at the end of their light cycle, whereas the remainder of the plants were placed in darkness immediately following their light cycle. Following 5 days of this treatment, seedlings were placed on agar plates and photographed, whereupon hypocotyl lengths were determined as described in Materials and Methods. The open bars represent the mean hypocotyl length of plants that did not receive end-of-day far-red treatment, whereas shaded bars represent plants that received end-of-day far-red treatment. Standard error bars are indicated. In cases where standard error bars are not visible, the error was not large enough to be detectable at this scale. The means represent the average hypocotyl lengths of 40 plants.


Mean Hypocotyl Length (mm)
10
7.5
5
2.5
0
WT
phyB
zap1-1
zap1-2
Genotype
-EOD FR
+EOD FR

germination, confirming previous results, whereas germination rates in all other genotypes was higher. In FR, both *phyA* and *phyAphyB* mutants germinated poorly, whereas *phyB* mutants had enhanced germination. Strikingly, *zap1-2* mutants also germinated poorly in FR, whereas *zap1-1* mutants displayed relatively normal responses (data not shown). These results suggest that the *zap1-2* allele disrupts phyA signaling to prevent germination in FR.

The results presented here suggest that *zap1* mutations disrupt phytochrome-dependent signaling. Reduced anthocyanin production and decreased germination in FR caused by the *zap1-2* mutation indicate that the phyA-dependent signaling pathway is disrupted, preventing normal physiological responses. This notion is supported by the increased hypocotyl length in FR possessed by both *zap1* mutants in the absence of zaprinast. Despite having no effect on EOD FR responses, both alleles of *zap1* caused plants to flower measurably later than comparable WT plants. Moreover, both mutants were less responsive to red light cues, resulting in increased hypocotyl lengths compared to WT plants. These data suggest that *zap1* mutations have effects on phyB-dependent responses as well. It is clear from these data that *zap1-1* is a weaker allele than *zap1-2*. This is indicated by the reduced responsiveness to FR displayed by *zap1-2* plants with regard to anthocyanin production, germination, and hypocotyl length. Nevertheless, the *zap1-1* mutation is sufficient to confer resistance to zaprinast despite having little effect on phytochrome-regulated responses other than hypocotyl elongation. Thus, it appears that the screen for zaprinast-resistant mutants allows the recovery of very weak alleles in addition to strong mutations that result in more dramatic loss of function.

Characterization of additional zaprinast-resistant mutants

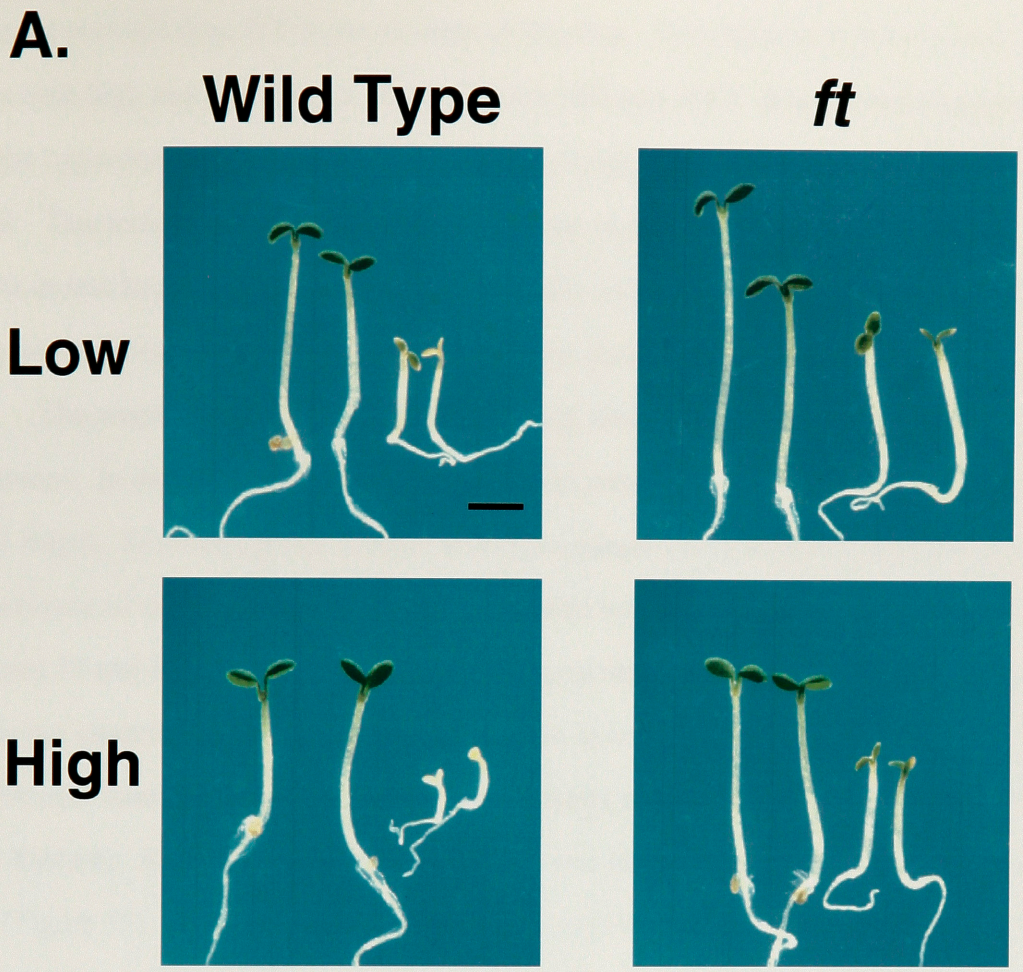
As described above, it was found that *zap1* mutants flower later than WT plants. It was therefore possible that a characteristic of mutants that are deficient in phytochrome signaling may be altered flowering time. For that reason, late-flowering mutants that have been previously described were obtained and screened for zaprinast resistance

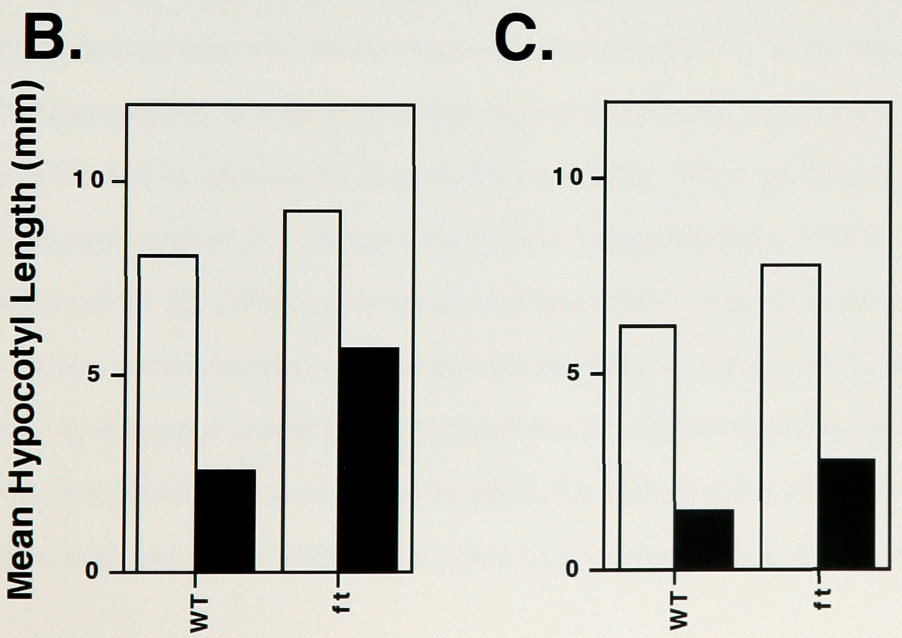
(Koornneef et al., 1991). Seedlings of late-flowering mutants were grown on media containing MS salts, 90 mM sucrose, and 175 μM zaprinast for 5 days under constant high fluence rate R (35 μmol m^{-2} s^{-1}). As before, mutants with elongated hypocotyls and green expanded cotyledons in the presence of zaprinast were considered zaprinast-resistant. Of the 11 late-flowering mutants that were tested, 2 mutants, designated *ft* and *fha*, were resistant to the drug. The resistance of these mutants was not as strong as that seen in *zap1* mutants, as the hypocotyls of mutant seedlings were significantly shortened by zaprinast, but not to the same degree as WT seedlings. In addition, the cotyledons of the zaprinast-resistant late-flowering mutants greened to a large degree in the presence of the drug, but not to the same degree as *zap1* mutants. The *ft* mutant was selected for further characterization.

Effects of ft *mutations on zaprinast-dependent inhibition of hypocotyl elongation*

The effect of zaprinast on hypocotyl elongation in *ft* plants was examined in more detail. As before, seedlings were grown under constant high fluence rate R (35 μmol m^{-2} s^{-1}) for 5 days. Under these conditions, zaprinast induced significant hypocotyl shortening in both WT and *ft* plants (Figures 36A and 36B), inhibiting hypocotyl elongation 76% in WT and 64% in *ft* seedlings (Table 6). In addition, *ft* mutants were more resistant than WT seedlings to the bleaching effects of zaprinast on cotyledons, although bleaching was still evident (Figure 36A). These data suggest that *ft* mutations confer limited resistance to zaprinast under these conditions. Zaprinast resistance was also tested under a lower fluence rate of R (16 μmol m^{-2} s^{-1}), with all other conditions remaining the same. Under these conditions, it was found that zaprinast did not cause a phenotype in WT seedlings as strong as that observed under higher fluence rate R. In WT plants, hypocotyl elongation was still significantly inhibited (68%), but not to the same degree, and cotyledons were only partially bleached. By contrast, *ft* mutants displayed much higher resistance to zaprinast under these conditions, as hypocotyl elongation was only inhibited 38%, and cotyledons were much greener (Figures 36A and

Figure 36: The effect of *ft* mutations on zaprinast sensitivity in red light. A. Seedlings were grown for 5 days in constant low fluence rate red light (16 μmol m^{-2} s^{-1}) or constant high fluence rate red light (35 μmol m^{-2} s^{-1}) on MS salts with 90 mM sucrose, with or without 175 μM zaprinast present in the medium for the duration of the experiment. The two seedlings on the left of each panel are untreated controls, and the two seedlings on the right were treated with 175 μM zaprinast. Scale bar = 2 mm. Seedlings were grown in the conditions described above, and were transferred to an agar plate on which they were photographed. The photographs were projected, and the hypocotyl length of each seedling was determined as described in Materials and Methods. B. Mean hypocotyl lengths of control (open bar) and zaprinast-treated (shaded bar) seedlings grown in constant low fluence rate red light. C. Mean hypocotyl lengths of control (open bar) and zaprinast-treated (shaded bar) seedlings grown in constant high fluence rate red light. In cases where standard error bars are not visible, the error was not large enough to be detectable at this scale. The means represent the average hypocotyl length of 30 seedlings.


A.
Wild Type
ft
Low
High


B.
C.
Mean Hypocotyl Length (mm)
10
5
0
WT
ft
10
5
0
WT
ft

36C; Table 6). In addition to zaprinast resistance in R, *ft* mutants also possessed slightly longer hypocotyls than WT in the absence of the drug. This difference was observed under both high and low fluence rate R (Figures 36B and 36C). It is interesting to note that the hypocotyl lengths of both WT and *ft* seedlings were shorter under high fluence rate R. This indicates that increased activity of the phytochrome signaling pathway results in less hypocotyl elongation, supporting the idea that zaprinast achieves its effect on hypocotyl elongation by increasing signal flow through the signaling pathway.

The sensitivity of *ft* plants to zaprinast was also assessed under other light conditions. In darkness, both WT and *ft* seedlings were etiolated, and hypocotyls were of equal length. Similarly, plants of both genotypes retained their etiolated appearance in the presence of zaprinast, and hypocotyl elongation was inhibited to the same degree (Figures 37 and 38A; Table 6). The insensitivity to zaprinast possessed by *ft* mutants in R was not observed in FR. Hypocotyl elongation in both WT and *ft* plants was significantly inhibited in the presence of zaprinast by approximately 70% (Figures 37 and 38B; Table 6). Moreover, cotyledon expansion was inhibited in WT and *ft* seedlings as well (Figure 37). In B, zaprinast inhibited hypocotyl elongation by approximately 60% in plants of both genotypes (Figures 37 and 38C; Table 6). This degree of inhibition by zaprinast in blue is consistent with results obtained earlier (Figure 17). In the absence of zaprinast, the hypocotyls of WT and *ft* seedlings were of comparable length when plants were grown in the dark or constant FR (Figures 38A and 38B). When seedlings were grown in B, the hypocotyls of *ft* seedlings were slightly longer than those of WT, although the reason for this difference is unknown (Figure 38C). From these data, it is clear that *ft* confers weak resistance to zaprinast, and resistance is observed in R only. This is interesting because *ft* was originally isolated as a late-flowering mutant, and flowering time is regulated to a large degree by phyB, the photoreceptor that controls most responses in R, and whose activity is required for zaprinast sensitivity in R (Goto et

Figure 37: The effect of *ft* mutations on zaprinast sensitivity in darkness, far-red light, and blue light. Seedlings were grown for 5 days in constant darkness, constant far-red light (5 μmol m^{-2} s^{-1}), or constant blue light (20 μmol m^{-2} s^{-1}) on MS salts with 90 mM sucrose, with or without 175 μM zaprinast present in the medium for the duration of the experiment. The two seedlings on the left of each panel are untreated controls, and the two seedlings on the right were treated with 175 μM zaprinast. Scale bar = 2 mm.

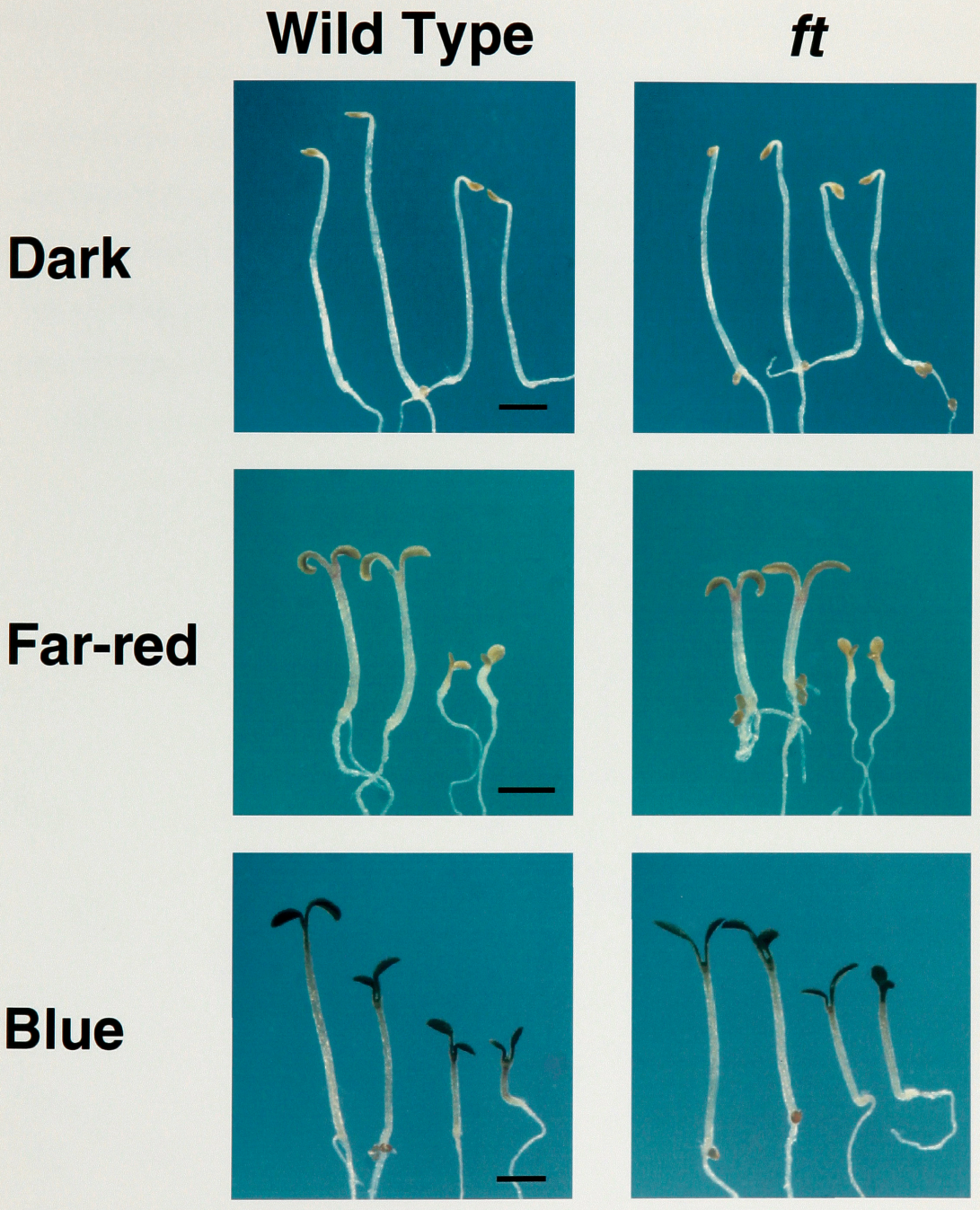

Wild Type
ft
Dark
Far-red
Blue

Figure 38: The effect of *ft* mutations on zaprinast-mediated inhibition of hypocotyl elongation in darkness, far-red light, and blue light Seedlings were grown for 5 days in constant darkness (A.), constant far-red light (5 μmol m^{-2} s^{-1}; B.), or constant blue light (20 μmol m^{-2} s^{-1}; C.) on MS salts with 90 mM sucrose, with or without 175 μM zaprinast present in the medium for the duration of the experiment. Seedlings were grown in the conditions described above, and were transferred to an agar plate on which they were photographed. The photographs were projected, and the hypocotyl length of each seedling was determined as described in Materials and Methods. Mean hypocotyl lengths of control (open bar) and zaprinast-treated (shaded bar) seedlings grown in the conditions described above. In cases where standard error bars are not visible, the error was not large enough to be detectable at this scale. The means represent the average hypocotyl length of 30 seedlings.

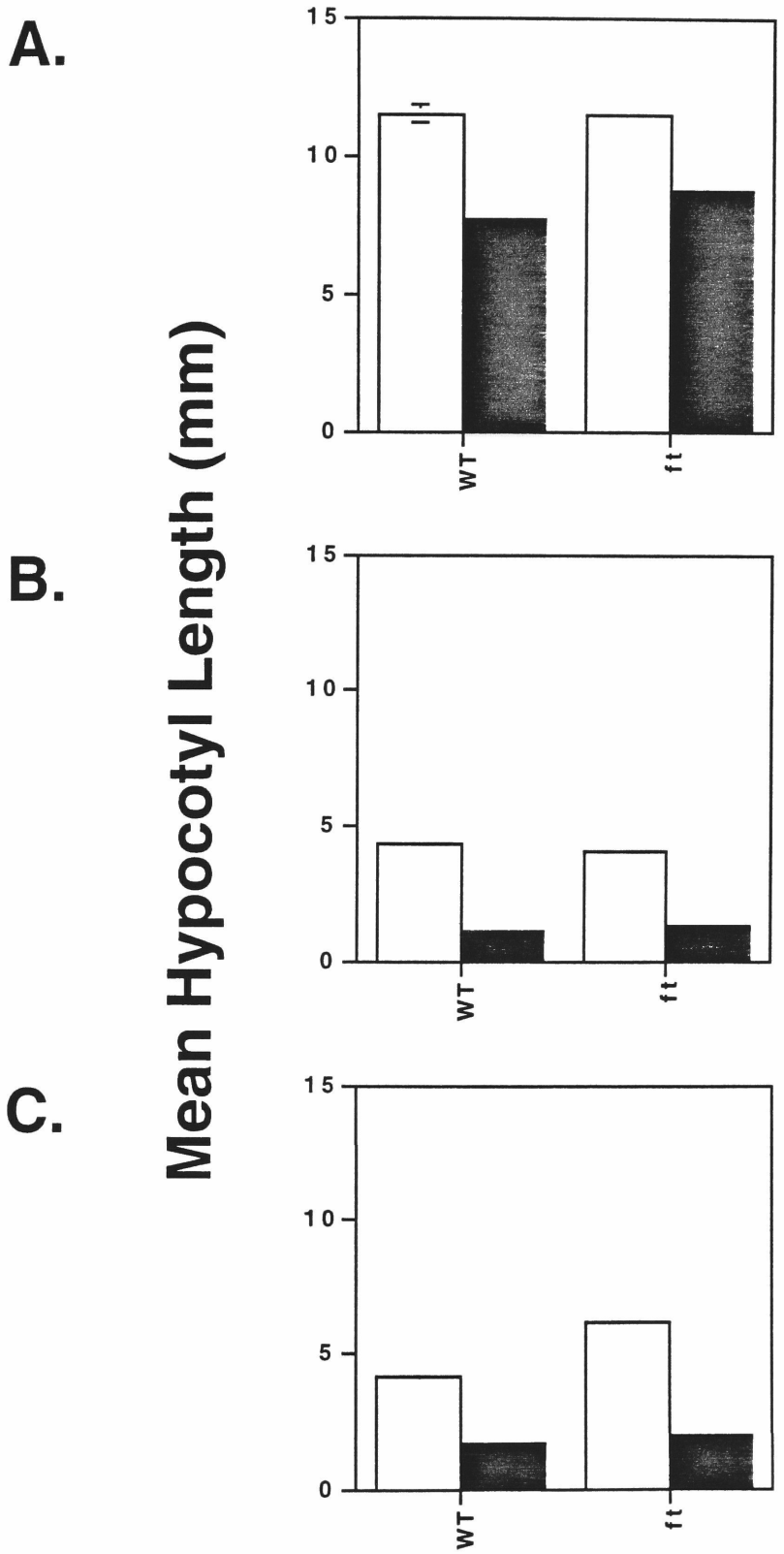

A.
B.
C.
Mean Hypocotyl Length (mm)
15
10
5
0
WT
ft

Table 6: Effect of zaprinast on hypocotyl elongation in the late flowering mutant *ft* under the light conditions listed. Seedlings were grown for 5 days in darkness or constant red (high; 35 μmol m^{-2} sec^{-1}), red (low; 16 μmol m^{-2} sec^{-1}), far-red (5 μmol m^{-2} sec^{-1}), or blue (20 μmol m^{-2} sec^{-1}) light. The hypocotyls of zaprinast-treated and untreated seedlings were measured, and the numbers listed in the table represent the percent inhibition by zaprinast relative to untreated controls.

Genotype	Dark	Red (High)	Red (Low)	Far-red	Blue
Wild type	33	76	68	74	60
ft	24	64	38	67	67

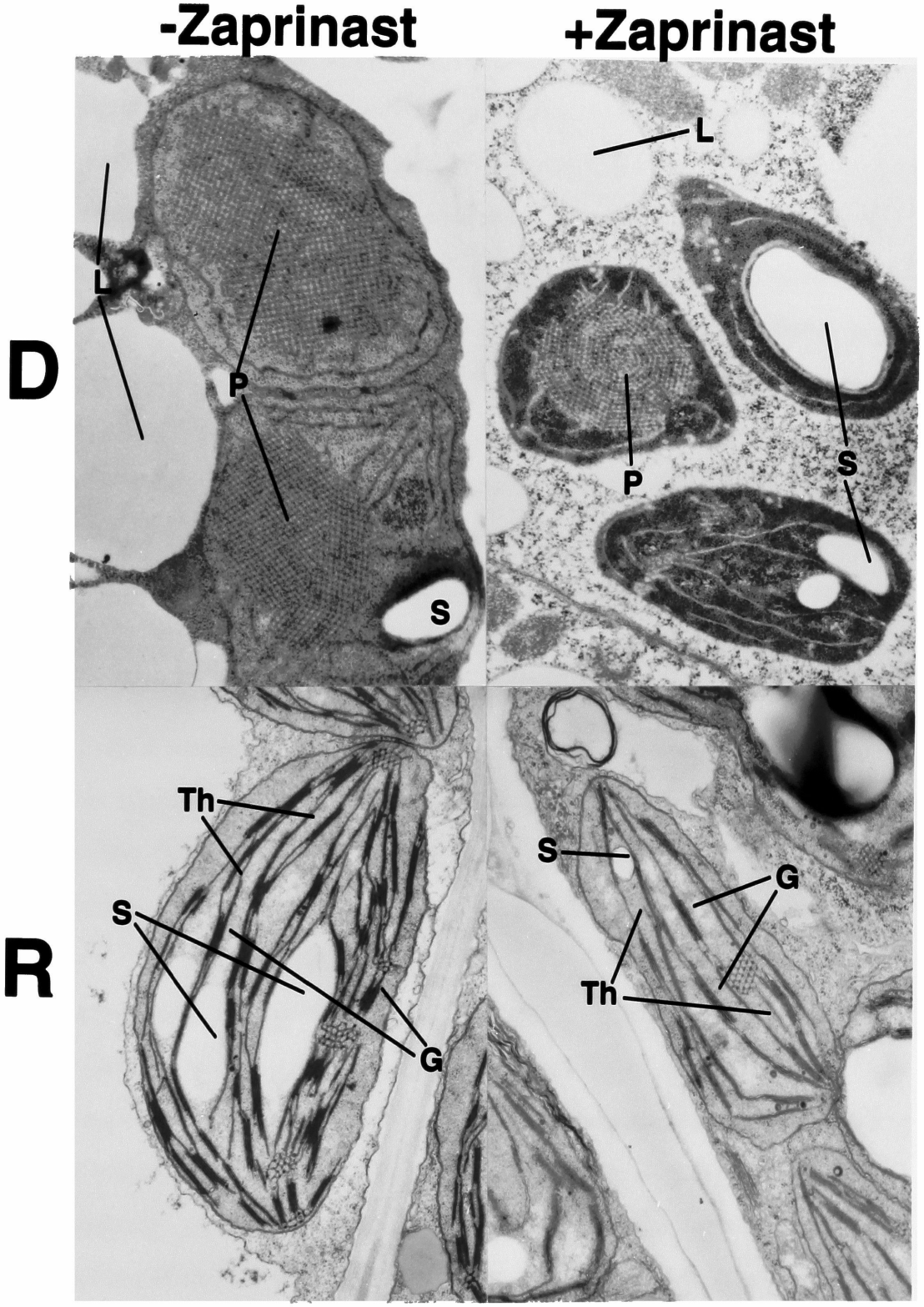
al., 1991; Koornneef et al., 1991). It is therefore possible that *ft* may affect a specific component of the signal transduction pathway regulated by phyB.

ft *inhibits zaprinast-dependent effects on chloroplast development*

The bleached-cotyledon phenotype caused by zaprinast in WT plants grown under constant high fluence rate R was shown to result from the inhibition of proper chloroplast development. Plastids of light-grown zaprinast treated seedlings had begun light-dependent development, as evidenced by the lack of a prolamellar body, but development was inhibited any further. Plastids of untreated seedlings developed chloroplasts with complex thylakoid membrane systems and numerous grana stacks. By contrast, plastids of zaprinast-treated seedlings displayed limited thylakoid membrane development and no obvious grana stacks (Figure 18). Unlike WT seedlings, *ft* seedlings green in the presence of zaprinast. Chloroplasts may therefore develop normally in this mutant. To examine this question, transmission electron microscopic analysis of plastid development was performed in *ft* plants.

In dark grown *ft* seedlings, the cells of zaprinast-treated and untreated seedlings are similar, possessing remnants of nutrient storage bodies like starch granules, lipid droplets, and protein bodies (data not shown). The etioplasts of treated and untreated seedlings are similar as well, containing well-developed prolamellar bodies and limited thylakoid membrane development (Figure 39). The appearance of the plastids in *ft* plants are quite similar to those observed in WT seedlings (Figure 18). The dark staining of the etioplast matrix observed in zaprinast-treated *ft* seedlings grown in the dark may be an artefact of fixation and not an intrinsic property of *ft* plastids, as a similar appearance has occasionally been observed in the plastids of WT seedlings (data not shown). The plastids of untreated light-grown *ft* mutants are virtually identical to those of their WT counterparts, containing well-developed thylakoid membrane systems and grana stacks (Figure 39). Plastids of light-grown, zaprinast-treated *ft* seedlings differed from their WT counterparts in that they possessed more developed thylakoid membrane systems and

Figure 39: The effect of *ft* mutations on the zaprinast-dependent inhibition of chloroplast development. Seedlings were grown for 5 days in constant high fluence rate red light (35 μmol m^{-2} s^{-1}) on MS salts with 90 mM sucrose, with or without 175 μM zaprinast present in the medium for the duration of the experiment. Seedlings were then fixed as described in Materials and Methods, and plastids were examined by transmission electron microscopy. Magnification: Dark: 20,000X; Red: 16,875X.


-Zaprinast
+Zaprinast
D
R
L
P
S
L
P
S
Th
S
G
S
G
Th

some grana (Figure 39). Nevertheless, the resistance to zaprinast conferred by the *ft* mutation did not completely eliminate the effects of the drug on phytochrome-dependent plastid development. Despite containing thylakoid membranes and grana, development of the plastids of zaprinast-treated seedlings is retarded compared to untreated controls, which possess much more well-developed chloroplasts.

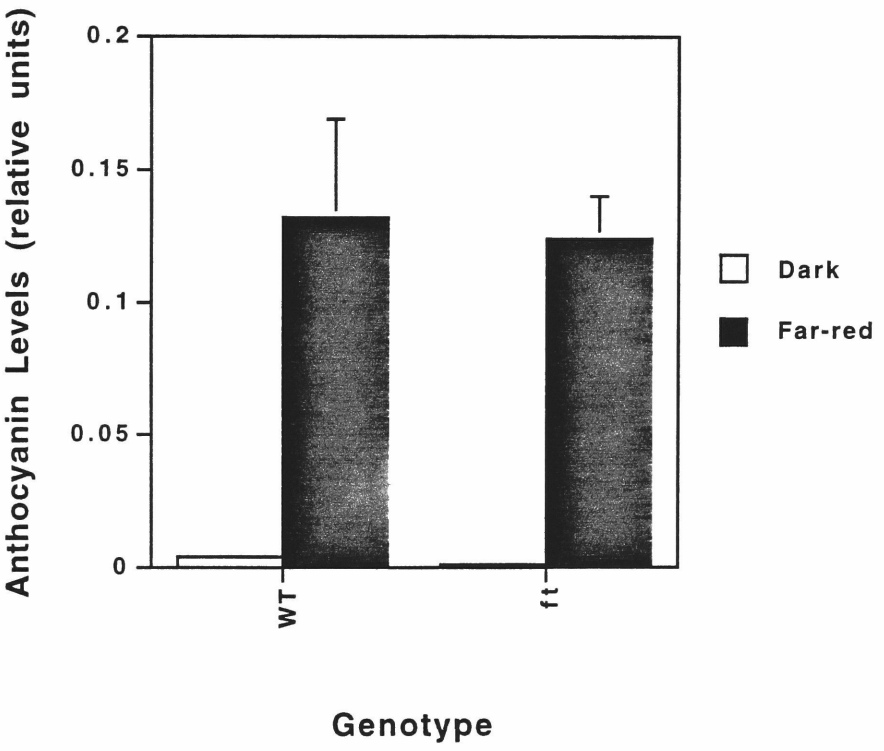
Effects of ft *mutations on anthocyanin accumulation*

As described earlier, accumulation of anthocyanin pigments in WT plants in FR is dependent on the activity of phyA and its associated signal transduction pathway. *phyB* mutants accumulate anthocyanin normally in FR, demonstrating that phyB is not required for that response (S. Barnes, personal communication). Thus, if *ft* mutants are deficient in phyB signaling, little or no effect on anthocyanin accumulation in FR would be expected. To test this hypothesis, WT and *ft* seedlings were grown in darkness in the presence of sucrose for 4 days before either being shifted to constant FR or maintained in darkness for an additional 3 days. Anthocyanins were subsequently extracted from dark-grown and FR-treated plants, and quantified spectrophotometrically. It was found that neither WT nor *ft* mutants accumulated detectable anthocyanins in darkness, whereas both produced detectable pigments in FR (Figure 40). *ft* plants seem to produce slightly less anthocyanin than WT, but the difference is statistically insignificant, demonstrating that *ft* mutations do not affect the phyA-dependent process of anthocyanin accumulation in FR.

Effects of ft *mutations on gene expression*

As described above, it may be possible to identify the regions of the signal transduction pathway that are affected by zaprinast-resistance mutations, based on the patterns of induction of marker genes for the individual branches of the phytochrome signaling pathway. *CHS* is used as a marker for cGMP-dependent pathway activity, and *CAB* is used as a marker for activity of the Ca^{2+}/CaM-dependent pathway. In the following experiments, WT and *ft* seedlings were grown in the dark for 4 days, and were

Figure 40: The effect of *ft* mutations on anthocyanin accumulation in far-red light. Seedlings were grown for 4 days in constant darkness in 1X MS salts containing 90 mM sucrose. Seedlings were subsequently treated with constant far-red light (5 μmol m^{-2} s^{-1}) or were maintained in darkness for an additional 3 days. Anthocyanins were extracted and measured as described in Materials and Methods. Open bars represent dark grown controls, and shaded bars represent plants treated with far-red light. The mean values of detectable anthocyanin of 50 seedlings in 3 independent experiments are presented. Standard error values are indicated.

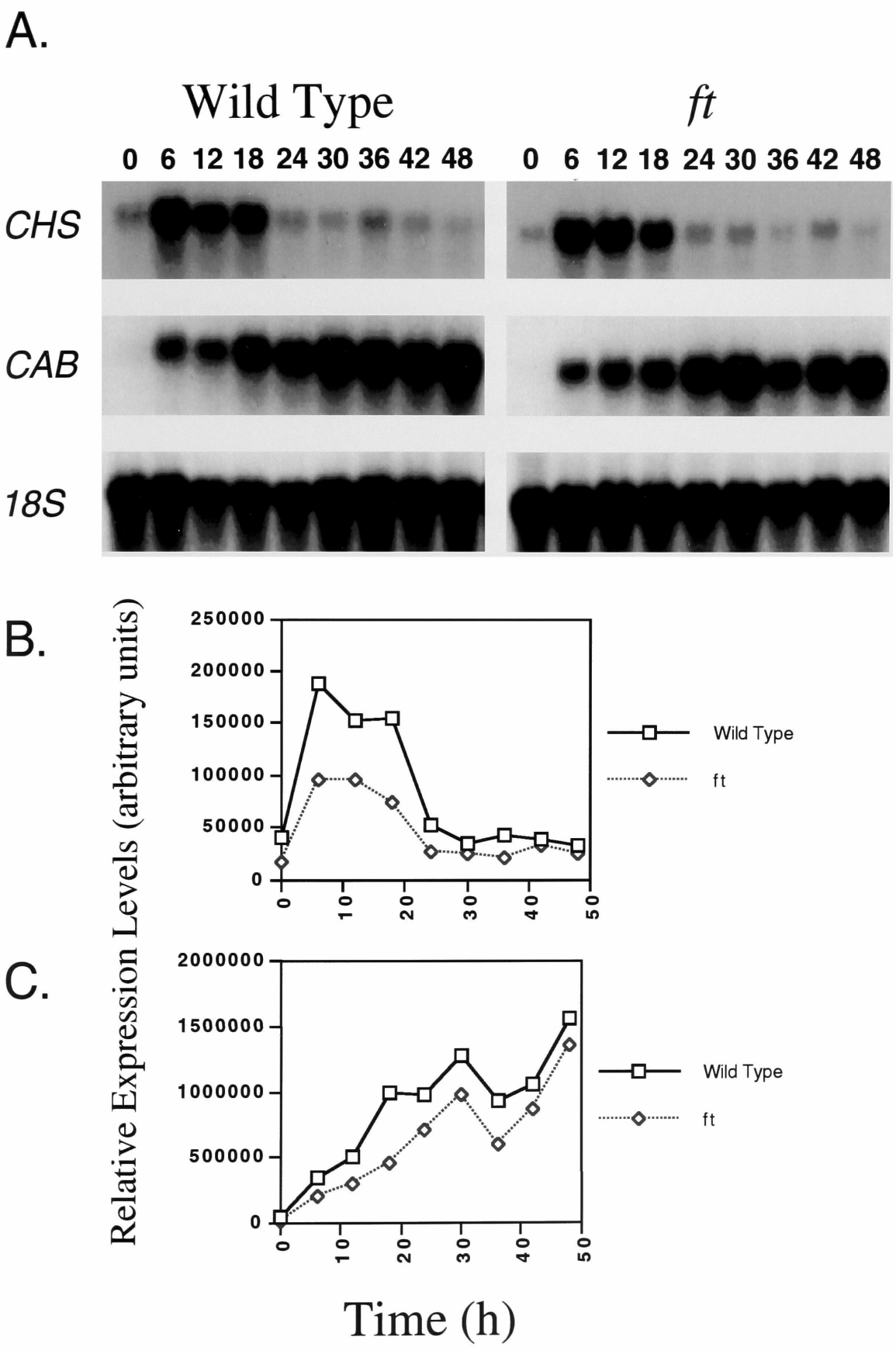

Anthocyanin Levels (relative units)
0.2
0.15
0.1
0.05
0
WT
ft
Dark
Far-red
Genotype

subsequently shifted to constant R or FR for the duration of the time course of induction. RNA isolated from seedlings harvested at the time points indicated was analyzed by gel blot hybridization analysis, and the levels of the different RNAs were determined by phosphorimage quantification. Both *CHS* and *CAB* mRNA levels were corrected based on values obtained from measurements of *18S* rRNA abundance to account for differences in loading.

WT and *ft* seedlings were treated with constant high fluence rate R (35 μmol m^{-2} s^{-1}) in the presence of 90 mM sucrose for 48 hours. Analysis of gene expression indicates that *CHS* is induced in both WT and *ft* seedlings with similar kinetics, achieving maximal levels within 6 hours, remaining elevated for an additional 12 hours, and declining to basal levels by 24 hours after the onset of illumination (Figure 41A). It is striking that the level of *CHS* expression in *ft* is only half of that observed in WT. *ft* mutations, therefore, cause a 2-fold reduction in the amount of detectable *CHS* mRNA in R (Figure 41B). Conversely, both *CAB* mRNA levels in WT and *ft* seedlings display similar kinetics, and achieve similar levels of induction (Figures 41A and 41C). In this experiment, *CAB* mRNA levels in *ft* seedlings are reduced relative to those observed in WT, but in additional experiments, little difference was observed (data not shown). Thus, *ft* mutations appear to specifically affect the expression of the targets of the cGMP-dependent pathway of phytochrome in R.

In a similar experiment, WT and *ft* seedlings were treated with constant FR (5 μmol m^{-2} s^{-1}) in the presence of 90 mM sucrose for 48 hours. Unlike the pattern of expression observed in R, *CHS* expression was induced to high levels in WT plants, achieving maximal expression within 12 hours, followed by a slow decline in expression, returning to basal levels by 48 hours after the onset of illumination. In *ft* plants, *CHS* expression shows very similar early kinetics to those observed in WT plants, and the levels of induction are comparable. Unlike WT plants, however, *CHS* levels remain higher for a longer period of time in *ft* seedlings, eventually returning to basal levels by

Figure 41: RNA gel blot analysis of the effect of *ft* mutations on red light-induced *CHS* and *CAB* gene expression in the presence of sucrose. A. Seedlings were grown for 4 days in darkness on 1X MS salts containing 90 mM sucrose. Seedlings were then transferred to constant red light (35 μmol m^{-2} s^{-1}). Samples were harvested over the next 48 hours at the times indicated (hours). RNA gel blots were sequentially hybridized with cDNAs of *CHS* and *CAB1*, and an *18S* rDNA. mRNA levels were determined by phosphorimage analysis, and the mRNA levels of *CHS* (B.) and *CAB* (C.) were normalized relative to *18S* rRNA levels.


A.
Wild Type
ft
0 6 12 18 24 30 36 42 48
0 6 12 18 24 30 36 42 48
CHS
CAB
18S
B.
C.
Relative Expression Levels (arbitrary units)
250000
200000
150000
100000
50000
0
2000000
1500000
1000000
500000
0
0 10 20 30 40 50
Wild Type
ft
Time (h)

48 hours after the onset of illumination (Figures 42A and 42B). By contrast, the levels and kinetics of *CAB* expression are virtually identical in WT and *ft* seedlings (Figures 42A and 42C). Once again, the effect of the *ft* mutation was restricted to the expression of a gene that is a target of the cGMP-dependent pathway of phytochrome.

Patterns of gene expression were also observed in R and FR in the absence of sucrose. Following illumination of WT and *ft* seedlings with high fluence rate R (35 $\mu mol\ m^{-2}\ s^{-1}$), *CHS* expression was rapidly induced, achieving maximal levels within 2 hours of illumination. Subsequently, *CHS* mRNA levels rapidly declined, returning to basal levels by 6 hours after the onset of illumination (Figure 43A). Similarly, the patterns of *CAB* gene expression in response to R was similar in both WT and *ft* seedlings (Figure 43A). The responses of WT and *ft* seedlings to FR (5 $\mu mol\ m^{-2}\ s^{-1}$) were also observed to be comparable. *CHS* expression was rapidly induced in both strains within 2 hours, and returned to basal levels soon thereafter (Figure 43B). Moreover, *CAB* expression in both WT and *ft* seedlings displayed similar kinetics and relative levels of induction (Figures 43B). Phosphorimage analysis of mRNA levels in these experiments confirm that little difference is observed between WT and *ft* plants with regard to *CHS* and *CAB* expression in R and FR in the absence of sucrose (data not shown).

These data suggest that the *ft* mutation has little effect on phytochrome-regulated gene expression, as the only effects on gene expression were observed in the presence of sucrose. In R, the *ft* mutation caused a two-fold reduction in *CHS* expression, whereas in FR, the mutation caused a significant delay in desensitization. Little effect on *CAB* expression was observed in either light treatment, suggesting that the mutation specifically affects the way sucrose interacts with the cGMP-dependent branch of the phytochrome signaling pathway to regulate gene expression. The disparate effects produced by *ft* on *CHS* expression in R and FR will be considered below (See Discussion). Nevertheless, the patterns of gene expression suggest a reason for the insensitivity of *ft* to zaprinast in R alone. The mutation caused a two-fold reduction in

Figure 42: RNA gel blot analysis of the effect of *ft* mutations on far-red light-induced *CHS* and *CAB* gene expression in the presence of sucrose. Seedlings were grown for 4 days in darkness on 1X MS salts containing 90 mM sucrose. Seedlings were then transferred to constant far-red light (5 μmol m^{-2} s^{-1}). Samples were harvested over the next 48 hours at the times indicated (hours). RNA gel blots were sequentially hybridized with cDNAs of *CHS* and *CAB1*, and an *18S* rDNA. mRNA levels were determined by phosphorimage analysis, and the mRNA levels of *CHS* (B.) and *CAB* (C.) were normalized relative to *18S* rRNA levels.

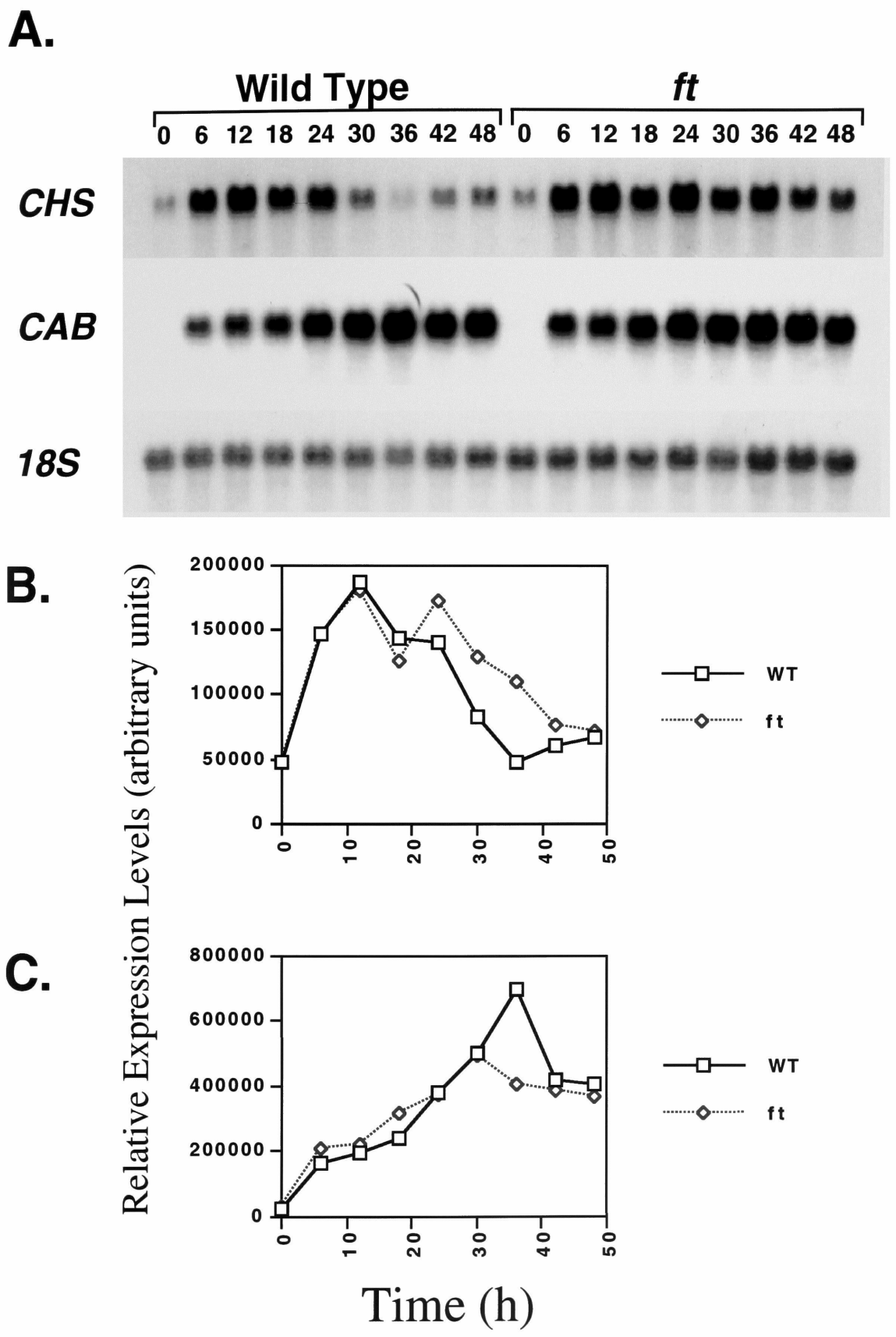
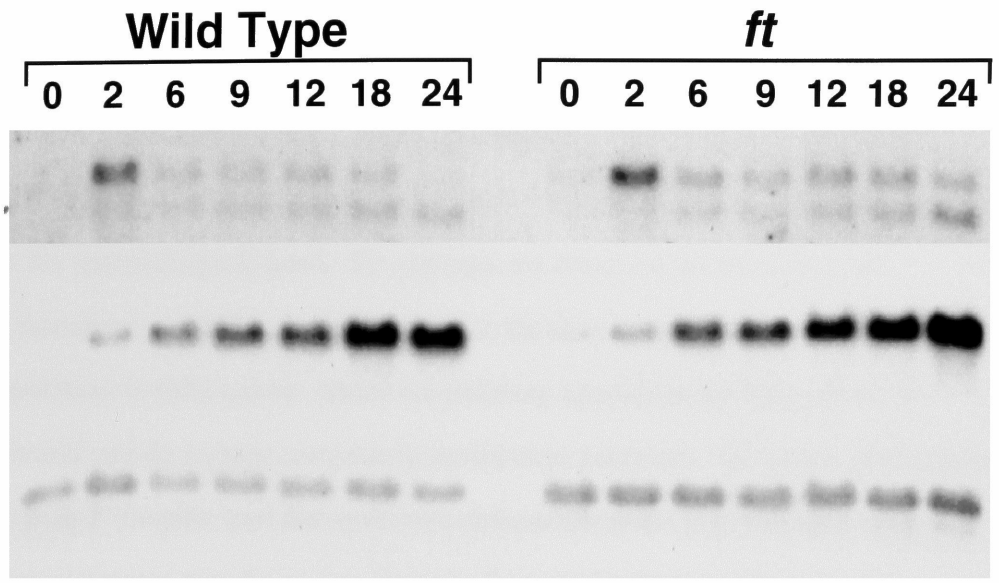
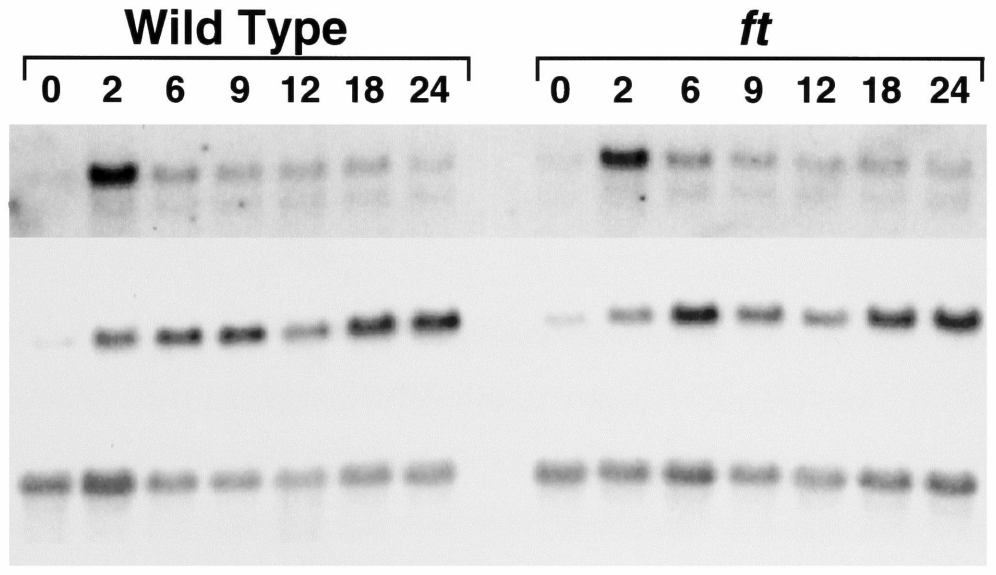

A.
Wild Type
ft
0 6 12 18 24 30 36 42 48
0 6 12 18 24 30 36 42 48
CHS
CAB
18S
B.
C.
Relative Expression Levels (arbitrary units)
200000
150000
100000
50000
0
800000
600000
400000
200000
0
0 10 20 30 40 50
WT
ft
Time (h)

Figure 43: RNA gel blot analysis of the effect of *ft* mutations on red and far-red light-induced *CHS* and *CAB* gene expression in the absence of sucrose. Seedlings were grown for 4 days in darkness on 1X MS salts. Seedlings were then transferred to constant red (35 μmol m^{-2} s^{-1}; A.) or far-red light (5 μmol m^{-2} s^{-1}; B.). Samples were harvested over the next 24 hours at the times indicated (hours). RNA gel blots were sequentially hybridized with cDNAs of *CHS* and *CAB1*, and an *18S* rDNA.

A.


Wild Type
ft
0 2 6 9 12 18 24
0 2 6 9 12 18 24
CHS
CAB
18S

B.


Wild Type
ft
0 2 6 9 12 18 24
0 2 6 9 12 18 24
CHS
CAB
18S

cGMP-pathway activity in R, and a weak insensitive phenotype was observed at the morphological level. Interestingly, reducing the fluence rate of R two-fold allowed WT seedlings to develop to the same degree in the presence of zaprinast as *ft* seedlings developed at the higher fluence rate. There is therefore a correlation between the level of cGMP-pathway activity leading to gene expression, and the degree of resistance to zaprinast at the morphological level. By contrast, no effect on the level of *CHS* expression was observed in FR, indicating that at the fluence rate used, the cGMP-dependent pathway is fully active. Since the pathway appears to be fully active, *ft* seedlings should and do remain completely sensitive to zaprinast. Given the phenotypes possessed by *zap1* mutants, and the molecular defects observed in *ft* mutants, it appears that zaprinast resistance may be an important indicator of defects in phytochrome pathway function or perturbed interactions between the phytochrome pathway and other signaling pathways with which it has synergistic interactions.

Chapter 5
Discussion

Regulation of gene expression by sucrose

In this study, the ability of sucrose to regulate the expression of phytochrome-responsive genes has been assayed in a light-responsive soybean cell culture. It was found that sucrose strongly induces the expression of chalcone synthase (*CHS*) and other anthocyanin biosynthetic genes (Figure 3 and data not shown) in the absence of light, and the kinetics of expression are reminiscent of those observed when phytochrome induces *CHS* expression (Bowler et al., 1994a). Specifically, induction of gene expression is very rapid, reaches a peak within 3-6 hours of induction, and declines thereafter. Experiments with the protein synthesis inhibitor cycloheximide demonstrated that *CHS* expression is induced by sucrose via a mechanism that does not require new protein synthesis, suggesting that a sucrose response pathway pre-exists in cells (Figure 4). It has previously been shown that induction of *CHS* expression by phytochrome is sensitive to the protein kinase inhibitor genistein (Bowler et al., 1994b). Experiments with this inhibitor indicated that induction of gene expression by sucrose was similarly affected, although the sensitivity of the sucrose signaling pathway to genistein was reduced (Figure 5).

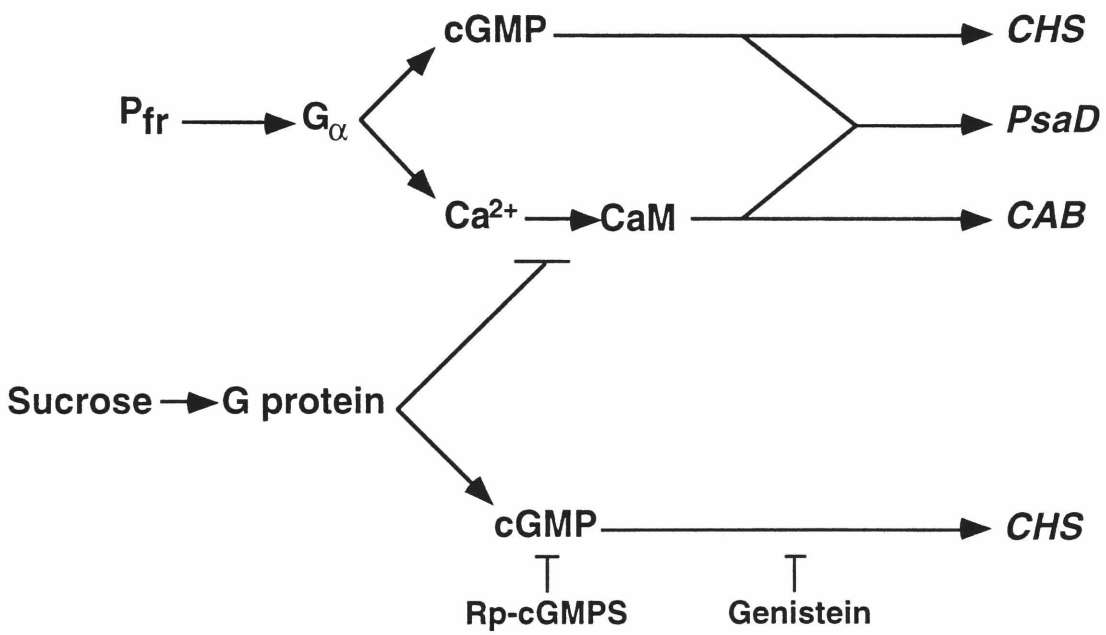
It was found that the cGMP-dependent phosphodiesterase inhibitor zaprinast was able to significantly delay the decline of *CHS* mRNA levels following prolonged stimulation by sucrose (Figure 6). These results suggest that cGMP turnover is involved in the mechanism of downregulating *CHS* expression, and, moreover, suggest that cGMP plays a role in the sucrose-mediated induction of *CHS* as well. It was subsequently demonstrated in microinjection experiments that the expression of a fusion of a multimer of the Unit I element of the parsley *CHS* promoter to β-glucuronidase (*GUS*) is inducible by sucrose in a cGMP-dependent manner. Moreover, microinjection experiments also

demonstrated that induction of gene expression by sucrose requires the activity of GTP-binding proteins (G proteins), although the particular class of G proteins involved remains undetermined (McGrath et al., manuscript in preparation).

It was also found that sucrose is a potent inhibitor of the phytochrome-dependent expression of genes that are regulated by the Ca^{2+}- and Ca^{2+}/cGMP-dependent branches of the phytochrome signaling pathway, *CAB* and *PSAD*, respectively (Figure 8). These data are consistent with those obtained by other groups that demonstrated that light-inducible gene expression may be suppressed by sugars (Sheen, 1990; Harter et al., 1993; Krapp et al., 1993; Jang and Sheen, 1994). The involvement of cGMP in the induction of *CHS* expression led to the hypothesis that repression of *CAB* and *PSAD* could be mediated by a cGMP-dependent reciprocal control mechanism that has recently been described (Bowler et al., 1994b). Co-incubation of cultures with genistein, a previously characterized inhibitor of cGMP-dependent reciprocal control, did not prevent sucrose repression of *CAB* and *PSAD* (Figure 9). Moreover, microinjection experiments confirmed that cGMP was not required for sucrose-mediated repression of *CAB* expression and plastid development. It was found, however, that G proteins were required for normal sucrose repression, much as they are in sucrose-mediated induction of gene expression (McGrath et al., manuscript in preparation).

From these data, the following model may be proposed to account for the effects of sucrose on gene expression in plants (Figure 44). Sucrose activates 1 or more G proteins within the cell that initiate 2 separate signaling events. First, following G protein activation, cGMP accumulates to high levels, and its signals are transduced through a genistein-sensitive component to induce the expression of anthocyanin biosynthetic genes like *CHS*. Second, a G protein activates a signaling pathway that results in the repression of the genes that are targets of the Ca^{2+}- and Ca^{2+}/cGMP-dependent branches of the phytochrome signaling pathway. The mechanism of G protein activation is unknown. Because sucrose uptake is mediated by a sucrose transporter which is a protein containing

Figure 44: Model of the sucrose signal transduction pathway.


cGMP
CHS
Pfr
Gα
PsaD
Ca2+
CaM
CAB
Sucrose
G protein
cGMP
CHS
Rp-cGMPS
Genistein

12 transmembrane spanning domains (Riesmeier et al., 1992), a G protein may be coupled to it in a manner analogous to coupling to serpentine receptors (Wilkie et al., 1992), and may be activated by sucrose uptake. Alternatively, an intracellular sucrose binding protein may interact with a G protein to stimulate its activity.

In *Escherichia coli* and *Saccharomyces cerevisiae*, sugar-regulated gene expression has been studied in the context of the phenomenon of catabolite repression. In both organisms, the presence of glucose in the medium has been shown to repress the expression of genes whose products are required for proper metabolism of other carbohydrates, like lactose and galactose (Saier, 1989; Gancedo, 1992). In *E. coli*, the catabolite repression system is very simple and exquisitely sensitive, utilizing allosteric control of enzyme activity and regulation of cAMP levels via a mechanism directly regulated by a system of phosphate transfer to regulate gene expression. In the absence of sugars recognized by sugar-specific enzyme II, phosphate groups are transferred from phosphoenolpyruvate (PEP) to enzyme I, which, in turn, transfers phosphate to HPr. Phosphate is then transferred to a complex of enzyme III and glucose (III^{glc}). Phosphorylated III^{glc} binds to adenylate cyclase, and activates it (Saier, 1989). Catabolite activating protein (CAP) binds cAMP, and is then able to bind as a dimer to a 22 bp CAP binding site, activating transcription of operons required for proper metabolism of a variety of sugars (de Crombrugghe et al., 1984). In the meantime, sugar permeases are active, allowing the entry of many different sugars into the cell (Saier, 1989).

With glucose present in the medium, however, HPr transfers its phosphate to enzyme II, which, in turn, transfers the phosphate to glucose. Because phosphorylated enzyme I, HPr, and III^{glc} are in equilibrium with each other, diversion of phosphate to glucose effectively dephosphorylates III^{glc}. This results in the inactivation of adenylate cyclase, and dephosphorylated III^{glc} binds to sugar permeases, reducing their affinities for their specific sugars (Saier, 1989). Loss of cAMP via phosphodiesterase-mediated hydrolysis renders CAP unable to bind its operator, and transcription is effectively shut

off (de Crombrugghe et al., 1984). At the same time, uptake of other sugars is considerably reduced as well (Saier, 1989). Through this simple mechanism of phosphate transfer, *E. coli* is able to maintain fine control of the transcription of sugar metabolic genes.

Catabolite repression in yeast is significantly more complicated than it is in *E. coli*. Repression is known to require the activity of hexokinases 1 or 2, as well as the DNA binding proteins CYC8 and TUP1 (Gancedo, 1992). Hexokinases must phosphorylate their target sugars in order to achieve catabolite repression, as loss of the ability to phosphorylate glucose strongly correlates with the loss of catabolite repression (Ma and Botstein, 1986; Ma et al., 1989). It is unclear, however, what role sugar phosphorylation plays in this process. CYC8 and TUP1 have been shown to associate in a high molecular mass complex (Williams et al., 1991), and both have been shown to mediate the formation of repressive chromatin structures (Cooper et al., 1994). Mutations of the *GRR1* gene have also been demonstrated to result in loss of catabolite repression. The wild type protein is part of a large complex that is localized to the cytoplasm, and it has been proposed that GRR1 is required for the generation of the intracellular signal that causes glucose repression (Flick and Johnston, 1991).

Derepression of genes that are inactivated by catabolite repression is mediated by a number of proteins, mutations of which result in the inability to reinduce the transcription of repressed genes. These proteins include the kinase CAT1 and CAT3, a protein that may act by stabilizing CAT1 or by preventing the function of a phosphatase that is antagonistic to it (Celenza and Carlson, 1986; Celenza and Carlson, 1989). HAP2, HAP3, and HAP4 appear to be transcription factors that associate in a complex in which HAP2 and HAP3 mediate DNA binding, and HAP4 stabilizes the complex or alters its conformation (Olesen et al., 1987; Forsburg and Guarente, 1989). SNF2, SNF5, and SNF6 are nuclear-localized proteins, but there is no evidence for DNA binding activity, and their mode of action in mediating derepression is not known (Gancedo, 1992).

Multicopy suppression screens to identify suppressors of catabolite repression identified a protein, MSN1, that is nuclear localized and is rich in acidic residues, but binds DNA weakly (Estruch and Carlson, 1990). Despite the large number of mutants that have been isolated, it is not clear how catabolite repression or derepression of inactivated genes is accomplished in yeast.

The components of the pathway through which sucrose stimulates the transcription of anthocyanin biosynthetic genes are currently unknown. In plants, it has been found that inhibition of protein phosphatases 1 and 2A blocks sucrose-inducible expression of a number of genes of sweet potato (Takeda et al., 1994). The results described in this study demonstrate that the protein kinase inhibitor genistein also inhibits sucrose-mediated induction of *CHS*. Because genistein is an isoflavonoid (4', 5, 7-trihydroxyisoflavone) and is itself a product of plant anthocyanin biosynthesis, it is possible that its effects may result from feedback inhibition, and not from an effect on kinases. Alternatively, it is possible that genistein defines a kinase that functions in a sucrose signaling pathway. The roles played by different transcription factors in sucrose-inducible gene expression will be considered below (see Synergistic interactions between phytochrome and sucrose).

The mechanism of sugar-mediated repression of gene expression is similarly unclear. It has been shown in an autotrophic cell culture of *Chenopodium rubrum* that the repression of *RBCS* correlates with increased levels of hexose phosphates and UDP-glucose, 2 predicted products of glucose metabolism (Krapp et al., 1993). By contrast, studies of photosynthetic gene expression in maize protoplasts have shown that treatment of cells with glucose-6-phosphate has no effect on gene expression, suggesting that hexose phosphates play no role in sugar repression (Jang and Sheen, 1994). The latter study suggests that hexokinase serves as a sensor that distinguishes sugar levels and transmits a signal that results in the repression of photosynthetic gene expression (Jang and Sheen, 1994). Data described here suggest that this hypothesis is unlikely, because in

soybean cells, the hexoses glucose and fructose were ineffective inhibitors of photosynthetic gene expression relative to sucrose (Figure 10). As described above, differences of cell culture and experimental methodology may explain this discrepancy, but the fact remains that under different conditions, sucrose and hexoses inhibit gene expression to different degrees.

Unlike catabolite repression in *E.coli*, in which the induction and repression of gene expression are tightly linked to phosphate transfer, these two processes are genetically separable in yeast (Gancedo, 1992), and biochemically-separable in plants. The isolation of two different classes of mutants in *S. cerevisiae* suggests that two different regulatory mechanisms function to maintain proper control of carbohydrate metabolism. The manner in which these two mechanisms interact is unknown. In plants, gene expression is inducible by cGMP, whereas repression of photosynthetic gene expression is mediated by a mechanism that does not require cGMP. Nevertheless, recent demonstration that both induction and repression of gene expression require the activity of G proteins suggests that the two processes may share common upstream steps.

Synergistic interactions between phytochrome and sucrose

It is clear that the abilities of phytochrome and sucrose to induce *CHS* expression separately is considerably reduced compared to that observed when the two act in concert. Phytochrome stimulates *CHS* expression only transiently, peaking within 2 hours, and rapidly desensitizing thereafter, in both R and FR. By contrast, in the presence of sucrose *CHS* mRNA levels are considerably higher, and expression persists for 18 hours in R and more than 24 in FR (Figure 11). Such a dramatic effect on gene expression cannot be attributed to an additive interaction alone. Instead, the two pathways appear to interact synergistically to regulate gene expression.

Mechanisms of transcriptional synergy appear to primarily occur at the level of interactions between transcription factors. It has been shown that transcriptional synergy that is mediated by ZEBRA, a non-acidic activator of the Epstein-Barr virus lytic cycle,

occurs because it facilitates interaction of the basic transcriptional apparatus with the TATA box. Moreover, it enhances interactions between the TFIID:TFIIA complex with TFIIB, thus enhancing the ability to initiate transcription (Chi et al., 1995). Similarly, it has been found that the *Drosophila* developmental regulators Hunchback and Bicoid synergistically interact through specific interactions with the TATA binding protein (TBP)-associated factors (TAF_{II}s) 60 and 110, respectively. Simultaneous interaction of both transcription factors with these two TAF_{II}s stimulates transcription to a much higher level than either accomplishes alone (Sauer et al., 1995). A third level of interaction is seen between the homeodomain-containing protein DORSAL and the helix-loop-helix transcription factor TWIST. In this case, TWIST enhances binding of DORSAL to its operator, and stronger activation of the promoter is seen, as evidenced by increased domains of target gene expression (Szymanski and Levine, 1995).

A fourth level of synergistic interaction is seen in which fibroblast growth factor (FGF), which acts through a RAS-dependent pathway, and cAMP synergistically regulate gene expression from the same cAMP responsive element. The synergistic interactions in this case are mediated by the transcription factors ATF-3 and c-Jun, and FGF promotes accumulation of both. Moreover, FGF promotes the formation of ATF-3:c-Jun heterodimers that bind to the response element and activate transcription (Tan et al., 1994). A similar situation may exist in the case of phytochrome and sucrose. Both synergistically stimulate transcription from the *CHS* promoter, and each may utilize the Unit I element of the parsley *CHS* promoter to activate transcription. This element contains binding sites for MYC- (CANNTG) and MYB-like (C/TAACG/TG) DNA binding proteins (Block et al., 1990). Moreover, the MYC-like site may also serve as a binding site for basic-leucine zipper (b-Zip) transcription factors (Weisshaar et al., 1991). It remains to be determined whether the Unit I element may be synergistically-activated by sucrose and phytochrome, and if so, which DNA binding elements are required for synergy.

The synergistic effect of sucrose and phytochrome on CHS expression raises intriguing possibilities regarding the mechanism of synergistic interaction. One possibility for increased mRNA levels is enhanced stability of *CHS* mRNA, as it has been shown that cAMP enhances the stability of nitric oxide synthase mRNA in its synergistic interactions with interleukin-1β (Kunz et al., 1994). Secondly, sucrose and phytochrome may utilize different transcription factors that interact synergistically at the level of the *CHS* promoter to enhance gene expression. This may be mediated by cooperative binding, interaction with TAF_{II}s, or facilitation of binding to the TATA box by the basic transcriptional machinery. Third, since both phytochrome and sucrose utilize cGMP to regulate *CHS* expression, interactions may occur upstream in the signal transduction pathway that enhance production of cGMP, or hyperactivate kinase or phosphatase activity. The expression of *CHS* in response to sucrose and phytochrome may provide an excellent model system in which to investigate synergistic interactions between signal transduction systems in higher plants.

ft: *a mutation that affects the synergistic integration of sucrose and phytochrome signals*

ft is a mutation of *Arabidopsis* that causes plants to flower later than WT in both short and long days (Koornneef et al., 1991). By virtue of the late flowering phenotype of *zap1* mutants, *ft* plants were tested for sensitivity to zaprinast, and it was found that they were weakly insensitive to the drug under high light fluence rates. Under low light fluence rates, *ft* plants were considerably more resistant to zaprinast than WT (Figure 36). Studies of gene expression in *ft* yielded surprising results. It was found that *CHS* expression in R in the presence of sucrose was reduced 2-fold compared to WT (Figure 41). In addition, expression of the same gene in FR in the presence of sucrose reached levels equivalent to WT, but the desensitization of *CHS* to FR was considerably delayed (Figure 42). In contrast, the expression of *CHS* in R and FR in the absence of sucrose was normal compared to WT (Figure 43). Under all conditions tested, *CAB* expression

displayed kinetics and expression levels equivalent to those observed in WT plants. These results suggest that the *ft* mutation affects the way in which phytochrome and sucrose interact to regulate the activity of the cGMP-dependent pathway of phytochrome, as measured by the expression of *CHS*.

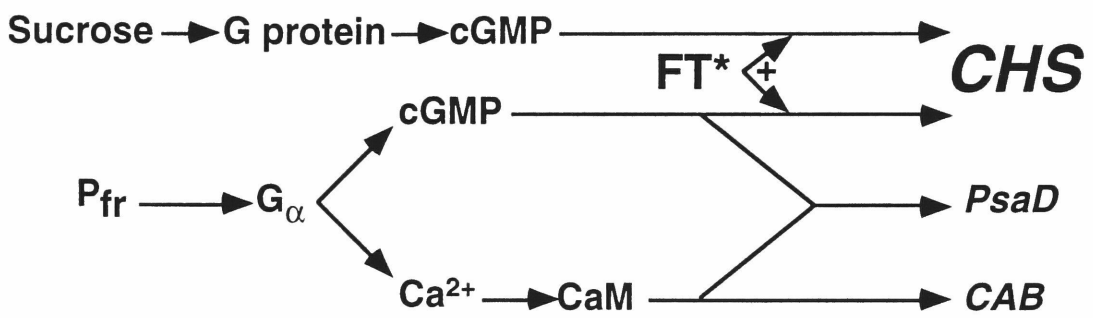
The observation that *ft* affects normal induction of *CHS* in R, whereas it blocks desensitization in FR is paradoxical. Such a mutation would not be expected to have two seemingly disparate effects. It is possible, however, to deduce a role for wild type FT protein, that would allow it to function in two different mechanisms. The recessive nature of the *ft* mutation suggests that it is deficient in a positively-acting factor that promotes flowering. In R, it is also deficient in a positively-acting factor that helps integrate signals from sucrose and phytochrome to regulate the expression of cGMP-responsive genes. Expression of *CHS* in response to sucrose or phytochrome alone is not significantly affected in *ft* mutants, suggesting that the wild type protein plays little role in either signaling pathway as it functions independently. Thus, one may predict that interaction of the two signaling pathways results in modification of FT, allowing it to enhance the expression of cGMP-responsive genes (Figure 45A).

Similarly, *ft* mutations result in the reduced ability to desensitize in FR, suggesting that the mutant is deficient in a factor that promotes desensitization. It is possible that a negative feedback mechanism that participates in desensitization of *CHS* expression in the context of synergistic phytochrome and sucrose activation requires the activity of FT to function properly. In this scenario, modified FT serves as the signal or regulates the production of one that is recognized by the negative feedback mechanism, resulting in its full activation and subsequent down-regulation of cGMP-dependent pathway activity and *CHS* expression (Figure 45B).

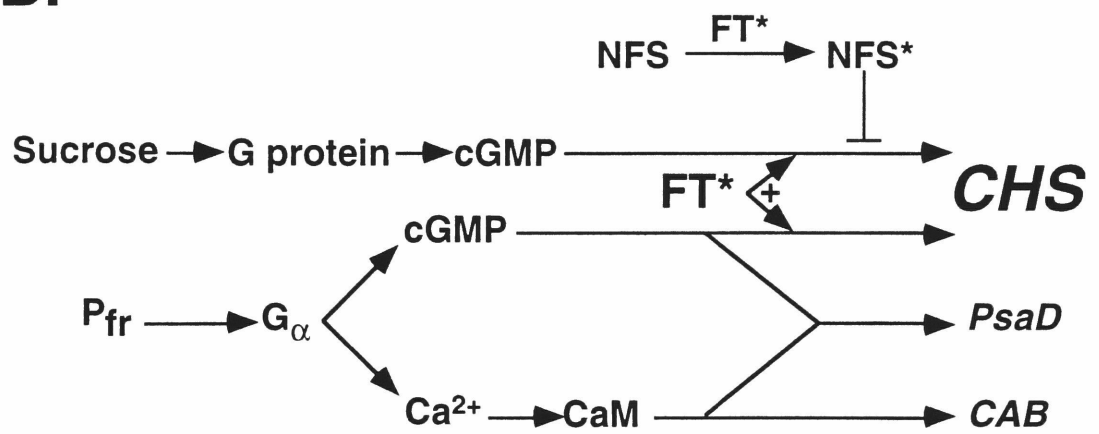
ft mutations would be predicted to cause similar reductions in *CHS* expression in FR as well as R, yet none are observed. This may be explained by the fact that a FR high irradiance response (HIR) produces much stronger responses than prolonged irradiation

Figure 45: A proposed role for FT in the synergistic regulation of gene expression by phytochrome and sucrose. A. A proposed mechanism by which FT acts to integrate the signals of sucrose and phytochrome. FT*: activated FT that has been modified by the sucrose and phytochrome signaling pathways. B. A proposed mechanism through which FT regulates desensitization of *CHS*. FT*: as above; NFS: inactive negative feedback system; NFS*: activated negative feedback system.

A.


Sucrose
G protein
cGMP
FT*
+
CHS
cGMP
Pfr
Gα
PsaD
Ca2+
CaM
CAB

B.


NFS
FT*
NFS*
Sucrose
G protein
cGMP
FT*
+
CHS
cGMP
Pfr
Gα
PsaD
Ca2+
CaM
CAB

with high fluence rate R (Mancinelli, 1994). Under FR HIR conditions, induction of *CHS* receives strong inputs from sucrose, but receives much higher levels of stimulation from phytochrome than may be observed in R. Consequently, a 2-fold reduction of expression caused by the *ft* mutation may be masked by increased stimulation of gene expression resulting from the FR HIR. This hypothesis may be tested in two ways. Most simply, induction of *CHS* expression may be performed with significantly reduced levels of FR, resulting in reduced stimulation of the cGMP-dependent signaling pathway by phyA. The concomitant reduction in *CHS* expression may then allow the loss of function caused by *ft* mutations to become visible at the level of gene expression. Second, *ft* may be crossed into a genetic background that results in reduced levels of *CHS* expression in FR in the presence of sucrose, such as *fhy1* (S. Barnes, personal communication). This, too, could allow a 2-fold reduction in mRNA levels to be observed by RNA gel blot analysis.

The *ft* mutation would also be predicted to affect the proposed negative feedback mechanism in R as well, yet no effect on desensitization is observed. This difference may be reconciled by the fact that desensitization in R and FR appears to occur by different mechanisms. In R, it is observed that following peak *CHS* expression between 12 and 18 hours after the onset of illumination, mRNA levels rapidly decline to basal levels by 24 hours. By contrast, *CHS* expression peaks between 18 and 24 hours in FR, and slowly desensitizes, returning to basal levels by 48 hours. Even in *phyA* mutants, expression of *CHS* in R rapidly declines after 18 hours, ruling out a role for phyA decay as a desensitization mechanism. Thus, there appears to be a mechanism through which activity of the cGMP pathway is dramatically reduced after 18 hours in R, that does not function in FR. Consequently, the FT-dependent negative feedback mechanism may not function until after 24 hours of constant stimulation, by which time, *CHS* expression in R has already returned to basal levels, thereby masking the penetrance of this aspect of the *ft* mutation.

Evidence for a negative feedback mechanism is indirect at best at this time. Experiments investigating the effects of zaprinast on gene expression demonstrated that in FR, mRNA levels of *CHS* remained higher for an extended period of time, but eventually returned to basal levels. Because *CAB* levels remained repressed over this time period, it is not likely that zaprinast degradation allowed desensitization to occur. Rather, it is possible that a desensitization mechanism that functions independently of a cGMP PDE exists in plants. Testing this hypothesis is not feasible in *Arabidopsis*, as specific mutants that alter desensitization of *CHS* in FR have not been identified. It has been found, however, that *CHS* levels decline in soybean cells after prolonged treatment with sucrose in the dark. Because this mechanism functions late in the time course of sucrose treatment, it is possible that it may be synthesized *de novo* after the onset of treatment. If this is the case, it may be possible to inhibit the synthesis of the components of the negative feedback system with cycloheximide. Because *Arabidopsis* mutants that are deficient in desensitization in FR are not available, proving the existence of such a mechanism is currently neither straightforward nor possible.

Based on these experiments, the role of FT in the promotion of flowering and the regulation of gene expression is becoming clearer. Recent work has demonstrated that plants grown in constant darkness are able to flower as long as their apical meristems are in contact with sucrose (Martinez-Zapater et al., 1995). These data suggest that proper transport of sucrose or transmission of sucrose-dependent signals to the meristem is an important factor in the induction of flowering. In genetic studies of 12 late flowering mutants, the *ft* mutation was proposed to disrupt processes that act very early to promote the transition of the apical meristem from vegetative growth to reproductive growth (Koornneef et al., 1991). If *ft* is deficient in the integration of sucrose signals with those of the flowering time regulator phyB, meristems of mutant plants may not receive enough of a factor that promotes the vegetative to reproductive transition, resulting in late flowering.

The role of ZAP1 in phytochrome-dependent signaling

Mutants that were resistant to the effects of zaprinast in constant R were isolated, and 4 were found to retain zaprinast resistance in the M3 generation. These 4 plants were derived from 3 separate pools of mutagenized seed, and the 2 plants that were selected for further characterization were found to be allelic. *zap1* mutants segregate in a manner consistent with single gene recessive mutations (Table 3). Adult *zap1* mutant plants are very different in appearance from their isogenic WT counterparts, displaying reduced apical dominance, large stature, and increased fertility (data not shown). Further testing demonstrated that *zap1* mutants were resistant to zaprinast in both R and FR, retaining the general sensitivity in darkness that is observed in plants of all other genotypes that have been tested. In addition, *zap1* mutants possess elongated hypocotyls relative to WT in the absence of zaprinast in R, FR, and B, with and without sucrose present in the medium (Figure 29, Figure 30, and Figure 32; data not shown). In W, however, *zap1* mutants are only slightly longer than WT in the absence of sucrose, and the hypocotyl lengths of WT and *zap1* plants are equivalent in darkness (Figure 35).

Because hypocotyl length is an accurate indicator of photoreceptor pathway activity (Koornneef et al., 1980), other physiological responses that are known to be regulated by phytochrome were examined in *zap1* mutants. It was found that the *zap1-2* mutant, which possesses a strong allele of *zap1*, produces reduced levels of anthocyanin in FR in the presence of sucrose (Figure 33). In addition, preliminary evidence indicates that *zap1-2* seeds do not germinate well in constant FR, while retaining normal germination in other light conditions (data not shown). Because these two responses are known to be regulated by phyA, the loss of function displayed by *zap1-2* plants suggests that their molecular lesion may partially disrupt phyA-dependent signaling. It was also found that both *zap1* mutants flowered later than WT plants, producing inflorescence meristems approximately 4 days later than WT, and producing 2 more rosette leaves during that time (Table 5). Because flowering time is known to be regulated in large part

by phyB, these data suggest that *zap1* mutations disrupt phyB signaling. It was found, however, that *zap1* mutants displayed normal increases in hypocotyl length in response to end-of-day FR treatment, another response that is regulated by phyB (Figure 35). Thus, *zap1* mutations may disrupt only a subset of phyB-regulated responses.

The phenotype of *zap1* mutants is entirely novel. Previously-identified loss-of-function mutants have specifically disrupted the photoreceptors in all but a few cases. In the cases of *fhy1* and *fhy3*, their insensitivity to light is confined to FR (Whitelam et al., 1993). Like *zap1* plants, *hy5* mutants are insensitive to R, FR, and B (Koornneef et al., 1980), but *hy5* plants, however, are sensitive to zaprinast in R, whereas *zap1* plants are resistant to the drug. Moreover, *zap1* plants are only modestly longer than WT plants in W, whereas *hy5* mutants are significantly longer (Koornneef et al., 1980). It is for that reason that *zap1* mutants were not previously isolated in screens for long hypocotyl mutations in W. Similarly, their hypocotyls are not as long as those of *fhy1* and *fhy3* in the absence of sucrose in FR, rendering them inconspicuous in a screen for FR-insensitive plants.

zap1 mutants also possess a phenotype that has not been observed before in long hypocotyl mutants. Specifically, they undergo normal photomorphogenesis in the light, despite retaining elongated hypocotyls. The apical hooks open, cotyledons open and expand, and radial expansion occurs in the hypocotyl. This phenotype is not observed in all previously-isolated mutations, in which the apical hooks may open, but cotyledon expansion is retarded (Koornneef et al., 1980). Moreover, radial expansion of the hypocotyls of *hy* mutants does not occur to the same degree as it does in *zap1* mutants (RBM, unpublished observation). There are two possible explanations why *zap1* mutants possess the phenotypes they do. First, the mutations may simply be leaky, and if some residual protein function remains, normal photomorphogenesis may occur to a large degree. Alternatively, the *zap1* mutation may affect only a subset of phytochrome-regulated processes. This possibility is more likely given the fact that some phyB-

regulated processes like flowering time are altered, whereas others like EOD FR responses appear normal. Mutations affecting only subsets of responses have been isolated in the ethylene response pathway of *Arabidopsis* (Ecker, 1995). In this system, disruptions of the ethylene receptor ETR1 cause complete loss of ethylene responsiveness (Bleecker et al., 1988), whereas mutations in the *EIN3* and *EIN6* genes only affect some processes controlled by ethylene (Lawton et al., 1994; Roman et al., 1995).

Based on the phenotypes of the two alleles of *zap1*, it is possible to tentatively assign a role for the wild type gene product in the phytochrome signal transduction pathway. The mutants are insensitive to zaprinast in both R and FR, much like *phyAphyB* double mutants. These data suggest that ZAP1 functions as a component in the pathways regulated by both phyA and phyB. These data are supported by the observation that in the absence of zaprinast, *zap1* mutants possess longer hypocotyls than WT plants in R and FR as well, with and without sucrose (Figure 29, Figure 30, and data not shown), indicating that they do not perceive these light signals as efficiently as WT plants. In addition, strong alleles of *zap1* produce less anthocyanin than WT plants and do not germinate as well in FR, indicating a defect in phyA responses. Finally, both *zap1* mutants flower later than WT, suggesting a defect in phyB responses, although they retain WT EOD FR responses. All of these data suggest that ZAP1 functions in the pathways regulated by both phyA and phyB, but appears to contribute more to phyA-dependent responses.

Isolation of novel mutations that affect phytochrome signaling

The lessons learned from the isolation of zaprinast-resistant mutants have implications for the isolation of new mutations that disrupt phytochrome signaling. It appears likely that the screen for loss-of-function mutations based on long hypocotyl length in W is saturated (Millar et al., 1994). Thus, novel screens must be developed. From the results described here, it is clear that drugs that have phytochrome-dependent effects may be used to isolate loss-of-function mutations, and it is likely that similar

approaches may be devised to allow the isolation of phytochrome-dependent gain-of-function mutations as well. Characterization of *zap1* mutants and other loss-of-function mutants like *fhy1* have demonstrated the utility of measuring the effect of a mutation on phytochrome-regulated physiological processes (Johnson et al., 1994). Genetic screens that take advantage of well-characterized phytochrome-dependent physiological responses in addition to the inhibition of hypocotyl elongation should be explored to allow the isolation of mutations that have more subtle effects. Mutations that confer subtle effects on phytochrome responses may yield significant insights into the mechanisms by which this photoreceptor system controls plant growth and development.

Conclusions

In this work, the mechanisms by which phytochrome and sucrose regulate a diverse group of responses ranging from germination to gene expression have been investigated. Pharmacological analysis of gene expression in a soybean cell culture has resulted in the discovery that sucrose induces the expression of anthocyanin biosynthetic genes via a cGMP-dependent signaling pathway. This observation coupled with similar observations made with regard to phytochrome signaling has profound implications for the way in which different signal transduction pathways function in the context of a global regulatory network. The observation that sucrose and phytochrome synergistically interact to regulate the expression of *CHS* raises questions of how the two pathways interact to achieve their combined effects. One possible insight has been gained through the analysis of the late flowering mutation *ft*, which was found to reduce the level of *CHS* expression in R, and to delay the desensitization of this gene in FR, when sucrose and phytochrome acted in concert. In addition, the development of a genetic screen based on sensitivity to the cGMP phosphodiesterase inhibitor zaprinast in R has resulted in the isolation of mutants that disrupt some phytochrome-regulated processes. Further

characterization of these mutants may yield significant insights into the nature of the phytochrome signal transduction pathway.

Chapter 6
Materials and Methods

Plant Material

Growth of *Arabidopsis thaliana*

Wild type *Arabidopsis thaliana* plants of the Landsberg *erecta* ecotype were used for all experiments. Seeds were obtained from laboratory stocks, or were purchased from Lehle Seeds (Round Rock, TX). The *Arabidopsis* mutants *hy1-1* (Koornneef et al., 1980), *phyB-5* (formerly *phyB-8-36* and *hy3-8-36*) (Koornneef et al., 1980), *hy4-2.23N* (Koornneef et al., 1980), *ft-1*, and *fha-1* (Koornneef et al., 1991) were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH). The mutant *phyA-201* (formerly *fre1-1*) (Nagatani et al., 1993) was provided by Dr. Akira Nagatani, *phyA-201phyB-5* was generated and provided by Dr. Andrew Millar (University of Warwick, UK), and *fhy1* (Whitelam et al., 1993)was kindly provided by Dr. Garry Whitelam (University of Leicester, UK).

For growth under aseptic conditions, seeds were sterilized as follows: Seeds are soaked in a solution of 2.6% sodium hypochlorite (Clorox) containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20) for 10 minutes. This solution was diluted 2 fold with sterile water, seeds were pelleted by 1 second centrifugation, and the supernatant was discarded. Seeds were subsequently rinsed at least 3 times with sterile water, discarding the supernatant each time after pelleting the seeds as described above. Seeds were resuspended in 0.15% low melting point agarose (SeaPlaque, FMC Bioproducts, Rockland, ME). Seeds were then plated on solid media (varies depending on the experiment, see below). For growth in soil, seeds were suspended in 0.15% low melting point agarose, and stored at 4° C for 24 hours prior to sowing in soil (Metromix 2000, Scotts Sierra Horticultural Products, Marysville, OH). The growth conditions in soil were variable depending on the experiment (see below).

Soybean cell culture

The SB-P cell culture of soybean (*Glycine max*) which was first described by Horn et al. (1983) was used for all experiments. Cells were grown under photomixotrophic conditions (atmospheric CO_2) in KN1 medium (1X MS salts (JRH Biosciences, Denver, PA), 15 mM sucrose, 0.1 mg/l thiamine (Sigma, St. Louis, MO), 0.2 mg/l kinetin (Sigma), and 1 mg/l α-naphthalene acetic acid (NAA; Sigma), pH 5.7; Lam et al., 1989). Cells were maintained in 50 ml cultures constantly shaking in constant white light (50 μmol m^{-2} sec^{-1}), and were subcultured (5 ml per 50 ml culture) every 2 weeks. 500 ml cultures for the experiments described herein were established by inoculating 500 ml KN1 medium with a 2 week old 50 ml culture, were grown ~10 days in the conditions described above, and were dark-adapted for 3-4 days prior to experimental treatments. All subsequent manipulations of dark-adapted cells were performed under green safelight conditions.

After dark-adaptation, large cultures were split, and solid sucrose, mannitol, glucose, or fructose was added to yield equimolar final concentrations. For dark experiments, time courses commenced immediately upon addition of sugar, whereas in red light experiments, cells were incubated for three hours in the dark after sugar addition, and were subsequently shifted to constant red light (50 μmol m^{-2} sec^{-1}). Samples were collected on filters by vacuum filtration and immediately frozen in liquid nitrogen.

Light conditions

Red light was generated by F48T12/2364/VHO fluorescent bulbs (Osram Sylvania, Danvers, MA; Parks and Quail, 1993), and filtered through 1 layer of #2793 red plexiglas (Laird Plastics, Hicksville, NY). Far-red light was generated by F48T12/232/VHO fluorescent bulbs (Osram Sylvania) and filtered through 1 layer of FRF red filter #067894 (West Lake Plastics, Lenni, PA; Parks and Quail, 1993). Blue light was generated by General Electric F20T12-B fluorescent bulbs (Tudor Electric,

New York, NY), and filtered through 1 layer of #2424 blue plexiglas (Dayton Plastics, Columbus, OH; Liscum and Hangarter, 1991). Fluence rates of red and blue light were measured with a LI-189 Quantum Radiometer (Li Cor, Lincoln, NE), and fluence rates of far-red light were measured with a IL1400A Radiometer/Photometer (International Light, Newburyport, MA).

RNA isolation and analysis

Total RNA from *Arabidopsis* seedlings and soybean cells was isolated by the following method (Lam et al., 1989). Tissue was frozen with liquid N_2 and ground to a fine powder with a mortar and pestle. The powder was transferred to 4.5 ml ATA extraction buffer (50 mM Tris-Cl, pH 8.0, 300 mM NaCl, 5 mM EDTA, pH 8.0, 2% sodium dodecyl sulfate (SDS), 2 mM aurintricarboxylic acid (ammonium salt; Sigma), and 0.1% (v/v) β-mercaptoethanol) and vortexed. 0.7 ml ice cold 3 M KCl was added, and the solution was incubated on ice for 15 minutes. Samples were centrifuged at 8800 xg for 20 minutes, and the supernatant was mixed with 2 ml 8 M LiCl. RNA was precipitated for 6 hours at 4° C. Samples were centrifuged at 8800 xg for 20 minutes, and the supernatant was discarded. The pellets were resuspended in 2 ml sterile distilled H_2O, and extracted with 1 ml borate-buffered phenol and 1 ml $CHCl_3$. Samples were centrifuged at 8800 xg for 15 minutes, and the aqueous phase was transferred to a new tube containing 0.2 ml 5 M NaCl and 6 ml 100% ethanol. Samples were precipitated at -80° C for 45 minutes, and were centrifuged at 8800 xg for 20 minutes. The supernatant was discarded, the pellets were washed with 1 ml 80% (v/v) ethanol, and the samples were centrifuged at 8800 xg for 5 minutes. Pellets were briefly air dried and were resuspended in 0.1-0.2 ml sterile distilled H_2O, depending on the size of the pellet. The concentration of total RNA was determined by measurement of optical density at 260 nm in an Ultrospec III spectrophotometer (Pharmacia LKB Biotechnology, Cambridge, UK).

Samples containing 10-20 μg total RNA were lyophilized and resuspended in loading buffer (50% (v/v) formamide, 6.5% (v/v) formaldehyde, 0.5X RNA gel running

buffer (see below), and 1/6 volume gel loading dye (50% (v/v) glycerol, 1 mM EDTA, pH 8.0, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF)), and heated to 65° C for 15 minutes. RNA was separated on 2.2 M formaldehyde gels that were run for approximately 4 hours in 1X RNA gel running buffer (0.1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.0), 40 mM sodium acetate, 5 mM EDTA (pH 8.0)) at 70 V essentially as described in Sambrook et al. (1989). Gels were stained with 1 μg/ml ethidium bromide in 1 liter 1X RNA gel running buffer for 20 minutes at room temperature, were destained in 1 liter 1x RNA gel running buffer for 3 hours at room temperature, and were subsequently photographed to assess equal loading. RNA was transferred to positively-charged nylon membranes (Boehringer Mannheim, Indianapolis, IN) in 10X SSC (1.5 M NaCl, 150 mM Na citrate) overnight. Blots were rinsed for 10 minutes in 2X SSC (300 mM NaCl, 30 mM Na citrate), air dried, and baked at 80° C for 1 hour.

The following DNA fragments were used as probes: a 1.1 kb Hin dIII fragment of soybean *CAB* (Walling et al., 1986), a 0.4 kb Hin dIII-Eco RI fragment of soybean *CHS* (Estabrook and Sengupta-Gopalan, 1991), a 0.8 kb Eco RI fragment of soybean *PsaD* (Dr. Hiroshi Yamagata, unpublished results), a 1.5 kb Eco RI fragment of *Arabidopsis* 18S ribosomal DNA (Takahashi et al., 1995), a 0.9 kb Pst I-Xba I fragment of *Arabidopsis CHS* (Feinbaum and Ausubel, 1988), and a 0.5 kb Ava II fragment of *Arabidopsis CAB1* (Leutweiler et al., 1986). Probes were labeled by random primed labeling using a Megaprime DNA labeling kit according to the manufacturers instructions (Amersham, Arlington Heights, IL). RNA gel blots were hybridized to labeled probes in hybridization buffer containing 6X SSC (900 mM NaCl, 90 mM Na citrate), 0.1% (w/v) SDS, 0.1% (w/v) non-fat dry milk, 50% (v/v) formamide, 7.5% (w/v) dextran sulfate, 100 mg/ml denatured salmon sperm DNA (Sigma), and 1X Denhardt's reagent, at 55° C for 12-18 hours. Blots were washed with washing buffer (3X SSC (450 mM NaCl, 45 mM Na citrate), 0.5% (w/v) SDS) at 55° C 2 times, 1 hour per wash. Blots were exposed to

XAR-5 film (Eastman Kodak, Rochester, NY) for 1-2 days with an intensifying screen at -80° C. Quantification of signals on RNA gel blots was performed on a Molecular Dynamics Phosphorimager (Sunnyvale, CA) according to the manufacturer's instructions.

RNA gel blots were routinely stripped for subsequent reprobing by the following method. Blots were vigorously shaken in boiling stripping buffer (0.1X SSC (15 mM NaCl, 1.5 mM Na citrate), 1% (w/v) SDS) for 15 minutes. The buffer containing the blots was boiled for 5 minutes in a microwave oven, and shaken for an additional 15 minutes. This step was then repeated. Subsequently, blots were thoroughly rinsed with distilled water, and prehybridized for 15-20 minutes at 55° C prior to subsequent hybridization with a new probe.

Chemical treatments of soybean cells

Genistein (Biomol, Plymouth Meeting, PA) was dissolved in DMSO to a final concentration of 74 mM, and was tested at various concentrations in a dose response experiment. In subsequent experiments, 125 μM genistein, which was found to be very effective at inhibiting sucrose induction of *CHS*, was used. Cycloheximide (Sigma) was dissolved in water to a concentration of 30 mg/ml and, was used at 30 μg/ml. Zaprinast, which was generously provided by Dr. Les Bell (Monsanto Co., St. Louis, MO), was dissolved in DMSO to 100 mM immediately prior to use, and was used at 100 μM. This concentration was found to be effective in both soybean cells (Bowler et al., 1994b) and animal cells (Tremblay et al., 1988).

Electron microscopic analysis

Six plants from each condition (Wild type or *ft*, +/- 175 μM zaprinast, grown for 5 days in darkness or constant red light (35 μmol m^{-2} sec^{-1})) were fixed in 3% glutaraldehyde in 100 mM cacodylate, pH 7.4 for 3 hours on ice in the refrigerator. This is to ensure that the dark grown plants are not exposed to light during fixation. The plants were then post-fixed in 2% osmium tetroxide in the same buffer for 2 hours on ice in the hood. All samples were treated *en bloc* with uranyl acetate (Farquhar and Palade,

1965), dehydrated in alcohol followed by treatment with propylene oxide. Subsequently, the plants were infiltrated and embedded in Spurr low viscosity resin per the vendor's instructions (Electron Microscopy Sciences, Fort Washington, PA).

Semi-thin sections were cut with glass knives, and stained with methylene blue-azure II (Richardson et al., 1960) for light microscopic evaluation. For ultrastructural study, 60 nm sections were made with a Diatome diamond knife on a Reichert-Jung Ultracut E microtome (Microscopical Optical Consulting, Inc., Valley Cottage, NY) and collected on Formvar-carbon coated copper grids. The thin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963), and were examined in a Jeol 100 CX electron microscope (JEOL, Peabody, MA) operated at 80 kv.

Genetic screen for zaprinast resistant mutants

800 ethyl methane sulfonate (EMS)-mutagenized seeds of the *Arabidopsis* ecotype Landsberg (*erecta*) (Lehle Seeds) were sterilized and resuspended in agar as described above, and were plated on 150 mm round plates containing solid medium (1X MS salts (JRH Biosciences), 90 mM sucrose, 175 μM zaprinast, 0.8% Bacto agar (Difco, Detroit, MI)). Seeds were vernalized at 4° C for 4 days, and were treated with white light (40 μmol m^{-2} sec^{-1}) for 30 minutes to induce germination. Seedlings were grown under constant high fluence rate red light (35 μmol m^{-2} sec^{-1}; see above) for 5 days. Mutants were selected as seedlings that possessed longer hypocotyls, open, expanded green cotyledons, and reduced agravitropism compared to the wild type, and were transferred to soil. Plants were grown at 20° C in a 16 hour:8 hour light:dark cycle until they set seed. Seeds were harvested, and were subsequently rescreened under identical conditions. Positive mutants were selected, transferred to soil, and allowed to set seed.

Genetic analysis of zaprinast resistant mutants

Zaprinast-resistant mutants were grown in soil to flowering, and were backcrossed to wild type Landsberg (*erecta*), with the wild type plant serving as the pollen donor. F_1 seeds of that cross were tested for zaprinast sensitivity as described above. The sensitive

plants were placed in low fluence rate white light (10 μmol m^{-2} sec^{-1}) to recover, at which time they were transferred to soil to set seed. F_2 seedlings of at least 12 individual F_1 plants were scored for zaprinast sensitivity as described above. A Chi-square Goodness of Fit test (Ambrose and Ambrose, 1981) was performed using the data obtained from this experiment to test the prediction that the mutants possessed single gene recessive mutations. To test for allelism, zaprinast-resistant mutants were grown in soil to flowering, and were crossed to each other as described in the text. F_1 seedlings of this cross were tested for zaprinast sensitivity as described above.

Hypocotyl length measurements

Seeds of different genotypes were sterilized and resuspended as described above, and were sown on 0.8% agar medium containing 1X MS Salts (JRH Biosciences) and 90 mM sucrose, with or without 175 μM zaprinast. Seeds were treated with white light (40 μmol m^{-2} sec^{-1}) for 30 minutes to induce germination, and were subsequently grown at 20° C for 5 days under the light conditions described in the text. 30 seedlings were selected at random from the populations on the plates for each condition (light+/-zaprinast; e.g. red+/-zaprinast) for each genotype, and were laid on the surface of agar plates. A scale bar was included on each plate. The plates were photographed, and the slides were projected on a screen. Hypocotyls of the projected seedlings were measured by hand with a ruler, and the actual hypocotyl length was determined based on the scale bar on each photographed plate. Mean hypocotyl lengths, standard deviations, and standard errors were calculated for each genotype under each condition using the spreadsheet program Microsoft Excel. Results were plotted as histograms using the graphing program Cricketgraph, and percent inhibition by zaprinast was calculated by the formula (1.00-(Length+zaprinast/Length-zaprinast)).

Anthocyanin assays

Seeds were sterilized and resuspended in agar as described above, and sown on 0.8% agar medium containing 1X MS salts and 90 mM sucrose. 175 μM zaprinast was

included in one experiment (see above). Seeds were vernalized at 4° C for 4 days, and were subsequently treated with white light (40 μmol m^{-2} sec^{-1}) to induce germination. Plates were wrapped in aluminum foil, and grown in darkness at 20° C for 4 days, at which time some plates were transferred to far-red light (5 μmol m^{-2} sec^{-1}) while controls were maintained in darkness for 3 days. Anthocyanins were extracted as previously described (Adamse et al., 1989). 3 sets of 50 seedlings for each sample in replicate experiments were placed in microcentrifuge tubes, and ground using a plastic grinding tool in 500 μl anthocyanin extraction buffer (1% (v/v) HCl in methanol). Samples were kept in darkness at room temperature overnight to extract the anthocyanins from the tissues. 333 μl of sterile distilled H_2O were added to each sample, and each sample was subsequently extracted with 500 μl $CHCl_3$. The absorbance of the aqueous phase was measured at 530 nm and 657 nm (Lange et al., 1971). Anthocyanin levels are expressed as (A_{530}-A_{657}/50 seedlings).

Gravitropism measurements

Seeds were sterilized and resuspended in agar as described above, and sown on 0.8% agar medium containing 1X MS salts with or without 175 μM zaprinast. Seeds were vernalized at 4° C for 4 days, and were wrapped in aluminum foil with no light pre-treatment. Seedlings were grown vertically on plates for 5 days in darkness. The plates were photographed, and the images projected on a screen. The angles relative to vertical of the hypocotyls of 200 seedlings were measured by hand with a protractor. Left of vertical was designated negative, and right of vertical was designated positive. The mean and standard deviations of each data set were determined using the spreadsheet program Microsoft Excel, and the number of seedlings at each angle were plotted as a histogram using the graphing program Cricketgraph. The degree of agravitropic growth is expressed by the standard deviation of the angles, as previously described (Liscum and Hangarter, 1993; Robson and Smith, 1996).

Flowering time measurements

50 seeds of each genotype were resuspended in 0.15% low melting point agarose, and were vernalized for 24 hours at 4° C. Subsequently, the seeds were sown in soil, and grown at 20° C under a light:dark cycle of 16 hours:8 hours. The plants were examined every day for the emergence of the inflorescence, and the day on which the inflorescence was first observed was designated as the number of days to flower (Koornneef et al., 1991). The number of rosette leaves was also determined. The mean number of days to flower and the mean number of rosette leaves at flowering was calculated for each genotype as well as the standard errors using the spreadsheet program Microsoft Excel. For the distribution of the number of plants flowering on each day, a histogram was plotted using the graphing program Cricketgraph.

End-of-day Far Red treatments

Seeds of each genotype were sterilized and resuspended in agar as described above, were sown on 0.8% agar containing 1X MS salts, and were vernalized for 4 days at 4° C. The plants were then grown for 3 days in a light:dark cycle of 8 hours:16 hours. Subsequently, half the plants of each genotype received a 15 minute treatment with far-red light (5 μmol m^{-2} sec^{-1}) prior to the shift to darkness, whereas the other half were immediately placed in darkness (Nagatani et al., 1991a). This treatment was carried out for 5 days. Subsequently, 40 seedlings for each treatment were laid on agar plates with a scale bar, and were photographed. The pictures were projected on a screen, and the hypocotyl lengths were measured by hand with a ruler. The actual hypocotyl length was determined based on the scale bar on each plate. The mean hypocotyl length, standard deviation, and standard error was calculated for each data set using the spreadsheet program Microsoft Excel. The results were presented as a histogram using the graphing program Cricketgraph.

Germination

Seeds were sterilized and resuspended in agar as described above, and were sown on plates containing 0.6% agar only. Plates were immediately wrapped in aluminum foil and placed at 4° C to imbibe and vernalize for 19 hours. The beginning of seed imbibition is measured from the time of the first rinse with sterile distilled H_2O during the seed sterilization process (Shinomura et al., 1994). Seeds are then grown in the dark, or under constant red (35 μmol m^{-2} sec^{-1}), far red (5 μmol m^{-2} sec^{-1}), or white light (40 μmol m^{-2} sec^{-1}) for 7 days, at which time, the degree of germination is assessed. A seed is considered to have germinated if, at the minimum, the radicle has penetrated the seed coat (Shinomura et al., 1994).

Bibliography

Adamse P, Peters JL, Jaspers PAPM, van Tuinen A, Koornneef M, Kendrick RE (1989) Photocontrol of anthocyanin synthesis in tomato seedlings: a genetic approach. Photochem Photobiol 50:107-111.

Ahmad M, Cashmore AR (1993) *HY4* gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. Nature 366:162-166.

Algarra P, Linder S, Thummler F (1993) Biochemical evidence that phytochrome of the moss *Ceratodon purpureus* is a light-regulated protein kinase. FEBS Lett. 315:69-73.

Ambrose H, Ambrose K (1981) A Handbook of Biological Investigation. Winston-Salem: Hunter Textbooks Inc.

Ang L-H, Deng X-W (1994) Regulatory Hierarchy of photomorphogenic loci: allele-specific and light-dependent interaction between the *HY5* and *COP1* loci. Plant Cell 6:613-628.

Audus L (1962) The mechanism of the perception of gravity by plants. Symp Soc Exp Biol 16:197-226.

Bagnall D, King R, Whitelam G, Boylan M, Wagner D, Quail P (1995) Flowering responses to altered expression of phytochrome in mutants and transgenic lines of *Arabidopsis thaliana* (L.) Heynh. Plant Physiol 108:1495-1503.

Barnes S, Nishizawa N, Quaggio R, Whitelam G, Chua N-H (1996a) Far-red light blocks greening of *Arabidopsis* seedlings via a phytochrome A-mediated change in plastid development. Plant Cell in press.

Barnes S, Quaggio R, Whitelam G, Chua N-H (1996b) *fhy1* defines a branchpoint in phytochrome A signal transduction pathways for gene expression. manuscript submitted.

Bartholomew DM, Bartley GE, Scolnik PA (1991) Abscisic Acid Control of *rbcS* and *cab* Transcription in Tomato Leaves. Plant Physiol 96:291-296.

Batschauer A, Gilmartin P, Nagy F, Schafer E (1994) The molecular biology of photoregulated genes. In: Photomorphogenesis in Plants (Kendrick R, Kronenberg G, eds), pp 559-600. Dordrecht: Kluwer Academic Publishers.

Beavo JA, Reifsnyder DH (1990) Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. TIPS 11:150-155.

Beggs C, Wellmann E (1994) Photocontrol of flavonoid biosynthesis. In: Photomorphogenesis in Plants (Kendrick R, Kronenberg G, eds), pp 733-751. Dordrecht: Kluwer Academic Publishers.

Bell C, Ecker J (1994) Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. Genomics 19:137-144.

Besnard-Wibaut C (1981) Effectiveness of gibberellins and 6-benzyladenine on flowering of *Arabidopsis thaliana*. Physiol Plant 53:205-212.

Bleecker A, Estelle M, Somerville C, Kende H (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science 241:1086-1089.

Block A, Dangl J, Hahlbrock K, Schulze-Lefert P (1990) Functional borders, genetic fine structure, and distance requirements of cis elements mediating light responsiveness of the parsley chalcone synthase promoter. Proc Natl Acad Sci USA 87:5387-5391.

Borthwick H, Hendricks S, Parker M, Toole E, Toole V (1952) A reversible reaction controlling seed germination. Proc Natl Acad Sci USA 38:662-666.

Borthwick HA, Hendricks SB (1960) Photoperiodism in plants. Science 132:1223-1228.

Bossen ME, Dassen HHA, Kendrick RE, Vredenberg WJ (1988) The role of calcium ions in phytochrome-controlled swelling of etiolated wheat (*Triticum aestivum* L.) protoplasts. Planta 174:94-100.

Bossen ME, Kendrick RE, Vredenberg WJ (1990) The involvement of a G-protein in phytochrome-regulated, Ca^{2+}-dependent swelling of etiolated wheat protoplasts. Physiol Plant 80:55-62.

Bowler C, Neuhaus G, Yamagata H, Chua N-H (1994a) Cyclic GMP and calcium mediate phytochrome phototransduction. Cell 77:73-81.

Bowler C, Yamagata H, Neuhaus G, Chua N-H (1994b) Phytochrome signal transduction pathways are regulated by reciprocal control mechanisms. Genes & Dev 8:2188-2202.

Boylan MT, Quail PH (1989) Oat phytochrome is biologically active in transgenic tomatoes. Plant Cell 1:765-773.

Boylan MT, Quail PH (1991) Phytochrome A overexpression inhibits hypocotyl elongation in transgenic *Arabidopsis*. Proc Natl Acad Sci USA 88:10806-10810.

Boylan M, Douglas N, Quail P (1994) Dominant negative suppression of *Arabidopsis* photoresponses by mutant phytochrome A sequences identifies spatially discrete regulatory domains in the photoreceptor. Plant Cell 6:449-460.

Bruce W, Quail P (1990) *cis*-Acting elements involved in photoreguation of an oat phytochrome promoter in rice. Plant Cell 2:1081-1089.

Bruce W, Deng X-W, Quail P (1991) A negatively acting DNA sequence element mediates phytochrome-directed repression of *PHYA* gene transcription. EMBO J 10:3015-3024.

Cabrera y Poch HL, Peto CA, Chory J (1993) A mutation in the *Arabidopsis DET3* gene uncouples photoregulated leaf development from gene expression and chloroplast biogenesis. Plant J 4:671-682.

Caspar T, Pickard B (1989) Gravitropism in a starchless mutant of *Arabidopsis*. Planta 177:185-197.

Castle LA, Meinke DW (1994) A *FUSCA* gene of Arabidopsis encodes a novel protein essential for plant development. Plant Cell 6:25-41.

Celenza J, Carlson M (1986) A yeast gene that is essential for release from glucose repression encodes a protein kinase. Science 233:1175-1180.

Celenza J, Carlson M (1989) Mutational analysis of the *Saccharomyces cerevisiae* SNF1 protein kinase and evidence for functional interaction with the SNF4 protein. Mol Cell Biol 9:5034-5044.

Chappell J, Hahlbrock K (1984) Transcription of defence genes in response to UV-light or fungal elicitor. Nature 311:76-78.

Chaudhury AM, Letham S, Craig S Dennis, ES (1993) *amp1*: A mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis, and precocious flowering. Plant J 4:907-916.

Cheng C-L, Acedo GN, Cristinsin M, Conkling MA (1992) Sucrose mimics the light induction of *Arabidopsis* nitrate reductase gene transcription. Proc Natl Acad Sci USA 89:1861-1864.

Chi T, Lieberman P, Ellwood K, Carey M (1995) A general mechanism for transcriptional synergy by eukaryotic activators. Nature 377:254-257.

Chory J, Peto CA, Ashbaugh M, Saganich R, Pratt L, Ausubel F (1989a) Different roles for phytochrome in etiolated and green plants deduced from characterization of *Arabidopsis thaliana* mutants. Plant Cell 1:867-880.

Chory J, Peto C, Feinbaum R, Pratt L, Ausubel F (1989b) *Arabidopsis thaliana* mutant that develops as a light-grown plant in the absence of light. Cell 58:991-999.

Chory J, Peto CA (1990) Mutations in the *DET1* affect cell-type-specific expression of light-regulated genes and chloroplast development in *Arabidopsis*. Proc Natl Acad Sci USA 87:8776-8780.

Chory J, Nagpal P, Peto CA (1991) Phenotypic and genetic analysis of *det2*, a new mutant that affects light-regulated seedling development in *Arabidopsis*. Plant Cell 3:445-459.

Chory J (1992) A genetic model for light-regulated seedling development in *Arabidopsis*. Development 115:337-354.

Chory J, Reinecke D, Sim S, Washburn T, Brenner M (1994) A role for cytokinins in de-etiolation in *Arabidopsis*. Plant Physiol 104:339-347.

Clack T, Mathews S, Sharrock R (1994) The phytochrome apoprotein family in *Arabidopsis* is encoded by five genes: the sequences and expression of *PHYD* and *PHYE*. Plant Mol Biol 25:413-427.

Cone J, Kendrick R (1985) Fluence-response curves and action spectra for promotion and inhibition of seed germination in wild type and long-hypocotyl mutants of *Arabidopsis thaliana* L. Planta 163:43-54.

Cooper J, Roth S, Simpson R (1994) The global transcriptional regulators, SSN6 and TUP1, play distinct roles in the establishment of repressive chromatin structure. Genes & Dev 8:1400-1410.

Datta N, Chen Y-R, Roux SJ (1985) Phytochrome and calcium stimulation of protein phosphorylation in isolated pea nuclei. Biochem Biophys Res Comm 128:1403-1408.

Datta N, Cashmore AR (1989) Binding of a pea nuclear protein to promoters of certain photoregulated genes is modulated by phosphorylation. Plant Cell 1:1069-1077.

de Crombrugghe B, Busby S, Buc H (1984) Cyclic AMP receptor protein: role in transcription activation. Science 224:831-838.

Dehesh K, Bruce W, Quail P (1990) A *trans*-acting factor that binds to a GT-motif in a phytochrome gene promoter. Science 250:1397-1399.

Dehesh K, Hung H, Tepperman J, Quail P (1992) GT-2: a transcription factor with twin autonomous DNA-binding domains of closely related but different target sequence specificity. EMBO J 11:4131-4144.

Dehesh K, Franci C, Parks BM, Seeley KA, Short TW, Tepperman JM, Quail PH (1993) Arabidopsis *HY8* locus encodes phytochrome A. Plant Cell 5:1081-1088.

Deikman J, Hammer P (1995) Induction of anthocyanin accumulation by cytokinins in *Arabidopsis thaliana*. Plant Physiol 108:47-57.

Deng X-W, Caspar T, Quail PH (1991) *cop1*: a regulatory locus involved in light-controlled development and gene expression in *Arabidopsis*. Genes & Dev 5:1172-1182.

Deng X-W, Quail PH (1992a) Genetic and phenotypic characterization of *cop1* mutants of *Arabidopsis thaliana*. Plant J 2:83-95.

Deng X-W, Matsui M, Wei N, Wagner D, Chu AM, Feldmann KA, Quail PH (1992b) *COP1*, an *Arabidopsis* regulatory gene, encodes a protein with both a zinc-binding motif and a G_{β} homologous domain. Cell 71:791-801.

Devlin P, Rood S, Somers D, Quail P, Whitelam G (1992) Photophysiology of the elongated internode (*ein*) mutant of *Brassica rapa*. Plant Physiol 100:1442-1447.

Dooner H, Robbins T, Jorgensen R (1991) Genetic and developmental control of anthocyanin biosynthesis. Annu Rev Genet 25:173-199.

Dynlacht BD, Weinzierl ROJ, Admon A, Tjian R (1993) The $dTAF_{II}80$ subunit of *Drosophila* TFIID contains β-transducin repeats. Nature 363:176-179.

Ecker JR (1995) The ethylene signal transduction pathway in plants. Science 268:667-675.

Edelman J , Hanson AD (1971) Sucrose suppression of chlorophyll synthesis in carrot callus cultures. Planta 98:150-156.

Estabrook EM, Sengupta-Gopalan C (1991) Differential expression of phenylalanin ammonia-lyase and chalcone synthase during soybean nodule development. Plant Cell 3:299-308.

Estruch F, Carlson M (1990) Increased dosage of the *MSN1* gene restores invertase expression in yeast mutants defective in the SNF1 protein kinase. Nucl Acids Res 18:6959-6964.

Fallon KM, Shacklock PS, Trewavas AJ (1993) Detection in vivo of very rapid red light-induced calcium sensitive protein phosphorylation in etiolated wheat (*Triticum aestivum*) leaf protoplasts. Plant Physiol 101:1039-1045.

Farquhar M, Palade G (1965) Cell junctions in amphibian skin. J Cell Biol 26:263-291.

Feinbaum RL, Ausubel FM (1988) Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol Cell Biol 8:1985-1992.

Finley D, Chau V (1991) Ubiquitination. Ann Rev Cell Biol 7:25-69.

Flick J, Johnston M (1991) *GRR1* of *Saccharomyces cerevisiae* is required for glucose repression and encodes a protein with leucine-rich repeats. Mol Cell Biol 11:5101-5112.

Forsburg S, Guarente L (1989) Identification and characterization of HAP4: a third component of the CCAAT-bound HAP2/HAP3 heteromer. Genes & Dev 3:1166-1178.

Frances S, White M, Edgerton M, Jones A, Elliott R, Thompson W (1992) Initial characterization of a pea mutant with light-independent photomorphogenesis. Plant Cell 4:1519-1530.

Furuya M (1989) Molecular properties and biogenesis of phytochrome I and II. Adv Biophys 25:133-167.

Furuya M (1993) Phytochromes: Their molecular species, gene families, and functions. Annu Rev Plant Physiol Plant Mol. Biol. 44:617-645.

Furuya M, Song P-S (1994) Assembly and properties of holophytochrome. In: Photomorphogenesis in Plants (Kendrick R, Kronenberg G, eds), pp 105-140. Dordrecht: Kluwer Academic Publishers.

Gancedo J (1992) Carbon catabolite repression in yeast. Eur J Biochem 206:297-313.

Gilmartin P, Memelink J, Hiratsuka K, Kay S, Chua N-H (1992) Characterization of a gene encoding a DNA binding protein with specificity for a light-responsive element. Plant Cell 4:839-849.

Goldschmidt EE, Huber SC (1992) Regulation of photosynthesis by end-product accumulation in leaves of plants storing starch, sucrose, and hexose sugars. Plant Physiol 99:1443-1448.

Goto N, Kumagai T, Koornneef M (1991) Flowering responses to light-breaks in photomorphogenic mutants of *Arabidopsis thaliana*, a long-day plant. Physiol Plant 83:209-215.

Graham IA, Denby KJ, Leaver CJ (1994) Carbon catabolite repression regulates glyoxylate cycle gene expression in cucumber. Plant Cell 6:761-772.

Green P, Kay S, Chua N-H (1987) Sequence-specific interactions of a pea nuclear factor with light-responsive elements upstream of the *rbcS-3A* gene. EMBO J 6:2543-2549.

Grimm R, Eckerskorn C, Lottspeich F, Zenger C, Rudiger W (1988) Sequence analysis of proteolytic fragments of 124-kilodalton phytochrome from etiolated *Avena sativa* L.: conclusions on the conformation of the native protein. Planta 174:396-401.

Grimm R, Gast D, Rudiger W (1989) Characterization of a protein-kinase activity associated with phytochrome from etiolated oat (*Avena sativa* L.) seedlings. Planta 178:199-206.

Hara I, Matsubara H (1980) Pumpkin (*Cucurbita* sp.) seed globulin VII. Immunofluorescent study on protein bodies in ungerminated and germinating cotyledon cells. Plant & Cell Physiol 21:247-254.

Harter K, Talke-Messerer C, Barz W, Schafer E (1993) Light- and sucrose-dependent gene expression in photomixotrophic cell suspension cultures and protoplasts of rape (*Brassica napus* L.). Plant J 4:507-516.

Harter K, Kircher S, Frohnmeyer H, Krenz M, Nagy F, Schafer E (1994) Light-regulated modification and nuclear translocation of cytosolic G-box binding factors in parsley. Plant Cell 6:545-559.

Haupt W, Weisenseel M (1976) Physiological evidence and some thoughts on localised responses, intracellular localisation and action of phytochrome. In: Light and Plant Development (Smith H, ed), pp 63-74. London: Butterworths.

Hershey H, Barker R, Idler K, Lissemore J, Quail P (1985) Analysis of cloned cDNA and genomic sequences for phytochrome: Amino acid sequences for two gene products expressed in etiolated *Avena*. Nucl Acids Res. 13:8543-8559.

Hiratsuka K, Wu X, Fukuzawa H, Chua N-H (1994) Molecular dissection of GT-1 from *Arabidopsis*. Plant Cell 6:1805-1813.

Horn ME, Sherrard JH, Widholm JM (1983) Photoautotrophic growth of soybean cells in suspension culture. Plant Physiol 72:426-429.

Hou Y, von Arnim AG, Deng X-W (1993) A new class of *Arabidopsis* constitutive photomorphogenic genes involved in regulating cotyledon development. Plant Cell 5:329-339.

Hunt RE, Pratt LH (1980) Partial characterization of undegraded oat phytochrome. Biochemistry 19:390-394.

Izawa T, Foster R, Chua N-H (1993) Plant bZIP protein DNA binding specificity. J Mol Biol 230:1131-1144.

Jackson J, Fuglevand G, Brown B, Shaw M, Jenkins G (1995) Isolation of *Arabidopsis* mutants altered in the light-regulation of chalcone synthase gene expression using a transgenic screening approach. Plant J 8:369-380.

Jang J-C, Sheen J (1994) Sugar sensing in higher plants. Plant Cell 6:1665-1679.

Johnson E, Bradley M, Harberd N, Whitelam G (1994) Photoresponses of light-grown *phyA* mutants of *Arabidopsis*. Plant Physiol 105:141-149.

Karrer EE, Rodriguez RL (1992) Metabolic regulation of rice α-amylase and sucrose synthase genes *in planta*. Plant J 2:517-523.

Kay S, Keith B, Shinozaki K, Chua N-H (1989a) The sequence of the rice phytochrome gene. Nucl Acids Res 17:2865-2866.

Kay S, Keith B, Shinozaki K, Chye M-L, Chua N-H (1989b) The rice phytochrome gene: Structure, autoregulated expression, and binding of GT-1 to a conserved site in the 5' upstream region. Plant Cell 1:351-360.

Kay S, Nagatani A, Keith B, Deak M, Furuya M, Chua N-H (1989c) Rice phytochrome is biologically active in transgenic tobacco. Plant Cell 1:775-782.

Kendrick RE, Kronenberg GHM, eds. (1994) Photomorphogenesis in plants. Dordrecht: Kluwer Academic.

Kim I-S, Bai U, Song P-S (1989) A purified 124-kDa oat phytochrome does not possess a protein kinase activity. Photochem Photobiol 49:319-323.

Koornneef M, Rolff E, Spruit CJP (1980) Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana* (L.) Heynh. Z Pflanzenphysiol Bd 100:147-160.

Koornneef M, Cone JW, Dekens RG, O'Herne-Robers EG, Spruit CJP, Kendrick RE (1985) Photomorphogenic responses of long hypocotyl mutants of tomato. J Plant Physiol 120:153-165.

Koornneef M, Hanhart C, van der Veen J (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. Mol Gen Genet 229:57-66.

Kraepiel Y, Rousselin P, Sotta B, Kerhoas L, Einhorn J, Caboche M, Miginiac E (1994) Analysis of phytochrome- and ABA-deficient mutants suggests that ABA degradation is controlled by light in *Nicotiana plumbaginifolia*. Plant J 6:665-672.

Krapp A, Hofmann B, Schafer C, Stitt M (1993) Regulation of the expression of *rbcS* and other photosynthetic genes by carbohydrates: a mechanism for the `sink regulation' of photosynthesis. Plant J 3:817-828.

Kubasek WL, Shirley BW, McKillop A, Goodman HM, Briggs W, Ausubel FM (1992) Regulation of flavonoid biosynthetic genes in germinating *Arabidopsis* seedlings. Plant Cell 4:1229-1236.

Kunz D, Muhl H, Walker G, Pfeilschifter J (1994) Two distinct signaling pathways trigger the expression of inducible nitric oxide synthase in rat renal mesangial cells. Proc Natl Acad Sci USA 91:5387-5391.

Lam E, Benedyk M, Chua N-H (1989) Characterization of phytochrome-regulated gene expression in a photoautotrophic cell suspension: possible role for calmodulin. Mol Cell Biol 9:4819-4823.

Lam H-M, Peng SS-Y, Coruzzi GM (1994) Metabolic regulation of the gene encoding glutamine-dependent asparagine synthetase in *Arabidopsis thaliana*. Plant Physiol 106:1347-1357.

Lange H, Shropshire W, Mohr H (1971) An analysis of phytochrome-mediated anthocyanin synthesis. Plant Physiol 47:649-655.

Lawton KA, Potter SL, Uknes S, Ryals J (1994) Acquired resistance signal transduction in *Arabidopsis* is ethylene independent. Plant Cell 6:581-588.

Leu W-M, Cao X-L, Wilson T, Snustad D, Chua N-H (1995) Phytochrome A and phytochrome B mediate the hypocotyl-specific downregulation of *TUB1* by light in *Arabidopsis*. Plant Cell 7:2187-2196.

Leutweiler L, Meyerowitz E, Tobin E (1986) Structure and expression of three light-harvesting chlorophyll *a/b*-binding protein genes in *Arabidopsis thaliana*. Nucl Acids Res 14:4051-4064.

Leyva A, Jarillo J, Salinas J, Martinez-Zapater J (1995) Low temperature induces the accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs of *Arabidopsis thaliana* in a light-dependent manner. Plant Physiol 108:39-46.

Li H, Altschmied L, Chory J (1994) Arabidopsis mutants define downstream branches in the phototransduction pathway. Genes & Dev 8:339-349.

Li H-m, Culligan K, Dixon R, Chory J (1995) *CUE1*: a mesophyll cell-specific positive regulator of light-controlled gene expression in *Arabidopsis*. Plant Cell 7:1599-1610.

Li J, Nagpal P, Vitart V, McMorris T, Chory J (1996) A role for brassinosteroids in light-dependent development of *Arabidopsis*. Science in press.

Liscum E, Hangarter RP (1991) *Arabidopsis* mutants lacking blue light-dependent inhibition of hypocotyl elongation. Plant Cell 3:685-694.

Liscum E, Hangarter R (1993) Genetic evidence that the red-absorbing form of phytochrome B modulates gravitropism in *Arabidopsis thaliana*. Plant Physiol 103:15-19.

Lissemore J, Quail P (1988) Rapid transcriptional regulation by phytochrome of the genes for phytochrome and chlorophyll a/b binding protein in *Avena sativa*. Mol Cell Biol 8:4840-4850.

Lopez-Juez E, Nagatani A, Buurmeijer WF, Peters JL, Furuya M, Kendrick RE, Wesselius JC (1990) Response of light-grown wild-type and *aurea*-mutant tomato plants to end-of-day far-red light. J Photochem Photobiol 4:391-405.

Ma H, Botstein D (1986) Effects of null mutations in the hexokinase genes of *Saccharomyces cerevisiae* on catabolite repression. Mol Cell Biol 6:4046-4052.

Ma H, Bloom LM, Walsh CT, Botstein D (1989) The residual enzymatic phosphorylation activity of hexokinase II mutants is correlated with glucose repression in *Saccharomyces cerevisiae*. Mol Cell Biol 9:5643-5649.

Mancinelli A (1983) The photoregulation of anthocyanin synthesis. In: Encyclopedia of Plant Physiology (Shropshire W, Mohr H, eds),Vol 16B, pp 640-661. New York: Springer-Verlag.

Mancinelli A (1994) The physiology of phytochrome action. In: Photomorphogenesis in Plants (Kendrick R, Kronenberg G, eds), pp 211-270. Dordrecht: Kluwer Academic Publishers.

Martinez-Zapater J, Roldan M, Ruiz-Garcia L, Gomez-Mena C, Salinas J (1995) Dark flowering only requires carbon availability at the apex. 6th International Conference on *Arabidopsis* Research 158 (Abstract).

Matile P (1975) The Lytic Compartment of Plant Cells. New York: Springer-Verlag.

McCormac A, Cherry J, Hershey H, Vierstra R, Smith H (1991) Photoresponses of transgenic tobacco plants expressing an oat phytochrome gene. Planta 185:162-170.

McCormac A, Wagner D, Boylan M, Quail P, Smith H, Whitelam G (1993) Photoresponses of transgenic *Arabidopsis* seedlings expressing introduced phytochrome B-encoding cDNAs: evidence that phytochrome A and phytochrome B have distinct photoregulatory functions. Plant J 4:19-27.

McGrath R, Wu Y, Neuhaus G, Chua N-H Sucrose regulates gene expression by multiple signal transduction pathways. manuscript in preparation.

McMichael RW, Lagarias JC (1990) Phosphopeptide mapping of *Avena* phytochrome phosphorylated by protein kinases in vitro. Biochemistry 29:3872-3878.

Millar AJ, Kay SA (1991) Circadian control of *cab* gene transcription and mRNA accumulation in *Arabidopsis*. Plant Cell 3:541-550.

Millar AJ, McGrath RB, Chua N-H (1994) Phytochrome phototransduction pathways. Annu Rev Genet 28:325-49.

Misera S, Muller A, Weiland-Heidecker U, Jurgens G (1994) The *FUSCA* genes of *Arabidopsis*: negative regulators of light responses. Mol Gen Genet 244:242-252.

Mita S, Suzuki-Fujii K, Nakamura K (1995) Sugar-inducible expression of a gene for β-amylase in *Arabidopsis thaliana*. Plant Physiol 107:895-904.

Mohr H (1994) Coaction between pigment systems. In: Photomorphogenesis in plants (Kendrick RE, Kronenberg GHW, eds), pp 352-375. Dordrecht: Kluwer Academic.

Muller-Rober BT, Kossmann J, Hannah LC, Willmitzer L, Sonnewald U (1990) One of two different ADP-glucose pyrophosphorylase genes from potato responds strongly to elevated levels of sucrose. Mol Gen Genet 224:136-146.

Nagatani A, Chory J, Furuya M (1991a) Phytochrome B is not detectable in the *hy3* mutant of *Arabidopsis*, which is deficient in responding to end-of-day far-red light treatments. Plant Cell Physiol 32:1119-1122.

Nagatani A, Kay S, Deak M, Chua N-H, Furuya M (1991b) Rice type I phytochrome regulates hypocotyl elongation in transgenic tobacco seedlings. Proc Natl Acad Sci USA 88:5207-5211.

Nagatani A, Reed JW, Chory J (1993) Isolation and initial characterization of *Arabidopsis* mutants that are deficient in phytochrome A. Plant Physiol 102:269-277.

Nagy F, Kay SA, Boutry M, Hsu M-Y, Chua N-H (1986) Phytochrome-controlled expression of a wheat Cab gene in transgenic tobacco seedlings. EMBO J 6:1119-1124.

Neales T, Incoll L (1968) The control of leaf photosynthesis by the level of assimilate in the leaf. Bot Rev 34:431-454.

Neuhaus G, Bowler C, Kern R, Chua N-H (1993) Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. Cell 73:937-952.

Olesen J, Hahn S, Guarente L (1987) Yeast HAP2 and HAP3 activators both bind to the *CYC1* upstream activation site, UAS2, in an interdependent manner. Cell 51:953-961.

Otto V, Schafer E (1988) Rapid phytochrome-controlled protein phosphorylation and dephosphorylation in *Avena sativa* L. Plant Cell Physiol 29:1115-1121.

Parks BM, Jones AM, Adamse P, Koornneef M, Kendrick RE, Quail PH (1987) The *aurea* mutant of tomato is deficient in spectrophotometrically and immunochemically detectable phytochrome. Plant Mol Biol 9:97-107.

Parks BM, Shanklin J, Koornneef M, Kendrick RE, Quail PH (1989) Immunochemically detectable phytochrome is present at normal levels but is photochemically nonfunctional in the *hy 1* and *hy 2* long hypocotyl mutants of *Arabidopsis*. Plant Mol Biol. 12:425-437.

Parks BM, Quail PH (1991) Phytochrome-deficient *hy1* and *hy2* long hypocotyl mutants of *Arabidopsis* are defective in phytochrome chromophore biosynthesis. Plant Cell 3:1177-1186.

Parks BM, Quail PH (1993) *hy8*, a new class of *Arabidopsis* long hypocotyl mutants deficient in functional phytochrome A. Plant Cell 5:39-48.

Pepper A, Delaney T, Washburn T, Poole D, Chory J (1994) *DET1*, a negative regulator of light-mediated development and gene expression in *Arabidopsis*, encodes a novel nuclear-localized protein. Cell 78:109-116.

Perisic O, Lam E (1992) A tobacco DNA binding protein that interacts with a light-responsive box II element. Plant Cell 4:831-838.

Peters JL, Schreuder MEL, Verduin SJW, Kendrick RE (1992) Physiological characterization of a high-pigment mutant of tomato. Photochem Photobiol 56:75-82.

Pratt L, Kidd G, Coleman R (1974) An immunochemical characterization of the phytochrome destruction reaction. Biochim Biophys Acta 365:93-107.

Pratt L, Stewart S, Shimazaki Y, Wang Y, Cordonnier M (1991) Monoclonal antibodies directed to phytochrome from green leaves of *Avena sativa* L. cross-react weakly or not at all with the phytochrome that is most abundant in etiolated shoots of the same species. Planta 184:87-95.

Quail P, Schafer E, Marme D (1973) Turnover of phytochrome in pumpkin cotyledons. Plant Physiol 52:128-131.

Quail PH (1991) Phytochrome: a light-activated molecular switch that regulates plant gene expression. Annu Rev Genet 25:389-409.

Quail P, Boylan M, Parks B, Short T, Xu Y, Wagner D (1995) Phytochromes: photosensory perception and signal transduction. Science 268:675-680.

Reed JW, Nagpal P, Poole DS, Furuya M, Chory J (1993) Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development. Plant Cell 5:147-157.

Reed JW, Nagatani A, Elich TD, Fagan M, Chory J (1994) Phytochrome A and phytochrome B have overlapping but distinct functions in Arabidopsis development. Plant Physiol 104:1139-1149.

Reynolds E (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208-213.

Richardson K, Jarrett L, Finke E (1960) Embedding in epoxy resin for ultra-thin sectioning in electron microscopy. Stain Tech 35:313-323.

Riesmeier J, Willmitzer L, Frommer W (1992) Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. EMBO J 11:4705-4713.

Robson P, Whitelam G, Smith H (1993) Selected components of the shade-avoidance syndrome are displayed in a normal manner in mutants of *Arabidopsis thaliana* and *Brassica rapa* deficient in phytochrome B. Plant Physiol 102:1179-1184.

Robson P, Smith H (1996) Genetic and transgenic evidence that phytochromes A and B act to modulate the gravitropic orientation of *Arabidopsis thaliana* hypocotyls. Plant Physiol 110:211-216.

Roman G, Lubarsky B, Kieber J, Rothenberg M, Ecker J (1995) Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: five novel mutant loci integrated into a stress response pathway. Genetics 139:1393-1409.

Romano C, Robson P, Smith H, Estelle M, Klee H (1995) Transgene-mediated auxin overproduction in *Arabidopsis*: hypocotyl elongation phenotype and interactions with the *hy6-1* hypocotyl elongation and *axr1* auxin-resistant mutants. Plant Mol Biol 27:1071-1083.

Romero LC, Sommer D, Gotor C, Song P-S (1991) G-proteins in etiolated *Avena* seedlings. FEBS Lett 282:341-346.

Romero LC, Lam E (1993) Guanine nucleotide binding protein involvement in early steps of phytochrome-regulated gene expression. Proc Natl Acad Sci USA 90:1465-1469.

Roth B, Goff S, Klein T, Fromm M (1991) *C1*- and *R*-dependent expression of the maize *Bz1* gene requires sequences with homology to mammalian *myb* and *myc* binding sites. Plant Cell 3:317-325.

Roux SJ ,Yguerabide J (1973) Photoreversible conductance changes induced by phytochrome in model membranes. Proc Natl Acad Sci USA 70:762-764.

Roux SJ (1984) Ca^{2+} and phytochrome action in plants. BioScience 34:25-29.

Saier M (1989) Protein phosphorylation and allosteric control of inducer exclusion and catabolite repression by the bacterial phosphoenolpyruvate:sugar phosphotransferase system. Microbiol Rev 53:109-120.

Salisbury F, Ross C (1985) Plant Physiology. Belmont, CA: Wadsworth Publishing Co.

Sambrook J, Fritsch E, Maniatis T (1989) Molecular Cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sauer F, Hansen S, Tjian R (1995) Multiple TAF_{II}s directing synergistic activation of transcription. Science 270:1783-1788.

Seeley K, Byrne K, Colbert J (1992) Red light-independent instability of oat phytochrome mRNA in vivo. Plant Cell 4:29-38.

Shacklock PS, Read ND, Trewavas AJ (1992) Cytosolic free calcium mediates red light-induced photomorphogenesis. Nature 358:753-755.

Shanklin J, Jabben M, Vierstra R (1987) Red light-induced formation of ubiquitin-phytochrome conjugates: identification of possible intermediates of phytochrome degradation. Proc Natl Acad Sci USA 84:359-363.

Shanklin J, Jabben M, Vierstra R (1989) Partial purification and peptide mapping of ubiquitin-phytochrome conjugates in oat. Biochemistry 28:6028-6034.

Sharma R, Lopez-Juez E, Nagatani A, Furuya M (1994) Identification of photo-inactive phytochrome A in etiolated seedlings and photo-active phytochrome B in green leaves of the *aurea* mutant of tomato. Plant J 4:1035-1042.

Sharrock RA, Quail PH (1989) Novel phytochrome sequences in *Arabidopsis thaliana*: structure, evolution, and differential expression of a plant regulatory photoreceptor family. Genes & Dev 3:1745-1757.

Sheen J (1990) Metabolic repression of transcription in higher plants. Plant Cell 2:1027-1038.

Shibaoka H (1994) Plant hormone-induced changes in the orientation of cortical microtubules: Alterations in the cross-linking between microtubules and the plasma membrane. Annu. Rev. Plant Physiol. Plant Mol Biol 45:527-544.

Shinomura T, Nagatani A, Chory J, Furuya M (1994) The induction of seed germination in *Arabidopsis thaliana* is regulated principally by phytochrome B and secondarily by phytochrome A. Plant Physiol 104:363-371.

Shirley B, Kubasek W, Storz G, Bruggemann E, Koornneef M, Ausubel F, Goodman H (1995) Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. Plant J 8:659-671.

Silflow , C Key J (1979) Stability of polysome-associated polyadenylated RNA from soybean suspension culture cells. Biochemistry 18:1013-1018.

Simon MI, Strathmann MP, Gautam N (1991) Diversity of G proteins in signal transduction. Science 252:802-808.

Sineshchekov V, Lamparter T, Hartmann E (1994) Evidence for the existence of membrane-associated phytochrome in the cell. Photochem Photobiol 60:516-520.

Smith H, Whitelam GC, McCormac AC (1991) Do the members of the phytochrome family have different roles? Physiological evidence from wild-type, mutant and transgenic plants. In: Phytochrome Properties and Biological Action (Thomas B, Johnson CB, eds),Vol H50, pp 217-236. Berlin: Springer-Verlag.

Smith H (1994) Sensing the light environment: the functions of the phytochrome family. In: Photomorphogenesis in Plants (Kendrick R, Kronenberg G, eds), pp 377-416. Dordrecht: Kluwer Academic Publishers.

Somers DE, Sharrock RA, Tepperman JM, Quail PH (1991) The *hy3* long hypocotyl mutant of *Arabidopsis* is deficient in phytochrome B. Plant Cell 3:1263-1274.

Somers D, Quail P (1995a) Phytochrome-mediated light regulation of *PHYA*- and *PHYB-GUS* transgenes in *Arabidopsis thaliana* seedlings. Plant Physiol 107:523-534.

Somers D, Quail P (1995b) Temporal and spatial expression patterns of *PHYA* and *PHYB* genes in *Arabidopsis*. Plant J 7:413-427.

Stockhaus J, Nagatani A, Halfter U, Kay S, Furuya M Chua N-H (1992) Serine-to-alanine substitutions at the amino-terminal region of phytochrome A result in an increase in biological activity. Genes & Dev 6:2364-2372.

Sun L, Doxsee RA, Harel E, Tobin EM (1993) CA-1, a novel phosphoprotein, interacts with the promoter of the *cab140* gene in *Arabidopsis* and is undetectable in *det*1 mutant seedlings. Plant Cell 5:109-121.

Susek R, Ausubel F, Chory J (1993) Signal transduction mutants of *Arabidopsis* uncouple nuclear *CAB* and *RBCS* gene expression from chloroplast development. Cell 74:787-799.

Szymanski P, Levine M (1995) Multiple modes of dorsal-bHLH transcriptional synergy in the *Drosophila* embryo. EMBO J 14:2229-2238.

Takahashi T, Gasch A, Nishizawa N, Chua N-H (1995) The *DIMINUTO* gene of *Arabidopsis* is involved in regulating cell elongation. Genes & Dev 9:97-107.

Takeda S, Mano S, Ohto M, Nakamura K (1994) Inhibitors of protein phosphatases 1 and 2A block the sugar-inducible gene expression in plants. Plant Physiol 106:567-574.

Tan Y, Low K, Boccia C, Grossman J, Comb M (1994) Fibroblast growth factor and cyclic AMP (cAMP) synergistically activate gene expression at a cAMP response element. Mol Cell Biol 14:7546-7556.

Taylor WC (1989) Regulatory interactions between nuclear and plastid genomes. Annu. Rev. Plant Physiol. Plant Mol Biol 40:211-233.

Thummler F, Dufner M, Kreisl P, Dittrich P (1992) Molecular cloning of a novel phytochrome gene of the moss *Ceratodon purpureus* which encodes a putative light-regulated protein kinase. Plant Mol Biol 20:1003-1017.

Tremblay J, Gerzer R, Hamet P (1988) Cyclic GMP in cell function. In: Advances in second messenger and phosphoprotein research (Greengard P, Robison GA, eds),Vol 22, pp 319-383. New York: Raven Press.

Tretyn A, Kendrick RE, Bossen ME (1990) The effect of a calcium-channel antagonist, nifedipine and agonist, Bay K-8644, on the phytochrome-controlled swelling etiolated wheat protoplasts. Physiol Plant 78:230-235.

Tsukaya H, Ohshima T, Naito S, Chino M, Komeda Y (1991) Sugar-dependent expression of the *CHS-A* gene for chalcone synthase from petunia in transgenic Arabidopsis. Plant Physiol 97:1414-1421.

van Tuinen A, Kerckhoffs L, Nagatani A, Kendrick R, Koornneef M (1995a) Far-red light-insensitive, phytochrome A-deficient mutants of tomato. Mol Gen Genet 246:133-141.

van Tuinen A, Kerckhoffs L, Nagatani A, Kendrick R, Koornneef M (1995b) A temporarily red light-insensitive mutant of tomato lacks a light-stable, B-like phytochrome. Plant Physiol 108:939-947.

Vierstra R, Quail P (1985) Spectral characterization and proteolytic mapping of native 120-kilodalton phytochrome from *Cucurbita pepo* L. Plant Physiol 77:990-998.

Viner N, Whitelam G, Smith H (1988) Calcium and phytochrome control of leaf unrolling in dark-grown barley seedlings. Planta 175:209-213.

von Arnim A, Deng X-W (1994) Light inactivation of *Arabidopsis* photomorphogenic repressor COP1 involves a cell-specific regulation of its nucleocytoplasmic partitioning. Cell 79:1035-1045.

von Wettstein-Knowles P (1968) Mutations affecting anthocyanin synthesis in tomato. II. Physiology. Hereditas 61:255-275.

Wagner D, Tepperman JM, Quail PH (1991) Overexpression of phytochrome B induces a short hypocotyl phenotype in transgenic *Arabidopsis*. Plant Cell 3:1275-1288.

Walling L, Drews GN, Goldberg RB (1986) Transcriptional and post-transcriptional regulation of soybean seed protein mRNA levels. Proc Natl Acad Sci. USA 83:2123-2127.

Wang T-Z, Cosgrove D, Arteca R (1993) Brassinosteroid stimulation of hypocotyl elongation and wall relaxation in pakchoi (*Brassica chinensis* cv Lei-Choi). Plant Physiol 101:965-968.

Wayne R, Hepler PK (1984) The role of calcium ions in phytochrome-mediated germination of spores of *Onoclea sensibilis* L. Planta 160:12-20.

Wayne R, Hepler PK (1985) Red light stimulates an increase in intracellular calcium in the spores of *Onoclea sensibilis*. Plant Physiol 77:8-11.

Wei N, Deng X-W (1992) *COP*9: a new genetic locus involved in light-regulated development and gene expression in *Arabidopsis*. Plant Cell 4:1507-1518.

Wei N, Kwok S, von Arnim A, Lee A, McNellis T, Piekos B, Deng X-W (1994) *Arabidopsis COP8*, *COP10*, *COP11* Genes are involved in repression of photomorphogenic development in darkness. Plant Cell 6:629-643.

Wei N, Chamovitz D, Deng X-W (1994) *Arabidopsis* COP9 is a component of a novel signaling complex mediating light control of development. Cell 78:117-124.

Weiss D, Halevy AH (1989) Stamens and gibberellin in the regulation of corolla pigmentation and growth in *Petunia hybrida*. Planta 179:89-96.

Weisshaar B, Armstrong GA, Block A, da Costa e Silva O, Hahlbrock K (1991) Light-inducible and constitutively expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. EMBO J 10:1777-1786.

Wester L, Somers D, Clack T, Sharrock R (1994) Transgenic complementation of the *hy3* phytochrome B mutation and response to *PHYB* gene copy number in *Arabidopsis*. Plant J 5:261-272.

Whitelam GC, Johnson E, Peng J, Carol P, Anderson ML, Cowl JS, Harberd NP (1993) Phytochrome A null mutants of Arabidopsis display a wild-type phenotype in white light. Plant Cell 5:757-768.

Wilkie T, Gilbert D, Olsen A, Chen X, Amatruda T, Korenberg J, Trask B, deJong P, Reed R, Simon M (1992) Evolution of the mammalian G protein alpha subunit gene family. Nature Genetics 1:85-91.

Williams F, Varanasi U, Trumbly R (1991) The CYC8 and TUP1 proteins involved in glucose repression in *Saccharomyces cerevisiae* are associated in a protein complex. Mol Cell Biol 11:3307-3316.

Williams S, Weatherwax S, Bray E, Tobin E (1994) *NPR* genes, which are negatively regulated by phytochrome action in *Lemna gibba* L. G-3, can also be positively regulated by abscisic acid. Plant Physiol 105:949-954.

Wong Y-S, Cheng H-C, Walsh DA, Lagarias JC (1986) Phosphorylation of *Avena* phytochrome *in vitro* as a probe of light-induced conformational changes. J Biol Chem 261:12089-12097.

Yu S-M, Kuo Y-H, Sheu G, Sheu Y-J, Liu L-F (1991) Metabolic derepression of a-amylase gene expression in suspension-cultured cells of rice. J Biol Chem 266:21131-21137.

Zhou S, Jones AM, Scott TK (1990) Phytochrome-mediated swelling of etiolated leaf protoplasts and its possible biological significance. Plant Cell Rep 9:435-438.

End