ANALYSIS OF PHYTOCHROME FUNCTION IN THE GENUS

NICOTIANA USING MUTANT AND TRANSGENIC PLANTS

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

Matthew Eric Hudson, BA (Cantab.)

Department of Biology
University of Leicester
October 1997
Acknowledgements

I would like to thank my supervisor, Harry, and the inhabitants of lab 322 over the period I have worked there, particularly Anthony Hall for his advice and friendly discussions. Paul Robson helped me with a number of techniques and was responsible for a portion of the measurements used in Figures 3.2, 3.6 and 5.1. I would also like to thank the technical staff of the department, especially Malcom Pratt for his assiduous maintenance of the R:FR cabinets. Many others in the Botany department have proved extremely helpful to me, none more so than Luis Mur and Paul Devlin.

I am grateful to Akira Nagatani for supplying the mAT-1 antibody, to Tim Kunkel and Eberhard Schäfer for supplying the PMTB antibody, and to Garry Whitelam for supplying the AS-32 antibody, and for his valuable help and advice. I would also like to thank Murray Grant for helpful discussions concerning sequencing strategies.

Finally, I would like to thank my collaborators and co-authors, Yvan Kraepiel, Michel Caboche, Éva Ádám and Ferenő Nagy. Their contributions are detailed in the text.
Abstract

Two allelic mutants of *Nicotiana plumbaginifolia* have been isolated, which display a hypocotyl which is long (*hlg*) when seedlings are grown in continuous white light. This can be accounted for by the decreased response of hypocotyl elongation rate in these mutants to red light. Both mutants are deficient in a phyB-like polypeptide that is immuno-detectable in the wild type; both have wild-type levels of a phyA-like polypeptide. These alleles are inherited in a partially dominant manner, and correspond to single base missense mutations in a gene highly homologous to *N. tabacum PHYB*, which codes for a phytochrome-B-type photoreceptor. When grown in white light, mature *hlg* mutants are not elongated with respect to the wild type; they also bolt and flower later. The shade-avoidance responses appear intact in these mutants.

The sensitivity of the *Nicotiana plumbaginifolia* wild type and *hlg* mutants to photoperiod were investigated. It was found that the *hlg* mutant shows later bolting than the wild type under long days, but not under short days. The endogenous rosette-leaf-movement rhythms of the mutants display a much greater amplitude than those of the wild type under long-day conditions, but the two behave indistinguishably under short-day conditions.

The *Nicotiana plumbaginifolia pew* mutants, which are deficient in phytochrome chromophore, were also investigated. They show diminished sensitivity to both red and far-red light, and flower at an earlier stage. The *pew3* mutant may belong to a previously uncharacterised class of phytochrome chromophore mutants.

Finally, tobacco plants were created which overexpress the native *PHYA* gene under the control of the 35S and native *PHYA* promoters. Both sets of transgenic plants displayed enhanced far-red sensitivity, and were dwarfed as light-grown adults. However, the reversed shade-avoidance effect characteristic of *Avena* phyA overexpressers was not seen. The plants overexpressing native *PHYA* were all delayed in flowering.
**Abbreviations**

B = blue light  
d = day  
DN = day neutral  
EOD = end of day  
FR = far red light  
GA = gibberellic acid  
HIR = High Irradiance Response (High Energy Reaction)  
kDa = Kilodalton  
LD = long day  
SD = short day  
LFR = Low Fluence Response  
M = Molar  
mol = Mole  
P = total phytochrome (Pr + Pfr)  
PAR = photosynthetically active radiation  
PCR = Polymerase Chain Reaction  
Pfr = FR absorbing form of phytochrome  
phyx = biologically active, holophytochrome x  
PHYx = phytochrome x apoprotein  
PHYx = phytochrome x gene  
Pr = R absorbing form of phytochrome  
R = red light  
R:FR = the photon fluence rate ratio of red to far red light in 10 nm band-widths centred on 660 nm and 730 nm  
s = second  
SE = standard error  
VLFR = Very Low Fluence Response  
W = white light  
W+FR = white light with supplementary far-red light  
wgt = wild type
Table of Contents

Chapter 1: Introduction

1.1 Plants and Light
1.1.1 Plant Development 1
1.1.2 Light 1
1.1.3 Light Responses and Photomorphogenesis 2
   1.1.3.1 Germination 2
   1.1.3.2 De-etiolation 3
   1.1.3.3 Phototropism 3
   1.1.3.4 Photoperiodism 3
1.1.3.5 Shade Avoidance 4

1.2 Phytochromes 6
   1.2.1 The General and Spectral Properties of Phytochrome 6
      1.2.1.1 The Phytochrome Chromophore 7
   1.2.2 The Response Modes of Phytochrome 9
1.2.3 The Phytochrome Family 10
   1.2.2.1 Phytochrome Genes 11
      1.2.2.1.1 Angiosperm Phytochrome Genes 11
      1.2.2.1.2 Lower Plant Phytochrome Genes 12
      1.2.2.1.3 Cyanobacterial Genes Showing Homology to Phytochrome. 13
   1.2.4 The Active Form of Phytochrome 14
1.2.5 Phytochrome Signal Transduction 14
   1.2.5.1 Microinjection of Phytochrome 15
   1.2.5.2 Non-phy Mutants Showing Aberrant Photomorphogenesis 15
   1.2.5.3 Mutational Analysis of Motifs Within Phytochrome Genes 15

1.3 Photomorphogenic Mutants 16
   1.3.1 Types of Photomorphogenic Mutants 16
   1.3.2 Chromophore Mutants 17
   1.3.3 Mutants Showing Photomorphogenesis in the Dark 18
1.3.4 Photoreceptor Mutants
- 1.3.4.1 Phytochrome B mutants
- 1.3.4.2 Phytochrome A mutants
- 1.3.4.3 Blue and UV-A photoreceptor mutants

1.3.5 Specific Signal Transduction Mutants

1.4 The Role of Phytochrome in Photoperiodism
- 1.4.1 Photoperiodic responses
- 1.4.2 The Circadian Clock
  - 1.4.2.1 The Clock and Phytochrome
- 1.4.3 Genetics of Clock and Photoperiodic Mutants
- 1.4.4 Effect of Phytochrome Mutations on Photoperiodism

1.5 Plants Transgenic for Phytochrome
- 1.5.1 Plants Overexpressing Phytochrome A
- 1.5.2 Plants Overexpressing Phytochrome B
- 1.5.3 Effect of Phytochrome Overexpression on Flowering Time
- 1.5.4 Plants Expressing PHY Promoters fused to Reporter Genes

1.6 The Genus *Nicotiana* as an Experimental Model

1.7 Outline of the Thesis

Chapter 2: Molecular and Genetic analysis of the *hlg* mutants and the *PHYB* gene of *Nicotiana plumbaginifolia*

2.1 Introduction

2.2 Results
- 2.2.1 Mutant isolation and preliminary characterisation
- 2.2.2 *hlg*-1 and *hlg*-2 are allelic
- 2.2.3 *PHYB* mRNA levels are normal in the *hlg* mutants
- 2.2.4 Both *hlg* alleles are deficient in a phyB-type apoprotein
2.2.5 *N. plumbaginifolia* PHYB has large introns 37
2.2.6 PHYB transcript is spliced normally in *hlg-1* and *hlg-2* 38
2.2.7 PCR from the PHYB gene 39
2.2.8 Homology of *N. plumbaginifolia* PHYB to tobacco PHYB and others 39
2.2.9 Mutations in the PHYB gene of *hlg-1* and *hlg-2* 40
2.2.10 Partial dominance of the *hlg* alleles 40
2.2.11 Second site mutation in the *hlg-2* line 41

2.3 Discussion 42
Figures 45

Chapter 3: Analysis of photophysiology and shade-avoidance in the phyB deficient
*hlg* mutants of *Nicotiana plumbaginifolia* 64

3.1 Introduction 65

3.2 Results 67
3.2.1 The aberrant hypocotyl elongation of *hlg* is specific to R wavelengths 67
3.2.2 The *hlg* mutants display a small response to continuous R 68
3.2.3 The *hlg* phenotype is more pronounced under low PAR W 69
3.2.4 Gravitropic randomisation by R is less pronounced in *hlg* 69
3.2.5 The *hlg* mutants show a normal shade-avoidance response 70
3.2.6 The *hlg* mutations affect chlorophyll level and A:B ratio 71
3.2.7 Leaf biomass is increased in *hlg* mutants 72
3.2.8 Early flowering shade-avoidance is normal in *hlg* mutants 73

3.3 Discussion 74
Figures 77
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter 4: Analysis of photoperiodism and endogenous rhythmicity in <em>Nicotiana plumbaginifolia</em>: the role of phytochrome B.</th>
<th>86</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Introduction</td>
<td>87</td>
</tr>
<tr>
<td>4.2 Results</td>
<td>89</td>
</tr>
<tr>
<td>4.2.1 <em>hlg</em> mutants are late flowering in LD and show a reduced response to SD</td>
<td>89</td>
</tr>
<tr>
<td>4.2.2 The free-running leaf movement rhythm is altered in <em>hlg-1</em></td>
<td>89</td>
</tr>
<tr>
<td>4.2.3 Rhythmic leaf movement is distinct from that caused by low R:FR</td>
<td>90</td>
</tr>
<tr>
<td>4.2.4 Leaf angle oscillations are induced by SD in the wild type</td>
<td>91</td>
</tr>
<tr>
<td>4.2.5 Low-fluence day-extensions cause loss of movement in the wt but not <em>hlg</em></td>
<td>91</td>
</tr>
<tr>
<td>4.3 Discussion</td>
<td>92</td>
</tr>
<tr>
<td>Figures</td>
<td>96</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 5: Molecular and physiological analysis of the partially etiolated in white light (<em>pew</em>) mutants of <em>Nicotiana plumbaginifolia</em></th>
<th>102</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Introduction</td>
<td>103</td>
</tr>
<tr>
<td>5.2 Results</td>
<td>105</td>
</tr>
<tr>
<td>5.2.1 All <em>pew</em> mutants are deficient in responses to R and FR</td>
<td>105</td>
</tr>
<tr>
<td>5.2.2 The phenotype of <em>pew</em> 1 and 3 is moderated by biliverdin</td>
<td>106</td>
</tr>
<tr>
<td>5.2.3 The <em>pew</em> mutants are pale yellow</td>
<td>107</td>
</tr>
<tr>
<td>5.2.4 The <em>pew</em> mutants flower at an earlier developmental stage</td>
<td>107</td>
</tr>
<tr>
<td>5.2.5 phyA and phyB are present in the <em>pew</em> mutants</td>
<td>108</td>
</tr>
<tr>
<td>5.2.6 Shade-avoidance in <em>pew</em> is 'leaky'</td>
<td>109</td>
</tr>
<tr>
<td>5.3 Discussion</td>
<td>110</td>
</tr>
<tr>
<td>Figures</td>
<td>112</td>
</tr>
<tr>
<td>Chapter 6: Analysis of native phyA function by overexpression in transgenic <em>Nicotiana tabacum</em> cv. SR-1</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>6.1 Introduction</td>
<td></td>
</tr>
<tr>
<td>6.2 Results</td>
<td></td>
</tr>
<tr>
<td>6.2.1 Construction of Transformation Vectors</td>
<td></td>
</tr>
<tr>
<td>6.2.2 Transformation and selection of recombinant lines</td>
<td></td>
</tr>
<tr>
<td>6.2.3 Molecular characterisation of recombinant lines</td>
<td></td>
</tr>
<tr>
<td>6.2.4 Response of transgenic seedlings to monochromatic R and FR</td>
<td></td>
</tr>
<tr>
<td>6.2.5 The shade avoidance response in the transgenic plants</td>
<td></td>
</tr>
<tr>
<td>6.2.6 The effect on flowering of native phyA overexpression</td>
<td></td>
</tr>
<tr>
<td>6.3 Discussion</td>
<td></td>
</tr>
<tr>
<td>Figures</td>
<td></td>
</tr>
<tr>
<td>Chapter 7: General Discussion</td>
<td></td>
</tr>
<tr>
<td>Chapter 8: Materials and Methods</td>
<td></td>
</tr>
<tr>
<td>Literature Cited</td>
<td></td>
</tr>
</tbody>
</table>
## Chapter 6: Analysis of native phyA function by overexpression in transgenic *Nicotiana tabacum* cv. SR-1

### 6.1 Introduction

### 6.2 Results

- 6.2.1 Construction of Transformation Vectors
- 6.2.2 Transformation and selection of recombinant lines
- 6.2.3 Molecular characterisation of recombinant lines
- 6.2.4 Response of transgenic seedlings to monochromatic R and FR
- 6.2.5 The shade avoidance response in the transgenic plants
- 6.2.6 The effect on flowering of native phyA overexpression

### 6.3 Discussion

### Figures

### Chapter 7: General Discussion

### Chapter 8: Materials and Methods

### Literature Cited
Chapter One

General

Introduction
1.1 Plants and light

1.1.1 Plant Development

Animals are motile, and can hence respond to their environment by means of adaptive behaviour and physiology. Since higher plants are all sessile organisms, they must, in contrast, adjust to their surroundings by means of adaptive development. Consequently, although the overall symmetry, growth pattern and histology of a plant species is determined by its genome, there is a considerable degree of phenotypic plasticity observable in the proportions of most plant structures. This plasticity is not entirely randomly determined, but is, to a large extent, a result of the plant responding to the circumstances under which it is growing. These responses require accurate environmental sensors in order to be advantageous to the plant. This thesis is concerned with the environmental sensor phytochrome. Phytochrome is a potent influence in determining the morphology of a plant throughout its life cycle, as it senses the single most important factor in plant growth - light.

1.1.2 Light

Plants are able to utilise wavelengths of electromagnetic radiation roughly analogous to the human visible spectrum to capture energy via photosynthesis. However, these wavelengths are not all equally effective. An absorption spectrum of a green plant photosynthetic organ, as seen in Figure 1.1, and the corresponding action spectrum for photosynthesis (Figure 1.1) demonstrate that red and blue wavelengths are the most efficiently absorbed by the plant and the most effective at driving photosynthesis. It is unsurprising, then, that the environmental photoreceptors in plants, phytochrome and cryptochrome respectively, respond to these wavelength bands (see Section 1.2.1).
1.13 Light responses and photomorphogenesis

Plants need to gather information on the light environment for several reasons. Since a seed may lie under conditions that are less than ideal, photoreceptors are often required to determine whether these conditions are viable. If the seed then germinates, the plant must use the energy resources it contains to become photoautotrophic. It must then compete successfully with other plants in its immediate environment, and it must also sense when conditions are favourable to flower and reproduce. All these processes require the ability to detect light separately from photosynthesis. Since they result in a change in the shape of the plant in response to light, they are collectively termed photomorphogenesis.

1.13.1 Germination

Many seeds will not germinate in the absence of light. Often such seeds form a 'seed bank' which lies in the soil awaiting the opportunity to germinate (Odum, 1965). Once soil is disturbed, such seeds will germinate, often in huge numbers. This
detection of light by seeds is often very sensitive, and it has long been known that seed germination is triggered by phytochrome action (Borthwick et al., 1952a, Butler et al., 1960). Since seed germination is an all-or-nothing response (unlike stem elongation or greening, for example) germination is perhaps the best known and most clear cut example of a phytochrome response. In the species exhibiting the classic germination response, such as lettuce, this clearly demonstrates the ability of phytochrome to act as a developmental switch (the inductive response, section 1.2.1 and 1.2.2).

1.13.2 De-Etiolation

The apparatus of photosynthesis is incomplete in an etiolated seedling. A photoreceptor is required in order to divert the seedling’s energy stores away from elongation (which increases the chances of encountering light for, for example, a seedling which has germinated beneath leaf litter), and towards the generation of light-harvesting structures, once a photosynthetically viable light environment has been encountered. This process of de-etiolation is perhaps the most important function of photoreceptors in plants which have a non-photosynthetic etiolated state, and involves both phytochromes and cryptochrome. Once the plant is de-etiolated, such responses continue to function in order to maintain a balance between light-seeking and light-harvesting requirements, in response to the level of photosynthetically active radiation (PAR) (see later sections).

1.13.3 Phototropism

Sensing the direction of incident light allows the plant to maximise the levels of incident PAR on its photosynthetic organs, by growing towards the light (phototropism). Phototropism is triggered solely by blue light in angiosperms, and is hence a cryptochrome response (for more detail, see Firn, 1994).

1.13.4 Photoperoidism

In combination with an endogenous rhythm, a photoreceptor is required to sense the photoperiod. Along with the perception of temperature variations, this
allows plants in non-tropical climate to sense accurately the time of year. This is particularly important for the regulation of flowering time. Photoperiodism is usually dependent on phytochrome responses (Borthwick et al., 1952b, Vince-Prue, 1994). Since this thesis is in part concerned with photoperiodic responses, a brief outline of the current state of knowledge of the role of phytochrome(s) in photoperiodism is given in Section 1.4.

1.1.3.5 Shade-Avoidance

The balance between red wavelengths (R, 650-700 nm wavelength, strongly absorbed by green plants (Figure 1.1) and far red wavelengths (FR) in the 700-750 nm region (strongly reflected and transmitted by green plants (Figure 1.1) allows a plant to detect the presence of other plants in its immediate environment. The red to far-red ratio (R:FR) is detected by phytochrome (Section 1.2.2, Figure 1.2). The shade-avoidance responses which occur in response to changes in R:FR are an important element of competition between higher plants in almost all ecosystems (Smith, 1994, 1995). Shade-avoidance responses classically include alterations in stem extension rates, flowering time, leaf angle and chlorophyll synthesis. Shade avoidance may also affect partitioning of assimilate to roots or tubers, apical dominance, leaf and cotyledon area and thickness. Not all species are shade avoiding, and the extent of shade-avoidance shown by a plant may affect its fitness in certain environments (Smith, 1994).

Shade-avoidance can be affected by the levels of different phytochromes present in a plant (Whitelam and Smith, 1991, Weller and Reid, 1993, Smith, 1995, Robson et al., 1996b, Section 1.3.4, Section 1.5, Chapters 3, 5 and 6). Manipulation of phytochrome levels can hence be utilised as a method of altering the shade-avoidance characteristics of a plant. It may therefore provide a means of affecting shade-avoidance responses in a way which will improve the characteristics of crop plants (Robson et al., 1996b). Consequently the shade-avoidance response is of great interest both to ecophysiologists and crop breeders, and has wide implications beyond the field of photomorphogenesis.
1.2 Phytochromes

1.2.1 The general and spectral properties of phytochromes

The phytochromes are the best characterised sensory photoreceptors in plants. They have been known since the 1940s, when studies revealed that the induction of various photomorphogenic responses by R could be cancelled by a pulse of FR given afterward (e.g. Borthwick et al., 1952a, b, Withrow et al., 1957). (For an exhaustive history of this subject, see Sage, 1992). The simple yet powerfully predictive interpretation was that the plant tissue used in these experiments contained a pigment which existed in two interconvertible forms. One of the forms of this pigment, present in dark grown seedlings, absorbed R, and could be regenerated by FR (Pr). The other stable form of the pigment absorbed FR, and was generated by R (Pfr) (Borthwick et al., 1954). The postulated photoreceptor could hence be detected by \textit{in vivo} spectrophotometric analysis of etiolated maize seedlings (Butler et al., 1959). Since the photoreversible pigment was present in substantial quantities in etiolated oat tissue, it was possible to purify it. By this means, the absorbance spectra of isolated, purified phytochrome in both Pr and Pfr forms were obtainable (Figure 1.2) and it could be determined that phytochrome had a tetrapyrrole chromophore (Siegelman et al., 1966, Rüdiger and Correll, 1969). The phytochrome protein could then be purified, although it was some time before such preparations were successfully used for molecular biology purposes (Vierstra and Quail, 1983). It was then possible to begin detailed characterisation of the protein. The protein isolated from oat was found to be a 124kDa, soluble globular protein which purified as a dimer. The polypeptide chain has been shown to be covalently attached to its chromophore by a cysteine residue (Lagarias and Rapoport, 1980), which when the gene was sequenced transpired to be C$_{321}$ (Hershey et al., 1985).
1.2.1.1 The phytochrome chromophore

The structure of the bilin to which phytochrome is covalently linked is shown in Figure 1.3. It is a linear tetrapyrrole most closely related in structure to phycocyanobilin, the photosynthetic pigment of the cyanobacteria, and has been termed phytochromobilin (POB). It is synthesized in the plastid from 5-aminolaevulinic acid (ALA) via the same pathway that produces haem. The specialised enzymes of POB synthesis are thought to convert haem IX to the 3E isomer of POB by the steps shown in Figure 1.3. It is thought that the photoconversion of Pr to Pfr involves the Z-E isomerisation of the chromophore, with rotation of the bond between the C and D rings of the tetrapyrrole (Figure 1.2). Isomerisation of the chromophore leads to a conformational shift in the apoprotein, which then transduces a signal to the cellular machinery (Rüdiger and Thümmler, 1994).

Functional phytochrome may be created by the assembly of transgenically produced phytochrome apoprotein with the cyanobacterial photosynthetic pigment phycocyanobilin (PCB) as its chromophore (Elich et al., 1989). This lends weight to the hypothesis that phytochrome may originally have evolved from a photosynthetic protein. By inhibiting chromophore synthesis using gabacline, it is possible to disrupt phytochrome function (Jones et al., 1986). More recently, well-known mutants have been found to be deficient in the biosynthesis of phytochromobilin (see section 3.2).
By using these inhibitors and mutants, and feeding intermediates such as biliverdin to plants, it has been possible to arrive at a putative biosynthetic pathway for the phytochrome chromophore (Terry and Kendrick, 1996, Figure 1.3).

Figure 1.3: A representation of the current knowledge of phytochrome chromophore (POB) biosynthesis from haem. The proposed enzymes which are deficient in biochemically characterised mutants of pea and tomato are indicated.
1.2.2 The response modes of phytochrome

While the discovery of phytochrome was facilitated by R-FR reversible responses such as seed germination, it quickly became apparent that phytochrome did not always act in this manner. The R-FR reversible, or 'inductive' responses became known as the low fluence responses, or LFRs. This was due to the relatively small (i.e. photosynthetically insignificant, \(1 - 1 \times 10^3 \mu\text{mol m}^{-2}\)) fluences of light required to induce them (see Figure 1.4 for a typical LFR action spectrum). While germination in lettuce seeds is clearly an LFR, germination in other species is often triggered by R or FR at much lower fluences (\(10^{-1} - 10^{-3} \mu\text{mol m}^{-2}\)), in an apparently irreversible fashion. This response became known as the VLFR or Very Low Fluence Response (see Quail, 1994 and Mancinelli, 1994 for a discussion of these terms).

Other aspects of de-etiolation, such as the inhibition of hypocotyl elongation, appeared to be controlled in yet another way. They are induced by FR (or, in an entirely separate and somewhat controversial response, R) in a manner dependent on the irradiance of continuously applied light, in an irreversible manner. The most commonly used term for these responses is the High Irradiance Response or HIR, with a distinction being drawn between the FR-HIR and the R-HIR, which have substantially different properties. An action spectrum for a response typical of a FR-HIR is shown in Figure 1.4. The R-HIR has a similar action spectrum to the LFR. The FR-HIR is the classic HIR, and does not show reciprocity, unlike most other responses discussed so far. Reciprocity is the equivalence of response to total light fluence; i.e. the response \(\rho\) is a linear function of irradiance \(I\) and time \(\rho \propto I t\). This is not seen in the FR-HIR which shows a requirement for continuous irradiation, or rapidly repeated pulses (Heim and Schäfer, 1984, Mancinelli, 1994). This unique requirement is almost certainly due to the dependence of this response on the light-labile phytochrome (Section 1.2.3) phyA (Section 1.3.4). The R-HIR can be largely induced by pulses of R (Heim and Schäfer, 1982) which may indicate that it is not a true HIR, but a series of inductive responses.

Finally, a fourth response mode of phytochromes, which is active in light grown plants, is R:FR perception or shade-avoidance (Section 1.1.5). The equilibrium between Pr and Pfr (See Section 1.2.1) in response to R:FR is such that phytochrome photoequilibrium (the ratio of Pfr to total phytochrome; Pfr/P) is most sensitive to
R:FR in the range found within vegetation canopies (Smith, 1982). This response, which must be mediated by a light-stable phytochrome (Smith, 1994), is able to distinguish changes in the characteristics of very high fluence rate light, and is hence unique. It is also persistent throughout the light-grown stage of the lives of most plants.

A well-known phytochrome response which is analogous to, and probably directly related to the shade-avoidance response is that to end-of-day FR irradiation (EOD-FR). A pulse of FR given at the end of a light period will convert most of the Pfr present back to Pr, effectively giving the same signal during the dark period as would growth under low R:FR. The EOD responses are easily studied, and display R:FR reversibility in a classical manner. Shade-avoidance itself cannot be studied in this way due to the requirement for continuous irradiation, but EOD responses are almost certainly closely related to the shade-avoidance response, and probably share the same mechanism (Smith, 1994). The similarities between the EOD-FR and shade-avoidance responses add weight to the argument that a light-stable phytochrome must be involved in both of these responses. For an in depth review of these responses, and some of the hypotheses concerning their mechanism, see Mancinelli (1994).

Figure 1.4: Action spectra of generalised inductive (LFR) and FR-HIR responses. (redrawn from Mohr, 1964)
1.2.3 The phytochrome family

Interpretation of phytochrome responses became increasingly complex as many paradoxes concerning the effects of the pigment were identified (Smith, 1975). The relatively recent revelation that 'phytochrome' is not a single entity, but a series of photoreceptors produced by a gene family (Sharrock and Quail, 1989) has resolved many of these apparent paradoxes. It is now possible to attribute certain responses to specific phytochromes (see section 1.3). In particular, the discovery that phytochrome A was "light-labile" (i.e. specifically degraded in the Pfr form), while other phytochromes so far identified are "light-stable", vindicated the conclusion of many previous workers that there must be two such pools of phytochrome (reviewed by Sage, 1992).

Shortly after the discovery of phytochrome, evidence arose that at least two sub-pools of phytochrome existed, one light-labile, the other light-stable (Downs et al., 1957, Butler and Lane, 1965). Subsequent spectrophotometric (Brockmann and Schäfer, 1982) and immunochemical (Abe et al., 1985, Shimazaki and Pratt, 1985, Tokuhisa et al., 1985) studies suggested, and later confirmed (Abe et al., 1989) that these sub-pools represented different phytochrome proteins, rather than separate pools of the same pigment. But the discovery of the phytochrome gene family allowed the powerful tools of modern genetics to be used for the analysis of phytochrome action.

1.2.3.1 Phytochrome genes

The phytochrome gene family has been most extensively characterised in Arabidopsis, where five genes have so far been identified (Sharrock and Quail, 1989, Clack et al., 1994). Phytochrome genes have also been isolated from other species too numerous to list in full. The number of known PHY genes is growing monthly, and now includes sequences from many pteridophytes and algae (for an exhaustive and up-to-date list of these genes, search a database such as Entrez or EMBL with phytochrome or PHY as a search term).
1.2.3.1.1 Angiosperm phytochrome genes

The Arabidopsis phytochrome genes identified thus far have been named \textit{PHYA-PHYE}. They all share several features: the molecular weight of their protein products is in the range 115-125 kDa, the amino acid sequences of these polypeptides share many regions of strong homology, and the position of the introns is highly conserved (Sharrock and Quail, 1989, Clack et al., 1994). A system of nomenclature for these genes and their products has been proposed, which has been widely adopted for angiosperm phytochromes and will be used throughout this thesis (Quail et al., 1994). The wild-type genes are written in italicised capitals (eg. \textit{PHYA}) while the mutant genes are referred to in lower-case capitals (e.g. \textit{phyA}). The apoprotein of a phytochrome is referred to by upper case text (e.g. PHYA) while the complete photoreceptor is referred to in lower case (e.g. phyA). The term 'phy' universally designates a phytochrome holoprotein, while the following letter is arbitrary, and is given in order of discovery. Since the Arabidopsis genes were usually the first to be isolated, \textit{PHY} genes from other species are (usually) named according to the Arabidopsis gene to which they show the strongest homology.

When the Arabidopsis genes are compared with phytochrome genes or gene fragments isolated from other species, the sub-divisions within the gene family become apparent, as the sequences can be analysed with computer software capable of representing the degree of relatedness by a dendrogram (Dehesh et al., 1991, Quail, 1994, Mathews and Sharrock, 1997). All angiosperms examined appear to have a \textit{PHYA} gene, which are more closely related to other \textit{PHYA} genes than they are to other phytochrome genes from the same species. Most species seem to contain only one \textit{PHYA} gene, exceptions being the polyploid \textit{Avena} (see Quail, 1994) and the allotetraploid \textit{Nicotiana tabacum} (Ádám et al., 1995). All of these \textit{PHYA} genes appear to be solely responsible for encoding "light-labile" phytochromes. In contrast, the "light-stable" phytochromes are encoded by many genes, designated by \textit{PHYB} to (so far) \textit{PHYF}. Some species, such as tomato, appear to have two \textit{PHYB} genes which are more similar to each other than they are to Arabidopsis \textit{PHYB} (Pratt et al., 1995). It is now considered that the \textit{PHYB} genes represent a sub-family, including \textit{PHYB1} and \textit{PHYB2} of tomato and \textit{PHYB} and \textit{PHYD} of Arabidopsis. The \textit{PHYC} and \textit{PHYE} genes
now known in several species seem to define two distinct lineages of genes. The
PHYF gene, so far found only in tomato, may be another member of a putative PHYC
sub-family (Pratt et al., 1997).

1.2.3.1.2 Lower plant phytochrome genes

Phytochrome genes have been isolated from many lower plants, including the
ferns (Adiantum, Anemia, Dryopteris and Onoclea) the mosses (Ceratodon and
Physcomitrella) and the algae (Mesotaenium and Mougeotia) (Reviewed by Wada et
al., 1997). Most of these genes seem to form a family of 'lower plant phytochromes'
which are distinct from the PHYA-F sub-types in higher plants (Mathews and
Sharrock, 1997). They appear to be mostly light-stable, and retain many of the
conserved features of higher plant phytochromes (Wada et al., 1997). However, one
gene isolated from the moss Ceratodon encodes a protein with an N-terminus highly
homologous to the chromophore-binding region of phytochrome, but a C-terminus
totally unlike that of any other phytochromes then isolated (Thümmler et al., 1992).
The C-terminus resembles a bacterial two-component regulator, and has sparked much
interest in phytochrome as a light-dependent protein kinase. However, it transpired
that Ceratodon also contains "conventional" phytochromes (Hughes et al., 1996).
Although another "phytochrome" resembling the Ceratodon phytochrome with the
aberrant C-terminus has since been isolated from Anemia (Wada et al., 1997), the
function of these proteins remains unknown.

1.2.3.1.3 Cyanobacterial genes showing homology to phytochrome

Recently, a chromatic adaptation mutant in the cyanobacterium Fremyella has
been characterised, and the mutated locus, rcaE, cloned (Kehoe and Grossman, 1996).
This gene bears striking homology to phytochromes, particularly Arabidopsis phyE, in
certain restricted regions. However, certain seemingly essential features of a
phytochrome, such as the chromophore attachment site, do not appear to be present in
the product of this gene. The genome sequencing project of the cyanobacterium
Synechocystis yielded a gene with more convincing similarities to phytochrome
(Kaneko et al., 1996). When expressed in E. coli, this gene yields a protein which
autoassembles with a PCB chromophore to form an adduct with two interconvertible forms, one absorbing maximally at 658nm and the other at 702nm (Hughes et al., 1997). The evidence for the existence of phytochrome systems outside the plant kingdom is growing increasingly strong.

1.2.4 The active form of phytochrome

The absorption spectrum of Pr corresponds strongly to the action spectrum of lettuce seed germination (Figure 1.1(c)). Pr is extremely abundant in seedlings grown in the dark, which are not affected in their morphology by inhibitors of phytochrome synthesis, or in phytochrome-deficient mutants. Many phytochrome responses can be completely cancelled out by a pulse of FR. The above evidence strongly implies that Pfr is the form which produces responses, or the 'active form'. To this day, no well-authenticated example of a response to Pr exists, although many have been postulated (recent examples include Shinomura et al., 1994, Liscum and Hangarter, 1993). It seems likely that a specific property of the Pfr form of phytochrome enables a signal to be initiated that leads to photomorphogenesis. This property is unknown, but it is hopefully conserved throughout phytochromes like the Pr-Pfr interconversion, and will provide a unifying factor in phytochrome signal transduction.

1.2.5 Phytochrome signal transduction

As phytochrome responses induced by R can often be cancelled by a pulse of FR, given up to several hours subsequently, the 'signal' from phytochrome is clearly transmitted to the cellular machinery much more slowly than that in, for example, human vision. However, a pulse of FR given hours later can be ineffective at cancelling a response, even if the response is not yet visible. There must therefore be a 'transduction chain' acting to transmit the signal from phytochrome to those proteins which produce the response. This signal transduction chain has proved much less amenable to scientific study than phytochrome itself. Biochemical approaches to the problem had for many years produced many candidate signalling molecules, but few firm conclusions (Roux, 1994). However, three major approaches, discussed below, are currently being used which have begun to penetrate this hitherto unsuccessful area
of phytochrome research. In addition, work using more recent innovations such as the yeast two-hybrid library system, which has not yet reached publication, may allow another angle of approach to this problem.

1.2.5.1 Microinjection of phytochrome

The development of the technique of microinjection in plants has allowed the introduction of oat phytochrome A into single cells of the phytochrome-deficient tomato mutant *aurea* (Neuhaus et al., 1993). The phytochrome causes de-etiolation of the cell into which it is injected in response to light, involving the activation of many genes. This process can be monitored using tomato plants expressing a plasmid-borne co-injected GUS reporter gene under the control of phytochrome regulated gene promoters. Co-injection of antagonists to well known signal transduction pathways show that calcium/calmodulin and cyclic GMP may both be involved in effecting phytochrome action (Bowler and Chua, 1994, Millar et al., 1994). However, this approach yields few details about the cellular machinery involved in this process. Additionally, the physiological relevance of this work is debatable, since although phyA can clearly mediate the de-etiolation responses studied, they can still occur in the absence of phyA in tomato (van Tuinen et al., 1995b). This shows that other phytochromes also play a role in these responses, perhaps by different pathways.

1.2.5.2 Non-*phy* mutants showing aberrant photomorphogenesis

A second approach has been to isolate genetic mutants which appear to contain a full set of functional phytochromes but are incapable of responding to certain light signals. Three groups of such mutants have been identified in Arabidopsis; the *cop/ det* mutants which show photomorphogenesis in the dark, mutants which are elongated in light, such as *hy5/elg/elf3/pef*, and the *fhy* mutants, which are specifically blocked in the response to FR light. Such mutants are discussed further in Section 1.3. Generally speaking, the more specific the response that is blocked, the more directly related to phytochrome signal transduction the mutant protein is likely to be. Unfortunately the more specific mutants, such as the *fhy* mutants, have not yet been
fully characterised at the molecular level. The mutants which have been characterised, such as det2, are often difficult to relate directly to phytochrome, being, in this case, a steroid synthesis enzyme (section 1.3.3).

1.2.5.3 Mutational analysis of motifs within phytochrome genes

Another approach to the analysis of phytochrome signal transduction, pioneered by Peter Quail and co-workers, has been to sequence multiple alleles of phytochrome mutants (Section 1.3.4). Mutants which possess a single base-pair point mutation which does not appear to affect protein stability may define motifs directly involved in 'passing on' a signal that the phytochrome is in the Pfr state to a reaction partner. Such motifs may then be used as 'bait' in the yeast two-hybrid system in order to characterise proteins which interact with them strongly. By this means the proteins involved in passing the signal from phytochrome to effectors such as transcription factors may eventually be characterised. This approach has yielded some significant results, notably that a C-terminal motif now colloquially known as the 'Quail box' appears to be important in this process (The current state of knowledge from these experiments is explained in Quail et al., 1995). However the elucidation of the phytochrome signal transduction mechanisms has thus far eluded workers using this method also.

1.3 Photomorphogenic mutants

1.3.1 Types of photomorphogenic mutants

Photomorphogenic mutants can be roughly divided into three categories. Firstly there are the mutants which display aberrantly small responses to all light stimuli. The classic example is the aurea mutant of tomato. However, many analogous mutants have been isolated since, including hy1, hy2 and hy6 in Arabidopsis, yg-2 in tomato, pcdl and 2 in pea and the pew mutants of N. plumbaginifolia, with which Chapter 4 of this thesis is concerned. All of these mutants are thought to have lesions affecting the enzymes of the phytochrome chromophore biosynthetic pathway (Terry, 1997) and are subsequently referred to as 'chromophore mutants' (Section 1.3.2).
Secondly, mutants exist which show a constitutive light response, one which occurs in the absence of the stimulus necessary in wild-type plants. Several such groups of mutants have been isolated as such in *Arabidopsis*, including the *cop* mutants (constitutively photomorphogenic, Deng et al., 1991) and the *det* mutants (*de-eriolated*, Chory et al., 1989). These mutants show extensive photomorphogenesis when grown in complete darkness. They are discussed further in Section 1.3.3.

Thirdly, mutants exist which show a particular deficiency in a response to one wavelength of light. For example, the *hy4* mutant of *Arabidopsis* shows a deficiency in the perception of blue light (B) (Koomneef et al., 1980) and is now known to have a mutant B photoreceptor, or cryptochrome (Ahmad and Cashmore, 1993). Such mutants are usually thought to either have a mutation in a photoreceptor gene (discussed in Section 1.3.4), or in a gene encoding a component of a signal transduction pathway (Section 1.3.5). They are consequently the most interesting class of mutants for those seeking to identify the specific roles of the many known plant photoreceptors. Those mutants which appear not to lack any photoreceptor are perhaps even more valuable, as they may help to reveal how the signal from the photoreceptor is transduced into action by the cellular machinery.

### 1.3.2 Chromophore mutants

The *aurea* mutant of tomato, which has been known for many years, came in the 1980s to be widely considered as a phytochrome mutant (Koomneef et al., 1985). It displays a severe phenotype, including an elongated hypocotyl under most light conditions, elongated internodes, pale leaves, slow accumulation of dry mass and an altered chlorophyll a:b ratio. The *yg-2* mutant in tomato shares a very similar phenotype. These loci define separate complementation groups, *aurea* being located on chromosome 1 and *yg-2* being on chromosome 12 (See Terry and Kendrick, 1996).

The *aurea* mutant was shown to lack spectrophotometrically (Koomneef et al., 1985) and immunochemically (Parks et al., 1987) detectable phyA. It was subsequently demonstrated that phyA was present in *aurea*, albeit in reduced levels (Sharma et al., 1993). Doubts that *aurea* was a mutant in the PHYA apoprotein gene were confirmed when the *PHYA* gene in tomato was mapped to chromosome 10 (van Tunien et al., 1997).
Two mutants of Arabidopsis, *hyl* and *hy2*, were isolated which showed an analogous phenotype to *aurea* and *yg-2* (Koornneef et al., 1980). Unlike the tomato mutants, however, the light responses of these mutants could be partially (*hy2*) or almost completely (*hyl*) restored by supplementing the growth medium with the PФБ precursor biliverdin IXα (BV) (Parks and Quail, 1991). Subsequently, biochemical studies have established that both *aurea* and the *pcd2* mutant of pea are unable to convert BV to PФБ, while both *yg-2* and the *pcdl* mutant of pea are unable to convert haem to BV (Terry and Kendrick, 1996, Weller et al., 1997, Weller et al., 1996).

Since *hy2* is only partially rescued by biliverdin, it may be analogous to *aurea* in that it could possess a mutation in an enzyme which converts BV to PФБ. Such a mutant would still be able to produce PФБ when supplied with BV at high concentrations, if the mutation in the enzyme did not completely ablate function. Such a 'leaky' mutant could have a mutated enzyme with a substantially higher Km than the wild type, which could only make appreciable quantities of product at high substrate concentrations. However, some product would still be made, and this would explain the phytochrome responses which are still visible in so called 'chromophore mutants' (Adamse et al., 1988, Whitelam and Smith, 1991, Kerckhoffs et al., 1992). If all the chromophore mutants isolated so far are 'leaky', it may be the case that a mutation creating plants completely unable to synthesise functional phytochrome would be lethal. Evidence to support this comes from the additive phenotype of *aurea* and *yg-2* (van Tuinen et al., 1996) which leads to extremely compromised plants, and the lethality of the *pewl pew2* double mutant in *N. plumbaginifolia* (Kraepiel et al., 1994a). All of these loci are thought to be involved in chromophore biosynthesis.

1.3.3 Mutants showing photomorphogenesis in the dark

Two groups, those of Joanne Chory and Xing-Wang Deng, began to isolate these mutants in the late 1980s and 1990s. Consequently the nomenclature is somewhat confusing, with *det* and *cop* (section 1.3.1) referring to the same phenotype, and certain *det* mutants being allelic to given *cop* mutants. To make matters even less clear, many *det* and *cop* alleles had been identified previously as seed coat colour mutants (*fusca* mutants). This thesis will not attempt a description of all the *det, cop*
and *fus* alleles, which can be found in Chory et al. (1996). Although such mutants were initially thought to be candidates for light-signal transduction mutants, it is now thought more likely that they define genes with a more global role in plant development. For example, *det2* has recently been identified as a steroid synthesis mutant (Li et al., 1996) and is hence unlikely to be directly involved in the transduction of light signals, but rather acts by disabling an entire signalling system. Also, *det1* appears to encode a repressor protein which prevents all light regulated genes being transcribed in darkness, and consequently is probably not a component of the signal transduction chain of any given photoreceptor. Although many schemes have been postulated for the inclusion of these mutants in phytochrome signal transduction (e.g. Chory et al., 1996) such hypotheses are easily criticised and are best treated as genetic models, which may or may not bear any resemblance to the biochemical interactions between the gene products.

1.3.4 Photoreceptor mutants

These represent the most useful class of mutants for the study of the physiological and ecological role of photoreceptors, as each can be studied in a background where all the others are present. Although we now know that Arabidopsis has at least five phytochromes (Clack et al., 1994), we only have confirmed, published mutants in two of them, and one blue light photoreceptor, despite exhaustive screens. It seems likely, therefore, that mutants in the remaining phytochromes are too subtle in their phenotype to be easily spotted in a wild-type background. As yet unpublished research on mutagenised *phyA phyB* double mutants in the laboratories of Garry Whitelam and Richard Kendrick has begun to detect such mutants in Arabidopsis and tomato. The remaining light stable phytochrome mutants are hence unlikely to remain elusive for long, given current interest in this field.

1.3.4.1 Phytochrome B mutants

The first photoreceptor mutant to be identified as such was the Arabidopsis *hy3* long hypocotyl mutant (Koornneef et al., 1980). This was identified as lacking the light-stable phytochrome B apoprotein (Somers et al., 1991) and subsequently lesions
in the phyB gene were identified in hy3 mutants, which were renamed phyB mutants (Reed et al., 1993). Analogous mutants to hy3 have since been identified in cucumber (López-Juez et al., 1992), Brassica rapa (Devlin et al., 1992), pea (Weller et al., 1995) and Sorghum bicolor (Childs et al., 1992, 1997, Foster et al., 1994). These mutants all appear to have a lesion affecting the phyB gene. The tomato mutant, tri, is also deficient in a phyB-type phytochrome, phyB1 (van Tuinen et al., 1995a). All these mutants share two main aspects of their phenotype. They all appear somewhat elongated under standard growth conditions (although this is more noticeable in hy3 or lh than it is in tri). All of the phyB-deficient mutants display a deficiency in the response to monochromatic R (McCormac et al., 1993, Devlin et al., 1992, Weller et al., 1995, Foster et al., 1994). It is consequently a matter of relative certainty that phyB mediates responses to R. The role of phyB in R:FR perception is more controversial, with some workers having stated that phyB mediates R:FR perception (Quail et al., 1994) while others have published studies disputing the assertion that only phyB is involved (Whitelam and Smith, 1991, Robson et al., 1993). This subject is discussed further in Chapter 3.

1.3.4.2 Phytochrome A mutants

Although phytochrome A is by far the most abundant phytochrome in etiolated tissue, and was the first to be genetically characterised (Hershey et al., 1985, 1987), mutants in this gene were not isolated until phyB mutants in Arabidopsis were well known (Whitelam et al., 1993, Parks and Quail, 1993, Nagatani et al., 1993). Surprisingly for mutants in such an apparently fundamental gene, phyA mutants show little obvious phenotype when grown in W (Whitelam et al., 1993). However, phyA mutants appear to be deficient in some of the more subtle phytochrome responses, including daylength perception (Whitelam and Harberd, 1994).

Mutants at the PHYA locus were only isolated when workers began screening M2 populations of Arabidopsis under continuous FR. While wild-type Arabidopsis is extremely sensitive to continuous FR, phyA mutants appear to be completely blind to FR when it is given to seedlings which have never otherwise been exposed to light (Whitelam et al., 1993). The so-called FR-HIR response mode of phytochrome
(Section 1.2.2) is completely absent in \textit{phyA} mutants (Whitelam and Harberd, 1994). So is the VLFR response (Casal, 1997), which can also involve a response to FR (although other wavelengths are effective) in seedlings not previously exposed to light. However, the cancellation of inductive responses by FR, and R:FR perception, seem to be intact in these mutants, so they are far from 'blind' to FR.

Interestingly, the shade-avoidance responses appear to be exaggerated in these mutants during de-etiolation under dense canopies (Yanovsky et al., 1995) implying a role for \textit{phyA} in light grown plants which is antagonistic to the shade avoidance response (see section 1.5). The mutants are affected in photoperiodism (Section 1.4.4) and various subtle anomalies exist in the way they perceive shade (Casal et al., 1997). Analogous mutants to \textit{phyA} have since been isolated in pea (\textit{fun1}, Weller et al., 1995) and tomato (\textit{fri}, Van Tuinen et al., 1995). All of these mutants lack responses to FR as etiolated seedlings.

### 1.3.4.3 B (and UV-A) photoreceptor mutants

The only well characterised B photoreceptor mutant, which is also somewhat deficient in UV-A responses, is \textit{hy4} (Koomneef et al., 1980). Deficient in hypocotyl elongation inhibition and cotyledon expansion in response to B, \textit{hy4} has normal phototropic responses. Fortunately, the availability of a T-DNA tagged allele of this mutant has allowed the locus of this mutation to be cloned (Ahmad and Cashmore, 1993). Previously poorly characterised, the B receptors had come to be known as cryptochromes due to their discovery in cryptogams. The \textit{HY4} locus consequently was named \textit{CRY1} (Lin et al., 1995b). The protein encoded by this gene is a 75kDa flavoprotein with some amino acid homology to bacterial DNA photolyases. Overexpression of the gene in tobacco increases sensitivity to B, UV-A and green light (Lin et al., 1995a). However its precise molecular mode of action remains obscure. A second \textit{CRY} gene, \textit{CRY2}, has been isolated on the basis of nucleotide homology (Lin et al., 1996), but mutants at this locus are not yet available, and its physiological role remains unknown (recently reviewed by Cashmore, 1997). Other B photoreceptor mutants have been described, but with the \textit{blu} mutants (Liscum and Hangarter, 1991) now having been re-evaluated as alleles of \textit{hy4} (Jenkins et al., 1995), the only remaining mutants available for study at the moment are the phototropic mutants.
These belong to two classes, the shoot phototropism or JK/nph mutants (Khurana and Poff, 1989, Liscum and Briggs, 1995) and the root phototropism or rpt mutants (Okada and Shimura, 1992). It is beyond the scope of this thesis to give a thorough description of these mutants, which have recently been reviewed by Briggs and Liscum (1997).

1.3.5 Specific signal transduction mutants

Many putative phytochrome signal transduction mutants have been isolated, mainly by a light specific long hypocotyl phenotype. Mutants which show a long hypocotyl in W or R but appear not to lack functional photoreceptors include elg (Halliday et al., 1996), elf3 (Zagotta et al, 1996) and the pef mutants (Ahmad and Cashmore, 1997). All of these mutants have been postulated to be phytochrome signal transduction mutants. The hy5 mutant, which shows a long hypocotyl under most light treatments, is also a candidate signal transduction mutant (Koomneef et al., 1980). Since elg, elf3, and the pef series have all been isolated relatively recently, they have not been thoroughly checked by other groups to confirm that they are genuine light-signal transduction mutants. The most thorough characterisation of any of these mutants is probably that of Halliday et al. (1996), who concluded that elg was not a phytochrome signal transduction mutant, but had a constitutively long hypocotyl. Although the mutants have apparently wild-type hypocotyl lengths in the dark, this was revealed not to be the case when they were crossed with gibberrellin mutants. Until such a thorough analysis is completed on the other mutants mentioned, it may be premature to describe them as signal transduction mutants.
1.4 The role of phytochrome in photoperiodism

1.4.1 Photoperiodic responses

Photoperiodism gave rise to the field of photomorphogenesis, as a direct result of the work of Garner and Allard in the 1920s and 1930s (Sage, 1992). Work on a spontaneous mutant of Maryland Narrowleaf tobacco, known as Maryland Mammoth, demonstrated that the flowering of the mutant was dependent on daylength (Garner and Allard, 1920). Unlike the wild-type, Maryland Mammoth would only flower in short days, and consequently grew much larger than the original cultivar during the summer months. Despite the demonstration in 1919 that this mutation corresponded to a monogenic dominant locus (Allard, 1919), it has yet to be characterised by molecular genetics.

Flowering of many plants transpired to be influenced by daylength (Garner and Allard, 1933). Terms were coined for the three groups identified by Garner and Allard; Short-Day (SD) plants (which only flower under SD), Long-Day (LD) plants (which only flower under LD), and Day-Neutral (DN) plants, in which flowering occurs independently of photoperiod. Subsequently, subsidiary groups were discovered, where flowering is dependent on LD and SD in a particular order. These plants are known as LD-SD or SD-LD plants as they require LD followed by SD or SD followed by LD respectively in order to flower. These groups have clearly evolved to differentiate between the spring and autumn equinoxes. In other species this is achieved via a temperature input to the flowering response. Some other minor groups have also been identified (Vince-Prue, 1975). The plants described in the introduction and body of this thesis are usually considered DN (tobacco species, tomato, potato, cucumber) with the exception of Arabidopsis (LD) and sorghum and Mammoth tobacco (SD).

The work of Hamner and Bonner (1938) demonstrated that SD plants are actually "long night" plants, in that a brief interruption of the night by a light pulse, or night-break, was sufficient to prevent flowering. This allowed the discovery of the wavelengths most active in preventing flowering (Parker et al., 1946) and the subsequent demonstration of phytochrome involvement by the demonstration of R-FR photoreversibility (Borthwick et al., 1952b, Downs, 1956).
Tuberisation in certain potato cultivars is an example of a response other than flowering which is controlled in a photoperiodic manner. It has recently been convincingly demonstrated by Jackson et al. (1996) that the induction of tuberisation by SD in potato can be made constitutive by the elimination of phyB using antisense RNA in transgenic plants. In other words, phyB Pfr must be necessary to inhibit tuberisation in LD. This SD tuberisation response appears to be directly analogous to the induction of flowering in SD plants. However, transformable SD plants with which to test the obvious implications of this result are rare, and the obvious one (Maryland Mammoth tobacco) has too long a generation time to be convenient for molecular genetic studies.

1.4.2 The circadian clock

Photoperiodic responses must clearly be dependent on some internal means of timing the length and period of light stimuli. That such a "clock", or endogenous oscillator, occurs in most higher organisms has been known for some time. Molecular analysis of the clock in some organisms is extremely advanced, most particularly in Neurospora and Drosophila (see Dunlap, 1996, for a review). While such organisms are used mainly due to their suitability for experimental analysis, the analysis of the circadian system in plants has a unique importance, since plant circadian clocks control the timing of harvest of many of the world's most important crops.

Externally, the presence of an oscillator in many plants is betrayed by the nyctinastic or "sleep" movements of leaves. Leaves and cotyledons of many plants display changes in angle which are rhythmic and roughly synchronous with the photoperiod under which they were entrained. These movements continue even when the stimulus is removed, i.e. when the plants are places in continuous light or darkness (Carre, 1996, Vince-Prue, 1994, Chapter 5). The oscillation of the movement of leaves and cotyledons is combined at the molecular level by the oscillation of the mRNA level of many genes, the best known of which in plants are the CAB or lhc genes. The mRNA levels of these genes display free-running oscillations in many species, including wheat, tobacco and Arabidopsis (Nagy et al., 1993, Oberschmidt et al., 1995, Kolar et al., 1995). The promoter of an Arabidopsis CAB gene has hence been fused to the LUC reporter gene to provide a bioluminescence rhythm, which can be
measured automatically (Millar et al., 1992). Screening of mutagenised lines containing the CAB::LUC fusion has yielded mutants which show aberrant rhythms (Millar et al., 1995). However, the screen is far from complete, and has yet to identify mutants analogous to frequency (frq) in Neurospora or period (per) and timeless (tim) in Drosophila. Also, while the leaf movement and CAB expression rhythms appear to be connected (Hicks et al., 1996) they have different periods (Millar et al., 1995). Together with evidence that two distinct circadian oscillators can control the same system (Roenneberg and Morse, 1993, Kolar et al., 1995) this makes the search for the mechanism of "the" circadian clock increasingly complicated.

1.4.2.1 The clock and phytochrome

An oscillator which is synchronised to the photoperiod under which an organism is grown must necessarily sense that photoperiod using a photoreceptor. Such an entrainment of the clock is essential for the perception of the timing of daylength. There is substantial evidence for the involvement of phytochrome in the entrainment of leaf movement rhythms (Simon et al., 1976) and CAB mRNA levels (Nagy et al., 1993, Kolar et al., 1995, Millar and Kay, 1997). However the complex nature of the light stimuli given to a plant during the entrainment process usually prevents the kind of analysis necessary to perform studies such as R-FR reversibility, unless a system is used where phase-shifting is easily induced and monitored in constant darkness (e.g. Simon et al., 1976).

Another method of analysing the effect of photoreceptors on the entrainment of circadian rhythms is to examine the alteration of the period length in constant light. Since the period of cab::luc bioluminescence in Arabidopsis is shorter in constant light than in constant darkness, previously entrained plants can be allowed to display their free-running rhythm in continuous light, and the period measured. The hyl mutant displays a longer period under these conditions, implying that phytochrome is involved in entrainment. The period of hyl is still influenced by light, however, and the plants are still entrained. Since hyl is not entirely phytochrome deficient (Whitelam and Smith, 1991) this may be a result of 'leakiness' of this mutant allele. However, there is strong evidence that blue-light receptors are also involved in
entainment. For a recent and authoritative review of this area of the field, see Millar and Kay, 1997.

1.4.3 Genetics of clock and photoperiodic mutants

Many mutants of Arabidopsis have been identified which show altered flowering responses. While none have been isolated which are analogous to the Maryland Mammoth mutation in tobacco (Section 1.4.1), several groups of mutants have been identified which affect flowering time and/or floral development. This rather complex field has recently been reviewed in some detail by Peeters and Koornneef (1996) and by Simon and Coupland (1996), and an exhaustive listing and description of these mutants is beyond the scope of this thesis.

These mutants can be classified either by phenotype, or where it is known, by the nature of the mutation. Mutants which cause late and early flowering in both long and short days are known. Some of these mutations have been characterised at the molecular level, and some (e.g. ag, ap2, co) have been shown to encode transcription factors (Simon and Coupland, 1996). However, the most interesting mutants from the point of view of photomorphogenesis and the circadian clock are those mutants which show pleiotropic effects on one or both of these. One example of such a mutant is elf3. The elf3 mutant is early flowering under both long and short days, and shows little response to photoperiod. It also shows a long hypocotyl phenotype, but it is not connected with phytochrome apoprotein or chromophore synthesis (Zagotta et al., 1996). It displays a severe dysfunction of circadian rhythmicity under certain conditions, including both cab::luc bioluminescence rhythms and cotyledon movements (Hicks et al., 1996). Consequently it may play a central role in the transduction of light signals, particularly those from blue light (Zagotta et al., 1996) to the cellular machinery.

1.4.4 Effects of phytochromes on photoperiodism

Phytochrome mutants in many species display altered photoperiodic responses, perhaps the best known being the hyl - hy4 mutants of Arabidopsis, the ma^R mutant of sorghum and the i^v mutant of pea. All of these mutants are early flowering, (Peeters
and Koornneef, 1996, Childs et al., 1995, Reid et al., 1996) and with the exception of hy4, they are all thought to lack at least one light-stable phytochrome, including phyB (Section 1.3.4). The lh and ein mutants of cucumber and Brassica respectively both show a small, but significant, early flowering phenotype (López-Juez et al., 1990, Rood et al., 1990). The ma3R mutant of sorghum demonstrates substantially early flowering in LD, and is almost DN, but does still respond somewhat to photoperiod, and the entrainment of the circadian alterations in mRNA level of the CAB and RBCS genes occurs normally in ma3R (Childs et al., 1995). The ma3R mutation does however cause a phase-shift in the diurnal regulation of gibberellic acid (GA) levels (Foster and Morgan, 1995).

While mutations which reduce phyB levels seem, in general, to cause early flowering, those which reduce phyA levels seem to have a more subtle phenotype. The Arabidopsis phyA mutants display no phenotype when grown under continuous W (Whitelam et al., 1993), and little obvious phenotype when grown in LD, other than marginally longer hypocotyls (Johnson et al., 1994). However, phyA seems to be important in the perception of daylength in Arabidopsis, as the mutants are considerably later flowering when grown in SD plus either night-breaks or low-fluence rate day extensions (Johnson et al., 1994, Bagnall et al., 1995). However the observation that the delay in flowering in high fluence rate LD is not as significant implies that another photoreceptor may substitute for phyA at higher fluence rates.

1.5 Plants transgenic for phytochrome

1.5.1 Plants overexpressing phytochrome A

The isolation of the PHYA gene from oat (Section 1.2.1) happened at roughly the same time as the emergence of routine methods for genetic transformation of many plant species. Consequently, transgenic plants expressing heterologous PHYA genes were rapidly produced, initially in tobacco (Keller et al., 1989, Kay et al., 1989) and tomato (Boylan and Quail, 1989). These plants all showed a 'dark green dwarf' phenotype. It was only when overexpression lines were generated which had a more subtle phenotype that the specific role of phyA began to be elucidated. Overexpression of phyA produces plants which are more sensitive than the wild type to both R and FR
(McCormac et al., 1991, 1993). It also causes a persistence of the FR-HIR into the light-grown plant, causing dwarfing under conditions where the shade-avoidance response would normally produce elongated plants (Whitelam et al., 1992, McCormac et al., 1992). This effect was later confirmed in potato, where reducing phyA levels using antisense RNA produced the opposite phenotype (Heyer et al., 1995). Oat phyA overexpression thus allows the production of plants in which the shade-avoidance response is disabled, and are hence more suitable for growth as high-density crops (Robson et al., 1996b).

Another consequence of the routine overexpression of biologically active phytochrome was that deletion, site directed mutagenesis and domain-swap analyses could be conducted. Experiments were conducted to determine which regions of the phytochrome apoprotein were important in determining the presence, type and intensity of response observed. Deletion analysis has allowed the localisation of chromophore lyase activity (Vierstra and Quail, 1986, Lagarias and Lagarias, 1989) and dimerisation activity (Cherry et al., 1993). Additionally many deletion constructs were made which completely abolished activity (Cherry et al., 1993) and even reduced native phytochrome responses (Boylan et al., 1994). Domain swapping experiments with other phytochromes have demonstrated that the N-terminus is essential for photoreversibility and differential light lability, and that the C-terminus determines the photosensory specificity of individual phytochromes (Wagner et al., 1996). The current state of knowledge from these experiments has recently been reviewed by Quail (1997).

1.5.2 Plants overexpressing phytochrome B

Transgenic Arabidopsis overexpressing phytochrome B have been available for some time (Wagner et al., 1991). These transgenic seedlings display an increased sensitivity to R (McCormac et al., 1993). A similar phenotype is observed when Arabidopsis phyB is overexpressed in transgenic tobacco (Halliday, 1996). Since increased R sensitivity is the direct inverse phenotype of the phyB mutants, which are insensitive to R (McCormac et al., 1993), this provides clear evidence of the dose-dependent role of phyB in R induced de-etiolation. Further evidence comes from the partial dominence of the hy3=phyB alleles (Koornneef et al., 1980), and the effect of
introducing extra copies of the *PHYB* gene into Arabidopsis (Wester et al., 1994). The light-grown phenotype of the phyB-overexpressing Arabidopsis plants is dwarfing combined with darker green colouration, similar to that observed in high-level phyA overexpressors. The role of Arabidopsis phyB in R-induced de-etiolation is hence perhaps the best characterised response attributable to a single phytochrome, with the possible exception of the FR-HIR. However, the role of phyB in R:FR perception in light-grown plants is less clear. The *phyB* mutants in Arabidopsis retain shade-avoidance responses (Whitelam and Smith, 1991, Robson et al., 1993) and phyB overexpressors show a decreased sensitivity to changes in R:FR (McCormac et al., 1993). Consequently while the *phyB* mutants superficially appear to have a constitutive shade-avoidance phenotype, the role of phyB in shade avoidance is far from clear. This subject is discussed further in Chapter 3.

1.5.3 Effect of phytochrome overexpression on flowering time.

Transgenic Arabidopsis overexpressing phyA flower slightly early under standard LD growth conditions (Bagnall et al., 1995). Under short days, they flower considerably earlier than usual (Bagnall et al., 1995). This is a marked phenotype and is distinct from the late flowering caused by lack of day-extension perception observed in the *phyA* mutants under certain conditions. Overexpression of phyA in DN *N. tabacum* causes a delay in flowering under LD and SD conditions, and the mild sensitivity to night breaks (NB) (which delay flowering) in DN tobacco is enhanced by phyA overexpression. Overexpression of phyA in SD tobacco causes late flowering in SD and a massive increase in NB sensitivity (Halliday, 1996). Consequently the role of phyA is likely to include daylength perception, as it has opposite effects in LD and SD plants grown under SD.

Arabidopsis phyB overexpressers flower earlier than the wild type (Bagnall et al., 1995), and early flowering is also observed in the Arabidopsis *phyB* mutant. EOD-FR treatment to remove the phyB Pfr restores the wild-type phenotype in the phyB overexpressing plants, suggesting that transgenic phyB Pfr present in the dark acts to accelerate flowering. However, these data contradict those from the *phyB* mutants, which are also early flowering. Also, EOD-FR treatment in the wild-type accelerates flowering through a shade-avoidance type response. Tobacco, in both DN and SD
varieties, overexpressing Arabidopsis phyB, flowers late under all conditions (Halliday, 1996). However SD tobacco particularly shows an increase in the delay in flowering in response to NB in response to phyB overexpression. It appears that phyB is also involved in daylength perception in both these species, and that the interplay between these phytochromes in determining flowering time may be complex.

Phytochrome C overexpression in tobacco does not seem to alter photoperiodic sensitivity in DN tobacco. In SD tobacco there may be a small increase in NB sensitivity, but the effect is slight and the phytochrome gene is heterologous, making it difficult to draw firm conclusions (Halliday, 1996).

1.5.4 Plants expressing PHY promoters fused to reporter genes

Tobacco, pea and Arabidopsis PHYA promoters have been fused to the GUS (and in one case the CAT) reporter genes and expressed in tobacco, petunia and Arabidopsis, respectively (Ádám et al., 1994, Komeda et al., 1991, Somers and Quail, 1995a). Tobacco and Arabidopsis PHYB::GUS fusions have also been expressed in their native backgounds (Ádám et al., 1996, Somers and Quail, 1995a). The levels of the GUS enzyme were reduced in light grown PHYA::GUS transformants when compared to dark-grown controls. However, PHYA promoter activity was still high in light-grown vascular tissue, roots and floral organs (Ádám et al., 1994). The downregulation of the PHYA promoter still occurred in the phyB (hy3), hy4, hy5, hy6 and phyA mutant backgrounds, suggesting either a novel phytochrome or multiple phytochromes are involved in this response (Somers and Quail, 1995b).

The pattern of PHYB promoter activity is markedly different from that of PHYA, although the spatial regulation of the two promoters appears to differ strongly only in pollen, where PHYB is more active (Somers and Quail, 1995b). The PHYB promoter is also down-regulated by W and R, apparently by its own gene product. This down-regulation is by no means as strong as that of the PHYA promoter, however. It also appears that PHYB promoter activity is up-regulated by phyA in response to FR or B (Somers and Quail, 1995b).
1.6 The genus *Nicotiana* as an experimental model

Since the work of Garner and Allard, and pioneers in other fields (notably virology) early this century, tobacco (*Nicotiana tabacum*) has become a widely used species in many branches of plant science. It produces a substantial number of seeds which germinate readily, it can be grown to maturity in most climates (with the aid of a heated greenhouse in temperate zones), and the generation time is around 3 months. It is self fertile and has large flowers on which sexual crosses are simple to perform. Its tissue is not woody, and the leaves are amenable to most biochemical and organelle extraction procedures. The plants are large enough to provide sufficient tissue for extraction of most substances, without being too large to grow in a greenhouse or controlled environment chamber. In addition tobacco is a host to many well-characterised pathogens, notably tobacco mosaic virus (TMV). More recently, tobacco was the first plant species for which genetic transformation became a routine procedure. It is a convenient species for the study of shade avoidance (as internodes are easily measured, and much literature exists already) and of flowering time (as it is a classic model species, and DN and SD varieties are available).

Despite these advantages, Arabidopsis has now become the experimental plant species of choice for most workers. This is largely because of the widespread use of genetic screening of mutagenised populations, for which Arabidopsis is ideally suited due to its small size and rapid generation time. The ploidy of *N. tabacum* (usually described as allotetraploid) makes it unsuitable for the isolation of recessive mutations, although it has played an important role in genetics through dominant alleles such as Maryland Mammoth and the *N* gene. Also, techniques for transformation of Arabidopsis have recently become available, although at the time the work for this thesis was begun it was not routine for some ecotypes. These ecotypes included the Landsberg line in which the *hy* mutants were isolated.

The sole advantage of *Nicotiana plumbaginifolia* over *Nicotiana tabacum* is that it is diploid, and so recessive mutations can be isolated from a second generation mutagenised population (*M*₂). Such an *M*₂ population requires large resources to generate in a plant of this size, as tens of thousands of individuals need to be grown to adulthood, and seed collected. The original rationale for such a project was based on the ease of genetic transformation of *N. plumbaginifolia* in comparison with (at the
time) Arabidopsis. An M₂ population of *N. plumbaginifolia* was produced at INRA, Versailles, and used to isolate the mutants described in this thesis. The INRA laboratories have now developed a transposon mutagenesis system (Houba-Herin et al., 1994) which has already allowed the cloning of the genes from isolated mutants (Marin et al., 1996). Consequently, although *N. plumbaginifolia* is not the most convenient species to work with, being subject to seed dormancy (see chapters 2, 3 and 7), it is possible to perform powerful genetic screens in exactly the same way as in Arabidopsis. It is possible to isolate mutants which have not been isolated in Arabidopsis due to the differences between the species. For example, the seed dormancy of *N. plumbaginifolia*, perceived as a problem by most plant biologists, allowed the isolation of the first ABA biosynthesis gene (Marin et al., 1996). This provides an illustration of how working with a less heavily researched species can prove scientifically rewarding.

### 1.7 Outline of the thesis

This thesis represents an attempt to combine the advances in knowledge about phytochrome gained from Arabidopsis mutants with that gained from photophysiology in tobacco, by the analysis of phytochrome mutants in a *Nicotiana* species. Utilising the mutant lines in *N. plumbaginifolia* generated at INRA Versailles together with the *N. tabacum* phytochrome A gene and promoter isolated by Éva Ádám and co-workers, this project addresses the problem of phytochrome function in *Nicotiana* using mutant and transgenic plants. In addition to the use of standard methods of seedling physiology and measurement of flowering time, the simulation of shade-avoidance conditions and the analysis of circadian leaf movements provide additional data on the responses mediated by different phytochromes in this genus.

This thesis begins by describing the molecular biology of the mutation in the *N. plumbaginifolia hlg* mutant, and goes on to investigate the physiological consequences of this lesion. The physiological effects of the *pew* phytochrome chromophore mutants are also investigated. Finally, the effect of overexpression of the native *PHYA* gene in *Nicotiana tabacum* is investigated.
Chapter Two

Molecular and genetic analysis of the *hlg* mutants and
the *PHYB* gene of *Nicotiana plumbaginifolia*
2.1 Introduction

Phytochromes are a family of biliproteins which exist in two forms (Pr and Pfr), interconvertible by red (R) and far-red (FR) light (please refer to chapter 1 for more details). They control numerous morphogenic and biochemical processes in plants, many of which are associated with the process of de-etiolation. Two classes of phytochrome have been recognised, corresponding to the light-stability of the photoreversible protein. Phytochrome A from oat, *Arabidopsis* and other plants has been characterised as a "light-labile" or type I phytochrome. Other phytochrome genes have been shown to code for light-stable or type II phytochromes, including the phytochrome B gene (PHYB) in *Arabidopsis*, and closely homologous genes isolated from numerous other plants (For in-depth reviews, see Whitelam and Harberd, 1994, Quail, 1994, Koornneef and Kendrick, 1994, and Chapter 1).

The functions of the phytochromes have been investigated with the aid of several mutants of different species. Some clearly have a lesion in one of the genes encoding the phytochrome apoproteins, while others appear to be deficient in the biosynthesis of the tetrapyrrole chromophore (Whitelam and Harberd, 1994). Phytochrome apoprotein mutants are characterised by deficiencies in photomorphogenic responses, and a partial dominance of the mutant allele (Whitelam and Harberd, 1994), which is almost certainly due to an effect of gene copy number on phytochrome level (Wester et al., 1994). Mutants in the *Arabidopsis* PHYB gene have been extensively characterised (Koornneef et al., 1980, Somers et al., 1991, McCormac et al., 1993, Reed et al., 1994) and are the only light-stable phytochrome mutants in which lesions within the native apoprotein gene have hitherto been identified and sequenced (Reed et al., 1993, Bradley et al., 1996). Mutants displaying a deficiency in a gel-blot band detectable with monoclonal antibodies to a phytochrome-B-like apoprotein have been isolated in cucumber (López-Juez et al., 1992), *Brassica rapa* (Devlin et al., 1992, 1997), pea (Weller et al., 1995) and *Sorghum bicolor* (Childs et al., 1992, Foster et al., 1994), but none has yet been characterised by the sequencing of a mutant gene. A tomato mutant, *tri*, deficient in a phyB-type apoprotein, has been isolated (van Tuinen et al., 1995a). Some alleles have aberrant PHYB-1 mRNAs (Kerckhoffs et al., 1996). Potato plants have also been generated in which the PHYB
gene expression has been ablated by antisense techniques. They show lower levels of 
\textit{CAB 1} gene expression than that characteristic of wild-type plants. The above mutants 
al so show a decreased sensitivity to continuous R as seedlings which manifests itself in 
white light (W) as a partially etiolated phenotype.

The \textit{PHYB} gene of Arabidopsis has an ortholog in the \textit{PHYD} gene, which is 
thought to belong to a sub-family of genes encoding light-stable phyB-like 
photoreceptors (Clack et al., 1994). Genes encoding phyB-like sub-family members 
have been isolated in potato (Heyer and Gatz, 1992) and \textit{N. tabacum} (Kern et al., 
1993). The phyB-like sub-family in tomato seems to be encoded by at least two genes, 
of which fragments encoding the region adjacent to the chromophore attachment site 
have been isolated by the polymerase chain reaction (PCR) (Pratt et al., 1995). The 
sequences of these PCR fragments of the two tomato genes are more closely related to 
each other than to the corresponding regions of the Arabidopsis genes \textit{PHYB} and 
\textit{PHYD}, and they have consequently been referred to as \textit{PHYB-1} and \textit{PHYB-2}. It has 
been suggested on the basis of this evidence that the phylogeny of the phyB-like sub­
family in the Solanaceae is at variance with that in Arabidopsis (Pratt et al., 1995). If 
this is the case then any division of function between light-stable phytochromes in 
Arabidopsis may not hold true in other plant families. It is consequently necessary to 
produce fully characterised phytochrome mutants in other species, to determine 
whether conclusions drawn about the role of different phytochromes in Arabidopsis are 
likely to be more widely applicable.

Here there are presented two allelic mutants of \textit{Nicotiana plumbaginifolia} 
which display an elongated hypocotyl as seedlings grown in W. Both mutants are 
shown to display an absence of immunodetectable PHYB apoprotein. The coding 
region of the wild-type \textit{PHYB} gene of \textit{N. plumbaginifolia} is sequenced, and shown to 
be transcribed in both the wild-type plants and both mutants. Genetic lesions, present 
in a \textit{PHYB}-like gene, are defined in both mutants. It is also demonstrated that both 
mutations, in accordance with the previously isolated Arabidopsis \textit{phyB} mutant alleles, 
are partially dominant.
CHAPTER 2  MOLECULAR BIOLOGY OF THE hlg MUTANT  36

Results

2.2.1 Mutant isolation and preliminary characterisation

The hlg mutant alleles were isolated at INRA, Versailles, by Yvan Kraepiel and co-workers. They were identified in an ethyl methanesulphonate (EMS) mutagenised M2 population of N. plumbaginifolia background pb1hd. The screen consisted of examining a population of seedlings under fluorescent W (20 μmol m⁻² s⁻¹) with a photoperiod of 16 h. Mutants were selected which displayed an elongated, or partially etiolated phenotype. Two groups of mutants were isolated, referred to as pew (partially etiolated in white light) and hlg mutants (hypocotyle long). The pew mutants are discussed further in Chapter 4. Of the hlg mutants, two complementation groups initially appeared to exist, hlg1 and hlg2. Two mutant lines, an example from ‘hlg1’ and ‘hlg2’, are investigated in this chapter.

2.2.2 ‘hlg1’ and hlg2’ are allelic, and henceforth are renamed hlg-1 and hlg-2

Figure 2.1 shows the distribution of hypocotyl lengths in the wild type, hlg-1 and hlg-2, and the F2 of a hlg-1 x hlg-2 cross. While the data from the F1 are not conclusive as to the allelism of hlg-1 and hlg-2 (section 2.2.8), these data demonstrate that the two loci are allelic. Over 500 F2 plants were surveyed visually, and none showed a wild-type phenotype. Figure 2.1 shows those hypocotyls which were measured.

2.2.3 PHYB mRNA is present at wild-type levels and spliced normally in the alleles hlg1 and hlg2, as is the phytochrome regulated CAB mRNA

The levels of phytochrome B transcript in the mutants was investigated by RNA gel blotting of mRNA extracted from light-grown leaf tissue of the mutant and wild-type plants at the rosette stage. A gene-specific radiolabelled probe was derived from an RT-PCR product amplified from wild-type N. plumbaginifolia cDNA using primers designed by homology to the published Nicotiana tabacum PHYB gene. It can
be seen from the poly(A) mRNA blots in Figure 2.2 that the level of \textit{PHYB} transcript in the mutants, when compared to the ubiquitin loading control, is equivalent to that in the wild type. It could not however be definitely stated from the results in Figure 2.2 that the sizes of the mRNAs are exactly identical, or that splicing has occurred normally.

It can be seen from the total RNA blot also shown in Figure 2.2 that the phytochrome regulated \textit{CAB 1} gene message (Castresana et al., 1987) is also the correct size and present at wild-type levels, in light-grown leaf tissue of both mutants at the mid-point of the light period.

\textbf{2.2.4 Both hlg alleles are deficient in a phyB-type apoprotein}

Figure 2.3 shows immunoblots of protein extracts from dark-adapted leaf tissue from the \textit{hlg} mutants and the wild type, which have either been maintained in darkness or exposed to continuous R for 24 h. It can be seen from the uppermost blot, probed with a monoclonal antibody to phyA (Holdsworth, 1987), that the mutants have a high phyA apoprotein level in the dark but that the protein is light-labile, indicating that it is photochemically active. Figure 2.3 also shows two blots which were probed with antibodies raised to \textit{N. tabacum} phyB, one (centre) polyclonal (PMTB, Kunkel et al., 1993) the other (bottom) monoclonal (mAT-1, López-Juez et al., 1992). Both blots show that a phyB-co-immunoreactive protein is detectable in the wild-type extracts, but not present at detectable levels in extracts from either mutant.

\textbf{2.2.5 \textit{N. plumbaginifolia} PHYB has large introns in conserved positions, analogous to those in potato PHYB}

The positions of introns within phytochrome genes of all types, particularly in higher plants, are highly conserved (Quail, 1994). Although the introns in most phytochrome genes are relatively small (100-500bp, Quail, 1994) those in potato \textit{PHYB} are unusually large (Heyer and Gatz, 1992, Quail, 1994). The use of the primers described
in section 2.2.6 and Chapter 8 to amplify genomic DNA from \textit{N. plumbaginifolia} suggests that this species has introns larger even than those in potato. The primers were designed by homology to the wild-type \textit{N. tabacum PHYB} cDNA to give products of 800bp, ± 50bp. In Figure 2.4, it can be seen that a PCR reaction deliberately tuned to high sensitivity and long products (hence the high background) delivers a product from genomic DNA amplified with primer pair 4 (Figure 2.5A, Chapter 8) which is approximately 1.2kb larger than that expected from cDNA (Figure 2.5B). This implies that the first, conserved intron in \textit{N. plumbaginifolia PHYB} is around 1.2kb in size, as opposed to 850bp in potato \textit{PHYB} and 88bp in Arabidopsis \textit{PHYB}. The second and third introns would be expected to fall between the primer pair 6, and the distance between these primers is so great that no product is visible in Figure 2.4. Hence the total intron length in this region of genomic DNA is likely to be 4kb or more, since fragments of this gene up to 3.5kb can be amplified routinely (observations not shown). The combined length of introns 2 and 3 in potato is 3250bp (Heyer and Gatz, 1992).

\textbf{2.2.6 PHYB transcript appears to be spliced normally in the \textit{hlg} alleles}

Six primer pairs, specific to, and producing overlapping fragments representing the entire coding region of the wild-type \textit{N. tabacum PHYB} gene (Kern et al., 1993, Figure 2.4A), were used to amplify fragments from a highly homologous \textit{N. plumbaginifolia PHYB}-like mRNA by RT-PCR (see Chapter 8, Materials and Methods). Fragment 4 spans the first intron, which is approximately 1.2kb in size by comparison of genomic DNA and RT-PCR derived fragments (Figure 2.5A, Section 2.2.5) Fragment 6 is likely to span multiple introns (Section 2.2.5)

It can be seen from Figure 2.5B that the sizes of the fragments are equivalent in the mutant alleles and the wild-type \textit{N. plumbaginifolia}, showing that the pre-mRNA is correctly spliced in both mutant alleles, and suggesting that any mutation lies in the amino acid code of the message in both cases. The dilution series is present in Figure 2.5C to confirm that the fragments were produced in response to the applied cDNA and
also to show that the reaction was partially quantitative. Identical results were obtained from dilution series of the applied mutant cDNAs.

2.2.7 The entire coding region of the PHYB gene in *N. plumbaginifolia* can be sequenced directly from PCR products, despite the presence of large introns

By designing PCR primers homologous to the published *N. tabacum* PHYB gene (Kern et al., 1993, Chapter 8) we were able to generate overlapping fragments of an entire *N. plumbaginifolia* PHYB-like gene coding region (Figure 2.5A). The four fragments which did not span introns were amplified directly from genomic DNA by PCR (Section 2.2.5) The two which did contain introns were too large to amplify efficiently from genomic DNA, as the introns seemed to be over 1kb in size (Section 2.2.5). Consequently we amplified the coding section of these regions by PCR from a population of first strand cDNAs (RT-PCR), the RNA having been isolated from light grown leaf tissue of the mutants and the wild type (Section 2.2.6). This produced 750 - 850bp fragments which we then purified and sequenced in both directions directly, using fluorescent dye terminator methods and dye labelled primers, using amplimers which carried T3 and T7 promoter sequences as tags on the 5' end. The sequence of the wild-type gene has been submitted to EMBL (Accession number pending). It is reproduced in Figure 2.6 in full, together with a conceptual translation.

2.2.8 The *N. plumbaginifolia* PHYB gene is highly homologous to tobacco PHYB, and is not clearly identifiable with PHYB-1 or PHYB-2 of tomato

The nucleotide sequence of the *N. plumbaginifolia* PHYB gene shares 97% homology with the *N. tabacum* PHYB gene. In Figure 2.7, the degree of relatedness of various phytochrome amino acid sequences to *N. plumbaginifolia* PHYB is displayed in a dendrogram, which was generated using PAUP (Phylogenetic Analysis Using Parsimony, © Smithsonian Institution, 1995) for the Macintosh. Bootstrap values based on an analysis of 100 trees are shown at each node. It can be seen in Figure 2.7 that the degree of relatedness of *N. plumbaginifolia* PHYB to tobacco PHYB and potato
**CHAPTER 2**  MOLECULAR BIOLOGY OF THE *hlg* MUTANT

*PHYB* is very high, hence the decision to refer to it as *PHYB*. Although it is more similar to tomato *PHYB-1* than it is to tomato *PHYB-2*, few definite conclusions can be drawn, as the tomato sequences are only partial, and are limited to the chromophore region of the protein. The node separating the group including *N. plumbaginifolia PHYB* and tomato *PHYB-1* is separated from tomato *PHYB-2* by a very short distance, and a bootstrap value of only 86%. Consequently it is not justifiable to identify the *N. plumbaginifolia* gene with either tomato *PHYB* gene.

2.2.9 Both *hlg-1* and *hlg-2* have a single base-pair substitution within the coding region of the *PHYB* structural gene

Knowing the nucleotide sequence of the wild-type *N. plumbaginifolia PHYB* coding region, the entire coding region in both mutants was then sequenced. It was found that the *hlg-1* allele contained a G-A single base substitution at coding base 1138, causing a substitution in the amino acid sequence of residue 380 (glycine) for arginine, which lies in a highly conserved region, as shown in Figure 2.8. The *hlg-2* allele was also found to have a single base substitution, A for T at coding base 1496, which changes residue 499 (leucine) to a stop codon (TAG) (Figure 2.8). The mutations were both detected in genomic DNA derived sequence. No other substitutions were detected in the remainder of the coding sequence. The mutations were clearly and unambiguously defined on both strands of the genomic-derived PCR product Fragment 3 (Figure 2.5A). Where possible, both dye-terminator and dye-primer chemistry were used to sequence both strands of the mutant sequence, in order to eliminate the characteristically different artefacts caused by either method (see Chapter 8, Materials and Methods).

2.2.10 Both alleles display a partially dominant effect on both the phyB-like apoprotein level and the inhibition of hypocotyl elongation by R

The effect of the *hlg-HLG* heterozygous condition can be seen in Figure 2.9A, the heterozygotes containing, by qualitative inspection, around 70% of the wild-type level of phyB-type antigen signal. This is in agreement with the dependence of phyB
level on gene dosage observed by Wester et al. (1994) and Devlin et al. (1997) in Arabidopsis and Brassica. The heterozygotes of both alleles display around 70% of the wild-type response to R in terms of the inhibition of hypocotyl elongation, as shown by the hypocotyl length frequency plot of the F1 in Figure 2.9B. Consequently it can be stated that the response to R appears to be a function of, and hence at least partly dependant on, the phyB level in the plants. This semi-dominant, dose-dependant behaviour is characteristic of mutations in phytochrome apoprotein genes (Koomneef et al., 1980, Whitelam et al., 1993, Wester et al., 1994).

2.2.11 The hlg-2 mutant shows a longer hypocotyl than the allelic hlg-1 due to aberrant germination. This phenotype is the result of a mutation at a second site

Figure 2.10A shows that separately generated wild-type and hlg-1 seed stocks of different ages show differing degrees of dormancy, even after treatment with GA3 and R. In contrast, even relatively fresh stocks of hlg-2 seed show little dormancy, and the germination of some stocks is considerably more rapid than those of the wild type or the other mutant allele. Consequently, the hlg-2 line seems to be affected in seed germination rate and dormancy, while the hlg-1 line does not.

Figure 2.10B shows the wild-type seeds and those of hlg-1 require R for germination, even after GA3 treatment in a mature seed stock. It can also be seen from Figure 2.10B that this requirement is not observed in hlg-2. Hence, not only is hlg-2 affected in seed dormancy, but also in the light requirement for germination, which appears to be lost in this line. These observations indicate the origin of the longer hypocotyls of hlg-2 under all treatments used in Chapter 3, as the seeds will germinate before the W pulse routinely used to synchronise germination. The aberrant germination of hlg-2 also explains the greater variance in the length of hlg-2 hypocotyls, as the germination of the seeds was not synchronised. Figure 2.10B also indicates the dark germination of the F2 of the hlg-2 x wt cross is no more than 25%, consistent with this being caused by a recessive mutation.

Table 2.1 shows the distribution of the hlg, heterozygote (intermediate) and wild-type hypocotyl lengths in the germinated seed of the F2 of a cross between hlg-2 and the wild type. In both the seedlings which germinated under R, and those which
germinated under R having not first germinated in darkness, there is segregation which approximates to a 1:2:1 ratio of these phenotypes. The $\chi^2$ values are consistent with this observation. This strongly suggests the influence of another effect of the mutagen, i.e. another genetic lesion which does not co-segregate with the $hlg$ locus.

2.3 Discussion

The $PHYB$ mRNA detected in both $hlg$-1 and $hlg$-2 appears to be present at wild-type levels (Figure 2.2) and spliced correctly (Figure 2.5B) in both of the mutant alleles. This is what would be expected of mutations causing a single base substitution in the mRNA which alters the protein sequence. Since a deficiency was observed in a phyB-like apoprotein which was detected by an antibody to the product of the $N.\ tabacum\ PHYB$ gene (Figure 3, López-Juez et al., 1992), and a mutation in a gene 97% homologous to the $N.\ tabacum\ PHYB$ gene throughout its length, it is concluded that the protein detected in the wild-type plants is the product of the gene sequenced. Since the mutants show aberrant photomorphogenesis (Chapter 3), it is also concluded that this protein is an active photoreceptor. Since this $N.\ plumbaginifolia\ PHYB$-like sequence is 92% homologous to the published fragment of tomato $PHYB$-1 and 88% homologous to the $PHYB$-2 fragment at the amino acid level (Figures 2.6, 2.7, Pratt et al., 1995) it is not clearly identifiable with either gene, and consequently it is referred to as $PHYB$, as it is highly homologous with the previously published $N.\ tabacum\ PHYB$ gene, and its product reacts with antibody raised to the $N.\ tabacum\ PHYB$ gene product. This does not, however, discount the possibility of the presence of additional $PHYB$-type loci in the genome of $N.\ plumbaginifolia$.

The $hlg$-2 allele is a missense mutation introducing a stop codon which truncates the open reading-frame in the $PHYB$ gene to less than half its normal length (Figure 2.8). The C terminus of $Avena$ phytochrome A must be present for it to be functional (Cherry et al., 1992), and the same is thought to be true of $Arabidopsis$ phytochrome B (Quail et al., 1995). Many apparently null alleles of $hy3$ in $Arabidopsis$ contain stop codons which truncate the C terminus of phyB (Reed et al., 1993, Bradley et al., 1996). The $hlg$-2 mutant plants are hence unlikely to produce any functional phytochrome from this gene, and can be regarded as null. A truncated protein would be
expected from such a stop-codon mutation; such a protein would not be detected using the mAT-1 monoclonal antibody, because this antibody was raised to a C-terminal epitope. The detection of such a protein with the polyclonal antibody PMTB was not possible due to non-specific binding of this antibody with proteins of a similar size.

The hlg-2 allele is not the only mutation carried by this mutant line, as demonstrated by the data in Figure 2.10 and Table 2.1. A secondary lesion is present which causes germination in the absence of a R stimulus and in seed which is relatively immature. This postulated second-site mutation in hlg-2 would be analogous to that reported by Kraepiel et al. (1994b) in the abal mutant, in that it causes early germination in a mutant which otherwise displays increased dormancy. However the hlg-2 mutant line has not been shown to have decreased drought tolerance, which is a primary characteristic of the aba mutants (Kraepiel et al., 1994b, Marin et al., 1996). It is almost certainly worthy of further study. This locus has been provisionally named rig (red independent germination).

The phyB apoprotein signal in the hlg-1 mutant is undetectable (Figure 2.3), which is an uncommon result of mutations causing single amino-acid changes in phytochromes (Xu et al., 1995, Reed et al., 1993, Quail et al., 1995, Wagner and Quail, 1995). It could be possible that the signal from the monoclonal antibody mAT-1 is absent due to alteration of the epitope rather than the absence of protein. However, since this result is repeated with the polyclonal antibody PMTB, the conclusion drawn is that the entire protein, if present, is present in non-immuno-detectable quantities in this mutant. The hlg-1 allele is an amino acid substitution in a region C-terminal to the chromophore attachment site, shown in Figure 4, which is highly conserved throughout plant phytochromes (Xu et al., 1995). A mutation in this region in the Arabidopsis PHYA gene is known to cause decreased stability of the phyA apoprotein (phyA-109; Xu et al., 1995, Figure 4). Furthermore, the amino-terminal chromophore-bearing domain is known to regulate the differential light-lability of phytochromes (Wagner et al., 1996). The hlg-1 mutation may define a motif which is involved in this process.

Some phyB apoprotein may possibly remain in the hlg-1 allele, but if so it is below the limits of sensitivity of our blotting method. It is however likely that phyB function is almost completely, if not entirely, ablated in hlg-1 as it shows no more response to R than hlg-2 (Chapter 3). The two alleles have an identical phenotype,
other than the germination of hlg-2 (Figure 2.10): this could be an effect of the absence of phyB which the putative small amount of phyB remaining in hlg-1 is sufficient to overcome. It is, however, far more likely to be the result of a secondary effect of the mutagen on this line, such as a mutation at another locus, since the germinating seed segregates for the long hypocotyl phenotype (Table 2.1). Also, both hlg-1 (Figure 2.10) and the chromophore mutant pewl (Kraepiel et al., 1994a), display, if any effect, increased dormancy and retarded germination compared to the wild type.

The amino acid which is substituted in the hlg-1 allele, residue 380, is not conserved throughout the plant kingdom like many of the neighbouring residues in the conserved motif which contains it, as shown in Figure 2.8. This residue is a cysteine in phytochromes from ferns and mosses to Arabidopsis, but a glycine in Arabidopsis PHYE, the PHYB genes from potato, N. tabacum and N. plumbaginifolia, and tomato PHYB-1 and PHYB-2 (Figure 2.8). Yet as this residue clearly plays an essential role in phyB apoprotein stability, it is tempting to speculate that the presence of glycine instead of cysteine has some functional significance.

The specific monoclonal antibody to the N. tabacum PHYB gene product, mAT-1, has also been used to characterise mutants deficient in phyB-like phytochromes in cucumber, tomato and pea (López-Juez et al., 1992, van Tuinen et al., 1995a, Weller et al., 1995). The cross-reactivity of the mAT-1 antibody between the phytochromes in which these mutants are deficient implies a conservation of secondary structure between these photoreceptors which is not found between them and any other phyB-like photoreceptors which these species may contain. This provides further justification for referring to the phytochrome gene mutated in the hlg mutants as PHYB. The hlg mutant phenotype at the seedling level correlates well with other known and putative phyB-deficient mutants (Chapter 3, Smith, 1995). It is therefore concluded that the hlg mutants are phytochrome B mutants.
Table 1. The segregation of the germination phenotype of hlg-2.

a). The segregation of the hypocotyl phenotype of hlg-2 in those F₂ seeds of the hlg-2 x wild type (wt) cross which germinate in R.

Seeds were treated with GA₃, then treated with R at a constant 20°C for 10 days. Hypocotyls were scored into three groups (wt, intermediate, hlg) by comparison with wt and hlg controls. The probability of a 1:2:1 ratio (P (unlinked)) was calculated using the chi-squared method.

b). The segregation of the hypocotyl phenotype of hlg-2 in those F₂ seeds of the hlg-2 x wt cross which do not germinate in darkness, but subsequently germinate in R.

Seeds were treated with GA₃, then left in darkness at a constant 20°C for 10 days. The germinated seeds were then removed, and the remaining, ungerminated seeds were treated with R at a constant 20°C for 10 days. Hypocotyls were then scored into three groups (wt, intermediate, hlg) by comparison with wt and hlg controls. The probability of a 1:2:1 ratio (P (unlinked)) was calculated using the chi-squared method.

<table>
<thead>
<tr>
<th>(a)</th>
<th>wt</th>
<th>intermediate</th>
<th>hlg</th>
<th>P (unlinked)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>26</td>
<td>60</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Expected</td>
<td>27.5</td>
<td>55</td>
<td>27.5</td>
<td>0.612</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(b)</th>
<th>wt</th>
<th>intermediate</th>
<th>hlg</th>
<th>P (unlinked)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>13</td>
<td>27</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Expected</td>
<td>13</td>
<td>26</td>
<td>13</td>
<td>0.944</td>
</tr>
</tbody>
</table>
Figure 2.1 Complementation test of *hlg-1* and *hlg2*.

Hypocotyl lengths of the wild type (unfilled bars), *hlg-1* (grey bars) and *hlg-2* (black bars) mutant alleles [top]; and the F$_2$ of crosses between wild type and *hlg-1* (grey bars) and between the wild type and *hlg-2* (black bars) [bottom], under constant R. Seedlings were grown under monochromatic R for seven days. Bars represent the frequency of hypocotyls in each size class.
Figure 2.2 RNA gel blots showing the PHYB and CAB 1 transcript levels in the wild type, hlg-1 and hlg-2 mutants.

A preparation of poly (A)+ RNA from greenhouse grown rosette stage wild-type and hlg-mutant plants was blotted and probed with radiolabelled PHYB cDNA (top), stripped and reprobed with a tobacco ubiquitin loading control for comparison (centre). A preparation of total RNA from the same plants was blotted and hybridised to a probe cloned from the light regulated N. plumbaginifolia CAB 1 gene (bottom). The levels of all three transcripts are indistinguishable from the wild type in the mutant alleles.
Figure 2.3. Protein gel bands showing abundance of phyb and phyb' in the wild type and mutant hlg-1 and hlg-2 plants.

The bands were subjected to SDS-PAGE, stained with Coomassie Brilliant Blue, and visualized under UV light. The wild type (wt) and the mutants hlg-1 and hlg-2 displayed different expression patterns for phyb and phyb' genes, indicating a role for these genes in the mutant phenotype.
Figure 2.3 Protein gel blots showing immunodetectable phyA and phyB levels in the wild type, *hlg-1* and *hlg-2* mutants.

Protein preparations from rosette stage dark-adapted plants either maintained in darkness or subsequently treated with red light for 24 h were separated by SDS-PAGE, semi-dry blotted, and probed with the AS-32 anti-phyA monoclonal antibody (Holdsworth, 1987) (top). The same protein preparations were hybridised to the polyclonal anti-tobacco phyB antibody PMTB (centre) (Kunkel et al., 1993) and the monoclonal anti-tobacco phyB antibody mAT-1 (bottom) (López-Juez et al., 1992). Both mutants lack immuno-detectable phyB, while possessing wild-type levels of phyA which is normally degraded under R.
CHAPTER 2 MOLECULAR BIOLOGY OF THE hlg MUTANT

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>hlg-1</th>
<th>hlg-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>R</td>
<td>D</td>
</tr>
</tbody>
</table>

**phyA**

**phyB**

**phyB**
Figure 2.4 PCR products from genomic DNA showing intron positions in *N. plumbaginifolia* *PHYB*.

The genomic DNA isolated from *N. plumbaginifolia* was used in a PCR reaction designed to create fragments of the largest possible size. Introns are present within primer pair 4 (approx. 1.3kb) and primer pair 6 (over 2.5kb).
Figure 2.5 (a). Diagram to show the PCR products corresponding to the full coding region of the \textit{PHYB} gene.

The \textit{PHYB} coding region is shown, together with the putative introns present in the genomic sequence (by comparison with the published potato \textit{PHYB} gene, Heyer and Gatz, 1992). Although introns were detected in the positions shown, their number and size has not been determined. Also shown are the PCR derived fragments used to sequence the coding region and to confirm transcript integrity.

(b). RT-PCR showing \textit{PHYB} transcript integrity in the wild type, \textit{hlg-1} and \textit{hlg-2} mutants.

Using the same RNA preparations as Figure 1a, six separate primer pairs forming overlapping fragments covering the whole \textit{PHYB} coding sequence (Figure 1a) were used to amplify the fragments shown by RT-PCR. Pairs 4 and 6 span intron(s). All fragments are of the expected size.

(c). Dilution series of the RT-mix from the wild-type RNA, for primer pair 4.

The dilution series is shown to indicate the response of the reaction to applied cDNA and was repeated for both mutants with identical results (not shown).
A) **N. plumbaginifolia PHYB**

intron no.1 introns 2, 3, etc

Fragment Produced by Primer Pair 3

B) Primer pair

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hlg-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hlg-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C) **dilution factor**

<table>
<thead>
<tr>
<th>dilution factor</th>
<th>1</th>
<th>0.4</th>
<th>0.2</th>
<th>0.1</th>
<th>0.025</th>
<th>0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.6 Sequence of wild-type *N. plumbaginifolia* PHYB, and deduced amino acid sequence of the protein.

The sequence shown was determined via direct sequencing of PCR products as described in Chapter 8. The products were derived from both genomic DNA and cDNA from mRNA. The sequence shown is effectively that of the cDNA, as the introns are excluded; however it was derived from genomic DNA in parts.
Figure 2.7 Phylogenetic tree showing relationship of *N. plumbaginifolia* phyB amino acid sequence to that of other selected phytochromes.

The tree was created by the neighbour-joining method, using the PAUP (Phylogenetic Analysis Using Parsimony) software developed by the Smithsonian Institution. Bootstrap values are indicated by each node, and distances are proportional to sequence similarity. All sequences were full length, except tomato *PHYB-1* and *PHYB-2*, which are partial sequences representing the chromophore binding region of the phytochrome apoprotein (Pratt, 1995).
Figure 2.8 Mutations in the phyB polypeptide of the hlg-1 and hlg-2 mutants.

The loci of the mutations hlg-1 and hlg-2 were detected by DNA sequencing and comparison to the wild-type gene. The above shows the change in the deduced amino acid sequence of the phyB polypeptide in both alleles. The position of the truncation of the phyB polypeptide in the hlg-2 mutant is indicated. The missense hlg-1 mutation is also shown together with a Clustal alignment of the conserved region in which this mutation lies, showing selected phytochrome sequences from disparate plant taxonomical groups. The phyA-109 mutation in the Arabidopsis PHYA gene, which also causes loss of immunodetectable protein, (Xu et al., 1995) is also shown.
Figure 2.9 (a). Levels of immuno-detectable phyB in the F₁ of crosses between the wild type, hlg-1, and hlg-2 mutant alleles.

Protein extracts prepared from wild-type plants, homozygous mutant alleles, and plants heterozygous for either, or both, hlg alleles were immunoblotted and hybridised to the mAT-1 anti-tobacco phyB monoclonal antibody (López-Juez et al., 1992). Heterozygous plants have a reduced protein level. The F₁ of the hlg-1 x hlg-2 cross has no detectable phyB.

(b). Hypocotyl lengths of the wild type (unfilled bars), hlg-1 (grey bars) and hlg-2 (black bars) mutant alleles [top]; and the F₁ of crosses between wild type and hlg-1 (grey bars) and between the wild type and hlg-2 (black bars) [bottom], under constant R.

Seedlings were grown under monochromatic R for 7 days. Bars represent the frequency of hypocotyls in each size class.
A)  

```
wt  hlg-1/HLG-1  hlg-2/HLG-2  hlg-1/hlg-1  hlg-2/hlg-2  hlg-1/hlg-2
```

**phyB**

B)  

```
<table>
<thead>
<tr>
<th>mid-point of size class (mm)</th>
<th>number of hypocotyls</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
</tr>
</tbody>
</table>

parents

F1

mid-point of size class (mm)
Figure 2.10 (a) Germination of the wild type, *hlg*-1 and *hlg*-2 in constant red light. The number of seeds germinated from 100 planted on water agar at time zero was plotted against the number of days after germination was induced by GA$_3$. Five seed stocks of different ages were used for each genotype. Each individual seed stock was produced under identical conditions for each genotype.

(b) The germination of wild type, *hlg*-1 and *hlg*-2, and the selfed F$_2$ of the *hlg*-2 x wt back cross, in darkness. The black bars represent the percentage of seeds of each genotype which had germinated after 10 days in darkness, after germination was induced by GA$_3$. Five individual seed stocks were used as above.
CHAPTER 2  MOLECULAR BIOLOGY OF THE hlg MUTANT

A)

![Graph showing percentage germination under constant R over days after GA3 treatment for wt, hlg-1, and hlg-2]

B)

![Bar chart showing percentage germination in D of wt, hlg-1, hlg-2, and hlg2 x wt F2]
Chapter Three

Analysis of photophysiology and shade avoidance in the phyB-deficient hlg mutants of *N. plumbaginifolia*. 
3.1 Introduction

A general opinion has developed that phyB plays a dominant role in mediating shade-avoidance responses (e.g. Koornneef and Kendrick, 1994, Quail, 1994) This conclusion is based on the superficial resemblance of phyB mutants to shade-avoiding plants. The shade-avoidance response represents a competitive phytochrome-mediated response in light-grown plants: it is characterised by early bolting and/or flowering, elongation of internodes and petioles, and an increase in the rate of overall elongation, in response to low R:FR (Smith, 1994, Chapter 1). As light-grown adults, previously isolated mutants deficient in phyB-like phytochromes appear phenotypically similar to wild-type plants grown in low R:FR when grown in high R:FR. They typically show a reduced response to low R:FR, but the responses are not eliminated (Whitelam and Smith, 1991, Smith et al., 1992, Devlin et al., 1992, Robson et al., 1993, van Tuinen et al., 1995) demonstrating that phyB is not necessary for shade-avoidance (Smith and Whitelam, 1997).

The phenotype of phytochrome B mutants of Arabidopsis, Brassica and cucumber is the converse of that displayed by tobacco plants overexpressing phytochrome A. Such transgenic plants display a shade-avoidance response which is reduced, or even reversed (McCormac et al., 1992). This alteration of shade-avoidance responses has now been shown to be potentially useful as a method of improving yields in high-density crops (Robson et al., 1996). Consequently any advance in the knowledge of genetics affecting the shade-avoidance response may potentially have applications in the field, as well as in the laboratory.

The PHYB gene has been overexpressed in Arabidopsis, under the control of a viral promoter (Wagner et al., 1991). The light-dependent short hypocotyl phenotype of these transformants is R specific. In addition, these transgenic lines were shown to have short hypocotyls relative to the wild type at all R to FR ratios (R:FR), and their sensitivity to changes in R:FR was not enhanced (McCormac et al., 1993). Tobacco which has been engineered to overexpress Arabidopsis phyB shows a somewhat dwarfed phenotype, with elevated leaf chlorophyll levels and delayed flowering (Halliday, 1996). The phenotypes of phyB overexpressing Arabidopsis and tobacco are therefore essentially the reverse of that observed in Arabidopsis phyB mutants, with
the curious exception that both phyB mutants and overexpressors in Arabidopsis are somewhat early flowering (Bagnall et al., 1995) [Please refer to the introduction for a more thorough discussion of the phenotypes created by phytochrome overexpression].

A tomato mutant, tri, deficient in a phyB-type apoprotein, has been isolated (van Tuinen et al., 1995a). Some alleles have aberrant PHYB-1 mRNAs (Kerckhoffs et al., 1996). Although these mutants appear to show a deficiency in the response to R as seedlings, this deficiency is described as temporary, and only seems apparent for the first two days of growth. The tri mutants, however, elongate more rapidly than wild-type plants when grown in W of high R:FR, although this phenotype is not as marked as in other mutants deficient in a phyB-like apoprotein (van Tuinen et al., 1995a). In contrast, potato plants in which the PHYB gene expression has been ablated by antisense techniques show a considerably more severe phenotype. They develop very elongated internodes in W, and show lower levels of CAB 1 gene expression than that characteristic of wild-type plants. They also display aberrant photoperiodic responses (Jackson et al., 1996).

Another physiological response which is affected in Arabidopsis phyB mutants is the effect of light on the gravitropic orientation of the hypocotyl (Liscum and Hangarter, 1993). The negative gravitropism of the hypocotyls of dark-grown Arabidopsis is "switched off" by a phytochrome response, which can be triggered by R or FR. Initial speculation that phyB Pr played a role in this response has since been discounted. This response is thought to be partly mediated by phyB Pfr in Arabidopsis (Robson and Smith, 1996).

This chapter concerns the physiological effects of the mutation of the PHYB gene in N. plumbaginifolia. It is shown that these mutants, in contrast to other mutants deficient in phyB-type phytochromes, do not show any component of the shade-avoidance response when grown in high R:FR, and retain normal responses to reduced R:FR.
3.2 Results

3.2.1 The aberrant hypocotyl elongation of *hlg* is specific to R wavelengths

In order to investigate wavelength specificity of the phenotype, mutant and wild-type seedlings were grown under continuous illumination using light-emitting diode (LED) R and FR sources, a broad-band B source and fluorescent W, along with dark-grown controls.

Figure 3.1A shows that the mutant hypocotyl phenotype is only visible in W and R. The hypocotyl is longer, root development is inhibited and the cotyledons are smaller and relatively pale in both alleles of the mutant grown in R, in comparison with the wild type. However the mutants are by no means blind to R, with hypocotyl hook opening, cotyledon expansion and greening occurring. The cotyledons of the mutants are rather smaller in R, but nonetheless expanded. The most obvious effect of the mutation, however, is to lessen the inhibition of hypocotyl elongation under R and, to some extent, W. Despite this, there is also some inhibition of hypocotyl elongation of the mutants in R relative to the dark control. Normal photomorphogenesis is seen in response to FR and B, and wild-type skotomorphogenesis in darkness. It is not possible to distinguish between the dark controls of the *hlg*-1 mutant and the wild-type hypocotyls. However, the *hlg*-2 mutant can be seen to have longer hypocotyls than the *hlg*-1 mutant under all conditions. Furthermore, the hypocotyls of the *hlg*-2 mutant allele are more elongated in comparison to the wild type in R than they are in the dark.

Figure 3.1B shows that, although the phenotype of the *hlg* mutants is visible in white light at the seedling stage, little difference is observed between the mutants and the wild type at the rosette stage. There are phenotypic differences observable in the mutants at this stage, however, notably in leaf angle as described in Chapter 4. There is occasionally also a slight difference visible in pigmentation, as seen in the photograph, with the mutants being somewhat darker. The mutants may have a more highly branched root system, and the longer hypocotyls of the mutants are still clearly visible in Figure 3.1B. However, we have not observed the long root hairs seen in Arabidopsis *phyB* mutants by Reed at. al. (1993) (data not shown).
3.2.2 The hlg mutants display an aberrantly small response to continuous R irradiance

Red light causes separate responses to very low fluence, low fluence, and high irradiance treatments (Mancinelli, 1994, Section 1.2.2). It can be seen from Figure 3.2A that the irradiance-dependent inhibition of hypocotyl elongation by R in the mutants is substantially reduced. The wild-type responds strongly and logarithmically to photon irradiances of R in the range 0.1-10 μmol m⁻² s⁻¹. The mutants, by contrast, appear to display a wild-type sensitivity to photon irradiances below 0.1 μmol m⁻² s⁻¹, in a response which is probably a phyA mediated VLFR (see Chapter 1). They do not appear to perceive R irradiances between 0.1 and 5 μmol m⁻² s⁻¹, where the line of the graph is flat. Beyond 5 μmol m⁻² s⁻¹, the mutants again begin to respond, possibly due to another phytochrome still present in the mutants.

The irradiance-dependent inhibition of hypocotyl elongation by FR in the wild type is considerably less sensitive than that which occurs in response to R (Figure 3.2A,B) in contrast to the situation in Arabidopsis, which is more sensitive to FR (McCormac et al., 1993). The hlg mutants appear to respond in exactly the same way as the wild-type plants to FR light, which is consistent with this response being mediated solely by phyA (Whitelam et al., 1993, McCormac et al., 1993).

At high photon irradiance a R response can still be seen in the mutant. This is consistent with the response to continuous R observed in Arabidopsis phyB mutants (McCormac et al., 1993) which also show a residual response to R. However the residual response to R in the hlg mutants is more noticeable than that observed in Arabidopsis phyB mutants by McCormac and co-workers. Phytochrome B mutants in sorghum, pea, Brassica and tomato are also all deficient in the response to R (see Chapter 1).

The hlg-2 mutant displays a longer hypocotyl than hlg-1 under all conditions, but its R and FR response curves have the same slope as the hlg-1 mutant rather than the wild type. Consequently it seems to lack R responsiveness in a way directly comparable to hlg-1, but also be constitutively long. The reason for this is likely to be the early germination phenotype of the hlg-2 mutant, which is probably caused by the second-site rig mutation contaminating the hlg-2 line, as discussed in Chapter 2.
3.2.3 The phenotype of the hlg mutants is more pronounced under relatively low PAR levels in W

The hypocotyl lengths of 7 day-old wild-type and hlg-1 seedlings grown under different PAR levels of fluorescent W are shown in Figure 3.3. The inhibition of hypocotyl length by W is saturated in the wild type at the lowest PAR level in the experiment (50 μmol m⁻² s⁻¹), and the hypocotyls are in fact slightly longer at 150 μmol m⁻² s⁻¹, probably due to increased growth at the higher PAR. Hypocotyl length in the wild type is identical to that seen in the highest R irradiance (10 μmol m⁻² s⁻¹) in Figure 3.2A. Consequently the inhibition of hypocotyl elongation can be saturated by R alone.

In the hlg-1 mutant, inhibition of hypocotyl elongation is far from saturated at 50 μmol m⁻² s⁻¹. At 150 μmol m⁻² s⁻¹, however, the hypocotyl length is barely distinguishable from that in the wild-type plants. Consequently another photoreceptor appears to be causing hypocotyl inhibition in hlg-1, but requires high fluence W in order to substitute for phyB in this role.

3.2.4 The randomisation of hypocotyl gravitropism by R pulses is triggered less efficiently in the hlg mutants

Wild-type Arabidopsis plants respond to R or FR light by ceasing to grow away from the gravity vector, causing the hypocotyls in the absence of a phototropic B signal to have a randomised appearance (Liscum and Hangarter, 1994, Robson and Smith, 1996). This randomisation is usually measured by determining the standard deviation of the angles of many hypocotyls to the vertical.

When this experiment is done in wild-type N. plumbaginifolia, there appears to be no effect of continuous R on hypocotyl gravitropism, with the hypocotyls being oriented away from the gravity vector as in the dark. This is another example of a photomorphogenic response which is markedly different in N. plumbaginifolia to that in Arabidopsis. However, when the plants are grown under pulsed R of 5 min 10 μmol m⁻² s⁻¹ every 4 h, an effect can be seen, as in Table 3.1. This effect is not as strong, and probably does not share the same cause, as that in Arabidopsis. Nonetheless it is
measurable, and provides another response to R which can be used to investigate mutants. The *pewl* mutant is described in Chapter 5. It is deficient in phytochrome chromophore, and consequently all phytochrome responses in this mutant are attenuated to a great degree. It can be seen from the data given in Table 1 that the randomisation response of wild-type hypocotyls to pulsed R appears to be undetectable in this mutant. Consequently this response is likely to be a phytochrome response, rather than being due to some other effect of the R pulses such as photosynthesis.

The allelic *hlg-1* and *hlg-2* mutants, which are deficient in phyB (Chapter 2) appear to show a much smaller randomisation response to R pulses. There is some noise in the data, causing an apparent difference between the dark and R values for *hlg-1*. However, this serves to demonstrate the smallness of the effect of R pulses in these mutants, which is considerably smaller than that observed in the wild type. Nonetheless, there still appears to be some effect of R in the mutants. This response cannot therefore be wholly attributed to phytochrome B.

3.2.5 The *hlg* mutants show none of the morphogenic aspects of shade-avoidance as adults grown in high R:FR, and show no deficiency in the response to low R:FR

When the rosette-stage *hlg* mutant alleles are grown in continuous high R:FR together with wild-type *N. plumbaginifolia*, as shown in Figure 3.4, both overall height and internode length in the mutants are slightly shorter than the wild-type plants, and petiole length appears identical in the wild type and in the mutants. This is not consistent with the phenotypes of other *phyB* mutants. The cucumber *lh* mutant and the *Brassica ein* mutant both show elongated internodes compared to wild type when grown in high R:FR (López-Juez et al., 1992, Devlin et al., 1992). Although the phenotype of the tomato *tri* mutant is more subtle, it is still somewhat elongated under W (van Tuinen et al., 1995a). Arabidopsis *phyB* mutants show both elongated petioles and more rapid bolt elongation (Robson et al., 1993).

The shade-avoidance response was activated by the replication of the conditions for high R:FR growth, with the addition of FR to achieve low R:FR while
maintaining the PAR, as described by Keiller and Smith (1989). Under these simulated canopy shade conditions, the \textit{hlg} mutants display a similar internode length and overall height to the wild type, and have longer petioles (Figure 3.4). Consequently the response of the plants to R:FR is not diminished, as can be demonstrated in most other \textit{phyB} mutants (e.g. Robson et al., 1993, Devlin et al., 1993). The morphogenic aspects of the response to low R:FR can actually be seen to a greater extent in the mutants than in the wild type, as the mutants are less elongated than the wild type in W, but similar in W+FR, and the petioles of the mutants are similar to those of the wild type in W, but longer in W+FR.

3.2.6 The \textit{hlg} mutations appear to have a small effect on the chlorophyll level and \textit{a:b} ratio in adult \textit{N. plumbaginifolia}.

At the point indicated as Week 10 in Figure 3.4, the plants were harvested, the chlorophyll in a 5mm leaf disc from each of the 10 plants measured, and the mean taken. The top panel of Figure 3.5 shows the total chlorophyll per unit leaf area in these plants. The wild type shows a reduction in the amount of total chlorophyll per unit leaf area under W+FR, hence low R:FR, conditions. The \textit{pewl} mutant, which is deficient in phytochrome chromophore (Chapter 5), shows reduced chlorophyll levels in all conditions. The \textit{hlg} mutants show wild-type levels of chlorophyll under W, but retain slightly more of their chlorophyll under W+FR conditions. These data are based on a small sample (10 plants), and could be due to random variation. However, it is possible that the \textit{hlg} mutants do have an effect on chlorophyll levels in shade-avoiding plants.

The lower panel of Figure 3.5 shows the mean molar ratio of chlorophyll \textit{a} to chlorophyll \textit{b} in the samples extracted, measured spectrophotometrically as described in Chapter 6. The effect of W+FR on the chlorophyll \textit{a:b} ratio is small in the wild-type plants, although this ratio can be altered by shade-avoidance responses (Smith, 1994). The chlorophyll \textit{a:b} ratio in the \textit{pewl} mutant is significantly higher under both treatments. This suggests that incomplete de-etiolation, as seen in the \textit{pewl} mutant, leads to a change in chlorophyll \textit{a:b} ratio, but that shade avoidance does not. The chlorophyll \textit{a:b} ratio in the \textit{hlg} mutants appears to be somewhat higher than that in the
wild type, but this result is unlikely to be significant given the size of the sample. This could indicate that phyB is partly involved in mediating changes in this ratio. However other phytochromes must be involved, as there is a much more pronounced effect in the pewl mutant.

3.2.7 Leaf biomass at harvest is increased in the hlg mutants

The plants described in Figure 3.4 were harvested at the termination of this experiment (shown as Week 10), and the fresh weights of leaves and stem determined. It can be seen from the upper panel in Figure 3.6 that the yield of leaf tissue from the wild-type plants was not significantly affected by growth under W+FR as opposed to that under W. However, the pewl chromophore-deficient mutant (Chapter 5) has a significantly reduced leaf biomass under W, and less than two thirds of this under W+FR. The stress induced by growth under W+FR, which the pewl mutant is clearly able to detect, may have a greater effect on this already stressed mutant. The hlg mutants clearly show an increase in leaf biomass when compared to the wild-type plants, particularly when grown in W. The W+FR yields are reduced, unlike those in the wild type, but still higher than in the wild type. A possible explanation for this is that the late flowering of the mutants caused more assimilate to be diverted to the leaves. In addition the rosette leaf number at bolting of the hlg mutants is higher (Chapter 4). However this cannot entirely explain the result because under W+FR, where the difference in flowering time is minimal, there also appears to be increased leaf biomass in the hlg mutants. This may be explained by the observation that the hlg mutants have more chlorophyll than wild-type plants under W+FR conditions (Figure 3.5).

The stem weight measurements of the wild type show a marked increase under W+FR, due to earlier bolting. This increase is not seen in pewl, where W+FR leads to an overall decrease in mass, and these plants do not go on to flower, but collapse and die. In the hlg mutants, the difference between W and W+FR stem weight measurements is more pronounced than that in the wild type. This may reflect the somewhat greater response to R:FR in these mutants mentioned elsewhere in this chapter. It may also partly explain the greater leaf biomass in these mutants in W, as
less energy has thus far been committed to the bolt than in the wild type. However, under W+FR conditions the mutants display a slightly greater yield in both stem and leaf weight. The most plausible explanation for this is that the later bolting of the mutants leads to later senescence, hence more chlorophyll is present over more of the growth period, allowing higher total photosynthesis.

3.2.8 The early flowering characteristic of the shade-avoidance response is intact in the *hlg* mutants

When the mutants are grown in high R:FR W with a 16h photoperiod, as shown in Figure 3.7, they are observed to be slightly later in bolting and flowering than the wild-type plants. This temporally late flowering phenotype has been confirmed as being also at a later developmental stage, by counting the rosette leaf number at bolting under both long and short days of high R:FR (Chapter 4). This difference is lost when FR is added to reduce the R:FR, and both the mutant alleles and the wild-type plants are substantially earlier to bolt and accelerated in bolt growth under a lower R:FR (Figure 3.7A). This result is confirmed by the number of days to first-bud opening shown in Figure 3.7B, which again demonstrates that the time of flowering is brought forward in the mutants in response to simulated canopy shade conditions, to a similar degree as in the wild type. The aspects of the shade-avoidance response which affect the timing of bolting and flowering are hence intact in the mutants. Indeed, because *hlg-1* and *hlg-2* show delayed flowering under high R:FR, but flower at the same time as the wild type under low R:FR, the acceleration of flowering in the mutants by simulated canopy shade is greater than that in the wild type. This slightly exaggerated shade-avoidance response is comparable to that seen in petiole elongation (Figure 3.4) and stem:leaf partitioning (Figure 3.6). The slight discrepancy between the time of bolting and flowering between the mutant alleles can be explained by the relative rapidity of the germination of many *hlg-2* seed stocks (Chapter 2).
3.3 Discussion

The \textit{hlg} mutant phenotype at the seedling level correlates well with most other known and putative phyB-deficient mutants (Smith, 1995). It displays a specific deficiency in the R responses, while retaining some responsiveness to R and having a wild-type phenotype in other wavelengths (Figures 3.1 and 3.2). It follows that what is thought to be the major role of phyB in dicotyledonous seedlings, de-etiolation in response to R, appears to be highly conserved among most species in which \textit{phyB} mutants have been generated. However, the tomato \textit{tri} mutant displays only a temporary insensitivity to R as a seedling. This is the mutant most analogous to the \textit{hlg} mutants as an adult plant, as the R:FR ratio effects are close to normal (van Tuinen et al., 1995a). When full-length sequences of the tomato \textit{PHYB-1} and \textit{PHYB-2} genes are considered, the gene sequenced in chapter 2 shares more homology with \textit{PHYB-1} (R.E. Kendrick, Wageningen University, personal communication). It is therefore likely that a conserved role for this B-type (or B1) phytochrome in the solanaceae is R perception early in development.

The gravitropic responses of \textit{N. plumbaginifolia} seedlings (Table 1) are clearly different from those observed in Arabidopsis (Liscum and Hangarter, 1994). Rather than an obvious and sustained loss of negative gravitropism induced by light, they appear to have a partial loss of negative gravitropism specific to pulsed R conditions. This may be relatively readily explained by recourse to the presumed role of starch granules in gravity perception. A phytochrome triggered response to photosynthetically negligible R pulses may induce the breakdown of starch, inducing a partial loss of gravitropic stimuli. Sustained irradiation with high fluence rate R will allow significant photosynthesis to occur, and the lost starch to be replaced, restoring the gravitropic response.

The phenotype of the \textit{hlg} mutants as adults is inconsistent with the phenotypes of all other known or suspected mutants deficient in phyB (Kendrick and Kronenberg, 1994, Smith, 1995). An obvious characteristic of the Arabidopsis and \textit{Sorghum} phyB mutants is that they are early flowering. This is not seen in the \textit{hlg} mutant alleles, which are late flowering (Figure 3.7 A, B, Chapter 4). This may reflect differences in the photoperiodic flowering responses of these species, rather than being directly attributable to the early-flowering shade-avoidance response. Unlike phyB-deficient
mutants of Arabidopsis, Brassica and tomato (Robson et al., 1993, Devlin et al., 1992, van Tuinen et al., 1995a) the hlg mutants do not display a lessened response of flowering time to changes in R:FR (Figure 3.7).

The shade-avoidance response is usually characterised by early bolting and flowering, elongation of internodes and petioles, and an increase in overall elongation rate during plant growth (Smith, 1994). The phenotype of the previously characterised phyB-deficient mutants shows a tendency, not seen in the wild types, to be similar in high R:FR to that in low R:FR. This means that many phyB-deficient mutants, notably ein and lh, display a highly elongated growth habit in normal growth conditions. Tomato tri mutants are less obviously elongated, but still noticeably so. Responses to changes in R:FR are still present in all of these mutants, but the difference in phenotype between plants grown in high R:FR and low R:FR is smaller than that in the wild type of each species (Whitelam and Smith, 1991, Smith et al., 1992, Robson et al., 1993, Devlin et al., 1992, van Tuinen et al., 1995). The hlg mutants do not have an elongated growth habit, and appear to have responses to R:FR which are, if anything, more sensitive than those of the wild-type plants.

The hlg mutation appears to have the effect of increasing the yield of leaf tissue by comparison to wild-type plants grown under the same conditions (Figure 3.6A). Rather than being due to effects on shade-avoidance, this effect is likely to be attributable to the later flowering of the mutants. This attribute of the mutants means they have more rosette leaves before they bolt (Chapter 4), less assimilate is diverted to bolt production (Figure 3.6B), and chlorophyll levels are maintained at a higher level for longer (Figure 3.5A). Whatever the mechanism of this effect, it may be a means of increasing yields in crops where delaying bolting is desirable or acceptable.

The results presented here (Figures 3.1B, 3.4 and 3.7) show that, in N. plumbaginifolia, phyB is not responsible for R:FR perception, unless such responses are mediated by another phyB-like phytochrome present in this species. However the phyB in which these alleles are deficient is important in mediating the response of the seedling to R, and is analogous in this respect, and in its reactivity to mAT-1, to the mutated photoreceptors in previously described phyB-deficient mutants. Qualitative observations suggest that, unlike Arabidopsis, wild-type adult N. plumbaginifolia does not display obvious elongation when grown under low PAR W. It is clear from the
data in Figure 3.3 that the inhibition of hypocotyl elongation by W in the wild type is saturated at a PAR level which is very low compared to most field conditions, and that the absence of phyB only has a noticeable effect at fluences below 150 μmol m\(^{-2}\) s\(^{-1}\). Consequently the shade-avoidance responses in the hlg mutants grown in PAR levels sufficient to sustain healthy growth may not be masked by constitutive elongation. Such constitutive elongation in other phyB-deficient mutants may be caused by the loss of R fluence rate perception. While the overexpression of phyB in Arabidopsis gives rise to plants which have a reduced sensitivity to small changes in R:FR (McCormac et al., 1993) these transgenic plants were shown to be much more sensitive to changes in R fluence rate. We can therefore propose that the role of phyB is limited to the perception of R irradiance, and that its primary function may be to sustain de-etiolation under conditions where PAR is low but R:FR is high. Such conditions could conceivably be caused by weather conditions or seasonal changes rather than canopy shade. A plant which responded to such conditions by elongation would be disadvantaged. Canopy shade may produce low R:FR combined with relatively high R fluences, as is the case in the W+FR cabinets used in this experiment. Under these conditions it is clear that the phyB discussed here is not the photoreceptor which perceives R:FR in *N. plumbaginifolia*. By inference, this may also be the case in other species (Smith and Whitelam, 1997).
Table 3.1: Standard deviations of hypocotyl angles from the vertical in the wild type, *hlg* and *pew* mutants grown under continuous R (10 μmol m$^{-2}$ s$^{-1}$), 5 min pulses of R (10 μmol m$^{-2}$ s$^{-1}$) every 4 h, or in darkness.

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th><em>pew</em></th>
<th><em>hlg-1</em></th>
<th><em>hlg-2</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>11</td>
<td>14</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>n</td>
<td>104</td>
<td>46</td>
<td>113</td>
<td>103</td>
</tr>
<tr>
<td>Red pulses</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>29</td>
<td>15</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>n</td>
<td>166</td>
<td>96</td>
<td>165</td>
<td>157</td>
</tr>
<tr>
<td>Cont. Red</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>10</td>
<td>14</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>n</td>
<td>118</td>
<td>110</td>
<td>127</td>
<td>112</td>
</tr>
</tbody>
</table>
Figure 3.1 (A). Seedling morphology of the wild type and the *hlg*-1 and *hlg*-2 mutants under different light conditions.

Seedlings were grown under fluorescent W, monochromatic B, R or FR, or in the dark for 7 days (see Experimental Procedures). The long hypocotyl phenotype of the mutant alleles is clearly visible in R, and visible to some extent in W. The mutants show a wild-type response to B and FR.

(B). Phenotype of the *hlg* mutants and the wild type grown in W for 60 days post germination.

The plants shown were grown in a greenhouse at high PAR and R:FR for 60 days before the roots were washed and the plants photographed. The mutants are not elongated, and display a more branched root system than the wild-type plants.
CHAPTER 3

PHYSIOLOGY OF THE *hlg* MUTANT

A) 

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>hlg-1</th>
<th>hlg-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

10mm

B) 

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>hlg-1</th>
<th>hlg-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

50mm
Figure 3.2: Hypocotyl length of the wild-type *N. plumbaginifolia* and hlg mutants under variable fluence rates of (A) R and (B) FR.

Seedlings were grown under monochromatic R or FR for 7 days under the fluence rates indicated on the x-axis. The axis before the break shows the dark control; the axis after the break is a logarithmic scale. The wild-type plants are represented by circles, hlg-1 by squares, and hlg-2 by triangles. The increased length of the hypocotyls of hlg-2 under all conditions is due to the earlier germination of this mutant. Error bars represent ± the standard error of the mean. At least 30 hypocotyls were measured per point.
Figure 3.3: Hypocotyl length of the wild-type *N. plumbaginifolia* and *hlg-1* mutant under variable PAR fluorescent W.

Seedlings were grown under fluorescent W for 7 days under the PAR indicated on the x-axis. The wild-type plants are represented by circles, and *hlg-1* by squares. Error bars represent ± the standard error of the mean. At least 30 hypocotyls were measured per point.
Figure 3.4: Changes in morphology of the wild type *N. plumbaginifolia* and *hlg* mutants in response to altered red/far red ratio (R:FR).

The following determinants of shade avoidance were measured: (A) Overall height, (B) First internode length, (C) Second internode length, (D) Petiole length. The above were measured for wild type *N. plumbaginifolia* (circles), the *hlg*-1 mutant (squares), and the *hlg*-2 mutant (triangles), grown in continuous W of high R:FR (filled symbols) or continuous W plus supplementary FR, giving low R:FR (open symbols). Plants were all grown in identical high R:FR conditions for 6 weeks until transfer to different R:FR cabinets (indicated as Week 6). Measurements are the mean of ten individuals and error bars represent the standard error of the mean.
Figure 3.5: (A). Graph to show levels of total chlorophyll per unit area in wild type *N. plumbaginifolia* and hlg mutants in response to altered red/far red ratio (R:FR).

(B). Molar ratio of chlorophyll a to chlorophyll b in plants under the same conditions. Unfilled bars represent high R:FR W conditions, filled bars represent low R:FR W+FR. Chlorophyll a and b content of 5mm leaf discs was measured spectrophotometrically in plants grown in the conditions described for Figure 3.4 at the age of 10 weeks. Error bars represent the standard error of the mean for 10 individuals.
CHAPTER 3

PHYSIOLOGY OF THE hlg MUTANT

Figure 3.6: (A). Graph to show leaf biomass of wild-type *N. plumbaginifolia* and hlg mutants in response to altered red/far red ratio (R:FR).

(B). Stem biomass of plants under the same conditions. Unfilled bars represent high R:FR W conditions, filled bars represent low R:FR W+FR. Stem and leaf fresh weights were determined in plants grown in the conditions described for Figure 3.4 at the age of 10 weeks. Error bars represent the standard error of the mean for 10 individuals.
Figure 3.7: (A). Changes in the time of bolting in response to altered red-far red ratio (R:FR) in the wild-type *N. plumbaginifolia* and *hlg* mutants.

The overall height of wild type *N. plumbaginifolia* (circles), the *hlg-1* mutant (squares), and the *hlg-2* mutant (triangles), grown in continuous W of high R:FR (filled symbols) or continuous W plus supplementary FR, giving low R:FR (open symbols) was measured in plants grown in a 16h photoperiod. Plants were all grown in identical high R:FR conditions for 42 days until transfer to different R:FR cabinets. Measurements are the mean of ten individuals and error bars represent the standard error of the mean.

(B). Time of flowering in response to altered red/far red ratio (R:FR) in the wild type *N. plumbaginifolia* and *hlg* mutants.

Flowering time was defined as the day when the first flower bud opened. The mean day of first flower bud opening in the wild type (unfilled bars), the *hlg-1* mutant (grey bars) and the *hlg-2* mutant (black bars) was measured in plants grown under the conditions described above. Measurements are the mean of ten individuals and and error bars represent the standard error of the mean.
Chapter Four

Analysis of photoperiodism and endogenous rhythmicity in *N. plumbaginifolia*: the role of phytochrome B.
4.1 Introduction

Most organisms amenable to investigation, including representatives of the plants, animals and prokaryotes, exhibit some form of endogenous rhythm. Since the day/night cycles of life on Earth are a dominant force in determining the priorities of existence for most organisms, the biological or circadian clocks of most organisms have become adjusted to run with a period of roughly 24 h. However, the input of a photoreceptor is necessary in order to synchronise this clock with the environmental light/dark cycle (Dunlap, 1996). In plants, it has been known for some time that the red/far red reversible photoreceptor family, the phytochromes, are involved in this process of entrainment (Simon et al., 1976).

The most obvious, and most economically important aspect of endogenous rhythmicity in plants is the control of the time of flowering in response to photoperiod (Vince-Prue, 1974). The input of photoreceptors in this process must occur at at least two points. The endogenous oscillator must be entrained to run concurrently with the diurnal cycle, and the duration of the photoperiod and/or the dark period must be perceived by a photoreceptor with reference to this oscillator. The phytochrome photoreceptor family is again involved in this process; in fact the perception of night-breaks and their effect on flowering time was one of the first phytochrome responses characterised (Borthwick et al., 1952b). That the entrainment and floral induction responses are separate, and controlled by phytochrome(s), has been demonstrated (Lumsden and Furuya, 1986, Lumsden, 1991).

The role of phytochromes in the perception of daylength has been studied in greater detail more recently, thanks to the availability of mutant and transgenic plants with altered phytochrome levels. Phytochrome A (phyA) has been implicated in the perception of daylength in Arabidopsis, a long day plant, as demonstrated by a deficiency in day-extension perception in the Arabidopsis phyA mutant (Johnson et al., 1994, Bagnall et al., 1995). The Arabidopsis phyB mutant is early flowering under all conditions, ascribed to loss of R:FR perception (Goto et al., 1991). The shade-avoidance response can affect flowering time, and this effect is usually daylength insensitive (Chapters 1 and 2). However the Arabidopsis phyB mutant is also affected in its perception of daylength (Bagnall et al., 1995). Current opinion is that multiple
phytochromes control the perception of daylength and timing of flowering in Arabidopsis (Peeters and Koornneef, 1996).

The overexpression of phyA from Avena and phytochrome B (phyB) from Arabidopsis in day-neutral (DN) and short-day (SD) tobacco can both cause increased sensitivity to night breaks (Halliday, 1996). However, the ablation of PHYB transcript in potatoes requiring SD for tuberisation renders tuberisation constitutive (Jackson et al., 1996). Consequently it may be that the native solanaceous phyB has a major role in the induction of SD dependent responses, despite the fact that monocot phyA can also act in this manner when transgenically overexpressed (Halliday, 1996).

The role of phytochrome in the entrainment of the circadian clock has also proved resistant to simple characterisation. Although this effect was shown to be inducible as a phytochrome-mediated low-fluence response (Simon et al., 1976), it has also been shown to be modulated by the very low fluence VLFR response mode of phytochrome (Nagy et al., 1993). The oscillation of CAB promoter activity and transcript levels has provided a means of studying the endogenous oscillator of plants from a molecular perspective. However, single phytochrome mutants appear to entrain this oscillation normally, and only the hyl mutant, which is deficient in phytochrome chromophore (Parks and Quail, 1991), displays the longer oscillation period expected from an entrainment deficient mutant. However it has been shown that blue-light receptors must also play a role in entrainment in this mutant (Millar and Kay, 1997).

Possibly the easiest manifestation of the endogenous rhythm in plants to observe is the nyctinastic or sleep movement of leaves. Despite being known for some time (Büning and Stern, 1930), these movements are still important in tying together observations of oscillations at the molecular level with plant physiology (Carré, 1996). They are present in many species and represent a method of studying endogenous rhythms which is entirely non-invasive.

The results presented here show that leaf movements display a free-running, endogenous rhythm in N. plumbaginifolia. They also demonstrate that the input of photoreceptors into the control of such leaf movements may be more complex than previously thought.
4.2 Results

4.2.1 The \textit{hlg} mutants flower at a later developmental stage than the wild type under LD, and show a reduced response to SD

The \textit{hlg} mutants show no obvious phenotype at the vegetative rosette stage, as demonstrated in Chapter 3. However, observation of plants at the point of bolting indicated that \textit{hlg} mutants bolt later than the wild type, and develop more rosette leaves before doing so. This leads to the \textit{hlg} mutants forming the abnormally bushy rosettes seen in Figure 4.1, which also shows the retarded bolt development caused by the late-flowering phenotype. This phenotype explains the greater leaf biomass and abnormal chlorophyll levels found in the mutants, as discussed in Chapter 3.

Table 4.1 shows the mean leaf number at bolting of plants grown under 18h daylength confirms the late flowering phenotype of the \textit{hlg} mutants. The wild-type plants bolt with a mean 14 leaves (to 2 s.f.), while the \textit{hlg} mutants bolt having developed a mean 16 leaves. This is inconsistent with the behaviour of the \textit{phyB} mutant in Arabidopsis, which bolts with fewer rosette leaves than the wild type under LD (Goto et al., 1991, Bagnall et al., 1995).

Despite being for all practical purposes DN, wild-type \textit{N. plumbaginifolia} shows a small response of bolting time to growth under SD conditions. The flowering response is delayed by SD by the developmental time equivalent of two rosette leaves (Table 1). This is equivalent to the extent to which flowering is delayed in the \textit{hlg} mutants with respect to the wild type under LD (Table 1). The response of the \textit{hlg-1} mutant to SD in terms of flowering time is negligible (due to growth space limitations, this experiment could not also be performed with \textit{hlg-2}) (Table 1). It follows that the response of flowering time to photoperiod in \textit{N. plumbaginifolia} appears to be eliminated in the absence of phytochrome B.
4.2.2 Leaf movement in *N. plumbaginifolia* maintains a free-running rhythm. This movement displays an altered amplitude in the *hlg-1* mutant after a 12 h light (L)/12 h dark (D) entrainment.

The movement of leaves in plants grown under continuous illumination can be followed by time-lapse or timed-interval photography (See Materials and Methods Chapter 7). Rosette stage plants were assayed in this way for endogenous leaf-movement rhythms after entrainment for two weeks in 12 h fluorescent light periods in a 24 h cycle. The results are presented in Figure 4.2. It can be seen that the leaves of the wild-type plants do display a slight oscillation in their angle, which seems to be damped relatively rapidly under continuous illumination. The leaves of the *hlg-1* mutant, however, display a strong rhythmic oscillation in angle, with a period of between 24 and 26 h. This effect explains the previous failure to measure leaf angle differences between the mutant and the wild type on a daily basis, despite qualitative observations that there was a clear difference.

4.2.3 Rhythmic leaf-angle movements are distinct from the acute alteration in leaf angle in response to R:FR

Leaf movement rhythms were also examined in the wild type and *hlg* mutant *N. plumbaginifolia* entrained as described above, but under a 12 h light period of low red:far red ratio (R:FR). The shade-avoidance response (Smith, 1995; Smith and Whitelam, 1997) can induce leaf angle alterations in response to lowered R:FR. Since some phyB mutants display altered sensitivity to R:FR, and show some aspects of shade avoidance under high R:FR conditions (Smith and Whitelam, 1997), it is feasible that the altered leaf angle phenotype observed in Figure 4.2 is caused by shade-avoidance rather than effects on endogenous rhythms. However, when the leaf angles of plants entrained under low R:FR cycles are monitored, no rhythmic oscillation is displayed, and the mutants and the wild-type plants behave indistinguishably (Figure 4.3). The mean leaf-angle to the horizontal of plants entrained under W + FR is much higher than that of those entrained under W (Figures 4.2 and 4.3). It seems likely, therefore, that the shade-avoidance response of leaf angle
overwhelms rhythmic movements, which are no longer seen in plants entrained under these conditions.

4.2.4 The free-running oscillation of leaf angle is induced in the wild-type by SD entrainment conditions

Wild-type plants entrained under fluorescent light periods of 8 hours, with a subsequent 16 h dark period, show a marked free-running oscillation in leaf angle (Figure 4.4). This is in contrast to the minimal leaf movement observed when the wild-type plants are entrained under 12h light/12h dark conditions (Section 4.2.2). It is therefore likely that the duration of the light period has an effect on the amplitude of leaf movements in the wild-type. This is more likely to be caused by photoperiodism than the lower PAR under SD, as the leaf movement rhythm is not affected by shade-avoidance (Section 4.2.3). In contrast to the situation in plants entrained in 12h L/12h D conditions (Figure 4.2) the hlg-1 mutant and wild-type plants display an identical oscillation (although the mutant has a slightly higher mean leaf angle) when entrained under an 8h light period (Figure 4.4). There is consequently a response of leaf movement to light period duration in the wild-type plants which is absent in the hlg-1 mutant.

4.2.5 Low-fluence fluorescent day-extensions cause loss of detectable leaf-movement in the wild-type plants but not in the hlg-1 mutant

The response of LD plants such as Arabidopsis to daylength can be investigated by means of growth in SD, with the light period supplemented by a daylength extension of photosynthetically insignificant fluence rate. By this means, the responses mediated by various photoreceptors can be defined without the complication of the effect of PAR levels on flowering time (see Goto et al., 1991, Halliday et al., 1994, Johnson et al., 1994, Bagnall et al., 1995). To investigate the possibility that the lack of response of leaf movement to the length of the light period in the hlg-1 mutant is only an indirect consequence of the absence of phyB, the response of leaf movement to day-extensions was investigated. The mutant and wild type were entrained under
8 h fluorescent light periods as before (Figure 4.4) except that the high fluence rate (120 μmol m\(^{-2}\) s\(^{-1}\)) 8h light period was followed by a low fluence rate (2 μmol m\(^{-2}\) s\(^{-1}\)) day extension, also of 8 h duration. The movement of the leaves was then followed under continuous fluorescent light as before. The results are presented in Figure 4.5.

It can be seen that just as in Figure 4.2, where the plants were entrained under a 12h light period, there is a clear difference in leaf angle oscillation between the mutant and the wild type in Figure 4.5. The wild type has an unmeasurable oscillation whereas that in the mutant is quite clear. However, there seems to be another effect of low-fluence-rate daylength extensions during the entrainment, which is equivalent to that of entrainment under W+FR conditions (Figure 4.3) except not as strong. The low-fluence-rate day-extensions seem to cause an overall increase in leaf angle in both the mutant and the wild-type, which reduces the amplitude of oscillations in the mutant relative to those under SD entrainment (Figure 4.4). However, this does not alter the conclusion that the sensitivity of leaf-angle oscillation to day-extensions appears to be mediated by phytochrome B.

4.3 Discussion

The wild-type *Nicotiana plumbaginifolia* (Viv.) used in these experiments is for practical purposes day-neutral. However, when examined sufficiently closely, many apparently day-neutral plants respond to photoperiod to some extent. While the flowering response may be unaffected by daylength in many species, responses such as cold acclimation, changes in bud dormancy and tuberisation may be induced by changes in photoperiod, particularly by SD (Vince-Prue, 1994). Also, slight alteration in the internode number or leaf number at bolting or flowering may be observed in plants which are outwardly day neutral (e.g. DN tobacco varieties as described by Halliday, 1996).

Such sensitivity to photoperiod is apparent in the wild-type *N. plumbaginifolia*. The flowering time, in terms of rosette leaf number at bolting, is slightly delayed under SD (Section 4.2.1). While no day-extension or night-break analysis was performed to confirm that this was not a photosynthetic effect, the PAR level given to the plants under SD was sufficient to make this unlikely. The cabinets necessary for
these growth conditions unfortunately could not deliver low-fluence rate day-extensions.

The hlg mutants appear to bolt after forming more leaves than the wild-type plants under LD (Section 4.2.1). This is a highly unusual phenotype for phyB mutants, which are usually early flowering (See chapters 1 and 3). It may be that phyB mutants in a DN background do not show early flowering, as the tomato tri mutant has no obvious flowering phenotype (based on qualitative observations). It may, however, be the case that solanaceous phyB plays a rather different role to that in Arabidopsis, Sorghum and other plants studied. The bolting of the hlg mutant plants was not significantly delayed under SD conditions compared to LD conditions, unlike that of the wild-type plants. This may indicate that the slightly later bolting of the wild type in SD is due to the absence of a LD signal of phyB Pfr. This would be at odds with observations of the flowering responses in photomorphogenic mutants of Arabidopsis, where phyB mutants were found to be early flowering in SD, rather than late in LD (Bagnall et al., 1995). These experiments also showed a retention of photoperiodic sensitivity in the Arabidopsis phyB mutant. Transgenic DN and SD tobacco overexpressing Arabidopsis phyB show delayed flowering (Halliday, 1996); however overexpression would not necessarily produce the opposite phenotype to a mutation in this context. It seems probable, therefore, that the mechanisms of photoperiodic perception are qualitatively different in Arabidopsis and N. plumbaginifolia.

The angle at which N. plumbaginifolia leaves are held is controlled by a free-running endogenous rhythm. This rhythm is visible when the wild-type plants are conditioned by entrainment under short (8h) days, but not when the entrainment conditions involve a 12h light period or day-extensions (see Results section). There is no evidence that the entrainment of the circadian oscillator itself is affected by the alteration in photoperiod, rather that the coupling of this rhythm to the movement of the leaves only occurs in SD. It therefore appears that this response is dependent on photoperiod, in the same manner as that of the flowering response of a SD plant, or the SD-dependent tuberisation response of Solanum andigena (Jackson et al., 1996).

The phytochrome-B deficient hlg-1 mutant appears to lack sensitivity of the leaf movement rhythm to photoperiod, which is observed in the wild-type plants. The
leaf oscillations of hlg-1 appear analogous to those which occur in the wild type entrained under SD. The most likely interpretation is that the condition of the wild-type plants under SD entrainment is the default pathway, and that the measurement of photoperiod is somehow disrupted in the mutant. Such a measurement of photoperiod can only occur by means of the interaction of a photoreceptor with an entrained endogenous oscillator. The oscillator is, in turn, dependent on a photoreceptor to entrain it, in synchrony with the environmental light/dark cycle. In the absence of phyB, the leaf movements of *N. plumbaginifolia* are synchronised with each other and with the light/dark cycle, but are not affected by the length of the light period. Consequently, in the hlg mutant, the oscillator is normally entrained by the photoperiod. This implies that another photoreceptor is responsible for the entrainment of this cycle, but that phyB is required in order for the endogenous oscillator to be used to determine daylength.

![Figure 4.6: The role of phyB in photoperiodic adjustment of *N. plumbaginifolia* leaf movement.](image)

The SD-dependent tuberisation response in *Solanum andigena* becomes constitutive when antisense RNA is used to reduce the level of native phyB (Jackson et al., 1996). The tuberisation response in *Solanum* may be directly analogous to the control of leaf movement amplitude in *N. plumbaginifolia*, and since the two species are closely related, may share an evolutionary origin. The later flowering of the wild-type *N. plumbaginifolia* in response to SD also becomes constitutive in the phyB mutant background (Section 4.2.1). It is therefore shown that the flowering response and the effect of photoperiod on leaf movement share a dependence on phyB to
perceive daylength. The mechanism of these two responses could consequently be the same. If this is the case, then the oscillation which allows the perception of daylength by phyB could be that directly visible in the leaf movements of the *hlg* mutants. This movement consequently affords many opportunities for further study of the mechanisms by which plants perceive daylength. The mutagenesis of *phyB* null backgrounds in *N. plumbaginifolia* would allow genetic screening to be performed for mutants which affect this oscillator in the leaves directly, rather than indirectly via photoreceptors. It may also be possible to analyse such responses in a more genetically tractable species in future.

It is possible to speculate about the benefits to the plant which may be conferred by SD induced leaf movement. These may include protection of the meristem from low temperatures, the capture of low-angle light or a role in senescence or cold-adaptation. Whatever the reason why such leaf movements may exist, however, they provide a readily measured response which must be controlled by a circadian oscillator. The presence of this oscillation in the leaves, where the process of photoperiodic sensitivity to flowering is known to occur (Vince-Prue, 1994) provides circumstantial evidence for its relation to the oscillator controlling the flowering response.
Table 4.1: The number of rosette leaves at bolting of *N. plumbaginifolia* wild type and *hlg* mutants grown under different photoperiods of simulated daylight. Photoperiods were either 8 h of light followed by 16 h of darkness (SD) or 18 h of light followed by 6 h of darkness (LD). Light was provided by daylight supplemented by HQI metal halide bulbs, or HQI metal halide bulbs alone. The PAR was at least 300 μmol m$^{-2}$ s$^{-1}$ throughout the photoperiod.

<table>
<thead>
<tr>
<th></th>
<th>wt (± SE)</th>
<th><em>hlg</em>-1 (± SE)</th>
<th><em>hlg</em>-2 (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaf No. at bolting, LD (± SE)</strong></td>
<td>13.8 (±0.20), n=29</td>
<td>16.1 (±0.24), n=30</td>
<td>16.5 (±0.22), n=28</td>
</tr>
<tr>
<td><strong>Leaf No. at bolting, SD (± SE)</strong></td>
<td>15.8 (±0.47), n=20</td>
<td>16.9 (±0.19), n=18</td>
<td>not done</td>
</tr>
</tbody>
</table>
Figure 4.1: Phenotype of *N. plumbaginifolia* wild type and *hlg* mutants at 9 weeks after germination, grown in high R:FR W with an 18 h light period.

Plants were typical of a large population, and show the increased leaf number and late bolting characteristic of the *hlg* mutants.
Figure 4.2: Changes in leaf angle to the horizontal over a 72 h period in *N. plumbaginifolia* wild type and the *hlg-1* mutant entrained in a 12 h light period.

Plants were 5-6 weeks old and entrained for at least two weeks under 12 h fluorescent W/ 12 h dark cycles. Measurements were conducted under constant fluorescent illumination. Each data point represents the mean of at least 20 leaf angles, from at least 10 individuals. Error bars represent the standard error of the mean.
Figure 4.3: Changes in leaf angle to the horizontal over a 72 h period in *N. plumbaginifolia* wild type and the *hlg-1* mutant entrained in a 12 h light period of low R:FR.

Plants were 5-6 weeks old and entrained for at least two weeks under 12 h fluorescent W plus additional FR (R:FR 0.1)/ 12h dark cycles. Measurements were conducted under constant fluorescent illumination. Each data point represents the mean of at least 20 leaf angles, from at least 10 individuals. Error bars represent the standard error of the mean.
Figure 4.4: Changes in leaf angle to the horizontal over a 72 h period in *N. plumbaginifolia* wild type and the *hlg-1* mutant entrained in an 8 h light period.

Plants were 5-6 weeks old and entrained for at least 2 weeks under 8 h fluorescent W/16 h dark cycles. Measurements were conducted under constant fluorescent illumination. Each data point represents the mean of at least 20 leaf angles, from at least 10 individuals. Error bars represent the standard error of the mean.
Figure 4.5: Changes in leaf angle to the horizontal over a 72 hour period in N. plumbaginifolia wild type and the hlg-1 mutant entrained in a 16 h light period, consisting of 8 h high PAR and an 8 h low PAR daylength extension.

Plants were 5-6 weeks old and entrained for at least 2 weeks under 8 h fluorescent W (150 µmol m$^{-2}$ s$^{-1}$) 8 h day-extension (1.5 µmol m$^{-2}$ s$^{-1}$) / 8 h dark cycles. Measurements were conducted under constant fluorescent illumination. Each data point represents the mean of at least 20 leaf angles, from at least 10 individuals. Error bars represent the standard error of the mean.
Chapter Five

Molecular and physiological analysis of the partially etiolated in white light (pew) mutants of *Nicotiana plumbaginifolia.*
5.1 Introduction

Phytochromes are involved in the regulation of many processes in plant development (see Chapter 1). Before the existence of multiple phytochromes was generally accepted, it was therefore expected that a phytochrome mutant would have a severely compromised phenotype, if indeed such mutants were viable at all. The expected phenotype of such a mutant would include failure to de-etiolate fully, low levels of chlorophyll in the light grown plant, and a severely elongated morphology. Consequently, the *aurea* mutant of tomato, along with the *yg-2* mutant which has a very similar phenotype, were widely thought to be phytochrome mutants, as they fitted closely with the expected characteristics of such mutants. The *aurea* locus has been used as a genetic marker in tomato for many years, but was relatively recently characterised as lacking in spectrophotometrically detectable phytochrome (Koomneef et al., 1985). The subsequent demonstrations that *aurea* was deficient in immunochemically detectable phyA (Parks et al., 1987, Sharrock et al., 1988) proved misleading, as the *fri* mutant of tomato, which almost certainly has a mutation in the *PHYA* gene, has a much more subtle phenotype (Van Tuinen et al., 1995b). Recently, Terry and Kendrick (1996) demonstrated that both *aurea* and *yg-2* are unable to synthesise phytochrome chromophore. This strongly suggests that these loci encode enzymes involved in the committed steps of phytochromobilin (PΦB) synthesis.

These observations fit neatly with those of Parks and Quail (1991) on the *hyl* and *hy2* mutants of Arabidopsis (Koomneef et al., 1980). They showed that the PΦB precursor biliverdin could rescue *hyl* (totally) and *hy2* (partially). The phenotypes of *hyl* and *hy2* are broadly similar to those of both *aurea* and *yg-2*. The *pcdl* and *pcd2* mutants of pea have also been shown to be deficient in PΦB synthesis (Weller et al., 1996, 1997). It appears from this information as if all these mutants fall into two classes; those unable to synthesise biliverdin IXα (*hyl, yg-2* and *pcdl*) and those deficient in the capacity to reduce biliverdin to PΦB (*hy2, aurea* and *pcd2*). Apart from the report of *hy6* in Arabidopsis (Chory et al., 1989), which has been the subject of some controversy as to whether this is, in fact, a separate locus (Terry, 1997), only two such mutant loci have thus far been identified in any one plant species.
The primary characteristic of all PΦB synthesis mutants identified so far is a severe lack of chlorophyll, causing an obvious yellow colouration (hence the name of *aurea*). Another sign of a chromophore mutation is a severe reduction in the sensitivity of seedlings to R (primarily mediated by phyB) and FR (primarily mediated by phyA) (Terry, 1997). Selective mutations in the structural genes for these phytochromes cause specific losses in the response to R or FR (McCormac et al., 1993). Consequently, the loss of both responses from a monogenic mutation is characteristic of a mutation affecting PΦB, the common chromophore of phyA and phyB. Another characteristic of chromophore mutants is the presence of immunodetectable phytochrome apoprotein (which is sometimes present at wild-type levels), but sharply reduced levels of spectrophotometrically detectable phytochrome (Koornneef et al., 1985, Parks et al., 1987, Sharrock et al., 1988, Parks and Quail, 1991).

The work of Kraepiel et al. (1994a) in the isolation of two *pew* loci in *Nicotiana plumbaginifolia*, appears, despite the conclusions drawn in that paper, to have provided two new such chromophore biosynthesis mutants, *pew1* and *pew2* (Kraepiel, 1994). The *pew* mutants were isolated from an EMS mutagenised M2 population of *N. plumbaginifolia* line Pbh1d. They displayed a partially etiolated phenotype in W, including pale cotyledons and long hypocotyls relative to the wild-type plants. Now that it is known that none of the phytochrome apoprotein mutants isolated to date display the pale, *aurea* like phenotype displayed by *pew1* and *pew2*, it seems likely that these are both chromophore deficient mutants. The explanation given for believing that *pew2* carried a mutation in the *PHYA* gene is no longer valid, now it is known that *phyA* mutants usually show little phenotype under W or R (Whitelam et al., 1993, van Tuinen et al., 1995b).

In addition, a third chromophore-type mutant, *pew3*, was isolated at INRA by Kraepiel and co-workers (Kraepiel, 1994). Consequently, three separate recessive mutants causing extensive loss of light responses are available in this species. A third class of chromophore deficient mutant has been predicted, based on the possible existence of a PΦB isomerase (Terry, 1997). If *pew3* represents a third chromophore biosynthesis locus, this would allow extensive analysis of the genetics of phytochrome chromophore biosynthesis in *Nicotiana plumbaginifolia*, since it would be the only
species in which a third such mutant was available. Data presented in this chapter will indicate that all three \textit{pew} mutants are deficient in chromophore biosynthesis, and that \textit{pew3} may represent a previously undescribed class of chromophore mutant.

5.2 Results

5.2.1 All three \textit{pew} mutants are deficient in the responses to red and far red light.

The mutants \textit{pew1}, \textit{pew2} and \textit{pew3} were grown in a range of fluence rates of R and FR, each provided by an LED light source (See Chapter 7). The hypocotyl lengths of each genotype and the wild-type plants were recorded after 7 days growth. The results are presented in Figure 5.1. It can be clearly seen that all three mutants display a substantially reduced response to both R and FR. While some of the mutants appear to respond slightly to FR, this effect may be due to a delay in germination caused by the growth of these phytochrome-deficient mutants in FR. It may also, however, represent 'leakiness' of the mutant alleles, something which is well documented in phytochrome chromophore biosynthetic mutants (Terry, 1997). Indeed, without some leakiness these mutants would probably be very difficult to grow, and the \textit{pew1 pew2} double mutant has been shown to be lethal under certain growth conditions (Kraepiel et al., 1994a). The \textit{pew3} mutant displays a somewhat shorter hypocotyl than the other mutants under all conditions (Figure 5.1). This could possibly be due to retarded germination, but is likely to be due to the generally slower growth of the \textit{pew3} mutant. In terms of photomorphogenesis under R and FR, all three mutants show the same phenotype of near-blindness to both wavelengths. This is typical of POB deficient mutants in all species (Terry, 1997) whereas mutants selectively deficient in a single phytochrome apoprotein tend to display a strong phenotype in only one of these light wavelengths (Whitelam and Harberd, 1994).
5.2.2 The phenotype of \textit{pew}1 and \textit{pew}3 is moderated by growth on biliverdin supplemented media

A demonstration of the rescue of \textit{pew}1 by supplementing the growth medium with biliverdin was given by Kraepiel et al (1994a). While presenting no data on \textit{pew}2, this paper stated that its growth on biliverdin media did not produce rescue. In our hands, the \textit{pew}1 and \textit{pew}3 mutants are rescued by the presence of biliverdin, while the \textit{pew}2 mutant is not. However, in concurrence with Kraepiel and co-workers, it has been found to be difficult to achieve a sufficiently high rate of germination of \textit{N. plumbaginifolia} seed on a biliverdin-supplemented medium to provide quantitative data. It is also difficult to achieve rescue in this species, with the pH of the medium being critical (between 7.5 and 8). Outside this range, \textit{N. plumbaginifolia} mutants are not rescued by biliverdin media, whereas the Arabidopsis \textit{hyl} mutant is rescued most efficiently at pH 5.8, and pH is much less critical in achieving uptake (data not shown). The combination of lack of biliverdin uptake at low pH and poor germination at high pH make it impossible to provide repeatable, quantitative evidence of biliverdin rescue in this species. The media used were checked using Arabidopsis \textit{hyl} and found to give strong, repeatable rescue at most pH values.

The qualitative effects of biliverdin on the phenotype of R-grown \textit{pew}1, \textit{pew}2 and \textit{pew}3 are shown in Figure 2. On this basis, the \textit{pew}1 and \textit{pew}2 mutants are analogous to \textit{hyl} and \textit{hy}2 in Arabidopsis, in that one shows clear rescue while the other has a small and ambiguous response to biliverdin (Parks and Quail, 1991). However, \textit{pew}3 is likely to be a previously uncharacterised type of mutant, unless it is analogous to the original \textit{hy}6 line in Arabidopsis (Chory et al., 1989). A technique was developed for isolation of large quantities of phycocyanobilin from \textit{Spirulina} cells in order to use this chromophore substitute in rescue experiments (see Chapter 7). However, at the pH required, this substance was too unstable to be of use, oxidising to a mauve colour within one hour.
5.2.3 All three pew mutants show a pale yellow phenotype.

The photograph of the pew mutants and the wild-type N. plumbaginifolia in Figure 5.3 was taken after 9 weeks of growth under high PAR conditions in a greenhouse with supplementary illumination. It shows that all three pew mutants are slower growing, paler and show longer petioles than the wild type. The pew3 mutant is considerably more slow growing than the other pew mutants, and its leaves show small, brown necrotic lesions when grown under these irradiation conditions. The pew mutants are all extremely difficult to grow under the relatively dim light of growth chambers. The pew3 mutant becomes much greener than the plant shown after bolting, and the lesions increase in size.

5.2.4 All three pew mutants flower at an earlier developmental stage than the wild type

The number of days taken from germination to bolting in all three pew mutants is greater than the number taken by the wild-type plants. However, developmental time may not be running at the same speed in the mutants and the wild type. For this reason, the number of rosette leaves at bolting is usually considered a truer measure of the developmental stage at which a plant flowers (Halliday et al., 1994). When this is used as a measure of flowering time, all three mutants are extremely early flowering (Figure 5.4) unlike the hlg mutants, which are late flowering by reference to both leaf number and time (Chapter 4). The early-bolting-in-leaf-number phenotype of the pew mutants is entirely in accordance with the phenotype of hy1 and hy2 in Arabidopsis (Goto et al., 1991). It can therefore be stated that these mutants are early flowering in terms of developmental time. The temporal lateness of bolting can be explained by the slow growth of the mutants, probably caused by chlorophyll deficiency (Chapter 3).

Chromophore mutants are expected to be severely early flowering under all growth conditions, as they are deprived of multiple phytochromes, and so would be expected to display the shade-avoidance response even when grown under high R:FR (see Chapters 1 and 3). A major component of this response in many plants is early bolting and flowering, and this has been shown to be a particularly marked effect in N.
plumbaginifolia earlier in this thesis (Chapter 3). In addition, such a mutant would be deprived of the ability to perceive daylength, and although the effects of such a deprivation are not entirely predictable, they may cause an increase in the oscillation frequency of the circadian clock (Chapter 1, Millar and Kay (1997)).

5.2.5 Both phytochrome A and phytochrome B apoproteins are present in all three pew mutants. The pew3 mutant is deficient in phyA

The level of phytochrome apoproteins in the pew mutants was investigated using monoclonal antibodies to phytochromes A and B. Protein was extracted from 9-week-old rosette-stage plants grown in a greenhouse and subsequently dark-adapted for three days. Another extract was prepared from such plants treated with a pulse of R, in order to study the degradation of phyA. The extracts were loaded on an equal-fresh-weight basis on SDS-PAGE gels which were blotted and Coomassie stained. The resulting band pattern is seen in Figure 5.5 A. The pew1 and pew2 mutants appear to have a similar protein content to the wild-type, but the pew3 mutant appears to be deficient in all the proteins seen on the gel. Figure 5.5 B shows a gel loaded on an equal-protein basis, by the Bio-Rad protein assay (See Chapter 7) which is somewhat prone to error (Figure 5.5 B).

Protein gel blots equivalent to those Coomassie stained and shown in Figure 5.5 were then blocked and probed with monoclonal antibodies, which were subsequently detected using chemiluminescence (See Chapter 7). The autochemilumographs produced are shown in Figure 5.6. The uppermost blot shown is one equivalent to that Coomassie stained in Figure 5.5A, loaded on an equal-fresh-weight basis. This has been probed with the anti-phyA monoclonal antibody as-32 (Holdsworth, 1987). The central blot represents a gel loaded on an equal-protein basis, as in Figure 5.5B, probed with the same antibody. It can be seen from these two blots that all three of the pew mutants possess detectable levels of PHYA. It is also clear that while the phyA in the wild-type plants has been degraded by the pulse of R, that in the pew mutants has not. This indicates that while the PHYA apoprotein is present in the pew mutants, it is biologically inactive, presumably because it does not have bound chromophore (Parks and Quail, 1991). The pew3 mutant can be seen to be
deficient in PHYA apoprotein in both blots, indicating that this deficiency is not a consequence of the generally lower level of protein present in this mutant (Figure 5.6A). However, Kraepiel (1994) did not find a deficiency in PHYA in the seedlings of pew3, suggesting that this effect may be restricted to the light-grown plant. The lower chemilumograph shows the level of immunodetectable phyB in the pew mutants and the wild type, as detected by the mAT-1 monoclonal antibody (López-Juez et al., 1992). This blot was loaded on an equal-protein basis (see above) and shows that the level of PHYB apoprotein per unit protein is roughly the same in the wild-type and all three pew mutants. The wild-type appears to have slightly more phyB in the dark; this is probably due to variation in loading (Figure 5.6B). Unlike phyA in pew3, therefore, phyB does not appear to be present at lower levels in dark-adapted pew3 plants. All the pew mutants clearly lack the response of hypocotyls to R shown to be phyB mediated in Chapters 2 and 3 (Figure 5.1). Consequently phyB is also, like phyA, likely to be inactive in the pew mutants due to lack of chromophore. However, the PHYB apoprotein appears to be present at wild-type levels in the pew mutants.

5.2.6 The pewl mutant has a 'leaky' phenotype in the shade-avoidance response

In common with the aurea mutant of tomato and the hyl and hy2 mutants of Arabidopsis (Whitelam and Smith, 1991), the pewl mutant retains a response to R:FR. This is demonstrated by the data in Figure 5.7, where the response to low R:FR results in faster elongation growth. However, this response was such that the pewl mutants grown under W + FR did not survive to flowering, but overextended and died. It can be seen that pewl bolts several days later than the wild type, consistent with the results shown in Figure 5.4.

The response of pewl to R:FR is also demonstrated by the harvest data in Chapter 3. This experiment was not performed with the pew 2 and pew3 mutants, partly due to problems with viability in low light levels.
deficient in PHYA apoprotein in both blots, indicating that this deficiency is not a consequence of the generally lower level of protein present in this mutant (Figure 5.6A). However, Kraepiel (1994) did not find a deficiency in PHYA in the seedlings of \textit{pew3}, suggesting that this effect may be restricted to the light-grown plant. The lower chemilumograph shows the level of immunodetectable phyB in the \textit{pew} mutants and the wild type, as detected by the mAT-1 monoclonal antibody (López-Juez et al., 1992). This blot was loaded on an equal-protein basis (see above) and shows that the level of PHYB apoprotein per unit protein is roughly the same in the wild-type and all three \textit{pew} mutants. The wild-type appears to have slightly more phyB in the dark; this is probably due to variation in loading (Figure 5.6B). Unlike phyA in \textit{pew3}, therefore, phyB does not appear to be present at lower levels in dark-adapted \textit{pew3} plants. All the \textit{pew} mutants clearly lack the response of hypocotyls to R shown to be phyB mediated in Chapters 2 and 3 (Figure 5.1). Consequently phyB is also, like phyA, likely to be inactive in the \textit{pew} mutants due to lack of chromophore. However, the PHYB apoprotein appears to be present at wild-type levels in the \textit{pew} mutants.

5.2.6 The \textit{pewl} mutant has a 'leaky' phenotype in the shade-avoidance response

In common with the \textit{aurea} mutant of tomato and the \textit{hyl} and \textit{hy2} mutants of Arabidopsis (Whitelam and Smith, 1991), the \textit{pewl} mutant retains a response to R:FR. This is demonstrated by the data in Figure 5.7, where the response to low R:FR results in faster elongation growth. However, this response was such that the \textit{pewl} mutants grown under W + FR did not survive to flowering, but overextended and died. It can be seen that \textit{pewl} bolts several days later than the wild type, consistent with the results shown in Figure 5.4.

The response of \textit{pewl} to R:FR is also demonstrated by the harvest data in Chapter 3. This experiment was not performed with the \textit{pew2} and \textit{pew3} mutants, partly due to problems with viability in low light levels.
5.3 Discussion

All three of the *pew* mutants display the expected characteristics of mutants deficient in the phytochrome chromophore. They are all deficient in the responses to both R and FR light (Figure 5.1). They are all somewhat yellow, have an elongated growth habit and are slow growing (Figure 5.4). The *pewl* and *pew3* mutants have their phenotypes moderated by supplementing the growth medium with biliverdin (Figure 5.2). However, the most persuasive evidence is that while the PHYA and PHYB apoproteins are present in these mutants (Figure 5.7), responses known to be attributable to phyA and phyB (i.e. the response of hypocotyl growth inhibition to FR and R respectively) are both missing in these mutants (Figure 5.1) and the level of phyA in the mutants is little affected by R (Figure 5.6).

These mutants were isolated as separate complementation groups. In some cases, as with the *hlg* mutants (Chapter 2), mistakes can be made in determining the complementarity status of mutants. All conclusions based on the phenotype of *pew3* must therefore be treated with caution, as it has not yet been conclusively determined that *pew3* represents a separate locus from *pewl* and *pew2*. However, the phenotype of *pew3* is distinct from that of the other two mutants. The *pewl* mutant is yellower and earlier flowering than *pew2*, although *pew2* displays poorer germination (M. Hudson, qualitative observations). In addition, both these mutants fail to display the severely retarded growth, photosensitive lesions and low protein levels observed in *pew3*. This could be due to a second-site mutation. However, no evidence has been found in the course of the investigation that these mutants may be allelic, unlike that for the *hlg* mutants. Consequently it can tentatively be concluded that the *pew* mutations may define three separate loci, all of which are required for normal synthesis of the phytochrome chromophore. Since *pewl* is rescued by biliverdin and shows a typical chromophore deficient phenotype, this mutant is likely to be analogous to *hyl* in Arabidopsis, *pcdl* in pea and *yg-2* in tomato; i.e. a putative haem oxygenase mutant (Terry, 1997). By the same reasoning, *pew2* should be analogous to *hy2* and *aurea*; a putative PΦB synthetase mutant. However, the biochemical nature of *pew3* is more speculative. The upstream enzyme from haem oxygenase in the plastidic PΦB synthesis pathway is ferrochelatase. A plastidic ferrochelatase mutant would be
expected to be severely compromised, as it would not only be deficient in phytochrome but also in plastidic haemoproteins. It would also be expected to accumulate protoporphyrin IX, which would be likely to cause severe phototoxicity. Such toxicity would destroy plastids in the light-grown plant, possibly leading to the downregulation of some nuclear encoded genes. These may include *PHYA*, which would account for the fact that etiolated *pew3* seedlings are not deficient in PHYA apoprotein (Kraepiel, 1994) whereas light grown plants are (Figure 5.6). A mutant in the putative РФВ isomerase (Terry, 1997), however, would be expected to have a less pleiotropic phenotype, as the lesion would specifically affect the synthesis of phytochrome chromophore.

Since *pew3* displays a compromised phenotype compared to the other *pew* mutants, and shows necrotic lesions on the leaves of healthy plants grown in strong light, *pew3* seems likely to be deficient in plastidic ferrochelatase. Experiments are currently under way, in collaboration with Chang-Kee Lim of the MRC toxicology unit in Leicester and Alison Smith of Cambridge University, to quantify the protoporphyrin IX level in the *pew3* mutant and to attempt transgenic rescue using an Arabidopsis plastidic ferrochelatase gene.
CHAPTER 5
ANALYSIS OF THE pew MUTANTS

Figure 5.1: Hypocotyl length of the wild-type N. plumbaginifolia and pew mutants under variable fluence rates of R and FR.

Seedlings were grown under monochromatic R or FR for seven days under the fluence rates indicated on the x-axes. The axis before the break in each case shows the dark control; the axis after the break is a logarithmic scale. The increased errors in the data for the hypocotyls of pew2 are due to the difficulties in synchronising germination in this mutant, due to the poor germination described by Kraepiel et al (1994a). Error bars represent the standard error of the mean. At least 30 hypocotyls were measured per point.
Figure 5.2: Effect of biliverdin supplemented media on the growth of the pew mutants and the wild type under red light.

The seedlings were germinated on BG-11 media buffered to pH 7.5 with MOPS, containing either biliverdin + DMSO (biliverdin supplement) or DMSO only (control medium). Growth was then continued under 10μmol m⁻² s⁻¹ R for 7 days. Inhibition of hypocotyl elongation and promotion of cotyledon expansion is clear in those pew1 and pew3 seedlings grown with a biliverdin supplement, but not on the control medium.
Figure 5.3: Phenotype of the pew mutants and the wild type grown in W for 9 weeks post germination.

The plants shown were grown in a greenhouse at high PAR and R:FR for nine weeks before the roots were washed and the plants photographed. The mutants are paler, smaller and have a more acute leaf angle, in addition to being somewhat less developed in terms of flowering.
Figure 5.4: Flowering time of *N. plumbaginifolia* wild type and *pew* mutants. The number of rosette leaves at bolting (filled bars), and the number of days to bolting (open bars) were measured in *N. plumbaginifolia* wild type and *pew* mutants grown under simulated daylight. The photoperiod was 18h of light followed by 6h of darkness. Light was provided by daylight supplemented by HQI metal halide bulbs, or HQI metal halide bulbs alone. The PAR was at least 300μmol m$^{-2}$ s$^{-1}$ throughout the photoperiod.
Figure 5.5: Coomassie stained protein blots of extracts from *N. plumbaginifolia* wild type and *pew* mutants.

Protein preparations from 9-week old dark adapted plants either maintained in darkness or subsequently treated with red light for 24 hours were separated by SDS-PAGE loaded either on a fresh-weight basis (A) or an equal-protein basis (B), semi-dry blotted onto PVDF membranes, and stained by the Coomassie method. This shows the marked deficiency of the *pew3* mutant in protein on a gram-fresh-weight basis.
Figure 5.6: Protein gel blots showing immunodetectable phyA and phyB levels in the N. plumbaginifolia wild type and pew mutants.

Protein preparations from 9-week old dark adapted plants either maintained in darkness or subsequently treated with red light for 24 hours were loaded either on a fresh-weight basis (top, Figure 5.5 A) or an equal-protein basis (centre, Figure 5.5 B), separated by SDS-PAGE, and semi-dry blotted. Equivalent blots to those shown in Figure 5.5 were then probed with the AS-32 anti-phyA monoclonal antibody (Holdsworth, 1987). An equal-protein loaded protein blot was also hybridised to the monoclonal anti-tobacco phyB antibody mAT-1 (bottom) (Lopez-Juez et al., 1992).
CHAPTER 5  ANALYSIS OF THE pew MUTANTS

Figure 5.7 Changes in the time of bolting in response to altered red-far red ratio (R:FR) in the wild type *N. plumbaginifolia* and pew1 mutant.

The overall height of wild type *N. plumbaginifolia* (circles) and the pew1 mutant (squares), grown in continuous W of high R:FR (filled symbols) or continuous W plus supplementary FR, giving low R:FR (open symbols) was measured in plants grown in a 16h photoperiod. Plants were all grown in identical high R:FR conditions for 42 days until transfer to different R:FR cabinets. Measurements are the mean of ten individuals and error bars represent the standard error of the mean.
Chapter Six

Analysis of native phyA function by overexpression in transgenic *Nicotiana tabacum* cv. SR-1
6.1 Introduction

Phytochrome A (phyA) is the sole known representative of the class of "light labile" or Type I phytochromes. It is produced in the Pr form, and degraded after conversion to the Pfr form. The vast majority of the phytochrome in any etiolated angiosperm is phyA (Quail, 1994). Although Avena and N. tabacum have several PHYA genes, they encode very similar proteins (see Chapter 1) and it is a justifiable simplification to state that, as far as we know, all plants have only one type of phyA. Because of its abundance in etiolated tissue, phyA was the first phytochrome to be isolated and the PHYA gene of Avena was the first phytochrome gene to be sequenced (See Chapter 1). The relative abundance of phyA can be taken to imply that its physiological role is proportionally important. The elegance and efficiency of the way phyA is specifically degraded, only its Pfr form, also suggests that phyA Pfr must play an important role for its abundance to be regulated so tightly.

It was not long after the isolation of the first PHYA gene (Hershey et al., 1985) that the first phyA overexpressing plants became available. In tomato and tobacco, the insertion of a PHYA gene under the control of the CaMV 35S promoter produced dwarfed, dark green plants (Keller et al., 1989, Boylan and Quail, 1989, Kay et al., 1989). This was in perfect correlation with expectations; if plants without phytochrome stimuli were pale and elongated, then plants which overproduce phytochrome should be dark-green dwarfs. These plants provided a tool for the analysis of altered and domain-swapped transgenic phytochromes, and have thus become important tools for phytochrome biologists (see Chapter 1). However, once it was known that there are several PHY genes in all the plants used for experimental purposes, the focus of research into the function of the various phytochromes shifted to null mutants in individual PHY genes.

The isolation of phyA mutants in Arabidopsis produced the unexpected observation that plants without phyA show little phenotype under normal growth conditions (Whitelam et al., 1993). Although, in the laboratory, responses such as the FR-HIR and VLFR could be shown to be mediated solely by phyA, it appeared that plants could be perfectly viable without these responses. While phyA has a role in the regulation of flowering time (Johnson et al., 1994, Bagnall et al., 1995) it does not appear to do anything essential to the plants survival which cannot be done by the
other phytochromes in its absence. The ability of the remaining phytochromes to compensate for the loss of one of their number by mutation has been referred to as "genetic redundancy"; this is discussed in Chapter 7. The one clue to a possible reason for the obvious selective pressure to retain phyA (it is found in all angiosperms examined, at a high abundance and with a highly conserved sequence) is the inability of the phyA mutant to de-etiolute under dense canopy shade (Yanofsky et al., 1995). It can be inferred from this result, and the insensitivity of phyA mutants to FR (Whitelam et al., 1993, van Tuinen et al., 1995) that phyA provides a means for the plant to sense the FR irradiance transmitted by dense canopies.

Using transgenic N. tabacum cv. Xanthi overexpressing Avena phyA, McCôrmac et al. (1992) showed that these plants showed an enhanced sensitivity to FR. This sensitivity persisted into the light grown plant, a result attributed to the use of the 35S promoter which, unlike the native PHYA promoter, is not down-regulated by light. The most obvious consequence of this persistent FR sensitivity was that plants grown under simulated canopy shade, with additional FR, were dwarfed relative to W grown controls. This situation was the reverse of that in the wild-type plants, in which shade-avoidance responses caused elongation of the stem in the plants grown under supplementary FR. The implications of a tool to reverse the shade-avoidance response are many; perhaps the most important is as a potential means for generating crops capable of increased yields when grown at high densities. It has since been shown that the responses of field crops to crowding can be altered in this way, producing an increase in harvest index (Robson et al., 1996). This work is, however, based on the use of the Avena PHYA gene in tobacco under the control of a viral promoter. While this is no barrier to the use of this method as a means of improving crop plants by biotechnology, it limits the conclusions which can be drawn from these experiments about the role of phyA in the natural environment.

The native PHYA gene of N. tabacum has been shown to produce three alternative transcripts, the relative abundance of which varies with developmental stage (Ádám, et al., 1994). It has also been demonstrated that the PHYA transcript is down-regulated by light, and that a certain upstream sequence is both necessary and sufficient for the down-regulation of a partial promoter fused to GUS (Ádám et al., 1995). The current state of knowledge of the tobacco gene family is reviewed by
Ádám et al. (1997). Because the 35S promoter produces a different expression pattern from that of the native phyA promoter (Ádám et al., 1995), and because the \textit{N. tabacum} and \textit{Avena} PHYA proteins do not share perfect homology, 35S driven \textit{Avena} phyA overexpressing plants are not the ideal tool to investigate the role of the native phyA in tobacco. For this reason, transgenic tobacco SR-1 lines were produced which carry an additional native \textit{PHYA} gene, under the control of either the 35S promoter or the native \textit{PHYA} promoter. Their creation and analysis is described in this chapter.

6.2 Results

6.2.1 Construction of Transformation Vectors

In order to overexpress the \textit{Nicotiana tabacum} cv. SR-1 gene in its native background, it was first necessary to obtain the gene itself. The gene was a gift from Feren\'c Nagy and \'{E}va Ádám of the Hungarian Academy of Sciences, Szeged, Hungary. The Szeged group also kindly prepared the expression cassettes pictured in Figure 6.1, and supplied them to Leicester cloned into the pBluescript vector. It was then necessary to excise the construct from its vector and clone it into the binary plant transformation vector pBin19. Excision was performed using a \textit{Sal I}/\textit{Sac I} partial digest, and the fragment was ligated into pBin19 cut with \textit{Sac I}/\textit{Xho I}. Correct construction of the binary vector was checked by restriction digestion, and the plasmid transferred into \textit{Agrobacterium} strain LBA4404 by triparental mating (see Chapter 7). The transgenic \textit{Agrobacterium} were confirmed as containing the intended plasmids by Southern blotting.

6.2.2 Transformation and selection of recombinant lines

Cultures of the \textit{Agrobacterium} strains described above was used to transform tobacco cv. SR-1 leaf explants as described in Chapter 7. The transgenic plants were selected on the basis of kanamycin resistance. Lines segregating 3:1 for kanamycin resistance were qualitatively examined after growth under FR. Of the several lines from each construct showing a short hypocotyl under FR, two lines containing each construct were selected for further study. These were the lines 2.6 and 2.10, containing
the 35S::PHYA construct, and the lines 2.4a and 3.21a, containing the PHYA::PHYA construct.

6.2.3 Molecular characterisation of recombinant lines

Protein was extracted from the adult light-grown wild-type and transgenic lines as described in Chapter 8. The anti-phyA AS-32 antibody and the anti-phyB mAT-1 antibody were used to probe protein gel blots. The results are shown in Figure 6.2. The 3.21a, 2.10 and 2.6 lines clearly contain more phyA than the wild-type plants. The 2.4a line does not clearly contain a different amount of phyA than the wild type. The level of phyB is identical in the wild type and all the transgenic lines.

6.2.4 Response of transgenic seedlings to monochromatic R and FR

The wild-type tobacco SR-1, the two 35S promoter lines and the two PHYA promoter lines were germinated and grown under monochromatic light of different fluence rates for 3 days. The hypocotyls of at least 25 individuals per point were then measured and the mean and standard error taken. Figure 6.3 shows that the sensitivity to FR of the 35S promoter lines 2.6 and 2.10 is significantly enhanced. The slope of the fluence rate/hypocotyl length graph is steeper in the transgenics, and saturation of the response is reached sooner. The response to R in the transgenic lines is also enhanced, although less significantly than that to FR. These results are entirely consistent with those achieved by other workers using the Avena PHYA gene driven by the 35S promoter.

Figure 6.4 shows that the response to FR is also made more sensitive by insertion of another copy of the PHYA gene under the control of its own promoter. Both PHYA promoter lines show greater FR sensitivity, although saturation of the response is not achieved within the range of fluence rates used, unlike that for the 35S promoter lines. It can be seen that the 3.21a line has a much stronger phenotype than the 2.4a line, which nonetheless still shows an enhanced FR sensitivity. The 3.21a line also appears to show increased sensitivity to R. The curve of the FR response in 3.21a has a different shape to those of the 35S promoter lines, suggesting that the nature of
the promoter may have an influence on the response to different fluence rates of continuous FR.

6.2.5 The shade-avoidance response in transgenic tobacco overexpressing native phyA

Wild-type tobacco SR-1 shows an increase in elongation growth in response to simulated canopy shade conditions. Plants were grown in either fluorescent W of 125μmol m^-2 s^-1 PAR, or in W of identical PAR supplemented with sufficient FR to lower the R:FR to 0.17. Illumination was continuous. In Figure 6.5 the response of wild-type plants to these conditions are shown. The plants were grown under identical conditions for 30 days, until the point where the stems of the plants begin to elongate, then placed in the W or W+FR cabinets. It can be seen that the plants under W+FR elongate more rapidly than those in W, particularly in the initial days after placement in the cabinets. The plants reached a greater height at the end of the experiment and the growth curve is altered in shape. This response represents the classic shade-avoidance response seen in most angiosperms in response to low R:FR.

When the growth of the transgenic plants containing the 35S::PHYA construct in W is considered, they are dwarfed with respect to the wild type (Figure 6.6). Elongation growth is reduced, and the plants reach a smaller overall height at the close of the experiment, relative to the wild-type SR-1 grown in W. The 35S::PHYA transgenic lines grown in W+FR are also dwarfed when compared to the wild-type plants grown in W+FR. Comparison of the transgenic lines 2.10 and 2.6 grown in W or W+FR shows, however, that they retain the response of increased elongation growth in response to low R:FR. In fact, this response is perhaps more marked in the transgenic plants that in is in the wild type. This is in direct contrast to the behaviour of tobacco which expresses the Avena PHYA3 gene under the control of the 35S promoter (McCormac et al., 1992, Robson et al., 1996). In these plants, a phyA expression level which causes minimal dwarfing is sufficient to practically cancel out the shade-avoidance response to low R:FR. This effect of Avena phyA overexpression, which has been termed "proximity conditional dwarfing", is clearly not caused by overexpression of the native N. tabacum phyA.
The transgenic plants carrying the *PHYA* promoter fused to the native *PHYA* coding sequence effectively carry an extra copy of the native gene. Consequently, the promoter would be expected to be down-regulated in light grown plants, unlike the 35S promoter. For this reason, any influence of *PHYA* promoter driven phyA overexpression in light grown plants represents evidence that the role of phyA is not restricted to etiolated plants, or to plants during a dark period. It can be seen by comparison of Figure 6.7 with Figure 6.5 that the insertion of the *PHYA::PHYA* construct causes the plants to be dwarfed under continuous illumination. This dwarfing is observed under both W and W+FR; indeed the effect of the *PHYA::PHYA* construct is similar to that of the 35S::*PHYA* construct, but less severe. The difference between the phenotype of the two constructs is probably due to their different phyA expression level (Figure 6.2) which itself is probably due to insertion of the transgene in different areas of the genome. Both 2.4a and 3.21a show a light-grown phenotype distinct from the wild type, so it is reasonable to conclude that phyA does play a role in morphogenesis of the light-grown plant, despite the down-regulation of the promoter by light.

6.2.6 The effect on flowering of overexpression of native phyA in tobacco

The experiments described above were continued until the plants flowered. Flowering was defined as the time when the first flower bud opened, and the petals were visible. At this point, the number of internodes formed before the terminal inflorescence was recorded. Unlike Arabidopsis (Goto et al., 1991, Halliday et al., 1994) it can be seen from Figure 6.8 that the flowering of wild-type SR-1 tobacco under continuous illumination is not affected by R:FR. The plants flower after the formation of 11 internodes, whether under W or W+FR. However, the number of internodes at flowering is strongly affected by the presence of an introduced phyA gene. All four of the transgenic lines flower significantly later than the wild-type, with the 2.10 and 3.21a lines forming more than twice the number of internodes before flowering.

It is also interesting to note that the transgenic plants carrying the *PHYA::PHYA* construct show a less marked phenotype than those carrying the
35S::PHYA construct in terms of the FR response (Figures 6.3 and 6.4) and the inhibition of elongation growth (Figures 6.6 and 6.7). However the effect on internode number at flowering of the PHYA::PHYA construct is, if anything, stronger than the effect of the 35S::PHYA construct. In addition, the 2.6 line, which is more strongly dwarfed than the 2.10 line (Figure 6.6) is not as strongly affected in flowering time (Figure 6.8). It seems likely therefore that the expression pattern of phyA is more important than overall phyA level in determining the effect on flowering time. Note also that the transgenic lines carrying the 35S::PHYA construct flower earlier under W+FR than under W, while the wild type does not. This is another instance of the shade-avoidance response in the native phyA overexpressors being enhanced.

6.3 Discussion

The N. tabacum PHYA1 gene is well characterised (Âdám et al., 1994). It is difficult to argue that this gene is not a true expressed gene which produces a photoreceptor. The plants created as part of this thesis overproduce a phyA which cross-reacts with an antibody specific to PHYA protein (Figure 6.2, Holdsworth, 1987). They also show a light dependant phenotype. The transgenic plants can hence be confidently said to overexpress a photoreceptor. In consequence, the differences in light-grown phenotype between the transgenic and wild-type plants are unlikely to be anything other than the consequence of the inserted PHYA gene.

The plants which carry the 35S::PHYA construct display a phenotype reminiscent of that described by the first workers to overexpress Avena phytochrome in tobacco (Keller et al., 1989). As light grown adults, they have relatively dark-green leaves and they are dwarfed, with a shorter distance between the internodes. In this respect, the phenotype of these plants is identical to that of overexpressers of Avena phyA. Another consistent phenotype is the enhanced response of the etiolated transgenic seedlings to FR (McCormac et al., 1992, Figure 6.4) and the relatively small effect on the R response. However, an overexpression level which is sufficient to cause obvious dwarfing of the W-grown adults is insufficient to cause any FR-dependent dwarfing (Figures 6.5 and 6.6). This is qualitatively different from the phenotype observed in transgenic tobacco (cv. Xanthi) overexpressing Avena phyA. In
the *Avena* phyA overexpressers, an expression level insufficient to cause dwarfing in W will cause strong inhibition of elongation growth in W+FR (Robson et al., 1996). Therefore, the transgenic SR-1 containing the 35S::PHYA construct are expressing a photoreceptor which causes an enhanced FR dependent growth inhibition in etiolated seedlings, just like *Avena* phyA. However, overexpression of the native phyA does not cause an enhanced FR dependent growth inhibition in light-grown adult plants. It is reasonable, therefore, to conclude that the effect of *Avena* phyA on shade-avoidance does not represent a role of native phyA in tobacco, but an artefact of the expression of an exogenous gene.

The insertion of the PHYA::PHYA minigene into tobacco cv. SR-1 produces an effect on elongation growth similar to that of the 35S::PHYA construct, although less strong. This demonstrates that the native phyA is active in determining the morphogenesis of the adult light-grown plant, despite being controlled by its own, light down-regulated, promoter. Consequently, although a FR-dependant dwarfing is not observed in the native phyA transgenics, native phyA is present in sufficient quantities in light-grown tissue to be important in light sensing. The PHYA::PHYA transgenics show a massive difference in flowering time (Figure 6.8), and a strong effect on elongation growth, despite the low level of phyA overexpression in 2.4a (Figure 6.2). This implies that the native promoter targets expression to areas where the phytochrome is morphologically active, making a low level of overexpression cause a strong effect on phenotype.

The wild-type *N. tabacum* used in these experiments does not show a response of internode number at flowering to R:FR. However, native phyA overexpression causes a marked increase in the number of internodes formed before the terminal inflorescence (Figure 6.8). Overexpression of *Avena* phyA also delays flowering (Halliday, 1996). It seems likely that phyA has an important role in the regulation of flowering in tobacco, since a low level of expression under the control of the native promoter (2.4a, Figure 6.8) can cause a significant alteration in this critical parameter of plant growth.

The 35S::PHYA transgenics show a response of internode number at flowering to R:FR, unlike the wild type (Figure 6.8). They also show a more marked difference in elongation growth in W and W+FR than do the wild-type plants (Figures 6.5 and
This is consistent with the behaviour of a phytochrome which is itself involved in mediating the elongation in response to shade. Such phytochromes have always previously been thought to be light-stable (Smith, 1994). However, the phyB absent from the hlg mutants is not required for R:FR perception (Chapters 2 and 3). Possibly the role phyB plays in shade-avoidance in Arabidopsis is partly taken by phyA in tobacco. Certainly the phenotype of the native tobacco phyA overexpressers is more consistent with that expected of overexpressers of a light-stable phytochrome.

Tobacco cv. SR-1 overexpressing Arabidopsis phyB behaves in a way more similar to the native phyA overexpressers than do Avena phyA overexpressers in terms of the response of light-grown plants to shade (G. C. Whitelam, pers. comm.).

The difference between the native phyA and Avena phyA in tobacco is likely to be one of stability. The phyA Pfr degradation systems are unlikely to work in exactly the same fashion and at the same efficiencies in the two species. Consequently it is not reasonable to expect the system for phyA Pfr degradation in tobacco to recognise and destroy Avena phyA Pfr at the same rate as the native protein. From the results outlined above, it would be reasonable to assume tobacco phyA Pfr is more stable in tobacco than Avena phyA Pfr. This conclusion can be drawn because the phenotype caused by native phyA overexpression is more similar to that expected of light-stable phytochrome overexpression than that of Avena phyA overexpressers. The FR sensitivity in light grown plants seen in Avena phyA overexpressers may be a consequence of production of large quantities of rapidly degraded phyA. The response seen in the native phyA overexpressers is probably due to the action maximum of this response being shifted to the extent that it is also triggered under fluorescent W (Figures 6.6 and 6.7). However the response threshold is not shifted sufficiently to affect the response to monochromatic R significantly in the transgenic seedlings (Figures 6.3 and 6.4). In order to provide further evidence for this hypothesis, it will be necessary to measure the action maxima for inhibition of elongation in the native phyA overexpressers and overexpressers of Avena phyA. It would also be desirable to determine the degradation kinetics of the two phyA proteins on illumination.
Figure 6.1: The t-DNA constructs used to transform tobacco in order to overexpress native phyA.

The flags represent the left and right borders of the t-DNA respectively. The Kanamycin resistance cassette was that contained by the pBin19 vector. The double 35S promoter was supplied by Eva Adam and Ferenc Nagy of the Hungarian Academy of Sciences in Szeged, who kindly made and supplied these constructs, as was the PHYA promoter, a 1.7kb upstream clone (Adam et al., 1994). The PHYA gene is the PHYA1 gene (Adam et al., 1995).
Figure 6.2: Protein gel blots showing immunodetectable phyA and phyB levels in the wild-type SR-1, 35S::PHYA and PHYA::PHYA transgenics.

Protein preparations from light-grown plants just prior to flowering were separated by SDS-PAGE, semi-dry blotted, and probed with the AS-32 anti-phyA monoclonal antibody (Holdsworth, 1987) (top). The same protein preparations were hybridised to the monoclonal anti-tobacco phyB antibody mAT-1 (bottom) (Lopez-Juez et al., 1992). The 3.21a, 2.10 and 2.6 lines have clearly higher levels of phyA than the wild-type, whereas phyB levels are unaffected.
Figure 6.3: Hypocotyl length of the wild-type SR-1 tobacco and 35S::PHYA transgenics under variable fluence rates of (left) R and (right) FR.

Seedlings were grown under monochromatic R or FR for three days under the fluence rates indicated on the x-axis. The axis before the break shows the dark control; the axis after the break is a logarithmic scale. The wild type plants are represented by circles, 2.6 by squares, and 2.10 by triangles. At least 25 hypocotyls were measured per point.
Figure 6.4: Hypocotyl length of the wild-type SR-1 tobacco and PHYA::PHYA transgenics under variable fluence rates of (left) R and (right) FR.

Seedlings were grown under monochromatic R or FR for three days under the fluence rates indicated on the x-axis. The axis before the break shows the dark control; the axis after the break is a logarithmic scale. The wild type plants are represented by circles, 3.21a by squares, and 2.4a by triangles. At least 25 hypocotyls were measured per point.
Figure 6.5: Changes in extension growth in response to altered red-far red ratio (R:FR) in the wild-type tobacco SR-1.

The overall height of wild type tobacco, grown in continuous W of high R:FR (filled symbols) or continuous W plus supplementary FR, giving low R:FR (open symbols) was measured in plants grown under continuous illumination. Plants were all grown in identical high R:FR conditions for 30 days until transfer to different R:FR cabinets. Measurements are the mean of ten individuals and error bars represent the standard error of the mean.
Figure 6.6: Changes in extension growth in response to altered red-far red ratio (R:FR) in the transgenic tobacco lines carrying the 35S::PHYA construct.

The overall height of the 2.6 and 2.10 lines, grown in continuous W of high R:FR (filled symbols) or continuous W plus supplementary FR, giving low R:FR (open symbols) was measured in plants grown under continuous illumination. Plants were all grown in identical high R:FR conditions for 30 days until transfer to different R:FR cabinets. Measurements are the mean of ten individuals and error bars represent the standard error of the mean.
**PHYA::PHYA**

- • 3.21a WL
- ■ 2.4a WL
- ○ 3.21a WL+FR
- □ 2.4a WL+FR

Figure 6.7: Changes in extension growth in response to altered red-far red ratio (R:FR) in the transgenic tobacco lines carrying the *PHYA::PHYA* construct.

The overall height of the 3.21a and 2.4a lines, grown in continuous W of high R:FR (filled symbols) or continuous W plus supplementary FR, giving low R:FR (open symbols) was measured in plants grown under continuous illumination. Plants were all grown in identical high R:FR conditions for 30 days until transfer to different R:FR cabinets. Measurements are the mean of ten individuals and error bars represent the standard error of the mean.
CHAPTER 6 TRANSFORMATION OF TOBACCO WITH NATIVE *PHYA* GENE

Figure 6.8: The number of internodes at flowering in response to altered red-far red ratio (R:FR) in the transgenic tobacco lines carrying the 3SS::*PHYA* construct, the *PHYA*::*PHYA* construct, and the wild type (SR-1).

The number of internodes to the opened terminal inflorescence was counted in plants grown under continuous illumination, either continuous W of high R:FR (open bars) or continuous W plus supplementary FR, giving low R:FR (grey bars). Plants were all grown in identical high R:FR conditions for 30 days until transfer to different R:FR cabinets. Measurements are the mean of ten individuals and error bars represent the standard error of the mean.
Chapter Seven

General Discussion
In recent years, the study of photomorphogenesis has had a definite centre in the model plant Arabidopsis. Like so many other fields of biology, photomorphogenesis is now reliant on genetics. The advantages of Arabidopsis for genetic research are manifold. Its genome is small, its generation time short, each plant takes up little space and its seeds are abundant and germinate readily. These practical advantages have led to Arabidopsis having the best mapped genome and the largest amount of available genetic sequence of any plant species; an advantage in itself. Since transformation of Arabidopsis became routine, it is the species of choice for almost all plant science.

The phytochrome genes themselves are named according to their degree of homology to the Arabidopsis PHY genes. The photomorphogenic mutants in Arabidopsis provide a range of phenotypes according to which mutants in other species can be placed in groups. However, the phenotypes of some photomorphogenic mutants in other species are not easily reconciled with those in Arabidopsis, notably the tri mutant of tomato (van Tuinen et al., 1995a). It is clear from this research, and indeed from all the great body of research that shows differences in phytochrome responses between species, that different species use different phytochromes for different purposes. Despite this, some types of phytochrome response appear to be present in all plants, for example the use of phyA to detect very low fluences of light (Casal et al., 1997). If the knowledge acquired from the study of photomorphogenesis is to be applied, other species must be investigated to confirm that conclusions drawn in Arabidopsis are not unique to this species or its immediate relatives (chapter 1).

The study of phytochrome responses in the genus Nicotiana provides an opportunity to work in a species well characterised in terms of both molecular biology and photomorphogenesis. The work contained in this thesis is part of a considerable body of information on the biology of Nicotiana. However, this is the first time the role of different phytochromes in this genus has been investigated using mutants, as well as transgenic plants. Most of the existing work on photomorphogenesis in Nicotiana represents investigations of transgenic tobacco plants overexpressing "phytochrome", usually phyA from Avena (see chapters 1 and 6). Arabidopsis phyB and phyC (Halliday, 1996) and Oryza phyA (Kay et al., 1989) have also been overexpressed in tobacco. However, despite the availability of the native N. tabacum PHYA and PHYB sequences (Adam et al., 1997), and homozygous diploid N. plumbaginifolia in which mutants can
be isolated (chapter 1), *Nicotiana* lines in which the expression level of the native phytochrome genes has been altered have not until now been available.

Isolation of phytochrome B mutants in *N. plumbaginifolia* has provided new information on which roles of this phytochrome are conserved between angiosperms, and which are variable between genera. The seedling-stage phenotype of the *phyB* mutants of Arabidopsis, cucumber, *Sorghum* etc. share the deficiency in R sensitivity observed in the *N. plumbaginifolia phyB* mutants (Chapters 2 and 3). It follows that de-etiolation in response to R remains likely to be a universal role of phyB, being conserved among many species. However, because all these plants use phyB as a R sensor does not mean that the signal from phyB is always interpreted in the same way.

It has already been stated that the phyB discussed in Chapter 2 is not required for the wild-type perception of R:FR in *N. plumbaginifolia*. This could be explained by the concept of genetic redundancy; i.e. the phyB investigated, and one or more other phytochromes, are capable of mediating a wild-type shade-avoidance response in the absence of the other photoreceptor(s). However, *phyB* mutants of Arabidopsis show altered R:FR responses, although these responses are still seen (Goto et al., 1991, Whitelam and Smith, 1991). Despite the fact that phyD and phyE are also involved in shade avoidance in Arabidopsis (Smith and Whitelam, 1997), phyB is therefore required for shade avoidance to function in a wild-type manner. This implies that the phyB investigated here in *N. plumbaginifolia* plays a less important role in shade avoidance than phyB in Arabidopsis, if it plays a role at all.

If phyB is not used as a R:FR sensor in *N. plumbaginifolia*, at least one other phytochrome must mediate the strong shade-avoidance responses of bolting, elongation and flowering observed in chapter 3. In fact, these responses are likely to be mediated by several phytochromes, central as they are to the fitness of the plant in a competitive environment (Smith and Whitelam, 1997). While at least one of these phytochromes is likely to be fully "light stable", one phytochrome which may play a previously uncharacterised part in the shade-avoidance response in tobacco species is phyA. The data in chapter 6 clearly show that the dwarfed phenotype of *N. tabacum* cv. SR-1 carrying an extra copy of the native *PHYA* gene can be partially rescued by growth under low R:FR conditions. This phenotype is the reverse of that observed in tobacco or Arabidopsis overexpressing *Avena* phyA (McCormac et al., 1992, Whitelam et al., 1992) where addition of FR to light of high R:FR causes a dwarfing of high level
overexpressers. Dwarfing in W, partially rescued by low R:FR, is consistent with the effect expected of overexpression of a light-stable phytochrome such as phyB, and indeed tobacco plants overexpressing Arabidopsis phyB show a similar phenotype, based on preliminary observations. It is difficult to argue that the phyA present in light-grown, wild-type tobacco under high R:FR does not also cause a significant inhibition of elongation, as the plants overexpressing native phyA under copies of the native promoter are also dwarfed under W, and partly rescued by low R:FR (chapter 6). Based on these experiments, the inhibition of elongation by native phyA present in the light-grown wild-type would be at least partially relieved by low R:FR. In *N. tabacum*, therefore, and, by inference, *N. plumbaginifolia*, shade-avoidance is likely to be mediated at least partially by phyA, if sufficient quantities of phyA are present in the light-grown plant.

The overexpression of oat phyA in tobacco or Arabidopsis causes a relatively large increase in the sensitivity of the plants to FR, and a small increase in R sensitivity (McCormac et al., 1992, 1993, Whitelam et al., 1992). The tobacco plants which overexpress native phyA display the same phenotype as oat phyA transformed plants when etiolated seedlings (chapter 6). Consequently, despite causing opposite effects in light-grown plants responding to low R:FR, overexpression of both these phytochromes has the same effect on the responses of etiolated seedlings to defined wavelengths of light. Etiolated mutant seedlings deficient in phyA are universally deficient in the responses to FR (chapter 1). The responses of etiolated phyB mutant seedlings to monochromatic light are also equivalent in the many species studied (chapters 1, 2 and 3) in that they are all relatively insensitive to continuous R. It seems that the seedlings of the genus *Nicotiana* are no different from all other angiosperm seedlings, in that the primary photoreceptors of seedling de-etiolation are phyA and phyB. The universal roles of phyA and phyB in etiolated seedlings seem to be mediation of the responses to very low-fluence R and FR, and the vast majority of the R response, respectively. There must, consequently, be a highly conserved mechanism for de-etiolation in the angiosperms, with the signal transduction pathways carrying these signals from the phytochromes to the cellular machinery also likely to be highly conserved. It is once de-etiolation has occurred, and the plants are autotrophic, that the ubiquitous sub-families of phytochrome (phyA, phyB etc.) begin to be used in different ways by different species. This is consistent with the observation that most epigeal dicotyledonous
seedlings appear superficially similar, and it is only once photoautotrophy is firmly established that the different developmental programs of, for example, tobacco and Arabidopsis, become apparent. Once this stage is reached, all species are following different morphogenic instructions, and it is consequently unsurprising to observe that the influence of phytochromes on this development is also variable between species.

The conservation of the steps prior to the establishment of autotrophy may be explained by their fundamental importance to all plant species. The priorities of most plant species during the process of de-etiolation coincide because the same processes are occurring; synthesis of chlorophyll and photosynthetic proteins and the formation of light harvesting structures. Once plants have generated this common machinery, their developmental programs diverge according to the requirements of their niche.

One aspect of phytochrome-mediated light sensitivity which varies perhaps more than any other between species is the response to photoperiod. Almost as many types of photoperiodic sensitivity have been characterised as species examined (Vince-Prue, 1974) with the one large group which behaves consistently internally being the day-neutral plants; those in which flowering time does not respond to photoperiod. The lack of an essential role for phyB in shade-avoidance may be an indirect result of the use of phyB in *N. plumbaginifolia* to perceive LD (chapter 4). It is possible that the perception of SD is compatible with the use of the same phytochrome to perceive R:FR in the adult, while the perception of LD is not. This would be compatible with the notion that the acceleration of flowering in SD or in low R:FR could share a constitutive mechanism which is blocked by a phyB Pfr signal. A LDP would be unable to use phyB to accelerate flowering in response to a Pfr signal at a given time, if the absence of that signal, indicating low R:FR, also acted to accelerate flowering. In order to differentiate between darkness at the end of a short day and low R:FR in a plant using all the light-stable phytochromes to perceive R:FR, the photoreceptor controlling floral induction would need to be either a blue-light receptor, or a phytochrome of which the Pfr form does not retain its activity indefinitely (e.g. phyA). In order for phyB to fulfill one of these criteria, it must either show dark reversion, or only freshly formed Pfr must be active to promote flowering. This is therefore likely to be the case in *N. plumbaginifolia*.

It is now thought that photoperiodic sensitivity in Arabidopsis is primarily mediated by phyA and CRY1 (Jackson and Thomas, 1997).
In Arabidopsis, a LDP, phyB is of limited importance for photoperiodism (Bagnall et al., 1995, Jackson and Thomas, 1997) and may thus be a sensor of R:FR and not daylength in the adult plant. In Sorghum, a SDP, phyB seems likely to be central to the perception of both photoperiod and R:FR (Childs et al., 1995) a situation also apparent in Solanum tuberosum ssp. andigena, which tuberises in response to SD (Jackson et al., 1996). In SD tobacco, overexpression of Arabidopsis phyB both causes dwarfing and increases NB sensitivity (Halliday, 1996). Adult N. plumbaginifolia, which acts somewhat like a LDP (chapter 4), appears to use phyB for the opposite purposes of Arabidopsis, i.e. as a sensor of photoperiod but not as a R:FR sensor. It may be for this reason that measurement of the photoperiodic sensitivity of N. plumbaginifolia phyB mutants reveals rhythmic deficiencies in leaf movement not previously discovered in other species.

The constitutive oscillation of N. plumbaginifolia phyB mutant leaves is an example of a default response to an absence of photoreceptor signal, usually only seen in the absence of the signal concerned (in this case, under SD). Such responses are rarely seen in phytochrome mutants as important responses tend to be shared between several photoreceptors. It may be because responses to photoperiod are not particularly important to the survival of N. plumbaginifolia (consisting, as they do, of alterations in leaf movement and a slight alteration in bolting time) that they are, unusually, under the control of a single photoreceptor. Alternatively, the response of leaf movement to photoperiod could be an example of a recently evolved response which has not yet had chance to diversify the mechanisms by which it may operate. Alteration of the phyB level, the rhythm of sensitivity and the extent to which bolting time is affected by photoperiod could all be conceivably affected by a single mutation. By contrast, the response of leaf angle to low R:FR is likely to be more critical to the survival of the plant, and mediated by more than one phytochrome. Perhaps part of the success of N. plumbaginifolia, which is now well established as a weed in India, could be explained by a rapid adaptation to different conditions using just such a range of subtle environmental responses.

In conclusion, the work set out in this thesis has strengthened the already convincing evidence that the roles of phytochromes in de-etiolation are highly conserved. However it also provides evidence of the diversity of phytochrome responses, and more than one example of phytochromes mediating different responses
in *Nicotiana* to those they are known to play a role in in Arabidopsis. It also provides a new phytochrome response, sensitivity of leaf movement amplitude to photoperiod, which may be useful in elucidating the links between phytochrome and the circadian clock if it can be characterised in a genetically more tractable species. Finally, it provides more data towards understanding the role of different phytochromes in a plant which is not Arabidopsis, nor closely related to it, and which could loosely be said to be a genus containing agriculturally important species. Consequently some of this work may help towards the goal of being able to use phytochrome transgenes to alter predictably plant architecture and environmental responses.

The work already set out in this thesis presents a number of opportunities for further research. Five new *hlg* mutants are now available, and the sequencing of the *PHYB* genes of these lines will identify new lesions, possibly increasing knowledge about the structure and function of the PHYB apoprotein. Further analysis of *hlg-1* and related mutants may define a role for serine proteases in the control of phytochrome stability. Further screening of *M₂* populations of *N. plumaginifolia* would allow the isolation of new photoreceptor mutants, particularly *phyA* null mutants, which would be central to further photobiological work in this species. Such populations could also be screened for leaf-movement mutants; this work could also be carried out in Arabidopsis once photoperiodic leaf movement is better characterised in this species.

The differences between *Avena* and tobacco phyA overexpression presents a unique opportunity to investigate further the influence of PHYA amino acid sequence on Pfr stability. Sequence comparison, domain swapping and site directed mutagenesis of the *PHYA* genes, followed by the creation of more transgenic tobacco lines, could be used to determine which features of the PHYA sequence determine the light lability of the protein. Conclusions from these experiments may help to elucidate the molecular basis of Pfr degradation and the differences between "light labile" and "light stable" phytochromes. Subsequent physiological experiments could tie-in these results with action spectra of responses in seedlings, answering long-standing questions about the role of phyA degradation in the perception of light.
Chapter Eight

Materials and Methods
6.1 Extraction procedures

6.1.1 Extraction of plant genomic DNA

The method used was based on that of Doyle and Doyle (1983). Tissue from young (1-5 cm long) leaves was ground in liquid nitrogen in a pestle and mortar. 10 ml DNA extraction buffer at 60 °C [100 mM Tris pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% w/v hexadecyl trimethylammonium bromide (CTAB), 0.2% v/v 2-mercaptoethanol] was added per gram of leaf tissue used, and the powder ground further in the extraction buffer. The contents of the mortar were transferred to a 50ml polycarbonate centrifuge tube, which was then incubated at 60 °C in a water bath for 30 min. Stabilised chloroform (10 ml) was then added, and the layers separated by centrifugation for 10 min at maximum speed in a benchtop centrifuge. The aqueous phase was removed, transferred to a clean tube, and one volume of propan-2-ol (isopropanol) added. The DNA was checked visually for precipitation in long stands on gentle agitation, then separated by centrifugation for 3 min in a benchtop centrifuge on maximum speed. The supernatant was removed by aspiration, and the pellet washed twice in 70 % absolute ethanol then once in 100 % absolute ethanol. After drying in vacuo, the pellet was resuspended in TE [10 mM Tris pH 8.5, 1 mM EDTA] and 10 μg ml⁻¹ RNAse A added. The tube was incubated for 30 min at 37 °C. The contents were adjusted to 250 mM sodium acetate pH 5.8, divided into aliquots in microcentrifuge tubes, 2.2 volumes of ethanol added, placed at -20 °C for 10 min, then centrifuged at maximum speed in a microcentrifuge for 20 min. The pellets were washed in 100 % ethanol and air dried. They were stored at -20 °C and resuspended in an appropriate buffer just prior to use. The DNA preparations prepared in this way were checked for high molecular weight DNA without significant shearing or degradation by agarose gel electrophoresis (section 6.2.1.1)
6.1.2 Isolation of plasmid DNA from \textit{E. coli}, and restriction enzyme digestion.

Plasmid DNA was isolated by the alkaline lysis method. A fresh colony of \textit{E. coli} strain DH5\textalpha{} (see section 6.6, Materials) was used to inoculate a 50 ml polycarbonate centrifuge tube containing 10 ml of nutrient broth (Oxoid) containing an appropriate level of the selective antibiotic (usually 200 \(\mu\)g ml\(^{-1}\) ampicillin or 100 \(\mu\)g ml\(^{-1}\) kanamycin as the monosulphate). The tube was incubated overnight at 37 °C with vigorous shaking. The tube was then transferred to a benchtop centrifuge and the bacteria pelleted at maximum speed and 4 °C for 5 min. The supernatant was removed by aspiration and the pellet resuspended gently in 200 \(\mu\)l GTE [25 mM Tris pH 8.0, 10 mM EDTA, 50 mM glucose], the tube being kept on ice. To this suspension was added 200 \(\mu\)l of lysis solution [0.2 M NaOH, 1 % w/v SDS] and the tube was gently inverted until the suspension cleared. The solution was then neutralised by addition of 300 \(\mu\)l cold 200 mM potassium acetate/acetic acid pH 4.8, gently agitated, and incubated on ice for 15 min. After centrifugation for 15 min at 4 °C as before, the clear supernatant was carefully removed and transferred to a microcentrifuge tube. An equal volume of isopropanol was added, the tube mixed by inversion, and the mixture centrifuged for 15 min at room temperature in a microcentrifuge. After removal of isopropanol from the pellet by washing in ethanol and briefly drying, the pellet was resuspended in 400 \(\mu\)l TE [10 mM Tris pH 8.5, 1 mM EDTA] and RNase A added from a frozen stock of 2 mg ml\(^{-1}\) in TE at a rate of 1:100. After 20 min of incubation at 37 °C, the enzyme was removed by chloroform extraction and the aqueous phase transferred to a clean microcentrifuge tube. Ethanol was added at a rate of 2.2 volumes per volume of reaction mixture, and sodium acetate pH 5.8 to 100 mM. The tube was incubated briefly at -20 °C before centrifugation for 15 min in a microcentrifuge. The supernatant was removed by aspiration, and the pellet washed in 70 % ethanol and vacuum dried. The purified plasmid was then redissolved in sterile deionised water, quantified by agarose gel electrophoresis against known standards, and stored at -20 °C.

The DNA was used for restriction enzyme digestion as follows. The enzyme (5 units) the supplied buffer (Gibco BRL or New England Biolabs) and approximately 1 \(\mu\)g of plasmid DNA were combined in a microcentrifuge tube and incubated at the
recommended optimum temperature for 1 h. Digests with more than one enzyme were conducted with reference to the manufacturers instructions. The results of these reactions were run on agarose gels after addition of sample buffer, as described in Section 6.2.1.1.

6.1.3 Isolation of *Agrobacterium* DNA

A single colony of *Agrobacterium* from a selective plate was used to inoculate 10 ml of nutrient broth (Oxoid) in a 50 ml polycarbonate centrifuge tube containing 100 μg ml⁻¹ of both kanamycin and rifampicin. This suspension was incubated at 28 °C with shaking until it reached an approximate A₅₉₅ of 0.8 (usually 24 to 36 h). The cells were collected by centrifugation in a benchtop centrifuge for 5 min. The supernatant was removed by aspiration and the pellet resuspended in 300 μl TE (see section 6.1.2). To this was added 100 μl of 5% v/v Sarkosyl (sodium lauryl sarkosine) and 100 μl of 2.5 mg ml⁻¹ Pronase solution in TE (previously self digested at 37 °C for 1 h). The mixture was gently agitated and incubated at 37 °C for 1 h. The lysate was partially sheared by repeated passage through a 1000 μl Gilson pipette tip. It was then extracted three times with phenol/chloroform (1:1) and once with chloroform. Nucleic acids were precipitated from the aqueous phase by addition of 2.2 volumes of ethanol and sodium chloride to 250 mM, brief incubation at -20 °C, and centrifugation for 20 min in a benchtop centrifuge. The resulting nucleic acid pellet was RNase treated and reprecipitated, and used for restriction digests, as described in Section 6.1.2. Plasmid DNA was visualised by radioactive probing of gel blots (Section 6.2.1).

6.1.4 Plant RNA extraction

Leaf tissue from plants grown to the rosette stage (4-6 weeks) in the greenhouse was ground in liquid nitrogen and total RNA was extracted by the following method. One gram of frozen tissue kept at -80 °C, or fresh tissue, preferably young leaves, was ground to fine powder in liquid nitrogen. Without allowing the powder to thaw, it was transferred from the mortar into a 50 ml disposable polycarbonate centrifuge tube containing 5 ml CHCl₃, 5 ml liquefied phenol washed in Tris buffer (Fisher, Loughborough, Leics, UK) and 10 ml extraction buffer [10 mM
Tris pH 8, 1 % w/v TNS (triisopropyl-naphthalenesulphonic acid, Kodak) 4 % w/v PAS (p-amino salicylic acid, BDH, Poole, Dorset, UK) 0.2 % v/v 2-mercaptoethanol] using a spatula cooled in liquid nitrogen. The mixture was vortexed, and the phases separated at 3000 RPM for 10 min in a benchtop centrifuge. The aqueous phase was removed and RNA precipitated by addition of 0.1 volumes DEPC treated 3M sodium acetate pH 5.4 and 2.2 volumes absolute ethanol. The mixture was allowed to stand for one hour at -20 °C before centrifugation at maximum speed for 20 min in a benchtop centrifuge. The pellet was washed in ethanol and resuspended in de-ionised water. One volume of DEPC treated 8 M lithium chloride was added and the mixture allowed to stand overnight at 0 °C. RNA was then recovered by centrifugation for 30 min at 4°C at full speed in a microcentrifuge. The pellet was washed twice in 70 % ethanol to remove LiCl then once in 100 % ethanol, and dried quickly in vacuo. The pellet was resuspended in de-ionised water and stored at -20 °C. This RNA was used as "total RNA" after quantitation by A260. Poly(A)+ RNA was isolated using the polyAtract mRNA isolation kit (Promega) precisely according to the instructions.

6.1.5 Preparation of phytochrome-selective plant protein extracts

Protein extracts were prepared from 7-day-old light- or dark-grown seedlings, or from leaf tissue of rosette stage greenhouse grown plants, by what is essentially the method of Somers et al. (1991). Frozen (snap frozen in N₂ (l) in the dark and kept at -80 °C), or fresh tissue in the case of light-grown samples, was powdered in liquid nitrogen in a pestle and mortar. Extraction buffer [50 % v/v ethylene glycol, 100 mM Tris pH 8.5, 150 mM (NH₄)₂SO₄, 10 mM EDTA, 60 mM Na₂SO₃, 2 µg ml⁻¹ aprotinin (Sigma, Poole, Dorset, UK), 1 µg ml⁻¹ leupeptin, 1 µg ml⁻¹ pepstatin, 10 mM iodoacetamide, 2 mM phenylmethysuphonylfouride (PMSF) (all from Sigma)] was added at the rate of 2 ml per gram of tissue, and the mixture ground further. The contents of the mortar were transferred to a 15 ml polycarbonate centrifuge tube. One equivalent of the extraction buffer volume of de-ionised water at 0 °C was then added, as was polyethyleneimine (Sigma) to a final concentration of 0.1 % from a 10 % stock. The tube was then vortexed and centrifuged at maximum speed in a benchtop centrifuge for 20 min. The supernatant was removed to a fresh tube, and 0.25 g
(NH₄)₂SO₄ per ml was added. The tube was agitated gently at 4 °C until the powder was all dissolved, then centrifuged at maximum speed in a benchtop centrifuge for 20 min. All of the supernatant was carefully removed by aspiration, and the pellet resuspended in resuspension buffer [25 % v/v ethylene glycol, 50 mM Tris pH 8.5, 5 mM EDTA, 30 mM Na₂SO₃, 2 μg ml⁻¹ aprotinin (Sigma, Poole, Dorset, UK), 1 μg ml⁻¹ leupeptin, 1 μg ml⁻¹ pepstatin, 10 mM iodoacetamide, 2 mM phenylmethysuphonylflouride (PMSF) (all from Sigma)] to 5 % of the supernatant volume. An equal volume of sample buffer [50 mM Tris pH 6.8, 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol, 0.002 % bromophenol blue] was added, the mixture vortexed, and snap frozen in liquid nitrogen. Extracts were stored at -80 °C until needed.

6.1.6 Extraction and quantitation of chlorophyll from leaf tissue

Chlorophyll was extracted by placing one 5mm disc from an adult leaf (cut using a cork borer), or ten seedlings, into a 1.5ml microcentrifuge tube. Into this tube 1ml of N,N′-dimethylformamide (DMF) was then added. The tissue was incubated with the solvent in darkness at 4°C for three days, after which time the tissue was completely bleached. The solvent was then placed in a silica cuvette and the absorbance measured at 663.8, 646.8 and 750 nm using a diode-array spectrophotometer. The following equations, developed by Porra et al. (1989), were then used to give readings of chlorophyll a, b and a+b content in μg ml⁻¹.

\[
\begin{align*}
\text{Chl } a &= (12.00 \, A_{663.8} - 3.11 \, A_{646.8}) - A_{750} \\
\text{Chl } b &= (20.78 \, A_{646.8} - 4.88 \, A_{663.8}) - A_{750} \\
\text{Chls } a+b &= (17.67 \, A_{646.8} + 7.12 \, A_{663.8}) - A_{750}
\end{align*}
\]

6.1.7 Extraction of phycoerythrobilin from cyanobacteria

The starting point for phycoerythrobilin preparation was 10 g of lyophilised *Spirulina* spp. cells (Sigma). These were incubated overnight, at room temperature, in darkness, with 150 ml deionised water. The suspension was then centrifuged at
10,000 rpm for 10 min in a high speed centrifuge. The dark blue supernatant was decanted and filtered through standard gauge Whatman filter paper. The cell pellet was re-extracted as before and the supernatants combined. Trichloroacetic acid (TCA) was then added to the resulting phycocyanin solution to a concentration of 1 % w/v. Phycocyanin was pelleted at 10,000 rpm for 20 min, and the supernatant discarded. The pellet was washed in methanol twice, to remove water. The pellet was resuspended in methanol (150 ml) and the resulting suspension refluxed for 18 h. Protein was then removed by centrifuging at 10,000 rpm for 20 min. The methanolic solution was evaporated to dryness in rotary evaporator or vacuum centrifuge. The bilins were redissolved in chloroform (5 ml), leaving any insoluble residue. The yield was then estimated by $A_{660}$, assuming $\varepsilon = 12,300$. Yield was approximately 10 $\mu$mol. Phycocyanobilin was then precipitated from the chloroform solution in aliquots by addition of 15 volumes of n-hexane. After centrifugation at 10,000 rpm for 10 min, the supernatant was discarded, and the pellet briefly dried under vacuum. Phycocyanobilin was stored dry in darkness at -20 °C.

6.2 Gel electrophoresis, blotting, hybridisation and detection

6.2.1 Deoxyribose Nucleic Acids

6.2.1.1 Separation of DNA fragments in agarose-TAE gels

The gel electrophoresis apparatus was set up as described by the manufacturer, filling the apparatus with 1xTAE buffer [50 x TAE buffer, per litre: 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 EDTA pH 8.0, adjusted to pH 7.2 and made up to 1 litre with deionised water]. Usually 1 % agarose (w/v) was added to 1 x TAE to a volume suitable for the gel apparatus being used. The agarose suspension was heated to boiling in a microwave, or until the mixture was homogeneous. The agarose solution was then allowed to cool to 60 °C before pouring into a gel mould. By increasing the concentration of agarose to up to 2.5 %, smaller molecular weight fragments could be resolved and by decreasing it slightly (down to 0.8 %) the resolution of larger fragments could be improved. The gel was placed into the
electrophoresis apparatus. To the samples was added 1/5 of the total volume of 5 x loading buffer (50 % glycerol, 10 mM EDTA, 0.1 % Orange G dye). After thorough mixing, 20 µl of each of the samples were then were pipetted into the 30 µl wells formed by the standard comb, for the usual DNA minigel. In a separate lanes on either side of the samples, 1 µg of DNA molecular weight markers ("1 Kb ladder", Gibco BRL) in 1 x loading buffer were loaded. The gel was connected to a power supply adjusted to give a potential gradient of 5 volts cm⁻¹. The gel was run until the Orange G dye front was 1 cm from the far end of the gel. On completion of electrophoresis the DNA was stained by immersing the gel in de-ionised water, to which 1 µl ml⁻¹ 1% w/v ethidium bromide had been added, for 30 min. The DNA was then visualised by placing the gel on a UV transilluminator (UVP, Upland, USA).

6.2.1.2 Immobilisation of DNA on charged nylon membranes

This method is an adaption of the technique described by Southern (1975). For the successful transfer of DNA fragments from an agarose gel to a nylon membrane (Hybond, Amersham) the DNA in the gel must first be depurinated, and then denatured. Depurination is achieved by submerging the gel in 0.125 M HCl for 20 min, or until the bromophenol blue marker dye turns from blue to yellow. The gel is then submerged in denaturation buffer [8.766 % w/v NaCl, 2 % w/v NaOH] for 30 min during which time the bromophenol blue will return to its original colour. The gel is finally neutralised by submerging it in neutralising buffer [8.766 % w/v NaCl, 6.05 % w/v Tris, adjusted to pH 7.5]. The pre-treated gel is transferred to a nylon membrane by constructing a transfer stack. The stack consists of a tray half filled with 10 x SSC, into which a flat sponge is half submerged. Onto the sponge 2 sheets of 3 mm filter paper squares, previously wet in 10 x SSC, are placed. The gel is then positioned on the filter paper, taking care not to introduce any air bubbles. A surround of clingfilm or Nescofilm is used to prevent 'short circuit' of capillary transfer, i.e. not through the gel. On to the gel a sheet of Hybond nylon membrane is placed, again taking care not to introduce air bubbles. The transfer stack is completed with two more pre-wet 3 mm filter paper squares and a 10 cm thick stack of absorbent tissue. A 750 g weight is placed on a glass plate on top of the stack. The stack is left overnight.
to transfer DNA. The transfer stack is dismantled and the membrane surface dried on blotting paper, before 'fixing' the DNA to the membrane using a Strata-linker (Stratagene, Cambridge, UK) on the 'autocrosslink' setting, with the membrane DNA side uppermost. The membrane is then stored in clingfilm before prehybridisation.

6.2.1.3 Generation of radiolabelled DNA probes by random-primer labelling

The DNA probes were prepared by the isolation of the desired sequence, by restriction digestion of plasmid DNA (Section 6.1.2) and agarose gel electrophoresis (Section 6.2.1.1). The desired band from the gel was excised and purified according to the instructions using the Qiagen gel extraction kit. The \textit{N. tabacum} ubiquitin gene used as a loading control was provided by Dr Luis Mur in the Leicester Botany Department. The \textit{PHYB} probe was the PCR product of primer pair 4 (Section 6.3.2). The \textit{CAB 1} gene was described by Castresana et al. (1987). All the probes used in this thesis were radiolabelled by the random primer method (Feinberg and Vogelstein, 1984). A 20 µl aliquot of DNA solution in de-ionised water, containing approximately 10-50 ng of the purified, double stranded DNA to be labelled, was prepared. The DNA was denatured by heating to 95 °C for 10 min before rapidly chilling on ice for 2 min. The denatured DNA was added to a tube containing 6 µl of 5 x oligo-labeling buffer \{oligo-labeling buffer was made up of solutions A, B and C in the ratio 2:5:3. \{Solution A: 625 µl 2 M Tris-HCl pH 8, 25 µl 5 M MgCl$_2$, 18 µl 2-mercaptoethanol, 5 µl each of 3 mM dATP, dGTP, dTTP (Promega, Southhampton, UK), 350 µl SDW\} \{Solution B: 2 M Hepes pH 6.6.\} \{Solution C: random hexonucleotides (Pharmacia Biotech.) suspended in TE at 90 OD$_{260}$ units/ml.\}} 0.5 µl BSA (Pharmacia, 10 mg/ml), 0.5 µl of Klenow DNA polymerase (Gibco BRL) and 2 µl of de-ionised water. Finally 1 µl of $\alpha$-$^{32}$P dCTP (specific activity 110 TBq mol$^{-1}$, conc 370 M bq ml$^{-1}$) (Amersham) was added and the labelling reaction incubated at 37 °C for 1 hour.

The unincorporated radionucleotides were then removed from the labelling reaction as follows. A 1 ml syringe barrel was plugged with polyallomer wool, and filled with a suspension of Sephadex G-50 in TE (Section 6.1.2). The Sephadex was dried by placing the column in a 50 ml centrifuge tube and centrifuging at 2000 rpm.
for 10 min. The syringe barrel was adapted to rest in a 1.5 ml screwtop microcentrifuge tube by pushing on a 0.5 ml microcentrifuge tube with a 17 g needle hole in the base. The 1.5 ml microcentrifuge tube was placed inside a 50 ml centrifuge tube, and the column rested in the larger microcentrifuge tube. The labelling reaction was applied to the top of the column and the labelled DNA collected in the 1.5 ml microcentrifuge tube by centrifugation at 2000 rpm for 5 min. The unincorporated nucleotides remained in the column.

6.2.1.4 Hybridisation and detection of radiolabelled probes

The method described below is an adaption of the method described by Church and Gilbert (1984). The filter was prehybridised for 5 h at 65 °C in Church buffer [0.5 M NaHPO₄ pH 7.4, 7 % SDS, 1 mM EDTA] in a Hybaid oven (Hybaid, Middlesex). The radioactively labelled probe (see Section 6.2.1.3) was denatured for 5 min at 95 °C and rapidly cooled on ice. The probe was added to the prehybridisation buffer, and hybridised to the membrane overnight at 65 °C. The filter was washed twice at 60 °C for 30 min in wash A (2 x SSC and 0.1 % SDS) and then twice with wash B (0.5 x SSC and 0.1 % SDS). The radioactive probe was detected by exposing the membrane to X-ray film (GRI, UK) at -70 °C in an X-ray cassette containing an intensifying screen on the film side. The period of exposure varied from one hour to one week and was estimated by rough counting of the radioactivity of the membrane using a hand-held Geiger monitor.

6.2.2 Ribose Nucleic Acids

6.2.2.1 Separation of RNA in denaturing agarose-MOPS-formaldehyde gels

Denaturing gels were prepared by first dissolving 1 g of agarose in 87 ml of SDW by heating in a microwave. The agarose solution was allowed to cool to 60 °C, at which point 10 ml of 10 x MOPS/EDTA buffer [0.2 M MOPS pH 7.0, 50 mM sodium acetate, 10 mM EDTA] and 5.1 ml 37 % formaldehyde were added. The agarose solution was poured into a gel tray and left to solidify for 1 h. The gel was
then placed in the electrophoresis apparatus, previously filled with 1 x MOPS/EDTA buffer. RNA samples were prepared by adjusting the RNA sample to 300 mM sodium acetate pH 5.4 and precipitating the RNA with 2.5 volumes of ethanol. The RNA was pelleted in a microcentrifuge as described in Section 6.1.4, and re-dissolved in denaturing loading buffer [50 % v/v formamide, 10 % v/v 10 x MOPS/EDTA buffer, 15 % v/v 37 % formaldehyde solution, 0.1 % w/v bromocresol green, 24.9 % v/v DEPC treated de-ionised water]. The RNA samples were then denatured by heating to 70 °C for 10 min. Prior to loading 1 µl of 1 mg/ml ethidium bromide was added to each sample. Gels were electrophoresed as described for DNA gels (Section 6.2.1.1) and RNA visualised directly by UV fluorescence.

6.2.2.2 Immobilisation of RNA on charged nylon membranes

The immobilisation of RNA from agarose gels onto membranes is achieved in the same manner as that described for DNA (Section 6.2.1.2) with the following change to the protocol. The denaturation, depurination and neutralisation steps are not required. They are replaced by soaking the gel in 50 mM NaOH in 1X SSC for 20 min, then twice in 10X SSC for 20 min, before capillary transfer.

6.2.3 Proteins

6.2.3.1 Separation of proteins by SDS-PAGE

Extracted protein was separated by SDS-PAGE after the method of Laemmli (1970). Discontinuous acrylamide gels were prepared by adding 7.5 ml 30% acrylamide [29.22 % w/v acrylamide, 0.78 % w/v bisacrylamide] 7.5 ml 4x resolving gel buffer [1.5 M Tris pH 8.8, 0.4 % w/v SDS] 60 µl 10% w/v ammonium persulphate (AMPS) and 10 µl TEMED to de-ionised water to give a total volume of 30 ml. This mixture was poured between the plates of a standard mini-gel apparatus and overlayed with iso-propanol, and when set gave a resolving gel with a 7.5 % concentration of acrylamide. The iso-propanol overlay was removed by gentle washing in de-ionised water, and the stacking gel (2.4 ml 30% acrylamide, 2.5 ml 8x stacking gel buffer
[1 M Tris pH 6.8, 0.8 % w/v SDS], 60 µl AMPS, 10 µl TEMED, de-ionised water to 20 ml) poured on top until the gap was full. A square-tooth comb was inserted and the gel put aside to set. Equal amounts of protein, determined by the Bio-Rad protein assay, were added to an equal volume of 'cracking buffer' [50 mM Tris pH 6.8, 2 % w/v SDS, 5 % v/v 2-mercaptoethanol, 10 % w/v glycerol, 0.002 % w/v bromophenol blue], heated to 100 °C for 2 min and loaded into each lane with a Gilson P20 pipette. Molecular weight markers (SDS-6H, Sigma) were loaded at either side of the samples in each gel. The gel tank was filled with 90 % de-ionised water and 10 % 10 x electrophoresis buffer [0.25 M Tris / 1.92 M glycine : pH 8.3, 1 % w/v SDS]. Electrophoresis was carried out at 75 V in the stacking gel and 125 V in the resolving gel.

6.2.3.2 Immobilisation of proteins on PVDF membranes

Protein was blotted onto Immobilon P PVDF membranes (Millipore) by the semi-dry method. Chromatography paper (3 mm, Whatman) soaked in Cathode buffer [25 mM Tris pH 9.4, 40 mM 6-aminohexanoic acid (AHA), 20 % v/v methanol] was placed on the graphite cathode base of a semi-dry blotter prewetted with Cathode buffer. The electrophoresed gel was removed from the glass plates, the stacking gel removed, and placed on this paper. On the gel was placed the membrane, presoaked in methanol (30s) de-ionised water (2 min), and then placed in Anode buffer II [25 mM Tris pH 10.4, 10 % methanol]. On the membrane was placed chromatography paper presoaked in Anode buffer I [0.3 M Tris pH 10.4, 10 % methanol]. The graphite anode of the blotter was placed on this paper. Current was applied at 150 mA per minigel for at least 30 min. The membrane was removed, and stained with Ponceau S followed by a distilled water wash, to check transfer and loading. Molecular weight markers were localised by ballpoint pen marks. The Ponceau stain was removed by washing in TBS-Tween [50 mM Tris pH 7.5, 150 mM NaCl, 0.1 % polystyrene sorbitan monolaurate (Tween 20)]. The membrane was then blocked by incubation for one
hour in either Boehringer Mannheim blocking reagent (10% in TBS Tween), or 5% w/v skimmed milk powder in TBS Tween.

6.2.3.3 Immuno-localisation of membrane-bound proteins

Immunohybridisation and chemiluminescent detection were carried out with reference to the instructions using the Boehringer Mannheim Chemiluminescence Western Blotting kit (Boehringer). The antibodies AS-32 (a gift from Garry Whitelam, as culture supernatant) mAT-1 (a gift from Akira Nagatani, as both purified immunoglobulin and culture supernatant) and PMTB (a gift from Tim Kunkel, as partially purified immunoglobulin) were dissolved at approximately 10 μg ml⁻¹ in TBS Tween with either 2% skimmed milk powder or 5% Boehringer Mannheim blocking reagent. The antibody solution (5ml) was placed into a sealed plastic bag with the blocked membrane, and incubated overnight at 4°C with gentle agitation. The membrane was removed and washed in two changes of TBS Tween and one of TBS for a maximum of 30 min. The membrane was then sealed in a plastic bag with 5 × the recommended concentration of peroxidase labelled antibody conjugate (Boehringer) dissolved in TBS Tween with either 2% skimmed milk powder or 5% Boehringer Mannheim blocking reagent. After one hour of incubation at room temperature, the membrane was removed and washed in three changes of TBS Tween for a maximum of 20 min. Premixed detection reagent (Boehringer) was then applied evenly to the membrane using a 1 ml pipette in a Petri dish. After 1 min of incubation, the membrane was exposed to X-ray film in the dark for 1 min. If background was too high, the membrane was washed for a further 15 min in TBS Tween and detection reagent reapplied. If signal was too low, the exposure time was increased to a maximum of one hour, and if this failed, the experiment was repeated with longer wash times and the omission of Tween-20 from binding solutions.
6.3 DNA sequencing

6.3.1 Generation of oligonucleotide primers homologous to target sequences

The DNA sequences of oligonucleotide primers capable of amplifying specifically regions of the *N. tabacum PHYB* gene by PCR were designed as follows. The DNA sequence of the *N. tabacum PHYB* gene was obtained via the World Wide Web from the Entrez database (http://ncbi.nlm.nih.gov). The Macintosh program MacVector was used to analyse the sequence to locate suitable primer sequences. The Macintosh program Amplify was then used to confirm which primers had unique sites in the *N. tabacum PHYB* gene which did not occur in other published phytochrome genes with the exception of potato *PHYB*. These oligonucleotide sequences were modified by the addition of T3 and T7 primer sequences at the 5' ends to enable dye-labelled primer sequencing. The sequences were then converted into oligonucleotides by the Protein and Nucleic Acid Chemistry Laboratory at the University of Leicester. Primers supplied as ammonia stocks were purified by precipitation in 70 % absolute ethanol and 0.2 M sodium acetate pH 5.8. The pellet was resuspended and the single stranded DNA concentration quantitated by $A_{260}$.

6.3.2 Primer sequences used to sequence the *PHYB* gene

The following primer pairs had the T3 primer sequence at the 5' end of the forward primer and the T7 primer sequence at the 5' end of the reverse primer. The specific parts of the primer sequences were:

pair 1 forward (1f)=AGCTTACTACTCTGGTAGAACTTCCC
pair 1 reverse (1r)=AAAGCAGGGTCCTCTGTTCTAGCAGG

2f=GCTGAGATCCTCACTCTGTTGGAAC
2r=AAGCCCCAGCAGCCTCTATTGAAC

3f=GGAAATATGGGTTCTTTGCATGC
3r=CCTCGGCAATTTAAATGAATCTC
4f=TGGTGATGCAGTTTGTGGTATGG
4r=ACGCAATATGGGGTGATCAG

5f=GAAGAAGGCTGTTTTTGTGGTGG
5r=TCTTAGACATCTCCTCTCACAAGC

6f=GCGACAGATTTGAGACAGAAAACAG
6r=CCATACTTGCTCTTTGTGTAACCC

6.3.3 Amplification of Genomic and cDNA by the PCR

Total RNA isolated as described above was converted to first strand cDNA using the Gibco BRL 3' RACE kit, according to the instructions. Primers described elsewhere in this section were used under the stated conditions to amplify by RT-PCR cDNA generated from the equivalent of 25 ng total RNA. This amount represented a dilution of 1 in the series (Figure 2c). PCR was conducted under mineral oil in 500 μl microcentrifuge tubes. The reaction mixture contained 5 μl 10 x PCR buffer [750 mM Tris pH 9.0, 200 mM (NH₄)₂SO₄, 0.1 % w/v Tween 20] 5 μl of dNTP solution [2 mM dATP, dCTP, dGTP and dTTP (Promega)], 5 μl 25mM MgCl₂, 100 pmol of each primer, and 5 units of *Thermus aquaticus* DNA polymerase (Advanced Biotechnologies, Surrey) in a total volume of 50 μl. Thermal cycling conditions were [94 ºC 5', 51 ºC 1', 72 ºC 3'] (1 cycle) [94 ºC 1', 63 ºC 1', 72 ºC 2'] (29 cycles), in a Perkin Elmer DNA Thermal Cycler (ABI, Warrington, Cheshire). Products were run on a 1.2 % agarose TAE gel.

PCR amplification of genomic DNA sequences was performed using the method described under RT-PCR, unless otherwise described in the figure legend, except that the cDNA was replaced by 100 ng genomic DNA per reaction.

To generate DNA fragments for direct sequencing, four consecutive PCR reactions for each primer pair were performed and afterwards pooled. All DNA for sequencing was generated by PCR from genomic DNA, unless an intron was present between the primer binding sites, when RT-PCR was used. The correct products were
purified by agarose gel electrophoresis and the Qiagen gel purification kit (Qiagen) with the protocol modified by a 15 min vacuum drying step after the ethanol column wash, and the DNA used for fluorescent di-deoxy sequencing as described below.

6.3.4 Determination and Analysis of DNA sequence

Purified fragments were sequenced directly using dye labelled primers and terminators supplied by ABI (Warrington, Cheshire, UK) according to the supplied instructions, with the exception that 100-200 ng of PCR derived DNA were used per reaction. The sequencing was performed from the T3 and T7 primer sites and primers internal to the products, and the extension products visualised on an ABI 377 automated sequencer (ABI, Warrington, Cheshire, UK) in the Protein and Nucleic Acid Chemistry Laboratory at the University of Leicester. Both strands of DNA were sequenced to completion, and ambiguities were resolved by comparison of sequences derived from dye-primer and dye-terminator chemistries. Analysis of raw sequence data was performed on a Macintosh computer using the programs Edit View (ABI), Sequence Navigator (ABI) and Gene Jockey (Biosoft). Multiple sequence alignments were performed using the Clustal algorithm.

6.4 Genetic Transformation

6.4.1 Genetic transformation of E. coli

Transformation competent cells of E. coli were generated by the following procedure. A fresh colony of E. coli strain DH5α from a nutrient agar plate was inoculated into 2.5ml nutrient broth (Oxoid) and incubated overnight with shaking at 37°C. This culture was added to 250ml nutrient broth + 20mM MgSO₄ and grown on to $A_{590}=0.5$ (typically 3 h). Cells were collected by centrifugation and resuspended in 100 ml TFB1 [30 mM KOAc pH 5.8, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂,
15 % w/v glycerol: sterilised by filtration]. The suspension was incubated at 0 °C for 5 min, then bacteria pelleted in a benchtop centrifuge at 4 °C. The pellet was resuspended in 10 ml TFBII [10 mM MOPS pH 6.5, 75 mM CaCl₂, 10 mM RbCl, 15 % w/v glycerol]. The suspension was aliquoted into microcentrifuge tubes (100 µl per tube) and incubated on ice for 10-60 min before snap-freezing in liquid nitrogen and storage at -80°C. These cells were transformed as follows. An aliquot was thawed on ice, and plasmid DNA added. The mixture was incubated on ice for 1 h, heated to 37°C for 45 s, returned to ice for 2 min, diluted 15 times into nutrient broth and incubated at 37°C with shaking for 20 min. This suspension was then plated onto selective media.

6.4.2 Genetic Transformation of *Agrobacterium tumifaciens*

The introduction of plasmids into *Agrobacterium* was achieved by triparental mating. Single colonies of *Agrobacterium* strain LBA4404, *E. coli* strain HB101::pRK2013 and *E. coli* strain DH5α carrying the binary plasmid of interest were placed in separate vials containing 5 ml nutrient broth with 100 µg ml⁻¹ kanamycin (*E. coli*) or 100 µg ml⁻¹ rifampicin (*Agrobacterium*). After overnight incubation with shaking at 37 °C (*E. coli*) or 28 °C (*Agrobacterium*), 100 µl of each suspension was inoculated onto each of three nutrient agar plates and mixed by spreading. After overnight incubation at 28 °C, the bacterial lawn from these plates was streaked onto each of three nutrient agar plates containing 100 µg ml⁻¹ kanamycin and 100 µg ml⁻¹ rifampicin. After two days incubation at 28 °C, the colonies of transconjugant agrobacterium were inoculated into nutrient broth + kanamycin and rifampicin and grown for two days at 28 °C. A portion of the broth was added to an equal volume of sterile 50 % glycerol, snap frozen in liquid nitrogen, and stored at -80 °C. The remainder was used to confirm the presence of the plasmid by DNA isolation (section 6.1.3) and Southern blotting (section 6.2.1).
6.4.3 Genetic Transformation of *Nicotiana tabacum*

A culture of the agrobacterium strain to be used for transformation (in this case LBA4404, carrying pHbin19 plus the appropriate insert) was set up as follows. Nutrient broth (Oxoid, 10 ml) containing 100 µg ml⁻¹ rifampicin and kanamycin, was inoculated with a fresh colony of *Agrobacterium* from a nutrient agar plate containing 100 µg ml⁻¹ rifampicin. The tube containing the broth was incubated for 24-48 h at 28°C with shaking, until visibly turbid. Using a sterile Gilson pipette tip, 100µl of the above culture was used to inoculate 10 ml of fresh nutrient broth containing 100 µg ml⁻¹ kanamycin. This culture was incubated overnight at 28 °C and used as described below.

Young leaves (5-10 cm long) of tobacco (*Nicotiana tabacum* var. SR-1) were harvested from healthy greenhouse grown plants. The leaves were sterilised by submerging for 1 min in 70 % industrial methylated spirit (IMS), which was then poured off and replaced with 1 % NaOCl, 0.1 % Tween 20. The leaves were incubated in the hypochlorite solution for 30 min, with gentle shaking. The hypochlorite solution was poured off, and the leaves washed with 5 changes of autoclaved de-ionised water. In a sterile laminar flow hood, the leaves were transferred one by one to a fresh, sterile 150 mm petri dish. After removal of the edges of the leaf and the midrib, the lamina was cut into approximately 1 cm squares, using an autoclaved scalpel. The squares were transferred to another 150 mm dish, containing 50 ml liquid MSO (MS salts (Sigma) with 3 % sucrose), using autoclaved 30 cm forceps. When enough leaf squares had been cut, 2.5 ml of the overnight *Agrobacterium* culture was added to this dish using a sterile pipette. The leaves were incubated in this medium at room temperature for 30 min, with gentle shaking.

Leaf squares were dried on sterile filter paper, and transferred, upside down (i.e. veins uppermost) to MSO plates covered with sterile filter paper. These plates were incubated for two days in fluorescent light at 25 °C. The fragments were then transferred, veins downwards, to MSD4x2 (MSO + NAA, BAP) plates containing 500 mg/l augmentin and 100 mg/l kanamycin. These plates were sealed with nescofilm and incubated in fluorescent light at 25°C. After 3-4 weeks, the shoots appearing from the callus at the leaf edges were cut off with an autoclaved scalpel, as
close as possible to the base but including no callus. The shoots were transferred to powder-rounds or magenta pots containing MSO without selection. When extensive roots were visible on the shoots, they were transferred to potting compost and grown at high humidity in a propagator until growth was observed, then normally, in a greenhouse, to seed.

Once the seed was isolated and dried for 1-2 weeks, it was sprinkled on MSO plates, with the omission of sucrose and the addition of 100 mg ml\(^{-1}\) kanamycin. The ratio of kanamycin resistant : kanamycin sensitive plants was calculated. Where this was 3:1, the T2 generation was grown to seed, and plants giving 100 % kanamycin resistant seed were treated as carrying homozygous single copies of the transgene. Where 16:1 segregation was observed in the T1 seed, plants from the T2 were grown on to seed. Kanamycin resistant plants derived from seed from the T2 which showed a 3:1 segregation were grown to seed, and those plants giving seed which was 100 % kanamycin resistant were treated as carrying homozygous single copies of the transgene.

6.5 Plant Growth Conditions

6.5.1 Experiments with seedlings on defined media

*Nicotiana plumbaginifolia* seeds were germinated using gibberellic acid (GA\(_3\), Sigma, Poole, Dorset, UK) to break dormancy. Seeds were incubated in 500 µg ml\(^{-1}\) GA\(_3\) for one hour before washing with three changes of deionised water. They were then surface dried on filter paper before being sown onto half strength MS medium (Sigma) with the omission of sucrose, solidified with 0.6% agarose. Seeds were allowed to imbibe water in the dark for 2 days before germination was synchronised by a 30 min pulse of fluorescent W. They were returned to darkness for 3-5 days to germinate before placement in monochromatic light treatments. The length of time allowed for seedlings to germinate depended on the age of the seed stock and was constant within any given experiment. The R and FR sources used have been described before by Robson and Smith (1996). These light sources consisted of a mixed array of R and FR LEDs obtained from Quantum Devices, Inc., Barneveld, WI,
USA. The R LEDs had an emission maximum of 665 nm and a half-bandwidth of 27 nm. The FR LEDs had an emission maximum of 730 nm and a half-bandwidth of 29 nm. The B source was cool white fluorescent light, filtered through blue perspex and 1 M CuSO₄. Germination experiments were carried out as before except that no synchronising light pulse was given, the incubation period was ten days under R or in darkness, and, for the % germination experiments only, the seeds were placed on water agar. Fluence rate measurements were made using an Analytical Devices personal spectrometer model PS2 00A (Analytical Spectral Devices, Boulder, CO). Where not stated in the figure, fluence rates were 8 μmol m⁻² s⁻¹ for R and FR, 3.5 μmol m⁻² s⁻¹ for B, and 20 μmol m⁻² s⁻¹ for W. Monochromatic light treatments lasted for seven days unless detailed otherwise. Tetrapyrrole rescue experiments were carried out on media made by addition of tetrapyrroles dissolved by NaOH addition to BG11 medium. The control medium was adjusted to the same pH as the tetrapyrrole medium.

*Nicotiana tabacum* seeds for monochromatic light response experiments were imbibed on ½ MS medium without sucrose at 4°C for 2 days. They were then placed at 20 °C for 6 h before a synchronising light pulse was given as above. After 2 days in darkness at 20 °C, the seedlings were placed in monochromatic light treatments for 3 days.

### 6.5.2 Growth of adult plants in controlled environments

The cabinets capable of delivering W together with FR were described in detail by Keiller and Smith (1989). Briefly, they consist of a temperature and humidity controlled growth chamber to which white light of high R:FR is supplied by Cool White fluorescent tubes. The R:FR can then be modified by addition of high irradiance FR to the fluorescent W. This is achieved by means of a 20 kW bank of tungsten-halogen bulbs, mounted above the fluorescent tubes, from which the visible wavelengths above 700 nm are removed by a perspex filter, and the long wavelength infra-red radiation is removed by a water window. *Nicotiana plumbaginifolia* seeds were germinated as before (see section 6.4.1), and seedlings grown under identical
conditions in W on compost for six weeks to produce strong rosette-stage plants before transfer into W or W+FR. *Nicotiana tabacum* seeds were germinated on compost and grown for 30 days before transfer to cabinets. Both plants were grown in 4" (10 cm) square pots. Fluence rates were 92 µmol m$^{-2}$ s$^{-1}$ PAR in both cabinets (for the *N. plumbaginifolia* experiment) and 125 µmol m$^{-2}$ s$^{-1}$ (for the *N. tabacum* experiment). The W only cabinet had a R:FR of 4.31 and the W + FR cabinet had a R:FR of 0.17. Plants for DNA, RNA and protein extractions and for sexual crosses were grown from seed treated with GA$_3$ as before in compost in a heated greenhouse kept at approximately 25 °C. Plants for the 18 h day bolting experiment were also grown from seed treated with GA$_3$ as before in compost in a heated greenhouse kept at approximately 25°C. The daylength was maintained at 18 h by supplementary illumination and the experiment was performed when the ambient daylength was less than 12 h. The plants were grown together and under identical conditions.

6.5.3 Sexual crossing of *Nicotiana plumbaginifolia*

In order to cross different genotypes of *N. plumbaginifolia*, plants were first grown to flowering in a heated greenhouse with supplementary illumination. A flower with a corolla of 1-1.5 cm in length was then taken, and the outer petals removed with a scalpel. Under a binocular microscope, the corolla tube was slit lengthways, and the unripe anthers were removed using fine tweezers. Adjacent flowers and buds were removed, and the emasculated flower labelled. After a period of three days in the greenhouse, emasculated flowers which had not abscised were pollinated using an anther from a mature flower of the desired genotype. A small paper bag was then loosely fixed to the stem holding the flower, and the seed was allowed to set and the pod to shatter. The seed was then dried in a warm dry room for several weeks before gibberellin treatment and germination.
6.5.4 Entrainment and analysis of endogenous rhythms

Seeds of *N. plumbaginifolia* were GA$_3$ treated as described earlier, and germinated on compost under continuous fluorescent illumination at 22 °C. Once a rosette was formed, the seedlings were transferred to entrainment conditions of different photoperiods under a controlled temperature of 22 °C. After 2-4 weeks of entrainment, the plants were placed under continuous cool-white fluorescent illumination in a room kept at 22 °C ± 2 °C. The plants were arranged in front of a black background with the largest two healthy leaves (usually the second pair of true leaves) at 90 ° to the viewing axis. A Nikon F-801 35 mm SLR camera fitted with a 60 mm Micro-Nikkor lens, loaded with Fuji Provia colour transparency film and mounted on a tripod, was used to record the angle of the leaves to the horizontal every two hours. The exposures were triggered by an MF-21 multi-control back (Nikon), which also recorded the exact time of each exposure on the film for future reference. The transparencies were then projected onto paper and the midribs of the leaves placed at 90 ° to the viewing axis traced, along with the lip of the pot (as a horizontal reference). The angles of two leaves from at least 10 different plants were then used to calculate the mean and standard error of the leaf angle to the horizontal. Measurements of angles were performed with the aid of Sigma-Scan software and a Wacom digitising tablet.
Literature cited
Borthwick, H.A., Hendricks, S.B., Parker, M.W., Toole, E.H. and Toole V.K.


from characterisation of *Arabidopsis thaliana* mutants. Plant Cell 1, 867-880


Hamner, K.C. and Bonner, J. (1938). Photoperiodism in relation to hormones as


the photoperiodic control of tuber formation in potato. Plant J. 9, 159-166.


mutants to changes in the phytochrome photoequilibrium during the daily photoperiod. Photochem. Photobiol. 56, 611-615.


McCormac, A.C., Wagner, D., Boylan, M.T., Quail, P.H., Smith, H. and


Quail, P.H. (1994). Phytochrome genes and their expression. In: see Kendrick and
Kronenberg (1994), pp 71-104


Rood, S.B., Williams, P.H., Pearce, D., Murofushi, N., Mander, L.N. and


