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DISTINCT FUNCTIONS OF TYPE-A AND TYPE-B PHYTOCHROMES IN
PHOTOPERIODIC CONTROL OF FLOWERING IN NICOTIANA SYLVESTRIS
AND BIOTECHNOLOGICAL APPLICATIONS OF
PHYTOCHROME TRANSGENES IN CHRYSANTHEMUM

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the
Degree Doctor of Philosophy in the Graduate School of
The Ohio State University

By
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1999

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Phytochromes are the primary photoreceptors involved in the photoperiodic control of flowering, but it remains unclear what light cues are sensed by individual phytochromes. The obligate long-day plant *Nicotiana sylvestris* with a critical daylength of about 12h was used as a model to determine the functions of phyA and phyB during daylength extensions following an 8h natural short-day.

Flowering in SUA2 (PHY-A1 underproducer) was delayed compared to WT when plants were subjected to 4 or 8h daylength extensions with incandescent (Inc) light (R/FR=0.7), or with 8h fluorescent (Flu) light (R/FR=3.3), suggesting a type-A phytochrome sensing FR is required for flowering. Under an 8h Inc extension, SCB35 (PHY-B1 co-suppressor) flowered at the same time as WT, but was substantially delayed with 8h Flu. However, SCB35 flowered much earlier, and SOB36 (PHY-B1 overreproducer) later than WT under a 4h Inc extension. The results indicate phy-B1 mediating R has a dual inhibitory/promotive function in the photoperiodic control of flowering. The cross SUA2 × SCB35 flowered much later than any other genotypes under an 8h extension with Inc, and did not flower under other low intensity light extensions, demonstrating that both type-A and type-B phytochromes sensing FR and R, respectively, are required for the promotion of flowering by an optimal mixture of R and FR in *N. sylvestris*.
In addition, the biotechnological application in height control using the phytochrome transgene was exploited. Plants of chrysanthemum (*Dendranthema grandiflora* Tzvelev) cv Iridon were genetically engineered to ectopically express a tobacco *PHY-B1* gene. Transgenic plants were shorter in stature and had larger branch angles than WT. Transgenic plants also phenocopied WT grown under a filter that selectively attenuated far-red wavelengths. Furthermore, the reduction in internode length in transgenic plants was nearly identical when treated with gibberellin A₃ or a gibberellin biosynthesis inhibitor, suggesting the reduction of growth by the expressed *PHY-B1* transgene did not directly involve gibberellins. In addition, transgenic plants delayed flowering by increasing the sensitivity to night-breaks. The commercial application of this biotechnology could provide an economic alternative to the use of chemical growth regulators, and thus reducing the production cost.
Dedicated to my wife, Yihong Zhao
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I wish to thank my advisor, James Metzger, for intellectual guidance, encouragement and enthusiasm which made my interesting phytochrome research and this dissertation possible, and for his patience in correcting both my stylistic and scientific errors.

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you.
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TABLE OF CONTENTS

Abstract ........................................................................................................................... ii
Dedication ....................................................................................................................... iv
Acknowledgment ......................................................................................................... v
Vita .................................................................................................................................. vii
List of Tables .................................................................................................................. x
List of Figures ................................................................................................................. xii
List of Abbreviations ...................................................................................................... xiv
Introduction .................................................................................................................... 1

Chapters:

1. Literature review ....................................................................................................... 4
   Photoperiod controls the induction of flowering .................................................... 4
   Photoperiodic timing mechanisms in SDP ............................................................ 8
   Photoperiodism and time measurement in LDP .................................................... 19
   Different but overlapping functions of individual members of phytochromes .... 27
   Phytochromes mediate photoperiodic induction of flowering: two theories ...... 33
   Multiple phytochromes are involved in the control of flowering ....................... 38
   Molecular evidence for the involvement of a circadian clock in photoperiodic timing mechanisms .......................................................... 47
   Current hypothesis .................................................................................................. 51
   Experimental subject - *Nicotiana sylvestris*, an obligate LDP ......................... 53

viii
Research strategy: creation of PHY-A1 and PHY-B1 mis-expressors through a reverse genetic approach.................................................................................................................. 57

2. Generation and characterization of transgenic tobacco plants........................................................................................................................... 62
   Materials and methods....................................................................................................................... 66
   Results.......................................................................................................................................... 73
   Discussion.................................................................................................................................... 90

3. Type-A and type-B phytochromes have distinct functions in the photoperiodic control of flowering in the obligate long-day plant Nicotiana sylvestris........................................................................................................... 95
   Materials and methods.................................................................................................................. 100
   Results.......................................................................................................................................... 103
   Discussion.................................................................................................................................... 118

4. Other observations on phytochrome control of seed germination and stem elongation in transgenic tobacco plants ......................................................................................................................................................................................... 130
   Materials and methods .................................................................................................................. 133
   Results and discussion ..................................................................................................................... 135

5. Modification of plant architecture in chrysanthemum through ectopic expression of a tobacco phytochrome B1 gene......................................................................................................................................................................................... 145
   Introduction.................................................................................................................................. 146
   Materials and methods..................................................................................................................... 150
   Results and discussion ..................................................................................................................... 156

6. General discussion.......................................................................................................................... 176

List of References............................................................................................................................ 185
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Major photoperiodic response groups of plants</td>
<td>6</td>
</tr>
<tr>
<td>1.2. Amino acid sequence identity between PHY-B like phytochrome genes</td>
<td>29</td>
</tr>
<tr>
<td>1.3. Summary of phytochrome mutants in higher plants</td>
<td>30</td>
</tr>
<tr>
<td>2.1. Summary of transgenic lines of <em>N. sylvestris</em> misexpressing phytochrome genes</td>
<td>74</td>
</tr>
<tr>
<td>2.2. Summary of transgenic lines of <em>N. tabacum</em> cv Maryland Mammoth misexpressing phytochrome genes</td>
<td>77</td>
</tr>
<tr>
<td>3.1. Determination of the critical daylength in <em>N. sylvestris</em></td>
<td>105</td>
</tr>
<tr>
<td>3.2. Flowering responses of transgenic plants under incandescent daylength extensions close to the critical daylength</td>
<td>107</td>
</tr>
<tr>
<td>3.3. Flowering responses of transgenic plants under 8 h incandescent and fluorescent daylength extensions</td>
<td>110</td>
</tr>
<tr>
<td>3.4. Flowering responses of various genotypes under different light treatments</td>
<td>112</td>
</tr>
<tr>
<td>4.1. Seed germination of SUA, SOB and SCB35 lines</td>
<td>135</td>
</tr>
<tr>
<td>4.2. Seed germination of SUA, SOB and SCB35 lines after the brief R exposures</td>
<td>136</td>
</tr>
<tr>
<td>4.3. Seed germination of MUA8 and MUA17 lines in <em>N. tabacum</em> cv Maryland Mammoth</td>
<td>138</td>
</tr>
<tr>
<td>5.1. Summary of Iridon transformation</td>
<td>156</td>
</tr>
<tr>
<td>5.2. Ectopic expression of the tobacco PHYB-1 gene in chrysanthemum plants results in shorter stems under both LD and SD conditions</td>
<td>158</td>
</tr>
</tbody>
</table>
5.3 Ectopic expression of the tobacco \textit{PHYB-1} gene in chrysanthemum plants results in higher chlorophyll levels but does not affect leaf areas................................................................. 160

5.4 Ectopic expression of the tobacco \textit{PHYB-1} gene in chrysanthemum plants results in changes in shoot architecture.......................... 163

5.5 Comparison of the effect of exogenous GA$_3$ and 2-chlorocholine chloride (CCC) on internode length of transgenic and wildtype chrysanthemum plants........................................................................ 168

5.6 Ectopic expression of the tobacco \textit{PHYB-1} gene in chrysanthemum plants increases the sensitivity of flowering to inhibition by short night-breaks.............................................................. 171
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Absorbance spectrum of the P_r and P_f forms of phytochromes</td>
<td>10</td>
</tr>
<tr>
<td>1.2. The action of light on a circadian clock</td>
<td>16</td>
</tr>
<tr>
<td>2.1. Continuous R, FR and white light used for phenotypic screening of transgenic lines</td>
<td>72</td>
</tr>
<tr>
<td>2.2. Hypocotyl length distribution of the representative T_1 SUA2 line under continuous FR</td>
<td>75</td>
</tr>
<tr>
<td>2.3. Hypocotyl length distribution of the representative T_1 SCB35 and SOB36 lines under continuous R</td>
<td>76</td>
</tr>
<tr>
<td>2.4. Seedling phenotype of the three T_2 homozygous SUA lines grown under various conditions</td>
<td>79</td>
</tr>
<tr>
<td>2.5. Hypocotyl length of the three T_2 homozygous SUA lines grown under various conditions</td>
<td>80</td>
</tr>
<tr>
<td>2.6. Molecular characterization of SUA lines</td>
<td>81</td>
</tr>
<tr>
<td>2.7. Seedling phenotype of T_2 homozygous SCB35 and four SOB lines grown under various conditions</td>
<td>83</td>
</tr>
<tr>
<td>2.8. Hypocotyl length of T_2 homozygous SCB35 and four SOB lines grown under various conditions</td>
<td>84</td>
</tr>
<tr>
<td>2.9. Molecular characterization of SCB35 and SOB lines</td>
<td>85</td>
</tr>
<tr>
<td>2.10. PCR analysis of the crosses of SUA2 and SCB35</td>
<td>87</td>
</tr>
<tr>
<td>2.11. Hypocotyl length of the crosses of SCB35 and SUA2 lines grown under continuous red and far-red light conditions</td>
<td>87</td>
</tr>
</tbody>
</table>
2.12. PCR amplification of *PHY-B1* cDNA in the four sense *PHY-B1* transgenic lines of Maryland Mammoth tobacco....................................................................................................................88

2.13. Western blot analysis of the two MUA lines...............................................................89

3.1. The light conditions used in photoperiodic treatments........................................102

3.2. Determination of photoperiodic competence in *N. sylvestris*.................................104

3.3. Flowering responses under natural daylength from January to May in Columbus, OH (40°N).................................................................................................108

3.4. Flowering responses of SUA2 under two different R/FR ratios during the 8 h high intensity light followed by the same low intensity Flu extension...............................................................116

3.5. Flowering responses of various genotypes under 17 h high intensity LD................117

3.6. A model for phytochromes control of flowering in the LDP *N. sylvestris*.................126

4.1. Plant heights of SUA2, SCB35 and SOB36 lines under 8 h Inc or Flu extensions.................................140

4.2. Stem elongation of *N. sylvestris* WT and transgenic plants grown in 17 h high intensity LD condition..................................................................................................................143

5.1. Comparison of spectral distribution (330 to 800 nm) of sunlight passing through a plastic photoselective filter that selectively attenuates wavelength of 700 to 800 nm and clear plastic.............................................................................................................154

5.2. Identification and molecular characterization of transgenic chrysanthemum plants.................................................................................................................................157

5.3. Shoot architecture of transgenic chrysanthemums expressing the *PHY-B1* gene........................................159

5.4. Transgenic chrysanthemum plants phenocopy wildtype plants grown under FR-depleted light................................................................................................................165
LIST OF ABBREVIATIONS FREQUENTLY USED

LDP, long-day plants
SDP, short-day plants
DNP, day-neutral plants
LD, long day
SD, short day
CDL, critical daylength
CNL, critical nightlength
W, white light
Inc. incandescent
Flu, fluorescent
R, red light (660nm wavelength)
FR, far-red light (730nm wavelength)
R/FR, the ratio of R (photon flux density 655-665 nm) to FR (725-735 nm)
PFD, photon flux density
PPFD, photosynthetic photon flux density (400-700 nm)
EOD, end-of-day
NB, night-break
phy, phytochrome (holoprotein)
phy, phytochrome mutants
WT, wild-type
phyA, B, C, etc., phytochrome A, B, C, etc.
PHY, apophytochrome, phytochrome apoprotein
PHYA, B, C, etc., apophytochrome family A, B, C, etc.
PHYA, B, C, etc., genes encoding PHYA, B, C, etc.
P_r, red light absorbing form of phytochrome
P_{fr}, far-red light absorbing form of phytochrome
$P_{\text{tot}}$, total amount of $P_r$ and $P_f$
$P_{rB}$, R-absorbing form of phyB
$P_{rB}$, FR-absorbing form of phyB
$P_{rI}$, R-absorbing form of phyI
$P_{rI}$, FR-absorbing form of phyI
phyI, physiological pool I of phytochrome (light labile)
phyII, physiological pool II of phytochrome (light stable)
VLFR, very low-fluence responses
LFR, low-fluence responses
HIR, high-intensity reaction
CCC, 2-chlorocholine chloride
DIF, difference between day and night temperatures
GA, gibberellin(s)
SUA, *N. sylvestris* underexpressing *PHY-AI*
SCB, *N. sylvestris* co-suppressing *PHY-B1*
SUA, *N. sylvestris* overexpressing *PHY-B1*
INTRODUCTION

The manipulation of flowering has been the focus of farmers, horticulturists and plant breeders for centuries. It was Garner and Allard who first discovered that photoperiod controlled flowering in many plants (Garner and Allard, 1920). This modest investment in the ten-year project at USDA (only $10,000) has brought billions of dollars of benefits to farmers, horticulturists, and plant breeders (Sage, 1992). To further understand the mechanism by which plants measure the daylength is necessary for the better manipulation of flowering.

Borthwick et al. (1952a) demonstrated the photoperiodic control of flowering was mediated by the R and FR-reversible phytochrome system. Phytochrome has two photoconvertible forms, \( P_r \) and \( P_{fr} \). Subsequent physiological studies showed that the presence of \( P_{fr} \) at some time and the loss of \( P_{fr} \) at other times during a 24 h day/night cycle are required for floral induction in both SDP and LDP (Thomas and Vince-Prue, 1997). Moreover, a circadian clock is definitely involved in the photoperiodic control of flowering. However, how plants sense the different light signals (R, FR, light-on, light-off, etc.) by phytochrome, and how these signals interact with the circadian clock remains unclear.

Recent molecular genetic studies in the model plant Arabidopsis have shown that phytochrome actually represents a family of chromoproteins with distinct but overlapping
functions (Mathew and Sharrock, 1997; Quail et al., 1995; Smith, 1995; Whitelam and Delvin, 1997). Similar members of phytochrome gene family have been since found in many other plants (Robson and Smith, 1997). Thus, the central question for the role of phytochromes in the timing mechanism is: which phytochrome(s) are involved, and what is the role of the phytochrome(s).

Forward and reverse genetic approaches are two powerful tools to dissect the functions of phytochromes in the control of photomorphogenesis. It is well established that phyA and phyB have distinct but overlapping functions in seedling de-etiolation: the inhibition of hypocotyl elongation through FR and R is mediated by phyA and phyB, respectively.

Unfortunately, the use of phytochrome mutants and transgenic plants has met limited success, mostly because all phytochrome mutants are from either quantitative LDP, quantitative SDP, or DNP (for example, Childs et al., 1997; Dehesh et al., 1993; Delvin et al., 1997; Hudson et al., 1997; Kraepiel et al., 1994; Lopez-Juez et al., 1992; Nagatani et al., 1991; Reed et al., 1993; Somers et al., 1991; van Tuinen et al., 1995a, 1995b; Weller et al., 1995, 1997). Therefore, to better understand the photoperiodic timing mechanisms, a qualitative (or obligate) LDP Nicotiana sylvestris was chosen to reveal the role of two major members of phytochrome, phyA and phyB (Metzger and Zheng, 1998).

The specific objectives of this dissertation were:

1. To generate and characterize transgenic N. sylvestris lines under- or over-expressing N. tabacum PHY-A1 or PHY-B1 genes that are altered in the inhibition of hypocotyl elongation under continuous red (R) and far-red light (FR).

2
2. To use these transgenic plants to determine which phytochromes (A1 or B1) are involved in the photoperiodic control of flowering, and to delineate their specific functions. This will allow testing current hypotheses regarding the functions of individual members of phytochrome family.

3. To observe other physiological responses in growth and development by altered expression of \textit{PHY-A1} and \textit{PHY-B1} in these transgenic plants.

4. To ectopically express the \textit{PHY-B1} gene in a commercially important SDP, chrysanthemum, to exploit the biotechnological potentials of using phytochrome transgenes in height control and productivity.
CHAPTER 1

LITERATURE REVIEW

I. Photoperiod controls the induction of flowering

The flowering process can be divided into four major stages - 1) floral induction (transition from vegetative to reproductive state); 2) evocation (formation of inflorescence and floral meristems); 3) flower organ formation (pattern organization); 4) development and maturation of floral organs (tissue differentiation) (Gasser, 1993; Okada and Shimura, 1994). The commonly used term “initiation” includes the first two stages with induction occurring in the leaf and evocation occurring in the shoot apex.

The first stage in which floral induction is determined is regulated by a combination of genotype and three environmental stimuli such as light (including fluence rate, light quality and duration), temperature, and water availability (McDaniel, 1994). Among these stimuli, light is important in ensuring that flowering occurs in appropriate time.

The flowering response to the daylength was discovered by two USDA pioneers Garner and Allard (1920) working on the daylength control of flowering in *Nicotiana tabacum* cv Maryland Mammoth and *Glycine max* cv Biloxi. Such a response of plants to the relative length of day and night is called photoperiodism. This discovery is generally
recognized as the beginning of plant photomorphogenesis research. Photoperiodism is a mechanism that enables living organisms to adapt to seasonal changes in their environment, as well as to variations in latitude. Therefore, it is a major factor for the control of plant growth, development and survival.

**Photoperiodic response groups**

Since the discovery of photoperiodism, there have been numerous studies on many plant species. There are three major photoperiodic response groups: (1) long-day plants (LDP) which only flower when the daylength is *greater* than a certain period termed critical daylength (CDL), while (2) short-day plants (SDP) which only flower when the daylength becomes *less* than the CDL, and (3) day-neutral plants (DNP) which have no photoperiodic requirements for the induction of flowering. Table 1.1 lists some of common plant species and their photoperiodic requirements.

It should be noted that the term "critical daylength" does not refer to the relative daylength but whether the days are *longer* or *shorter* than a certain length in order to flower. In some cases, the CDL for SDP is longer than that for LDP. For example, a CDL of 15-16 h for some chrysanthemum and Japanese morning glory (*Pharbitis nil*) is longer than the CDL of 11.5 h for LDP *Hyoscyamus niger* (Table 1.1).

In addition, plants differ in respect to the strictness of dependence on daylength, showing an obligate or qualitative photoperiodic response in which there is an absolute requirement for a particular daylength (SD or LD). Other plants exhibit a facultative or quantitative response in which a particular daylength promotes flowering, but the plants
<table>
<thead>
<tr>
<th>Groups</th>
<th>Species</th>
<th>Critical Daylength (h)</th>
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<tbody>
<tr>
<td><strong>SDP Qualitative</strong></td>
<td><em>Chenopodium album</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Glycine max cv Biloxi</em></td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td><em>Nicotiana tabacum cv Maryland Mammoth</em></td>
<td>14</td>
</tr>
<tr>
<td></td>
<td><em>Pharbitis nil cv Violet</em></td>
<td>15-16</td>
</tr>
<tr>
<td></td>
<td><em>Xanthium strumarium</em></td>
<td>15.5</td>
</tr>
<tr>
<td><strong>Quantitative</strong></td>
<td><em>Chenopodium aristatum</em></td>
<td></td>
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<td></td>
<td><em>Dendranthema grandiflora cv White Wonder</em></td>
<td>16</td>
</tr>
<tr>
<td></td>
<td><em>Sorghum bicolor</em></td>
<td></td>
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<tr>
<td><strong>LDP Qualitative</strong></td>
<td><em>Anagallis arvensis</em></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td><em>Hyoscyamus niger (annual)</em></td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td><em>Lolium temulentum Ceres</em></td>
<td>14-16 (1 cycle)</td>
</tr>
<tr>
<td></td>
<td><em>Nicotiana sylvestris</em></td>
<td>unknown</td>
</tr>
<tr>
<td><strong>Quantitative</strong></td>
<td><em>Arabidopsis thaliana (early summer races)</em></td>
<td>4-5</td>
</tr>
<tr>
<td></td>
<td><em>Brassica alba</em></td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td><em>Hordeum vulgare (spring strains)</em></td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td><em>Lolium temulentum Ba 3081</em></td>
<td>14</td>
</tr>
<tr>
<td></td>
<td><em>Pisum sativum</em></td>
<td>unknown</td>
</tr>
<tr>
<td><strong>DNP</strong></td>
<td><em>Nicotiana tabacum cv Wisconsin 38</em></td>
<td></td>
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<tr>
<td></td>
<td><em>Solanum lycopersicum L.</em></td>
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</tr>
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</table>

Table 1.1. Major photoperiodic response groups of plants (adapted from Thomas and Vince-Prue, 1997)
will eventually flower even under non-inductive conditions (Table 1.1). For example, Arabidopsis, currently used as a model plant in developmental biology research, and pea are classified as the quantitative LDP, while *N. sylvestris* is an example of a qualitative LDP.

**Measurement of flowering**

Before a detailed discussion of photoperiodic timing mechanism in the control of flowering can commence, it is necessary to evaluate the methods for the quantitative measurement of flowering, since different methods might affect the conclusion drawn from the data. Thomas and Vince-Prue (1997) have summarized several methods frequently used: 1) the percentage of plants in any given treatment that flowered within some arbitrary period of time; 2) the number of days taken to reach some arbitrary stage of flower development, such as to the appearance of visible flower buds or to an open flower; 3) the number of nodes or leaves when the flower bud becomes visible or when flower is open. In some plants such as the SDP *Xanthium*, floral stage is assigned as an index, while in the LDP *Lolium* apex length is used.

Unfortunately, all of these methods can be misleading because they do not necessarily show that the observed effects are specific to the floral induction process which occurs in the leaf rather than in the shoot apex. The most troublesome factor is that the vegetative growth rate can be influenced by different light treatments. This could affect the measurement that expresses flowering as the time required to reach a certain developmental stage, such as days to the appearance of visible flower buds and open flower, or as a floral stage index. The number of leaves has been suggested for use especially in determinate plants, since the apex must continue to produce leaves until the
plants are induced (Thomas and Vince-Prue, 1997). Alternatively, the number of nodes can be used for both determinate and indeterminate plants. However, this is also problematic because the rate of leaf initiation can be reduced by far-red light (FR) (Smith, 1975). Indeed, when flowering was measured by days to the visible flower buds, extending SD with FR was not sufficient to promote flowering in Arabidopsis (Metzger and Zheng, 1998). But if leaf number was used to measure flowering, this extension treatment significantly reduced the number of leaves produced for flowering, leading to a different conclusion. This suggests that even leaf number is not always a precise measurement. The combination of these methods might help to analyze the flowering responses, and indeed this approach is used in this dissertation.

Perhaps the only precise method to determine the floral induction is the expression of the genes early in response to the induction signal. This would allow one to distinguish between floral induction, evocation and subsequent developmental processes. One gene, CO (CONSTANS) in Arabidopsis, and its homologues in other species, could be a candidate. CO gene encodes a transcriptional factor whose activity is induced under inductive (LD) condition and subsequently activates the expression of floral-meristem-identity genes (Coupland, 1997; Putterill et al., 1995; Simon et al., 1996).

II. Photoperiodic timing mechanisms in SDP

The length of darkness rather than light is measured in SDP

Since the absolute SDP will not flower at daylengths longer than the CDL, it appears the absolute length of the day is the controlling factor. However, if the long
inductive dark period (12-16 h) is interrupted by a brief red light (R) especially in the middle of dark period, many SDP including *Pharbitis*, *Xanthium*, and *Glycine* did not flower (review. Thomas and Vince, 1997). The experiments, called night-break (NB), have clearly demonstrated that the plants measure the length of night rather than of day. Thus, the SDP could be defined as the plants which will not flower if the dark period is shorter than the critical night length (CNL).

But this does not mean the light during the day is not important. Instead, the spectrum (light quality) during the light period is also important for SDP: R is more effective than FR for flowering. For example, in *P. nil* and *Chenopodium rubrum*, seedlings are induced by a single inductive dark period preceded and followed by continuous fluorescent light (R/FR= 9) (King, 1974). However, low intensity FR (1 h or longer) given about 9 h prior to darkness (around the subjected dawn) completely inhibited flowering in *P. nil*, and this inhibition could be reversed if two additional hours were added to the critical dark period (King, 1974).

**Night-break action is controlled by the phytochrome system**

The inhibition by NB-R can be readily reversed by a subsequent FR treatment, indicating the involvement of the R and FR-reversible pigment, termed phytochrome (Borthwick et al. 1952a). Light information is perceived by at least three different photoreceptors: phytochromes absorbing R and FR, cryptochromes, the blue/UV-A receptors, and a UV-B-absorbing pigment (Kendrick and Kronenberg, 1994). Of these three, phytochrome is the predominant and best-characterized photoreceptor family.

Phytochrome is a chromoprotein (designated as phy, see Quail et al., 1994), with
two monomers. Each monomer contains the apoprotein (designated as PHY) and a linear tetrapyrrole chromophore. The chromophore is synthesized from heme and attached to the cysteine residue on the N-terminal domain of the apoprotein. Due to the conformational changes in the chromophore, phytochrome exists in two photoconvertible forms, R-absorbing form (Pr) and FR-absorbing form (Pfr). Pr, upon absorption of a R photon, is converted to Pfr which, if irradiated by FR, can be converted back to Pr. Since, for the most part, the absorbance spectra of Pr and Pfr overlap (Fig. 1.1), a mixed light results in a photoequilibrium in which the rate of conversion of Pr to Pfr is balanced by the reverse reaction. The proportion of Pr and Pfr thus depends on the wavelength.

![Absorbance spectrum of the Pr and Pfr forms of phytochrome](image)

**Figure 1.1. Absorbance spectrum of the Pr and Pfr forms of phytochrome** (copied from Chory, 1997). Pr, red light absorbing form. Pfr, far-red light absorbing form.
Pr and Pfr forms of phytochrome have different absorbance properties, as shown in Fig. 1.1. Pr, strongly absorbs the red wavelengths (peak at about 660 nm) and also weakly in the blue region (380 nm). This is in contrast to Pfr which strongly absorbs at FR (peak at about 730 nm) and also weakly in the blue region of the spectrum (400 nm).

Since NB-R is inhibitory to flowering while NB-FR has no effect, it has been deduced that phytochrome is synthesized during the dark period as Pr and also that it is the biologically inactive form. On the other hand, Pfr is generally believed to be the only biologically active form.

Phytochrome mediates three types of physiological responses in terms of the fluence (review, Chory, 1997). (1) FR-HIR (far-red light high irradiation reactions), which are elicited by prolonged or continuous irradiation by fluences >10 mmol m^-2, is not R/FR reversible, and is involved in the control of pigment synthesis, stem elongation, leaf expansion, de-etiolation, and flowering in some species. (2) LFR (low-fluence response), which requires at least 1 mmol m^-2, exhibits classical R/FR reversibility and is involved in the control of seed germination, photoperiodic flowering induction, stem elongation and leaf expansion. (3) VLFR (very low fluence response), which is super-sensitive to low fluence down to 0.1 nmol m^-2, is not R/FR reversible (probably because FR produces enough Pfr to saturate response) and is involved in the control of germination, seedling development and expression of various genes (including phytochrome).

Further studies of NB-FR reversibility have shown that newly generated Pfr following a NB-R treatment is not stable since the inhibition of flowering can not be reversed if plants are irradiated by FR later than certain periods after NB-R. For example,
in *Xanthium*, FR given immediately after NB-R almost completely restored flowering but had no effect after about 40 min darkness, while in *Pharbitis*, NB-FR reversibility was lost within 2 min (Thomas and Vince, 1997). It also suggested that the action of Pfr to prevent flowering appears to occur very rapidly following the formation of Pfr.

**End-of-day (EOD) FR response**

During the day, a considerable fraction of phytochrome would be in the Pfr since the ratio of Pfr/Ptot is 0.6 in natural daylight (R/FR=1.2 at noon) and 0.8 in R or white fluorescent light (high R/FR) as used in many experiments (Smith, 1982). However, at EOD, the ratio of R/FR declines to less than 0.7-0.9, leading to lower Pfr (about 50% of Ptot, together with the decline in total solar irradiance (Smith, 1982). Therefore, it is assumed that this light cue maybe used by plants to signal the beginning of the dark period. If it is true, the treatment of EOD-FR should promote flowering. Indeed, for a typical SDP, *P. nil*, EOD-FR (1 h or longer) substituted for fluorescent light just prior to the 12 h darkness significantly enhanced flowering, and also shortened by 2-3 h the critical dark period required for flowering (King, 1974).

However, this is too simplistic since a brief FR at the end of an 8 h light period (much shorter than the critical daylength of 15-16 h) inhibited flowering of *P. nil* (King, 1974). Moreover, the inhibitory effect could be reversed by a subsequent brief exposure to R, indicating the involvement of the phytochrome system (King, 1974). The inhibition of flowering by FR provided at the end of photoperiods (usually 2-4 h) that are much shorter than the critical daylength has also been reported for many other SDP such as *Chenopodium, Begonia boweri, Lemna paucicostata, Xanthium*, sorghum, rice and
chrysanthemum (Thomas and Vince-Prue, 1997 and reference therein). When using 24 h light/dark cycles, the inhibition of flowering by EOD-FR is only observed when a short photoperiod is combined with a long dark period, and if the duration of the dark period is close to the CNL. flowering is promoted by EOD-FR (Thomas and Vince-Prue, 1997).

Notably the inhibition by EOD-FR in Chenopodium still occurred after as long as 50 h from the end of the light period, suggesting that the existing $P_g$ during the main light period is relatively stable (Thomas and Vince, 1997).

One interpretation of these results is that there are two different pools of $P_g$: the pre-existing pool of $P_g$ which is stable, and another, the newly produced $P_g$ from NB-R which is unstable. The possible functions of these two physiological pools of phytochrome in the control of flowering has been proposed in the two pool theory (Takimoto and Saji, 1984) and will be discussed in detail later. But in brief, the light labile pool (Pool I) is inhibitory if perceiving NB-R during the dark period, while the light stable Pool II is promotive to flowering. This leads to the paradox of why the loss of $P_g$ has different actions in the photoperiodic control of flowering.

The phytochrome system is not the timer for measuring the critical nightlength

The central question about photoperiodic timing mechanisms in SDP is how plants measure the CNL. Since the phytochrome system is involved in both NB-R and EOD-FR reactions, can phytochrome itself measure the length of the dark period?

For many years, it was assumed that the time is measured by a "hourglass" system, in which time measurement stops after a CNL has been measured and a minimum duration of light is needed to re-start dark time-keeping, i.e. to turn the
"hourglass" over (Thomas and Vince-Prue, 1997). Since phytochrome undergoes dark reversion of Pfr to Pr, it was proposed that time measurement is analogous to an hourglass in that CNL is determined by the rate of dark reversion, i.e. the time required for the level of Pfr to fall below a critical threshold level.

However, the concept that the phytochrome reversion process is part of time measurement turned out to be incorrect. First, the dark reversion property is thought to be limited to the phytochrome of dicotyledonous plants, and absent in dark-grown seedlings of the Graminae which still show photoperiodic response (Thomas and Vince-Prue, 1997). Second, dark reversion is very rapid, with a half-life of 8 min, compared to about 100 h observed in dark-grown plants and 1-2 h in plants exposed to light. When plants are subjected to EOD-FR, Pfr levels are rapidly reduced before transfer to darkness but the CNL and maximal effects of NB are unaffected (Thomas and Vince-Prue, 1997). Third, as discussed above, there is a diurnal cycle in sensitivity to R and FR, and even EOD-FR can be inhibitory (after very short light period) or promotive (when the light period is close to the critical daylength). This can not be explained by an "hourglass" mechanism initiated by the EOD-FR signal. Instead, it is possible that the processes associated with flower induction are promoted when EOD-FR signal coincides with the time of day when FR is promotive or has a neutral effect on flowering (Metzger and Zheng, 1998).

A circadian clock is involved in the time measurement but needs phytochrome to entrain

Originally, the inhibition by NB was thought to be due to the possibility that NB divided the long night into two periods of darkness, each of which would be shorter than
the CNL. However, this hypothesis does not appear to be correct, since if a NB is applied
during a 40-48 h dark period to either *Pharbitis* or *Xanthium*, the maximal inhibition of
flowering (NBmax) is always observed 8-9 h after the beginning of the long night,
despite the remaining 30-40 h of darkness longer than the CNL (Salibury and Ross,
1991). Clearly, the NB action is related in time to the beginning of the dark period. Thus,
photoperiodically sensitive plants must possess both a clock to measure the time and
photoreceptors such as phytochrome to sense the light-to-dark or dark-to-light transitions.

Many physiological experiments using non-24 h cycles have demonstrated that
the clock to measure the time is indeed a circadian clock. For example, in *Pharbitis*,
NBmax always occurred at 6-12 h and 22-36 h after the beginning of the 48, 64 or 72 h of
dark period and thus displayed a near 24 h-rhythm of NBmax (Takimoto and Hamner,
1965). Similar circadian rhythmic responses to NB-R have been reported in other SDP,
*Glycine*, *Xanthium* and *Chenopodium* (review, Takimoto and Saji, 1984; Thomas and
Vince-Prue, 1997).

The difference between the "hourglass" clock and the circadian clock is that the
circadian clock restarts nightlength measurement spontaneously after completing each 24
h cycle, whereas the "hourglass" is incapable of restarting nightlength measurement in
prolonged darkness (Thomas and Vince-Prue, 1997).

The observed circadian rhythm is coupled to an underlying endogenous
oscillatory process called a circadian oscillator or central oscillator (Fig. 1.2). The
activity of circadian clocks is best revealed under constant light or darkness. Under such
conditions, the central circadian oscillator often continues to run at least for several
cycles, and the rhythm is called to be *free running*. The *period* (the time for an oscillation
to make a complete cycle and return to the original starting position) is then close to, but not exactly, 24 h. Therefore, the term circadian (from the Latin for "about one day") is generally used. The period is affected by temperature so that timekeeping can continue accurately under different temperature conditions. Thus, the clock mechanism is temperature compensated, and within 15-30°C. the clock is better compensated. The photoperiodic timing mechanism for many plants is also fairly temperature insensitive (Thomas and Vince-Prue, 1997).

**Figure 1.2. The action of light on a circadian oscillator and coupling to circadian rhythms.** This figure is adapted from McClung (1998), Millar and Kay (1997) and Thomas and Vince-Prue (1997). In this example, the rhythm of *CAB* transcription and other two rhythms (A and B) are shown coupled to the same circadian oscillator. The components are discussed in detail in Section VII. PHY, phytochromes. CRY, cryptochromes. ELF, early flowering 3. TOC, timing of *CAB*. *CAB*, chlorophyll a/b binding proteins. CCA1, Circadian Clock Associated 1. LHY, Late elongated hypocotyl.
Because the period of the free running rhythm is not exactly 24 h, the rhythm has to be entrained (synchronized) to a 24 h cycle by the light signal. Thus, the assembly of the circadian clock requires three components: the input pathways, central oscillator, and output pathways (Millar and Kay, 1997). The input pathways transmit environmental signals (mainly light through photoreceptors such as phytochrome and cryptochrome) to the central oscillator, which has been shown in mammalian systems to be a negative feedback loop that generates the timing signals. The timing signals are then transmitted to cells via the output pathways that result ultimately in the overt rhythms that can be measured experimentally, like \textit{CAB} transcription (Fig. 1.2).

The term \textit{phase} is used for any point or stage in the cycle, and thus different rhythms have different phase relationships with the entraining light signals, resulting in peaks of activity at different times of day. Phase shift (phase delay or advance) can be observed in experiments in which a relatively short exposure is given at different phases of the free-running rhythm in constant darkness. The phase action of light is considered to be on the oscillator (Fig. 1.2).

**General conclusion about the time measurement in SDP**

In summary, there is a diurnal cycle in the sensitivity to R and FR in 24 h cycle. R given during the main light period tends to promote flowering, while FR given at the same time inhibits flowering. However, R becomes increasingly inhibitory if given during an inductive long night, with \textit{NBmax} 8-9 h after the light-off signal (equal to the middle of the 16 h dark period).

The photoperiodic time measurement in SDP was first suggested by Bünning in
1936 to be dependent on an endogenous circadian oscillator. The key point in Büning's hypothesis is the proposal that there are two phases in a 24 h cycle, a 12 h photophile (light-requiring) and a 12 h skotophile (dark-requiring) phase. Thus, light is required during the photophile phase, and light given during the skotophase inhibits flowering, consistent with a requirement of critical night-length.

Following this hypothesis, the external coincidence model has been proposed (Thomas and Vince-Prue, 1997). This model assumes there is a single photoperiodic rhythm and that light has a direct effect by preventing the induction of flowering in SDP when it is coincident with a particular light-sensitive phase of this rhythm. Thus, light has two actions, first as an entraining or rephasing signal and second as a photoperiodic inhibitor, whether induction occurs or not depends on the coincidence of an external light signal with an internal light-sensitive phase of a circadian rhythm.

This model can explain how an oscillation with a periodicity 24 h can measure the CNL in some typical SDP including *Pharbitis*, *Xanthium* and *Chenopodium* (Thomas and Vince-Prue, 1997). These SDP are one-cycle plants which can be induced by one SD cycle. In this model, the transfer to light at dawn initiates the rhythm, and the light-sensitive phase occurs about 14-15 h after the light-on signal so that in real time NBmax always occurs 14-15 h after light-on. Thus, a light-on signal is sufficient to induce flowering under these conditions unless a second pulse of light is given at the light-sensitive phase. The rhythm continues to run until about 5-6 h after the light-on when the rhythm becomes suspended for as long as plants stay in continuous light. The light-to-dark transition (dusk, or light-off signal) releases the rhythm from its suspended state. Thus, NBmax always occurs about 8-9 h after dusk or transfer to darkness, as would be
predicted form the original light-on rhythm. Therefore, the flower induction in SDP depends on whether or not the light-sensitive phase is reached before the dawn signal is experienced.

III. Photoperiodism and time measurement in LDP

Photoperiodic timing mechanism is less well characterized in LDP than in SDP and thus is much less well understood (For reviews, see Salibury and Ross, 1969; Vince-Prue, 1983; Deitzer, 1984; Thomas and Vince-Prue, 1997). However, limited studies have revealed the following characteristics of photoperiodism in LDP. Before the detailed discussion, the daylength extension which is used typically in experiments for LDP should be introduced here. LDP requires a light period longer than the CDL to flower, and thus the short day (typically 8 h) is usually extended by a period of low intensity light in order to distinguish the photoperiodic effect from the photosynthetic inputs in the flower induction process.

A high-irradiance response (HIR) rather than photosynthesis is important for flower induction during the main light period.

The responses of many LDP are frequently of a semiquantitative nature over a wide range of irradiances and durations of light. For example, when Brassica campestris plants were grown in 12 h photoperiods, the percentage of plants flowering increased with increasing irradiance. These results seemed to indicate a requirement for photosynthesis, suggesting that the realization of flowering requires a supply of
assimilates either from concurrent photosynthesis or stored assimilates.

However, accumulating evidence suggests that involvement of photosynthetic processes is unlikely. First, for equal total irradiance, a long period of low intensity was more effective than a short period of high intensity light (Friend, 1968). Second, for many LDP, only low intensity light is required for daylength extension. SD of same total energy plus sucrose cannot substitute for the LD effect. Third, in the absolute LDP _Anagallis arvensis_, the removal of CO$_2$ during the daylength extension inhibited flower initiation but flowering continued to increase at irradiances higher than the saturation value for CO$_2$ uptake (Brulfert et al., 1985). Fourth, not all LDP show irradiance dependence. For example, in _Lolium temulentum_ Ceres only the duration seems important and, for a day extension with tungsten filament light (a mixture of R and FR), the response was saturated at only 1.0 W m$^{-2}$ (Evans, 1976). It is evident that the timekeeping mechanism in LDP is not dependent on exposure to high intensity light, and in fact, the immediate products of this HIR rather than the photosynthetic product(s) is (are) critical.

**A mixture of R and FR is optimal for flowering**

In general, incandescent (Inc) light (a mixture of R and FR) is more effective than fluorescent (Flu) light (high R/FR) or FR alone to induce the flowering. In many LDP, for example, _Lolium temulentum_, _Hordeum vulgare_, petunia and _H. niger_, daylength extensions with 8 h Inc light (R/FR=1) at low fluences have been repeatedly shown to be more effective in promoting flowering than 8 h of light from Flu lamps which contain a lot of R and virtually no FR (Deitzer et al., 1979; Downs and Thomas, 1982; Thomas and Vince-Prue, 1997). In addition, 8 h extension with Flu light results in morphological
malfuncti on in flower development (failure of filament elongation) (Downs and Thomas, 1982), or even the failure to flower in some LDP such as carnation, lettuce and *Lolium* unless FR is added (Vince et al., 1964).

Action spectrum for the daylength extension has shown the peak for the induction at 700-720 nm in many LDP (Thomas and Vince, 1997). This has been interpreted as follows: since both P$_r$ and P$_{fr}$ absorb the wavelengths between 600 and 750 although with varying efficiencies (Fig. 1.1), the wavelengths of 700-720 nm would establish the optimal ratios of P$_r$ and P$_{fr}$. In other words, this action spectrum suggested the maximal promotion of flowering by the optimal mixture of P$_r$ and P$_{fr}$ during the daylength extension. However, this interpretation turned out to be incorrect, because LDP have different sensitivities to R and FR during the extension (will be discussed next) and therefore the wavelengths of 700-720 nm would allow plants to have FR or R being perceived during the early and later parts of the daylength extension.

**The timing of the response to R and FR is different during day extension**

Not only the mixture of R and FR, but also the timing of when the plants are subjected to the R or FR treatment during the extension is important for flowering in LDP. Vince (1965) observed 8 h (R+FR) and 7 h FR + 1 h R extension following 8 h SD strongly promoted flowering in the quantitative LDP *L. temulentum* (Ba 3081), while plants extended with 8 h R or 7 h R + 1 h FR remained vegetative. Furthermore, the maximal promotive effect in the obligate LDP *L. temulentum* Ceres was achieved by FR during the first part, or by R during the second part of 16 h daylength extension, while the maximal inhibitory effect by FR and R was the opposite sequence (Evans, 1976). These
results are consistent with that of Vince (1965) in which an 8 h R (0:00 to 8:00 h) treatment before the beginning of 8 h natural SD promoted flowering more than 8 h R extension from 16:00 to 24:00 h. The treatment of 7 h FR + 1 h R at the end of 16 h photoperiod can be considered as being at the beginning of the subsequent photoperiod, indicating that R is promotive during the main light period.

The conclusion that FR is much more effective in the early part of daylength extension than the later part, while R is opposite (inhibitory in the early part but promotive in the later part of the extension) was further supported by the promotion of flowering by FR interruption during the R extension. Vince (1965) found that a 2 h FR interruption gave a maximal promotion of flowering in *L. temulentum* (Ba 3081) at 3 or 4 h after EOD, and this coincided with the maximal inhibition of 2 h R when inserted into a daylength extension with FR at this time. Similar results were obtained with several other LDP including *L. temulentum* Ceres, *Petunia* and *Fuchsia* where flowering was promoted by inserting FR at early part of R extension compared with at later part (Thomas and Vince-Prue, 1997).

Perhaps the most interesting result to support the idea about inhibitory effect of R in the early part of extension is the promotion of flowering by dark interruption. In *L. temulentum* (Ba 3081), Holland and Vince (1971) interrupted a 9 h R extension following 8 h SD with various durations of darkness. They found such dark interruption promoted flowering and the effect was proportional to the duration of darkness at least up to 3 h. Moreover, 10 min FR received prior to darkness increased the effect by an amount equal to that resulting from about 45 min of darkness. Furthermore, the promoting effect of this brief FR could be reversed by a subsequent exposure to R.
These results suggested that the loss of Pfr during the early part of daylength extension and presence of Pfr during the later part are required for flower induction in LDP. This is similar to SDP which require the loss of Pfr at some time and the presence of Pfr at another. This indicates that the timing mechanism in LDP is possibly a mirror image of that in SDP. This will be discussed in detail later.

The sensitivity to FR during daylength extension displays a circadian rhythm

Indeed, similar to SDP, circadian rhythms are clearly involved in time measurement in LDP. Rhythmic responses in the promotion of flowering to added FR of 4-6 h over the whole period of 40 to 72 h of Flu light following 8 h natural SD have been reported in the LDP *L. temulentum* (Ba 3081) (Vince-Prue, 1975), *H. vulgare* (Deitzer et al., 1979) and Arabidopsis (Deitzer, 1984). In *Lolium*, the rhythmic response to 4 h FR peaked at 8-10 and 35 h after the beginning of the photoperiod. In *H. vulgare*, a circadian rhythm in the response to added 6 h FR was also evident, with a maximal promotion of flowering occurring at about 16 and 40 h from the beginning of photoperiod. Moreover, 6 h FR added between 15 and 21 h after the beginning of 72 h light gave about 50% of the promotion gained by adding FR for the whole of the experiment (Deitzer et al., 1982). In both cases of *Lolium* and *H. vulgare*, the rhythm clearly continued over at least two 24 h cycles, and thus indicating the involvement of a circadian clock.

EOD-FR has no apparent effect on flowering

It is expected that when the subjective daylength is close to the critical daylength, EOD-FR should promote flowering in LDP, similar to SDP. Downs et al. (1958) found
that EOD-FR enhanced flowering and stem elongation of *Anethum graveolens*, a quantitative LDP, and that this could be reversed by a subsequent exposure to R.

However, in another LDP, *H. niger*, although EOD-FR given following inductive photoperiods promoted flowering, the promoting effect of EOD-FR was found to be greater during the second half of a 20 day LD treatment than in the first half. This suggests that the main effect of EOD-FR is on post-inductive floral extension and flower development rather than on induction, since the induction is largely complete after the first 10 LD (Downs and Thomas, 1982). This is consistent with the known effects of EOD-FR to promote stem elongation in a number of plants where flowering is not daylength dependent (Downs et al., 1957). Thus, it appears that in terms of floral induction there are no apparent effects of EOD-FR given under both inductive and non-inductive daylength conditions, although the flowering response can be reduced by EOD-R (Downs and Thomas, 1982).

**NB-R responses and FR reversibility for LDP are not clear-cut**

Unlike many SDP, only a few LDP species (*H. vulgare* and *H. niger*) can be induced by a single NB of less than 30 min during marginally inductive long nights (Borthwick et al., 1948; Parker et al., 1950). Longer (1 to 2 h) exposures of R or Flu light can promote flowering in some LDP, however (Downs, 1956; Downs and Thomas, 1982). In addition, only limited studies show that the inductive effects can be partially reversed by subsequent FR in *H. vulgare* and *H. niger*, and no action spectrum for NB reversal in LDP has been published (Thomas and Vince-Prue, 1997).

Nevertheless, there are a few reports about the circadian rhythm in response to
NB-R in the LDP *Sinapis. Hyoscyamus* and *Lolium* (Thomas and Vince-Prue, 1997). In *Sinapis*, 8 h fluorescent light used as NB in the 40 and 64 h dark period gave two peaks (at about 10 and 34 h) and three peaks (at 6, 30, 58 h), respectively (Kinet, 1972; Kinet et al., 1973). Similarly, two peaks of promotive action were obtained using a 2 or 8 h NB in a 40 h dark period in *Lolium* (Perilleux et al., 1994; Vince-Prue, 1975). This indicates the response to an 8 h NB is similar to that of a 2 h NB. From that limited information, it appears there is a circadian rhythm in response to NB in LDP.

It should be noted that blue light is most effective for NB in *Sinapis* and perhaps other cruciferous plants. When species from other families are tested, daylength extension with blue light are generally not very effective (Thomas and Vince-Prue, 1997, and references therein). Although phytochrome absorbs blue light, the action of blue light is believed to be due to the receptor cryptochrome rather than phytochrome. This is because the absorbance of blue light by $P_r$ and $P_{fr}$ is low relative to R and FR (Fig. 1.1). Moreover, both $P_r$ and $P_{fr}$ absorbs blue light equally well (Fig. 1.1), and thus the $P_{fr}/P_{tot}$ ratios will unlikely be affected. Indeed, in Arabidopsis, a typical cruciferous LDP, irradiance with 589 nm light alone was ineffective in promoting flowering, but the addition of blue light was inductive. These two treatments established approximately the same $P_{fr}/P_{tot}$ ratios and cycling rates between $P_r$ and $P_{fr}$ (Mozley and Thomas, 1995).

From these limited studies, it is concluded that phytochrome is the photoreceptor for the NB-R in most LDP, and that the blue light effect in the Cruciferae LDP is not due to the phytochrome system but rather by the action of the distinct blue light receptors.
Photoperiodic time measurement in LDP as compared to SDP

Similar to SDP, a circadian clock is involved in the measurement of the critical daylength in LDP since they displayed the circadian rhythms to NB-R and FR during the daylength extension as discussed before. The similarity in NB-R rhythms between LDP and SDP suggested a similar timing mechanism for LDP; for example, a light exposure promoted flowering in the LDP *Lolium* at times when it inhibited flowering in the SDP *Glycine* (Hsu and Hamner, 1967). The light-on signal initiated the rhythm and the only difference was whether the second pulse of light promoted (LDP) or inhibited (SDP) flowering. But it is difficult to test whether the dusk (light-off) signal released the rhythm as in SDP, as altering the duration of the photoperiod can itself alter the flowering response in LDP. On the other hand, the mechanism is not that simple, since the critical nightlength which is the controlling factor for SDP is not crucial for LDP. In LDP, the spectral composition rather than absolute period of night is the critical factor. In addition, due to the fact that the promotion of flowering by both NB-R and FR during daylength extensions displayed circadian rhythms, flowering in LDP may be controlled by two rhythms, one in which R promotes flowering and the other in which R inhibits flowering (Vince-Prue and Takimoto, 1987).

Regardless of the difference and similarity of timing mechanisms between SDP and LDP, the basic question comes to what are the specific roles of phytochrome in the time measurement. As discussed next, which members of phytochrome family sense light-on, light-off and NB-R signal will be addressed.
IV. Different but overlapping functions of individual members of phytochromes

Phytochrome apoproteins are encoded by a gene family with multiple members

Recent molecular genetic studies have revealed that phytochrome apoproteins are encoded by a gene family with at least five members (PHYA, B, C, D & E) in Arabidopsis (Sharrock et al., 1989; Clack et al., 1994) and possibly as many as 9-13 members in tomato genome (Hauser et al., 1995). Phytochrome genes have been cloned from 11 higher plants and 6 lower plants (Xu et al., 1995).

Presumably, phytochrome A, abundant in darkness but unstable in light, belongs to Pool I, and phytochrome B and other members, relatively stable either in light or darkness, are regarded as Pool II (Furuya, 1993; Quail et al., 1995; Smith, 1995). To date, except for phyB which can be translocated from cytoplasm (in darkness) to nucleus after the exposure to R (Sakamoto and Nagatani, 1996a; Yamaguchi et al., 1999), the other phytochromes are likely in the cytoplasm (Pratt et al., 1994).

Both PHYA and PHYB contain two significant domains: N-terminal domain (about 70 KD) responsible for photosignal perception, while the C-terminal domain (about 55 KD) mediates the intramolecular signal transduction which subsequently activates the signaling pathways leading to a wide range of photomorphogenic events (Quail et al., 1995; Xu et al., 1995; Wagner & Quail, 1995). Even in the N-terminal region, there are two subdomains with one (residues 6-12) responsible for attenuating phytochrome responses and the other (residues 13-62) necessary for conformational stability and biological activity (Jordan et al., 1995a). More recently, PHYA and PHYB domain exchange experiments in which the N-terminal domain of PHYA is fused to the
C-terminal domain of PHYB, and vice versa, has shown that N-terminal domains specify the photosensory functions, while the activity of the C-terminal domains is controlled by a common biochemical mechanism (Sakamoto and Nagatani, 1996b; Wagner et al., 1996).

In Arabidopsis, phytochrome members share only about 50% identity, except for PHYB and PHYD which are about 80% identical at amino acid level, (Clack et al., 1994; Mathew and Sharrock, 1997; Sharrock and Quail, 1989). The phytochrome family is thus divided into three subgroups: PHYA, PHYB and PHYC (Mathew and Sharrock, 1997). The PHYB subgroup consists of thee members: PHYB, PHYD, and PHYE.

However, molecular phylogenetic analysis shows the three closely related species in the Solanaceae family, tomato, tobacco and potato, have slightly different phytochrome members than for Arabidopsis (Pratt et al., 1997). In tomato, there are two PHYB-like genes and one newly discovered PHYF gene (Hauser et al., 1995). Indeed, the PHYB-like genes are more identical to each other than PHYB and PHYD in Arabidopsis, and thus are designated as PHYB-1 and PHYB-2 (Hauser et al., 1995; Pratt et al., 1997; and see Table 1.2). A similar situation exists for two PHYB-like genes in tobacco and potato (Adam et al., 1997; Kern et al., 1993; Pratt et al., 1997; and see Table 1.2). Nevertheless, neither the PHYB1 nor PHYB2 are the orthologs of either PHYB or PHYD in Arabidopsis (Pratt et al., 1997; and see Table 1.2). Thus, a progenitor B PHYTOCHROME might have been duplicated independently in both the Brassicaceae and Solanaceae after these two groups of plants diverged from one another (Pratt et al., 1997). The recently identified PHYB-like gene encoded by the HLG locus in Nicotiana plumbaginifolia (Hudson et al., 1997) shares much higher sequence identity with PHYB1.
in tobacco, tomato and potato than **PHYB2** (Table 1.2), indicating it is very closely related to the **PHY-B1** in *N. tabacum*.

<table>
<thead>
<tr>
<th></th>
<th>NpB</th>
<th>PotB1</th>
<th>TomB1</th>
<th>PotB2</th>
<th>TomB2</th>
<th>AtB</th>
<th>AtD</th>
</tr>
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<tbody>
<tr>
<td>NtB1</td>
<td>97</td>
<td>92</td>
<td>93</td>
<td>87</td>
<td>90</td>
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<td>91</td>
<td>92</td>
<td>87</td>
<td>89</td>
<td>78</td>
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<td></td>
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<tr>
<td>PotB1</td>
<td></td>
<td>94</td>
<td>83</td>
<td>84</td>
<td>76</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>TomB1</td>
<td></td>
<td></td>
<td>89</td>
<td>91</td>
<td>78</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>PotB2</td>
<td></td>
<td></td>
<td></td>
<td>97</td>
<td>82</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>TomB2</td>
<td></td>
<td></td>
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<td>83</td>
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<tr>
<td>AtB</td>
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<td></td>
<td></td>
<td></td>
<td>82</td>
</tr>
</tbody>
</table>

**Table 1.2. Amino acid sequence identity between **PHY-B** like phytochrome genes.**
The percentage of identity between two genes at the amino acid sequence level is shown. The program used is SIM - Alignment Tool for protein sequences (http://www.expasy.ch/tools/sim-prot.html). The potato **PHYB2** (PotB2) and tomato **PHYB2** (TomB2) are partial sequences with 210 and 220 AA, respectively, and all the others are full length sequences with around 1130 AA. Sequences examined (and their Accession Numbers) are: *Nicotiana tabacum** PHY-B1** (NtB1, L10114), *N. plumbaginifolia** PHYB** (NpB, CAA74992), potato **PHYB1** (PotB1, S51538), potato **PHYB2** (PotB2, AAD01517), tomato **PHYB1** (TomB1, CAA05293), tomato **PHYB2** (TomB2, AAC49299), Arabidopsis thaliana **PHYB** (AtB, X17342) and A. thaliana **PHYD** (AtD, X76609).

**Different but overlapping functions of individual phytochrome members revealed through the studies of mutants and transgenic plants**

As shown in Table 1.3, phytochrome mutants have been isolated from a variety of species. In the meantime, the transgenic plants expressing phytochrome genes have also been generated (Robson and Smith, 1997). These have provided a powerful means to
<table>
<thead>
<tr>
<th>Phytochrome</th>
<th>Species (mutant)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>phyA</td>
<td><em>Arabidopsis thaliana</em> (<em>phyA, or phy2, fre1, hy8</em>)</td>
<td>Dehesh et al. (1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nagatani et al. (1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Parks and Quail (1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whitelam et al. (1993)</td>
</tr>
<tr>
<td></td>
<td><em>Nicotiana plumbaginifolia</em> (<em>pew2)</em></td>
<td>Kraepiel et al. (1994)</td>
</tr>
<tr>
<td></td>
<td><em>Pisum sativum</em> L. (<em>fun1</em>)</td>
<td>Weller et al. (1997)</td>
</tr>
<tr>
<td></td>
<td><em>Solanum lycopersicum</em> L. (<em>tri</em>)</td>
<td>van Tuinen et al. (1995a)</td>
</tr>
<tr>
<td>phyB</td>
<td><em>Arabidopsis thaliana</em> (<em>phyB, or hy3</em>)</td>
<td>Nagatani et al. (1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reed et al. (1993)</td>
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<td></td>
<td></td>
<td>Somers et al. (1991)</td>
</tr>
<tr>
<td></td>
<td><em>Brassica rapa</em> (<em>ein</em>)</td>
<td>Delvin et al. (1992; 1997)</td>
</tr>
<tr>
<td></td>
<td><em>Cucumis sativus</em> (<em>lh</em>)</td>
<td>Lopez-Juez et al. (1992)</td>
</tr>
<tr>
<td></td>
<td><em>Solanum lycopersicum</em> L. (<em>tri</em>)</td>
<td>Kerckhoffs et al. (1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>van Tuinen et al. (1995b)</td>
</tr>
<tr>
<td></td>
<td><em>Nicotiana plumbaginifolia</em> (<em>hlg</em>)</td>
<td>Hudson et al. (1997)</td>
</tr>
<tr>
<td></td>
<td><em>Pisum sativum</em> L. (<em>lv</em>)</td>
<td>Weller et al. (1995)</td>
</tr>
<tr>
<td></td>
<td><em>Sorghum bicolor</em> (<em>ma3R</em>)</td>
<td>Childs et al. (1992; 1997)</td>
</tr>
<tr>
<td>phyD</td>
<td><em>Arabidopsis thaliana</em> (<em>phyD</em>)</td>
<td>Aukerman et al. (1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delvin et al. (1999)</td>
</tr>
<tr>
<td>phyE</td>
<td><em>Arabidopsis thaliana</em> (<em>phyE</em>)</td>
<td>Delvin et al. (1998)</td>
</tr>
<tr>
<td>Chromophore</td>
<td><em>Arabidopsis thaliana</em> (<em>hy1, hy2, hy6</em>)</td>
<td>Chory et al. (1989)</td>
</tr>
<tr>
<td>biosynthesis</td>
<td></td>
<td>Parks et al. (1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Parks and Quail (1991)</td>
</tr>
<tr>
<td></td>
<td><em>Nicotiana plumbaginifolia</em> (<em>pew1</em>)</td>
<td>Kraepiel et al. (1994)</td>
</tr>
</tbody>
</table>

Table 1.3. Summary of phytochrome mutants in higher plants
Note: a, *pew2* mutant has not been well characterized but is believed to be a putative *phyA* mutant.
dissect the roles of individual phytochromes in photomorphogenesis starting from seed germination, seedling de-etiolation, to vegetative development including stem elongation, leaf development and shade-avoidance, and finally culminating in reproductive development (Robson and Smith, 1997; Smith, 1995; Whitelam and Harberd, 1994). This section discusses the roles of phytochromes in seed germination, seedling de-etiolation and vegetative development, while the functions of individual members of phytochrome in the photoperiodic control of flowering will be fully discussed in the Section VI.

**Seed germination** Photocontrol of seed germination in *Lactuca sativa* was one of the very first R/FR reversible responses observed (Borthwick et al., 1952b) and led to the discovery of phytochrome (Butler et al., 1959). Recent molecular genetic studies have shown that in Arabidopsis, phyB plays a primary role in the induction of seed germination, while phyA induces germination under continuous FR (Reed et al., 1994; Shinomura et al., 1994). The phyA dependent germination can be prevented by R-absorbing form of phyB (Shinomura et al., 1994). Furthermore, seed germination induced by LFR and VLFR is mediated by phyB and phyA, respectively (Shinomura et al., 1996). Other phytochromes are also involved in the control of seed germination since the phyA phyB double mutants responded to light treatments (Poppe and Schafer, 1997).

The mechanism of photostimulated seed germination is likely acting through GA, since R can be circumvented by the application of GA in Arabidopsis and lettuce seeds (Toyomasu et al., 1998; Yamaguchi et al., 1998). Moreover, R induces the expression of the gene encoding GA 3β-hydroxylase converting inactive GA$_{20}$ to biologically active GA$_{1}$ (Toyomasu et al., 1998; Yamaguchi et al., 1998). It is likely that different GA 3β-
hydorxylase genes are regulated by distinct phytochrome members including phyB (Yamaguchi et al., 1998).

*Seedling de-etiolation*  It is now well established that the two major members of the phytochrome family, phyA and phyB, have overlapping functions in the seedling de-etiolation process, since both phyA and phyB act to inhibit the hypocotyl elongation and promote cotyledon opening and greening (Quail et al., 1995; Smith, 1995; Whitelam and Delvin, 1997). But the photosensory functions for phyA and phyB are different: phyA mediates FR while phyB perceives R (Quail et al., 1995; Smith, 1995; Whitelam and Delvin, 1997). The other phytochrome members apparently have a minor role in the seedling de-etiolation process (Smith, 1995; Whitelam and Delvin, 1997). In addition, phyB is also active in blue light-mediated hypocotyl elongation by interacting with the blue light receptor cryptochrome (Casal and Mazzella, 1998; Hennig et al., 1999; Neff and Chory, 1998).

The mechanism for light-mediated inhibition of hypocotyl elongation has been shown to inhibit the third endocycle which is present in the dark-grown hypocotyls (Gendreau et al., 1998). More specifically, phyA mediating FR completely, and phyB sensing R, to a lesser extent, suppress the third endocycle (Gendreau et al., 1998).

*Vegetative development*  The light environment can dramatically modulate vegetative development, especially the low R/FR ratio-mediated shade avoidance response. The EOD-FR can also stimulate the shade avoidance response, resulting in morphological and physiological changes in plants such as the promotion of stem elongation, inhibition of leaf development, enhancement of apical dominance, and the acceleration of flowering (Smith, 1994, 1995). The stem elongation is the most dramatic
(Smith, 1994). It has been demonstrated that phyB-subgroup phytochromes (phyB, phyD and phyE) are responsible for shade avoidance response by mediating low R/FR ratios or the EOD-FR response in Arabidopsis, Brassica rapa, pea, and cucumber (Aukerman et al., 1997; Delvin et al., 1992, 1997, 1998, 1999; Lopez-Juez et al., 1990, 1992; Smith and Whitelam, 1997; Weller et al., 1995). PhyA is not involved in this process, although the ectopic expression of phyA can lead to constitutive suppression of shade avoidance response through increasing the sensitivity to FR (for example, Halliday et al., 1997; Robson et al., 1996).

However, it appears the phyB1 orthologs in tobacco and tomato do not mediate the low R/FR ratio or EOD-FR responses (Hudson et al., 1997; van Tuinen et al., 1995a). Instead, the phyB1 seems to be the R sensor, rather than a sensor for the R/FR ratio (Hudson et al., 1997). Whether it is true for phyB1 in all of the Solanaceae species or other plants remains to be determined.

V. Phytochromes mediate photoperiodic induction of flowering: Two theories

Phytochromes in leaves perceive the photoperiodic signals

Grafting and other physiological experiments have shown that the leaves measure daylength and under inductive condition activate an unknown long range signaling pathway from leaves to the shoot meristem tissue (in the past referred to as flower promoters or inhibitors) that results in flower induction (Bernier, 1993; O'Neil, 1992; Zeevaart, 1976). Recent biochemical studies have shown G-protein, cGMP, calcium and
Calmodulin are at least some of the signaling components that mediate the light response (Millar et al., 1994). Molecular genetic studies have also revealed the COP complex as the negative component for photomorphogenesis since the cop mutants in the dark behaved as WT plants in the light (McNellis and Deng, 1995). In addition, many genes regulating flowering time have been identified in Arabidopsis such as ELF, EMF, LD, CO and TFL (Coupland, 1997; Koornneef and Peeters, 1997; Okada and Shimura, 1994), and some genes in pea (Murfet and Reid, 1993). Two of them, LD (Lee et al., 1994) and CO (Putterill et al., 1995), have been cloned. CO has been shown to respond to light signals of R and blue wavelengths and act as a transcriptional factor to inhibit the expression of flower-meristem-identity genes such as LFY (Coupland, 1997; Guo et al., 1998; Simon et al., 1996). Despite many efforts to dissect flower induction, the photosensory functions of phyA and phyB involved in the photoperiodic control of flowering remain unclear (for recent reviews see Smith, 1995; Whitelam and Delvin, 1997).

The puzzles about the involvement of Pfr in the timing mechanism of SDP and LDP

The measurement of the length of the dark period is precise to within 30 minutes after transition to darkness for SDP (Lumsden and Vince-Prue, 1984). However, it is not clear exactly how phytochromes perceive the light-on signal to initiate the photoperiodic rhythm and the light-off signal to allow dark-time measurement to begin despite numerous physiological and biochemical studies (Vince-Prue, 1983; Thomas and Vince-Prue, 1997).

It is generally accepted that both the rapid decrease of Pfr after transition to dark
period and a remaining threshold level of Pfr during the dark period are required for photoperiodic induction for SDP (Lang, 1965). Moreover, the newly produced Pfr, which is unstable from a NB-R in the night and the existing Pfr (relatively stable) during the light period appear to have different roles, with the former being inhibitory while the latter promotive. The apparent paradoxical roles of Pfr in floral induction in SDP has led to postulation of the existence of two physiological pools of phytochromes: Pool I and Pool II being unstable and stable, respectively (Takimoto and Saji, 1984; Thomas and Vince-Prue, 1997).

Similarly, in LDP, the response to R and FR during the daylength extension is different with R being promotive and FR inhibitory during the later part of extension, while the responses are reversed during the early part of the extension. This indicates Pfr at early part of the extension is inhibitory while promotive during the later part. The latter may coincide with the fact that NB-R promotes the flowering in some LDP, suggesting Pfr is promotive at this time.

Clearly, for both SDP and LDP, the presence of Pfr is required for flowering at some time during the diurnal cycle, while at other times the loss of Pfr is required. In addition, time measurement is under the control of a circadian clock. To explain these observations and thus the action of phytochromes in photoperiodic timing mechanism, two theories have been proposed sequentially.

**Two pool theory**

In the two-phytochrome-pool theory (Takimoto & Saji, 1984; Thomas & Lumsden, 1984), Pfr I (unstable) is under the control of circadian rhythm, and thus can
measure EOD signal. Furthermore, PrI responds to R in darkness, while PrII (stable) is favorable for floral induction at any time during the dark period and responds to FR in darkness but is not influenced by the circadian clock.

This theory can explain R/FR reversibility and some other physiological phenomena in SDP. However, there are several lines of evidence arguing against the proposed roles of phytochrome in this model. First, neither the transcription of PHYA genes (A1 and A2) in tobacco (Adam et al., 1994), nor the regulation of protein levels are under the control of the circadian clock (Anderson et al., 1997). Second, the loss of Pool II phytochromes (phyB-1) in potato disrupted the NB-R inhibition of tuber formation (Jackson et al., 1996), indicating a possible role for PrII in the inhibition by NB-R. It is possible that both PrI and PrII are involved in NB-R response since the ectopic expression of oat PHYA and Arabidopsis PHYB in the SDP N. tabacum cv Maryland Mammoth increased the NB-R sensitivity (Halliday et al., 1997). Thus, the hypothesis that only PrI responds to NB-R might not be valid. Third, PrB is inhibitory to flowering rather than favorable as proposed, since the phyB mutants in Arabidopsis, sorghum and pea flowered earlier under both SD and LD conditions (Childs et al., 1992, 1997; Goto et al., 1991; Reed et al., 1994; Weller et al., 1995).

**One pool theory**

The one pool theory where a single pool of phytochrome is sufficient for time measurement has also been proposed (Thomas, 1991). The theory is based on the assumption that phyA (Pool I) levels are very low in light-grown plants because of continuous Pr destruction and inhibition by light of phyA synthesis (Rombach et al.,
Therefore phyA is probably not the photoreceptor for the detection of EOD (Thomas, 1991). It is then proposed that Pool II phytochromes alone can detect EOD in SDP (Thomas, 1991) if differentially stable Pfr-receptor complexes exist (Thomas and Vince-Prue, 1987).

There are two possibilities for the origins of the stable and unstable phytochromes from a single pool (Pool II) with differential stability. One is that a mixture of stable Pfr:Pfr homodimers and unstable Pfr:Pr heterodimers would detect light-dark transitions and yet maintain a pool of stable Pfr in the dark period using one member of Pool II phytochrome (Brockmann et al., 1987). A recent observation that phyB in oat, unlike in Arabidopsis, is not completely stable (Wang et al., 1993), raises the possibility that phyB alone mediates the photoperiodic induction. The actions of hetero- and homodimers of phytochrome have also been discussed recently (Furuya and Schafer, 1996). Despite the fact that Pfr/Ptot ratios established by R/FR treatments correlate with the stabilities in vitro of homodimers and heterodimers, this hypothesis requires that the cell can distinguish these two dimers. Moreover, whether heterodimers exist in vivo is unknown.

The alternative possibility is based on the fact that Pool II phytochrome is made up of at least two different light-stable phytochromes (Sharrock and Quail, 1989). It is argued that the modification of N-terminus can significantly alter the stability of the PHYB gene product to undergo the light-to-dark transition, while other members (e.g. PHYC) could be relatively stable (Thomas, 1991). In support of this idea is the observation that phyB in light-grown oat is also unstable, although relatively more stable than phyA (Wang et al., 1993).

However, this theory has its own problems. First, although phyA levels are very
low in light-grown plants such as oat and Arabidopsis, usually under the limits of
detection for immunological techniques (Wang et al., 1993; Quail et al., 1995). But this
does not mean that phyA has no physiological functions in light-grown plants. Moreover,
the threshold level for phyA to have physiological response may be much lower than the
detection limit using currently available tools. Supporting this is the fact that phyA
mutations in pea and Arabidopsis did affect the photoperiodic responses in these species
(Johnson et al., 1994; Weller et al., 1997). Second, there is no evidence to support the
existence of such unstable Pfr:Pr complexes in vivo. Third, phyC has been suggested by
some investigators to have only minor functions (review, Smith, 1995), and even the
existence of phyC in other plants such as tomato is questioned (Pratt et al., 1997).

Taken together, the two-pool theory is more consistent with recent molecular
genetic studies than the one-pool theory, but the specific roles of individual
phytochromes needs to be determined. Indeed, multiple phytochromes are involved in
this process, as discussed in detail in the following section.

VI. Multiple phytochromes are involved in the control of flowering

As in seed germination, seedling de-etiolation and vegetative development, the
studies using phytochrome mutants and transgenic plants have similarly provided
important insights into the two basic questions: which phytochrome(s) are involved and
what role do the individual phytochromes play in the photoperiodic control of flowering
(Metzger and Zheng, 1997; Smith, 1995; Whitelam and Delvin, 1997; Whitelam and
Harberd, 1994).
Phytochrome A is required for day length extension but the photosensory function is not clear

Johnson et al. (1994) showed that Arabidopsis *phyA-1* mutant flowered at essentially same time as WT (ecotype Landsberg erecta) under continuous Flu light (PPFD=87 μmol m⁻² s⁻¹, R/FR=5.82). When plants were subjected to continuous Flu light plus FR (same fluence rate but R/FR=0.12), the same promotive effect of flowering was observed in *phyA-1*, indicating phyA is not responsible for the supplementary high-fluence-rate FR.

When plants were subjected to 8 h SD of high fluence rate Flu light, there was no difference between WT and *phyA-1* in the flowering times as measured by either the number of days to bolting or the number of rosette leaves at bolting. However, when 8 h photoperiod was extended with low-fluence-rate Inc light (PPFD=2 μmol m⁻² s⁻¹), *phyA-1* plants flowered 20 days later and with nine leaves more than WT plants (Johnson et al., 1994). These results suggest phyA is necessary for the perception of low-fluence-rate Inc daylength extension. However, using 8 h high-fluence-rate Flu extensions, *phyA-1* displayed only a very small late-flowering phenotype compared to WT, implying that phyA only affects flowering times in the perception of FR. The effect of a phyA deficiency under low-fluence-rate Flu light extensions was not examined.

The delayed flowering response of Arabidopsis *phyA-1* under 8 h low-intensity Inc light following 8 h high-intensity Flu light has also been reported by Bagnall et al. (1995). Moreover, a transgenic line in the Arabidopsis ecotype Nossen overexpressing *PHYA* flowered earlier than WT under either 8 h SD of fluorescent light, or SD extended with 10 min low-fluence-rate FR or Inc light, or 10 min FR + 10 min R at EOD (Bagnall...
et al., 1995). The fact that the phyA overexpressors flowered early and approached a day-neutral response supports the idea that phyA is required for daylength extension with FR or Inc light.

Similarly, flowering in the quantitative LDP pea phyA mutant (*funl*) was delayed under LD condition (8 h natural SD followed by a 10 h extension of a mixture of Inc and Flu lights), a phenotype similar to WT plants under 8 h SD (Weller et al., 1997). Detailed studies showed that WT flowered earlier in response to a 16 h low-fluence-rate Inc light (R/FR=0.6) extension following 8 h natural SD, but *funl* plants were essentially unresponsive to the extension, showing a similar growth habit in both SD and extended LD conditions. These indicates that pea phyA plays a similar role as Arabidopsis phyA, being the primary phytochrome responsible for the detection of a FR-rich photoperiod extension. It was suggested that phyA controls flowering through control of either the synthesis or the transport of a flowering inhibitor (Weller et al., 1997).

However, in contrast to Arabidopsis phyA which specifically mediates Inc light extension (Johnson et al., 1994), pea phyA tends to sense both Inc and Flu light extensions. The *funl* mutant plants were similarly unresponsive to 16 h extension with either low-intensity (10 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) or high-intensity Flu light (150 \(\mu\)mol m\(^{-2}\) s\(^{-1}\), R/FR=4.8) extension, which contains essentially no FR (Weller et al., 1997). Although phyA protein in the EMS-mutagenized *funl* mutant is undetectable by the antibody, how much phyA is deficient is unclear without knowing its genetic nature of the mutant. Therefore, the observation that *funl* plants still flowered earlier when subjected to Inc than Flu extensions can be explained that the remaining phyA or other phytochromes are involved in this process.
Moreover, the Arabidopsis phyA signaling mutant *fhy1* seedlings bolted at the same time as WT seedlings but had three more leaves at this time under low-intensity extended conditions, indicating the slight delay of flowering (Johnson et al., 1994). A similar result using phyA signal transduction mutant *fhy3* in Arabidopsis has been reported (Bagnall et al., 1995). These results suggest that phyA pathway is involved in the perception of daylength extension.

**Phytochrome B is involved in flowering but its role(s) are not clear**

Phytochrome B has been shown to be inhibitory in the LDP, Arabidopsis, barley and pea, and the SDP sorghum (Bagnall et al., 1995; Childs et al., 1997; Goto et al., 1991; Reed et al., 1993; Reed et al., 1994; Weller et al., 1997). The Arabidopsis phyB mutant flowered substantially earlier with or without NB-R (1 h light in the middle of 16 h night) treatments (Goto et al., 1991; Reed et al., 1993, 1994), and also in both SD and LD (Goto et al., 1991; Bagnall et al., 1995). These results suggest an inhibitory role of phyB in flowering in Arabidopsis.

Similarly, the phyB mutant *lv* in the quantitative LDP pea (Weller et al., 1995) flowered earlier than WT in 8 h SD conditions (Weller and Reid, 1993; Weller et al., 1997). Moreover, it was shown that there was no difference in flowering times in *lv-l* when subjected to low-intensity daylength extensions with either Inc or Flu, indicating the loss of responses to the promotion of flowering by FR (Weller et al., 1997).

Recently, an early flowering mutant BMDR-1 of the quantitative LDP barley (*H. vulgare* L.) has been shown to have lower levels of phyB in the light-grown plants but normal levels in the dark as WT (Hanumappa et al., 1999). The mutant flowered at the
same time under both continuous or 12 h Flu and those supplemented with FR. However, the mutant flowered earlier than WT under all treatments (Hanumappa et al., 1999; Principe et al., 1992). Thus the destabilization of phyB in the mutant appears to make BMDR-1 insensitive to photoperiod (Hanumappa et al., 1999).

Furthermore, an early flowering mutant \((ma)^R\) of the quantitative SDP sorghum flowered earlier under a wide range of photoperiods (12 to 24 h LD), and the early maturity gene encoded by the \(Ma\) locus has been determined to be the \(PHYB\) gene (Childs et al., 1992, 1997). This indicates a general inhibitory role of phyB in the SDP.

In addition, the recent observation that transgenic potato with greatly reduced phyB through antisense suppression tuberized in both SD and LD, and lost sensitivity to an inhibitory NB-R treatment (Jackerson et al., 1996). The ability for antisense \(PHYB\) plants to disrupt the SD requirement for tuber formation indicates that phyB mediates an inhibitory role in photoperiodic induction, at least in the induction of tuber formation. Furthermore, this has led to the suggestion that phyB controls flowering by modulating the production of a graft-transmissible inhibitor (Jackson and Thomas, 1997).

In total, the evidence suggests that phytochrome B plays an inhibitory role in floral induction in both LDP and SDP. However, the overexpression of \(PHYB\) in Arabidopsis retained a daylength response like WT, when 8 h main photoperiod with Flu light was extended with Inc light. In addition, a 15 min or 3 h extension gave somewhat earlier flowering of phyB overexpressors than WT, inconsistent with the inhibitory role of phyB (Bagnall et al., 1995). Moreover, the mutation of phyB in the SDP sorghum did not remove the requirement for a 10 to 12 h dark period to express the earliest possible floral initiation (Childs et al., 1995). Thus it seems that postulated role of phyB as a
general inhibitor of flowering might be too simplistic.

Indeed, in contrast to the above "classic" phyB which senses R/FR, the likely phyB1 ortholog encoded by the HLG locus in the DNP N. plumbaginifolia is a sensor for R rather than for the R/FR ratio, and is also required for the detection of photoperiod (Hudson et al., 1997; Hudson and Smith, 1998).

The hlg mutant exhibited delayed flowering under 16 h of high R/FR, although it flowered at the same time as WT under 16 h of low R/FR (Hudson et al., 1997). Further studies showed that this DNP in fact responds to photoperiod, with a delay in flowering (about two rosette leaves) under 8 h SD compared with 18 h LD, but the flowering response of the hlg plants is negligible (Hudson and Smith, 1998). The results suggest that the phy-B1 ortholog has a promotive effect on flowering under LD at high R/FR and is required for the photoperiodic response in N. plumbaginifolia. This may be because the phy-B1 has a different function in tobacco and tomato (Hudson et al., 1997; van Tuinen et al., 1995b) than phyB in Arabidopsis, Brassica rapa, pea and cucumber which is responsible for the EOD-FR or low R/FR ratio-mediated shade avoidance response (Delvin et al., 1992, 1997; Lopez-Juez et al., 1990, 1992; Nagatani et al., 1991; Reed et al., 1993; Smith and Whitelam, 1997; Somers et al., 1991; Weller et al., 1995), as discussed above.

**Multiple phytochromes are involved in the photoperiodic control of flowering**

Night-breaks in the middle of the dark period have been shown to be effective to inhibit the flowering of many SDP and to promote the flowering in some LDP (Thomas and Vince-Prue, 1997). Under 8 h SD without NB, Arabidopsis phyA plants flowered at
the same time as WT, whereas phyB and the double mutant phyA phyB flowered at the same time but substantially earlier than WT. A 1 h NB with Flu light in the middle of a 16 h long night accelerated flowering in WT by about 6 days and with 6 fewer rosette leaves, but only accelerated flowering in phyA by about 2 days and with just 4 fewer leaves compared with SD. The NB accelerated the flowering in phyB mutant by about 3 days, whereas in phyA phyB by just 2 days. The phyA phyB produced more rosette leaves than the phyB single mutant (Reed et al., 1994). The observations that the phyA and phyA phyB mutants retained a residual NB response indicate the involvement of another photoreceptor in this process in addition to phyA and phyB.

The involvement of phyA, phyB and phyC in the inhibition of flowering by NB has also been reported in transgenic tobacco plants. Halliday et al. (1997) showed that ectopic expression of Arabidopsis PHYC in SDP cv Hicks MM (Maryland Mammoth gene was introgressed into cv Hicks) also substantially increased the delay of flowering under 30 min NB of high-intensity Flu (138 μmol m⁻² s⁻¹) centered at 8 h into the 16 h dark period, similar to plants ectopically expressing an oat PHYA or Arabidopsis PHYB. Similar to the findings in Arabidopsis, this indicates multiple phytochromes are involved in the control of flowering likely through modulating the sensitivity to NB.

In Arabidopsis, EOD-FR treatments accelerated flowering response (Bagnall et al., 1995). The phyA, phyB mutants, and phyA phyB double mutants retained the early flowering response to EOD-FR treatment (Halliday et al., 1994). Moreover, the effect of EOD-FR on flowering time in phyA phyB could be almost completely abolished if the FR treatment was followed by R, suggesting the operation of novel phytochromes in perceiving R to reverse the EOD-FR response. Clearly, phyB and other phytochromes are
involved in the perception of EOD-FR (Halliday et al., 1994).

The involvement of multiple phytochromes in the photoperiodic control of flowering has also been demonstrated using chromophore biosynthesis mutants that have lower levels of the holoproteins for all phytochromes. There are three classes of chromophore biosynthesis mutants in Arabidopsis, hyl, hy2 and hy6 (Chory et al., 1989; Parks et al., 1989; Parks and Quail, 1991). The hyl, hy2 and hy6 single mutants (Goto et al., 1991; Koornneef et al., 1995), as well as the hyl hy2 double mutants (lack phyB and reduced phyA and other phytochromes) flowered earlier than WT (Koornneef et al., 1995). Moreover, hy2 hy3 double mutants flowered earlier than hy2 and hy3 (phyB mutant) alone (Halliday et al., 1994). In addition, flowering times are altered in the chromophore synthesis mutant pewi in the DNP N. plumbaginifolia with a two-week delay in LD (Kraepiel et al., 1994). These results suggest the involvement of one or more phytochromes in addition to phyB, but the specific roles of the other phytochrome (phyC, D and E) remain unclear until the specific mutants for these phytochromes are isolated. It was argued that the other phytochrome species (phyC, D, and E and etc.) have uncertain and probably only minor functions (Smith, 1995).

Recent advances have shown that phytochromes D and E, together with phyB, also mediate the early flowering response to low R/FR. A deletion in the PHYD gene of the Ws ecotype of Arabidopsis resulted in the lack of the PHYD apoprotein, and the phyD mutant did not affect the flowering in terms of rosette leaves at the onset of flowering (Aukerman et al., 1997). However, the phyB phyD double mutants did lead to a decrease of rosette leaves, compared with WT and phyB mutants (Aukerman et al., 1997; Delvin et al., 1999). Therefore, phyD, which is believed to have been recently duplicated
from phyB in Arabidopsis. acts in conjunction with phyB in mediating EOD-FR.
Furthermore, *phyA phyB phyD* triple mutants had elongated petioles and displayed an enhanced elongation of internodes in response to EOD-FR, indicating the involvement of phyC and/or phyE in the perception of low R/FR characteristic of shade avoidance signal (Delvin et al., 1999).

Indeed, *phyB phyE* double mutants flowered earlier than did phyB mutants, although the phyE mutant itself was indistinguishable from WT plants (Delvin et al., 1998). Furthermore, compared with *phyA phyB* double mutants, *phyA phyB phyE* triple mutants in the late flowering co background flowered earlier under 8 h control conditions but displayed only a very modest additional response to EOD-FR. Nevertheless, they retained a small but significant residual promotion of internode elongation, a phenocopy of the response of *phyA phyB* to EOD-FR treatments following 8 h SD (Delvin et al., 1998). This result also suggests the involvement of other phytochromes in the process of elongation, in addition to phyA, phyB and phyE.

Neither the phyD nor phyE single mutants displayed a distinguishable phenotype compared to WT, but both phyD and phyE in either the phyA phyB or phyB background showed an enhanced flowering response to EOD-FR (Aukerman et al., 1997; Delvin et al., 1998), demonstrating that phyD and phyE are functionally redundant to phyB.

There have been no reports of phyC mutants, and *phyB phyD phyE* triple mutants or *phyA phyB phyD phyE* quadruple mutants. However, based on the available results, it was concluded that phyB, phyD and phyE, which are placed within the phyB-subgroup, are mainly responsible for the shade avoidance response by detecting low R/FR ratios and function to promote the elongation and flowering at low R/FR ratio. In this regard.
phyB plays the major role and phyD and phyE have minor roles (Aukerman et al., 1997; Delvin et al., 1998).

VII. Molecular evidence for the involvement of a circadian clock in photoperiodic timing mechanisms

As discussed earlier, it has been long recognized that the photoperiodic control of flowering in SDP and LDP is also mediated by an endogenous circadian clock entrained or synchronized by daily changes (mainly the light, delayed by a dusk signal and rephased by a dawn signal). This concept is based on many observations that a circadian rhythm in the sensitivity to R and FR exists in flowering process (Thomas and Vince-Prue, 1997).

Most organisms have evolved circadian clocks to time biological processes such as metabolic, developmental and behavioral events, rather than simply responding on a daily basis to acute cues produced by light/dark transitions. Recent advances in dissecting the internal circadian clock have been made using a reporter construct (cab2::Luc) in transgenic plants. The transcription of the CAB gene encoding the chlorophyll a/b binding protein has a 24 h rhythm, and when the expression of the luciferase (Luc) gene is induced by the promoter of the CAB gene, the bioluminescence after the spraying of the substrate luciferin can be readily observed.

This noninvasive method has allowed isolation of many toc (timing of CAB) mutants from Arabidopsis (Millar et al. 1995a). One of the mutants, toc1, which is a short-period mutant with a period of 21 h compared with 24.7 h in WT, displayed an
early flowering phenotype under 8 h SD in a 24 h cycle, with a reduction in the photoperiodic control of flowering (Somers et al., 1998b). This mutant has been shown to be a very likely component of the central oscillator, rather than being deficient in the light input pathways into the oscillator (Somers et al., 1998b). Furthermore, when the 24 h cycle was changed to 21 h cycle, tocl recovered the normal photoperiodic responses in flowering (Somers et al., unpublished data). Furthermore, some late flowering mutants also showed a correlation between flowering time and defects in circadian rhythms, with a slow clock (28 h) (Carre, 1996).

Since Arabidopsis responds to both R and FR with 2 peaks of sensitivity during a day in the R background, the sensitivity to R and FR is under the control of a circadian clock (Carre, 1996). Therefore, the early flowering phenotype of the short-period mutant (toc1) under 24 h cycle is likely because after several cycles the light signal in the 8 h SD is regarded as the NB signal during the internal dark period in tocl mutants and thus flowering is promoted. This explains why tocl mutants recovered the normal photoperiodic response if the cycle length was changed to the period length (21 h). Most importantly, the findings are consistent with the external coincidence model described earlier for SDP time measurement.

Studies on the other two closely related genes likely encoding clock components have also supported the participation of circadian clock in the control of flowering. Overexpression of CCA1 (CIRCADIAN CLOCK ASSOCIATED 1) in Arabidopsis disrupted the circadian rhythms in CAB transcription and leaf movement, and caused late flowering (Wang et al., 1998). In an independent approach, Schaffer et al. (1998) have isolated a Ds-tagged lhy (late elongated hypocotyl) mutant, which is a functionally LHY
overreproducer, similarly disrupted circadian rhythms and delayed flowering.

Taken together, it is clear that the circadian clock plays a critical role in the photoperiodic determination of flowering that has long been inferred from numerous physiological experiments in both SDP and LDP.

On the other hand, the circadian clock itself is synchronized by the light inputs which are mediated by phytochrome and the blue light receptor cryptochrome (Anderson et al., 1997; Somers et al., 1998a). The importance for this input pathway has been demonstrated by an early flowering mutant *elf3* in Arabidopsis (Hicks et al., 1996). This mutant is photoperiodic insensitive and also exhibits a conditionally aberrant circadian rhythm in leaf movement and *CAB* transcription (under constant illumination but not SD or constant dark conditions), indicating ELF3 more likely a block in the input pathways into the central oscillator. The long hypocotyl phenotype under all wavelengths of light (most notably under blue and green light) of *elf3* suggested a role for ELF3 in the phototransduction pathway (Zagotta et al., 1996). When flowering-time was analyzed under 9 h SD or 18 h LD using Flu light, *elf3* was epistatic to *hy4*, which is deficient in the blue light receptor, and *elf3 hy2* double mutants flowered earlier than either single mutant while still exhibiting photoperiodic insensitivity, indicating other photoreceptors (such as cryptochromes) at least partially regulate the signal transduction pathways controlling photoperiodism (Zagotta et al., 1996).

It has been demonstrated that the central oscillator and the light input pathways regulate many rhythmic responses including flowering, and phytochrome has been shown to sense the light signals and thus shift the phase of the circadian rhythm (review, Lumsden, 1991). However, the specific roles of phytochrome and other photoreceptors
have not been clear until recently. Using the *cab2::*Luc reporter construct, Miller et al. (1995b) were first able to detect the prolonged period (26.5 h) in the *Arabidopsis* phytochrome chromophore mutant *hyl*, compared with 25.0 h of WT under constant R, indicating the participation of phytochrome in the entrainment of the circadian clock. Additional detailed tests using complete loss-of-function alleles and overexpressors of specific phytochromes (*phyA, phyB, phyA phyB* mutants, and *PHYA* and *PHYB* overexpressors) have revealed the phytochromes involved in this process and their specific roles (Anderson et al., 1997). These results have shown that, in etiolated and green seedlings, there are similar phytochrome and circadian clock signaling pathways. (Anderson et al., 1997). Different phytochromes have distinct and overlapping roles in *CAB2* regulation: in etiolated seedlings, phyA has no role in the circadian response but phyB positively regulates the level of expression of the circadian oscillations, while the other three phytochromes (phyC, D, E) make only minor quantitative contributions to the circadian response (Anderson et al., 1997). On the other hand, in green seedlings, both phyA and phyB individually do not appear to make significant contributions to the circadian response, and therefore one or more of the remaining phytochromes must contribute significantly to the overall amplitude of the circadian oscillations (Anderson et al., 1997). Similar studies using *phyD* and *phyE* mutants will reveal how these phytochromes mediate the light in the circadian response.

More recently, Somers et al. (1998b) showed that the different spectra that entrain the clock are mediated by different photoreceptors: phyB responsible for high-fluence-rate R control, while phyA and cryptochrome 1 (CRY1) for low-fluence-rate R control. Thus the distinct functional roles for phyA and phyB in the regulation of *CAB2*
expression imply that phyA and phyB operate through different phototransduction pathways. But the independent pathways from phyA and phyB must converge at or before interaction with the transcriptional factor CGF-1/GT-1 at the level of *CAB2* promoter to induce the circadian response (Anderson et al., 1997).

In summary, it has been clearly demonstrated that phytochromes are involved in the perception of light inputs that entrain the clock in the regulation of *CAB* transcription and leaf movement. However, several questions regarding the roles of phyA and phyB in the photoperiodic control of flowering have to be addressed. Which phytochrome senses R or FR in the flower induction? In particular, which phytochrome mediates the NB-R response in Arabidopsis that is under the control of a circadian clock (Carre, 1996)? Is there a correlation between the perception of R or FR by phyA (or phyB) and the changes in clock entrainment leading to the corresponding flowering response? How much cross-talk is there between the input or output pathways, or even some components of the circadian clock and phytochrome signal transduction pathways.

**VIII. Current hypothesis**

As discussed above, it has been demonstrated that multiple phytochromes including phyA and phyB are involved in the photoperiodic control of flowering. Moreover, the circadian clock that is entrained by phytochromes and other photoreceptors is involved in the time measurement. However, the roles of the two major members phyA and phyB in photoperiodic control of flowering remain unclear.

It is essential to first determine which phytochrome(s) are involved in this
complex process. The assignment of the roles of phyA and phyB will enable us to test the current hypothesis. Moreover, the classification about the roles of phytochromes will help plant breeders more easily manipulate the flowering by modifying the photoperiodic requirements using the transgenic approach which is important for agriculture.

Based on physiological, genetic and molecular studies, the hypothesis is proposed as follows: phytochrome A senses FR and is required for daylength extension, while phytochrome B mediates R and has either inhibitory or promotive effects in the control of flowering depending the timing of R during the photoperiod.

This hypothesis can explain the different responses of the LDP to the timing of R and FR. Although in light-grown plants, phyA levels could be very low due to the long exposure to light which suppresses the \( \text{PHYA} \) expression (Quail et al., 1995), it is possible that phyA mediates the FR response in LDP in a way which does not require high phytochrome levels (Thomas and Vince-Prue, 1997). The inability of light-grown phyA mutant plants to sense the daylength extension with FR-rich Inc light clearly demonstrates the involvement of phyA in WT plants (Johnson et al., 1994; Weller et al., 1997). Thus, phyA can specifically act as a sensor for FR, either alone or in a mixture of R and FR. There are possibilities regarding the role of phyA in the promotion of flowering by FR. One is that the slight but significant reduction in \( P_{\text{R}} \text{A} \) could account for the FR response. In this case, \( P_{\text{R}} \text{A} \) is the active form and acts to inhibit flowering. The alternative possibility is that \( P_{\text{F}} \text{A} \) is the active form and promotes flowering. Thus, the fluctuation in the sensitivity to FR during the day/night cycle is likely a fluctuation of phyA signaling components that entrains the circadian clock.

On the other hand, phyB and maybe other Pool II phytochromes are proposed to
mediate R. If phyB mediates R for flower induction in LDP, the changing sensitivity to added R could be a response to phyB. The fact that phyB itself is not absolutely stable has been observed in light-grown oat plants (Wang et al., 1993) and in etiolated seedlings of Arabidopsis treated with a R pulse (Anderson et al., 1997). Moreover, in the LDP barley, the destabilization of phyB in light-grown plants has been suggested to be responsible for the early flowering response in the mutant BMDR-1 (Hanumappa et al., 1999). It is also possible that the perception of R results in the fluctuation in phyB signaling components that interacts with the clock components.

Therefore, the changing sensitivity to FR and R during the daylength extension in LDP may be simply a reflection of the changing responses of phyA and phyB, respectively. In other words, phyA sensing FR is required during the early part of daylength extension, while phyB mediating R is required for the later part of extension, and phyA and phyB acting together to have a maximal promotion of flowering under a mixture of R and FR.

IX. Experimental subject – *Nicotiana sylvestris*, an obligate LDP

Photoperiodic requirements of *N. sylvestris*

The use of obligate LDP or SDP will better address this question, since current phytochrome mutants are in backgrounds of two quantitative LDP Arabidopsis and pea, a quantitative SDP sorghum, and many other DNP such as cumcuber, tomato, and *N. plumbaginifolia*. In particular, Arabidopsis phytochrome mutants are in Ler genetic background, which is an early flowering ecotype and not sensitive to photoperiod.
Moreover, unlike most other LDP that display a fluctuation in the sensitivity to R and FR (Thomas and Vince-Prue, 1997), LD-induced promotion of flowering in Arabidopsis (Ler) is only achieved when daylength extensions contain FR while R has an inhibitory effect (Bagnall et al., 1992; Bagnall et al., 1995).

To obtain a better understanding of phytochrome control of flowering in the Nicotiana system, the qualitative (absolute or obligate) LDP N. sylvestris is used as a model in this thesis research project. N. sylvestris plants will not flower under 8 h SD and will flower following treatments of 10-14 cycles of 16 h LD after 3-4 month of growth (Neale et al., 1990). The qualitative LDP can be maintained vegetative with a rosette habit for almost indefinitely under 8 h SD before the treatments begin, and therefore all the flowering responses are related to the photoperiodic induction, excluding any effects from the developmental stages.

N. sylvestris is one of the two diploid progenitors of the tetraploid N. tabacum L. (Okamuro and Goldberg, 1985), most of varieties of which are DNP except for N. tabacum cv. Maryland Mammoth, a monogenic recessive mutant behaving as a SDP. The other parental species, N. tomentosiformis, is a SDP. Thus, it is likely that there are two distinct (LD and SD) pathways in N. tabacum such that tobacco will flower at any daylength (Metzger and Zheng, 1998). In addition, grafting experiments using the LDP N. sylvestris, DNP N. tabacum and SDP Maryland Mammoth tobacco have revealed the existence of both a flower promoter and an inhibitor (Lang, 1989). This makes the Nicotiana system an attractive model for the research of photoperiodic control of flowering.
Phytochrome gene family and their expression patterns in *Nicotiana*

*PHYA*- and *PHYB*-like phytochrome genes in *N. tabacum* have been cloned (Adam et al., 1993; Kern et al., 1993) and their expression studied (Adam et al., 1994, 1995, 1996). Since *N. tabacum* is derived from the two parental diploid species, *N. sylvestris* and *N. tomentosiformis*, the two *PHYA* genes may have individually originated from each of the parental species (Adam et al., 1993, 1997). Without the isolation of *PHYA*-like genes in *N. sylvestris* or *N. tomentosiformis*, the other possibility that both *PHY-A1* and *A2* genes can exist in any of the two species can not be excluded. However, the partial upstream cDNA sequences of *PHY-A1* and *A2* share relatively high (about 89%) homology (Adam et al., 1993). In addition, the *PHY-A1* shared high (about 90%) sequence homology with *PHYA* from the closely related species, tomato and potato at the amino acid level (Hauser et al., 1995; Heyer and Gatz, 1992a; Pratt et al., 1997). This indicates that *PHY-A1* and *A2* coding regions probably have a high sequence homology.

For the *PHYB* subgroup, the situation is more complex. In the three closely related Solanaceae species, tobacco, potato and tomato, there are two *PHYB*-like genes, *B1* and *B2*. Neither *PHYB1* nor *B2* are the orthologs of either Arabidopsis *PHYB* or *PHYD* (Adam et al., 1997; Hauser et al., 1995; Pratt et al., 1997; also see Table 1.2). PhyB1 seems to have slightly different functions in that they do not appear to measure the low R/FR ratios or EOD-FR which is characteristic of Arabidopsis phyB, phyD or phyE (Hudson et al., 1997; Pratt et al., 1997; Smith and Whitelam et al., 1997; van Tuinen et al., 1995b).

In *N. tabacum*, there are two *PHYB*-like genes (*PHY-B1* and *PHY-B2*) although *PHY-B2* has not been sequenced. It is also possible there are more than two *PHYB*-like
genes (Adam et al., 1997; Kern et al., 1993). Indeed, the likely PHY-B1 ortholog encoded by the HLG locus has been identified in N. plumbaginifolia (Hudson et al., 1997).

Therefore, PHYB genes are a complicated subgroup in the various tobacco species. There are some possibilities for the origin of PHYB genes in the tetraploid N. tabacum. Does each parental species like N. sylvestris have their own PHYB1 (and PHYB2) genes, or just one of them? Does N. sylvestris have additional PHYB-like gene? Again, the isolation and characterization of all PHYB genes in N. sylvestris will answer these questions.

**Phytochrome gene expression in N. tabacum**

The transcriptional regulation of both PHYA and PHYB-like phytochrome genes in N. tabacum has been determined (Adam et al., 1994; Adam et al., 1996; Adam et al., 1997). Both PHY-A1 and PHY-A2 genes are transcribed and expressed in a similar manner: they are down regulated by the light, with a developmentally regulated and organ and tissue-specific pattern, but are not regulated by the circadian clock (Adam et al., 1994, 1997). The highest levels of PHY-A1 and PHY-A2 mRNA are in seedlings and in the reproductive organs (gynoecium and andronoecium) of fully developed plants, while the expression levels are about 10-fold lower in other organs such as stem, leaf and root (Adam et al., 1994, 1995). Notably the expression in both stem and petiole is restricted to the vascular rings (primary and secondary phloem and companion cells), indicating a role of phy-A1 and phy-A2 in regulating the translocation of assimilates, as well as photoperiodic signals (Adam et al., 1997).

The spatial expression pattern of PHY-B1 is similar to PHY-A1 and PHY-A2, relatively high in seedlings and in stems, petiole, leaves and various flower organs (Adam
et al., 1996). The similar localization of PHY-B1 promoter activity (in phloem and companion cells of stem, petiole and leaf) indicates a possible role for phy-B1 in the control of translocation of assimilate or signaling molecules as well (Adam et al., 1997). The transcription is, similar to tobacco PHYA genes, in that it is not regulated by a circadian rhythm (Adam et al., 1996). However, the expression of PHY-B1 has several characteristics distinct from those exhibited by PHY-A1. First, in contrast to the tobacco PHYA genes and Arabidopsis PHYA and PHYB genes (Adam et al., 1994; Somers et al., 1995), the expression of PHY-B1 gene is below detection level in roots or root tips (Adam et al., 1996). Second, the transcription is not significantly down-regulated by light, similar to potato PHYB-1 genes (Heyer and Gatz, 1992b), indicating that, unlike PHYA genes, phytochrome does not play a major role in regulating the transcription of the PHYB-1 genes. Third, the abundance of the PHY-B1 transcript does not seem to be regulated developmentally; it is comparable at all developmental stages examined (Adam et al., 1996).

XII. Research strategy: creation of PHY-A1 and PHY-B1 mis-expressors through a reverse genetic approach

Currently, the two experimental approaches often utilized to define the functions of phytochromes are 1) conventional or forward genetics (mutant); and 2) reverse genetics i.e. transgenic mis-expressors (Whitelam & Harberd, 1994). Physiological analysis of mutants deficient in phytochrome synthesis or signaling is a useful tool to identify the roles of phyA and phyB. These mutants exist in Arabidopsis, Brassica rapa,
Pisum, Sorghum, Cucumis sativus, and Solanum lycopersicum (Table 1.3). But no mutants specifically deficient in phyA or phyB in the obligate N. sylvestris have been found. In the DNP N. plumaginifolia, there are two reports about phytochrome mutants: a putative chromophore biosynthesis mutant pewl and a possible phyA mutant pew2 (Kraepiel et al., 1994), and the PHY-B1-like hlg-1 and hlg-2 mutants (Hudson et al., 1997).

Since phytochrome apoproteins expressed by phytochrome transgenes can be attached to the endogenous chromophore, and become spectrophotometrically and biologically functional in plants, the transgenic approach has provided some important insights into functions of phyA and phyB (Whitelam and Harberd, 1994; Robson and Smith, 1997). In this thesis project, transgenic expression of sense and antisense PHY-A1 and PHY-B1 genes will be used to delineate the roles of type-A and type-B phytochromes in photoperiodic timing mechanisms.

Under-expression of PHY-A1 and PHY-B1 genes

The powerful approaches to suppress the expression of PHY-A1 and PHY-B1 genes could be at the three levels: 1) Down-regulation of transcription through antisense RNA and co-suppression or so-called ‘‘sense gene silencing’’ (review, Meyer and Saedler, 1996). 2) Posttranscriptional stability of mRNA or directly blocking translation, including antisense RNA (Bourque, 1995), antisense oligonucleotides (usually 20 bases designed as drugs), co-suppression (Meyer and Saedler, 1996), and ribozymes (Barinaga, 1993). 3) Protein function such as anti-sense protein (Owen et al., 1992), microinjection of antibody, or expression of trans-dominant negative mutant form of proteins.
Anti-sense protein (usually antibody) can specify and react with the corresponding phytochrome and thus causes phytochrome inactive. There is one report on the inhibition of phytochrome through anti-sense protein (Owen et al., 1992).

Antisense RNA can suppress gene expression via coupling sense RNA to interfere the stability, export and translation of the corresponding phytochrome mRNA. To date, only two reports in which PHYA and PHYB1 expression is suppressed in potato by antisense RNA have been published (Heyer et al., 1995; Jackson et al., 1996).

In potato, the antisense PHYB1 does not suppress PHYA and PHYB2 gene expression (Jackson et al., 1996; Pratt et al., 1997). Similarly, since PHY-A1 and PHY-B1 share about 57% nucleic acid sequence identity, it is reasonable to expect that the expression of PHY-A1 and PHY-B1 as antisense constructs will likely suppress the expression of the corresponding PHYA1 and PHYB1 genes without affecting the expression of PHYB1 and PHYA1, respectively.

Co-suppression, or sense gene silencing, was first discovered during an attempt to overexpress a CHS (chalcone synthase) gene in petunia but resulted instead in a null phenotype due to the suppression of the CHS expression (Napoli et al., 1990; van der Krol et al., 1990). It was revealed that both the transgene and the endogenous gene had become "co-suppressed". Further studies have shown that gene silencing falls into two classes: transcriptional and posttranscriptional regulation (Meyer and Saedler, 1996). Nuclear run-on assays can distinguish whether the silencing is due to transcriptional or postranscriptional (for example, Park et al., 1996; van Blokland et al., 1994).

Transcriptional silencing can be triggered by either trans silencing in which homologous promoters present at different genomic locations in a single transgenic plant,
or "positional effects" (Matzke and Matzke, 1995; Park et al., 1996). The positional effects result from the chromosomal position into which a transgene inserts and the sequence surrounding the insertion site regulate the turnover of gene expression (Matzke and Matzke, 1995; Park et al., 1996).

In contrast, posttranscriptional silencing often, but not always, occurs when the transgene transcripts in the nucleus are abundant, and it is triggered at the level of mRNA processing, localization, and/or degradation, resulting in the turnover of mRNA (Meyer and Saedler, 1996).

There are some limitations to antisense suppression or sense gene silencing. Quite often the "null" mutants can not be obtained or no suppression can be observed. It may be necessary to select more appropriate promoters, or combine with certain enhancers, and/or modify signals for RNA transport and stability (Bourque, 1996).

**Over-expression of PHY-B1 gene**

Once individual phytochrome gene is overexpressed in tobacco plants, the effects of overproduction of either phy-A1 or phy-B1 on floral induction can be examined and therefore their roles can be determined. This strategy has also been applied in defining physiological functions of phyA, phyB and phyC (Halliday et al., 1997; Robson and Smith, 1997; Wagner et al., 1991). Since only the full-length PHY-B1 cDNA is available, *PHY-B1* was overexpressed in transgenic plants and the effects of overproduced *PHY-B1* gene product (using the CaMV 35S promoter) on the photoperiodic control of flowering in the *N. sylvestris* species was similarly assessed.

This strategy has two main limitations. One is the reliability in assigning the roles
to the overexpressed phytochrome, since the observed physiological behavior of these transgenic plants are not the simple opposite of the behavior of the corresponding phytochrome null mutants (Whitelam and Harberd, 1994). The overproduced phytochrome will almost certainly have pleiotropic effects on plant growth and development. The other is that the CaMV 35S promoter drives the genes to express in vascular tissues (especially in the stem) and not necessary in leaves where site of perception of photoperiod resides.
CHAPTER 2

GENERATION AND CHARACTERIZATION OF TRANSGENIC TOBACCO PLANTS

Abstract

In this study, antisense *Nicotiana tabacum* *Nt-PHY-A1* and sense and antisense *Nt-PHY-B1* genes were transferred into two tobacco species, the LDP *Nicotiana sylvestris* and the SDP *Nicotiana tabacum* cv Maryland Mammoth. The putative transgenic *N. sylvestris* mis-expressing *PHY-A1* or *PHY-B1* were characterized in detail. Three *PHY-A1*-underexpressing lines (SUA2, 7 and 11), one *PHY-B1* co-suppression line (SCB35) and four *PHY-B1* overexpression lines (SOB1, 36, 40 and 50) were obtained. SUA seedlings had longer hypocotyls and unseparated cotyledons under continuous FR (FRc), but displayed normal phenotype under continuous white light, dark and R (Rc). RNA blot analysis showed the *PHY-A1* mRNA levels were dramatically reduced but *PHY-B1* mRNA was unaffected in the three SUA lines, indicating they are *PHY-A1* underproducers. SCB35 seedlings showed longer hypocotyls while SOB (1, 36, 40 and 50) lines shorter hypocotyls under Rc, but both exhibited normal phenotype under FRc,
white light and dark. SCB35 had much lower while SOB lines much higher levels of
*PHY-B1* mRNA, but the *PHY-A1* mRNA expression was unaffected, suggesting that
SCB35 is the *PHY-B1* co-suppressor, while SOB lines are *PHY-B1* overproducers.
Compared to WT, the crosses of SUA2 and SCB35 had longer hypocotyls under Rc,
similar to SCB35, and longer hypocotyls under FRc, similar to SUA2. Taken together,
the results showed that, similar to other plant species, type-A and type-B phytochromes
have distinct but overlapping functions in the seedling photomorphogenesis, with *phy-A1*
and *phy-B1* independently mediating FR-induced and R-induced de-etiolation process in
*N. sylvestris.*
Introduction

The R and FR-absorbing phytochromes are probably the best characterized photoreceptors involved in the perception of regulatory light signals in plants (Kendrick and Kronenberg, 1994). Phytochromes are encoded by a gene family with five members, PHYA through PHYE, in Arabidopsis (Clack et al., 1994; Sharrock and Quail, 1989). Phylogenetic analysis has shown that they belong to three subgroups: PHYA, PHYB and PHYC subgroups. PHYB-subgroup consists of three members: PHYB, PHYD and PHYE, but PHYB and PHYD are more closely related members since they share higher (about 80%) amino acids identity than other phytochromes (about 50%) (Mathews and Sharrock, 1997).

Similar PHYA genes were isolated from the Solanaceae; however, the PHYB-like genes are slightly different from PHYB-subgroup in Arabidopsis (Pratt, 1997). In tomato, two PHYB-like genes and a new phytochrome gene (PHYF) have been isolated (Hauser et al., 1995; Pratt et al., 1997). The two PHYB-like genes share much higher amino acids identity (about 91%) than other members, and are thus designated as PHYB1 and PHYB2. Similarly, in potato, PHYB1 and PHYB2 have been isolated (Heyer and Gatz, 1992b; GeneBank accession number AAD01517 for PHYB2). In Nicotiana tabacum, there are two PHY-B like genes, PHY-B1 and PHY-B2, and it is possible that there are additional members of this family (Adam et al., 1997; Kern et al., 1993). PHY-B1 has been sequenced and studied at the transcriptional level, while PHY-B2 has not been sequenced (Adam et al., 1997; Kern et al., 1993).

The phytochrome mutants and transgenic plants have provided a powerful means to dissect the roles of individual phytochromes played in plant photomorphogenesis.
Accumulating evidence shows phyA and phyB have distinct but overlapping functions in seedling photomorphogenesis. PhyA is responsible for the FR-mediated seedling de-etiolation, and phyB for R-mediated de-etiolation (Quail et al., 1995; Smith, 1995; Whitelam and Delvin, 1997).

However, very few phytochrome mutants in Nicotiana species have been identified. In N. plumbaginifolia, the hlg mutants are deficient in a PHYB-like gene, which is likely a PHY-B1 ortholog (Hudson et al., 1997; Chapter 1). pew1 was determined to be a chromophore synthesis mutant, and pew2 which was not genetically characterized is believed to be a putative phyA mutant (Kraepiel et al., 1994).

The DNP tetraploid N. tabacum system is an ideal model for the photoperiodic research (Metzger and Zheng, 1998). It was derived from two parental species, the LDP N. sylvestris and the SDP N. tomentosiformis. In addition, there is a mutant N. tabacum cv Maryland Mammoth which is a SDP and is believed to be deficient in the LD pathway (Long, 1989; Metzger and Zheng, 1998). As a first step toward gaining an understanding of individual phytochrome members in the control of photomorphogenesis in the tobacco plants, N. sylvestris and N. tabacum cv Maryland Mammoth were chosen to be transformed with the antisense Nt-PHY-A1, and sense and antisense PHY-B1 genes. The generation and characterization of the phytochrome mis-expressors in N. sylvestris are reported in this chapter.
Materials and Methods

Plant Material and General Growth Conditions

Wild-type *Nicotiana sylvestris* and *N. tabacum* cv Maryland Mammoth, and transgenic plants were germinated and planted in 15 cm pots containing a commercial soilless media (Metro-Mix 360; Scotts-Sierra Horticultural Products Company, Marysville, OH). The plants were grown in a greenhouse with a temperature setpoint of 25°C. Natural photoperiods were extended to 14 h using light from 1000 W high-pressure sodium vapor lamps (80-100 μmol m⁻² s⁻¹ PAR) from 7:00-10:00 h and 16:00-21:00 h. The LDP *N. sylvestris* and the transgenic plants were induced to flower under 14 h daylength conditions, while the SDP Maryland Mammoth and the transgenic plants grown under 8 h daylength chambers.

Construction of Antisense PHY-Al and Sense and Antisense PHY-Bl Expression Cassettes

A 2.3kb *EcoRI* fragment of a partial *Nt-PHY-Al* cDNA (Adam et al., 1993), which shared 62% homology at the nucleotide level with the corresponding *Nt-PHY-Bl* region (Kern et al., 1993), was blunt-ended and ligated in the antisense orientation into *SmaI* site in the pUC18 vector, resulting in the pUA2.3. Then 2.3kb *BamHI/SacI* fragment containing the antisense *PHY-Al* from pUA2.3 was subcloned into *BamHI/SacI* sites in the binary vector pBI121 which has a CaMV 35S promoter and Nos terminator. The resulting plasmid is designated as pAA2.3.
For the sense \(\text{PHY-Bl}\) cassette under the control of single 35S promoter, a 4kb \(\text{BamHI/Sacl}\) fragment of a full length \(\text{Nt-PHY-Bl}\) cDNA sharing 59% nucleic acid sequence homology with \(\text{PHY-AI}\) (Adam et al., 1993; Kern et al., 1993) was directly cloned into \(\text{BamHI/Sacl}\) sites of \(\text{pBI121}\), resulting in the \(\text{pSB}\). For the construction of the enhanced \(\text{PHY-Bl}\) expression cassette, a 4kb \(\text{BamHI/Clal}\) fragment of a full length \(\text{Nt-PHY-Bl}\) cDNA (Kern et al., 1993) was blunt-ended and ligated in the sense orientation into the \(\text{Smal}\) site in the \(\text{pJIT117A2}\) vector containing a CaMV 35S promoter and dual enhancers minus the chloroplast-targeting peptide sequence (Guerineau et al., 1988). The resulting \(\text{pJB}\) was cut by \(\text{KpnI}\), and a 5.5kb \(\text{KpnI}\) fragment containing the \(\text{Nt-PHY-Bl}\) cDNA, 35S promoter and terminator was subcloned into the \(\text{KpnI}\) Site in the binary vector \(\text{pBIN19}\).

For the antisense \(\text{PHY-Bl}\) constructs, both the full-length \(\text{PHY-Bl}\) cDNA (Kern et al., 1993) and its 2.4kb \(\text{EcoRV}\) fragment encoding the C-terminal domain were used. The 2.4kb fragment was ligated in the antisense orientation into \(\text{Smal}\) site of \(\text{pUC18}\), resulting in the plasmid \(\text{pUB2.4}\). The \(\text{BamHI/Sacl}\) fragment from the \(\text{pUB2.4}\) was then subcloned into \(\text{BamHI/Sacl}\) sites of the vector \(\text{pBI121}\), giving rise to the plasmid \(\text{pAB2.4}\). A 4kb \(\text{BamHI/Clal}\) fragment of a full length \(\text{Nt-PHY-Bl}\) cDNA was blunt-ended and ligated in the antisense orientation into \(\text{Smal}\) site in the \(\text{pJIT117A2}\) vector containing a CaMV 35S promoter and dual enhancers minus the chloroplast-targeting peptide sequence (Guerineau et al., 1988). Then the subcloning was essentially the same as the \(\text{pESB}\) construction described above.
Transformation of Tobacco Plants

The resulting plasmids, pAA2.3, pSB, pESB, pAB2.4, and pEAB were transferred into the *Agrobacterium tumefaciens* strain LBA4404 using the freeze-thaw method (An et al., 1993). Agrobacterium-mediated transformation was described by Horsch et al. (1985), except that plants were generated from leaf discs cultured on the MS (Murashige and Skoog, 1962) medium containing BA (1 mg L\(^{-1}\)) and NAA (0.1 mg L\(^{-1}\) for *N. sylvestris*, or no NAA for Maryland Mammoth tobacco), and selection was based on carbenicillin (500 mg L\(^{-1}\)) and kanamycin (100 mg L\(^{-1}\) for *N. sylvestris* and 200-250 mg L\(^{-1}\) for Maryland Mammoth tobacco).

Phenotypic Screening of Transgenic Lines

Putative lines underexpressing *PHY-AI* and lines over- or under-expressing *PHY-Bl* were screened by the altered hypocotyl length grown for 12 to 14 d under continuous far-red light (FRc) and continuous red light (Rc), respectively. Seeds were spread on one half strength MS medium without sucrose, put in the darkness at 4°C for 2 d and then exposed to white light for 1 to 2 d to synchronize germination. The seeds were then transferred to Rc or FRc. The T\(_1\) transgenic lines showing seedling phenotype of longer hypocotyls under FRc (for antisense *PHY-AI* transformants), longer hypocotyls under Rc (for *PHY-Bl* antisense or sense *PHY-Bl* transformants), or shorter hypocotyls under Rc (for sense *PHY-Bl* transformants) were selected. The kanamycin-resistant T\(_1\) seedlings from the selected lines were grown and selfed to obtain the T\(_2\) seeds. Homozygous T\(_2\) seeds were determined by the uniform kanamycin-resistance, and their seedling phenotype were verified under continuous darkness, white light, Rc and FRc.
PCR Analysis

PCR was used to amplify the tobacco *PHY-A1* and *PHY-B1* cDNA to identify the putative transgenic plants. Genomic DNA was extracted from young leaves of the T₂ homozygous lines by the CTAB-method (McGarvey and Kaper, 1991). For *PHY-A1*, two primers were used to amplify a 0.68kb genomic DNA fragment and a 0.48kb transgenic cDNA fragment. The 22 mer forward primer AA1 was 5' CGT GAG TAA AAT GTT GGA ATT A 3', and the 22 mer reverse primer AA2 was 5' CTT CGC ACA CTC TTG ACC AGT C 3'. For *PHY-B1*, two primers were used to amplify a 1.2kb cDNA fragment. The forward primer SB1 (5' GAA TGG TAT ACG CTT TAC AA 3') was specific for *Nt-PHY-B1* cDNA, while the reverse primer SB2 was based on the pBI121 vector sequence: 5' TCC AGC CGA ATT CCC CGA TA 3'. PCR was conducted under the following conditions for 30 cycles: 94°C, 1 min; 52°C (for *PHY-A1*) or 55°C (for *PHY-B1*), 1 min; 72°C, 1 min. An additional 7 min extension at 72°C was performed after the last cycle.

RNA Blot Analysis

Total RNA was extracted from 10-day old dark-grown seedlings and young leaves of light-grown plants under dim green safe light using the method described by Logemann et al. (1987). Ten μg of total RNA was fractionated on a 1.2% formaldehyde agarose gel and then transferred onto a nylon membrane. The probe was labeled by PCR (Sambrook et al., 1989). The primers AA1 and AA2 used for PCR screening of *PHY-A1* antisense plants were also used to label a 0.48kb *PHY-A1* cDNA probe which shared 57%
nucleic acid sequence homology with \textit{PHY-B1} cDNA. The \textit{PHY-B1} probe was a 0.3kb \textit{PHY-B1} cDNA fragment (showing about 48% nucleotide sequence identity with \textit{PHY-A1} gene) generated using a forward primer SB1 used in PCR screening of transgenic plants and a reverse primer BP2 (5' ACC TGT AGT ATT CTC ACT TG 3'). PCR labeling was carried out using the same conditions as described above except $\alpha(\text{32P})$-dATP was incorporated. The hybridization was performed as described (Sambrook et al., 1989).

\textbf{Western Blots}

Total proteins containing phytochromes were extracted from 7-day old dark grown seedlings or young leaves of 4-week old light-grown plants under dim green safe light using the hot SDS sample buffer extraction method (Wang et al., 1991; 1992). Total proteins were estimated by Bradford's method using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). About 50 $\mu$g of total proteins was separated on the 7.5% SDS-PAGE gel (Laemmli, 1970), transferred to the PVDF membrane through electrotransfer using a Hoefer Transphor Electrophoresis Unit (Pharmacia Biotech, San Francisco, CA) according to the manufacturer's instructions. The chemiluminescent method using Phototope®-Star Western Blot Detection Kit (New England BioLabs, Beverly, MA) was performed to detect the phytochrome proteins. The antibodies used were P25, a mouse monoclonal antibody against phytochromes from dark-grown tissue in pea (Cordonnier et al., 1986), and AS-32, a rabbit monoclonal antibody against phytochrome A (Halliday et al., 1997). A polyclonal antipeptide antibody was raised by injecting the rabbits with a 13 amino acid residue peptide (\textit{PHY-B1}: 1120 NH$_2$-DLPMTTRGRSGKSLG-COOH$_{1132}$) by Zymed Company (South San Francisco, CA).
Crossing of SUA2 and SCB35 transgenic lines

The T₂ homozygous SUA2 and SCB35 plants were grown under 14 h LD conditions to induce flowering. About 4 days before anthesis, the anthers of SUA2 were removed and the stigma was pollinated with pollens from SCB35 plants, and vice versa. The plants were then isolated from each other and subsequently initiating flower buds were removed.

Light Sources

For the screening of the seedling phenotypes, cool white fluorescent light had a ratio of R to FR (PFD 655-665nm / PFD 725-735 nm) of 6.2, and a PPFD of 35.6 μmol m⁻² s⁻¹ (Fig. 2.1). Rc was obtained by wrapping two 20W cool white fluorescent tubes with two layers of the red cellulose membrane, resulting in a R/FR ratio of 5.12, and a PPFD of 2.7 μmol m⁻² s⁻¹ (Fig. 2.1). FRc was obtained by filtering light from a 250 W halogen spotlight through a CBS-750 Far Red filter (Carolina Biological Supply Co., Burlington, NC), giving a R/FR at 0.008 and the PFD (700-750 nm) of 2.1 μmol m⁻² s⁻¹ (Fig. 2.1).
Figure 2.1. Continuous R, FR and white light used for phenotypic screening of transgenic lines. W, cool white fluorescent light; R, red light; FR, far-red light.
Phenotypic Screening of Transgenic T₁ Tobacco Plants

Agrobacterium-mediated transformation resulted in more than 10 lines for each plasmid vector in *N. sylvestris* (Table 2.1) except for the pSB plasmid which gave rise to only one line (SOB1) due to unexpected overheating in the incubator. The T₁ seedlings showing longer hypocotyls under FRc for antisense *PHY-A1* construct were designated as SUA (sylvestris underexpressing *PHY-A1*), and those displaying shorter and longer hypocotyls under Rc for sense and antisense *PHY-B1* construct were designated as SOB and SUB, respectively. The seedlings showing longer hypocotyls under Rc for sense *PHY-B1* construct were likely PHY-B1 co-suppressors and thus designated as SCB (sylvestris co-suppressing *PHY-B1*).

Chi-square ($\chi^2$) test showed that SUA1, 2, 7, 11 and 13 lines, and SUB8, 18, 20 and 35 segregated 3:1 for kanamycin-resistance to kanamycin-sensitive. Similarly, SOB1, 36, 40 and 50, and SCB35 also showed a similar 3:1 segregation pattern.
| Construct      | Plasmid | T₀ Lines | Putative T₁ | T₁ HL (%) | Kan<sup>R</sup>:kan<sup>S</sup> |
|---------------|---------|----------|-------------|-----------|----------------|---|
| Antisense A1  | pAA2.3  | 11       | SUA1        | 138       | 33:12          |
|               |         |          | SUA2        | 142       | 47:14          |
|               |         |          | SUA7        | 133       | 32:10          |
|               |         |          | SUA11       | 117       | 37:11          |
|               |         |          | SUA13       | 143       | 32:10          |
|               |         |          | SUA16       | 140       | 34: 8          |
| Sense B1      | pSB     | 1        | SOB1        | 79        | 36:12          |
|               | pESB    | 13       | SCB33       | 111       | 36:17          |
|               |         |          | SCB35       | 148       | 63:22          |
|               |         |          | SOB36       | 73        | 35:10          |
|               |         |          | SOB40       | 75        | 22: 8          |
|               |         |          | SOB50       | 76        | 37:11          |
|               |         |          | SOB53       | 81        | 36: 7          |
| Antisense B1  | pAB2.4  | 16       | SUB8        | 120       | 37:10          |
|               | pEAB    | 20       | SUB35       | 113       | 35:11          |
|               |         |          | SUB52       | 135       | 41:11          |

Table 2.1. Summary of transgenic lines of *N. sylvestris* mis-expressing phytochrome genes. Note: <sup>a</sup> the percentage of the T₁ average hypocotyl length relative to that for WT. For *PHY-A1* transgenic plants, the condition was FRc while for *PHY-B1* transgenic plants was Rc. 40 to 50 seedlings were measured. HL, hypocotyl length; Kan<sup>R</sup>, kanamycin-resistant; Kan<sup>S</sup>, kanamycin-sensitive.

In addition, hypocotyl length distribution of the representative T₁ SUA2 line showed a 3:1 segregation of long:short hypocotyls under FRc (Fig. 2.2). The 3:1 segregation of long:short hypocotyls for SCB35 and of short:long hypocotyls for the
representative SOB36 line was also apparent (Fig. 2.3). These indicate the SUA2, SCB35 and SOB36 lines have a single locus of T-DNA insertion.

Moreover, when both short and tall seedlings of SOB1 and SOB36 were transferred to kanamycin-containing medium, the short seedlings survived, while those tall seedlings died 15 days after they were transferred. This result suggested the short hypocotyl phenotype of SOB1 and SOB36 lines co-segregated with the neomycin phosphotransferase II gene which confers kanamycin resistance. Thus, the kanamycin-resistant T1 seedlings from the putative lines were selected to grow and selfed to obtain the T2 homozygous lines for further characterization.

Figure 2.2. Hypocotyl length distribution of the representative T1 SUA2 line under continuous FR. WT seedling had an average hypocotyl length of 10.2 mm. SUA2 seedlings averaged 12.3 mm and segregated 43:17 for long (>11.0 mm):short (<11.0 mm) hypocotyls. The χ² for the 3:1 segregation was 0.36. Empty box, wild-type; solid box, SUA2; WT, wild-type.
Figure 2.3. Hypocotyl length distribution of the representative T1 SCB35 and SOB36 lines under continuous R. A, Hypocotyl length distribution of SCB35. WT seedling had an average hypocotyl length of 10.1 mm. SCB35 seedlings averaged 14.9 mm. but segregated 46:14 for long (>13.0 mm):short (<13.0 mm) hypocotyls. The $\chi^2$ for 3:1 segregation was 0.09. Empty box, wild-type; solid box, SCB35. B, Hypocotyl length distribution of SOB36. WT seedlings averaged 12.5 mm of hypocotyl length, while SOB36 averaged 9.2 mm but segregated 44:16 for short (<10.5 mm):long (>10.5 mm) hypocotyls. The $\chi^2$ for 3:1 segregation was 0.09. Empty box, wild-type; solid box, SOB36. Note: Experiments in A and B were carried out at different times.
A total of 5 putative *PHY-BI* underexpressing lines (SUB8, 18, 20, 35 and 52) in *N. sylvestris* were also obtained. They segregated 3:1 for kanamycin-resistance and had slightly longer hypocotyls than WT under Rc (Table 2.1). The kanamycin-resistant T$_1$ seedlings were also selected, transferred to greenhouse and then selfed to get the T$_2$ homozygous seeds for further characterization.

The transformation of the Maryland Mammoth tobacco plants was also successful, resulting in at least several lines for each construct (Table 2.2). However, very few T$_1$ transgenic lines exhibited the characteristic phenotype (Table 2.2). Some of the kanamycin-resistant T$_1$ plants were grown in the 8 h SD growth chamber to induce flowering and selfed in order to obtain the T$_2$ homozygous lines.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Plasmid</th>
<th>T$_0$ Lines</th>
<th>Putative T$_1$</th>
<th>T$_1$ HL (%)$^a$</th>
<th>Kan$^R$:kan$^S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisense A1</td>
<td>pAA2.3</td>
<td>14</td>
<td>MUA8</td>
<td>123</td>
<td>41:0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MUA17</td>
<td>130</td>
<td>65:16</td>
</tr>
<tr>
<td>Sense B1</td>
<td>pESB</td>
<td>4</td>
<td>MSB31</td>
<td>Not</td>
<td>Not</td>
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<td></td>
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<td>pAB2.4</td>
<td>10</td>
<td>MUB8</td>
<td>109</td>
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<tr>
<td></td>
<td>pEAB</td>
<td>17</td>
<td>MUB32</td>
<td>115</td>
<td>42:12</td>
</tr>
</tbody>
</table>

Table 2.2. Summary of transgenic lines of *N. tabacum* cv Maryland Mammoth misexpressing phytochrome genes Note: $^a$, the percentage of the T$_1$ average hypocotyl length relative to that of WT. For *PHY-AI* transgenic plants, the condition was FRc, while for *PHY-BI* transgenic plants was Rc. 40 to 50 seedlings were measured. HL, hypocotyl length; Kan$^R$, kanamycin-resistant; Kan$^S$, kanamycin-sensitive.
Characterization of SUA Lines

Three T2 homozygous lines of SUA (2, 7, 11) were obtained, while the other two SUA lines (SUA1 and SUA13) were sterile. When T2 homozygous seedlings were grown under continuous dark, white light, Rc and FrC for 7 days, SUA2, 7, and 11 showed longer hypocotyls and unseparated cotyledons under FrC (Fig. 2.4, and Fig. 2.5). However, no alterations in hypocotyl or cotyledon phenotypes were observed under Rc and continuous white light (Fig. 2.4), indicating the specific down-regulation of the PHY-A1 gene in the three SUA lines, but with little or no effect on the expression of the PHY-B1 gene. No phenotypic difference between SUA lines and WT under continuous dark (Fig. 2.4), suggesting the skotomorphogenesis was normal in the SUA lines.

PCR analysis showed the PHY-A1 transgene was incorporated into the genome of the three SUA lines (Fig. 2.6A). RNA blot analysis showed the expression of antisense PHY-A1 resulted in the suppression of endogenous PHY-A1 gene expression, thus demonstrating that the longer hypocotyls and unseparated cotyledons in the three lines were due to the downregulation of the PHY-A1 gene (Fig. 2.6B). Moreover, consistent with the unaltered hypocotyl and cotyledon phenotypes for the three SUA lines under Rc and continuous white light, the expression of the PHY-B1 gene was also not affected (Fig. 2.6C).
Figure 2.4. Seedling phenotype of the three T<sub>2</sub> homozygous SUA lines grown under various conditions. Seedlings were grown for 7 days. WT, wild-type; D, R, FR, W: continuous dark, red, far-red and white fluorescent light, respectively.
Figure 2.5. Hypocotyl length of the three T<sub>2</sub> homozygous SUA lines grown under various conditions. Seedlings were grown for 7 days. Values represent the average of 40 seedlings for each genotype under each treatment. The error bars represent the standard deviation. WT, wild-type; D, R, FR, W: continuous dark, red, far-red and white fluorescent light, respectively.
Figure 2.6. Molecular characterization of SUA lines. A, PCR amplification of both antisense \textit{PHY-AI} cDNA (0.48kb) and endogenous \textit{PHY-AI} genomic DNA (0.68kb) containing an intron. P, plasmid pAA2.3. B, RNA blot analysis using a 0.48kb \textit{PHY-AI} cDNA probe. Both the antisense RNA (lower band) and the endogenous \textit{PHY-AI} mRNA (upper band) were hybridized. The loading of total RNA was shown by ethidium bromide staining of 25S rRNA. C, RNA blot analysis using a 0.3kb \textit{PHY-BI} cDNA probe. The loading of total RNA was shown by ethidium bromide staining of 25S rRNA. WT, wild-type; 2, 7, 11: three SUA lines.
Characterization of SOB and SCB35 Lines

One T2 homozygous SCB35 line and four SOB lines were obtained. When T2 homozygous seedlings were grown under continuous dark, white light, Rc and FRc for 7 days, SCB35 had longer hypocotyls and partially separated cotyledons under Rc while four SOB lines showed shorter hypocotyls and well-separated cotyledons (Fig. 2.7, and Fig. 2.8). However, neither the hypocotyl nor the cotyledon phenotypes were altered under FRc, suggesting the expression of the sense PHY-B1 gene specifically affect the seedling development under Rc.

PCR analysis showed the PHY-B1 transgene was incorporated into the genome of the SCB35 and four SOB lines (Fig. 2.9A). RNA blot analysis confirmed the expression of sense PHY-B1 resulted in higher endogenous levels of the PHY-B1 mRNA in the SOB lines, while in SCB35 both endogenous and transgene PHY-B1 mRNA expression were suppressed (Fig. 2.9B), a phenomenon called co-suppression (Matzke and Matzke, 1995; Meyer and Saedler, 1996). The results demonstrate that the longer hypocotyls in SCB35 and shorter hypocotyls in SOB lines are due to the down-regulation and up-regulation of the PHY-B1 gene expression, respectively. Moreover, consistent with unaffected seedling hypocotyl and cotyledon phenotypes for both SCB35 and SOB lines under FRc, the expression of the PHY-A1 gene was unaffected (Fig. 2.9C).
Figure 2.7. Seedling phenotype of T2 homozygous SCB35 and four SOB lines grown under various conditions. Seedlings were grown for 7 days. WT, wild-type; 35, SCB35; 36, 40, 50: four SOB lines; D, R, FR, W: continuous dark, red, far-red and white fluorescent light, respectively.
Figure 2.8. Hypocotyl length of T$_2$ homozygous SCB35 and four SOB lines grown under various conditions. Seedlings were grown for 7 days. Values represent the average of 40 seedlings for each genotype under each treatment. The error bars represent the standard deviation. WT, wild-type; D, R, FR, W: continuous dark, red, far-red and white fluorescent light, respectively.
Figure 2.9. Molecular characterization of SCB35 and SOB lines. A, PCR amplification of *PHY-B1* DNA (1.2kb). B, RNA blot analysis using a 0.3kb *PHY-B1* cDNA probe. Both the transgenic RNA (slightly lower position) and endogenous *PHY-B1* mRNA (slightly upper position) were hybridized. The loading of total RNA was shown by ethidium bromide staining of 25S rRNA. C, RNA blot analysis using a 0.48kb *PHY-A1* cDNA probe. The loading of total RNA was shown by ethidium bromide staining of 25S rRNA. P, plasmid pESB; WT, wild-type; 35. SCB35; 1, 36, 40, 50: four SOB lines;
Due to the lack of the \textit{PHY-BI} genomic DNA sequences, several primers for \textit{PHY-BI} cDNA were designed to amplify the \textit{PHY-BI} transgene and endogenous \textit{PHY-BI} genomic sequence in putative SUB lines. However, both WT and transgenic SUB lines were amplified (data not shown). Thus, I was not able to confirm the incorporation of transgenic antisense \textit{PHY-BI} cDNA into the genomes of the transgenic SUB lines. As a result and also because of the weak phenotypes in the putative SUB lines (Table 2.1), they were not characterized further. It is obvious that more specific primers are needed to amplify the \textit{PHY-BI} cDNA and genomic DNA in the SUB lines.

\textbf{Characterization of the Crosses of SCB35 and SUA2 Lines}

As shown previously, SUA lines specifically underexpressed the \textit{PHY-AI} gene without affecting the \textit{PHY-BI} expression. Similarly, SCB35 down-regulated the \textit{PHY-BI} gene expression without an effect on the expression of \textit{PHY-AI}. In order to obtain a genotype in which both \textit{PHY-AI} and \textit{B1} were down-regulated, SCB35 and SUA2 were crossed.

PCR analysis showed both \textit{PHY-AI} and \textit{B1} genes were incorporated into the genome of both crosses, SUA2 × SCB35 and SCB35 × SUA2 (Fig. 2.10). When the seedlings of the crosses and their parents were grown under Rc and FRc, both crosses had longer hypocotyls than WT under Rc, similar to SCB35 (Fig. 2.11). Under FRc, similar to SUA2, both crosses displayed longer hypocotyls than WT under FRc (Fig. 2.11). This indicates that both crosses are functionally deficient in perceiving the R and FR, a characteristic of \textit{phyA phyB} double mutants in Arabidopsis (Smith et al., 1997).
Figure 2.10. PCR analysis of the crosses of SUA2 and SCB 35. A, PCR amplification of both antisense \textit{PHY-A1} cDNA (0.48kb) and endogenous \textit{PHY-A1} DNA (0.68kb) containing an intron. B, PCR amplification of the sense \textit{PHY-B1} cDNA (1.2kb). Since the reverse primer was based on the vector sequence, no amplification occurred from WT and SUA2 DNA samples. WT, wild-type; 2, SUA2; 35, SCB35; 2x35, SUA2xSCB35; 35x2, SCB35xSUA2; P, plasmid pAA2.3 and pESB in A and B, respectively.

Figure 2.11. Hypocotyl length of the crosses of SCB35 and SUA2 lines grown under continuous red and far-red light conditions. Seedlings were grown for 7 days. Values represent the average of 30 seedlings. The error bars represent the standard deviation. WT, wild-type; Rc, FRc: continuous red and far-red light, respectively.
Preliminary Characterization of Transgenic Maryland Mammoth Tobacco Plants

Due to the fact that very few putative transgenic lines of Maryland Mammoth tobacco were obtained based on exhibiting an altered response to Rc or FRc, only a few plants were characterized. PCR analysis showed the PHY-B1 transgene was incorporated into the genomes of four sense PHY-B1 plants (Fig. 2.12). PCR analysis was not performed in the putative antisense PHY-B1 plants, due to unknown sequences of the genomic PHY-B1 DNA.

Figure 2.12. PCR amplification of PHY-B1 cDNA in the four sense PHY-B1 transgenic lines of Maryland Mammoth tobacco. A 1.2kb band was amplified from the plasmid pESB containing the PHY-B1 cDNA and all of the four sense PHY-B1 lines (31, 35, 37, 42) but not from wild-type (WT).

The putative antisense PHY-A1 lines in Maryland Mammoth (MUA8 and 17, see Table 2.2) were characterized by Western blot analysis using the monoclonal anti-phyA antibody, AS-32 (Fig. 2.13). This antibody recognized the phyA apoprotein in N. plumbaginifolia (Hudson et al., 1997). Although this antibody did not react with phyA-like phytochrome in N. sylvestris, it recognized the phyA from the tetraploid Maryland Mammoth tobacco (data not shown). In these two MUA lines, the PHYA-like protein level was lower than WT, consistent with the longer hypocotyls under FRc (Table 2.2). The other two lines (4 and 19) did not show decreased PHYA-like protein levels, consistent with same hypocotyl length under FRc. However, due to the limitations in time, these two MUA lines were not characterized further.
**Figure 2.13. Western blot analysis of the two MUA lines.** A, Western blot detection result. B, Total protein on the PVDF membrane was stained by black ink to show the loading. About 50 μg of total protein extracted from dark-grown seedlings were separated on 7.5% SDS-PAGE gel and transferred to the PVDF membrane. The antibody AS-32 was used to detect the PHYA-like protein. WT, wild-type. 4, 8, 17 19: MAA4, MUA8, MUA17, MAA19, respectively.
DISCUSSION

It is now well established that phyA and phyB have distinct but overlapping functions in the control of seedling de-etiolation process (Quail et al., 1995). The inhibition of hypocotyl elongation by prolonged FR is under the control of phyA, operating via the FR high-irradiance response, while the inhibition by R is controlled by phyB (Quail et al., 1995; Smith, 1995; Whitelam and Delvin, 1997).

Seedlings deficient in phyA or overexpressing the PHYA transgene exhibited longer or shorter hypocotyls than WT under FRc, respectively (Boylan and Quail, 1991; Dehesh et al., 1993; Halliday et al., 1997; Keller et al., 1989; Parks and Quail et al., 1993; Weller et al., 1997). The reduced sensitivity to FRc provides a simple but efficient screening method for the putative PHY-A1 underproducers. Three T2 homozygous SUA lines (2, 7, 11) were obtained through this screening procedure. Hypocotyl elongation was reduced under FRc but unaffected under Rc and continuous white light (Fig. 2.4; Fig. 2.5). They also exhibited unseparated cotyledons under FRc (Fig. 2.4), a phenotype consistent with phyA-deficient mutants in Arabidopsis and pea (Parks and Quail, 1993; Weller et al., 1997). RNA blot analysis showed these three lines had reduced levels of endogenous PHY-A1 mRNA without affecting the expression of the PHY-B1 mRNA (Fig. 2.6). The PHY-A1 protein level was not analyzed due to the lack of a specific antibody, but the elongated hypocotyls under FRc and reduced PHY-A1 mRNA levels strongly indicate that these three lines are phy-A1 underproducers. The WT seedling phenotype exhibited by the three SUA lines under continuous darkness (Fig. 2.4) indicates that skotomorphogenesis is not affected. Therefore, phy-A1 is the predominant
phytochrome mediating FR-induced de-etiolation process in *N. sylvestris*, but is not essential for seedling photomorphogenesis under continuous R and white light.

Red light induced inhibition of hypocotyl elongation is reduced or enhanced in seedlings deficient in phyB or overexpressing *PHYB* genes (Delvin et al., 1997; Halliday et al., 1997; Hudson et al., 1997; Lopez-Juez et al., 1992; Nagatani et al., 1991; Parks et al., 1993; Reed et al., 1993; Wagner et al., 1991; Weller et al., 1995). Through screening the hypocotyl length of seedlings grown under Rc, four independent *PHY-B1* overexpression lines (SOB1, 36, 40 and 50) and one *PHY-B1* co-suppression line (SCB35) were obtained. Under both Rc and continuous white light, the SOB lines exhibited enhanced inhibition of hypocotyl elongation, while SCB35 displayed reduced inhibition (Fig. 2.7; Fig. 2.8). In addition, FRc did not affect hypocotyl elongation of these lines (Fig. 2.7; Fig. 2.8), suggesting a specific effect on the *PHY-B1* gene. Northern blot analysis confirmed that SOB lines had higher and SCB35 lower levels of *PHY-B1* mRNA, respectively, but the level of *PHY-A1* mRNA was unaffected (Fig. 2.9). Due to the unavailability of specific PHY-B1 antibody, and the failure of anti-PHY-B1 peptide antibody to recognize the PHY-B1 protein, the protein levels in these lines were not determined. However, the *PHY-B1* mRNA levels in SCB35, WT and SOB lines correlated well with the long, intermediate and short hypocotyls in the three groups of plants. Therefore, it is likely that functional phy-B1 protein is reduced in SCB35 or increased in four of SOB lines. Thus, phy-B1 appears to play a major role in seedling photomorphogenesis under continuous R and white light in *N. sylvestris*, similar to the photosensory functions of phyB in many other plants species (Quail et al., 1995; Smith, 1995; Whitelam and Delvin, 1997; Whitelam and Harberd, 1994).

91
However, SCB35 is by no means blind to Rc, with green and partially separated cotyledons (Fig. 2.7). This is different from Arabidopsis phyB mutants which display unseparated cotyledons under Rc (Nagatani et al., 1991; Reed et al., 1993; Somers et al., 1991), but it is consistent with hlg mutants in N. plumbaginifolia (Hudson et al., 1997).

The HLG-encoded PHYB-type phytochrome which shares 91% identity of amino acid sequence with Nt-PHY-B1 (Table 1.2) is likely an ortholog of Nt-PHY-B1 in N. plumbaginifolia. The other interpretation for the greening and partially separated cotyledons is that the PHY-B1 mRNA is not completely suppressed (Fig. 2.9), or the protein is not completely eliminated in SCB35. Although the protein level is not determined due to the lack of a specific antibody, it has been shown that co-suppression does not always completely eliminate the gene expression but exhibit a relatively wide range of suppression (for example, Que et al., 1997). Moreover, the cotyledons in SCB35 are only partially separated. Thus, it is likely that the observed seedling phenotype in SCB35 is due to incomplete suppression of PHY-B1 gene expression.

The progeny of the crosses of SUA2 and SCB35 displayed longer hypocotyls under both Rc and FRc (Fig. 2.11). However, the crosses behaved as SUA2 under FRc, and as SCB35 under Rc, suggesting that there are no antagonistic or synergistic effects on the inhibition of hypocotyl elongation under either Rc or FRc (Fig. 2.11).

It has been shown in the tetraploid N. tabacum, there are two PHYA genes (A1 and A2), and more than two PHYB genes (Adam et al., 1993; Kern et al., 1993). Since N. tabacum is derived from the two parental diploid species, N. sylvestris and N. tomentosiformis, the two PHYA genes may have individually originated from each of the parental species, or they may have existed in both species. In other words, N. sylvestris
may have one or both of PHYA genes. Due to the lack of PHY-A2 cDNA sequence, these two possibilities could not be distinguished. The PHY-A1 cDNA probe used in this study hybridized well with the RNA from WT and SUA lines, indicating PHY-A1 likely exists in *N. sylvestris*. However, the partial upstream sequences of PHY-A1 and A2 share relatively high (about 89%) sequence homology (Adam et al., 1993). In addition, PHY-A1 cDNA share high sequence homology with PHYA gene products from closely related species, tomato and potato (Hauser et al., 1995; Heyer and Gatz, 1992a). This indicates that PHY-A1 and A2 coding regions probably have a high sequence homology.

Undoubtedly, the isolation of PHYA gene(s) in *N. sylvestris* will provide the conclusive evidence about the involvement of PHY-A1 and/or A2 in *N. sylvestris* and SUA lines. Nevertheless, PHY-A1 mRNA is clearly reduced, and PHY-A2 is also likely reduced in SUA lines if it exists in the genome of *N. sylvestris*, based on the likely high sequence homology between these two genes and on the seedling phenotypes of SUA lines grown under FRc.

For the PHYB subgroup, the situation is more complex. In the three closely related species in the Solanaceae family, tobacco, potato and tomato, there are two PHYB-like genes, *B1* and *B2* (Pratt et al., 1997; also see Table 1.2 in Chapter 1). Neither PHYB1 nor B2 is the ortholog of either Arabidopsis PHYB or PHYD (Adam et al., 1997; Hauser et al., 1995; Pratt et al., 1997). PhyB1 orthologs in tomato and *N. plumbaginifolia* appear to have slightly different functions in that they apparently do not sense R/FR ratios or EOD-FR which is characteristic of Arabidopsis phyB or phyD (Hudson et al., 1997; Pratt et al., 1997; Smith and Whitelam et al., 1997; van Tuinen et al., 1995b).
In *N. tabacum*, there are two *PHYB*-like genes (*PHY-B1* and *PHY-B2*) although *PHY-B2* has not been sequenced, and it is possible there are more than two *PHYB*-like genes (Adam et al., 1997; Kern et al., 1993). Indeed, the likely *PHY-B1* ortholog encoded by the *HLG* locus has been identified in *N. plumbaginifolia* (Hudson et al., 1997). Therefore, the *PHYB* genes are complicated in the various tobacco species. There are several possible origins of *PHYB* genes in the tetraploid *N. tabacum*. Does each parental species have their own *PHYB1* and *PHYB2* genes, or just one of them? Does it have additional *PHYB*-like gene? The isolation and characterization of all *PHYB* genes in *N. sylvestris* will reveal the expression of other *PHYB*-like genes in the SCB35 and SOB lines. Nevertheless, the strong phenotypic correlation with the altered *PHY-B1* mRNA levels suggests that the functional phy-B1 protein levels have been down-regulated in the SCB35 line and up-regulated in the SOB lines.

Overall, the *PHYA* and *PHYB* genes are very complex in the tobacco genomes. However, the generation of SUA lines which are at least partially deficient in phy-A1, and the SOB lines and SCB35 that are overproduced and partially deficient in the functional phy-B1, respectively, has provided the compelling evidence to support the highly conserved functions of phyA- and phyB-like phytochromes in the de-etiolation process in plants. Furthermore, these transgenic plants will provide the powerful tools for the functional dissection of the phyA- and phyB-like phytochromes in the photoperiodic control of flowering in the obligate LDP *N. sylvestris.*
CHAPTER 3

TYPE-A AND TYPE-B PHYTOCHROMES HAVE DISTINCT FUNCTIONS IN THE PHOTOPERIODIC CONTROL OF FLOWERING IN THE OBLIGATE LONG-DAY PLANT NICOTIANA SYLVESTRIS

Abstract

Phytochromes are red (R) and far-red light (FR) photoreceptors in plants that function in assessing daylength information in the photoperiodic control of flowering, but it remains unclear what regulatory light cues are sensed by individual phytochromes. Toward a better understanding of the roles of two major phytochromes (phyA and phyB) in the photoperiodic control of flowering, the obligate long-day plant Nicotiana sylvestris with a critical daylength of about 12 h was used as a model. The three representative homozygous transgenic lines were used to study the flowering responses under different daylength extensions following 8h natural light: SUA2 (PHY-A1 underproducer), SCB35 (PHY-B1 co-suppressor), SOB36 (PHY-B1 overproducer) and the cross, SUA2 × SCB35.
FR alone did not promote flowering in any genotype but the addition of FR to fluorescent (Flu) light (R/FR=3.3) greatly promoted flowering, suggesting that it is a necessary but not sufficient component in the daylength extension for maximum flower promotion. Flowering in SUA2 was delayed compared to WT when plants were subjected to 8 h extension with low intensity Flu light. Flowering in SUA2 were also dramatically delayed under 4 or 8 h daylength extensions with low intensity Inc light (R/FR=0.7). The results suggest that the type-A phytochrome likely mediates the FR response and that PrA1 is promotive to flowering.

Under an 8 h extension with Inc, SCB35 flowered at the same time as WT, but was substantially delayed with 8 h Flu. The effects of the two treatments were reversed when SOB36 and WT were compared. However, SCB35 flowered earlier, and SOB36 later, than WT under a 4 h Inc extension. In total, the results indicate the response to R in the daylength extension is mediated by a type-B phytochrome and that R is a necessary and sufficient component of the daylength extension. The results also indicate that phy-B1 has a dual inhibitory/promotive function in the photoperiodic control of flowering.

WT plants flowered earlier if the early part of Flu extension was interrupted by 2 h darkness following 30 min FR than those continuous 8 h Flu extension, indicating that R at this time is inhibitory while FR is promotive to flowering. Flowering in both SOB36 and SCB35 delayed compared to WT, but SOB36 flowered earlier than SCB35 under either 8 h Flu extension or 2 h dark interruption. In addition, a 30 min followed by 2 h dark-interruption induced SCB35 to flower, compared to 2 h dark interruption. These results support the action of R mediated by phy-B1 is likely inhibitory at early part of extension and FR mediated by phy-A1 is promotive.
The cross SUA2 x SCB35 flowered much later than any other genotypes under an 8 h extension with Inc, and did not flower under other low intensity light extension treatments. The results demonstrate that both phy-A1 and phy-B1 are required during the daylength extension for the optimal flowering.

Taken together, the results show that a mixture of R and FR is optimal for the promotion of flowering, and that both type-A and type-B phytochromes sensing FR and R, respectively, are required in the long-day plant *N. sylvestris*.

**Introduction**

Plants sense the daylength information through photoreceptors. One of the most important is phytochrome. Phytochrome has two photoconvertible forms: the red light-absorbing form Pr, and far-red light absorbing form Pfr which is generally believed to be the only active form (Kendrick and Kronenberg, 1994).

Numerous physiological studies have shown that for both LDP and SDP, there is a diurnal fluctuation in the requirement for the presence or absence of Pfr for flowering. For SDP, Pfr during the day is promotive, but becomes inhibitory during the middle of the inductive dark period (Thomas and Vince-Prue, 1997). LDP are the mirror image of SDP in that high levels of Pfr are usually inhibitory to flowering during the early part of daylength extension but become promotive later (Thomas and Vince-Prue, 1997). The paradox that both the absence and presence of Pfr can be either promotive or inhibitory to flowering led to the hypothesis that two different pools of phytochrome with differential
stabilities were involved in the photoperiodic timing mechanisms (Takimoto and Saji, 1984).

Recent molecular genetic studies have revealed that phytochrome apoproteins are encoded by a gene family, \textit{PHYA} through \textit{PHYE} in Arabidopsis, and perhaps more phytochrome genes exist in other plants such as tomato (Mathews and Sharrock, 1997; Pratt et al., 1997). Thus it was proposed that individual phytochromes have distinct functions in the photoperiodic timing mechanisms and other photomorphogenic aspects (Quail et al., 1995; Thomas and Vince-Prue, 1997; Whitelam and Delvin, 1997).

The flowering responses in genetic mutants deficient in phyA, phyB or other phytochromes, as well as transgenic plants ectopically expressing the phytochrome genes have supported the notion that multiple phytochromes are involved in the photoperiodic control of flowering (Whitelam and Delvine, 1997). However, the mutants or transgenic plants are in the quantitative LDP (Goto et al., 1991; Johnson et al., 1994; Weller et al., 1995, 1997) or SDP (Childs et al., 1997; Halliday et al., 1997) or even DNP (Hudson et al., 1997). None of the experimental subjects have been in plants with an obligate LD or SD photoperiodic requirement. Nevertheless, the results suggest that phyA is required for sensing the daylength extension in the LDP Arabidopsis and pea (Johnson et al., 1994; Weller et al., 1997). Furthermore, in pea at least phyA controls the synthesis or the transport of a flower inhibitor (Weller et al., 1997). On the other hand, phyB is inhibitory to flowering in both LDP Arabidopsis and pea and SDP sorghum (Childs et al., 1997; Reed et al., 1994; Weller et al., 1995). In addition, the disruption of the SD-required tuber formation in the antisense \textit{PHYB} potato plants has led to the suggestion that phyB also...
controls the production of a graft-transmissible flowering inhibitor (Jackson et al., 1996; Jackson and Thomas, 1997).

However, the idea that phyA and phyB control the photoperiodic flower induction through regulating the inhibitor may be too simplistic (Metzger and Zheng, 1998). Recently, phyB has been shown to promote the flowering in the DNP tobacco *N. plumbaginifolia* (Hudson et al., 1997). The fact that both Pfr absence and presence in LDP and SDP can alternatively promote or inhibit the flowering depending on the time of the day/night cycle is not easily explained by phytochrome control of the production or transport of inhibitors (Metzger and Zheng, 1998).

Therefore, it is necessary using a different system to dissect the functions of individual phytochromes in the photoperiodic timing mechanisms. In this study, the obligate LDP *N. sylvestris* was chosen as a model plant. The transgenic plants underexpressing *PHY-A1* (SUA2), overexpressing *PHY-B1* (SOB36) and co-suppressing *PHY-B1* (SCB35) characterized in detail (Chapter 2) were used to study the flowering responses under different photoperiodic conditions.
Materials and Methods

Plant Material and Growth Conditions

Three groups of T₂ transgenic *N. sylvesteris* plants mis-expressing *PHY-AI* or *PHY-BI* genes characterized in Chapter 2 were used: the PHY-AI underproducer (SUA2), PHY-BI overproducer (SOB36), and the PHY-BI co-suppressor (SCB35). The cross SUA2 × SCB35 was also used in some experiments. Seeds were germinated and planted in 15 cm pots containing a commercial soilless media (Metro-Mix 360; Scotts-Sierra Horticultural Products Company, Marysville, OH). The plants were grown and maintained under non-inductive 8 h daylength condition in a greenhouse with a temperature setpoint of 25°C. After approximately two months of growth, the plants were transferred to the photoperiodic treatments.

Photoperiodic Treatments

Most of the photoperiodic experiments were carried out in the greenhouse. The 8 h SD main light period was obtained by covering the benches with a black cloth from 16:30 h to 8:30 h. During the main light photoperiod, supplemental light from high pressure sodium lamp (PPFD=80-100 μmol m⁻² s⁻¹) was provided when the intensity of sunlight outside of the greenhouse fell below 300 W m⁻². For daylength extension experiments, the 8 h SD was extended by low intensity light from fluorescent lamp (Flu), incandescent lamps (Inc) or FR from 16:30 h to 19:30, 20:30 or 0:30 h. For the dark-interruption experiments, an 8 h Flu extension was interrupted by a 2 h dark period (19:30 to 21:30 h). In some cases, the Flu lamps were turned off at 19:00 h, and the plants
were subjected to a 30 min FR treatment before the 2 h dark period. A 17 h LD was provided by high intensity light from the high pressure sodium lamps from 6:00-10:00 h and 16:00-23:00 h.

In some instances, the experiments were done in the growth chambers to test the effects of FR during the 8 h main light period. In one chamber, only the Flu lamps (PPFD=200.8 μmol m⁻² s⁻¹, R/FR=6.3) were turned on, and in another chamber the Inc lamps were added to the Flu lamps to give a R/FR ratio at 1.4 and PPFD at 240.7 μmol m⁻² s⁻¹. Both were followed by the same 8 h Flu light treatment (PPFD=46.3 μmol m⁻² s⁻¹, and R/FR=6.3). The temperature in the growth chambers was set at 24°C.

Measurement of Flowering

Flowering percentage, flowering time and leaf number were used to measure the level of flower induction. Leaf number was defined as the number of leaves at least 10 cm long for the old leaves and at least 3 cm long for the young leaves. At the beginning of each experiment, the number of leaves was recorded for each plant. During the course of the experiments, shoot tips were examined daily for the presence of flower buds. Once the flower buds appeared, the number of leaves produced during the intervening time from the start of the treatment until flower bud formation was determined. In one experiment where both WT and SCB35 were subjected to natural SD from January 5 (9.4 h daylength) to May 11 (14.2 h), the appearance of the first open flower was used to measure the flower induction and the number of total nodes was recorded.
**Light Sources**

For photoperiodic treatments, 30 W cool white Flu lamps (R/FR= 3.3, PPFD = 2.3 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)), or 60 W Inc lamps (PPFD= 2.6 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), R/FR= 0.7) and FR were used for daylength extension (Fig. 3.1). FR was obtained by wrapping the Inc lamps with the plastic filter, giving R/FR= 0.15 and photon flux density 700-800 nm= 1.02 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (Fig. 3.1). The 1000 W high-pressure sodium vapor lamps gave PPFD of 78 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), with a R/FR at 2.4. All the light intensity and the R/FR ratios were measured by a Model LI-1800 Portable Spectroradiometer (LiCor Inc, Lincoln, NE).

![Graph showing photon flux density vs wavelength for Flu, Inc, and FR](image)

**Figure 3.1. The light conditions used in photoperiodic treatments.** Flu, fluorescent light. Inc, incandescent light. FR, far-red light.
Results

Determination of Photoperiodic Competence and Critical Daylength

In many plants, there is a juvenile phase (a period of vegetative growth) in which plants are incapable of responding to floral "signal" and thus can not be induced to flower. In *N. sylvestris*, the juvenile phase is about two months, and after reaching this stage plants need the photoinductive condition (8 to 12 LD) presumably to produce a sufficient amount of a hormone-like, flower inducing agent ("florigen"), and then begin forming flowers very rapidly, i.e. they have attained the floral state (Lang, 1989).

The developmental stage was expressed as leaf numbers. Under 14 h LD in 24 h cycles. *N. sylvestris* plants became competent to perceive the photoperiodic stimulus only after development of 6 leaves (Fig. 3.2). This is equal to about 2 months after germination, consistent with the observations of others (Lang, 1989). Once photoperiodic competence was achieved, further increases in age did not affect the sensitivity of plants to LD (Fig. 3.2). Thus, plants were not used in experiments until they had attained at least 6 leaves.
Figure 3.2. Determination of photoperiodic competence in *N. sylvestris*. A total of 6 to 7 WT plants at the various stages were transferred from 8 h SD to 14 h LD. The days to the visible flower buds were recorded. Values are the means and the bars represent the standard deviation. The leaves are defined in the Materials and Methods.

*N. sylvestris* is an obligate LDP, which means it will not flower if the daylength is shorter than the *critical daylength*. This photoperiodic behavior seems to be relatively independent of temperature, with a range of a maximum 30°C day and minimum 15°C night temperature (Lang, 1989). However, its critical daylength is unknown. Since the response of most LDP to photoperiod treatments depends on how close the length of the experimental treatment is to the critical daylength (Thomas and Vince-Prue, 1997), it was necessary to determine the critical daylength in *N. sylvestris*. Plants were subjected to daylength extensions with 3, 4 and 5 h low intensity light from Inc lamps following an 8 h main light period. Results in Experiment 1 showed that 12 and 13 h photoperiods induced all of the plants to flower at the same time, and about the same number of leaves developed (Table 3.1). However, under 11 h daylength, only 67% plants flowered and those plants that flowered required longer time with more leaves to form visible flower
buds (Table 3.1). In the second experiment, although plants flowered later under 12 h daylength than that in Experiment 1 (Table 3.1), it was consistent that a lower percentage of plants flowered and larger leaf numbers developed for the plants that have formed flower buds under 11 h photoperiods than Experiment 1 (Table 3.1). Therefore, it is concluded that *N. sylvestris* has a critical daylength at about 12 h. The overall delay in flowering for Experiment 2 may have been due to the lower light intensity during the 8 h main light period in Experiment 2 (August to November) compared to Experiment 1 (June to August), indicating that the light intensity also affects the flowering process (Thomas and Vince-Prue. 1997).

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Daylength</th>
<th>Percentage</th>
<th>Days to visible flower buds</th>
<th>Increase in leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>extensions</td>
<td>(%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>3 h Inc</td>
<td>67</td>
<td>41.0±14.7 ^a</td>
<td>19.0±8.0 ^a</td>
</tr>
<tr>
<td></td>
<td>4 h Inc</td>
<td>100</td>
<td>27.2±3.1 ^b</td>
<td>11.8±0.4 ^a</td>
</tr>
<tr>
<td></td>
<td>5 h Inc</td>
<td>100</td>
<td>25.3±1.7 ^b</td>
<td>12.0±1.8 ^a</td>
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<tr>
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<td>3 h Inc</td>
<td>20</td>
<td>67.0</td>
<td>31.0</td>
</tr>
<tr>
<td></td>
<td>4 h Inc</td>
<td>100</td>
<td>43.6±9.6</td>
<td>19.8±2.8</td>
</tr>
</tbody>
</table>

Table 3.1. Determination of the critical daylength in *N. sylvestris* For each treatment, 5-6 WT and 4 transgenic plants were used. Means followed by the same letter in a column within the Experiment 1 are not significantly different at P=0.05. In Experiment 2, only one out of 5 WT plants under 3 h Inc condition flowered, and thus no statistic test was performed. Percentage, the percentage of plants that have flowered after about 3 months; Inc, incandescent light.
SCB35 Accelerated but SOB and SUA Lines Delayed Flowering at Photoperiods Close to the Critical Daylength

WT and the transgenic lines, SUA2, SCB35 and SOB36, did not flower after 3 months under 8 h SD (data not shown), but they responded differently when subjected to photoperiods close to the critical daylength (Table 3.2). After 3 months of being subjected to 3 h daylength extension from Inc light, only 20% WT plants flowered, while all of SCB35 plants flowered (Table 3.2). Plants of both SUA2 and SOB36 did not flower (Table 3.2). Under 12 h photoperiod, all WT and SCB35 plants flowered, but SCB35 flowered much earlier than WT and developed fewer leaves (Table 3.2). Flowering in SOB36 was delayed compared to WT (Table 3.2). Flowering under 12 h photoperiods was even further delayed in SUA2: only 50% SUA2 plants formed flower buds, and the plants that were induced required longer time and more leaves (Table 3.2). The promotion of flowering in SCB35 and the delay in SOB36 under both 11 and 12 h photoperiods suggest that phy-B1 is inhibitory to flower induction. Conversely, the delay of flowering in SUA2 indicates that phy-A1 is required for the promotion of flowering under daylength extension close to the critical daylength using Inc light.
<table>
<thead>
<tr>
<th>Extension</th>
<th>Genotype</th>
<th>Percentage (%)</th>
<th>Days to visible flower buds</th>
<th>Increase in leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h Inc</td>
<td>WT</td>
<td>20</td>
<td>67.0</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>SUA2</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td></td>
<td>SCB35</td>
<td>100</td>
<td>32.3±3.3</td>
<td>17.8±1.0</td>
</tr>
<tr>
<td></td>
<td>SOB36</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>4 h Inc</td>
<td>WT</td>
<td>100</td>
<td>43.6±9.6</td>
<td>19.8±2.8</td>
</tr>
<tr>
<td></td>
<td>SUA2</td>
<td>50</td>
<td>58.5±9.2</td>
<td>26.0±1.4</td>
</tr>
<tr>
<td></td>
<td>SCB35</td>
<td>100</td>
<td>31.3±2.4</td>
<td>16.3±0.5</td>
</tr>
<tr>
<td></td>
<td>SOB36</td>
<td>100</td>
<td>56.0±12.2</td>
<td>24.5±1.9</td>
</tr>
</tbody>
</table>

Table 3.2. Flowering responses of transgenic plants under incandescent daylength extensions close to the critical daylength. For each treatment, 5 WT and 4 transgenic plants were used. Means ± standard deviations followed by the same letter in a column in the 4 h Inc experiment are not significantly different at P=0.05. Under 3 h Inc condition, only one out of 5 WT plants flowered, and thus no statistical analysis was performed. Percentage, the percentage of plants that have flowered after about 3 months; Inc, incandescent light.

When WT and SCB35 were grown in a greenhouse under natural photoperiods beginning January 5, 1999 (about 9.4 h daylength), SCB35 plants flowered substantially earlier than WT, with the first open flower appearing around April 7. This was 17 days from March 21, a day with a daylength of 12 h (Fig. 3.3). In contrast, the first open flower appeared in WT plants around May 3, 42 days from March 21 (Fig. 3.3). Since the plants required 15-20 days to open the first flower from the formation of the visible flower buds (data not shown), SCB35 plants had been induced to form the flower buds before March 21, the 12 h daylength, while WT plants just began to respond to the
inductive LD. The other measurement of flowering using the number of nodes led to the same conclusion that SCB35 flowered earlier than WT under this natural SD condition. This result is consistent with the previous 3 or 4 h Inc extension experiments (Table 3.2), supporting that phy-B1 has an inhibitory role in photoperiodic control in nature. The inhibition of flowering by phy-B1 is consistent with other findings that phyB is inhibitory to flowering in both LDP Arabidopsis, barley and pea, and SDP sorghum (Bagnall et al., 1995; Childs et al., 1997; Goto et al., 1991; Hanumappa et al., 1999; Reed et al., 1994).

**Figure 3.3. Flowering responses under natural daylength from January to May in Columbus, OH (40°N).** Five young WT and SCB35 seedlings were transferred to the greenhouse without any daylength extension, beginning from January 5 (a daylength of 9.4 h) to May 11, 1999 (a daylength of 14.2 h). The days from March 21 (with a daylength of 12 h) to the first open flower and the number of nodes were recorded. The nodes were counted from at least 10 cm long basal leaves to the node below the terminal bud. Values represent the means ± standard deviations. Values with the same letter above the column are not statistically different at P=0.05.

**SCB35 Delayed Flowering under 8 h Fluorescent Light Extensions while SUA2 Delayed Flowering under both Fluorescent and Incandescent Light Extensions**

In order to more fully understand the functions of phy-A1 and phy-B1 during an inductive daylength, the flowering responses of various genotypes were investigated.
using 8 h daylength extensions of different spectral compositions. Results showed that under 8 h extensions (a total photoperiod of 16 h) with either Inc or Flu, WT, SOB36 and SUA2 lines flowered earlier, compared to 3 or 4 h extensions (Table 3.2 and 3.3).

In sharp contrast to the early flower induction under 3 or 4 h Inc extension (Table 3.2), flowering was dramatically delayed in SCB35 compared to WT under the 8 h Flu daylength extension (Table 3.3). Only 75% of SCB35 plants flowered by the end of 3 month experimental period, and those that flowered required, on average, 36 more days and produced 19 more leaves than WT (Table 3.3). Moreover, SOB36 flowered at the same time as WT. Since the Flu light contains a much larger proportion of R and very little FR than Inc light (Fig. 2.1), R seems to be a necessary and sufficient component for the daylength extension and this response is likely mediated by phy-B1.

Previously, it has been shown that phy-B1 is inhibitory to flowering at 11 or 12 h photoperiods. Thus, phy-B1 has a dual inhibitory/promotive function depending on the light during the daylength extension, inhibitory at the Inc extension close to the critical daylength, while promotive at the Flu extension above the critical daylength.

Under 8 h Inc extension, WT plants flowered slightly earlier (about 5 days) than those under Flu extension, although the number of leaves produced was the same (Table 3.3). This is consistent with many other observations that mixtures of R and FR (such as Inc, R/FR=0.7) are the most effective in promoting flowering in LDP (Thomas and Vince-Prue, 1997). However, both SCB35 and SOB36 flowered at the same time as WT plants under 8 h Inc extension (Table 3.3). These results show that the overexpression or co-suppression of PHY-B1 in SOB36 or SCB35 does not greatly affect the flower
induction under an Inc daylength extension that creates photoperiods longer than the critical daylength.

<table>
<thead>
<tr>
<th>Extension</th>
<th>Genotype</th>
<th>Percentage (%)</th>
<th>Days to visible flower buds</th>
<th>Increase in leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 h Flu</td>
<td>WT</td>
<td>100</td>
<td>23.0±2.0 a</td>
<td>11.0±1.0 a</td>
</tr>
<tr>
<td></td>
<td>SUA2</td>
<td>100</td>
<td>25.8±3.1 b</td>
<td>12.0±1.6 a</td>
</tr>
<tr>
<td></td>
<td>SCB35</td>
<td>75</td>
<td>59.0±27.2 c</td>
<td>29.7±14.2 b</td>
</tr>
<tr>
<td></td>
<td>SOB36</td>
<td>100</td>
<td>23.3±2.6 a</td>
<td>11.8±1.7 a</td>
</tr>
<tr>
<td>8 h Inc</td>
<td>WT</td>
<td>100</td>
<td>18.0±0.8 a</td>
<td>10.8±1.0 a</td>
</tr>
<tr>
<td></td>
<td>SUA2</td>
<td>100</td>
<td>24.0±1.4 b</td>
<td>11.3±1.0 a</td>
</tr>
<tr>
<td></td>
<td>SCB35</td>
<td>100</td>
<td>21.0±2.4 a</td>
<td>11.3±0.5 a</td>
</tr>
<tr>
<td></td>
<td>SOB36</td>
<td>100</td>
<td>20.3±2.2 a</td>
<td>10.8±1.5 a</td>
</tr>
</tbody>
</table>

Table 3.3. Flowering responses of transgenic plants under 8 h incandescent and fluorescent daylength extensions A total of 5 WT and 4 plants of each transgenic line (SUA2, SCB35 and SOB36) were grown under 8 h SD and then transferred to 8 h Inc or Flu extensions following 8 h SD. Percentage (%), the percentage of plants which have formed the flower buds after 3 months of the treatments. Values are the means ± standard deviations. Values followed by the same letters in a column are not statistically different at P=0.05. Flu, cool white fluorescent light. Inc, incandescent light.

When the flowering response of SUA2 under 3 or 4 h Inc extension and 8 h Inc extension were compared (Table 3.2 and 3.3), SUA2 plants flowered much faster under 8 h Inc extension than 3 or 4 h Inc extensions. This is likely due to the remaining phy-A1 levels in the SUA2 plants. When flowering responses of WT and SUA2 under 8 h Flu extension were compared, SUA2 plants flowered slightly later (about 3 days) than WT, although the numbers of the leaves that developed were the same (Table 3.3). When
plants were subjected to an 8 h Inc daylength extension, WT plants flowered 5 days earlier than with an 8 h Flu extension (Table 3.3), consistent with many physiological observations that the addition of FR promotes flowering in LDP (Thomas and Vince-Prue, 1997). However, the promotion of flowering in SUA2 did not occur (Table 3.3). This result indicates that phy-A1 is responsible for the promotion of flowering by FR.

Since the leaf numbers in the different genotypes (except for SCB35) were not statistically different under 8 h Inc and Flu daylength extensions (Table 3.3), the flowering responses under these treatments were repeated during November to April. In addition, 8 h FR extension, 7.5 h Flu extension followed by 30 min EOD-FR, and 2 h dark-interruption experiments were also carried on. The results are summarized in Table 3.4.

Under 8 h FR extensions, none of the genotypes flowered (Table 3.4), indicating that FR is not a sufficient component of an inductive daylength extension. In contrast, WT, SOB36 and SUA2 plants flowered under 8 h Flu daylength extensions (Table 3.4), supporting that R is a necessary and sufficient component for day extension.

Furthermore, it has been shown that some LDP such as Lolium are differentially sensitive to R and FR during the daylength extension with different sensitivity: During the early part of extension R is inhibitory while FR is promotive, and their effects during the later part are reversed (Thomas and Vince-Prue, 1997). The effects of R and FR during the early part can also be demonstrated using dark-interruption experiments (Holland and Vince, 1971). Thus similar dark-interruption experiments were performed in N. sylvestris and the results were consistent with the notion that the presence of Pfr at this time is inhibitory to flower induction. Providing a 2 h dark-interruption significantly
promoted flowering in WT, compared to a continuous 8 h Flu extension (Table 3.4). Further promotion of flowering was obtained if a 30 min FR treatment was provided prior to the dark period. indicating that R and FR during this phase of the daylength extension is inhibitory and promotive to flowering, respectively.

The flowering responses of SOB36 and SCB35 plants under 8 h continuous Flu extension and dark-interruption were quite different (Table 3.4). SOB36 plants flowered at the same time as WT plants under 8 h Flu extension and dark-interruption treatments, but flowered later than WT plants pretreated with 30 min FR before the dark-interruption. In contrast, flowering in SCB35 was always delayed compared to WT under these conditions (Table 3.4). These results indicate that phy-B1 is not simply either promotive or inhibitory.

Table 3.4. Flowering responses of various genotypes under different light treatments
A total of 7 or 8 plants for WT and SUA2, SCB35 or SOB36 transgenic line, and 5 plants of the cross SUA2 × SCB35 were grown under 8 h SD and then transferred to different light conditions. Percentage (%), the percentage of plants which have formed the flower buds to the total number of plants after 5 months of the treatments. Values are the means ± standard deviations. Flu, cool white fluorescent light; Inc, incandescent light; 7.5 Flu + 0.5 FR, 7.5 h Flu extension followed immediately by 30 min FR; 2 D, extended by 3 h Flu followed by 2 h dark period and then 3 h Flu extension; 0.5 FR + 2 D, 8 h photoperiod extended by 2.5 h Flu, then 30 min FR before 2 h dark period, and finally 3 h Flu. None of genotype flowered after 4 months under 8 h SD and 8 h FR extensions and thus these data are not shown.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genotype</th>
<th>Percentage (%)</th>
<th>Days to visible flower buds</th>
<th>Increase in leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 Flu</td>
<td>WT</td>
<td>100</td>
<td>70.6±6.2</td>
<td>25.8±3.0</td>
</tr>
<tr>
<td></td>
<td>SUA2</td>
<td>75</td>
<td>141.3±15.1</td>
<td>52.5±2.7</td>
</tr>
<tr>
<td></td>
<td>SCB35</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>SOB36</td>
<td>100</td>
<td>74.9±8.0</td>
<td>27.3±3.2</td>
</tr>
<tr>
<td></td>
<td>SUA2 X SCB35</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>7.5 F + 0.5 FR</td>
<td>WT</td>
<td>100</td>
<td>53.5±6.3</td>
<td>18.6±2.1</td>
</tr>
<tr>
<td></td>
<td>SUA2</td>
<td>25</td>
<td>155.0±0</td>
<td>65.5±0.7</td>
</tr>
<tr>
<td></td>
<td>SCB35</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>SOB36</td>
<td>100</td>
<td>87.9±8.9</td>
<td>33.4±4.2</td>
</tr>
<tr>
<td></td>
<td>SUA2 X SCB35</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>2 D</td>
<td>WT</td>
<td>100</td>
<td>57.0±13.6</td>
<td>22.8±4.1</td>
</tr>
<tr>
<td></td>
<td>SUA2</td>
<td>43</td>
<td>138.3±27.2</td>
<td>52.0±7.9</td>
</tr>
<tr>
<td></td>
<td>SCB35</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>SOB36</td>
<td>100</td>
<td>85.6±22.4</td>
<td>36.0±9.5</td>
</tr>
<tr>
<td></td>
<td>SUA2 X SCB35</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>0.5 FR + 2 D</td>
<td>WT</td>
<td>100</td>
<td>39.5±6.3</td>
<td>15.9±2.2</td>
</tr>
<tr>
<td></td>
<td>SUA2</td>
<td>100</td>
<td>136.7±8.7</td>
<td>54.9±4.5</td>
</tr>
<tr>
<td></td>
<td>SCB35</td>
<td>100</td>
<td>95.6±27.2</td>
<td>38.6±12.7</td>
</tr>
<tr>
<td></td>
<td>SOB36</td>
<td>100</td>
<td>61.9±19.9</td>
<td>24.5±6.9</td>
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<tr>
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<td>SUA2 X SCB35</td>
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<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>8 Inc</td>
<td>WT</td>
<td>100</td>
<td>28.9±2.5</td>
<td>10.6±1.1</td>
</tr>
<tr>
<td></td>
<td>SUA2</td>
<td>100</td>
<td>82.0±3.3</td>
<td>35.3±1.5</td>
</tr>
<tr>
<td></td>
<td>SCB35</td>
<td>100</td>
<td>29.1±3.4</td>
<td>11.4±0.7</td>
</tr>
<tr>
<td></td>
<td>SOB36</td>
<td>100</td>
<td>37.6±2.5</td>
<td>13.5±0.9</td>
</tr>
<tr>
<td></td>
<td>SUA2 X SCB35</td>
<td>100</td>
<td>133.0±2.3</td>
<td>60.4±1.7</td>
</tr>
</tbody>
</table>

Table 3.4. Flowering responses of various genotypes under different light treatments
A 30 min FR treatment immediately before the 3 h dark-interruption induced all of SCB35 plants to flower, while no flowering was observed if the plants were subjected to an 8 h Flu extension that was continuous or interrupted with a 2 h dark period (Table 3.4). This indicates that the promotion of flowering by FR is likely mediated by phy-A1. The slight promotion of flowering in SUA2 plants pretreated with a 30 min FR prior to the 2 h dark-interruption, compared to the 2 h dark-interruption or 8 h continuous Flu extension (Table 3.4), suggests that there is sufficient phy-A1 remaining for an attenuated response to FR. This also supports the earlier conclusion that R is inhibitory and FR is promotive during the early part of extension. In addition, 30 min FR added at the end of the 7.5 h Flu extension promoted flowering in WT but not in SUA2 plants, indicating FR at this time also promotes flowering. Taken together, these results support that FR is sensed by phy-A1 during the extension.

Under 8 h Inc extension, SUA2 plants flowered much later (53 days) and produced about 25 more leaves than WT (Table 3.4). The delay was much greater than the previous experiment (Table 3.3). On the other hand, when plants were subjected to 8 h Flu extension, flowering in SUA2 was dramatically delayed, with only 75% plants forming flower buds. All WT plants flowered within 5 months from the start of the treatments. There was also a delay of flowering compared to SUA2 under 8 h Inc extension (Table 3.4). The inconsistency with the earlier observations under both 8 h Flu and Inc extension (Table 3.3) might be due to the slight deficiency in FR perception during the 8 h SD period. Indeed, compared to the previous experiment (June to August, see Table 3.3), there was an overall delay of flowering in WT, SCB35, SOB36 and SUA2
plants, likely due to the lower intensity during the main 8 h photoperiod (August to November).

Thus, another experiment with two different R/FR ratios during the 8 h SD period followed by the same Flu extension was setup in the growth chambers since light intensity can be more easily controlled inside the chambers. WT plants flowered earlier under a low R/FR ratio (1.4) than with a high R/FR ratio (6.3), suggesting the addition of FR during the main light period also promotes flowering (Fig. 3.4). There was a delay of flowering in SUA2 under high R/FR main light period. However, SUA2 plants flowered later than WT when the main light period had a R/FR ratio of 1.4, and phenocopied WT plants grown under high R/FR ratio (Fig. 3.4). This indicates that phy-A1 is also involved in the perception of FR during the main light period as well as the daylength extension.

The Cross SUA2 x SCB35 Dramatically Delayed Flowering under Low Intensity Extensions

Since phy-A1 and phy-B1 appear to sense FR and R, respectively, and seem to have the opposite functions in the control of flower induction at least during the early part of extension (3 or 4 h), the cross SUA2 x SCB35 (Chapter 2) were also subjected to various daylength extensions.

Regardless of the treatments, the greatest delay in flowering in any genotype was the cross SUA2 x SCB35 (Table 3.4). Under an 8 h Inc extension, flowering in the cross SUA2 x SCB35 was delayed nearly two-fold over that of SUA2 (Table 3.4). Similar results were obtained with 30 min FR plus 2 h dark-interruption (Table 3.4).
Figure 3.4. Flowering responses of SUA2 under high and low R/FR ratios during the 8 h high intensity light period followed by the same low intensity Flu extensions. A. Days to visible flower buds. B. Increase in leaf number when plants formed flower buds. Five plants of both WT and SUA2 were subjected to the high R/FR (R/FR=6.3, PPFD=200.8 μmol m⁻² s⁻¹) and low R/FR (R/FR=1.4, PPFD=240.7 μmol m⁻² s⁻¹) followed by low intensity Flu (R/FR=6.3, PPFD=46.3 μmol m⁻² s⁻¹) extensions. Values represent the means ± standard deviations. Means followed by the same letter in a column within a treatment are not significantly different at P=0.05.
These suggest that both phy-A1 and phy-B1 are required for maximal flowering induced by low intensity daylength extension and they have a synergistic effect.

In another preliminary experiment, the flowering responses of the cross and other genotypes were investigated under high intensity light extensions. Under 17 h high intensity LD conditions, SCB35 and SOB36 flowered at the same time as WT plants, while in SUA2 and the cross SUA2 × SCB35 flowering was significantly delayed by about 4-5 days and 2-3 leaves (Fig. 3.5). This indicates phy-A1 perhaps plays a more important role than phy-B1 under such a light conditions.

**Figure 3.5. Flowering responses of various genotypes under 17 h high intensity LD**

A total of 7 plants for each genotype were grown under 8 h SD and then transferred to the 17 h high intensity LD greenhouse. Values are the means and the vertical bars represent the standard deviations. Values with the same letters shown above the column are not statistically different at P=0.05.
Discussion

The rosette plant *N. sylvestris* is an obligate LDP that will not flower if the daylength is shorter than a critical daylength of about 12 h (Table 3.1). The use of this type of LDP has the advantage over quantitative LDP such as Arabidopsis and pea in that plants can be maintained under non-inductive SD (e.g. 8 h) for quite a long period and then readily induced by the LD (Fig. 3.2).

Both R and FR are Necessary Components for Flowering, but Only R is Sufficient

Consistent with many physiological studies (Thomas and Vince-Prue, 1997), daylength extensions with FR alone are not sufficient for flower induction while R is a necessary and sufficient component for the day extension in the LDP *N. sylvestris* (Table 3.3 and 3.4). However, the addition of FR dramatically promotes flowering (Table 3.3; Table 3.4), indicating that, although not sufficient, FR is a necessary component of the daylength extension for maximal flowering. This is consistent with the general conclusion that a mixture of R and FR (like in Inc) is optimal for flower induction in the LDP such as *Hordeum vulgare, Lolium temulentum, Hyoscyamus niger* (Thomas and Vince-Prue, 1997).

The diurnal sensitivity to R and FR has been observed in some LDP including *Lolium* (Vince-Prue, 1975), *H. vulgare* (Deitzer et al., 1979) and Arabidopsis (Deitzer, 1984). FR promotes and R inhibits the flowering if given during the early part of daylength extension, while R is promotive and FR inhibitory if given during the later part of daylength extension (Thomas and Vince-Prue, 1997). It appears that for *N. sylvestris* FR is generally promotive for flower induction, since WT plants flowered earlier if FR is
added either during the 8 h main light period (Fig. 3.4), 2.5 h after Flu extension before the dark-interruption, or even at the end of 7.5 h Flu extensions (Table 3.4). However, it is not certain at what time during the 24 h day/night cycle FR is maximally promotive. Further experiments to discern a possible diurnal fluctuation in the sensitivity to FR are necessary.

On the other hand, it seems that R is inhibitory to flowering during the early part of the daylength extension, but becomes promotive during the later part of the extension in *N. sylvestris* (Tables 3.2, 3.3 and 3.4). These results agree well with early physiological studies of LDP including *Lolium* (Evens, 1976; Holland and Vince, 1971; Vince, 1965), *Petunia* and *Fuchsia* (Thomas and Vince-Prue, 1997). In *Lolium*, a 2 h interruption with darkness promoted flowering compared with the uninterrupted red light extension following the 8 h main light period, and the effect was proportional to the duration of darkness at least up to 3 h (Holland and Vince, 1971; Vince, 1966). Moreover, 10 min FR received prior to darkness increased the effect by an amount equal to that resulting from about 45 min of darkness, and the promotive effect of this brief FR could be reversed by a subsequent exposure to R (Holland and Vince, 1971). The mechanism for the dark-interruption is not clear. It is possible that the dark reduces the level of inhibitory Pfr through dark reversion of Pfr to Pr or the degradation of Pfr, and the added FR simply further removes the Pfr (Thomas and Vince-Prue, 1997). Another experiment varying the sequences of R and FR during the extensions has also shown that the extension of 7 h FR followed by 1 h R promoted flowering in *Lolium temulentum* (Ba 3081) as strongly as an 8 h R+FR extension, but remained vegetative under an extension with 7 h R followed by 1 h FR plants (Vince, 1965). In addition, in *Lolium* when an 11 h FR extension was
interrupted by 2 h R at various times, the maximal promotion of flowering was observed when R was provided in the final 2 h of the extension period (Vince, 1965). This is consistent with the night-break induction of flowering in LDP (Thomas and Vince-Prue, 1997). Clearly, R becomes promotive during the later part of the extension.

Although no direct test for the promotive effects of R during the later part of extension in *N. sylvestris* has been performed, the 8 h Flu extension induced flowering, while R at photoperiods close to the critical daylength (equal to the early part of extension) was inhibitory to flowering (Table 3.2; Table 3.3; Table 3.4). These observations suggest that R at the later part of extension should be promotive.

**Phy-A1 is Required for Daylength Extension and is Likely a Sensor of FR**

The use of transgenic *N. sylvestris* plants underexpressing *PHY-A1* (SUA2), overexpressing *PHY-B1* (SOB36) and co-suppressing *PHY-B1* (SCB35) has yielded some useful information on role of individual phytochromes in photoperiodic control of flowering.

Phy-A1 is required for daylength extension, and likely mediates FR similar to the photosensory function of phy-A1 in seedling de-etiolation process (Chapter 2). This is consistent with the role of phyA in daylength perception in Arabidopsis and pea (Johnson et al., 1994; Weller et al., 1997). Results have also shown that phy-A1 is responsible for the perception of FR during the 8 h SD, in addition to both the early and later part of extension (Table 3.2; Table 3.3; Table 3.4; Fig. 3.4). It is likely that FR converts P_R-A1 to P_A1, and that P_A1 is the active form to promote the flowering under the experimental conditions.
There is a difference between pea and Arabidopsis phyA in sensing the daylength extension. In pea, the phyA mutant *fun1* plants are deficient in perception of either high or low intensity Flu extensions (Weller et al., 1997), while in Arabidopsis, the *phyA* mutants flowered at the same time as WT under high intensity Flu extension (Johnson et al., 1994). Thus, it is believed that Arabidopsis phyA is specifically sensing FR-rich light extension but pea phyA is responsible for the perception of daylength extensions with both high and low R/FR ratios.

In this study, such a discrepancy also occurred. One experiment showed that SUA2 plants were insensitive to the addition of FR during the extension, behaving as WT plants under Flu extensions (Table 3.3), while another experiment showed there was also a delay for SUA2 under 8 h Flu extension, compared to WT (Table 3.4). The discrepancy between these two experiments is likely due to the different light intensity during the 8 h natural light period. In nature, sunlight has higher light intensity from August to November (the first experiment, Table 3.3) than from November to March (the second experiment, Table 3.4). This resulted in the overall higher levels of FR during the first experiment than the second. All of genotypes including WT, SUA2, SCB35 and SOB36 flowered later in response to lower intensity light during the second experiment (Table 3.3; Table 3.4). Indeed, the addition of FR during the main light period promotes flowering in WT (Fig. 3.4). Thus, the delay of flowering in SUA2 under Flu extension is likely due to the reduction of total P_rAl in SUA2 plants which is at least partially deficient in phy-A1 (Chapter 2), rather than to the reduced perception of R through phy-A1.
The delay of flowering in SUA2 under 8 h Flu extensions (Table 3.4; Fig. 3.4) can be explained as follows. Although very little FR is contained in Flu, earlier studies have shown that even the pure R can not convert all of Pr to Pfr (about 20% of Ptot at the photoequilibrium exists as Pr), indicating that there are significant levels of PrAl after irradiation by R (Smith, 1982). In fact, the estimated PrAl under Flu conditions in this study is about 30%, based on the Pfr and R/FR ratios curve (Smith, 1982). Since phytochrome is synthesized as Pr, and, as discussed above, PrAl positively activates flower induction, the delayed flowering in SUA2 under Flu extensions is because of the lower levels of PrAl in SUA2 than WT (Chapter 2). The addition of FR led to the conversion of PfrAl to PrAl, slightly increasing the absolute PrAl level, and SUA2 plants would be expected to respond to the FR signal through remaining PfrAl and thus promote flowering, but still flower later than WT (Table 3.2, 3.3, and 3.4; Fig. 3.4). The fact that 30 min FR added at the end of 7.5 h Flu extension promotes flowering in WT but not SUA2 (Table 3.4), demonstrating the perception of FR by phy-Al. In addition, the ectopic expression of PHYA in transgenic tobacco plants increased the sensitivity to FR and resulted in the shorter stems (Jordan et al., 1995; Robson et al., 1996), indicating that phyA can be a sensor for FR in light grown plants.

**Phy-B1 Mediates R during the Extension and can be either Inhibitory or Promotive to Flowering Depending on the Time of the Extension**

Similar to its photosensory functions in seedling development, phy-B1 likely mediates R-induced processes in the light-grown mature plants, since flowering in SCB35 was substantially delayed under 8 h Flu extension compared to WT (Table 3.3,
3.4). In other words, PfrB1 is the active form in the flower induction process.

The apparent positive action of phy-B1 in flower induction is in sharp contrast to the inhibition of phyB in flowering in both LDP, Arabidopsis, barley and pea, and the SDP sorghum (Childs et al., 1997; Goto et al., 1991; Hanumappa et al., 1999; Weller et al., 1995). The promotion of flowering by phyB has been observed recently in the DNP \textit{N. plumbaginifolia} (Hudson et al., 1997; 1998). The mutation in a \textit{PHYB}-like gene encoded by the \textit{HLG} locus resulted in the delay of flowering in \textit{N. plumbaginifolia}. The \textit{HLG}-encoded phyB shares higher (91-97%) amino acid identity with the phyB1 orthologs than phyB2 orthologs (87-89%) in \textit{N. tabacum}, tomato and potato (see Table 1.2 in Chapter 2). This suggests that the phyB in \textit{N. plumbaginifolia} is likely an ortholog of \textit{N. tabacum} phy-B1. Therefore, it appears that the \textit{Nicotiana} phyB1 has different functions in flower induction than phyB in Arabidopsis, barley, pea and sorghum (Childs et al., 1997; Goto et al., 1991; Hanumappa et al., 1999; Weller et al., 1995). In addition, the photosensory functions of phyB1 in light-grown plants are also different. Unlike phyB mediating R/FR ratios or EOD-FR in other plants including Arabidopsis, pea, cucumber and \textit{Brassica rapa} (Delvin et al., 1992, 1997; Lopez-Juez et al., 1992; Nagatani et al., 1991; Somers et al., 1991; Reed et al., 1993; Weller et al., 1995), phy-B1 in the three closely related species in the Solanaceae, tobacco, potato and tomato, is unlikely the sensor for the end-of-day FR or low R/FR ratio (Hudson et al., 1997; Smith and Whitelam, 1997; van Tuinen et al., 1995b). In this study, similar to the \textit{hlg} mutant in \textit{N. plumbaginifolia}, flowering in SCB35 was accelerated at low R/FR ratios compared with high R/FR ratios (Table 3.3, 3.4; Fig. 3.4).
On the other hand, phy-B1 can be inhibitory to flowering if the extension period is close to the critical daylength, i.e. about 12 h (Table 3.2; Fig. 3.3). Since phy-B1 is likely a sensor for R, the early flowering response of SCB35 can be explained by the inhibition of R mediated by phy-B1. Previously it has been shown that phy-B1 is required for 8 h daylength extension with Flu light, and it can be concluded that phy-B1 can have a dual inhibitory/promotive function during the day extension: inhibitory during the early part of the extension (before the critical daylength) and then becomes promotive during the later part of the extension (beyond the critical daylength). This model can explain many earlier physiological studies using the classical LDP, *Lolium, H. niger* and *H. vulgare* that FR is promotive and R inhibitory during the early part of extension while becomes the opposite during the later part of extension (Thomas and Vince-Prue, 1997).

Both phy-A1 and phy-B1 are required for the maximal promotion of flowering during daylength extension

The dramatic delay of the cross SUA2 x SCB35 under low intensity light extensions (Table 3.4) demonstrates both phy-A1 and phy-B1 mediating FR and R, respectively, are required for the maximal promotion of flowering. Thus, a mixture of R and FR can be interpreted in this way: phy-A1 mediates FR while phy-B1 perceives R, and an optimal ratio of PfAl to PfB1 is established. Since flowering was dramatically suppressed in the cross SUA2 x SCB35 under 8 h Inc extension or 30 min FR plus 2 h dark-interruption, compared to either SUA2 or SCB35 (Table 3.4), it is likely that phy-A1 and phy-B1 act through two distinct pathways in the photoperiod control of flowering. The different cellular localizations of phyA and phyB in Arabidopsis, i.e.
phyA is localized in cytoplasm (Pratt, 1994) while phyB can be translocated to nucleus after exposure to R (Sakamoto and Nagatani, 1996a; Yamaguchi et al., 1999), also indicates that phyA and phyB have different pathways. Thus, an alternative explanation for the promotion of flowering by dark-interruption could be: 2 h dark period reduces the amount of inhibitory P_{fr}B1, and the FR treatment converted P_{fr}A1 to the promotive P_{r}A1, rather than through dark reversion of P_{fr} to P_{r} (Thomas and Vince-Prue, 1997). However, the other explanation may be possible that the dark-interruption acts to falsely signal the dusk and dawn, and thus the circadian rhythm is involved (Thomas and Vince-Prue, 1997).

The slight delay of flowering in the cross SUA2 x SCB35 under high intensity extensions (Fig. 3.5) could be due to the fact that the expression of both PHY-A1 and PHY-B1 genes were only partially suppressed. The remaining levels of phy-A1 and phy-B1 are perhaps capable of perceiving the minimal photons of R and FR under such a condition. The other possibility that other phytochromes are involved in the flower induction can not be excluded. like in Arabidopsis (Whitelam and Delvin, 1997).

A Model for Photoperiodic Control of Flowering by Phytochromes

Based on the discussion above, a model for the roles of phy-A1 and phy-B1 in the photoperiodic control of flowering in the obligate LDP N. sylvestris is proposed (Fig. 3.6). In this model, FR is mediated by phy-A1 and is generally promotive to flowering during the main light period, and both the early and later part of the daylength extension. On the other hand, R is sensed by phy-B1 and can be inhibitory to flowering during the first part of the extension but becomes promotive during the second part of extension. At
Figure 3.6. A model for phytochromes control of flowering in the LDP *N. sylvestris*. The 8 h extension period is divided into early and later parts. Phy-A1 and phy-B1 have distinct functions: phy-A1 mediating FR promotes flowering, while phy-B1 mediating R inhibits flowering during the early part but promotes at the later part of extension. CDL, critical daylength; R, red light; FR, far-red light.

what point of time during the 16 h light period or even the whole 24 h day/night cycle, FR is most promotive (or inhibitory) remains unclear. But it can be tested by investigating the flowering responses to FR added during the 8 or 16 h Flu (or R) extension period. Similarly, the effects of R at other times during the 24 h day/night cycle can be determined by interrupting the FR extension with R.
It has been demonstrated that blue light has the effect on flowering in Arabidopsis and other cruciferous plants and can also be perceived by phytochromes with a low absorbance value (Fig. 1.1 in Chapter 1) (Thomas and Vince-Prue, 1997). Although light from Flu lamps in this study contains a considerable proportion of blue light (Fig. 3.1), the direct involvement of blue light mediated by phytochromes is perhaps not profound in *N. sylvestris*. First, blue light is reported so far to be effective in the cruciferous plants including Arabidopsis, not in other plants (Thomas and Vince-Prue, 1997). Second, both P_r and P_f absorbs blue light equally well (Fig. 1.1), and thus the P_f/P_tot ratios will unlikely be affected. Indeed, irradiance with 589 nm alone was ineffective to promote the flowering in Arabidopsis but the addition of blue light was inductive. The 589 nm alone and 589 nm plus blue light treatments established approximately the same P_f/P_tot ratios and cycling rates between P_r and P_f (Mozley and Thomas, 1995), demonstrating that the effect of blue light is not due to phytochrome. In fact, blue light receptors CRY1 and CRY2 redundantly mediate the promotion of flowering by blue light in Arabidopsis (Guo et al., 1998; Mockler et al., 1999). In addition, both phyB and CRY1/CRY2 seem to maintain photoperiodic sensitivity at the early developmental stage (between 1 and 7 days postgermination) in Arabidopsis (Mockler et al., 1999).

It has been demonstrated that the clock is involved in the control of flowering in both SDP and LDP (Thomas and Vince-Prue, 1997). In this study, there are three possible situations in which a circadian clock is involved in the photoperiodic control of flowering. First and most important, the observation that phy-B1 mediates R with opposite functions depending on the time of day can be explained by the involvement of an endogenous circadian clock. It has been show that Arabidopsis phyB is responsible-
for high-fluence-rate R control of the clock period length (Somers et al., 1998b). The phy-B1 ortholog in *N. plumbaginifolia* have also been shown to mediate the light inputs to the internal clock which controls leaf movement (Hudson and Smith, 1998).

Second, the promotion of flowering by dark-interruption may not only because of the lower levels of Pfr through either dark-reversion (Thomas and Vince-Prue, 1997) or lower levels of inhibitory PfrB1 being converted from PfrB1 by R, but also due to the involvement of the circadian clock. The dark interruption may generate false dusk and dawn signals within the treatment cycle, which could have unexpected effects on the circadian rhythms in light sensitivity (Thomas and Vince-Prue, 1997).

Third, blue light in the Flu lamps may also act as a light input to affect the circadian clock. Somers et al. (1998a) have shown that the blue light control of period length of *CAB* transcription rhythm is independent of phyB but depends on both phyA and CRY1. Notably CRY2 which mediates blue light-induced promotion of flowering to antagonize phy-B mediated inhibition in Arabidopsis (Guo et al., 1998) has little effect on the control of the clock period length (Somers et al., 1998a). The short-period mutant (*toc-1*) flowered earlier and the long-period mutant (*toc-7*) later than WT under a 24 h daily cycle, indicating that both *CAB* transcription and flowering rhythms are controlled by the same clock (Somers et al., 1998b; Somers et al., unpublished data). It has been shown that CRY1 and CRY2 act redundantly to promote flowering in blue light (Mockler et al., 1999) and that perhaps CRY2 is involved in LD while CRY1 in SD pathways (Bagnall et al., 1996). There may be some kind of interaction between CRY1 and CRY2 phototransduction pathways which together regulate flowering in Arabidopsis. It will be

128
interesting to investigate the effects of cryptochromes on flowering in the obligate LDP *N. sylvestris* and the interaction between cryptochromes and phytochromes.

In addition, phyA also mediates blue light and low-fluence-rate R control of the period length in Arabidopsis (Somers et al., 1998a). Indeed, the synergistic signaling by phyA and CRY1 has effects on circadian clock-regulated catlase gene expression (Zhong et al., 1997). Therefore, in addition to lower levels of the active PrA1, the other possible reason for the delayed flowering in SUA2 under Flu extension conditions is that the reduction of phy-A1 in SUA2 plants might affect blue light or low-fluence-rate R control of the clock in *N. sylvestris*.

If both phy-A1 and phy-B1 affect the circadian clock and they operate through two distinct pathways, how do phy-A1 and phy-B1 signaling pathways interact with each other and have the inputs into the clock? The understanding of the photoperiodic timing mechanisms in the LDP *N. sylvestris* needs to address this question through the isolation of components of the phototransduction pathways and the clock.

In conclusion, the use of transgenic *N. sylvestris* plants mis-expressing *PHY-A1* and *PHY-B1* genes has shed new light on the mechanisms for photoperiodic control of flowering in LDP. These transgenic materials thus promise to serve as an important tool to study how the LDP senses the daylength information during the whole day/night cycle and how the light input pathway interacts with the circadian clock in order to switch to the reproductive development. Likewise, the generation of the obligate SDP in *Nicotiana* (and other species) using the same reverse genetic approach will reveal the timing mechanisms in SDP. It is anticipated that the photoperiodic timing mechanisms in both LDP and SDP will be elucidated.
CHAPTER 4

OTHER OBSERVATIONS ON PHYTOCHROME CONTROL OF SEED GERMINATION AND STEM ELONGATION IN TRANSGENIC TOBACCO PLANTS

Abstract

Seed germination and stem elongation were investigated using transgenic lines mis-expressing PHY-A1 and/or PHY-B1 genes in N. sylvestris and N. tabacum cv Maryland Mammoth. Preliminary results showed that these two photomorphogenic aspects were also under phytochrome control. Phy-B1 mediated the R promotion of germination in N. sylvestris, while phy-A1 was required for Maryland Mammoth tobacco seeds to germinate in the dark.

The retention of elongation response to low R/FR ratios in SCB35 and SOB36 plants, similar to WT, supported that phy-B1 is not a sensor for low R/FR ratio. Together with the dual inhibitory/promotive function of phy-B1 in flowering (Chapter 3) and other published work (Hudson et al., 1997; van Tuinen et al., 1995b), phy-B1 is proposed to be a sensor for R. On the other hand, under 17 h high intensity LD, SCB35 had longer, while SOB36 and SUA2 had shorter internodes than WT, but the cross SUA2 × SCB35
had the same internode length as WT. These results suggest that phy-A1 and phy-B1 antagonistically regulate stem elongation: phy-A1 promotes but phy-B1 inhibits elongation. This is different from the control of hypocotyl elongation where phy-A1 and phy-B1 mediating FR and R independently inhibit hypocotyl elongation.

Introduction

Phytochromes control photomorphogenesis throughout the entire life cycle of plants, beginning with seed germination and seedling de-etiolation to vegetative and reproductive development (Kendrick and Kronenberg, 1994). Previously, phy-A1 and phy-B1 were shown to have distinct photosensory functions in de-etiolation (Chapter 2) and photoperiodic control of flowering (Chapter 3) in *N. sylvestris*. In this study, the control of seed germination and stem elongation by phytochromes were investigated.

Photocontrol of seed germination in *Lactuca sativa* was one of the very first R/FR reversible responses observed (Borthwick et al., 1952b) and led to the discovery of phytochrome (Butler et al., 1959). Recent molecular genetic studies have shown that in Arabidopsis, phyB plays a primary role in the induction of seed germination, while phyA induces germination under continuous FR (Reed et al., 1994; Shinomura et al., 1994). The phyA dependent germination can be prevented by R-absorbing form of phyB (Shinomura et al., 1994). Furthermore, seed germination induced by LFR and VLFR is mediated by phyB and phyA, respectively (Shinomura et al., 1996). Other phytochromes are also involved in the control of seed germination since the *phyA phyB* double mutants
responded to light treatments (Poppe and Schafer, 1997). In this chapter, the well-characterized SUA, SOB and SCB35 lines of *N. sylvestris*, and two putative *PHY-A1* underexpressing lines of *N. tabacum* cv Maryland Mammoth (MUA8 and MUA17) were chosen to study the phytochrome control of seed germination in *Nicotiana*.

Stem elongation is another aspect of photomorphogenesis controlled by phytochromes. It is well established that R-rich light environment (high R/FR ratio) inhibits stem elongation while FR-rich environment (low R/FR ratio) is stimulatory (Smith, 1994). The collective response to low R/FR ratios is referred to as the shade-avoidance syndrome, one of which is dramatic and rapid stem elongation (Smith, 1994; 1995). PhyA is not involved in this process, although the ectopic expression of *PHYA* can lead to constitutive inhibition of elongation by increasing the sensitivity to FR (for example. Robson et al., 1996; Halliday et al., 1997). In contrast, phyB-subgroup phytochromes (phyB, D and E) are responsible for the shade-avoidance response in Arabidopsis. *Brassica*, pea and cucumber by mediating the low R/FR ratio or end-of-day FR response (Smith and Whitelam, 1997). However, it appears that phy-B1 orthologs in *N. plumbaginifolia* tobacco and tomato do not mediate the low R/FR ratio or EOD-FR response (Hudson et al., 1997; Pratt et al., 1997; van Tuinen et al., 1995b; Smith and Whitelam, 1997). *N. sylvestris* is a rosette LDP that does not elongate or flower under SD unless it is transferred to LD. Flowering has been shown previously to be under the control of at least phy-A1 and phy-B1 in *N. sylvestris*, and the control of stem elongation by phytochromes is investigated using those phytochrome mis-expressing lines and reported here.
Materials and Methods

Plant Material and Growth Conditions

Three groups of transgenic lines in *N. sylvestris* were used: SUA (2, 7, and 11), SOB (1, 36, 40 and 50), and SCB35 (characterized in Chapter 3). Two antisense *PHY-A1* transgenic *N. tabacum* cv Maryland Mammoth lines (MUA8 and MUA17) which were putative PHY-A1 unproducers characterized in Chapter 2 were also used in germination studies. General growth conditions were described in Chapter 3. Photoperiodic treatments were described in Chapter 3.

Measurement of Stem Elongation

Plant heights were measured with a ruler every week after the initiation of the photoperiodic treatments until plants reached the mature stage as defined as 10 days after the first flower opened. Heights were measured from the node subtended from a leaf at least 10 cm long to the terminal bud. The number of nodes was counted as described in Materials and Methods in Chapter 3.

Germination Test

Between 50 and 100 seeds of transgenic *N. sylvestris* lines were spread onto wet 3MM filter paper in a 100 x 15mm Petri dish, sealed with Parafilm, and then transferred to continuous darkness, or subjected to R and FR treatments. MUA8 and MUA17 seeds were germinated on the one half strength MS (Murashige and Skoog, 1962) medium
without any sucrose. After 5 to 7 days, percentage of germination was assessed; seeds were considered germinated when the radicle visibly protruded the seed coat.

**Light Sources**

For stem elongation with photoperiodic treatments, light condition was described in Chapter 3. For seed germination experiments, R, FR and white lights were described in Chapter 2.
Results and Discussion

Seed Germination

Three groups of transgenic *N. sylvestris* seeds were germinated under different light conditions (Table 4.1). Results showed that *N. sylvestris* WT seeds did not germinate under continuous darkness, and the alteration of either phy-A1 or phy-B1 did not affect dark germination (Table 4.1). However, while FR failed to promote germination, continuous R promoted germination with a similar germination percentage to continuous white light (Table 4.1). This suggests R alone is sufficient to promote seed germination. With the exception of SUA11, where lower percentage germination occurred, no effects of the reduced *PHY-A1* expression was observed in the seed germination responses mediated by FR and R.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Dark (%)</th>
<th>FRc (%)</th>
<th>Rc (%)</th>
<th>WLc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0/81 (0)</td>
<td>0/80 (0)</td>
<td>93/100 (93.0)</td>
<td>102/109 (93.6)</td>
</tr>
<tr>
<td>SUA2 7</td>
<td>0/64 (0)</td>
<td>0/72 (0)</td>
<td>70/70 (100)</td>
<td>107/112 (95.5)</td>
</tr>
<tr>
<td>SOB1 36</td>
<td>0/58 (0)</td>
<td>0/77 (0)</td>
<td>55/60 (91.7)</td>
<td>85/86 (98.8)</td>
</tr>
<tr>
<td>SCB35</td>
<td>0/63 (0)</td>
<td>0/69 (0)</td>
<td>67/72 (93.1)</td>
<td>75/84 (89.3)</td>
</tr>
</tbody>
</table>

**Table 4.1.** Seed germination of SUA, SOB and SCB35 lines Seed germination was asessed 5 days after the start of the treatments. WT, wild-type; Dark, Rc, FRc, WLc: Continuous dark, red, far-red and fluorescent white light, respectively.
Although R promoted germination in WT compared to FRc or darkness, SOB lines and SCB35 seemed to have no great effect on germination under continuous R or white light (Table 4.1). However, when the seeds which have been subjected to continuous dark or FR treatments for 5 days were exposed to R for 5 min and then transferred to continuous darkness for 8 days, SOB lines promoted while SCB35 inhibited the germination, compared to WT (Table 4.2). This suggests the promotion of seed germination by brief R exposure is mediated by phy-B1. The SUA lines did not respond well to the R exposure, except for SUA2 which germinated as well as WT (Table 4.2).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>FRc (%)</th>
<th>Dark (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>15.0</td>
<td>95.1</td>
</tr>
<tr>
<td>SUA2</td>
<td>13.9</td>
<td>98.4</td>
</tr>
<tr>
<td>7</td>
<td>3.4</td>
<td>15.7</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>SOB1</td>
<td>81.8</td>
<td>100.0</td>
</tr>
<tr>
<td>36</td>
<td>56.9</td>
<td>85.2</td>
</tr>
<tr>
<td>40</td>
<td>85.7</td>
<td>95.1</td>
</tr>
<tr>
<td>50</td>
<td>69.0</td>
<td>100.0</td>
</tr>
<tr>
<td>SCB35</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.2. Seed germination of SUA, SOB and SCB35 lines after the brief R exposures. The same seeds which were treated with FRc and continuous dark for 5 days in Table 4.1 were exposed to 5 min R and then transferred to continuous darkness for additional 8 days. Seed germination was expressed as the percentage of germinated seeds to the total number of the seeds.
This preliminary result is consistent with Arabidopsis phyB which is responsible for the promotion of germination through low fluence R response (Shinomura et al., 1994; Shinomura et al., 1996). It is not clear about the role of phy-A1 in the control of germination in *V. sylvestris*, which can not germinate in the dark or FR (Table 4.1). Further experiments using different photon flux densities and/or irradiation times are necessary to investigate the functions of phy-A1 or phy-B1 (light perception or the interaction with GA) in the control of germination.

In contrast to *N. sylvestris*, high percentage of germination of WT Maryland Mammoth tobacco seeds were obtained in continuous darkness or FR (Table 4.3). However, there was slightly less germination of seeds under FRc than darkness (Table 4.3). In contrast, seeds of the two putative PHY-A1 underproducers, MUA8 and MUA17, did not germinate under continuous darkness (Table 4.3), indicating that phy-A1 is required for germination in the dark. Moreover, MUA8 and MUA17 seeds germinated at a lower percentage than WT seeds in continuous FR (Table 4.3), suggesting phy-A1 mediates FR in germination. The lower percentage of germination in MUA8 and MUA17 is consistent with poor germination in Arabidopsis phyA mutants (Shinomura et al., 1994).

Although germination was reduced in WT seeds under continuous FR compared to dark, there was a significant promotion of germination for MUA8 and MUA17 in continuous FR compared to dark (Table 4.3). This implies that phy-A1-mediated FR action inhibits germination. More detailed studies of MUA8 and MUA17 are needed to understand the photocontrol of germination by phy-A1.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Continuous Dark (%)</th>
<th>Continuous FR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Maryland Mammoth)</td>
<td>87/95 (91.6)</td>
<td>95/122 (77.8)</td>
</tr>
<tr>
<td>MUA8</td>
<td>0/93 (0)</td>
<td>12/157 (7.6)</td>
</tr>
<tr>
<td>MUA17</td>
<td>0/105 (0)</td>
<td>20/195 (10.3)</td>
</tr>
</tbody>
</table>

Table 4.3. Seed germination of MUA8 and MUA17 lines in *N. tabacum* cv Maryland Mammoth  Seeds were germinated on the 1/2 MS medium without sucrose for 7 days. The germination percentage is given in the parenthesis. WT, wild-type

Stem elongation

The regulation of the shade avoidance syndrome in plants is a major function of phytochromes. One of the most dramatic response to shade is the rapid stimulation of stem elongation which places an advantage for plants under the shade to compete more effectively for light (Smith, 1994). The shade avoidance response can be stimulated by low R/FR ratio and/or end-of-day FR in many plants (Smith, 1994; Smith and Whitelam, 1997). The use of phytochrome mutants and transgenic plants has shown that the phyB-subgroup of phytochromes - but not phyA- are responsible for the perception of low R/FR ratio or EOD-FR (Smith and Whitelam, 1997). The phyB-subgroup phytochromes include phyB, D and E (Mathews and Sharrock, 1997). Among these, phyB plays a predominant role in the shade avoidance response (Aukerman et al., 1997; Delvin et al., 1999; Whitelam and Smith, 1997). A similar role for phyB has also been reported for pea,
cucumber and *Brassica* (Delvin et al., 1995; Lopez-Juez et al., 1992; Weller et al., 1995; Smith and Whitelam, 1997).

However, the *PHYB*-like phytochrome mutant *hlg* in *N. plumbaginifolia* (Hudson et al., 1997) and the phy-B1 mutant *tri* in tomato (van Tuinen et al., 1995b) maintained the response to low R/FR ratio or EOD-FR, indicating a different role for phyB in these two species from that in Arabidopsis.

In order to better understand the functions of phytochromes in the control of stem elongation, the phy-A1 underproducer, phy-B1 over- or co-suppressors in *N. sylvestris* were subjected to 8 h low intensity Flu or Inc extension following 8 h natural SD. Results showed that while SUA2 plants reached the same height as WT plants, SCB35 plants were shorter under Flu extension but taller under Inc extension (Fig. 4.1). SOB36 plants were shorter at early stage of development, but after flower induction, the extension growth was accelerated and reached the same height as WT when the plants were mature (Fig. 4.1).
Figure 4.1. Plant heights of SUA2, SCB35 and SOB36 lines under 8 h Inc or Flu extensions. A total of 4 plants for each genotype were transferred from 8 h SD to 8 h inc extension (A), or Flu extension (B). Height was measured every week after the treatment until the mature stage (10 days after the first open flower).
The observation that SCB35 plants under high R/FR ratios (Flu light extension) did not behave WT plants under low R/FR (Inc extension) is in sharp contrast to the phyB mutants in Arabidopsis, pea, cucumber and Brassica which phenocopied WT plants under low R/FR or EOD-FR treatments and exhibited a constitutive elongation response under both high and low R/FR ratios (Delvin et al., 1995; Lopez-Juez et al., 1992; Weller et al., 1995; Smith and Whitelam, 1997). Instead, the result suggests that phy-B1 is not the sensor for the low R/FR ratio. This is consistent with phy-B1 orthologs in tomato and N. plumbaginifolia (Hudson et al., 1997; van Tuinen et al., 1995b). Molecular phylogenetic analysis indicates that neither PHYB1 nor PHYB2 from the three closely related Solanaceae species, tomato, tobacco and potato could be considered orthologs of either Arabidopsis PHYB or PHYD (Pratt et al., 1997). Thus, a progenitor B phytochrome might have been duplicated independently in both the Brassicaceae and Solanaceae after these two groups of plants diverged from one another (Pratt et al., 1997). Therefore, it is possible that phy-B1 in the Solanaceae has different functions from phyB in Arabidopsis and Brassica. The conclusion that phy-B1 can have a dual inhibitory/promotive function in flower induction in the LDP N. sylvestris (Chapter 3) also supports this idea.

Transgenic plants were also subjected to 17 h LD conditions where high intensity high pressure sodium lamps were used for the daylength extension, with a R/FR ratio (2.4) higher than Inc (0.7) but lower than Flu light (3.3). Under such a condition, SCB35 plants were taller and SOB36 plants shorter than WT (Fig. 4.2A), and the average lengths of top 5 to top nine nodes followed same patterns (Fig. 4.2B), consistent with the earlier observation that phy-B1 is required for the inhibition of stem elongation in a mixed R and FR light environment. Similarly, SUA2 and the cross SUA2 × SCB35 achieved nearly
the same heights as WT plants (Fig. 4.2A). However, SUA2 and the cross developed more nodes to induce the flowering (see Fig. 3.5 in Chapter 3) and the internode lengths would be shorter. Indeed, when the average internode lengths were compared, similar to SOB36, SUA2 plants had the shorter internodes than WT, and the cross SUA2 SCB35 had the same internode length as WT (Fig. 4.2). The shorter internodes in SUA2 are consistent with the observations of the pea phyA mutants fun1 (Weller et al., 1997), indicating that phy-A1 acts to promote stem elongation in mature N. sylvestris plants under high intensity LD conditions. This is different from the function of phy-A1 to inhibit hypocotyl elongation in de-etiolation process, suggesting that phy-A1 may operate through two distinct signaling pathways in N. sylvestris. Dual signaling pathways of phyA have been demonstrated in pea in which there are two genetically separable pathways controlling seedling de-etiolation and photoperiodism, respectively (Weller et al., 1997). Perhaps, the combination of inhibition of stem elongation by phy-B1 and the promotion by phy-A1 leads to the same stem elongation in the cross SUA2 × SCB35 as WT plants. Therefore, it appears that phy-A1 and phy-B1 act in an antagonistic way to control the stem elongation in N. sylvestris.
Figure 4.2. Stem elongation of *N. sylvestris* WT and transgenic plants grown in 17 h high intensity LD condition. A total of 7 plants for each genotype were transferred from 8 h SD to 17 h LD. A, Average heights of mature plants. B, Average internode lengths of plants. The internode immediately below the terminal bud was designated as No. 1 internode. The length of five internodes from No.5 to No.9 from the shoot tip was measured and the average internode length was represented in this figure. The bars represent standard deviations. Values with the same letter above the column are not statistically different at P=0.05.
Conclusion

Photomorphogenesis including seed germination, seedling de-etiolation, vegetative growth and flowering is important for the life cycle in plants (Hendrick and Kronenberg, 1994). Phytochromes are clearly involved in these aspects and the functions of individual phytochromes can be revealed through transgenic plants mis-expressing phytochrome genes in *N. sylvestris*. Results suggest phy-B1 is at least partially responsible for R-mediated promotion of germination. It is also supported that phy-B1 in tobacco and tomato have different functions from phyB in Arabidopsis: phy-B1 is not the sensor for low R/FR ratio or EOD-FR, and thus not responsible for the shade avoidance response (Smith and Whitelam, 1997). Further detailed studies using these transgenic materials will elucidate the mechanisms by which individual phytochromes modulate the growth and development in *N. sylvestris*.
CHAPTER 5

MODIFICATION OF PLANT ARCHITECTURE IN CHRYSANTHEMUM
THROUGH ECTOPIC EXPRESSION OF A TOBACCO PHYTOCHROME B1 GENE

Abstract

Height control is a major consideration during the commercial production of chrysanthemum (*Dendranthema grandiflora* Tzvelev). We have addressed this problem through a biotechnological approach. Plants of chrysanthemum cv Iridon were genetically engineered to ectopically express a tobacco phytochrome B1 gene under the control of the CaMV 35S promoter. The transgenic plants were shorter in stature and had larger branch angles than WT plants. The leaves of the transgenic plants were greener in color due to higher levels of chlorophyll. Transgenic plants also phenocopied WT plants grown under a filter that selectively attenuated far-red wavelengths. Furthermore, the reduction in internode length resulting from the ectopic expression of *PHY-B1* was nearly identical in plants treated with gibberellin A$_3$ or 2-chlorocholine chloride, an inhibitor of gibberellin biosynthesis. This suggests that the reduction of growth by the expressed *PHY-B1* transgene did not directly involve gibberellins. In addition, transgenic plants
delayed flowering by increasing the sensitivity to night-breaks. The commercial application of this biotechnology could provide an economic alternative to the use of chemical growth regulators, and thus reducing the production cost.

Introduction

Height control is a major consideration during the production of agronomic and horticultural crops. Reduction of height through genetic means or cultural practices often provides economic advantages. First, cultivars of agronomic crops with shorter stems often exhibit higher harvest indices by re-directing the partitioning of assimilates such that there is an increase in the proportion of shoot biomass accumulating in the seed, thereby resulting in increased yields (Loomis and Conner, 1992). Second, some cereal crops such as wheat have a serious lodging (falling over) problem due to vigorous stem growth under the conditions of high fertility or unfavorable weather (Loomis and Conner, 1992). Vegetable transplants with tall and weak stems are prone to physical damage and lodging after transplanting, and shorter and strong stems help to resist lodging (Garner and Bjorkman, 1996). Third, shorter stems increase the ornamental values of floricultural crops grown in greenhouse such as chrysanthemum, poinsettia, and Easter lily crops and therefore height control is an economic necessity for growers (Gianfagna, 1995).

Height control in greenhouse crops is achieved by several approaches. The most common practice is the use of chemical growth regulators (Dole and Wilkins, 1999). Most commercial growth retardants reduce stem growth by inhibiting GA biosynthesis or
metabolism, and thus reduce the endogenous levels of one or more biologically active GAs (Gianfagna, 1995). However, this method has limitations. Application of growth retardants adds significantly to the cost of crop production both in terms of material and labor. Furthermore, there are increasingly stringent governmental regulations for their use in nonfood crops and they are not permitted on crops destined for human consumption.

Cultural methods are also used to control the height of greenhouse crops. For example, manipulation of the daily greenhouse thermoperiod by maintaining the night temperature higher than the day temperature is an effective cultural alternative to the use of chemical growth regulators for many crops (Dole and Wilkins, 1999). However, this so-called "-DIF" method of growth regulation can not be exploited in certain geographical regions or at certain times of the year when it is impractical or deleterious to plant growth to raise the greenhouse night temperature higher than the day temperature (Dole and Wilkins, 1999).

Mechanical stimulation (brushing) of plants can limit undesirable stem elongation (Garner and Bjorkman, 1996). However, it is often impractical to be implemented on a commercial large scale, since how much, how long, and when to treat should be carefully investigated for each growing season before its application (Garner and Bjorkman, 1996).

The third approach of stem growth control is the development of cultivars with reduced stature through conventional breeding (Loomis and Conner, 1992). While this approach lowers grower economic inputs compared to the use of cultural methods or growth regulators, the development of new cultivars with shorter stems is a slow and
expensive process. As a result, the breeding approach to the control of plant stature has been exploited primarily for field-grown crops.

Biotechnology provides an attractive alternative to conventional breeding in that traits controlled by single genes can be quickly incorporated in existing cultivars, thereby greatly reducing the time and costs of breeding. Genetic engineering of phytochrome genes has provided a potential means to control the vegetative growth and reproductive development (Robson and Smith, 1997). The ectopic expression of an *PHYA* gene in tobacco and potato plants significantly inhibited stem elongation of mature plants (Halliday et al., 1997; Heyer et al., 1995; Jordan et al., 1995; Keller et al., 1989; McCormac et al., 1992), and increased harvest index through hypersensitivity to FR (Robson et al., 1996). Likewise, the ectopic expression of Arabidopsis or rice *PHYB* gene in Arabidopsis led to reduced extension growth of light-grown seedlings (McCormac et al., 1993; Wagner et al., 1991). In addition, the ectopic expression of Arabidopsis *PHYB* in tobacco and potato inhibited stem elongation of mature plants as well (Halliday et al., 1997; Thiele et al., 1999).

Recently, a *PHYB*-like phytochrome encoded by the *HLG*-locus in *Nicotiana plumbaginifolia* has been shown to be a sensor for R rather than the R/FR ratio as is the case for *PHYB* in Arabidopsis, *Brassica rapa* and cucumber (Smith and Whitelam, 1997; Hudson et al., 1997). In *hlg* mutants, the response to low R/FR ratios is not attenuated but the response to R is altered (Hudson et al., 1997). The *HLG*-encoded *PHYB* shares higher homology at the amino acid level with the *PHYB*-1 orthologs (91-92%) from tomato and potato than *PHYB*-2 (87-89%), and it is 97% identical to the *PHY-B1* in *N. tabacum* (Adam et al., 1997; Hudson et al., 1997; Kern et al., 1993; Pratt et al., 1997). Since R-rich
light has been shown to inhibit the stem elongation (Smith, 1994; Smith, 1995), we were particularly interested in the inhibition of stem elongation and other modifications in the plant architecture through the ectopic expression of the tobacco *PHY-B1* gene in chrysanthemum.

In addition, most chrysanthemum varieties are SD plants, which, depending on cultivar, flower when daylength is shorter than about 14.5 h (Larson, 1992). For the commercial production of chrysanthemum, a certain amount of vegetative growth is required before flowering is induced. Vegetative conditions are maintained by providing a NB of low intensity light in the middle of an otherwise inductive long night. R is the most effective light for NB (Thomas and Vince-Prue, 1997), but for the commercial in chrysanthemum, a 2 h NB in the summer or 4-5 h in the winter using incandescent light which contains a mixture of R and FR is applied (Larson, 1992).

Phytochrome is clearly involved in the NB-R action (Thomas and Vince-Prue, 1997). The ectopic expression of oat *PHYA* or Arabidopsis *PHYB* in a short day tobacco variety greatly inhibited the flowering using NB with fluorescent light (Halliday et al., 1997). On the other hand, the suppression of *PHYB* expression through antisense RNA in potato disrupted the NB-inhibition of tuber formation (Jackson et al., 1996). Based on the effect of altering phyB levels on the response to NB, we hypothesized that the ectopic expression of the tobacco *PHY-B1* could increase the sensitivity of NB in chrysanthemum, and thus have the practical effect of reducing the amount of light energy during the NB required for maintaining the plants in the vegetative state.
MATERIALS AND METHODS

Plant Materials and Growth Conditions

Rooted cuttings of the chrysanthemum (*Dendranthema grandiflora* Tzvelev) cultivar, Iridon, were obtained from Yoder Brothers, Inc. (Pendleton, SC) and were maintained in the greenhouse as stock plants from which plants for experiments were generated through vegetative propagation. The cut ends of shoot tips from vegetative stock plants were dipped in a 1 mg mL\(^{-1}\) solution of NAA, and then planted in 10 cm pots containing a commercial soilless media (Metro-Mix 360; Scotts-Sierra Horticultural Products Company, Marysville, OH). The plants were grown in a greenhouse with a temperature setpoint of 25°C. Natural photoperiods were extended to 17 h using light from 1000 W high-pressure sodium vapor lamps (80-100 \(\mu\)mol m\(^{-2}\)s\(^{-1}\) PAR) from 6:00-10:00 h and 16:00-23:00 h.

Construction of 35S::Nt-PHY-B1

The plasmid pESB was constructed as described in Chapter 2 to drive the expression of a full length *Nt-PHY-B1* cDNA under the control of a CaMV 35S promoter with dual enhancers. Then it was transferred into two *Agrobacterium tumefaciens* strains, LBA4404 and EHA105 using the freeze-thaw method (An et al., 1993).
Plant Transformation

Plants were regenerated from leaf discs and young internode segments on MS (Murashige and Skoog, 1962) medium containing BA (0.5 mg L\(^{-1}\)) + NAA (1.0 mg L\(^{-1}\)) and BA (1.0 mg L\(^{-1}\)) + NAA (1.0 mg L\(^{-1}\)), respectively. The plant transformation procedure was modified from Horsch et al. (1985). *Agrobacterium tumefaciens* cells harboring pESB were grown for 2 d on YEB liquid medium with 50 mg L\(^{-1}\) kanamycin, and 100 mg L\(^{-1}\) streptomycin (for LBA4404) or 25 mg L\(^{-1}\) rifampicin (for EHA105). Prior to the transformation, the cultures were centrifuged at 4,000 rpm for 5 min and the pellet was suspended with 10 mL of sterile MS medium. Leaf discs (1.0 cm\(^2\)) or internode segments (0.5 cm long) were co-incubated with the *Agrobacterium* cultures for 3 d on the regeneration medium without any antibiotics. The infected explants were then blotted with 3MM paper to remove excess *Agrobacterium*, and transferred to regeneration medium supplemented with 50 mg L\(^{-1}\) of kanamycin and 500 mg L\(^{-1}\) of carbenicillin. Kanamycin-resistant shoots were regenerated in 4 to 5 weeks. The shoots were cut off and transferred onto root-inducing medium (0.1 mg L\(^{-1}\) of NAA) also containing both kanamycin and carbenicillin at the same concentrations used in shoot regeneration. After about 3 weeks, plants with well-developed root systems were planted in pots and transferred to the greenhouse as described previously.

PCR and RNA Blot Analyses

PCR was used to amplify the tobacco *PHY-B1* cDNA to identify the putative transgenic plants. Genomic DNA was extracted from young leaves of the kanamycin-
resistant lines by the CTAB-method (McGarvey and Kaper, 1991). The primers SB1 and SB2 described in Chapter 2 were used to amplify a 1.2kb fragment.

Total RNA was extracted from young leaves of chrysanthemum plants (Logemann et al., 1987), fractionated on a 1.2% formaldehyde agarose gel, and then transferred onto a nylon membrane. A 0.3kb PHY-B1 cDNA probe used for the hybridization was amplified from Nt-PHYB-1 cDNA (Kern et al., 1993) and labeled by PCR as described in Chapter 2.

Characterization of the Transgenic Plants

Plant heights were measured from the media surface to the shoot tip using a ruler. The branching pattern of shoots was analyzed in plants in which apical dominance was released by removing the apical 1-cm of the shoot ("pinched") 3 weeks after transplanting the cuttings. The pinched plants were grown under LD for three weeks and then to SD. After 2 months, the angles between the main stem and the three oldest branches were measured using a protractor. The canopy diameter was determined by measuring the maximum horizontal extension of the branches. The canopy height was measured using a ruler from the media surface to the top of the plants. Leaf areas were determined using a Model 3100 Area Meter (LiCor Inc, Lincoln, Nebraska, USA).

Chlorophyll levels and areas were measured in leaves at two different ages. Two leaf discs, each of 0.26 cm², were punched from the lamina of leaf number 1 (the youngest leaf at least 2 cm long) and leaf number 5 and placed in 5 mL of N, N-dimethylformamide. After 2 days in the dark at 4°C, 1 mL of the extract was removed and the absorbance at 664 and 647 nm was measured, respectively. Total chlorophyll
levels were determined using the method of Moran (1982). Each treatment contained five plants and the experiment was repeated once.

The effects of reducing FR wavelength on the growth of WT and transgenic plants were compared. Plants from 3-week-old cuttings were grown in 10-cm pots in the greenhouse under a plastic film containing a dye that selectively absorbs radiation of 700 to 800 nm (Mitsui Chemical Corp., Japan). This filter resulted in an increase in the R/FR ratio (photon flux density 655-665nm/photon flux density 725-735nm) at noon on a clear day in January from 1.20 to 1.73 as measured by a Model LI 1800 Portable Spectroradiometer (LiCor Inc, Lincoln, NE) (Fig. 5.1). Control plants were grown under a clear plastic film that provided a PPFD nearly the same as the FR filter (151 vs. 125 μmol m⁻² s⁻¹ for the clear plastic and FR filter, respectively), but did not affect the R/FR ratio. Temperatures were also nearly the same in both treatments. Stem heights were measured every 3 d following transfer. Each treatment contained 5 plants, and the experiment was repeated once.
Figure 5.1. Comparison of spectral distribution (330 to 800 nm) of sunlight passing through a plastic photoselective filter that selectively attenuates wavelength of 700 to 800 nm and clear plastic. Measurements were made in a greenhouse at noon on cloudless day in January.
The role of GA in the phytochrome control of stem elongation was investigated by comparing the effects of CCC and exogenous GA$_3$ on stem elongation in transgenic and WT plants. Cuttings of various genotypes were prepared and grown in the greenhouse under a 17 h photoperiod as described above. After 3 weeks, the pots were drenched with 100 mL of a 9.5 mM solution of a commercial preparation of CCC. After 10 d the CCC drench was repeated. Ten microliters of a solution containing 10 µg of GA$_3$ dissolved in 10% (v/v) aqueous acetone plus 0.01% Tween 20, were applied to the shoot tips of plants every 2 d. Stem heights of the plants were measured every 3 d for 15 d. The number of nodes was counted from the node closest to the media surface to the node that had the youngest 1 cm long leaf. Each treatment contained five plants.

**Flowering Responses of Transgenic Chrysanthemum Plants**

Flowering was induced by subjecting the plants to SD. Inductive conditions were achieved by covering the plants with a black shade cloth from 16:30 h to 8:30 h. The effects of NB on flowering was investigated by providing a low intensity (PPFD of 1.2 µmol m$^{-2}$ s$^{-1}$) red light from 0:00 h to 0:30 h. The R was obtained from two 20 W cool white fluorescent lamps wrapped with one layer of red cellulose film that provided a R/FR of 3.34. Each treatment contained 8 plants. The number of days to the appearance of visible flower buds and the first open flower were recorded.
RESULTS AND DISCUSSION

Generation and molecular characterization of transgenic Iridon plants

Our preliminary studies have shown that one chrysanthemum cultivar, Iridon, had the best \textit{in vitro} regeneration capacity using the intermediate molar ratio of BA to NAA at 0.41 and 0.82 for leaf and internode explants, respectively (data not shown). We chose Iridon for further transformation studies using LBA4404 and the hypervirulent strain EHA105. A number of kanamycin-resistant plants were generated (Table 5.1), but PCR analysis showed that only EHA105-mediated transformation facilitated the integration of \textit{Nt-PHY-B1} cDNA into the chrysanthemum genome. Four independent transgenic lines, LE31, 32, 46 and 56, were obtained from a total of 32 kanamycin-resistant plants (Fig. 5.2A). Since one of the primers was based on the vector sequence, no band was amplified from WT plants. These results are consistent with other work that comparing the effectiveness of various \textit{Agrobacterium} strains in obtaining stable transformants (Renou et al., 1993; Urban et al., 1994).

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textit{Agrobacterium} & \multicolumn{3}{|c|}{Leaf disc} & \multicolumn{3}{|c|}{Internode} \\
\cline{2-7}
 & No. of explants & Kan\textsuperscript{R} plants & PCR-positive & No. of explants & Kan\textsuperscript{R} plants & PCR-positive \\
\hline
LBA4404 & 43 & 8 & NO & 34 & 4 & NO \\
EHA105 & 48 & 11 & 2 & 36 & NO & NO \\
109 & 21 & 2 & & & & \\
\hline
\end{tabular}
\caption{Summary of Iridon transformation}
\end{table}

Only the highly virulent \textit{Agrobacterium} strain EHA105-mediated transformation from leaf disc explants resulted in a total of 4 PCR-positive plants from two experiments. Kan\textsuperscript{R}, kanamycin-resistant.
Figure 5.2. Identification and molecular characterization of transgenic chrysanthemum plants. A) The PCR amplification of *Nt-PHY-B1* transgene from kanamycin-resistant lines: lane 1. 1kb ladder marker; lanes 2 and 3, without and with plasmid pESB, respectively; lane 4. wild type; lanes 5-11, transgenic lines regenerated from leaf explants mediated by Agrobacterium EHA105 strain, LE11, 31, 32, 45, 46, 50, 56. B) RNA blot analysis. A fragment of *Nt-PHY-B1* cDNA was used as a probe to detect the expression of transgene. Shown are LE31 and LE32 with high levels of expression of *PHY-B1* gene. C) The loading of 5 µg total RNA was shown by ethidium bromide staining. A three fold loading (15 µg) for WT (WT1) was also included. WT, wild-type.

Our goal was to express the tobacco phytochrome B1 gene at high levels in chrysanthemum plants, and thus a strong cauliflower mosaic virus (CaMV) 35S promoter with dual enhancers (Guerineau et al., 1988) was chosen to constitutively drive the expression of the *Nt-PHY-B1* transgene at high levels. Northern blot analysis showed
high expression of the \textit{Nt-PHY-B1} transgene in two of the four transgenic lines, LE31 and LE32 (Fig. 5.2B). No \textit{Nt-PHY-B1} message was detected in WT plants, suggesting low sequence homology between the tobacco and chrysanthemum B-type phytochrome genes. The growth habits of these two transgenic lines were characterized further.

**Transgenic plants are shorter and contain higher levels of chlorophyll**

Plants of transgenic lines, LE31 and LE32, were shorter than WT plants, with a 13 to 25\% reduction of stem height (Table 5.2, Fig. 5.3A). The reduction of the height was due to the reduction of internode elongation, with a 16 to 28\% reduction of average internode length, compared to WT plants (Table 5.2).

<table>
<thead>
<tr>
<th>Photoperiod</th>
<th>Genotype</th>
<th>Stem Height (cm)</th>
<th>Number of Nodes</th>
<th>Internode length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD Wild-type</td>
<td>11.4±1.2\textsuperscript{a}</td>
<td>18.8±0.4\textsuperscript{a}</td>
<td>6.1±0.6\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td>LE31</td>
<td>8.5±0.8\textsuperscript{b}</td>
<td>18.2±0.8\textsuperscript{a}</td>
<td>4.6±0.3\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>LE32</td>
<td>9.4±0.3\textsuperscript{b}</td>
<td>18.4±0.6\textsuperscript{a}</td>
<td>5.1±0.2\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>SD Wild-type</td>
<td>13.7±1.0\textsuperscript{a}</td>
<td>21.5±1.2\textsuperscript{a}</td>
<td>6.4±0.3\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td>LE31</td>
<td>11.0±1.4\textsuperscript{b}</td>
<td>21.1±1.6\textsuperscript{a}</td>
<td>5.2±0.4\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>LE32</td>
<td>11.9±0.8\textsuperscript{b}</td>
<td>22.0±1.1\textsuperscript{a}</td>
<td>5.4±0.4\textsuperscript{b}</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2. Ectopic expression of the tobacco \textit{PHYB-1} gene in chrysanthemum plants results in shorter stems under both LD and SD conditions. Plants were vegetatively propagated from cuttings under 17 h LD. After 3 weeks, half of the plants were transferred to SD. Plant heights, number of nodes, and internode lengths were determined after 15 and 28 d for LD- and SD-treated plants, respectively. Values represent the average ± standard deviation of 5 and 8 plants for LD- and SD-treated plants, respectively. Values within a photoperiodic treatment in a column followed by the same letter are not significantly different at \( P=0.05 \).
Figure 5.3. Shoot architecture of transgenic chrysanthemums expressing the *PHY-B1* gene. A) A picture showing shorter stems of transgenic plants than wild-type (WT) plants grown under LD for 15 days. B) A picture taken three weeks after the plants were pinched. Note that the angles for LE31 and LE32 were much larger than WT and that transgenic plants appeared wider and shorter.
The leaves of LE31 and LE32 appeared slightly greener than WT plants. Comparative analysis of the three genotypes showed that LE31 and LE32 had higher total chlorophyll levels in both young and mature leaves than WT plants, while there was no difference in the leaf areas (Table 5.3). The increase of leaf chlorophyll in transgenic plants is consistent with lower chlorophyll content in PHYB mutants of Arabidopsis (Reed et al., 1993), and PHY-B1 antisense potato plants (Jackson et al., 1996).

<table>
<thead>
<tr>
<th>Leaf Age</th>
<th>Genotype</th>
<th>Total Chlorophyll (mg g fresh wt.(^{-1}))</th>
<th>Leaf Area (cm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>Wild-type</td>
<td>0.91±0.13(^{a})</td>
<td>4.46±0.64(^{a})</td>
</tr>
<tr>
<td></td>
<td>LE31</td>
<td>1.28±0.14(^{b})</td>
<td>4.47±0.88(^{a})</td>
</tr>
<tr>
<td></td>
<td>LE32</td>
<td>1.28±0.15(^{b})</td>
<td>4.69±0.61(^{a})</td>
</tr>
<tr>
<td>Mature</td>
<td>Wild-type</td>
<td>1.39±0.11(^{b})</td>
<td>15.61±1.38(^{b})</td>
</tr>
<tr>
<td></td>
<td>LE31</td>
<td>1.70±0.26(^{c})</td>
<td>15.26±1.38(^{b})</td>
</tr>
<tr>
<td></td>
<td>LE32</td>
<td>1.67±0.22(^{c})</td>
<td>15.08±1.97(^{b})</td>
</tr>
</tbody>
</table>

Table 5.3. Ectopic expression of the tobacco PHYB-I gene in chrysanthemum plants results in higher chlorophyll levels but does not affect leaf areas. Plants were vegetatively propagated from cuttings were grown in a greenhouse for 5 weeks under LD conditions. Young and mature refer to the first and fifth youngest leaves, respectively. Values represent the mean ± standard deviation of 5 plants. Values in a column followed by the same letter are not significantly different at P=0.05.
Since there was no difference in leaf areas among the three genotypes, individual leaves of the transgenic plants contained higher absolute amounts of chlorophyll. Recently Thiele et al. (1999) have shown quantitatively similar increases in leaf chlorophyll levels resulting from the heterologous expression of Arabidopsis \textit{PHYB} in potato. In this case the higher chlorophyll levels resulted in higher rates of photosynthesis, and as a consequence higher tuber yields.

\textbf{Transgenic plants exhibit altered shoot architecture}

The light environment controls many aspects of shoot development and canopy structure. Most of these responses are due to changes in the R/FR ratio, and are therefore collectively termed the shade avoidance response (Smith, 1994; 1995). One manifestation of the shade avoidance response in many species is the orthotropic growth habit of leaves when plants are subjected to light environments with low R/FR ratios; this presumably represents an adaptation to maximize the interception of light in crowded conditions (Whitelam and Johnson, 1982). However, the ectopic expression of the \textit{Nt-PHY-B1} gene did not affect leaf angles in plants grown under thin or dense spacing (data not shown). This is consistent with the observation that changes in leaf angle in response to the R/FR ratio were unaltered in \textit{hlg} mutants of \textit{N. plumbaginifolia}, which are deficient in a phytochrome closely related to \textit{PHY-B1} of \textit{N. tabacum} (Hudson and Smith, 1998).

Phytochrome is also involved in the control of lateral branching (Smith, 1994; Smith, 1995; Smith and Whitelam, 1997). Indeed, a decrease in lateral branching is another important component of the shade-avoidance response in many species (Smith, 1994; Smith, 1995; Smith and Whitelam, 1997). However, we observed no difference in
the number of lateral branches between the transgenic and WT plants (data not shown), indicating apical dominance is unaffected by the ectopic expression of the *PHY-B1* gene.

Nevertheless, we did observe another alteration in the architecture of the transgenic plants. Mature plants that were pinched to promote the outgrowth of axillary buds appeared wider than WT plants (Fig. 5.3B). Indeed, the canopy diameters of both LE31 and LE32 were greater than WT plants (Table 5.4). The ratio of the diameter to the height of LE31 and LE32 was significantly higher than that of WT plants (Table 5.4). The basis for the increase in canopy diameter and reduced canopy height in the transgenic plants could be the larger branch angles. Indeed, the angles of lateral branches relative to the main stem of LE31 and LE32 were significantly larger than WT plants (Table 5.4).

Shoot architecture is often altered as the competition for light increases (Harper, 1977). In general, branching patterns are optimized to reduce both self-shading and shading by neighbors. Reduced lateral branching and/or an orthotropic growth habit of the branches can minimize shading by neighbors. A more plagiotropic growth habit of the branches, which is characteristic of many plants growing in open communities, maximizes the area available for the interception of light. To our knowledge, there are no reports demonstrating the involvement of specific phytochrome(s) in the regulation of branch angles in dicots. However, the action of phytochrome in the control of shoot zenith angles (the angles of tillers with respect to the vertical) in two grass species, *Lolium multiflorum* and *Paspalum dilatatum*, has been demonstrated. In these two species shoot zenith angles increase with higher R/FR ratios (Casal et al., 1990; Gibson et al., 1992). These grasses when grown in the absence of nearby neighbors have shoots with a more plagiotropic growth habit than plants growing in crowded communities. As
a consequence, the plants are able to intercept a higher proportion of incident radiation per plant (Casal et al., 1986). Thus one function of the tobacco phy-B1 may be to control branch angle in response to changing light environments.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Canopy Diameter (cm)</th>
<th>Canopy Height (cm)</th>
<th>Diameter to Height Ratio</th>
<th>Branch Angle (degrees)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>36.5±3.2 a</td>
<td>32.6±1.1 a</td>
<td>1.12±0.13 a</td>
<td>33.5±7.2 a</td>
</tr>
<tr>
<td>LER31</td>
<td>43.2±3.9 b</td>
<td>29.1±2.6 b</td>
<td>1.50±0.27 b</td>
<td>62.6±3.3 b</td>
</tr>
<tr>
<td>LE32</td>
<td>40.8±2.4 b</td>
<td>29.2±1.3 b</td>
<td>1.40±0.08 b</td>
<td>65.6±3.3 b</td>
</tr>
</tbody>
</table>

Table 5.4. Ectopic expression of the tobacco *PHYB-1* gene in chrysanthemum plants results in changes in shoot architecture. Young plants (each line with 4-5 plants) were pinched to release apical dominance and grown under 17 h LD for 3 weeks before being transferred to 8 h SD. The diameter, height and branch angles of 4-5 flowering plants were measured after 2 months grown under SD. Values represent the mean ± standard deviation of 4-5 plants. Values in a column followed by the same letter are not significantly different at P=0.05.

Transgenic chrysanthemums phenocopy WT plants grown under the FR-depleted light

To investigate the mechanism of the inhibition of stem growth in transgenic chrysanthemums, plants were grown in a light environment in which the R/FR of sunlight at noon was increased from 1.20 to 1.73 using a spectrally selective plastic filter (Fig. 5.1). As in the previous experiment, plant heights of both LE31 and LE32 were less than
WT plants under control conditions (clear plastic film) and phenocopied WT plants grown in FR-depleted light (Fig. 5.4). However, no further reduction in the growth of the transgenic plants was achieved by subjecting them to FR-depleted light, suggesting that the tobacco phy-B1 is unlikely a sensor for the shade avoidance response.

Another characteristic of plants grown under FR-depleted light environments is an increased level of chlorophyll per unit area (McMahon and Kelly, 1995). This can be regarded as a result of the removal of shade conditions which result in the retardation of leaf development including leaf area growth, chloroplast development and chlorophyll synthesis (Smith, 1994; Smith, 1995). Similarly leaves of WT chrysanthemum plants grown under the spectral filter exhibited roughly 20% higher chlorophyll levels than when grown under control conditions (Fig. 5.4B). The higher chlorophyll levels in WT leaves in response to the increase in the R/FR ratio were not statistically different from those observed in the transgenic plants grown under the clear plastic. Likewise under natural light both LE31 and LE32 contained more chlorophyll than WT plants (Fig. 5.4B). However, subjecting LE31 and LE32 to the reduced FR-light environment did not result in any further increases in chlorophyll levels (Fig. 5.4B). Again, this indicates the tobacco phy-B1 is not a mediator for the shade-avoidance response.
Figure 5.4. Transgenic chrysanthemum plants phenocopy wildtype plants grown under FR-depleted light. Plants were transferred to boxes covered with clear plastic (control) or a plastic photoselective filter (see Fig. 1) 3 weeks after vegetative propagation. Plants were maintained in a greenhouse in which the natural photoperiod was extended to 17 h. A) Stem heights measured every 3 d. Each point represented the mean of the five plants; vertical bars indicate the standard errors of the means. B) Total chlorophyll levels measured at 15 days after transfer to the photoselective filters. Columns with the letter are not statistically different at P=0.05.
There are two possible reasons for the increase in chlorophyll concentrations in the transgenic plants. First, a reduction in leaf surface area or an increase in the numbers of palisade cells per unit area will effectively result in an increase in the concentration of chlorophyll per unit leaf area. The application of growth retardants or FR-depleted filters has this effect (Crittendon, 1966; McMahon and Kelly, 1995). Alternatively, the green color of transgenic leaves might be the result of an increase in the absolute level of chlorophyll per cell. Although the leaf areas of transgenic plants did not change (Table 5.3), we could not distinguish these two possibilities without investigating the leaf anatomic structures.

It is generally believed that the B-type phytochromes measure the R/FR ratios at least for the shade avoidance response and end-of-day FR effect (Smith and Whitelam, 1997). However, the fact that increasing the R/FR ratio of the prevailing light environment provided no additional effect on both stem length and total chlorophyll levels in transgenic plants indicates that the plants are responding to the absolute amount of the FR-absorbing form of phytochrome (P₅₇) rather than the proportion of P₅₇ to P₅₉. Similarly, mustard seedlings measure the amount of P₅₇, not the ratio of P₅₇/P₅₉ for light-dependent anthocyanin synthesis (Schmidt and Mohr, 1982). Furthermore, the tobacco phy-B1 ortholog encoded by the HLG locus in N. plumbaginifolia has been shown to be a R sensor rather than a sensor of the R/FR ratio in light-grown plants (Hudson et al., 1997). A similar conclusion has been drawn that the tomato homologue phy-B1 does not play a significant role in the shade avoidance response (Smith and Whitelam, 1997; van Tuinen et al., 1995b). Therefore, the tobacco phy-B1 is likely a sensor for R, and its
ectopic expression in transgenic chrysanthemum plants increased the sensitivity to R only, resulting in the inhibition of stem elongation.

Expression of *PHY-B1* reduces growth by a mechanism that does not directly involve GA

Previously, we showed that both LE31 and LE32 plants behaved as WT plants grown under FR-depleted light. GA has been proposed to partially mediate the regulation of chrysanthemum plant height under different R/FR ratios (Rajapakse and Kelly, 1991). Supporting this idea are the observations that transgenic tobacco plants overexpressing an oat *PHYA* gene had reduced levels of endogenous GA (Jordan et al., 1995), while a *PHYB*-type mutant in sorghum (*ma3R*) had higher levels (Beall et al., 1991; Childs et al., 1997). Therefore, we investigated whether GA mediates the action of the tobacco phy-B1.

Endogenous GA levels were reduced by treating with CCC, an inhibitor of GA biosynthesis (Sponsel, 1995). This treatment resulted in a reduction in internode length of both WT and the transgenic plants (Table 5.5). However, the mean internode length of LE31 and LE32 plants treated with CCC was still shorter than CCC-treated WT plants (Table 5.5). Moreover, absolute differences in mean internode length between transgenic and WT plants were nearly the same (1.3 and 1.5 mm for LE31, and 0.9 and 1.1 mm for LE32) in both control and CCC treatments (Table 5.5). Exogenous GA3 treatments fully restored growth in both WT and transgenic plants to that of plants treated with GA3 alone. Nevertheless, exogenous GA3 failed to reverse the reduction in stem length caused by the expression of tobacco *PHY-B1* gene (Table 5.5).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genotype</th>
<th>Mean Internode Length (mm)</th>
<th>Mean Difference from WT Plants (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>WT</td>
<td>6.0±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LE31</td>
<td>4.7±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>LE32</td>
<td>5.1±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9</td>
</tr>
<tr>
<td>GA</td>
<td>WT</td>
<td>11.1±0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LE31</td>
<td>9.9±0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>LE32</td>
<td>9.9±0.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.2</td>
</tr>
<tr>
<td>CCC</td>
<td>WT</td>
<td>4.8±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LE31</td>
<td>3.3±0.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>LE32</td>
<td>3.7±0.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.1</td>
</tr>
<tr>
<td>GA+CCC</td>
<td>WT</td>
<td>11.2±0.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LE31</td>
<td>9.9±0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>LE32</td>
<td>9.9±0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Table 5.5. Comparison of the effect of exogenous GA<sub>3</sub> and 2-chlorocholine chloride (CCC) on internode length of transgenic and wildtype chrysanthemum plants. Plants were vegetatively propagated from cuttings and grown in the greenhouse under 17 h LD. After 3 weeks plants received either a soil drench of a 9.5 mM solution of CCC, 10 μg of GA<sub>3</sub> applied to the shoot tips every 2 d for a total of 7 treatments, or a combination of the CCC and GA<sub>3</sub> treatments. Values represent the mean ± S.E. of 5 plants. Internode lengths were determined 15 d after the treatments began. Means followed by the same letter are not significantly different at P=0.05. WT, wild-type.
Regardless of the treatment, the average internode lengths of plants of both transgenic lines were always about 1.2 mm less than WT plants subjected to the same treatment (Table 5.5). In other words, the combined effect of transgenic expression and growth retardant treatment on stem growth was the sum of their effects when applied individually. One interpretation of this observation is that GA does not mediate the R-induced inhibition of stem elongation in the transgenic plants. A similar rationale was used to argue against a possible role of GA in mediating phytochrome control of hypocotyl growth in mustard (Mohr and Appuhn, 1962). In addition, transgenic plants increased the branch angle (Table 5.4) and did not change the leaf area (Table 5.3), characteristics different from the growth retardant treatments. There are other independent lines of experimentation that provide corroborating evidence for this conclusion. First, endogenous GA levels were not altered in either the cucumber \( lh \) mutant or the pea \( lv \) mutant, which are deficient in B-type phytochromes (Lopez-Juez et al., 1995; Weller et al., 1994) or the \( PHYB \) mutant of Arabidopsis (Reed et al., 1996). On the other hand, exogenous GA\(_3\) does not fully restore hypocotyl growth in Arabidopsis \( PHYB \) overexpressors (Wagner et al., 1991). Second, it has been shown in several instances that GA and phytochrome regulate distinct cellular mechanisms contributing to growth. In \textit{Thlaspi arvense}, most of GA-induced petiole growth and stem growth can be attributed to an increase in cell number, while phytochrome regulation of growth in these tissues appear to be restricted to controlling the length that the cells ultimately attain (Metzger, 1988; Metzger and Dusbabek, 1991).
Expression of \textit{Ni-PHY-B1} increased the sensitivity of night-breaks to inhibit flowering

It has been demonstrated that R is the most effective wavelength for NB to inhibit the SD plants flowering under inductive long night and phyB is among the photoreceptors mediating the NB-R response (Halliday et al., 1997; Jackson et al., 1996; Thomas and Vince-Prue, 1997). Since we have previously shown that the tobacco phy-B1 mediated R in the inhibition of stem elongation, we then tested whether the phy-B1 also mediated the NB-R inhibition of flowering in the SD plant chrysanthemum. Plants were grown under 8 hr natural SD plus 30 min NB of low intensity R (1.2 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)).

Results showed that under SD flower induction of LE31 and LE32 was not significantly delayed compared to WT (Table 5.6). Under NB-R, WT plants delayed the flower bud formation and flowering by 3 and 16 days, respectively, compared with SD conditions. However, the NB-R had a much larger effect on flowering in the transgenic plants: there was an additional 4 to 5 and 17-20 day delay in the time to appearance of visible flower buds and flower bud opening, respectively.

This appears to agree with the finding that phyB mediates NB-R inhibition of flowering since transgenic SD tobacco plants expressing an Arabidopsis \textit{PHYB} gene dramatically delayed the flowering under higher intensity fluorescent light (Halliday et al., 1997). The slight delay for the inhibition of flowering in our transgenic plants is possibly due to the very low levels of R used in the NB treatment, since WT plants delayed by only 3 and 16 days in the time to flower bud formation and further development, respectively, compared to the SD control conditions (Table 5.6). This is true for chrysanthemum in that a longer duration (2-5 h) of low intensity light or a short
<table>
<thead>
<tr>
<th>Photoperiodic treatment</th>
<th>Genotype</th>
<th>Days to appearance of flower buds</th>
<th>Days to first open flower</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD</td>
<td>Wild-type</td>
<td>25.1±1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.4±1.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>LE31</td>
<td>25.3±1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63.5±1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>LE32</td>
<td>25.5±1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.8±3.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NB-R</td>
<td>Wild-type</td>
<td>28.4±0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76.0±3.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>LE31</td>
<td>33.1±2.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>95.6±6.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>LE32</td>
<td>32.3±1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>93.3±5.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table 5.6.** Ectopic expression of the tobacco *PHYB-1* gene in chrysanthemum plants increases the sensitivity of flowering to inhibition by short night-breaks. Plants were vegetatively propagated from cuttings and grown in the greenhouse under 17 h LD. After 3 weeks plants were transferred to SD or SD plus a 30-min night-break of red light from [0:00 h to 0:30 h]. The number of days to the appearance of visible flower buds and to the opening first flower were recorded. Values represent the means ± standard deviation of 8 plants per treatment. Means followed by the same letter are not significantly different at P=0.05.

A period of high intensity light is required to have maximal suppression of flowering (Larson, 1992; Thomas and Vince-Prue, 1997). Alternatively, our transgenic plants might not accumulate sufficient high levels of the tobacco phy-B1 protein to greatly increase the sensitivity to R, although we have not confirmed it.

However, the slight delay for transgenic plants in the time to the appearance of visible flower buds observed in the transgenic plants indicates that the initiation of flower primordia occurred about the same time as WT, since transgenic plants had a greater delay (13 to 15 days) from the flower bud formation to the opening of first flower, compared with 4 to 5 days delay in the time to flower bud formation (Table 5.6).
coupled with the much greater effect on the time to flower bud opening suggests that the primary effect of the ectopic expression of phy-B1 on flowering is on inflorescence development rather than floral induction. This is not unexpected since inflorescence development is a growth process, and we have previously shown that stem growth is reduced in the transgenic plants. Nevertheless it is still possible that under different NB conditions (e.g. higher PFD and/or longer duration), an effect on inductive processes would be more apparent.

**Biotechnological implications**

Biotechnology has several advantages over the conventional breeding approach for crop improvement. First, desirable genes, including those from other species, can be transferred into existing cultivars without affecting other valuable traits. Secondly it is a relatively rapid process (in our case the production of transgenic chrysanthemum plants took less than one year). and thereby reducing the cost of breeding new cultivars.

Genetic engineering of phytochrome genes has significant potential applications in both fundamental research and crop production (Robson and Smith, 1997). Ectopic expression of oat PHYA in tobacco resulted in the suppression of the shade avoidance response, thereby increasing the leaf-harvest index (Robson et al., 1996). Thiele et al. (1999) also found that the heterologous expression of Arabidopsis phyB in potato reduced stem elongation and higher chlorophyll levels. This latter effect resulted in an increase in photosynthetic performance and subsequent higher tuber yields (Thiele et al., 1999). We demonstrated here that plant architecture can be modified in other agriculturally useful ways through the ectopic expression of a B-type phytochrome gene.
Management of plant stature is a significant activity during the production of many greenhouse crops. Currently control of plant height is achieved through the use of chemical growth retardants or cultural methods such as negative DIF and mechanical stimulation (Dole and Wilkins, 1999; Garner and Bjorkman, 1996; Gianfagna, 1995). But all of these methods have disadvantageous features that result in higher costs of production. The ectopic expression of the tobacco PHYB-1 gene in crops provides an alternative technology that could potentially reduce the economic inputs required for management of plant height. Using this strategy, we were able to generate plants that exhibited a reduction in stature comparable to that using a growth regulator at the recommended rate (Tables 5.2 and 5.5).

In addition, since no synthetic chemical growth regulators for height control are labeled for use in the crops that are destined for human consumption, the extension of this technology into such crops may have potential applications in other areas of agriculture production as well. In many cases, shorter stems often result in a greater proportion of the assimilates produced by the plant to be partitioned into the harvestable parts of the plant such as seeds instead of stems (Loomis and Conner, 1992; Robson et al., 1996). This, coupled with the possibility that the increased chlorophyll levels observed in the transgenic plants could lead to higher net photosynthetic rates (Thiele et al, 1999) raise the possibility that overall increases in the yield of many agronomic and horticulture crops may be achieved using this technology.

Branching pattern is also a major consideration for plant architecture during crop production. The surrounding light environment regulates the two components of plant architecture that affect crop competition and productivity, branching pattern and stem
height. Plants have evolved the phytochrome system to adapt to the continuous change of light environment. Low R/FR induces the shade-avoidance syndrome (dramatic stem elongation and inhibited branching or tillering) which allows the leaves at higher positions to compete for the light (Smith, 1994; Smith, 1995). The assumption that phytochrome-mediated responses have adaptive value in nature has been experimentally tested using phytochrome mutants and transgenic plants (Schmitt et al., 1995). First, longer stems are an advantage for plants in dense stands but disadvantageous for uncrowded plants. Second, more profusely branched plants with shorter stems are more successful in open habitats or at low densities (Casal and Smith, 1989). This has been also supported by earlier observations that larger shoot zenith angles (Casal et al., 1986) maximized the exposure of photoreceptive tissues to sunlight.

In many cases, shorter stems often result in a greater proportion of the assimilates produced by the plant to be partitioned into the harvestable parts of the plant such as seed instead of stems or reproductive organs instead of stems (Loomis and Conner, 1992; Robson et al., 1996). Commercially desirable changes in branching patterns are usually achieved by manual pruning, so it is likely that genetic approaches to the control of branching patterns would also provide economic advantages for growers. Indeed, monocultures of tomato plants with a prostrate growth habit (larger branch angles) have increased total and marketable yield and are notably easier to harvest, compared with plants of upright growth habit (Ozminkowski et al., 1990).

Therefore, transgenic chrysanthemum plants having shorter stems and larger branch angles than WT plants at low density could reduce the height, increase canopy area, and thus expose lower leaves to more sunlight. The extension of this biotechnology
into the commercial production of chrysanthemums and other floricultural, vegetable and agronomic crops and fruit trees may significantly benefit growers by reducing the costs associated with management of plant architecture. In addition, plants with this type of architecture can cover more ground surface and thus may be very ideal for landscape purpose. It is anticipated that deeper understanding of the functions of phytochromes in the light regulation of vegetative and reproductive development will make significant contributions to crop improvement.
CHAPTER 6

GENERAL DISCUSSION

The photoperiodic timing mechanisms in plants have been the focus for almost 80 years since the discovery of photoperiodism by Garner and Allard in 1920. It was Bothwick et al. (1952a) that demonstrated the photoperiodic control was mediated by the R and FR-reversible phytochrome system with two photoconvertible forms, Pr and Pfr. Subsequently, it was demonstrated by grafting experiments that leaves serve as a main source of graft-transmissible, hormone-type messengers or flower hormones (so called "florigen") that target the shoot apex where flower initiation occurs (Zeevaart, 1976). Numerous physiological studies have found a paradox for both SDP and LDP in that the presence of Pfr at some time and the absence of Pfr at other times during a 24 h day/night cycle are required for floral induction (Thomas and Vince-Prue, 1997; Zeevaart, 1976). Moreover, a circadian clock which is entrained by phytochrome is definitely involved in the photoperiodic control of flowering (Lumsden, 1991; Thomas and Vince-Prue, 1997). Recently, molecular genetic studies using the Arabidopsis as a model have shown that phytochrome actually represents a family of chromoproteins with distinct but overlapping functions in photomorphogenesis (Quail et al., 1995; Smith, 1995; Whitelam and Delvin, 1997). However, how plants sense the different light signals (R, FR, light-on, light-off,
NB-R, etc.) by the phytochrome family, and how the phototransduction pathways interact with each other and with the circadian clock remains unclear.

Based on the distinct properties of different phytochrome, light-labile pool (mainly phyA) and light-stable pool (phyB, C, E and maybe others), two-pool (Takimoto and Saji, 1984) and one-pool (Thomas, 1991) theories have been proposed. Unfortunately, the proposed functions of phytochrome in either of the two theories proved to be inconsistent with the studies of some phytochrome mutants in the quantitative LDP, SDP or DNP, such as Arabidopsis, pea, sorghum and tobacco (Childs et al., 1997; Goto et al., 1991; Hudson et al., 1997; Johnson et al., 1994; Reed et al., 1994; Weller et al., 1995; Weller et al., 1997).

The roles of phytochrome A and B in the control of flowering have been suggested to modulate the flowering inhibitor(s). For example, phyA serves as a positive regulator of flowering through the control of either the synthesis or transport of a graft-transmissible inhibitor (Weller et al., 1997). In contrast, phyB acts as a negative regulator of flowering likely through modulating the production of a graft-transmissible inhibitor (Jackson and Thomas, 1997).

However, the idea that both phyA and phyB control the flowering through regulating the level or transport of an inhibitor may be too simplistic (Metzger and Zheng, 1998). The fact that R and FR can alternatively promote or inhibit flowering in both SDP and LDP depending on the time of the day/night cycle is not easily explained by phytochrome control of inhibitor levels.

Reverse genetic approach has allowed the generation of transgenic *Nicotiana sylvestris* plants mis-expressing tobacco *PHY-A1* or *PHY-B1* genes (Chapter 2), and the
use of these transgenic plants would allow addressing the central question: which phytochrome(s) are involved, and what is the role of the phytochrome(s) in the timing mechanism. The results support that both pools of phytochrome, namely phy-A1 and at least phy-B1 are involved, but their functions are clearly different from the proposed two pool theory (Takimoto and Saji, 1984).

**Type-A phytochrome sensing FR is required for daylength extension**

Similar to pea and Arabidopsis phyA (Johnson et al., 1994; Weller et al., 1997), the type-A phytochrome (phy-A1) in *N. sylvestris* is required for daylength extensions, indicating a highly conserved function of phyA in LDP. Consistent with the classic LDP such as *Lolium*, FR effectively promotes flowering in *N. sylvestris* during the early part of the 8 h daylength extension (Chapter 3). The reduced sensitivity to FR in both seedling and mature plants of transgenic SUA2 (underexpressing *PHY-A1*) suggests that type-A phytochrome is the sensor for FR in photomorphogenesis throughout plant growth and development.

The biggest challenge for phy-A1 as a FR sensor is the delayed flowering of SUA2 under Flu light conditions, compared to WT (Chapter 3). The interpretation for this is that phy-A1 is only partially reduced in SUA2 and thus under such conditions the active P_A1 is much lower than WT. If a phy-A1 knockout mutant can be generated, it will be interesting to observe the flowering response of such a mutant under R or Flu light conditions. Will such a null mutant respond to the promotion of FR?

On the other hand, the role of phy-A1 appears to be required during the whole 16 h period (8:30 to 0:30 h), and it is difficult to determine at what time of the light period or
even the 24 h cycle phy-A1 (namely FR) has the maximal promotion of flowering. If an inducible system to control the \textit{PHY-A1} expression can be created in \textit{N. sylvestris}, then the effects of FR mediated by phy-A1 will be fine-tuned. For example, in the glucocorticoid-inducible system (Aoyama et al., 1995), the antisense RNA suppression or sense overexpression of \textit{PHY-A1} gene could be turned on by spraying the leaves with the chemical solution of a synthetic glucocorticoid such as dexamethasone (DEX) which itself has no effects on plant growth and development. However, an elegant inducible system is need in which turning-off of the system is allowed.

\textbf{Type-B phytochrome sensing R has a dual inhibitory/promotive function in the control of flowering}

Similar to the role of phy-B1 in seedling de-etiolation, phy-B1 is also a sensor of R, rather than of R/FR ratios, in the light-grown plants. There are several lines of evidence to support this conclusion. First, flowering in the PHY-B1 co-suppressor (SCB35) was dramatically suppressed under 8 h Flu light extensions, and retained the response to low R/FR ratios (Chapters 3 and 4). Second, mutations on the \textit{PHYB1} orthologs in another tobacco (\textit{N. plumbaginifolia}) and closely related species tomato retained the response to low R/FR ratio or EOD-FR (Hudosn et al., 1997; van Tuinen et al., 1995b). The phy-B1 ortholog in \textit{N. plumbaginifolia} has been shown to be the R sensor (Hudson et al., 1997). Third, the ectopic expression of the \textit{PHY-B1} gene in chrysanthemum resulted in the same amount of inhibition of stem elongation under two different R/FR ratios (Chapter 5).
In contrast to phyB in LDP (Arabidopsis, barley, pea) and SDP (sorghum) which is inhibitory to flowering (Childs et al., 1997; Goto et al., 1991; Hanumappa et al., 1999; Weller et al., 1995), the phy-B1 in *N. sylvestris* has a dual function in the photoperiodic control of flowering: phy-B1 inhibits flowering during the early part but becomes promotive at the later part of the 8 h daylength extension. It has been shown that the phy-B1 ortholog in the DNP (*N. plumbaginifolia*) is promotive to flowering, but it is a DNP and therefore no photoperiodic treatments similar to this study could be carried out in this species. It will be interesting to observe the effects of the phy-B1 knockout on flowering in *N. sylvestris* and other tobacco LDP. Again, the use of the obligate LDP significantly helps to understand the mechanism of phytochrome control of flowering.

The inhibition of flowering by R during the early part of daylength extension is consistent with many other physiological data that R at this time is inhibitory but becomes promotive to flowering during the later part of the extension (Thomas and Vince-Prue, 1997). It can now be concluded that the inhibition of R during early part of extension is mediated by phy-B1 in *N. sylvestris*. And in fact, phy-B1 mediating R during the later part of extension is likely promotive to flowering, since SCB35 flowered earlier in 3 or 4 h extensions, but under the 8 h Flu extension flowering was dramatically suppressed (Chapter 3).

On the other hand, is it possible that Arabidopsis exhibits a fluctuation of sensitivity to R for flower induction, rather than simply inhibited by R and promoted by FR? To the best of my knowledge, there is no report so far doing similar photoperiodic experiments to this dissertation. One simple test can be expressing the *PHY-B1* gene which encodes a R sensor under the control of an inducible system in Arabidopsis *phyB*,
phyB phyD, or phyB phyE mutant backgrounds and investigate the flowering responses under the photoperiodic treatments similar to this study.

Flowering promoters and inhibitors in the photoperiodic floral induction

Due to the fact that phyA mutants in Arabidopsis and pea delayed flowering in daylength extensions (Johnson et al., 1994; Weller et al., 1997), phyA under such conditions probably generates the flowering inhibitor. Indeed, in pea, phyA has been shown to modulate the synthesis or transport of a flowering inhibitor (Weller et al., 1997). Since the phy-A1 deficient plants (SUA2) also delayed the flowering under daylength extension, is this true for *N. sylvestris* and other LDP plants? Although the LDP such as *Lolium* displays a fluctuation in the sensitivity to FR during a 24 h cycle, similar rhythmic patterns have not been reported in pea and Arabidopsis. In *N. sylvestris*, it has been shown that FR is promotive during the 8 h main light period and 8 h daylength extensions (Chapter 3). Although further experiments are needed to study the effects of FR during this 16 h period and maybe the 24 h day/night cycle in order to determine at what part of the cycle FR has the maximal promotion, phy-A1 must be involved in either the synthesis of a flowering promoter or regulation of the levels of a flowering inhibitor.

On the other hand, *N. sylvestris* clearly shows a fluctuation of the sensitivity to R, being inhibitory during the early part of extension but promotive at later part of extension. Thus, phy-B1 likely generates flowering promoters during the early part of extension and then flowering inhibitors. This is different from the phyB in potato in which phyB controls the synthesis or transport of a flowering inhibitor (Jackson et al., 1997).
To support the involvement of both flowering promoters and inhibitors, grafting experiments are necessary. It has been shown that *N. sylvestris* can produce a graft-transmissible promoter under inductive LD and induce flowering of DNP Trapezond tobacco as a grafting partner under LD (Lang, 1989; Lang et al., 1977). But if under non-inductive SD, *N. sylvestris* produced an inhibitor, and transported it to its grafting partner, where it suppressed flowering in Trapezond tobacco (Lang et al., 1977).

Therefore, it is likely that type-B phytochrome produces graft-transmissible flowering promotive or inhibitory substances, depending on the time of the day. But the grafting experiments are necessary to prove it.

**LDP measure the sensitivity to R and FR, through type-B and type-A phytochrome, respectively, other than the actual length of the day or night**

The fact that phy-B1 can be either inhibitory or promotive to flowering and the possibility that it can produce either the flowering promoters or inhibitors can be explained by the involvement of a circadian clock. As discussed in detail in Chapter 1, light signals can be sensed by phytochromes and other photoreceptors, and then transmitted via the light input pathways to entrain or synchronize the circadian clock. On the other hand, if the light signals perceived by phytochromes are coincident with the promotive or inhibitory phase of flowering rhythm, flowering will be promotive or inhibitory.

It has been suggested that in the regulation of *CAB* transcription in Arabidopsis which is under the control of a circadian clock, phyA and phyB operate through two independent phototransduction pathways and the two pathways then converge to induce
the circadian response of *CAB* transcription (Anderson et al., 1997). Coincidentally, there are likely two distinct pathways for phy-A1 and phy-B1-mediated flowering control, since the cross SUA2 × SCB35 flowered much later than SUA2 while SCB35 flowered at the same time as WT under 8 h Inc extension (Chapter 3). Thus, it is speculated that phyA generates a flowering-promotive pathway, and phy-B1 operates through another pathway in which the promotive and inhibitory signals can be produced and transported depending on the time of the day. These two independent flowering-related pathways might interact with a component(s) in the oscillator, and the decision to flower is dependent on whether the combined action of phy-A1 and phy-B2 pathways coincides with the phase of the circadian rhythm. To test this hypothesis, a *CAB::LUC* construct can be transformed into transgenic *N. sylvestris* mis-expressing phytochrome genes, and the circadian response of *CAB* transcription can be observed under different photoperiodic treatments. Second, the isolation of the clock component(s) which interact with the phy-A1 and phy-B1 phototransduction pathways will help determine whether light signals coincide with the phase of the circadian rhythm. But this is difficult due to the lack of detailed information regarding the clock components. Third, mutants specifically deficient in either phy-A1 or phy-B1 pathways can be isolated. SUA2, SCB35 or SUA2 × SCB35 can be mutagenized (for example through EMS or fast neutron), and the putative suppressors can be screened under different photoperiodic conditions such as 8 h Flu or Inc extensions.

Most importantly, the significant implication of the finding in this research is that the LDP may not necessarily measure the relative length of day or night, as being proposed for almost eighty years. The possibility for this time measurement mechanism
can be supported by the earlier observations that in *Sinapis* and *Lolium*, a 2 to 8 h NB given during the long dark period (40 or 64 h) promoted flowering (Thomas and Vince-Prue, 1997). Traditionally, LDP is thought to flower when the daylength is longer than a critical period, or when the nightlength is shorter than a critical period. However, the NB experiments showed the timing of R is crucial, despite the fact that the 2 to 8 h lights which are used for NB are clearly shorter than the critical daylengths for these two LDP (*Sinapis* and *Lolium*). Based on these experiments and the finding in this research using *N. sylvestris*, it is possible that the photoperiodic timing mechanism for LDP is that LDP times the sensitivity to R and FR during a 24 h day/night cycle by type-B and type-A phytochrome, respectively, and do not necessarily measure the absolute length of day.
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190


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202

