Phytochromes and the Photocontrol of Flowering

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by

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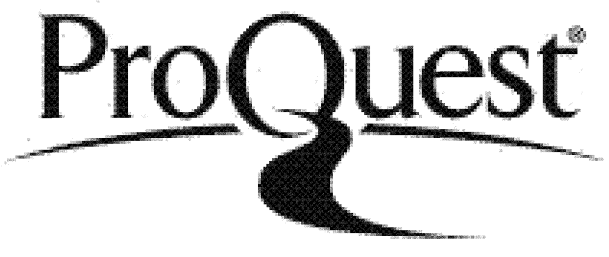

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Abstract

Analysis of photomorphogenic mutants and transgenic plants provides further insights into the roles of individual phytochrome species. The presence of a significant early-flowering response to low R/FR ratio has been revealed in *Arabidopsis phyB* mutants, that are also homozygous for a late-flowering mutation. This firstly, implicates at least one other novel phytochrome species, in addition to phytochrome B, in the low R/FR ratio-mediated early-flowering response. Secondly, identifies features that are likely to represent a loss-of-function mutant in this novel phytochrome species.

Examination of the *Arabidopsis elg* mutant, a putative novel phytochrome loss-of-function mutant, defines *ELG* as a novel gene that influences elongation growth. What is more, *ELG* appears to act independently of phytochrome and GA. However, H4S seedlings overexpress the *Arabidopsis HAT4* transgene, which is proposed to be down-regulated by a novel phytochrome. Thus, physiological analysis of *Arabidopsis* H4S seedlings reveal characteristics that may be representative of seedlings with a deficiency in a novel phytochrome species.

The physiological effects of phytochrome overexpression have also been examined in DN and SD *N. tabacum*, cv. Hicks. Allelic series overexpressing phytochrome A, phytochrome B and phytochrome C were generated for this purpose. These analyses provide a full characterisation of a phytochrome B-overexpression phenotype in tobacco, previously unreported. They also provide the first evidence that phytochrome C is a functional photoreceptor, and the first description of a phytochrome C-overexpression phenotype.

DN and SD *N. tabacum* differ only with respect to the *MM* locus, which confers SD photoperiodicity. Hence, these plants are ideally suited for the comparative analysis of phytochrome overexpression on photoperiodic perception. Alterations in the NB-mediated flowering response in transgenic plants suggests that phytochrome A- and B-overexpression disrupt photoperiodic timimg. However, the behaviour of *N. tabacum* overexpressing phytochrome C suggests that the phytochrome C transgene may specifically affect *MM* transduction.

Abbreviations

B	Blue
cM	centimorgan
cm	centimetre
DN	Day-neutral
d	Day
EOD	End-of-day
FR	Far red
GA	Gibberellic acid
h	Hour
HIR	High irradiance response
kD	Kilodalton
LD	Long-day
LFR	Low fluence response
min	minute
MM	Maryland mammoth allele
NB	Night break
PAR	Photosynthetically-active radiation
PCR	Polymerase chain reaction
Pfr	Far red light-absorbing form of phytochrome
PHY	Phytochrome encoding gene, wild type allele
phy	Phytochrome encoding gene, mutant allele
phy	Holophytochrome
Pr	Red light-absorbing form of phytochrome
R	Red
R/FR ratio	The photon fluence rate ratio of red to far red light in 10 nm band widths centred on 660nm and 700 nm
RBCL	Ribulose bisphosphate oxygenase
RBCS	Ribulose bisphosphate carboxylase
SD	short day

SE	standard error
UV	Ultra-violet
VLFR	Very low fluence response
W	White

Contents

Chapter 1 Introduction

1.1 The perception of light

The sessile nature of plants has resulted in the evolution of highly sensitive systems for perceiving environmental changes and eliciting adaptive responses. As plants are photoautotrophs, light is arguably the most influential environmental factor. Not only does light provide the source of energy for manufacturing organic molecules in photosynthesis, but it is also an important environmental signal for some crucial decisions in plant development. There are a multitude of growth and developmental responses initiated or directed by light, which are known collectively as *photomorphogenesis*. These photomorphogenic responses are regulated by several classes of photoreceptors: the phytochromes, blue (B) light photoreceptor(s), UV-A photoreceptor(s), UV-B photoreceptor(s), and possibly others. Of these, the phytochromes have been the most extensively characterised. Phytochrome functions at all stages in the plant life cycle providing the means of detection and interpretation of changes in light quantity and quality. Such a sophisticated interaction mechanism, amongst other things, allows the adjustment of germination, growth and development to environmental changes, and the synchronisation of growth and/or reproduction in response to seasonal changes in daylength.

1.2 A history of phytochrome

The initiation and subsequent development of photomorphogenesis as a field of research is a direct consequence of work carried out by Garner and Allard, (USDA), in the early part of the century (Vince-Prue, 1975; Sage, 1992). An interest had arisen in a giant strain of *Nicotiana tabacum*, named Maryland Mammoth, that had appeared spontaneously in a field of Maryland Narrowleaf tobacco in 1906. Work that followed

established that flowering time in Maryland Mammoth, and hence, the period of the time spent in the vegetative state, was determined by daylength (Garner and Allard, 1920). Unlike the wild type tobacco, Maryland Mammoth required short days to flower. Crosses made between Maryland Mammoth and the day-neutral cultivar of *N. tabacum* showed that the short-day response was dominant and involved a single allele (Allard, 1919). The short-day requirement for flowering in Maryland Mammoth was, therefore, probably controlled by a single gene. The term *photoperiodism* was introduced for the response of organisms to the relative length of day and night. Further work revealed that different plants had different day length requirements for flowering. Having found that plants fell into at least three groups, Garner and Allard, (1933), coined the terms short-day (SD) plants, long-day (LD) plants and day-neutral (DN) plants. A characteristic of most SD plants is that a brief irradiation given during an inductive dark period has an inhibitory effect on flowering (Hamner and Bonner, 1938). This "night break-" (NB-) mediated inhibition of flowering provided a means of probing the mechanism of photoperiodic flowering and determining the spectrum of light necessary for activation of the photoreceptor (Parker *et al.*, 1946; Borthwick *et al.*, 1952a).

Concurrent work at the USDA, was the investigation of the germination characteristics of lettuce seed. This led to the discovery that germination induced by short wavelength light could be abolished by subsequent exposure to light of a longer wavelength (Flint, 1934). The maximum sensitivity for promotion of germination, at about 660 nm, was found to be very similar to that for the inhibition of flowering by NBs in SD plants. Therefore, the two responses were probably under control of the same photoreceptor (Parker *et al.*, 1946; Borthwick *et al.*, 1954). This work naturally progressed to the classic experiment where the red (R) light-promotion of germination was shown to be reversible by far-red (FR) light (Borthwick *et al.*, 1952b). Hendricks, hence, proposed that the pigment controlling germination existed in two interconvertible forms, one absorbing R and the other absorbing FR (Borthwick *et al.*, 1952b). The R/FR reversibility of absorbance maxima in tissue extracts confirmed these initial findings (Butler *et al.*, 1960) and the pigment was named *phytochrome* from the Greek: *phyto*=plant, *khrōma*=colour (USDA, 1960). The R-absorbing form became known as

Pr and the FR-absorbing form was named Pfr (Borthwick and Hendricks, 1961).

1.3 Phytochrome structure

The phytochrome molecule has two moieties: a polypeptide and a covalently-linked, linear tetrapyrrole chromophore (Quail, 1994; Jones and Edgerton, 1994). The polypeptide comprises two domains joined by a protease sensitive hinge region: a globular NH_2-terminal domain (≈ 70 kDa) and a more elongated COOH-terminal domain (≈ 55 kDa). The chromophore is located in the globular domain, and is attached approximately 321 residues from the amino terminus to a cysteine residue via a thioether linkage. *In vivo*, phytochrome exists as a homodimer. The site of dimerisation is contained within the carboxy-terminal region.

The phytochrome holoprotein can exist in two forms: Pr, the R light-absorbing form, and Pfr, the FR light-absorbing form (Rüdiger and Thümmler, 1994). Pr absorbs maximally in the R at about 660 nm and Pfr absorbs maximally in the FR at about 730 nm. R irradiation stimulates chromophore Z-E isomerisation involving rotation at the double bond connecting the C and D rings of the tetrapyrrole. This photoconversion of the chromophore induces a conformational change in the protein moiety. The photochromic properties of phytochrome provide the means for it to act as a molecular switch that invokes a response in the active (Pfr) form. In the "classical" phytochrome response, activation by Pfr can be subsequently "turned off" by conversion of phytochrome to the inactive (Pr) form.

1.4 The phytochrome gene family

Phytochrome comprises a family of photoreceptors, the number and diversity of which vary from species to species. Initial work in *Arabidopsis thaliana* revealed the existence of five phytochrome genes designated *PHYA*, *PHYB*, *PHYC*, *PHYD* and

PHYE (Sharrock and Quail, 1989). All five genes have since been isolated and sequenced (Sharrock and Quail, 1989; Cowl *et al.*, 1994; Clack *et al.*, 1994). Three of the genes, *PHYA*, *PHYB* and *PHYC* have been mapped to chromosomes 1, 2 and 5, respectively (Chang *et al.*, 1988).

As early as 1965, Hillman suggested the presence of two pools of phytochrome to account for the action of phytochrome that was not spectrophotometrically detectable. It is now recognised that there are indeed two pools of phytochrome. They comprise Type I phytochrome that is light labile, and Type II phytochrome that is light stable (see Quail, 1991; Furuya, 1993; Smith, 1995). Type I phytochromes are abundant in etiolated seedlings and are rapidly down regulated upon exposure to light. Protein micosequencing data have established that Type I phytochrome is encoded by phytochrome A genes. Type II phytochromes are low abundance phytochromes in which the Pfr form is relatively stable under light conditions. Phytochromes B, C, D and E represent species in the light-stable phytochrome pool (Somers *et al.*, 1991; Clack *et al.*, 1994).

The sequences of phytochromes A-C, are equally divergent from each other, with the polypeptides exhibiting (approx.) 52% amino acid sequence identity (Sharrock and Quail, 1989; Clack *et al.*, 1994). Phytochrome E apoprotein shows a slightly higher degree of relatedness to phytochromes B and D, at 55% and 55%, respectively, although the most striking similarity is between phytochromes B and D that have 80% amino acid sequence indentity (Clack *et al.*, 1994). The relative divergence of the phytochrome protein sequences is consistent with the suggestion that each phytochrome may play a discrete role in photomorphogenesis (eg. Smith and Whitelam, 1990).
The first phytochrome A gene homologues were cloned and sequenced in oat (Hershey *et al.*, 1984, 1985). Since then *PHYA* genes have been reported in several species including: zucchini (Sharrock *et al.*, 1986), pea (Sato, 1988), rice (Kay *et al.*, 1989), maize (Christensen and Quail, 1989), potato (Hayer and Gatz, 1992a) and tobacco (Adam *et al.*, 1993). Phytochrome B genes have been isolated in rice (Dehesh *et al.*, 1991), potato (Hayer and Gatz, 1992b) and tobacco (Kern *et al.*, 1993).

Recent research reports the presence of an even more complex gene family in tomato (Hauser *et al.*, 1994; Pratt, 1995). Five phytochrome sequences have been

isolated: *PHYA*, *PHYB1*, *PHYB2*, *PHYE* and *PHYF*, and the existence of up to eight additional phytochromes including a putative *PHYC* orthologue has been demonstrated (Hermsmeier *et al.*, 1995). The *PHYB1* and *PHYB2* genes of tomato are proposed to form a subfamily of the *PHYB* gene in a manner that is similar to that of the *PHYB*, *PHYD* and *PHYE* genes of *Arabidopsis*. A possible explanation for this is based on the proposed early evolution of the *PHYA* and the *PHYC* genes and a single precursor for the *PHY-B,-D* and *-E* genes (Clack *et al.*, 1994). Subsequent duplication of the precursor may have given rise to a number of closely related genes including the *PHYB1* and *PHYB2* genes of tomato, and the *PHYB*, *PHYD* and *PHYE* genes of *Arabidopsis*.

A family of phytochrome genes has also been identified in *Sorghum*. Sequences with high homology to the *PHYA*, *PHYB* and *PHYC* genes of *Arabidopsis*, and a novel phytochrome sequence, *PHYF*, have been cloned (Hermsmeier *et al.*, 1995). Furthermore, low stringency Southern Blotting indicates the presence of up to five other putative phytochrome genes.

1.5 The phytochrome response modes

Phytochrome has been shown to be influential in all developmental stages in the life of a plant. In many species the germination characteristics of the seed are profoundly influenced by both phytochrome status established during seed development, and by phytochrome interaction with light following imbibition. Phytochrome action also influences the development of the seedling and is responsible for the initiation of chlorophyll formation in the transfer to the photoautotrophic state. In light-grown plants, changes in the mode of growth and the timing of flowering can be mediated via phytochrome responses to a changing light environment. Furthermore, the synchronisation of events such as seed germination, the initiation of flowering and plant growth, and breaking of bud dormancy in response to changes in day length, have been shown to be controlled by phytochrome. The different facets of phytochrome action are discussed first, with regard to the types of response modes utilised, and secondly with

regard to the ecological significance of the response modes.

1.5.1 Phytochrome function in seed germination and etiolated seedlings

Phytochrome acts via a number of distinguishable response modes in the imbibed seed and the etiolated seedling. Three response modes, have been identified in these developmental stages: (1) the *very low fluence response*, or VLFR; (2) the *low fluence response*, or LFR; and (3) the *high irradiance response*, or HIR. These response modes are characterised by the following parameters: fluence response curves, red/far-red (R/FR) reversibility, fluence rate dependence and reciprocity. Reciprocity requires the same degree of response to be elicited when the light duration and the photon fluence rate are reciprocally altered, whilst maintaining the same total number of photons in a given treatment.

The germination responses of seed populations have been shown to be biphasic for increasing photon fluence in some species (eg. Cone *et al.*, 1985; VanDerWoude, 1985; Rethy *et al.*, 1987; Botto *et al.*, 1996). The two phases of these responses are the VLFR and the LFR. Phytochrome operating in a VLFR is effective in bringing about a response to very low fluences of light (10^{-9} to 10^{-7} mol m^{-2}). Only a very small amount of Pfr is required to initiate such a response, as saturation occurs at very low fluences (Blaauw *et al.*, 1968; Mandoli and Briggs, 1981). In this situation FR, as well as R light, can generate enough Pfr to effect a response (Mandoli and Briggs, 1981; Botto *et al.*, 1996). This means that it is not possible for the VLFR to be fully R/FR reversible. In addition, the very low fluences required for this response make it difficult to test for reciprocity.

Given that VLFRs are saturated at extremely low fluences, it is probable that they represent a facet of phytochrome action that can initiate a response in conditions where light is severely limiting. Such conditions may exist under sufficient soil cover or very dense vegetative shading. Recently, phytochrome A has been implicated in VLFR seed germination in *Arabidopsis* (Botto *et al.*, 1996).

LFRs are characteristically induced by intermediate fluences

(10^{-6} to 10^{-3} mol m^{-2}) and they exhibit full R/FR reversibility and full reciprocity (Kendrick and Kronenberg, 1994). The LFR, therefore, represents the classical phytochrome response where an effect of a pulse of R light can be nullified by a subsequent pulse of FR light. There is extensive documentation of LFR responses, which include germination, inhibition of hypocotyl elongation, promotion of cotyledon expansion, chloroplast formation and anthocyanin synthesis. There is now convincing evidence that LFRs are mediated by phytochrome B as well as other phytochrome species in the stable phytochrome pool (Botto *et al.*, 1995).

The HIR contrasts with the LFR and VLFR, as it requires prolonged light exposures in order to realise maximum expression of the response (Mancinelli and Rabino, 1978). The HIR does not show red/far-red (R/FR) reversibility and does not appear to exhibit reciprocity. HIRs can be initiated in the etiolated seedling by FR, R and B light (Hartmann, 1966; Beggs *et al.*, 1980; Holmes *et al.*, 1982). It is likely that there is more than one mechanism of action, however, all HIRs appear to be fluence rate-dependent (Heim and Schäfer, 1984).

A defining characteristic of the FR HIR appears to be a requirement for continuous irradiation. It has been demonstrated that for a given fluence, uninterupted irradiation effects a much greater response in seedlings than discrete light pulses (Heim and Schäfer, 1984). This requirement for continuous irradiation is thought to be due to phytochrome action via cycling (eg. Johnson and Tasker, 1979). As there is some degree of overlap in the Pr and Pfr absorption spectra range, cycling between Pr and Pfr establishes a dynamic equilibrium that reflects the wavelength and the photon fluence rate. Wavelengths that are most effective in FR HIR are those that maintain phytochrome conversions at equilibrium. This is achieved by FR wavelengths, which favour the photoconversion to Pr, as Pfr destruction is dependent on its concentration (Frankland, 1972). The rate of phytochrome cycling is thought to be an important component of this response as there is an obvious fluence rate-dependency. Thus, it has been proposed that message transduction is initiated by a product of the phytochrome cycling process (Johnson and Tasker, 1979).

In the FR HIR, the requirement for continuous irradiation probably reflects an inherent instability of phytochrome Pfr. This is certainly true of phytochrome A, which

is light-labile. Indeed, analysis of mutants and transgenic plants has implicated phytochrome A as the species operational in the HIR (see Whitelam and Harberd, 1994; Smith 1995). Phytochrome A-mediated responses include germination and inhibition of hypocotyl elongation (McCormac *et al.*, 1991; 1992; Johnson *et al.*, 1994; Reed *et al.*, 1994; Shinomura *et al.*, 1994).

It is still unclear whether R HIRs are mediated by light-labile phytochrome, light-stable phytochrome, or by both via different mechanisms of action. The maintenance of some R HIRs after de-etiolation does suggest that stable phytochrome species can mediate this form of response (Beggs *et al.*, 1980). Furthermore, demonstration that continuous irradiation can be substituted by repeated pulses of red light suggests that such a response could be mediated by multiple inductions of Pr to Pfr (Heim and Schäfer, 1982). However, the fluence rate dependence of this response remains unexplained.

The VLFR, LFR and HIR represent mechanisms that are, collectively, sensitive to a wide range of light conditions that are encountered in the natural environment. Via these response modes phytochrome is able to initiate germination, and promote many aspects of the seedling development. This includes, inhibition of hypocotyl extension, the production of chorophyll, the expansion of cotyledons and the development of the first leaves. The cumulative effect is a transformation from an etiolated to a photoautotrophic state.

1.5.2 Phytochrome function in the light-grown plant

When the seedling emerges from the soil phytochrome is influential in adjusting the growth and development of the plant in a manner that befits the prevailing light environment. Several strategies operate which include growth and development that minimises shading by neighbouring plants, and synchronisation of developmental phases to match seasonal variations in light conditions. These two strategies are referred to as the *shade avoidance response* and *photoperiodism.*

Shade avoidance responses are initiated in shade-sensistive species via proximity perception of other vegetation, whether that be in the form of canopy shade or lateral reflection from neighbouring plants (eg. Smith and Morgan, 1983; Casal and Smith, 1989; Smith, 1990, 1992, 1994a; Gilbert *et al.*, 1995). Indeed, it appears that phytochrome is well equipped to respond to these differences in light quality. The relationship between R/FR ratio and Pfr/P (where P = Pr+Pfr) is such that the greatest rate of change in Pfr/P, and therefore the greatest sensitivity to light quality changes, occurs in the R/FR ratio range found under vegetation canopies (Smith, 1982). As vegetation absorbs very little radiation in the FR region of the spectrum, this means that a large proportion of incident FR is transmitted through or scattered by leaves from neighbouring vegetation (Smith, 1994a). The resultant decrease in R/FR ratio changes the phytochrome Pfr/P and triggers a redirection of resources into stem elongation at the expense of leaf and root development. An extensive range of shade avoidance responses have been documented which include: increased internode and petiole extension; decreased branching; reduced leaf area and leaf thickness; impaired chlorophyll development and accelerated flowering (Smith, 1994a). The shade avoidance responses are most effectively utilised by those species that have a shade intolerant growth strategy. An opposing, shade tolerant strategy is utilised by other species where competition is substituted with improved photosynthetic efficiency and reduced energy expenditure, as a result of slower growth rates.

In many plants, end-of-day far-red (EOD FR) treatments can induce developmental responses that are similar to those induced by continuous low R/FR ratio. This is easily explained, as both light treatments result in the reduction of Pfr for a period of time. The EOD responses are also R/FR reversible, saturate at low fluences and exhibit full reciprocity, in a similar manner to the LFR in etiolated seedlings (Smith, 1994a). It is therefore, possible that the R/FR, the EOD and the LFR responses of light grown plants are different manifestations of the same response. Phytochrome with a Pfr form that is relatively stable under continuous light is required for the initiation of responses to low R/FR ratio. Additional support for the EOD FR having the same mechanism comes from the observation that Pfr is stable for long periods into the dark period (Downs *et al.*, 1957). Indeed, recent work has implicated phytochrome B as one

of the light-stable phytochromes that function in these shade avoidance responses (see Whitelam and Harberd, 1994).

1.5.3 Photoperiodism

Over a wide range of lattitudes, daylength is probably the single environmental factor that provides a reliable source information about seasonal changes. The ability to measure daylength means that growth and development can be synchronised to coincide with environmental conditions that are the most favourable for reproduction or the availability of resources. This is accomplished by the interaction of phytochrome with an endogenous oscillator that primarily acts as a timer. Responses initiated in this way are called photoperiodic responses. The most extensively characterised photoperiodic response is floral induction. Plants can be divided into three broad categories according to their flowering responses to daylength: (1) SD plants, which have a requirement for dark periods over a specific length either to initiate or accelerate flowering; (2) LD plants, which have a requirement for dark periods under a specific length either to initiate or accelerate flowering; and (3) DN plants, where flowering is independent of dark period length (Vince-Prue, 1975).

The complexity of the photoperiodic response means that the precise nature of the response mechanism is still unclear. Much of the investigative work has been carried out on SD species where a single inductive photoperiodic cycle can induce flowering. Therefore, the photoperiodic mechanisms involved in such SD plants are by far the best understood. In SD plants the day length is measured not by the length of the photoperiod, but by the length of the dark period. Thus, flowering occurs when the night length exceeds a critical length of time. In resonance experiments where SDs of a fixed length are followed by increasing periods of darkness, flowering demonstrates a circadian rhythimicty which correlates with dark period length (Hamner and Takimoto, 1964). The involvement of phytochrome in photoperiodic control of flowering can be most clearly demonstrated in experiments where a R light NB, given during an otherwise inductive dark period, leads to a delay in flowering time. The effects of the R

light can be reversed by subsequent exposure to FR light (Downs, 1956). When the NB is given at intervals throughout a dark period, again flowering inhibition displays a 24 hour (approx.) rhythmicity. This suggests that although phytochrome may be the primary instigator of this response, the timing and degree of the response are influenced by the phase of the underlying rhythm at the point of phytochrome action (Vince-Prue, 1994). Thus, in the natural environment, if sufficient time has elapsed so that the light signal (dawn) coincides with the appropriate phase of the rhythm, Pfr will stimulate flowering.

Timing of the dark period, however, involves not one, but at least two discrete actions of light. In addition to the direct action of light on the circadian timer, light is also required to set the phase of the circadian timer at the end of the photoperiod (dusk). Indeed, these two actions of light have been demonstrated in *Pharbitis nil* (Lumsden and Furuya, 1986). Separation of the NB-induced phase shifting of the photoperiodic response and the NB-induced inhibition of flowering reveals different roles for light in these two responses. Phytochrome is also implicated in the control of this underlying oscillator. R light-stimulated phase-shifting of the rhythm with R/FR reversibility characteristics has been demonstrated in a number of species (Lumsden, 1991). However, a VLFR has been shown to phase-shift the circadian cycling of *CAB* mRNA in wheat (Nagy *et al.*, 1993). As phytochrome A has been implicated in the VLFR (Botto *et al.*, 1996), this suggests that phytochrome A may fulfil this function in wheat. It also appears that phytochrome is influential in controlling the length of the circadian rhythm. This is evident in *Arabidopsis*, where R light is not as effective in shortening the period of *CAB* mRNA oscillation in *hy1* seedlings as it is in wild type seedlings (Millar *et al.*, 1995).

In SD species, there is evidence that Pfr is also required for flowering promotion. However, this Pfr functions in a manner that is entirely independent of the circadian timer. In this case, the removal of Pfr by EOD FR prevents or delays flowering (Lumsden, 1991; Vince-Prue, 1994). In addition, when FR pulses are given at intervals throughout the duration of an inductive night, the extent of flowering inhibition gradually reduces. The non-rhythmic decay of FR-inhibition of flowering suggests that this phytochrome Pfr does not interact with an endogenous timer. Also,

the requirement for Pfr well into the dark period suggests that this Pfr is stable in darkness.

Photoperiodic flowering control in LD species is much less clearly defined. It would seem, however, that many LD plants demonstrate a quantitative relationship between the duration and/or irradiance of light and the level of response (Thomas, 1991; Vince-Prue, 1994). Plants that are controlled primarily by the photoperiod are referred to as "light-dominant" plants. In contrast SD plants, where control is derived from light interactions with the dark period, are referred to as "dark-dominant" plants. In LD plants there seems to be a requirement for Pfr in flowering promotion (Thomas and Vince-Prue, 1984). However, this appears to be at odds with the observations that Pfr removal by a FR light NB is generally ineffective or promotory. In some SD and LD species there is a parallel in terms of the interaction of light with a circadian oscillator (Deitzer *et al.*, 1982). Generally, in SD species, a rhythmic pattern of flowering inhibition is seen in response to R pulses given at intervals during an inductive night, whilst in LD species, the same effect can be achieved by FR pulses. In many LD plants, however, short NBs are ineffective. NBs of longer duration are required to stimulate an increased flowering response (Carr-Smith *et al.*, 1989). This type of response has the characteristics of a FR HIR and could therefore be attributed to action of a light-labile phytochome (Thomas, 1991).

Differences in the photoperiodic behaviour of LD plants may be a reflection of a variety of response modes employed by different LD species. Elucidation of these questions requires further investigation.

1.5.4 Co-action between phytochrome and the blue light photoreceptors

Physiological experiments conducted in a wide range of species have demonstrated co-action or synergism between phytochrome and the blue light photoreceptors (Attridge *et al.*, 1984; Drumm-Herrel and Mohr, 1984; Fernbach and Mohr, 1990; Casal and Boccalandro, 1995). Besides phytochrome, there are other photomorphogenically active pigments in plants that absorb in the B and UV-A region

of the spectrum (see Senger and Schmidt, 1994). As phytochrome also absorbs blue and UV light in addition to R and FR light, it has been difficult to separate responses initiated by the B- and UV-absorbing receptors (B/UV) and those responses initiated by phytochrome. However, it does seem that in many plants concominant absorption of light by B/UV receptors may be required for the establishment or the maintenance of some Pfr-stimulated responses (Mohr, 1994). This interdependence of the photoreceptor action has been demonstrated for a number of responses, including anthocyanin synthesis, hypocotyl inhibition and phototropic growth.

1.6 Analysis of photomorphogenic mutants

The identification of specific roles for individual phytochrome species has been aided significantly in recent years by the physiological and molecular characterisation of photomorphogenic mutants. There are three classes of such mutants: (1) those in which the mutation is within the phytochrome gene; (2) those in which the mutation affects chromophore biosynthesis or attachment; and (3) those in which the mutation affects a component of the transduction pathway. Extensive characterisation of photomorphogenic mutants has enabled the identification of discrete roles for a number of photoreceptors.

1.6.1 Phytochrome A-deficient mutants

Several *Arabidopsis* mutants have been isolated that have been shown to lack spectrophotometrically detectable phytochrome A, are deficient in immunochemically detectable phytochrome A polypeptide and are deficient in *PHYA* transcript (Nagatani *et al.*, 1993; Parks and Quail, 1993; Whitelam *et al.*, 1993). Each of the mutants carries a lesion in the phytochrome A gene. The recently selected *fri* mutants of tomato have reduced levels of spectrophotometrically detectable phytochrome in etiolated seedlings and are severely depleted in immunochemically detectable phytochrome A (van Tuinen

et al., 1995a). Both *phyA* and *fri* mutants are selectively deficient in the FR-mediated inhibition of elongation growth in etiolated seedlings, and therefore appear normal under R or white (W) light conditions. An almost complete absence of germination in *phyA* under continuous FR has also implicated phytochrome A in FR-mediated germination (Johnson *et al.*, 1994; Reed *et al.*, 1994; Shinomura *et al.*, 1994; Devlin *et al.*, submitted). Furthermore, the absence of a germination response in *phyA* seeds to a pulse of FR light implies that phytochrome A is active in VLFR-mediated germination (Johnson *et al.*, 1994; Botto *et al.*, 1996).

When grown under continuous W the *phyA* and *fri* mutants resemble wild type plants (Nagatani *et al.*, 1993; Parks and Quail, 1993; Whitelam *et al.*, 1993; van Tuinen *et al.*, 1995a). In addition, the *phyA* mutants demonstrate normal responses to low R/FR ratio and EOD FR light treatments (Johnson *et al.*, 1994). These observations led to the suggestion that the role for phytochrome A in light-grown plants is relatively minor. However, there is evidence that phytochrome A plays a significant role in plants grown under photoperiodic conditions (Johnson *et al.*, 1994). *Arabidopsis* is a quantitative LD plant that flowers earlier under long days than short days. The *phyA* mutants display a dramatically reduced ability to perceive inductive low fluence rate, incandescent day extensions, given after a short photoperiod. In addition to this, when grown under short days *phyA* seedlings are unresponsive to W light NBs, which very effectively accelerates flowering in wild type seedlings (Reed *et al.*, 1994). These observations suggests that phytochrome A may have a role in photoperiod perception in *Arabidopsis*, although the precise role of phytochrome A in photoperiodic flowering is still unclear. Interpretation of the photoperiodic behaviour of the *phyA* mutant is hampered by the lack of understanding of photoperiodic responses in LD species. Much more is known about the mechanisms of SD photoperiodic flowering, unfortunately no phytochrome A-deficient mutants are available in SD species.

Further evidence of phytochrome A action in light-grown plants comes from the observation of reduced inhibition of hypocotyl elongation in *phyA* seedlings, relative to wild type seedlings, grown in light/dark cycles (Johnson *et al.*, 1994). The hypocotyls of wild type and *phyA* seedlings grown under continuous W are indistinguishable (Whitelam *et al.*, 1993). It is, therefore, possible that a re-accumulation of phytochrome

A in the dark period is responsible for an enhanced sensitivity to W for hypocotyl growth inhibition. Another role for phytochrome A-mediated hypocotyl growth inhibition in de-etiolated seedlings has also been identified. In this instance, *phyA* seedlings display reduced hypocotyl inhibition, when compared with wild type seedlings, under continuous low R/FR ratio light. This implies that phytochrome A mediates hypocotyl growth inhibition under these conditions. However, the phytochrome A-mediated inhibition of hypocotyl elongation is not normally observed in wild type seedlings due to a second antagonistic phytochrome-mediated response. A reduction of phytochrome B Pfr by low R/FR ratio light leads to a reduction of hypocotyl growth inhibition resulting in enhanced hypocotyl elongation.

The *phyA* mutations exhibit partial dominance (Whitelam *et al.*, 1993). This is reflected by the relative amounts of FR-mediated hypocotyl elongation inhibition in seedlings with differing gene copy number. Seedlings homozygous for the mutation (*phyAphyA*), seedlings heterozygous for the mutation (*phyAPHYA*) and wild type (*PHYAPHYA*) seedlings display long, intermediate and short hypocotyl length in FR, respectively. This suggests a close relationship between the degree of response and the level of gene product.

1.6.2 Phytochrome B-deficient mutants

Mutants that lack phytochrome B or a light-stable phytochrome B-like protein have been reported for several species. These include the *Arabidopsis phyB* (=*hy3*) mutants (Nagatani *et al.*, 1991a; Somers *et al.*, 1991), the *lh* mutant of cucumber (López-Juez *et al.*, 1992), the *ein* mutant of *Brassica rapa* (Devlin *et al.*, 1992), the *tri* mutant of tomato (van Tuinen *et al.*, 1995b), the *lv* mutant of pea (Weller *et al.*, 1995a), and the ma_3^R mutant of *Sorghum* (Childs *et al.*, 1991, 1992). Analysis of several alleles of *phyB*, the *ein* and the ma_3^R mutant have confirmed that these mutations are contained within the phytochrome B structural gene (Reed *et al.*, 1993; P.F. Devlin and P.W. Morgan, pers comm.).

Mutants with a deficiency in phytochrome B display an elongated hypocotyl phenotype under R and W but not in FR (Koornneef *et al.*, 1980; Adamse *et al.*, 1987; Devlin *et al.*, 1992; van Tuinen *et al.*, 1995b). Also, the *phyB*, *lh, ein* and *tri* mutants all display reduced cotyledon expansion and reduced pigmentation (Adamse *et al.*, 1987; Devlin *et al.*, 1992; Chory, 1992; van Tuinen *et al.*, 1995b). These observations suggest that phytochrome B is involved in some aspects of seedling de-etiolation.

Phytochrome B has also been found to be instrumental in the promotion of *Arabidopsis* seed germination, both in the dark and in response to R light. Comparative analysis of the germination characteristics of *Arabidopsis phyB*, *phyA* and wild type plants has revealed that stored phytochrome B is responsible for the initiation of seed germination in dark-grown seedlings (Shinomura *et al.*, 1994). The lack of a germination response in *phyB* mutants to a pulse of R light has also implicated phytochrome B in a LFR-mediated seed germination (Shinomura *et al.*, 1994; Devlin *et al.*, submitted).

Light grown phytochrome B-deficient mutants display an elongated phenotype. They exhibit increased stem and petiole elongation and increased apical dominance. Furthermore, the extension growth seen in wild type plants following EOD FR treatments is absent or severely diminished in *phyB*, *lh*, and *ein* mutants (López-Juez *et al.*, 1990; Nagatani *et al.*, 1991; Devlin *et al.*, 1992). These mutants also have a reduced ability to respond to low R/FR ratio (Whitelam and Smith, 1991; Devlin *et al.*, 1992). Observations such as these have led to the suggestion that phytochrome B plays a significant role in these aspects of photomorphogenesis (Smith and Whitelam, 1990; Whitelam and Smith, 1991). However, shade-avoidance responses are not completely absent in phytochrome B-deficient mutants. For instance, it has been shown that the *phyB* and *ein* mutants have a normal response with respect to leaf area and specific stem weight (Robson *et al.*, 1993; P.F. Devlin, pers comm.). In addition, although *phyB* has an early flowering phenotype, there is a small, but detectable acceleration of flowering in response to low R/FR ratio (Whitelam and Smith, 1991; Robson *et al.*, 1993). This suggests that although phytochrome B has a role in the shade-avoidance responses of the light-grown plant, it is not the only player. To add weight to the argument, recent work with the *tri* mutant has shown that it responds to EOD FR

treatment with an increase in plant height that is quantitatively similar to the wild type (van Tuinen *et al.*, 1995b).

In many plants, phytochrome control of flowering is achieved by both responses to light quality and photoperiod. The flowering responses of *phyB* or *phyB*-like mutants have been studied in several species. The *ein* mutant and the *lh* mutant both flower early when compared to their respective wild types, but the effects are small (Rood *et al.*, 1990b; López-Juez *et al.*, 1990). The *phyB* mutant displays marked early flowering under both LD and SD conditions (eg. Goto *et al.*, 1991). The early flowering nature of these plants has been largely attributed to a reduction in their ability to regulate flowering in response to changes in R/FR ratio (see Whitelam and Harberd, 1994). Furthermore, it appears that photoperiodic control of flowering is not overtly affected in *phyB* mutants, as they are responsive to changes in daylength (Goto *et al.*, 1991). However, *Arabidopsis* is a quantitative LD plant, and most of the physiological studies on photoperiodic time-keeping have been performed with single cycle SD plants. The are no phytochrome B mutants of qualitative SD species, but the photoperiodic flowering behaviour of the ma_3^R mutant of *Sorghum bicolor*, a quantitative SD plant has been investigated. This mutant was originally described as a photoperiod insensitive mutant (see Childs *et al.*, 1995). It is now apparent that although ma_3^R plants flower early under LD conditions, they are nevertheless still daylength responsive (Childs *et al.*, 1995). Furthermore, the absence of phytochrome B in ma_3^R does not effect timing of the circadian oscillation of the light-harvesting chlorophyll a/b binding protein gene (*CAB*) and ribulose 1,5-bisphosphate carboxylase gene (*RBCS*) transcript levels (Childs *et al.*, 1995). However, these findings do not preclude the operation of phytochrome B in other circadian pathways which may influence flowering time. Thus, the precise role of phytochrome B in photoperiodic responses remains unclear, however, what is clear, is that other phytochromes are also involved.

It has been speculated that there may be a relationship between phytochrome B and gibberellic acid (GA) in the regulation of growth. A link was suggested following the subsequent discovery of phytochrome deficiencies in mutants with defective GA

metabolism. The *ein* mutation in *Brassica rapa* results in elevated levels of GAs (Rood *et al.*, 1990a). The ma_3^R mutation in *Sorghum* results in a phase shift in the diurnal regulation of GAs (Foster and Morgan, 1995). In addition, the *lh* mutant of cucumber and the *lv* mutant of pea have been shown to be hypersensitive to applied GAs (Reid and Ross, 1988; Weller *et al.*, 1994).

Like the *phyA* mutations, the *phyB* and *ein* mutations are semi-dominant (Koornneef *et al.*, 1980; P.F. Devlin, pers comm.). For example, the hypocotyls of light-grown seedlings heterozygous for the *phyB* mutation (*phyB/PHYB*) are of intermediate length, between those of the long, mutant homozygotes (*phyB/phyB*) and the short, wild type homozygotes (*PHYB/PHYB*). As increased hypocotyl inhibition correlates with gene copy number, this implies a close relationship between the phenotype and the abundance of the gene product. Furthermore, the gene dosage of the maturity alleles of *Sorghum* has been shown to correlate with levels of immunochemically recognised phytochrome B (Foster *et al.*, 1994).

1.6.3 Mutants deficient in phytochromes A and B

The examination of responses in *Arabidopsis* mutants homozygous for *phyA* and *phyB* has not only yielded additional information on the roles of phytochromes A and B, but has provided an insight into the roles of novel phytochrome species. The observation that *phyAphyB* seedlings are longer than either of the monogenic mutants under continuous R is revealing (Reed *et al.*, 1994). This suggests a role for phytochrome A in the promotion of hypocotyl inhibition under R. The response is not observed in the monogenic *phyA* mutant as the effect is masked by the much greater response mediated by phytochrome B.

Analysis of the *phyAphyB* double mutant seeds has also confirmed the action of a novel phytochrome in germination (Devlin *et al.*, submitted). The *phyAphyB* seeds are unresponsive to a brief pulse of R light, but they display a marked promotion of germination in response to continuous R. Furthermore, the continuous R can be substituted for by R light pulses. This suggests that another stable phytochrome is

active in germination that has a requirement for prolonged red light pulses.

1.6.4 Blue light receptor mutants

The *hy4*, (*blu* = *hy4*), mutant of *Arabidopsis* is selectively deficient in B light-stimulated responses. Etiolated mutant seedlings display normal inhibition growth under R and FR, but reduced growth inhibition under B light (Koornneef *et al.*, 1980; Ahmad and Cashmore, 1993; Jenkins *et al.*, 1993; Liscum and Hangarter, 1991), which suggests a deficiency in the blue light-sensing pathway. Indeed, the *HY4* (=*CRY1*)gene, isolated by gene tagging, has been shown to encode a protein that has the properties of a flavin-type, blue light photoreceptor (Ahmad and Cashmore, 1993).

1.6.5 Mutants deficient in chromophore synthesis

The *hy1* and *hy2*, (*hy6* = *hy1*), mutants of *Arabidopsis* have been shown to contain wild type levels of immunochemically detectable phytochrome (Chory *et al.*, 1989; Parks and Quail, 1989), that has no detectable spectral activity (Koornneef *et al.*, 1980; Parks *et al.*, 1989; Chory *et al.*, 1991). These findings led to the speculation that mutations at the *hy1* and *hy2* loci affect chromophore synthesis or attachment. Further analysis has demonstrated a disruption of chromophore synthesis in *hy1* and *hy2*. These mutants are blocked at a stage of phytochromobilin synthesis prior to the formation of biliverdin IXa (Parks and Quail, 1991). "Rescue" experiments, have demonstrated a restoration of a wild type phenotype in *hy1* and *hy2* seedlings grown on medium containing biliverdin IXa, a precurser of phytochromobilin. As each of the phytochrome species is believed to share the same phytochromobilin chromophore, it is likely that *hy1* and *hy2* cause a functional deficiency in all phytochromes.

Arabidopsis hy1 and *hy2* seedlings display a pleiotropic phenotype that is consistent with a global deficiency in phytochrome. Seedlings have a long-hypocotyl phenotype when grown under W, R or FR light conditions. The adult mutant plants

have a spindly appearance, characterised by elongated petioles, fewer, smaller leaves and increased apical dominance (Koornneef *et al.*, 1980; Chory *et al.*, 1989). Another feature of these mutants is their pale yellow/green colour, which has been attributed to a dysfunction of the photosynthetic apparatus (Chory *et al.*, 1989).

It also appears that the *hy1*, *hy2* mutants are not completely deficient in phytochrome, but are, in fact, severely depleted in functional phytochrome. This has been demonstrated by the elicitation of phytochrome-mediated responses in each mutant. Responses include the promotion of germination under R, increased elongation growth and flowering acceleration in response to a reduction in R/FR ratio (Cone, 1985; Whitelam and Smith, 1991).

Other chromophore, or putative chromophore mutants include the *aurea* (*au*) mutant of tomato, the (*partially etiolated* in *W* light) *pew* mutant of *Nicotiana plumbaginifolia*, and the (*phytochrome chromophore deficient*) *pcd-1* mutant of pea (Parks and Quail, 1987; Kraepiel *et al.*, 1994; Weller *et al.*, 1995b). Like the *hy1* and *hy2* mutants, these mutants also have elongated hypocotyls when grown under W, and are pale green in colour. Several lines of investigation have suggested that the *au* mutant phenotype is a result of chromophore deficiency (eg. Parks *et al.*, 1987; Sharrock *et al.*, 1988; Reed *et al.*, 1992). However, recent work provides conclusive evidence that the *au* mutant is, in fact, blocked in the synthesis of phytochromobilin (Terry *et al.*, 1995).

Etiolated *pew* mutant seedlings have severely depleted levels of spectrophotometrically-detectable phytochrome, whilst maintaining reasonable levels of immunodetectable phytochrome polypeptide (Kraepiel *et al.*, 1994). Furthermore, biliverdin partially restores the wild type phenotype in *pew* mutant seedlings (Kraepiel *et al.*, 1994). Likewise, etiolated seedlings of *pcd-1* have no spectrophotometrically detectable phytochrome, and crude extracts from *pcd-1* have been shown to acquire spectral activity after incubation with an analogue of phytochromobilin (Weller *et al.*, 1995b). It is, therefore, likely that the *pew* and the *pcd-1* mutations also interfere with chromophore synthesis.

1.6.6 Putative phytochrome transduction chain mutants

There are very few mutants that are thought to define positively acting components of phytochrome signalling pathways. However, the *hy5*, *fhy1-1* and *fhy3-1* mutants of *Arabidopsis* are believed to represent mutants in this class. The *hy5* mutant seedlings display a reduced hypocotyl inhibition response under R, FR and B light. This mutant, therefore, may encode a component that acts downstream of phytochrome and a blue light receptor, although it is possible that it may operate in a parallel pathway (Chory, 1992; Ang and Deng, 1994).

The *fhy1-1* and *fhy3-1* seedlings exhibit reduced sensitivity in hypocotyl inhibition growth when grown under FR, but not R or W light. The *fhy1-1* and *fhy3-1* mutations have also been shown to complement *phyA*, and the *fhy1-1* and *fhy3-1* mutant seedlings have normal levels of spectrophotometrically and immunochemically detectable phytochrome A (Whitelam *et al.*, 1993). Therefore, *fhy1-1* and *fhy3-1* probably identify genes that affect the function of components down-stream of phytochrome A. Furthermore, analysis has revealed that *fhy1-1* does not exhibit the full range of aberrant physiological responses displayed by *phyA*. The *fhy1-1* seeds have been shown to germinate in response to continuous FR, a response not seen in *phyA*. The *fhy1-1* seedlings also exhibit a wild type flowering response to low fluence rate, incandescent day extensions, that is absent in *phyA* seedlings (Johnson *et al.*, 1994). As *fhy1-1* is only operational in some of the phytochrome A-mediated responses it follows that the phytochrome A signal transduction pathway has at least two branches.

A second class of mutants are proposed to define negatively-acting components of photoreceptor signalling pathways. These mutants display some of the characteristics of light-grown plants when grown in the dark. In *Arabidopsis*, mutations in several different loci lead to this phenotype. These include the (*de-etiolated*) *det* mutants (Chory *et al.*, 1989, 1991; Cabrera y Poch *et al.*, 1993), and the (*constitutive photomorphogenic*) *cop* mutants (Deng *et al.*, 1991; Wei and Deng, 1992; Hou *et al.*, 1993; Wei *et al.*, 1994). The *cop* and *det* mutations cause dark-grown plants to develop short hypocotyls, open and expanded cotyledons and true leaves. These seedlings also

accumulate anthocyanin and activate a number of normally light-regulated genes when grown in the dark. These include the *CAB* gene, the *RBCS* gene and the ribulose bisphosphate oxygenase gene (*RBCL*) (Chory *et al.*, 1993). Epistasis analysis is suggestive that *COP* and *DET* are functional in the transduction of the phytochromes and blue light receptors (see Chory *et al.*, 1993). However, an additional aspect of the *det1* phenotype is the inappropriate differentiation of protoplasts into chloroplasts in *det1* roots, furthermore the *det1* phenotype can be phenocopied by wild type plants with high cytokinin levels (Chory and Peto, 1990; Chory *et al.*, 1993). It has been proposed that *DET1* is involved in the repression of cell-type-specific genes in the light, but, *DET1* also appears to act as an intermediate, linking light and cytokinin pathways. The COP1 protein has been proposed to function as a light-inactivatable master switch that represses photomorphogenic processes in the dark (Deng *et al.*, 1992). However, it is not clear from this model why *cop* mutations are deleterious or lethal in the light. All the *cop* and *det* loci have also been independently defined as *FUSCA* loci (Castle and Meinke, 1994; McNellis *et al.*, 1994; Miséra *et al.*, 1994). *Arabodopsis fusca* mutants have been so named for their purple seeds, which result from an inappropriate accumulation of anthocyanin in the embryonic cotyledons. Recent work has revealed that *FUSCA* genes perform critical functions before germination (Castle and Meinke, 1994). It has, therefore, been suggested that as FUSCA proteins, DET and COP may carry out their functions prior to seedling germination and that partial gene activity is responsible for the aberrant growth of mutant plants (Castle and Meinke, 1994).

1.6.7 Mutants with defective GA metabolism

A number of photomorphogenic mutants including ma_3^R, *ein*, *lh* and *lv*, (see above), have also been shown to be defective in GA metabolism. Indeed these mutants were originally identified as putative GA mutants (Reid and Potts, 1986; Reid and Ross, 1988; Rood *et al.*, 1990a, 1990b). Therefore, it is possible that other elongated mutants with aberrant GA metabolism may also have mutations that intefere with phytochrome biosynthesis or phytochrome signal transduction.

Several slender or elongated mutants have been described that are defective in various aspects of GA metabolism. Amongst these are the *procera* (*pro*) mutant of tomato (Jones, 1987),the *slender* (*sln*) mutant of barley (Lanahan and Ho, 1988), the *cry*^{s} *la* mutant of pea (Potts *et al.*, 1985), the *slender* (*sln*) mutant of pea (Reid *et al.*, 1992) and the *spindly* (*spy*) mutant of *Arabidopsis* (Jacobsen and Olszewski, 1993). These mutants can be divided into two classes, a GA-responsive class, consisting of the *pro, sln* pea and *spy* mutants that respond to GA and/or GA biosynthesis inhibitors (Jones, 1987; Reid *et al.*, 1992; Jacobsen and Olszewski, 1993), and a GA-unresponsive class, which includes the *sln* barley and the *cry*^{s} *la* mutants whose phenotypes are unaffected by changing GA levels (Potts *et al.*, 1985; Lanahan and Ho, 1988; Croker *et al.*, 1990). All these mutants can be phenocopied by their respective wild type plants treated with GA. They have a slender appearance with elongated hypocotyls and/or stems, pale leaves and increased apical dominance. These are also some of the characteristics displayed by seedlings with mutations affecting phytochrome function.

1.7 Analysis of transgenic plants

The isolation and cloning of phytochrome genes paved the way for a proliferation of activity in the analysis of phytochrome structure and function using molecular biological techniques. The transgenic expression of a variety of phytochrome gene fragments has made it possible to probe the structural and chemical properties of phytochrome, including: chromophore-protein interactions, dimerisation and the function of domains within the phytochrome molecule. The construction of transgenic plants has also been used to analyse the activity and function of phytochrome species. Temporal and spatial expression patterns, and the control of expression has been investigated with the use of promoter-reporter gene constructs. Further elucidation of the roles played by phytochromes has been achieved with analysis of plants overexpressing individual phytochrome genes.

1.7.1 Phytochrome structure/function

The examination of plants transgenically expressing either whole or partial phytochrome cDNA constructs, has greatly facilitated the investigation of phytochrome domain function. The attachment of the chromophore to the amino terminus is an autocatalytic process requiring lyase activity (Vierstra and Quail, 1986; Langaris and Langaris, 1989). The use of transgenically expressed phytochromes with a series of deletions has made possible the prediction of the region in which lyase activity is located (Cherry and Vierstra, 1994).

A major function of the carboxy terminus in all the phytochromes, is dimerisation. Two complementary techniques have been employed in the location of the dimerisation site. A technique using C-terminus deletions of oat phytochrome expressed in tobacco has identified the dimerisation site as a region between residues 920 and 1095 (Cherry *et al.*, 1993). An alternative approach has involved the identification of C-terminus fragments which when inserted into the deleted dimerisation site of lambda repressor (*cI*), restore dimerisation. This work has led to the proposal of a dimerisation site for oat phytochrome A in a region around the residue 750 (Edgerton and Jones, 1993).

The photoinduced conformational change in the chromophore results in concomitant changes in the conformation of the chromophore pocket which, in turn, affects the secondary structure of the protein. The amino terminus is thought to play an essential role in stabilizing the Pfr conformation. Initial work revealed changes in the spectral properties and enhanced dark reversion both in phytochrome lacking residues in the amino terminus, and in phytochrome with amino-terminal epitopes blocked by monoclonal antibodies (Cordonnier *et al.*, 1985; Lumsden *et al.*, 1985; Vierstra and Quail, 1985). Subsequent analysis of transgenics demonstrated that plants overexpressing oat phytochrome A lacking residues 7-69 did not display the characteristic dark green, dwarfed, overexpression phenotype (Cherry *et al.*, 1993). Furthermore, if oat phytochrome A lacking the amino-terminal 52 residues is overexpressed in *Arabidopsis*, endogenous phytochrome function is blocked under continuous FR (Boylan *et al.*, 1994). These findings provide valuable information on

the role of the amino terminal region in stabilising PfrA conformation under FR light (Cherry *et al.*, 1993; Jones and Edgerton 1994).

There is evidence that the C-terminus is important for phytochrome function. The absorption spectra of plants expressing phytochrome with various C-terminal truncations is similar to that of wild type plants. However, these plants fail to display an overexpression phenotype (see Edgerton and Jones, 1993). This does not rule out the possibility that absence of phenotype may result from indirect conformational changes in the amino region. Recent work has identified a number of *Arabidopsis* mutants with mis-sense mutations clustered in a small portion of the *PHYA* or the *PHYB* C-terminus (Wagner and Quail, 1995; Xu *et al.*, 1995). It appears that these mutants produce phytochrome molecules that are fully competent in photoperception but are defective in signal transduction. Thus, a region, identified as that between residues 624 and 777 in the C-terminus, has a common role in phytochromes A and B in the intermolecular signal transfer to downstream signalling intermediates.

Pfr appears to be degraded by the ubiquitin proteolytic pathway (Cherry and Vierstra, 1994). The finding that transgenic oat Pfr expressed in tobacco becomes modified by ubiquitin and rapidly degraded, suggests the conservation of degradation mechanisms between monocots and dicots.

1.7.2 Phytochrome promoter analysis

The assembly of constructs containing the promoter regions of phytochrome A or B genes fused to reporter genes has enabled the detailed analysis of spatial and temporal expression patterns. Pea *PHYA-GUS* and tobacco *PHYA-GUS* and *-CAT* constructs were introduced into petunia and tobacco, respectively (Komeda *et al.*, 1991; Adam *et al.*, 1994). High expression in the vascular tissue and at the root tip was observed in dark-grown tobacco seedlings and in the shoot apex in both transgenics. On transfer to light, expression was shown to be markedly down-regulated. In light-grown seedlings, tobacco *PHYA-GUS* transgene expression has also been identified in roots, phloem cells and in floral tissues (Adam *et al.*, 1994).

The activity of *PHYA* and *PHYB* promoters fused to *GUS* in *Arabidopsis* reveals a marked difference between the two in expression level, photoregulation, and to a lesser extent spatial distribution (Somers and Quail, 1995a). *PHYB-GUS* expression was higher in seeds which is consistent with the view that dark germination is mediated by phytochrome B (Reed *et al.*, 1994; Shinomura *et al.*, 1994). The well documented *de novo* synthesis of phytochrome A in dark-imbibed seeds can also be measured in terms of increasing *PHYA-GUS* activity in *Arabidopsis* embryos (Somers and Quail, 1995a). Higher levels of *PHYB* promoter activity was also found in mature pollen, otherwise the *PHYA-GUS* and *PHYB-GUS* transgenes appear to have similar spatial expression patterns.

There are quite marked differences in the temporal expression patterns of the two promoters (Somers and Quail, 1995b). In dark-grown seedlings the *PHYA* promoter is 20 times more active than the *PHYB* promoter. Light-grown seedlings exhibit a massive reduction in *PHYA-GUS* expression and a significant reduction in *PHYB-GUS* expression, though the activity of the *PHYA* promoter activity remains higher than that of *PHYB*. These results are in agreement with the earlier observations of higher levels of *PHYA* mRNA, when compared with *PHYB* mRNA levels, in both light and dark-grown seedlings (Sharrock and Quail, 1989; Clack *et al.*, 1994). These findings suggest that the relative abundances of phytochromes A and B is both a function of transcription rate and Pfr degradation rate.

An additional observation in these transgenic plants is the increase in *PHYA* and *PHYB* promoter activity following transfer to the dark (Somers and Quail, 1995b). Raised phytochrome levels at such a time may serve to enhance sensitivity to light that interrupts or terminates a period of darkness. This adaptation may be essential for photoperiodic timing.

1.7.3 Phytochrome photoregulation

Photoregulation of *Arabidopsis PHYA* and *PHYB* genes has also been examined using promoter-*GUS* constructs. In shoots, a reduction in the activity of

PHYA-GUS occurs in R or W light (Somers and Quail, 1995b). This suggests that a light-stable photoreceptor is primarily responsible for the down-regulation of phytochrome A. However, the behaviour of *PHYA* promoter activity in a range of mutant backgrounds is not supportive of this proposition. *PHYA-GUS* activity in the *phyA*, *phyB*, *hy4*, *hy5* and *hy6* mutants proved to be no different from that in wild type plants. No clear conclusions can be drawn from these results, especially as light mediated reduction in the *PHYA* promoter activity in *hy6* may have resulted from the action of a phytochrome species present at very low levels (Chory *et al.*, 1989). Therefore, control of phytochrome A expression could either require the cooperation of two or more photoreceptors, or the action of a novel photoreceptor (possibly a species of phytochrome).

The photoregulation of phytochrome B appears to operate through the light-modulated control of gene transcription (Wester *et al.*, 1994). An absence of *PHYB* photoregulation in light-grown *phyB* seedlings indicates that phytochrome B is responsible for its own down-regulation in R and W light (Somers and Quail, 1995b). What is clear, however, is that *PHYB* expression is differentially controlled under differing wavelengths. An increased *PHYB-GUS* activity in wild type seedlings under continuous FR or B light is absent in the *phyA* mutant seedlings (Somers and Quail, 1995b). This suggests that phytochrome A is necessary for the FR and B light induction of *PHYB* activity. The sensitivity of phytochrome B expression to different wavelengths suggests that expression levels may be a crucial factor in *PHYB*-mediated responses. The activity of the *PHYB* promoter varies greatly between the extremes of monochromatic R and FR irradiation. Thus, in the natural environment the opposing effects of R and FR light on *PHYB* transcription will reflect the ambient R/FR ratio. The ecological significance of a phytochrome A-mediated stimulation of phytochrome B expression under vegetative shade could be the enhancement of seedling sensitivity to R in conditions where light is limiting.

1.7.4 Overexpression of phytochrome transgenes

The analysis of transgenic plants expressing elevated levels of specific phytochrome transgenes has confirmed roles for phytochrome species deduced from the analysis of phytochrome mutants. Transgenic phytochrome overexpressors have been generated in tomato, tobacco, *Arabidopsis* and potato, using cereal or dicot *PHYA* or *PHYB* cDNAs as the transgenes (Furuya, 1993; Smith, 1994b; Heyer *et al.*, 1995). *Agrobacterium*-mediated transformation methods have been successful in overexpressing phytochromes A and B that, in the majority of cases, are under the control of the cauliflower mozaic virus (CaMV) 35S promoter. Each of these transgenes produce phytochrome apoproteins that bind to the endogenous chromophore, and are functionally active in their host plants (Furuya, 1993; Smith, 1994b).

Confirmation of roles for phytochrome species can be straightforward under conditions where the transgenic phenotype is the antithesis of the mutant phenotype. However, problems with interpretation may occur when this is not the case. Exaggerated levels of a particular gene product can potentiate a number of secondary physiological effects which may result from abnormal persistence of the transgene product. Also, transgenes under the control of the CaMV 35S promoter are expressed in most tissues which may not represent the normal spatial distribution of the phytochrome species. These factors combined, have the potential to affect the temporal and spatial activity of phytochrome. Interpretation of mutant physiology is less complex, but problems do arise when the absence of a response is masked by the action of another phytochrome. This can occur when responses are affected by two or more phytochrome species acting in a parallel pathways.

Transgenic overexpression of phytochrome A enhances photosensitivity to FR and R light (McCormac *et al.*, 1991; Whitelam *et al.*, 1992; Heyer *et al.*, 1995). Etiolated wild type seedlings exhibit a photon fluence rate-dependent inhibition of hypocotyl elongation under FR. Overexpression of phytochrome A has the effect of enhancing this response in transgenic plants (McCormac *et al.*, 1991; Whitelam *et al.*, 1992; Heyer *et al.*, 1995). Such a response represents the antithesis of that displayed by phytochrome A mutants, and is consistent with phytochrome A being responsible for

the FR HIR response mode. Upon de-etiolation, wild type seedlings respond to supplementary FR light by the enhancement of elongation growth, regulated by light stable phytochromes. However, light-grown tobacco and *Arabidopsis* seedlings overexpressing monocot phytochrome A have been shown to display fluence rate-dependent inhibition of elongation growth in response to supplementary FR (McCormac *et al.*, 1991; Whitelam *et al.*, 1992). This has been attributed to the persistence of the FR HIR, mediated by transgenic phytochrome A, which is maintained at levels not normally present in de-etiolated wild type seedlings. Not all transgenic plants expressing introduced phytochrome A demonstrate a persistence of the FR HIR once de-etiolated. The latter appears to be the case in transgenic potato expressing introduced potato cDNA under the control of the CaMV 35S promoter (Heyer *et al.*, 1995). Light-grown transgenic potato do not appear to have elevated levels of phytochrome A. Thus, it has been proposed that difference in abundance in light-grown material may be related either to expression levels, or to enhanced stability and/or activity of monocot phytochrome A in a dicot host, as native phytochrome A seems to be efficiently degraded in potato. Indeed the action of the introduced native transgene in potato may be more representative of the action of the endogenous phytochrome.

There also appears to be a role for phytochrome A in R light-mediated responses. Enhanced sensitivity to R has been reported in etiolated seedlings of tobacco, *Arabidopsis* and potato overexpressing transgenic phytochrome A (McCormac *et al.*, 1991; Whitelam *et al.*, 1992; Nagatani *et al.*, 1993; Schäfer *et al.*, 1994). Some plants, where transgenic protein levels remain high after transfer to W light conditions, also display increased photosensitivity when grown under these condition. These plants are characterised by their dark-green, dwarfed growth habit under these conditions (Keller *et al.*, 1989; Cherry *et al.*, 1991; Boylan and Quail, 1989). Other phenotypical traits include increased chlorophyll content, reduced apical dominance and delayed leaf senescence.

Alteration of the levels of phytochrome A has a marked effect on flowering time. *Arabidopsis* seedlings overexpressing an oat phytochrome A transgene flower earlier than wild type seedlings, whereas *phyA* mutants flower late. Along with the fact

that phytochrome A mutants are insensitive to low fluence day extensions and W light NBs (Johnson *et al.*, 1994; Reed *et al.*, 1994; Bagnall *et al.*, 1995), these observations suggest that phytochrome A may be involved in daylength perception in *Arabidopsis*. Interpretation of phytochrome overexpression in LD plants such as *Arabidopsis* can be hampered by the paucity of knowledge of LD photoperiodic flowering. Therefore, it would probably be beneficial to study the effects of phytochrome A overexpression in plants with a SD requirement for flowering. However, with the exception of potato, which propagates primarily by vegetative means, there are no transgenic SD species available expressing introduced phytochrome A.

Transgenic plants expressing phytochrome B cDNAs, under the control of the CaMV 35S promoter, have been generated in *Arabidopsis* (Wagner *et al.*, 1991; Wester *et al.*, 1994). Etiolated transgenic seedlings display increased hypocotyl inhibition under R light. This phenotype is the antithesis of that of *Arabidopsis phyB* seedlings, which display reduced sensitivity to R. These observations implicate phytochrome B in R light-mediated seedling de-etiolation. Analysis of mutants and transgenics has also linked *PHYB* gene copy number to the level of response (Koornneef *et al.*, 1980; Wester *et al.*, 1994). Increased hypocotyl growth inhibition correlates with an increasing number of gene copies, which suggests that level of response is directly linked with the amount of phytochrome B present. It appears that phytochrome B controls hypocotyl elongation by changing cell size, rather than affecting cell number (Nagatani *et al.*, 1991; Reed *et al.*, 1993; Wester *et al.*, 1994). Adult *Arabidopsis* plants overexpressing phytochrome B display a light conditional, dark-green, dwarfed phenotype (Wagner *et al.*, 1991; McCormac *et al.*, 1993; Wester *et al.*, 1994; Bagnall *et al.*, 1995). This contrasts with the elongated phenotype of the light-grown *phyB* mutant which resembles the shade-avoidance phenotype displayed by wild type plants grown under low R/FR ratio light. These observations implicate phytochrome B in the control of plant elongation growth and development during the photoperiod. These characteristics, coupled with the light-stable qualities of PfrB have led to the proposal that phytochrome B plays an important role in the mediation of shade avoidance responses to low R/FR ratio light.

Analysis of *Arabidopsis* seedlings overexpressing phytochrome B has revealed

a possible role for PfrB in the promotion of flowering during the inductive dark period (Bagnall *et al.*, 1995). Seedlings expressing transgenic PhyB protein flower earlier than wild type seedlings, whilst removal of PfrB by an EOD FR light treatment restores the wild type phenotype. However, these observations are not consistent with those made on the *phyB* mutants that also flower earlier than wild type plants. As with the phytochrome A-overexpressors, it may be beneficial to study the effects of phytochrome B-overexpression in SD species. As yet, however, there are no SD species available that overexpress transgenic phytochrome B.

1.7.5 Antisense-reduction of endogenous phytochrome

A reduction in the level of endogenous phytochrome expression can either be selected for in mutant screens, or, it can be achieved with the introduction of homologous transgenes in the antisense orientation. Recent work contrasted the over- and underexpression phenotypes of potato with native phytochrome A transgenes in the sense and antisense orientation, respectively (Heyer *et al.*, 1995). The photophysiology of sense and antisense plants was largely reciprocal and consistent with the observed physiological responses in previously characterised mutants and transgenics. However, unlike the phytochrome A-deficient mutants of *Arabidopsis* and tomato (Nagatani *et al.*, 1993; Reed *et al.*, 1994; van Tuinen *et al.*, 1995a), the antisense potato has a reduced sensitivity to R. This may reflect a larger role for phytochrome A in R-mediated responses in etiolated potato.

1.8 The thesis

The application of biochemical and molecular biological techniques have led to the amalgamation of a vast amount of information on phytochrome function. However, the complexity of the many physiological and developmental responses means that the process of defining roles for each phytochrome species is far from conclusive. Indeed, in some aspects of photomorphogenesis, such as photoperiodism, knowledge of phytochrome function is very scant. Both mutant and transgenic strategies have been utilised in this thesis to provide further insights into the components of phytochrome action.

The availability of mutants defective in phytochromes A and B have provided a valuable insight into the function of these two phytochrome species throughout the life cycle of a plant. The absence of mutants deficient in phytochromes C or E has so far precluded similar, direct investigation of phytochrome C or E function. However, further analysis of *Arabidopsis phyB* mutants has been very revealing, not only in enhancing our understanding of phytochrome B function, but also defining an operational framework for a novel, light-stable, phytochrome species.

The establishment of a role for another phytochrome species provided the opportunity to predict the phenotype of and, therefore, a screen for a mutant of this novel phytochrome. The required phenotypical characteristics are displayed by the *elongated* (*elg*) mutant of *Arabidopsis*. However, characteristation of the *elg* mutant demonstrated that, in fact, the phenotype was unrelated to phytochrome, but rather, identified a novel gene operational in elongation growth responses that acts both independently of phytochrome and GA.

A phenotype that results from deficiencies specifically in the novel phytochrome could result either from a mutation within the novel phytochrome gene or disruption of the transduction pathway. It has been proposed that *HAT4* gene expression of *Arabidopsis* is negatively regulated by a novel phytochrome (Carabelli *et al.*, 1996). Analysis of H4S transgenic plants, overexpressing the introduced *HAT4* gene has provided an extensive characterisation of the putative novel phytochrome mutant

phenotype.

In recent years much effort has been dedicated to the exploration of the physiological roles of phytochrome through the study of mutant and transgenic LD plants. As a consequence a range of phytochrome A-mediated and phytochrome B-mediated responses to incident light quality and quantity have been established. Phytochrome also plays a large role in the timing of certain responses to seasonal changes. The mechanisms involved in photoperiodic timing of events, such as flowering, have been extensively studied. Many experiments have been conducted with SD species that required a single inductive dark period to initiate flowering. As a result the nature of the photoperiodic flowering response in SD species is much better understood than that in LD species. Apart from the ma_3^R mutant of *Sorghum bicolor* which lacks a phytochrome B homologue (P.W. Morgan, pers comm.), there are no SD plant phytochrome-deficient mutants. Furthermore, there are no SD plants overexpressing transgenic phytochromes.

One method of probing the function of phytochromes in SD photoperiodism is to compare the effects of phytochrome overexpression on flowering in plants with and without SD-requirement for flowering. DN and SD *Nicotiana tabacum* cv. Hicks were chosen for this work, firstly as the DN and SD plants are genetically identical in every respect other than at the *MARYLAND MAMMOTH* locus, which confers photoperiodicity, allowing a direct comparison of responses, and secondly, *N. tabacum* is very amenable to transgenic manipulation. A property of most SD plants is that a NB given during an inductive night can delay flowering. This feature was exploited to investigate the effects of phytochrome A-, B- and C-overexpression on flowering time.

The creation of *N. tabacum* expressing phytochrome transgenes also provided the opportunity to establish the physiological attributes of each overexpression phenotype. These observations are of particular interest as a phytochrome B-overexpression phenotype has not yet been reported in tobacco. What is more, a phenotype associated with phytochrome C-overexpression has not yet been reported for any species.

Chapter 2 Analysis of *Arabidopsis thaliana* mutants reveals the action of a novel phytochrome(s), in addition to phytochrome B in the flowering response to low R/FR ratio

2.1 Introduction

Our knowledge of the phytochromes has been greatly enhanced by the analysis of mutants with deficiencies in phytochrome species (see Whitelam and Harberd, 1994). In *Arabidopsis* three classes of phytochrome mutant have been identified. The first class comprises the long-hypocotyl, *hy1* (*hy6=hy1*) and *hy2* mutants that were selected on the basis of their failure to display normal inhibition of hypocotyl elongation in response to white (W) light (Koornneef *et al.*, 1980; Chory *et al.*, 1989). These mutants, are severely deficient in spectrally detectable phytochrome (Chory *et al.*, 1989; Parks and Quail, 1989) as a result of a blockage in chromophore synthesis prior to the formation of biliverdin II (Parks and Quail, 1991). Thus, both *hy1* and *hy2* are depleted in photochemically active light-labile and light-stable phytochromes (Chory *et al.*, 1989; Parks *et al.*, 1989; Parks and Quail, 1991).

The second class of mutants have mutations in genes that are functional in phytochrome signalling pathways. The *fhy1-1* and *fhy3-1* mutants are believed to represent mutations in phytochrome A signal transduction. Both mutants have characteristics in common with phytochrome A-deficient mutants, yet they retain wild type levels of spectrophotometrically and immunochemically detectable phytochrome A (Whitelam *et al.*, 1993). However, it is also clear that *fhy1-1* does not display all the phytochrome A-mediated responses. This indicates that phytochrome A transduction has at least two branches, and that *FHY1* is involved in one of these branches (Johnson *et al.*, 1994).

The third class of phytochrome-related mutants carry lesions in the structural

genes that encode phytochrome apoproteins. The *phyA* mutants, formerly known as *hy8*, *fre1* and *fhy2*, are specifically deficient in *PHYA* transcripts and immunochemically detectable phytochrome A (Nagatani *et al.*, 1993; Parks and Quail, 1993; Whitelam *et al.*, 1993). Furthermore, the *phyA* mutations have been shown to be due to structural alterations in the *PHYA* gene (Dehesh *et al.*, 1993; Nagatani *et al.*, 1993; Whitelam *et al.*, 1993).

The *Arabidopsis phyB* (*hy3=phyB*) mutant, which displays a long hypocotyl in W light (Koornneef *et al.*, 1980), has been shown to lack immunochemically detectable phytochrome B (Nagatani *et al.*, 1991; Somers *et al.*, 1991). Further analysis of a number of alleles showed that they contain mutations within the *PHYB* structural gene (Reed *et al.*, 1993). Compared with wild type seedlings, etiolated *phyB* seedlings display an extended hypocotyl in red (R) or W light, but not in far-red (FR) light (Koornneef *et al.*, 1980). The *phyB* mutant is also defective in the greening process, producing less chlorophyll and fewer chloroplasts per mesophyll cell than wild type plants (Chory *et al.*, 1992). Light-grown *phyB* plants have very elongated stems, petioles, and leaves and display increased apical dominance (Koornneef *et al.*, 1980; Chory *et al.*, 1989; Reed *et al.*, 1993). The *phyB* mutant is deficient in end-of-day (EOD) FR light elongation responses and shows an attenuated shade-avoidance response to low R/FR ratio (Nagatani *et al.*, 1991., Whitelam and Smith, 1991; Robson *et al.*, 1993). Similar behaviour has been reported for the *lh* (*long hypocotyl*) mutant of cucumber (Whitelam and Smith, 1991; López-Juez *et al.*, 1992) and the *ein* (*elongated internode*) mutant of *Brassica rapa* (Devlin *et al.*, 1992), both of which lack immunochemically detectable phytochrome B.

The phenotype of light-grown *phyB* mutant plants is similar to the shade-avoidance phenotype displayed by wild type plants in response to low R/FR ratio conditions. This and the presence of a severely attenuated shade-avoidance response in *phyB* plants, has led to the suggestion that phytochrome B plays a significant role in this aspect of photomorphogenesis (see Smith and Whitelam, 1990; Whitelam and Smith, 1991). Shade-avoidance responses are not completely absent in the *phyB* mutant, and recently it has been shown to respond normally to low R/FR ratio with respect to leaf area and specific stem weight (Robson *et al.*, 1993).

One of the most obvious responses of *Arabidopsis* plants to low R/FR ratio conditions is a marked acceleration of flowering, displayed both in terms of the time of floral initiation and the number of rosette leaves at flowering (Whitelam and Smith, 1991). It has been noted that *phyB* causes an early flowering phenotype (Goto *et al.*, 1991; Whitelam and Smith, 1991). Nevertheless, *phyB* shows a slight acceleration of flowering in response to a reduction in R/FR ratio (Whitelam and Smith, 1991; Robson *et al.*, 1993). Since this slight, early-flowering response to low R/FR ratio is observed in a phytochrome B null mutant, the participation of another photoreceptor in the response is implied (Robson *et al.*, 1993). The promotive effect of low R/FR ratio on the flowering of *phyB* plants appears small and is relatively difficult to assess because of the already early-flowering phenotype of the *phyB* mutant phenotype.

Several late-flowering mutants of Landsberg *erecta* ecotype of *Arabidopsis* have been isolated (Koornneef *et al.*, 1991). The *fca*, *fwa* and *co* mutants were selected for late flowering under long-day (LD) photoperiods. Flowering time in the *fca* mutant is sensitive to vernalization, whereas this sensitivity is reduced in the *fwa* and *co* mutants (Martinez-Zapater and Somerville, 1990; Koornneef *et al.*, 1991; Bagnall, 1992, 1993). Flowering in these mutants is affected by R/FR ratio, being earlier in plants grown under fluorescent plus incandescent light than in plants grown under fluorescent light only (Martinez-Zapater and Somerville, 1990; Bagnall, 1992, 1993). The effect of the lower R/FR ratio is reported to be greatest in the vernalization-sensitive *fca* mutant (Martinez-Zapater and Somerville, 1990; Bagnall, 1992, 1993), and it has been reported that much of the effect of R/FR ratio on flowering time in *fca* disappears following a vernalization treatment (Bagnall, 1993).

In this chapter, the effects of very low R/FR ratio on flowering time of *Arabidopsis* plants that are homozygous for *hy2* or *phyB* phytochrome-related mutations or the *fca*, *fwa*, and *co* late-flowering mutations, have been investigated. In addition,the effects of R/FR ratio have been studied in plants that are homozygous for one or both of the phytochrome-related mutations and one of the late flowering mutations. All of the late-flowering mutations show a marked acceleration of flowering in response to low R/FR ratio conditions. Also, whereas plants doubly homozygous for *phyB* and a late-flowering mutation also show a marked response to low R/FR ratio,

plants that are triply homozygous for *hy2*, *phyB* and a late-flowering mutation are early flowering and insensitive to low R/FR ratio. These findings implicate phytochrome B and at least one other phytochrome species in the perception of R/FR ratio light signals.

2.2 Experimental procedures

2.2.1 Plant material

Arabidopsis thaliana wild type, ecotype Landsberg *erecta* was used in this work, together with the *hy2-1* (To76) and *phyB-1* (=*hy3-1*) (Bo64), phytochrome-related mutants (Koornneef *et al.*, 1980; Reed *et al.*, 1993), and the *fca*, *fwa* and *co-3* late-flowering mutants (Koornneef *et al.*, 1991).

Mutants doubly homozygous for *hy2* or *phyB* and a late-flowering mutation were initially isolated as late-flowering, long-hypocotyl plants in F_2 generations derived from crosses of monogenic *hy2* and *phyB* mutants with monogenic late-flowering mutants. The phenotype (long hypocotyl but later flowering than the monogenic phytochrome-related mutants) was rechecked in the F_3 progeny and nonsegregating (for both long hypocotyl and late-flowering = homozygous) lines were maintained by selfing. Triple mutants were obtained by crossing mutants doubly homozygous for *hy2* and a late-flowering mutation with mutants doubly homogous for *phyB* and the same late-flowering mutation. Putative triple mutants in the F_2 generation were selected on the basis of an extreme long-hypocotyl phenotype. Nonsegregating lines were crossed to each of the three monogenic mutants to demonstrate allelism. This also served as a check for the genetic composition of the parental double mutants. The *hy2phyB* double mutant had a more extreme long-hypocotyl phenotype than either the *hy2* or the *phyB* monogenic mutant and was confirmed to be a double mutant by allelism tests with both parental lines.

2.2.2 Growth conditions

Seeds were sown in Petri dishes on 1% (w/v) agar containing BG11 mineral salts (Stanier *et al.*, 1971) and chilled for 4 d at 4°C. Seeds were germinated and grown at 21 to 22°C under continuous W fluorescent light (photon fluence rate, 400-700 nm, = 100 μmol m^{-2} s^{-1}). The seedlings were selected for uniform appearance, were transplanted into 5 cm pots containing a compost:sand (3:1) mixture and were grown for a further 7 d under the same light conditions. On day 8 seedlings were transferred to R/FR ratio growth cabinets.

2.2.3 Measurement of flowering

Flowering was scored daily, and flowering time was defined as the number of days from sowing for the first petal to become visible in the expanded floral bud. Flowering time was both measured in terms of days and in terms of the number of rosette leaves at flowering time.

2.2.4 Light sources

The R/FR ratio treatment cabinets were the same as those described in detail by Keiller and Smith (1989). The high R/FR ratio cabinet (cool-W fluorescent light) provided a photon fluence rate (400-700 nm) of 100 μmol m^{-2} s^{-1} and a R/FR ratio of 6.81. The low R/FR ratio cabinet (cool-W fluorescent light, supplemented with FR) provided the same photon fluence rate (400-700 nm) but a R/FR ratio of 0.13. All light measurements were made using a LI 1800/12 spectroradiometer (Li-Cor, Lincoln, NE).

2.3 Results

2.3.1 The effect of R/FR ratio on flowering time and leaf number *hy2*, *phyB* and *hy2phyB* mutant seedlings

Flowering time was measured in days and rosette leaf number, in seedlings grown under continuous high or a low R/FR ratio light. Wild type seedlings flower at around 19 d with (approx.) 8 leaves when grown under high R/FR ratio conditions (Fig. 2.1). Under low R/FR ratio light flowering is accelerated to 14 d with a halving of the rosette leaf number to (approx.) 4 (Fig. 2.1). When grown in high R/FR ratio, the *hy2*, *phyB* and *hy2phyB* mutants all exhibit an early-flowering phenotype which resembles that displayed by the wild type plants under low R/FR ratio. The *hy2*, *phyB* and *hy2phyB* mutants flower at around 13 d, with (approx.) 3, 4 and 2 rosette leaf means respectively (Fig. 2.1). When grown under low R/FR ratio light there is not a significant acceleration in flowering time or leaf number in *hy2*, *phyB* or *hy2phyB* (Fig. 2.1). However, it is noteworthy that the *hy2phyB* double mutant flowers earlier than either the *hy2* or the *phyB* monogenic mutant, in terms of rosette leaf number produced (Fig. 2.1(b)). The additive effect in the *hy2phyB* mutants implies, the *hy2* mutant must have an amount of active phytochrome B. The earlier flowering of seedlings carrying both the *hy2* and the *phyB* mutations, in comparison to seedlings carrying only the *phyB* mutation also implies another phytochrome species other than phytochrome B is operational in this response to low R/FR ratio.

2.3.2 The effect of R/FR ratio on flowering time and leaf number in seedlings homozygous for the *fca*, *fwa* or *co-3* late-flowering mutation

The late-flowering mutants *fca*, *fwa* and *co-3*, flower significantly later than wild type plants when grown under both high and low R/FR ratio light, both in terms of days to flower and rosette leaf number (Figs. 2.1-2.4). Indeed, under both low and high R/FR ratio, each of the late-flowering seedlings produce more than twice the

number of leaves of wild type seedlings grown under the same conditions (Figs. 2.1-2.4(b)). However, the accelerated flowering response displayed by wild type seedlings when grown under low R/FR ratio light (Fig. 2.1), is also seen in seedlings with a late-flowering mutation (Figs. 2.2-2.4). The *fca*, *fwa* and *co-3* mutant seedlings flower at (approx.) 34, 29 and 32 d, respectively, under high R/FR ratio as opposed to (approx.) 19, 20 and 22 d under low R/FR ratio (Figs. 2.2-2.4(a)). Likewise, rosette leaf number of *fca*, *fwa* and *co-3* seedlings grown under high R/FR ratio is (approx.) 17, 16 and 19 leaves, respectively, whilst under low R/FR ratio leaf number is significantly less at (approx.) 11, 9 and 11 (Figs. 2.2-2.4(b)). These observations show that all three late-flowering mutations have the ability to respond to reduced R/FR ratio with an acceleration of flowering time.

2.3.3 The effect of R/FR ratio on flowering time and leaf number in seedlings homozygous for *hy2*, *phyB* and *hy2phyB* and a late-flowering mutation

Seedlings grown under high R/FR ratio, that are doubly homozygous for *hy2* or *phyB* and one of the late-flowering mutations flower at a time that is intermediate, between wild type plants and the late-flowering mutants (Figs. 2.1-2.4(a)). In the same growth conditions, rosette leaf number in the mutants seedlings appears to be about the same (6-8 leaves) as that in wild type seedlings (Figs. 2.1-2.4(b)). However, under conditions of reduced R/FR ratio, both the *hy2*/ and the *phyB*/late-flowering mutants display a clear early flowering phenotype, which is significant both for days to flower and rosette leaf number (Figs. 2.2-2.4).

Under high R/FR ratio, seedlings that are triply homozygous for the *hy2phyB* mutations and one of the late-flowering mutations flower earlier than either of the digenic mutants, homozygous for the *hy2* or *phyB* and a late-flowering mutation (Figs. 2.2-2.4). In addition, when these triple mutants are grown under low R/FR ratio conditions, either a very small early-flowering response, or no response is observed (Figs. 2.2-2.4). A small acceleration in flowering is displayed by the *fca* mutants that

also carry the *hy2* and *phyB* mutations (Fig. 2.2). However, this response is smaller than that displayed by either *fcahy2* or *fcaphyB*. In the *fwahy2phyB* and the *co-3hy2phyB* seedlings, a reduction in R/FR ratio does not stimulate a further reduction in flowering time (Figs. 2.3, 2.4). Deficiency of an early-flowering response to low R/FR in these late-flowering/*hy2phyB* mutants, which probably have negligable levels of phytochrome, confirm that this response is phytochrome-mediated.

These experiments also demonstrate the presence of a marked early-flowering response to low R/FR ratio in seedlings homozygous for either the *hy2* or the *phyB* mutations. All seedlings that are homozygous for a late-flowering mutation and either the *hy2*, or the *phyB* mutations flower earlier in low R/FR ratio than high R/FR ratio light (Figs 2.2, 2.3 and 2.4). This low R/FR ratio-stimulated, early-flowering-response is striking in the *fwaphyB* seedlings, as well as in the wild type and *fwa* seedlings shown in Fig. 2.3(c).The examination of these phytochrome mutants in late-flowering backgrounds has, therefore, revealed pronounced early-flowering responses to low R/FR ratio. The extent of this response would be extremely difficult to gauge from analysis of either monogenic mutant. These observations suggest that firstly, *hy2* contains some functional phytochrome B, and secondly, another phytochrome in addition to phytochrome B can accelerate flowering in response to low R/FR ratio.

2.4 Discussion

The induction of flowering in *Arabidopsis* leads to the transition from vegetative to reproductive development within the apical meristem. Since this transition leads to a cessation of rosette leaf initiation, flowering time and rosette leaf number are correlated. The Landsberg *erecta* ecotype of *Arabidopsis*, in keeping with many other LD plants, can be induced to flower earlier under low R/FR ratio light conditions. The flowering response to low R/FR ratio is manifest both in terms of flowering time and leaf number, indicating that the early flowering results from a reduction in the period of vegetative development. The phytochrome-related *hy2* and *phyB* mutants flower very early, both in terms of flowering time and leaf number when grown under high R/FR ratio

conditions. In fact, these mutants grown under high R/FR ratio are phenotypically similar to wild type seedlings grown under reduced R/FR ratio light. However, unlike wild type seedlings, the *hy2* and *phyB* seedlings are not very responsive to low R/FR ratio light, which suggests that phytochrome B acts in this apsect of the shade-avoidance response. Seedlings that are doubly homozygous for the *hy2* and *phyB* mutations also show little response to low R/FR ratio, but it is noteworthy that the double mutant flowers slightly earlier, with fewer leaves than either of the monogenic mutants. This apparent additive effect of the *hy2* and *phyB* mutations implies that seedlings carrying the *hy2* mutation alone must possess some functional phytochrome B. Previous physiological observations had suggested that *hy2* retains some active phytochrome (Cone, 1985; Whitelam and Smith, 1991). The earlier flowering of seedlings carrying both the *hy2* and *phyB* mutation, compared with seedlings carrying only the *phyB* mutation, also indicates that in the absence of phytochrome B the reduction in activity of another phytochrome, as a consequence of the *hy2*-related chromophore deficiency, also leads to early flowering.

The late-flowering monogenic *Arabidopsis* mutants *fca*, *fwa* and *co-3* have a delayed flowering response in LD growth conditions (Koornneef *et al.*, 1991). This delayed flowering response is also seen when seedlings carrying these mutations are grown in continuous W light. All three of the late-flowering mutations show a marked acceleration in flowering when grown in low R/FR ratio conditions, which in terms of both flowering time and leaf number is of more or less the same magnitude as that seen in wild type seedlings. Thus, the late-flowering phenotype of these mutants is not abolished by growth under low R/FR ratio light. Nevertheless, under low R/FR ratio conditions seedlings carrying the *fca*, *fwa* or *co-3* mutations still flower later than wild type seedlings under the same conditions. It has been reported that the flowering of the *co-3* mutant is unaffected by R/FR ratio and that only the *fca* mutant shows a significant decrease in flowering time in response to low R/FR ratio (Bagnall, 1993). This conclusion is based on a comparison of flowering times for seedlings grown under fluorescent W light (R/FR ratio approx. 5.0) or under fluorescent W light with supplementary incandescent light (R/FR ratio approx. 1.0). In this study, the low R/FR ratio light source was composed of fluorescent W light with supplementary FR and

produced a R/FR ratio of only 0.13. Under these conditions it was not possible to detect a gradient of responsivity in the various late-flowering mutants. Several other late-flowering mutants of Landsberg *erecta*, including *gi-3*, *fha*, *fe*, *fve*, *fd*, and *ft* (Koornneef *et al.*, 1991) also show an equally pronounced flowering response to low R/FR (K.J. Halliday; G.C. Whitelam, unpublished data).

The observed responses of these late-flowering mutants to extremely low R/FR ratio suggests that this particular phytochrome effect acts to a large extent independently of the factors that control responsiveness to vernalization and daylength, which are differentially affected in these late-flowering mutants. Since mutations in many genes can result in late-flowering without abolishing flowering completely, it has been suggested that multiple parallel pathways control flowering (Koornneef *et al.*, 1991).

Seedlings that are doubly homozygous for *hy2*, or *phyB* and one of the late-flowering mutations, flower early in high R/FR ratio conditions, compared with seedlings carrying the late-flowering mutation alone. Growth of these double mutants under low R/FR ratio light leads to a significant acceleration in flowering and a reduction in leaf number. Thus, it seems that combining the phytochrome-related *hy2* or *phyB* mutations with a late-flowering mutation has, in effect, unveiled a significant shade-avoidance response. One may assume that in the wild type genetic background flowering is so early in phytochrome B-deficient mutants that a reduction in the level of the active form of other (possibly light-stable) phytochromes by lowering R/FR ratio has little or no physiological effect. The *hy2* mutation would be expected to have a more pronounced effect on the active form of other phytochromes, thereby enabling only a slight physiological effect to be observed in the absence of phytochrome B. In a late-flowering genetic background, flowering is not saturated for earliness in the absence of phytochrome B, thus allowing the effects of low R/FR ratio to be observed.

Seedlings that are triply homozygous for *hy2*, *phyB*, and one of the late-flowering mutations and grown under high R/FR ratio conditions, flower earlier than double mutants carrying only one phytochrome-related mutation. Furthermore, when these triple mutants are grown under low R/FR ratio conditions, either a very reduced early-flowering response or no response is observed. The loss of this shade-avoidance response in triple mutants that lack phytochrome B and that are phytochrome

chromophore-depleted indicates that phytochromes are the only mediators of the flowering reponse to low R/FR ratio light. Thus, the observed flowering responses to low R/FR ratio in the phytochrome B-deficient mutants can be attributed to the action of another, or other phytochrome species and not to the action of some other R- and FR-absorbing photoreceptor.

The observations reported here implicate phytochrome B as a mediator of the early-flowering component of the shade-avoidance syndrome in *Arabidopsis* and also provide strong evidence for the involvement of at least one other phytochrome species in this response. Other authors have reported the failure of phytochrome B-deficient mutants to display elongation growth responses to EOD FR light treatments, but have noted attenuated elongation growth responses of these mutants to R/FR ratio conditions (López-Juez *et al.*, 1990; Whitelam and Smith, 1991; Devlin *et al.*, 1992). It has also been shown that phytochrome B-deficient *Arabidopsis* show unmodified retention of some growth responses to low R/FR ratio, namely the reduction in leaf area and specific stem weight (Robson *et al.*, 1993). Thus, although the presence of phytochrome B appears to be correlated with the ability of seedlings to respond to low R/FR ratio by elongation, it is apparently unnecessary for growth responses to low R/FR ratio that involve radial expansion. Shade-avoiding plants that are exposed to vegetation shade channel their resources into elongation growth at the expense of radial or lateral expansion. Thus, the responses to low R/FR ratio that are retained in *phyB* mutants are considered to be related to the shade-avoidance syndrome (Robson *et al.*, 1993).

The phytochrome-mediated acceleration of flowering, partly attributable to phytochrome B but also displayed in the absence of phytochrome B, can be considered to represent an important component of the shade-avoidance syndrome. In fact, early flowering could be considered to represent the ultimate manifestation of shade avoidance. It is now evident that this and other aspects of the shade-avoidance syndrome are mediated by multiple phytochrome species, only one of which is phytochrome B.

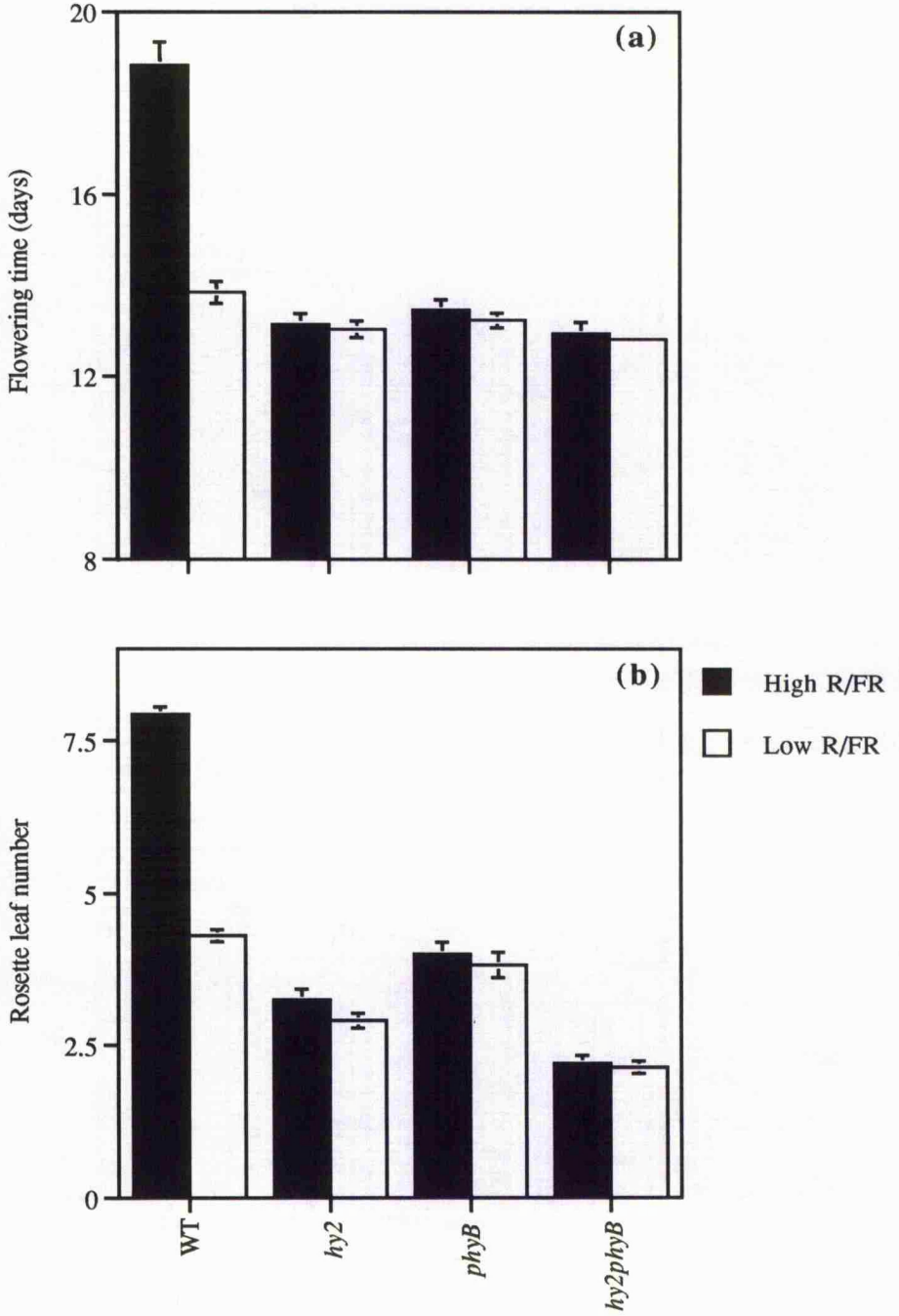


Figure 2.1 Flowering time measured in days (**a**) and number of basal rosette leaves (**b**) of Laer WT, *hy2*, *phyB* and *hy2phyB* seedlings grown in high R/FR ratio (closed bars) and low R/FR ratio (open bars) continuous light. Data represent the means from 20 plants and the error bars are SE.

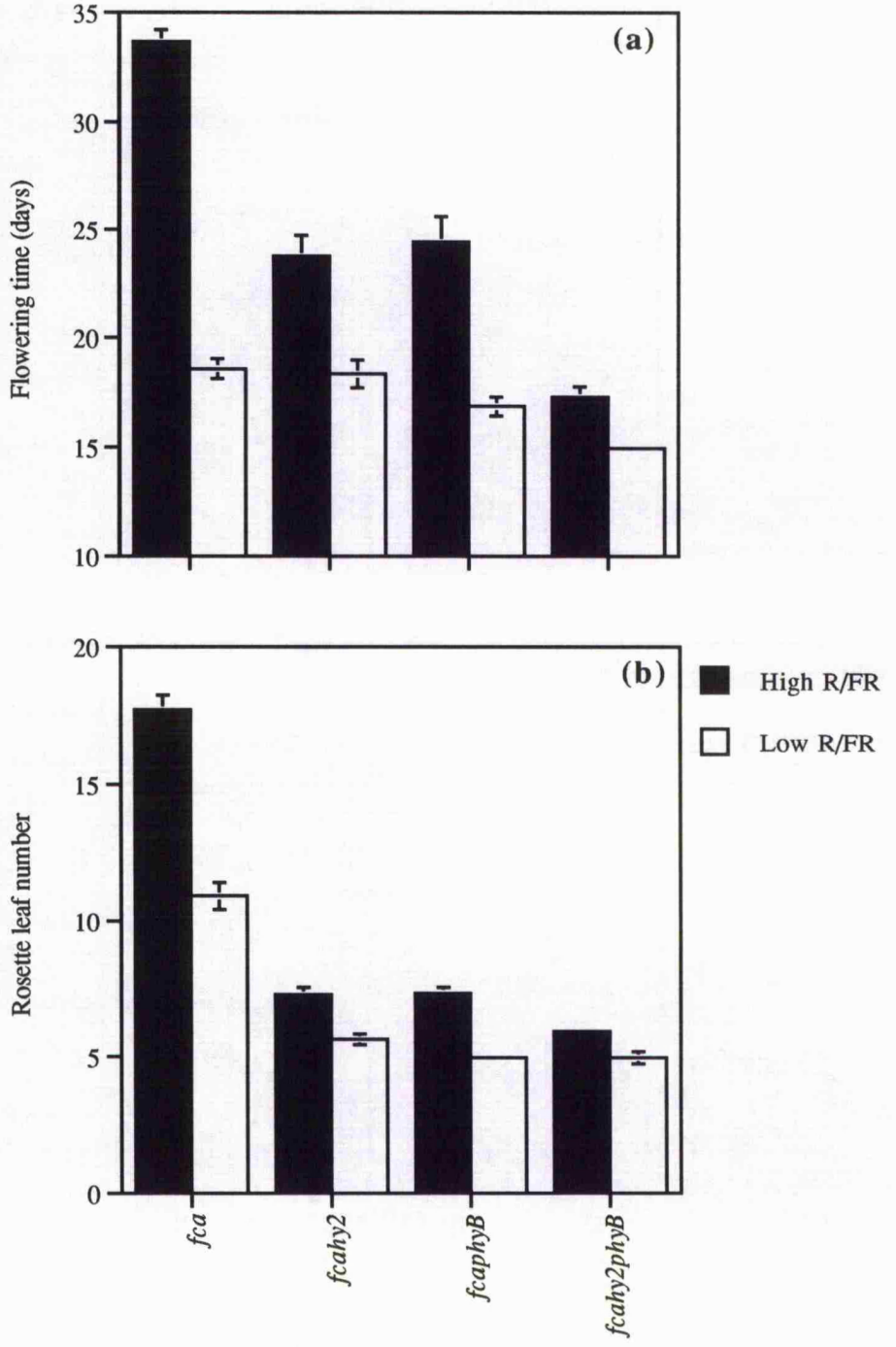


Figure 2.2 Flowering time measured in days (**a**) and number of basal rosette leaves (**b**) of *fca*, *fcahy2*, *fcaphyB* and *fcahy2phyB* seedlings grown in high R/FR ratio (closed bars) and low R/FR ratio (open bars) continuous light. Data represent the means from 20 plants and the error bars are SE.

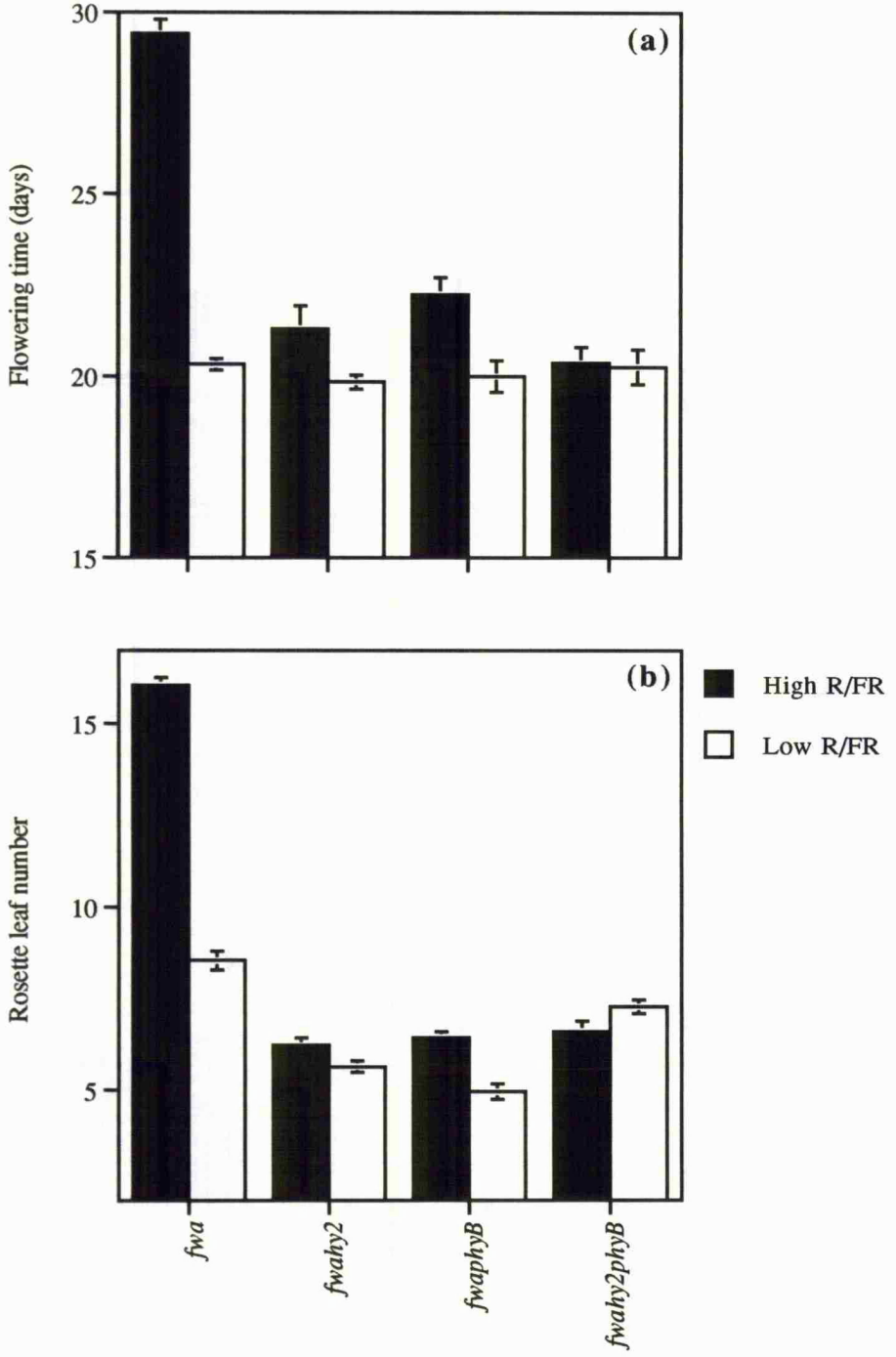
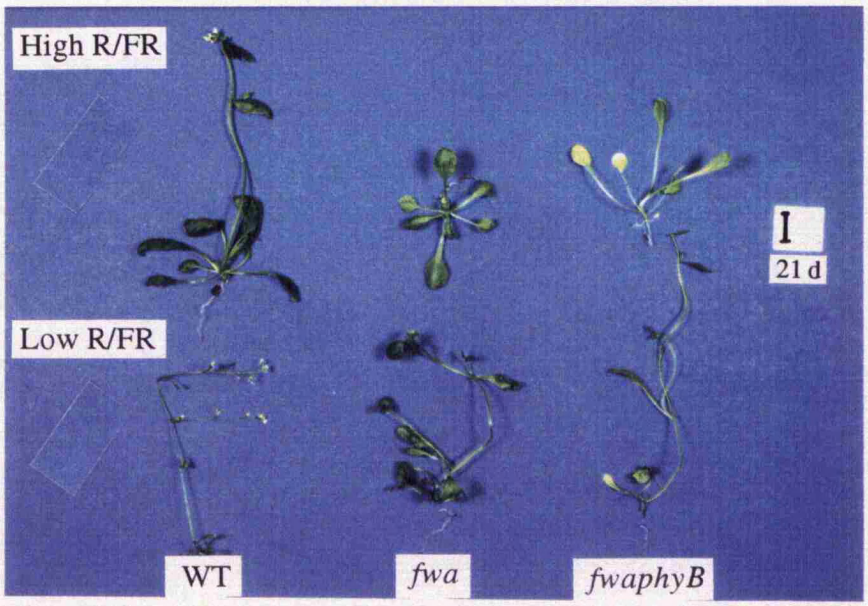


Figure 2.3 Flowering time measured in days (**a**) and number of basal rosette leaves (**b**) of *fwa*, *fwahy2*, *fwaphyB* and *fwahy2phyB* seedlings, and phenotype of WT, *fwa*, and *fwaphyB* seedlings (**c**) grown in high R/FR ratio (closed bars) and low R/FR ratio (open bars) continuous light. Data represent the means from 20 plants and the error bars are SE.

(c)



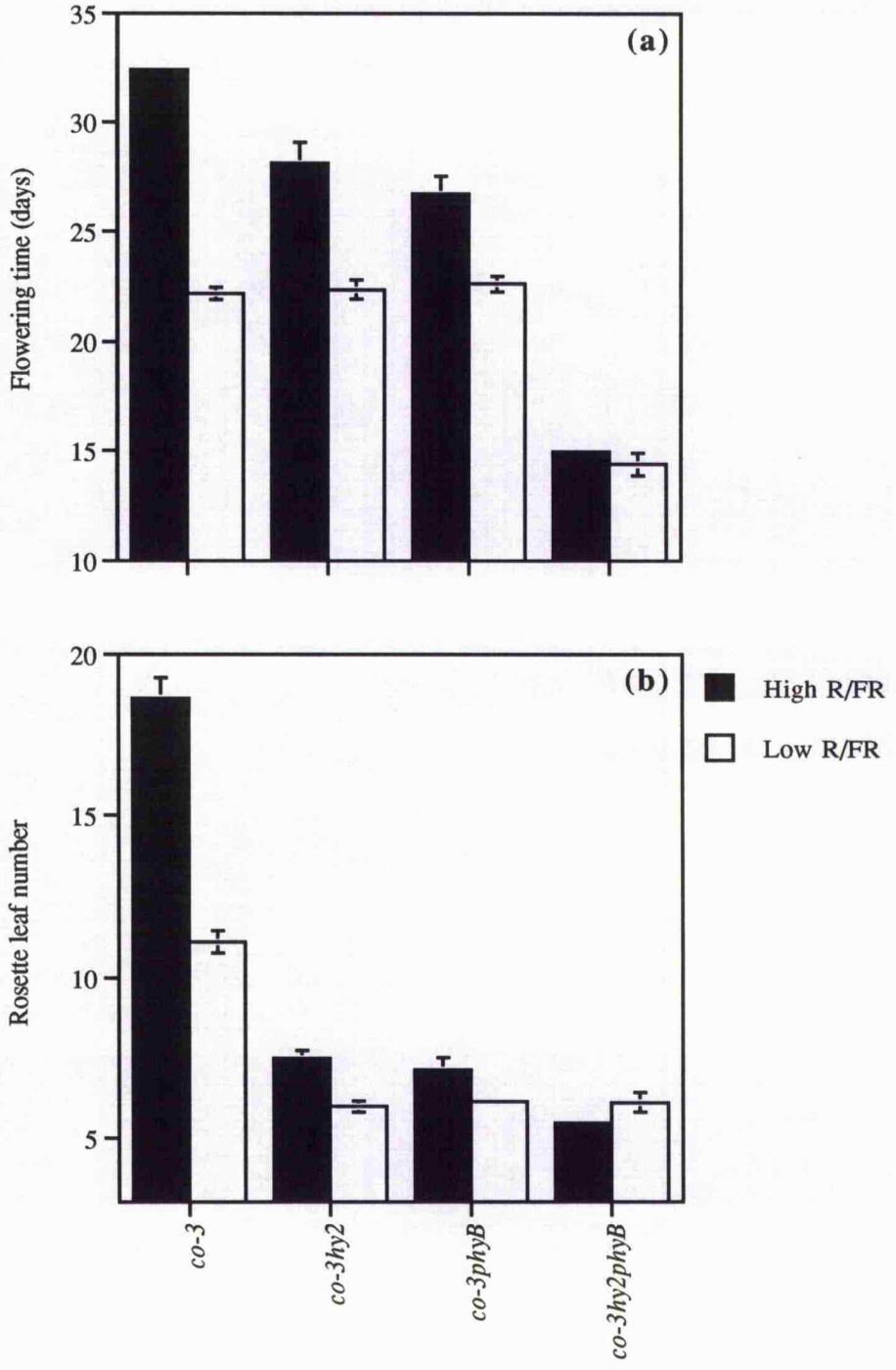


Figure 2.4 Flowering time measured in days (**a**) and number of basal rosette leaves (**b**) of *co-3*, *co-3hy2*, *co-3phyB* and *co-3hy2phyB* seedlings grown in high R/FR ratio (closed bars) and low R/FR ratio (open bars) continuous light. Data represent the means from 20 plants and the error bars are SE.

Chapter 3 Physiological analysis of the *ELONGATED* mutant and the H4S transgenic mutant of *Arabidopsis thaliana*

3.1 Introduction

The influential role of phytochrome in adjusting the vegetative morphology and the timing of flowering in response to changing light quality in the plant is well documented (see Smith and Whitelam, 1990). This adjustment to changes in light quality can be readily observed when shade-avoiding plants are grown in canopy shade conditions (low R/FR ratio) where they respond by growing in an elongated fashion and display increased apical dominance, the so-called "shade-avoidance response".

The phytochrome B-deficient *phyB* mutant of *Arabidopsis*, the *lh* mutant of cucumber and the *ein* mutant of *Brassica rapa* all display a phenotype that is very similar to the shade-avoidance phenotype of wild type plants grown in low R/FR ratio light conditions. Light-grown *phyB*, *lh* and *ein* have increased stem and petiole elongation, and *phyB* and *lh* are early flowering (Adamse *et al.*, 1987; Goto *et al.*, 1991; Devlin *et al.*, 1992; Chory *et al.*, 1992). In addition, unlike their wild type plants, the *phyB*, *lh* and *ein* mutants do not respond significantly to EOD FR light treatments (López-Juez *et al.*, 1990; Nagatani *et al.*, 1991; Devlin *et al.*, 1992) and all three mutants respond poorly to low R/FR ratio light treatments (Whitelam and Smith, 1991; Devlin *et al.*, 1992). One of the most clear examples of this is the marked decrease in the flowering response to low R/FR ratio light seen in the *Arabidopsis phyB* mutant when compared to its isogenic wild type (Whitelam and Smith, 1991). This phenotypic mimicry, by phytochrome B-deficient mutants, of the wild type growth habit in shade conditions and their lack of response to low R/FR ratio light conditions and EOD FR light treatments has led to the speculation that phytochrome B is responsible for the shade-avoidance response. However, there is evidence to indicate that shade-avoidance responses are not completely absent in the *Arabidopsis phyB* mutant with respect to leaf area and specific

stem weight (Robson *et al.*, 1993). Also, the retention of an accelerated flowering response in low R/FR light conditions in the *phyB* mutant, which is clearly observed in seedlings homozygous for both the *phyB* mutation and a late-flowering mutation (*co-3*, *fca*, *fwa* or *gi3*), infers that although there may be a role for phytochrome B in this response this role is not exclusive (Halliday *et al.*, 1994 and Chapter 2). Thus, a novel phytochrome(s) is also implicated in the accelerated flowering response in low R/FR light conditions. There are several possible means by which this novel phytochrome(s) can be identified and two complementary approaches can be taken. (1) The identification of the mutant from a screen designed to select for the mutant phenotype. Such a screen could involve the mutagenesis of *phyAphyB*/late-flowering seed and selection of early flowering phenotype that is unresponsive to low R/FR ratio. (2) The identification of the mutant by the analysis of mutants selected from other screens, where the mutation does not map to the phytochrome B locus, with characteristics in common with a predicted phenotype. Such a mutant would be expected to have a light-conditional, constitutive shade avoidance phenotype and would therefore be spindly in appearance, have an early flowering nature and show a reduction in the accelerated flowering response to low R/FR ratio. Mutants with such a phenotype are likely to represent a range of mutations. These include: mutations in a novel phytochrome gene; mutations in the signal transduction pathway of a novel phytochrome; or indeed, mutations entirely unconnected to phytochrome function.

The *elg Arabidopsis* mutant and the transgenic H4S *Arabidopsis* mutant are plants that display the necessary phenotypic criteria that could be linked to defective function or signal transduction of a novel phytochrome that operates in the *Arabidopsis* shade-avoidance response. Both mutants have a somewhat elongated growth habit and flower early when compared with wild type plants.

The *elg* (*elongated*) mutant has recently been isolated from EMS-mutagenised populations of the *Arabidopsis ga4* (*gibberellic acid* sensitive) mutant (Koornneef and van der Veen, 1980). The *ga4* mutant is a gibberellic acid- (GA-) deficient mutant that is blocked in the 3 ß-hydroxylation of GAs. The total pool of active GAs is, therefore, reduced due to the absence of 3 ß-hydroxyl GAs (Talon *et al.*, 1990). The *ga4* mutant is phenotypically dwarfed, has reduced apical dominance and is darker green than the wild

type. An EMS mutagenesis of *ga4* was originally performed to identify mutants that would restore the wild type phenotype. The screen was, therefore designed to select for mutants in GA biosynthesis or its transduction chain. The *elg* mutant does not have a wild type phenotype, but it is elongated and early flowering, and therefore has many features in common with both phytochrome mutants and constitutive GA response mutants. The *la cry*^{s} mutant of pea, the *procera* mutant of tomato, the *sln* mutant of barley and the *spy* mutant of *Arabidopsis* have been classified as GA response mutants (Reid and Ross, 1993; Jones, 1987; Lanahan and Ho, 1988; Jacobsen and Olzewski, 1993). These mutants display a phenotype that resembles that of a wild type plant that has been treated with GA. Thus, when compared to their isogenic wild types these mutants are much paler, have an elongated morphology, increased apical dominance and early flowering.

The *elg* mutation could therefore be disruptive of phytochrome and/or GA function, and analysis was carried out with a view to examining both these parameters. This work was carried out in collaboration with Corrie Hanhart and Maarten Koornneef (Wageningen Agricultural University, The Netherlands).

The transgenic H4S (*HAT4* sense) *Arabidopsis* mutant overexpresses the *HAT4* transgene.The H4S mutant, provided by Schena *et al.*, (Stanford University Medical Center, California, USA) has been transformed with an expression vector comprising the constitutive cauliflower mosaic virus 35S promotor fused to the *HAT4* cDNA in the sense orientation. The H4S plants have a 10-fold elevation of *HAT4* transcript levels (Schena *et al.*, 1993).

The *HAT4* gene encodes an *Arabidopsis* homeodomain (HD) protein. Several lines of evidence suggest that homeodomain proteins are transcription factors that are responsible for sequence-specific recognition of DNA (Laughton, 1991). Homeodomain proteins isolated in animal systems have been found to control several developmental pathways acting as molecular switches which control the fate of the cells during development (Wright, 1991; McGinnis and Krumlauf, 1992). HAT4 is also a member of a subfamily of HD proteins that contain a novel 90 amino acid segment, a homeo domain-leucine zipper (HD-Zip) motif which has not been identified in animal systems. The uniqueness of the HD-Zip to higher plants is suggestive of the fact that it

may mediate pathways that couple development to the environment (Carabelli *et al.*, 1993).

Evidence of the light dependent regulation of the *HAT4* gene comes from the analysis of *Athb-2* mRNA levels in *Arabidopsis* plants, as *Athb-2* and *HAT4* represent the same gene, (Carabelli *et al.*, 1993). Further work with *Arabidopsis* phytochrome mutants links the regulation of the gene more implicitly with the action of phytochrome and significantly with the action of a novel phytochrome operational in shade-avoidance responses (Carabelli *et al.*, 1996). A low R/FR- or EOD FR-stimulated increase in *HAT4* transcript levels is detectable not only in wild type plants and in the *phyA* mutant, but also in the *phyB* mutant and the *phyA phyB* double mutant (Carabelli *et al.*, 1996). However, the steady state levels of *HAT4* mRNA in *phyB* and *phyAphyB* seedlings are slightly higher than those in wild type seedlings. The nature of the *HAT4* gene response to low R/FR and EOD FR in the *phyB* mutants is reminiscent of the flowering response in *phyB* mutants where the plants flower early but retain the ability to respond to these light treatments. This evidence strongly suggests that *HAT4* gene transcription may be under the control of a novel phytochrome operating in these aspects of the shade-avoidance response. If this supposition is true, the novel phytochrome would be influential in the down-regulation of the *HAT4* gene product, therefore, one would expect that the overexpression of HAT4 would phenocopy deficiency of the novel phytochrome.

Physiological analysis was carried out in order that characteristics may be identified that define the mutant phenotype of a plant lacking in this novel phytochrome.

3.2 Experimental Procedures

3.2.1 *Elg* mutant isolation, genetic characterisation and the construction of double mutants

(carried out by Corrie Hanhart and Maarten Koornneef, Department of Genetics, Wageningen Agricultural University, The Netherlands)

Seeds of the *Arabidopsis ga4-1* mutant (Koornneef and van der Veen, 1980) were treated with 10 mM EMS as described elsewhere (Koornneef *et al.*, 1982). Genetic mapping was performed as described by Koornneef and Stam, 1992. The program JOINMAP (Stam, 1993) was used to calculate the map position of *elg*, using the *elg* recombination data and data for markers *bp*, *cer2* and *ap2* that were used previously to construct the linkage map for chromosome 4 (Koornneef, 1994). Mutants digenic for *elg* and *hy1-1*, *hy2-1* and *phyB* (=*hy3-1*) were identified as plants with an extremely long hypocotyl phenotype in the F2 and F3 progenies, derived from the respective mutant crosses.

The *ga1-2elg* double mutant was isolated from F3 lines homozygous for *elg* and segregating for gibberellin-responsive dwarfs, which is a defining characteristic of the *ga1* mutant phenotype (Koornneef and van der Veen, 1980).

Physiological experiments were performed with a homozygous *elg* genotype obtained from the original mutant backcrossed three times with Landsberg *erecta* wild type (Laer WT).

3.2.2 Source and construction of H4S

H4S seeds were obtained from Mark Schena (Stanford University Medical Center, Stanford, California, USA). H4S plants, homozygous for the *HAT 4* transgene were created by the transformation of *Arabidopsis* Columbia wild type plants with an expression vector containing the CaMV 35S promoter linked to the *HAT 4* cDNA in the sense orientation (Schena *et al.*, 1993). The expression plasmid p35S-*HAT 4* was introduced into *Agrobacterium tumifaciens* strain LBA4404 (Clontech) by electroporation and plant transformation was acheived by *Agrobacterium*-root incubation (Schena *et al.*, 1993).

3.2.3 Growth conditions

For measurements of hypocotyl length in the dark and in R, FR, B and W light, Landsberg *erecta* WT and *elg* seeds were sown in 10 x 1 x 1.5 cm plexiglass troughs filled with finely sieved 3:1 compost:horticultural silver sand mix and cold treated for 5 d at 4°C in darkness. For experiments with H4S, seeds were sown in 9 cm Petri dishes on 0.8% (w/v) agar containing BG11 mineral salts (Stanier *et al.*, 1971) and chilled at 4°C for 5 d. The seeds were stimulated to germinate by a 15 min W treatment on day 5 and grown for a further day in darkness. The seedlings were then either kept in the dark or transferred to continuous R, FR, B or W.

For measurements of hypocotyl length in WT, *elg*, *ga1* and *elgga1* the seeds were sown in 9 cm Petri dishes on 0.8% (w/v) agar containing MS mineral salts (Murashige and Skoog, 1962) plus 10^{-4} M gibberellic acid to stimulate germination in *ga1* and *elgga1*. After chilling for 5 d the seeds were exposed to 15 min W, kept in darkness for 1 d and then transferred to MS media plates where they were grown for a further 4 d.

In experiments with mature plants, seeds were sown in 9 cm Petri dishes on 0.8% (w/v) agar containing MS (for experiments with *elg*) or BG11 (for experiments with H4S) mineral salts, and chilled for 5 d at 4°C. Seeds were germinated and grown in continuous W (photon fluence rate 80 μmol m^{-2} s^{-1} in the range of 400-700 nm). Seedlings were then selected for uniformitiy and transferred to 5 cm pots containing a peat compost:horticultural silver sand (3:1) mixture and grown for a further 7 d in the same light conditions. On day 8 the seedlings were transferred to the appropriate experimental light treatments. For all experiments temperature was maintained between 22°C and 24°C.

3.2.4 Measurement of hypocotyl length, petiole length and flowering time

All hypocotyl lengths were determined from calibrated projected photographic slides. Petiole lengths were measured with a ruler. Flowering time was measured and/or as the number of leaves (over 5 mm in length) in the basal rosette present at first flower bud formation. Sample sizes for all experiments were between 20 and 30 plants.

3.2.5 Light sources

The monochromatic R light was obtained from light emitting diodes (LEDs) supplied by Hansatech (Norwich, UK). The R LEDs have an emission maximum at 665 nm and provide a fluence rate of 12 μmol m^{-2} s^{-1} at the seedling level. The FR monochromatic light was obtained from FR-emitting LEDs (Quantum Devices, Inc. Barneveld, WI. USA) with an emission maximum of 730 nm and a fluence rate of 25 μmol m^{-2} s^{-1}. Blue light (photon fluence rate 2 μmol m^{-2} s^{-1}) was provided by the output of a bank of Philips TLAK 40W fluorescent tubes filtered through one layer of primary blue cinemoid. Fluence rates were measured at seedling level with an Analytical Spectral Devices personal spectrometer, model number PS2 000A.

The R/FR ratio light (Zeta) cabinets used were the same as those described by Keiller and Smith (1989). In experiments with *elg* the high R/FR ratio cabinet provided a photon fluence rate (400-700 nm) of 80 μmol m^{-2} s^{-1} and a R/FR ratio of 5.63. The low R/FR cabinet provided a similar photon fluence rate (400-700 nm) of 87 μmol m^{-2} s^{-1} and a R/FR ratio of 0.23 . In experiments with H4S the high R/FR ratio cabinet provided a photon fluence rate (400-700nm) of 94 μmol m^{-2} s^{-1} and a R/FR ratio of 5.39. The low R/FR cabinet provided the photon fluence rate (400-700 nm) of 89 μmol m^{-2} s^{-1} and a R/FR ratio of 0.27.

The short-day (SD), SD extension (EXT), long-day (LD), 10 h photoperiod and EOD FR growth conditions were provided by Fitotron 600 growth cabinets (Fisons Scientific Apparatus, Loughborough, UK). The SD light conditions comprised 8 h of cool-W fluorescent light (photon fluence rate 400-700 nm 144 μmol m^{-2} s^{-1}) followed by 16 h of darkness. The SD EXT conditions comprised 8 h of cool-W fluorescent light (photon fluence rate 400-700 nm 142 μmol m^{-2} s^{-1}) followed by 8 h of incandescent light (photon fluence rate 400-700 nm 2 μmol m^{-2} s^{-1}), and then 8 h of dark. For experiments using a combination of SDs and SDs+EOD FR treatments, the SD cabinet described above were utilised. Seedlings receiving EOD FR were given a 15 min FR light treatment (under the FR LEDs described above), at the end of the 10 h light period.

3.3 Results

3.3.1 *elg* mutant isolation and genetic characterisation

(Work carried out by Corrie Hanhart and Maarten Koornneef, Department of Genetics, Wageningen Agricultural University, The Netherlands)

A mutant with a slightly longer hypocotyl and total plant length compared to the parental genotype was selected from a M2 progeny obtained by treating the *ga4-1* mutant with 10 mM ethylmethanesulphonate (EMS) for 24 h at 24°C. Backcrossing of this mutant with Laer wild type followed by selfing resulted in the identification of the mutant phenotype in the wild type background, which was characterised by a slightly elongated hypocotyl, increased plant height and, compared to the wild type, leaves with longer petioles that were more narrow and somewhat epinastic. This pleiotropic phenotype segregated in a monogenic fashion. Based on F2 segregation data and on measurements on homozygous genotypes (Table 3.1) it was concluded that the mutant phenotype is partially dominant. Allelism tests with the *hy1-hy5* mutants indicated that

this mutant was not allelic with any of these long hypocotyl mutants. The gene symbol *elg* (*elongated*) was proposed for this new locus.

From analysis of an F2 population derived from the cross of the *elg* mutant with the chromosome 4 linkage tester line W8 (homozygous for *bp*, *cer2* and *ap2*) the location of *elg* on this chromosome could be established. When testing the progeny of 30 *elg* F2 homozygotes close linkage of *elg* to the markers *cer2* and *ap2* on chromosome 4 was observed (Fig. 3.1). The finding that the recombinants between *elg* and *ap2* and between *elg* and *cer2* also had a cross-over between *cer2* and *ap2* confirmed the location of *elg* between these two loci.

3.3.2 Photocontrol of hypocotyl elongation in *elg* seedlings

Seedlings were grown for 5 d in darkness or under continuous R, FR, B or W light, after which hypocotyl lengths were determined. For seedlings grown in the dark, the hypocotyls of the *elg* mutant were indistinguishable from those of wild type seedlings (Fig. 3.2). However, for seedlings grown under R, FR, B or W the hypocotyls of the *elg* mutant were significantly longer than those of the wild type (Fig. 3.2). The most marked difference in hypocotyl length was seen in R-treated seedlings. Thus, *elg* seedlings appear to display a light-conditional long hypocotyl phenotype.

3.3.3 Photocontrol of petiole length in *elg* seedlings

The length of petioles, in the second leaf pair, was measured for plants grown under 10h light/14 h dark photoperiods and for plants grown under the same conditions but in which a 15 min FR treatment immediately preceded the transition to darkness each day. For both wild type and *elg* seedlings, this EOD FR treatment led to a significant increase in petiole length compared with seedlings that did not receive the FR treatment (Fig. 3.3(b)). Furthermore, although *elg* seedlings possess significantly longer petioles than wild type seedlings under both light regimes, they respond to FR in

a manner that is qualitatively similar to wild type seedlings (Fig. 3.3(b)). The long-petiole phenotype is also displayed by *elg* seedlings when grown under continuous light (Fig 3.3(a)). Thus, the accelerated growth habit of *elg* is independent of these changes in the light environment.

3.3.4 Photocontrol of flowering time in *elg* seedlings

Seedlings of the *elg* mutant flower significantly earlier than wild type seedlings under a range of experimental light regimes. Flowering time was recorded as rosette leaf number at first bud formation in plants grown under SD (8 h light, 16 h dark) and SD with an incandescent day extension (8 h light, 8 h incandescent light, 8 h dark) photoperiods, and for seedlings grown under continuous light conditions that differ in R/FR ratio. For seedlings grown in light/dark cycles, *elg* mutant seedlings flower earlier than wild type seedlings in both SDs and SDs with an incandescent day extension (Fig. 3.4). Despite this early flowering behaviour, *elg* mutants still display a marked response to photoperiod in which flowering is delayed under SDs.

When grown in continuous light of high or low R/FR, *elg* seedlings flower earlier than wild type seedlings irrespective of light quality (Fig. 3.5). However, *elg* seedlings do respond to a reduction in R/FR ratio, in a similar fashion to wild type seedlings, with a marked acceleration of flowering (Fig. 3.5).

3.3.5 The interaction of *elg* and the *hy1*, *hy2* and *phyB* mutations

Arabidopsis mutants digenic for *elg* and *hy1*, *elg* and *hy2*, or *elg* and *phyB* mutations were created in order to investigate the relationship between *ELG* and phytochrome regulated developmental changes. The *elghy1*, *elghy2* and *elgphyB* all have longer hypocotyls than their respective monogenic mutants (Fig. 3.6). These data indicate that the *elg* mutation acts additively with the different phytochrome-related mutations to increase hypocotyl length. This additivity suggests that the *elg* mutation

defines a developmental pathway that is independent of phytochrome B and is either influential in another phytochrome pathway, (as *hy1* and *hy2* are leaky), or is entirely separate from the phytochrome-regulated pathways.

3.3.6 Interaction of *elg* and the *ga1* and *ga4* mutations

(Part of this work was carried out by Corrie Hanhart and Maarten Koornneef, Department of Genetics, Wageningen Agricultural University, The Netherlands)

In order to establish if the *ELG* gene encodes a component that is functional in either GA biosynthesis or GA signal transduction, mutants digenic for *elg* and the GA-deficient *ga1* or *ga4* mutations were created and analysed. The *elg* mutation is not able to completely suppress the effects of GA deficiency, caused by the *ga1* mutation. As with *ga1*, seeds of the digenic *elgga1* mutant still require applied GA for germination (data not shown). Nevertheless, plants that are digenic for the *elg* mutation and the *ga1* mutation or the *elg* and *ga4* mutations are significantly more elongated than the monogenic *ga1* or *ga4* plants, measured as the length of the cauline stem from the rosette to the first silique (Fig. 3.7). Monogenic *elg* plants show a similar increased stem extension in comparison with wild type seedlings (Fig. 3.7). Thus, the *elg* mutation appears to lead to increased elongation of stems regardless of the GA status of the plant.

The *elgga1* double mutant was used to study the effect of the *elg* mutation on plant responsiveness to applied GA. Topical GA applications, at concentrations ranging from 0 to 10 μg per plant, were given to plants in 10 μl drops at 11 days after sowing. Elongation growth was measured as the total plant height on growth cessation. Wild type and *elg* plants do not respond to the single GA applications across the range of concentrations used (Fig. 3.8). The *ga1* mutant responds to the GA applications with increases in elongation growth that reflect the concentration of applied GA (Fig. 3.8). The *elgga1* double mutant also responds to the applied GA with an increase in stem elongation growth (Fig. 3.8). Furthermore, although the *elgga1* double mutant is more elongated that the *ga1* mutant following GA application, the response of the two

genotypes to GA appears to be very similar (Fig. 3.8). Thus, it seems that applied GA and the *elg* mutation act in an additive manner.

The dwarf phenotype of the *ga1* mutant is displayed in dark grown seedlings as a short hypocotyl compared with wild type seedlings (Fig. 3.9). Significantly, the additive nature of the *elgga1* double mutant is also observed for dark grown seedlings. Thus, digenic *elgga1* mutants grown in the dark have longer hypocotyls than the monogenic *ga1* mutants. This indicates that the *elg* mutation can confer an elongated phenotype independently of the action of light.

3.3.7 Photocontrol of hypocotyl elongation in H4S seedlings

Landsberg *erecta* (Laer) wild type, *phyA*, *phyB*, Columbia (Col) wild type and H4S seedlings were grown for 5 d in darkness or under continuous R, FR or W, after which hypocotyl lengths were determined. For seedlings grown in the dark, R and W the hypocotyls of H4S are equivalent to those of its wild type (Fig. 3.10). As previously reported, *phyB* mutant hypocotyls are longer when compared to Laer wild type hypocotyls in R and W, with a more pronounced difference seen in the R treatment. Likewise, *phyA* mutant hypocotyls are longer when compared to Laer wild type hypocotyls in FR (Fig. 3.10). H4S seedlings displayed longer hypocotyls in R when compared to those of its wild type, though the extent of this hypocotyl elongation was less marked than in the *phyB* mutants (Fig. 3.10). Thus, H4S seedlings appear to have a less severe "*phyB*-type", light-conditional, long-hypocotyl phenotype in R.

3.3.8 Photocontrol of petiole elongation in H4S seedlings

Petiole lengths of the first leaf pair were measured for plants grown under 10 h light, 14 h dark, SD photoperiods and for plants grown under SDs with a 15 min light treatment given at the end of the photoperiod (EOD FR). The H4S and the *phyB* seedlings display increased petiole length when compared their respective wild type seedlings in SD photoperiods (Fig. 3.11). The application of EOD FR treatments

stimulates an increase in petiole extension in H4S, Col wild type and Laer wild type seedlings. This is evident when these seedlings are compared with those that did not receive FR treatments (Fig. 3.11). In contrast, *phyB* mutant seedlings show a reduction in petiole length when grown in SD+EOD FR conditions, when compared to *phyB* seedlings grown in SDs (Fig. 3.11). Thus, when grown under SDs, seedlings with elevated levels of HAT4 display an elongated petiole phenotype which resembles that of *phyB* seedlings. It also appears that HAT4 overexpression does not suppress the EOD FR-stimulated elongation of petioles.

3.3.9 Photocontrol of leaf shape in H4S seedlings

The leaf shape of the first leaf pair was measured as length/width (L/W) ratio in plants grown under continuous W, and in SD and 16 h light, 8 h dark (LD) photoperiods. An incremental increase in L/W ratio was observed in Laer WT, *phyB*, Col wild type and H4S seedlings with each successive decrease in photoperiod (Fig. 3.12). In all treatments, however, the H4S and *phyB* seedlings display a greater L/W value when compared with their wild type seedlings (Fig. 3.12). Therefore, although both H4S seedlings and *phyB* seedlings have an elongated appearance, they respond in a wild type manner to conditions of decreased photoperiod. Thus, seedlings with increased levels of HAT4 appear to have a qualitatively wild type response to changes in photoperiod.

3.3.10 Photocontrol of flowering time in H4S seedlings

Flowering time was recorded as number of days from germination and rosette leaf number at flower bud formation in plants grown under continuous light conditions with either a high or a low R/FR ratio, and for plants grown under SD photoperiods with or without a 15 min EOD FR light treatment. When grown under high R/FR ratio in continuous light both the H4S and the *phyB* mutants flower early in comparison to

their isogenic wild types (Figs. 3.11, 3.13 and 3.14). Despite this early flowering phenotype the H4S and the *phyB* seedlings still show an accelerated flowering response under low R/FR conditions, though the response is markedly less than the wild type response (Fig. 3.13). When grown under SDs the H4S plants are also early flowering but, significantly, the accelerated flowering response to EOD FR treatments which is observed in Laer wild type, Col wild type and *phyB*, is absent in the H4S plants (Figs. 3.11 and 3.14). This suggests, that the levels of HAT4 overexpression in H4S seedlings is sufficient to accelerate flowering to such an extent that EOD FR treatments are ineffective. However, the transgenic HAT4 levels in H4S are not sufficient to abolish the low R/FR ratio-stimulated early-flowering response in H4S seedlings. As HAT4 is thought to be down-regulated by a novel phytochrome under low R/FR ratio and EOD FR (Carabelli *et al.*, 1996) the behaviour of H4S seedlings under these conditions may be representative of a loss-of-function mutant in a novel phytochrome species.

In summary, etiolated H4S seedlings display reduced inhibition of hypocotyl elongation under R. The light-grown seedlings have an elongated, early flowering phenotype that is reminiscent of the *phyB* phenotype. What is more, like *phyB* seedlings, H4S seedlings have a reduced early-flowering response to low R/FR ratio. However, unlike *phyB* seedlings, H4S seedlings do not display an early-flowering response to EOD FR treatments.

3.4 Discussion

The *elg* mutant

Arabidopsis seedlings homozygous for the *elg* mutation display a pleiotropic phenotype characterised by an elongated growth habit (hypocotyls, petioles, leaves and stems), increased apical dominance and early flowering. This phenotype resembles that of wild type plants grown under shade conditions and, therefore, it is possible that the *elg* phenotype may represent a mutation affecting the function of a novel phytochrome known to function in the shade-avoidance response. Another feature of the *elg* mutant is

the intermediate phenotype displayed by the heterozygote, a characteristic that is shared by the *phyA* and the *phyB* mutants (Koornneef *et al.*, 1980; Whitelam *et al.*, 1993). The *elg* phenotype is also reminiscent of wild type plants subjected to GA application and could, by the same measure, reflect a malfunction with regard to GA. The photoresponses of the *elg* mutants and the relationship between *elg* and photomorphogenic and gibberellin synthesis mutants were investigated in order to gain some insight into the relatedness of the *ELG* gene to the novel phytochrome and/or GA.

The photophysiological analyses indicate that the *elg* mutation is unlikely to disrupt phytochrome function. Under a wide range of light treatments that have previously been used to diagnose dysfunction of phytochrome-mediated signalling, the *elg* mutant was found to respond in a qualitively wild type manner. Thus, although *elg* seedlings have elongated petioles they respond to EOD FR treatments with a further increase in petiole elongation, a response thought to be under the control of phytochrome B (Somers *et al.*, 1991; Reed *et al.*, 1992, 1993). Also, despite a marked early flowering phenotype, the *elg* mutant responds to low R/FR ratio light with a further acceleration of flowering. This accelerated flowering response to low R/FR ratio is greatly reduced in mutants deficient in phytochrome B, implicating phytochrome B as one, but not the sole phytochrome involved in this response (Whitelam and Smith, 1991; Robson *et al.*, 1993; Halliday *et al.*, 1994 and Chapter 2). Furthermore, the *elg* mutant maintains a qualitatively wild type flowering response to varying photoperiods, as flowering is substantially delayed in *elg* seedlings grown under SDs. Both phytochromes A and B have been implicated in the control of plant responses to changes in the length of photoperiod (Goto *et al.*, 1991; Johnson *et al.*, 1994; Reed *et al.*, 1994; Childs *et al.*, 1995).

Analysis of the *elghy1*, *elghy2* and *elgphyB* digenic mutants reveals that they all have longer hypocotyls than their respective monogenic parent plants. Therefore, in this respect, the *elg* mutation acts additively with the *hy1*, the *hy2* and the *phyB* mutations. This suggests that *ELG* acts independently of phytochrome B and is either functional in another phytochrome pathway, (as *hy1* and *hy2* mutants are leaky), or that it acts in a manner that is entirely separate from phytochrome.

The observation that the long hypocotyl phenotype of *elg* seedlings is light-

conditional suggests that the action of *ELG* in regulating flowering and elongation growth is dependent upon activation of light-signalling pathways; in other words ELG appears to act down-stream of photoreceptor action. That the phenotype of *elg* mutants grown in the dark is indistinguishable from that of the wild type is central to the view that the *elg* phenotype is light-conditional. However, it is significant that in the dark the *elg* mutation acts additively with the *ga1* mutation. Thus, the *elgga1* digenic mutant displays a long hypocotyl in the dark compared with the monogenic *ga1* mutant. This suggests that ELG is active in darkness in *ga1* seedlings where its action leads to an inhibition of hypocotyl elongation. Thus, photoreceptor activation is not a pre-requisite for the display of the *elg* phenotype. Presumably, for wild type seedlings grown in the dark the action of the *ELG* gene product does not lead to a detectable inhibition of elongation which is why *elg* hypocotyls are no more elongated than wild type hypocotyls.

Possible interactions between the *elg* mutation and GA action have also been studied. Unlike the paclobutrazol-resistant *spy* mutation (Jacobsen anf Olszewski, 1993) the *elg* mutation cannot fully suppress the effects of the *ga1* mutation on seed germination. However, in common with *spy*, *elg* can partially suppress some of the effects of GA-deficiency on elongation growth, although not to the same extent as *spy* (Jacobsen and Olszewski, 1993). Despite the fact that *elgga1* seedlings are more elongated than *ga1* seedlings, both genotypes respond in a very similar way to a single application of GA. Thus, applied GA and the *elg* mutation act additively suggesting that *ELG* acts independently of GA action.

To summarise, a novel mutant of *Arabidopsis* has been described that displays a constitutive shade-avoidance phenotype that could represent deficiences in a novel phytochrome that functions in the low R/FR ratio response or could represent deficiencies in GA function. However, the action of the *ELG* gene product in control of flowering and elongation growth appears to be mediated through a transduction pathway that is separate from the photoreceptor-mediated or GA-mediated signal transduction chains. Therefore, *elg* neither identifies a novel phytochrome active in the shade-avoidance response, nor a component involved in GA function, but clearly represents a factor influential in elongation growth independent of phytochrome or GA.

The H4S transgenic mutant

The transgenic *Arabidopsis* H4S mutant, that overexpresses the *HAT4* (*Athb-2*) gene, has a pleiotropic phenotype with many features in common with phytochrome mutants. The most striking characteristic of the H4S mutant is its early flowering nature and elongated growth habit affecting hypocotyls, petioles, leaves and stems, a phenotype that resembles the shade-avoidance phenotype of wild type plants (Schena *et al.*, 1993). The *HAT4* gene encodes an HD-Zip protein, a putative transcriptional regulator and is, therefore, a likely candidate for a signal-transduction component (Carabelli *et al.*, 1993; Schena *et al.*, 1993). In addition, it has been shown that *HAT4* transcript levels are enhanced under low R/FR and EOD FR not only in wild type, but in phytochrome A- and phytochrome B-deficient seedlings (Carabelli *et al.*, 1996). Interestingly, the physiological analysis of *phyB* demonstrates that although it has a constitutive early flowering phenotype it still responds to low R/FR ratio with a further acceleration of flowering (Whitelam and Smith, 1991; Robson *et al.*, 1993; Halliday *et al.*, 1994 and Chapter 2). This implies that an unidentified phytochrome(s) in addition to phytochrome B also functions in the early flowering response to low R/FR ratio. It is possible, therefore, that this novel phytochrome is responsible for the down-regulation of *HAT4* gene product. If this premise is correct, the phenotype of H4S seedlings, which have elevated levels of the *HAT4* gene product, could resemble that of a mutant deficient in this novel phytochrome. Physiological analysis of H4S seedlings define characteristics that may be representative of a novel phytochrome mutant deficient in aspects of the shade-avoidance response.

When hypocotyl de-etiolation responses are examined, the H4S seedlings respond in a manner that is qualitatively similar to that of *phyB* mutants, with reduced hypocotyl inhibition in R light. This suggests that the *HAT4* gene product operates in a pathway that induces seedling de-etiolation under R. In this respect, it does not seem likely that H4S seedlings would be behave in a similar manner to seedlings with a deficiency in a novel phytochrome. The *phyAphyB* mutant seedlings are saturated for loss of hypocotyl growth inhibition when grown under R light (Reed *et al.*, 1993; P.F. Devlin, pers comm.). It, therefore, follows that in *Arabidopsis*, R-mediated inhibition of hypocotyl elongation is controlled by phytochromes A and B. In etiolated wild type,

phyA, *phyB* and *phyAphyB* seedlings, *HAT4* mRNA levels fall in response to R light (Carabelli *et al.*, 1996). However, as each of the mutant seedlings also have higher basal levels of *HAT4* transcript, it is possible that phytochromes A and B can also down-regulate *HAT4* in these conditions (Carabelli *et al.*, 1996). Therefore, the reduced R-mediated inhibition of hypocotyl growth displayed by H4S seedlings would probably not be a characteristic of seedlings with deficiencies in a novel phytochrome species.

Examination of the H4S elongated growth habit was carried out in photoperiods with or without an EOD FR with a focus on petiole length. Seedling growth under these conditions is revealing. Although the H4S petioles are more elongated than wild type petioles, they respond in a manner consistent with wild type plants when grown both SDs and SDs with an EOD FR treatment. In both wild type seedlings and H4S seedlings EOD FR treatments stimulate a further elongation of petioles. In *phyB* seedlings there is a reduction of petiole length. However, these observations are not consistent with those in other experiments where *phyB* seedlings maintain the same petiole length when grown in photoperiods with or without an EOD FR (Nagatani *et al.*, 1991; Devlin *et al.*, 1996). These apparent differences in the behaviour of *phyB* seedlings are likely to reflect differences in growth conditions. In these experiments the *phyB* seedlings were very small. The observed EOD FR-mediated reduction in petiole length probably represents a necessary re-channelling of resources from vegetative to reproductive development. Therefore, whilst both H4S and *phyB* seedlings have elongated petioles, only H4S seedlings retain the ability to respond to EOD FR treatments with a further extension of petioles. With regard to this response, phytochrome action appears to be more severely effected in the *phyB* seedlings when compared to H4S seedlings. Whilst increased HAT4 levels correlate with reduced inhibition of petiole growth, they do not reduce the effectiveness of the EOD FR-mediated petiole-elongation response.

The elongated growth habit is also reflected in the leaf shape, as the H4S seedlings produce leaves that are more slender in appearance than those of their wild types. The H4S seedlings, like the *phyB* seedlings, behave in a way that is qualitatively similar to that of the wild type with respect to differences in leaf shape observed in

continuous light, SD or LD photoperiods. These observations are not unexpected as both H4S and *phyB* seedlings behave in a similar manner with respect to petiole length, which is a component of leaf shape. Thus, it appears that elevated HAT4 levels result in a change in leaf shape. However, the shape of H4S seedling leaves is altered in a wild type manner to changes in photoperiod length.

A prominent feature of the H4S phenotype is its early flowering nature. In a similar manner to *phyB*, H4S flowers early with respect to its wild type and displays a reduced early-flowering response to low R/FR ratio. The constitutive early flowering phenotype seen in *phyB* seedlings coupled with the fact that they remain responsive to low R/FR ratio (Whitelam and Smith, 1991; Halliday *et al.*, 1994; Chapter 2), implies that both phytochrome B and a novel phytochrome are active in the control of flowering in response to low R/FR ratio. A plant with deficiencies in this novel phytochrome species would be expected to share these phenotypical attributes with *phyB*. Certainly H4S seedlings fulfil this requirement. If *HAT4* is down-regulated by a novel phytochrome species in the light one would expect the H4S and *phyB* phenotypes to be additive. However, if *HAT4* regulation is also controlled by phytochrome B, this may not be the case.

The early flowering phenotype of H4S seedlings is also seen under photoperiodic conditions but, significantly, the H4S seedlings fail to show the accelerated flowering response to EOD FR treatments. Even though *phyB* seedlings are early flowering, a small acceleration in flowering can be stimulated by EOD FR. This response is qualitatively similar to that displayed by wild type plants. Therefore, it is possible that increased levels of HAT4 present in the H4S seedlings are effective in blocking the early flowering response to an EOD FR treatment. If this is the case, the photoreceptor responsible for the down-regulation of *HAT4* will mediate an early-flowering response to EOD FR treatments. This response is thought to be controlled by a novel phytochrome in addition to phytochrome B. Therefore, with regard to this response, it is likely that the H4S phenotype reflects the behaviour of a loss-of-function mutant in a novel phytochrome.

In summary, the *Arabidopsis HAT4* gene has a multitude of features that are suggestive that it may be a component of a novel phytochrome pathway. The *HAT4*

gene product is a HD-Zip protein and a putative transcriptional regulator and, therefore, a good candidate for such a role. In addition, expression of *HAT4* is rapidly induced by EOD FR and is R-reversible in light-grown wild type, *phyA*, *phyB* and *phyAphyB* seedlings. This suggests that in de-etiolated seedlings *HAT4* is down regulated by a novel phytochrome Pfr. If this supposition is correct, the phenotype of transgenic H4S seedlings, which overexpress HAT4, may be representative of seedlings with a deficiency in this novel phytochrome. In fact, the phenotypical changes evident in adult H4S plants are consistent with those expected of plants deficient in a novel phytochrome in many aspects of the phenotype. H4S plants are elongated in appearance and exhibit a reduced early-flowering response to low R/FR, and a loss of this response to EOD FR treatments. However, the etiolated seedling displays a reduction of R-mediated hypocotyl growth inhibition. This response is thought to be exclusively under the control of phytochromes A and B. Therefore, it is likely that H4S seedlings are not representative of a novel phytochrome loss-of-function mutant in this respect.

Futher work on the identification of mutants and transduction chain mutants in this novel phytochrome could be accomplished by the selection of a loss-of-function mutant using a screen based on the H4S phenotype. Alternatively, another mutagenesis approach could be employed using seed transformed with the *HAT4* gene promoter fused to a resistance marker gene. Due to the low expression levels of *HAT4* one would expect these seedlings would not confer any degree of resistance. However, mutagenised seeds may lead to mutations that cause an upregulation in *HAT4*, and increased resistance as a result of enhanced promoter activity. Resistant plants are likely to carry mutations in the novel phytochrome or in its transduction pathway.

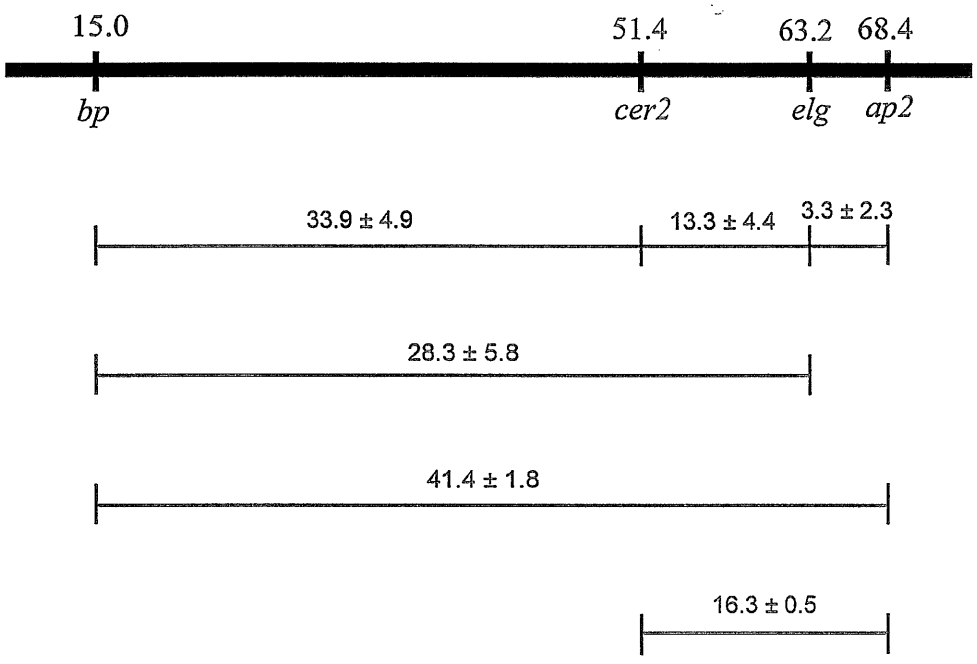


Figure 3.1 Location of the *elg* locus on chromosome 4; distances are given in cM. Estimates of recombination percentages with their standard deviation are indicated under the chromosome bar. (Hanhart and Koornneef)

Genotype	Plant length (cm) to 1st silique	Plant length (cm) Total	Length/Width of largest rosette leaf
ELG/ELG	10.6 ± 0.6	30.0 ± 0.6	2.48 ± 0.07
ELG/elg	15.0 ± 0.3	31.6 ± 0.6	3.19 ± 0.09
elg/elg	15.5 ± 0.2	34.7 ± 0.7	3.42 ± 0.08

Table 3.1 Plant length and leaf shape of the *elg* mutant in comparison to the wild type and heterozygote. Data represent the means from 20-30 plants. Standard errors are shown. (Hanhart and Koornneef)

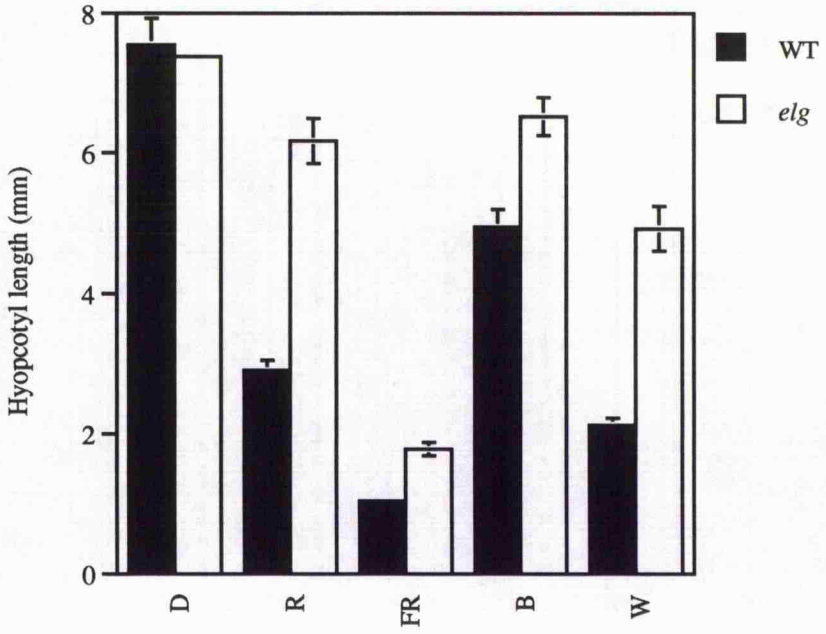


Figure 3.2 Hypocotyl length of WT (closed bars) and *elg* (open bars) seedlings after 5 d growth in continuous darkness (D), red (R), far-red (FR), blue (B) and white (W) light. Data represent the means from 20-30 seedlings and the error bars are SE.

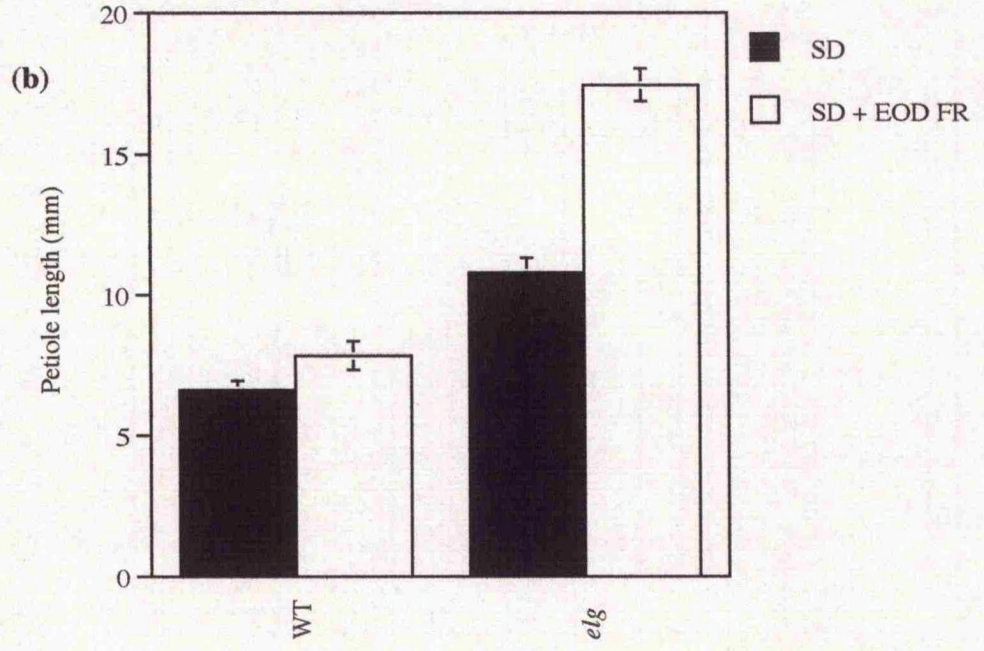


Figure 3.3 Petiole length of **(a)** WT and *elg* seedlings grown under continuous light and **(b)** second leaves of WT and *elg* seedlings grown under SD, 10h light, 14 h dark (closed bars), and under SD + 15 min EOD FR light treatments (open bars). Data represent the means from 20-30 plants and the error bars are SE.

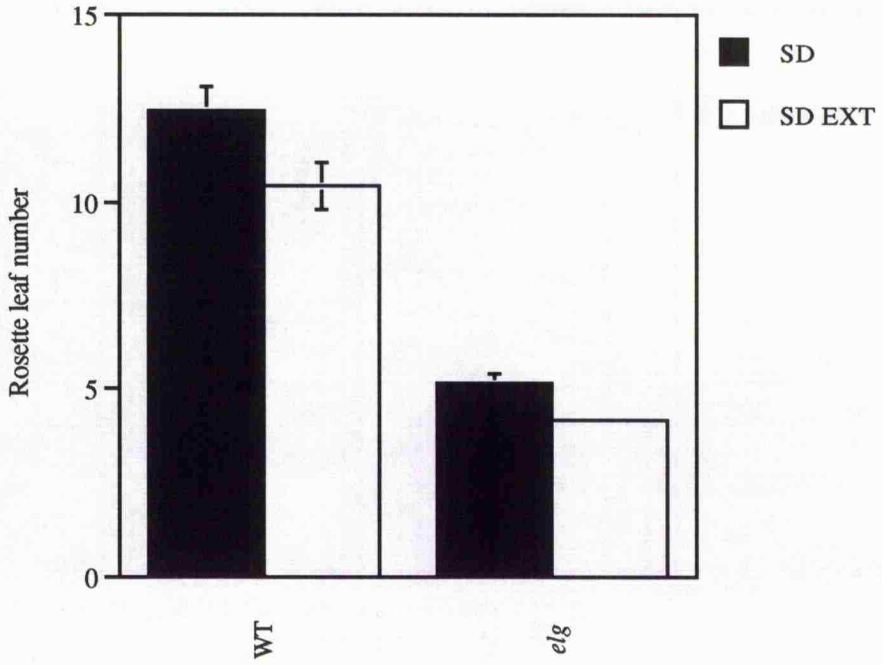


Figure 3.4 Flowering time measured as basal rosette number in WT and *elg* seedlings grown under SD, 10 h light, 14 h dark (closed bars) and under SD EXT, 10 h light, 8 h incandesent light, 6 h dark (open bars). Data represent the means from 20-30 plants and the error bars are SE.

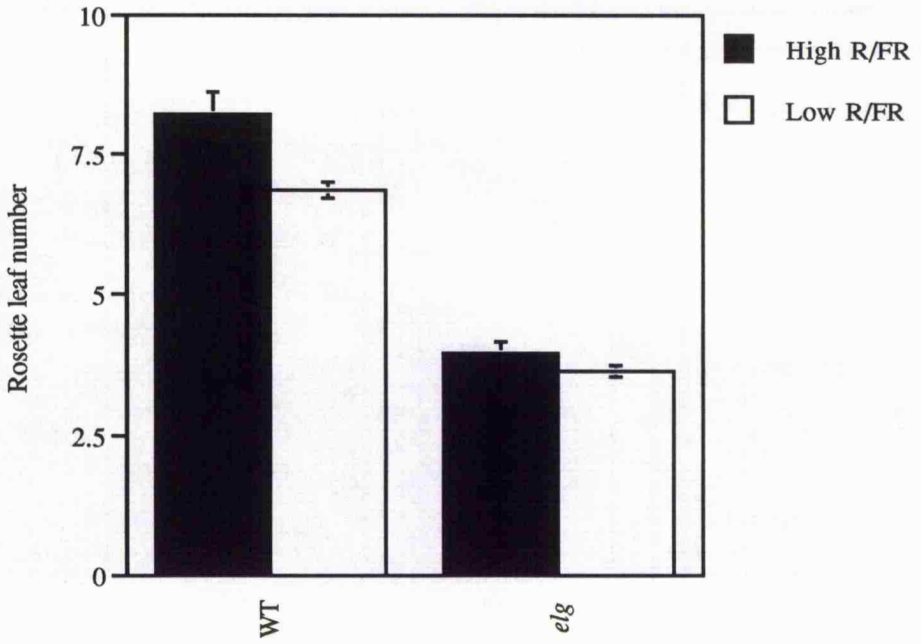


Figure 3.5 Flowering time measured as basal rosette number in WT and *elg* seedlings grown under high R/FR ratio (closed bars) or low R/FR ratio (open bars) continuous light. Data represent the means from 20-30 plants and the error bars are SE.

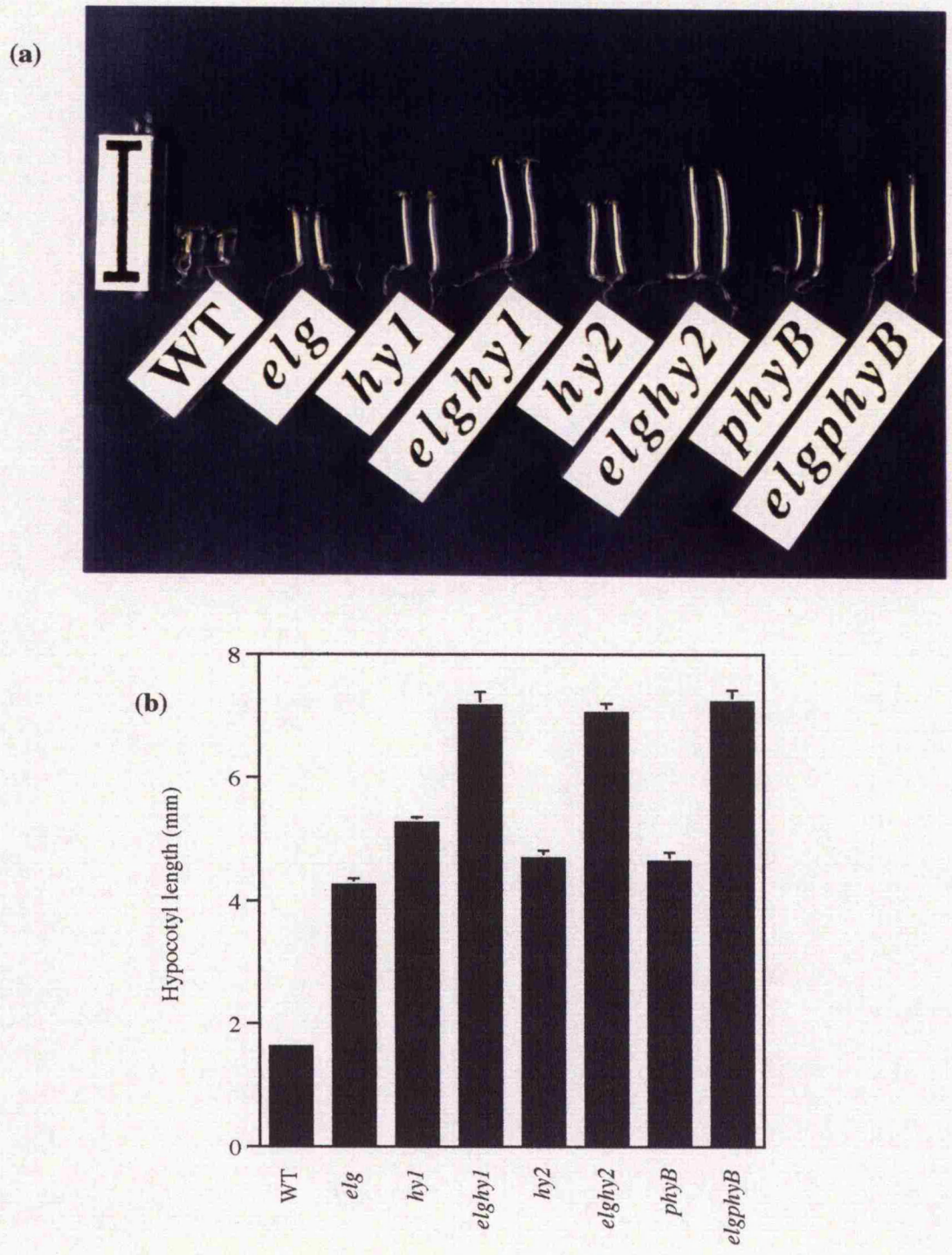


Figure 3.6 Phenotype (**a**) and hypocotyl length (**b**) of WT seedlings and *elg*, *hy1*, *elghy1*, *hy2*, *elghy2*, *phyB* and *elgphyB* mutant seedlings after 5 d growth in continuous W light. Data represent the means of 25 plants and the error bars are SE.

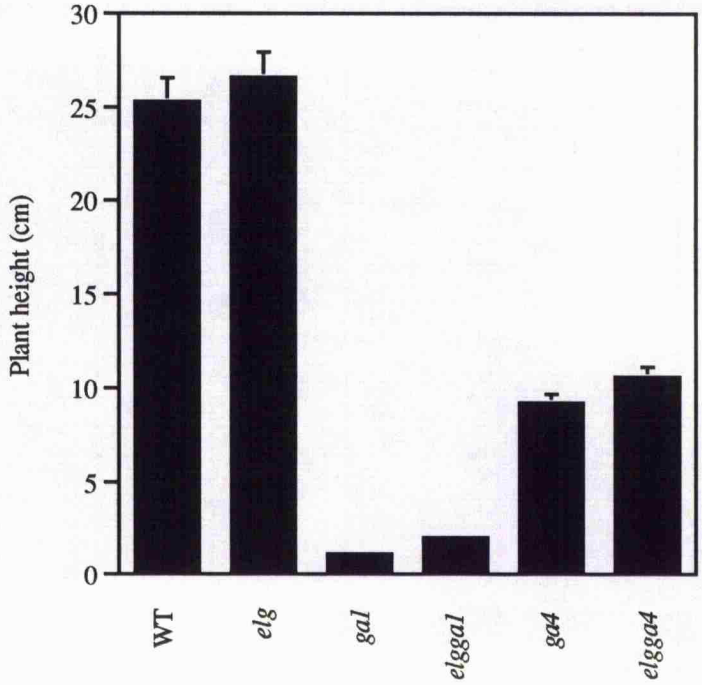


Figure 3.7 Plant height measured after cessation of growth in WT, *elg*, *ga1*, *elgga1*, *ga4* and *elgga4* seedlings grown in continuous W light. Data represent the means from 20-30 plants and the error bars are SE. (Hanhart and Koornneef)

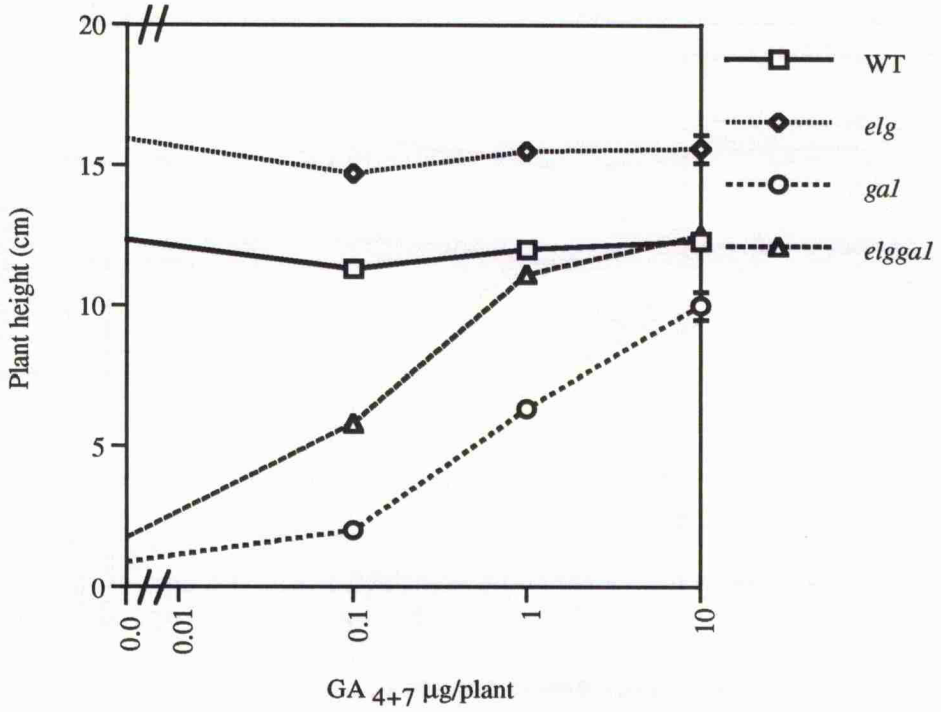


Figure 3.8 Plant height measured from basal rosette to first silique in WT, *elg*, *ga1* and *elgga1* seedlings following the application of GA (4+7) at day 11. Data represent the means from 20-30 plants and the error bars are SE. (Hanhart and Koornneef)

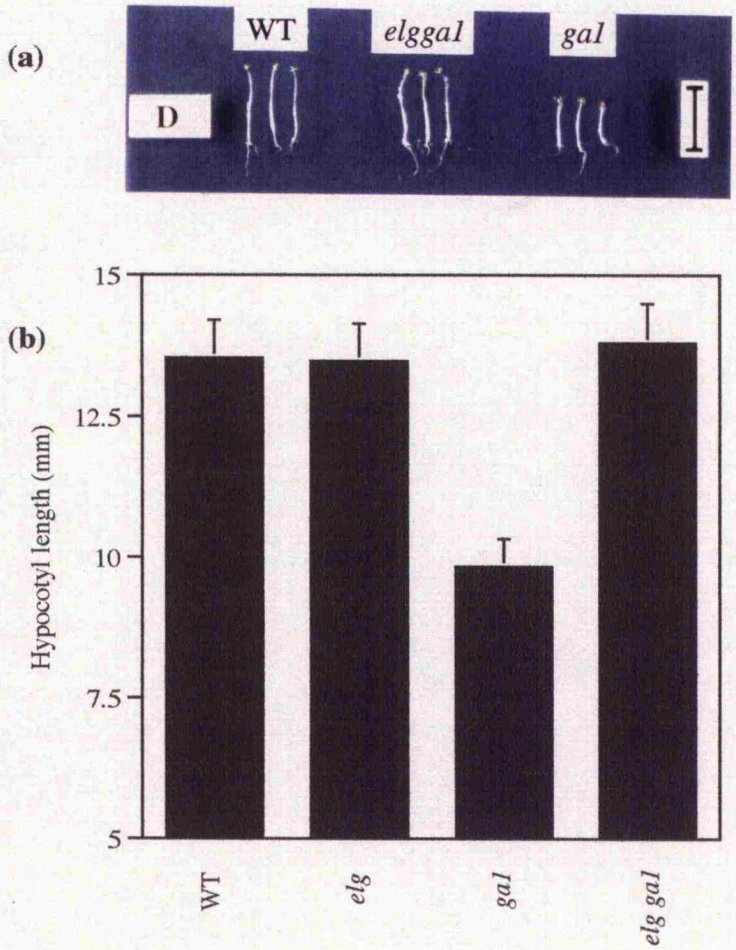


Figure 3.9 Phenotype of WT, *elggal* and *gal* seedlings (**a**) and hypocotyl length of WT, *elg*, *gal* and *elggal* seedlings (**b**) after 4 d growth in the dark. Data represent the means from 16-20 plants and error bars are SE.

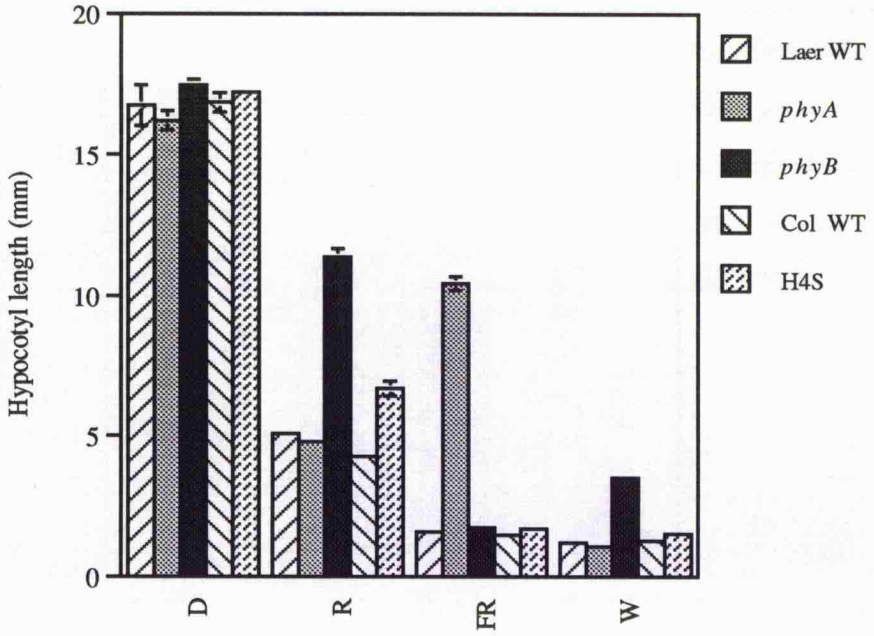
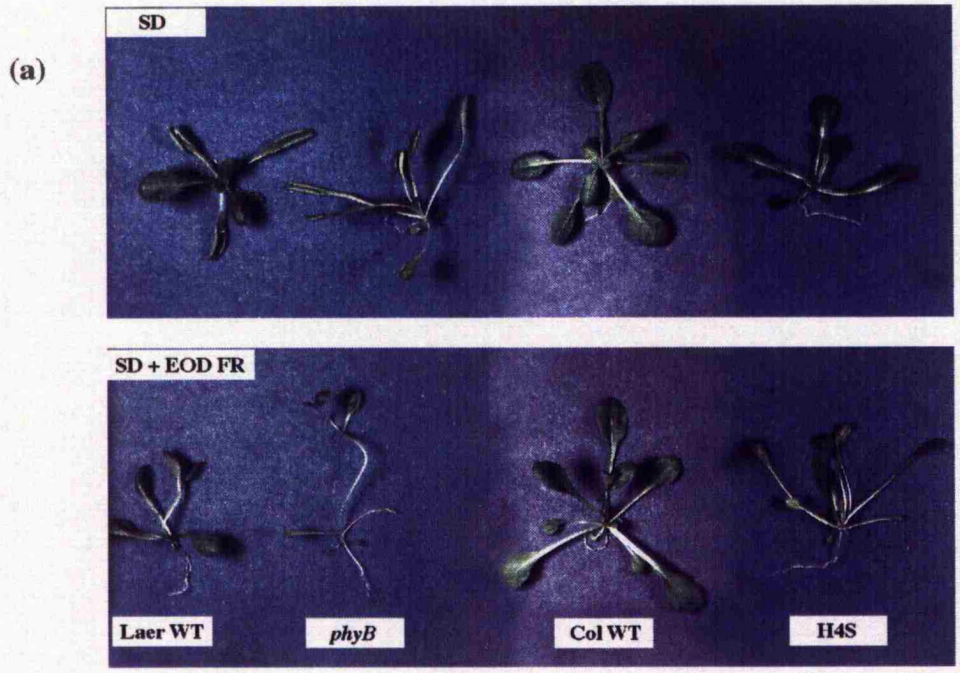


Figure 3.10 Hypocotyl length of Laer WT, *phyA*, *phyB*, Col WT and H4S seedlings after 5 d growth in darkness (D), red (R), far-red (FR) and white (W) light conditions. Data represent the means from 20 plants and the error bars are SE.



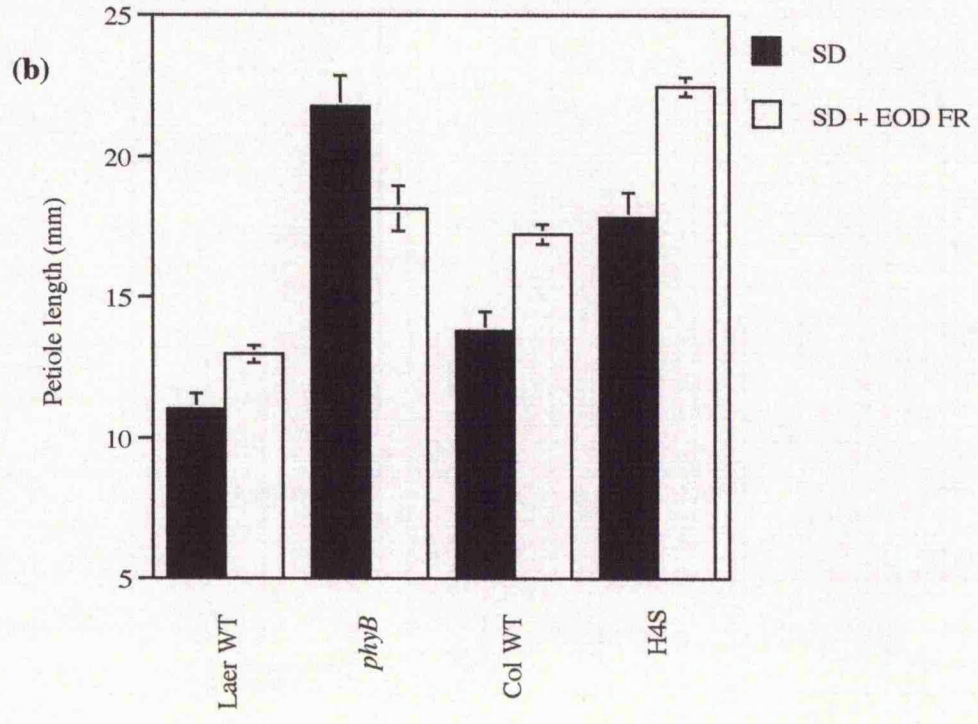


Figure 3.11 Phenotype (**a**) and petiole length of second leaf pair(**b**) of Laer WT, *phyB*, Col WT and H4S seedlings measured at 14 d SD, 10 h light, 16 h dark (Filled bars) and SD + 15 min EOD FR (open bars). Data represent the means from 20 plants and the error bars are SE.

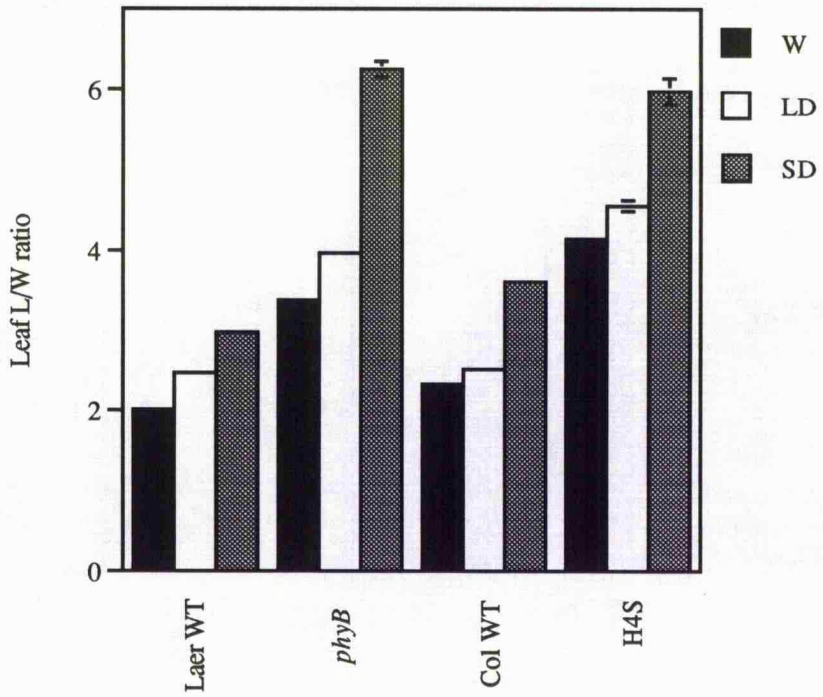


Figure 3.12 Leaf length/width ratio (L/W) of the first leaf pair of Laer WT *phyB*, Col WT and H4S seedlings measured at 14 d in continuous W (closed bars), LD, 16 h light, 8 h dark (open bars) and SD, 10 h light, 14 h dark (hatched bars). Data represent the means from 20 plants and the error bars are SE.

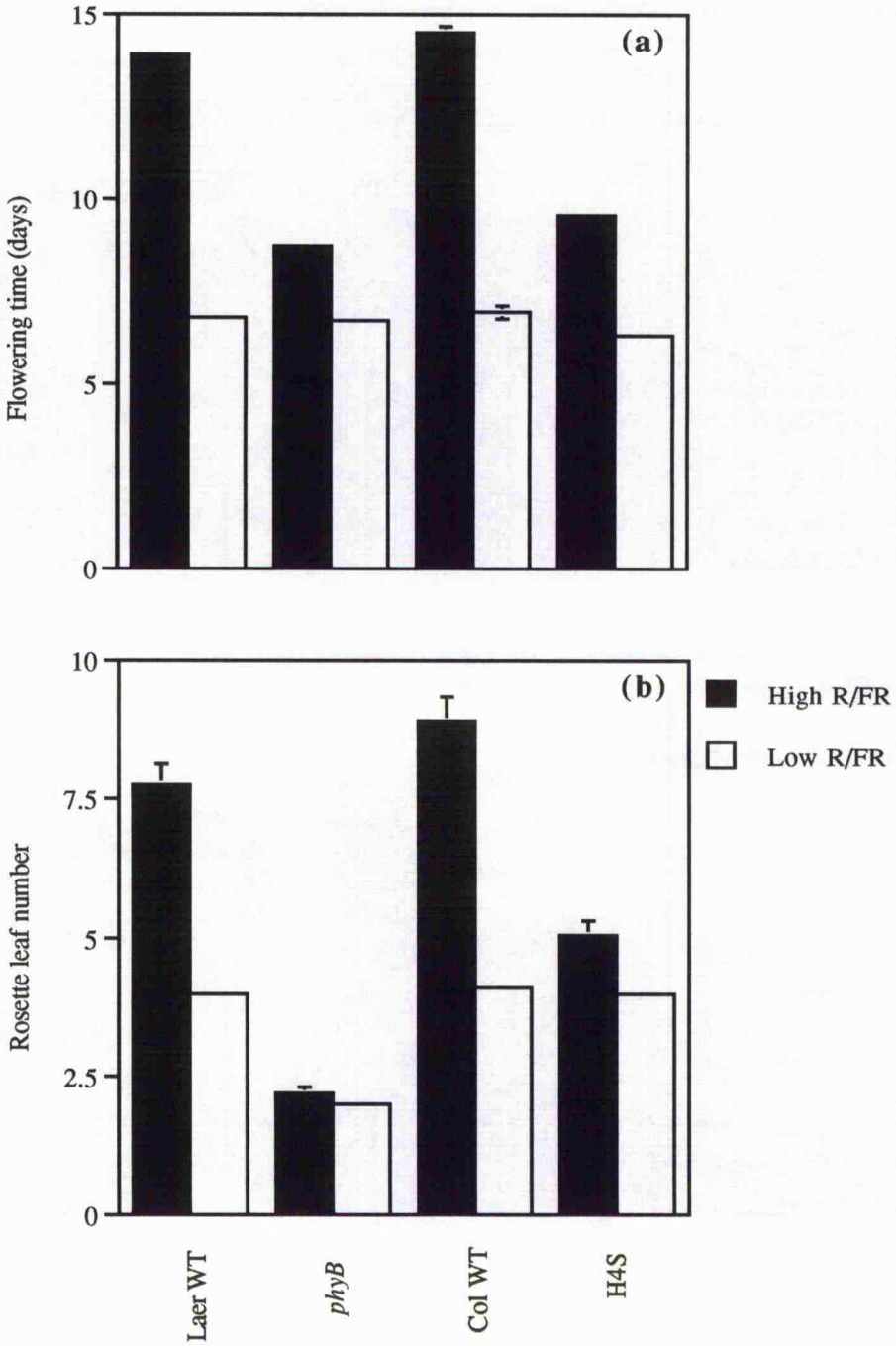


Figure 3.13 Flowering time measured in days **(a)** and number of basal rosette leaves **(b)** in Laer WT, *phyB*, Col WT and H4S seedlings grown under high R/FR (closed bars) and low R/FR ratio (open bars) continuous light. Data represent the means from 20 plants and the error bars are SE.

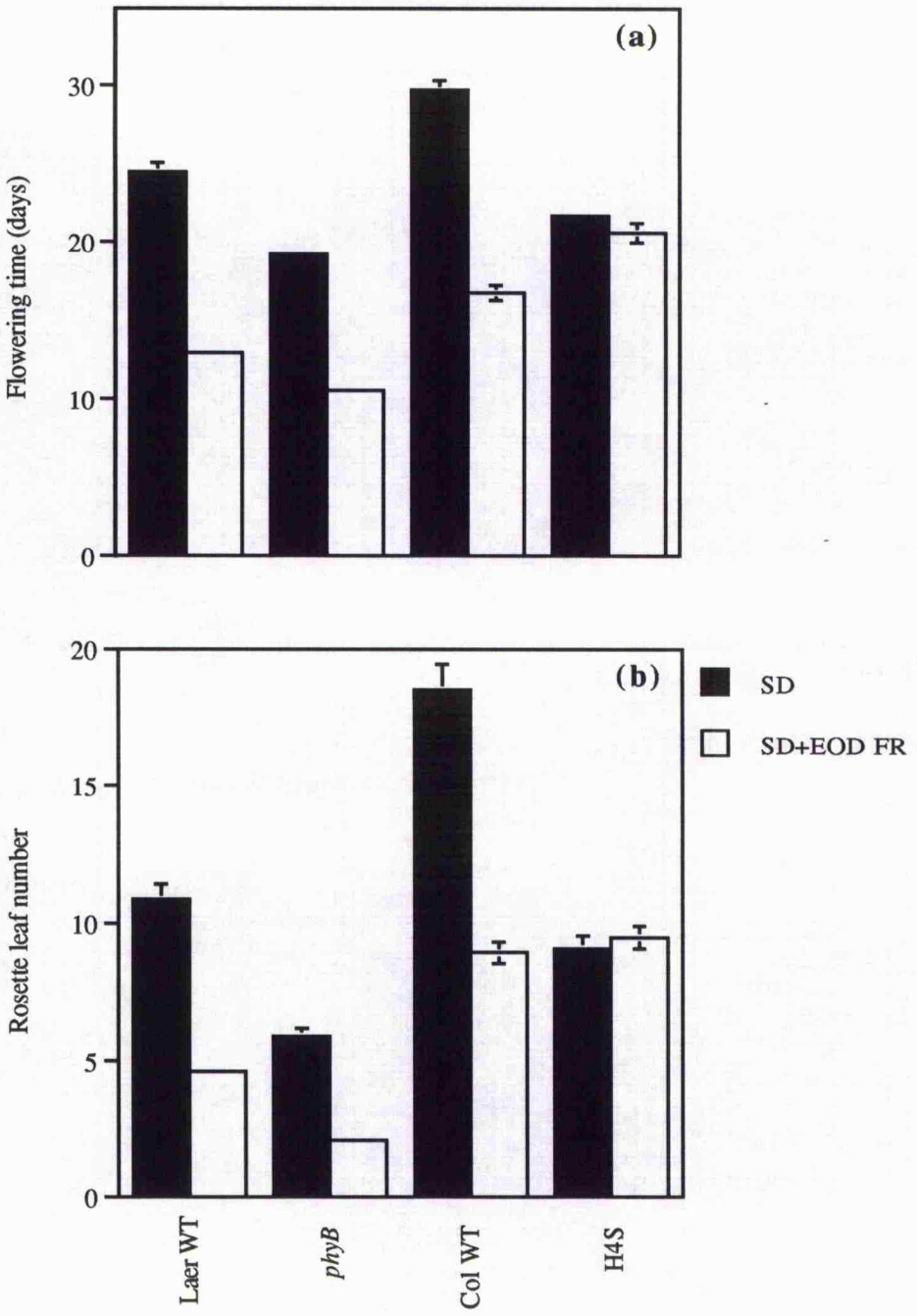


Figure 3.14 Flowering time measured in days (**a**) and number of basal rosette leaves (**b**) of Laer WT, *phyB*, Col WT and H4S seedlings grown in SD, 10 h light, 14 h dark (closed bars) and SD + 15 min EOD FR (open bars). Data represents the means from 20 plants and the error bars are SE.

Chapter 4 Molecular characterisation of DN and SD *Nicotiana tabacum* overexpressing phytochrome A, B or C

4.1 Introduction

Our understanding of phytochrome function has been greatly assisted by the analysis of transgenic plants that express increased amounts of a particular phytochrome species. The construction of transgenic plants has been made possible with the cloning of phytochrome genes from various plant species (see Smith, 1994b). Analysis of transformed plants expressing elevated levels of these phytochrome-encoding cDNAs is proving a valuable complementary approach to mutant analysis. Three types of photomorphogenic mutants have been identified: (1) those in which the lesion is within the phytochrome gene, resulting in the partial or complete absence of a specific phytochrome; (2) those in which the lesion affects chromophore biosynthesis or function; and (3) those in which the lesion affects a downstream component in one or more phytochrome transduction pathways (see Whitelam and Harberd, 1994). Loss-of-function phytochrome photoreceptor mutants characteristically display "light insensitivity" under light conditions in which the particular phytochrome species is normally operational. In contrast, when transgenically expressed, the introduced phytochrome species confers a "light hypersensitive" phenotype. Several *Arabidopsis* mutants specifically deficient in phytochrome A have been reported (Nagatani *et al.*, 1993; Parks and Quail, 1993; Whitelam *et al.*, 1993). These mutants were identified because they display reduced sensitivity to continuous FR, with respect to growth inhibition under FR (Nagatani *et al.*, 1993; Parks and Quail, 1993; Whitelam *et al.*, 1993). In contrast, transgenic *Arabidopsis* overexpressing a heterologous phytochrome A display enhanced sensitivity to continuous FR (Boylan and Quail, 1991; Whitelam *et al.*, 1993). *Arabidopsis* phytochrome B mutants display a reduced sensitivity in continuous R which results in an elongated hypocotyl phenotype under these

conditions. Again this contrasts with transgenic plants where phytochrome B-overexpression induces a short-hypocotyl phenotype under R light (Wagner *et al.*, 1991; McCormac *et al.*, 1993).

Analysis of the complementary, and sometimes contrasting aspects of responses in the *phyA* and *phyB* mutants and their corresponding transgenics has provided much information on the photosensory roles of these phytochrome species. For instance, it is well known that etiolated wild type seedlings demonstrate a FR HIR-mediated elongation growth inhibition and that upon de-etiolation, this response to FR is lost due to the rapid degradation of a light-labile phytochrome A (Beggs *et al.*, 1980; Holmes and Schäfer, 1981). Indeed it was the selective lack of sensitivity of *phyA* mutants to FR that led to the proposal that the FR HIR is specifically mediated by phytochrome A. When grown under FR light, *phyA* seedlings do not display the inhibition of hypocotyl elongation that is evident in wild type plants. Analysis of transgenic plants served to confirm this role for phytochrome A in the FR HIR. The phenotype of etiolated seedlings overexpressing phytochrome A complement that of *phyA*, as these seedlings exhibit exaggerated FR-mediated hypocotyl growth inhibition (McCormac *et al.*, 1991, 1992; Whitelam *et al.*, 1992). Examination of mutants and transgenic plants have also identified a role for phytochrome A in light-grown plants. When grown under R light, the *Arabidopsis phyAphyB* mutant seedlings have longer hypocotyls than the *phyB* seedlings, whilst seedlings overexpressing phytochrome A display increased sensitivity to R (Reed *et al.*, 1994; Whitelam *et al.*, 1992). These findings suggests a role for phytochrome A in hypocotyl inhibition under R.

The shade-avoidance responses of light-grown plants are thought to be a function of the light-stable phytochrome pool. Much has been learned about the function of phytochrome B, in this respect, with analysis of the *Arabidopsis phyB* null mutant. The *phyB* mutants display a constitutively elongated phenotype which suggests that phytochrome B may play a role in the elongation responses to low R/FR ratio light. Reassuringly, *Arabidopsis* overexpressing phytochrome B grow in a dwarfed habit, which lends support to this finding (McCormac *et al.*, 1993). It should also be noted that like the transgenic overexpressors, the phytochrome B mutants respond to low R/FR ratio, which implicates at least one other species of phytochrome in the shade

avoidance responses (Whitelam and Smith, 1991; Robson *et al.*, 1993; Halliday *et al.*, 1994; McCormac *et al.*, 1993).

Where possible, comparisons are usually made between mutants and transgenics in the same species to reduce the probability of misinterpretation of phenotypical features which may represent different functions in different species. In practice, however, many plant species are not equally suitable for both mutagenesis and transgenic manipulation. It is also the case, that many aspects of the overexpression phenotype are conserved between species. For instance, the introduction of heterologous and homologous phytochrome A genes into *Arabidopsis*, tobacco and tomato confer a light-independent dwarfed phenotype (Boylan and Quail, 1989, 1991; Kay *et al.*, 1989; Keller *et al.*, 1989). Characteristics that are conserved between transgenic plants overexpressing particular phytochrome species also provide very useful information about global phytochrome function in higher or lower plant species.

There are several criteria for selection of the species most suitable for transformation. Important factors are, the compatability of the plant species to genetic manipulation techniques and regeneration from transformed cells, as well as the identification of photophysiological traits in the species, that warrant investigation. Techniques using *Agrobacterium tumefaciens* transformation of *Arabidopsis* have been developed over recent years and are now relatively straight forward (Clarke *et al.*, 1992). Alternative methods of transformation, involving direct transgene delivery, are frequently used. However, *Agrobacterium*-mediated transfer is by far the most simple and, therefore, still the preferred method. Many species, particularly the graminaceous monocotyledons, are not easily transformed using *Agrobacterium*, though intensive work in this area has led to the recent development of such a system in rice (Hiei *et al.*, 1994). However, explants such as leaf discs of solanaeous species such as *Nicotiana tabacum, Petunia hybrida* and tomato, are easily transformed with high efficiency using *Agrobacterium*-mediated DNA transfer (Horsch *et al.*, 1985; McCormick *et al.*, 1986).

The choice of promoter is also of prime importance as it is essential that the transgene is expressed to a sufficiently high level to produce an exaggerated phenotype. Phytochrome overexpression has been achieved in many plants by the use of the CaMV 35S promoter (Boylan and Quail, 1989; Kay *et al.*, 1989; Keller *et al.*, 1989; Wagner

et al., 1991). However, with the use of the "constitutive" CaMV 35S promoter, there is the likelihood of transgene expression within cells that do not normally express the native phytochrome gene. This ectopic expression of transgenes may result in abnormal plant photophysiology. In situations such as these a degree of caution must be exercised in aportioning precise functions to the introduced phytochrome species. Indeed, some aspects of the *phyA* mutant phenotype, and the phenotype of *Arabidopsis* seedlings expressing an oat phytochrome A cDNA appear to be at odds with one another. When grown under continuous W, seedlings overexpressing phytochrome A display a dwarfed phenotype when compared with wild type seedlings (Whitelam *et al.*, 1992), whilst *phyA* seedlings are indistinguishable from wild type seedlings (Whitelam *et al.*, 1993). Likewise, there are also difficulties with the interpretation of flowering time in *phyB* mutant seedlings and *Arabidopsis* seedlings overexpressing phytochrome B, that both appear to flower early under long day conditions (Goto *et al.*, 1991; Whitelam and Smith, 1991; Bagnall *et al.*, 1995). One possible solution to this problem may be to utilise phytochrome promoters so that the amplified expression of the transgene could be retained within the cell types that express the native phytochrome. Recent work expressing a phytochrome B cDNA sequence fused to a phytochrome B promoter fragment, in *Arabidopsis*, has been successful in achieving transgene overexpression and an exaggerated phenotype (Wester *et al.*, 1994). However, it is noteworthy that the phytochrome B-overexpression phenotype appears to be very similar in plants where the transgene is under the control of the native promoter or the CaMV 35S promoter.

The effects of phytochrome overexpression have largely been studied in long-day (LD) plants. Analysis of the photophysiology of LD plants has yielded much information on the roles of phytochrome-mediated responses to changing light quality in germination, de-etiolation, vegetative growth and development, and flowering. However, very little is known about the involvement of phytochrome in the photoperiodic control of flowering. Much of the work on photoperiodism has been carried out with short-day (SD) species that can be induced to flower by a single inductive photoperiod. Using many such plants, flowering responses to a multitude of light treatments that alter photoperiodic perception have been tested. As a result the mechanisms involved in photoperiodic flowering in SD plants are more clearly

understood than those in LD plants (see Vince-Prue, 1994). Few phytochrome mutants in SD plants have been isolated and few SD species are readily transformable. However, the availability of day-neutral (DN) and SD *N. tabacum* provides the opportunity to compare the action of phytochrome in a SD plant with phytochrome action in a photoperiod-insensitive plant. By subjecting the SD plants to a night-break (NB) during an inductive night it is possible to delay flowering. The involvement of a particular phytochrome in daylength perception can, therefore, be tested by assessing the impact of phytochrome overexpression on NB perception. Transgenic DN and SD tobacco overexpressing phytochromes A, B and C have been created for this purpose and to establish the physiological attributes of each overexpression phenotype. This is of particular interest, as effects of phytochrome B transgenic expression have not been reported for tobacco. Also, a phenotype associated with phytochrome C-overexpression has not yet been established.

This chapter focuses on the tranformation and molecular characterisation of DN and SD *N. tabacum* cv. Hicks, with oat phytochrome A cDNA, *Arabidopsis* phytochrome B cDNA and *Arabidopsis* phytochrome C gDNA, each under the control of the CaMV 35S promoter.

4.2 Experimental procedures

4.2.1 Transformation of DN and SD *N. tabacum* with full-length clones of phytochromes A, B and C

Nicotiana tabacum cv. Hicks with different day length sensitivities were obtained from Vern Sisson, USDA, Oxford, USA. The DN and SD genotypes differ with respect to a single locus, *MARYLAND MAMMOTH* (*MM*) which confers SD photoperiodicity. The SD genotype was created by backcrossing the *MM* allele in *N.tabacum* cv. Maryland Mammoth into the DN cv. Hicks (Vern Sisson pers. comm.), therefore, except at the *MM* locus, DN and SD *N. tabacum* are isogenically identical.

Both DN and SD *N. tabacum* were transformed with oat phytochrome A,

Arabidopsis phytochrome B and *Arabidopsis* phytochrome C DNA clones. The oat phytochrome A construct, (contained in pFY122), comprises a hybrid of "type 5" and "type 3" cDNA clones that differs from the type 3 polypeptide by just 3 amino acid sequences (Rogers *et al.*, 1989). The *Arabidopsis* phytochrome B fragment was generated by PCR from a phytochrome B clone in lambda EMBL3 (Somers *et al.*, 1991). The *Arabidopsis* phytochrome C fragment comprises a full-length gDNA clone isolated from a genomic DNA library in lambda fix (Cowl *et al.*, 1994). The phytochrome A, B and C gene constructs were each cloned into the vector pROK2 to produce the integrated vectors pROKA, pROKB and pROKC, respectively. Each of these genes is positioned in the sense orientation and is under the control of the CaMV 35S promoter. The manipulation of constructs and cloning was carried out by Dao-Xing Xie at Leicester University. The pROK2 vector carries the dominant marker gene which encodes neomycin phosphotransferase-II (*npt-II*), confering kanamycin resistance. The pROK A, B and C vectors function were transformed into the *Agrobacterium tumefaciens* strain LBA4404.

Transformation of plant material was achieved by co-cultivation of leaf discs with *Agrobacterium.* Adventitious shoot and root production was stimulated by placing the leaf discs on MSD x 4 (MSO plus napthalene acetic acid 0.1 mg l^{-1} and 6-benzylaminopurine 1.0 mg l^{-1}) medium to promote shoot development, followed by a transfer to MSO medium to encourage root development. All media preparations contained kanamycin (100 $\mu g\ ml^{-1}$) to favour the growth of transformed explants. Futher details of the transformation procedure and details of the growth media used are contained in Draper *et al.* (1988). After rooting, a number of primary transformants were selected and transferred to soil.

4.2.2 Selection of *N. tabacum* lines overexpressing phytochromes A, B and C

Seed (T1) from the primary transformants sown on kanamycin MSO medium displayed kanamycin resistance ratios of approximately 3:1, 15:1 and 63:1 (resistant:sensitive). Kanamycin resistance at these frequencies provided the potential for selection of transgenics with insertions at one, two or three independently segregating loci. Segregating populations were also examined with respect to hypocotyl growth inhibition under R and/or FR monochromatic light conditions. Lines homozygous for the transgene were selected from T2 generation plants that produced seed with 100% kanamycin resistance.

4.2.3 DNA extraction and PCR analysis

Genomic DNA was extracted from 0.25 g fresh weight of *N. tabacum* leaf material when the plants were at the 2-4 leaf stage. The tissue was then placed in an Eppendorf tube and ground to a slurry in 0.5 ml of 2 x CTAB isolation buffer (100 mM Tris HCl, 1.4 M NaCl, 20 mM EDTA, 2% hexadecyltrimethyl ammoniumbromide, pH 8.0). This was incubated at 60°C for 20 min. An equal volume of 0.5 ml chloroform:isoamylalcohol (24:1) was then added and the tube was mixed gently until no further extraction of the pigment from the aqueous layer was observed (approx. 5 min). Following 10 min centrifugation the aqueous phase was transferred to a fresh tube and DNA was precipitated with 2 volumes of ice-cold ethanol, pelleted and resuspended in 300 µl of water. RNase was added to a final concentration of 5 $\mu g\ ml^{-1}$ and the mixture was incubated for 20 min at room temperature. The DNA sample was extracted three times with phenol/choroform. The DNA was then precipitated with 1/5 th volume sodium acetate (4M) and 2 x volume of ethanol at -80°C and spun down. Finally the pellet was washed with 70% ethanol, dried and resuspended in 50 µl of water.

Presence of the transgene in the selected lines was confirmed using PCR amplification. Genomic DNA extracts from wild type plants were used as the negative controls, and plasmids containing the oat phytochrome A (pFY122), and the *Arabidopsis* phytochrome B (pBSB) and C (pBSC) transgenes, were used as positive controls. A 20 µl PCR reaction mixture comprised 2 µl of 10 x PCR buffer (Promega), 2 µl of 25 mM $MgCl_2$ (Promega), 2 µl of 2 mM dNTPs, 2 µl of DNA extract, 0.5 µl of each of the primers (100 ng μl^{-1}), 0.5 µl of Taq DNA polymerase (Promega, 5 units μl^{-1}) and 10.5 µl of water. PCR was then performed in a thermocycler for 30 cycles of 91°C for 1 min, 57°C for 1 min, 72°C for 1 min, plus another 5 seconds per cycle. Primers were designed from the sequences of oat phytochrome A (AP3) cDNA (Hershey *et al.*, 1985), *Arabidopsis* phytochrome B cDNA (Sharrock and Quail, 1989), and *Arabidopsis* phytochrome C gDNA (Cowl *et al.*, 1994, EMBL accession number Z32538). The oat phytochrome primers comprised OA1, 5' CGCCTTCTGGCTATCA GATG 3' (positioned at 1473-1494) and OA2, 5' CGAGGAAGCATTGCTACTGT 3' (positioned at 2468-2487). The *Arabidopsis* phytochrome B primer pair were B3, 5' GCTTGTTCCAGCAAGGACTACT 3' (positioned at 2340-2361) and B4, 5' GCT GCTATGGAACATGTCT 3' (positioned at 3416-3398). The *Arabidopsis* phytochrome C primers were PHYC5, 5' ATGTCATCGAACACTTCACGAAG 3' (positioned at 3348-3370) and CST30, 5' GGAGCTCTCAAAGTAGATCCAG 3' (positioned at 4270-4290).

4.2.4 Protein extraction and immunoblot analysis

Protein was extracted from etiolated seedlings or from light-grown tissue harvested from plants either at the 2 or the 5 leaf stage. Tissue used for immunoblot analysis of the oat phytochrome A transgene was taken from light-grown plants that had been dark-adapted for 3 d prior to extraction. Plant tissue was homogenised using a pestle and mortar with 1 ml g^{-1} of extraction buffer, (50% ethanediol, 100 mM Tris HCl, 150 mM $(NH_4)_2$ SO_4, 10 mM EDTA, pH 7.9) with a reducing agent, 60 mM

Na_2SO_3, and protease inhibitors (2 μg ml^{-1} aprotinin, 1 μg ml^{-1} pepstatin, 2 mM PMSF and 10 mM iodoacetate). After addition of an equal volume of water, polyethyleneimine (10 μl ml^{-1}) was then added to the sample which was mixed thoroughly. After centrifugation for 20 min, the supernatant was poured on to finely powdered $(NH_4)_2SO_4$ (0.25 g ml^{-1}) and gently shaken for 20-30 min at 4°C to precipitate proteins. Following centrifugation, the pellet was then dissolved (50 μl per ml of supernatant) in resuspension buffer (0.5 x extraction buffer plus Na_2SO_4 and protease inhibitors at the same concentrations). At this point a protein concentration assay was carried out using the microplate assay protocol in the DC Protein Assay Kit (Bio-Rad). A third volume of 3 x SDS sample buffer was then added to each sample which was heated at 100°C for 2 min before placing in ice.

Equal amounts of protein (100 μg) were separated on a 9% SDS-acrylamide gel and electroblotted on to nitrocellulose membrane (Hybond-C, Amersham International, Amersham, Bucks., UK). Uniformity of protein loadings was confirmed by total protein staining of blots with 0.2% Ponceau in 3% trichloroacetic acid. Phytochrome bands were detected by the incubation of blocked blots with anti-phytochrome monoclonal antibodies. The oat phytochrome A was stained by the mouse monoclonal antibody LAS41-mouse IgG (1:200 dilution), raised against purified phytochrome A from etiolated oats (Holdsworth, 1987). In immunoblots with LAS41, 50 ng of purified oat phytochrome A was used as a positive control. *Arabidopsis* phytochrome B and phytochrome C were detected by the monoclonal antibodes B1, B7, B8 (1:1000 dilution) and C1, C11 and C13 (1:500 dilution) (Somers *et al.*, 1991). These antibodies were raised in mice and are selective for phytochromes B and C (Somers *et al.*, 1991). For blots identifying phytochrome B, the antibodies were either used together or B7 was used on its own as it was found to have a more specific affinity for *Arabidopsis* phytochrome B. Bands were visualised by secondary incubation with alkaline-phosphatase-conjugated goat anti-mouse-immunoglobulin antibodies and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) staining.

4.2.5 Growth conditions

For the growth of seedlings under R and FR light seeds were sown in 9 cm Petri dishes on 0.8% (w/v) agar containing MS mineral salts (Murishige and Skoog, 1962) and chilled at 4°C for 5 d. The seeds were stimulated to germinate by a 15 min white light treatment on day 5 and grown for a further day in darkness. The seedlings were then transferred to continuous R or FR light.

In experiments with light-grown plants, seeds were sown in 9 cm Petri dishes on 0.8% (w/v) agar containing MS and chilled for 5 d at 4°C. Seeds were germinated and grown in continuous W light (photon fluence rate 144 μmol m^{-2} s^{-1} in the range of 400-700 nm). Seedlings were then selected for uniformitiy and transferred to 3 cm pots containing a peat compost:horticultural silver sand (3:1) mixture. The temperature was maintained between 22 and 24°C in all experiments.

4.2.6 Measurement of hypocotyl length

Hypocotyl lengths were determined from calibrated projected photographic slides. This was achieved by measuring the projected images with a ruler and scaling the measurements down.

4.2.7 Light sources

In experiments designed to assess the hypocotyl growth inhibition in transgenic seedlings, narrow band R and FR light was used. Narrow band R light was obtained from light emitting diodes (LEDs) supplied by Hansatech (Norwich, UK). The R LEDs have an emission maximum at 665 nm and provide a fluence rate of 12 μmol m^{-2} s^{-1} at the seedling level. The FR light was obtained from FR-emitting LEDs (Quantum

Devices, Inc. Barneveld, WI. USA) with an emission maximum of 730 nm and a fluence rate of 25 μmol m^{-2} s^{-1} at seedling level.

De-etiolated plants were grown in Fitotron 600 growth cabinets (Fisons Scientific Apparatus, Loughborough, UK). Continuous light was provided by a bank of cool-W fluorescent tubes (photon fluence rate 400-700 nm 144 μmol m^{-2} s^{-1}).

4.3 Results

4.3.1 T1 seedlings in the phytochrome A and phytochrome B transgenic lines segregate for reduced hypocotyl length under R and FR light, respectively

Oat phytochrome A cDNA, *Arabidopsis* phytochrome B cDNA and *Arabidopsis* phytochrome C gDNA, each under the control of the CaMV 35S promoter, were introduced into SD and DN *N. tabacum*. First generation, T1 seedlings, segregated for kanamycin resistance as the selective marker is linked to each of the phytochrome transgenes. The T1 populations segregating at 3:1 (resistant:sensitive) on kanamycin displayed a range of hypocotyl lengths. This was most prominent in the lines arising from transformations with phytochromes A or B, when grown under FR or R light, respectively (Figs. 4.1 and 4.2). The range in hypocotyl lengths in each T1 population could be roughly divided into three classes: long, medium and short hypocotyls in the ratio 1:2:1. It is possible that this ratio reflects the number of wild type:heterozygous transgene:homozygous transgene seedlings expected from the To selfing (Figs. 4.1 and 4.2). Progeny of phytochrome C primary transformants segregating for kanamycin resistance at a ratio of 3:1, did not demonstrate this obvious range of hypocotyl lengths (data not shown). This may be a reflection of transgenic phytochrome C expression levels, or a small role for phytochrome C in the control of hypocotyl growth.

4.3.2 T2 seedlings homozygous for the phytochrome C transgene display inhibition of hypocotyl elongation under R

Several lines, homozygous for phytochrome transgenes were selected from T2 seedlings that displayed 100% kanamycin resistance. Seedlings in T2 lines expressing the phytochrome C transgene appear to display enhanced sensitivity with respect to inhibition of hypocotyl when grown under R light. Hypocotyls of the DN lines DN C1, DN C2 and DN C4, and the SD lines SD C1, SD C3 and SD C2 are shorter than those of wild type seedlings by small, but significant amounts (Fig. 4.3). In addition, hypocotyl length of seedlings in the different phytochrome C-overexpression lines falls between narrow range of values (Fig. 4.3). This may represent similar levels of transgenic phytochrome C in the seedlings.

4.3.3 Lines homozygous for the phytochrome A or the phytochrome B transgene display differential inhibition of hypocotyl elongation

Seedlings in the T2 generation, homozygous for either the phytochrome A or the phytochrome B transgene show an enhanced inhibition of hypocotyl growth when compared to wild type plants when grown under FR and R, respectively (Figs. 4.4 and 4.5). Furthermore, different transgenic lines exhibit this growth inhibition to differing degrees. Under FR, seedlings in the DN phytochrome A transgenic lines DN A1, DN A4 and DN A3 display weak, intermediate and strong inhibition of hypocotyl elongation, respectively (Fig. 4.4). Likewise, the SD lines SD A7, SD A19 and SD A4 demonstrate a similar range of hypocotyl growth inhibition under FR (Fig. 4.4). Seedlings in the SD A7 line display the weakest and SD A4 seedlings display the strongest inhibition of hypocotyl elongation. Under R light, the DN phytochrome B transgenic line DN B7 exhibits a moderate inhibition of hypocotyl elongation, whereas DN B5 has a more marked inhibition response (Fig. 4.5). Again , seedlings in the SD lines SD B2, SD B13 and SD B5 display weak, intermediate and strong inhibition of hypocotyl growth, respectively, when grown under R (Fig. 4.5).

4.3.4 Hypocotyl inhibition is linked with transgene copy number in transgenic lines expressing phytochrome A or B

The transgenic lines DN A3, SD A4 , DN B5 and SD B5 not only demonstrate the most severe inhibition responses in each series, but they also may represent lines with multiple, unlinked insertions of the transgene. Segregation for kanamycin resistance in the T1 generation from the lines DN A3, SD A4 and SD B5, is consistent with insertions at two independent loci (Fig. 4.6). The T1 generation of the line DN B5 segregate at a ratio expected from insertions at three independent loci (Fig. 4.6). In contrast, the transgenic lines displaying either weak or intermediate inhibition of hypocotyl elongation, in each series, represent single locus insertions of the transgene (Fig. 4.6).

4.3.5 Hypocotyl inhibition is not linked with transgene copy number in transgenic lines expressing phytochrome C

The range in hypocotyl length displayed by transgenic seedlings expressing phytochrome A or B is not seen in seedlings expressing transgenic phytochrome C. However, these seedlings also represent lines that were selected as single and double locus insertions in the T1 generation (Fig. 4.6). Seedlings in the DN C1 and DN C2 lines have single locus transgene insertions. Kanamycin selection ratio in the T1 generation of the SD C1 and SD C3 lines is indicative of transgene segregation at two loci. Interestingly, the SD C2 T1 seedlings segregate at a ratio of 30:1. This may either represent the presence of two gene loci, or the presence of three gene loci with linkage between two of them. The T1 generation seedlings in the DN C4 line segregate at a ratio expected for transgene at three independent loci.

4.3.6 Presence of the phytochrome transgene is confirmed by PCR

Confirmation of the presence of the appropriate transgene in each of the lines was achieved using PCR (Figs. 4.7(a), (b) and (c)). For the oat phytochrome A transgene, internal primers amplified a 1014 bp fragment in the DN A1, DN A4, DN A3, SD A7, SD A19 and SD A4 transgenic lines (Fig. 4.7(a)). The PCR fragment is also amplified from the transgene fragment in pFY122 (+ve), but is not amplified from endogenous phytochrome genes in either DN or SD wild type genomic DNA extracts (Fig. 4.7(a)). Likewise, *Arabidopsis* phytochrome B transgene internal primers amplified a 1058 bp fragment in the positive control (pBSB) and in the DN B7, DN B5, SD B2, SD B13 and SD B5 lines, but not in wild type plants (Fig. 4.7(b)). Transgene presence in lines expressing the phytochrome C transgene is also confirmed by the PCR amplification of a 942 bp fragment in the DN C1, DN C2, DN C4, SD C1, SD C3 and DN C2 transgenic lines (Fig. 4.7(c)). Primers, that are homologous to fragments within the *Arabidopsis* C gDNA sequence demonstrate PCR amplification of a fragment in the pBSC (+ve), but not in wild type plants (Fig. 4.7(c)).

4.3.7 Increased inhibition of hypocotyl elongation correlates with increased levels of transgene product

Anti-phytochrome monoclonal antibodies have been produced that selectively detect oat A phytochromes (Holdsworth, 1987), *Arabidopsis* phytochrome B or *Arabidopsis* phytochrome C (Somers *et al.*, 1991). The oat phytochrome A-specific monoclonal antibody (LAS41) which readily recognises purified oat phytochrome A and does not cross-react with tobacco phytochrome A from wild type seedlings (Fig. 4.8). LAS41 also fails to recognise transgenic protein in the DN A1 and SD A7 seedlings, but does detect oat phytochrome A in the DN A4, DN A3, SD A19 and SD A4 seedlings. For these assays protein was extracted from etiolated or dark-adapted tissue to maximise phytochrome A content. It is significant that seedlings in the lines with detectable transgenic protein also display either intermediate or severe hypocotyl

growth inhibition (Figs. 4.4 and 4.8). Seedlings where LAS41 failed to recognise the transgenic protein display only a mild inhibition of hypocotyl elongation (Figs. 4.4 and 4.8). This suggests that the transgene product is present in these tissues, but at levels below the limit of detection by LAS41.

For analysis of *Arabidopsis* phytochrome B levels a combination of B1, B7 and B8 monoclonal antibodies detect the total amount of phytochrome B expressed in wild type and transgenic lines. The monoclonal antibody B7, however, reacts specifically with *Arabidopsis* phytochrome B and does not cross-react with wild type phytochrome B. Immunoblots using both a combination of monoclonal antibodies, and B7 on its own, have provided useful information on transgene protein levels. Immunoblots using all three antibodies demonstrate that plants in all the transgenic lines have higher levels of phytochrome B than wild type plants (Fig. 4.9(a)). The most marked increase in transgenic protein level is seen in the lines that also display moderate or severe inhibition of hypocotyl elongation (DN B7, DN B5, SD B13 and SD B5) (Figs.4.5 and 4.9). This trend is also seen when immunoblots are stained with B7, which can detect phytochrome B in the transgenic lines DN B7, DN B5, SD B13 and SD B5 (Fig. 4.9(b)). Immunoblots for etiolated (data not shown) and light-grown seedlings are indistinguishable from each other.

The monoclonal antibodies C1, C11 and C13 when used together have a specificity for *Arabidopsis* phytochrome C and, therefore, do not cross-react with wild type tobacco phytochrome C (Fig 4.10(b)). Immunoblots show a reaction with phytochrome C antigen in each of the transformed lines (DN C1, DN C2, DN C4, SD C1, SD C3 and SD C2), confirming transgene overexpression. Immunoblots carried out on tissue extracts from etiolated, or young, light-grown seedlings expressing transgenic phytochrome C also demonstrate a correlation between transgene expression and a decrease in hypocotyl length (Figs. 4.3 and 4.10(b)). A similarity in the levels of detectable phytochrome C apoprotein in these seedlings could account for the similarity in hypocotyl growth inhibition.

4.3.8 Transgene product levels vary during plants development

In some of the transgenic lines there is evidence for developmentally-linked changes in transgene expression level. The oat phytochrome A (DN A3) and *Arabidopsis* phytochrome C (DN C4) lines show the most dramatic changes. Immunoblots of etiolated seedling extracts in both of these lines demonstrate a relatively high level of transgenic protein (Fig. 4.8(a)). Seedlings in the DN A3 line also demonstrate a marked inhibition of hypocotyl elongation which may also reflect the high transgene product level at this stage (Fig. 4.4). However, immunoblots of plant extracts harvested at the 5 leaf stage reveal a lower relative level of protein (Fig. 4.8(c)). This apparent reduction in transgenic protein is quite striking when compared to the transgenic phytochrome A levels of the DN A4 extract on the same blot (Fig. 4.8(c)). Therefore, it would seem that unlike DN A3 seedlings, DN A4 seedlings maintain a high level of expression of transgenic protein in light-grown plants. This also appears to be the case for the SD A19 and SD A4 lines, where transgene product is detectable in seedlings at the same growth stage (Fig. 4.8(d)).

In a similar fashion to DN A3, transgenic protein levels fall off as the plant matures in the DN C4 line as well. Immunoblots of tissue extracted from DN C4 seedlings at the two leaf stage reveal the presence of the protein, although at a lower level than in etiolated tissue (Fig. 4.10(a) and (b)). By the 5 leaf stage the transgenic protein has fallen to such a level that it escapes detection (Fig. 4.10(c)). Thus, in the transgenic lines DN A3 and DN C4 expression of the transgene-encoded protein appears to be developmentally regulated. In both cases a there is a reduction in transgene expression in the adult plant.

In summary, the transgenic lines selected in the DN and SD *N. tabacum* represent allelic series of transformants overexpressing oat phytochrome A, *Arabidopsis* phytochrome B and *Arabidopsis* phytochrome C. Seedlings overexpressing phytochrome A display increased hypocotyl elongation inhibition when under FR and the extent of inhibition is related to the level of transgenic protein. Seedlings overexpressing phytochrome B exhibit enhanced hypocotyl elongation inhibition when grown under R. The severity of the inhibition response also correlates

with increased transgenic protein levels. The most severe phytochrome A- and phytochrome B-overexpression lines are also thought to represent multiple loci insertions of the transgene.

Seedlings overexpressing phytochrome C also demonstrate increased hypocotyl elongation inhibition, when grown under R. This inhibition in hypocotyl growth is relatively small, but significant, which may either reflect the level of transgene expression or the minor role that phytochrome C has in this particular response. The transgenic phytochrome C DN and SD allelic series represent single and multicopy transgene insertions.

Finally, the plants in the DN A3 and DN C4 lines do not maintain an even level of transgenic protein, but one that falls after the seedling stage. Therefore, both these lines appear to demonstrate developmentally-linked regulation of transgene-encoded protein levels.

4.4 Discussion

The introduction of heterologous phytochromes, each under the control of the CaMV 35S promoter, leads to an overexpression of transgenic apoproteins that correlates with a short-hypocotyl phenotype. This indicates that these transgenically expressed apoproteins attach the chromophore and are biologically active. The selected lines with introduced oat phytochrome A cDNA, *Arabidopsis* phytochrome B cDNA and *Arabidopsis* phytochrome C gDNA represent allelic series in both DN and SD *N. tabacum.*

Presence of the phytochrome transgene has been confirmed with PCR amplification and expression of the transgene-encoded protein has been confirmed with immunoblotting and physiological analysis for several transgenic lines expressing phytochromes A, B and C. The hypocotyls of seedlings in each of the lines expressing heterologous phytochrome exhibit an enhanced sensitivity to light when de-etiolating. Seedlings overexpressing the oat phytochrome A transgene, when grown under FR light, display a greater hypocotyl growth inhibition than wild type seedlings grown

under the same conditions. This increased sensitivity to hypocotyl growth inhibition by FR is consistent with previous reports on hypocotyl responses in *Arabidopsis* and tobacco expressing heterologous phytochrome A (Boylan and Quail, 1991; McCormac *et al.*, 1992 and 1993; Nagatani *et al.*, 1993). Furthermore, inhibition has been shown to have a fluence-rate dependency that is consistent with the action of phytochrome A in the FR HIR (Whitelam *et al.*, 1992). The antithesis is seen in the *Arabidopsis phyA* mutant and the tomato *fri* mutant, where hypocotyl growth is insensitive to continuous FR (Nagatani *et al.*, 1993; Parks and Quail, 1993; Whitelam *et al.*, 1993, van Tuinen, 1995a). The monoclonal antibody LAS41, which has specificity for oat phytochrome A, is able to recognise the transgene product in the DN A4, DN A3, SD A19 and SD A4 lines. These are the lines that display the most marked hypocotyl growth inhibition responses. However, transgenic protein is not detected on immunoblots from extracts of seedlings in the lines displaying the weakest growth inhibition, (DN A1 and SD A7). Although the FR-stimulated short-hypocotyl phenotype is not striking in either DN A1 or SD A7, it is, nonetheless present. Thus, it is likely that the lack of transgene product immunoblot staining reflects a transgene product level that falls below the limit of detection.

Transgenic lines that overexpress *Arabidopsis* phytochrome B have been identified using both a combination of monoclonal antibodies, B1, B7 and B8, and the use of B7 on its own. When used together, B1, B7 and B8 identify increased levels of phytochrome B in the transgenic seedlings when compared to wild type seedlings. The monoclonal antibody, B7 that is specific for *Arabidopsis* phytochrome B, selectively recognises the transgene product in immunoblots of the more severe overexpression lines, DN B7, DN B5, SD B13 and SD B5. Seedlings in all the lines overexpressing *Arabidopsis* phytochrome B, (DN B7, DN B5, SD B2, SD B13 and SD B5), exhibit enhanced inhibition of hypocotyl elongation when grown under R light. Observations such as these have also been made in *Arabidopsis* overexpressing *Arabidopsis* or rice phytochrome B cDNAs when grown under continuous R and/or W light (Wagner *et al.*, 1991; McCormac *et al.*, 1993; Wester *et al.*, 1994). In addition, this increased hypocotyl elongation inhibition under R has been shown to be fluence rate dependent in wild type and overexpression lines (McCormac *et al.*, 1993). These findings have led

to the suggestion that native phytochrome B may be operating in a R HIR mode in the de-etiolating seedling. The converse is seen in the *Arabidopsis phyB* mutants, that display an elongated phenotype in R or W (Koornneef *et al* ., 1980; Goto *et al.*, 1991; Reed *et al.*, 1993). This response is also observed in other species with deficiencies in phytochrome B-like or light-stable phytochromes. Examples include the *lh* mutant of cucumber, (López *et al.*, 1992), the *ein* mutant of *Brassica rapa* (Devlin *et al.*, 1992), the ma_3^R mutant of sorghum (Childs *et al.*, 1991, 1992) and the *tri* mutant of tomato (van Tuinen *et al.*, 1995b).

Lines overexpressing *Arabidopsis* phytochrome C have been identified using a combination of C1, C11 and C13 monoclonal antibodies that selectively recognise the *Arabidopsis* phytochrome C protein. These lines are of particular interest as, to date, phytochrome C-overexpression has not been reported. Seedlings overexpressing phytochrome C, like seedlings overexpressing phytochrome B, display increased hypocotyl growth inhibition when grown under continuous R light. It could, therefore, be speculated, that the increased sensitivity to R light in the newly germinated seedling, conferred by phytochrome C-overexpression, may reflect the function of native phytochrome C in the de-etiolation response to R light. It may be the case that phytochromes A, B and C have similar roles to play in this respect. Alternatively, as the effect on hypocotyl inhibition is only small, phytochrome C may not regulate hypocotyl elongation growth in a direct manner. Phytochrome C-overexpressors produce enlarged cotyledons (see chapter 5). Thus, it is possible that phytochrome C action promotes cotyledon expansion and seedlings with larger cotyledons are more "robust" and, therefore, have somewhat shorter, thicker hypocotyls.

Although increased phytochrome expression correlates with increased hypocotyl inhibition in lines expressing the phytochrome A, B or C transgenes, this effect is most striking in seedlings overexpressing phytochrome A or B. Seedlings in each phytochrome A or B transgenic lines display inhibition of hypocotyl growth to an extent that corresponds with transgene expression level. The dose-linked severity of phenotype is also very clearly seen in the segregating T1 populations. Selfing of the primary phytochrome A or B transformants produces seedlings that segregate for long medium and short hypocotyl length at ratios representative of wild type homozygous,

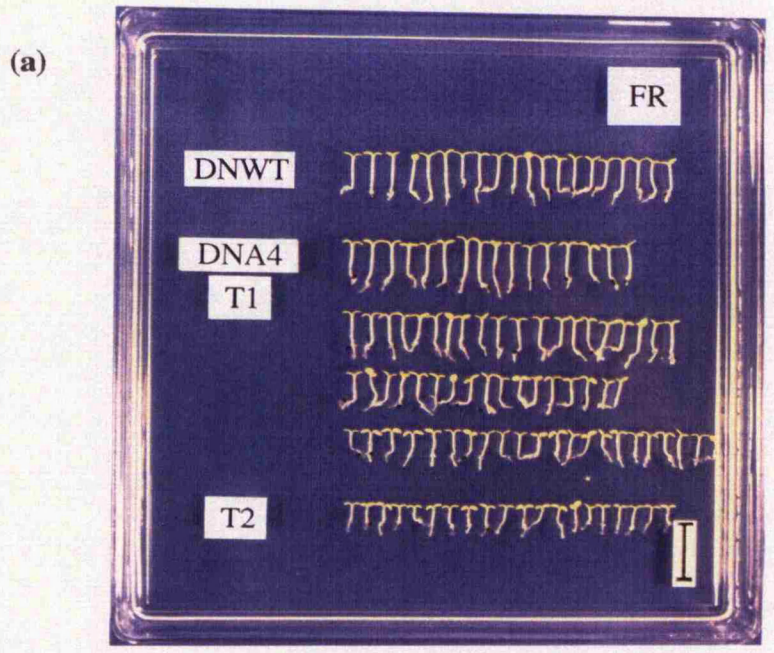
transgene heterozyous and transgene homozygous segregants. This gene dosage effect of phenotype is also seen in *Arabidopsis* wild type seedlings and seedlings either heterozyygous or homozygous for the *phyA* and *phyB* mutations (Koornneef *et al.*, 1980; Whitelam *et al.*, 1993). The heterozogotes, (*phyAPHYA* and *phyBPHYB*) have hypocotyl lengths that are intermediate between those of the homozygous mutant, (*phyAphyA* and *phyBphyB*) and the wild type, (*PHYAPHYA* and *PHYBPHYB*). This gene dosage dependence indicates there is a close relationship between phenotype and the abundance of the gene product. A strong correlation between transgene-encoded protein levels and severity of phenotype has also been reported in *Arabidopsis* expressing oat phytochrome A (Boylan and Quail, 1991; Whitelam *et al.*, 1992), and in *Arabidopsis* expressing rice and *Arabidopsis* phytochrome B (McCormac *et al.*, 1993). Other work demonstrating transgenic complementation of *hy3* (*phyB*) by the introduction of a phytochrome B minigene, also links hypocotyl growth inhibition to phytochrome levels in *phyB*, wild type and phytochrome B-overexpressors (Wester *et al.*, 1994).

The obvious range of differences in hypocotyl length in seedlings overexpressing phytochrome A or phytochrome B is not seen in transgenics overexpressing phytochrome C. Although increased levels of phytochrome C do appear to enhance the sensitivity of the seedling to R, the extent of inhibition is not as marked as in seedlings with increased levels of phytochrome A or B. This may be a reflection of the relative amounts of transgenic phytochrome or may reflect a more minor role in phytochrome C inhibition of hypocotyl elongation in this response to R light.

Immunoblots of extracts from seedlings at the etiolated, 2 leaf and 5 leaf growth stages indicate, for most transgenic lines, overexpression of the transgene is held at a fairly constant level over this period of growth. It must be noted that the presence of transgenic protein could not be measured in the DN A1 and SD A7 lines, as levels were not sufficient for detection. However, in the DN A3 and DN C4 lines there is evidence for developmentally-linked changes in transgenic protein levels. Transgene product levels appears to "fall-off" in both of these transgenic lines as the plants mature. Reduction in transgene expression has been linked to methylation of the introduced transgene or its promoter sequence (see Finnegan and McElroy, 1994; Meyer, 1995).

Furthermore, the frequency of transgene inactivation correlates with gene copy. Trans-inactivation of genes has been shown occur in instances when there is more than one copy of a T-DNA insertion with high sequence homology (Matzke *et al.*, 1989; Matzke and Matzke, 1990 and 1991). Although single copy transgenes can be inactivated, a higher frequency of transgene inactivation is associated with multiple copy insertions. It also appears that the relative chromosomal locations of the transgenes is an important factor in their epistatic interaction, as the degree of inactivation differs according to the transgene allelic positions (Matzke and Matzke, 1991). What is more, depending on the location of the transgene, expression may be influenced by developmental regulators. It is, therefore, perhaps no coincidence that the DN A3 and DN C4 lines which exhibit a developmentally-linked reduction of transgenic protein appear to have transgene insertions at two or three independent loci. There are obvious benefits from the selection of transformants with multiple T-DNA insertions as they tend to yield high transgene-encoded protein levels, however, temporal and/or spatial transgene inactivation should be expected in some transformed lines.

In summary, DN and SD *N. tabacum* lines transformed with oat phytochrome A cDNA, phytochrome B cDNA and phytochrome C gDNA are represented by allelic series with differential expression levels. Transgenic seedlings overexpressing phytochrome A grow with enhanced sensitivity to hypocotyl elongation inhibition under continuous FR. Seedlings expressing transgenic phytochrome B or C display increased hypocotyl growth inhibition when grown under R. All seedlings in the phytochrome C-overexpression lines appear to exhibit inhibition of hypocotyl elongation to about the same degree, which could be a reflection of similar levels of transgenic protein at this developmental stage. The extent of this response in seedlings overexpressing phytochrome A or B has been shown to correlate with levels of heterologous phytochrome. Finally, with the exception of DN A3 and DN C4 where transgenic proteins appear to be down-regulated in the light-grown plant, all testable transgenic lines demonstrate stable integration and expression of the transgene.



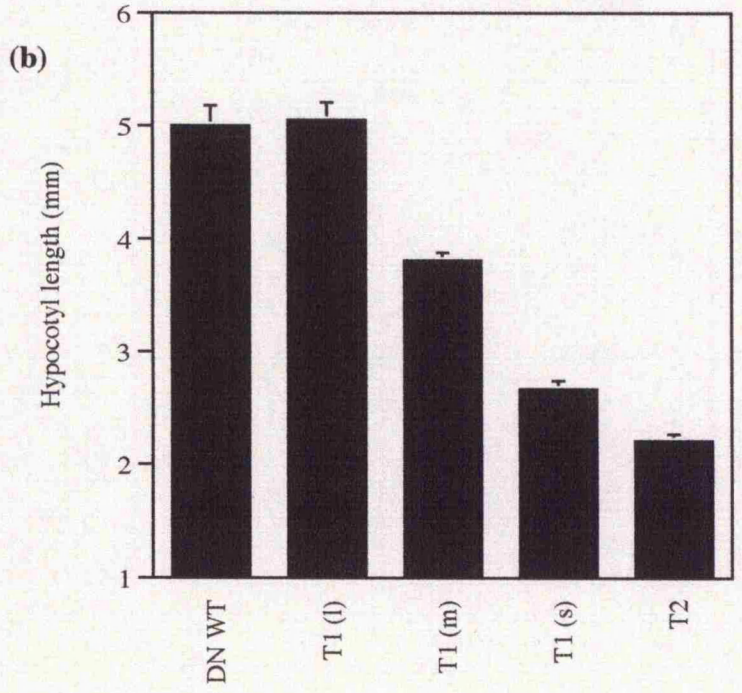
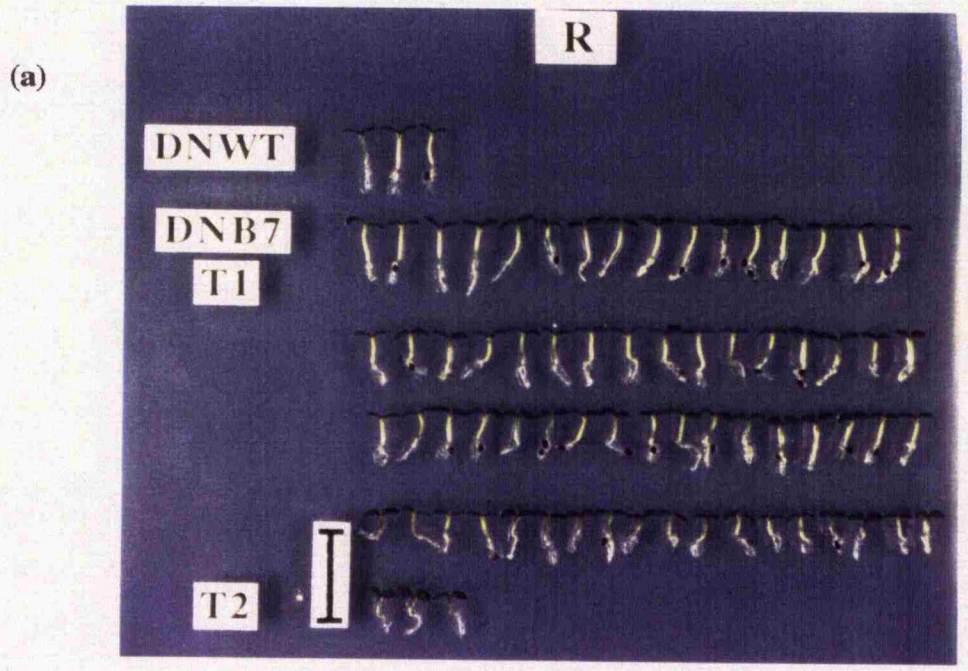


Figure 4.1 Phenotype **(a)** and hypocotyl length measurements **(b)** of DN WT, T1 segregating population, T1 long (l), T1 (m) and T1 short (s), derived from the To, DN A4 line, and T2 homozygous DN A4 seedlings, grown for 7 d under FR light. Data represent the means of over 16 seedlings and the error bars are SE.



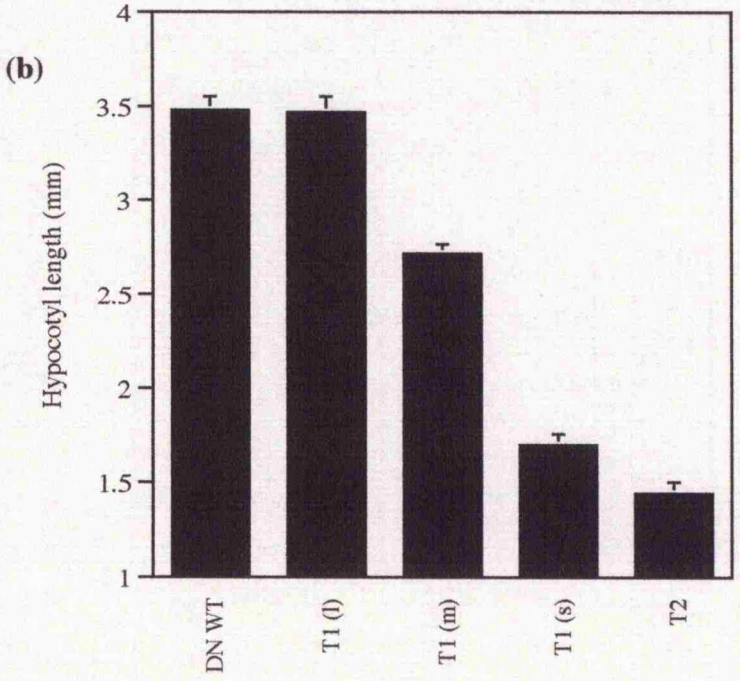


Figure 4.2 Phenotype (**a**) and hypocotyl length measurements (**b**) of DN WT, T1 segregating population, T1 long (l), T1 (m) and T1 short (s), derived from the To, DN B7 line, and T2 homozygous DN B7 seedlings, grown for 7 d under R light. Data represent the means of over 16 seedlings and the error bars are SE.

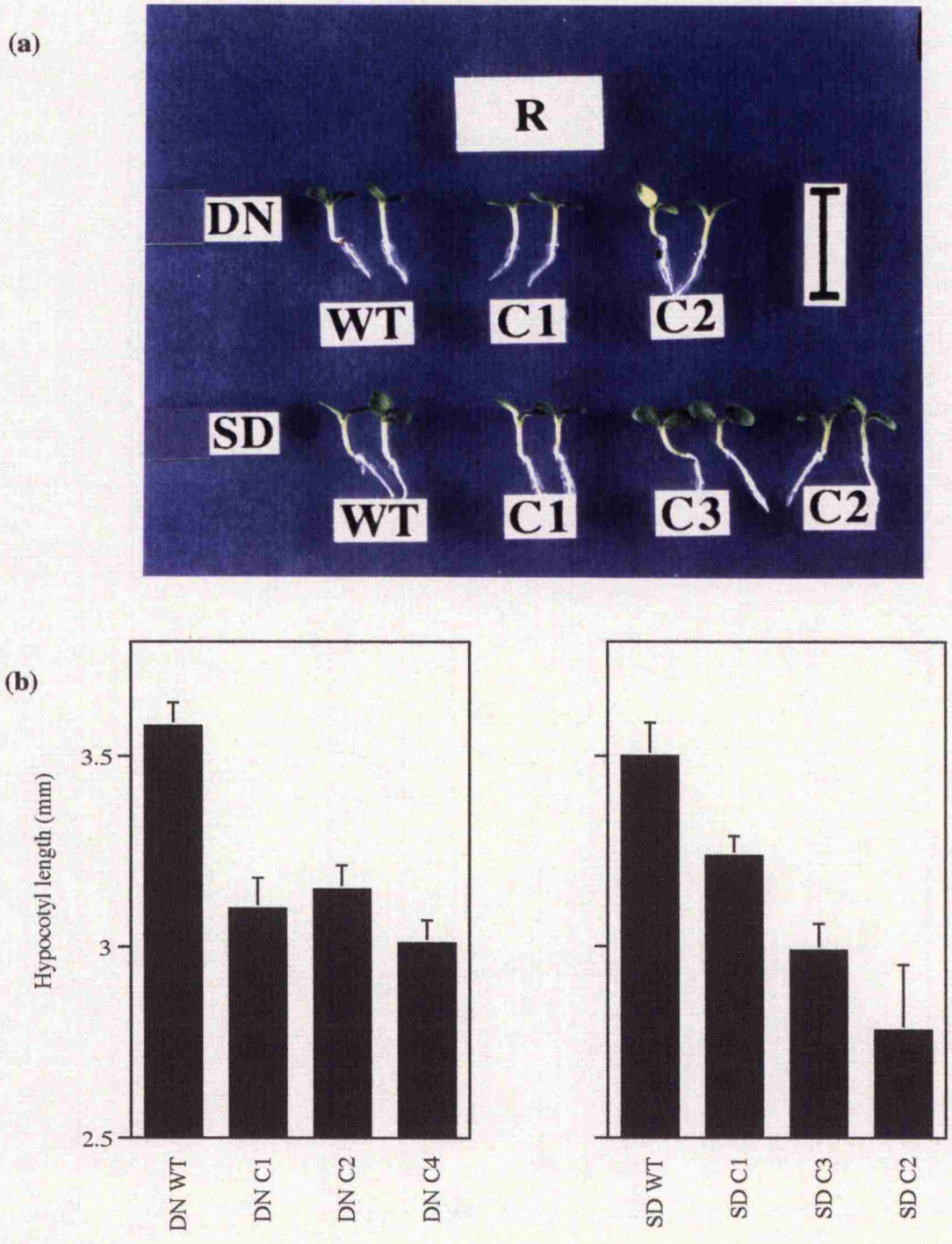


Figure 4.3 Phenotype (DN C4 not shown) **(a)** and hypocotyl length measurements**(b)** of DN WT, DN C1, DN C2, DN C4, and SD WT, SD C1, SD C3, SD C2 seedlings after 7 d growth under continuous R light. Data represent the means from 20 seedlings and the error bars are SE.

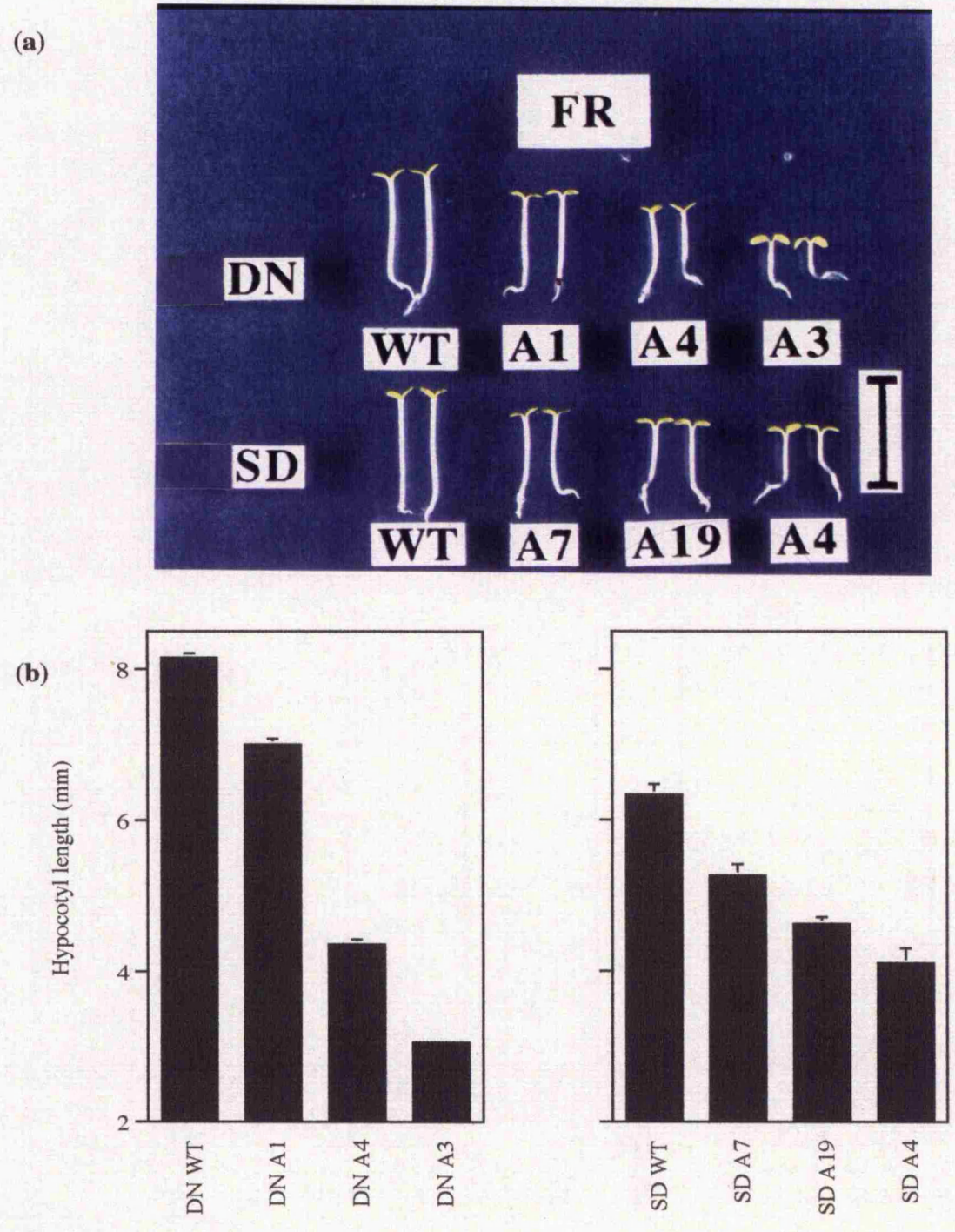


Figure 4.4 Phenotype (**a**) and hypocotyl length measurements (**b**) of DN WT, DN A1, DN A4, DN A3, and SD WT, SD A7, SD A19, SD A4 seedlings after 7 d growth under continuous FR light. Data represent the means from 20 seedlings and the error bars are SE.

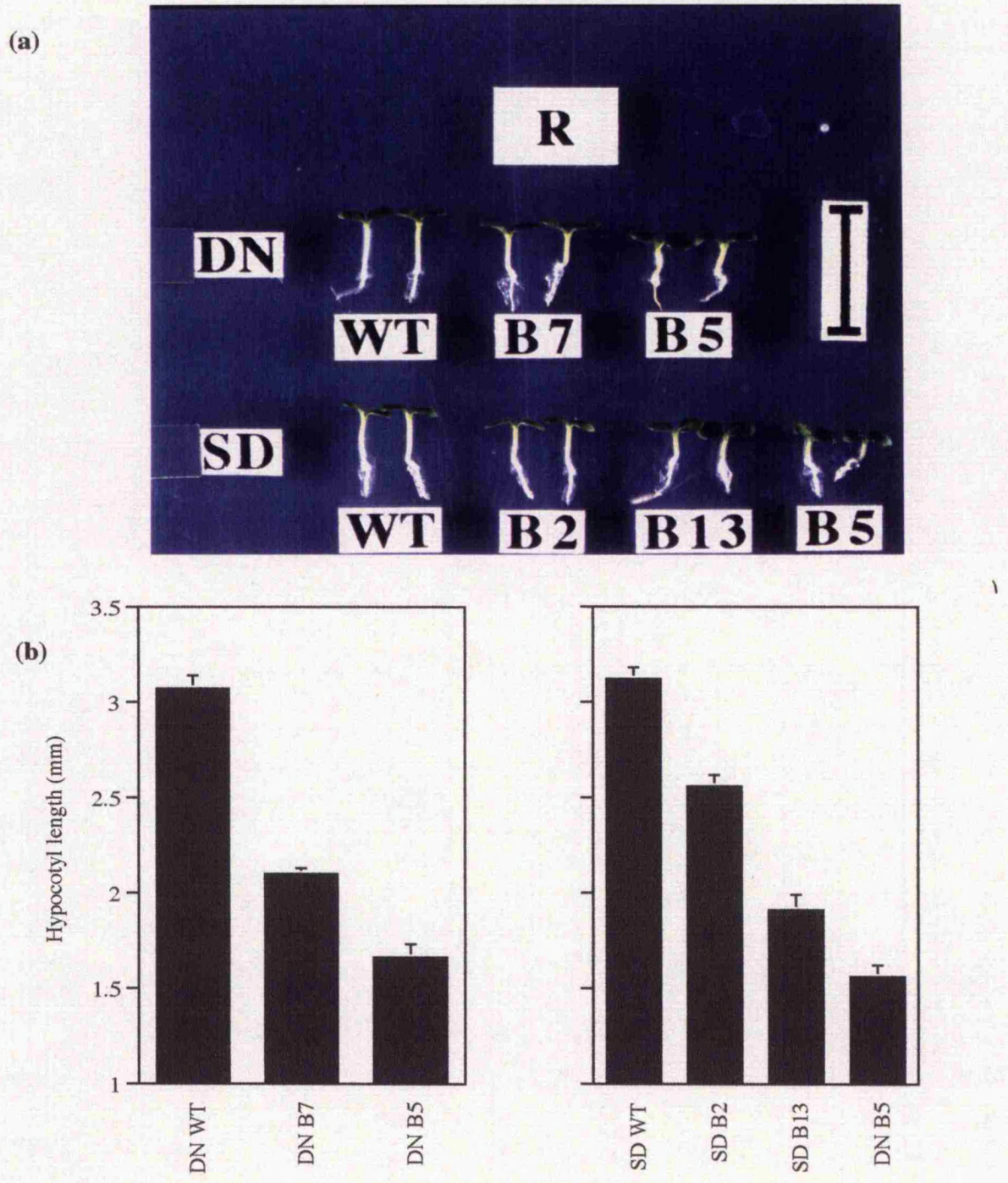


Figure 4.5 Phenotype **(a)** and hypocotyl length measurements **(b)** of DN WT, DN B7, DN B5, and SD WT, SD B2, SD B13, SD B5 seedlings after 7 d growth under continuous R light. Data represent the means from 20 seedlings and the error bars are SE.

Transgenic *N. tabacum* Line	T1 generation kanamycin segregation ratio (resistant:sensitive)
DN A1	3:1
DN A4	3:1
DN A3	13:1
SD A7	3:1
SD A19	3:1
SD A4	12:1
DN B7	3:1
DN B5	75:1
SD B2	3:1
SD B13	3:1
SD B5	17:1
DN C1	3:1
DN C2	3:1
DN C4	79:1
SD C1	10:1
SD C3	12:1
SD C2	30:1

Figure 4.6 Kanamycin segregation of the T1 generation of DN and SD transgenic lines overexpressing phytochrome A, B or C. Segregating populations represent over 200 seedlings.

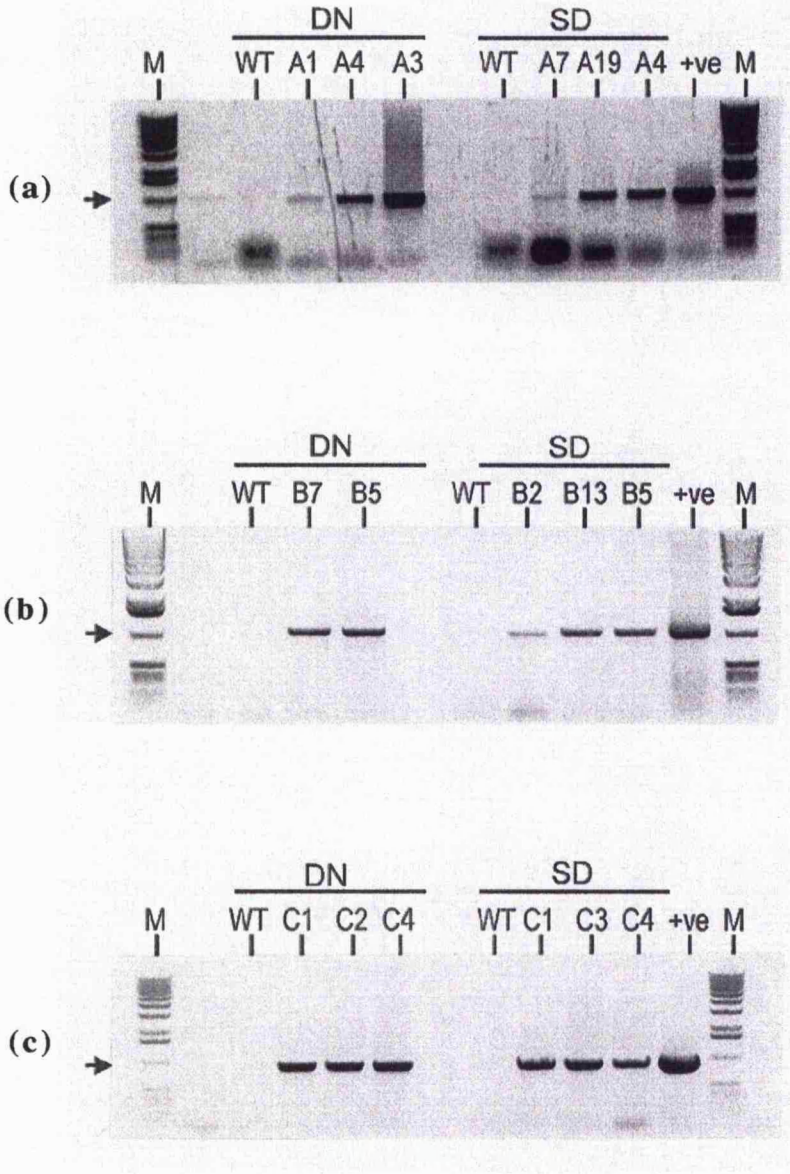


Figure 4.7 PCR amplification of transgene fragments from DN and SD *N. tabacum,* transformed with phytochrome A, B or C. **(a)** illustrates PCR amplification of an oat phytochrome A 1058 bp fragment in DN A1, DN A4, DN A3, SD A7, SD A19, SD A4 and pFY122 (+ve); **(b)** illustrates PCR amplification of an *Arabidopsis* phytochrome B 1014 bp fragment in DN B7, DN B5, SD B2, SD B13, SD B5 and pBSB (+ve); **(c)** illustrates PCR amplification of an *Arabidopsis* phytochrome C 942 bp fragment in DN C1, DN C2, DN C4, SD C1, SD C3, SD C2 and pFYBSC (+ve). No such fragments were obtained using wild type DNA as a template.

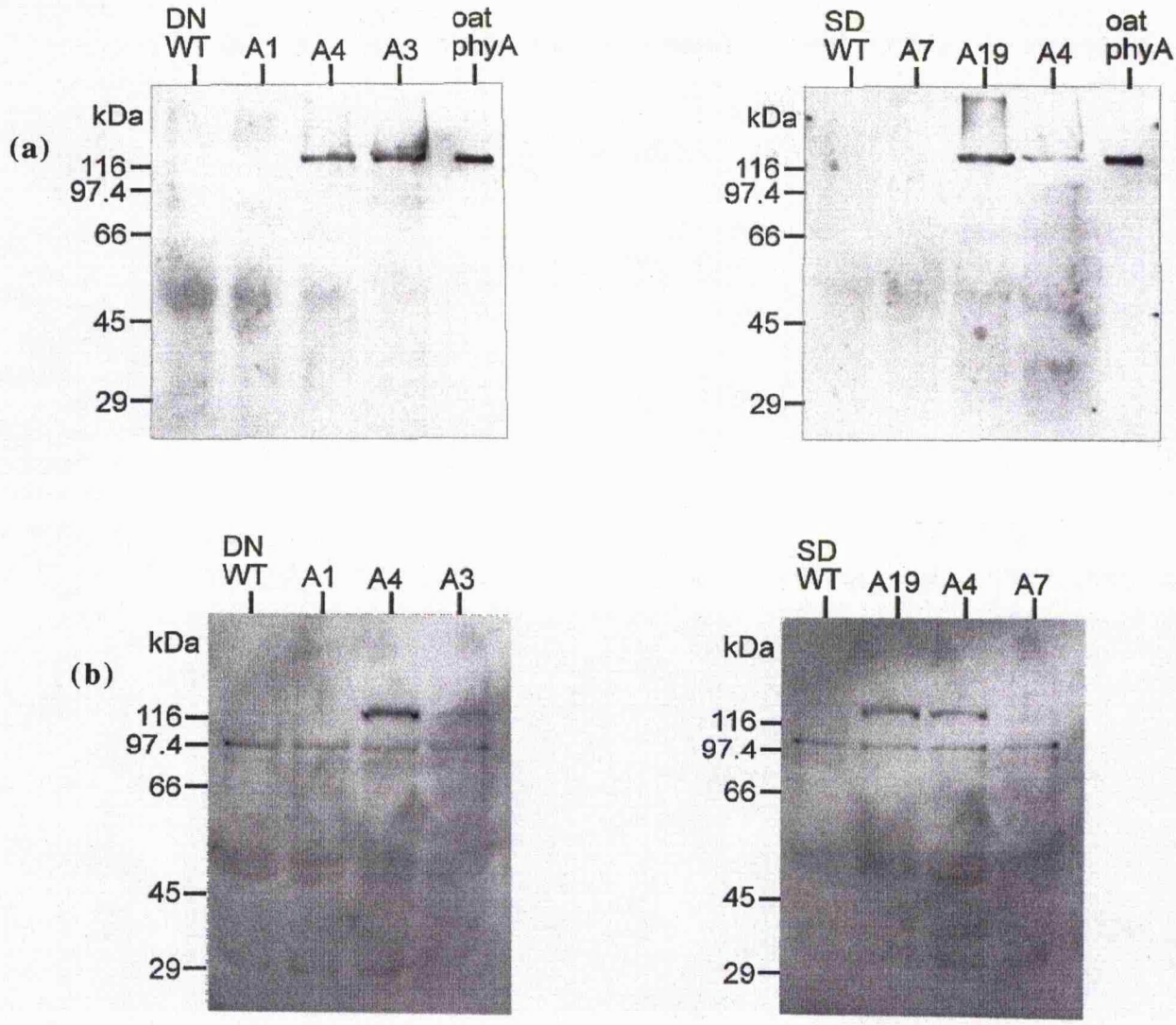
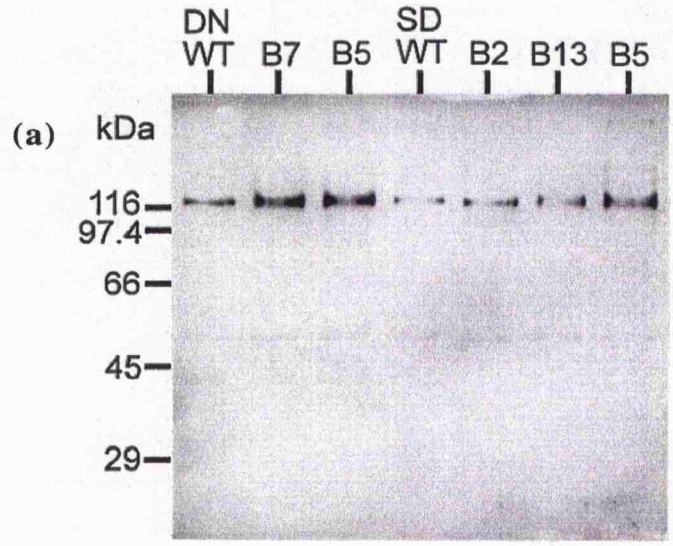


Figure 4.8 Immunoblot analysis of oat phytochrome A protein from DN A1, DN A4, DN A3, SD A7, SD A19, SD A4 seedlings homozygous for the transgene, and wild type *N. tabacum* seedlings. Crude protein extracts (100 μg per lane) were prepared from (**a**) 5 d old etiolated seedlings, or (**b**) light-grown plants at the 5 leaf stage that had been dark adapted for 3 d. All blots were probed with the monoclonal antibody LAS41.



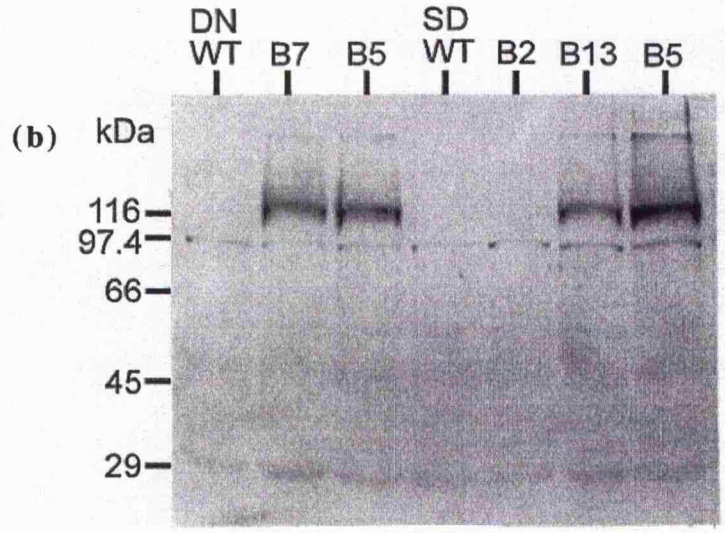


Figure 4.9 Immunoblot analysis of *Arabidopsis* phytochrome B protein from DN B7, DN B5, SD B2, SD B13, SD B5 seedlings homozygous for the transgene, and wild type *N. tabacum* seedlings. Crude protein extracts (100 μg per lane) were prepared from light-grown plants at the 5 leaf stage. The blots were either probed with a combination of the monoclonal antibodies, B1, B7 and B8 (**a**), or just B7 (**b**).

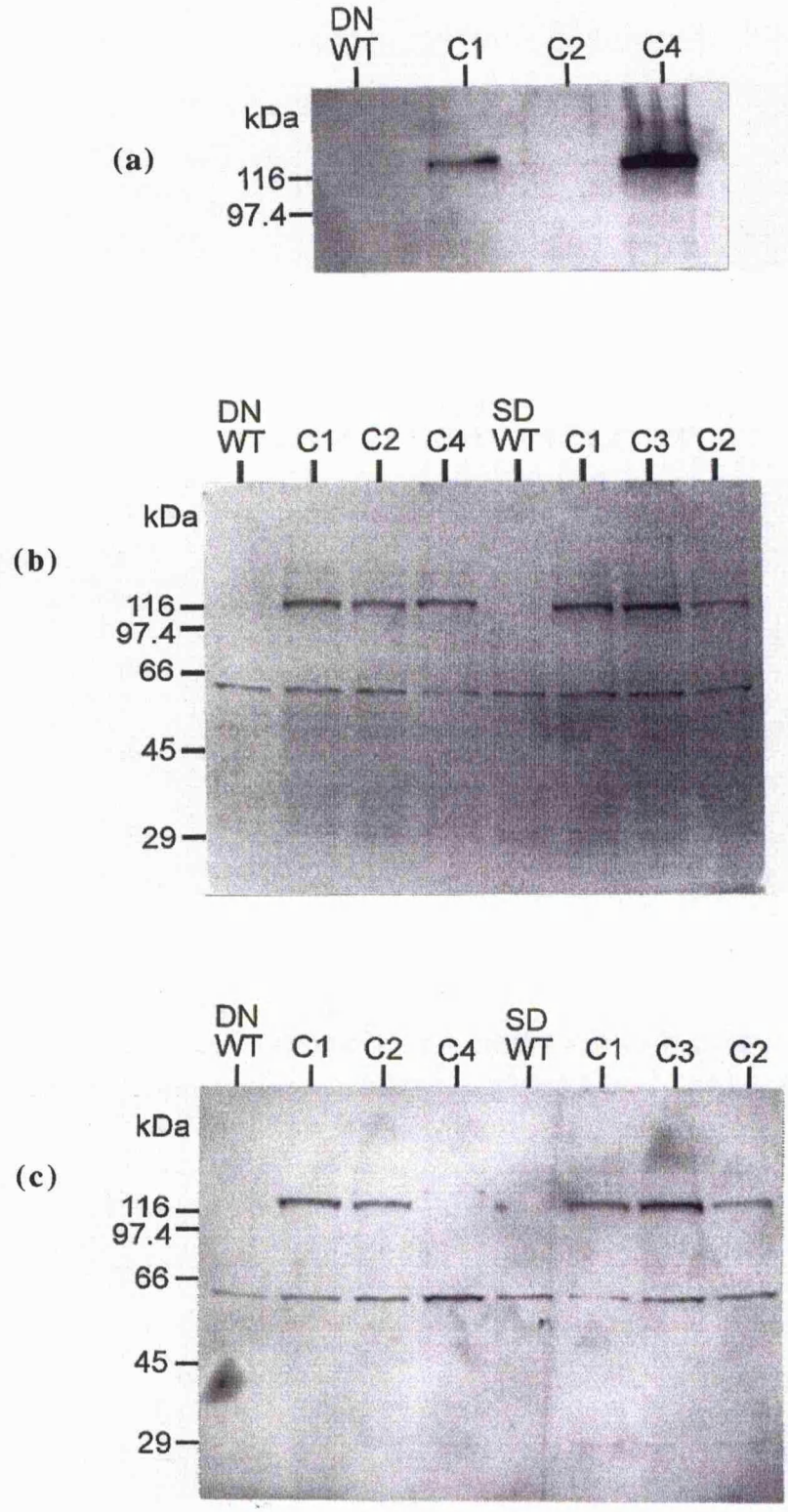


Figure 4.10 Immunoblot analysis of *Arabidopsis* phytochrome C protein from DN C1, DN C2, DN C4, SD C1, SD C3, SD C2 seedlings homozygous for the transgene, and wild type *N. tabacum* seedlings. Crude protein extracts (100 µg per lane) were prepared from **(a)** 5 d old etiolated seedlings; **(b)** light-grown plants at the 3 leaf stage; **(c)** light-grown at the 5 leaf stage. All blots were probed with the a combination of the monoclonal antibodies, C1, C11 and C13.

Chapter 5 Vegetative physiology of DN and SD *Nicotiana tabacum* overexpressing phytochrome A, B or C

5.1 Introduction

The assignment of roles to individual phytochrome species has been greatly facilitated in recent years by the selection of photomorphogenic mutants and the production of transgenic plants overexpressing phytochrome species. Analysis of these plants has provided information on the specific roles of phytochrome species in vegetative development and flowering responses. It has also provided information concerning the response modes via which phytochrome action is mediated (see Smith, 1995).

There are obvious benefits from analysing mutants that are null for a particular phytochrome species or have defective function in that phytochrome. However, there can be problems with the interpretation of phytochrome function when more than one phytochrome species can elicit the same response via different pathways. Transgenic plants overexpressing phytochrome species can, to some extent, overcome these problems, but there may be other hazards associated with the interpretation of the overexpression phenotype. The successful production of transgenic plants expressing elevated levels of introduced phytochrome genes under the control of the CaMV 35S promoter prompted extensive use of this promoter. In these plants expression is driven at a high level in most cells, which does not reflect the expression patterns of endogenous phytochrome. In addition, these foreign phytochromes may not be subject to the same developmental and light-mediated regulation as endogenous species. However, even when the CaMV 35S promoter has been used, the phenotype of the transgenics overexpressing a phytochrome species is often the antithesis of the null mutant, which greatly facilitates interpretation. For example, hypocotyl growth of *Arabidopsis phyA* mutants is completely insensitive to continuous FR, but responds in a wild type manner to R and W (Nagatani *et al.*, 1993; Parks and Quail 1993; Whitelam

et al., 1993). The antithesis is seen in *Arabidopsis* transformed with oat phytochrome A cDNA, that displays a greatly enhanced sensitivity of hypocotyl growth inhibition to FR (Boylan and Quail, 1991; Whitelam *et al.*, 1992). Further analysis of transgenic seedlings showed that hypocotyl elongation was inhibited by FR wavelengths in a fluence-dependent manner consistent with the high irradiance response (HIR) response mode (Whitelam *et al.*, 1992). Experiments such as these have confirmed the role of phytochrome A in the FR HIR. In fact, there is evidence to suggest that a FR HIR persists in light-grown *Arabidopsis* and tobacco (9A4) which overexpress phytochrome A. For although these transgenic seedlings respond in a wild type fashion to FR pulses given at the end of the photoperiod with an increase in elongation growth, this response is reversed when FR is given over a prolonged photoperiod (McCormac *et al.*, 1992; Whitelam *et al.*, 1992). There is, however, unlikely to be an extensive role for endogenous phytochrome A in the vegetative development of the light grown plant as the *phyA* mutants are indistinguishable from wild type plants when grown in W light.

Detailed physiological analyses have revealed that phytochrome A also controls hypocotyl elongation in light-grown seedlings. Light grown *phyA* mutants grown under photoperiodic conditions have longer hypocotyls than those of wild type seedlings grown under the same conditions (Johnson *et al.*, 1994). The phenomenon is not observed in *phyA* seedlings grown under continuous W (Whitelam *et al.*, 1993). This suggests that phytochrome A reaccumulates during dark periods, and has an inhibitory effect on hypocotyl elongation when the seedlings are exposed to light. There is other evidence that phytochrome A can influence the development of the light-grown plant. Under R and W, the hypocotyl length of the *phyAphyB* double *Arabidopsis* mutant is longer than that of either monogenic mutant (Reed *et al.*, 1994). This suggests that phytochrome A may have an inhibitory role in hypocotyl elongation under these conditions. Support for this comes from analysis of transgenic seedlings, where phytochrome A overexpression leads to a marked sensitivity in hypocotyl inhibition under continuous R (Whitelam *et al.*, 1992). This may refect the ability of transgenically expressed phytochrome A to function in a HIR mode under R.

As the phenotype of plants overexpressing phytochrome A is not always the antithesis of the null mutant it is not possible to assign functions or roles to particular

phytochromes based solely on the phenotype. For instance, *phyA* mutants are phenotypically identical to wild type plants when grown under continuous W, whilst plants overexpressing phytochrome display a dwarfed growth habit (McCormac *et al.*, 1992). However, sometimes the incongruous behaviour of the transgenics can provide a further insight into the characteristics of phytochrome function. An example is the afore-mentioned, regulation of hypocotyl elongation by R light in etiolated *Arabidopsis*. Whereas *phyA* seedlings respond in a wild type manner with inhibition of hypocotyl growth under R light (eg. Whitelam *et al.*, 1993), *Arabidopsis* overexpressing phytochrome A display enhanced inhibition (Whitelam *et al.*, 1992). In fact, a loss of R-mediated hypocotyl growth inhibition is only displayed by *phyA* seedlings which are also deficient in phytochrome B (Reed *et al.*, 1994). As this response is strongly controlled by phytochrome B, the action of phytochrome A is masked and, therefore, not detectable in the monogenic *phyA* mutant.

Similar opposing photophysiological responses are also displayed by the transgenic plants expressing phytochrome B cDNA and phytochrome B-deficient mutants. Transgenic *Arabidopsis* seedlings overexpressing phytochrome B display a selective increase in their sensitivity to R, with respect to the inhibition of hypocotyl elongation (McCormac *et al.*, 1993). This contrasts with the selective lack of responsiveness to R displayed by the *Arabidopsis phyB* mutant and other phytochrome B-like mutants, including the *lh* mutant of cucumber and the *ein* mutant of *Brassica rapa* (Adamse *et al.*, 1987; Goto *et al.*, 1991; Devlin *et al.*, 1992). The analysis of hypocotyl inhibition reponses of phytochrome B-deficient mutants and B-overexpression lines in relation to R photon fluence rate has implicated phytochrome B in a R HIR in the etiolated seedling (McCormac *et al.*, 1993).

Like the transgenics overexpressing phytochrome A cDNAs, light-grown phytochrome B-overexpressors have a dwarfed phenotype characterised by short hypocotyls, short internodes and reduced overall height. The *phyB* mutants and *phyB*-like mutants, in contrast, have an elongated phenotype that is similar to the shade-avoidance phenotype displayed by wild type plants in response to low R/FR ratio. Also, unlike their wild types, the *phyB*, *lh* and *ein* mutants do not show significant growth promotions to EOD FR (López-Juez *et al.*, 1990; Nagatani *et al.*, 1991; Devlin

et al., 1992). The photophysiological responses of these plants led to the suggestion that phytochrome B is responsible for the R/FR reversible low fluence response.

Further analysis has shown that phytochrome B-deficient mutants are indeed able to respond to low R/FR ratio in a number of ways. Growth responses to low R/FR ratio have been shown for *phyB* with respect to leaf area, specific stem weight and flowering time and for *lh* with respect to hypocotyl length (Smith *et al.*, 1992; Robson *et al.*, 1993; Halliday *et al.*, 1994 and Chapter 2). It has, therefore, been proposed that a novel phytochrome(s), in addition to phytochrome B, has a major role in this response.

As for plants overexpressing phytochrome A, plants overexpressing phytochrome B are not always the antithesis of the null mutant. The *Arabidopsis phyB* mutant flowers early under LDs (Goto *et al.*, 1991; Whitelam and Smith, 1991), whereas, seedlings overexpressing transgenic phytochrome B are also early flowering under these conditions (Bagnall *et al.*, 1995). These observations suggest that phytochrome B has a role to play in the flowering response, but they also serve to emphasise that a degree of caution must be exercised when interpreting an overexpression phenotype.

Clearly much can be learned from the phenotype of transgenic plants overexpressing phytochrome genes, particularly when the null mutant is also available. Often the transgenic phenotype will confirm predicted roles for phytochrome species deduced from the analysis of mutants. Equally, some of the phenotypical characteristics of the transgenic plant may not represent the normal function of the particular phytochrome. Not only is it possible that ectopically-expressed transgenic phytochrome may not reflect its normal spatial and temporal expression, it may also have the capacity to mimic the function of other phytochrome species. This could lead to confusion if little is known about the phytochrome in question, but could also provide useful additional information about a phytochrome that is already reasonably well-characterised.

In this chapter the vegetative phenotypes of DN and SD *N. tabacum* expressing heterologous phytochrome A and B cDNAs and phytochrome C gDNA are examined. From each of the phytochrome transformations a number of transgenic lines have been

selected to represent different levels of transgene expression. Particular attention is paid to the transgenic plants overexpressing phytochromes B and C. This work represents the first complete characterisation of a phytochrome B-overexpression phenotype in tobacco. This work also identifies for the first time, characteristics associated with phytochrome C-overexpression and provides evidence that phytochrome C functions as a photoreceptor.

5.2 Experimental procedures

5.2.1 The creation of *Nicotiana tabacum* transgenic lines overexpressing phytochrome A, B and C

The DN (2326) and the SD (22NF) *Nicotiana tabacum* cv. Hicks were obtained from Vern Sisson, USDA, Oxford, USA. The DN and SD cultivars differ with respect to one locus, *MARYLAND MAMMOTH* (*MM*). The *MM* allele, that was originally in *N. tabacum* cv. Maryland Mammoth, was introduced into the DN cv. Hicks by a standard backcrossing procedure, to produce the Hicks SD cv. (Vern Sisson pers. comm.). The DN and SD plants are isogenically identical in every respect other than at the *MM* locus. Both DN and SD *N. tabacum* were transformed with the oat phytochrome A cDNA, the *Arabidopsis* phytochrome B cDNA and the *Arabidopsis* phytochrome C gDNA, each under the control of the "constitutive" CaMV 35S rRNA promoter. For both DN and SD *N. tabacum*, transformants were selected to represent a range in transgene expression levels in the A, B and C lines. For details of transformation procedures and selection methods see Chapter 4.

5.2.2 Growth conditions

For measurements of hypocotyl elongation in darkness (D), R, FR, B and W

light, and cotyledon area in R, all seeds were sown in 9 cm Petri dishes on 0.8% (w/v) agar containing MS mineral salts (Murashige and Skoog, 1962) and chilled at 4°C for 5 d. The seeds were stimulated to germinate by a 15 min W treatment on day 5 and grown for a further day in darkness. The seedlings were then either kept in darkness or transferred to continuous R, FR, B or W for 7 d.

In experiments with mature plants, seeds were sown in 9 cm Petri dishes on 0.8% (w/v) agar containing MS mineral salts and chilled for 5 d at 4°C. Seeds were germinated and grown in continous W (photon fluence rate 99 μmol m^{-2} s^{-1} in the range of 400-700 nm). Seedlings were then selected for uniformity and transferred to 5 cm pots containing a compost:horticultural silver sand (3:1) mixture and grown for a further 14 d in the same light conditions. On day 14 the seedlings were transferred to the appropriate experimental light treatments.

5.2.3 Measurement of hypocotyl length, internode length, petiole length, plant height, cotyledon area, leaf area and leaf angle

All hypocotyl lengths were determined from calibrated projected photographic slides. Internode length, petiole length and plant height were measured with a ruler. Cotyledon and leaf area were measured using a leaf area meter. Leaf angle between opposing leaves was measured using a protractor. Sample sizes for all experiments were between 10 and 20 plants.

5.2.4 Measurement of leaf chlorophyll content

Pigments were extracted by immersing leaf discs in dimethylformamide (DMF) at a ratio of DMF:tissue of 100:1 (v/w). Samples were placed in the dark at 4°C for 1 d after which time a spectrophotometric reading was taken at 664 and 667 nm.

Chlorophyll concentration was calculated as μg 10 mm^{-2} using the following formula:

$$(7.04 \times A_{664}) + (20.27 \times A_{647}) \div \text{(leaf disc area)}.$$

5.2.5 Light sources

The monochromatic light sources are as described in chapter 3 in all respects other than the fluence rates supplied by the R and the FR LEDs, which were 20 and 12 μmol m^{-2} s^{-1}, respectively.

In experiments using continuous high and low R/FR ratio light, Fitotron 600 growth cabinets (Fisons Scientific Apparatus, Loughborough, UK) were used. The high R/FR ratio cabinet (cool-W fluorescent light) provided a photon fluence rate (400-700 nm) of 99 μmol m^{-2} $s^{-1}$and a R/FR ratio of 5.76. The low R/FR ratio cabinet (cool-W fluorescent light supplemented with FR) provided a photon fluence rate (400-700 nm) of 91 μmol m^{-2} $s^{-1}$and a R/FR ratio of 0.08.

Plants in experiments carried out under greenhouse conditions were grown in daylight supplemented with continuous W light provided by Son-T plus 400 lights (Phillips).

5.3 Results

5.3.1 Photocontrol of hypocotyl elongation in seedlings overexpressing phytochrome A, B or C

Seedlings were grown for 5 d in darkness or under continuous R, FR, B or W light, after which hypocotyl lengths were determined. For seedlings grown in the dark, the hypocotyls of the lines overexpressing phytochromes A, B and C were largely indistinguishable from those of wild type seedlings (Fig. 5.1). The exceptions being

DN A4, DN B5 and the DN C-overexpression lines that display shorter hypocotyls when grown in darkness (Fig. 5.1(a)(i), (b)(i) and (c)(i)). For lines overexpressing phytochrome A, seedlings demonstrate increased inhibition of hypocotyl elongation under FR, R, B and W when compared to wild type seedlings, this inhibition being more marked in the lines with higher transgenic protein expression levels (Fig. 5.1(a) and Chapter 4). For lines overexpressing phytochrome B, seedlings display a short-hypocotyl phenotype in R, B and W, the degree of inhibition of hypocotyl elongation being more pronounced in the seedlings with higher transgene expression levels (Fig. 5.1(b) and Chapter 4). The most severe B-overexpressors, DN B5 and SD B5 have shorter hypocotyls than their wild types under FR (see Fig. 5.1(b)(iii)). For lines overexpressing phytochrome C there appears to be no clearly identifiable alteration in hypocotyl length when compared with wild type seedlings in FR and B (Fig. 5.1(c)). However, with the exception of DN C3 that has a long hypocotyl in W, all the C-overexpression lines show a small but significant reduction in hypocotyl length when compared with wild type seedlings in R and W (Fig. 5.1(c)).

Thus, all the overexpression lines appear to demonstrate light-mediated increased inhibition of hypocotyl elongation, and the severity of this inhibition correlates positively with the level of transgenic protein present in the seedlings (see Chapter 4). All the lines overexpressing phytochrome A display an enhanced sensitivity to R, FR, B and W that results in increased hypocotyl growth inhibition under these conditions. The increased inhibition of hypocotyl elongation is also a response common to all the B-overexpression lines grown under R, B and W, whereas, with the exception of DN C3, the C-overexpression lines display this response under R and W.

5.3.2 Photocontrol of cotyledon expansion in seedlings overexpressing phytochrome B or C

Cotyledon area was measured 5 d after germination in plants grown under continuous R. When compared with wild type seedlings, both DN B7 and DN B5 lines display an increase in cotyledon area (Fig. 5.2). Cotyledon area was not measured in

the SD transgenics overexpressing phytochrome B. This increase in cotyledon area is not seen in seedlings in the DN C1 and SD C1 lines, that display a cotyledon size that is comparable to that of wild type seedlings (Fig. 5.3). However, seedlings in the DN C2, DNC4, SD C3, and SD C2 lines do display an increase in cotyledon expansion under R, when compared with wild type seedlings (Fig. 5.3). Thus, increased expression of either phytochrome B or phytochrome C can mediate a R-induced increase in cotyledon expansion in *N. tabacum*.

5.3.3 Photocontrol of leaf area in plants overexpressing phytochrome A, B or C

Leaf area was measured in both wild type and transgenic plants grown under continuous W. Plants of the A-overexpression lines show a reduction in leaf area when compared to wild type plants (Fig. 5.4). This reduction in leaf area is quite marked in plants with the higher transgene product expression levels, whereas there is no effect on leaf expansion in the weak overexpression line DN A1 (see Chapter 4) that has a leaf area which is indistinguishable from that of its wild type (Fig. 5.4). Plants overexpressing phytochrome B also produce leaves that are smaller than those of wild type plants (Fig. 5.5). Here also, smaller leaf size correlates positively with higher transgenic protein level (see Chapter 4). The reduced leaf area response displayed in the adult B-overexpressors is the antithesis of the increased cotyledon area response displayed by the de-etiolating seedlings (Figs. 5.5 and 5.2).

In contrast to both phytochrome A- and phytochrome B-over expressing lines plants overexpressing phytochrome C display an increase in leaf area when compared to wild type plants (Fig. 5.6). The degree of leaf enlargement may be linked to the severity of the overexpression line, though, in the young plant there is probably only a small difference in levels of transgenic protein (see Chapter 4). It appears, therefore, that increased levels of phytochrome A and phytochrome B mediate an inhibition of leaf expansion in adult *N. tabacum*, whereas, increased levels of phytochrome C lead to an increase in leaf expansion.

5.3.4 Photocontrol of leaf angle in plants overexpressing phytochrome A, B or C

The angle made between a leaf pair was measured in plants grown for either 21 d under high R/FR ratio, or 20 d under high R/FR ratio followed by 1 d under low R/FR ratio. When grown under high R/FR ratio, plants overexpressing high levels of phytochrome A or B have an increased leaf angle in comparison with wild type plants (Figs. 5.7 and 5.8). The degree of increase in leaf angle also corresponds to the level of transgenic protein in both the DN and the SD lines (Figs. 5.7, 5.8 and Chapter 4). Although this response is quite marked in the more severe overexpression lines, the weakest overexpression lines, DN A1, SD A7 and DN B2 display a wild type-leaf angle response under high R/FR ratio light.

Wild type plants demonstrate a marked reduction in leaf angle following the transfer to low R/FR ratio. Again, plants in the weakest overexpression lines (DN A1, SD A7 and SD B2), behave in a similar manner to wild type plants in this respect (Figs. 5.7 and 5.8). However, the more severe A- and B-overexpressors demonstrate a smaller reduction in leaf angle than that displayed by wild type plants when transferred to low R/FR ratio light (Figs. 5.7 and 5.8). In fact, the leaf angle response to low R/FR in the severe DN B-overexpressors is either marginal (in DN B5) or absent (in DN B7) (Fig. 5.8).

When leaf angle is measured under high and low R/FR ratio, C-overexpression lines largely behave in a manner that is more or less indistinguishable from wild type plants. Although there appears to be a slight increase in leaf angle, with respect to wild type plants, in SD C3 and SD C2 plants grown under high R/FR ratio, all of the lines display a "wild type " reduction in leaf angle under low R/FR ratio (Fig. 5.9).

These data indicate that for *N. tabacum* lines expressing higher levels of phytochromes A and B, growth in high R/FR ratio conditions leads to an increased angle between opposing leaves. The dramatic reduction in leaf angle observed in wild type plants upon exposure to a period of low R/FR ratio is much less marked in plants expressing high levels of phytochromes A an B, and is completely absent in the severe B-overexpression line, DN B7. Phytochrome C-overexpression in *N. tabacum* appears

to stimulate an increase in leaf angle under high R/FR ratio (in SD C3 and SD C2), however, plants in the C-overexpression lines, behave in a wild type manner with respect to leaf angle responses in low R/FR ratio.

5.3.5 Photocontrol of internode length, leaf shape and leaf chlorophyll levels in plants overexpressing phytochrome B

N. tabacum overexpressing phytochrome B display a dark green, dwarfed phenotype with altered leaf size and shape when compared to wild type plants. This dwarfed growth habit is characterised by the production of internodes that are markedly shorter than the corresponding internodes in the wild type plant, measured here in plants grown in continuous W (Fig. 5.10(a) and (b)). There is a graded increase in hypocotyl growth inhibition across both the DN and SD expression series. In the DN lines, DN B7 and DN B5 show intermediate and strong inhibition responses, respectively (Fig. 5.10(a)). In the SD lines, SD B2 shows the weakest response, whilst DN B13 and DN B5 display intermediate and severe responses, respectively (Fig. 5.10(b)). As well as a reduction in leaf size reported in section 5.3.3, the B-overexpressors have altered leaf shape. This change in leaf shape was measured as leaf length/width (L/W) ratio in plants grown under continuous W. The L/W ratio was smaller in the DN B7 and DN B5 transgenic lines in comparison with wild type plants (Fig. 5.11). This reduction in L/W ratio means that the leaves produced in the B-overexpression lines are relatively broader as well as smaller (Fig. 5.5). In addition to these parameters, chlorophyll content is also affected in the B-overexpressors. Compared to wild type plants leaf chlorophyll content is increased in the DN B7 and the DN B5 plants when grown under continuous W (Fig. 5.12). With regard to both L/W ratio and chlorophyll content the DN B2 transgenics are indistinguishable from wild type plants. Thus, the presence and severity of characteristics associated with phytochrome B-overexpression is intimately linked to the level of the transgene product (see Chapter 4).

5.3.6 Photocontrol of vegetative growth in DN plants overexpressing phytochrome A, B or C, grown under greenhouse conditions

Under greenhouse conditions, plants in the A- and the B-overexpression lines display a dwarf growth habit that is characterised by a short-internode phenotype. It is represented here as 5th internode length, which is significantly reduced in the DN A4, DN B7 and DN B5 transgenic plants, when compared to wild type plants (Fig. 5.13). Plants in the DN A3 line, produce internodes comparable to those of wild type plants (Fig. 5.13). Likewise, the length of the 5th internode in the C-overexpression lines is also indistinguishable from the 5th internode of wild type plants (Fig 5.13).

When leaf area is examined there is only a very small effect in the plants overexpressing phytochrome A (Fig. 5.14). These observations contrast with those made for plants grown under W light, where leaf area is reduced in plants with high levels of transgenic phytochrome (Fig. 5.4). The reduction in leaf size observed in DN B-overexpression lines grown under W (Fig. 5.5) is also seen in plants grown under greenhouse conditions. Plants in the DN B7 and DN B5 lines produce leaves that are significantly smaller than wild type leaves (Fig. 5.14). In contrast, the C-overexpression lines produce leaves that are significantly larger than those of the wild type (Fig. 5.14). This "large leaf" phenotype is also observed in C-overexpression lines grown under W (Fig. 5.6) so it appears to be a consistent feature of *N. tabacum* overexpressing phytochrome C.

In addition to these parameters, plant height was measured over a time course in the greenhouse-grown plants. The more severe A- and B-overexpressors DN A4 and DN B5 maintained a dwarfed growth habit throughout their life (Figs 5.15 and 5.16). The difference in height of the wild type plants and DN B5 is fairly constant along the timecourse. However, the difference in height between the wild type and DN A4 become larger as the plants mature (Fig 5.15). This suggests that perhaps expression levels of the transgene may be controlled by a developmental factor. The same argument could be made for DN B7, that grows initially in a dwarfed habit but demonstrates a gradual increase in stem extension, and achieves the same final height as the wild type plant (Fig. 5.15). However, this growth pattern may not necessarily be due to a

decrease in transgene expression, but may reflect a level of transgenic phytochrome B that falls short of a threshold required to inhibit the final bolting response. Plants in the DN A3 line maintain the same height as wild type plants throughout the timecourse (Fig. 5.15), which suggests that the high levels of transgene protein overexpression observed in the young plant (Chapter 4) fall-off substantially in the adult plant. This lack of a phenotype in the DN A3 line is also seen with respect to internode length, where the mature transgenic plants are, again, indistinguishable from wild type plants (Fig. 5.13).

Plants overexpressing phytochrome C are taller than wild type plants (Figs. 5.15 and 5.16). The DN C4 transgenics are marginally taller than wild type plants throughout their life. The DN C2 trangenics initially show the same rate of stem extension as wild type plants, but, unlike wild type plants, they display a sudden increase in stem extension from around 46 d (Fig. 5.15). Again, the differental growth response in DN C2 may reflect developmental regulation of the transgene with the production of higher levels of transgene protein in the more mature plant. However, it may equally reflect an enhanced sensitivity of the later internodes to transgenically expressed phytochrome C. In DN C4 seedlings, the relatively high levels of transgenic protein seen young seedlings cannot be detected in the adult plant (see Chapter 4). This may reflect the lack of a more severe adult phenotype (Figs. 5.15 and 5.16). The moderately increased height of plants in the DN C4 line is not reflected by a corresponding increase in internode length. This is probably because the difference in height represents an accumulation of marginally longer internodes (Fig. 5.13). Moreover, the internode data imply that plants in the DN C2 lines also have internodes that are the same length as wild type plants (Fig. 5.13). However, when the internode measurements were taken on day 40, neither internode length nor plant height, in DN C2 plants, are significantly different from that of the wild type. The increased elongation growth in DN C2 is not actually realised until day 46 and, therefore, internodes produced after this time would be expected to be longer when compared to those of the wild type.

To summarise, A-, B- and C-overexpression in *N. tabacum* can have a profound effect on de-etiolation and mature plant vegetative morphology. All

transgenics display a light-mediated increase in hypocotyl elongation inhibition. Etiolated seedlings overexpressing phytochrome A demonstrate this response to FR, R and B light. Etiolated seedlings overexpressing phytochrome B display enhanced hypocotyl inhibition under R and B light. Etiolated seedlings overexpressing phytochrome C only exhibit increased elongation inhibition in response to R light. It also appears that phytochrome B and phytochrome C overexpression can both enhance R-mediated cotyledon expansion.

The overexpression of phytochromes A and B in the de-etiolated plant also causes dwarfing, which is characterised by reduced internode elongation and reduced leaf size. The overexpression of phytochrome C appears to have the opposite effect, stimulating the production of a larger plant, which is characterised by increased stem height and increased leaf area (see Fig. 5.16).

5.4 Discussion

The generation of transgenic SD and DN *N. tabacum* expressing introduced phytochromes A, B and C provided a means of investigating the impact of phytochrome overexpression on the many aspects of vegetative plant growth.

Each of the lines in each of the overexpression series were selected on the basis of severity of hypocotyl inhibition (see Chapter 4). What is more, plants in the line with the most severe hypocotyl inhibition also have the highest levels of transgenic apoprotein. The link between transgene product level and severity of the physiological response has been an extremely useful aid in defining the phenotypical characteristics of the overexpression phenotypes.

Physiological analysis of plants in the transgenic lines provides a comprehesive characterisation of a phytochrome B-overexpression phenotype in *N. tabacum*, previously unreported. This work also provides the first evidence that phytochrome C functions as a photoreceptor, and the first evidence of physiological characteristics that can be attributed to phytochrome C transgenic overexpression.

The effect of overexpression of phytochrome A, phytochrome B and

phytochrome C *N. tabacum* transgenic lines was examined with respect to hypocotyl growth under R, FR, B and W light. Seedlings in the phytochrome A-overexpression lines display an increased sensitivity to FR, R, B and W, with enhanced inhibition of hypocotyl elongation under these conditions. This increase in hypocotyl inhibition has previously been reported for phytochrome A-overexpressing *Arabidopsis* and tobacco seedlings grown under FR (Boylan and Quail, 1991; Whitelam *et al.*, 1992; McCormac *et al.*, 1992). Elongation growth inhibition under FR is under the control of phytochrome A, operating via a FR HIR (Boylan and Quail, 1991; Whitelam *et al.*, 1992; McCormac *et al.*, 1992). The reciprocal response is seen in *Arabidopsis phyA* mutants which lack the ability to inhibit hypocotyl elongation under FR (Nagatani *et al.*, 1993; Parks and Quail, 1993; Whitelam *et al.*, 1993). The increased inhibition of hypocotyl elongation of the phytochrome A-overexpression lines under R, (and W), has also been reported for *Arabidopsis* and potato (Whitelam *et al.*, 1992; Heyer *et al.*, 1995). In addition, it has been observed that the *Arabidopsis phyAphyB* double mutant is longer than *phyB* under R (Reed *et al.*, 1993). All these observations suggest that phytochrome A may also have an inhibitory role in hypocotyl elongation under R.

Enhanced hypocotyl growth inhibition is displayed by etiolated seedlings in the phytochrome B-overexpression lines grown under R, and W. This response is also observed in *Arabidopsis* seedlings overexpressing the phytochrome B transgene (McCormac *et al.*, 1993). The reciprocal reponse is seen in the *Arabidopsis phyB* mutant, that displays a reduced R light-mediated inhibition of hypocotyl elongation (Koornneef *et al.*, 1980). The data presented here also show that the most severe phytochrome B-overexpression lines can respond to FR with increased inhibition of hypocotyl growth. However, these observations are not consistent with those previously reported on phytochrome B-overexpression in *Arabidopsis* (McCormac *et al.*, 1993; Wester *et al.*, 1994). The apparent response to FR, may be a reflection of the persistence of a high enough level of PfrB to initiate the response. It could also could be a trivial consequence of a delay in germination of the transgenic seed. Indeed, the reduced hypocotyl length displayed by some of the overexpression lines in darkness is thought to represent a delay in germination time of the transgenics with respect to wild type seed.

Examination of the hypocotyl elongation responses in transgenic lines can be particularly valuable in establishing responses specific to a narrow waveband light. There is, however, a drawback in measuring hypocotyl length after a set period of time. No allowances can be made for the late-germinating seed, which will, as a result, display delayed hypocotyl extension. Efforts to reduce the inaccuracies of this method included the synchronisation of germination using a prolonged cold treatment of seed batches, and the analysis of replicates. Accuracy could have been further improved by collecting the data over a time course, though this method requires a large amount of space and is very time-consuming.

Both the A- and the B-overexpressors demonstrated an inhibition of hypocotyl elongation when grown under B. As phytochrome also absorbs light in the B region of the spectrum a growth response under B is not unexpected. The increased growth inhibition displayed by the A-overexpressors is likely to reflect transgenically expressed phytochrome A functioning in a HIR. The hypocotyl elongation inhibition seen in the seedlings overexpressing phytochrome B may be a direct consequence of the formation of PfrB under B. However, it may also represent the coaction of phytochrome and the UV/B photoreceptor. Analysis of *Pinus sylvestris* has shown that the inhibition of axis (hypocotyl) elongation under B is only possible in the presence of Pfr (Fernbach and Mohr, 1990). It appears that in *P. sylvestris* the UV/B activity amplifies the response to Pfr. Likewise, in *Cucumis sativus*, inhibition of hypocotyl elongation is only realised in the presence of Pfr (Shinkle and Jones, 1988). Therefore, it is possible that the growth inhibition displayed by seedlings expressing transgenic phytochrome B is a PfrB-stimulated response that has been enhanced by the activity of the UV/B photoreceptor.

In the C-overexpression lines, although the DN lines appear to be shorter in darkness, the DN- and the SD-overexpression lines both show the same trends when grown under the range of light treatments. The C-overexpressors (with the exception of SD C3) show an enhanced hypocotyl inhibition response in R (and W), which implies that phytochrome C may also play a part in the mediation of hypocotyl elongation inhibition. Furthermore, as phytochrome C action has not been previously reported, these data provide evidence that phytochrome C is an active photoreceptor.

In addition to hypocotyl elongation, phytochrome B-overexpression also affects cotyledon expansion under R. The DN phytochrome B transgenics, display a marked increase in cotyledon area under these conditions. Likewise, in addition to hypocotyl elongation inhibition, cotyledon area is also increased in C-overexpression lines when grown under R. This suggests that phytochrome C may act in a similar manner to phytochrome B in de-etiolation, enhancing hypocotyl growth inhibition and cotyledon expansion in R light. However, it is also noteworthy that the hypocotyls of seedlings overexpressing phytochrome C are only marginally shorter than wild type hypocotyls. Phytochrome B has been shown to exercise this control by reducing cell elongation growth (Reed *et al.*, 1993). Therefore, it is possible that phytochrome C may affect other aspects of cell expansion, the effects of which are more obvious in the cotyledons.

The impact of overexpression of all three phytochromes is also very much evident in the adult plant. In *N. tabacum*, leaf morphology is very sensitive to the changing light environment with quite dramatic differences in leaf size, shape, and leaf angle reflecting the quality and the quantity of the light under which the plant is growing. Both the A- and the B-overexpression lines display a reduction in leaf area when the plants are grown under continuous W. This contrasts with plants in the C-overexpression lines that display an increase in leaf area, relative to wild type plants, when grown under the same conditions.

Upon transfer from high to low R/FR ratio, wild type *N. tabacum* respond in a quite dramatic manner with a rapid reduction in the angle between opposing leaf pairs. Under high R/FR ratio conditions, plants in the A- and the B-overexpression lines display an increase in leaf angle when compared with wild type plants. In this respect the behaviour of these transgenic plants is the antithesis of the "shade avoidance" phenotype. Furthermore, although the ability of A and B transgenics to respond to a reduction in R/FR ratio is maintained, the extent of this response is significantly less than that of wild type plants. The behaviour of the C-overexpression lines, again contrasts with that of the A- and the B-overexpression lines, which, for the most part, display wild type leaf angle responses. It is noteworthy that the *N. tabacum* lines expressing phytochrome A maintain the ability to respond to low R/FR not only with

respect to leaf angle, but with respect to leaf area, petiole and internode length (data not shown). This contrasts with plants in the 9A4 *N.tabacum* line which overexpress phytochrome A. 9A4 plants display a "reversed shade avoidance response" under low R/FR ratio (McCormac *et al.*, 1992), a phenomenon that is thought to represent the persistence of the FR HIR in the light-grown plant (McCormac *et al.*, 1991; 1992). The A-overexpressors used in this study, therefore, probably represent less severe overexpression lines. The increased leaf angle in high R/FR ratio displayed by the overexpressors, together with the less marked reduction in leaf angle following the transfer to low R/FR ratio is probably due to the higher basal level of Pfr present under both light conditions.

Further analysis of plants in the lines expressing the phytochrome B cDNA show that they display a dark green dwarf phenotype, characterised by increased leaf chlorophyll content, reduced internode length and a reduction in leaf L/W ratio. This phenotype is reminiscent of that displayed in lines overexpressing phytochrome A (Boylan and Quail, 1989, 1991; Kay *et al.*, 1989; Keller *et al.*, 1989) which suggests that the action of phytochrome A and phytochrome B can affect similar cellular processes, although they may act via distinct mechanisms.

A direct comparison of the physiological attributes of plants in the DN transgenic lines expressing introduced A, B and C transgenes and DN wild type plants was carried out under greenhouse conditions. Many of the characteristics, observed in the transgenic lines grown under W light conditions, are also maintained under greenhouse conditions. The plants overexpressing higher levels of phytochromes A and/or B assume a dwarf phenotype, which is measured as a reduction in internode length and plant height, and, in the B-over expressors, a reduction in leaf area. The plants overexpressing phytochrome C, in contrast, are taller than wild type plants and produce larger leaves. These observations may reflect the synergistic roles for these phytochromes in the control of these aspects of vegetative plant development.

The monitoring of these plants over a period of time has also uncovered developmental changes in plant physiology that are probably linked to developmental fluctuations in transgene expression levels. The dwarfed phenotype of the DN A4 plants becomes more severe as the plants mature. This may either reflect a gradual

increase in the levels of transgenic protein, or sensitivity to transgenic protein. The DN A3 line has been shown to possess high levels of transgenic protein at the etiolated seedling stage, but a relatively low level of the transgene product at the later stages of development (see Chapter 4). The change in transgene expression levels is reflected by a change in the severity of the overexpression-phenotype. The DN A3 transgenics display a marked hypocotyl inhibition phenotype at the seedling stage, but behave very much like wild type plants during their adult life.

The behaviour of DN B7 plants suggests that either transgene expression is reduced, or there is a reduction in sensitivity to the transgenic protein in the later developmental stages. In these plants a dwarfed growth habit is maintained until bolting, after which they attain the same final height of wild type plants.

Etiolated seedlings in the DN C4 line have high transgenic protein levels. However, levels fall off in the young plant stage and are undetectable in the adult plant (see Chapter 4). This is also reflected in the phenotype, as young DN C4 seedlings display very pronounced hypocotyl expansion under R when compared to wild type seedlings, but the adult plants, are only marginally taller than wild type plants. Interestingly, plants in the DN C2 line, that have a lower level of transgenic protein than DN C4 at the etiolated seedling stage (see Chapter 4), show a quite dramatic increase in stem elongation when mature. This could be as a result of increased sensitivity of the more mature plant to transgenic phytochrome C, or the enhancement of transgene expression in the older plant. However, this has not been measured due to the difficulties in obtaining sufficient good protein or mRNA from mature *N. tabacum* tissue extracts. Developmental changes in transgene expression levels have not previously been reported for phytochrome-overexpression lines. This may be due to the selection for and the subsequent molecular and physiological characterisation of the transgenics within a narrow developmental period. The developmental changes in the levels of transgene expression observed in this study are likely to represent effects imposed upon the transgene by virtue of its position of insertion in the chromosome.

The analysis of transgenic lines with differing levels of heterologous phytochrome expression has been an extremely useful method of establishing phenotypical characteristics that can be linked directly with phytochrome

overexpression. The degree of response elicited by phytochrome overexpression has been shown to be implicitly related to the level of phytochrome expression, and is seen as a graded response across the overexpression series. Confirmation of the phenotypical characteristics has also been possible in some of the transgenic lines that demonstrate a loss or a gain of response at a developmental stage where there is a corresponding rise or fall in transgene expression levels.

To summarise, the overexpression of phytochrome A, B and C in *N. tabacum* can have a profound effect on plant vegetative morphology. In the etiolated seedling stage, phytochrome A overexpression appears to be active in the inhibition of hypocotyl elongation under FR, R and B and W. Phytochrome B-overexpression has an inhibitory effect on hypocotyl elongation in R and B light. Cotyledon expansion is also enhanced by phytochrome B-overexpression under R light. Likewise, phytochrome C-overexpression inhibits hypocotyl elongation and increases cotyledon expansion under R. Not only does this demonstrate that phytochrome C is a functional photoreceptor, it suggests that it may act in a similar manner to phytochrome B in the de-etiolation of seedlings in response to R.

Adult plants overexpressing higher levels of phytochrome A and B have a dwarfed phenotype that is characterised by a reduction in internode and stem length and a reduction in leaf size. Although phytochrome A and B overexpression have similar pleiotropic effects, their native roles are thought to be very different. The phytochrome C-overexpression phenotype contrasts strongly with that of the A- and B-overexpression phenotype, as the plants are taller and have larger leaves than wild type plants. These observations could represent contrasting roles for endogenous phytochrome B and phytochrome C in these aspects of vegetative development.

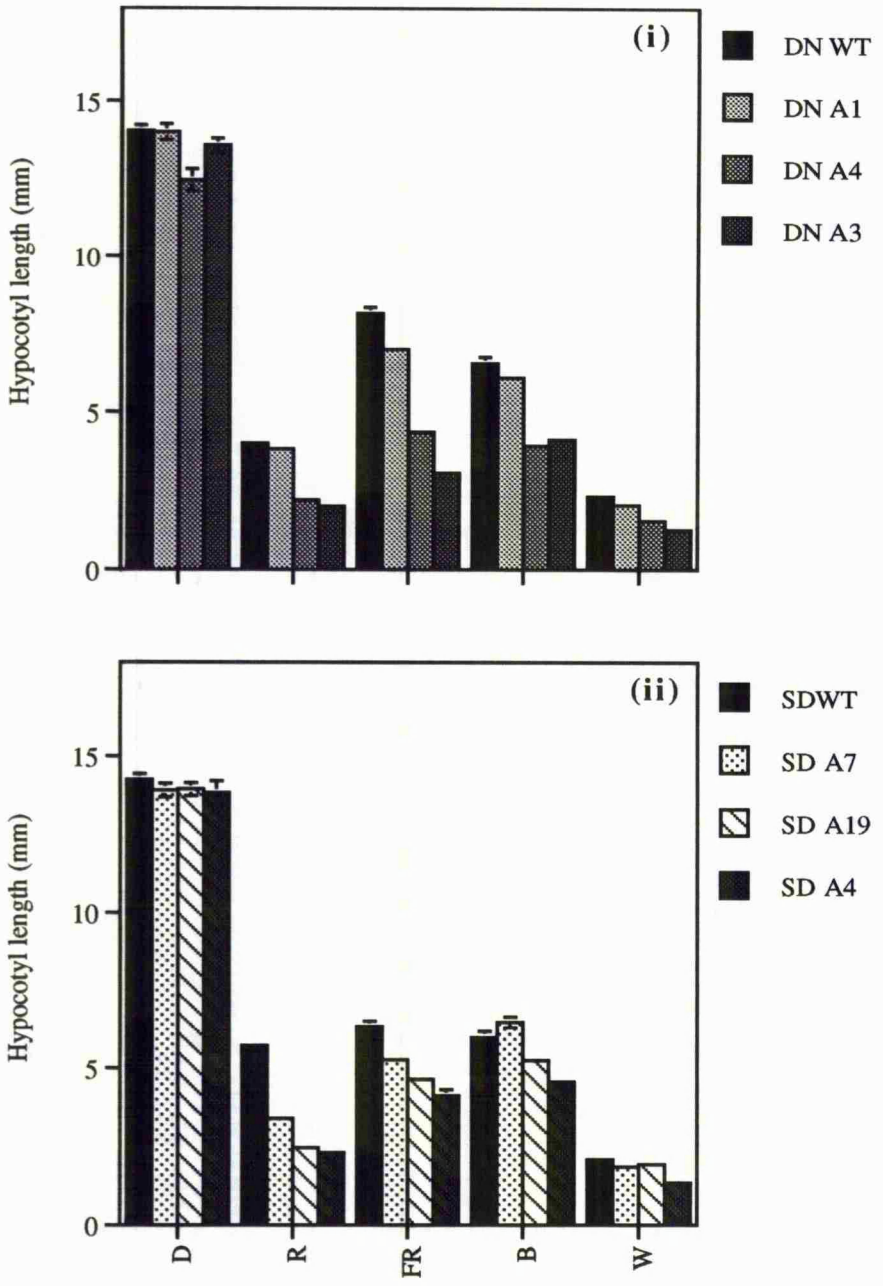
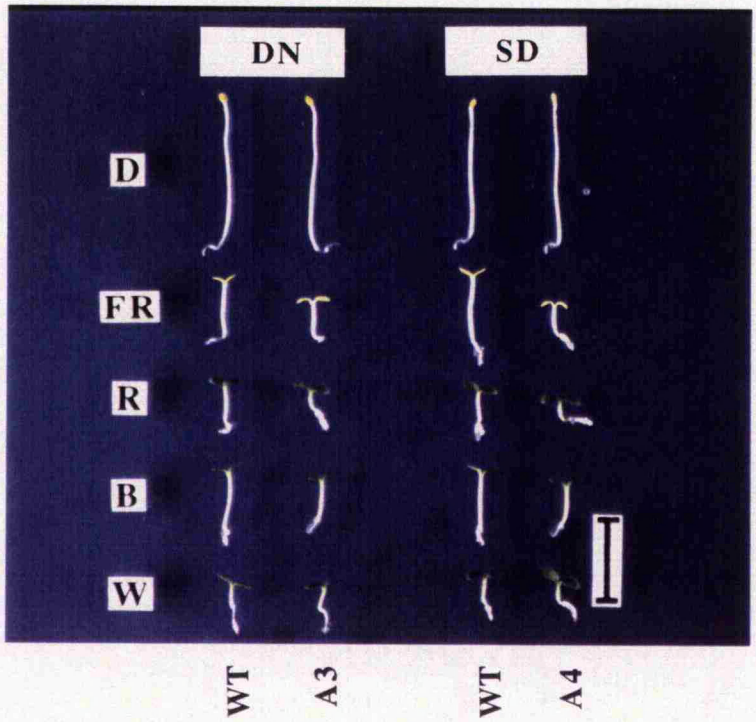


Figure 5.1(a) Hypocotyl length of **(i)** DN WT, DN A1, DN A4 and DN A3 seedlings; **(ii)** SD WT, SD A7, SD A19 and SD A4 seedlings; and phenotype of **(iii)** DN WT, DN A3, SD WT and SD A4 seedlings, grown for 7 d in continuous darkness (D), red (R), far-red (FR), blue (B) and white (W) light. Data represent the means from 20 seedlings and the error bars are SE.

(iii)



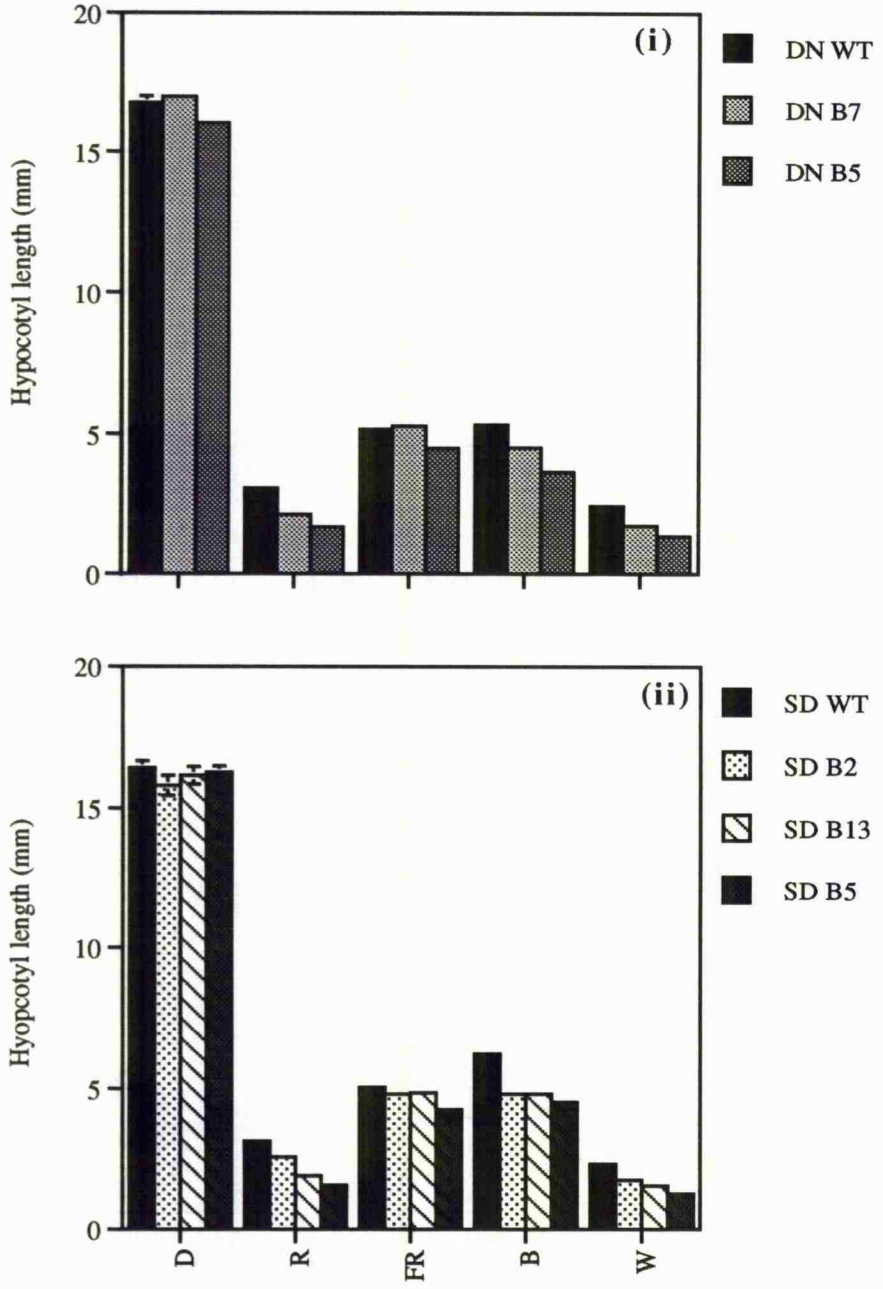
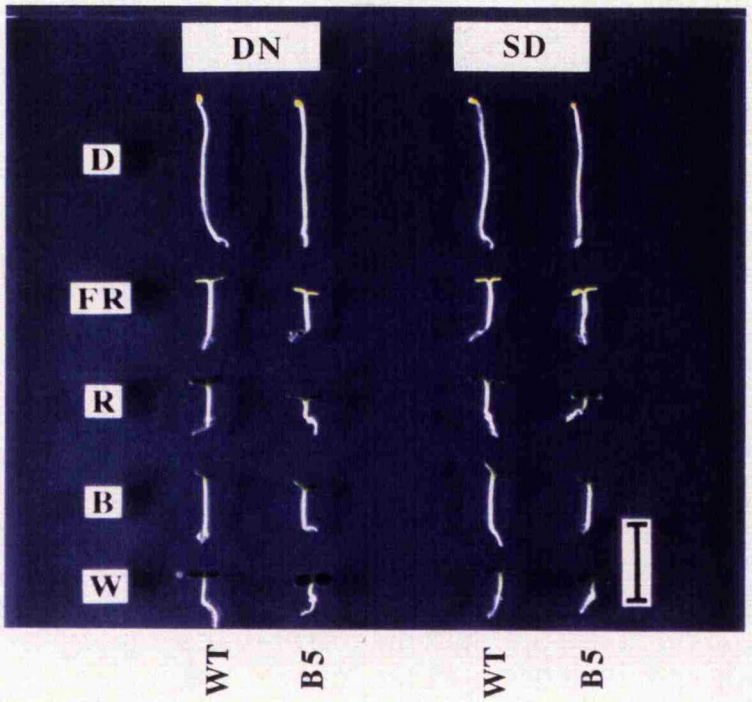


Figure 5.1(b) Hypocotyl length of **(i)** DN WT, DN B7 and DN B5 seedlings; **(ii)** SD WT, SD B2, SD B13 and SD B5 seedlings; and phenotype of **(iii)** DN WT, DN B5, SD WT, SD B5 seedlings, grown for 7 d in continuous darkness (D), red (R), far-red (FR), blue (B) and white (W) light. Data represent the means from 20 seedlings and the error bars are SE.

(iii)



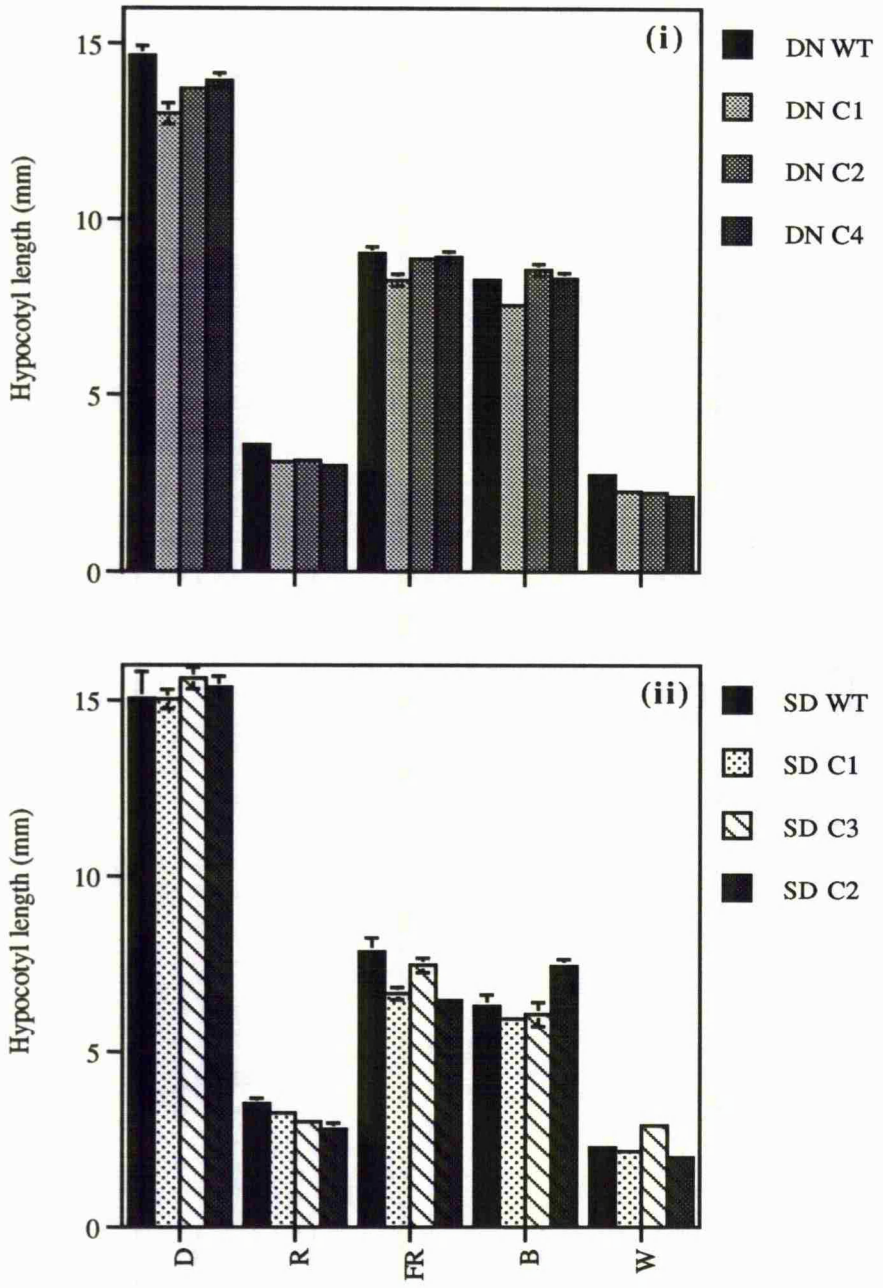
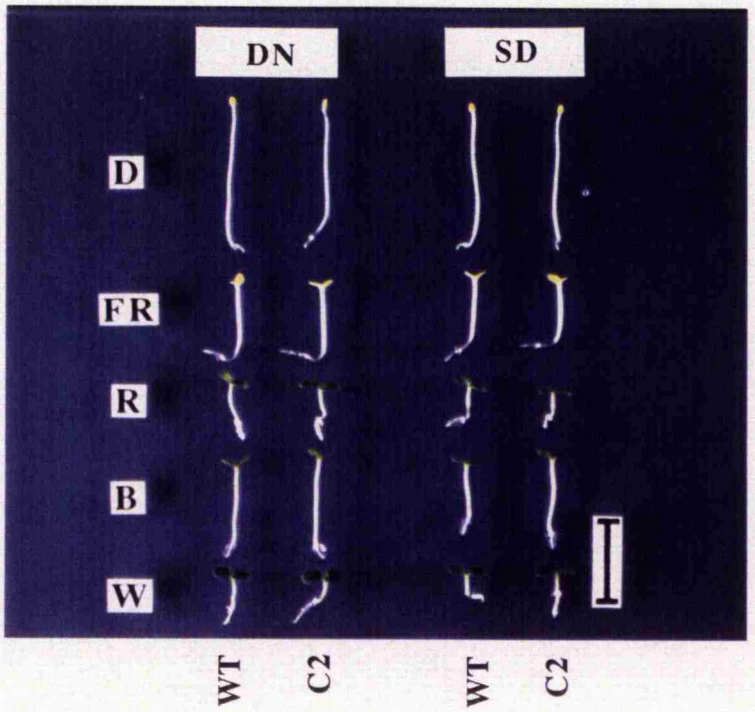


Figure 5.1(c) Hypocotyl length of **(i)** DN WT, DN C1, DN C2 and DN C4 seedlings; **(ii)** SD WT, SD C1, SD C3 and SD C2 seedlings; and phenotype of **(iii)** DN WT, DN C2, SD WT and SD C2 seedlings, grown for 7 d in continuous darkness (D), red (R), far-red (FR), blue (B) and white (W) light. Data represent the means from 20 seedlings and the error bars are SE.

(iii)



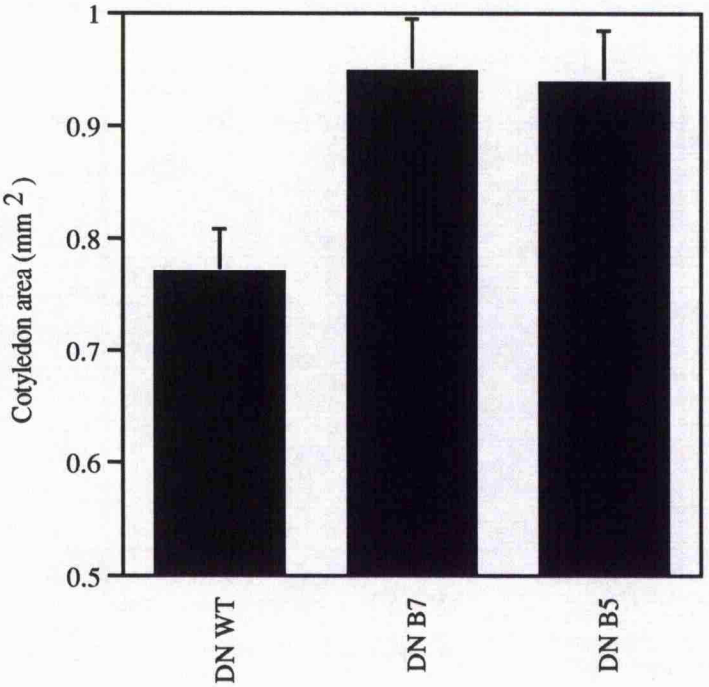


Figure 5.2 Cotyledon area of DN WT, DN B7 and DN B5 seedlings after 5 d growth under continuous R light. Data represents the means of 20 seedlings and the error bars are SE.

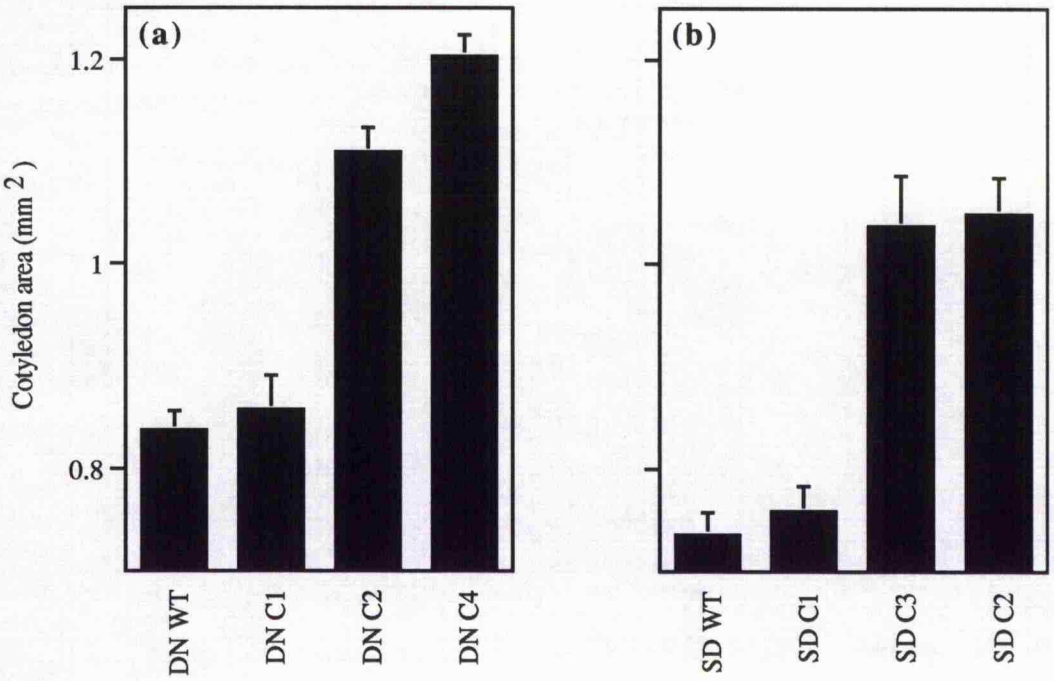


Figure 5.3 Cotyledon area of (**a**) DN WT, DN C1, DN C2 and DN C4; (**b**) SD WT SD C1, SD C3 and SD C2 seedlings after 5 d growth in continuous R light. Data represents the means of 20 seedlings and the error bars are SE.

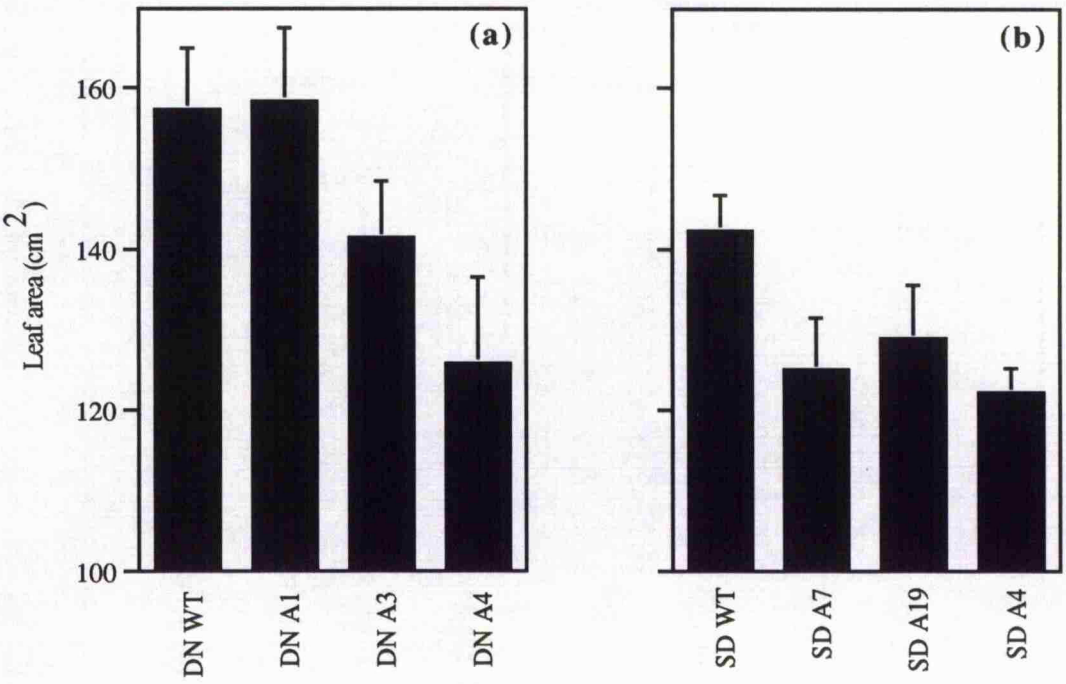


Figure 5.4 Area of fourth leaf of **(a)** DN WT, DN A1, DN A3 and DN A4; **(b)** SD WT, SD A7, SD A19 and SD A4 plants after 33 d growth in continuous W light. Data represents the means of 12 plants and the error bars are SE.

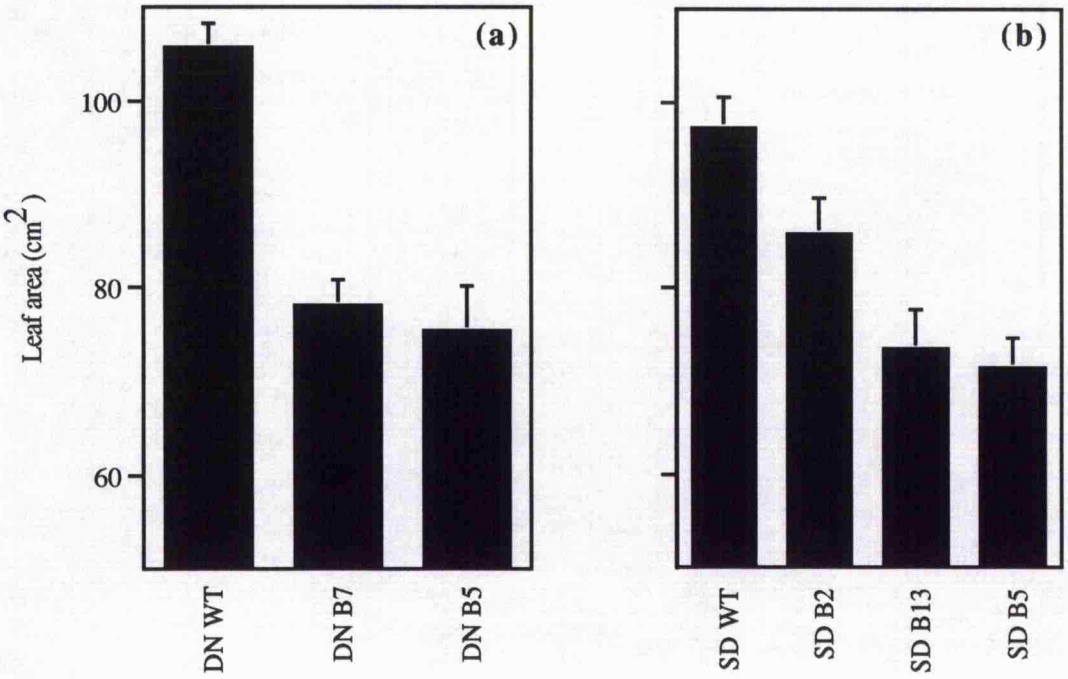


Figure 5.5 Area of second leaf of (**a**) DN WT, DN B7 and DN B5; (**b**) SD WT, SD B2, SD B13 and SD B5 plants after 32 d growth in continuous W light. Data represents the means of 12 plants and the error bars are SE.

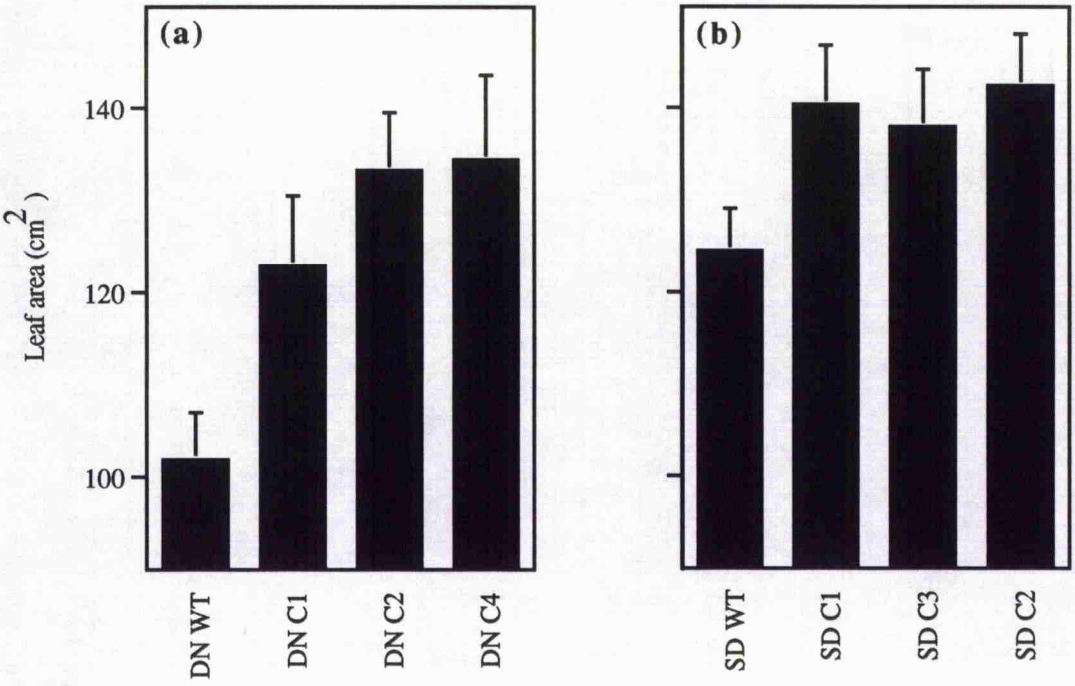


Figure 5.6 Area of third leaf of (**a**) DN WT, DN C1, DN C2 and DN C4; (**b**) SD WT, SD C1, SD C3 and SD C2 plants after 33 d growth in continuous W light. Data represents the means of 12 plants and the error bars are SE.

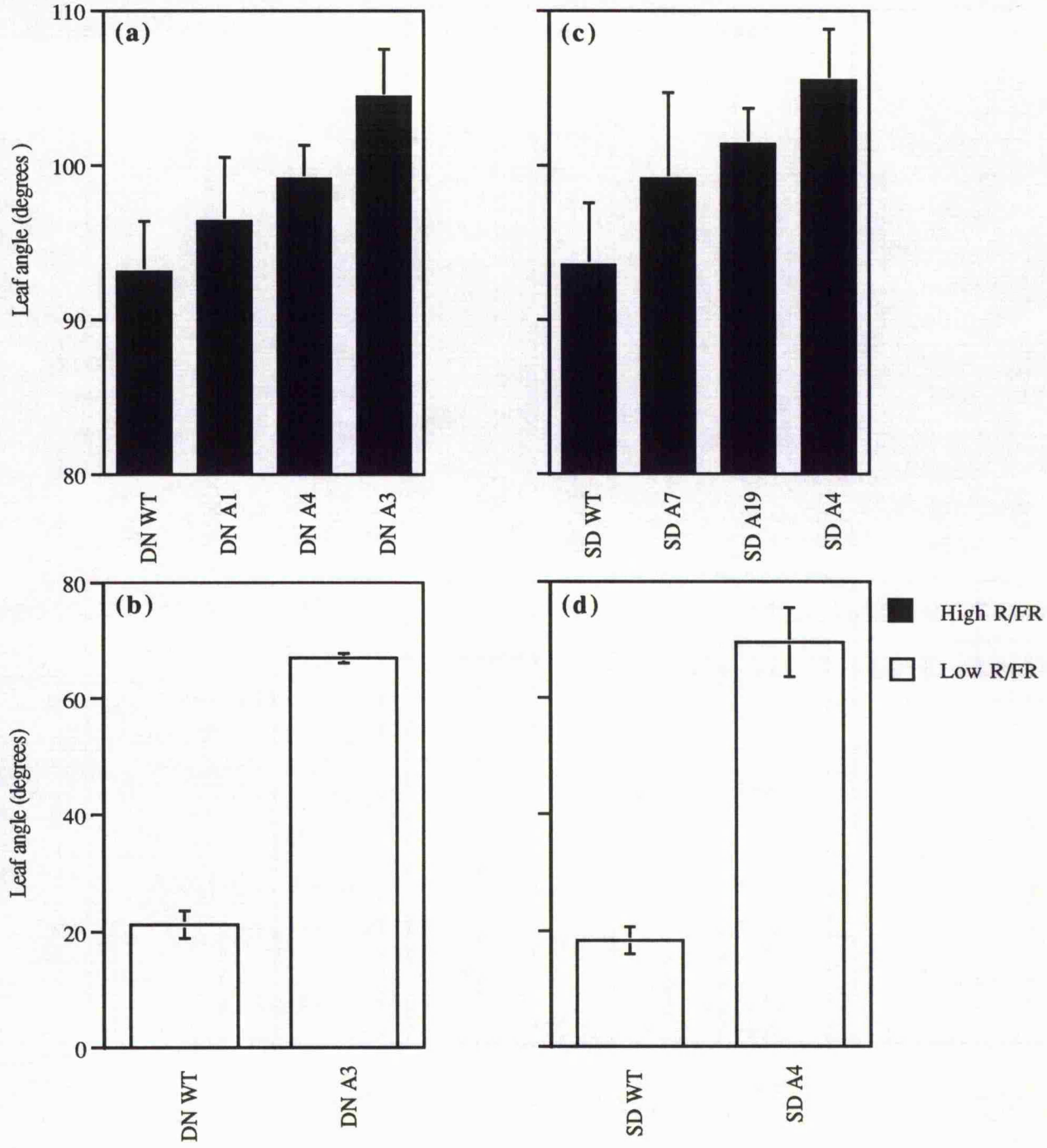


Figure 5.7 Leaf angle between the second leaf pair of **(a)** DN WT, DN A1, DN A4, and DN A3; **(c)** SD WT, SD A7, SD A19 and SD A4 plants grown for 21 d under high R/FR ratio light (closed bars) and **(b)** DN WT and DN A3; **(d)** SD WT and SD A4 plants grown for 20 d under high R/FR ratio, followed by 1 d under low R/FR ratio light (open bars). Data represent the means from 12 plants and the error bars are SE.

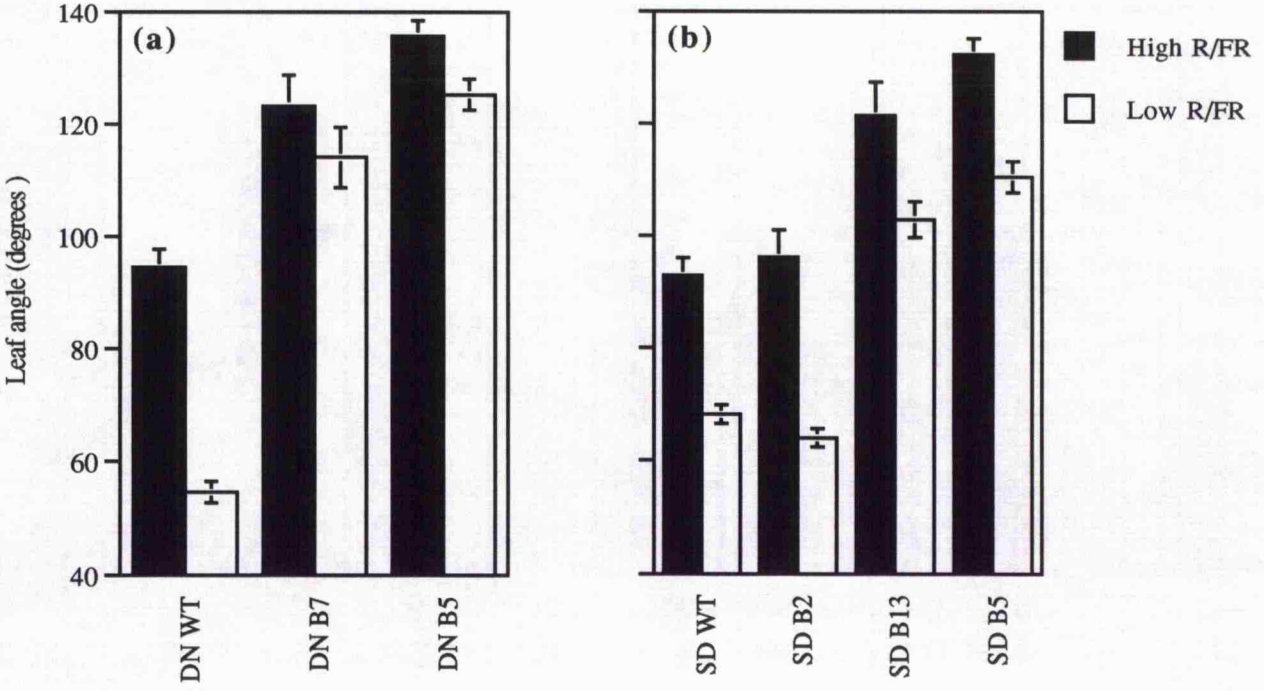


Figure 5.8 Leaf angle between the first leaf pair of **(a)** DN WT, DN B7 and DN B5; **(b)** SD WT, SD B2, SD B13 and SD B5 plants grown for 21 d under high R/FR ratio light (closed bars) and for 20 d under high R/FR ratio, followed by 1 d under low R/FR ratio light (open bars). Data represent the means from 12 plants and the error bars are SE.

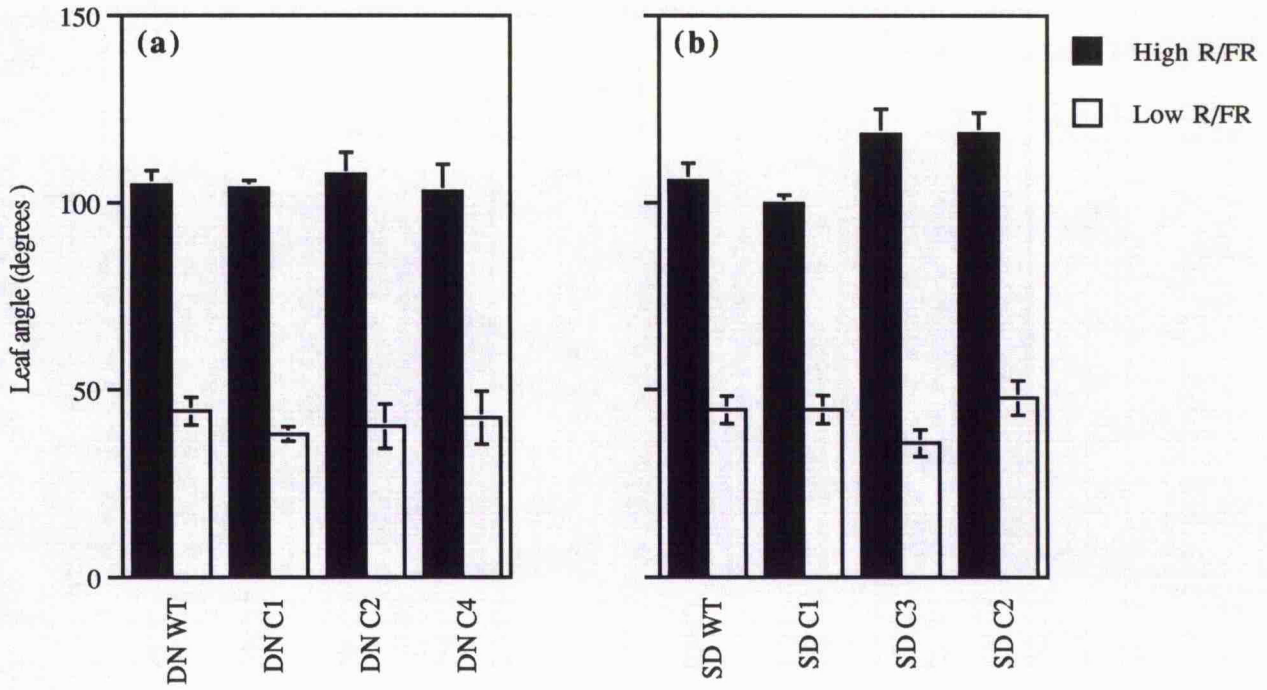


Figure 5.9 Leaf angle between the first leaf pair of (**a**) DN WT, DN C1, DN C2 and DN C4; (**b**) SD WT, SD C1, SD C3 and SD C2 plants grown for 21 d under high R/FR ratio light (closed bars) and for 20 d under high R/FR ratio, followed by 1 d under low R/FR ratio light (open bars). Data represent the means from 12 plants and the error bars are SE.

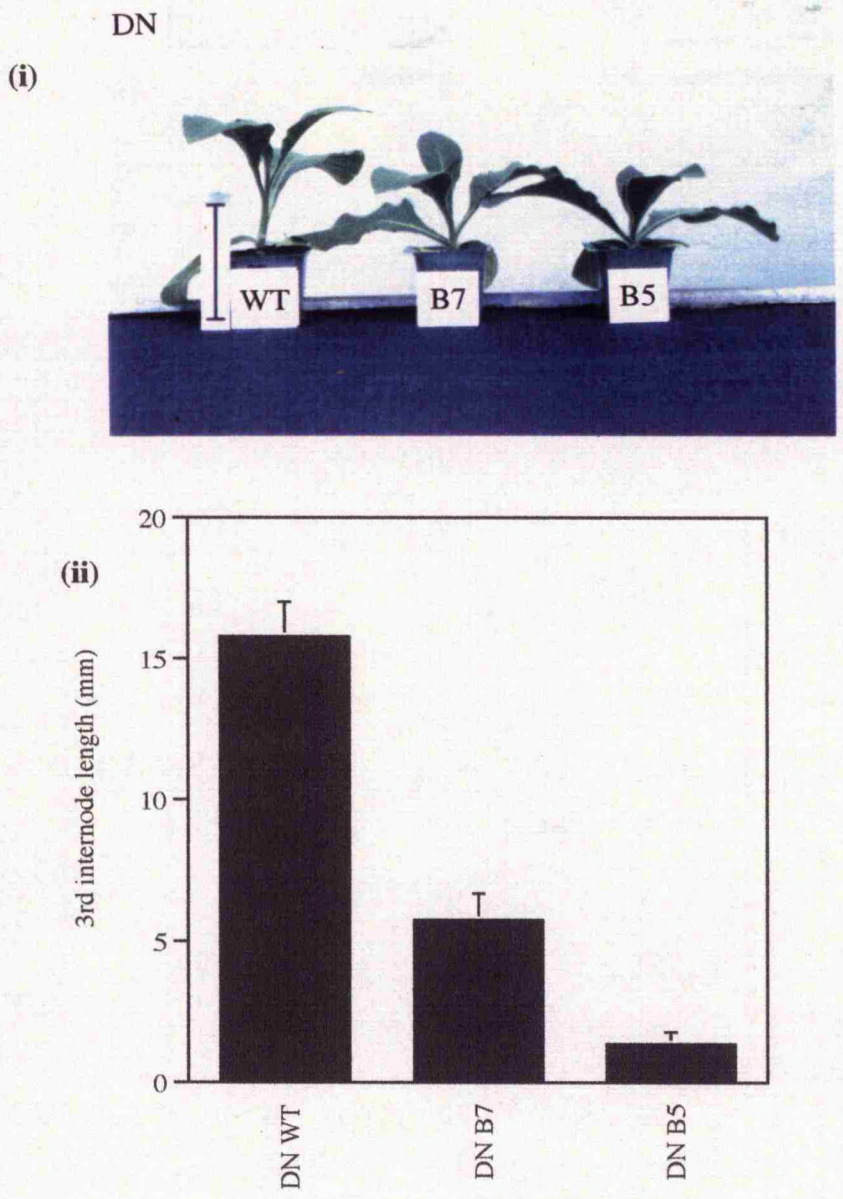


Figure 5.10(a) Phenotype **(i)** and third internode length **(ii)** of DN WT, DN B7 and DN B5 plants after 32 d growth in continuous W light. Data represent the means from 16-20 plants and the error bars are SE.

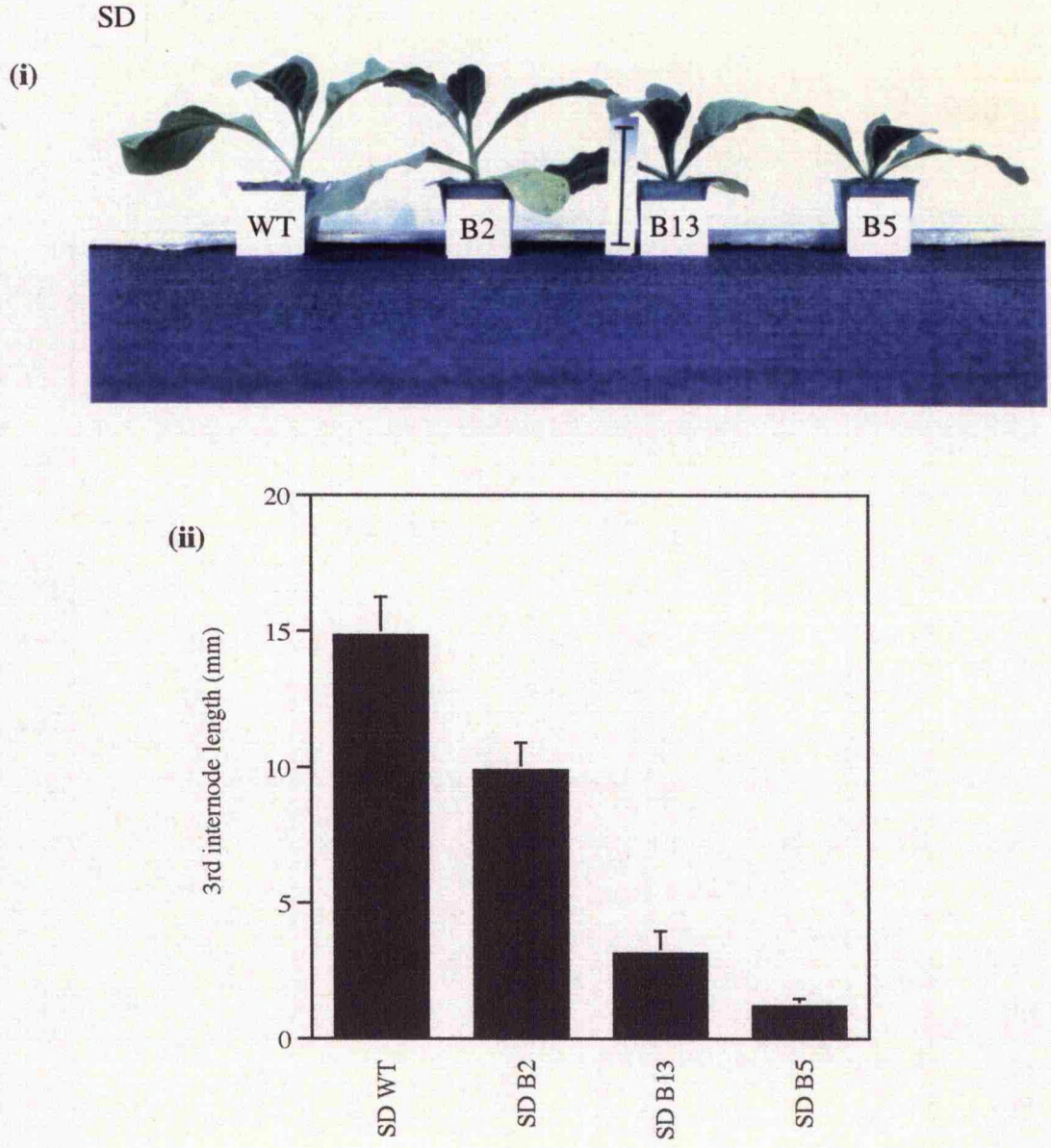


Figure 5.10(b) Phenotype **(i)** and third internode length**(ii)** of SD WT, SD B2, SD B13 and SD B5 plants after 32 d growth in continuous W light. Data represent the means from 16-20 plants and the error bars are SE.

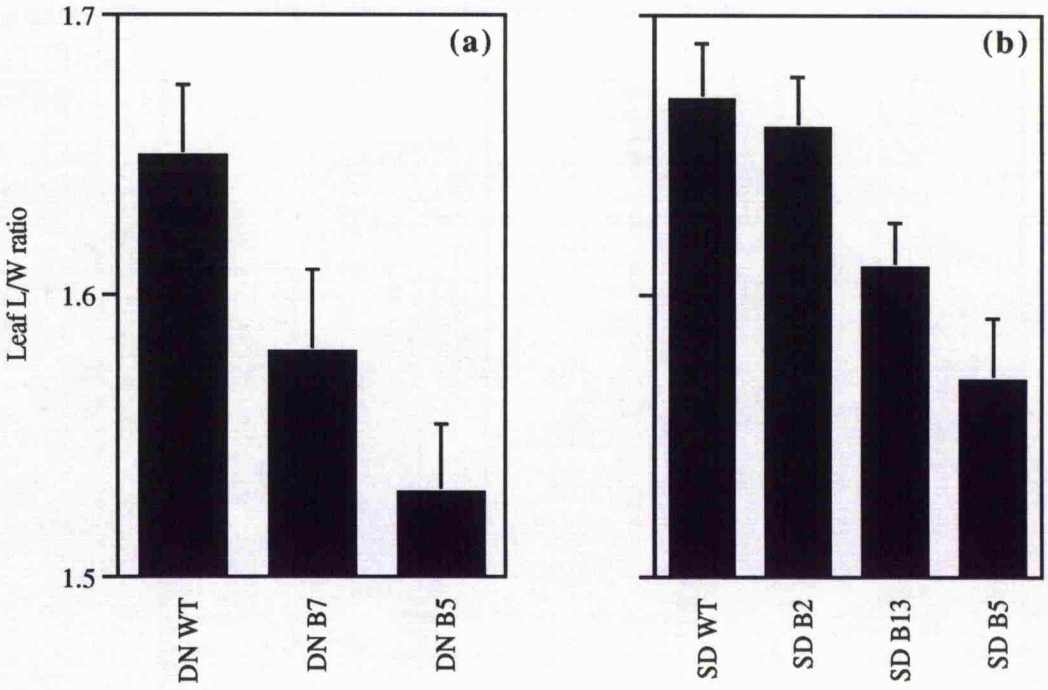


Figure 5.11 Leaf length/width (L/W) ratio of (**a**) DN WT, DN B7 and DN B5; (**b**) SD WT, SD B2, SD B13 and SD B5 plants measured after 32 d growth in continuous W light. Data represent the means from 16-20 plants and the error bars are SE.

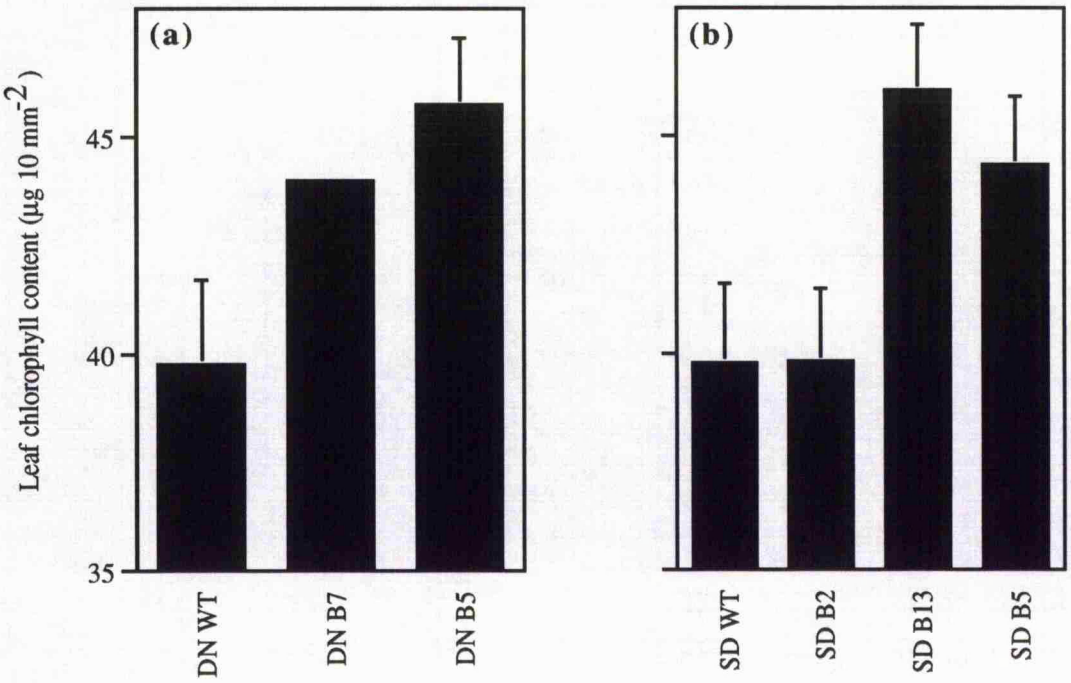


Figure 5.12 Leaf chlorophyll content of **(a)** DN WT, DN B7 and DN B5; **(b)** SD WT, SD B2, SD B13 and SD B5 plants measured after 28 d growth in continuous W light. Data represent the means from 12 plants and the error bars are SE.

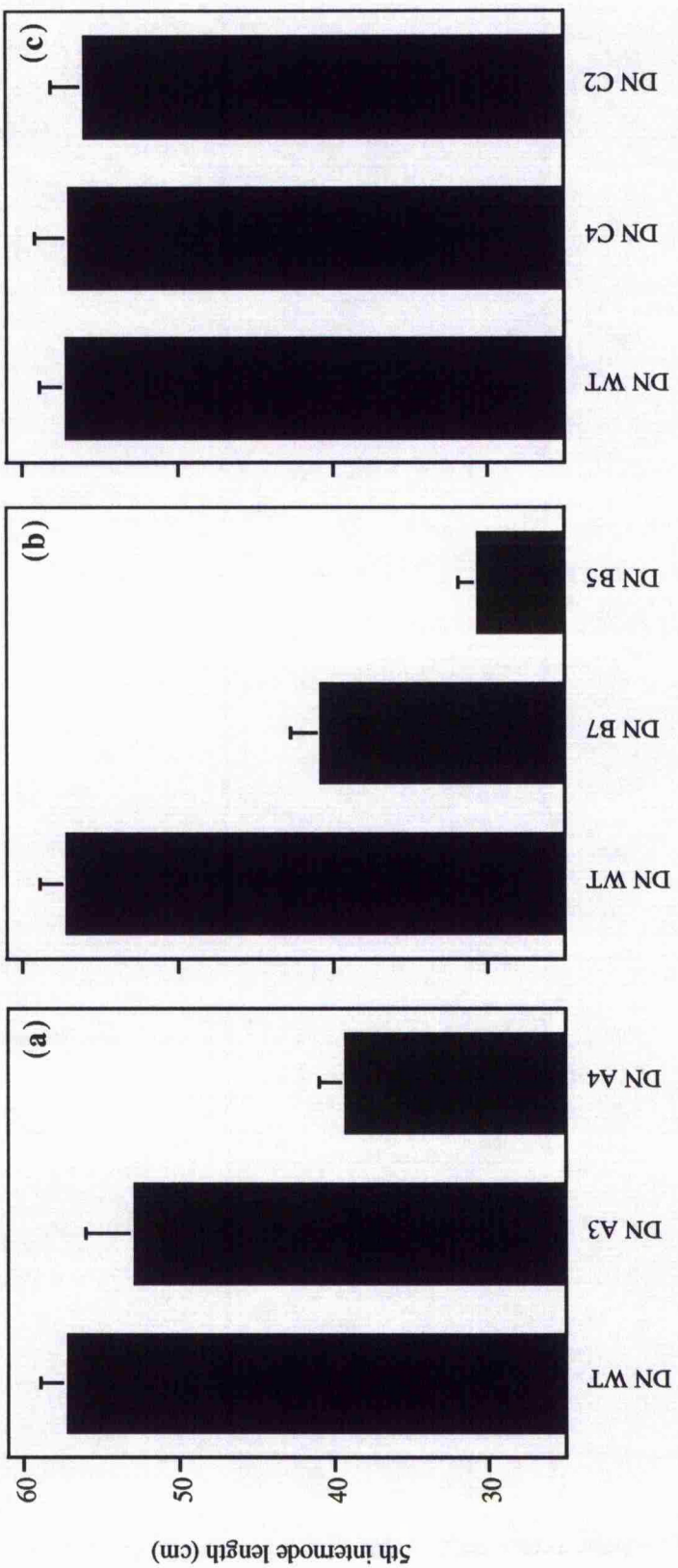


Figure 5.13 Fifth internode length of (**a**) DN WT, DN A3 and DN A4; (**b**) DN WT, DN B7 and DN B5; (**c**) DN WT, DN C4 and DN C2 plants measured after 40 d growth under greenhouse conditions. Data represent the means from 12 plants and the error bars are SE.

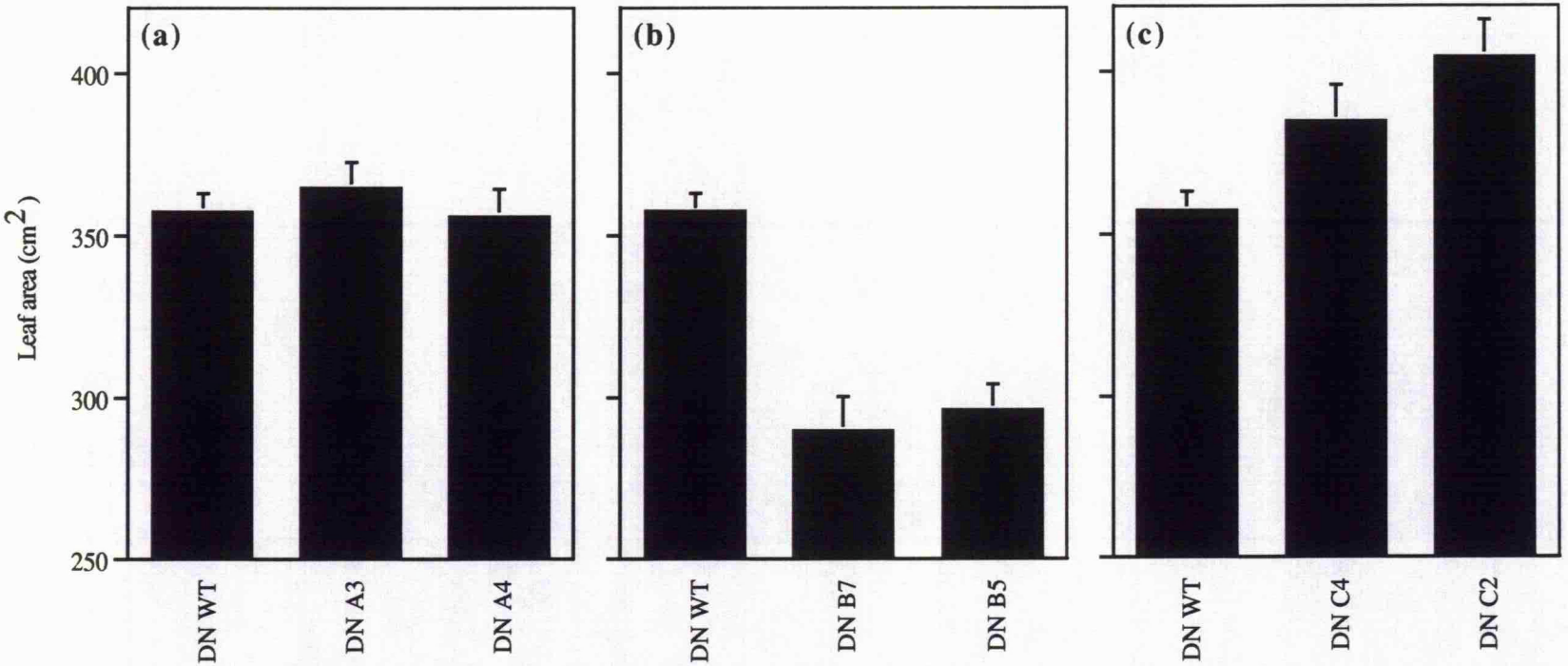


Figure 5.14 Area of fourth leaf of (**a**) DN WT, DN A3 and DN A4; (**b**) DN WT, DN B7 and DN B5; (**c**) DN WT, DN C4 and DN C2 plants measured after 40 d growth under greenhouse conditions. Data represent the means from 12 plants and the error bars are SE.

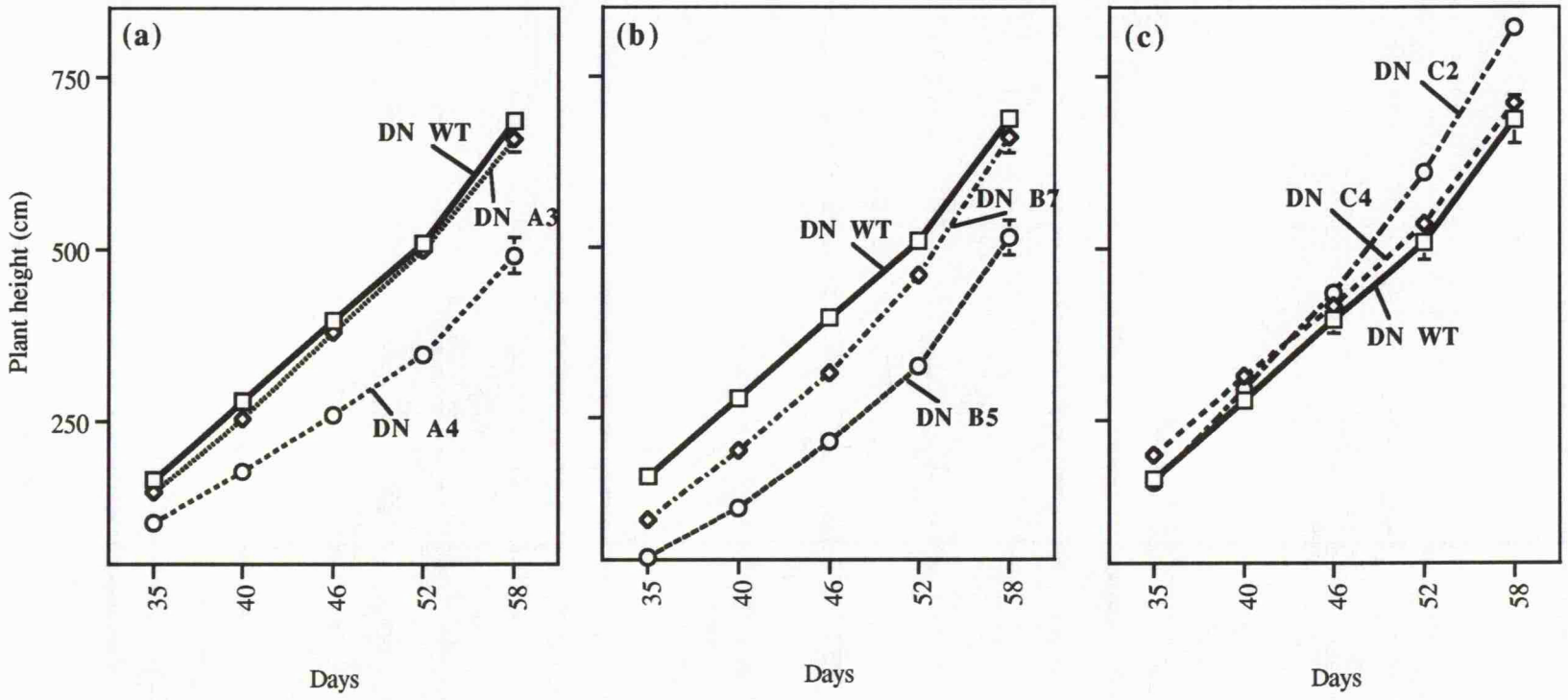


Figure 5.15 Plant height measured over a 35 to 58 d time course, of **(a)** DN WT, DN A3 and DN A4; **(b)** DN WT, DN B7 and DN B5; **(c)** DN WT, DN C4 and DN C2 plants grown under greenhouse conditions. Data represent the means from 12 plants and the error bars are SE.

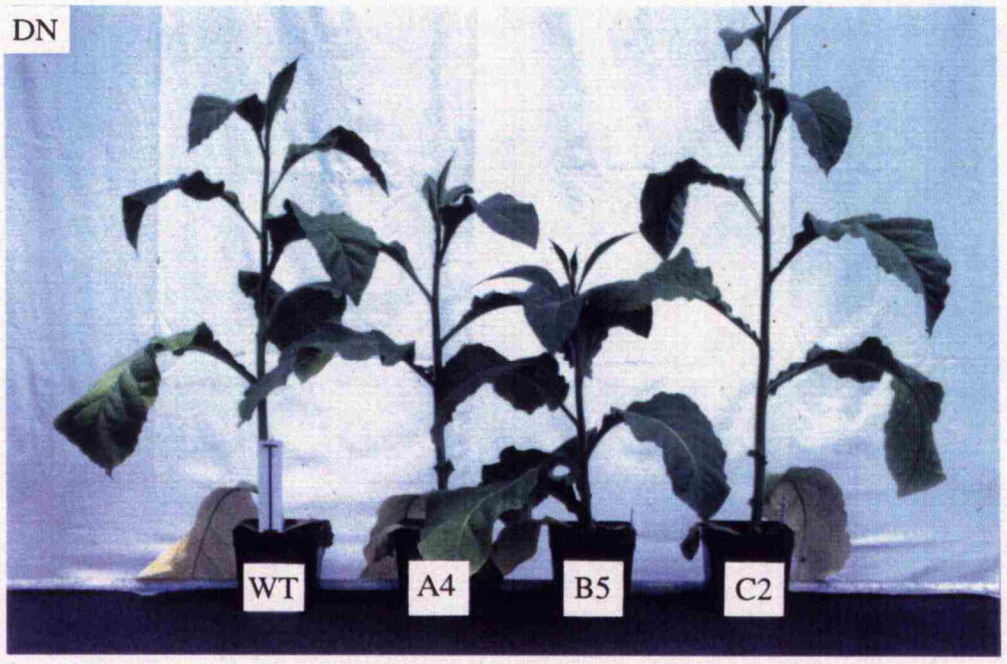


Figure 5.16 The phenotypical characteristics of DN WT, DN A4, DN B5 and DN C2 plants grown under greenhouse conditions.

Chapter 6 Flowering physiology of SD and DN *Nicotiana tabacum* overexpressing phytochrome A, B or C

6.1 Introduction

Flowering is controlled by multiple internal genetic factors and multiple external factors including temperature, water availability and the light environment. In a changing environment it is important that the plant is able to regulate the time of reproduction in response to favourable or unfavourable conditions. In precisely this way phytochrome can influence the onset of flowering in many species in relation to changes in light quality (see Smith, 1995). For instance, in *Arabidopsis* it has been shown that both phytochrome B, and a novel phytochrome(s), are responsible for an acceleration of flowering, mediated by low R/FR ratio light conditions that simulate canopy shade (Whitelam and Smith, 1991; Halliday *et al.*, 1994; Chapter 2 and Chapter 3). In this situation plants adopt a growth strategy that channels more resources into stem elongation, at the expense of leaf formation. It is the shade-avoiding species that make maximum use of this strategy. Under these conditions flowering is accelerated, which may be a safety mechanism, as the survival of the plant may be under threat in conditions where light is a limiting factor .

Many plants also flower at a particular time of year. The time of flowering and, hence, the timing of the life cycle of the plant, may be designed to benefit from optimal food resources, light, temperature, or to avoid competition from other species. The timing of flowering in response to photoperiod also enables the sychronisation of flowering within a species which may be important for reproductive purposes. There may also be a requirement for seeds to be set and or dormancy to be initiated at a certain time of year.

The ability to stimulate flowering in response to changing photoperiods is under the control of phytochrome, although little is understood of its exact role in this process (see Thomas, 1991; O'Neill, 1992). This is quite ironic as it was the early work on

photoperiodic control of flowering that ultimately led to the discovery of phytochrome. Experiments by Garner and Allard, in the 1920's, initiated a rapid development of knowledge in photoperiodism. Much of their work focussed on a tobacco mutant, Maryland Mammoth that, unlike the wild type tobacco is photoperiodic, requiring short days (SDs) to flower. Subsequent work on photoperiodic flowering control in SD plants revealed that flowering could be prevented if an inductive dark period was interrupted by a pulse of light (night-break). R light was found to be the most effective in the night-break (NB) response, and in some cases the effect of R could be cancelled by a FR light pulse (Parker and Hendricks, 1946). The phenomenon of R/FR reversibility was identified in other developmental and physiological processes and led to the formulation of the proposal that the photoreceptor, (phytochrome) has two photoreversible forms, Pr and Pfr (Borthwick *et al.*, 1952b).

Plants can be catagorised according to their photoperiodic requirements for flowering. Three main catagories exist: the first are SD plants, where flowering occurs or is accelerated when the length of the dark period exceeds a critical value; the second are long-day (LD) plants, where flowering occurs or is accelerated when the length of the dark period falls below a critical value (Thomas, 1991); and the third are day-neutral (DN) plants, where day length does not play a regulatory role in flowering (Vince-Prue, 1975).

Photoperiodic flowering control has been extensively studied in SD species, and as a consequence, the mechanism of action in SD plants is more thoroughly understood than that in LD plants. It is now generally accepted that photoperiodic time measurement involves the interaction of phytochrome with an endogenous circadian timer (Vince-Prue and Takimoto, 1987). Broadly speaking, in SD species, timing of the duration of darkness is the critical factor for induction of flowering and this involves two discrete actions of light. This has been demonstrated in *Pharbitis nil*, where light was shown to differentially affect flowering and phasing of the circadian timer (Lumsden and Furuya, 1986). In the natural environment, the "light off" signal at dusk sets the phase of the rhythm at the end of the photoperiod. During the dark period the circadian timer oscillates between insensitivity and sensitivity to light. Flowering initiation occurs when the "light on" signal at dawn coincides with a light-insensitive

phase of the rhythm.

Evidence for the involvement of phytochrome in the measurement of night length came initially from NB experiments, such as those carried out by Parker and Hendricks (1946), mentioned above. Later experiments describe rhythmic flowering responses to NBs given over a dark period, revealing the interaction of phytochrome with an endogenous rhythm (see Vince-Prue, 1994). There is evidence that phytochrome also adjusts the phase of the rhythm. Phase-shift experiments in *Pharbitis nil* demonstrate R/FR reversibility, which suggests that this role may be fulfilled by phytochrome (Lumsden, 1991). Interestingly, in wheat, the circadian cycling of *CAB* mRNA can be phase-shifted by a VLFR (Nagy *et al.*, 1993). Phytochrome A has been implicated in the VLFR in *Arabidopsis* (Botto *et al.*, 1996). Therefore, it is possible that this response is mediated via phytochrome A in wheat.

As well as setting the phase of the rhythm, entrainment of the circadian timer may also involve an adjustment in the length of the circadian cycle. Examination of the photoperiodic cycling of *CAB* transcript levels in *Arabidopsis* wild type and *hy1* seedlings suggest that phytochrome may also be influential in this response. When grown under R light, *CAB* expression cycles are longer in *hy1* seedlings than wild type seedlings (Millar *et al.*, 1995). These observations suggest that one or more phytochromes have a role(s) in altering the period of the rhythm.

Phytochrome species that are active in the various apects of rhythm entrainment have not yet been identified. However, it is probable that phytochromes which interact with the circadian rhythm either have light-labile characteristics, or have a Pfr which is unstable in the dark. Effective measurement of the dark period requires that the Pfr present at the end of the light period must decline at a sufficient rate so as not to interfere with the ciradian rhythm. A rapid decline in Pfr would also appear to be a necessary requirement of the phytochrome that sets the phase of the rhythm, as dark time measurement can begin within an hour of transfer to darkness (Lumsden and Vince-Prue, 1984). The proposal that phytochrome A may mediate this response in wheat (Nagy *et al.*, 1993), is consistent with this suggestion.

There also appears to be a quite separate requirement for Pfr in the promotion of flowering in SD plants, and this function is independent of the circadian rhythm. The

need for Pfr can be demonstrated by giving an EOD FR light treatment which has been shown to prevent flowering in many SD species (Vince-Prue, 1983). In some plants, this Pfr-requirement for flowering, can be satisfied during the light period if the photoperiod is of sufficient duration and/or of a sufficiently high fluence rate (Vince-Prue, 1983). However, if this requirement for flowering is not fulfilled during the preceding photoperiod, the Pfr-dependent process can continue for a long time into the inductive night. This is clearly demonstrated in the SD plant *Chenopodium rubrum*, where the inhibitory effect of a FR pulse on flowering has been shown to persist well into the inductive dark period (Cumming *et al.*, 1965). The effectiveness of the FR pulse gradually diminishes in a linear fashion, which illustrates that the Pfr-requiring response is independent of the circadian rhythm. The persistance of this Pfr species well into the dark period also demonstrates that it is very stable in darkness.

A number of photoperiodic studies have also been carried out with LD species, but the findings have been more complex and, therefore, the role of phytochrome in photoperiodism in LD plants is much less clear. In many cases a brief NB will have little effect on flowering, as there is a requirement for long daily photoperiods to initiate the reponse (see Vince-Prue and Takimoto, 1987; Thomas, 1991). What is more, NBs, where effective, have a promotory, rather than an inhibitory effect on flowering, and the maximum effect achieved by FR.

Direct evidence for the involvement of phytochrome in photoperiodic floral control has come from the analysis of phytochrome mutants and plants overexpressing phytochrome transgenes. The *phyA* mutants of *Arabidopsis*, a LD plant, show a lack of sensitivity to low fluence day extensions and W light NBs, conditions that stimulate flowering in wild type plants (Johnson *et al.*, 1994; Reed *et al.*, 1994). Furthermore, under SD (8 h) photoperiods *phyA* mutants flower somewhat later than wild type plants (Johnson *et al.*, 1994). In complementary experiments it has been shown that transgenic *Arabidopsis* seedlings that overexpress a *PHYA* cDNA also show reduced daylength sensitivity. However, in this case the transgenic seedlings flower earlier under SD conditions than wild type seedlings (Bagnall *et al.*, 1995). These observations suggest a role for phytochrome A in photoperiodic control in *Arabidopsis*.

Although phytochrome B function has been shown to be implicitly linked with

flowering (Chapter 2) it is unclear as to whether it can regulate flowering via a photoperiodic mechanism. The *phyB Arabidopsis* mutant and the *lh* mutant of cucumber, that lacks a phytochrome B-like phytochrome, both flower earlier than their respective wild type plants (Rood *et al.*, 1990b; López-Juez *et al.*, 1990). This early flowering response of *phyB* persists when grown under either LD or SD conditions (Goto *et al.*, 1991). However, the *phyB* mutants retain the ability to respond to a change in daylength. Interestingly, transgenic *Arabidopsis* overexpressing *PHYB* cDNAs are also early flowering (Bagnall *et al.*, 1995). In these plants, Pfr reduction with an EOD FR light treatment leads to a pronounced delay in flowering. It has, therefore, been proposed that, in *Arabidopsis*, PfrB acts in the dark period to promote flowering (Bagnall *et al.*, 1995).

The ma_3^R mutant of *Sorghum bicolor*, a quantitative SD plant, lacks the *PHYB* homologue (P.W. Morgan, pers comm.). The ma_3^R mutant seedlings also display an altered photoperiodic flowering response. Not only do they flower earlier than wild type plants in LDs, they are less responsive to daylength changes (Childs *et al.*, 1995). However, the means by which this phytochrome operates in wild type plants to control these aspects of flowering behaviour is yet to be determined.

Clearly much can be learned of the role of phytochrome in photoperiodism using phytochrome mutants and transgenic plants overexpressing phytochrome. To date, most of the this work has been carried out in *Arabidopsis* and other LD species. The analysis of SD species, however, has provided the most coherent picture of how phytochrome may be involved in photoperiodism (see Vince-Prue, 1994).

In this chapter, DN and SD *Nicotiana tabacum* cv. Hicks (obtained from Vern A. Sisson, United States Department of Agriculture, Oxford, USA.) were used to investigate roles of phytochrome species in photoperiodic flowering. SD *N. tabacum* differ from DN *N. tabacum* with respect to a single locus, *MARYLAND MAMMOTH* (*MM*). The SD plant was created by backcrossing the *MM* allele in *N. tabacum* cv. Maryland Mammoth into the DN *N. tabacum* cv. Hicks (V.A. Sisson pers. comm.). Therefore, except at the *MM* locus, the DN and the SD *N. tabacum* are isogenically identical. The *MM* gene, confers photoperiodicity, transforming *N. tabacum* from a

DN plant to a SD plant.

Both DN and SD *N. tabacum* have been transformed with heterologous phytochrome A and B cDNAs, and phytochrome C gDNA (see Chapter 4) with a view to assessing the impact of phytochrome overexpression on photoperiodic flowering. The photoperiodicity of the SD *N. tabacum* makes it possible to probe the photoperiodic sensitivity of the plants. This is facilitated by the fact that SD *N. tabacum* respond in a quantitative way to NBs. When 30 min NBs are given half way through an otherwise inductive night, flowering is delayed. The work in this chapter utilises this characteristic to explore the effect of phytochrome overexpression on NB sensitivity in plants grown in SD photoperiods. A comparative analysis of plants grown either in SDs without a NB, or continuous W light provides a further insight into the roles of phytochromes A, B and C in daylength sensing.

6.2 Experimental procedures

6.2.1 The creation of *Nicotiana tabacum* transgenic lines overexpressing phytochrome A, B or C

DN and SD *Nicotiana tabacum* cv. Hicks, were obtained from Vern A. Sisson, USDA, Oxford, USA. Both the DN and the SD cultivars have been transformed with the oat phytochrome A cDNA, the *Arabidopsis* phytochrome B cDNA and the *Arabidopsis* phytochrome C gDNA, each under the control of the "constitutive" CaMV 35S promotor. Transformants were selected with a range in transgene expression levels in each of the A, B and C lines. For details of the DN and SD *N. tabacum* cultivars, the transformation procedures and selection methods see Chapter 4.

6.2.2 Growth conditions

Conditions of germination, young seedling growth and mature plant growth in both growth room and greenhouse facilities are as for Chapter 5.

6.2.3 Measurement of flowering time and plant height

Flowering time was measured as the number of days and the number of leaves formed (above 2 cm width) at appearance of the first fully expanded corolla. Plant height was measured with a ruler. The sample sizes for all experiments was 12 plants.

6.2.4 Light Sources

For experiments using SD photoperiods and SDs with a 30 min NB given 8 h into a 16 h dark period, growth rooms were used. The photoperiods were provided by cool-W fluorescent tubes that provided a photon fluence rate (400-700 nm) of 138 μmol m^{-2} s^{-1} in the SD growth room and 139 μmol m^{-2} s^{-1} in the SD+NB growth room. The temperature range in the growth rooms was between 21 and 24°C.

Plants in experiments carried out under greenhouse conditions were grown in daylight supplemented with continuous W light provided by Son-T plus 400 lights (Phillips).

6.3 Results

6.3.1 Photocontrol of flowering time in DN *N. tabacum* overexpressing phytochrome A, B or C, grown under greenhouse conditions

Both flowering time and number of leaves produced at flowering time was assessed in DN plants grown under greenhouse conditions. Both the A- and B-overexpressors flower late and produce more leaves than wild type plants under these conditions (Figs. 6.1 and 6.2). In contrast, the DN C2 plants flower slightly earlier than wild type (Fig. 6.1). This early-flowering phenotype, however, is not observed in DN C4 plants that flower at the same time as wild type plants (Fig. 6.1). In addition, both DN C4 and DN C2 plants flower with a similar leaf number to wild type plants (Fig. 6.2). Under these conditions the dwarfed growth habit, displayed by plants in the A- and B-overexpression lines (see Chapter 5), correlates with late flowering, whilst the enlarged phenotype displayed by DN C2 plants (see Chapter 5) correlates with early flowering.

6.3.2 The influence of NBs on leaf number and plant height in DN and SD *N. tabacum* overexpressing phytochrome A, B or C

For these experiments plants were grown under SD, 8 h photoperiods with and without a 30 min W light NB, 8 h into the 16 h dark period. In *N. tabacum*, the length of the vegetative developmental stage can be measured in terms of leaf number and, to a lesser extent, in terms of plant height at flowering time. When DN or SD wild type plants are grown in SD or in SD+NB photoperiodic conditions, they achieve about the same height (Figs. 6.3 and 6.4). However, when grown under SDs+NBs, SD plants exhibit an increase in leaf number that is significantly greater than that of SD plants grown under SDs (Figs. 6.3 and 6.4). Likewise, DN plants grown under SD+NB conditions, display a small, but significant increase in leaf number compared with DN

plants grown under SDs photoperiods (Figs. 6.3 and 6.4). These observations indicate, firstly, that NBs appear to increase amount of time spent in the vegetative development not only in SD *N. tabacum*, but to a lesser extent in DN *N. tabacum*. Secondly, in wild type plants, NBs do not extend the length of the vegetative phase sufficiently such that it can be measured in terms of increased plant height.

The height of phytochrome A- and B-overexpressors is more variable than that of wild type plants. Their final height is in part a consequence of the length of time spent in the vegetative state, and in part a consequence of the overexpression phenotype. In the DN A-overexpression lines, plant height and leaf number are not markedly different from that of the wild type plants grown under SDs (Fig. 6.3(a)). When grown under SD+NB conditions, with the exception of DN A4 plants, height and leaf number of the transgenic plants are again similar to that of wild type plants (Fig. 6.3(a)). In response to NBs, plants in the DN A4 line are slightly taller than wild type plants and have over twice the number of leaves. Therefore, it appears that in DN A4 plants, the period of vegetative development is extended sufficiently under SDs+NBs so as to affect an increase in plant height, as well as leaf number.

Examination of DN plants overexpressing phytochrome B grown under SD photoperiods reveals that DN B7 plants are shorter than wild type plants, whilst DN B5 plants are the same height as wild type plants (Fig. 6.3(b)). Under the same conditions DN B7 leaf number is the same as in the wild type, whilst DN B5 leaf number is greater than that of the wild type. This suggests that flowering is sufficiently delayed in DN B5 plants, that plant height, as well leaf number, is increased. Both DN B7 and DN B5 plants, essentially display similar characteristics when grown under SD+NB conditions, although leaf number is greater in both transgenic lines (Fig. 6.3(b)). This suggests that the NBs stimulated a further extension of the vegetative phase of development in these plants.

It should be noted that plants in the DN A4 and DN B5 lines display a pronounced dwarfed growth habit when they are grown under continuous light (see Chapter 5). Thus, a reduced photoperiod appears to delay flowering sufficiently in these plants to extend the growing period, and hence, plant height.

Plants in the DN C-overexpression lines, all achieve a wild type height when

grown in SDs, both with and without a NB (Fig. 6.3(c)). These observations contrast with those made in plants grown under greenhouse conditions where DN C-overexpressors have an enlarged growth habit (see Chapter 5). When grown under either SD or SD+NB conditions the DN transgenics are also indistinguishable from wild type plants with regard to leaf number (Fig. 6.3(c)). These observations indicate that phytochrome C-overexpression in DN *N. tabacum* does not influence these aspects of vegetative development under SDs. What is more, NBs do not have a discernible effect on the length of the vegetative phase.

In SD photoperiods, plants in the SD transgenic lines overexpressing phytochrome A or B, (with the exception of SD A7 plants), are shorter in stature, but have a larger number of leaves than wild type plants (Figs. 6.4(a) and (b)). Plants in the SD A7 line are weak overexpressors (see Chapter 4) and when grown under either SD or SD+NB conditions, they are indistinguishable from wild type plants (Fig. 6.4(a)).

When grown under SD+NB photoperiods the vegetative period of growth is extended in all the SD plants expressing sufficient levels of phytochrome A or B (Fig. 6.4(a) and (b)). In fact, in the more severe overexpression lines NBs induced a very marked extension of the vegetative period, such that some plants grew to twice the height of wild type plants and produced over twice as many leaves (Figs. 6.4(a) and (b)). It is noteworthy that increased leaf production and, therefore, short-internodes are a characteristic of all the transgenic plants overexpressing phytochrome A or B, irrespective of final plant height.

SD plants in the different lines overexpressing phytochrome C, when grown under SD photoperiods display some variation in plant height, however, there does not appear to be a particular trend (Fig. 6.4(c)). Overall, plants in the SD C-overexpression lines achieve around the same height as wild type plants (Fig. 6.4(c)). If leaf number is examined, although again there is some variation, SD transgenics essentially behave in a wild type manner under SDs (Fig. 6.4(c)). When the SD transgenics are examined under SD+NB conditions, they behave like wild type plants with respect to plant height (Fig 6.4(c)). However, plants in the SD C3 and SD C2 transgenic lines produce a greater number of leaves than SD wild type plants grown under SD+NB conditions

(Figs. 6.4(c)). This suggests that the length of time spent in the vegetative state is prolonged in these SD plants overexpressing phytochrome C.

6.3.3 The influence of NBs on flowering time in DN *N. tabacum* overexpressing phytochrome A, B or C

In *N. tabacum*, the time taken to flower can be fairly accurately quantified as leaf number, which reflects the length of the vegetative phase, or it can be quantified as the number of days to flower formation. Under SD photoperiods, DN wild type plants flower between 71 and 81 d, whereas under SDs with a NB they flower between 85 and 89 d (Fig. 6.5(a), (b) and (c)). These observations are consistent with those made for leaf number. In DN wild type plants, flowering time is slightly delayed and, therefore, the length of the vegetative period is slightly extended in response to NBs (Fig. 6.3(a)).

In the DN A-overexpressors grown under SD conditions, flowering occurs at the same time as wild type plants (Fig. 6.5(a)). When grown under SD+NB conditions, however, flowering is severely retarded in the most severe overexpression line DN A4, at 168 d (Fig. 6.5(a) and Chapter 4). In the less severe overexpression lines DN A1 and DN A3 (Chapter 4) flowering time is not significantly different from that of wild type plants (Fig. 6.5(a)). Thus, phytochrome A-overexpression in DN A4 plants appears to accentuate the NB-stimulated delay in flowering, displayed in the wild type and less severe A-overexpression lines. For all these transgenics, leaf number and, therefore, the time spent in vegetative development corresponds with flowering time (Fig. 6.3(a)).

DN plants overexpressing the phytochrome B transgene flower significantly later than DN wild type plants when grown under both SD and SD+NB photoperiods and this is represented as an increase in days to flowering and leaf number (Figs. 6.5(b) and 6.3(b)). DN B7 and DN B5 plants flower at 75 and 102 d under SDs and 103 and 130 d under SD+NB conditions, respectively (Fig. 6.5(b)). Thus, the delay in

flowering observed in DN plants overexpressing phytochrome B appears to occur regardless of whether the plants receive NB treatments. However, the extent of the delay is more marked in plants subject to NBs.

When the DN C-overexpressors are grown in SDs and SDs+NBs they flower at around the same time as wild type plants (Fig. 6.5(c)). These plants also demonstrate a wild type response in leaf number under the same conditions (Fig. 6.3(c)). Therefore, unlike A- and B-overexpression, C-overexpression does not have an effect on the flowering time in DN *N. tabacum* grown in either SD or SD+NB conditions.

6.3.4 The influence of NBs on flowering time in SD *N. tabacum* overexpressing phytochrome A, B or C

Under SD photoperiods, SD wild type plants flower between 85 and 89 d, whereas under SDs with a NB they flower between 109 and 120 d (Figs. 6.6(a), (b) and (c)). A concomitant increase in leaf number confirms an extended vegetative phase of development in these plants grown under SD+NB conditions (Figs. 6.4(a), (b) and (c)). The NB clearly has a strong delaying action on flowering in SD *N. tabacum.*

Plants in the SD A7 and SD A19 lines are indistiguishable from wild type plants when grown in SD photoperiods (Fig. 6.6(a)). Under the same conditions, SD A4 plants exhibit a small delay in flowering time, when compared with wild type plants (Fig. 6.6(a)). However, this delay does not affect leaf number (Fig.6.4(a)). When grown under SD+NB photoperiods, compared with SD wild type plants, a very marked delay in flowering time is displayed, not only by the SD A4, but also by the SD A19 transgenics (Fig. 6.6(a)). SD A4 and SD A19 plants flower at 177 and 161 d respectively, compared to 120 d for the wild type plants. Here a marked increase in leaf number is also seen in both SD A4 and SD A19 transgenics (Fig. 6.4(a)). Thus, phytochrome A-overexpression can delay flowering in SD *N. tabacum* under SD conditions, though, the delay in flowering in response to a NB is much more striking. Therefore, it appears that phytochrome A-overexpression increases the sensitivity of the plant to NBs.

When grown under SDs the two most severe SD B-overexpression lines, SD B13 and SD B5, (see Chapter 4) flower later than wild type plants (Fig. 6.6(b)). Under SDs+NBs, the delay in flowering time is significantly greater than that of wild type plants, and is not only seen in the two more severe B-overexpression lines, but also in the weaker B-overexpression line SD B2 (Fig. 6.6(b)). This delay in flowering under SD+NB photoperiods is very marked in the SD B13 and SD B5 lines which flower at 188 d and 168 d respectively, as opposed to 110 d in wild type plants (Figs. 6.6(b)). Again for all these plants, the flowering time is reflected by a corresponding increase in leaf number (Fig. 6.4(b)). As in the DN transgenic lines, phytochrome B-overexpression in the SD lines can cause a delay in flowering time in both SD and SD+NB conditions, although the delaying effect of the NB is more effective.

When the C-overexpression lines are grown under SDs, plants in the SD C1 and SD C3 flower at 91 d and 94 d respectively, marginally later than wild type plants that flower at 85 d. (Fig. 6.6(c)). This delay in flowering, of some 6 to 9 d, is not reflected by an increase in leaf number (Fig. 6.4(c)). When grown under SDs+NB, however, all the SD C-overexpression lines exhibit a delay that is more severe than that displayed by wild type plants grown under the same conditions (Fig. 6.6(c)). DN C1, DN C3 and DN C2 flower at 116, 127 and 132 d, respectively, whilst wild type plants flower at 110 d. This delay is represented as an increase in leaf number in the SD C3 and SD C2 lines (Fig. 6.4(c)). It is noteworthy that the delay in flowering displayed in SD C-overexpression lines is not seen in the DN C-overexpression lines. This suggests that, either the DN lines do not overexpress the transgene to a level that stimulates a recognisible response, or that phytochrome C-overexpression specifically affects flowering in SD *N. tabacum*.

In summary, when grown under greenhouse conditions, (in continuous light), DN plants expressing high levels of the phytochrome A and the phytochrome B transgene display a delay in flowering, whilst, DN plants expressing the phytochrome C transgene can be early flowering (see Chapter 5). Under these conditions there is a marked difference in the growth habit of the A- and B-overexpressors that display a "dwarfed" phenotype, and the growth habit of the C-overexpressors that display an "enlarged" phenotype.

When grown under SD and SD+NB photoperiods the phenotypes of all the transgenic lines is much less striking. The elongated phenotype of the DN C-overexpression lines, seen under greenhouse conditions, is completely absent in plants grown under photoperiodic conditions. Also, the dwarfed phenotype associated with A- and B-overexpression, although present, is much less severe when plants are grown under photoperiodic conditions. Interestingly, the short-internode growth habit of the A- and B-overexpressors is maintained in SD lines where the vegetative period has been extended considerably by a NB. Although under these conditions the transgenics can grow quite tall and are, therefore, no longer dwarfed in appearance they, nonetheless, maintain the short-internode growth habit associated with an A- or B-overexpression phenotype.

Plants in the severe overexpression line, DN A4, display a greatly enhanced delay in flowering in response to NBs. In SD *N. tabacum*, overexpression of phytochrome A has only a marginal effect on flowering in the most severe overexpression line, SD A4, when grown in SDs. However, in response to NBs the two most severe overexpresion lines, SD A19 and SD A4, display a marked delay in flowering. The overexpression of phytochrome B can delay flowering in both DN and SD *N. tabacum* under SDs either with or without a NB. However, this delay does appear to be more marked in response to NBs. In contrast, phytochrome C-overexpression only causes a delay in flowering in the SD *N. tabacum*, and this delay is most striking in response to NBs. The DN transgenics overexpressing phytochrome C behave in a "wild type" manner under both SD and SD+NB conditions. This may either be a reflection of the transgenic phyC levels, which may be insufficient to stimulate a response in the DN plants, or may reflect the ability of phytochrome C-overexpression, to directly interact with the *MM* gene in SD *N. tabacum*.

6.4 Discussion

The SD *N. tabacum* used in this work differs from DN *N. tabacum* with respect to a single locus, *MM*, that confers SD photoperiodicity. Therefore, at a physiological level the DN and the SD *N. tabacum* probably differ only with respect to their photoperiodicity. These characteristics provide a useful model system for the direct comparison of flowering, and the corresponding effects on vegetative development, in DN and SD tobacco.

A feature of SD *N. tabacum* is that NBs, given at an appropriate time through the dark period, can have a quantitative effect, rather than a qualitative effect on flowering. Thus, NBs can be used as a means of delaying flowering. This characteristic has been exploited to investigate the behaviour of transgenic plants expressing heterologous phytochrome species. It would be expected that plants overexpressing a species of phytochrome capable of interacting with an endogenous circadian rhythm, are likely to display altered flowering time in response to NBs. For this purpose a series of overexpression lines have been generated from both DN and SD *N. tabacum*, transformed with phytochromes A and B cDNAs, and phytochrome C gDNA. Analysis of plants overexpressing phytochrome A, B or C under non-photoperiodic and photoperiodic conditions provide further evidence for the involvement of individual phytochrome species in the control of flowering. In addition, examination of the relationship between flower time, leaf number and plant height establishes the effect of altered flowering on vegetative development.

The characteristics of DN wild type and transgenic plants grown under greenhouse conditions have been examined. Under these conditions the defining features of the overexpression phenotypes are quite pronounced. Plants overexpressing phytochromes A or B display a dwarfed phenotype characterised by reduction in internode and stem length (see Chapter 5). The phytochrome B-overexpressors, in addition have reduced leaf area. In contrast, plants in the phytochrome C-overexpression lines are larger than wild type plants (see Chapter 5). This largeness can be measured in terms of increased stem length and increased leaf area. Likewise, when flowering time is examined, the dwarfed phytochrome A- and phytochrome B-

overexpressors are delayed with respect to wild type plants. This delay in flowering is also reflected by the increase in the number of leaves produced by these transgenics at flowering time. Again, contrasting with the A- and B-overexpression lines, plants in the larger C-overexpression line, DN C2, flower earlier than wild type plants. The early flowering, however, is not severe enough to shorten the vegetative phase of these plants, as the number of leaves produced are the same as wild type plants. Thus, in DN *N.tabacum* grown under continuous W light, there appears to be a correlation between plant size and flowering time. Decreased plant size, displayed by the phytochrome A and B transgenic plants, correlates with delayed flowering, whilst increased plant size, displayed by the phytochrome C transgenic plants, correlates with accelerated flowering.

When grown under short photoperiods the phenotype described for greenhouse grown DN plants is either absent or less marked. Plants in the DN C-overexpression lines do not display the enlarged phenotype seen under greenhouse conditions, as they are indistinguishable from wild type plants when grown under SDs. Likewise, the dwarfed phenotype displayed by the DN phytochrome A-overexpression lines under greenhouse conditions is not seen in plants grown under 8 h photoperiods. However, the severely dwarfed phenotype of the DN transgenics expressing high levels of phytochrome B is displayed under SDs, but is less severe. In both the A and the C DN transgenics, loss of the overexpression phenotype under SDs corresponds with loss of the altered flowering response also observed under greenhouse conditions. The DN B-overexpression lines, however, maintain both the dwarfed phenotype and the delayed flowering response under SDs.

A feature of the SD A- and B-overexpression lines is the large increase in the height of plants that flower very late in response to NBs. Transgenic plants, expressing high levels of phytochromes A or B display the characteristic dwarfed phenotype when grown under continuous light and/or SDs. However, when these plants are grown under SD+NB conditions the vegetative growth period is extended, therefore, plants can achieve a final height that is much greater than that of the wild type plants. What is apparent is the leaf number per length of stem is greater in transgenic plants when compared with wild type plants. Thus, the resultant reduction in internode length, a

principle characteristic of the dwarfed phenotype displayed by plants overexpressing phytochromes A or B, is also maintained in SD *N. tabacum* which grow very tall under SD+NB conditions.

Flowering time has also been compared in the DN and SD plants overexpressing phytochrome A, B or C, grown under SD and SD+NB conditions. This experiment was designed to allow a direct comparison of flowering time in plants without a photoperiodic sensitivity (DN) with those with a photoperiodic sensitivity (SD) to be observed under the same conditions. Therefore, it is interesting that wild type DN plants do, in fact, display a slight sensitivity to NB, as they flower between 9 and 13 d later than DN plants grown under SDs. This delay in flowering is also reflected by a small increase in leaf number in these plants. Thus, it appears that the DN *N. tabacum* does have a mild sensitivity to NBs.

Sensitivity to NBs is exaggerated in the most severe A-overexpressor, DN A4. When grown under SD+NB conditions, these plants flower 78 d later and produce over twice the number of leaves as wild type plants. Here A-overexpression appears to have the ability to greatly enhance the NB-sensitivity of the delayed flowering response in DN *N. tabacum*. The other transgenic lines in the series, DN A1 and DN A3, essentially have a wild type response to NBs. However, this may be a consequence of their comparatively low levels of transgenic phytochrome. All of the plants in these DN transgenic lines behave in a wild type manner when grown under both SD and SD+NB conditions.

Phytochrome B-overexpression delays flowering in DN *N. tabacum* under SDs and increases this delay in flowering under SDs+NBs. Flowering is delayed by up to 30 d under SDs and up to 44 d under SD+NB conditions. It appears that, although phytochrome B-overexpression in DN *N. tabacum* affects flowering time in SDs, this delay is enhanced by NBs.

In contrast, in the DN transgenic lines, flowering time is unaltered by phytochrome C-overexpression. All plants in the lines overexpressing phytochrome C flower at the same time and produce the same number of leaves as wild type plants grown under both SD, and SD+NB photoperiods.

When wild type SD plants are grown under SDs they flower between 85 and 89

d. Under SD+NB conditions, flowering occurs between 110 and 120 d, a delay of 25 to 31 d. Phytochrome A-overexpression in SD *N. tabacum* is effective in delaying further flowering under SDs, but this is only seen in the most severe A-overexpression line, SD A4, that flowers 14 d later than wild type plants. However, the NB-induced delay in flowering is extremely marked in the two lines with the highest transgene levels, SD A19 and SD A4, that flower 41 and 58 d, respectively, later than wild type plants. The NBs are effective in delaying flowering time in these transgenic lines for more than twice the time of the NB-induced delay in wild type plants. The late-flowering nature of the transgenics is also reflected by a corresponding increase in the number of leaves produced through the extended vegetative phase. Thus, it appears that although phytochrome A-overexpression can delay flowering time in SD *N. tabacum* grown in SDs, the delay is more marked in response to NBs. Furthermore, the NB-induced delay in flowering is displayed, not only by SD, but also by DN *N. tabacum* overexpressing phytochrome A.

Similarly, SD *N. tabacum* overexpressing higher levels of phytochrome B flower late in SDs and SDs+NBs. SD lines overexpressing the phytochrome B transgene flower up to 11 d later than wild type plants under SDs and up to 78 d later than wild type plants under SD+NB photoperiods. Again this delay in flowering is reflected by a corresponding increase in the number of leaves produced at flowering time. This late-flowering pattern is very reminiscent of that displayed in the DN transgenics. Both DN and SD *N. tabacum* overexpressing phytochrome B flower late in SDs, and they both have an exaggerated late-flowering response to NBs.

Plants in the SD C-overexpression lines flower slightly later than wild type plants when grown in SDs. However, this late-flowering-response is more pronounced when the plants are grown under SDs with a NB. Flowering is slightly delayed with respect to wild type plants in SD C1 and SD C3 plants, but this tendency is not a feature of plants in the SD C2 line. This observation may be significant as these plants certainly have the most pronounced response to NBs. Therefore, it is uncertain whether these data are a true reflection of phytochrome C-overexpression-effects in SD plants grown under SDs.

All the plants in the SD phytochrome C-overexpression series display a delay in

flowering when compared with wild type plants grown under SD+NB conditions. The most significant delay is displayed by plants in the SD C2 lines, which flower 22 d later than wild type plants. The extent of the NB-induced late flowering can also be measured as an increase in the number of leaves produced in the SD C3 and SD C2 transgenics. In these plants flowering is sufficiently late for this response to be measured in terms of an extended vegetative phase of development. As flowering time is unaffected in the DN transgenic plants it is possible that phytochrome C-overexpression may have a specific role in photoperiodic flowering in the SD plant.

Analysis of the transgenic DN and SD *N. tabacum* identifies differences in the effects of phytochrome A-, phytochrome B- and phytochrome C-overexpression on flowering time. When flowering is examined in DN *N. tabacum* grown under non-photoperiodic (greenhouse) conditions, the overexpression of phytochrome A or B delays flowering, whereas the overexpression of phytochrome C enhances flowering. In these plants, the timing of flowering appears to be linked with phenotypical characteristics. In the A- and B-overexpressors, a dwarfed phenotype correlates with delayed flowering. This contrasts with the C-overexpressors, where an enlarged phenotype correlates with early flowering.

In SD transgenics, phytochrome A-overexpression causes a delay in flowering under SDs and greatly enhances the sensitivity of the plant to NB which results in a massive delay in flowering. Likewise, in DN plants that are mildly sensitive to NBs, high levels of transgenic phytochrome A appear to amplify this sensitivity to NBs. Therefore, it is possible that phytochrome A may play a principle role in the timing of the dark period and hence in the timing of flowering in response to photoperiod. If this is the case, then phytochrome A would interact with the circadian rhythm during the light sensitive phase of the rhythm to inhibit flowering. The enhanced delay in flowering displayed by SD transgenics grown in SDs may represent a reduced NB effect, although, it may equally be due to a phase shift in the rhythm caused by the persistence of transgenic phytochrome A at the beginning of the dark period. If the latter is true, phytochrome A would be responsible for the setting of the rhythm at dusk.

There is evidence for the involvement of phytochrome A in photoperiodic perception in *Arabidopsis*, a LD plant (Johnson *et al.*, 1994; Reed *et al.*, 1994; Bagnall

et al., 1995). Responses mediated by phytochrome species are not likely to be identical in SD and LD species. However, it is possible that mode of action via which phytochromes mediate photoperiodic responses, is similar in both SD and LD plants. The *Arabidopsis phyA* mutants not only have a reduced ability to perceive otherwise inductive low fluence rate, indandescent day extensions, but also do not perceive W light NBs (Johnson *et al.*, 1994; Reed *et al.*, 1994). Futhermore, *Arabidopsis* seedlings that overexpress a *PHYA* cDNA flower earlier under SDs than wild type seedlings (Bagnall *et al.*, 1995). There is also indirect evidence that phytochrome A may be involved in phase-shifting of the circadian rhythm. A VLFR-mediated phase-shift in the rhythmic expression of the *CAB* gene has been demonstrated in wheat (Nagy *et al.*, 1993). These observations, together with the strong likelihood that phytochromes which interact with the circadian oscillator have light-labile qualities, or are unstable in their Pfr form in the dark (Vince-Prue 1994), lend support to the proposition that phytochrome A is active in photoperiodic time measurement in *N. tabacum*.

Phytochrome B-overexpression delays flowering in SDs and enhances this delay in response to NBs, in both DN and SD *N. tabacum*. It is clear that phytochrome B-overexpression can effect a delay in flowering irrespective of whether plants are grown under photoperiodic conditions or continuous light. However, phytochrome B-overexpression also appears to enhance NB-sensitivity in both DN and SD *N. tabacum*. This could be due to an interaction of PfrB and the circadian rhythm, although, it is thought that this action is likely to be fulfilled by a phytochrome with light-labile characteristics, or a Pfr that is short-lived in the dark. It is, therefore, unclear from these experiments whether phytochrome B controls flowering solely via mechanisms that are independent of day length, or whether it also has a role in the control photoperiodic flowering.

When behaviour is examined in other plants that are either deficient in, or have exaggerated levels of phytochrome B, the nature of the any observed changes in flowering to photoperiod are also ill-explained. For example, the *phyB* mutant of *Arabidopsis* which has a marked early phenotype, displays an alteration in photoperiodic flowering (eg. Goto *et al.*, 1991). The *phyB* mutants retain the ability to respond to changes in photoperiod. However, early-flowering does appear to be more

marked in plants grown under SDs, in comparison to those grown under LDs. *Arabidopsis* seedlings that overexpress phytochrome B are also early flowering (Bagnall *et al.*, 1995). In the case of these transgenic seedlings, reduction of Pfr with an EOD FR, delays flowering. Similar treatments, in wild type and *phyB* seedlings, accelerate flowering. This suggests that, in *Arabidopsis*, PfrB acts during the dark period to promote flowering.

Examination of photoperiodic flowering in the phytochrome B-deficient ma_3^R mutant of *Sorghum bicolor*, a SD plant, has revealed a reduced ability of these plants to perceive LDs (Childs *et al.*, 1995). However, the absence of phytochrome B does not affect the phase of *CAB* and *RBCS* mRNA circadian oscillation. It is, of course, possible that the circadian timer involved in photoperiodic flowering in *Sorghum*, acts independently of these rhythms. As with the *phyB* mutants, the ma_3^R mutants still respond to photoperiods, but photoperiodic perception is nonetheless altered. Thus, in *N. tabacum*, as in other species, although phytochrome B status can profoundly influence vegetative development and as a consequence, flowering, its precise role in photoperiodism remains unclear.

Phytochrome C-overexpression appears to directly interfere in SD photoperiodic flowering. Transgenic phytochrome C expression has no effect on the flowering characteristics of DN *N. tabacum*, which essentially display wild type behaviour. Likewise, phytochrome C-overexpression in the SD plants does not appear to substantially affect flowering when grown under SD photoperiods. However, transgenic plants grown under SD+NB conditions display an increased delay in flowering when compared to wild type plants. There are two possible interpretations of the C-overexpression effects. Firstly, the confinement of the response to the SD plants may simply be a reflection of the levels of transgenically-expressed phytochrome. It is possible that in the DN plants, transgenic phytochrome C is not present at levels that are sufficient to mount a response. Secondly, as the enhanced delay in flowering is only displayed by the SD transgenics, it may actually reflect the ability of phytochrome C-overexpression to interact specifically with *MM* gene function and, therefore, may be intimately connected to the SD photoperiodic time sensing mechanism.

The data presented here suggest that phytochromes A and C and possibly phytochrome B are influential in photoperiodic flowering. However, their precise roles in this aspect of photomorphogenesis is far from clear. Further analysis could reveal whether phytochrome overexpression specifically interacts with the circadian timer. This could be achieved by subjecting transgenic plants to NBs at different time intervals throughout the dark period using fluence rates sufficiently low as not to be recognised by wild type plants. If phytochrome overexpression can interact with the circadian rhythm, changes in flowering time would either exhibit a circadian rhythmicity, or reflect alterations in the phase of the rhythm. Complementary experiments, using phytochrome deficient plants, could also test whether the native phytochrome has a role in the timing of flowering in response to NB. The loss of a NB effect would implicate that phytochrome in dark-timing. However, whilst the maintenance of a NB response would implicate other phytochromes in this response, it would not exclude the possibility of that particular phytochrome acting in a similar capacity.

Modification of the DN, SD plant model used in these analyses, could yield additional information on the roles for phytochrome in photoperiodic perception. SD *N. tabacum* cv. Hicks require several consecutive inductive dark periods given for promotion of flowering. This, coupled with their large size imposes restrictions on the experimental design. Smaller SD plants, with a shorter life cycle and which also require a single inductive dark period to stimulate flowering would be ideally suited to this type of analysis. However, such SD plants that are deficient in or overexpress phytochromes are not available.

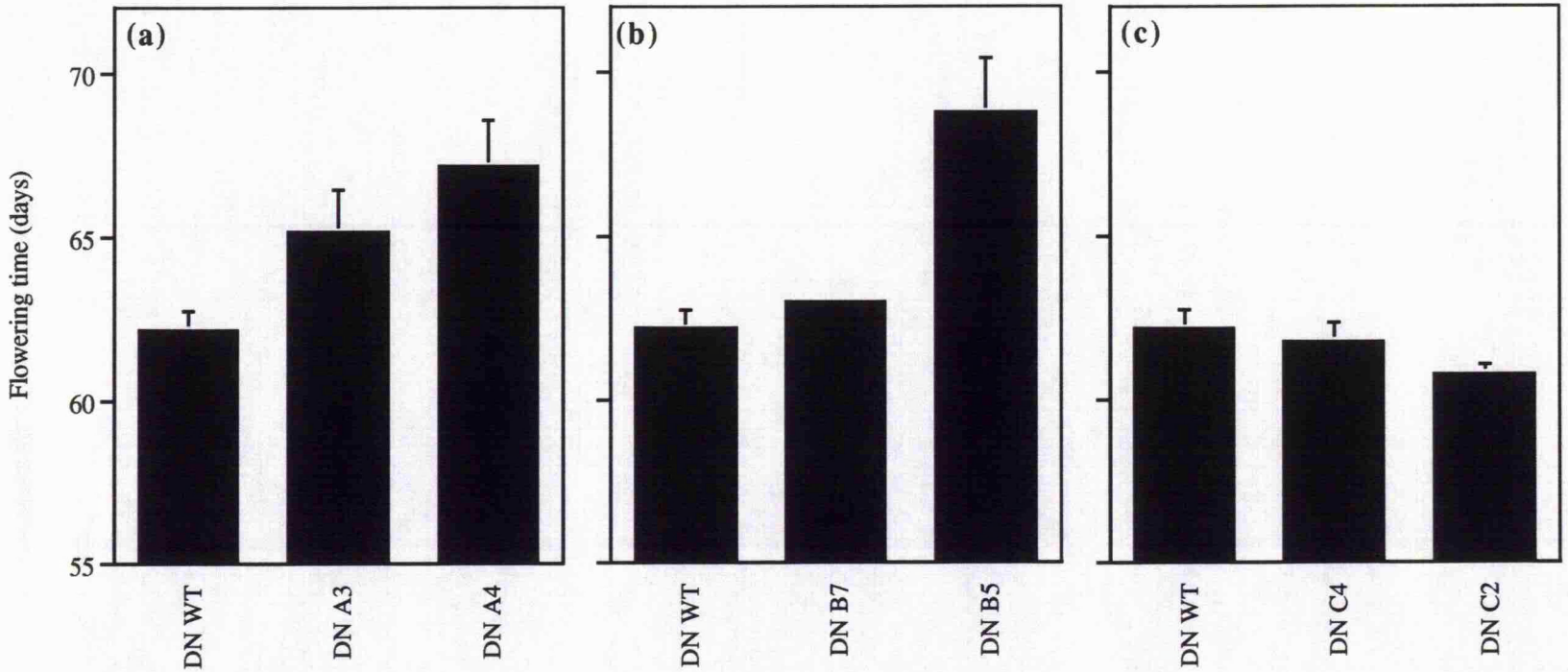


Figure 6.1 Flowering time of **(a)** DN WT, DN A3 and DN A4; **(b)** DN WT, DN B7 and DN B5; **(c)** DN WT, DN C4 and DN C2 plants grown under greenhouse conditions. Data represent the means from 12 plants and the error bars are SE.

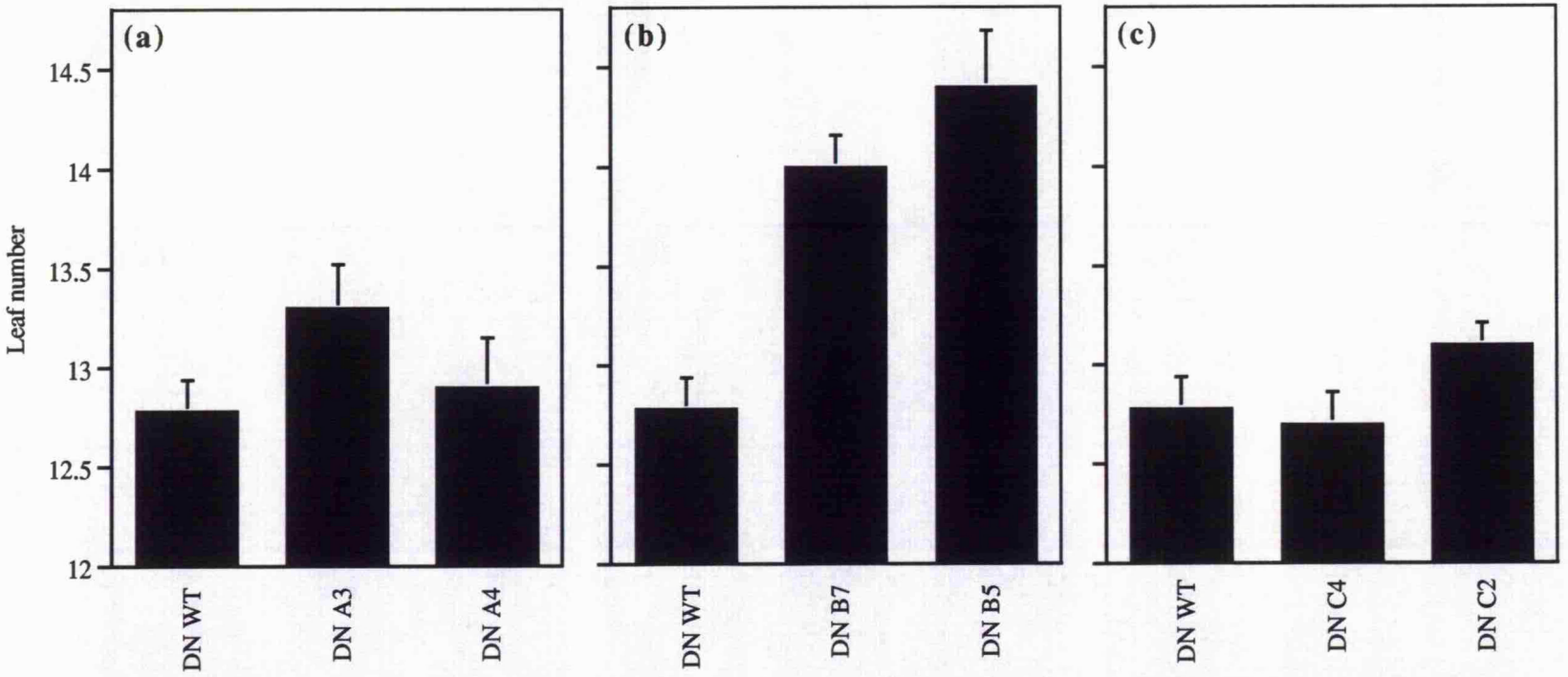


Figure 6.2 Leaf number at flowering time of **(a)** DN WT, DN A3 and DN A4; **(b)** DN WT, DN B7 and DN B5; **(c)** DN WT, DN C4 and DN C2 plants grown under greenhouse conditions. Data represent the means from 12 plants and the error bars are SE.

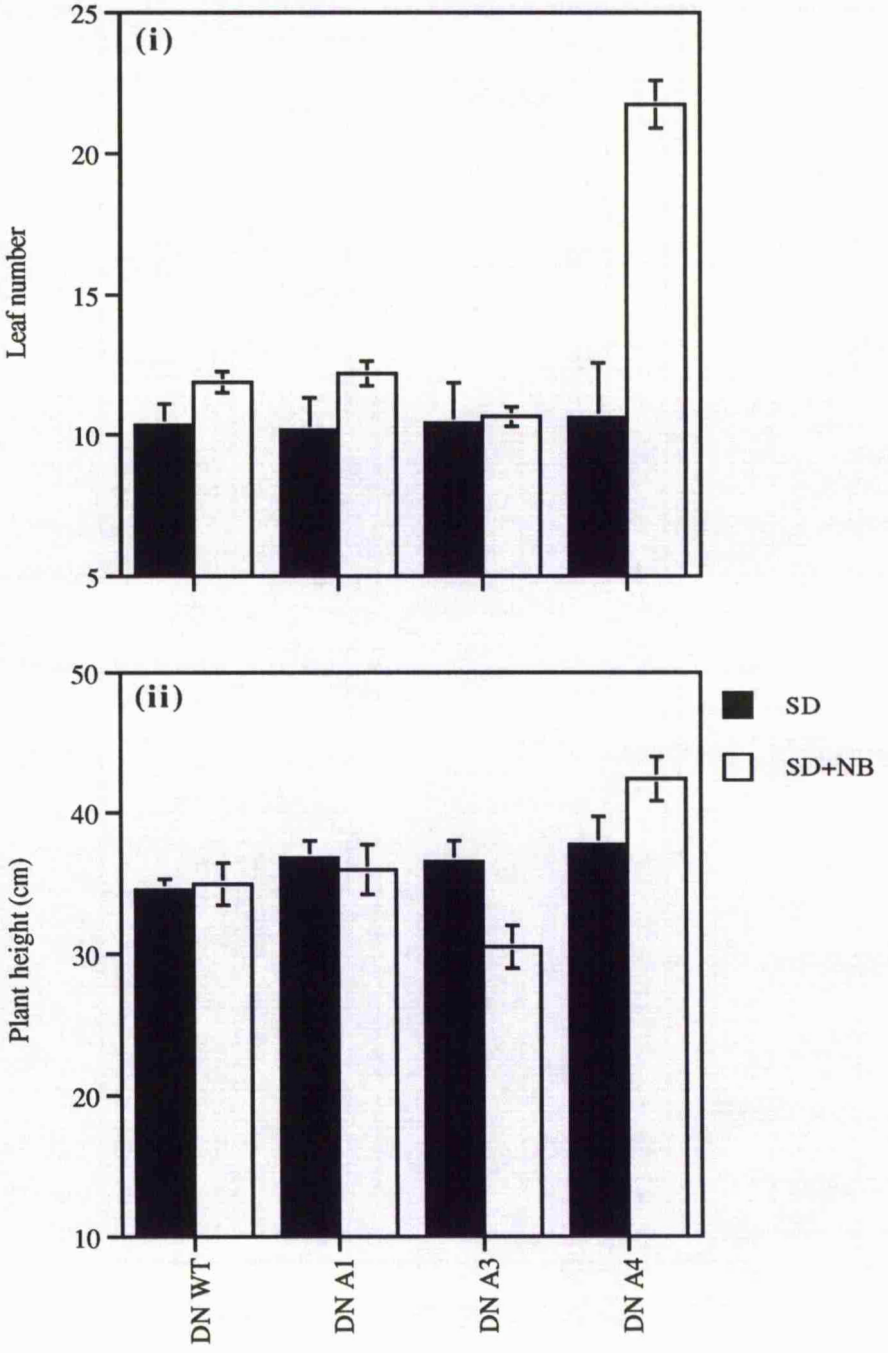


Figure 6.3(a) Leaf number **(i)** and plant height **(ii)** at flowering time in DN WT, DN A1, DN A3 and DN A4 plants grown under 8 h light, 16 h dark, SD photoperiods (closed bars) and under SDs with a 30 min W light NB given 8 h through the dark period (open bars). Data represent the means from 12 plants and the error bars are SE.

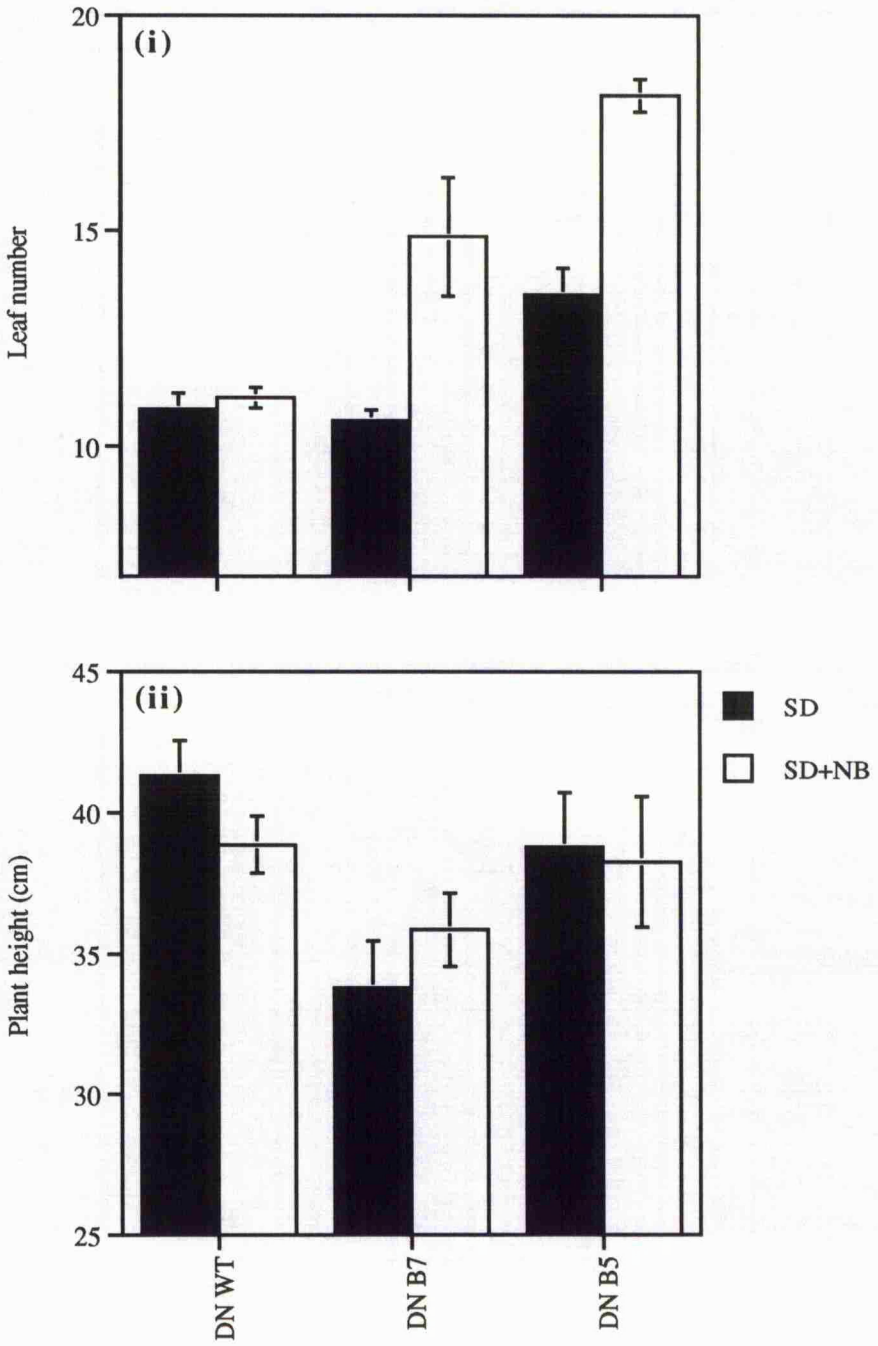


Figure 6.3(b) Leaf number (**i**) and plant height (**ii**) at flowering time in DN WT, DN B7 and DN B5 plants grown under 8 h light, 16 h dark, SD photoperiods (closed bars) and under SDs with a 30 min W light NB given 8 h through the dark period (open bars). Data represent the means from 12 plants and the error bars are SE.

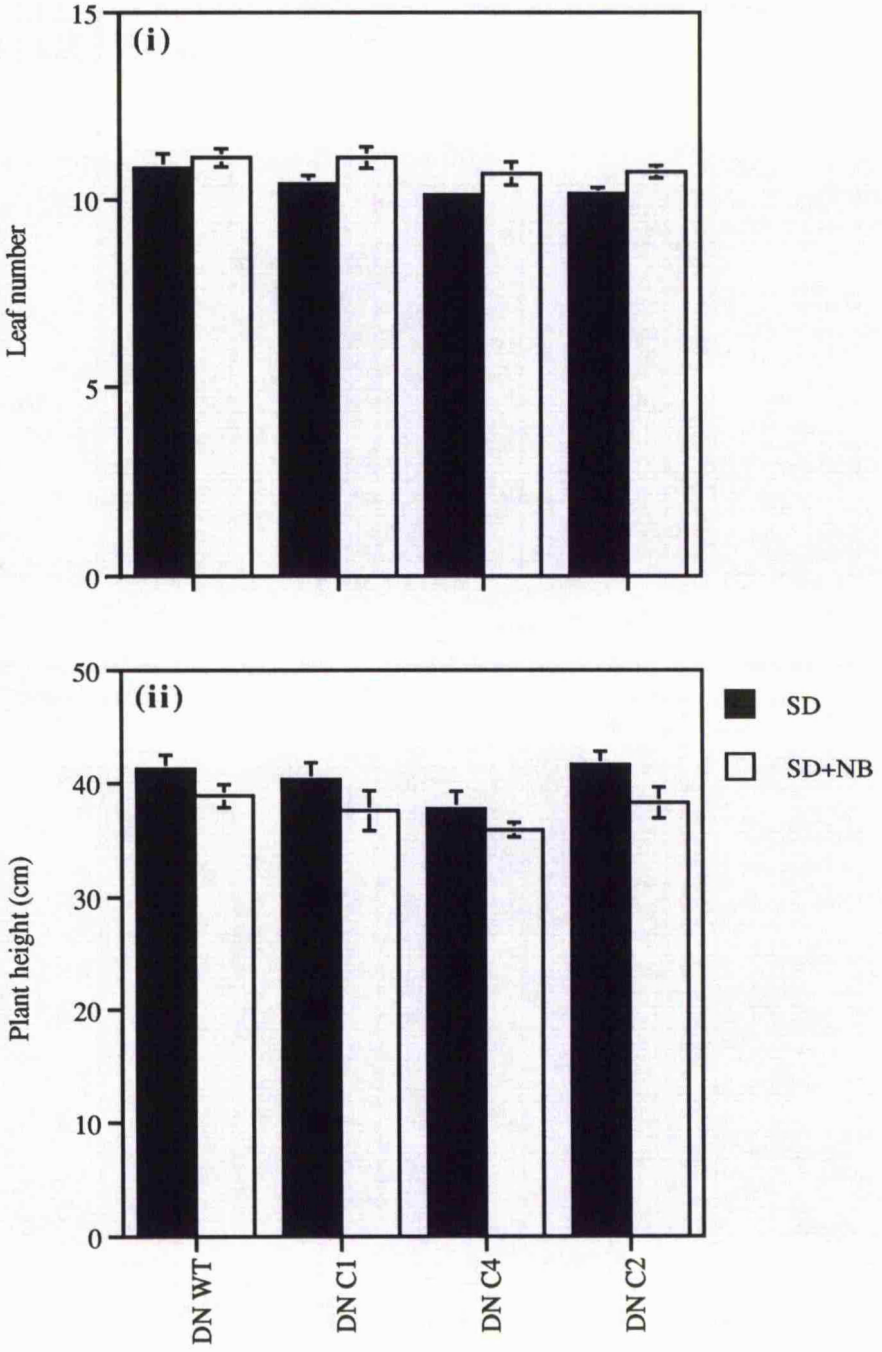


Figure 6.3(c) Leaf number (**i**) and plant height (**ii**) at flowering time in DN WT, DN C1, DN C4 and DN C2 plants grown under 8 h light, 16 h dark, SD photoperiods (closed bars) and under SDs with a 30 min W light NB given 8 h through the dark period (open bars). Data represent the means from 12 plants and the error bars are SE.

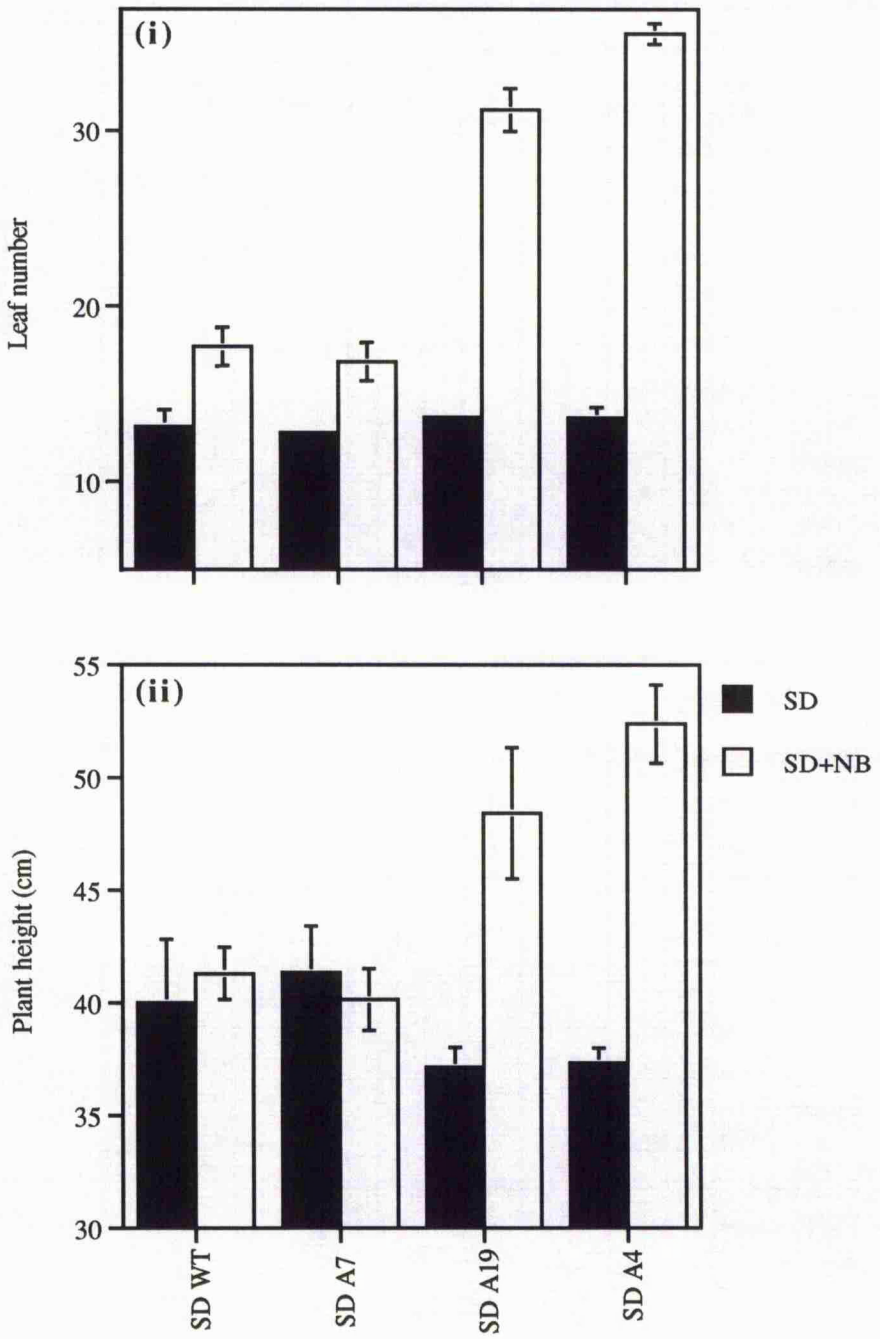


Figure 6.4(a) Leaf number (**i**) and plant height (**ii**) at flowering time in SD WT, SD A7, SD A19 and SD A4 plants grown under 8 h light, 16 h dark, SD photoperiods (closed bars) and under SDs with a 30 min W light NB given 8 h through the dark period (open bars). Data represent the means from 12 plants and the error bars are SE.

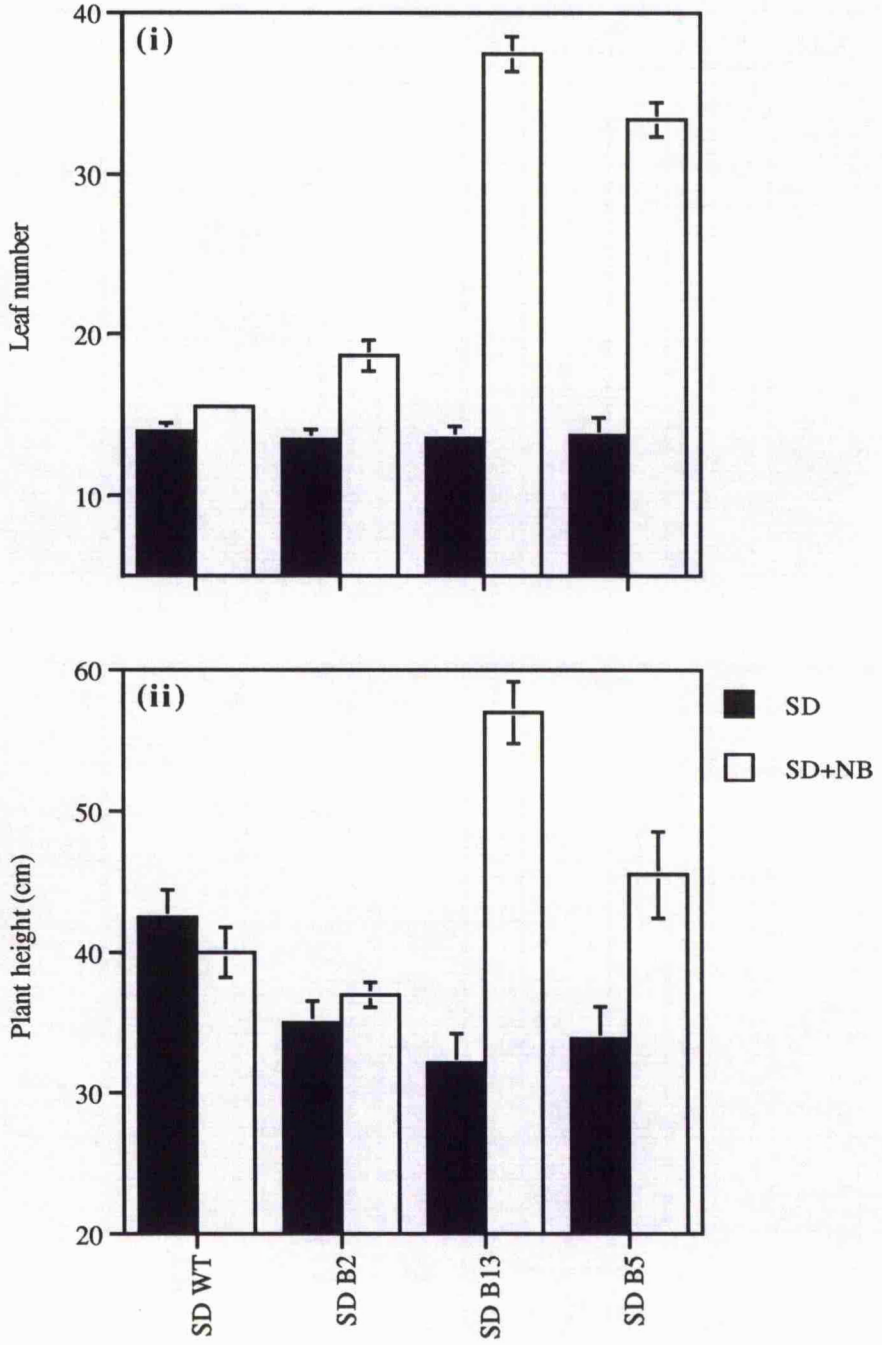


Figure 6.4(b) Leaf number **(i)** and plant height **(ii)** at flowering time in SD WT, SD B2, SD B13 and SD B5 plants grown under 8 h light, 16 h dark, SD photoperiods (closed bars) and under SDs with a 30 min W light NB given 8 h through the dark period (open bars). Data represent the means from 12 plants and the error bars are SE.

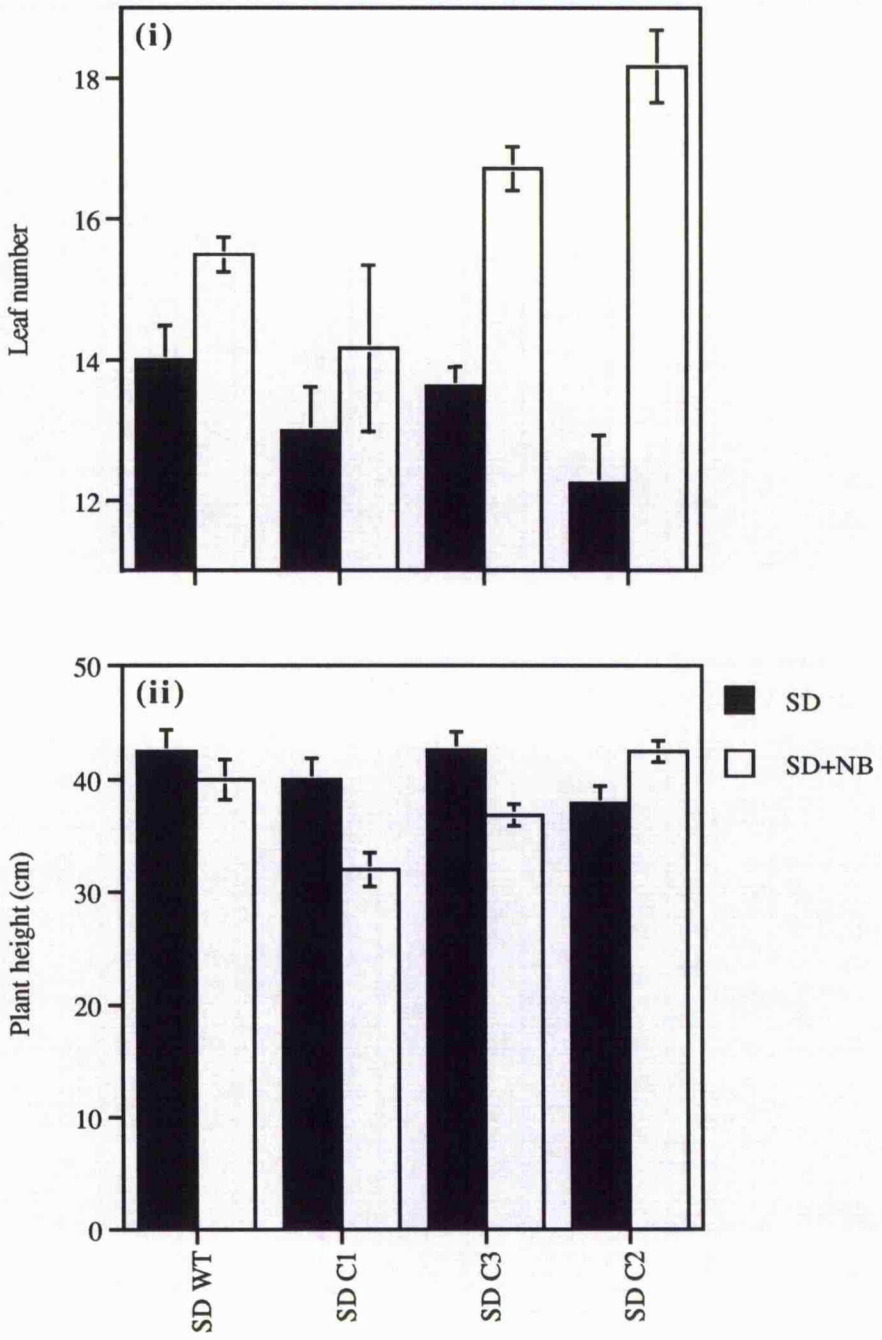
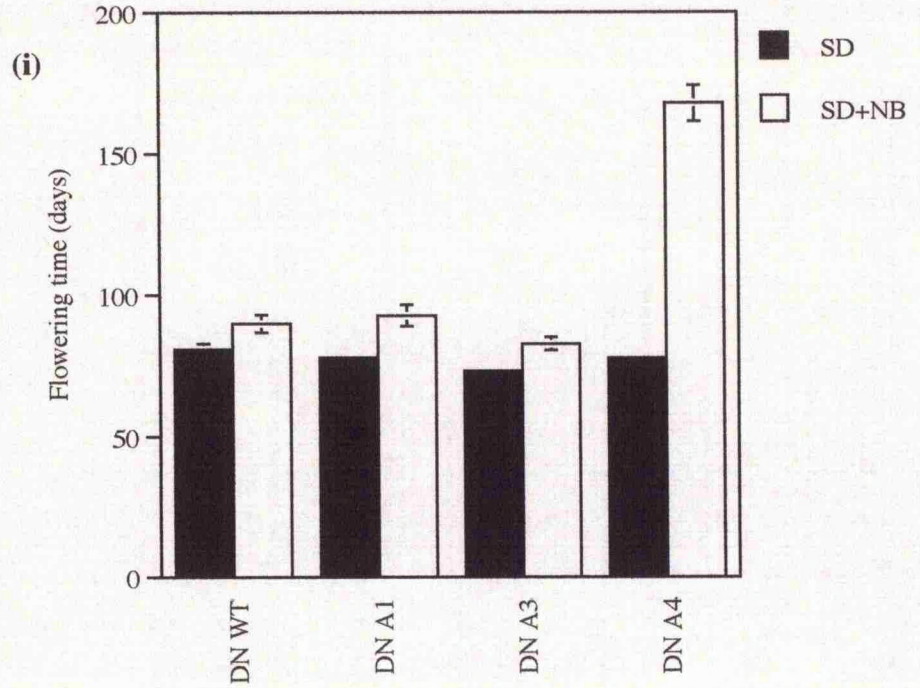


Figure 6.4(c) Leaf number (**i**) and plant height (**ii**) at flowering time in SD WT, SD C1, SD C3 and SD C2 plants grown under 8 h light, 16 h dark, SD photoperiods (closed bars) and under SD with a 30 min W light NB given 8 h through the dark period (open bars). Data represent the means from 12 plants and the error bars are SE.



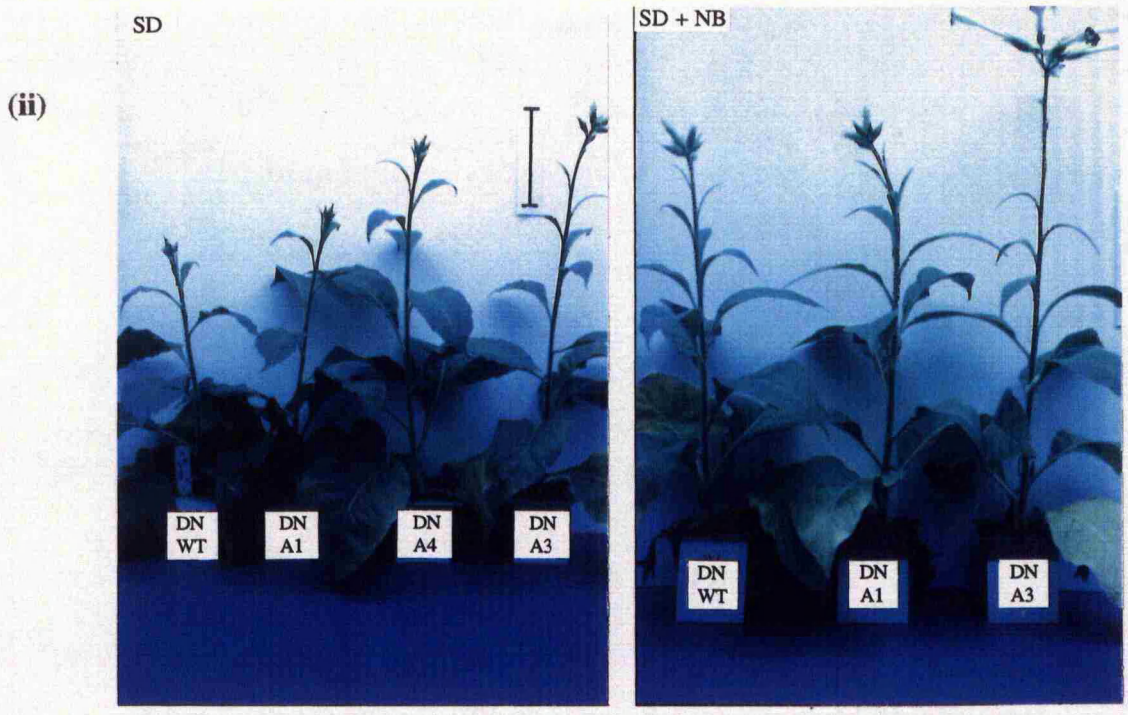
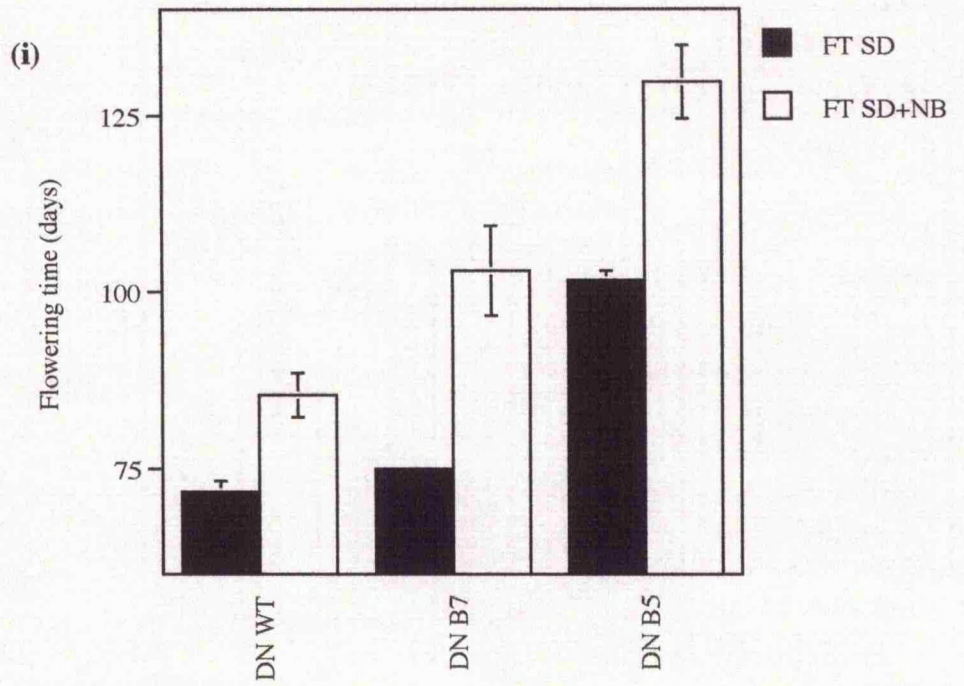


Figure 6.5(a)(i) Flowering time of DN WT, DN A1, DN A3 and DN A4 plants grown under 8 h light, 16 h dark SD photoperiods (closed bars) and under SDs with a 30 min W light NB given 8 h through the dark period (open bars). Data represent the means from 12 plants and the error bars are SE.
(ii) The relative developmental stages of DN A1, DN A4 and DN A3 at DN WT flowering time in plants grown, either in SD, or SD+NB photoperiodic conditions (DN A4 is not shown).



(ii)

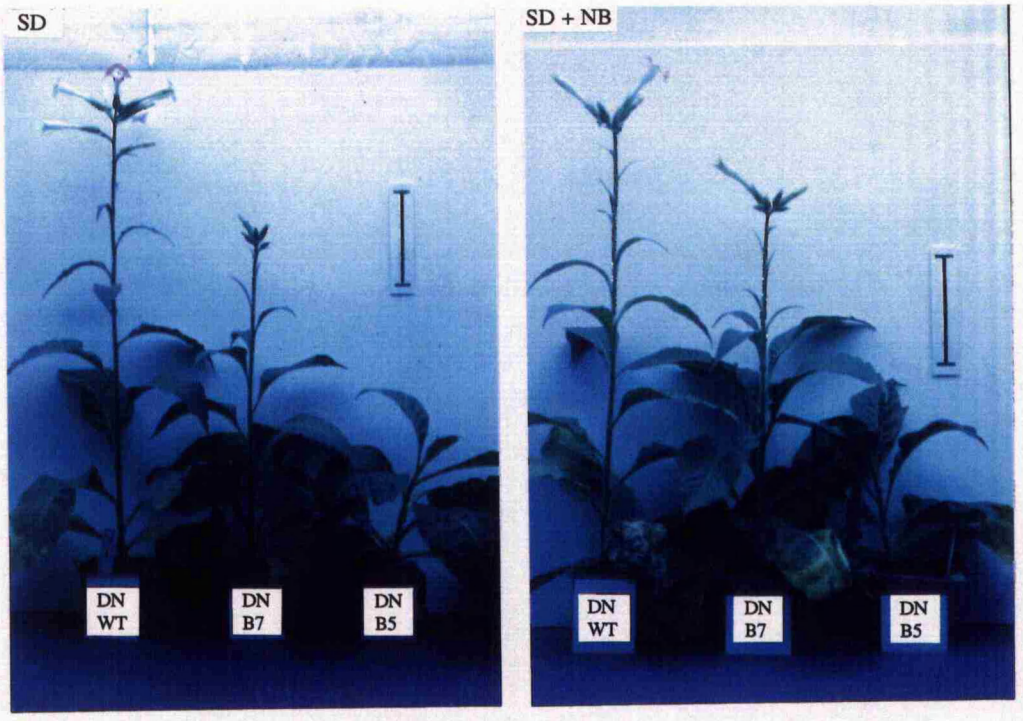
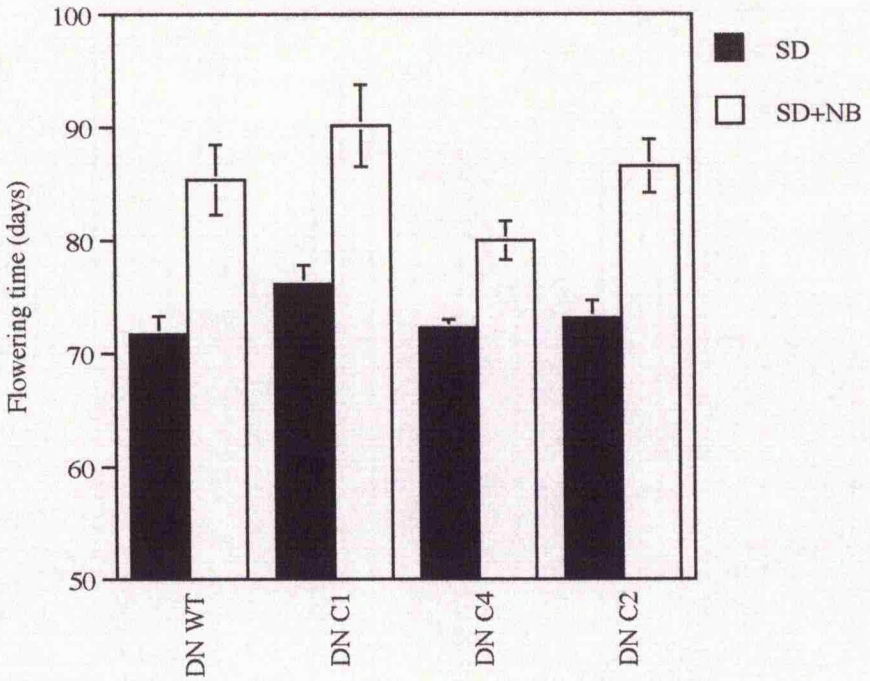


Figure 6.5(b)(i) Flowering time of DN WT, DN B7 and DN B5 plants grown under 8 h light, 16 h dark SD photoperiods (closed bars) and under SDs with a 30 min W light NB given 8 h through the dark period (open bars). Data represent the means from 12 plants and the error bars are SE. **(ii)** The relative developmental stages of DN B7 and DN B5 at DN WT flowering time in plants grown, either in SD, or SD+NB photoperiodic conditions.

(i)



(ii)

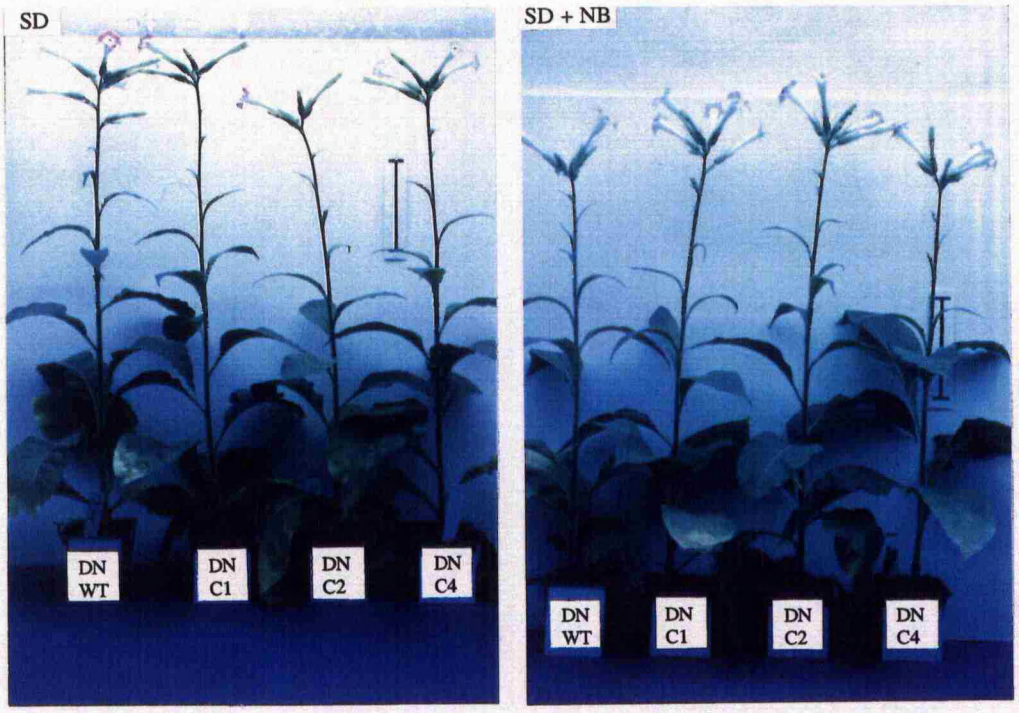
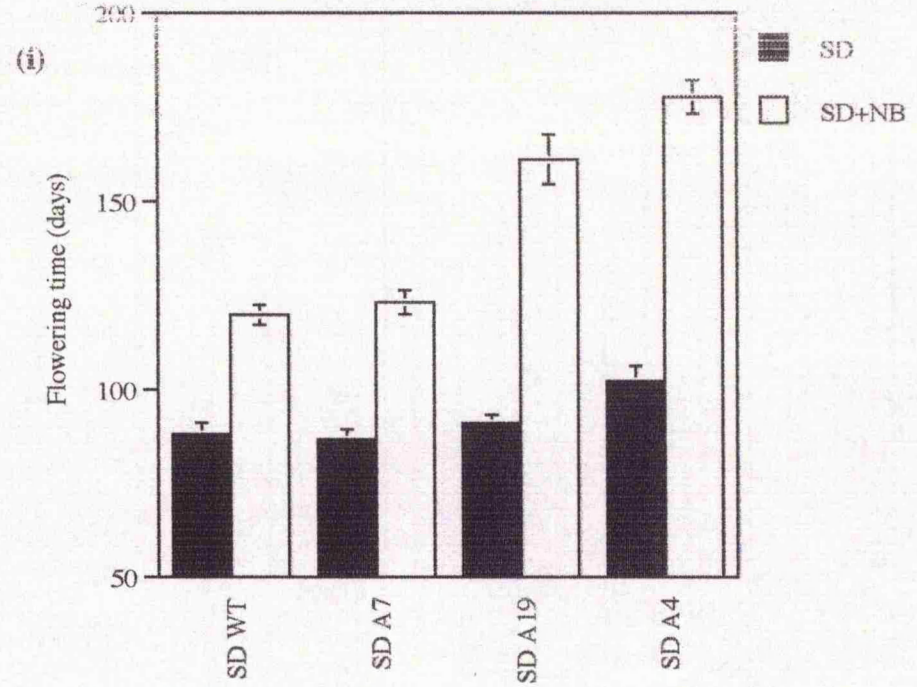


Figure 6.5(c) (i) Flowering time of DN WT, DN C1, DN C4 and DN C2 plants grown under 8 h light, 16 h dark SD photoperiods (closed bars) and under SDs with a 30 min W light NB given 8 h through the dark period (open bars). Data represent the means from 12 plants and the error bars are SE.

(ii) The relative developmental stages of DN C1, DN C4 and DN C2 at DN WT flowering time in plants grown, either in SD, or SD+NB photoperiodic conditions.



(ii)

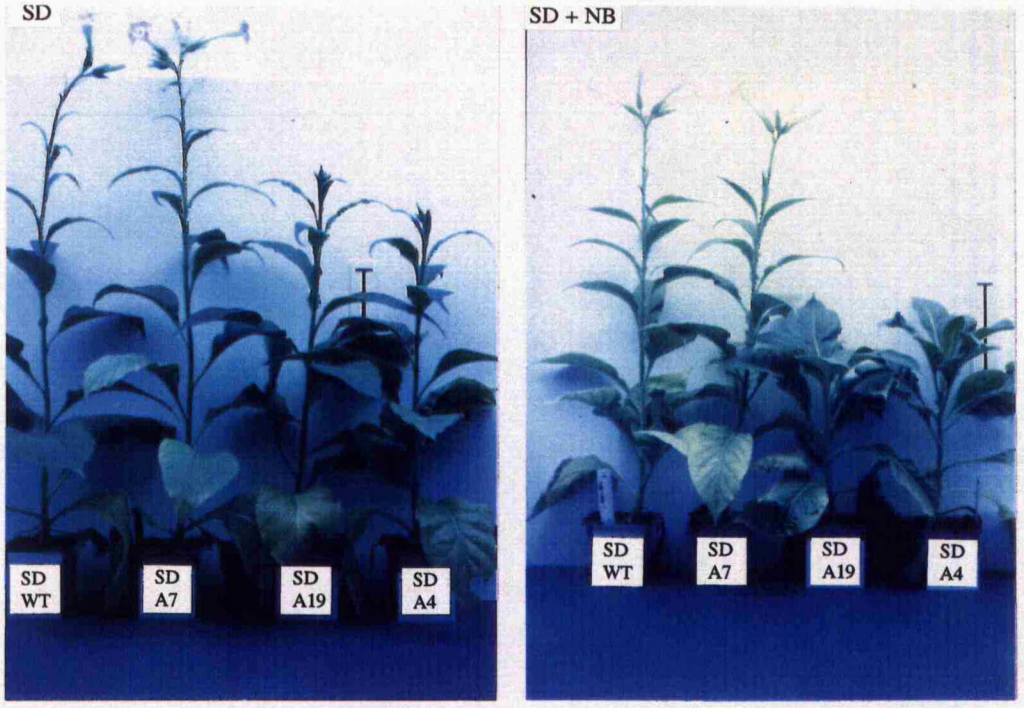
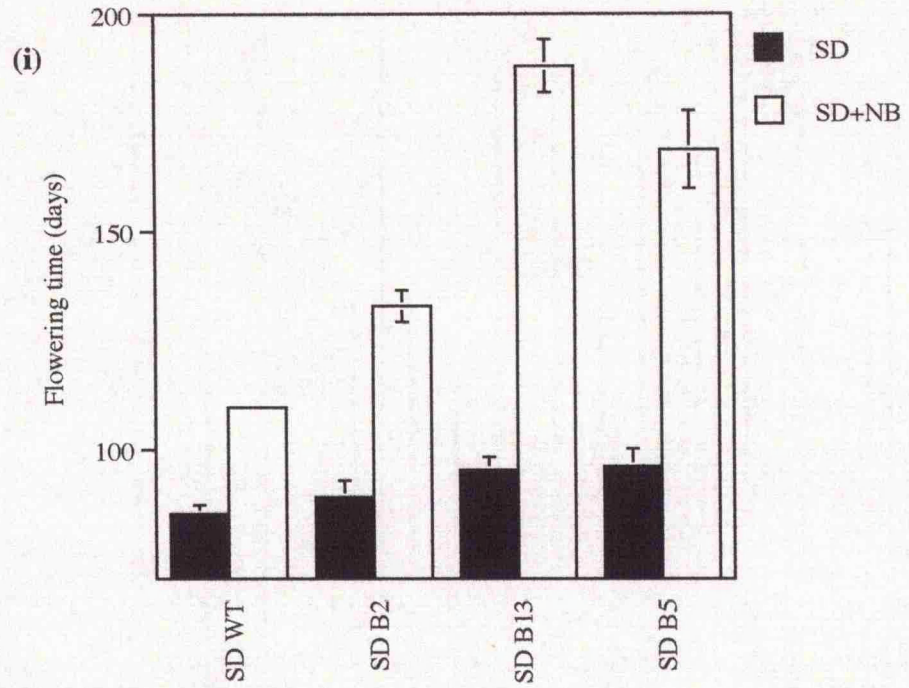


Figure 6.6(a)(i) Flowering time of SD WT, SD A7, SD A19 and SD A4 plants grown under 8 h light, 16 h dark SD photoperiods (closed bars) and under SDs with a 30 min W light NB given 8 h through the dark period (open bars). Data represent the means from 12 plants and the error bars are SE.
(ii) The relative developmental stages of SD A7, SD A19 and SD A4 at SD WT flowering time in plants grown, either in SD, or SD+NB photoperiodic conditions.



(ii)

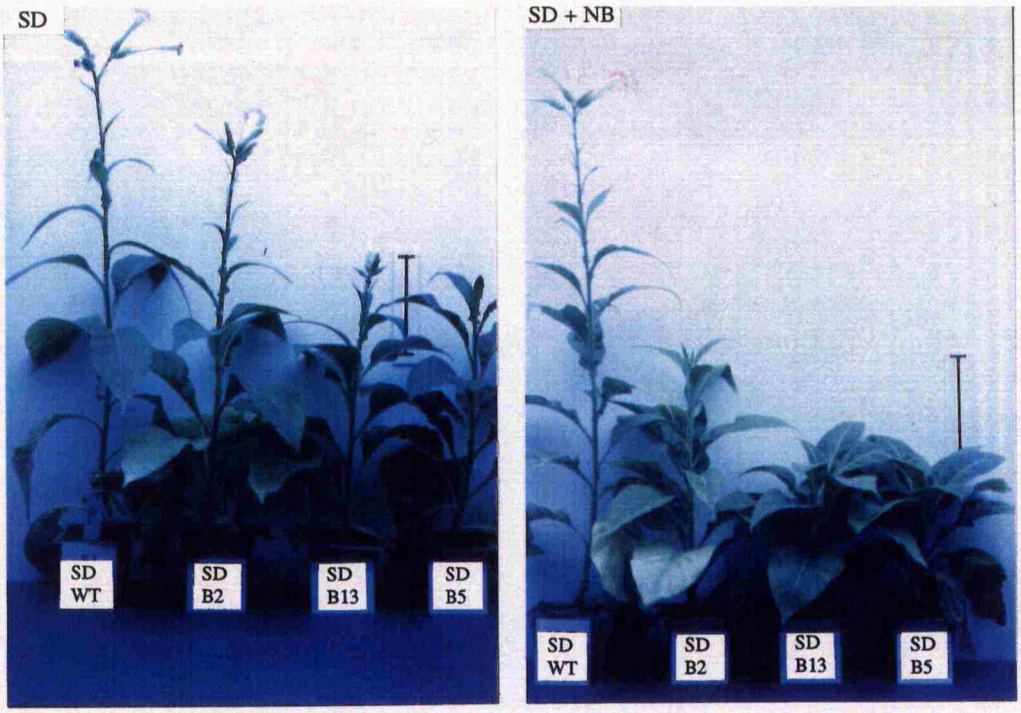
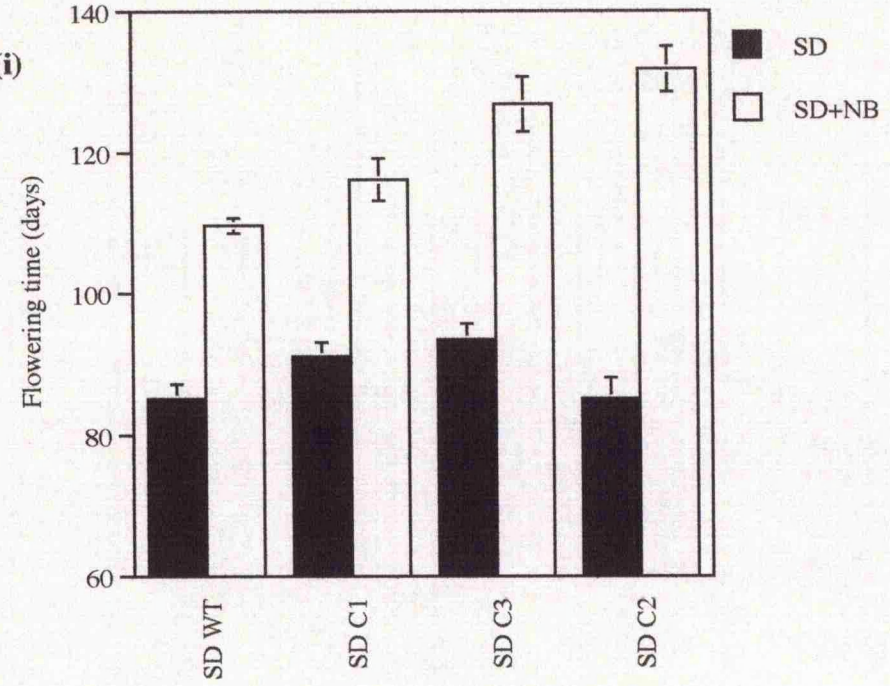


Figure 6.6(b)(i) Flowering time of SD WT, SD B2, SD B13 and SD B5 plants grown under 8 h light, 16 h dark S) photoperiods (closed bars) and under SDs with a 30 min W light NB given 8 h through the dark period (open bars). Data represent the means from 12 plants and the error bars are SE.

(ii) The relative developmental stages of SD B2, SD B13 and SD B5 at SD WT flowering time in plants grown, either in SD, or SD+NB photoperiodic conditions.

(i)



(ii)

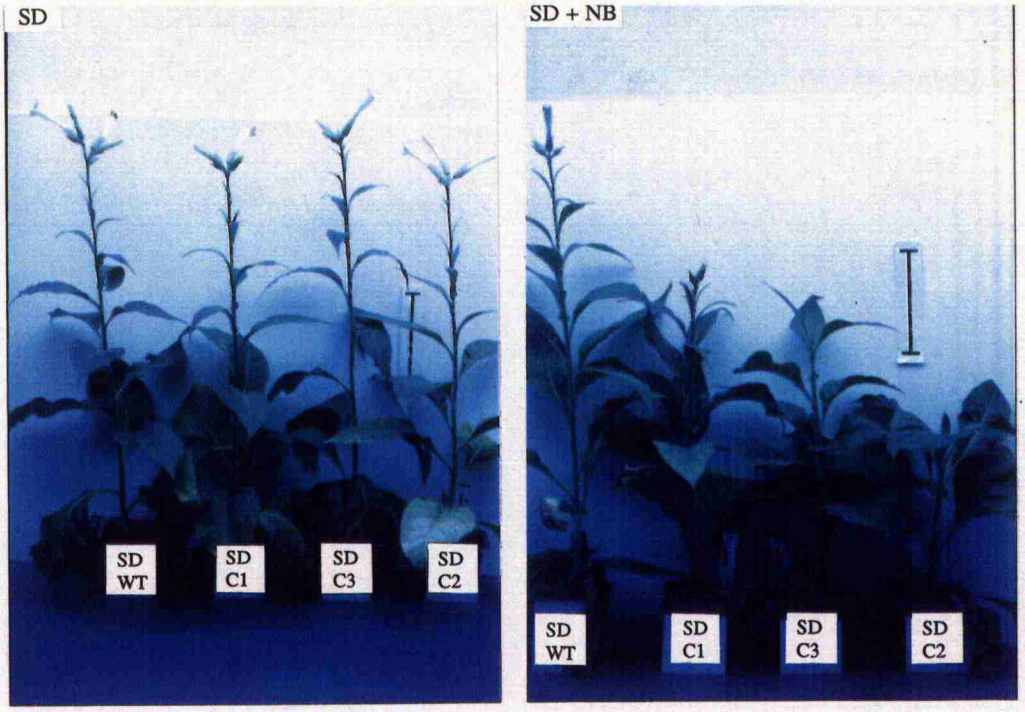


Figure 6.6(c)(i) Flowering time of SD WT, SD C1, SD C3 and SD C2 plants grown under 8 h light, 16 h dark SD photoperiods (closed bars) and under SDs with a 30 min W light NB given 8 h through the dark period (open bars). Data represent the means from 12 plants and the error bars are SE.

(ii) The relative developmental stages of SD C1, SD C3 and SD C2 at SD WT flowering time in plants grown, either in SD, or SD+NB photoperiodic conditions.

Chapter 7 Final Discussion

Phytochrome perception of variations in the light environment endows the plant with the capacity to strategically adapt to its surroundings and adjust the timing of events to seasonal changes. For many years, the numerous and complex phytochrome-regulated responses were interpreted in terms of a single phytochrome photoreceptor. Though, the differential behaviour of phytochrome was explained in terms of two independently acting pools of phytochrome as early as 1965 (Hillman), it is only relatively recently that a number of different members of the phytochrome gene family have been identified (Sharrock and Quail, 1989; Somers *et al.*, 1991; Clack *et al.*, 1994). The existence of multiple species of phytochrome, together with the diversity of phytochrome responses and response modes, led to the proposition that different molecular species of phytochrome have discrete functions (Sharrock and Quail, 1989; Smith and Whitelam, 1990).

The establishment of roles for individual phytochrome species has been greatly advanced through the analysis of photomorphogenic mutants and transgenic plants that overexpress individual phytochrome species (see Whitelam and Harberd, 1994). Both mutant-based and transgenic approaches have been used here to further elucidate roles for different phytochrome species in flowering responses to both low R/FR ratio and photoperiod, and in developmental vegetative responses to low R/FR ratio light.

W light-grown *Arabidopsis phyB* mutants, like phytochrome B-deficient mutants in other species, display a constitutively elongated phenotype reminiscent of wild type plants grown under low R/FR ratio light. Also, they lack wild type elongation growth responses to EOD FR and respond poorly to low R/FR ratio light (Nagatani *et al.*, 1991; Whitelam and Smith, 1991). The light-stable nature of phytochrome B (Furuya, 1993), together with these observations, suggest a major role for phytochrome B in the adjustment of growth and development to changes in light quality. However, aspects of the *phyB* phenotype led to the suggestion that these "shade-avoidance" responses were not mediated by phytochrome B alone. It has been shown that *phyB* has a normal low R/FR ratio response with respect to leaf area and

specific stem weight (Robson *et al.*, 1993). Furthermore, *phyB* mutant seedlings also respond to low R/FR ratio light with an acceleration in flowering time (Whitelam and Smith, 1991; Robson *et al.*, 1993). Since *phyB* seedlings are early flowering, the degree of response is much less than that displayed by wild type seedlings grown under the same conditions. This flowering response was analysed here in the *Arabidopsis* *hy2*, *phyB* and *hy2phyB* mutants, also homozygous for the *co-3*, *fwa* or *fca* late-flowering mutations. Each of the late-flowering mutations is effective in prolonging the phase of vegetative development without affecting the ability of the plant to respond to changing R/FR ratio. Examination of these plants grown under continuous high and low R/FR ratio light revealed a significant flowering response in both *hy2* and *phyB*, which was absent in *hy2phyB*. This not only indicates the presence of some functional phytochrome B in the *hy2* mutant, but also implicates another phytochrome(s), in addition to phytochrome B in the perception of R/FR ratio light signals. As the flowering response to low R/FR ratio is unaltered in both *Arabidopsis phyA* mutants (Johnson *et al.*, 1994), and in *phyAphyB* mutants compared with *phyB* mutants (Devlin *et al.*, 1996), it follows that this response must be under the control of a novel phytochrome(s). In addition, it would appear that the novel phytochrome may function in a similar way to phytochrome B. Therefore, it could be speculated that this novel phytochrome may be phytochrome D, which has high sequence homology with phytochrome B (Clack *et al.*, 1994).

Establishment of a role for a novel phytochrome made possible the prediction of a phenotype for a loss-of-function mutation in this phytochrome. Such a mutant would, therefore, probably resemble the phytochrome B-deficient mutant, with a light-conditional, early flowering phenotype and an elongated growth habit. The *elg* mutant of *Arabidopsis* has many phenotypical traits in common with this predicted phenotype. However, extensive physiological analysis of the *elg* mutant, suggests that the ELG gene, in fact, acts independently of phytochrome function. The *elg* mutant responds in a wild type fashion with elongation growth in response to EOD FR treatments. Likewise, with regard to flowering responses to low R/FR ratio light and changes in photoperiod *elg* also maintains a qualitatively wild type response. However, the long hypocotyl phenotype of the *elgga1* double mutants compared to the *ga1* mutants, grown in the

dark provides convincing evidence that *elg* can function in a manner that is independent of light. What is more, the additivity of *elg* and *ga1* mutations also suggests that *elg* may act independently of GA. This is supported by the fact that the *elg* mutation cannot suppress the effects of *ga1* on germination, and both *elgga1* and *ga1* respond in a similar way to the application of GA. Thus, *ELG* defines a novel gene that influences elongation growth, but is neither operational in responses initiated by light perception nor those involving GA transduction.

Arabidopsis seedlings that overexpress the *HAT4* (=*Athb-2*) gene are early flowering and have an elongated appearance (Schena *et al.*, 1993). The *HAT4* gene of *Arabidopsis* encodes a putative transcriptional regulator protein (Carabelli *et al.*, 1993; Schena *et al.*, 1993). *HAT4* expression is enhanced by both EOD FR treatments and by low R/FR ratio light in wild type seedlings (Carabelli *et al.*, 1996). What is more, these same light treatments also cause a dramatic increase in *HAT4* transcript levels in *phyA*, *phyB* and *phyAphyB* mutant seedlings. It has, therefore, been suggested that expression of the *HAT4* gene is down-regulated by a novel phytochrome (Carabelli *et al.*, 1996). As a novel Pfr is proposed to down-regulate the *HAT4* gene, it is possible that HAT4 is a component of its signal transduction. If this is the case, the phenotype of H4S plants, which express elevated levels of the *HAT4* transgene product could resemble that of a loss-of-function mutant in this novel phytochrome species.

Physiological analysis of transgenic H4S seedlings, revealed a number of characteristics that would probably be displayed by seedlings deficient in a novel phytochrome. However, this does not appear to be the case for all aspects of H4S seedling behaviour. H4S seedlings display a reduced R-mediated inhibition of hypocotyl growth that is qualitatively similar to that of *phyB* seedlings. The *phyAphyB* mutant seedlings demonstrate a complete loss of R-mediated inhibition of hypocotyl growth (Reed *et al.*, 1993; Devlin *et al.*, 1996). These observations suggest that, in *Arabodopsis*, this response is mediated exclusively by phytochromes A and B. Therefore, the phenotype of de-etiolated H4S seedlings probably does not represent the action of a novel phytochrome. Indeed, phytochromes A and B have both been implicated in the modulation of the *HAT4* transcript at this stage of seedling development (Carabelli *et al.*, 1996).

The adult phenotype of a plant with deficiencies in a novel light-stable phytochrome is likely to have characteristics in common with the *phyB* mutant (see Chapter 2). Indeed, light-grown H4S and *phyB* seedlings have many phenotypical similarities. Both H4S and *phyB* seedlings have an elongated appearance, are flower early, and display a reduced early-flowering response to low R/FR ratio. However, a difference in the behaviour of the *phyB* and H4S seedlings is observed in response to EOD FR treatments. Although *phyB* and H4S are early flowering, EOD FR treatments can stimulate even earlier flowering in *phyB* seedlings but not in H4S seedlings. In addition, both *phyB* and *H4S* seedlings have elongated petioles, but H4S responds to EOD FR treatments in a qualitatively wild type manner, with further petiole lengthening. In contrast, *phyB* seedlings respond to EOD FR treatments with a reduction in petiole length. These data appear to be at odds with reports in which *phyB* seedlings petioles achieve the same length under photoperiods with or without EOD FR treatments (Nagatani *et al.*, 1991; Devlin *et al.*, 1996). Under the experimental conditions used here, the *phyB* mutants are particularly small plants and, therefore, have limited resources. Thus, a further acceleration of flowering in response to EOD FR treatments may be at the expense of leaf development. Early-flowering and elongated petioles are phenotypical features common to both H4S and *phyB* seedlings. However, the ability to enhance these responses to EOD FR treatments appears to be differentially affected in H4S and *phyB* seedlings.

The analysis of existing mutants with a promising phenotype is one way of identifying loss-of-function mutants in this novel phytochrome. Alternative methods include the selection of seedlings with early flowering characteristics from mutagenised seed. The utilisation of seed homozygous for *phyAphyB* and a late-flowering mutation (eg. *co-3*, *fca* or *fwa*) would facilitate the detection of an early flowering response that is not mediated by phytochromes A or B. Isolation of the monogenic mutant could then be achieved by backcrossing with the wild type parent plant. Identification of such a mutant could also combine mutagenic and transgenic strategies. The first step would require the stable incorporation of a construct, comprising the *HAT4* gene promoter fused to a resistance marker, into the *Arabidopsis* genome. Low abundance of the transcript under high R/FR ratio light would probably mean that the resistance marker is

not likely to be very active. The second step would require mutagenesis of the transformed seed. It then follows, that seedlings with increased *HAT4* promoter activity and therefore, enhanced resistance, may have mutations in the novel phytochrome gene, in genes that control chromophore biosynthesis or attachment, or in genes that encode components of the transduction pathway.

Analysis of phytochrome mutants and transgenics overexpressing phytochrome DNA constructs has yielded valuable information on phytochrome responses to changing light quality in germination, de-etiolation, vegetative growth and development, and flowering. However, there is only a scant understanding of the role of phytochrome in the photoperiodic control of flowering. To date, much of the work on the physiology of photoperiodism has been carried out with SD species. As a consequence of this, the photoperiodic flowering mechanisms of SD plants are better defined than those of LD plants (see Vince-Prue, 1994). Transgenic DN and SD *Nicotiana tabacum* cv. Hicks overexpressing phytochromes A, B and C were generated: first, to allow the direct comparison of affects of phytochrome overexpression on the photoperiodic mechanism in a SD plant with a DN plant; and secondly, to establish the range of physiological characteristics that comprise each overexpression phenotype.

An allelic series representing a range of phytochrome A-, B- and C-overexpression phenotypes were selected in the DN and SD tobacco. PCR amplification of the transgene fragments confirmed the incorporation of the phytochrome transgenes in all the lines. Furthermore, detection of the transgenic proteins by immunoblot staining and the identification of physiological traits confirmed the presence and biological activity of the introduced phytochromes in the host plants.

For all of the lines, phytochrome overexpression correlates with inhibition of hypocotyl elongation under appropriate light conditions. This is seen most clearly in seedlings overexpressing phytochrome A or B grown under continuous FR and R light, respectively. In the segregating T1 populations the severity of hypocotyl inhibition correlates with gene copy number. The gene dosage effect on hypocotyl inhibition is similar to that seen in wild type *Arabidopsis* and mutants heterozygous and

homozygous for the *phyA* or *phyB* mutation (Koornneef *et al.*, 1980; Whitelam *et al.*, 1993). This is thought to reflect the close relationship between the abundance of phytochrome and the severity of phenotype.

Both phytochrome A- and B-overexpression plants display a transgenic protein dose-linked increase in hypocotyl growth inhibition under R, W and B light. These observations are thought to reflect the overlapping roles of native phytochromes A and B in de-etiolation. Phytochrome C-overexpression lines show a small, but significant increase in hypocotyl growth inhibition under R and W light. The elicitation of such a small response may reflect, either the levels of transgenic protein, or a relatively minor role for phytochrome C in this response. Enhanced cotyledon expansion under R is characteristic of seedlings expressing both the phytochrome B and the phytochrome C transgenes. This suggests complementary roles for phytochromes B and C in this aspect of R-mediated de-etiolation. It is also possible that the small increase in hypocotyl growth inhibition displayed by seedlings overexpressing phytochrome C is not a direct consequence of elongation growth inhibition, but rather, a change in cell shape. Increased cell expansion could account for increased cotyledon area and perhaps the development of shorter, thicker hypocotyls.

Phytochrome overexpression also has an impact on the phenotype of the adult plant. The phenotye of mature plants expressing the phytochrome A transgene is consistent with that reported for tobacco and other host plants (Boylan and Quail, 1989; Keller *et al.*, 1989 McCormac *et al.*, 1991; Whitelam *et al.*, 1992). Phytochrome A-overexpression levels correlate with a dwarfed growth habit, characterised by reduction in internode length. These transgenic plants, however, do not demonstrate the "reversed" shade-avoidance observed in tobacco and *Arabidopsis* (McCormac *et al.*, 1991; Whitelam *et al.*, 1992). This enhanced inhibition growth to supplementary FR has been interpreted in terms of the maintenance of the FR HIR in light-grown seedlings. Absence of the reverse response may mean that here, the transgenic phytochrome is either not maintained at sufficient levels in the light-grown plant, or it is not sufficiently active.

Adult transgenic plants overexpressing phytochrome B also have a dwarfed growth habit. Phenotypical features include reduced internode length, broader and

shorter leaves with reduced area and increased leaf chlorophyll content. These observations are consistent with those for B-overexpression phenotype in *Arabidopsis* (eg. McCormac *et al.*, 1993; Wester *et al.*, 1994; Bagnall *et al.*, 1995). This overexpression phenotype is largely the antithesis of the phytochrome B-deficient phenotype (see Whitelam and Harberd, 1994), confirming a major role for phytochrome B in the control of growth and development of the light-grown plant.

A marked contrast is seen in the phytochrome C-overexpression phenotype, where plants are taller and have more expanded leaves than wild type plants. The recent observation that *Arabidopsis* seedlings overexpressing phytochrome C also have larger leaves than wild type seedlings is supportive of these findings (P.H. Quail, pers comm.). The different actions of transgenically expressed phytochrome B and phytochrome C may represent roles for the native phytochromes, where PfrB inhibits cell elongation and PfrC enhances cell expansion and/or cell elongation.

The DN and SD *N. tabacum* are particularly amenable to comparative analysis as they differ only with respect to a single locus, *MM* that confers SD photoperiodicity. A useful characteristic of SD *N. tabacum* is that a NB, given half way through an otherwise inductive night, has a delaying effect on flowering. This feature was exploited to investigate the effects of phytochrome overexpression on this photoperiodic flowering response.

Following the commencement of the experiments it became clear that the DN plants also displayed a small NB-dependent delay in flowering. However, in comparison the SD plants exhibit a marked NB-dependent delay in flowering. In addition, SD *N. tabacum* have an absolute requirement for SD photoperiods for floral initiation, whereas DN *N. tabacum* do not have a photoperiodic requirement for flowering.

Phytochrome A-overexpression causes a large delay in flowering in plants from the most severe DN overexpression line, and in plants from each of the SD lines, when grown in photoperiods with a NB. This enhanced sensitivity to a NB suggests that phytochrome A may have the ability to interact with the circadian timer and, therefore, may have a role in the timing of the dark period. Support for this proposal comes from evidence for the involvement of phytochrome A in photoperiodic perception in the LD

plant, *Arabidopsis* (Johnson *et al.*, 1994; Reed *et al.*, 1994; Bagnall *et al.*, 1995). Such a response also requires that the species of phytochrome is unstable, or becomes de-sensitized in its Pfr form, which is of course characteristic of phytochrome A.

Phytochrome B-overexpression has the effect of delaying flowering in both DN and SD *N. tabacum* under inductive SD conditions. Flowering is also further delayed in transgenic plants subjected to NBs. Therefore, phytochrome B-overexpression appears to have a delaying effect on flowering in *N. tabacum*, regardless of the light conditions. However, it clearly has the capacity to enhance the effect of NBs. Evidence for a role for phytochrome B in photoperiodic perception comes from the analysis of ma_3^R, a phytochrome B mutant of *Sorghum*, which is a quantitative SD plant. Although, ma_3^R mutant seedlings can perceive photoperiods, sensitivity to changing photoperiods is greatly reduced, when compared to wild type plants (Childs *et al.*, 1995). It has also been demonstrated that both the chorophyll a/b binding protein mRNA and the ribulose bisphosphate carboxylase small subunit mRNA cycle in a circadian fashion in ma_3^R seedlings (Childs *et al.*, 1995). Thus, phytochrome B is clearly not involved in the control of the rhythmic cycling of these photosynthetic components in ma_3^R. Thus, in *N. tabacum*, it is uncertain whether phytochrome B-overexpression interacts with the circadian timer, as it is equally possible that increased levels of phytochrome B may delay flowering as a consequence of a prolonged period of vegetative development.

Phytochrome C-overexpression appears to have a direct effect on photoperiodic flowering in *N. tabacum*. It could be predicted that overexpression of a phytochrome that interacts with the cicadian timer would have no effect in DN plants, but would have an effect in SD plants. Indeed, in plants overexpressing phytochrome C an enhanced NB-induced delay in flowering is only observed in the SD plants. Therefore, the confinement of the altered response to the SD transgenic lines may mean that the phytochrome C transgene specifically interacts with *MM* gene transduction.

NB experiments have proved useful in identifying the species of phytochrome that are likely to be involved in the timing of the photoperiod. However, further refinement of this type of experiment could yield more detailed information on the precise roles of the different phytochrome species in photoperiodic flowering. For

example, R light NBs given at intervals throughout an inductive night could establish whether the response is rhythmic. Moreover, if the NBs are given at fluences too low to effect a response in wild type plants, any measurable response could be attributed to the transgenically expressed phytochrome species.

Useful information could also be gained from analysis of phytochrome mutants and/or transgenics in other SD species. In some ways the SD *N. tabacum* is not an ideal species with which to work. The plants are large, and they have a long life-cycle requiring large experimental growth areas for long periods. Furthermore, multiple short-day photoperiods are required to stimulate flowering. A more ideal species would be a small SD plant with a short life cycle that could be induced to flower with a single inductive dark period.

The production of plants expressing high levels of transgenic phytochrome can yield much information on the roles of the phytochromes. However, there may be problems associated with the ectopic expression of phytochrome. Alteration in spatial and temporal expression could effect phenotypical changes that are uncharacteristic of the phytochrome species in question. Generally, the interpretation of an overexpression phenotype is based on knowledge of phytochrome function from analysis of phytochrome mutants. Ideally a comprehensive analysis would be achieved using both phytochrome null mutants and transgenic plants with exaggerated phytochrome levels. However, the tobacco used in this study is a tetraploid, which means that a mutant screen would not have been feasible. Therefore, the interpretations of the transgenic phenotypes were based, to a large extent, on the roles of phytochrome previously defined by analysis of phytochrome mutants in other species.

In summary, the work presented in this thesis defines a possible role for a novel light-stable phytochrome, identifies a mutation that affects elongation growth independently of both phytochrome and GA transduction, and provides further insights into the physiological roles of phytochromes A, B and C in vegetative development and photoperiodism.

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