Photoregulation by the phytochrome family:

A physiological study of transgenic plants.

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

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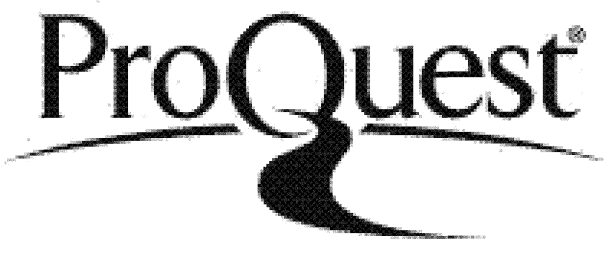
May, 1993



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STATEMENT OF ORIGINALITY

The accompanying thesis submitted for the degree of Doctor of Philosophy entitled *Photoregulation by the phytochrome family: A physiological analysis of transgenic plants* is based on work conducted by A.C. McCormac in the Department of Botany of the University of Leicester mainly during the period between 1st February, 1990 and 1st February, 1993.

All work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

Signed: A.C. McCormac Date: 11th May 1993

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Publications

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

McCormac, A.C., Whitelam, G.C., Boylan, M.T., Quail, P.H., Smith, H. (1992) Contrasting responses of etiolated and light-adapted seedlings to red:far-red ratio: A comparison of wild-type, mutant and transgenic plants has revealed differential functions of members of the phytochrome family. *J. Plant Physiol.* **140**, 707-714

McCormac, A., Whitelam, G., Smith, H. (1992) Light-grown plants of transgenic tobacco expressing an introduced oat phytochrome A gene under the control of a constitutive viral promoter exhibit persistent growth inhibition by far-red light. *Planta* **188**, 173-181

McCormac, A.C., Boylan, M.T., Wagner, D., Quail, P.H., Smith, H., Whitelam, G.C. (1993) Photoresponses of transgenic *Arabidopsis* seedlings expressing introduced phytochromeB-encoding cDNAs: Evidence that phytochrome A and phytochrome B have distinct photoregulatory functions. *The Plant Journal* **4**, (in press)

McCormac, A.C., Smith, H., Whitelam, G.C. (1993) Expression of oat-*phyA*-cDNA in a suspension cell culture of transgenic tobacco: a single-cell system for the study of phytochrome function. *J. Exp. Bot.* (in press)

McCormac, A.C., Smith, H., Whitelam, G.C. (1993) Photoregulation of germination in seed of transgenic lines of tobacco and *Arabidopsis* which express an introduced cDNA encoding phytochrome A or phytochrome B. *Planta* (in press)

Whitelam, G.C., McCormac, A.C., Boylan, M.T., Quail, P.H, (1992) Photoresponses of *Arabidopsis* seedlings expressing an introduced oat *phyA* cDNA: persistence of etiolated plant type responses in light-grown plants. *Photochem. Photobiol.* **56**, 617-621

Acknowledgements

I wish to thank the following for invaluable assistance during the time of this study: firstly, the Agriculture and Food Research Council who funded this project; Professor Harry Smith for initially obtaining the above grant and encouraging me in the writing of this thesis, and also for his role as a highly tolerant supervisor; Dr. Garry Whitelam for extensive discussion of ideas and for liaising with research groups in the U.S.A.; the research groups of Professor Peter Quail and Professor Richard Vierstra, especially Drs. Joel Cherry, Margaret Boylan and Doris Wagner, for their generous co-operation regarding the use of the transgenic lines produced in their laboratories; Mike Jackson and Malcolm Pratt for vigilant maintenance of growth cabinets and light boxes; Sue Ogden for helping with the hand-drawn diagrams; Graham Benskin and all co-workers at the Botanical Gardens for conscientious care of plants; Stuart, Lesley, Becks, and especially Ginger for much appreciated support throughout the three years it took to complete this project, and my parents for continuous support throughout the whole of my education.

Abbreviations

AHA = 4-amino-5-hexynoic acid

ALA = 5-aminolevulinic acid

BAP = 6-benzylaminopurine

cab (*cab*) = chlorophyll-*a/b*-binding protein (gene)

CaMV 35S = cauliflower mosaic virus 35 S promoter

2,4 D = 2,4 Dichloro-phenoxy-acetic acid

DMF = N,N-dimethylformamide

(c)DNA = (complementary) deoxyribonucleic acid

EOD-FR = end-of-day by irradiation by far-red

FR = far-red light

GA = gibberellin

HIR = high irradiance response

IAA = indole-3-acetic acid

IPA = N^6-(Δ^2Isopentenyl)-adenine

LF = low fluence response

MOPS = 3[N-Morpholino] propane-sulfonic acid

MS salts/medium = Murashige and Skoog standard culture medium

NAA = naphthalene acetic acid

PAGE = polyacrylamide gel electrophoresis

PAR = photosynthetically active radiation

phyA(B, C, D, E) = gene encoding phytochrome A (B, C, D, E) apoprotein

PMSF = phenylmethylsulphonylfluoride

P_r = the red-absorbing form of phytochrome

P_{fr} = the far-red-absorbing form of phytochrome

$P_{tot} = P_r + P_{fr}$

P_{fr}/P_{tot} = phytochrome photoequilibrium

R = red light

R:FR = the quantum ratio of red and far-red light

rbcS (*rbcS*) = ribulose-1,5-bisphosphate carboxylase small subunit (gene)

(m)RNA = (messenger)ribonucleic acid

SEM = standard error of the mean

SDS = sodium dodecyl sulphate

Tris-HCl = [Tris(hydroxymethyl)aminomethane]-HCl

VLF = very low fluence response

WL = white light; WL+FR = white light supplemented with far-red radiation

WT = wild-type

Abstract

Chapter 1 reviews the evidence, to date, that the individual molecular species of phytochrome have distinct physiological roles and explains why the use of transgenic plants, which express an introduced phytochrome-encoding sequence, is adopted in this thesis as a novel approach to test this hypothesis.

Chapter 2 describes the diversity of photomorphogenic responses of etiolated and light-grown wild-type plants.

Chapters 3 to 6 detail the photophysiological responses of transgenic tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* seedlings which, through expression of a transgene, accumulate supra-wild-type levels of either phytochrome A or phytochrome B; comparison is made with an *Arabidopsis* mutant specifically deficient in phytochrome B. The results indicate that endogenous phytochromes A and B have reciprocal and independent photosensory roles in seedling development, mediating responsiveness to continuous far-red and red, respectively. Persistent expression, in transgenic plants, of phytochrome A is seen to severely impair the shade-avoidance syndrome of light-grown plants, thus phytochrome A is discounted as the photoreceptor for red:far-red ratio perception.

A transgene encoding phytochrome A is expressed in horseradish (*Armoracia lapathifolia*) in order to supress the normal shade-avoidance responses, with implications for improved crop yield as discussed in Chapter 10.

Transgenic overexpression of phytochrome A in *Arabidopsis* (but not in tobacco) results in enhanced sensitivity of the greening response to red light, relative to wild-type and also phytochrome B-overexpressing seedlings (see Chapter 7).

Germination behaviour of transgenic *Arabidopsis* seed indicates a role for phytochrome B in mediating dark-germination. The action of a heterologous phytochrome A in photoregulation of germination in transgenic tobacco seed is less readily interpreted (see Chapter 8).

Expression of a heterologous sequence encoding phytochrome A in the *aurea* mutant of tomato fails to rescue wild-type phenotype (see Chapter 9).

Chapter 11 characterizes a single-cell system for the study of phytochrome activity.

Chapter 12 discusses the potential for, and caveats against, the use of transgenic plants in investigations of phytochrome function.

Chapter 1

General introduction

Phytochrome: a photochromic molecule

Phytochrome is a family of photoreceptors unique to photoautotrophic eucaryotes. The crucial property of the phytochromes, on which their biological activity is dependent, is their photochromicity, i.e. the phytochrome molecule undergoes reversible photoisomerisation between two stable yet interconvertible forms P_r and P_{fr} . P_r and P_{fr} have different (but overlapping) absorption spectra (Butler *et al.,* 1964; Holdsworth and Whitelam, 1987): the P_r form absorbs maximally in the red region of the spectrum [wavelength of maximum absorption (λ_{max})= 667nm]; the P_{fr} form absorbs maximally in the far-red region of the spectrum (λ_{max} =730nm). These unique reversible spectral changes provide the basis for a spectrophotometric assay [$\Delta(\Delta A)$[1]] which facilitated the subsequent purification of the protein and physiochemical characterization of the molecule. The interconversion of the phytochrome molecule by appropriate radiation of the P_r and P_{fr} forms is by virtue of the associated chromophore group. The phytochrome chromphore is a linear tetrapyrrole, phytochromobilin, covalently attached to apophytochrome via a thioether linkage at a peptidyl cysteine residue located in the

[1] $\Delta(\Delta A)$: the difference in absorbance of the red irradiated form (A667nm - A730nm) and the far-red irradiated form (A667 - A730nm)

NH_2-terminal part of the apoprotein (see Whitelam and Smith, 1988 for review). The phytochrome chromophore belongs to a family of several plant pigments (phycobilins) whose structure closely resembles biliverdin (Brown *et al.*, 1981; 1990); bile-pigment–protein complexes such as phytochrome also include phycocyanin and phycoerythrin.

The difference in spectral properties between P_r and P_{fr} forms reflects chemical differences both within the chromphore and within the apoprotein. The data pertaining to the molecular events in the tetrapyrrole chromophore resulting from irradiation of phytochrome have been subject to recent comprehensive reviews (Schaffner *et al.*, 1990; 1991; Rüdiger and Thümmler, 1991; Thomas and Johnson, 1991; Rüdiger, 1992). One manifestation of differences in the apoprotein structures of P_r and P_{fr} is differential sensitivity of the two forms to proteolysis in crude extracts. For example, studies of limited proteolysis of 124 kDa oat phytochrome with either endogenous proteases from oat (Jones *et al.*, 1985) or with various exogenous endoproteases (Lagarias and Mercurio, 1985) have shown that proteolytic cleavage of a 6-10 kDa NH_2-terminal fragment occurs more rapidly in the P_r- rather than P_{fr}-form, suggesting that sites are more exposed in the far-red-irradiated phytochrome species. Studies on binding of monoclonal antibodies located to epitopes within the NH_2-terminal 6 kDa subdomain also show higher affinities for P_r (Cordonnier *et al.*, 1985; Vierstra and Quail, 1986; Holdsworth and Whitelam, 1987). Clearly there are several sites within the NH_2-terminal 6-10 kDa of the phytochrome polypeptide which undergo light-induced conformational changes. Furthermore, these sites are conserved in phytochrome from a range of plant sources (Vierstra *et al.*, 1984; Vierstra and Quail, 1985). Monoclonal antibodies which exhibit higher affinities for P_{fr} than P_r have also been mapped to regions in the chromophore-bearing domain (Partis and Thomas, 1985; Shimazaki *et al.*, 1986). Other

major regions of photoconversion-induced conformational changes are found around the central hinge region at the junction of the principal NH_2- and COOH-terminal domains (Quail, 1991). There is also evidence that, upon P_r --> P_{fr} transformation, conformational changes to the protein surface in the peptide chain adjacent to the chromophore group include a corresponding movement of the chromophore (Sundqvist and Björn, 1983; Ekelund *et al.,* 1985; Hahn *et al.,* 1984; Thümmler *et al.*, 1985). Photointerconversion of phytochrome is therefore known to elicit a multiplicity of changes in the structure and interaction of the chromophore and protein moieties; however the precise nature of the light-induced changes in the phytochrome molecule which determine the respective physiological activities of the P_r and P_{fr} forms remains to be resolved.

Phytochrome action in the light-grown plant

Studies on photochemistry of the phytochrome molecule have been almost entirely restricted to phytochrome extracted from etiolated plants; the direct characterization of the phytochrome pool from light-grown plants has been impeded principally by the low abundance [typically 50-100 times less than etiolated plants of the same species (Hunt and Pratt, 1979; Pratt, 1982)]. Light-grown tissues also contain chlorophyllous pigments which, through screening and fluorescence effects, have prevented conventional spectrophotometric assays. Sensitive immunochemical techniques have overcome some of the problems regarding detection in green-plant extracts. In contrast, physiological studies of phytochrome regulation of photomorphogenic reactions have emphasised the role in the light-grown plant. For example, early work of Hendricks and Borthwick using action spectra techniques to investigate photomorphogenetic reactions, used light-grown plants (Borthwick *et al.*, 1952a) as did Downs (Downs *et al.*, 1957) who demonstrated the effect of changing the proportions of P_r and P_{fr} at the beginning of the dark period on

the growth behaviour of light-grown beans.

Phytochrome functions at all stages of the life-cycle, acquiring information on the nature of the light environment and providing the plant with the capacity to adapt to fluctuations in the supply of radiant energy. Phytochrome is involved in two principal adaptational phenomena in light-grown plants: proximity perception leading to shade-avoidance reactions, and photoperiod perception leading to the induction of flowering and bud dormancy. Light-grown plants have the capacity to perceive the ratios of red and far-red radiation in the incident light (Holmes and Smith, 1975; Smith and Holmes, 1977d). Substantial changes in the spectral energy distribution in the 400-800nm wavelength region of natural daylight occur both daily (i.e. sunset and sunrise) and under vegetation canopies. Selective attenuation of radiation by chlorophyllous tissues results in large decreases in the blue and red wavebands, and to a lesser extent in the green, while far-red wavelengths are largely reflected or transmitted. The net result is a reduction in the red:far-red (R:FR) quantum ratio from the values observed in natural daylight (Holmes and Smith, 1977a, 1977b). Detection of neighbouring or shading vegetation is ecologically important as it confers the capacity for shade avoidance reactions and thus contributes to the ability of individual plants to compete for the resource of light (Smith, 1982; Casal and Smith, 1989). The shade-avoidance strategy generally tends to redirect development in shade such that extension growth is favoured, thereby placing the young leaves in a better lit stratum within the canopy. For shade-avoiding species, reductions in R:FR ratio elicit a syndrome of developmental responses including enhanced internode and petiole extension growth, reduced leaf development, enhanced apical dominance and reduced branching, reduced leaf epinasty, reduced chlorophyll production and large changes in the distribution in assimilates between leaves, stems and roots (Morgan and Smith, 1978; 1979; 1981a, b; Holmes and Smith, 1977d; Smith, 1982; 1986; Child *et al.*, 1981; McClaren and Smith, 1978; Smith and Morgan, 1983; Smith and Whitelam, 1987; Kieller and Smith, 1989;

Casal and Smith, 1989). R:FR ratio perception has been shown from extension growth responses to be effectively compensated for changes in fluence rate over a wide range (Morgan *et al.*, 1980; 1981; Smith and Hayward, 1985; Child and Smith, 1987; Smith, 1990). However, the rate of photosynthesis below values of photosynthetically active radiation (PAR) of approximately 60 μmol m^{-1} s^{-1} drastically limits the capacity for young seedlings to respond to low R:FR by increased extension growth (McClaren and Smith, 1975; Smith and Hayward, 1985). The inference therefore is that waiting for overhead shading effects to elicit a R:FR ratio growth response would be poorly effective compared to proximity detection of neighbours as a means for plants to gauge anticipatory responses to incipient shading while overhead PAR is still high.

To distinguish experimentally between effects of light quantity and light quality it is necessary to vary each factor independently. Developmental responses similar to those found in shaded plants can be evoked merely by changing the quality of light in the absence of any substantial drop in the quantity of light. To vary R:FR while maintaining total light quantity (400-800nm) constant inevitably implies a non-constant supply of PAR (400-700nm). One compromise is to use fluorescent light for a constant spectrum and fluence rate of PAR to which FR from incandescent lamps or special fluorescent tubes is added. Such a set-up enables an almost full-range of R:FR to be simulated while keeping PAR constant (Holmes and Smith, 1975; 1977d); however restrictions of light-sources mean that this technique, as yet, operates within the light-limiting range of photosynthesis. For plants grown in controlled environments in which R:FR ratio of the actinic radiation throughout the daily photoperiod is varied whilst the total PAR is held constant, an inverse linear relationship between P_{fr}/P_{total} and growth elongation-response is observed (Morgan and Smith, 1976; 1978). Such responses have been observed in a wide range of species, and when taken together and normalized (Smith, 1982) the slope of the response-versus-P_{fr}/P_{tot} lines for extreme shade-avoiding species are seen to be very steep whereas for herbs obtained from woodland shaded sites

the slopes are shallow, thereby emphasising the ecological siginificance of R:FR ratio perception. Shade tolerant species clearly perceive low R:FR but responses are muted. A similar inverse linear relationship of stem growth rate has also been observed for the state of phytochrome established at the end of the daily light period (Vince-Prue, 1977; Kasperbauer, 1971; Morgan and Smith, 1978). Seedlings given a short period of FR at end-of-day commonly show marked enhancement of extension growth which may be "reversed" by a subsequent R pulse; repeated R/FR reversibility is usually observed and reciprocity is fulfilled.

Evidence favouring the conclusion that phytochrome is the photoreceptor responsible for perception of R:FR light quality include: (a) a comparison of the spectral characteristics of the natural environment with those of the phytochrome photoconversion; (b) the establishment of quantitative relationships between plant response and calculated phytochrome photoequilbria in green tissues; (c) the demonstration of rapid and repeatable R/FR reversibility of plant responses in simulated natural conditions. The phytochrome photoequilibrium (measured in etiolated material) is closely correlated with the ratio of the quantum flux in the R and FR wavelength bands in broad spectrum (400-800nm) radiation (assuming that the photon fluence rate is sufficient to drive the photoconversion to equilibrium), and this relationship approximates to a rectangular hyperbola (Holmes and Smith, 1977c). From this simple quantitative relationship it can be seen that the phytochrome state shows greatest sensitivity to spectral changes in the range R:FR = 0 --> 1.0 which is the range found in the natural environment, thereby supporting the role of phytochrome as the photoreceptor of vegetative shade.

The role of phytochrome in photoperiod perception has been recognized for 50 years, but its precise function is poorly characterized. The classic demonstration of phytochrome action in photoperiods is the light break experiment in which the induction of flowering in short-day plants may be prevented by interrupting an inductive

long-night with a brief light exposure and this effect typically shows R/FR reversibility (Thomas, 1991; O'Neill, 1992). The action of phytochrome in photoperiod perception in long-day plants is less readily demonstrated.

Phytochrome regulation of development in etiolated seedlings: different modes of physiological action

It has been proposed that high levels of phytochrome accumulated in dark-grown seedlings provide the capacity for extremely sensitive perception of light thus allowing emerging seedlings to react rapidly when their apical regions approach the surface of the soil (Sharrock and Quail, 1989; Smith and Whitelam, 1990). This proposed "antenna" function of phytochrome in etiolated seedlings is supported by the observation that de-etiolation responses (stem inhibition/ leaf development) are generally saturated at relatively low doses of light, i.e. low $[P_{fr}]$, suggesting that etiolated phytochrome functions to sensitively detect the presence of light, rather than the photochromicity of phytochrome acting to detect the relative levels of R and FR light, as observed in light-grown plants. Phytochrome regulation of development is manifested in a number of physiologically-distinguishable modes. In the imbibed seed and etiolated seedlings, different modes of phytochrome action are characterised by: different fluence-response curves; R/FR reversibility characteristics; reciprocity relationships; dependence on fluence rate. These response modes are known as: (a) the very low fluence (VLF) response; (b) the low fluence (LF) response; (c) the high irradiance response or HIR. In the VLF response-mode, saturation is reached at extremely low fluences (typically 10^{-3}-10^{-1} μmol m^{-2} R) and P_{fr} concentrations; consequently FR-reversibility is not possible since even FR establishes sufficient P_{fr} to induce the response. VLF responses can only be detected in seeds or seedlings which contain near zero levels of P_{fr}, i.e. have been grown in absolute darkness. LF responses exhibit the classical characteristics of

phytochrome-mediated responses, as established by Hendricks and Borthwick and co-workers, and can be observed in the photoresponses of light-grown plants as well as etiolated seedlings. LF responses are saturated at moderate R fluences (typically $1\text{-}10^3$ μmol m^{-2}), reversible by FR and normally exhibit full reciprocity. HIRs are only readily observed in imbibed seeds and etiolated seedlings; in de-etiolating seedlings HIRs become less evident and eventually cannot be detected or assume different spectral characteristics (e.g Beggs *et al.*, 1980). HIRs in etiolated seedlings require prolonged irradiation treatments and are dependent on fluence rate [typically saturated at fluence rates of 5 μmol m^{-2} s^{-1} (Hartmann, 1966) and 30 μmol m^{-2} s^{-1} (Beggs *et al.*, 1980)], i.e. reciprocity is not fulfilled.

High irradiance responses in dark-grown plants are normally seen to have action maxima [although strictly speaking such data for fluence-rate dependence cannot generate true action spectra (Shropshire, 1972; Schäfer and Fukshansky, 1982)] in the blue (425-480nm) and far-red (705-730nm) wavebands (Hartmann, 1967a) with either a shoulder (e.g. Mohr, 1957; Siegelman and Hendricks, 1957) or a peak (e.g. Jose and Vince-Prue, 1977; Beggs *et al.*, 1980) in the red waveband (600-690nm). Responses to blue or far-red light vary with both age (Black and Shuttleworth, 1974; Evans *et al.*, 1965) and previous light treatments (Black and Shuttleworth, 1974; Evans *et al.*, 1965; Siegelman and Hendrick, 1958; Beggs *et al.*, 1980; Holmes and Schäfer, 1981). Beggs *et al.* (1980) showed that whereas hypocotyl growth in dark-grown *Sinapis alba* seedlings was inhibited by the blue, red and far-red wavebands, seedlings which had been grown under continuous white light were strongly inhibited only by radiation in the 550-700nm waveband. Two 5 minute pre-irradiations of previously dark-grown plants with red light were adequate to reduce, substantially, the subsequent response of seedlings to blue and far-red. In contrast to induction responses (as characteristic of low fluence reactions) which are effected by a brief red light irradiation and which can be reversed by subsequent irradiation with far-red, high irradiance-type responses require prolonged/

continuous irradiations. Anthocyanin formation in dark-grown *Sinapis alba* L. seedlings and inhibition of hypocotyl elongation in response to continuous 654nm R could be substituted for by hourly 5 minute light pulses where the total fluence over the irradiation period was the same (Schäfer *et al.*, 1981); these pulses were partially or fully reversible by subsequent FR pulses. However, for continuous 715nm FR almost none of the effects could be substituted for by 5 minute hourly pulses from the same light-source (Schäfer *et al.*, 1981). The authors therefore concluded that the R peak in action spectra for continuous light responses is predominantly due to a phytochrome induction-response activity (Beggs *et al.*, 1980; Schäfer *et al.*, 1981) whereas the response under continuous FR represents a true HIR.

In contrast to the action spectra of LF responses which closely agree with the absorption spectrum of the P_r phytochrome form, the action spectrum of etiolated seedlings to continuous irradiation shows no similarity to any known pigment absorption spectrum in the FR waveband. However, strong evidence exists that phytochrome is the functional pigment in the red to far-red region of the spectrum (Hartmann, 1966; Schäfer, 1975; 1976; Mancinelli and Rabino, 1975; Johnson and Tasker, 1979; Wall and Johnson, 1983). The observed effects of prolonged irradiation are not only a function of the light absorption in the photoreceptor pigment, but also a function of the temporal changes of the photoreceptor and signal transfer system involved in regulation of growth. In the phytochrome pool of etiolated seedlings, the P_{fr} form is very much more unstable than P_r (Butler *et al.*, 1963); irreversible destruction, or "decay" of phytochrome is a process which occurs up to 100 times faster in P_{fr} than P_r. P_r and P_{fr} have quite broad and overlapping absorption spectra and therefore under continuous irradiation by light of wavelengths which are absorbed by both P_r and P_{fr} forms, a dynamic equilibrium is set-up so that a photostationary state of the relative proportions of P_r and P_{fr} is maintained at a constant value depending on the wavelength of the monochromatic

light-source. Rate-of-loss of total phytochrome due to P_{fr}-specific destruction is therefore seen to be proportional to the photostationary state established by the wavelength of constant irradiation (Kendrick and Frankland, 1968; Frankland, 1972). Over a period of time, absoiute concentrations of P_{fr} (the presumed physiologically-active form) are highest under steady-state conditions which establish relatively low P_{fr}/P_{total}. The classic dual-wavelength experiment of Hartmann provided the first direct experimental evidence for the role of phytochrome as the photoreceptor for FR-HIR: in this technique the far-red peak of action for controlling inhibition of hypocotyl extension growth in etioiated seedlings of *Lactuca sativa* was shown to correspond to an optimal effectiveness of P_{fr}/P_{total} = approx 0.03 (Hartmann, 1966). Also consistent with this concept, correlation between P_{fr}-specific degradation-rate and the induction of photomorphogenic responses has been demonstrated in a number of experimental situations (Lange *et al.*, 1971; Oelze-Karow and Mohr, 1973; Schopfer and Oelze-Karow, 1971). The precise position of the long-wavelength HIR action maximum will be determined by the kinetic properties of the phytochrome system of the particular plant species. According to spectrophotometric measurements *in vivo,* seedlings of monocotyledonous species, in contrast to dicotyledonous species, demonstrate a high rate of disappearance of P_{totai} through P_{fr} destruction in the FR region of the spectrum: at wavelengths longer than 720nm, even at high fluence rates, P_{fr} destruction in dicots is negligible (Schäfer *et al.,* 1976), however in *Avena* mesocotyl tissues there is a low level of saturation with respect to P_{fr} (of the order of 1% of total phytochrome) and hence this process can be saturated by light (10-100 μmol m^{-2} s^{-1}) up to 730nm (Butler *et al.*, 1963; Pratt and Briggs, 1966; Schäfer *et al.*, 1975). Therefore in tissues of monocot plants the decrease in P_{fr} ieveis under monochromatic irradiation of wavelengths between 680nm and 730nm will parallel the change in photoequilibrium. The action spectrum for

the high fluence rate inhibition of extension growth in etiolated seedlings of *Avena* shows a trough at 730nm (Schäfer *et al.*, 1982), in marked contrast to the action peak at 716nm in *Sinapis alba* (Beggs *et al.*, 1980). Beyond 730nm, P_{fr} destruction in monocot species does appear rate-limited by the P_{fr} concentration (Schäfer *et al.*, 1975) and under these conditions the phytochrome system of *Avena* in the 720-800nm region (Schäfer *et al.*, 1982) resembles the "high irradiance" mode of the dicot system operating in the 700-730nm region (Beggs *et al.*, 1980).

The photoreceptor for blue light action

The photoreceptor for action in the blue waveband of the spectrum, in both the high-irradiance-response of etiolated seedlings and photoresponses of light-grown plants, remains largely uncharacterized. Some aspects of blue light action appear consistent with phytochrome as the responsible photoreceptor (Hartmann, 1966; 1967a, b; Wildermann *et al.*, 1978; Beggs *et al.*, 1980) but the weight of evidence indicates that the blue light response is due to a separate photoreceptor, probably a flavin molecule. Research in this area has recently been reviewed by Galiand (1992).

Is P_{fr} the active form?

Central to almost every attempt to interpret complex physiological data in terms of phytochrome function is the assumption of P_{fr} as the physiologicaily-active form, while P_r is generaliy regarded as lacking a direct biological function. The concept of P_{fr} as the sole active form was initiated through early demonstrations that red light induced physiological effects in dark-imbibed seeds and dark-grown seedlings, whereas far-red light had no effect except to reverse a prior R irradiation (Borthwick *et al.*, 1952b;

1954). The sensitivity of response such that substantial developmental changes were elicited in response to small amounts only of red light suggested that the molecular form produced by the light treatment, i.e. P_{fr}, actively induced the change; the alternative scenario in which P_r acted to inhibit the developmental changes was clearly untenable as the removal of a large proportion of an active inhibitor, requiring high doses of light, would be necessary for the observation of a significant response. Other evidence cited as supportive of P_{fr} activity comes mainly from correlations between spectrophotometrically-determined phytochrome leveis *in vivo* and physiological responses. For dark-grown seedlings given brief light treatments, reports of such positive correlations between response and P_{fr} concentration are principally described by log-linear relationships (Mandoli and Briggs, 1981). In the light-grown plant, stem extension growth measured over a period of days is related in a linear fashion (as opposed to a log-iinear relationship) to the P_{fr}/P_{total} ratio calculated from the spectral distribution of the actinic radiation (Morgan and Smith, 1976; 1978; Child and Smith, 1987) and this relationship has been observed over a wide range of species (Morgan and Smith, 1979; Smith, 1982). Transducer measurements of growth-rate in mustard seedlings have shown responses of light-grown plants to a change in R:FR to be very rapid; transducer methodology allows growth-rate to be directly related to P_{fr} concentration because during the short period between light treatment and measurement of response it is unlikely that there is a substantiai change in total levels of phytochrome. However, such evidence can only, at best, be considered circumstantial. There also exist equally established examples of responses in etiolated seedlings which lack correlation with P_{fr} concentration (Chon and Briggs, 1966; Briggs and Chon, 1966). Detailed analysis of the phytochrome role in the high irradiance repsonses of dark-grown seediings to continuous light have also led some to conclude that under these conditions, P_{fr} cannot be the sole component responsible for the action of phytochrome (Holmes and Schäfer, 1981; Johnson

and Tasker, 1979; Schäfer, 1975). The interpretation of the linear relationship of growth-response in light-grown plants with P_{fr}/P_{total} as evidence for a direct P_{fr} role is subject to the assumption that P_{fr} concentration is directly proportional to P_{fr}/P_{total}. A difficulty with this concept is the accumulation of photoconversion intermediates under continuous irradiation. Direct and indirect estimates of intermediate accumulation have indicated that intermediate levels are a function of fluence rate (Smith *et al.*, 1988; Smith and Fork, 1992) and, by extension, steady-state P_{fr} concentration (assuming P_{total} remains constant) must also be a function of fluence rate. However, growth responses to R:FR ratio have been shown to be compensated for fluence rate over a wide range (Morgan *et al.*, 1981b; Smith and Hayward, 1985; Child and Smith, 1987; Smith, 1990). A paradox is therefore presented for the interpretation of growth-response simply in terms of P_{fr} concentration. The assumption of P_{fr} as the biologically-active form of phytochrome has provided a useful working hypothesis for the interpretation of physiological data. However, in view of important anomalies, as outlined above, and in the absence of an *in vitro* system in which biological activity can be directly associated with purified phytochrome, it is not possible to definitively conclude whether or not P_{fr} possesses unique biological activity.

Evidence for discrete pools of phytochrome from molecular properties of phytochrome in etiolated and light-grown plants

Physiological and spectrophotometric data and, more recently, immunochemical evidence has indicated the existence of more than one type of phytochrome. Early studies of the time-dependent loss of FR-reversibility of R-induced responses provided evidence for both physiologically-labiie (Borthwick *et al.*, 1952a; 1954; Hillman, 1967) and physiologically-stable (Downs, 1955; Downs *et al.*, 1957; Saji *et al.*, 1983)

phytochrome. *In vivo* spectrophotometric studies have shown that destruction of P_{fr} following the irradiation of etiolated tissue demonstrates biphasic kinetics, namely a rapid first order decay resulting in the loss of most of the P_{fr} followed by a significantly slower decay of the remainder, and thereby also indicate the existence of a labile and a more stable pool of phytochrome (Heim *et al.*, 1981; Brockman and Schäfer, 1982). The transition from fast destruction to the slow destruction that predominates in light-grown tissues becomes evident when P_{fr} levels reach 3-4% of dark-grown plants (Heim *et al.*, 1981). Brockman and Schäfer (1982) showed that the size of the stable pool in *Amaranthus* seedlings was maintained constant throughout seedling development. Spectrophotometric studies have also shown that the difference spectrum of phytochrome prepared from light-grown shoots of oat is distinct from that of phytochrome found in etiolated seedlings (Tokuhisa and Quail, 1983). However, the differences are only slight and there is no apparent change in the spectral properties of phytochrome partially purified from iight-grown pea and spinach (Lane *et al.*, 1963; Shimazaki *et al.*, 1981). Tokuhisa and Quail (1983) presented the first direct evidence that extracts of fully green oat tissue contains two pools of phytochrome: a greatly reduced level of etiolated-tissue form and a second, immunochemically-distinct form. Further experiments confirmed these observations (Shimazaki and Pratt, 1985; Tokuhisa *et al.*, 1985) and extended them to a dicot species (Abe *et al.*, 1985). The accumulated data led to the concept of two operationally-defined species of the photoreceptor: Type I and Type II. Type I is an abundant form in etiolated tissue but is rapidly degraded as P_{fr} *in vivo*, and is the molecuie that has been purified and characterised extensively *in vitro*. Type II is of low abundance in etiolated tissue (1-2% of Type I levels) but is apparently stable as P_{fr} and is therefore constitutively present regardless of light treatment, and is more readily detectable in green tissue where Type I is depleted. There is also some evidence that Type II phytochrome is heterogeneous: Pratt and co-workers using monoclonal antibodies have

shown green oats to contain two polypeptides which are immunochemically distinct from one another and from Type I phytochrome.

Comparisons of immunological activity were carried out in the presence of a denaturing agent, thereby eliminating tertiary structure which may be affected by post-translational modification. The complete amino-acid sequence for phytochrome from several etiolated sources has been derived from the corresponding nucleic acid sequence (oat, Hershey *et al.,* 1985; zucchini, Sharrock *et al.*, 1986; pea, Sato, 1988; rice, Kay *et al.*, 1989a; maize, Christensen and Quail, 1989). Microsequence analysis of fragments of highly purified Type II phytochrome from pea compared with the full-length amino acid sequence of pea Type I phytochrome provided the first unequivocal evidence for the difference in primary structure between Type I and Type II (Furuya, 1989). The evidence was therefore clearly for different genes encoding the various phytochrome species as opposed to the Type I and Type II divergence resulting from differential post-translational modifications.

Phytochrome is encoded by a family of genes

Initial data indicated that pea (Sato, 1988) and rice (Kay *et al.*, 1989a) contained only a single phytochrome gene per haploid genome. Multiple phytochrome genes were identified in the hexaploid genome of oat (Hershey *et al.*, 1985) but these genes encoded almost identical polypeptides (>98% identity). The first evidence for a family of divergent phytochrome genes was obtained in *Arabidopsis thaliana.* Sharrock and Quail (1989) screened for plant genomic sequences that cross-hybridised under low stringency conditions with nucieic acid probes derived for coding sequences of etiolated-tissue phytochrome. Southern hybridisation analysis of *Arabidopsis* DNA first suggested that this smallest of known plant genomes contains 4-5 *phy* genes (Sharrock and Quail, 1989). Full-length cDNA clones for genes designated *phyA, phyB* and *phyC* have been sequenced and

mapped to chromosomes 1, 2 and 5, respectively (Chang *et al.,* 1988; Quail, 1991). The three sequences are equally divergent from each other, the polypeptides exhibiting 50% amino acid sequence identity in each pairwise combination. The *phyA*-encoded polypeptide from this dicot is more closeiy reiated to all early published sequences for monocots and dicots (65-80% identity) (Christensen and Quail, 1989; Hershey *et al.*, 1985; Kay *et al.*, 1989a; Sato, 1988; Sharrock *et al.*, 1986) than the *phyB* or *phyC* sequences within its own *Arabidopsis* genome (50% identity). Previous sequences from other species have been categorised as members of the *phyA* subfamily. Within the group of *phyA* sequences, the three monocot sequences are highly related (88-89% identity) and the three dicot sequences are less related to each other (78-79% identity); comparisons across the monocot/dicot line are 63-65% identity. Protein microsequencing data has established that Type I phytochrome of etiolated seedlings is encoded by *phyA* (polypeptide product, phytochrome A). A more recently characterised sequence from the monocot rice is more closely related to the *Arabidopsis phyB* gene (73% amino acid identity) than to *phyA* in the rice genome, and is therefore assigned to the *phyB* subfamily (Dehesh *et al.,* 1991). Purified fragments of pea Type II phytochrome (Abe *et al.*, 1989) show greater sequence relation to rice and *Arabidopsis phyB* than *phyA* or *phyC*. In addition to *phyA, phyB* and *phyC*, the *Arabidopsis* genome contained two, more divergent sequences, *phyD* and *phyE*.

Cucurbita and *Avena* phytochrome A sequences are 65% homologous at the nucleotide and amlno acid level, but this sequence conservation is not evenly distributed. Most of the NH_2-terminal two-thirds of the aligned polypeptide chains exhibit localised regions of high conservation, while the extreme NH_2-terminal and COOH-terminal one-third are less homologous. The distribution of sequence conservation along the polypeptide is very simiiar for the intra-*phyA* and intra-*phyB* subfamily comparisons (i.e. monocot A versus dicot A, and monocot B versus dicot B). Similar regions of phytochrome A, phytochrome B and phytochrome C also are either highly conserved or prone to substitutions, and the hydropathy profiles for the three polypeptide speciles are

very similar over their entire lengths. The most highly conserved stretch of consecutive amino acids is at the chromophore attachment site. A region NH_2-terminal to the chromophore-attachment site is also very highly conserved and is directly involved in forming the hydropathic cavity in which the chromophore is postulated to reside in the native phytochrome molecule. A high degree of conservation of those regions of the polypeptide which interact directly with the chromophore is expected to give almost identical spectral properties of phytochrome from the divergent plant species and between the products of the different gene subfamilies. Immunoblot analysis with monoclonal antibodies raised against etiolated pea phytochrome recognized phytochrome in species as diverse as the moss *Physcomitrella* and algae *Mongeotia*, *Mesotaenium* and *Chlamydomonas* (Cordonnier *et al.*, 1986), thereby demonstrating a highly conserved domain even on the nonchromophore-bearing carboxyl half of phytochrome. There is, however, notable deviation from this conserved phytochrome structure at the ends of the *phyB*-encoded polypeptide in the form of amino- and carboxy-terminal extensions, and this feature is itself conserved between rice and *Arabidopsis* phytochrome B polypeptides as a relatively extensive hydrophilic NH_2-terminal subdomain.

The absence of any striking sequence homology with any protein in the data bases suggests that phytochrome may have a mechanism of action different from that of other known regulatory molecules (Partis and Grimm, 1990), although there has been one suggestion that a COOH-terminal section of phytochromes share sequence homologies with bacterial sensor proteins (Schneider-Poetsch, 1992). Alignment of all available phytochrome polypeptide sequences has not provided any definitive information on possible action mechanisms.

Pairwise combinations show *phyA, phyB* and *phyC* in *Arabidopsis thaliana* to be equally related to one another at 45-52% identity; this level of sequence conservation indicates significant structural heterogeneity and distant evolutionary origins. Sharrock and Quail (1989) compiled a phylogenetic tree which proposed a tripartite branching of

the three major phytochrome species (*phyA, phyB, phyC*) from a percursor gene, followed by subsequent divergence of the gene subfamilies. Rice and *Arabidopsis phyB* polypeptides are more closely related to one another (73% identity) than are monocot and dicot *phyA* sequences (63-65% identity); therefore, it appears that *phyB* has evolved more slowly than *phyA*. It is also suggested that trifurcation was an ancient evolutionary event, with gene duplication giving rise to *phyA, phyB* and *phyC* lines before the divergence of monocots and dicots and therefore predicts that ail angiosperm species have genes in each of these subfamiles. Until recently, phytochromes of the more primitive cryptogamic plants such as algae, mosses and ferns have not been studied at a molecular level, but the notion that the diversion of the various *phy* genes was an ancient evolutionary event has stimulated interest in the molecular biology of lower plant phytochromes. Phytochrome has been immunochemically-detected in many cryptogamic gametophytes and sporophytes and sequences homologous to known phytochrome genes have been found in organisms as early as the green alga *Mougeotia* (Winands *et al.,* 1992). The phytochrome of cryptogamic plants appears to be more related to *phyB* of angiosperms than *phyA* or *phyC* (Hanelt *et al.*, 1992; Winands *et al.,* 1992) perhaps suggesting that *phyA* is an evolutionary modification of an earlier Type II progenitor.

Photoregulation of expression of the phytochrome gene family

In addition to encoding divergent polypeptides, the *phyA, phyB* and *phyC* genes are expressed in different ways, quantitatively and qualitatively. Light-grown tissues contain 1-3% of the initial etiolated tissue concentration of total phytochrome (Hunt and Pratt, 1979) and previously this modulation of phytochrome levels has been accounted for strictly at the protein level in terms of the disparate turnover rate of P_r and P_{fr}. Implied in this view is a constant rate of *de novo* synthesis. However, a second level of control operates at the concentration of translatable mRNA. In monocot seedlings, rate of

phytochrome synthesis in the dark depends on the light pretreatment and appears to be under phytochrome control (Gottmann and Schäfer, 1982a;1983). Phytochrome levels in dicots, in contrast, are regulated prlmarily by differences in degradation rates for P_r and P_{fr}; reaccumulation of phytochrome in etiolated *Cucurbita* seedlings indicates that the rate of P_r synthesis is not greatly affected by light (Schäfer *et al.*, 1972; Oelze-Karow *et al.*, 1976; Schäfer, 1978; Jabben *et al.*, 1980) and shows no obvious controi by phytochrome. In etiolated seedlings of oat a R pulse leads to a detectable reduction in phytochrome mRNA within 15-30 min, and levels are reduced 50% within 50-60 min and 95% within 2 hours (Colbert *et al.*, 1983; 1985). Northern analysis has similarly shown that maize *phy* transcripts are down-regulated 2-3 fold in the etiolated seedlings after a R pulse and show a >10-fold reduction in continuous white light. This R-effect is reversed by a subsequent FR pulse (oat; Colbert *et al.*, 1983; 1985; rice, Kay *et al.*, 1989a). The light effect was demonstrated to result from rapid transcriptional regulation (Lissemore and Quail, 1988; Kay *et al.*, 1989a), and is, in large part, limited to reduced transcription of *phyA* genes (Lissemore and Quail, 1988). In dicots, down-regulation of *phyA* RNA is less pronounced (Lissemore *et al.*, 1987; Sato, 1988) and, in the case of *Arabidopsis* appears to require the presence of continuous white light.

It appears that a single transcript for *phyA* is produced in oat (Lissemore and Quail, 1988) and rice (Kay *et al.*, 1989a). In *Arabidopsis thaliana*, however, the *phyA* probe hybridised to more than one RNA transcript, the smaller of which was strongly down-regulated by white light whereas the higher molecular weight transcript became clearly visible only in the white light-irradiated sample. In pea (*Pisum sativum*) a single-copy *phyA* gene generates three distinct transcripts initiated from different start sites within a complex promoter (Sato, 1988); the levels of these three transcripts in dark-grown plants show various sensitivities to light, namely: RNA1 is strongly reduced by white light to almost zero levels and also shows R/FR reversible effects, RNA2 is slowly reduced to approximately 55% of dark levels under white light, RNA3 is elevated

after transfer to light. The main translation product of RNA3 should be identical to the products of RNA1 and RNA2. A complex promoter structure has also been found upstream of the *phyA* gene of *A. thaliana*. The existence of upstream reading frames (URFs) in the pea (*phyA*) (Sato, 1988) and *A. thaliana* (*phyA, phyB* and *phyC*) (Sharrock and Quail, 1989) 5'-leader sequences provides the potential for complex transcriptional regulation. Monocot *phyA* 5'-leaders do not contain URFs (Hershey *et al.*, 1985; Kay *et al.*, 1989a; Christensen and Quail, 1989), perhaps suggesting that the phytochrome family in dicots is subject to a wider range of regulation potentiai.

In light-grown pea seedlings, a FR pulse leads to enhanced content of *phyA* transcripts RNA1 and RNA2 when seedlings were transferred to darkness; this FR effect was reversible by subsequent R irradiation (Furuya *et al.*, 1991). There is some suggestion, therefore, that Type I mRNA in pea is regulated by a physiologically-stable pool of P_{fr} (i.e. Type II phytochrome) (Furuya, 1989; Furuya *et al.*, 1991). *PhyB* and *phyC* mRNAs are less abundant than the *phyA* mRNA in dark-grown tissues (5-10% in *A. thaliana*). In rice, whereas *phyA* is negatively regulated by light, *phyB* is constitutively expressed irrespective of light treatments and consequently *phyA* and *phyB* transcripts are equaily abundant in fuliy green tissue. *Arabidopsis phyB* mRNA levels are also unaffected by light [or show a small transient increase following transfer to white iight (Sharrock and Quail, 1989)], and therefore this mode of low level, constitutive expression appears evolutionarily conserved among *phyB* genes. There is, however, some indication in green oat shoots of autoregulation of Type II phytochrome levels: 60% higher phytochrome levels (immunochemically distinct from Type I phytochrome) result after end-of-day far-red irradiations than after end-of-day red, and this far-red effect is reversible by red light (Stewart *et al.*, 1992).

Phytochrome-regulation of gene expression

Phytochrome is responsible for many light-regulated events in lower and higher plants ranging from chloroplast orientation to control of morphogenesis (Mohr and Shropshire, 1983). Phenomena investigated in lower plants such as phytochrome-mediated turning of chloroplasts in *Mougeotla* do not involve altered patterns of gene expression. This is in contrast to the major changes in gene-activity associated with de-etiolation processes and changes in morphogenetic patterns in higher plants. Studies of phytochrome regulation have primarily been conducted in de-etiolating angiosperm species and it is well documented that, in response to light, phytochrome regulates the expression of a wide array of plant genes (Harpster and Apel, 1985; Thompson *et al.*, 1985; Tobin and Silverthorne, 1985; Kuhlemeir *et al.*, 1987), including its own gene family (Colbert *et al.*, 1983). Of the few light-repressible genes investigated, the most in-depth characterised are the *phyA* genes. Several conserved sequence elements have been identified by comparison of the various monocot *phyA* promoters, suggesting that these common regions may be regulatory elements involved in *phy* expression. Advances in gene transfer methodology have facilitated attempts to define critical *cis*-acting elements in the promoters of rice and oat *phyA* genes responsible for the autoregulation of expression (Bruce and Quail, 1990; Bruce *et al.*, 1991; Dehesh *et al.*, 1990). The evidence suggests that the rice *phyA* and oat *phyA* promoters contain two positive synergistically-acting elements, and that phytochrome represses transcription through a single independent negatively-acting sequence element that is the target at the terminus of the P_{fr}-triggered transduction chain. The most throughly studied of the light-inducible genes encode the chlorophyll a/b binding proteins of photosystem I and photosystem II (*cab*) and the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS*). Expression of *cab* and *rbcS* multigene families in etiolated seedlings of monocots and dicots is controlled by phytochrome at the level, at least in part, of transcription (Apel, 1979; Berry-Lowe and

Meagher, 1985; Tobin and Silverthorne, 1985). *In vitro* "run-off" transcription assays suggest that phytochrome regulates the rate of initiation of RNA transcripts, although there is evidence that phytochrome can also regulate the process of gene-expression at other levels such as mRNA stability (Schäfer *et al.*, 1986). Significant progress has been made in identifying the *cis*-acting sequence elements involved in the expression of the *cab* and *rbcS* genes (Benfrey and Chua, 1989; Gilmartin *et al.*, 1990; Kuhlemeir *et al.*, 1987; Nagy *et al.*, 1988); studies on the wheat *cab-1* promoter in transgenic tobacco plants have identified a 268 bp region responsible for phytochrome action (Nagy *et al.*, 1987), and the analysis of genes negatively regulated by phytochrome action in *Lemna gibba* has identified a promoter region required for phytochrome responsiveness (Okubara *et al.*, 1993). The identification of the *trans*-acting factors that interact with these *cis*-elements is the next area of intensive research.

The transduction pathway for the phytochrome signal

The signal transducer path linking the light sensor to transcription has remained concealed in a black box; no enzymatic activity or specific protein- or nucleic acid-binding activity has been assigned to phytochrome. Much of the research towards elucidating the components of the transduction chain between photoconversion and deveiopmentai change has been focused upon the phytochrome regulation of gene expression in etiolated plant tissue. Kinetic analysis of phytochrome-regulated transcription suggests that, in some cases, the phytochrome effects are very rapid. The observed change in translatable *phyA* mRNA level in etiolated oats is one of the most rapid [15 minute lag following a 5 second R pulse (Colbert *et al.*, 1983)] phytochrome-induced alteration in any cellular mRNA yet recorded. The rapidity with which P_{fr} initiates repression of *phyA* transcription in monocots, without the need for new protein synthesis (Lissemore and Quail, 1988), indicates that the transduction chain from cytoplasm to nucleus may be

short, with all necessary components pre-existing in the cell before P_{fr} formation. A direct effect of P_{fr} on the nuclear DNA seems unlikely because of the absence of detectable movement of phytochrome into the nucleus (McCurdy and Pratt, 1986a; 1986b; Nagatani *et al.*, 1988; Pratt, 1986) and the inability of purified phytochrome (phytochrome A) to bind to the *phyA* promoter (Quail, 1991), suggesting that a secondary messenger cellular component carries the signal from P_{fr} to the promoter. Also, in *Mougeotia* phytochrome-mediated chloroplast rotation does not involve altered gene expression, and effects on electrical properties of membranes in higher plants are among the fastest known phytochrome responses (Roux, 1986); such evidence is not consistent with direct effects of phytochrome on the nucleus, unless multiple primary mechanisms are proposed.

Involvement of calcium (Poovaiah and Reddy, 1987; Roux and Serlin, 1987; Lam *et al.*, 1989), protein phosphorylation cascades (Otto and Schäfer, 1988; Singh and Song, 1990; Dooshi *et al.*, 1992), inositol lipids (Boss and Morré, 1990; Morré *et al.,* 1990) and G-protein (Romero *et al.*, 1991) has been implicated in light- and P_{fr}-dependent processes. Calcium ions serve as important regulatory elements in stimulus-response coupling in both piants and animals and were first proposed to help couple the photoactivated form of phytochrome to various photomorphogenetic changes by Haupt and Weisenseel (1976); the evidence for this is all indirect (Tretyn *et al.*, 1991). P_{fr} has been found to affect Ca^{2+} influx in isolated maize protoplasts (Das and Sopory, 1985); changes in the concentration of Ca^{2+} has a role in stimulus-response coupling via one or more Ca^{2+}-binding proteins. The best studied of these proteins, calmodulin, is associated with stimulation of a number of different enzymes in plant cells (Poovaiah and Reddy, 1987; Lam *et al.*, 1989). At least two enzymes' activities in pea nuclei are known to be stimulated by P_{fr} in a Ca^{2+}-dependent fashion (Roux, 1992). Protein targets of Ca^{2+} action in plant cells also include phospholipid-dependent and phospholipid-independent protein kinases (Elliot and Kokke, 1987; Harmon *et al.,* 1987; Roux *et al.*, 1990). Some

reports of phosphorylation of *Avena* phytochrome *in vitro* raised the possibility that phytochrome might itself be a protein kinase (Wong *et al.*, 1989), however further studies failed to support this hypothesis (Grimm *et al.*, 1989; Kim *et al.*, 1989). During the early events of transduction of signals across the plasma membrane, phosphoinositides (PPIs) play an important role in many animal systems. It has also been observed that in some fast responses of plants, various inositides are involved (Morse *et al.*, 1987a, 1987b; Acharya *et al.*, 1991; Memon and Boss, 1990). Influence of P_{fr} in PPI turnover has been demonstrated in etiolated leaves of maize (Guron *et al.*, 1992) and is one possible initial early biochemical event in phytochrome-mediated signal transduction.

Sequestering of phytochrome in irradiated seedlings

Immunochemical studies (targetted to phytochrome A) in etiolated seedlings of monocot and dicot species (Coleman and Pratt, 1974a, 1974b; Saunders *et al.,* 1983) have shown that phytochrome in the inactive red-absorbing form (P_r) is accumulated as a soluble molecule, diffusely distributed throughout the cytosol. In contrast, in irradiated seedlings, the P_{fr} form is redistributed, or sequestered, into numerous discrete areas as large membraneless molecular aggregates in the cytoplasm (McCurdy and Pratt, 1986a, b; Mackenzie *et al.*, 1975; Pratt and Marmé, 1976; Saunders *et al.*, 1983). In etiolated oat seedlings, sequestering is completed at room temperature within 5-10 seconds following red irradiation (Pratt and Marmé, 1976; McCurdy and Pratt, 1986a, 1986b); far-red reversal of this process, however, has a half-life time of approximately 1 hour (Mackenzie *et al.*, 1975). In the dicots pea (Saunders *et al.,* 1983) and soybean (*Glycine max* L.) (Cope and Pratt, 1992), R-mediated redistribution of immunochemically-detectable phytochrome requires several minutes at room temperature but is reversed rapidly following irradiation with far-red. The time courses for red light-enhanced sequestering are very similar, in each species, to those measured

for the change in pelletability of phytochrome (Pratt and Marmé, 1976; McCurdy and Pratt, 1986b; Cope and Pratt, 1992) and therefore it appears that these two effects are different manifestations of the same intracellular event.

The relationships between phytochrome location and the subcellular sites of its action are, as yet, far from clear. There is no good evidence that P_{fr} relocates to any identifiable subcellular compartment nor binds to any molecular transduction partners. If the sequestering reactions were part of a transduction chain leading to phytochrome-mediated response, they would be expected to display more rapid time courses than do even the fastest responses. In soybean and pea, red light-induced sequestering is notably slower than rapid phytochrome-mediated responses, and the differences in the rates between monocot and dicot species are also inconsistent with the hypothesis that this event is a primary step in the mode of action of phytochrome (Cope and Pratt, 1992). There is some evidence that ubiquitin is present in these sequestered areas of phytochrome (Speth *et al.*, 1987) and is also found conjugated to pelletable phytochrome after photoconversion to P_{fr} (Jabben *et al.*, 1989). Studies of the light-induced degradation of phytochrome in oat seedlings suggest that ubiquitination of phytochrome may be an intermediate step in degradation (Vierstra and Quail, 1986). This might indicate that redistribution of phytochrome within the cytosol is an initial step leading to the enhanced phytochrome turnover observed in irradiated etiolated seedlings.

Studies of phytochrome function using mutant genotypes: transduction chain mutants

Efforts towards establishing the link between phytochrome and its transduction chain would be substantially aided by a reliable *in vitro* system in which phytochrome could be shown to exert some biologically meaningful action. The absence of such a system has focused attention on genetic approaches to this problem, aiming specifically at the isolation of mutations in trans-acting factors that interact with promoters of

light-regulated genes (Schäfer, 1987; Karlin-Neuman and Tobin, 1987; Chory and Ausubel, 1987). Transduction chain mutants wouid be expected to have pleiotropic defects for phytochrome-mediated responses, unless the *trans*-acting factors are extremely gene specific in which case the mutants isolated will be response-mutants. Two basic aberrant phenotypes have been selected as carrying potential mutations in transduction pathway components: (a) mutants with phenotypes in the light that at least partially resemble dark-grown seedlings, (b) mutants that develop in darkness as if they were exposed to light. Included in the first class are the long hypocotyl mutants *hy5* of *Arabidopsis thaiiana* (Koornneef *et al.,* 1980) and the (slender) *lv* mutant of pea (Reid and Ross, 1988). These plants have approximately normal phytochrome levels (Somers *et al.*, 1991; Nagatani *et al.*, 1990) and appear, therefore, to be mutated in a component which acts downstream of phytochrome in the light signal transduction pathway. Because *cab* genes are expressed normally with respect to phytochrome regulation in the *Arabidopsis hy5* mutant (Sun and Tobin, 1990) the genetic lesion appears unlikely to be early in any primary transduction chain common to ail phytochrome molecular species. The etiolated seedlings of the *lv* mutant show an almost wild-type response to continuous far-red light, similarly suggesting that not all phytochrome responses are affected by the *lv* gene-product.

A different group of mutants have been identified in *Arabidopsis*; designated *det* (Chory *et al.*, 1989a; Chory and Peto, 1990; Hou *et al.*, 1993) these mutants initiate de-etiolation in absolute darkness resulting in short hypocotyls, expanded leaves and accumulation of anthocyanin. Another set of phenotypically similar mutants designated *cop* and which map to loci different from *det* have also been isolated (Deng *et al.*, 1991). Both *cop* and *det* mutants display dramatic pleiotropic effects at the morphological, cellular, subcellular and gene-expression levels, and in particular an array of genes normally induced by light are fully or partially activated in the dark. It is proposed, therefore, that the *det* and *cop* gene-products normally suppress the light developmental pathway in darkness and that light-activated regulatory photoreceptors reverse this action.

Mutations may not oniy lead to the absence of a particular photoreceptor and/or response but may, alternatively, result in an amplified response. A tomato mutant called high pigment (*hp*) contains a normai population of phytochrome and was initially classified as an anthocyanin response mutant, having about 10-fold higher levels of anthocyanin than wild-type under phytochrome control. However, the *hp* mutant shows pleiotropic effects that also are associated with phytochrome such as reduced plant height under red light and more chlorophyli, indicating that *hp* is a mutant with an enhanced sensitivity to phytochrome itself (Peters *et al.*, 1989; 1992). In tomato seedlings blue light appears to function by amplifying the phytochrome response-transduction-chain (Drumm-Herrel and Mohr, 1982) and it appears that the *hp* mutation mimics this action. On the basis of its recessive (loss of function) nature it is proposed that the phytochrome action in dark-grown seedlings is under the constraint of the *hp* gene-product.

Studies of phytochrome function using mutant genotypes: photoreceptor mutants

In addition to photomorphogenetic mutants resulting from a defective transduction chain, photoreceptor mutants also would be expected to exhibit strongly pleiotropic phenotypic effects since all responses regulated by the photoreceptor should be modified. Lesions within the photoreceptor structural gene have the power to affect a specific member of the phytochrome famiiy and thus such photomorphogenetic mutants have potential utility for distinguishing the physiological function of the different phytochromes. The existence of multigene families with multiple regulatory mechanisms has been characterized as a molecular genetic basis for phenotypic plasticity in several plant and animal systems (Smith, 1990a). The identification of multiple phytochrome genes suggests that the diversity of response in plant light perception that occurs throughout plant development may reflect heterogeneity of receptor structure and differential patterns of expression wihin a family of receptor genes. Physiological studies can distinguish between

physiologically-stable and -labile phytochrome pools. However, since Type I and Type II phytochrome pools display very similar absorption spectra, it is essentially impossible to decide purely from physiological data of wild-type plants which phytochrome species is responsible for a particular response. A comparison of the responses of a photoreceptor mutant with the isogenic wiid-type wiil provide direct evidence on the role of the deleted component in the mutant; the anaiysis of photomorphogenetic mutants has provided the first definitive link between defined individual phytochrome species and a specific photoresponse.

Of the putative photoreceptor mutants identified to date, one of the best characterized is the *aurea* (*au*) mutant of tomato. Isolated during selection of gibbereilin (GA) deficient mutants because of its GA-requirement for germination; light-grown plants of *aurea*, however, show a long-hypocotyl phenotype and reduced chlorophyll content (Koornneef *et al.*, 1981). Spectrophotometric and immunochemical assays revealed that dark-grown seedlings of the *aurea* mutant contain less than 5% of the phytochrome of its isogenic wild-type (Koornneef *et al.,* 1985; Parks *et al.*, 1987). The *au* mutant shows the predicted pleiotropic phenotype characterized, in comparison with wild-type, by reduced chloroplast development, seed germination, anthocyanin accumulation and inhibition of hypocotyl elongation (Koornneef *et al.*, 1985). The iight-grown tissues of the *au* mutant, such as flower petals and norflurazon-bleached leaves, contain 30-50% of the *in vivo* spectrophotometrically-detectable phytochrome (Adamse *et al.*, 1988c); immunochemical data indicate that *aurea* has normal phytochrome B levels (R. Kendrick and M. Furuya, unpublished data, cited in: Quail, 1991). Wild-type and *aurea* plants exhibit a quantitatively similar physiological response in terms of enhanced stem elongation to end-of-day far-red treatments (Adamse *et al.*, 1988c; Lopez-Juez *et al.*, 1990b) and also an apparently normal shade-avoidance response to a reduction in R:FR quantum ratio (Whitelam and Smith, 1991). These results led to the hypothesis that the *au* mutant lacks light-labile (Type I) phytochrome, presumably the

phytochrome A homolog, but contains at least a partial functional pool of light-stable (Type II) phytochrome. Proposed explanations to account for the germination behaviour of the tomato *au* mutant (Koornneef *et al.*, 1985) and the allelic *V au* mutant (Lipucci Di Paola *et al.*, 1988) have invoked a light-stable and a light-labile pool of phytochrome performing different roles. Wild-type tomato seeds show 97% dark-germination but are fully inhibited by continuous far-red, whereas both mutant lines display low levels of dark germination that are increased by continuous red, white and far-red light. Lipucci Di Paola *et al.* (1988) proposed that wild-type seeds contain sufficient P_{fr} to account for the high dark-germination but the mutants require a light treatment to produce P_{fr} (presumably Type II phytochrome) and thus stimulate germination. Further it has been proposed that inhibition of wild-type seed germination by continuous far-red is a high irradiance reaction mediated specifically by Type I phytochrome (Peters, 1992) and it is this pool of phytochrome that is deficient in the mutants. The exact molecular nature of the *au* mutation has yet to be determined. The evidence appears to indicate that the lesion is not located within the *phy* structural gene. The apparent homolog of *phyA* in tomato maps to a chromosome location distinct from the *aurea* locus (R, Sharrock, unpublished data, cited in: Reed *et al.*, 1992). The mutant produces wild-type levels of phytochrome mRNA in the etiolated seedlings that can be translated *in vitro* to produce an apparently normal polypeptide product (Sharrock *et al.*, 1988). The inability of *au* etiolated seedlings to accumulate phytochrome has been attributed to *in vivo* instability of the apoprotein; it seems unlikely that this is due to failure of chromophore insertion because (a) spectraliy-active phytochrome is measured in light-grown plants indicating that chromophore synthesis occurs, and (b) etiolated oats (Elich and Lagarias, 1987) and peas (Jones *et al.*, 1986) grown in the presence of inhibitors of chromophore biosynthesis accumulate near normal levels of phytochrome apoprotein. However, more recent data favouring the hypothesis of a chromophore deficiency in *aurea* has revealed that the mutant in fact contains 10-20% of wild-type phytochrome A apoprotein levels, but that

this protein is mostly spectrally inactive (E. Lopez and R. Sharman, unpublished data, cited in : Reed *et al.*, 1992).

A number of photomorphogenetic mutants of *Arabidopsis thaliana* have been isolated and, of these, etiolated seedlings of the long hypocotyl mutants *hy1, hy2* and *hy6* contain greatly reduced levels of spectrophotometrically-detectable phytochrome, but have near normal levels of immunodetectable phytochrome apoprotein (Koornneef *et al.*, 1980; Parks *et al.*, 1989; Chory *et al.*, 1989b). The mutants have been attributed to accumulate photochemically non-functional phytochrome. However *hy1, hy2* and *hy6* do contain some functional phytochrome, as evidenced by the demonstration that photocontrol of seed germination remained under phytochrome control (although sensitivity to red irradiation was reduced compared to wild-type) (Cone and Kendrick, 1985). Furthermore, the light-grown seedlings of these three mutant classes, under continuous irradiation showed more or less the same response to R:FR ratio as do wild-type seedlings with respect to elongation (Whitelam and Smith, 1991). It has been speculated that a functional light-stable form of phytochrome is the most iikely candidate for control of these responses in the mutants (Cone and Kendrick, 1985; Whitelam and Smith, 1991). Chory *et al.* (1989b) have shown that although etiolated *hy1* and *hy6* mutants display poor photoregulation of expression of *cab* and certain other genes compared to wild-type, plants grown at high fluence rate white iight accumulate wild-type levels of *cab* transcripts. This has been interpreted to indicate that in de-etiolated plants, phytochrome is no longer involved in regulating these genes. A similar conclusion was reached by Jenkins and Smith (1985) based on experiments with fuily greened pea seedlings exposed to different amounts of supplementary far-red. These observations could be interpreted as evidence that the photocontrol of *cab* gene expression is a property of a discrete, labile phytochrome pool that is depleted during de-etiolation. It has been demonstrated that exogenously supplied biliverdin (a chromophore precursor) can rescue the wild-type phenotype and also re-establish wild-type levels of spectrally-active phytochrome in *hy1*

and *hy2* (Parks and Quail, 1991) and *hy6* (J. Chory, unpublished data, cited in Quail, 1991). These results are consistent with the mutants being defective in components of the chromophore biosynthetic pathway. The likelihood therefore that the *hy1, hy2* and *hy6* mutations affect all phytochromes in *Arabidopsis* makes them of limited utility in dissection of the different phytochrome pathways.

The *Arabidopsis* long hypocotyl *hy3* mutant contains normal amounts of light-labile phytochrome in etiolated tissue, but is deficient in the light-stable phytochrome pool due to a specific absence of the *phyB* polypeptide-product; phytochrome C is detected at normal levels (Somers *et al.*, 1991). The *hy3* mutation has been mapped to the *phyB* structural gene in *Arabidopsis* (Reed *et al.*, 1993) and is therefore one of the few proven examples of a genic photoreceptor mutant. The *lh* mutant of cucumber appears to be analogous to the *Arabidopsis hy3* mutant. Dark-grown seedlings of *lh* exhibit R/FR photoreversibility of hypocotyl elongation and cotyledon expansion (Adamse *et al.*, 1987; Adamse *et al.*, 1988a) but light-grown *lh* seedlings are reported to display virtually no elongation growth response to end-of-day far-red irradiations (Adamse *et al.*, 1988a; Lopez-Juez *et al.*, 1990a) and a much attenuated shade-avoidance elongation response to reductions in R:FR ratio of continuous irradiation (Whitelam and Smith, 1991). Etiolated *lh* seedlings contain normal levels of spectrally-active light-labile phytochrome but both etiolated and light-grown seedlings of *lh* iack a light-stable protein, of approximately the correct size for a phytochrome, that can be immmunochemically detected in the isogenic wild-type using a monoclonal antibody raised against tobacco phytochrome B (Kendrick and Nagatani, 1991; Lopez-Juez *et al.*, 1992). A mutant gene that increases gibberellin production in a rapid cycling line of *Brassica rapa* (*ein/ein*) (Rood *et al.*, 1990) also results in an elongated phenotype under red and white light. Like *hy3* and *lh*, *ein* mutant plants have been shown to contain a normal immunodetectable phytochrome A population, but lack a normal immunoreactive polyppeptide which, in wiid-type parent plants, reacts with monoclonal antibodies raised against tobacco phytochrome B (Devlin *et al.*, 1992); it

is therefore hypothesised that the *ein* mutation represents an altered *phyB* structural gene.

Another possible photoreceptor mutant is an allele of the sorghum MA3 locus ($ma3^R$); this mutation causes day length-independent flowering, increased GA production, and elongated stems, leaf sheaths and leaf blades. It also causes reduced red light-induced anthocyanin production as well as delayed de-etiolation in response to red light (Childs *et al.*, 1991). A protein, thought probably to correspond to phytochrome, is missing in the ma3 strain (Childs *et al.*, 1991).

Thus, photomorphogenetic mutants are promising tools for the dissection of phytochrome-medlated responses, but lack of a response may result from a modified photoreceptor moiecule, signal transduction chain or final response itself. Because for many of the putative photoreceptor mutants there is little information available on the precise genetic lesions in the various candidate mutants, interpretation of the phenotypes of the mutants is difficult and this especially true for the *aurea* mutant of tomato. In the *hy1*, *hy2* and *hy6 Arabidopsis* mutants, the nature of the mutation appears likely to affect all molecular forms of phytochrome, and futhermore these mutants retain some small measure of spectrally-active phytochrome so that phytochrome responses are not completely eliminated; interpretation of the mutant phenotype is therefore very complex. Strains that are identified as mutated within a specific *phy* structural gene should significantly aid the determination of which light responses were mediated by that particular phytochrome species. Retention of a normal photoresponse in the absence of a photoreceptor would be strong evidence for non-involvement of that photoreceptor in mediating the response in question. Conversely, loss-of-response in photoreceptor deficient mutants demonstrates a requirement for the phytochrome, but is not exclusive of an indirect effect through possible interaction with another photoreceptor species. The study of mutants, taken in isolation, cannot provide unequivocal evidence for phytochrome function.

Until very recently, the isolation of mutant classes has revealed a pronounced bias for the selection of mutations affecting the light-stable phytochrome B species. Inorder to effectively use mutant lines for the dissection of function of the phytochromes it is obviously necessary to have genotypes representing the various molecular forms. Selection of desirable mutant genotypes requires some form of readily scored (i.e. preferably visual) handle, and uncertainty in predicting the phenotypes of individual *phy* mutants may mean that the methods used for screening mutagenised stocks are effectively blind to certain groups of mutants. Alternative strategies are thus clearly required.

Transgenic plants: potential tools for the study of phytochrome function?

The lack of suitable *phyA* mutant genotypes has stimulated several laboratories to adopt an alternative approach, namely to introduce and express cloned cDNA copies of *phy* genes into transgenic plants, leading to accumulation of elevated ("overexpressed") levels of the particular phytochrome species. Expression of oat or rice wild-type *phyA* cDNA sequences in tobacco (Keller *et al.*, 1989; Kay *et al.*, 1989), tomato (Boylan and Quail, 1989) and *Arabidopsis* (Boylan and Quail, 1991) leads to the synthesis of spectrally and biologically active monocot phytochrome in the dicot tissue with the induction of a dwarf phenotype. These results have established that: (a) the dicot phytochromobilin attaches normally so that typical red/far-red photoreversible absorption changes are observed spectrophotometrically, and the chromophore is not limiting since the additional monocot phytochrome accumulates without affecting normal levels of the endogenous dicot phytochrome A; (b) the monocot phytochrome A recognizes dicot transduction chain components causing, what appears superfically to be, an exagerated expression of its normal mode of inhibition of elongatlon growth; (c) phytochrome A is capable of functioning in fully green tissue; (d) since the monocot phytochrome decreases in light, it is recognized at least partially by the dicot machinery responsible for selective turnover

of P_{fr} (Cherry *et al.*, 1991).

The availability of cloned phytochrome sequences suggests an enormous potential for innovative research, for example providing the opportunity to probe the functional importance of various regions of the polypeptide by the use of directed *in vitro* mutagenesis and expression of the mutant molecule in a biologically-active system. Preliminary results have indicated that transgenic plants provide an *in vivo* system for the assay of heterologous phytochrome action and therefore may represent an important advance in the field of phytochrome research. However, interpretation of the results from transgenic plants is complicated by the potential for artifacts arising from expression of a particular phytochrome at abnormally high levels, or in tissues in which it is, perhaps, not normally expressed. Thus it cannot be assumed that the observed phenotypes reflect the endogenous function of the transgenically-encoded phytochrome species. Careful physiological analysis of such transgenic plants coupled with interpretation of the results with regard to wild-type plants and conventional photoreceptor mutants is therefore a necessary step in order to determine the magnitude in practise of such caveats, and hence establish the utility of transgenic plant technology in furthering the study of phytochrome as a muitifaceted family of photoreceptors.

Thus, the primary objective of this thesis is to describe a study of the photophysiological responses of transgenic plants overexpressing a specific phytochrome, and to attempt to relate the results in terms of phytochrome action to the established evidence from wild-type plants and also mutant genotypes, where available. In such an assessment of the utillty, at a practical level, of transgenic plants, it is to be remembered that the transgenic lines used in this study represent an, arguably, crude state of the technology in that the transgene is expressed under the control of a constitutive viral promoter and the introduced phytochrome sequence is often derived from a heterologous plant source. Future approaches could benefit from overexpression of homologous sequences which may also retain upstream sequences defining celi-specific and/or

light-sensitive expression characteristics. The above interpretations of the activity of the introduced phytochrome would then aim to provide further evidence for the function of endogenous phytochromes, especially with a view to extending the evidence for the assignment of distinct photophysiological roles to the individual molecular species of phytochrome. The ultimate aim of a study such as this is to shed light on the "black box" of the phytochrome transduction pathway(s): establishing transgenic plant technology alongside conventional studies of phytochrome function would open a powerful novel approach which has the potential to significantly advance the long-awaited identification of the molecular mechanism of phytochrome activity.

Chapter 2

Contrasting responses of etiolated and light-adapted seedlings to red: far-red ratio : a comparison of wild-type and mutant plants.

Introduction

Physiological studies have revealed that major differences in the mode of response to the phytochrome photoequilibrium are observed in the photoresponses of etiolated seedlings compared to light-grown plants. The photoresponses of etiolated seedlings have been intensively studied under conditions of continuous monochromatic irradiation and a fluence-rate-dependent peak of responsiveness can typically be obtained in the far-red (far-red high irradiance response) as well as red wave-band (Jose and Vince-Prue, 1977; Beggs *et al.*,1980; Holmes *et al.,* 1982). In green plants, however, growth responses are directly related to the value of the estimated photoequilibrium (P_{fr}/P_{tot}) established by the red:far-red (R:FR) quantum ratio of the actinic radiation (Morgan and Smith 1976; 1978; 1981a; 1981b). Described in this chapter are the growth responses of dark-grown seedlings, both in their initial etiolated state and as they progress to the fully de-etiolated state, when the P_{fr}/P_{tot} value is changed by the addition of high fluence rates of FR radiation to a background of white light.

Phytochrome comprises an heterogeneous population of several distinct molecular species. In the past, it has been found useful to divide this population broadly into two pools on the basis of stability in the P_{fr} form and relative abundance in dark-grown and

light-grown plants (Abe *et al.*, 1985; Tokuhisa *et al.*, 1985). Type I phytochrome is accumulated to high levels in etiolated seedlings but is sensitive to light-mediated degradation. Type II describes the low abundance, light-stable species which form the predominant constituents of the phytochrome pool in green plants. The physiological transition from the etiolated to light-adapted state in plants is therefore associated with a change in the total abundance and relative composition of the phytochrome pool.

The photophysiological transition from the etiolated to de-etiolated state is compared here for the wild-type (WT) genotypes of mustard (*Sinapis alba* L.), *Arabidopsis thaliana* and tomato (*Lycopersicon esculentum* L.), and contrasted with those of long hypocotyl mutant lines. Associated with the long-hypocotyl *aurea* mutation of tomato is a deficiency in immunodetectable levels of Type I phytochrome in the etiolated seedlings. Light-grown tissues of *aurea*, however, appear to contain approx. 50% spectrophotometrically-active phytochrome levels of wild-type light-grown plants, and physiological studies also suggest the presence of functional Type II phytochrome (Koornneef *et al.*, 1985; Parks *et al.*, 1987; Adamse *et al.*, 1988c; Lopez-Juez *et al.*, 1990b). In contrast, the *hy3* long hypocotyl mutant of *Arabidopsis thaliana* contains apparently normal quantities of spectrophotometrically-active phytochrome in etiolated seedlings (Koornneef *et al.*, 1980; Parks *et al.*, 1989), but lacks the *phyB* gene-product (Somers *et al.*, 1991; Reed *et al.*, 1993) which constitutes a major component of the Type II phytochrome pool of WT plants.

Materials and methods

Plant material

Seeds of white mustard (*Sinapis alba* L.) (Asgrow, Leicester, U.K.) were sown onto water-saturated potting-compost (Petersfield, Leicester, U.K. No. 2 commercial range) and grown for 2 days in the dark at 25^{o}C prior to light-treatments.

The *Arabidopsis thaliana* (L.) Heynh *hy3* mutant and its isogenic wild-type, Landsberg *erecta,* were isolated by Dr. M. Koornneef (University of Wageningen, The Netherlands) and obtained from Dr. N. P. Harberd (IPSR, Norwich, U.K.). Seeds were sown onto water-saturated compost and maintained at 4^{o}C in darkness for 4 days. Germination was stimulated by a white light pulse followed by a 1 day dark-incubation at 25^{o}C, before transfer to the light treatments.

Seeds of the *aurea* mutant (V *au*) of tomato *Lycopersicon esculentum* Mill. and its isogenic wild type cultivar UC-105, were isolated and obtained from Dr. M. Lipucci Di Paola (University of Pisa, Italy) (Lipucci Di Paola *et al.*, 1988). Seeds were imbibed on filter paper saturated with 15 mM KNO_3 for 1 day and then transferred to compost for a further 3 days in darkness at 25^{o}C, prior to light treatments.

Measurement of growth-rate and anthocyanin content

Seedling hypocotyl lengths of tomato and mustard were measured on a daily basis using a ruler to an accuracy of 0.5 mm. Dark-grown seedlings were measured under dim green safe-light. *Arabidopsis* seedlings were measured from projections of calibrated photographic transparencies giving a resolution of 0.1mm. Growth rate (mm/ 24 h) was calculated from the overall increase in hypocotyl length between two consecutive measurements. All data presented are the mean of three independent trials of a minimum of

10 and maximum of 30 seedlings each. Error bars represent the standard error of the mean; where error bars are not visible they are smaller than the symbol size. Anthocyanin was extracted by boiling whole seedlings in a solution of propanol:HCl:water (18:1:81) for 5 min. Absorption of the extract was measured at 535 nm (Lange *et al.*, 1971).

Light measurement

Fluence rates and spectral distributions of light sources were recorded using a LI-COR LI-1800 spectroradiometer (Lincoln, Nebraska, USA). Photosynthetically active radiation (PAR) was measured as total fluence rate between 400nm and 700nm. R:FR quantum ratio was calculated as the ratio of fluence rates over the 654-664nm and 724-734nm wavelength intervals. Phytochrome photoequilibria (P_{fr}/P_{tot}) calculations were performed by the method of Hayward (1984) using a computer programme which integrated the spectral photon-fluence-rate data with the absorption coefficients and quantum efficiencies of photoconversion for the red-absorbing (P_r) and far-red-absorbing (P_{fr}) forms of oat Type I phytochrome.

Light sources

White light (WL) was provided by eight 40 W cool white fluorescent tubes (PAR = 65 μmol m^{-2} s^{-1}; R:FR = 6.8; P_{fr}/P_{tot} = 0.71). R:FR ratio of this background WL was reduced by the addition of supplemental FR, supplied by eight quartz-halogen lamps using 4 cm of water and one layer of red (No. 14) and green (No. 24A) Cinemoid sheet (Rank Strand, Isleworth, Middlesex, U.K.) as filters (PAR = 65 μmol m^{-2} s^{-1}; R:FR = 0.18; P_{fr}/P_{tot} = 0.44).

Results

Mustard

Transfer of etiolated mustard seedlings from darkness to continuous irradiation with white light (WL) produced a rapid (within 24 h) and marked inhibition of the dark-grown hypocotyl growth-rate (Fig. 2.1b). The degree of this initial light-mediated inhibition of growth was increased by the presence of supplementary far-red radiation (WL+FR) (Fig. 2.1b), and therefore after the initial 24 h period of irradiation, seedlings exposed to WL+FR (R:FR = 0.18) were significantly shorter than those treated with WL alone (R:FR = 6.8) (Fig. 2.1a). However, as the period of light-treatment was extended, growth rate of seedlings under low R:FR ratio was accelerated relative to those under WL alone (Fig. 2.1b). By day 6 of the light treatment, plants exposed to WL+FR were markedly taller than their counterparts under WL (Fig. 2.1a). The response to FR was therefore seen to change as the period of illumination progressed; etiolated seedlings (less than 1 day light treatment) responded to supplementary FR by increased inhibition of growth while light-adapted seedlings displayed FR-mediated acceleration of growth-rate.

In previously dark-grown seedlings of mustard, light-mediated accumulation of anthocyanin within the initial one-two days of irradiation reached higher levels under the FR-supplemented source (R:FR = 0.18) than under WL-alone (R:FR = 6.8) (Fig. 2.2). Following this period however, levels of total anthocyanin in the seedlings declined and this decline occurred more rapidly in the plants under the low R:FR ratio light source. Final anthocyanin levels in the light-adapted seedlings were therefore higher under WL than WL+FR (Fig. 2.2).

Arabidopsis thaliana

The transfer of etiolated seedlings of wild-type (WT) *Arabidopsis* to WL resulted in a decrease in growth rate relative to those maintained in darkness. For a transient period (up to 12 h from start of light treatment) this growth inhibition was more marked in the presence of supplementary FR (Fig. 2.3a, b). Subsequent to the initial 12 h period of light treatment, growth rate of seedlings under WL+FR was accelerated relative to those under WL alone (Fig. 2.3b), and by day 6 hypocotyl length under WL+FR exceeded that under WL (Fig. 2.3a).

Hypocotyl growth-rate of the etiolated seedlings of *hy3* was also reduced for an initial period in response to FR supplementation of the background WL (Fig. 2.4b). Following 1 day of light treatment, however, growth rates of *hy3* seedlings under the WL and WL+FR sources were almost identical (Fig. 2.4b); in contrast to WT, light-adapted seedlings of *hy3* did not response to low R:FR ratio by an acceleration of growth rate. After 6 days of light treatment the absolute hypocotyl lengths of seedlings grown under WL+FR were therefore still significantly shorter than those under WL (Fig. 2.4a).

The dark growth rates of the WT and *hy3* seedlings were observed to be indistinguishable (compare Fig. 2.3a, 2.4a; Koornneef *et al.*, 1980). The light-adapted seedlings of *hy3* displayed an accelerated growth rate relative to WT under white light, (compare Fig. 2.3a, b, 2.4a, b; Koornneef *et al.*, 1980). Light-mediated inhibition of elongation growth-rate in etiolated seedlings of *hy3* (Fig. 2.4b - day 1), however, was not reduced relative to the response of etiolated WT seedlings (Fig. 2.3b - 12 h) under the respective light-sources.

Tomato

The growth rates of de-etiolating seedlings of WT tomato were indistinguishable under WL

and WL+FR light-sources for up to 3 days following the initial transfer from darkness to the light environments (Fig. 2.5a, b). After 3 days of light-adaptation, however, hypocotyl growth under the low R:FR ratio treatment was accelerated relative to that under WL (Fig. 2.5a, b).

When measured after the first day of light treatment, supplementary FR was seen to encourage a small increase in total levels of anthocyanin compared to WL alone (Fig. 2.7). Subsequently, the decline in total anthocyanin levels with tissue aging in the tomato seedlings progressed faster under the low R:FR light-source. In addition, anthocyanin synthesis in the newly emerging leaves (Fig. 2.7 - day 6) was markedly higher under WL than WL+FR, and hence total anthocyanin content of light-adapted seedlings was higher under the high R:FR ratio light-source.

Following transfer of the dark-grown seedlings of the *aurea* genotype to the light environments an accelerated rate of hypocotyl growth under WL+FR, compared to WL, was immediately (within 24 h) apparent (Fig. 2.6b). The *aurea* response to phytochrome photoequilibrium did not change significantly as the seedlings progressed from the etiolated to light-adapted state, ie. growth rate under WL+FR (P_{fr}/P_{tot}=0.44) exceeded that under WL (P_{fr}/P_{tot}=0.71) by approx. 2.5mm / 24 h throughout the period of light treatment (Fig. 2.6b). Tissues of *aurea* did not accumulate anthocyanin to a detectable level under any of the conditions used here.

Discussion

Response of etiolated plants to far-red-supplementation of white light

FR-mediated growth-inhibition and anthocyanin-synthesis in etiolated seedlings under monochromatic light sources has been well documented and characterised as the FR-high irradiance response (FR-HIR) (Beggs *et al.*, 1980; Koornneef *et al.* ,1980; Lange *et al.*, 1971). Dark-grown seedlings of WT mustard and *Arabidopsis*, which were transferred to WL, responded to the presence of supplementary FR radiation by a further depression of extension growth rate (Fig. 2.1, 2.3) and increased anthocyanin synthesis (Fig. 2.2), relative to the response to WL alone. Changing the value of P_{fr}/P_{tot} by simultaneous irradiation with FR during exposure to WL appears, therefore, to elicit a response in de-etiolating seedlings which resembles the direction of response seen in the high irradiance reaction to FR (i.e. synergistic to the R-induced low fluence reactions). Etiolated seedlings of WT tomato are not sensitive to FR-mediated growth-inhibition under monochromatic FR (Koornneef *et al.*, 1985) and did not show enhanced growth-inhibition under FR-supplemented WL (Fig. 2.5).

Etiolated seedlings of the *hy3* mutant of *Arabidopsis,* transferred to WL, showed an inhibition of growth in response to supplementary FR radiation (Fig. 2.4), a response which was comparable in magnitude to that briefly displayed by isogenic WT seedlings (Fig. 2.3); *hy3* is also known to retain an apparently unimpaired response to monochromatic FR (Koornneef *et al.*, 1980). This suggests that the phytochrome species mediating the response of etiolated seedlings to FR is present as a normal population in the *hy3* mutant. In *aurea*, etiolated seedlings displayed an accelerated growth-rate in response to supplementary FR-irradiation of WL (Fig. 2.6); this is in contrast to the isogenic WT in which no acceleration of growth occurred under low R:FR ratio prior to a 3 day period of light adaptation (Fig. 2.5).

Response of light-adapted seedlings to far-red-supplementation of white light

Following a period of light adaptation, seedlings of WT mustard, *Arabidopsis* and tomato responded to supplementary FR radiation of WL with an acceleration of growth rate (Fig. 2.1, 2.3, 2.5) and depletion of anthocyanin levels (Fig. 2.2, 2.7), and this result is consistent with a direct relationship of response with the P_{fr}/P_{tot} status as is typical of light-grown plants (Morgan and Smith 1976; 1978; Whitelam *et al.,* 1979). Hypocotyl growth of *aurea* was similarly accelerated under low R:FR ratio (Fig. 2.7) (see also Whitelam and Smith, 1991), which is consistent with the view that the phytochrome population mediating the response of de-etiolated seedlings to R:FR ratio is functional in this mutant.

White light-mediated inhibition of hypocotyl extension rate in the light-adapted seedlings (2 or more days under continuous white light) of the *hy3* mutant was severely diminished compared to WT, and no effect of supplementary FR-radiation on the relative growth rates of *hy3* seedlings was observed (Fig. 2.4). Whitelam and Smith (1991) did detect a growth response to R:FR ratio in the mature plants of *hy3*, but the response was much attenuated compared to WT. A deficiency of a light-stable phytochrome which is responsible for a component of the growth response to low R:FR ratio is therefore implied.

Concluding remarks

It has been demonstrated here that under conditions of continuous white-light irradiation there are key differences between etiolated and light-adapted seedlings in the phytochrome-mediated response to added FR radiation. The criteria for photoregulation of growth in de-etiolating seedlings therefore differ from those relating to more established plants. It may be argued that an ecological significance for the extreme sensitivity of etiolated seedlings to far-red light lies in the requirement for the emerging seedlings to make a rapid transition from the heterotrophic to the autotrophic state, even in the event of emergence from the soil in to leafy shade (Holmes *et al.,* 1982; Smith and Whitelam, 1990).

When etiolated WT plants were initially transferred to white light, additional FR-radiation mediated a strong inhibition of elongation growth. Continued irradiation by light sources initiating removal of Type I phytochrome was associated with a time-dependent loss of this responsiveness to FR. The *hy3* mutant retained a full WT-like responsiveness to FR in the etiolated state, despite the absence of the *phyB* gene-product. However, whereas growth acceleration under the FR-supplemented source was suppressed in the etiolated seedlings of WT tomato prior to a period of light adaptation, etiolated seedlings of *aurea* responded to FR as seen in the light-adapted WT seedlings. Deficiency in Type I phytochrome, but not the light-stable phytochrome B species, was therefore associated with an impaired response of the etiolated seedlings to FR.

Together, these results indicate that the qualitative differences that exist between etiolated and light-adapted plants in the photomorphogenic sensitivity to FR radiation may be a function of the molecular components of the respective phytochrome pools.

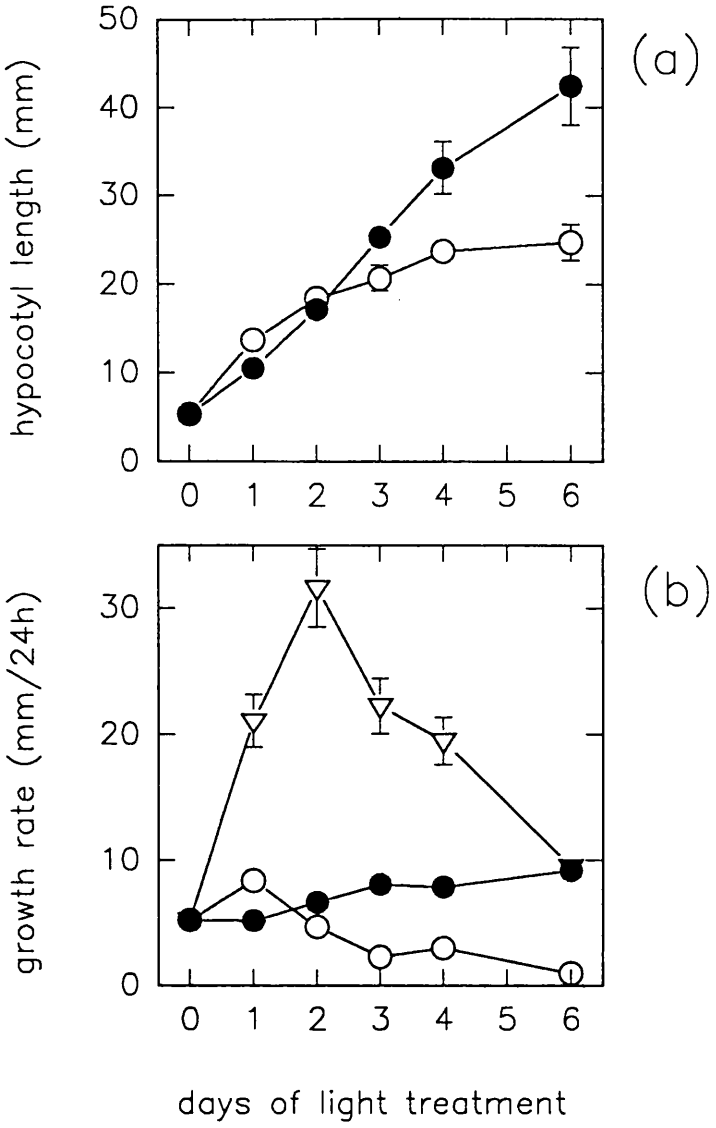


Figure 2.1 (**a**) hypocotyl growth of seedlings of mustard (*Sinapis alba* L.) under continuous irradiation by white light (WL) (*open circle*) and white light with supplementary far-red (WL+FR) (*solid circle*). Seedlings had been grown for 2 days in darkness prior to transfer to the light environments at day 0. (**b**) the change in hypocotyl growth-rate (averaged over the preceeding 24 h period) as the time of light treatment progressed under WL (*open circle*) and WL+FR (*solid circle*) or continued darkness (*open triangle*).

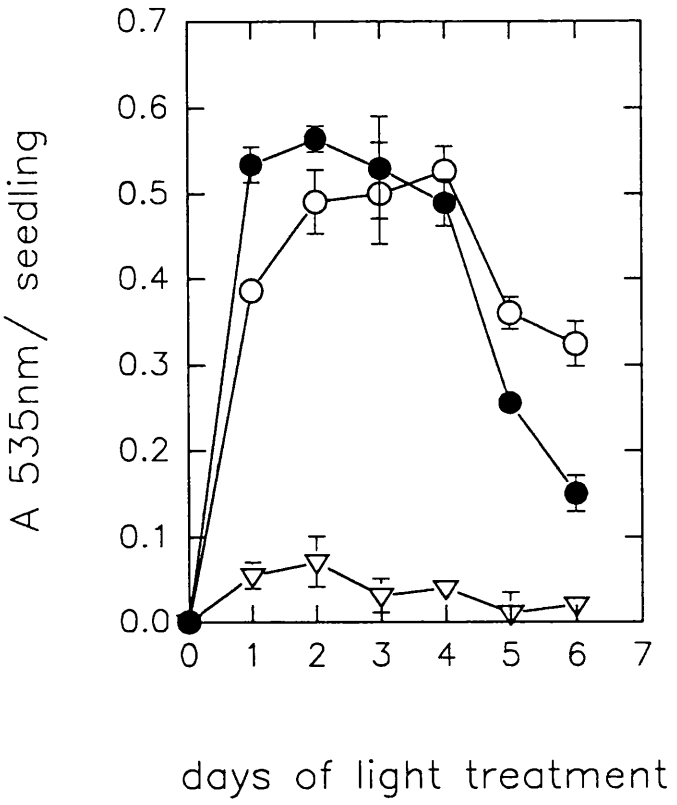


Figure 2.2 Total anthocyanin accumulation in seedlings of mustard (*Sinapis alba* L.) under continuous irradiation by white light (WL) (*open circle*) and white light with supplementary far-red (WL+FR) (*solid circle*) or maintained in darkness (*open triangle*). Seedlings had been grown for 2 days in darkness prior to transfer to the light environments at day 0. Anthocyanin levels in seedling extracts were measured as absorbance at 535 nm (A 535nm).

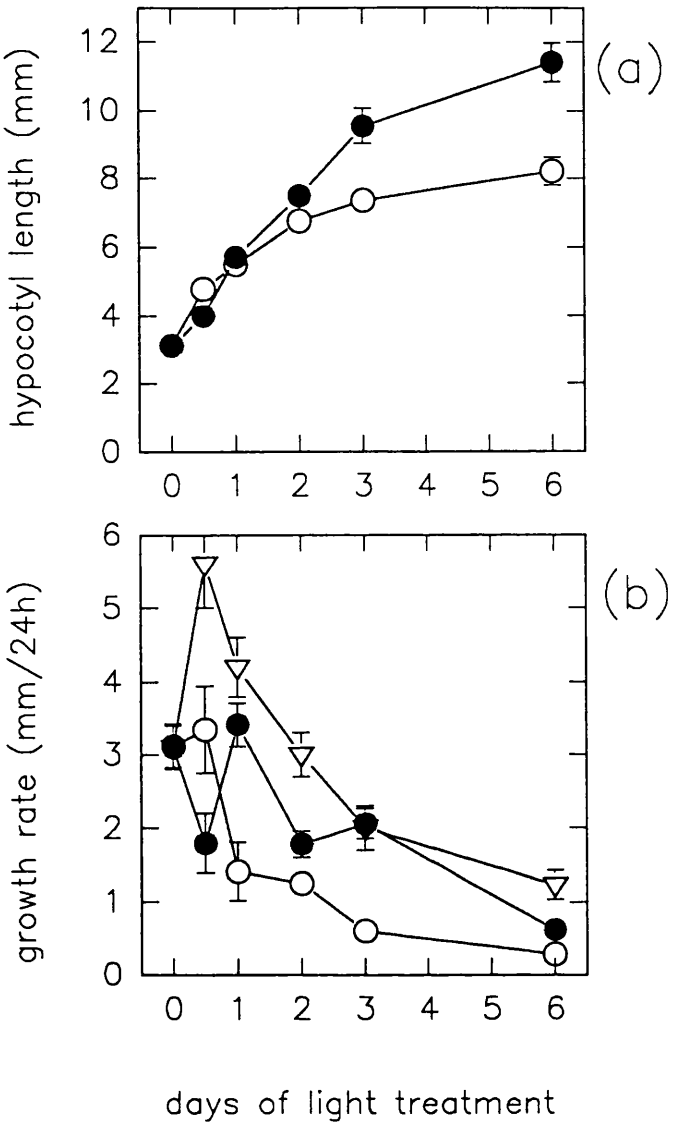


Figure 2.3 (**a**) hypocotyl growth of seedlings of wild-type *Arabidopsis thaliana* (Landsberg *erecta*) under continuous irradiation by white light (WL) (*open circle*) and white light with supplementary far-red (WL+FR) (*solid circle*). Seedlings had been grown for 1 day in darkness prior to transfer to the iight environments at day 0. (**b**) the change in hypocotyl growth-rate (averaged over the preceeding 24 h period) as the time of iight treatment progressed under WL (*open circle*) and WL+FR (*solid circle*) or continued darkness (*open triangle*).

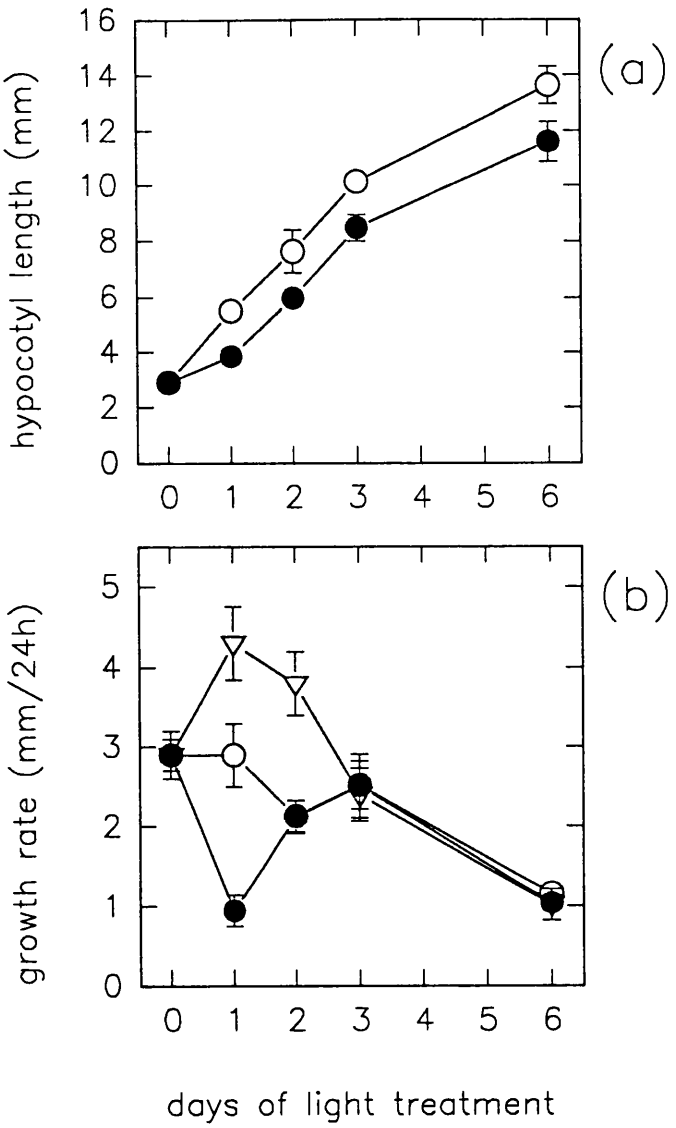


Figure 2.4 (**a**) hypocotyl growth of seedlings of the *hy3* mutant of *Arabidopsis thaliana* under continuous irradiation by white light (WL) (*open circle*) and white light with supplementary far-red (WL+FR) (*solid circle*). Seedlings had been grown for 1 day in darkness prior to transfer to the light environments at day 0. (**b**) the change in hypocotyl growth-rate (averaged over the preceeding 24 h period) as the time of light treatment progressed under WL (*open circle*) and WL+FR (*solid circle*) or continued darkness (*open triangle*).

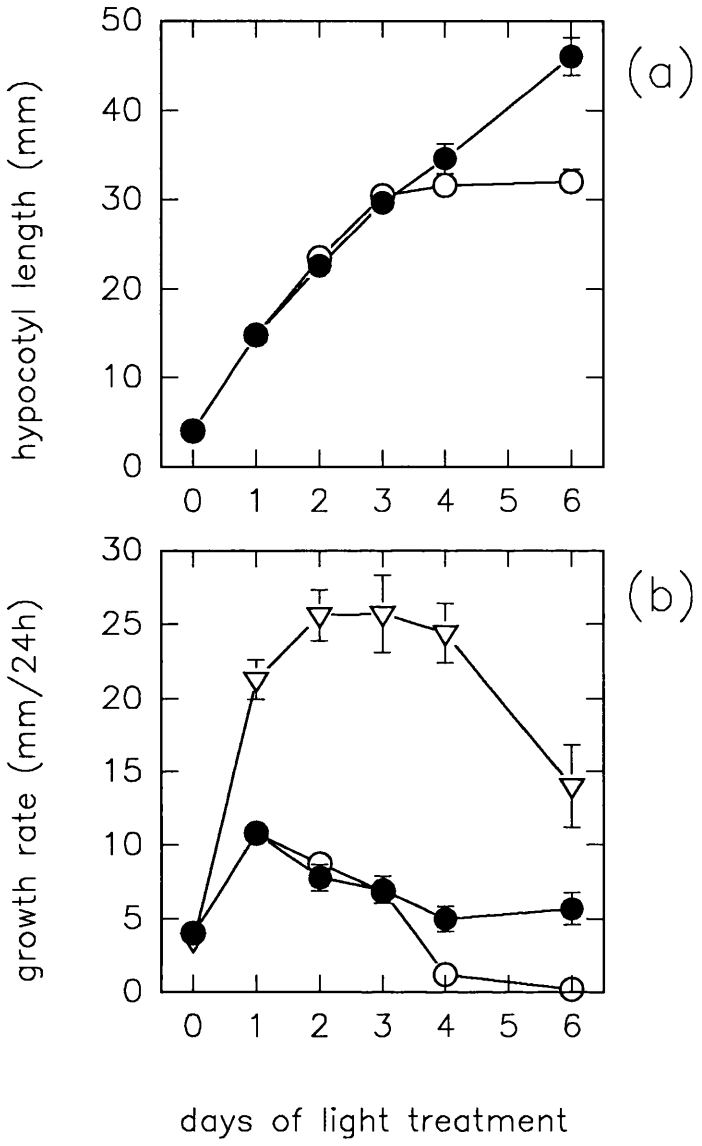


Figure 2.5 (**a**) hypocotyl growth of seedlings of wild-type tomato (cultivar UC105) under continuous irradiation by white light (WL)(*open circle*) and white light with supplementary far-red (WL+FR) (*solid circle*). Seedlings had been grown for 4 days in darkness prior to transfer to the light environments at day 0. (**b**) the change in hypocotyl growth-rate (averaged over the preceeding 24 h period) as the time of light treatment progressed under WL (*open circle*) and WL+FR (*solid circle*) or continued darkness (*open triangle*).

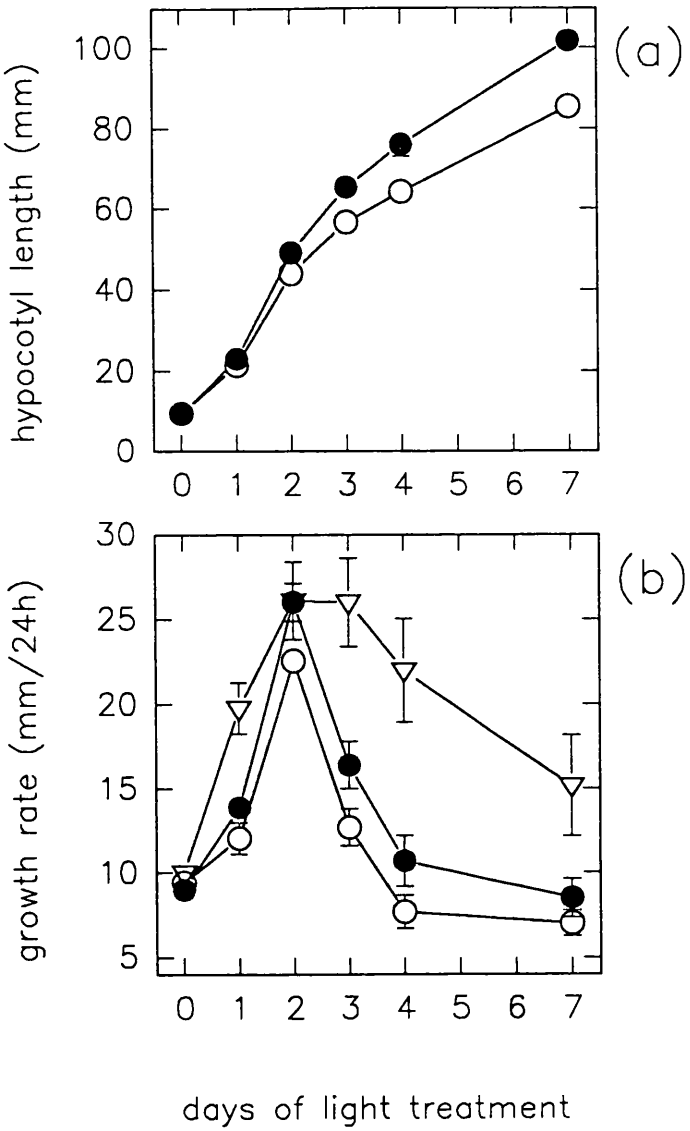


Figure 2.6 (**a**) hypocotyl growth of seedlings of the *aurea* mutant of tomato under continuous irradiation by white light (WL) (*open circle*) and white light with supplementary far-red (WL+FR) (*solid circle*). Seedlings had been grown for 4 days in darkness prior to transfer to the light environments at day 0. (**b**) the change in hypocotyl growth-rate (averaged over the preceeding 24 h period) as the time of light treatment progressed under WL (*open circle*) and WL+FR (*solid circle*) or continued darkness (*open triangle*).

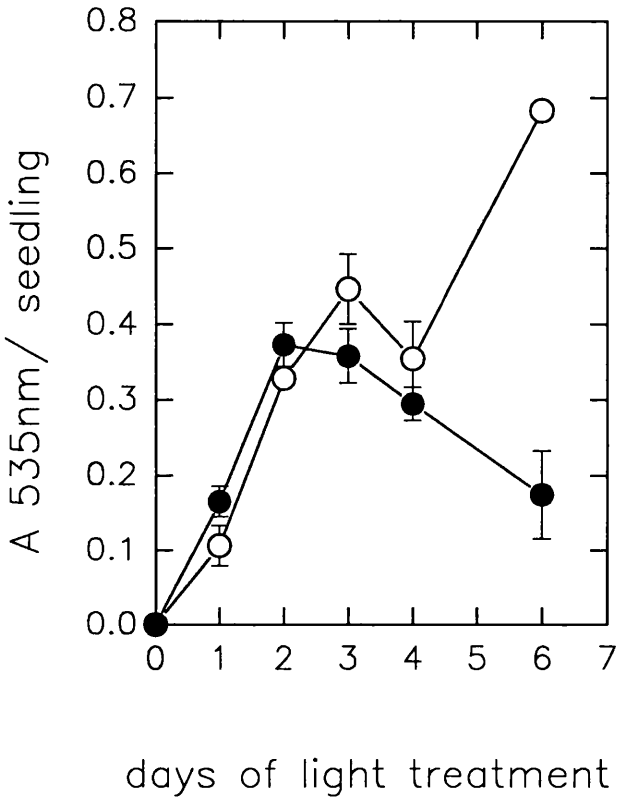


Figure 2.7 Total anthocyanin accumulation in seedlings of wild-type tomato (cultivar UC-105) under continuous irradiation by white light (WL) (*open circle*) and white light with supplementary far-red (WL+FR) (*solid circle*). Seedlings had been grown for 4 days in darkness prior to transfer to the light environments at day 0. The measurement at day 6 includes the synthesis of anthocyanin in the newly emerged true leaves. Anthocyanin levels in seedling extracts were measured as absorbance at 535 nm (A 535nm).

Chapter 3

Photoresponses of transgenic tobacco plants constitutively expressing oat *phyA*-cDNA.

Introduction

Perception of the light environment and photoregulation of development of the plant throughout its life-cycle has been the subject of extensive physiological studies, and these have characterised a number of distinct modes of photoregulation attributable to the phytochrome photoreceptor. A major change in the mode of response to the phytochrome photostationary status coincides with the transition from the etiolated to de-etiolated state. Etiolated tissues display a responsiveness to prolonged irradiation which shows dependence on the prevailing photon fluence rate. Action spectra for such high irradiance responses (HIRs) are commonly reported to show a peak of activity in the far-red wave-band (705-730nm) (far-red-HIR) as well as the red (600-690nm) (Jose and Vince-Prue 1977; Beggs *et al.*, 1980; Holmes and Schäfer 1981). In contrast, photoresponses in de-etiolated plants demonstrate a linear relationship with the estimated phytochrome photoequilibrium (Morgan and Smith 1976; 1978; Whitelam *et al.,* 1979) and are independent of fluence rate (Morgan *et al.,* 1981; Child and Smith 1987; Smith 1990b). Photocontrol in light-adapted plants is therefore predominantly sensitive to the R:FR quality of the actinic radiation, as opposed to the etiolated situation where the level of total irradiance is the primary factor (Holmes *et al.,* 1982). Such multiplicity of control appears irreconcilable on the basis of a single photoreceptor. One proposal is that the divergent physiological response modes are attributable to specific activities of individual

members of the phytochrome family (Sharrock and Quail 1989; Smith and Whitelam 1990; Smith *et al.,* 1991). Definitive evidence to support this concept has, to date, been difficult to achieve. In this chapter the question of a photophysiological role for etiolated-type phytochrome, distinct from that of the light-stable phytochromes, is addressed by the analysis of transgenic tobacco plants that constitutively express an introduced cereal-cDNA encoding phytochrome A.

Comparison of the *phyA*-derived amino-acid sequences with protein microsequencing data of phytochrome purified from etiolated tissues (Hershey *et al.,* 1985; Grimm *et al.,* 1988; Jones and Quail, 1989) indicates that the polypeptide product of the *phyA* gene (phytochrome A) constitutes the light-labile (or Type I) phytochrome which is accumulated to high levels in etiolated seedlings but is a minor component, only, of the phytochrome pool of light-grown plants (see Furuya, 1989; Quail, 1991 for reviews). *phyA* cDNA cloned from etiolated tissues of oat (*Avena sativa* L.) has been introduced, under the control of a constitutive viral promoter, into tobacco to produce a transgenic line expressing high levels of an etiolated-type phytochrome in both the dark-grown and, to a lower steady-state level, the light-grown plants (Keller *et al.,* 1989; Cherry *et al.*, 1991). Therefore, in contrast to WT plants in which levels of phytochrome A in light-adapted tissues are depleted to the limits of detection (Wang *et al.,* 1991), the transgenic tobacco plants retain a significant level of the light-labile molecular species. In common with similar such transformations of tomato with oat-*phyA*-cDNA (Boylan and Quail, 1989) and tobacco with the rice-derived *phyA*-cDNA (Kay *et al.*, 1989b; Nagatani *et al.*, 1991), alterations of the transgenic green-plant morphology were observed (notably a dwarfed phenotype) and were interpreted as indicating the presence of abnormally high levels of physiologically-functional P_{fr} established in the light-grown plants as a consequence of the expression of the introduced cDNA. Foliar application of gibberellic acid (GA_3) can alleviate the dwarf-habit of the oat-*phyA*-transformed tobacco plants, and this observation has raised the question of

whether increased gibberellin levels can suppress the physiological activity of the heterologous phytochrome (J.R. Cherry and R.D. Vierstra, Plant Gene Expression Center/ USDA, USA, personal communication); this proposal is further addressed here.

In dicotyledonous plant species light-stimulated degradation of total levels of the phytochrome pool of etiolated seedlings is subject to the photostationary state as established by irradiance quality (Kendrick and Frankland, 1968; Frankland, 1972). The relationship of rate-of-loss with phytochrome photoequilibrium has been proposed as a central mechanism for the reconciliation of the etiolated FR-HIR with total P_{fr} levels (eg. Hartmann, 1966). Phytochrome destruction in monocotyledonous species, including oat, is saturated at such low levels of P_{fr} (Butler *et al.,* 1963; Pratt and Briggs, 1966; Schäfer *et al.,* 1975) that under standard "high" energy light-sources (Schäfer *et al.,* 1975) the kinetics of P_{tot} degradation appear to be independent of the photoequilibrium; this coincides with an absence of a high-irradiance growth-inhibition at 730nm in cereals (Schopfer *et al.,* 1982; Schäfer *et al.,* 1982). The stability of the heterologous oat-encoded phytochrome in the transgenic tobacco seedlings under irradiation by various R:FR light-sources, was compared here with that observed for the native phytochrome of etiolated WT tobacco seedlings and the Type I phytochrome pool of etiolated oat seedlings.

Materials and methods

Plant material

Plants used were of the wild-type *Nicotiana tabacum* L., cv. *xanthi* and an homozygous isogenic line which, using standard *Agrobacterium* procedures had been transformed with oat-*phyA*-cDNA fused to the cauliflower mosaic virus 35S promoter (Keller *et al.,* 1989). Expression of the introduced cDNA has been shown to result in the accumulation of high levels of spectrophotometrically-active, light-labile phytochrome in etiolated and, to a lower steady-state level, light-grown tissues (Cherry *et al.*, 1991). The transgenic seed-stock used in these experiments was derived from the primary transformant line described as "9A" by Keller *et al.* (1989) and the homozygous line "9A4" by Cherry *et al.* (1991).

Light measurements and P_{fr}/P_{tot} calculations

Fluence rates and spectral distributions of light sources were recorded by placing the cosine-corrected remote probe of a calibrated LI-1800 spectroradiometer (LI-COR, Lincoln, Nebraska, U.S.A.) horizontally at plant height. Photosynthetically active radiation (PAR) was measured as total fluence rate between 400nm and 700nm. R:FR quantum ratio was calculated as the ratio of fluence rates over the 654-664nm and 724-734nm wavelength intervals. Phytochrome photoequilibria values (i.e. P_{fr}/P_{tot}) were calculated from corrected spectrophotometric measurements (Butler *et al.,* 1964) of an irradiated sample of purified etiolated oat phytochrome.

Light sources

Red light (R) (total fluence rate 600-700 nm = 4.8 μmol m^{-2} s^{-1}) was provided by filtering the radiation from Thorn EMI (Birmingham, UK.) Deluxe Natural 40-W fluorescent tubes through 1 cm-deep copper sulphate solution (1.5% w/v) and one red (Number 14) Cinemoid sheet (Rank Strand, Isleworth, Middlesex, UK). Far-red (FR) (total fluence rate 700-800nm stated in text) as used for seedling hypocotyl-growth experiments and phytochrome degradation was provided by water-cooled 100-W incandescent bulbs with a black acrylic filter (Plexiglas Type FRF 700; West Lake Plastics, Lem., Penn., USA).

Two pairs of R:FR-treatment cabinets of similar design were used, one was used at low and one at high irradiances. In both pairs of cabinets, background white light (WL) was provided by banks of fluorescent tubes, and additional FR by filtering the radiation from interspersed banks of incandescent tubes through combinations of red and green plastic filters, the radiant infra-red being removed by flowing "water windows". The design allows for uniform levels of PAR (400-700 nm) and wide ranges of R:FR ratio. In the "high-PAR cabinets", background WL was provided by filtering the radiation from 24 500-W Phillips (Turnhout, Belgium)7785R quartz-halogen lamps through 4 cm of cooled flowing water, one layer (3 mm) of red (Number 4400), and one layer of green (Number 6600) Perspex (SBA, Leicester, UK.). In the "low-PAR cabinets", WL was from eight 40-W Cool White fluorescent tubes, and FR from eight quartz-halogen lamps using 4 cm of water and a single layer of red (Number 14) and green (Number 24A) Cinemoid as filters. Changes in fluence rate whilst maintaining a constant R:FR ratio were achieved by altering the distance from the overhead light source (i.e. shelf height) within the growth cabinet. The cabinets providing WL and FR for the end-of-day experiments of mature light-grown plants are described above as the "high PAR cabinets". The arrangement of the R:FR ratio cabinets used for the induction of nitrate reductase activity and

phytochrome degradation are described above as the "low PAR cabinets". The spectra, R:FR ratios and corresponding photoequilibria values are shown in figure 3.1. The corresponding integrated fluence rates for PAR are given in the text.

Inhibition of hypocotyl growth-rate under continuous R and FR

Dry seed were sown in a horizontal line half-way up a piece of water-saturated 10 · 12 cm^2 Whatmann 3MM paper (Maidstone, Kent, UK.). This was placed on a 3 mm-thick perspex plate and held vertically in a water-filled growth tray so that the bottom edge of the paper was submerged to a depth of 1 cm. The seed were allowed to imbibe for 24 h in the dark at 25°C and then transferred to 4°C for 10 days, still maintained in darkness. The seed were then either germinated for 4 days in WL at 25°C ("light-grown seedlings" - Fig. 3.3) or maintained in darkness at 25°C for a further 5 days ("dark-grown seedlings" - Fig. 3.3). Subsequently, seedlings were placed in continuous darkness, or under continuous irradiation by R or FR light-sources for 48 h. In the case of dark-grown seedlings, owing to non-uniformity of germination times, hypocotyl length was measured (under illumination by a dim green light) before and after the light treatments, and growth was expressed as the increase in length; only seedlings between 3 and 6 mm in length at the beginning of the light treatments were included. Light-grown seedlings were measured only after the R or FR treatment period and growth was expressed as absolute hypocotyl length.

Inhibition of seedling hypocotyl growth under various fluence rates of FR

Etiolated: Seed was sown onto 1% (w/v) aqueous agar in the dark and germination was stimulated after 1 day by a WL-pulse. Seedling growth was continued for 2 days in darkness at 25°C before transfer to continuous FR irradiation at the fluence rates

indicated for a further 7 days.

Light-Grown: Seed was sown onto water-saturated potting-compost and germinated for 5 days under continuous WL (PAR = 130 μmol m^{-2} s^{-1}). Seedlings were then subjected to four successive 10 h WL day / 14 h night cycles in which the night-treatment consisted of either (a) 14 h darkness (end-of-day P_{fr}/P_{tot} = 0.71), (b) 15 min end-of-day FR (establishing P_{fr}/P_{tot} < 0.01) followed by 13 h 45 min. darkness or (c) 14 h continuous FR. Where the fluence rate of this prolonged FR treatment was varied, all seedlings received 15 min end-of-day high irradiance FR (40 μmol m^{-2} s^{-1}) to rapidly establish photoequilibrium.

Hypocotyl lengths were measured at the end of the treatment period from projections of calibrated photographic transparencies.

Hypocotyl growth of de-etiolating seedlings under FR-supplemented WL

Seed were sown onto sterile 1 % (w/v) aqueous agar and after 1 day germination was stimulated by a brief WL irradiation. Seedlings were grown for a further 2 days in darkness at 25°C before entering the light treatments. A solution of 100 μM gibberellin$_3$ (GA_3) was made in 0.01% (v/v) Tween-20. From day 0 of the light treatment this was applied every 2 days as a fine spray to the cotyledons. Non-GA_3-treated plants received complementary foliar applications of 0.01% Tween-20.

Photomorphogenetic responses of mature light-grown plants

Seed was sown onto the surface of water-saturated potting-compost (Petersfield, Leicester, U.K., No. 2 commercial range) and germinated under WL. Plants were grown for a further 4 weeks (i.e. when the third true leaf had expanded) under continuous WL

(PAR=130 μmol m^{-2} s^{-1}) at 25 °C. At this stage, plants were transplanted into 300 cm^3 pots and transferred to the indicated light-treatments in temperature and humidity-controlled growth-cabinets.

For end-of-day experiments, the day-period consisted of 12 h of WL (PAR = 75 μmol m^{-2} s^{-1}) followed by a 12 h night period. End-of-day treatments consisted of 15 min FR (100 μmol m^{-2} s^{-1}) immediately after the day-time WL, followed by 11 h 45 min darkness. The prolonged treatment was provided by continuing the FR-irradiation throughout the 12 h night. Control plants received no light-treatment subsequent to the day-time WL.

Plants subjected to high and low R:FR ratio treatments were maintained under continuous irradiation by WL and WL+FR light-sources respectively.

All measurements of the plants were recorded 2 weeks after the beginning of the light-treatments (when the seventh leaf-pair was expanding). Plant height, internode length and petiole length were measured using a ruler divided to an accuracy of 1 mm. Internode length was measured above the 9th leaf; petiole length and leaf angle were measured from the 5th leaf-pair. Leaf area measurements were recorded using a LI-COR LI-3050A portable area meter. Total dry weight was measured in leaves subjected to 48 h at 80°C. Chlorophyll was extracted from 0.16 cm^2 leaf discs by immersion in 1 cm^3 N, N-dimethylformamide for 48 h in the dark at 4°C. Absorbance was read at 664 nm and 647 nm, and chlorophyll a and chlorophyll b concentrations were calculated according to Moran (1982).

Induction of nitrate reductase activity

Seed was sown onto sterile 1% (w/v) aqueous agar containing 15mM potassium nitrate and the seedlings grown for two weeks under continuous irradiation by WL (PAR = 55 μmol m^{-2} s^{-1}; P_{fr}/P_{tot}=0.71) or WL+FR (P_{fr}/P_{tot}=0.44). Nitrate reductase activity

was determined by the colorimetric method described by Whitelam and Johnson (1980).

Immunochemical analysis of phytochrome levels

Etiolated seedlings were grown for 10 days on a layer of filter paper (Whatmann 3MM) overlaying 1% (w/v) sterile aqueous agar in unsealed petri-dishes. At various time intervals during irradiation, seedlings were homogenised in buffer (1 $cm^3\ g^{-1}$fr. wt.) of 40% (v/v) ethylene glycol in 100 mM [Tris(hydroxymethyl)aminomethane]-HCl (pH 7.8) containing 140 mM ammonium sulphate, 10 mM EDTA, 20 mM sodium sulphite and 5 mM phenylmethylsulphonyl fluoride. After clarification by centrifugation, the supernatant was boiled for 2 minutes with a half volume of 3x strength sodium dodecyl sulphate (SDS) sample buffer. Equal volumes were separated on an 8% SDS-polyacrylamide gel and electroblotted onto nitrocellulose (Hybond-C, Amersham International). Uniformity of protein loadings was confirmed by total protein staining of the blots with 0.2% ponceau in 3% trichloroacetic acid. Independent assay of the heterologous oat-phytochrome in the transgenic tobacco extracts was achieved by use of anti-phytochrome monoclonal antibodies which specifically recognised the mononcotyledon-encoded protein (antibody LAS41-mouse IgG, raised against purified phytochrome from etiolated oats). The wild-type tobacco extracts were probed with anti-phytochrome mononclonal antibodies which were cross-reactive with both monocotyledon and dicotyledon phytochromes (antibody LAS32-mouse IgG, raised against oat phytochrome) (Holdsworth, 1987). Bands were visualized by secondary incubation with alkaline-phosphatase-conjugated goat anti-mouse immunoglobulin antibodies followed by 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrates. The blots were scanned using a GS Hoefer densitometer (Hoefer Scientific Instruments, San Francisco, CA.) and the relative band intensities quantified by integration of the peak area.

Results

Photoregulation of hypocotyl growth in etiolated seedlings

The original transgenic line of tobacco was shown by Keller *et al.* (1989) to contain elevated levels of spectrophotometrically-active phytochrome compared to wild-type. Immunoblot analysis of the etiolated seedlings of the homozygous accession used in these experiments also demonstrated increased levels of total phytochrome, and extracts of the dark-grown transgenic seedlings contained a band which was recognised by a monoclonal antibody specific for monocotyledon-type phytochrome (Fig. 3.2), thereby confirming that the high levels were a direct result of expression of the introduced oat-*phyA*-cDNA.

There was no measurable difference between the actual rates of hypocotyl extension in the dark-grown seedlings of the WT and transgenic tobacco (2.9 $\pm$ 0.667 and 2.4 $\pm$ 0.567 mm/ day respectively in 5-6 day old seedlings). Elongation of the hypocotyls of etiolated WT and transgenic seedlings was strongly inhibited under continuous R (total photon fluence rate 600-700nm=4.8 µmol m^{-2} s^{-1}) (Fig. 3.3) or continuous FR (total photon fluence rate 700-800nm=11 µmol m^{-2} s^{-1}) (Fig. 3.3, 3.4). The degree of this FR-mediated inhibition was dependent on the photon fluence rate of the light-source such that exposure of etiolated WT seedlings to continuous FR at a fluence rate of 0.01 µmol m^{-2} s^{-1}, or lower, did not lead to a significant reduction in hypocotyl elongation relative to seedlings maintained in darkness (Fig. 3.4). The transgenic seedlings proved to be more sensitive than WT to low fluence rates of FR; continuous FR at a fluence rate of 0.15 µmol m^{-2} s^{-1} led to a level of inhibition of the dark-growth rate in the transgenic seedlings which was achieved in the WT only at fluence rates approximately half an order of magnitude higher (Fig. 3.4). Because of the increased sensitivity of the transgenic seedlings towards FR, it is evident that the short hypocotyl phenotype of the transgenic plants is most apparent at lower FR photon fluence rates, that is, under light-limited

conditions (cf. Nagatani *et al.*, 1991).

Photoregulation of hypocotyl growth in light-grown seedlings

Following growth under continuous WL for 4 days, WT tobacco seedlings exposed to continuous R or FR irradiation for 48 h demonstrated R-mediated growth-inhibition but showed little or no response to continuous FR relative to dark controls (Fig. 3.3). Inhibition of hypocotyl growth in transgenic seedlings however, even following several days of adaptation to white light, remained sensitive to both R and FR light sources (Fig. 3.3).

Transgenic and WT seedlings which had been grown under continuous WL for 5 days responded to four successive cycles of end-of-day FR (establishing $P_{fr}/P_{tot} < 0.01$) by a promotion of hypocotyl growth relative to those transferred directly from WL to darkness (i.e. end-of-day $P_{fr}/P_{tot} = 0.71$) (Fig. 3.5). This response to end-of-day FR in WT seedlings was not altered qualitatively or quantitatively under continuous irradiation by FR throughout the 14 h night period. However, hypocotyl growth of transgenic light-grown seedlings, when exposed to prolonged 14 h FR, was markedly inhibited relative to the 15 min end-of-day FR treatment and also relative to seedlings which received no end-of-day irradiation. The degree of this inhibition under prolonged FR was dependent on the fluence rate (Fig. 3.5); light-grown seedlings appeared to require a slightly higher fluence rate for saturation of response ($\geq$ 40 μmol m^{-2} s^{-1}; Fig. 3.5) than seen for the etiolated transgenic seedlings (approx. 2 μmol m^{-2} s^{-1}; Fig. 3.4).

Morphological responses of mature light-grown plants to FR

Plants of WT and transgenic tobacco were grown for 2 weeks under 12 h days of white light irradiation. WT plants which were exposed to FR as a 15 min end-of-day treatment,

establishing end-of-day P_{fr}/P_{tot} = 0.03 (compared to end-of-day P_{fr}/P_{tot} = 0.71 in control plants which received no irradiation following the WL)) exhibited typical shade-avoidance responses, notably increased stem height (Fig. 3.6a) through internode elongation (Fig. 3.6b), extended petioles (Fig. 3.6c) and increased leaf erectness (Fig. 3.6d). Plants of the transgenic line similarly showed increased extension growth when given a 15 min end-of-day FR pulse, relative to the control plants which entered the night-time dark period without end-of-day FR (Fig. 3.6a-d). The proportional increase in growth of the transgenic plants treated to end-of-day FR, relative to the controls, was approximately threefold greater than the corresponding response in the WT, and the absolute growth-increase in the transgenic plants also exceeded WT (Fig. 3.6a-d).

When the end-of-day irradiation was extended to provide continuous FR irradiation throughout the 12 h night period, WT plants still responded by increased elongation growth and an elevated leaf angle compared with the dark controls (Fig. 3.6a-d); the magnitude of the measured response was, in fact, approximately two-fold greater under this prolonged FR treatment as compared with the 15 min end-of-day irradiation. However, little or no growth acceleration relative to the dark controls was observed in transgenic plants which received 12 h continuous FR treatments (Fig. 3.6a, b), and the changes in petiole extension and leaf angle seen in response to a brief end-of-day FR-pulse were also absent under the prolonged FR treatment (Fig. 3.6c, d).

Regulation of seedling hypocotyl growth in response to R:FR ratio

Transfer of etiolated seedlings of WT and transgenic tobacco from darkness to continuous WL resulted in a large decrease in elongation growth-rate of the hypocotyls relative to seedlings maintained in darkness (Fig. 3.7b, 3.8b). When etiolated seedlings of WT tobacco were initially placed under WL they responded to supplementary FR irradiation (WL+FR) by a small increase in the level of light-mediated growth-inhibition relative to seedlings

subjected to WL-alone (Fig. 3.7 -day 1). Subsequently, however, growth rate was accelerated under WL+FR relative to WL (Fig. 3.7b) and seedlings under WL+FR eventually out-grew their WL-grown counterparts (Fig. 3.7a -day 6). In contrast, the transgenic seedlings maintained a depressed extension rate under WL+FR throughout the growth period (Fig. 3.8b) and the final hypocotyl length was therefore shorter than those under WL-alone (Fig. 3.8a).

Exogenously-applied GA_3 did not effect the dark-growth rate but did stimulate increased hypocotyl growth under the two light environments in both the WT (compare Figs. 3.7a/b with 3.7c/d) and transgenic tobacco seedlings (compare Figs. 3.8a/b with 3.8c/d). Qualitatively, response of GA_3-treated material to the R:FR light regime remained largely unperturbed relative to the untreated seedlings: etiolated seedlings of WT tobacco treated with GA_3 showed FR-mediated growth-inhibition and this was superseded by FR-mediated growth-acceleration following 1 day of light treatment (Fig. 3.7c, 3.7d); GA_3-treated seedlings of the transgenic tobacco displayed a persistence of FR-mediated growth-inhibition under the FR-supplemented white-light source (Fig. 3.8c, 3.8d).

Levels of nitrate reductase activity in light-grown seedlings in response to R:FR ratio

A comparison of total nitrate reductase activity in wild-type and transgenic seedlings, grown for several days under WL, showed the transgenic seedlings to accumulate significantly higher levels (Fig. 3.9). A reduction in R:FR ratio, achieved by the addition of supplementary FR wavelengths, served to exaggerate this difference between WT and transgenic seedlings. In the tissues of the transgenic tobacco, increased levels of nitrate reductase activity were measured under WL+FR relative to seedlings under WL-alone; no such stimulation under low R:FR ratio was seen in the wild-type seedlings (Fig. 3.9).

Morphological responses of mature light-grown plants to R:FR ratio

The transgenic genotype was associated with a distinctive morphology of the mature light-grown plant when compared to that of the WT, most notable being the reduced stem height (Table 3.1 and Cherry *et al.,* 1991). This was due to a reduction in internode extension-growth (Fig. 3.10a) rather than internode number, as no difference in the number of leaves was apparent. The transgenic plants had shorter petioles (Fig. 3.10b) and increased levels of total chlorophyll per unit of leaf area (Table 3.1). The leaf area of the transgenic plants was also reduced with a corresponding decrease in total fresh weight compared to WT plants grown under the same light environment, but a more dense lamina was measured as increased fresh weight per unit area (Table 3.1). Those traits apparently unaffected by expression of the oat-*phyA*-cDNA included leaf dry-weight density (Table 3.1) and the chlorophyll a:b ratio (Fig. 3.12).

Wild-type plants displayed a large developmental response to a reduction in R:FR ratio [achieved by the addition of FR radiation to the background white light (WL+FR)]. Under the highest fluence rate studied (PAR = 160 μmol m^{-2} s^{-1}) WT plants responded to the decreased R:FR ratio (WL, R:FR=6.8; WL+FR, R:FR=0.07) with an approximately threefold increase in internode length (Fig. 3.10a) and petiole extension (Fig. 3.10b), and decreased levels of total chlorophyll (51.6 μg cm^{-2} and 28.8 μg cm^{-2} in WL and WL+FR respectively). In addition, the angle of leaf orientation became more vertical by approx. 50^{o} and this was a very rapid response, occurring in established leaves within 24 h of the transfer of WT plants to WL+FR. In contrast, plants of the transgenic line (growing at PAR = 160 μmol m^{-2} s^{-1}) showed little or no growth acceleration under the low R:FR treatment (Fig. 3.10a, b), there was no change in leaf angle and total chlorophyll levels decreased only 20%, in contrast to 45% in the WT.

Wild-type plants growing under WL showed a significant trend towards increasingly elongated internodes and petioles as the fluence rate was decreased from PAR=

160 to 55 μmol m^{-2} s^{-1}; those under the FR-supplemented light-source showed no significant response to corresponding decreases in PAR fluence rate (R:FR ratio was consistent at each fluence rate) (Fig. 3.10a, b). But consistently, under each value of PAR, extension growth of WT plants was increased under the low R:FR ratio light source. Plants of the transgenic line under WL displayed a similar growth trend to WT, i.e. increased elongation growth under decreasing fluence rates, but maintained strong inhibition of growth even under reduced fluence rates of the FR-supplemented light source (Fig. 3.10a, b). Under light conditions corresponding to PAR=55 μmol m^{-2} s^{-1}, supplementary FR radiation resulted in a significant depression of total plant height (Fig. 3.11), internode length (Fig. 3.10a) and petiole length (Fig. 3.10b) in the transgenic plants. This FR-mediated inhibition of extension growth was also observed in a separate experiment in which FR was added to a background of WL with a PAR value of 20 μmol m^{-2} s^{-1} (WL, R:FR=5.0; WL+FR, R:FR=0.27) (Fig. 3.10a, b).

Both WT and transgenic tobacco lines exhibited identical decreases in chlorophyll a:b ratio under R:FR=0.07 compared to R:FR=6.8 (Fig. 3.12). This response was unaffected, in both lines, by changes in fluence rate corresponding to PAR values between 55 and 160 μmol m^{-2} s^{-1}. A R:FR ratio of 0.27 delivered at a PAR value of 20 μmol m^{-2} s^{-1}, however, proved ineffective in altering the chlorophyll a:b ratio relative to plants grown under WL, and again this result was consistent between WT and transgenic lines (Fig. 3.12).

Light-mediated destruction of phytochrome in etiolated seedlings

Immunodetectable levels of phytochrome within etiolated oat seedlings declined rapidly under continuous irradiation by broad-band R or FR light sources (Fig. 3.13a and Fig. 3.14a). Native tobacco phytochrome, measured in dark-grown wild-type tobacco seedlings, was also removed rapidly under R but total levels appeared to be maintained

under continuous FR (Fig. 3.13b and Fig. 3.14b). In the transgenic tobacco seedlings, R-mediated destruction of the oat-encoded phytochrome A was measurable (Fig. 3.13c), although with a notably extended half-life compared to the native tobacco etiolated phytochrome [R: wild-type $t_{1/2}$ = 41 minutes (Fig. 3.14b); transgenic $t_{1/2}$ = approx. 4.5 hours (Fig. 3.14c)] (see also Cherry *et al.*, 1991). Continuous FR irradiation brought about no detectable decrease in the total levels of the heterologous oat-encoded protein (Fig. 3.13c and 3.14c).

Changes in immunodetectable phytochrome in the seedlings of the two tobacco genotypes were also studied under continuous irradiation by white light (WL) (PAR = 25 μmol m^{-2} s^{-1}) and FR-supplemented WL (WL+FR); these light sources established estimated photoequilibria values of 0.71 (WL) and 0.44 (WL+FR). Under continuous WL, the rate of disappearance of the native-tobacco phytochrome (measured in WT etiolated seedlings) and oat-encoded phytochrome in the etiolated transgenic tobacco seedlings, (Fig. 3.13b and 3.13c) proved to be very similar to the respective rates seen under R light [WL: wild-type $t_{1/2}$ = 46 minutes (Fig. 3.14b); transgenic $t_{1/2}$ = approx. 4.7 hours (Fig. 3.14c)]. The apparent degradation rates of both the native-tobacco and the foreign-oat phytochromes were significantly reduced by the addition of FR radiation to the WL background [WL+FR: wild-type $t_{1/2}$ = 78 minutes (Fig. 3.14b); transgenic $t_{1/2}$ = 9.5 hours (Fig. 3.14c)]. The apparent rate-of-loss of phytochrome was calculated as the gradient of the logarithmic relationship between total phytochrome levels remaining and times of irradiation (Fig. 3.14b and 3.14c). These relative rate constants for the native and oat-encoded phytochrome populations of the tobacco seedlings were fitted to a linear relationship with the photoequilibrium value established under each light source (Fig. 3.15); such a linear relationship is consistent with a dependence of phytochrome proteolytic turnover on the proportion of total phytochrome in the P_{fr} form.

Discussion

In WT plants, exposure to WL and the consequent de-etiolation lead to a massive decrease in the levels of Type I phytochrome. In the transgenic tobacco plants the use of a strong constitutive promoter to drive expression of a foreign phytochrome gene presumably disrupts the normal down-regulation of phytochrome accumulation, allowing the persistence of high levels of phytochrome A in the light-grown plants. Total levels of phytochrome in the *phyA*-transformed plants have been shown to be nine-fold higher than in wild type plants, even in the light-grown tissues (Cherry *et al.*, 1991). Therefore the heterologous oat phytochrome, despite being light-labile, is expressed at sufficiently high levels as to form the main component of the phytochrome pool of the transgenic plants. The results presented in this paper strongly indicate that this heterologous phytochrome is also dominant with respect to photoregulation of development and provide significant evidence on the physiological role of phytochrome A.

Etiolated seedlings of WT and transgenic genotypes, when grown in darkness displayed comparable rates of hypocotyl extension, despite a 5-fold higher level of phytochrome in the etiolated transgenic seedlings (Cherry *et al.*, 1991). As concluded from similar studies of phytochrome deficient mutants (Adamse *et al.*, 1988a; 1988b; 1988c) it appears that the concentration of P_r does not directly influence extension rate in etiolated seedlings. Continuous, low fluence rate R or FR radiation inhibited hypocotyl extension in etiolated seedlings of both the WT and transgenic lines. The FR-mediated growth-inhibition of the WT tobacco seedlings was shown to be fluence-rate dependent, as is consistent with the established data for the FR-HIR of etiolated seedlings (e.g. Beggs *et al.*, 1980). Extension-growth of the etiolated transgenic seedlings was similarly inhibited under continuous FR in an irradiance-dependent manner, but sensitivity to low fluence rates of FR appeared to be enhanced in the presence of the elevated levels of phytochrome A. The physiological action of the heterologous phytochrome A therefore appeared to

function in a co-operative manner with the response mediated by the native etiolated-type phytochrome.

Exposure of WT seedlings to white light led to the loss of the FR-mediated inhibition of extension growth (although R continued to be inhibitory) as reported for mustard (Beggs *et al.,* 1980) and radish (Jose and Vince-Prue, 1979). In contrast, de-etiolated seedlings of the transgenic line displayed persistent sensitivity to inhibition of extension growth in response to prolonged FR irradiation. Hypocotyl extension growth of light-grown transgenic seedlings was accelerated by a 15 min end-of-day FR pulse but inhibited when this FR irradiation was continued for 14 h periods. The duration of the FR-treatment was therefore critical to the manner of the response in the transgenic light-grown seedlings, and the level of inhibition under the prolonged FR treatment was shown to be dependent on fluence rate. The oat-phytochrome A-mediated response to continuous FR in the transgenic seedlings, therefore, displayed an irradiance dependency, similar to that characterised in the etiolated plants. The reduced sensitivity to low fluence rates in the light-adapted seedlings compared to the response of etiolated seedlings was probably due to decreased total levels of the light-labile oat protein in the green tissues (Cherry *et al.*, 1991). Mature green plants of the transgenic tobacco showed a divergence of response to brief end-of-day and prolonged FR-irradiations which was comparable to that described for hypocotyl growth in the young seedlings, i.e. the growth-acceleration seen in response to 15 min of end-of-day FR was inhibited when irradiation from the same FR source was continued for 12 h; this result demonstrated that, even in well established plants, the manner of response which was mediated by the heterologous phytochrome A pool was critically dependent on the duration of the radiation. Unlike the response of young seedlings, however, prolonged FR failed to significantly inhibit growth-rate of the mature plants relative to control plants which received no FR irradiation.

Neither hypocotyl growth of light-grown seedlings or elongation growth in mature

green plants of the WT genotype demonstrated a sensitivity to FR-mediated growth-inhibition such as that displayed in the transgenic light-grown plants. However, prolonged FR irradiation of the mature WT plants elicited an enhancement of the end-of-day acceleration of growth, a response which is not readily explained in terms of stable (Type II) phytochrome. The phenomenon of so-called "persistent promotion" of extension growth during the photoperiod in response to a prior FR irradiation has been demonstrated in mustard (Casal and Smith 1988a; 1989) and is proposed as a possible mechanism relating to the growth promotion of the WT tobacco under prolonged FR. Such an effect may also interact with the phytochrome-mediated responses of the transgenic plants and may therefore explain the failure of prolonged FR to inhibit the growth of the mature plants as effectively as in the young seedlings.

Plants of the WT and transgenic lines when subjected to a brief end-of-day FR pulse developed the morphological traits characteristic of the classic shade-avoidance-response of light-grown plants (Casal and Smith 1989). The marked degree of this alleviation of the dwarf-habit of the transgenic plants, relative to the response in the WT, suggests that the pool of oat-encoded light-labile P_{fr}, as well as the tobacco light-stable P_{fr}, was capable of sustaining some measure of growth-inhibition during a period of darkness. The display of a response to this form of end-of-day treatment cannot, therefore, be regarded as specific proof of an active Type II phytochrome population (Adamse *et al.* 1988a).

The characteristic responses of light-grown plants to variations in R:FR ratio have been extensively described and shown to be mediated by phytochrome (Smith, 1982; Casal and Smith, 1989). In particular, shade-avoiding species exhibit marked acceleration of internode and petiole elongation when exposed to broad-band radiation rich in FR, as present within vegetation canopies, and such responses show an apparently linear relationship with the estimated photoequilibrium (P_{fr}/P_{tot}) (Morgan and Smith, 1976; 1978; 1981b). The WT tobacco in this study exhibited a strong shade-avoidance response,

with up to a threefold increase in extension rate at R:FR=0.07, compared to that at R:FR=6.8. The extension growth of the transgenic line, however, was not stimulated by low R:FR ratio. This could be interpreted most simply in terms of the ninefold excess of total phytochrome in the de-etiolated transgenic plants compared to WT (Cherry *et al.,* 1991). Moreover, reduction of the R:FR ratio by supplementary FR irradiation of WL mediated an inhibition of elongation growth, and also increase in nitrate reductase activity (contrast with Whitelam *et al.,* 1979), in the light-grown plants of the transgenic tobacco. The response of the oat-*phyA*-transformed tobacco to the photoequilibrium status of the heterologous phytochrome A was therefore inconsistent with the response of WT light-grown plants to the P_{fr}/P_{tot} ratio of Type II phytochrome. In no other case has a depression of extension rate by low R:FR ratio been reported in green plants; even shade-tolerating species normally show weak extension-rate increases in response to low R:FR (Smith and Morgan, 1983). Inhibition of growth when low R:FR ratio is attained by simultaneous irradiation with FR during exposure to WL was, however, observed as a transient reaction in the etiolated seedlings of WT tobacco (Fig. 3.7), mustard and *Arabidopsis* (see Chapter 2 of this thesis). Whereas this FR-mediated inhibition response was lost in WT seedlings following a period of de-etiolation, the transgenic tobacco seedlings exhibited a persistently depressed hypocotyl growth-rate under the FR-supplemented source. The exogenous application of gibberellin A_3 accelerated overall growth-rate but did not qualitatively interfere with the relative response to FR in either the etiolated or light-adapted seedlings of the WT or transgenic tobaccos. Therefore imitation of the WT phenotype by the application of plant hormones to the transgenic plants did not suppress the photoregulatory effects of the introduced phytochrome A. The persistence of phytochrome A in light-grown plants is therefore proposed to facilitate the continuation of photoresponses normally observed only in etiolated seedlings. Such responses appear to operate antagonistically to normal stimulation of extension growth by supplementary FR, nullifying, or even reversing, shade-avoiding responses. On this basis

it might be speculated that, during normal development, removal of phytochrome A during de-etiolation is important because it presumably allows one, or more, other phytochrome species to operate as the R:FR sensor for shade-avoidance and neighbour detection.

Severe inhibition of stem elongation growth and high levels of total chlorophyll accumulation could be interpreted as an exaggerated manifestation of the de-etiolation response (i.e. Type I specific) or merely due to the high levels of total P_{fr}, irrespective of molecular type. However, one component of the shade-avoidance syndrome that was unaffected by the expression of the oat-*phyA*-cDNA in the transgenic tobacco was the chlorophyll a:b ratio. This result suggests that introduced phytochrome A could not interfere with all the phenomena that are regulated by phytochrome in the light-grown plant. By extension, this implies that another form of phytochrome is responsible.

Measurement of total phytochrome levels in irradiated etiolated tissues showed that, over a limited period of time at least, the wavelength of the light-treatment significantly affected the relative immunodetectable levels of the WT tobacco phytochrome pool; higher total levels persisted under irradiation by low R:FR ratio light-sources. Levels of the heterologous oat-encoded phytochrome A in the transgenic tobacco seedlings were similarly dependent on the R:FR ratio of irradiation, in contrast to the phytochrome pool of etiolated oat seedlings which appeared equally sensitive to removal under R or FR light sources. Without knowledge of the respective synthesis rates, these measurements cannot definitively identify a relationship of rate of degradation with photoequilibrium, and the implications for the mode of response mediated by the heterologous phytochrome population in the transgenic plants are necessarily restricted. However, so far as they extend, these observations appear compatible with the predictions of Hartmann (1966) for the properties of the phytochrome pool mediating FR-action in etiolated seedlings of dicotyledonous species.

Concluding Remarks

Compared to WT, the etiolated photophysiology of the oat-*phyA*-transformed tobacco was not qualitatively altered. The enhanced sensitivity to low fluence rates of FR in the transgenic etiolated seedlings is considered compatible with the native etiolated response-mode and supports the conclusion that the phytochrome A species of phytochrome can mediate an irradiance-dependent response to FR within etiolated tissues.

Whilst de-etiolation of WT seedlings led to a loss of FR-mediated growth-inhibition, transgenic light-grown plants continued to display an irradiance-dependent response under continuous FR. An apparent insensitivity of the growth responses of transgenic light-grown plants to reductions in R:FR ratio, and even a reversal of the WT shade-avoidance-response under FR-enriched light source, emphasised that the manner of response mediated by the heterologous phytochrome A was antagonistic to Type II phytochrome action.

The constitutive population of oat-phytochrome A therefore imposed a mode of photoregulation which differed markedly from the characteristic responses of light-grown WT plants to the photoequilibrium, but which displayed some of the properties of the response mode normally restricted to etiolated seedlings (Mancinelli 1980) i.e. (a) a strong action of broad-band FR (b) a critical dependence of response on the prolonged delivery of the FR radiation, and (c) a failure of reciprocity. Although on this evidence alone the photoresponse of the light-grown transgenic plants cannot confidently be interpreted as the classic high irradiance response, these observations do appear to strongly implicate phytochrome A in the irradiance-dependent response-mode of etiolated seedlings.

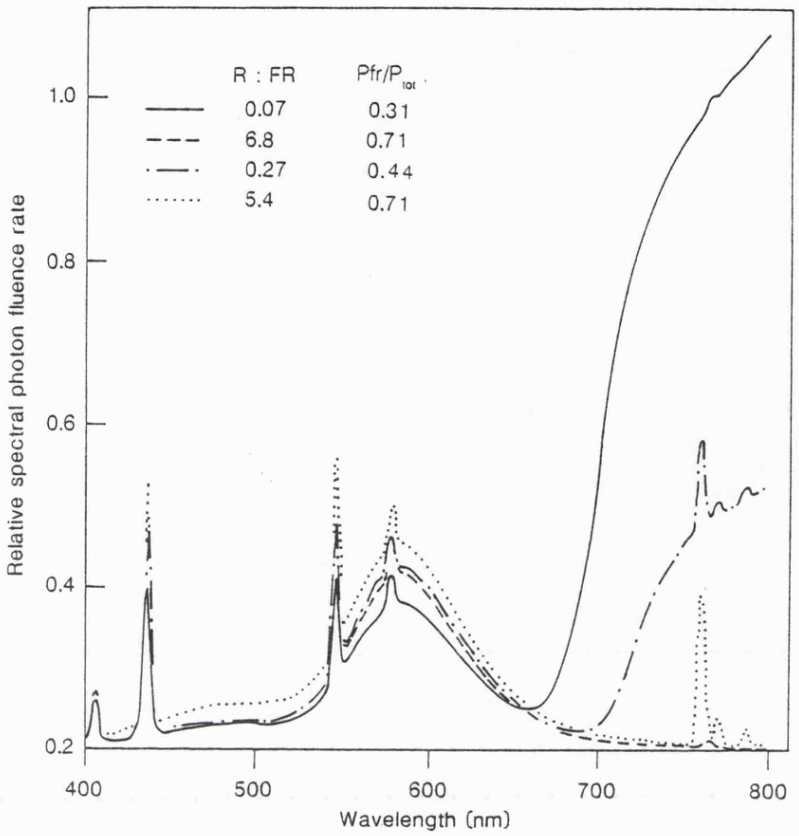


Figure 3.1 Spectral photon distributions of the light sources of the red (R):far-red (FR) ratio treatment cabinets. R:FR ratios of 6.8 (WL; *dashed lines*) and 0.07 (WL+FR; *solid lines*) were delivered by the "high-PAR cabinets" described in Materials and Methods. R:FR ratios of 5.4 (WL; *dotted lines*) and 0..27 (WL+FR; *dashed-dotted lines*) were delivered by the "low-PAR cabinets" described in Materials and methods. R:FR ratio was calculated as the ratio of photon fluence rates witihn the 654-664 nm and the 724-734 nm wavebands. Fluence rates of photosynthetically active radiation (PAR) are given in the text.

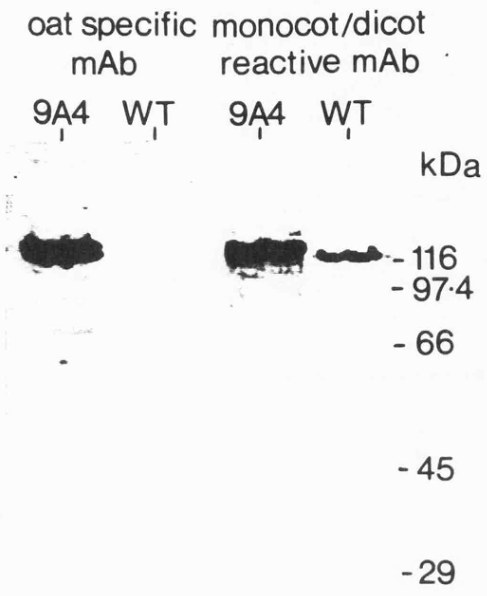


Figure 3.2 Detection of oat-encoded phytochrome in transgenic tobacco by immnuoblot analysis. Extractions of etiolated wild-type tobacco (WT) and a transgenic line expressing oat-*phyA*-cDNA (9A4) were separated by SDS-polyacrylamide gel electrophoresis. The resulting electroblots were incubated with monoclonal antibodies (mAb) either specific to oat phytochrome or capable of recognising both the introduced oat phytochrome and the native tobacco phytochrome. Samples loaded in each lane contained equal amounts of total protein. Minor bands detected are considered to be degradation products of the major band phytochrome.

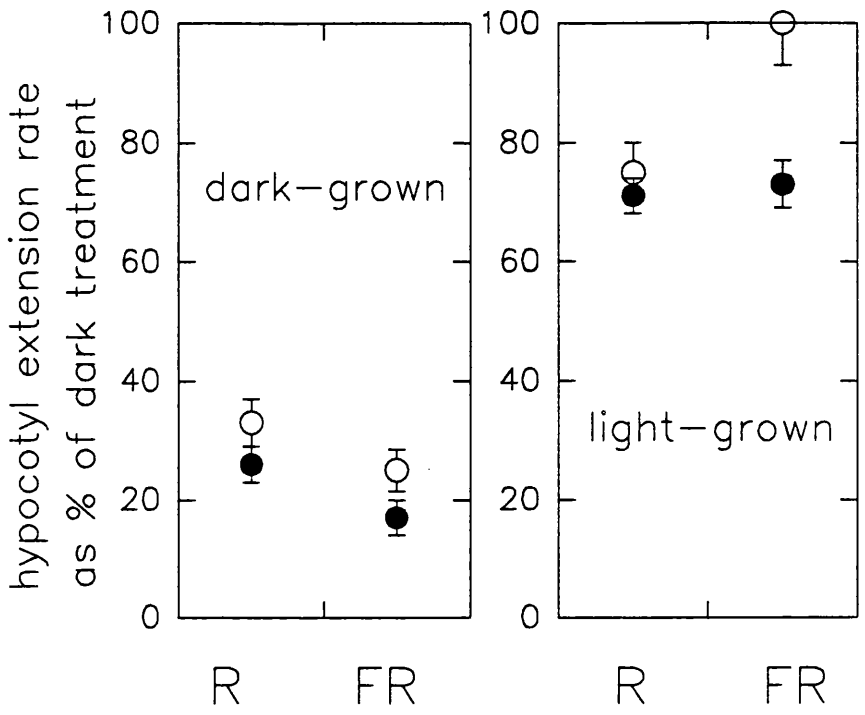


Figure 3.3 Relative effectiveness of continuous low fluence red (R; 600-700nm=4.8 μmol m^{-2} s^{-1}) and far-red (FR; 700-800nm=11 μmol m^{-2} s^{-1}) irradiation for the inhibiton of hypocotyl extension-growth in dark-grown and white light-adapted (WL-grown) seedlings of wild-type (*open circles*) and transgenic (*solid circles*) tobacco. Seedlings were grown for 48 h under continuous irradiation by the R or FR light sources and the growth over this period was compared with that of control seedlings which had been placed in darkness for the same period. Values are the means ± SE of 5 independent replicates of 20 seedlings each.

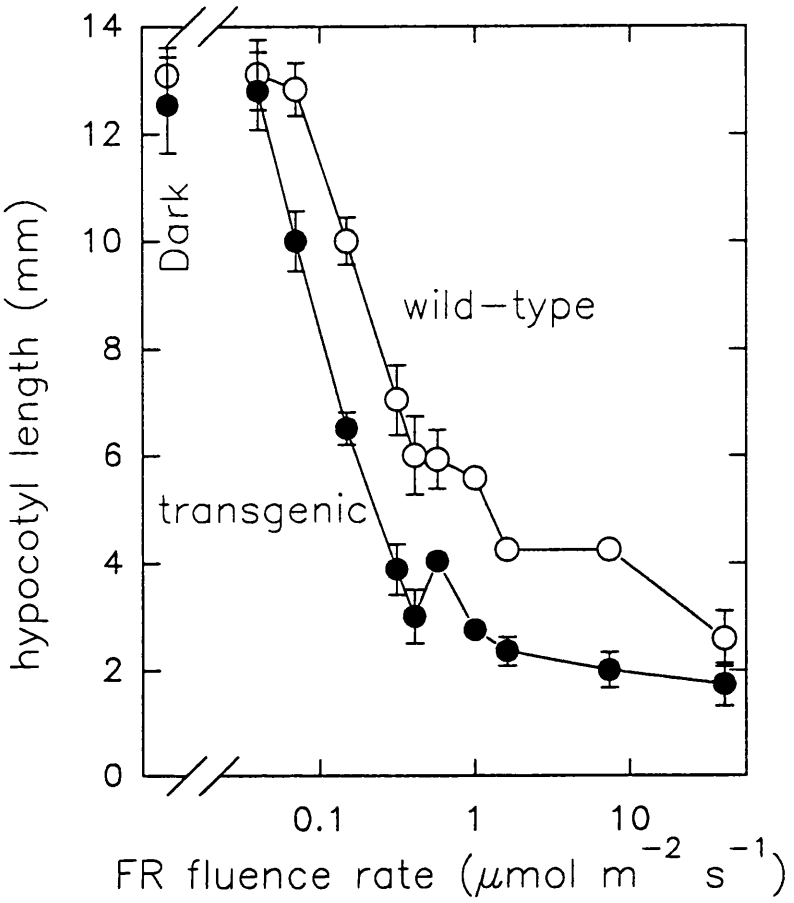


Figure 3.4 Fluence-response relationship of inhibition of hypocotyi-elongation in etiolated seedlings of wiid-type (WT) (*open circles*) and transgenic (*solid circles*) tobacco exposed to continuous far-red radiation (FR). Seedlings were grown on 1% (w/v) agar for 3 days in darkness and then exposed for a further 7 days to FR before measurement of hypocotyl lengths. Values are the mean ± SE of 45 seedlings.

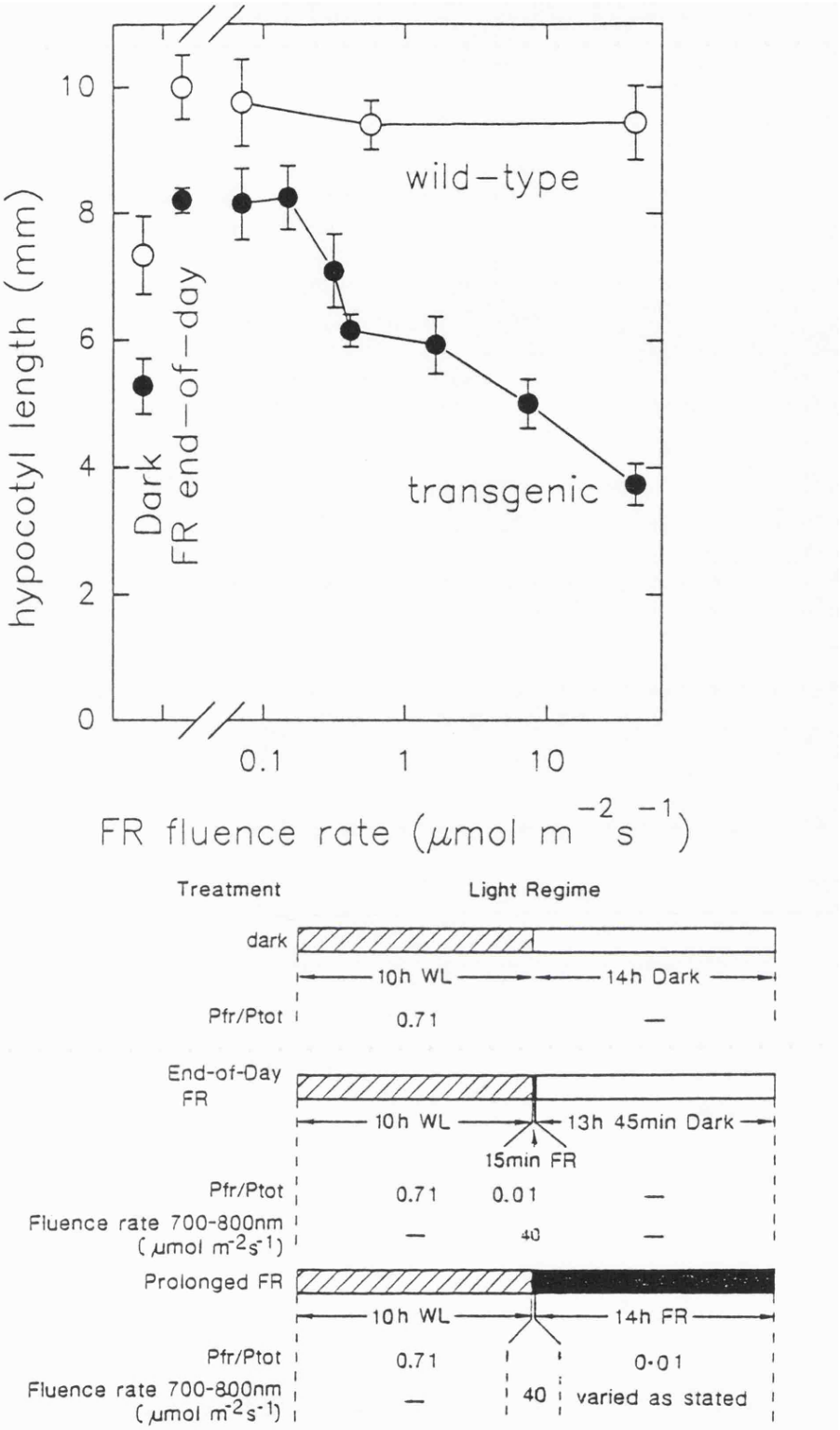
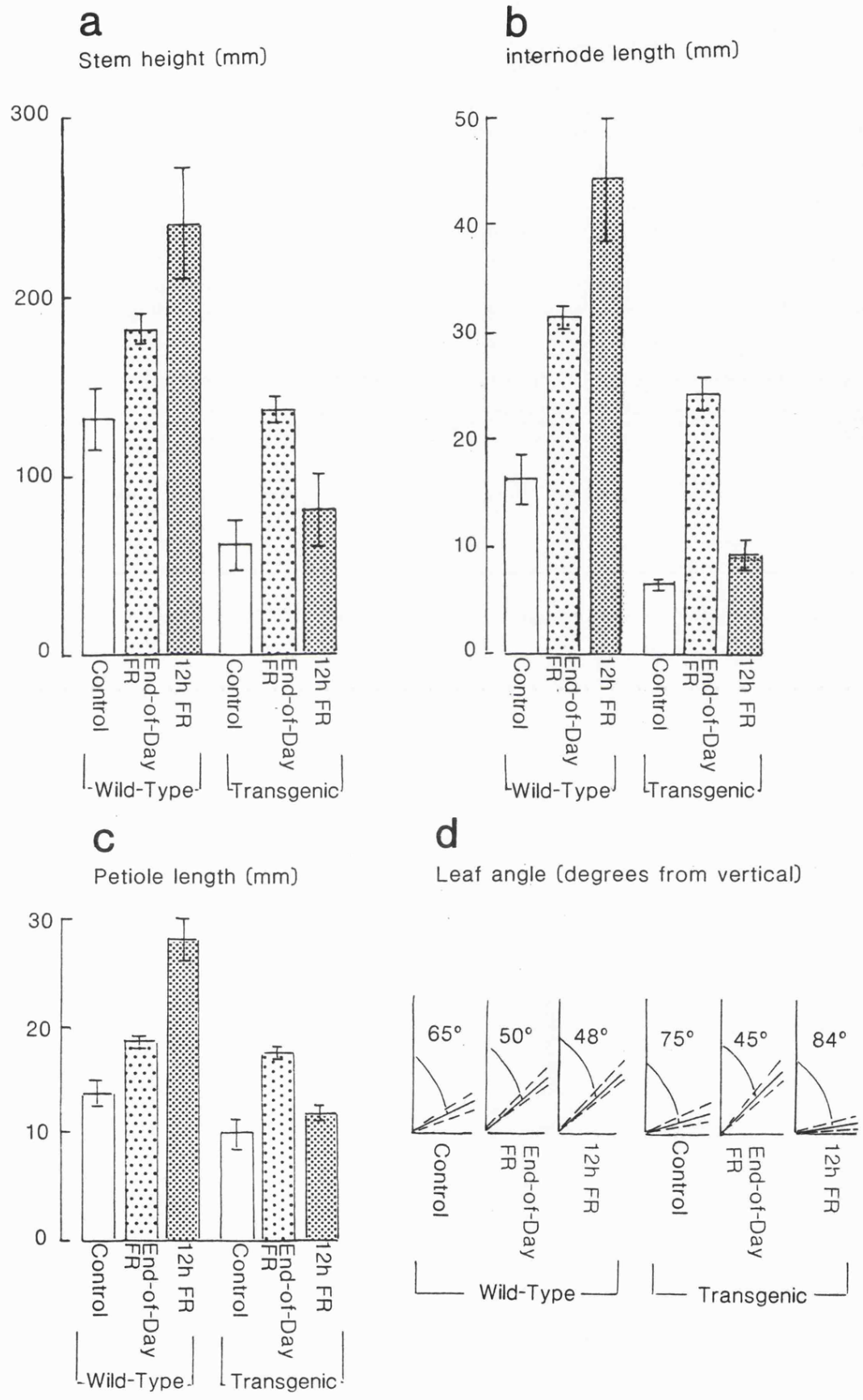


Figure 3.5 Effect of end-of-day far-red (FR) and prolonged FR on hypocotyl elongation in light-grown seedlings of wild-type (WT) (*open circles*) and transgenic (*solid circles*) tobacco. Seedlings were grown on potting compost for 5 days under continuous white light (WL) (PAR = 130 μmol m^{-2} s^{-1}) and then subjected to four successive day/night cycles. The day consisted of 10h WL; night treatments consisted of either: 14 h darkness ("dark"); 15 min FR followed by 13 h 45 min darkness ("end-of-day"); or 14 h continuous FR. Fluence rate response curves are shown for the prolonged 14 h FR treatment. Values are the mean ± SE of 45 seedlings. A schematic representation of the light regimes is shown above.

Figure 3.6 Effect of an end-of-day far-red (FR) pulse or prolonged delivery of FR on (**a**) stem height, (**b**) internode length (**c**) petiole length and (**d**) the angle of leaf erectness in wild-type and transgenic tobacco plants. All plants received a 12 hour day of white light (WL) (R:FR = 6.8; PAR = 75 μmol m^{-2} s^{-1}). Control plants entered the 12 hour dark period without further treatment. The FR end-of-day treatment consisted of a 15 minute pulse (10^5 μmol m^{-2}) immediately following the WL, followed by 11 h 45 min darkness. The prolonged treatment consisted of 12 hours continuous FR (700-800 nm = 100 μmol m^{-2} s^{-1}) throughout the night period. Plants were measured after 14 consecutive days of treatment. All values are the mean ± SE of 30 plants.


a
Stem height (mm)
300
200
100
0
Control
End-of-Day FR
12h FR
Wild-Type
Transgenic
b
internode length (mm)
50
40
30
20
10
0
c
Petiole length (mm)
d
Leaf angle (degrees from vertical)
65°
50°
48°
75°
45°
84°

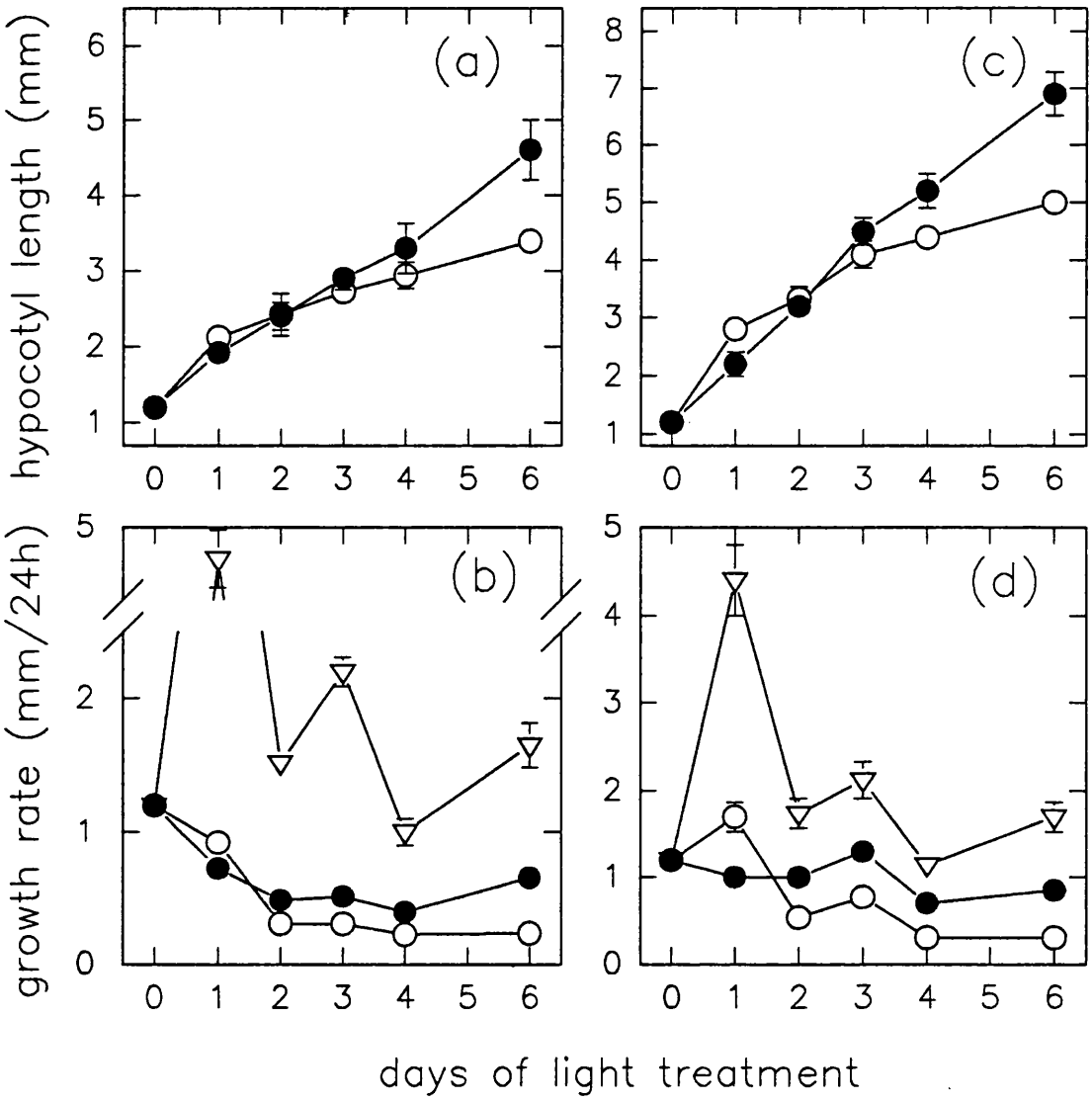


Figure 3.7 (**a**, **c**) hypocotyi growth of seedlings of wild-type (WT) tobacco treated with a solution of 0.01% Tween-20 (**a**) or 100μM gibberellin$_3$ in 0.01% Tween-20 (**c**) under continuous irradiation by white light (WL) (*open circles*) or white light with supplementary far-red (WL+FR) (*solid circles*). Seedlings had been grown for 3 days in darkness prior to transfer to the respective light environments at day 0.

(**b**, **d**) the change in hypocotyl growth rate (averaged over the preceeding 24 h period) in etiolated seedlings of WT tobacco treated with a solution of 0.01% Tween-20 (**b**) or 100μM gibberellin in 0.01% Tween-20 (**d**) as the time of light treatment progressed under WL (*open circles*), WL+FR (*solid circles*) or continued darkness (*open triangles*).

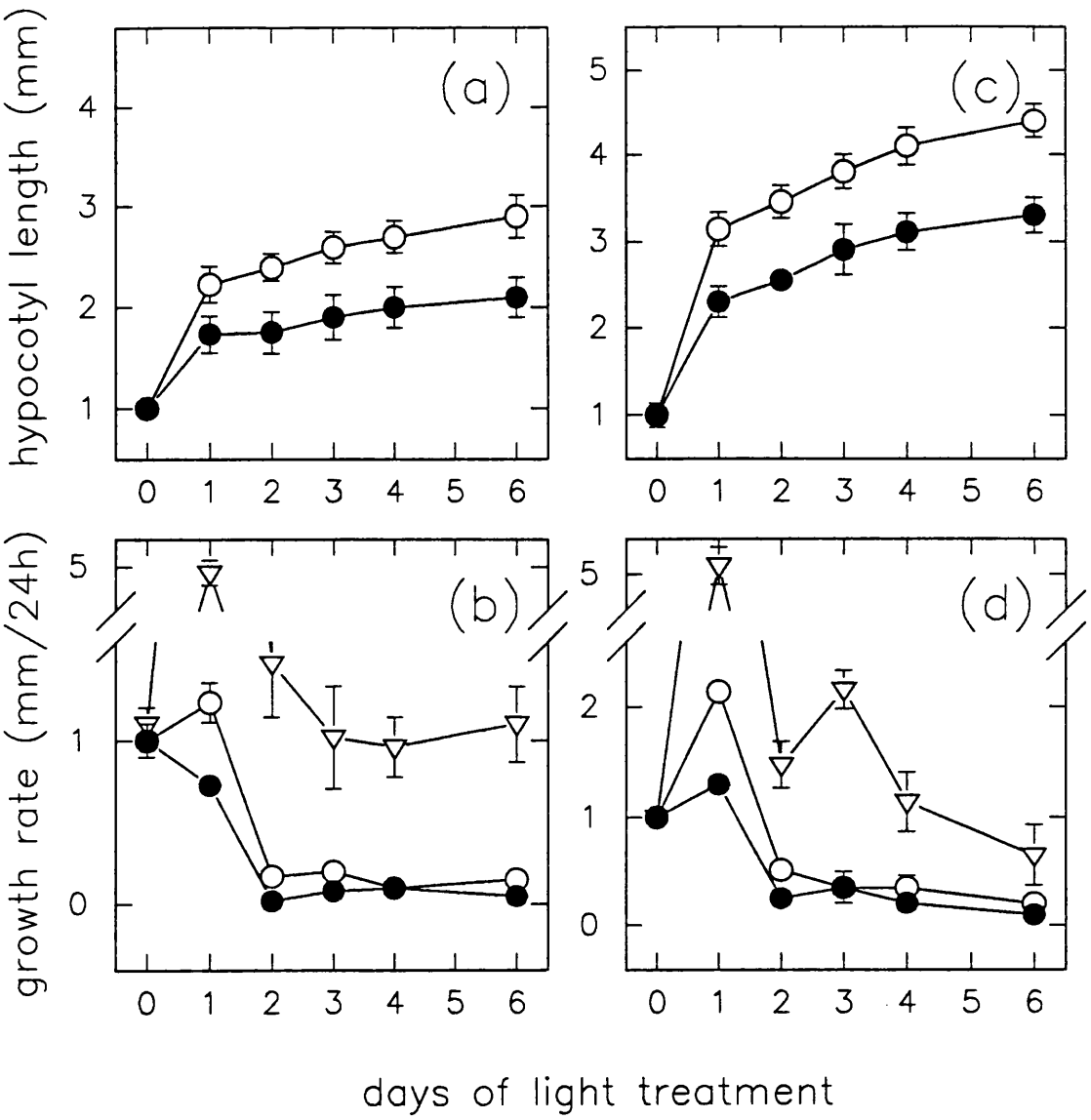


Figure 3.8 (**a**, **c**) Hypocotyl growth of seedlings of transgenic tobacco treated with a solution of 0.01% Tween-20 (**a**) or 100μM gibberellin$_3$ in 0.01% Tween-20 (**c**) under continuous irradiation by white light (WL) (*open circles*) or white light with supplementary far-red (WL+FR) (*solid circles*). Seedlings had been grown for 3 days in darkness prior to transfer to the respective light environments at day 0.

(**b**, **d**) The change in hypocotyl growth rate (averaged over the preceeding 24 h period) in etiolated seedlings of transgenic tobacco treated with a solution of 0.01% Tween-20 (**b**) or 100μM gibberellin in 0.01% Tween-20 (**d**) as the time of light treatment progressed under WL (*open circles*), WL+FR (*solid circles*) or continued darkness (*open triangles*).

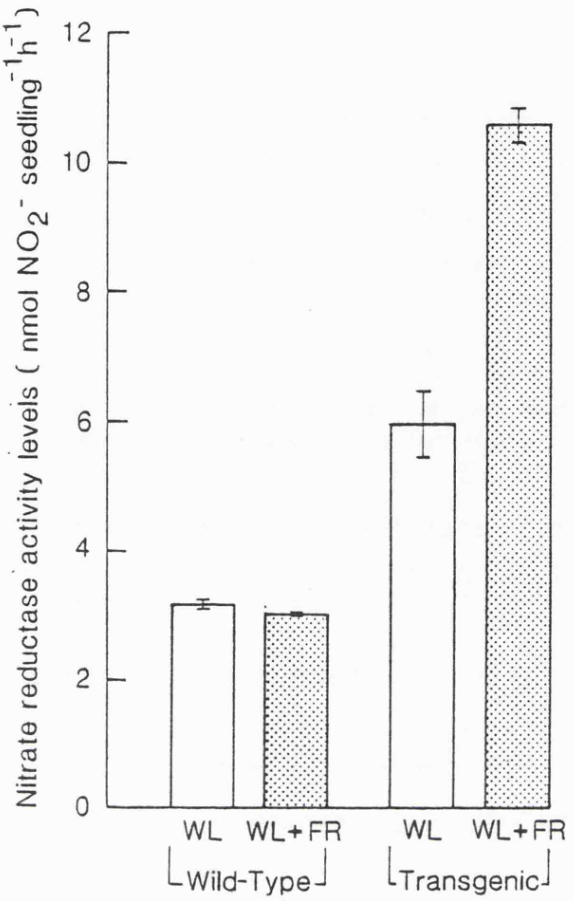
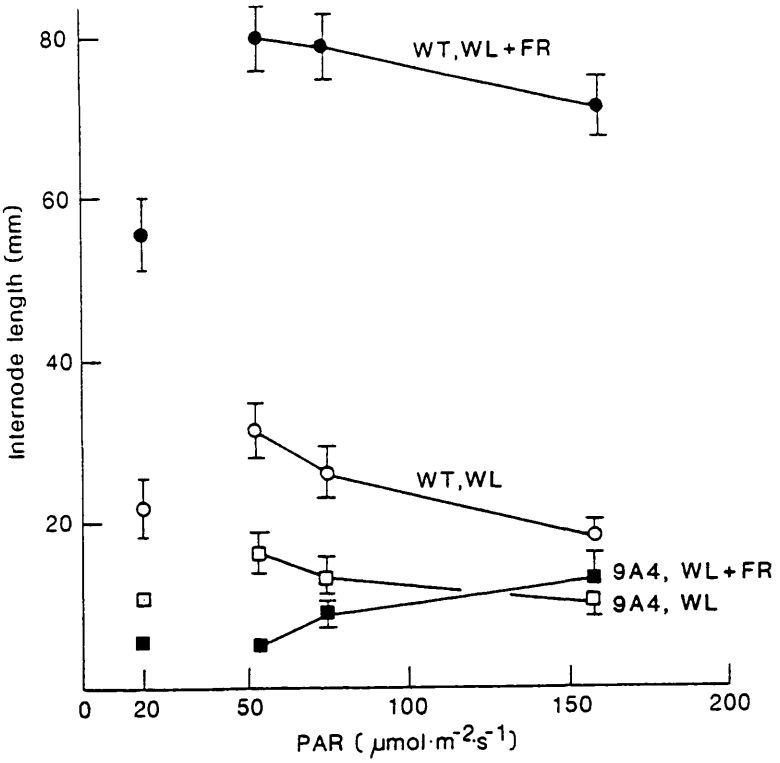


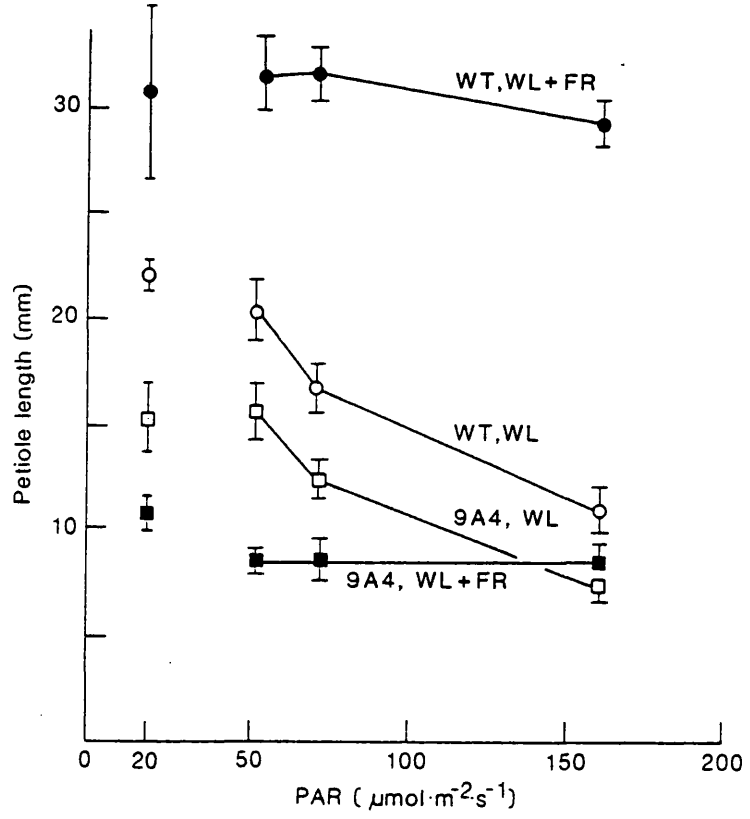
Figure 3.9 Induction of nitrate reductase activity in light-grown seedlings of wild-type (WT) and transgenic tobacco under white light (WL) and white light with added far-red radiation (WL+FR). Seedlings were grown for 2 weeks under continuous illumination by the respective light sources and the growth substrate contained 15 mM KNO_3 throughout this period. Results shown are the means ±SE of 5 replicates from a single representative trial.

Figure 3.10 (**a**) Internode and (**b**) petiole extension growth responses of wild-type (WT) (*circles*) and transgenic (*squares*) tobacco plants grown under continuous irradiation by white light (WL) (*open symbols*) or white light with added far-red radiation (WL+FR) (*solid symbols*).Two sets of irradiation cabinets were used: one set delivered fluence rates of photosynthetically active radiation (PAR) (400-700nm) of 55, 75 or 160 μmol m^{-2} s^{-1} at an R:FR ratio of either 6.8 (*open symbols*) or 0.07 (*solid symbols*); the other set of cabinets delivered a PAR value of 20 μmol m^{-2} s^{-1} at an R:FR ratio of either 5.4 (*open symbols*) or 0.27 (*solid symbols*). General conditions of growth were not uniform between the two sets of cabinets and hence direct comparison of mean values from each has no significance. (**a**) internode length was measured above the ninth leaf. (**b**) petiole length was measured from the fifth leaf pair. Measurements were made after two weeks growth in the respective treatments. Values shown are the mean ± SE of five replicates of five plants each.

(a)


Internode length (mm)
80
60
40
20
WT,WL+FR
WT,WL
9A4, WL+FR
9A4, WL
0
20
50
100
150
200
PAR (μmol·m-2·s-1)

(b)


Petiole length (mm)
30
20
10
WT,WL+FR
WT,WL
9A4, WL
9A4, WL+FR
0
20
50
100
150
200
PAR (μmol·m-2·s-1)

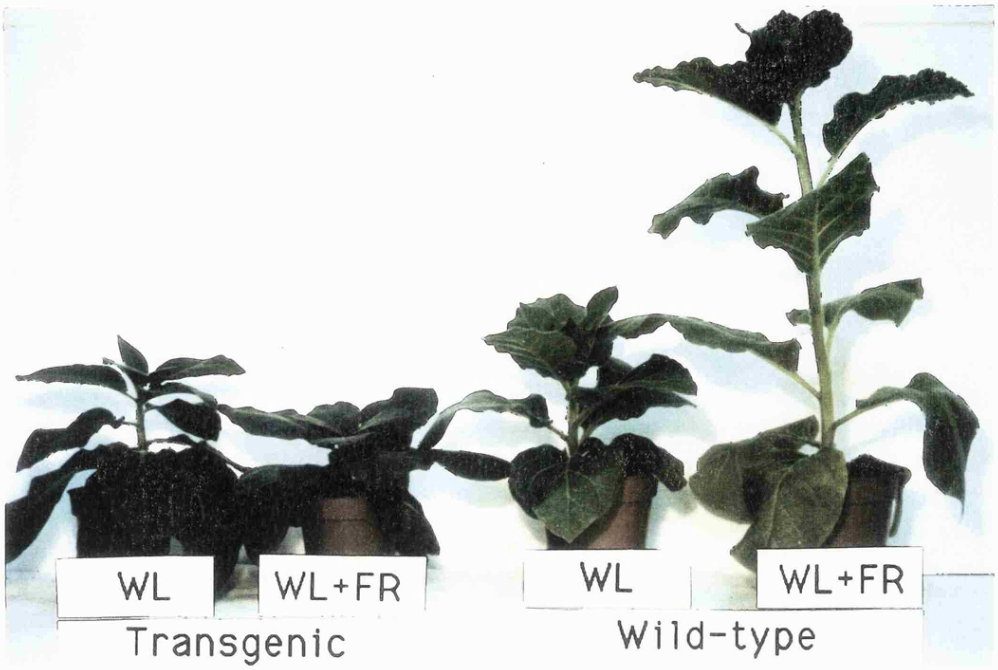


Figure 3.11 Stem extension growth of light-grown plants of wild-type and transgenic tobacco in response to a reduction in the red (R):far-red (FR) ratio. Transgenic plants constitutively expressed an introduced oat-*phyA*-cDNA. Plants were grow for 4 weeks under white light and then for a further 2 weeks under continuous irradiation by white light (WL, R:FR = 6.8) or white light enriched with far-red (WL+FR, R:FR = 0.07). Photosynthetically active radiation for both light sources was 50 μmol m^{-2} s^{-1}.

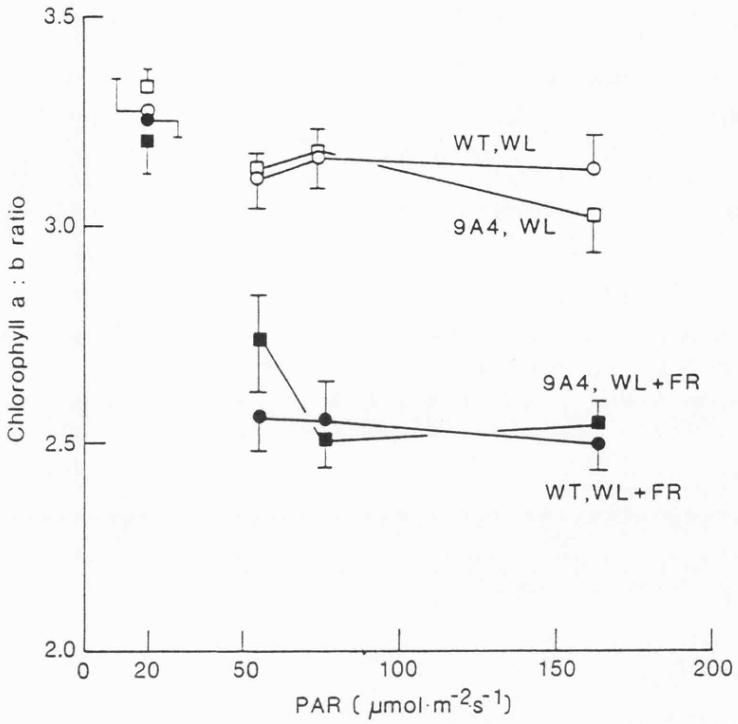


Figure 3.12 Chlorophyll a:b ratio in leaves of wild-type (WT) (*circles*) and transgenic (*squares*) tobacco grown under continuous irradiation by white light (WL) (*open symbols*) or white light with added far-red radiation (WL+FR) (*solid symbols*). Two sets of irradiation cabinets were used: one set delivered fluence rates of photosynthetically active radiation (PAR) (400-700nm) of 55, 75 or 160 μmol m^{-2} s^{-1} at an R:FR ratio of either 6.8 (*open symbols*) or 0.07 (*solid symbols*); the other set of cabinets delivered a PAR value of 20 μmol m^{-2} s^{-1} at an R:FR ratio of either 5.4 (*open symbols*) or 0.27 (*solid symbols*). Total chlorophyll was extracted from the fifth leaf pair after two weeks growth in the respective treatments, and the ratio of chlorophyll a:b calculated from the absorption coefficients. Values shown are the mean ± SE of five replicates of five plants each.

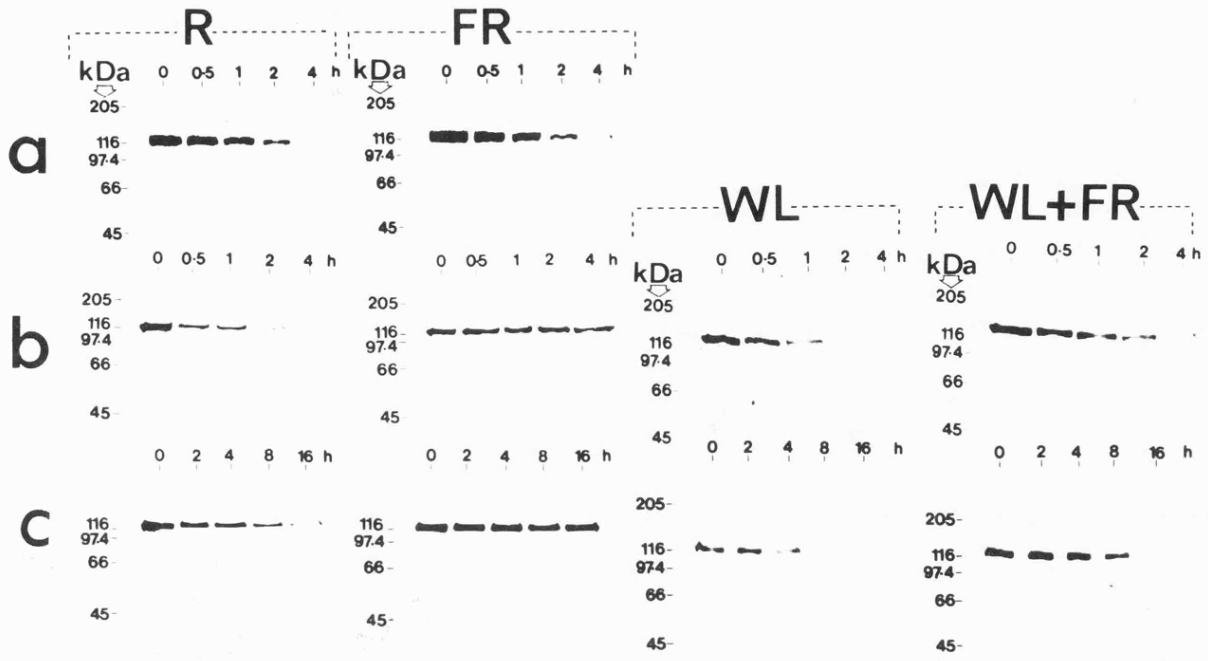


Figure 3.13 Immunoblot analyses of phytochrome decay in etiolated seedlings of (**a**) oat (5 day old), (**b**) wild-type tobacco (10 day old), and (**c**) oat-*phyA*-transformed tobacco (10 day old) under continuous irradiation from red (R) or far-red (FR) light-sources, and irradiation by white-light (WL) (R:FR = 6.8) or far-red-supplemented white-light (WL+FR) (R:FR = 0.18). At the times of irradiation shown, crude extracts were prepared and these were resolved on 8% SDS-polyacrylamide gels and electroblotted onto nitrocellulose. All lanes within each blot were loaded with equivalent levels of total protein. Phytochrome detection in the oat and transgenic tobacco samples used monoclonal antibodies which specifically recognized monocotyledonous phytochrome. Wild-type tobacco extracts were incubated with monoclonal antibodies which cross-reacted with monocotyledonous and dicotyledonous phytochromes.

Figure 3.14 Loss of total phytochrome in etiolated seedlings of (**a**) oat, (**b**) wild-type tobacco, and (**c**) oat-*PhyA*-transformed tobacco under continuous irradiation by R (*open circles*) or FR (*solid circles*) light-sources, and irradiation by white light (P_{fr}/P_{tot} = 0.71) (*open squares*) or white light with supplementary far-red (P_{fr}/P_{tot} = 0.44) (*solid squares*). The logarithmic values of the relative intensities of the immunodetected bands (shown in Figure 3.13) were plotted versus the time of irradiation, and the linear regression relationship calculated. The half-life time of degradation under each treatment was estimated from this regression line as the time taken for 50% of the original phytochrome level to be lost.

Figure 3.15 Relationship between apparent degradation-rate-constant and phytochrome photoequilibrium (P_{fr}/P_{tot}) for wild-type tobacco etiolated phytochrome (*open circles*) and the introduced oat-encoded phytochrome A in etiolated seedlings of transgenic tobacco (*solid circles*). In order to plot all data on a single scale, rate constant values shown are not relative between the wild-type and transgenic lines. Rate constants were calculated from the data in Figure 3.14 as the gradient of the log-linear relationship. P_{fr}/P_{tot} under each light-source was estimated as described in *Materials and methods.*

Figure 3.14

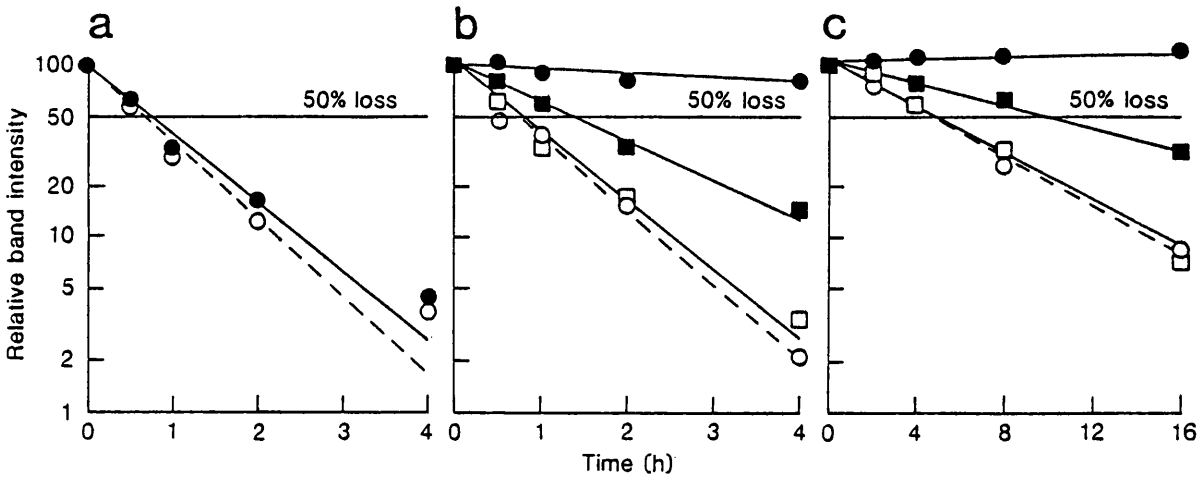


Figure 3.15

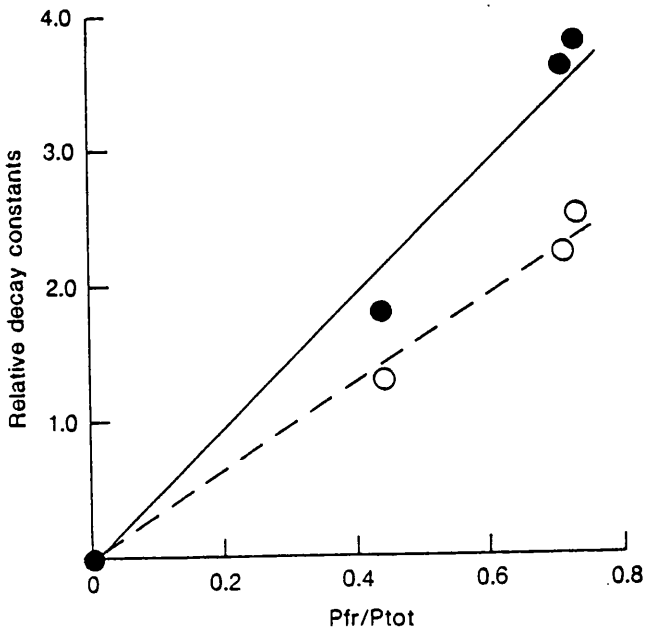


Table 3.1 Comparison of the morphology of the mature light-grown plants of wild-type (WT) *N. tabacum* and a transgenic line expressing oat-*phyA*-cDNA. Plants were grown under continuous white light at the fluence rates of photosynthetically active radiation (PAR) indicated. Measurements were taken when the plants were approximately seven weeks old. All leaf measurements were taken from the fifth leaf pair which was fully expanded at the time of measurement. Values shown are the mean (± SE) of five replicates of five plants each.

Character	PAR (μmol m^{-2} s^{-1})	Genotype			
		WT		Transgenic	
Stem height (mm)	160	143.1	(9.82)	88.2	(2.34)
	75	186.7	(10.33)	91.1	(8.28)
	55	205.5	(6.20)	88.4	(9.11)
Leaf area (cm^2)	160	67.2	(4.54)	44.2	(2.51)
	75	79.1	(5.32)	56.4	(2.39)
	55	75.9	(4.97)	56.8	(3.43)
Total leaf fr. wt. (mg)	160	1977.8	(136.25)	1465.0	(99.77)
	75	2196.6	(92.57)	1703.3	(157.25)
	55	1979.8	(143.10)	1651.0	(109.97)
Leaf fr. wt. density (mg cm^{-2})	160	29.2	(0.99)	32.3	(0.77)
	75	27.1	(0.93)	30.2	(0.78)
	55	26.0	(1.11)	29.6	(0.74)
Total leaf dry wt. (mg)	160	300.1	(24.51)	226.8	(17.47)
	75	204.1	(11.24)	136.7	(10.68)
	55	139.7	(9.59)	100.4	(5.69)
Leaf dry wt. density (mg cm^{-2})	160	4.5	(0.25)	5.0	(0.25)
	75	2.6	(0.13)	2.4	(0.16)
	55	1.9	(0.08)	1.7	(0.08)
Total chlorophyll (μg cm^{-2})	160	51.6	(3.05)	71.5	(3.11)
	75	72.0	(2.57)	83.0	(3.01)
	55	61.1	(2.21)	70.0	(2.78)

Chapter 4

A comparison of three independent lines of transgenic tobacco expressing oat-*phyA*-cDNA.

Introduction

Transgenic tobacco plants which constitutively express oat-*phyA*-cDNA have been reported to display aberrant photoresponses namely (a) an increased sensitivity of the etiolated seedlings to far-red (FR)-mediated growth-inhibition (b) persistence of FR-mediated growth-inhibition in the light-adapted seedlings (c) an inverted shade-avoidance-response of the light-grown plants to reductions in R:FR ratio (Chapter 3 of this thesis; McCormac *et al.,* 1991). These results were discussed with a view to attributing the physiological effects of the transgenic genotype to the action of the heterologous phytochrome A. However, since the results were obtained from the product of an isolated transformation event (Keller *et al.,* 1989), the possibility must also be considered that an indirect effect of the introduced cDNA (e.g. causing an insertional mutation in the host plant genome) was responsible for the change from wild-type physiology. The ability to reproduce these results between a number of transgenic lines created from independent transformation events would favour the conclusion of a direct role for the product of the introduced cDNA in mediating the transgenic phenotype. A quantitative relationship between the levels of response in the individual transgenic lines and the respective levels of expression of the transgene would also provide strong support for this conclusion. One interpretation of the impaired response to low R:FR ratio in the transgenic plants proposed that it was simply a function of the high levels of total phytochrome (McCormac *et al.,* 1991). Transgenic lines expressing the same

phytochrome-encoding cDNA, but to a lower level than in this original line, could provide a means to test such an idea.

In order to address the questions discussed above, additional independent transgenic tobacco lines expressing oat-*phyA*-cDNA have been generated and their photophysiological characteristics are compared here both with wild-type and with the other previously characterised transgenic line.

Materials and methods

Plasmid constructions

A copy of the uninterrupted coding sequence of the oat-*phyA* gene was kindly supplied by P.H. Quail (U.C., Berkeley, CA., USA.) as a full length cDNA clone contained within the pUC19-based plasmid, pFY122 (Boylan and Quail, 1989). pFY122 was digested with *Eco*RI, releasing the 3' end of the phytochrome cDNA, and the end was rendered blunt with the klenow fragment of DNA polymerase I. The resulting DNA was cleaved with *Bam*H1. The 3.5kbp fragment containing the full-length *phyA*-cDNA was gel purified and subcloned into pROK2, which had been digested with *Bam*H1 and *Sma*1, to generate plasmid pRFY1. pROK2 is a binary system cloning vector based on pBin19 (Bevan, 1984) and contains a polylinker flanked 5' by the promoter region and 3' by the downstream polyadenylation signals from the CaMV 35S transcript, a wide host range RK2 origin of replication, a bacterial selection marker and a plant kanamycin-resistance coding sequence under the control of the CaMV 35S promoter and 3' sequences. In pRFY1 the complete phytochrome coding-sequence was operably linked to the CaMV 35S pomoter and 3' polyadenylation signals.

pRFY1 was mobilized from *Escherichia coli* strain XL1-blue into *Agrobacterium tumefaciens* strain 2260, using pRK2013 in *E. coli* strain HB101 for plasmid mobilization in a triparental conjugation (Ditta *et al.*, 1980). The *Agrobacterium* acceptor strain contained a chromosomally-located rifampicin resistance gene. Conjugates were selected, after 24 h of growth at 28 ^{o}C on non-selective medium, by plating onto LB agar (10 g l^{-1} Tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl, 12 g l^{-1} agar) containing 100 µg ml^{-1} kanamycin, 100 µg ml^{-1} ampicillin, 200 µg ml^{-1} rifampicin. Restriction-enzyme digestion coupled with Southern blot analysis of total DNA from conjugate *Agrobacterium* colonies was used to check that the *phyA* cDNA was maintained in

the binary vector and also to confirm its orientation. Total DNA was extracted from a 2 day old culture of a kanamycin-resistant colony of *Agrobacterium* and digested with either *Sac*1 or *Xba*1. Fragments were separated on an 0.8% agarose gel and blotted onto a nylon membrane (Hybond-N, Hybond, Amersham, UK.). The resulting blot was probed with a full-length radiolabelled (^{32}P) cDNA probe (obtained as a 3.5 kbp fragment from *Eco*R1/*Bam*H1 digestion of pFY122), and was washed under high stringency conditions: 0.5X SSC, 0.1% (w/v) sodium dodecyl sulphate (SDS) (20X SSC = 3.0M NaCl, 0.3M NaCitrate pH 7.0). [Blotting and hybridisation protocols were followed from "Membrane transfer and detection methods", Amersham International plc., Amersham, U.K.]. The pattern of restriction fragments which hybridised with the probe was compared with the restriction maps of the oat-*phyA*-CDNA (Hershey *et al.*, 1985) and the *Agrobacterium* vector; this showed the digestion pattern to be consistent with the cDNA having the correct orientation, with respect to the vector-linked promoter, for sense expression (Fig. 4.1).

Transformation of plants

Constructions were mobilized into the plant genome via *A. tumefaciens* infection of *N. tabacum* (cv. xanthi) leaf discs, in a method after Horsch *et al.*, (1985). Leaves of mature tobacco plants were surface sterilised in a 10 % (v/v) solution of domestic bleach for 15 min. Cut leaf discs were soaked in a 1/50 dilution of an overnight culture of the *Agrobacterium* strain containing the oat-*phyA*-cDNA vector (pRFY1). Infected leaf discs were placed onto plates of MSD4 X2 medium [MS salts (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 0.1 mg l^{-1} naphthaleneacetic acid, 1.0 mg l^{-1} 6-benzylaminopurine] and incubated under a low light intensity at 25^{o}C for 2 days. Subsequently, leaf pieces were transferred to fresh plates of MSD4 X2 containing 100 mg l^{-1} kanamycin and 400 mg l^{-1} Augmentin, and were incubated further until shoot regeneration occurred. When shoots reached approx. 1 cm length they were excised and

inserted into MS medium (supplemented with 30 g l^{-1} sucrose, 7 g l^{-1} agar) containing 100 mg l^{-1} kanamycin. Shoots in which root development was resistant to kanamycin were transplanted in to potting compost and grown under continuous irradiation from white fluorescent lights.

Plant RNA extraction

Potted transformed plants were grown to approximately the 10-leaf stage and 100 mg of leaf tissue was harvested and frozen in liquid nitrogen. Total RNA was extracted in a method after Verwoerd *et al.* (1989). The tissue was ground and added to 500 µl of extraction buffer (50 % v/v phenol, 0.05M LiCl, 50 mM Tris.HCl pH 8.0, 5mM EDTA, 0.5% w/v SDS) heated to 80^{o}C, and this was vortexed for 30 seconds. A half volume of chloroform: isoamylalcohol (24:1) was added, vortexed, and the top aqueous phase collected by centrifugation. An equivalent volume of 4M LiCl was added, and RNA was precipitated overnight at -20 ^{o}C and pelleted by centrifugation. The pellet was cleaned by dissolving in water followed by re-precipitation with 0.1 vol. 3M sodium acetate (pH 6.0) and 2 vols. ethanol. The pellet was dried at room temperature under vacuum and dissolved in 12 µl water.

Dot-blot analysis of RNA

Dot-blot analysis of total RNA was used to screen primary transformants for expression of the oat-*phyA*-cDNA. RNA samples were added to 3 vols. of formamide: formaldehyde (37% solution): 10x MOPS buffer (25:8:5) and heated at 65^{o}C for 5 min; (10x MOPS buffer = 0.2 M 3-[N-Morpholino] propane-sulfonic acid, 0.05M sodium acetate pH 7, 0.01M Na_2 EDTA). The samples were dotted onto Hybond-N and, when dried, cross-linked over a uv light-source for 2 min. Blots were probed with a radiolabelled (^{32}P) 650bp fragment

from the 5' end of oat-*phyA*-cDNA obtained by a *Sac*1/ *Nco*1 digestion of pFY122. Hybridisation conditions were 50% formamide in 5X SSPE containing 5X Denhardts, 0.5% SDS at 45^{o}C (20X SSPE = 3.6 M NaCl, 0.2M NaPhosphate pH 7.7, 0.02M Na_2EDTA; 50X Denhardts = 1% w/v Ficoll, 1% w/v polyvinylpyrrolidone, 1% w/v bovine serum albumin). Wash conditions were 1X SSPE, 0.1% SDS at 45^{o}C. [Blotting and hybridisation protocols were followed from "Membrane transfer and detection methods", Amersham International plc., Amersham, U.K.].

RNA gel analysis

Northern blot analysis was used to test the progeny of transformed plants for expression of the introduced oat-*phyA*-cDNA and also to confirm that the mRNA product of the transgene was full-length compared to the native oat transcript. RNA samples (12 µl) were added to 25 µl formamide, 5 µl 10x MOPS buffer and 8 µl formaldehyde. The samples were heated at 65^{o}C for 5 min, chilled on ice and added to 5 µl RNA sample buffer (50% v/v glycerol containing 0.1 mg ml^{-1} bromophenol blue). Samples of total RNA were run on a 1.2% agarose-formaldehyde gel. The resulting blot was probed with the *Sac*1/*Nco*1 650bp fragment of oat-*phyA*-cDNA. Hybridisation and wash conditions were as described above for dot-blots.

Immunoblot analysis

The accumulation of the mature polypeptide product of the oat-*phyA*-cDNA in transgenic tobacco plants was assessed by immunoblot analysis using anti-phytochrome monoclonal antibodies. Seeds from transformed plants which were no longer segregating for kanamycin resistance were sown onto a layer of filter paper overlaying 1% (w/v) aqueous agar in petri dishes. Germination was induced by a white light pulse to the seeds 1

day after sowing and the seeds were left to germinate in complete darkness. When the etiolated seedlings were 10 days old, phytochrome extractions were performed as described in chapter 3 of this thesis (*Materials and methods: Immunochemical analysis of phytochrome levels*"). Extracts were separated on an 8% SDS-polyacrylamide gel and electroblotted onto nitrocellulose (Hybond-C, Amersham, UK.). Blots were probed with monoclonal antibodies which were either (a) cross-reactive with both native etiolated tobacco and oat-encoded phytochrome species, or (b) specific for oat-encoded phytochrome. Relative band intensities on the developed blots were measured using a GS Hoefer densitometer (Hoefer Scientific Instruments, San Fransisco, CA., USA).

Plant material

Three homozygous transgenic lines of tobacco expressing oat-*phyA*-cDNA were used. Two lines (referrred to as Y_{10} and Y_{12}) were the result of transformations described above and were characterised with respect to the levels of transgene expression in the *Results and discussion* section of this chapter. A third transgenic line, which also expressed oat-*phyA*-cDNA under control of the CaMV 35S promoter, was developed by Keller *et al.* (1989) and contained 5-fold wild-type levels of total phytochrome in the etiolated seedlings (Cherry *et al.,* 1991); this line is referred to as 9A4 in the following text. The isogenic wild-type (WT) line was *N. tabacum* cv. xanthi. A heterozygous line was formed from a cross of 9A4 with a tobacco line containing a WT complement of phytochrome genes; plants of 9A4 were de-masculated and pollinated by plants of *N. tabacum* var. SR1 which were homozygous for a transgenic GUS marker gene. The resulting F1 progeny were checked for heterozygosity by screening for the GUS marker using standard histochemical techniques (Pearse, 1972). It was assumed that the heterozygote had an intermediate level of expression of the heterologous phytochrome gene (i.e. 2.5-fold WT etiolated levels). A corresponding F1 line of WT cv. xanthi crossed with the GUS-transformed SR1 line was

also produced and was assumed to contain single-fold levels of WT etiolated phytochrome.

Light-sources and plant measurements

Broad-band red (R) and far-red (FR) light sources were provided by a construction of lights and filters which are described in detail in Chapter 3 of this thesis (*Materials and methods; Light sources*). The construction of the cabinets providing the R:FR ratio treatments has also been detailed in Chapter 3 (*Materials and methods; Light sources: "low PAR cabinets").* Growth measurements were recorded in the manner described in Chapter 3 for seedling hypocotyl growth (*Materials and methods: "Inhibition of seedling hypocotyl growth under various fluence rates of FR*") and mature plant morphology (*Materials and methods: Photomorphogenetic responses of mature light-grown plants*).

Results and discussion

Construction of transgenic lines

The structural gene of oat-*phyA*, fused to the constitutive CaMV 35S promoter, was introduced into tobacco using *Agrobacterium*-mediated transformation of leaf discs. Kanamycin-resistant shoots were grown under continuous white light. Expression of the oat cDNA in these primary transformants was assessed by hybridisation of a radiolabelled 650bp oat-*phyA*-cDNA probe with dot blots of total RNA extracts. The oat-cDNA probe preferentially hybridised with extracts from a transgenic tobacco line known to constitutively express oat-*phyA*-cDNA (9A4, Cherry *et al.*, 1991) as compared to extracts from WT tobacco (Fig. 4.2a) and thus confirmed the probe to be specific for the heterologous mRNA. Two transformants were identified which hybridised strongly with the probe (Fig. 4.2a). Plants of the two positive transformants, and one kanamycin-resistant plant not expressing the oat cDNA, were allowed to self-fertilize. The progeny segregated for kanamycin resistance and antibiotic-resistant seedlings were grown-on in potting compost under continuous white light. These plants were tested for expression of oat-*phyA*-cDNA by RNA gel analysis of total RNA from leaf extracts using the 650bp oat-cDNA probe (Fig. 4.2b). Gel analysis showed that the band recognised by the oat-*phyA*-cDNA probe in the RNA extracts of the transformed tobaccos had approximately the same mobility as that in extracts of etiolated oat seedlings (Fig. 4.2b). This suggested that the heterologous mRNA corresponded to the same molecular size as the native mRNA, i.e. it was full length. Plants corresponding to extracts with a strongly hybridising band were continually self-fertilised and selected on kanamycin until the population no longer showed segregation.

Immunoblot analysis was used to check that the transformed plants expressing the mRNA product of the introduced oat-*phyA*-cDNA accumulated the full-length oat-encoded

protein. Blots of protein extracts from etiolated seedlings of the homozygous lines were probed with 2 different species of monoclonal antibody raised against etiolated oat phytochrome. One antibody species recognised both dicot. and monocot. forms of phytochrome while the other was specific for the oat-encoded protein. Figure 4.3 shows the result of an immunoblot of extracts of the transgenic tobacco lines. A single band was detected in the extracts of WT tobacco seedlings, approximating to a molecular mass of 120 kDa. This band was not detected in blots incubated with the oat-specific monoclonal antibody. In extracts of the transgenic tobacco lines expressing oat-*phyA*-mRNA (Y_{10} and Y_{12}) two phytochrome species were apparent, only the larger of which was detected by the oat-phytochrome-specific monoclonal. Seedlings of a transgenic line which did not express the heterologous mRNA (Y_5) contained only the WT species of phytochrome. From the relative intensities of the two immunodetected bands in the transgenic plant extracts it was estimated that the Y_{10} and Y_{12} transgenic plants contained approx. 2.5-fold and 3-fold levels, respectively, of total WT etiolated phytochrome.

Response of etiolated seedlings to far-red

WT tobacco and four independent transgenic lines containing varying levels of total etiolated phytochrome all showed strong levels of inhibition of hypocotyl elongation growth under continuous FR, and this response was dependent on the fluence rate of the light received. At the lowest (700-800nm = 0.1 μmol m^{-2} s^{-1}) and highest (700-800nm = 40 μmol m^{-2} s^{-1}) of the fluence rates tested there was little measurable difference in growth rate between the five lines (Fig. 4.4a). Under intermediate fluence rates of FR (1.62 - 0.55 μmol m^{-2} s^{-1}) the three transgenic lines which expressed introduced oat-*phyA*-cDNA (9A4, Y_{12} and Y_{10}) were significantly shorter than the WT seedlings; the growth-response of a transgenic line (Y_5), which although kanamycin resistant did

not express the product of the mRNA or protein product of the oat cDNA to a detectable level, was not significantly different from WT (Fig. 4.4a). Under the lower range of fluence rates (0.1-0.4 μmol m^{-2} s^{-1}), however, only the line which expressed the transgene to the highest level (9A4) was significantly inhibited relative to WT (Fig. 4.4a). Data from the five homozygous genotypes shown in Fig. 4.4a, and also from a heterozygous line formed by a F1 cross of the 9A4 transgenic line with a tobacco possessing a WT complement of phytochrome genes, was plotted as a function of the estimated relative levels of total phytochrome (P_{tot}). At a given fluence rate of FR the degree of inhibition of the dark growth-rate demonstrated a linear relationship with the estimated values of P_{tot} in the etiolated seedlings (Fig. 4.4b). This result is consistent with a graded response to continuous FR mediated by phytochrome A. Fairly small increases in levels of phytochrome A were therefore able to produce a significantly dwarfed phenotype under continuous FR radiation. The observtaion of a graded response to [P_{tot}] in an HIR under broad-band FR is not readily interpreted in terms of the Hartmann dichromatic experiments which related response under continuous FR to an optimum P_{fr}/P_{tot} (Hartmann, 1966). Subjecting *phyA*-overexpressing plants to a similar arrangement of dual monochromatic light-sources, however, may provide a test for this model.

Response of etiolated seedlings to red

Hypocotyl growth of etiolated seedlings was inhibited under continuous R light and this appeared dependent on fluence rate. Of the four transgenic lines, only the highest level expresser of the heterologous phytochrome (9A4) demonstrated an increased sensitivity to R compared to WT (Fig. 4.5a). In relation to P_{tot} levels (as measured in the etiolated seedlings) sensitivity to R displayed an apparent threshold of responsiveness (Fig. 4.5b),

in contrast to the continuum of response seen under continuous FR light (Fig. 4.4b). Also in contrast to the FR-mediated response, relatively high levels of expression of the transgene (> 3-fold wild-type P_{tot}) were necessary in order to produce a phenotypic effect under R irradiation.

Responses of light-grown seedlings to far-red

Transgenic and WT seedlings which had been grown under continuous white light for 5 days received end-of-day FR irradiation treatments which consisted of either a 15 min pulse followed by 13 h 45 min darkness, or continuous FR throughout the 14 h night-period; these FR-treatments were delivered over four successive days and during the intermittent 10 h day-periods seedlings were irradiated by white light. In the three transgenic lines expressing a constitutive population of oat phytochrome A (9A4, Y_{12}, Y_{10}), elongation growth of seedings under the continuous 14 h FR-irradiation treatment was inhibited relative to seedlings which received an end-of-day FR pulse (Fig. 4.6a). Light-grown seedlings of the lines which contained WT levels of phytochrome (WT, Y_5) showed no significant effect of continuous FR compared to 15 min end-of-day treatments. The growth-inhibition response of light-grown seedlings under continuous FR appeared to be saturated by relatively low levels of expression of the heterologous phytochrome A (as measured from the levels accumulated in the etiolated seedlings) (Fig. 4.6b).

Response of light-grown plants to R:FR ratio

The elongation growth response of plants growing under a low R:FR quantum ratio is well documented in many shade-avoiding species including tobacco. Transgenic tobacco plants constitutively expressing oat phytochrome A have already been reported to exhibit an impaired response to reductions in R:FR ratio and, in fact, FR-enriched radiation served

to inhibit elongation growth of the transgenic plants (McCormac *et al.*, 1991). Plants of the transgenic lines Y_{10} and Y_{12} displayed similarly inverted shade-avoidance-responses under continuous irradiation by the FR-supplemented light-source in both the light-adapted seedlings (Fig. 4.7a) and mature green plants (Fig. 4.7b, c; Fig. 4.8). Levels of transgene expression which produced only a 2-3 fold increase in total levels of etiolated phytochrome were therefore sufficient to cause a FR-mediated growth-inhibition in the light-grown plants.

The mature light-grown plants of the 9A4 transgenic line displayed a dwarfed phenotype relative to WT (Fig. 4.9a; Cherry *et al.*, 1991; McCormac *et al.*, 1991). However in the Y_{10} and Y_{12} lines no significant effect of the transgenic genotype on green-plant morphology was seen in plants growing under continuous white light (WL) (Fig. 4.7b, c, 4.9a). Under FR-supplemented WL (WL+FR), plants of all three oat-*phyA*-expressing transgenic lines were markedly dwarfed compared to WT, (Fig. 4.7a, b, c, 4.9b) and the elongation growth responses of the mature plants under low R:FR (Fig. 4.7b, c) suggested a graded response with respect to levels of expression of the heterologous phytochrome A. Under irradiation by light of high R:FR ratio, therefore, high levels of expression of the heterologous phytochrome were necessary in order to produce a phenotypic effect. In contrast, the phenotype of plants growing under continuous irradiation from a low R:FR ratio light-source was sensitive to even low levels of oat-*phyA* expression.

Conclusions

All three transgenic lines of tobacco expressing introduced oat-*phyA*-cDNA exhibited impaired shade-avoidance-responses to a reduction in the R:FR quantum ratio of continuous irradiation. The transgenic lines also demonstrated a persistent FR-mediated inhibition of growth in the light-grown plants under prolonged end-of-day FR-irradiations and in response to supplementary FR-irradiation of background white light. In contrast, lines containing WT levels of phytochrome displayed typical shade-avoidance extension-growth under FR. The nature of the growth-response to irradiations by FR and low R:FR ratio light-sources therefore segregated consistently between WT and transgenic lines according to expression of introduced oat-*phyA*-cDNA. In the etiolated seedlings of the WT and transgenic lines, the sensitivity of the growth-response to low fluence rates of continuous FR appeared to be graded with respect to levels of expression of the introduced oat-cDNA. This result is consistent with a direct relationship of FR-mediated response with concentrations of the heterologous phytochrome A. These results add strong support for the conclusion that the photophysiological characteristics of the oat-*phyA*-transformed plants (as described in detail in Chapter 3 of this thesis) are directly mediated by the product of the transgene.

The two transgenic lines whose production has been described here (Y_{10} and Y_{12}) expressed the introduced oat-*phyA*-cDNA to a lower level than in the originally characterised "9A4" line. In these low-level expressers of the transgene, a dwarfed phenotype relative to WT plants was seen under a WL+FR source, but not under WL (or R). This result demonstrates that levels of the heterologous phytochrome which were insufficient to mediate inhibition of growth under high R:FR ratio were effective under the FR-supplemented light-source. It is therefore concluded that the impaired shade-avoidance-response of oat-*phyA*-transformed plants reflects the response to low R:FR ratio specifically mediated by the constitutive phytochrome A population, as opposed to a simple effect of high levels of total P_{fr}.

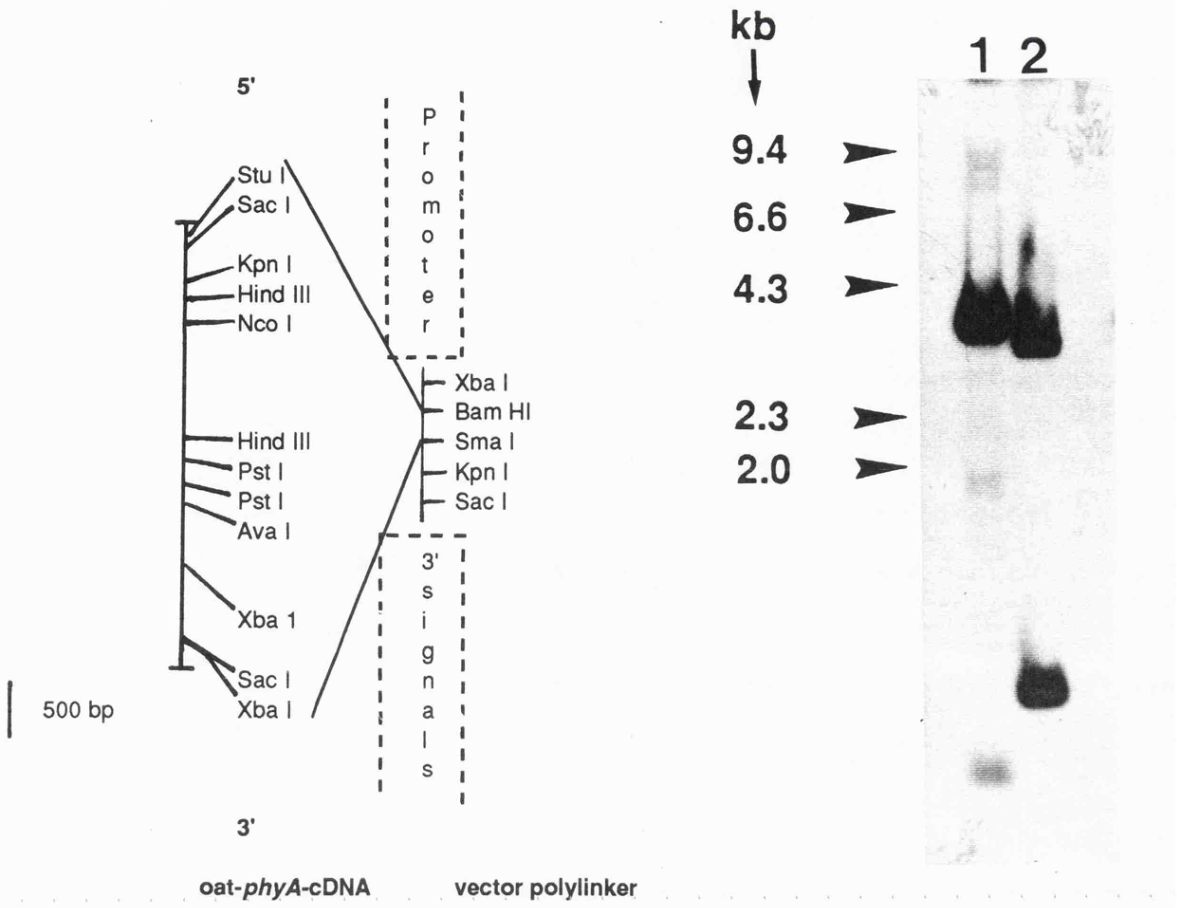
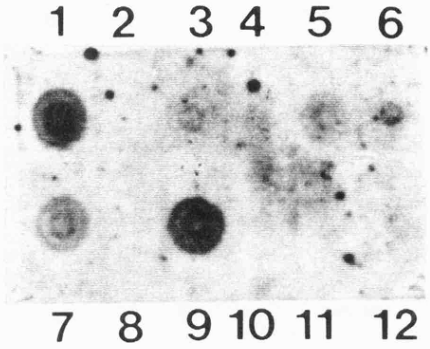


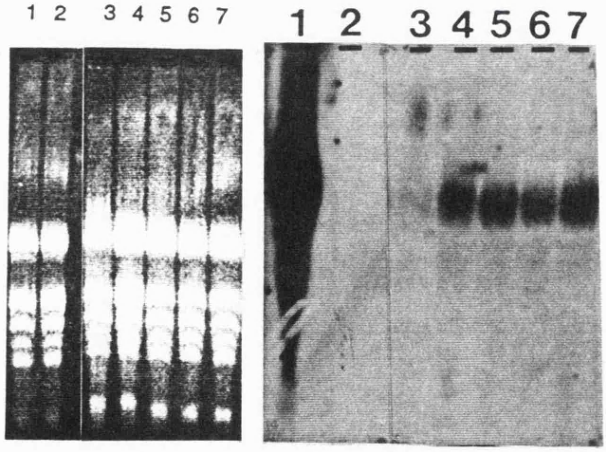
Figure 4.1 Southern blot analysis of the *Agrobacterium* strain used for transformation of tobacco plants. Total DNA extracted from the *Agrobacterium* culture was digested with *Sac*1 (lane **1**) or *Xba*1 (lane **2**) and run on an 0.8% agarose gel. Blots of the denatured DNA were hybridised with a radiolabelled probe of full length oat-*phyA*-cDNA. The resulting hybridisation pattern confirmed that the oat-*phyA*-cDNA was contained within the *Agrobacterium* binary vector in the correct orientation, with respect to the promoter and 3' signals, for transcription in th 5' --> 3' direction.



(a)

Figure 4.2 Northern analysis of transgenic tobacco plants to test for expression of oat-*phyA*-cDNA.

(**a**) Dot-blot analysis of RNA extracts from primary transformants. Each dot contained approx. 30 µg total RNA extracted from leaf tissues of light-grown plants of: (**1**) a transgenic tobacco line (9A4) known to express a high level of oat-*phyA*-mRNA, (**2**) wild-type tobacco, (**3-12**) putative expressers of introduced oat-*phyA*-cDNA.



(b)

(**b**) RNA gel analysis of a seedling population segregating for expression of introduced oat-*phyA*-mRNA. Total RNA extracts were made from: (lane **1**) etiolated oat seedlings, (lane **2**) wild-type tobacco, (lanes **3-7**) progeny of a primary transformant expressing oat-*phyA*-mRNA. Samples containing approx. 30 µg total RNA were run on a denaturing gel, stained with ethidium bromide (*left*) and the resulting blot was hybridised with a radiolabelled probe (*right*). The probe used for the dot-blots (a) and RNA-gel-blots (b) was a 650bp fragment from the 5' end of oat-*phyA*-cDNA generated by a *Sac*1/ *Nco*1 digestion.

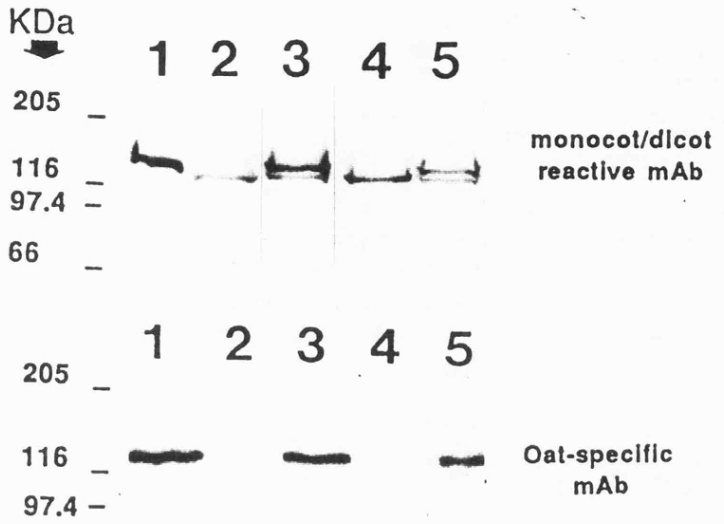


Figure 4.3 Immunoblot analysis of phytochrome in etiolated seedlings of homozygous transgenic tobacco lines. Protein extracts were made from dark-grown seedlings of: oat (lane **1**), wild-type tobacco (lane **2**), transgenic tobacco lines Y_{12} (lane **3**) and Y_{10} (lane **5**) which accumulated oat-*phyA*-mRNA, and transgenic tobacco line Y_5 (lane **4**) which was kanamycin resistant but did not express a detectable level of oat-*phyA*-mRNA. Extracts were separated on an 8% SDS-polyacrylamide gel and the resulting blots were probed with monoclonal antibodies which were either cross-reactive with both monocot. and dicot. phytochrome species (*top*) or were specific for oat-encoded phytochrome (*bottom*).

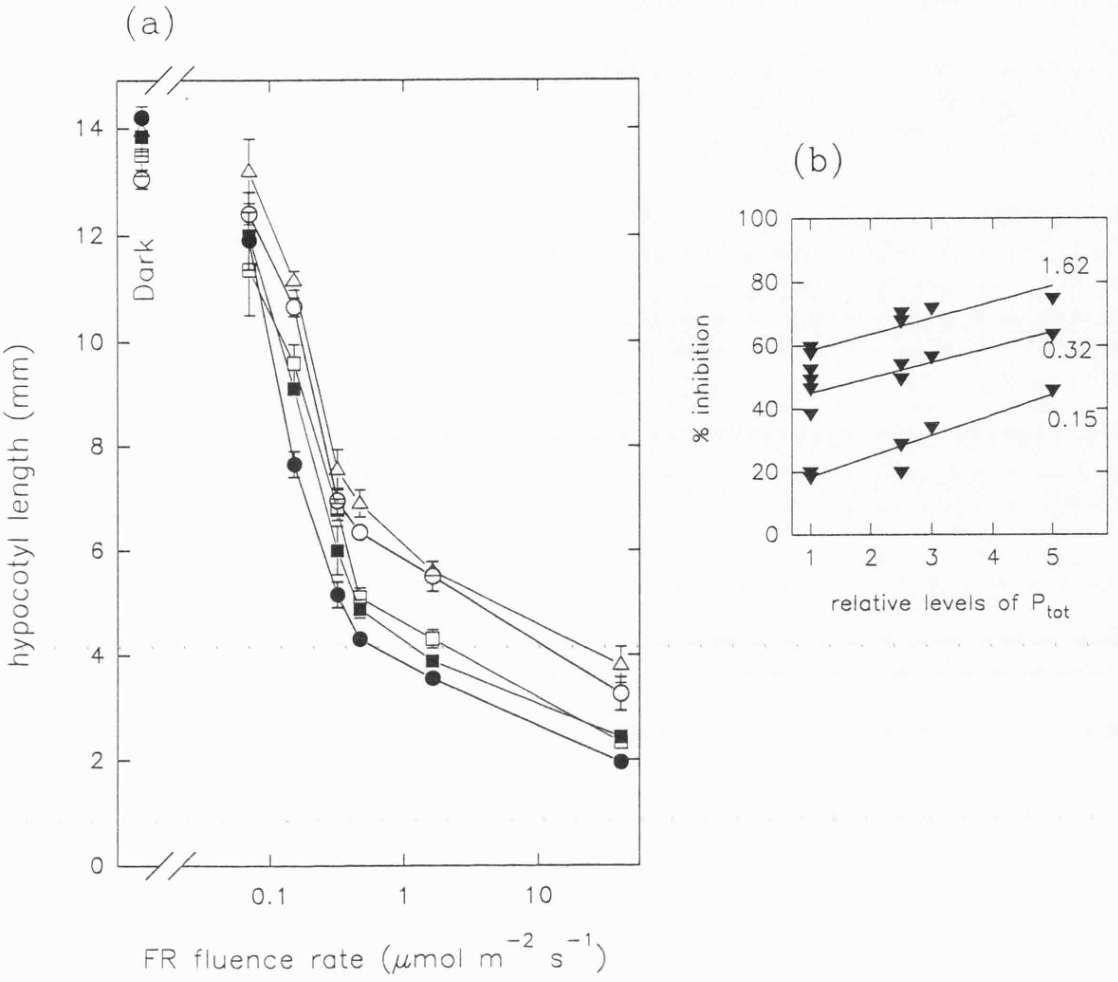


Figure 4.4 (**a**) Fluence-rate-response relationships for inhibition of hypocotyl growth in etiolated seedlings under continuous far-red (FR) radiation. Dark-grown seedlings of wild-type (WT) tobacco (*open circles*) and the transgenic line Y_5 (*open triangles*) contained WT levels of total phytochrome in the etiolated seedlings. Homozygous transgenic lines Y_{10} (*open squares*), Y_{12} (*solid squares*) and 9A4 (*solid circles*) expressed introduced oat-*phyA*-cDNA and thereby contained 2.5-fold, 3-fold and 5-fold total levels, respectively, of WT etiolated phytochrome. Each value is the mean ± SEM of 30 seedlings.

(**b**) Inhibition of the dark hypocotyl growth-rate under a given fluence rate of FR [indicated on the graph (μmol m^{-2} s^{-1})] is shown relative to the respective levels of total phytochrome (P_{tot}) in the etiolated seedlings of each line. The results were calculated from the data in Fig. 4.4a and also include data from a heterozygous line derived from 9A4, corresponding to intermediate levels of P_{tot}.

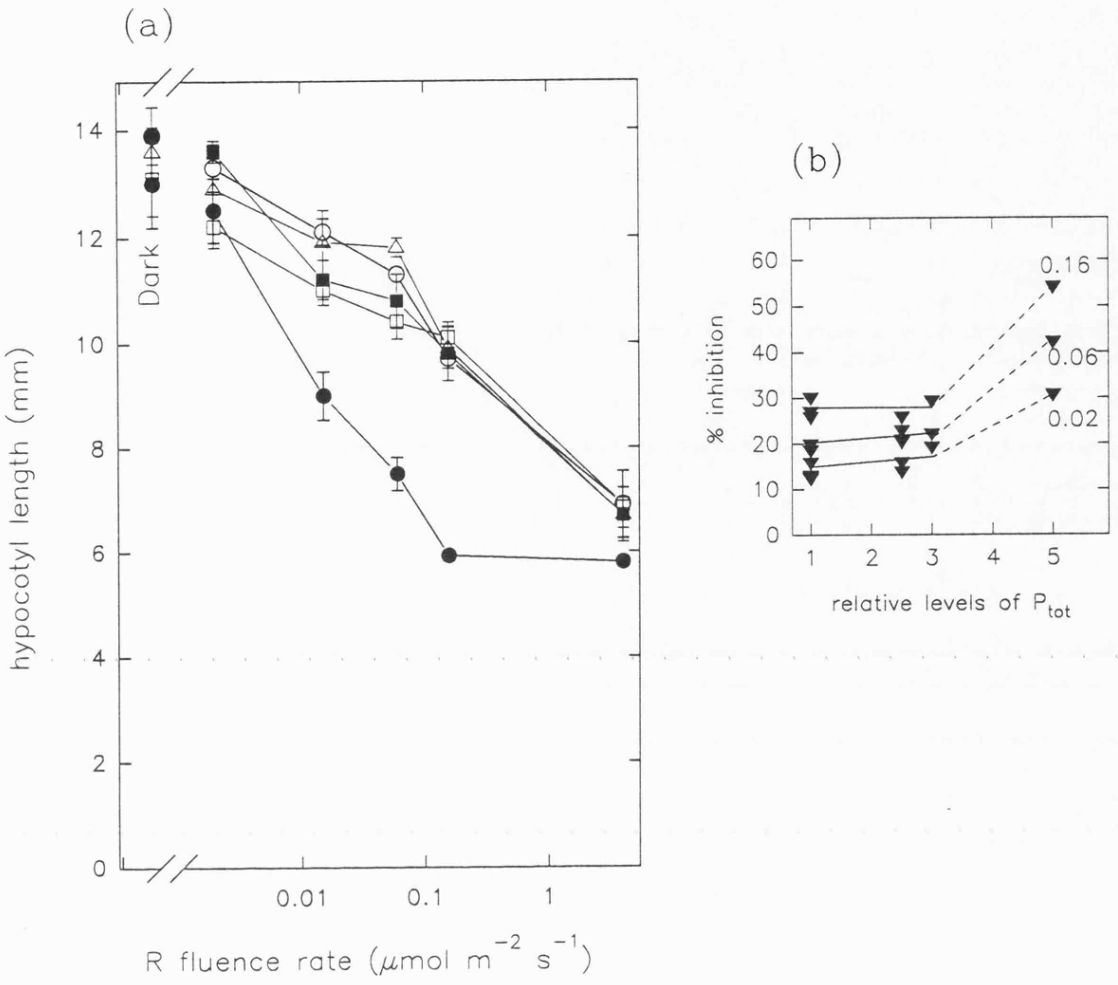


Figure 4.5 (**a**) Fluence-rate-response relationships for inhibition of hypocotyl growth in etiolated seedlings under continuous red (R) radiation. Dark-grown seedlings of wild-type (WT) tobacco (*open circles*) and the transgenic line Y_5 (*open triangles*) contained WT levels of total phytochrome in the etiolated seedlings. Homozygous transgenic lines Y_{10} (*open squares*), Y_{12} (*solid squares*) and 9A4 (*solid circles*) expressed introduced oat-*phyA*-cDNA and thereby contained 2.5-fold, 3-fold and 5-fold total levels, respectively, of WT etiolated phytochrome. Each value is the mean ± SEM of 30 seedlings.

(**b**) Inhibition of the dark hypocotyl growth-rate under a given fluence rate of R [indicated on the graph (μmol m^{-2} s^{-1})] is shown relative to the respective levels of total phytochrome (P_{tot}) in the etiolated seedlings of each line. The results were calculated from the data in Fig. 4.5a and also include data from a heterozygous line, derived from 9A4, corresponding to intermediate levels of P_{tot}.

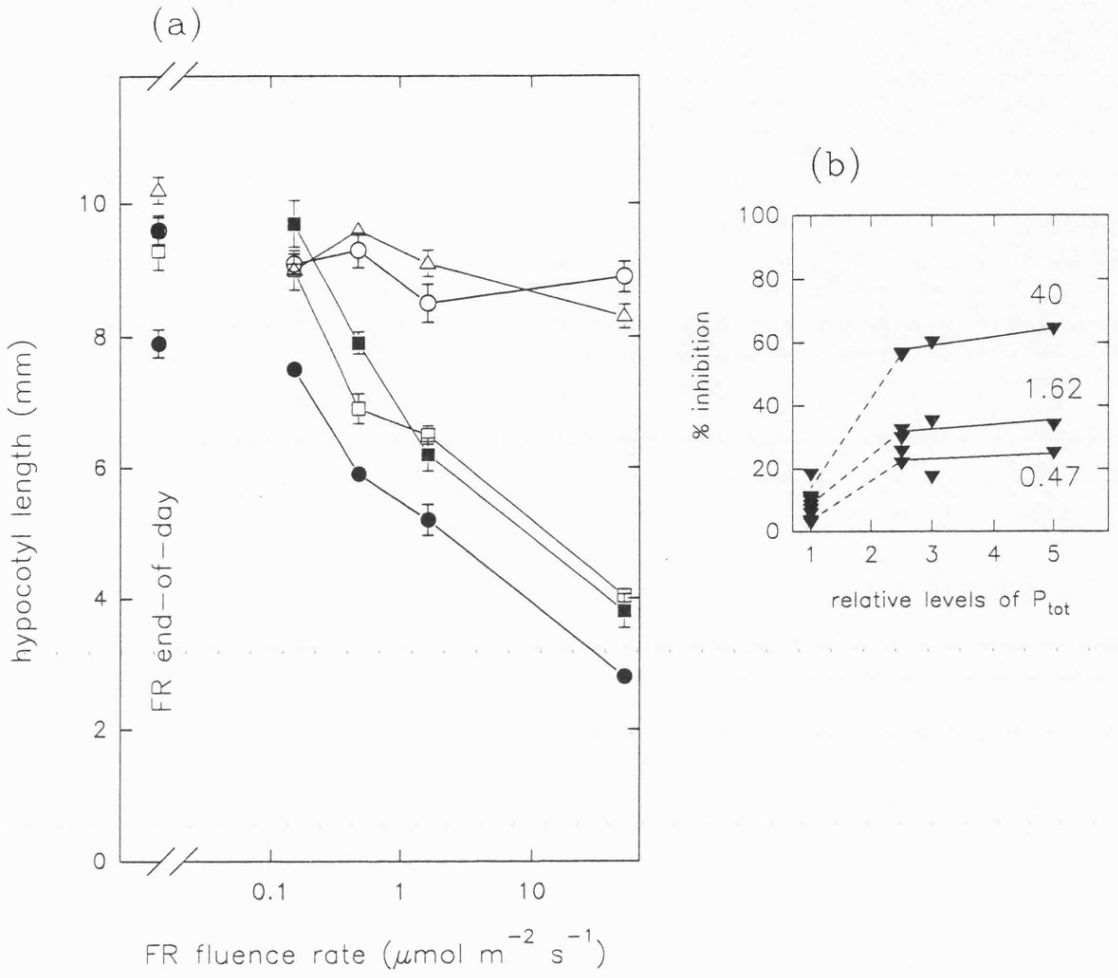


Figure 4.6 Growth-response of light-grown seedlings to far-red (FR). Light-grown seedlings received end-of-day FR irradiation as either a 15 min pulse followed by 13 h 45 min darkness (FR-end-of-day) or were irradiated by continuous FR throughout the 14 h night period. (**a**) Hypocotyl growth is shown for various fluence rates of the continuous FR treatment. Wild-type (WT) tobacco (*open circles*) and the transgenic line Y_5 (*open triangles*) contained WT levels of total phytochrome. Homozygous transgenic lines Y_{10} (*open squares*), Y_{12} (*solid squares*) and 9A4 (*solid circles*) constitutively expressed introduced oat-*phyA*-cDNA. Each value is the mean ± SEM of 30 seedlings. (**b**) Inhibition of hypocotyl-growth under continuous FR [at the fluence rates indicated on the graph (μmol m^{-2} s^{-1})] was calculated relative to seedlings which received an end-of-day FR pulse. Levels of FR-mediated growth-inhibition in the light-grown seedlings are plotted versus the relative levels of total phytochrome (P_{tot}) measured in the etiolated seedlings. The results were calculated from the data in Fig. 4.6a and also include data from a heterozygous line derived from 9A4, corresponding to intermediate levels of P_{tot}.

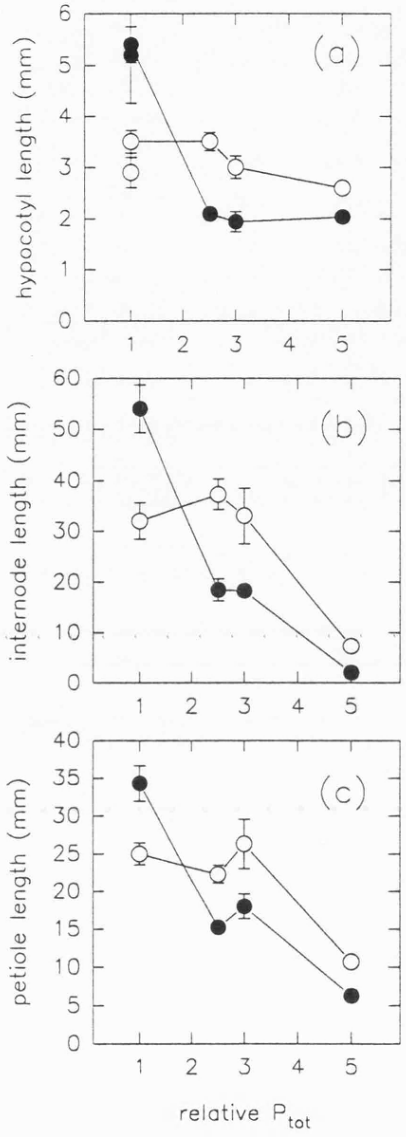


Figure 4.7 Response of light-grown plants of wild-type (WT) tobacco and three oat-*phyA*-transformed lines to red (R):far-red (FR) ratio. Plants were grown under continuous irradiation by white light (R:FR = 6.8) (*open symbols*) or white light with added far-red radiation (R:FR = 0.27) (*solid symbols*). The morphological measurements are plotted against the relative levels of total phytochrome (P_{tot}) measured in the etiolated seedlings of each line: WT plants [and plants of the control transgenic line Y_5 : (a) only] correspond to a relative P_{tot} value of 1; the transgenic lines expressing oat-*phyA*-cDNA, Y_{10}, Y_{12} and 9A4, correspond to relative P_{tot} values of 2.5, 3 and 5, respectively. Hypocotyl length (**a**) was measured in the light-grown seedlings; internode length (**b**) and petiole length (**c**) was measured in the mature green plants. Each value is the mean ± SEM of 10 plants.

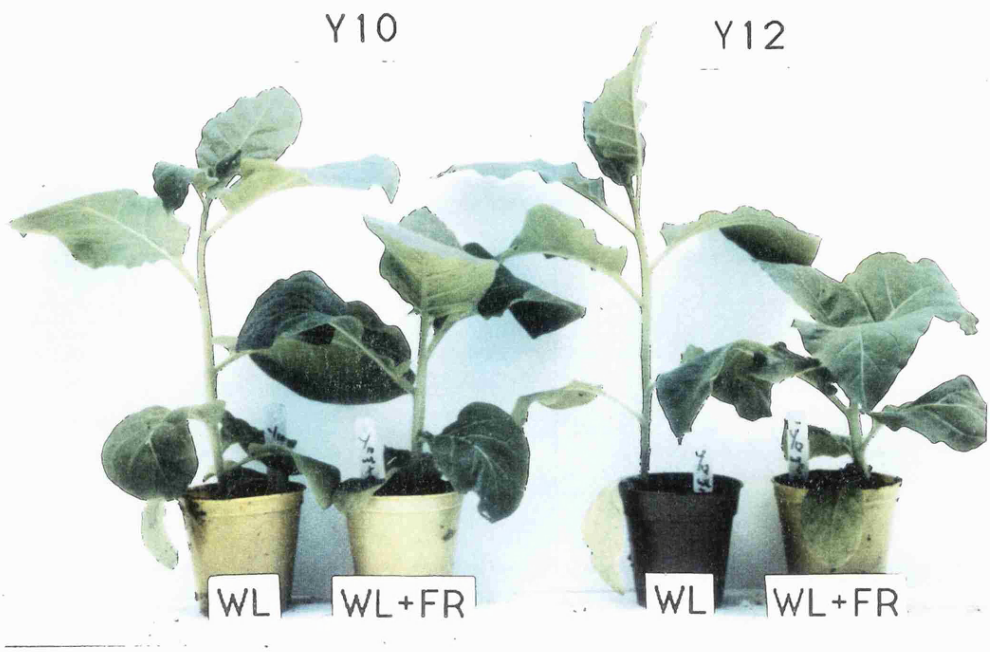


Figure 4.8 Response of transgenic tobacco plants to red (R):far-red (FR) quantum ratio. Plants of Y_{10} and Y_{12} express oat-*phyA*-cDNA under the control of the constitutive CaMV 35S promoter. The plants were grown under continuous irradiation by white light (WL) (R:FR = 6.8) or white light with added far-red (WL+FR) (R:FR = 0.27).

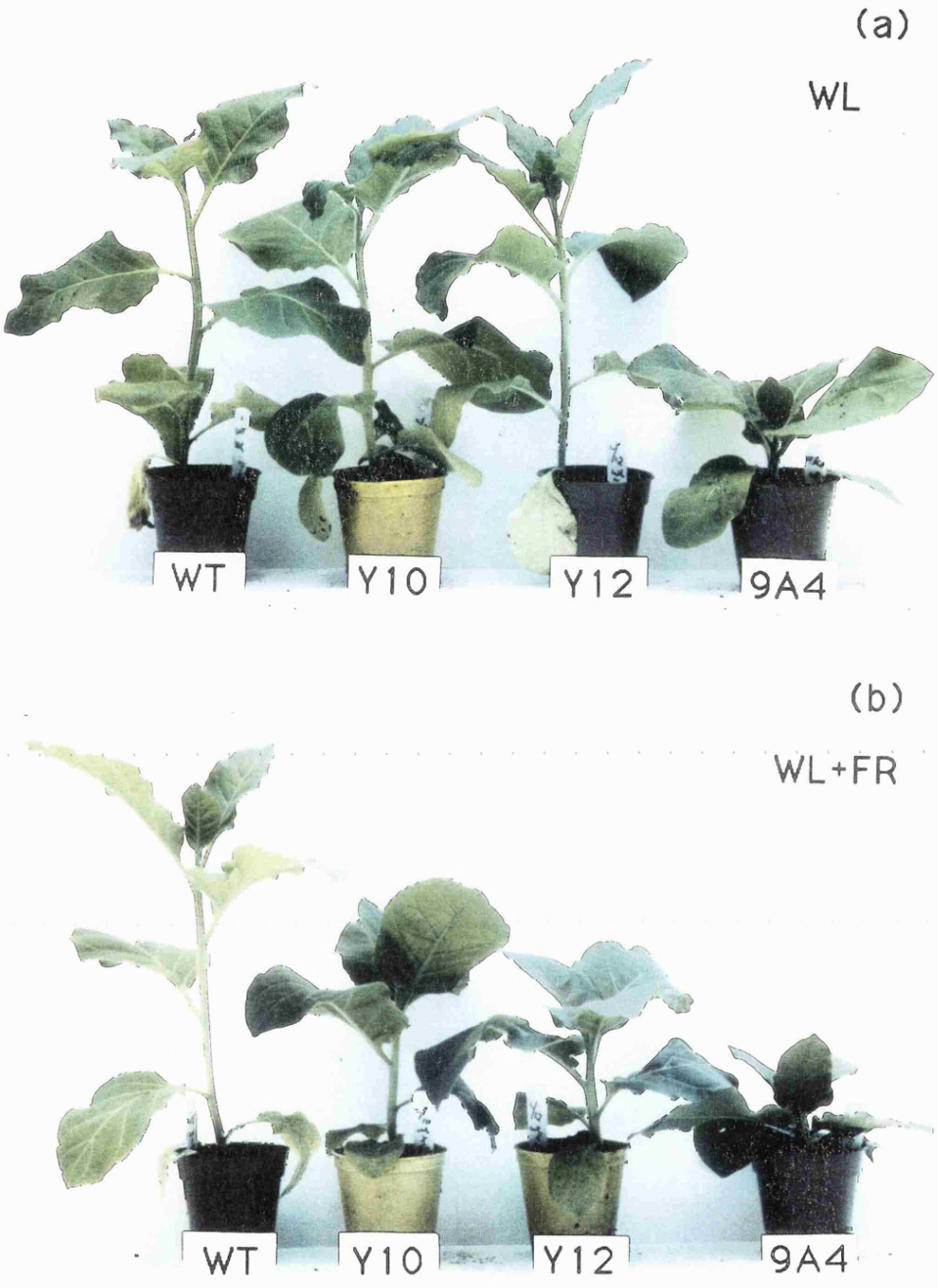


Figure 4.9 Light-mediated phenotypes of three transgenic lines of tobacco constitutively expressing oat-*phyA*-cDNA. The relative levels of transgene expression were measured in the etiolated seedlings where they caused a 2.5-fold (Y_{10}), 3-fold (Y_{12}) or 5-fold (9A4) increase in total levels of etiolated phytochrome compared to wild-type (WT). Plants were grown under (**a**) white light (WL) (R:FR = 6.8) or (**b**) white light with added far-red (WL+FR) (R:FR = 0.27).

Chapter 5

Photoresponses of transgenic *Arabidopsis* seedlings expressing introduced cDNAs encoding phytochrome A or phytochrome B: Evidence that phytochrome A and phytochrome B have distinct photoregulatory functions.

Introduction

Phytochrome comprises a family of immunochemically-distinguishable molecular species (Abe *et al.*, 1985; Tokuhisa *et al.,* 1985; Wang *et al.,* 1991) encoded by at least three divergent genes, namely *phyA*, *phyB* and *phyC* (Sharrock and Quail, 1989; Dehesh *et al.*, 1991). The gene product of *phyA* (phytochrome A) has been identified as light labile (Type I) phytochrome which accumulates to high levels in etiolated tissues but is at much lower levels in light-grown plants (Sharrock and Quail, 1989; Somers *et al.*, 1991; Wang *et al.*, 1991). The product of the *phyB* gene (phytochrome B) (Sharrock and Quail, 1989; Somers *et al.,* 1991; Wagner *et al.*, 1991), and probably the *phyC* gene (Sharrock and Quail, 1989), is light stable; light stable phytochromes comprise the Type II pool which predominates in extracts from green plants.

The proposal that individual members of the phytochrome family have distinct regulatory roles (Sharrock and Quail, 1989; Smith and Whitelam, 1990) is acquiring credence through the physiological analysis of photoreceptor-deficient mutants

(Adamse *et al.*, 1988a; 1988b; 1988c; Kendrick and Nagatani, 1991; Somers *et al.*, 1991). Reports that expression of a foreign phytochrome-encoding sequence in tomato (Boylan and Quail, 1989), tobacco (Keller *et al.*, 1989; Cherry *et al.*, 1991; Kay *et al.*, 1989; Nagatani *et al.*, 1991) and *Arabidopsis* (Boylan and Quail, 1991; Wagner *et al.*, 1991) leads to the accumulation within these plants of spectrally-active foreign phytochrome and a light-dependent dwarf-phenotype (indicative of biological activity of the foreign phytochrome) have offered another experimental tool for investigating the roles of different phytochromes. The analysis of transgenic plants overexpressing individual phytochrome species provides a complementary approach to the use of phytochrome-deficient genotypes.

A detailed photophysiological analysis of tobacco plants expressing oat phytochrome A has revealed that some aspects of their photomorphogenesis are disturbed, namely shade-avoidance-responses to reductions in R:FR ratio are disabled and the light-grown plants exhibit persistance of FR-mediated growth-inhibition (see: Chapter 3 & 4 of this thesis; McCormac *et al.*, 1991; 1992b). In the case of these transgenic tobacco plants, the constitutive expression of a foreign phytochrome A led to the maintenance of a response mode that was normally restricted to etiolated seedlings (see Chapter 2 of this thesis; Beggs *et al.*, 1980; Holmes *et al.*, 1982; McCormac *et al.*, 1992a). Here evidence is presented that this aberrant photomorphogenetic behaviour as the result of the accumulation of a foreign phytochrome A population is not restricted to tobacco, but is also displayed in transgenic *Arabidopsis* seedlings constitutively expressing an introduced oat-*phyA*-cDNA.

Phytochrome B-encoding cDNAs (isolated from green plants of rice and *Arabidopsis*) have been introduced into *Arabidopsis* (Wagner *et al.*, 1991) and, like constitutive overexpression of a foreign *phyA*-cDNA, are seen to cause a pronounced inhibition of hypocotyl extension growth in light-grown seedlings. Action of the phytochrome A and phytochrome B molecular species can therefore impinge on similar

cellular processes. However, a more detailed characterisation of the *phyB*-transformed plants suggests different photosensory roles for phytochrome A and the light-stable phytochrome species.

One potential caveat regarding the interpretation of the responses of transgenic plants is the likelihood of expression of the chimeric CaMV 35S promoter/cDNA construct within cell types which lack expression of the native phytochrome gene. The extent of such ectopic expression is difficult to assess due to the dearth of information concerning the tissue-specific regulation of phytochrome genes. Aberrations of plant photophysiology, such as those reported in the *phyA*-transformed lines, cannot, therefore, be unequivocally attributed to the physiological action of the introduced phytochrome as opposed to the abnormal effects of ectopic expression. In the *phyB*-transformed lines, the introduced cDNA has been fused to the same constitutive CaMV 35S promoter (Wagner *et al.*, 1991) as utilised for the transgenic *phyA*-cDNA constructs (Boylan and Quail, 1991). This fact therefore renders both of the species of foreign phytochrome susceptible to the same potential loss of regulation of expression and thus encourages a direct comparison of the photophysiologies of these two sets of transgenic plants. The results presented in this chapter examine the photophysiology of the transgenic *Arabidopsis* seedlings overexpressing introduced phytochrome B, and are compared with those obtained for phytochrome A-overexpressers.

Materials and methods

Plant material and growth conditions

Seeds used were of the wild type (WT) *Arabidopsis thaliana* (L. Heynh.), ecotype Nossen, and two homozygous isogenic lines, 21K15 and 13K7, that have been transformed with oat-*phyA*-cDNA, under the control of the cauliflower mosaic virus (CaMV) 35S promoter as described by Boylan and Quail (1991), and also two homozygous transgenic lines, RBO and ABO, that have been transformed with the rice-*phyB*-cDNA or the *Arabidopsis-phyB*-cDNA, respectively, also under the control of the constitutive CaMV 35S promoter as described by Wagner *et al.* (1991). The *hy3* mutant of *Arabidopsis* (Koornneef *et al.*, 1980) is specifically deficient in the *phyB* gene-product (Somers *et al.*, 1991; Reed *et al.*, 1993), and its isogenic WT ecotype is Landsberg *erecta*.

For experiments involving the photoresponses of etiolated seedlings, seeds were sown on 1% (w/v) agar contained in 10x1x1.5 cm high Plexiglas troughs, and chilled for 4 days at 4^{o}C in darkness. Where appropriate, Norflurazon was added to the molten agar to a concentration of 2 mg l^{-1}. This herbicide inhibits the synthesis of carotenoids thereby permitting the photodestruction of chlorophyll pigments. Seeds were encouraged to germinate by a saturating WL pulse (Cone and Kendrick, 1985) and grown for a further 24 h in darkness at 25^{o}C. Seedlings were then transferred to continuous irradiation from the appropriate light source for a further 3 days.

For experiments involving light-grown seedlings, seeds were sown onto water-saturated potting compost, chilled for 4 days at 4 ^{o}C and then germinated under white light (WL). Seedlings designated to receive the end-of-day treatments were grown for 2 days under continuous WL (photosynthetically active radiation 400-700nm = 130 µmol m^{-2} s^{-1}). Seedlings were then grown under 4 successive cycles of 10 h WL/ 14 h dark and, where appropriate, end-of-day far-red radiation [establishing phytochrome

photoequilibrium (P_{fr}/P_{tot}) <0.01] was provided immediately following the WL period. Seedlings to be subjected to a range of red:far-red quantum ratios (R:FR) were grown for 1 day under WL and then transferred to continuous irradiation by the appropriate R:FR light source.

Light sources

Red light (R) for the continuous irradiation of etiolated seedlings (600-700nm, photon fluence rate as indicated in figures) was provided by filtering the radiation from Thorn EMI (Birmingham, UK) Deluxe Natural 40-W fluorescent tubes through 1 cm deep copper sulphate solution (1.5% w/v) and one layer of red (No. 14) Cinemoid (Rank Strand, Isleworth, Middlesex, UK). The far-red (FR) light source used for the continuous irradiation of etiolated seedlings (photon fluence rate as indicated in figures) and for the end-of-day irradiation of light-grown seedlings (photon fluence rate 700-800nm = 40 μmol m^{-2} s^{-1}) was provided by the output of water-cooled 100-W incandescent bulbs filtered through a black acrylic filter (Plexiglas Type FRF 700; West Lake Plastics, Lem, Penn., USA).

The R:FR ratio treatment cabinets used were of the same design as described in detail in Chapter 3 of this thesis (*Materials and methods: light sources,"high PAR cabinets"*). In order to provide a gradient of supplementary FR radiation, against a uniform background of WL (photosynthetically active radiation = 60 μmol m^{-2} s^{-1}), a selective filter was constructed of a 1 cm deep layer of a copper sulphate solution of various concentrations (0-2% w/v).

Light measurements and photoequilibria calculations

Fluence rates and spectral distributions of light sources were measured using a LI-1800

spectroradiometer (LI-COR, Lincoln, Nebraska, USA) as described in chapter 3 of this thesis (*Materials and methods: Light measurements and P_{fr}/P_{tot} ccalculations*). Photosynthetically active radiation (PAR) was measured as total fluence rate between 400nm and 700nm. The quantum ratio of red and far-red wavelengths (R:FR) was calculated as the ratio of photon fluence rates over the 654-664 nm and 724-734 nm wavelength intervals. Phytochrome photoequilibria values (P_{fr}/P_{tot}) were calculated from corrected spectrophotometric measurements (Butler *et al.,* 1964) of an irradiated sample of purified etiolated-oat phytochrome.

Measurement of seedling hypocotyl length and petiole growth

Hypocotyl lengths and leaf dimensions were determined from projections of calibrated photographic transparencies of the whole seedlings and excised leaves, respectively. Petiole growth was calculated as the length:width ratio of the leaf, where length was measured from the tip of the leaf to the base of the petiole and width was determined from the broadest part of the lamina. Data are given as the mean values derived from three replicate experiments which consisted of a minimum of 15 seedlings each. Error bars represent the standard error of the mean; where none are shown, error was smaller than the symbol size.

Results and discussion

The aim of this study was to investigate the photophysiological effects of the expression of an introduced heterologous (rice) or homologous (*Arabidopsis*) phytochrome B-encoding cDNA in transgenic plants of *Arabidopsis,* and the results are compared with the photophysiology of transgenic plants expressing introduced oat-phytochrome A-encoding cDNA. The transgenic lines referred to as RBO (transformed with rice *phyB*-cDNA) and ABO (transformed with *Arabidopsis phyB*-cDNA) contained approx. 2.5-fold and 15-fold levels, respectively, of total phytochrome B relative to WT, as measured from immunoblots of extracts from light-grown seedlings (Wagner *et al.*, 1991). The results are compared with the photoresponses of the *hy3* mutant of *Arabidopsis*, which specifically lacks the *phyB* gene-product (Somers *et al.*, 1991; Reed *et al.*, 1993). Transgenic *Arabidopsis* seedlings which constitutively express an introduced oat-*phyA*-cDNA (21K15, 13K7) (Boylan and Quail, 1991) contained approximately 4-fold levels of total phytochrome in the etiolated seedlings compared to wild-type.

Response of etiolated seedlings to red light

Under prolonged exposure to R (photon fluence rate, 600-700 nm, 4 μmol m^{-2} s^{-1}), inhibition of hypocotyl elongation was clearly more pronounced in the *phyB*-transformed lines (ABO and RBO) and an oat-*phyA*-transformed line (21K15), than in the isogenic WT (Fig. 5.1a). As fluence rate was reduced, R-irradiation became progressively less effective in the inhibition of WT hypocotyl growth; at a fluence rate of 0.06 μmol m^{-2} s^{-1} growth of WT was not significantly inhibited relative to seedlings maintained in darkness; hypocotyl elongation in all three transgenic lines, however, remained severely inhibited at this fluence rate (Fig. 5.1a). Therefore, the growth difference between transgenic and WT seedlings was exaggerated as the fluence rate was reduced from 4 to

0.06 μmol m^{-2} s^{-1}, confirming that constitutive expression of *phyA*- or *phyB*-encoding cDNA leads to a light-dependent dwarf-phenotype. At R fluence rates below approx. 0.08 μmol m^{-2} s^{-1} seedling growth in both the RBO and ABO lines was dependent on fluence rate (Fig. 5.1a). Response of the *phyA*-transformed seedlings, however, remained saturated at fluence rates of R as low as 0.03 μmol m^{-2} s^{-1}, below which effectiveness of R declined steeply (Fig. 5.1a).

Application of the bleaching herbicide Norflurazon to etiolated seedlings did not alter the relationship of growth-inhibition with fluence rate from that measured in the untreated seedlings (data not shown). Screening effects by chlorophyllous pigments did not, therefore, appear important to the R-mediated response in the WT or transgenic seedlings.

At all fluence rates of R tested, hypocotyl extension-growth of the *hy3* mutant failed to be significantly inhibited relative to dark growth (Fig. 5.1b). In contrast, growth of the isogenic WT was significantly inhibited at R fluence rates above 0.01 μmol m^{-2} s^{-1}. The loss of sensitivity to R in *hy3* [Koornneef *et al.* (1980) detected a much attenuated R-response in *hy3*] suggests that the native phytochrome B in WT seedlings is responsible for a R-mediated response that is dependent on fluence rate. The increased sensitivity to low fluence-rate R conferred by the elevated levels of total phytochrome B in the RBO and ABO seedlings supports this conclusion. Conversely, the absence of R-responsiveness in *hy3* with normal phytochrome A levels (Somers *et al.*, 1991) indicates that endogenous phytochrome A cannot mediate the R-induced effects, at least in the absence of phytochrome B. The marked sensitivity of the growth-response of the *phyA*-transformed line to very low fluence rates of R (Fig. 5.1a) demonstrates that transgenically-overexpressed levels of phytochrome A can, however, be highly active in mediating a response to R. This apparently anomolous result could be due to either the sustained high levels of phytochrome A in the transgenic irradiated seedlings in contrast to the rapid light-mediated destruction of the native phytochrome A pool (Boylan and Quail,

1991), and/or ectopic expression of the molecule over-coming an intrinsic lack of activity of phytochrome A in mediating this response to R in the native cellular context of the WT plant.

As in the *phyA*-transformed lines (Boylan and Quail, 1991), the short-hypocotyl phenotype of the *phyB*-transformed lines can be induced by brief pulses of R-irradiation (Fig. 5.2). Over the total growth period, 5 min irradiations of R at 4 h intervals provided a total fluence of 18.9 mmol m^{-2}; this light treatment resulted in a final inhibition of seedling dark-growth which was comparable to that achieved, in the respective *Arabidopsis* lines, under continuous irradiation by 0.1 μmol m^{-2} s^{-1} R (total fluence 25.9 mmol m^{-2}) (Fig. 5.1a). Thus, continuous irradiation was not essential for R-action and the response displayed essentially full reciprocity. Moreover, the effects of the R pulses were fully reversed by immediately subsequent FR in each case (Fig. 5.2). These results indicate that a transgenically-overexpressed population of phytochrome B acts to inhibit hypocotyl elongation in a manner characteristic of low fluence responses.

Response of etiolated seedlings to far-red light

Continuous FR irradiation (photon fluence rate, 700-800nm, 40 μmol m^{-2} s^{-1}) resulted in marked inhibition of growth of the etiolated seedlings of both cultivars of WT *Arabidopsis*, transgenic lines expressing introduced cDNAs encoding phytochrome A (21K15) or phytochrome B (RBO and ABO), and the mutant *hy3* line (Fig. 5.3a, b). The response required prolonged irradiation; 10 min irradiations with FR at 4 h intervals, providing a total fluence of 151 mmol m^{-2}, did not elicit inhibition of dark-growth (Fig. 5.2), whereas delivery of an equivalent total fluence by continuous FR (0.6 μmol m^{-2} s^{-1}) resulted in approx. 30% inhibition in WT seedlings (Fig. 5.3a, b). The response mediated by continuous FR was dependent on fluence rate (Fig. 5.3a, b), as is characteristic of an HIR (Mancinelli, 1980). As the photon fluence rate of FR was reduced

below a value of about 40 μmol m^{-2} s^{-1}, the inhibition of hypocotyl elongation was progressively less pronounced. Exposure of etiolated WT seedlings to continuous FR at a fluence rate of 0.14 μmol m^{-2} s^{-1}, or lower, did not lead to a significant reduction in hypocotyl elongation compared to seedlings maintained in darkness (Fig. 5.3a). The photon fluence rate-response curve appeared to be discontinuous and could be resolved into two regions with different slopes. Similar discontinuous photon fluence rate response curves have been reported for the FR inhibition of mustard hypocotyl growth (Heim and Schäfer, 1984).

In the case of the etiolated seedlings of the oat-*phyA*-transformed 21K15 line, a FR photon fluence rate of only 0.5 μmol m^{-2} s^{-1} was sufficicent for the saturation of inhibition of hypocotyl elongation by continuous FR; only for FR photon fluence rates below this value was the extent of the response in the transgenic seedlings reduced (Fig.5.3a). Exposure of 21K15 seedlings to a photon fluence rate of 0.05 μmol m^{-2} s^{-1} did not lead to a significant reduction in hypocotyl elongation compared to seedlings maintained in darkness. The mean hypocotyl length of 21K15 seedlings exposed to continuous FR at an apparently saturating photon fluence rate of 0.5 μmol m^{-2} $s^{-1}$was not significantly different to that of WT seedlings that had been exposed to a FR photon fluence rate that was about two-orders of magnitude higher. Furthermore, elongation of the hypocotyls of the *phyA*-overexpressing seedlings was substantially inhibited by a FR photon fluence rate of 0.14 μmol m^{-2} s^{-1}, a fluence rate that was not inhibitory to the elongation of WT hypocotyls. The photon fluence-rate-response curve observed for 21K15 seedlings did not appear to show a discontinuity; the slope of the curve under subsaturating FR-fluences was similar to the steep region of the discontinuous curve observed for WT seedlings. It is clear that the transgenic seedlings displayed an increased sensitivity towards continuous FR, an assumed consequence of the increased accumulation of phytochrome A in these seedlings.

The sensitivity of the growth response to low fluence-rate FR was not enhanced in

either of the *phyB*-transformed lines (Fig. 5.3a), nor was it impaired in the phytochrome B-deficient seedlings of *hy3* (Fig. 5.3b) (see also Koornneef *et al.*, 1980). In fact, if anything, the response to the intermediate range of FR fluence rates (i.e. 1-10 μmol m^{-2} s^{-1}) appeared slightly impaired in the etiolated seedlings of ABO (Fig. 5.3a), possibly indicating an action of the elevated phytochrome B population which was antagonistic to the native phytochrome of etiolated seedlings.

The data presented here are consistent with the hypothesis that the light-stable phytochromes are not active in the response of etiolated seedlings to continuous FR. In contrast, etiolated seedlings of *phyA*-transformed *Arabidopsis* (Fig. 5.3a; Whitelam *et al.*, 1992) and tobacco (McCormac *et al.*, 1992b) have been shown to exhibit a shift in the sensitivity of the growth-inhibition response towards lower fluence rates of continuous FR, thereby strongly implicating phytochrome A as the photoreceptor for the irradiance-dependent response to FR of etiolated seedlings.

Under the lower range of FR fluence rates (< 0.3 μmol m^{-2} s^{-1}) and also in darkness, the etiolated seedlings of ABO appeared slightly shorter than WT (Fig. 5.3a). This was shown to reflect a reduction in the dark growth-rate of ABO hypocotyls following a saturating WL pulse (1 mol m^{-2}) given to the pre-imbibed and chilled seed (Fig. 5.4a, c; 5.4e, g). A saturating pulse of FR, 24 h subsequent to the WL irradiation, proved ineffective in reversing this inhibition (Fig. 5.4c, g). Replacement of the WL treatment with exposure to FR, however, did restore the dark growth-rate of ABO (Fig. 5.4c, g) seedlings to that of WT (Fig. 5.4a, e). Neither WT (Fig. 5.4a, e), RBO (Fig. 5.4b, f) nor the *phyA*-transformed seedlings (Fig. 5.4d, h) showed such diversity of relative growth-rates as a consequence of the light treatment delivered to ungerminated seed. Etiolated seedlings of the *phyA*-transformed line showed reduced growth relative to WT under all of the treatments; reduced dark growth was not observed in previous experiments (Fig. 5.1a; Whitelam *et al.*, 1992) and is attributed here to poor vigour of the particular seed lot. It is therefore proposed that a high level of light-stable P_{fr} (the

far-red absorbing form of phytochrome) in ABO seed was able to influence subsequent seedling growth.

Phytochrome levels in etiolated seedlings under red or far-red light

The levels of total immunodetectable phytochrome of the heterologous oat-*phyA*-encoded species in etiolated transgenic tobacco seedlings have previously been reported to be sensitive to the wavelength of the light-source, i.e. rapid loss of phytochrome occurred in seedlings under continuous R but not continuous FR (McCormac *et al.,* 1992b). Levels of oat-encoded phytochrome A were measured in etiolated seedlings of 21K15 which had been subjected to 24 h continuous R or FR irradiation (Fig. 5.5). Dark-grown levels were seen to be depleted in the seedlings exposed to R but not FR. In this respect, therefore, the heterologous phytochrome displayed light-labile characteristics similar to that of the native etiolated phytochrome of dicotyledonous species (Clarkson and Hillman, 1967). However, significant levels of oat-encoded protein were still present in the transgenic seedlings, even after 24 h under a R light-source; following such a light treatment, phytochrome A in WT seedlings is normally removed to below the limits of detection. This result may be explained by high levels of expression of the introduced cDNA in the transgenic *Arabidopsis* seedlings and suggests that, although light-labile, the heterologous phytochrome A can persist at a significant level in the light-grown tissues. Boylan and Quail (1991) also reported that very high levels of the heterologous phytochrome A were maintained within the transgenic light-grown plants.

Response of light-grown seedlings to far-red light

Fully de-etiolated seedlings of WT, which were subjected to four consecutive day-night cycles consisting of 10 h WL followed by 14 h darkness (end-of-day P_{fr}/P_{tot} = 0.71)

showed a significant increase in hypocotyl elongation in response to 15 min end-of-day FR treatments (establishing end-of-day $P_{fr}/P_{tot} < 0.01$) (Fig. 5.6a, b). The FR-mediated increase in hypocotyl growth in WT seedlings was not significantly altered when the FR treatment was extended from a 15 min end-of-day pulse to continuous irradiation throughout the 14 h night period (Fig. 5.6a, b). Thus for WT seedlings the growth-promoting effect of end-of-day FR was unaffected by the duration of FR exposure, indicating that following de-etiolation these seedlings were no longer responsive to prolonged FR. Such a loss of response to prolonged FR following light treatments has been documented in several species (e.g. Beggs *et al.*, 1980; Jose and Vince-Prue, 1979).

Seedlings of both of the oat-*phyA*-expressing transgenic lines (21K15, 13K7) also showed a substantial increase in hypocotyl elongation in response to a 15 min end-of-day FR treatment (Fig. 5.6a). However, in contrast to the situation in WT seedlings, extending the end-of-day FR treatment from 15 min to 14 h led to a significant inhibition of elongation growth in the transgenic seedlings (Fig. 5.6a). This growth inhibition was clearly dependent on the duration of FR irradiation since a brief exposure of transgenic seedlings to FR at the end-of-day promoted a growth acceleration. Thus, for the transgenic seedlings which constitutively expressed foreign phytochrome A, de-etiolation did not lead to a loss of responsiveness to prolonged FR. The growth-inhibitory effect of 14 h FR given to the de-etiolated 21K15 seedlings at the end of 10 h periods of white light was dependent upon the photon fluence rate of the FR radiation (Fig. 5.7). For this experiment, the four 14 h periods of FR were each preceeded by 15 min of high photon fluence rate FR in order to ensure that photoequilibrium was attained. As was the case for etiolated seedlings of 21K15, the inhibition of hypocoty elongation by prolonged FR in the de-etiolated seedlings was apparently saturated by a FR fluence rate of about 0.5 $\mu mol\ m^{-2}\ s^{-1}$, and only for fluence rates below this value was the extent of inhibition of hypocotyl elongation reduced. Thus, for the oat-*phyA*-transformed *Arabidopsis* seedlings, de-etiolation, which did not lead to a loss of

responsiveness to prolonged FR, did not appear to affect photon fluence rate dependency of FR action. The light-grown seedlings of transgenic Arabidopsis therefore displayed a similar sensitivity towards the fluence rates of prolonged FR as in the etiolated seedlings. This is in contrast to the response of oat-*phyA*-transformed tobacco seedlings which showed reduced sensitivity to FR in the de-etiolated state (Chapter 3 of this thesis; McCormac *et al.*, 1992a). Such a result may be explained by the persistently high levels of oat phytochrome A even following the growth of the transgenic *Arabidopsis* seedlings in the light [Boylan and Quail, 1991 (*Arabidopsis*) cf. Cherry *et al.*, 1991 (tobacco)].

A pronounced acceleration of growth in response to end-of-day FR was also seen in the seedlings of the *phyB*-transformed RBO and ABO lines (Fig. 5.6b). In proportion to the growth of seedlings which received no end-of-day irradiation, the response to FR appeared larger in the *phyB*-transgenic plants than in WT, i.e. the FR-treated seedlings of ABO, RBO and WT showed a 3.1-, 2.4- and 1.2-fold increase in growth respectively. However, the absolute increase in hypocotyl length in FR end-of-day treated seedlings over that of continuous WL-treated seedlings was similar in all three of these *Arabidopsis* lines (i.e. approx. 4 mm). The discrepancy in absolute hypocotyl length between WT and *phyB*-transgenic seedlings which had received end-of-day FR can be fully accounted for by the difference in the growth which occurred during the 10 h day. This observation implies that the FR-mediated growth-rate during the 14 h night period was not significantly different between the transgenic and WT seedlings. With the implication of the almost total removal of P_{fr} in all three lines by the FR treatment, the response is consistent with the regulation of growth rate by levels of the P_{fr} form of phytochrome B . The increase in growth during the night in WT plants which received no end-of-day FR compared to WT plants under continuous WL indicates a partial loss of P_{fr}-controlled inhibition of growth during the 14 h dark period. In contrast, the phytochrome B overexpressing lines receiving no end-of-day FR did not show increased growth during the night period. Thus, phytochrome B P_{fr} appears to have sustained the inhibition of hypocotyl elongation over

the extended dark period, indicating stability of the active form of this molecule in the transgenic lines. Like WT, the light-grown seedlings of RBO and ABO showed no sensitivity to prolonged FR (Fig. 5.6b); in other words, the duration of irradiation was not critical to the response of the *phyB*-transformed plants. The response of the phytochrome B-overexpressing plants was therefore in marked contrast to the oat-*phyA*-transformed plants of tobacco and *Arabidopsis* which, even following several days of light-growth, continued to display a FR-mediated growth-inhibition which was dependent on the duration and fluence rate of irradiation.

Thus, the presence of elevated levels of phytochrome B in the transgenic plants did not interfere with the transition from the irradiance-dependent response of etiolated seedlings, to the fluence-rate-independent response of green plants to FR. This result contrasts the situation in the *phyA*-transgenic plants where a constitutive population of phytochrome A elicited a persistence of the response mode normally restricted to etiolated seedlings. These data strongly support the already popular view that light-labile phytochrome A, specifically, is responsible for the FR-mediated response of etiolated seedlings, while the action of phytochrome B appears to be compatible with the photoresponses of light-adapted plants.

Response of light-grown seedlings to R:FR ratio

De-etiolated seedlings of both *phyA*-transgenic lines, 13K7 and 21K15, showed substantially reduced hypocotyl elongation, compared to WT seedlings, when maintained under high R:FR, provided by continuous white fluorescent light (Fig. 5.8a, b, c). Exposure of WT seedlings to a low R:FR ratio (achieved by the addition of FR radiation to the background of white light) led to a marked increase in the elongation growth of the hypocotyls (Fig. 5.8a), characteristic of the shade-avoidance-syndrome (Casal and Smith, 1989). In contrast, exposure of *phyA*-transgenic seedlings to the FR-supplemented

light-source led to a significant reduction in hypocotyl elongation compared to seedlings under high R:FR ratio (Fig. 5.8b, c). Thus, the short hypocotyl phenotype of the transgenic seedlings was more exaggerated under the low R:FR conditions used here. A similar depression of extension growth by low R:FR has also been reported for light-grown transgenic tobacco plants that also express oat-*phyA*-cDNA (see Chapters 3 & 4 of this thesis; McCormac *et al.*, 1991b). The depression of extension growth by low R:FR, achieved by the addition of supplementary FR to WL, is a characteristic, transientally-displayed response of etiolated seedlings of several species, including *Arabidopsis thaliana* (McCormac *et al.,* 1992a), but for fully de-etiolated plants there are no other cases where a depression of extension growth by low R:FR has been reported.

De-etiolated seedlings of both the RBO and ABO *phyB*-transformed lines showed pronounced inhibition of hypocotyl extension, compared to WT seedlings, when maintained under continuous WL (R:FR = 6.8; P_{fr}/P_{tot} = 0.71). Reduction of the R:FR ratio (and hence P_{fr}/P_{tot} ratio) by the addition of increasing quantities of FR radiation to a uniform background of WL caused a marked acceleration of extension growth in the WT seedlings, including hypocotyl length (Fig. 5.9a) and elongation of the petioles (Fig. 5.9b). Seedlings of the RBO and ABO lines similarly responded to a decrease in the P_{fr}/P_{tot} ratio by increased elongation growth of the hypocotyl and petioles (Fig. 5.9a, b). This is contrasted to the inverted shade-avoidance response of the transgenic plants expressing an heterologous *phyA*-encoding cDNA (Fig. 5.8b, c).

Although the mode of response to R:FR ratio in the *phyB*-transformed lines appeared qualitatively consistent with that of light-grown WT plants, at all values of R:FR ratio tested *phyB*-transformed seedlings were shorter than WT, and the growth of ABO seedlings was reduced, yet further, relative to that of RBO seedlings. The reduced growth of the transgenic plants relative to WT, even under conditions establishing low photoequilibrium values, appeared superfically correlated with total levels of phytochrome B (i.e., ABO>RBO>WT). The characteristically elongated phenotypes of

phytochrome-deficient mutants (Koornneef *et al.*, 1980; 1985; Adamse *et al.*, 1988a; 1988c), and the sensitivity of etiolated seedlings to very low fluences of R, is widely accepted as convincing evidence for the active role of Pfr (as opposed to the red-absorbing form of phytochrome, P_r) in growth regulation (see: Smith, 1983 for a review). On this assumption, a direct relationship of the shade-avoidance responses of light-grown plants with levels of P_{fr} is suggested by the linearity of the plots of response versus P_{fr}/P_{tot} ratio (Morgan and Smith, 1976; 1978). However, calculations of absolute [P_{fr}] from the relative levels of phytochrome B in the light-grown seedlings of the WT and two transgenic lines [ie. WT: RBO: ABO = approx. 1: 2.5: 15 (Wagner *et al.*, 1991)] resulted in poor superimposition of the three sets of data (Fig. 5.10a, b). Notably, promotion of extension growth in ABO seedlings was seen at calculated absolute values of [P_{fr}] which corresponded to saturated growth-inhibition in the WT and RBO lines (Fig. 5.10a, b). Thus, the relative growth responses between the three lines could not be directly related to calculated absolute levels of phytochrome B P_{fr}. Indeed, at face value, the data imply that with increasing phytochrome B level (achieved via transgene expression) there is an apparent decrease in the efficiency of phytochrome B (on a per mole basis) in inhibiting hypocotyl elongation. These findings are open to at least two interpretations. Firstly, ectopic expression of the introduced cDNA may result in a substantial proportion of the total phytochrome population of transgenic plants being present in cells which lack the facility for the transduction of the phytochrome signal. Phytochrome levels measured in whole tissue extracts of transgenic plants would therefore exaggerate the size of the functional phytochrome pool relative to WT. The direct comparison of isogenically-transformed lines, which express the introduced cDNA to varying levels, would effectively by-pass this potential inaccuracy (see Chapter 6 of this thesis). Secondly, the inability of high levels of phytochrome B-P_{fr} to inhibit extension growth effectively at low R:FR ratios may reflect a requirement for the action of a second

molecular species of light-stable phytochrome. Circumstantial evidence for such a conclusion is provided by the light-stable characteristics of the phytochrome C of *Arabidopsis* (Sharrock and Quail, 1989; Somers *et al.*, 1991), and the retention of an attenuated shade-avoidance response in the phytochrome B-deficient mutant, *hy3* (Whitelam and Smith, 1991).

Conclusions

Expression of phytochrome A- or phytochrome B-encoding cDNAs in transgenic plants results in two contrasting sets of light-mediated responses. Whereas phytochrome A-overexpressing lines exhibit increased sensitivity to low fluence rates of both R and FR, phytochrome B-overexpressing lines display enhanced responsiveness to R only. Sensitivity to FR has been demonstrated in each one of a number of independent transgenic lines expressing *phyA*-cDNA, including two discrete lines of transgenic *Arabidopsis* (Boylan and Quail, 1991; Whitelam *et al.*, 1992; McCormac *et al.*, 1993) and three lines of transgenic tobacco (Keller *et al.*, 1989; McCormac *et al.*, 1992b; Chapter 4 of this thesis). In comparison, out of seven independent transgenic lines of *phyB*-cDNA-expressing *Arabidopsis* which have been studied, all seven have shown specifically increased sensitivity to R and not to FR (chapter 6 of this thesis). The pattern of responsiveness to R and FR reported here therefore appears to segregate consistently with the species of cDNA. This failure of elevated levels of phytochrome B to enhance responsiveness to FR reinforces the conclusion that the FR-mediated growth-inhibition observed in *phyA*-transformed lines is an innate function of the phytochrome A species not possessed by phytochrome B, and is not a simple consequence of ectopic expression of the introduced *phyA* cDNA. Conversely, the lack of R-responsiveness of the phytochrome B-deficient *Arabidopsis hy3* mutant establishes the complementary pair of conclusions: (a) that endogenous phytochrome B is necessary to mediate inhibition of hypocotyl elongation in response to R irradiation; and (b) that endogenous phytochrome A is poorly, if at all, effective (at least in the absence of phytochrome B) in mediating the growth response to R. Thus, taken together, these data (summarised schematically in Table 5.1) indicate that phytochromes A and B mediate reciprocal sensitivities to R and FR, at least in the context of the WT plant, with phytochrome A mediating responses to continuous FR (FR-HIR) and phytochrome B mediating responses to continuous R. The observation that

overexpression of phytochrome B leads to increased sensitivity to R is consistent with this conclusion and indicates that plants exhibit a continuum of phenotypic expression in response to increasing levels of phytochrome B. On the other hand, the observation that overexpression of phytochrome A also enhances sensitivity to R while endogenous phytochrome A does not appear to mediate responsiveness to R is superficially inconsistent with these data. However, this effect may be the result of the sustained high level and/or ectopic expression of the phytochrome A molecule in the transgenic plants. These data imply in turn that phytochrome A does not lack an intrinsic capacity to mediate R-induced responses, thus suggesting the possibility that the apparent inactivity of endogenous phytochrome A to R may result from either the known lability of phytochrome A-P_{fr} or topographical localisation in unresponsive tissues or cells in the context of the WT plant. Regardless of the mechanisms, however, the data presented here are consistent with the growing evidence from established physiological studies with WT plants (e.g., Beggs *et al.,* 1980; Holmes *et al.,* 1982) and conventional mutants (e.g., Adamse *et al.,* 1988a; 1988c) that phytochromes A and B have distinct photoregulatory roles in plant growth and development.

The conclusions made from the above data, that phytochromes A and B have reciprocal and independent photosensory roles in mediating responsiveness to continuous FR and R, respectively, have been confirmed by the characterisation of a recently isolated class of *Arabidopsis* mutants, *hy8* (Parks and Quail, 1993); *hy8* mutants are specifically-deficient in the phytochrome A molecular species (probably due to a lesion in the *phyA* structural gene) and show the converse of the responsiveness of *hy3* to continuous FR and R (Table 5.1).

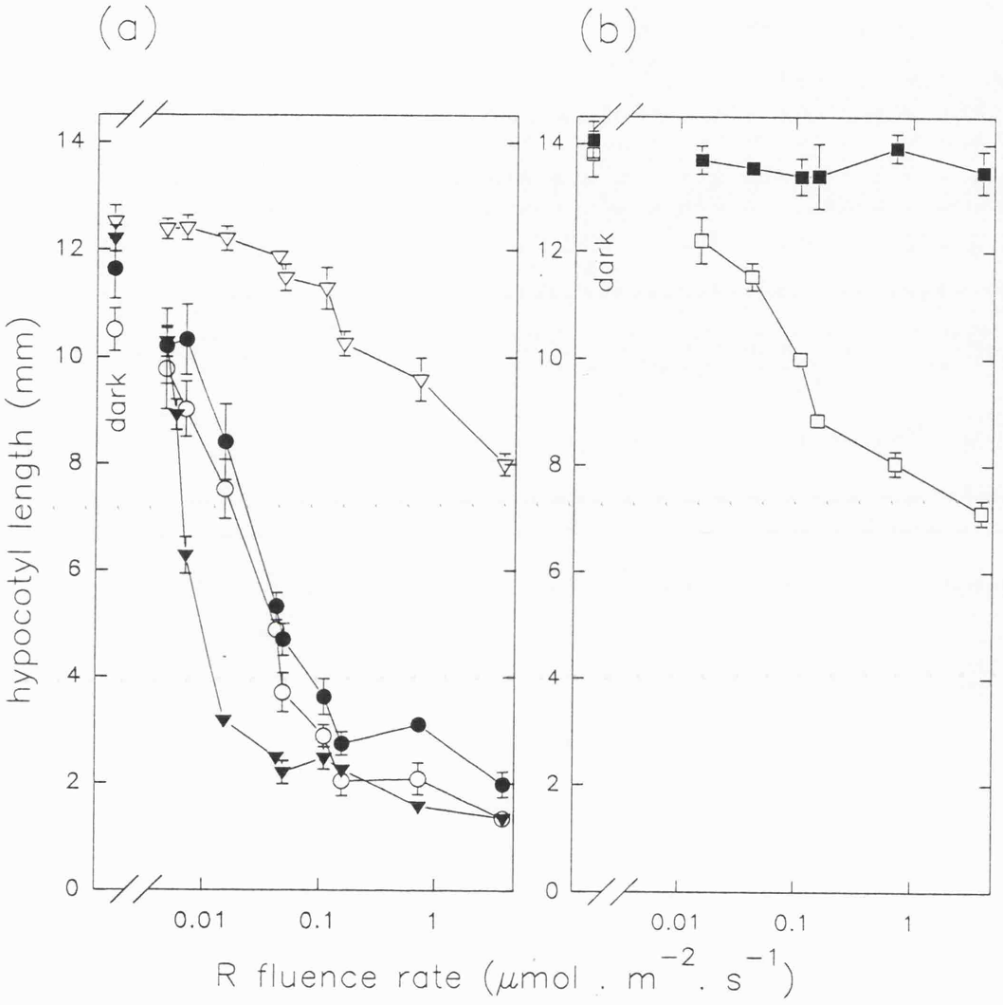


Figure 5.1 Inhibition of hypocotyl elongation growth under continuous red light (R). Photon fluence rate response curves are shown for etiolated seedlings of: (**a**) wild-type (Nossen) (*open triangle*), *phyB*-transformed lines RBO (*solid circle*) and ABO (*open circle*), *phyA*-transformed line 21K15 (*solid triangle*); (**b**) wild-type (Landsberg) (*open square*), *hy3* (*solid square*). Seedlings were grown in darkness for 1 day and then exposed to continuous broad-band R irradiation for a further 3 days prior to measurement of hypocotyl lengths. Results are the mean ± SEM from 3 independent experiments.

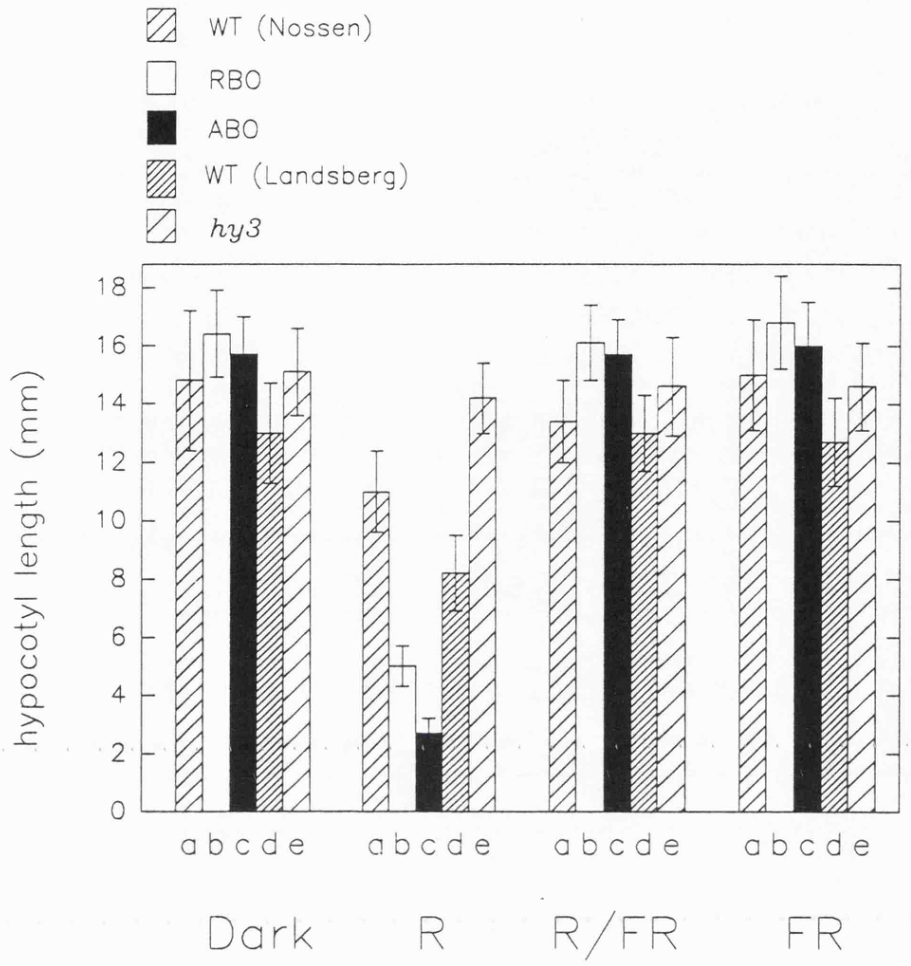


Figure 5.2 Inhibition of hypocotyl elongation growth under intermittent irradiation by red or far-red sources. Hypocotyl growth is shown for etiolated seedlings of (**a**) wild-type (Nossen), *phyB*-transformed lines (**b**) RBO and (**c**) ABO, (**d**) wild-type (Landsberg), and (**e**) *hy3*. Seedlings were germinated in darkness for 36 h and were subsequently maintained under continuous darkness (dark) or received pulse irradiation by red light (3 μmol m^{-2} s^{-1}) or far-red light (12 μmol m^{-2} s^{-1}). Light treatments consisted of: 5 min red light alone (R); 5 min red immediately followed by 10 min far-red (R/FR); or 10 min far-red alone (FR), and were delivered at 4 h intervals for 3.5 days. Values are the mean ± SEM of 50 seedlings.

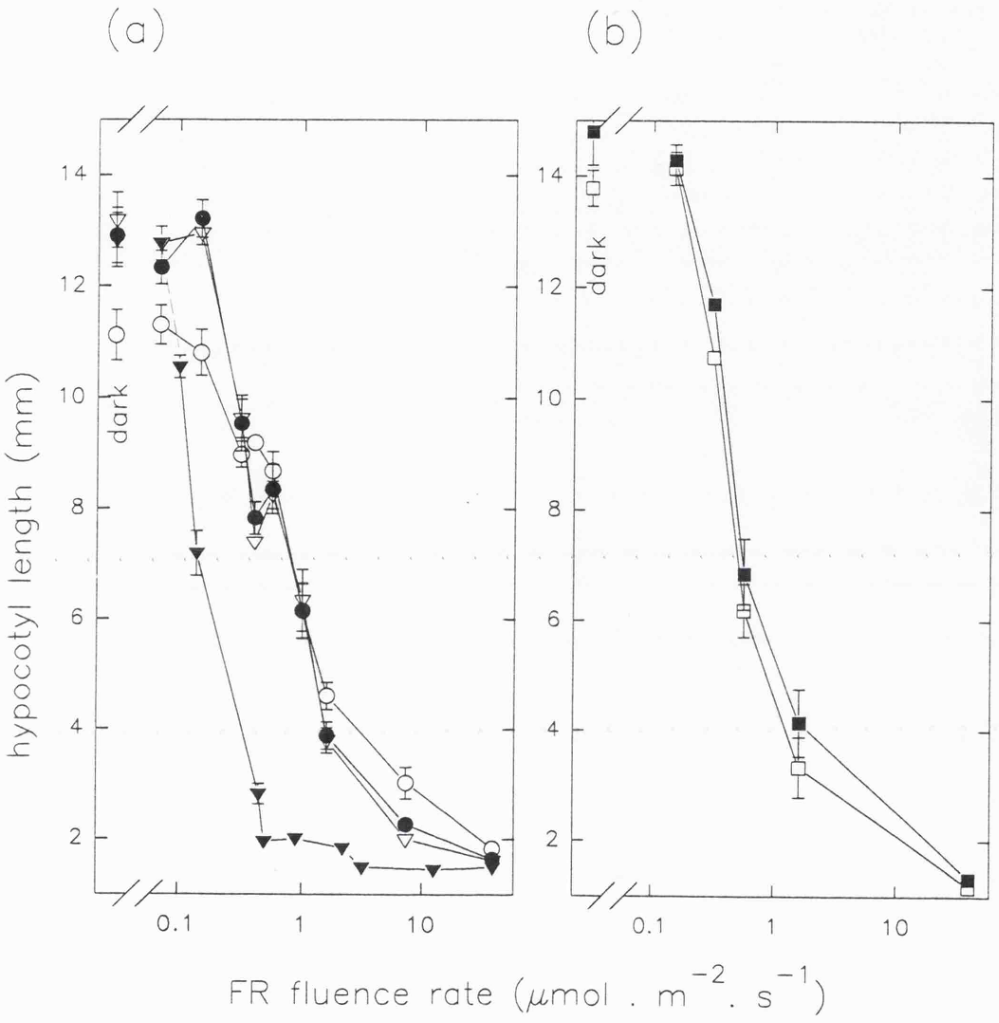


Figure 5.3 Inhibition of hypocotyl elongation growth under continuous far-red (FR) irradiation. Photon fluence rate response curves are shown for etiolated seedlings of: (**a**) wild-type (Nossen) (*open triangle*), *phyB*-transformed lines RBO (*solid circle*) and ABO (*open circle*), *phyA*-transformed line 21K15 (*solid triangle*); (**b**) wild-type (Landsberg) (*open sqquare*), *hy3* (*solid square*). Seedlings were grown in darkness for 1 day and then exposed to continuous broad-band FR irradiation for a further 3 days prior to measurement of hypocotyl lengths. Results are the mean ± SEM from 3 independent experiments.

Figure 5.4 The effect of light treatments to the ungerminated seed on the subsequent hypocotyl growth-rate (a-d) and final hypocotyl length (e-h) of dark-grown seedlings of wild-type (**a**, **e**), *phyB*-transformed lines RBO (**b**, **f**) and ABO (**c**, **g**), and *phyA*-transformed line 21K15 (**d**, **h**). The pre-imbibed and chilled seeds were exposed to a saturating pulse (1 mol m^{-2}) of white light (WL) or far-red (FR). Half of the seeds exposed to WL received a FR pulse 24 h later (WL/ FR). Seedlings were grown in complete darkness except during measurement under a dim green light. Hypocotyl lengths were measured at 24 h intervals and growth rate was calculated from the difference between consecutive measurements on day 2 and day 3 (d2-d3), and day 3 and day 4 (d3-d4). Final hypocotyl lengths were measured after 4 days growth.

hypocotyl growth rate (mm/24h)
final hypocotyl length (mm)
WL
WL/ FR
FR
d2–d3
d3–d4
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

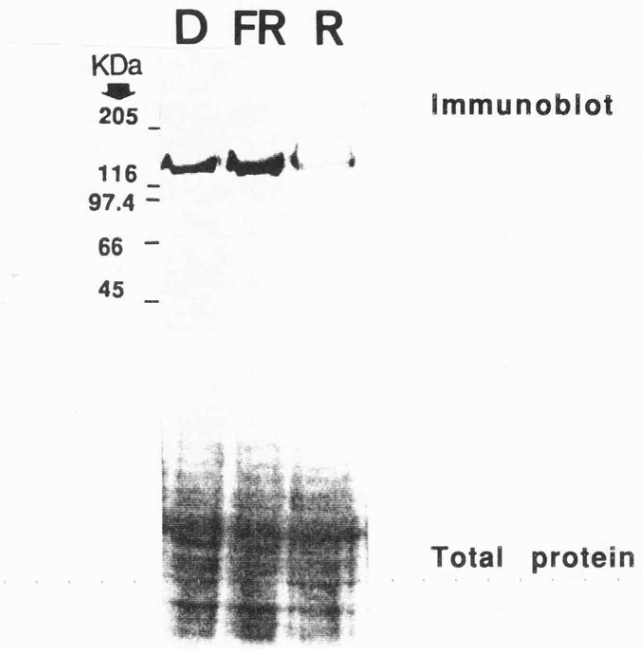


Figure 5.5 The effect of continuous red (R) or far-red (FR) irradiation on levels of oat-encoded phytochrome A in the etiolated seedlings of transgenic *Arabidopsis* (21K15). Seeds was sown onto 1% w/v agar and germinated in the dark for 4 days. Seedlings were irradiated for 24 h by broad band R (4 μmol m^{-2} s^{-1}) or FR (40 μmol m^{-2} s^{-1}) light-sources, or were maintained in the dark. Extracts were made at the end of the irradiation period and probed for phytochrome using a monoclonal antibodies raised against etiolated oat phytochrome.

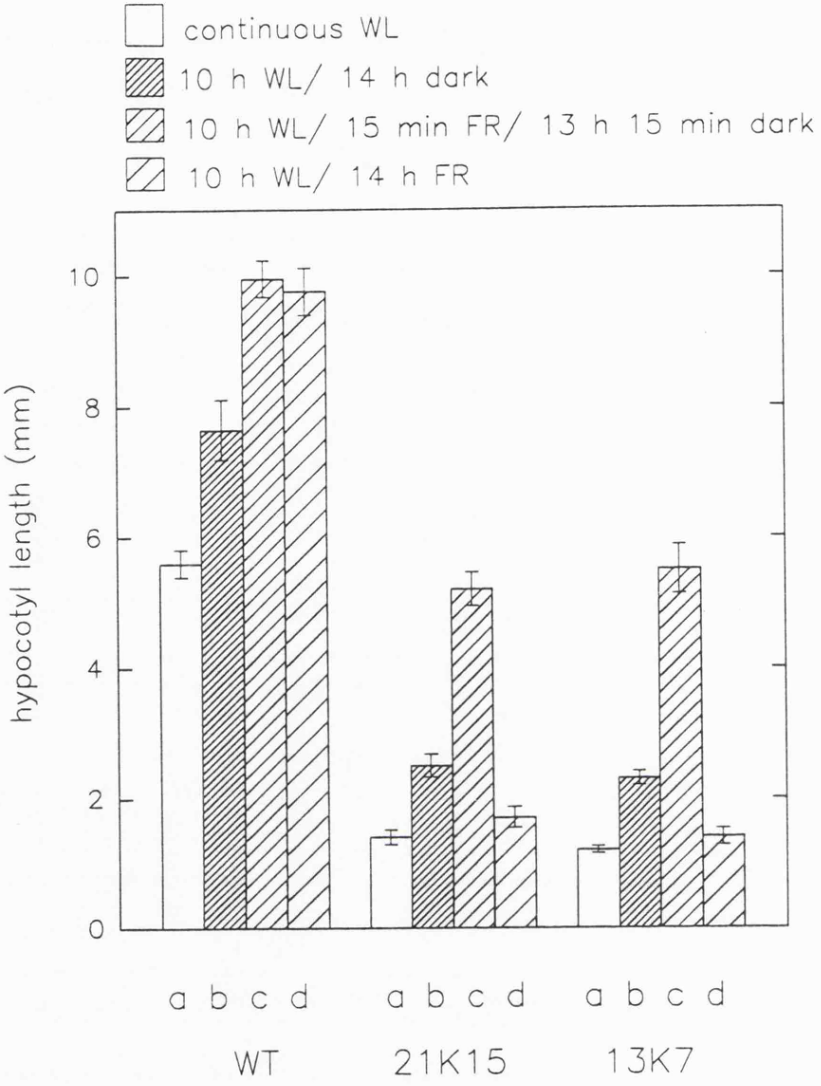


Figure 5.6a Effect of end-of-day far-red (FR) on hypocotyl elongation growth in light-grown seedlings of wild-type and *phyA*-transformed 21K15 and 13K7 lines. Seedlings were grown in continuous white light (WL) for 2 days prior to growth for 4 days under day/ night cycles. The day consisted of 10 h WL; night treatments consisted of (**a**)14 h WL; (**b**) 14 h darkness; (**c**) 15 min FR followed by13 h 45 min darkness; (**d**) 14 h FR.

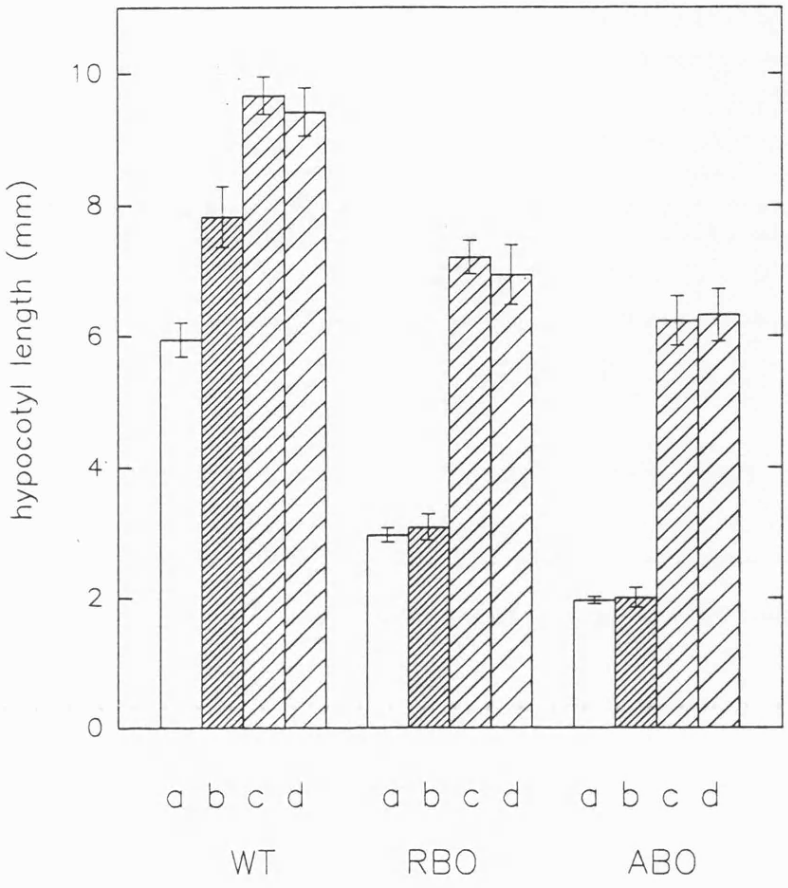


Figure 5.6b Effect of end-of-day far-red (FR) on hypocotyl elongation growth in light-grown seedlings of wild-type and *phyB*-transformed RBO and ABO lines. Seedlings were grown in continuous white light (WL) for 2 days prior to growth for 4 days under day/ night cycles. The day consisted of 10 h WL; night treatments consisted of (**a**)14 h WL; (**b**) 14 h darkness; (**c**) 15 min FR followed by13 h 45 min darkness; (**d**) 14 h FR.

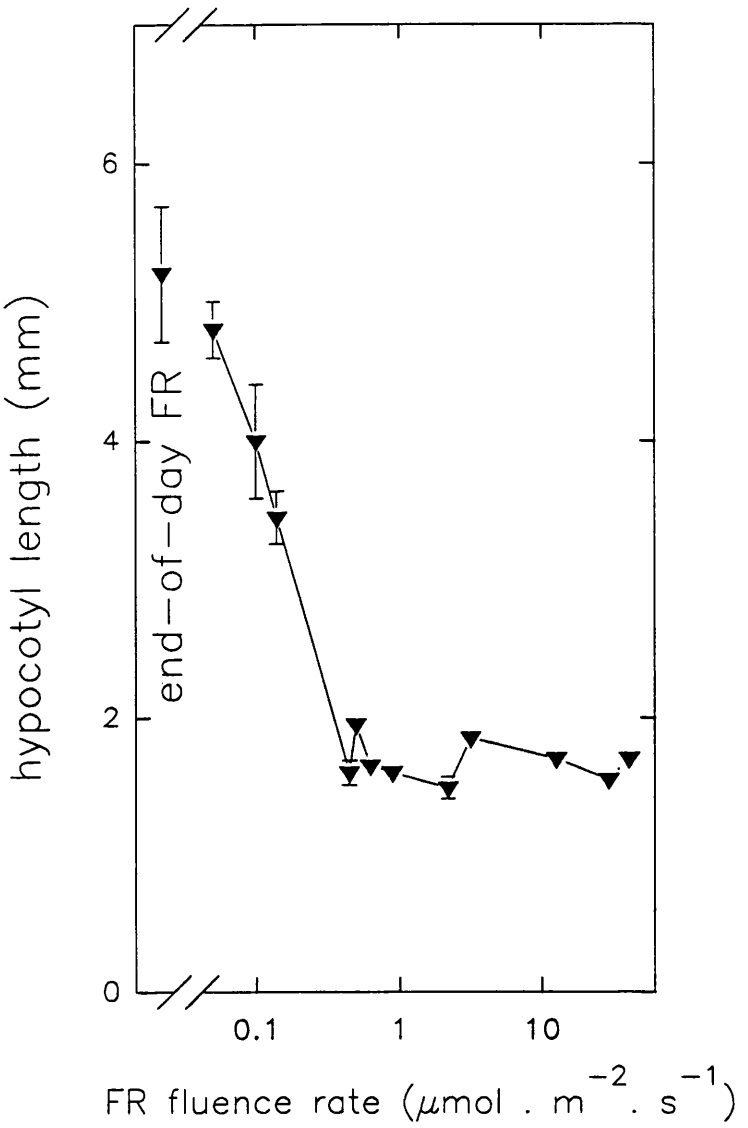


Figure 5.7 Photon fluence rate response relationship for the effect of prolonged far-red (FR) on hypocotyl elongation in light-grown seedlings of *phyA*-transformed *Arabidopsis* (21K15). Seeds were grown for 2 days in continuous white light (WL) and then for 4 days in cycles of 10 h WL followed by 14 h continuous FR at different photon fluence rates. In all cases, the 10 h WL period was foilowed by 15 min of high photon fluence rate FR (40 μmol m^{-2} s^{-1}) to ensure rapid attainment of photoequilibrium. For the data value marked as "end-of-day FR" seedlings received 15 min end-of-day FR followed by 13 h 45 min darkness.

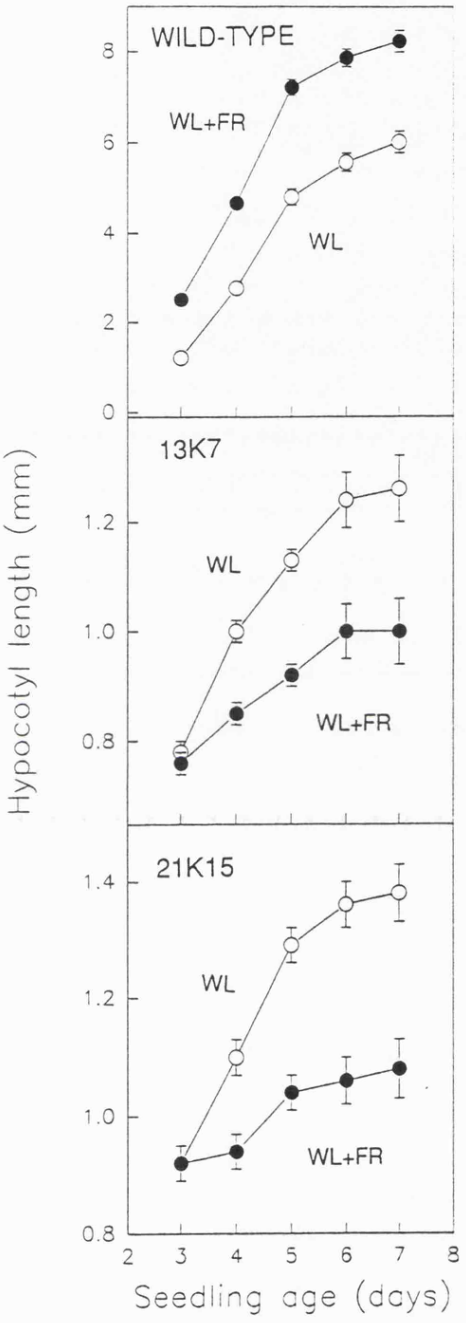


Figure 5.8 Hypocotyl lengths of light-grown seedlings of (**a**) wild-type (WT), (**b**) *phyA*-transgenic 13K7, and (**c**) *phyA*-transgenic 21K15 *Arabidopsis* grown under white light (WL, R:FR = 6.8) (*open symbols*) or WL with added far-red radiation (WL+FR, R:FR = 0.07) (*solid symbols*).

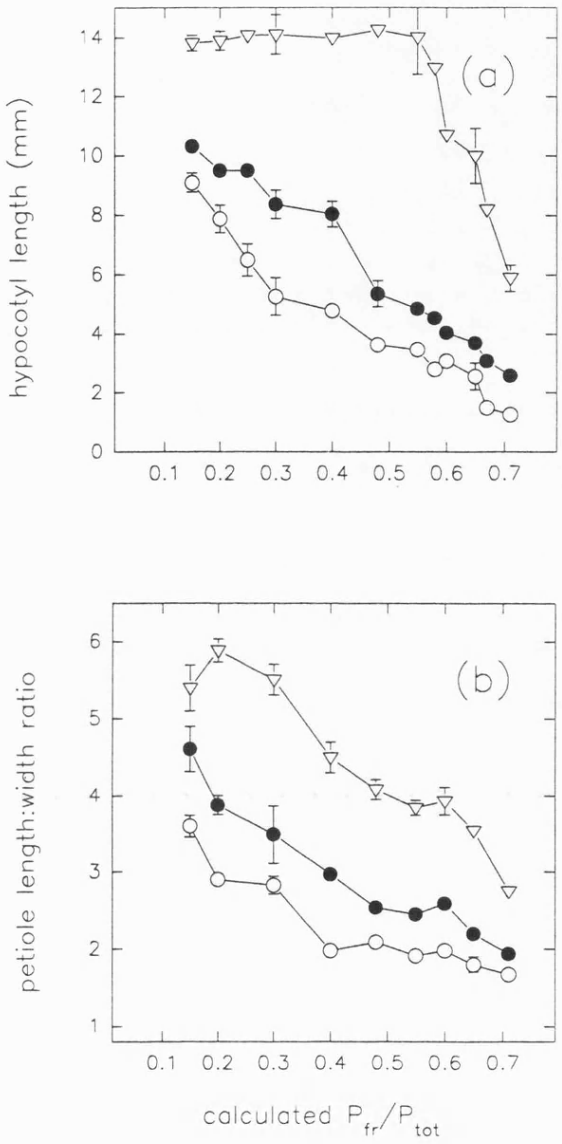


Figure 5.9 Growth responses of light-grown seedlings to the photoequilibrium (P_{fr}/P_{tot}) established by the red:far-red quantum ratio (R:FR) of an overhead light-source. (**a**) hypocotyl length and (**b**) petiole extension growth (measured as the ratio of total length over leaf-width) were measured in seedlings of wild-type (*open triangle*) and *phyB*-transformed lines RBO (*solid circle*) and ABO (*open circle*). Various quantities of supplementary FR radiation were added to a background of continuous white light (photosynthetically active radiation, 400-700nm = 60 μmol m^{-2} s^{-1}) in order to reduce R:FR. The phytochrome photoequilibrium (P_{fr}/P_{tot}) established under each R:FR ratio was calculated from spectrophotometric measurements of an irradiated sample of purified etiolated-oat phytochrome.

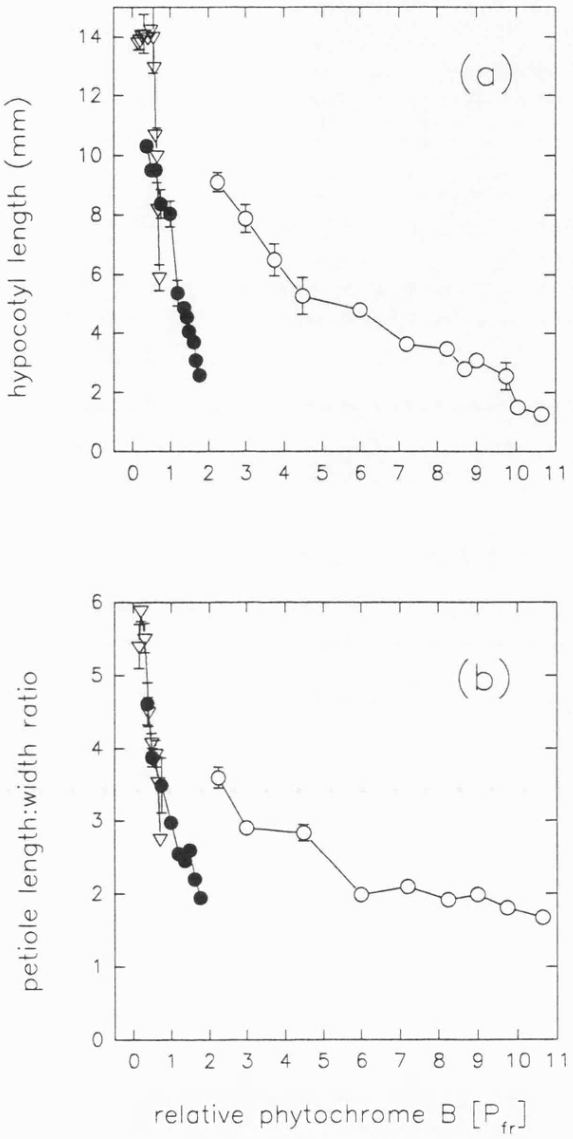


Figure 5.10 (**a**) hypocotyl growth and (**b**) petiole extension growth of light-grown seedlings of wild-type (*open triangle*) and *phyB*-transformed lines RBO (*solid circle*) and ABO (*open circle*) shown as a function of calculated absolute levels of the far-red absorbing form (P_{fr}) of phytochrome B. Growth measurements are those shown in Fig. 5.9. The corresponding values of phytochrome B [P_{fr}] were calculated from the phytochrome photoequilibrium (see Fig. 5.9) using the estimated relative levels of total phytochrome B in the three lines i.e. wild-type: RBO: ABO = 1: 2.5: 15 (Wagner *et al.*, 1991).

Table 5.1. Schematic summary of the responsiveness of mutant and transgenic lines of *Arabidopsis* to continuous red (R) or continuous far-red (FR) irradiation.

Arabidopsis line	Phytochrome levels[1,2]	Responsiveness[2]	
		R	FR
wild-type	$A^{+}B^{+}$	+	+
AOX[3]	$A^{++}B^{+}$	+ +	+ +
BOX[3]	$A^{+}B^{++}$	+ +	+
hy3	$A^{+}B^{-}$	-	+
hy8[4]	A-B+	+	-

[1]A = phytochrome A; B = phytochrome B.

[2]+ = wild-type; ++ = enhanced; - = deficient.

[3]AOX = transgenic phytochrome A overexpresser;
BOX = transgenic phytochrome B overexpresser

[4](Parks and Quail, 1993)

Chapter 6

A comparison of the photoresponses of transgenic lines of *Arabidopsis* expressing introduced phytochrome B-encoding cDNAs.

Introduction

The physiological analysis of transgenic *Arabidopsis* lines expressing an introduced phytochrome B-encoding cDNA has demonstrated an increased sensitivity of response to red light (Wagner *et al.*, 1991; McCormac *et al.*, 1993). The interpretation of the photophysiological characteristics of the transgenic plants in terms of a direct effect of the overexpressed phytochrome B population can be supported by the repetition of results between numerous independent transformed lines. The responses to red and far-red light of etiolated seedlings of eight such transgenic lines, expressing introduced rice-*phyB*-cDNA or *Arabidopsis*-*phyB*-cDNA, are compared here with WT and with each other.

A comparison of the responses of *phyB*-transgenic plants to reductions in the R:FR ratio with those of wild-type (WT) plants revealed poor quantitative agreement of response with the calculated levels of total phytochrome B-[P_{fr}] (Chapter 5 of this thesis; McCormac *et al.*, 1993). A possible explanation for this result was proposed to be the (assumed) ectopic expression of the chimeric CaMV 35S promoter/*phyB*-cDNA construct within the transgenic plants, resulting in a discrepancy between total levels of phytochrome in transgenic seedlings and the size of the physiologically-active pool. For example, under the control of the constitutive 35S promoter, expression of the introduced

phyB-cDNA may result in the accumulation of phytochrome B in cells which do not express the endogenous *phyB*-gene; such cell types may not possess the capacity for transduction of the phytochrome signal, or the phytochrome molecule itself may be inactive due to lack of chromophore-binding or instability of the polypeptide. Transgenic plants expressing the same introduced 35S/cDNA construct, but to different levels, are subject to the same potential for ectopic expression. The work described here attempts to establish the relationship between the levels of phytochromeB-$[P_{fr}]$ in the various isogenic transgenic lines and the response of those lines to continuous R and to R:FR ratio. Two species of *phyB*-cDNAs, isolated from green plants of rice and *Arabidopsis*, were used to generate the different transgenic lines. It is conceivable that the heterologous rice-cDNA-encoded and homologous *Arabidopsis*-cDNA-encoded phytochrome species exhibit different levels of physiological activity within the *Arabidopsis* host plants. The transformed lines containing each species of introduced cDNA are therefore considered here as two separate sets of plants.

Materials and methods

Plant material

Seeds used were of wild-type (WT) *Arabidopsis thaliana* (L. Heynh) ecotype Nossen, and eight homozygous isogenic lines that had been transformed with either rice-*phyB*-cDNA or *Arabidopsis-phyB*-cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Wagner *et al.,* 1991). Transgenic lines expressing rice-*phyB*-cDNA are referred to as RBO (see also chapter 5 of this thesis; McCormac *et al.,* 1993), A7, K18, and H3. Transgenic lines expressing *Arabidopsis-phyB*-cDNA are referred to as ABO (see also Chapter 5 of this thesis, McCormac *et al.*, 1993), Z15, 12X and D2. The levels of expression of the introduced *phyB*-cDNA were quantitated by the relative levels of immunodetectable total phytochrome B in the light-grown plants. Seeds of each transgenic line (kindly supplied by D. Wagner and P.H. Quail, U.C. Berkeley, CA., USA.) were germinated on kanamycin-supplemented medium and grown-on in potting compost for the production of seed to be used in these experiments.

Experimental treatments and conditions of plant growth were as descibed in Chapter 5 of this thesis (*Materials and methods, Plant material and growth conditions*).

Measurements of seedling hypocotyl length and petiole extension growth were made in the way described in Chapter 5 of this thesis (*Materials and methods, Measurement of seedling hypocotyl length and petiole growth*).

Light sources

Light sources providing broad-band red (R) or far-red (FR) were the same as those described in chapter 5 of this thesis (*Materials and methods, Light sources*). The R:FR ratio treatment cabinets used were of the same design as described in detail in Chapter 3 of

this thesis *(Materials and methods, light sources, "high PAR cabinets")*. In order to provide a gradient of supplementary FR radiation against a uniform background of white light (photosynthetically active radiation 400-700nm = 60 μmol m^{-2} s^{-1}), a selective filter was constructed of a 1 cm deep layer of copper sulphate solution of various concentrations (0-2% w/v). Phytochrome and photoequilibria values (P_{fr}/P_{tot}) were calculated from corrected spectrophotometric measurements (Butler *et al.*, 1964) of an irradiated sample of purified etiolated-oat phytochrome.

Results and discussion

The aim of this study was to investigate the relative photophysiological effects of expression of an introduced phytochrome B-encoding cDNA in several isogenically-transformed lines of *Arabidopsis*. Different levels of expression of either an introduced heterologous (rice) or homologous (*Arabidopsis*) *phyB*-cDNA within each transgenic line produced seedlings with a range of levels of total phytochrome B. The levels of expression of the heterologous *phyB*-cDNA have been quantified by the relative levels of total phytochrome B in whole-plant extracts of the light-grown plants. The transgenic lines expressing rice-*phyB*-cDNA, A7, RBO, K18 and H3, contained levels of immunodetectable phytochrome B in the respective ratios of 1: 2 : 3 : 3[1]; compared to WT these transgenic lines contained 1.25-fold (A7), 2.5-fold (RBO) and 3.8-fold (K18, H3) levels of total phytochrome B[1]. The transgenic lines expressing *Arabidopsis-phyB*-cDNA, Z15, 12X, ABO and D2, contained levels of immunodetectable phytochrome B in the ratio of 3 : 4 : 6 : 12[1] respectively; compared to WT these transgenic lines contained 7.5-fold (Z15), 10-fold (12X), 15-fold (ABO) and 30-fold (D2) levels of total phytochrome B[1] (D. Wagner, U.C. Berkeley, CA., USA., personal communication).

Response of etiolated seedlings to far-red light

Hypocotyl growth of the etiolated seedlings of WT and transgenic lines was markedly inhibited under continuous FR (photon fluence rate 700-800 nm = 40 μmol m^{-2} s^{-1}) compared to dark growth rate (Fig. 6.1a, b). The growth-response was dependent on the fluence rate of the radiation received; all of the rice-*phyB*-transformed lines and

[1] N.B. These values were taken from preliminary data of immunoblots and work is currently in progress to refine them. Therefore all interpretations need to be made with the reservation that calculations of total phytochrome B and phytochrome B [P_{fr}] may be subject to error of, as yet, unknown magnitude.

Arabidopsis-phyB-transformed lines used here showed a relationship of growth-inhibition with FR fluence-rate which was not significantly different from WT (Fig. 6.1a, b). Therefore, even a 30-fold increase in levels of total WT phytochrome B (as measured in the *Arabidopsis-phyB*-transformed line, D2) failed to mediate an enhanced sensitivity of response to low fluence-rates of FR.

Response of etiolated seedlings to red light

Hypocotyl growth of the dark-grown seedlings of the WT and *phyB*-transgenic lines was inhibited under continuous R (photon fluence rate 600-700 nm = 4 μmol m^{-2} s^{-1}). All of the phytochrome B-overexpressing lines, with the exception of Z15 (which expressed the introduced cDNA to the lowest level of the set of four *Arabidopsis-phyB*-transformed lines), were dwarfed under R light compared to WT seedlings, and exhibited increased sensitivity of the growth-response to low fluence rates of R (Fig. 6.2a, b).

The four *Arabidopsis* lines transformed with rice-*phyB*-cDNA, under a given fluence rate of R, displayed essentially similar levels of inhibition to one another. The three lines expressing the introduced *Arabidopsis-phyB*-cDNA, which showed a dwarfed phenotype relative to WT (i.e. 12X, ABO, D2), also did not display significantly different fluence rate-response relationships compared to one another (Fig. 6.2a). Thus, within the range of levels of phytochrome B exhibited by the transgenic lines used here, no significant correlation could be drawn between the degree of R-mediated response and relative levels of phytochrome B overexpression.

Therefore, while it seems clear that a transgenically-overexpressed population of phytochrome B can mediate response to R, it has not been possible to demonstrate a continuum of phenotypic expression in response to increasing levels of phytochrome B. This result is in apposition to the evidence from the *hy3* (*phyB*) mutation of *Arabidopsis* which displays semi-dominance, i.e. the *hy3/HY3* heterozygote has a long hypocotyl,

intermediate in length between WT and *hy3/hy3*. The simplest interpretation of semi-dominance of a loss-of-function allele is that the WT allele is needed in two copies to confer the WT phenotype and there is a quantitative relationship between level of gene-product and phenotype. Thus, the genetic evidence tends to argue that hypocotyl length is linearly related to phytochrome B [P_{fr}], at least where phytochrome B levels are being reduced relative to WT. Possible explanations for the apparent lack of relationship of the response in transgenic plants with calculated levels of phytochrome B may involve the range of phytochrome B levels which were tested. Most simply the relationship with R fluence rate of the transgenic seedlings may correspond to a saturated phytochrome B-mediated response. A possible alternative is that the relationship of R-mediated response with phytochrome B is not linear; a logarithmic relationship, for example, would require a 10-fold incremental increase in phytochrome B levels (cf. a maximum of 4-fold difference within the transgenic lines used here) in order for a significant phenotypic effect to be observed. Indeed, much of the early data of the response of dark-grown seedlings to brief light treatments (e.g. Hillman, 1965; 1966; Loercher, 1966) has been subsequently shown to describe a relationship of response with P_{fr} concentration which could be plotted on a log-linear basis (Mandoli and Briggs, 1981). Anthocyanin synthesis in mustard, however, has been reported to show a biphasic-linear relation to P_{fr} levels (Drumm and Mohr, 1974; Steinitz *et al.*, 1979). This form of a relationship is expressed as a much greater degree of sensitivity to changes in [P_{fr}] at low [P_{fr}] than at higher [P_{fr}], and would also, therefore, be consistent with the observed sensitivity of R-response in the *hy3/hy3*, *hy3/HY3* mutant genotypes and *phyB*-transformed seedlings compared to WT, but an absence of a detectable effect of increasingly higher levels of phytochrome B for comparisons within the trangenic lines. A third type of relation of response with levels of P_{fr} has been shown to require a critical threshold value of P_{fr} and hence no graded response was observed in this situation

(Oelze-Karow and Mohr, 1970; 1973).

Response of light-grown seedlings to R:FR ratio

Light-grown plants of all eight transgenic lines expressing introduced rice-*phyB*-cDNA or *Arabdiopsis-phyB*-cDNA responded to reductions in R:FR ratio (achieved by the addition of increasing quantities of FR radiation to a background of white light) by an acceleration of elongation growth of the hypocotyls (Fig. 6.3a, 6.4a) and petioles (Fig. 6.3c, 6.4c). The growth response to R:FR ratio of the transgenic plants expressing introduced rice-*phyB*-cDNA were not significantly different between the four individual lines (Fig. 6.3a, c). The levels of phytochrome B-P_{fr} were calculated under each R:FR light treatment according to the relative levels of total phytochrome B in each line. Figures 6.3b and 6.3d show an apparently significant difference in the growth responses of the four transgenic lines to a given level of calculated phytochrome B-[P_{fr}]. The growth responses to R:FR ratio of the transgenic lines expressing *Arabidopsis-phyB*-cDNA did appear to show some degree of divergence between the four lines (Fig. 6.4a, c). Calculations of relative P_{fr} levels indicated that this divergence of response could be partially, in the case of hypocotyl length (Fig. 6.4b), or fully, in the case of petiole growth (Fig. 6.4d), related to levels of expression of the introduced *phyB*-cDNA.

In general it was not possible accurately to relate phenotypic response as a continuous function of levels of phytochrome B-P_{fr} between the individual transgenic lines. Calculations of P_{fr} levels under each light source were based on photoequilibria values measured in a purified sample of etiolated oat-phytochrome. Therefore the relationship in Figures 6.3a, c and 6.4a, c would only be strictly true if the phytochrome molecules responsible for the modulation of the growth responses were exposed directly to the actinic radiation. Within the plant the photoreceptor molecules are unlikely to be

located solely in the outer epidermal layers, and therefore the presence of chlorophyll in the internal cells will modify the perceived spectral photon distribution. The effects of self-absorption do not appear significantly to affect the linearity of the relationship of response with phytochrome status, but differences in the level of chlorophyll through which light is filtered affect the actual photoequilibrium established (Morgan and Smith, 1978). Therefore it is conceivable that differences in internal chlorophyll screening effects could influence the relative positions of the gradients in Figure 6.3b, d and 6.4b, d. As a result of the linearity of the plots of response versus P_{fr}/P_{tot} ratio (Morgan and Smith, 1976; 1978), it has generally been assumed that the shade-avoidance-responses are directly related to levels of P_{fr}. However if instead of a linear relationship with P_{fr} concentration, a logarithmic relationship (for example) was a more accurate representation of the relationship of response with levels of the transgenically overexpressed phytochrome B-Pfr, it might be expected that increases in total phytochrome B of an order of magnitude would be required to produce an incremental effect on phenotype. Such a scenario would be consistent with the rice-*phyB*-cDNA-expressing lines which displayed a range of expression of only 1.3- to 3.8-fold WT-levels and showed no significant divergence of phenotype from one another (Fig. 6.3a, c). In contrast, in the transgenic lines expressing introduced *Arabidopsis-phyB*-cDNA overexpression of phytochrome B was in the range of 7.5- to 30-fold WT-levels and associated with these levels was a significant phenotypic effect between the individual transgenic lines (Fig. 6.4a, c); the data in figure 6.4d seem to suggest a log-linear relationship. Another possibility is that the inability to relate response to levels of phytochrome B may be an indication that one or more light-stable phytochrome species, in addition to phytochrome B, are responsible for mediating the responses of light-grown plants to R:FR ratio.

The attempt described above to quantify the responses of light-grown plants to reductions in R:FR ratio in terms of levels of phytochrome B has been based on the

assumption that P_{fr} is the sole active molecular form. Although this appears to be a widely accepted concept generated by the early experiments of Hendricks and Borthwick (Borthwick *et al.,* 1952a) there is little direct evidence for this conclusion in light-grown plants, and in fact for continuous light treatments the argument has been advanced that there is some form of interactive and opposing action of P_{fr} and P_r in light-grown plants (Smith, 1981; 1983; 1986); such a scenario would seem to fit well with the data in figure 6.3, i.e. response is related to the ratio of P_r:P_{fr} irrespective of P_{total}, but is not consistent with the results in figure 6.4.. It is known that at high fluence rates short-lived photoconversion intermediates between P_r and P_{fr} accumulate to significant proportions (Kendrick and Spruit, 1972; 1973; Smith *et al.*, 1988; Smith and Fork, 1992) and hence at constant R:FR the concentration of P_{fr} will be considerably lower at high, than at low, fluence rates. On this premise, growth response to R:FR ratio, if determind solely by P_{fr} concentration, should be confounded by changes in P_{fr} level mediated by fluence rate, whereas shade-avoidance responses have been shown to be compensated for fluence-rate changes (Child and Smith, 1987; Smith and Hayward, 1985; Smith, 1990b); it should be noted, however, that all measurements of photointermediates have been restricted to phytochrome A and attempts to extend such observations to the light-stable phytochrome species have so far been unsuccessful (Smith and Fork, 1992). Interpretation of the results presented in this chapter may be viewed as adding further support for the conclusion that R:FR ratio perception is not a simple function of absolute P_{fr} concentration but involves the interaction of some other active component of the phytochrome system.

Conclusions

It has been previously reported that overaccumulation of phytochrome B through the expression of a transgene leads to increased sensitivity of the etiolated seedlings to R light, while response to FR light remains unchanged from WT. Here, this result has been shown to be a consistent trait in several independent lines of transgenic *Arabidopsis*.

A comparison of the phenotype of transgenic plants with WT and a *phyB*-mutant (*hy3*) suggested a continuum of R-mediated response with respect to levels of phytochrome B (McCormac *et al.*, 1993; Chapter 5 of this thesis). From a comparison of the phenotype between several isogenic transgenic lines, however, growth-response under R did not appear to be directly related to the levels of expression of the introduced *phyB*-cDNA. The shade-avoidance-responses of light-grown plants to R:FR ratio, also, did not conform to a simple linear function of phytochrome B-P_{fr} levels. Since the introduced *phyB*-cDNA in all transgenic lines studied here were expressed under the control of the same viral promoter it seems reasonable to assume that even in the event of ectopic expression of the introduced phytochrome in physiologically-inactive cells, the relative levels of expression in physiologically-active cells would be proportional to the overall levels of phytochrome expression between the transgenic lines. It cannot, however, be ruled out that the introduced polypeptide was selectively unstable in certain cell-types, and in this case the phytochrome levels measured in whole seedling extracts may not reflect the relative size of the physiologically-active pools between transgenic plants. Another conceivable failing of this transgenic plant system may be that, in the presence of phytochrome B levels in excess of wild-type, a secondary component of the phytochrome-system may be limiting to the ultimate induction of physiological response. In the absence of evidence to substantiate the above "trivial' explanations for the observed lack of correlation of response in the transgenic plant with calculated levels of phytochrome B [P_{fr}], it is equally valid to consider interpretations of the data on the

premise that the necessary assumptions for unrestricted activity of the measured phytochrome pool hold true. These results suggest a more complex system for phytochrome-mediation of response, possibly in terms of the mathematical relationship describing the relationship of response with phytochrome B-P_{fr} or, perhaps, involving the co-action of one or more other light-stable molecular species of phytochrome. Alternatively these results may be interpreted as adding to the evidence in conflict with the concept of P_{fr} as the sole active component of the phytochrome system.

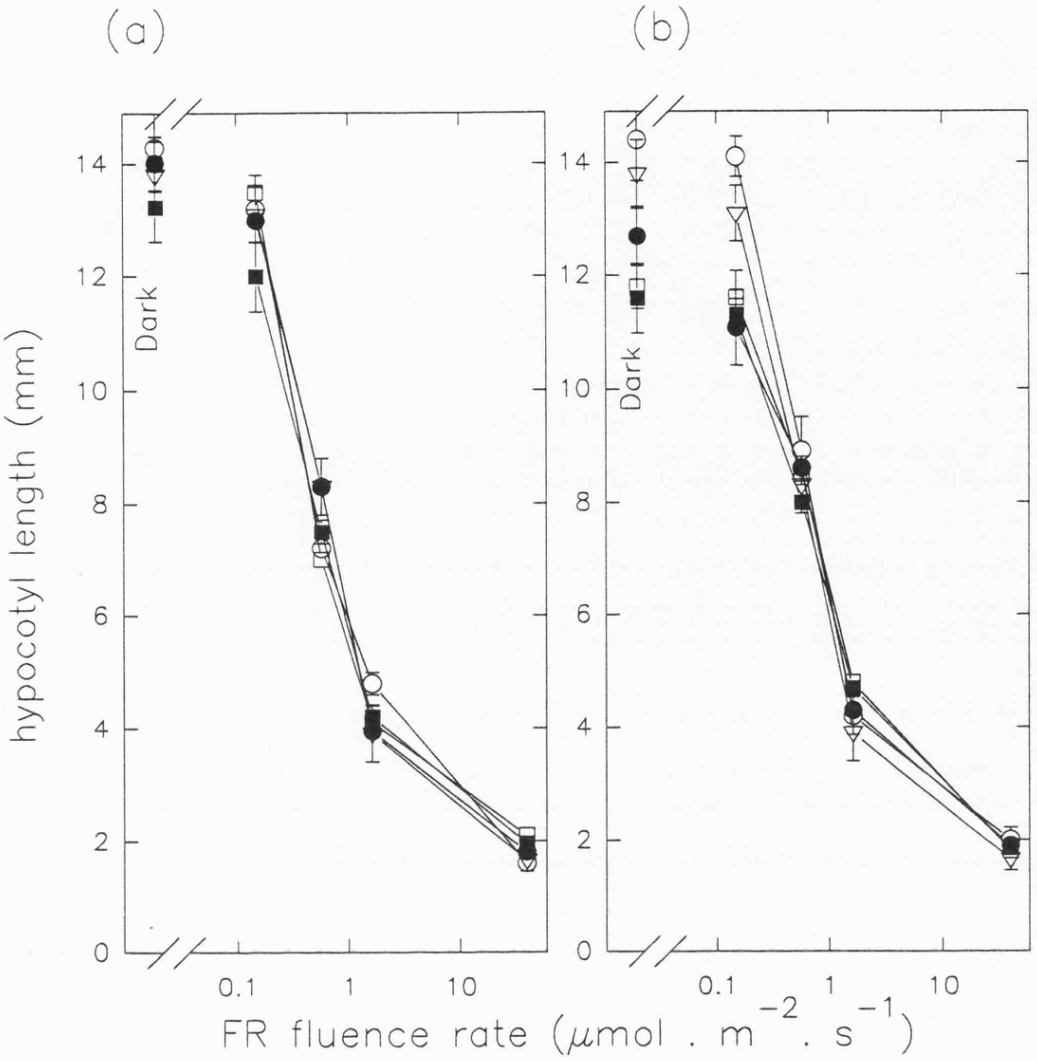


Figure 6.1 Inhibition of hypocotyl elongation growth under continuous far-red light (FR). Photon fluence rate response curves are shown for etiolated seedlings of: (**a**) wild-type (Nossen) (*open triangle*), rice-*phyB*-cDNA transformed lines A7 (*open circle*), RBO (*solid circle*), K18 (*open square*) and H3 (*solid square*); transgenic lines contained 1.25-fold (A7), 2.5-fold (RBO) and 3.8-fold (K18, H3) levels of total phytochrome B relative to WT: (**b**) wild-type (Nossen) (*open triangle*), *Arabidopsis-phyB*-cDNA transformed lines Z15 *(open circle*), 12X (*solid circle*), ABO (*open square*) and D2 (*solid square*); transgenic lines contained 7.5-fold (Z15), 10-fold (12X), 15-fold (ABO) and 30-fold (D2) levels of total phytochrome B relative to WT (D. Wagner, personal communication). Seedlings were grown in darkness for 1 day and then exposed to continuous broad-band FR irradiation for a further 3 days prior to measurement of hypocotyl lengths. Results are the mean ± SEM from 3 independent experiments.

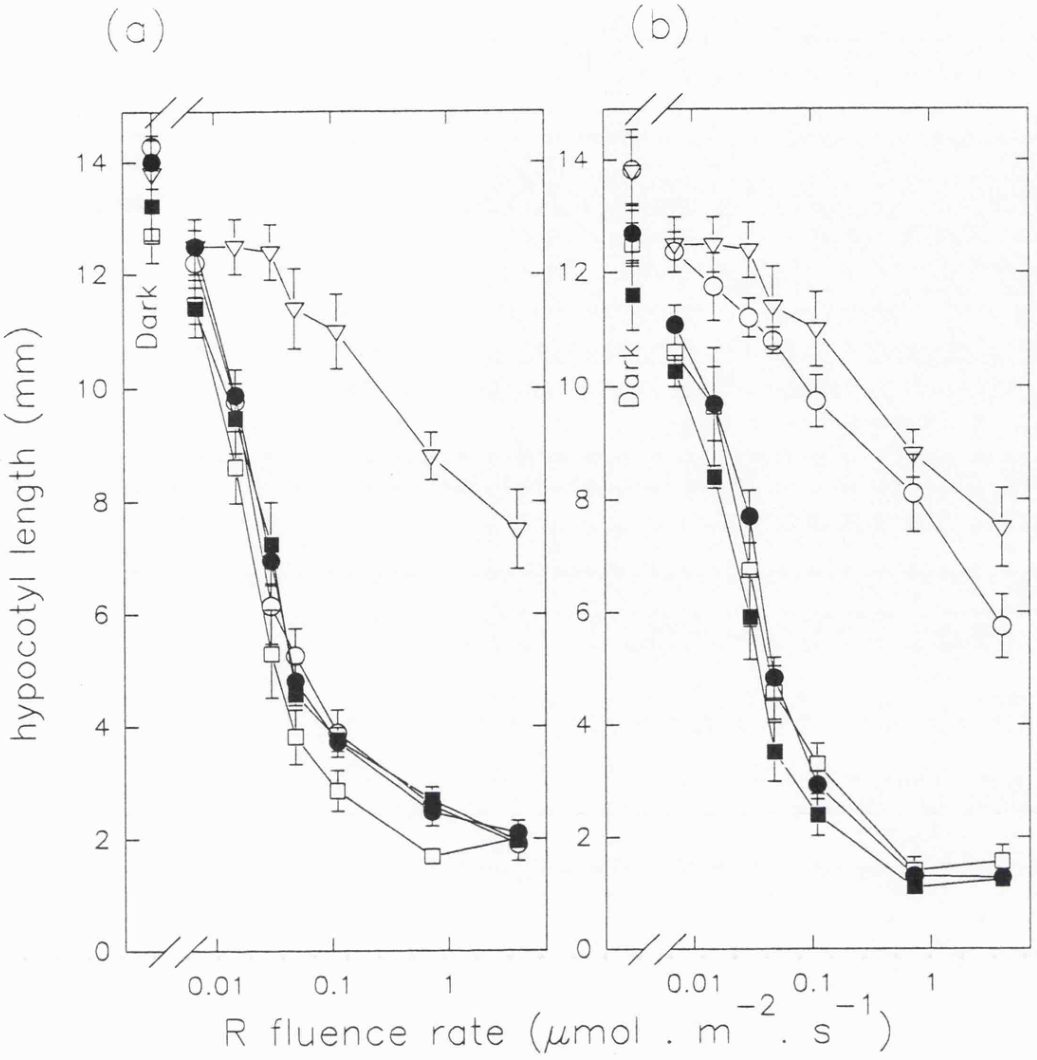


Figure 6.2 Inhibition of hypocotyl elongation growth under continuous red light (R). Photon fluence rate response curves are shown for etiolated seedlings of: (**a**) wild-type (Nossen) (*open triangle*), rice-*phyB*-cDNA transformed lines A7 (*open circle*), RBO (*solid circle*), K18 (*open square*) and H3 (*solid square*); transgenic lines contained 1.25-fold (A7), 2.5-fold (RBO) and 3.8-fold (K18, H3) levels of total phytochrome B relative to WT: (**b**) wild-type (Nossen) (*open triangle*), *Arabidopsis-phyB*-cDNA transformed lines Z15 (*open circle*), 12X (*solid square*), ABO (*open square*) and D2 (*solid square*); transgenic lines contained 7.5-fold (Z15), 10-fold (12X), 15-fold (ABO) and 30-fold (D2) levels of total phytochrome B relative to WT (D. Wagner, personal communication). Seedlings were grown in darkness for 1 day and then exposed to continuous broad-band R irradiation for a further 3 days prior to measurement of hypocotyl lengths. Results are the mean ± SEM from 3 independent experiments.

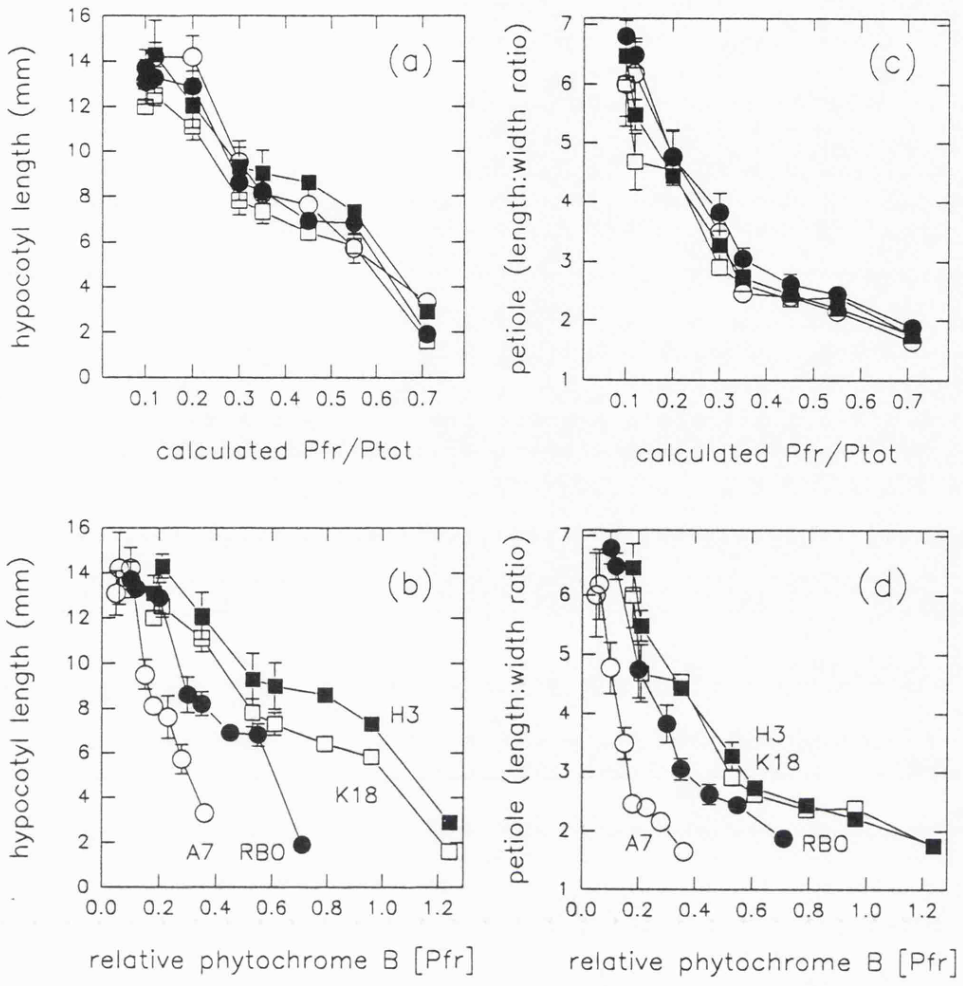


Figure 6.3 Growth of light-grown seedlings in response to the phytochrome photoequilbrium (P_{fr}/P_{tot}) established under different red:far-red quantum ratios. Hypocotyl length (**a**), and petiole extension growth (measured as the ratio of length over leaf-width) (**c**), were measured in seedlings of rice-*phyB*-transformed lines A7, (*open circle*), RBO (*solid circle*), K18 (*open square*) and H3 (*solid square*). Hypocotyl growth (**b**) and petiole extension growth (**d**) in light-grown seedlings expressing rice-*phyB*-cDNA are shown as a function of calculated relative levels of the far-red absorbing form (P_{fr}) of phytochrome B. Growth measurements are those shown in (a) and (c). The corresponding values of phytochrome B [P_{fr}] were calculated from the phytochrome photoequilibrium using the estimated relative levels of total phytochrome B in the four lines ie. A7: RBO: K18: H3 = 1: 2: 3: 3 (D. Wagner, personal communication).

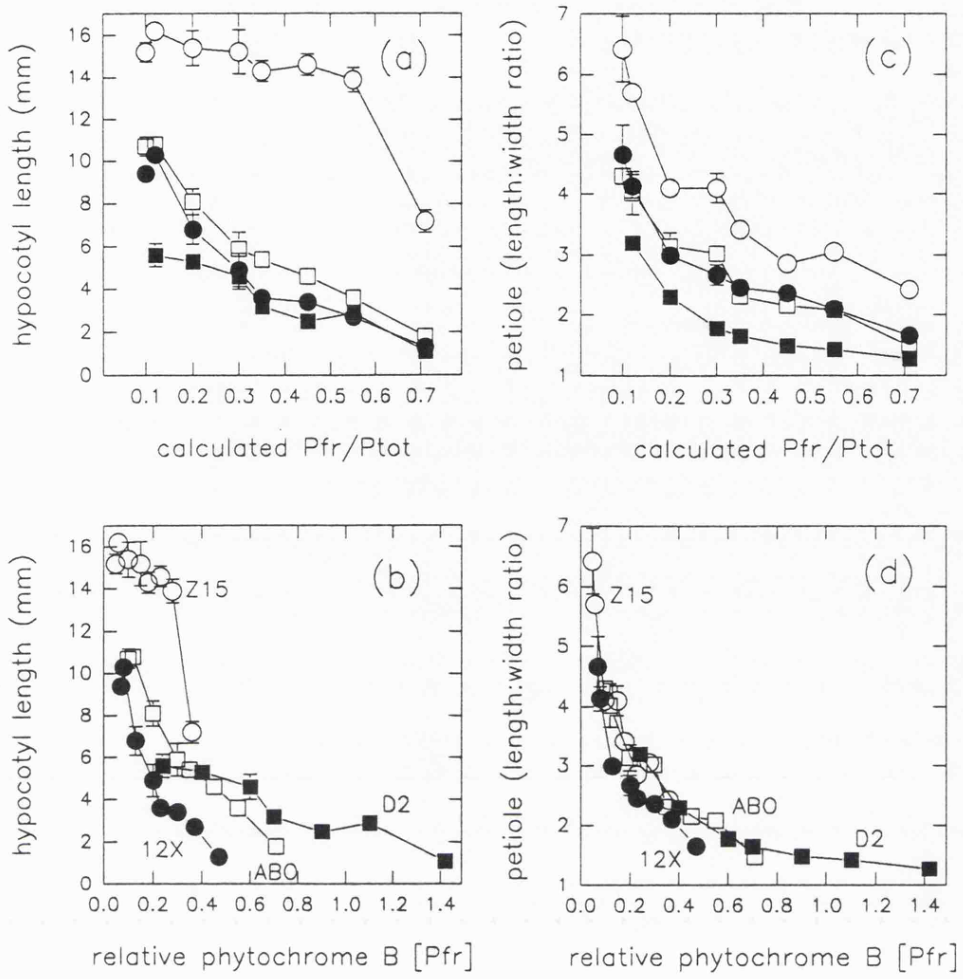


Figure 6.4 Growth of light-grown seedlings in response to the phytochrome photoequilbrium (P_{fr}/P_{tot}) established under different red:far-red quantum ratios. Hypocotyl length (**a**), and petiole extension growth (measured as the ratio of length over leaf-width) (**c**), were measured in seedlings of *Arabidopsis-phyB*-transformed lines Z15, (*open circle*), 12X (*solid circle*), ABO (*open square*) and D2 (*solid square*). Hypocotyl growth (**b**) and petiole extension growth (**d**) in light-grown seedlings expressing *Arabidopsis-phyB*-cDNA are shown as a function of calculated relative levels of the far-red absorbing form (P_{fr}) of phytochrome B. Growth measurements are those shown in (a) and (c). The corresponding values of phytochrome B [P_{fr}] were calculated from the phytochrome photoequilibrium using the estimated relative levels of total phytochrome B in the four lines ie. Z15: 12X: ABO: D2 = 3: 4: 6: 12 (D. Wagner p. comm).

Chapter 7

Phytochrome-regulation of greening in transgenic seedlings of *Arabidopsis thallana* and tobacco expressing an introduced phytochrome-encoding cDNA

Introduction

The lag in chlorophyll accumulation that occurs upon transfer of etiolated seedlings to continuous white light can be reduced, or eliminated, by a previous light pulse. This response, described as potentiation of greening, is regulated by phytochrome and has been observed in all angiosperm species so far studied (e.g. Briggs *et al.*, 1988; Horwitz *et al.*, 1988; Kasemir *et al.,* 1973; Ken-Dror and Horwitz, 1990; Virgin *et al.,* 1972). The properties of the greening response to red light (R) indicate that phytochrome-regulation operates in both the low fluence (LF) and very low fluence (VLF) response ranges (Raven and Shropshire, 1975; Briggs *et al.*, 1988; Ken-Dror and Horwitz, 1990). Greening involves, in addition to chiorophyll accumulation, the regulation of abundance of numerous chloropiast polypeptides, and there is substantial evidence that phytochrome mediates the photoregulation of expression of such genes (Ellis, 1981). For the transcript of the *cab* gene (encoding chiorophyil a/b binding protein), which might be expected to be closely linked to the greening process, both LF and VLF responses have been identified (Kaufman *et al.,* 1985).

Studies of photoreceptor-deficient mutants have demonstrated variously impaired greening responses. The *aurea* mutant of tomato, which possesses a severely depleted pool

of phytochrome in the etiolated seedlings (Sharrock *et al.,* 1988), is reported to have lost the VLF response to R, and also displays full FR reversibility of response as opposed to WT seedlings in which the VLF component of the R-induced response was not reversible by FR (Ken-Dror and Horwitz, 1990). The *hy1* and *hy2* mutants of *Arabidopsis*, containing a spectrophotometricaiiy-defective phytochrome pool (Parks *et al.,* 1989; Parks and Quaii, 1991), similarly showed weak potentiation of greening in response to a FR pulse, compared to WT (Lifschitz *et al.,* 1990).

Dissection of phytochrome-mediated photoresponses through physiological studies of conventional mutants can be usefully complemented by the parallel analysis of transgenic plants which contain supra-wiid-type levels of a specific phytochrome molecular species, encoded by an introduced cDNA (McCormac *et al.*, 1993; Parks and Quail, 1993). Transgenic *Arabidopsis* plants have been produced which express an introduced phytochrome-encoding cDNA and hence contain elevated levels of total phytochrome A (Boylan and Quail, 1991) or phytochrome B (Wagner *et al.*, 1991). An heterologous *phyA*-cDNA has also been introduced into tobacco, resulting in the accumulation of an enlarged phytochrome A pool in the etiolated seedlings (Keller *et al.*, 1989; Cherry *et al.,* 1991). These transgenic lines are used to test the roles of phytochromes A and B in mediating the potentiation-of-greening response in etiolated seedlings.

Materials and methods

Plant material

Arabidopsis thaliana: Seeds used were of the wild-type (WT) *Arabidopsis thaliana* (L. Heynh) ecotype Nossen, and four homozygous transgenic lines; 21K15 and 13K7 had been transformed with oat-*phyA*-cDNA under the control of the constitutive cauliflower mosaic virus (CaMV)35S promoter as described by Boylan and Quail (1991), and expression of the introduced phyA-cDNA results in an etiolated phytochrome pool approx. four-fold that of etiolated WT seedlings; transgenic lines RBO And ABO express introduced rice-*phyB*-cDNA and *Arabidopsis-phyB*-cDNA, respectively, under the control of the CaMV 35S promoter (Wagner *et al.*, 1991) and contain approx. 2.5-fold and 15-fold WT levels, respectively, of total phytochrome B in the light-grown plants. Seed was sown densely onto a layer of filter paper (3MM; Whatmann, Maidstone, Kent, UK.) overlaying 1% (w/v) aqueous agar in petri dishes. *Arabidopsis* seed was left to imbibe in darkness at 4^{o}C; after 4 days, seed was transferred to 25^{o}C and given a 30 min pulse of white light. Seeds were allowed to germinate for 3 days in absolute darkness.

Nicotiana tabacum: Seeds of tobacco used were WT (*N. tabacum*, var. Xanthi) and an homozygous transgenic line (9A4) which expressed an introduced oat-*phyA*-cDNA (Keller *et al.*, 1989) and contained approx. 5-fold WT levels of light-labile phytochrome in the etioiated seedlings (Cherry *et al.*, 1991). Seed was sown densely onto a layer of filter paper (3MM) overlaying 1% (w/v) aqueous agar in petri dishes and left to imbibe in darkness for 1 day. Subsequently seeds were irradiated for 30 min by white light and grown for a further 5 days in absolute darkness.

The red or far-red light treatments (maximum 15 min duration) for the potentiation of greening were given to the etiolated seedlings, followed by 4 h darkness (25^{o}C). The seedlings were subsequently allowed to green for 3 h under white light.

Light sources

Red light (R) (maximum photon fluence rate 600nm-700nm = 4 μmol m^{-2} s^{-1}) was provided by filtering the radiation from Thorn EMI (Birmingham, UK) Deluxe Natural 40-W fluorescent tubes through 1cm deep copper sulphate solution (1.5% w/v) and one layer of red (Number 14) Cinemoid (Rank Strand, isleworth, Middlesex, UK.). The far-red (FR) light source (photon fluence rate 700nm-800nm = 40 μmol m^{-2} s^{-1}) was provided by the output of water-cooled 100-W incandescent bulbs filtered through a black acrylic filter (Plexiglas Type FRF 700; West Lakes Plastics, Lem, Penn., USA). White light was provided by cool white fluorescent tubes, total photon fluence rate 400nm-700nm = 130 μmol m^{-2} s^{-1}.

Chlorophyll extraction and measurement

Whole seedlings [approx. 300 mg/assay *(Arabidopsis)*; approx. 600 mg/assay (tobacco)] were harvested, weighed and freeze-dried. The dried seedlings were placed in N,N-dimethyiformamide (DMF) at 600 μg fresh weight ml^{-1} DMF, and left at 4°C in darkness for a minimum of 2 days. The extracts were assayed for chlorophyll by measurement of absorbance coefficients according to Moran (1982). The fresh weight of 100 seedlings was measured for each trial and chlorophyll content per seedling was calculated on the basis of average seedling fresh-weight.

Results

Arabidopsis

Chlorophyll accumulation under continuous white light (WL) in the absence of a light pretreatment (i.e. dark control seedlings) was slightly higher in the transgenic *Arabidopsis* lines compared to the isogenic WT seedlings (Fig. 7.1). Lifschitz *et al.* (1990) reported that a R fluence of 1 mmoi m^{-2} saturated the potentiation-of-greening response in *Arabidopsis*. Foiiowing a saturating R pulse (3.6 mmol m^{-2}) chlorophyll accumulation in all four transgenic lines significantly exceeded that in WT; phyB-transformed ABO seedlings contained almost 2-fold WT chlorophyll levels after 3 h under WL (Fig. 7.1). R-mediated potentiation of greening in barley (Briggs *et al.*, 1988), tomato (Ken-Dror and Horwitz, 1990) and *Arabidopsis* (Lifschitz *et al.*, 1990) is reversible by FR. However, a FR pulse does not return the response to dark-levels. FR-mediated levels of greening correspond to a saturated VLF R-response in barley (Briggs *et al.*, 1988) and tomato (Ken-Dror and Horwitz, 1990), but Lifschitz *et al.* (1990) reported that the response of *Arabidopsis* to FR exceeded the response to a R pulse in the VLFR range (0.42 μmol m^{-2}). In this study of *Arabidopsis*, a FR-pulse resulted in a significant increase in greening rate compared to dark control seedlings (Fig. 7.1), and in each WT and transgenic line the response to FR was not significantly different from that to R in the 0.1-1.0 μmol m^{-2} range (Fig. 7.2a). The FR-mediated potentiation response observed here, therefore, can be fully explained through saturation of the P_{fr} requirement of the VLF-response mode. The inductive effect of a FR pulse, relative to dark-control seedlings of the same line, was of a comparable magnitude in the WT seedlings and the transgenic lines 21K15, 13K7 and RBO. The transgenic line ABO, however, showed a poor response to FR compared to the dark treatment. This effect in the ABO line appeared to be due to a high level of chlorophyll

accumulation in the dark-control seedlings as opposed to a low absolute level of chlorophyll accumulation in the FR-treated seedlings (Fig. 7.1). Dark-grown seedlings of ABO have previously been reported to exhibit a reduced hypocotyl extension-rate compared to WT seedlings, and this effect was shown to be dependent on the R:FR ratio of the light treatment delivered to the ungerminated seed (McCormac *et al.*, 1993); it was suggested that high levels of phytochrome B P_{fr} (encoded by the introduced cDNA) accumulated in the seed and were able to mediate a persistent growth-inhibition effect in the etiolated seedlings. It may be possible that a similar accumulation of stable P_{fr} in the ABO seed was able to mediate a small greening-response, effectively causing near saturation of the VLF response, in the absence of a light pulse to the etiolated seedlings.

Fluence-response curves for potentiation of rapid greening by a pulse of R (Fig. 7.2a) show that all four transgenic lines of *Arabidopsis* displayed increased levels, compared to WT, of chlorophyll accumulation in response to R ≥ 1 μmol m^{-2}. However, when chlorophyll levels were expressed relative to the dark control seedlings of each line (Fig. 7.2b), the R dose-response relationship of the *phyB*-transformed lines, RBO and ABO, were not seen to be notably different from WT. On this relative scale, the response of the oat-*phyA*-transformed lines, 21K15 and 13K7, to a R pulse in the 10-300 μmol m^{-2} range was 1.5- to 2-fold greater than in WT seedlings (Fig. 7.2b). The response to R of seedlings of WT and also the two *phyB*-transformed lines was saturated only at fluences exceeding 1000 μmol m^{-2} (Fig. 7.2a). Transgenic lines expressing oat-*phyA*-cDNA appeared to be saturated by a potentiating R pulse of approx. an order of magnitude lower, i.e. 100 μmol m^{-2}; a 10-foid increase in fluence led to no further increase in chlorophyll accumulation (Fig. 7.2a, b). In the *phyA*-transformed lines (13K7 and 21K15) maximum sensitivity of response to increasing R fluence occurred in the range 1-100 μmol m^{-2}, compared to 1-1000 μmol m^{-2} in the RBO and ABO lines, and 10-1000 μmol m^{-2} in WT (Fig.7.2b). The gradient of the dose-response relationship was therefore notably steeper in the *phyA*-transformed lines.

Tobacco

The greening response of tobacco seedlings demonstrated a clear R-mediated potentiation effect (Fig. 7.3). A far-red pulse was also partially effective in accelerating subsequent chlorophyll accumulation under white light. Chlorophyll levels accumulated after 3 h continuous WL were measured In seedlings of WT and oat-*phyA*-transformed tobacco; following a R (3.6 mmol m^{-2}) or FR (36 mmol m^{-2}) light pretreatment, no significant difference in the levels of rapid greening were observed between the WT and transgenic genotypes (Fig. 7.3). Fluence-response curves were constructed over 5 orders of magnitude, and also failed to show any significant difference between the R-mediated response of the WT and transgenic tobacco lines (Fig. 7.4). Tobacco seedlings, in common with previously studied angiosperm species (Briggs *et al.*, 1988; Ken-Dror and Horwitz, 1990; Horwitz *et al.*, 1988; Raven and Shropshire, 1975) demonstrated a biphasic dose-response relationship comprising both VLF and LF components. In both tobacco lines the VLF mode was saturated at approx. 1 µmol m^{-2}, and a near saturated LF R-response was seen at 3.6 mmol m^{-2} (Fig. 7.4). The level of rapid greening following a FR pulse (Flg. 7.3) corresponded to the R-mediated response at a fiuence of approx. 2 µmol m^{-2} (Fig. 7.4) and was therefore consistent with a saturated VLF response.

Discussion

Higher levels of phytochrome A or phytochrome B, achieved through expression of an introduced cDNA, in seedlings of transgenic *Arabidopsis* resulted in an increased capacity for rapid chlorophyll accumulation. It therefore appears that, in the transgenic seedlings, both light-labile and light-stable molecular species of phytochrome could mediate some component of the greening process which, directly or in directly, influenced total chlorophyll accumulation. Phytochrome A-transformed *Arabidopsis* lines demonstrated an increased sensitivity, compared to WT, of the dose-response relationship to R. Studies of conventional mutants of tomato (Ken-Dror and Horwitz, 1990) and *Arabidopsis* (Lifschitz *et al.*, 1990) have shown the effects of a deficiency in the phytochrome pool of etiolated seedlings to be directed at the VLF response. In contrast, the augmented phytochrome A population of the oat-*phyA*-transformed *Arabidopsis* seedlings mediated a rapidly saturated LF response, but no significant change in the sensitivity of the VLF component was observed. Phytochrome B-transformed seedlings showed a fluence-response range which was not markedly different from WT. One interpretation of these results is that potentiation of greening is mediated predominantly by the phytochrome A photoreceptor species. The results of similar studies of the *hy3* mutant of *Arabidopsis* have also shown that, although overall greening capacity is reduced in the absence of phytochrome B (Lifschitz *et al.*, 1990), the relative VLF and LF components of the phytochrome-mediated response are conserved. Such differential effectivenesses of phytochromes A and B may be due to the specific physiological activities of the two molecular species or may reflect the relative abundances; phytochrome A forms by far the larger component of the total phytochrome pool of dark-grown seedlings, typically 50-100 times the levels of light-stable phytochromes (Jabben *et al.*, 1980; Rombach *et al.*, 1982).

The effects of expression of an introduced phytochrome-encoding cDNA on the rapid greening response in transgenic *Arabidopsis* seedlings were not repeated in the results of a

transgenic line of tobacco expressing introduced oat-*phyA*-cDNA. Overall greening capacity of the transgenic tobacco seedlings was not altered significantly from WT and no increased sensitivity of the response to R, over a wide range of fluences, could be demonstrated. The apparent discrepancy of the phytochrome A-mediated greening response in the transgenic seedlings of *Arabidopsis* and tobacco may suggest a difference in the molecular species of photoreceptor responsible for the same response in either plant species, or may indicate that the same oat-encoded phytochrome A population exhibits differential sensitivities to R within *Arabidopsis* and tobacco seedlings. Similar marked differences in the sensitivity to R of oat-*phyA*-transformed tobacco and *Arabidopsis* seedlings have been noted for the photoinhibition of hypocotyl elongation growth; whereas transgenic *Arabidopsis* seedlings exhibit a strongly dwarfed phenotype compared to WT under continuous irradiation by low fluence rates of R (McCormac *et al.,* 1993), corresponding transgenic tobacco genotypes display only a very weak, at best, increase in the WT sensitivity to growth-inhibition by R (Chapter 4 of this thesis; Tennessen *et al.*, 1992).

The interpretation of the photoresponses of transgenic plants in terms of phytochrome function in WT plants is subject to phytochrome-null mutant-genotypes establishing the complementary set of photoresponses. For example, the short hypocotyl phenotype of oat-*phyA*-transformed *Arabidopsis* seedlings (Boylan and Quail, 1991; McCormac *et al.*, 1993) suggested a role for phytochrome A in R-mediated inhibition of elongation growth in etiolated seedlings. The growth response of the *hy3* mutant, however, demonstrates that the endogenous phytochrome A population (at least in the absence of phytochrome B) does not mediate a significant R-activity (Koornneef *et al.,* 1980; McCormac *et al.*, 1993). A new mutant class of *Arabidopsis* specifically deficient in the *phyA* gene-product is currently being characterised (*hy8*; Parks and Quail, 1993; *fre*; Nagatani *et al.*, 1993; *fhy*; Whitelam *et al.,* 1993), and should provide the means to test the proposed role of endogenous phytochrome A in the

rapid greening response of etiolated seedlings. Initial results have shown the *fre* mutants of Nagatani *et al.* to display an apparently normal potentiation of greening response following a saturating R pulse (Nagatani *et al.*, 1993) and thus would appear to be in apposition to the role for phytochrome A suggested by the responses of the transgenic *Arabidopsis* plants; whether or not the dose-response relationship to subsaturating R fiuences is altered in these mutants [as observed in *aurea* (Ken-Dror and Horwitz, 1990)], however, remains to be determined.

For the biosynthesis of chlorophyll, δ-aminolevulinic acid formation has been found to be the rate-limiting step and this reaction has been implicated as responsible for the lag period during the greening process (Beale, 1971; Schneider, 1973; Nadler and Granick, 1970). However, it seems likely that the incorporation of chlorophyll into the thylakoid membranes does not only depend on the concentration of chlorophyll but also on the concentration of the apoprotein of the complex, namely the chlorphyll *a/b* binding protein (cab). Thus the greening process is not necessarily only determind by the rate of chiorophyil synthesis alone but probably depends also on the rate of synthesis of the cab protein, and thus the induction of cab mRNA activity might be an additional rate-limiting step in the assembly of a functional chloroplast. A treatment of red light induces the increase of template activity for the apoprotein of the light-harvesting chlorophyll *a/b* protein (Apel, 1979) and also stimulates the appearance of the light-harvesting δ–aminolevulinic-acid-forming enzyme (Masoner and Kasemir, 1975; Klein *et al.*, 1977); thus phytochrome seems to affect the synthetic pathways of both the chlorophyll and cab apoprotein constituents of the light-harvesting chlorophyll *a/b* complex. On the above premises it might be expected that the overexpressed phytochrome molecular species would need to mediate R-enhanced rates of both chlorophyll and cab-protein synthesis inorder to elicit an increase in the overall greening reaction. These observations stress that further investigations are required to clarify the actions of the individual phytochromes on the sequence of reactions leading to assembly of the thylakoid structure.

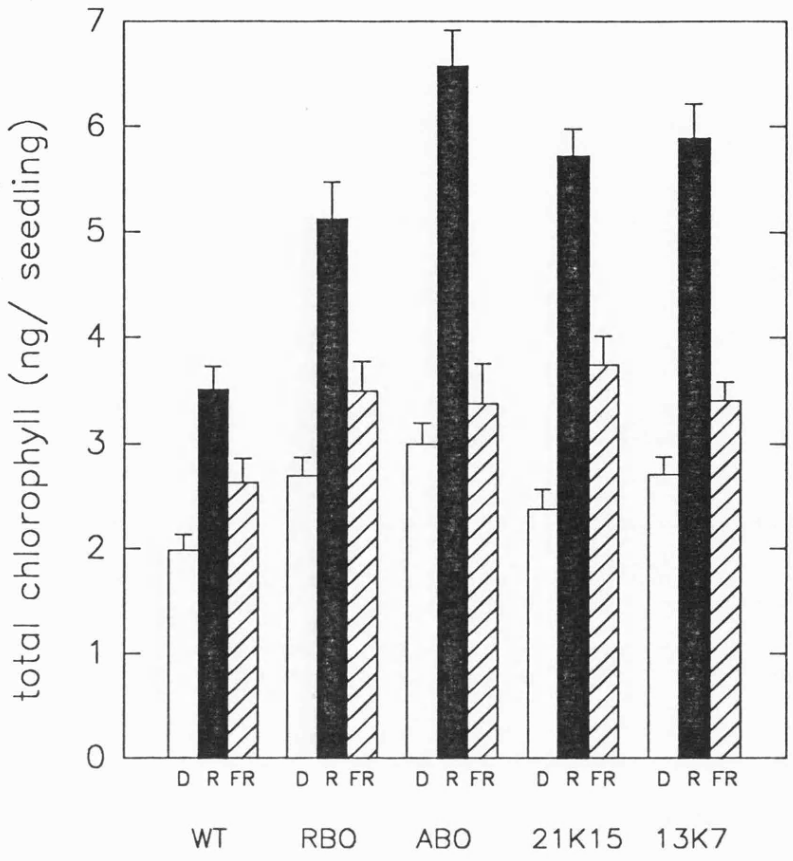


Figure 7.1 Potentiation of greening by a pulse of red light (R) or far-red light (FR) in wild-type (WT) and transgenic seedlings of *Arabidopsis*; RBO and ABO expressed an introduced rice-*phyB*-cDNA and *Arabidopsis*-*phyB*-cDNA, respectively; 21K15 and 13K7 expressed an introduced oat-*phyA*-cDNA. 3 day-old dark-grown seedlings received a 15 min pulse of R (600-700nm=4 μmol m^{-2} s^{-1}) or FR (700-800nm=40 μmol m^{-2} s^{-1}) or were maintained in darkness (D); seedlings were returned to darkness for 4 h prior to transfer to white light. Chlorophyll accumulation was measured after 3 h under continuous white light (400-700nm=130 μmol m^{-2} s^{-1}). Values are the mean ±SEM of six experiments.

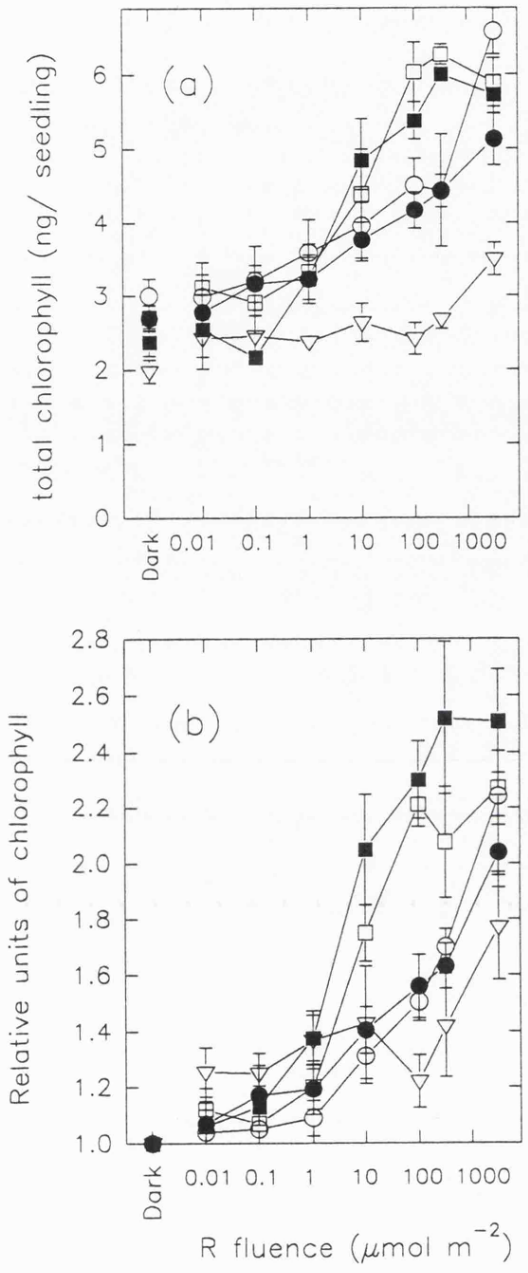


Figure 7.2 Fluence-response curves for potentiation of rapid greening by a pulse of red light (R) in wild-type *Arabidopsis* (*open triangles*), *phyA*-transformed lines 13K7 (*open squares*) and 21K15 (*solid squares*), and *phyB*-transformed lines RBO (*solid circles*) and ABO (*open circles*). 3 day-old dark-grown seedlings received a R pulse of the fluence shown [or were maintained in darkness (dark)]. Seedlings were returned to darkness for 4 h prior to transfer to white light. Chlorophyll was measured after 3 h under continuous white light and the results are shown either as (**a**) absolute ng of chlorophyll calculated per seedling on the basis of average seedling fresh weight, or (**b**) relative levels meaured in proportion to the dark-control seedlings within each trial. Values are the mean ±SEM of six experiments.

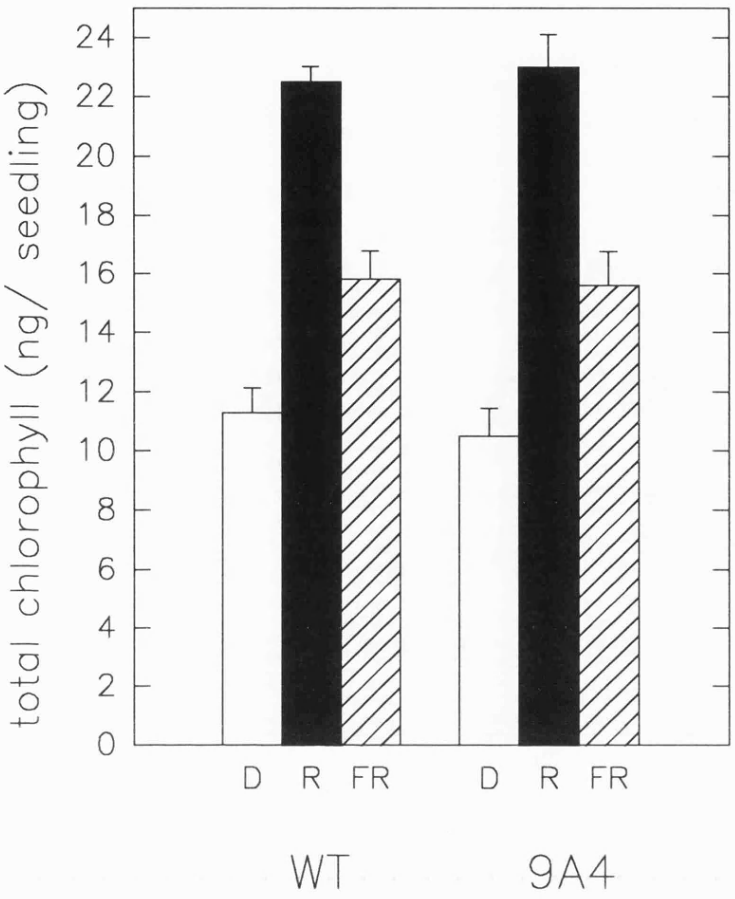


Figure 7.3 Potentiation of greening by a pulse of red light (R) or far-red light (FR) in wild-type (WT) and oat-*phyA*-transformed (9A4) genotypes of tobacco. 5 day-old dark-grown seedlings received a 15 min pulse of R (600-700nm=4 μmol m^{-2} s^{-1}) or FR (700-800nm=40 μmol m^{-2} s^{-1}) or were maintained in darkness (D); seedlings were returned to darkness for 4 h prior to transfer to white light. Chlorophyll accumulation was measured after 3 h under continuous white light. Values are the mean ±SEM of six experiments.

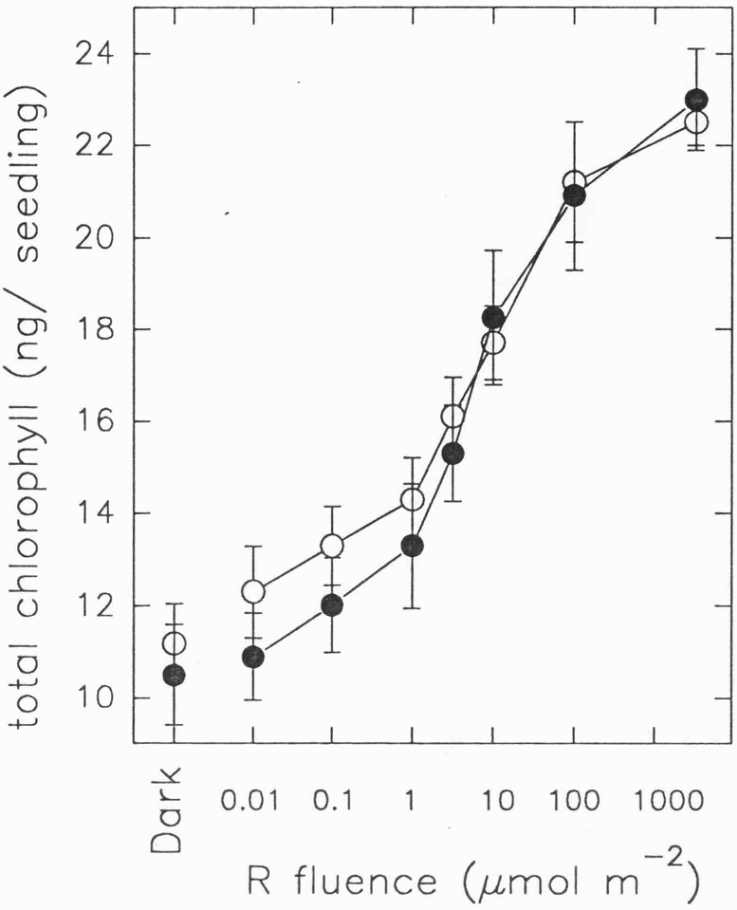


Figure 7.4 Fluence-response curves for potentiation of rapid greening by a pulse of red light (R) in wild-type (*open circles*) and *phyA*-transformed genotypes (*solid circles*) of tobacco. 5 day-old dark-grown seedlings received a R pulse of the fluence shown [or were maintained in darkness (dark)]. Seedlings were returned to darkness for 4 h prior to transfer to white light. Chlorophyll was measured after 3 h under continuous white light and the results are shown either as (**a**) absolute ng of chlorophyll calculated per seedling on the basis of average seedling fresh weight, or (**b**) relative levels meaured in proportion to the dark-control seedlings within each trial. Values are the mean ±SEM of six experiments.

Chapter 8

Photoregulation of germination in seed of transgenic lines of tobacco and *Arabidopsis* which express an introduced cDNA encoding phytochrome A or phytochrome B.

Introduction

Light has a key regulatory role in the germination behaviour of seeds of several plant species (Toole, 1973). Whether a given irradiation results in promotion or inhibition of germination in a seed batch depends on a number of interacting factors including (a) genotype between plant species (e.g. Hilton, 1982) or within a species (Frankland, 1976), (b) environmental conditions of seed maturation, harvest, storage and imbibition (McCullough and Shropshire, 1970; Cone and Spruit, 1983; Gettens-Haynes and Klein, 1974), (c) timing of light treatment with respect to imbibition, (d) red (R): far-red (FR) quality of irradiation, (e) duration and fiuence rate of irradiation. In addition, the light requirement to trigger germination varies between individual seeds within a batch and this variation conforms to a Normal distribution (Frankland, 1976); percentage germination of a sample population therefore provides an incremental response to a light stimulus, rather than a discrete all-or-nothing response, as is observed in a single seed.

The promotory effect of light is mediated by the plant photoreceptor phytochrome, operating in its far-red-absorbing molecular form (P_{fr}). P_{fr} may be present in the

dark-imbibing seed through rehydration of inactive P_{fr}; the R:FR quality of the radiation experienced during seed maturation can affect the levels of P_{fr} in the dry seed sufficiently to elicit a significant effect on the rate of dark germination (McCullough and Shropshire, 1970; Gettens-Haynes and Klein, 1974). P_{fr} can also be formed in the imbibed seed by R-mediated photoconversion of P_r to P_{fr}. Photoinhibition of germination appears to be complex. In its simplest form inhibition of germination can be brought about by FR irradiation resulting in the photoconversion of pre-existing P_{fr} to P_r. Prolonged irradiation with high photon fiuence rates can also impose a high level of inhibition of germination. It has been proposed that the rate of phytochrome photointerconversion, or cycling, is an important factor (Bartley and Frankland, 1982), but the mechanism through which this irradiance-dependent photoinhibition operates is unclear. In *Sinapis arvensis* germination is stimulated by a brief pulse of R, but inhibition of germination can subsequently be imposed by prolonged, high-irradiance light, even after the R-pulse effect has escaped from FR-reversibility (Bartley and Frankland, 1982). The low-fluence-induction and high-irradiance-inhibition effects therefore appear to be separate sequential events, and there is some evidence that they govern distinct physiological processes relating, respectively, to gene-expression within seed tissues and radicle emergence (B. Frankland, Queen Mary College, London., personal communication).

Three immunochemically-distinguishable molecular species of phytochrome have been detected in the embryo-containing portions of unimbibed seed of *Avena*, one of which corresponds to the light-labile phytochrome A species (encoded by the *phyA* gene) which accumulates in etiolated seedlings, and two to the light-stable forms which predominate in green plants, probably including the *phyB* gene-product (Wang *et al.*, 1992). There is some evidence, from the physiological study of photoreceptor-deficient mutants (e.g. Adamse *et al.*, 1988a; 1988b; Kendrick and Nagatani,1991; Whitelam and Smith, 1991) and transgenic plants which express introduced phytochrome-encoding cDNAs (McCormac

et al., 1993), that the individual phytochrome species have distinct photoregulatory roles in seedling photomorphogenesis. Transgenic expression of phytochrome-encoding cDNAs is used here, in an attempt to assess the importance of the members of the phytochrome family in the induction and inhibition events in seed germination. Expression of the introduced cDNA is under the control of the CaMV 35S promoter which acts as a strong constitutive promoter in most organs of transgenic plants, including seed embryo tissues (Benfrey *et al.*, 1990).

Materials and methods

Plant material

Seeds of *Nicotiana tabacum* L. (cv. xanthi) were obtained from wild-type (WT) and an homozygous isogenic transgenic line. The transgenic line had been transformed with the coding sequence of the oat-*phyA*-gene fused to the cauliflower mosaic virus 35S promoter (Keller *et al.*, 1989); expression of this construct results in the accumulation of supra-wild-type levels of spectrophotometrically-active phytochrome in the etiolated and light-grown plants (Cherry *et al.*, 1991). Seed batches were collected from plants grown under natural daylight supplemented with radiation from sodium vapour lamps. All experiments were conducted on the seed within three months of harvest. Seed was sown onto 1 % (w/v) aqueous agar in petri dishes and allowed to imbibe in darkness for 1 day (25^{o}C) before being transferred to the appropriate light treatments. Seeds were allowed to germinate for the times stated and germination was scored by visually assessing radicle emergence. Each datum point was obtained from a minimum of 4 trials each consisting of at least 50 seeds.

Seeds of *Arabidopsis thaliana* L. were obtained from wild-type and four homozygous isogenic lines, derived from the ecotype Nossen. Two of these transgenic lines, 21K15 and 13K7, had been transformed with oat-*phyA*-cDNA fused to the CaMV 35S promoter (Boylan and Quail, 1991). The transgenic lines RBO and ABO had been transformed with rice-*phyB*-cDNA and *Arabidopsis-phyB*-cDNA, respectively, also under the control of the CaMV 35S promoter (Wagner *et al.*, 1991). In each line, expression of the introduced cDNA resulted in the accumulation of a spectrophotometrically-active population of the heterologous phytochrome species. The long hypocotyl mutant of *Arabidopsis*, *hy3*, (Koornneef *et al.*, 1980) is specifically deficient in the *phyB* gene-product (Somers *et al.*, 1991; Reed *et al.*, 1993), and its isogenic WT is Landsberg *erecta*. Seed batches were

collected from plants growing under continuous irradiation by either white light (R:FR = 6.8) or white light with supplementary far-red radiation (R:FR = 0.07), and were used in experiments within three weeks of harvest. Dry seed was sown onto 1% (w/v) aqueous agar in petri dishes and incubated in complete darkness (25 ^{o}C) for 6 days. Germination was scored by assessing radicle emergence.

Light sources

White light (WL) was provided by Cool White fluorescent tubes and supplementary far-red (FR) was provided by filtering the radiation from quartz-halogen lamps through 4 cm of water and a single layer of red (Number 4400) and green (Number 6600) Perspex (SBA, Leicester, UK). Changes in fluence rate whilst maintaining a constant R:FR ratio were achieved by altering the distance from the light source. Broad-band FR light was provided by water-cooled 100-W incandescent bulbs with a black acrylic filter (Plexiglass Type FRF 700; West Lake Plastics, Lem, Penn., USA). Narrow wavebands were obtained by filtering the light from a halogen lamp (Thorn lighting Ltd., Birmingham, UK) through interference filters of approximately 20nm bandwidth at 50% of transmission maximum. The total fiuence delivered was varied by changing the duration and/ or fluence rate of the radiation; fluence rate was reduced by using layers of muslin.

Fluence rates and spectral distribution of light sources were recorded using the cosine-corrected remote probe of a calibrated LI-1800 spectroradiometer (LI-COR, Lincoln, NA., USA). Red (R): FR ratios were calculated as the ratio of fluence rates over the 654-664nm and 724-734nm wavelength intervals.

Results

Inhibition of germination of transgenic tobacco seed under continuous irradiation

Seed of wild-type (WT) and oat-*phyA*-transformed tobacco were sown onto 1% (w/v) aqueous agar and immediately placed under continous irradiation by white light (WL) (R:FR = 6.8; total photon fluence rate 400-700nm = 60 μmol m^{-2} s^{-1}) or white light with supplementary far-red radiation (WL+FR) (R:FR = 0.07; total photon fluence rate 400-700nm = 60 μmol m^{-2} s^{-1}, 700-800nm = 185 μmol m^{-2} s^{-1}). Germination of WT seed was strongly inhibited under the FR-supplemented source, in contrast to the transgenic seed batch which maintained a high level of germination under continuous low R:FR ratio irradiation (Fig. 8.1). Increasing the fiuence rate of the low R:FR ratio source (total photon fluence rate 400-700nm = 160 μmol m^{-2} s^{-1}; 700-800nm = 500 μmol m^{-2} s^{-1}), whilst maintaining the R:FR ratio at 0.07, resulted in inhibition of germination in the trangenic seed lot as well as in the WT (Fig. 8.1).

The level of inhibition of germination in response to continuous irradiation by light of low R:FR ratio [provided by FR-supplemented white light (WL+FR) or broad-band FR light-sources] was compared with the response to a 15 min pulse from the same light source. Dark-imbibed seeds were given a saturating pulse of white light and then returned to darkness. At various intervals of dark-incubation following this WL treatment, seeds received the stated low R:FR light treatment. Seeds which received no light treatment following the WL pulse showed approx. 90% germination in both WT and transgenic seed batches. Subsequent irradiations establishing a low P_{fr}/P_{tot} caused an inhibition of this WL-induced germination-response in both genotypes, and this inhibitory effect was gradually lost as the intervening period of dark incubation was extended. Such time-dependent loss of R/FR reversibility indicates completion of P_{fr} action. In WT seed, continuous irradiation by WL+FR or broad-band FR mediated a level of

inhibition which exceeded that in response to a 15 min pulse from the same light-source (Fig. 8.2a, 8.2b). In contrast, transgenic tobacco seed, which had received a WL pulse and was subsequently irradiated by the low R:FR ratio light-sources, showed a higher level of germination under continuous irradiation by the FR-supplemented light-source (WL+FR) than seed which received a 15 min pulse from the same low R:FR source (Fig. 8.2c); in other words, prolonged irradiance was less effective than a saturating pulse in causing photo-inhibition of germination in the transgenic seed. Germination of the transgenic seed under continuous broad-band FR did not significantly exceed that resulting from a FR pulse, but neither was it inhibited (Fig. 8.2d), in contrast to the behaviour of WT (Fig. 8.2b).

Within each genotype, under each low R:FR ratio light source, the time-dependent escape from the inhibition mediated by the pulse and continuous irradiation treatments followed almost parallel time courses (Fig. 8.2a-d).

Dose-response and action spectra of germination in transgenic tobacco seed

Figure 8.3 shows the fluence-response relationships for the induction of germination in WT and *phyA*-transformed tobacco seed by R light of 666nm. The fluence-response curves are biphasic reflecting low fluence (LFR) (10^2 - 10^4 μmol m^{-2}) and very low fluence (VLFR) (10^{-2} - 10 μmol m^{-2}) reactions. Biphasic fluence relationships have been observed for the light-induction of germination in several plant species (e.g. Small *et al.*, 1979; Blaauw-Jansen, 1983; Kendrick and Cone, 1985), although in these studies the observation of a VLFR mode required a light and/or temperature pretreatment in order to deplete endogenous P_{fr} levels. Over the range of total fluences 10^2 - 10^4 μmol m^{-2}, the germination response of the tobacco seed lots did not appear to be affected by R fluence-rate (Fig. 8.3); an immediately subsequent FR pulse (730nm, 1 mol m^{-2}) was effective in depressing the R-mediated response (Fig. 8.3). Such reciprocity and R/FR

reversibility of response confirmed that the observed R-induction of germination was mediated by the low energy reactions of phytochrome. Both WT and transgenic seed displayed a marked sensitivity of the germination response to fluences of R between 10^2 and 10^4 μmol m^{-2}, above which germination became saturated. No increased sensitivity to R of the germination response in the *phyA*-transformed genotype, relative to WT, was apparent. In the particular transgenic seed batch shown in figure 8.3, a lower level of absolute germination was achieved over the 10^{-2} - 10 μmol m^{-2} range compared to WT.

Irradiation of imbibed seed with a saturating pulse of FR light (730nm) stimulated germination of WT and transgenic seed above that of dark germination levels (Fig. 8.4). Comparison of the levels of FR-stimulated germination with the VLFR response to R (Fig. 8.3) indicates that the latter was saturated by very low levels of P_{fr}. By implication, both WT and transgenic seed batches contained very low levels of pre-existing P_{fr} in the dark-imbibed seed. Saturating pulses of light at wavelengths below 710nm, setting progressively higher levels of P_{fr}/P_{tot}, resulted in increasing levels of germination (Fig. 8.4). Germination of transgenic seed did not show increased sensitivity to lower values of P_{fr}/P_{tot}, relative to WT.

Fluence-response relationships for the induction of germination were constructed for a range of wavelengths in the red-range of the spectrum (544-707nm) (Fig. 8.5). In order to construct action spectra, the standard response was taken as the fluence which resulted in 70% and 55% absolute germination in WT and transgenic seed lots, repectively; these germination values corresponded to the response at a fluence of $10^{2.5}$ μmol m^{-2} of the optimum wavelength (Fig. 8.5). The reciprocal of the fluences which gave this standard response were plotted in action spectra normalized to 100% for peak effectiveness (Fig. 8.6). These action spectra agree, broadly, with published action spectra for germination in other seed species (e.g. Cone and Kendrick, 1985; Small *et al.* 1979). Both WT and transgenic seed batches showed maximum effectiveness at approx. 670nm. There was no enhancement in the relative sensitivity of the transgenic seed batch

to subsaturating fluences of wavelengths approaching the FR waveband; the activity of wavelengths longer than 700nm in both seed genotypes was less than 1% of the peak activity (Fig. 8.6). The band width of the peak of effectiveness of the transgenic seed response was slightly narrower than WT. Wavelengths shorter than the action maxima showed a steady decline in photon effectiveness for the germination response of both WT and transgenic seed. However, in the 550-650nm waveband a shoulder of activity was apparent for the germination response of the transgenic seed compared to the WT action spectrum.

Dark germination of seed of transgenic Arabidopsis

The dark germination of seed from transgenic lines of *Arabidopsis* containing an introduced phytochrome A-encoding cDNA (lines 21K15 and 13K7; Boylan and Quail, 1991) or phytochrome B-encoding cDNA (lines RBO and ABO; Wagner *et al.,* 1991) was compared with wild-type. Seed was set and matured on plants growing under continuous irradiation from two alternative light sources establishing two extremes of R:FR ratio, namely white light (R:FR = 6.8) or white light with supplementary FR (R:FR = 0.07). The level of germination for seed imbibed in complete darkness was scored for each genotype for the two pretreatments. Seed which had been irradiated during development with light of high R:FR ratio (establishing high P_{fr}/P_{tot}) demonstrated a very high level of dark germination; this result was measured in all transgenic and WT lines (Table 8.1). Germination of WT seed was fully inhibited by a long wavelength FR pulse delivered to the imbibed seed (Table 8.2), confirming that dark-germination was sensitive to levels of P_{fr} and demonstrated the R/FR reversibility of the low energy reactions of phytochrome. WT seed which had been irradiated with the low R:FR ratio light source during maturation displayed much reduced dark-germination compared to seed matured under white light (Table 8.1). Dark germination was similarly inhibited in seed of the transgenic lines

overexpressing phytochrome A (Table 8.1); seed of 13K7 and 21K15 which had matured under low R:FR showed 82% and 72% inhibition, respectively, of the response to the high R:FR ratio pretreatment, compared to 85% inhibition in WT. Seed of the phytochrome B overexpressing line ABO germinated fully in darkness regardless of the light pretreatment (Table 8.1). Irradiation of the imbibed seed with 749nm FR was able to fully inhibit germination (Table 8.2), thus confirming that control of ABO seed germination was sensitive to levels of P_{fr}. Germination of seed of the RBO line, matured under low R:FR ratio, showed 40% inhibition relative to the white-light pretreatment; this inhibitory effect was notably diminished compared to the effect in the WT and phytochrome A-overexpressing lines (Table 8.1 and see above). The *hy3* mutant of *Arabidopsis* is deficient in the phytochrome B molecular species (Somers *et al.*, 1991); dark germination of *hy3* seed was severely depressed relative to WT, even in seed batches matured under white light (Table 8.1).

Discussion

The germination behaviour in response to inductive and inhibitory light treatments was investigated in seed lots of transgenic genotypes containing an introduced phytochrome-encoding cDNA. A number of notable differences in the photoresponsiveness of these transgenic seeds compared to the corresponding isogenic wild-type seed were observed but which are not easily interpreted in terms of the action of the introduced phytochrome molecular species. Any attempts to interpret these results in terms of elevated levels of the phytochrome species encoded by the introduced cDNA make the assumption that the heterologous phytochrome is expressed in the seed and is accumulated in cells where it might be expected to control germination. Information of the activity of the CaMV (35S) promoter, used to drive expression of the introduced *phy*-cDNAs, suggests that transcription will occur in the seed embryo (Benfrey *et al.,* 1990), but this point remains to be demonstrated directly.

Seed of WT tobacco demonstrated very low levels of germination under continuous irradiation by a white light source supplemented with FR radiation (WL+FR) (R:FR=0.07). In contrast, seed of a transgenic tobacco genotype containing introduced oat-derived sequences encoding phytochrome A demonstrated markedly higher germination rates (Fig. 8.1; Fig. 8.2a cf. Fig. 8.2b). The simplest interpretation of these data is that, under continuous irradiation by the WL+FR light source, insufficient levels of P_{fr} were accumulated in the WT seed to promote germination, but elevated levels of total phytochrome in the transgenic seed produced a concentration of P_{fr} which was adequate for germination induction. Inhibition of germination could be induced in the transgenic seed by continuous irradiation, but required higher irradiances than WT. This appears to be consistent with the proposal of opposing actions for P_{fr} and light-dependent cycling-rate (Bartley and Frankland, 1982), although assuming this scenario, the dramatic effect on the germination of the transgenic seed batch mediated by an only three-fold change in light

intensity seems suprising. Seed of both WT and oat-*phyA*-transformed seed batches could be induced to show very high levels (>90%) of germination by a pulse of white light, and a large proportion of this WL-effect could be reversed by an immediately subsequent pulse of light establishing low P_{fr}/P_{tot}. In the case of the WT tobacco seed, prolonged irradiation by WL+FR or broad band FR resulted in a significantly greater degree of inhibition than mediated by a 15 min pulse from the same light-source. Thus, the inhibition of germination observed under continuous irradiation of low R:FR cannot be explained simply in terms of the photoequilibrium value established; photoinhibition of seed germination which is dependent on the length of the irradiation period (independently of total fluence) has been reported in a number of seed species (Hartmann, 1966; Hendricks and Toole, 1968; Bartley and Frankland, 1982). Such an inhibitory effect of continuous irradiation over and above that of a saturating pulse was most notable under the FR-supplemented white light source (Fig. 8.2a), and to a lesser degree under broad-band FR (Fig. 8.2b). This result supports the proposal that the phytochrome cycling rate, which would be expected to be higher under FR-supplemented white light than FR-alone, is important in the irradiance-dependent inhibition of germination. The parallel time courses followed for the time-dependent escape from inhibition as mediated by the pulse and continuous irradiation treatments (Fig. 8.2a-d) would seem to suggest that the inhibitory actions of these two forms of irradiance impinged on simultaneous events, as opposed to temporally-distant processes as demonstrated in *Sinapis* (Bartley and Frankland, 1982).

Photoinhibition of germination in seed under prolonged light exhibits a number of characteristics typical of the "high irradiance reactions" (HIRs) of seedling photomorphogenesis, namely a lack of reciprocity of response, and an action peak at around 710-720 nm (Hartmann, 1966; Hendricks *et al.,* 1968; Bartley and Frankland, 1982). There is an increasing body of evidence that the FR-HIR mode of seedling photomorphogenesis is specifically mediated by the phytochrome A molecular species (Whitelam *et al.,* 1992; McCormac *et al.*, 1991; 1992b; 1993). The *aurea* mutation in

tomato is associated with a deficiency in type I phytochrome (Parks *et al.*, 1987; Lopez-Juez *et al.,* 1990b) and, in contrast to WT, germination of the seeds of *aurea* is not inhibited by prolonged FR (Lipucci Di Paola, 1988; Koornneef *et al.*, 1985). This observation has led to suggestions that the high irradiance inhibition of seed germination is mediated by phytochrome A (Peters, 1992). On this premise it might be expected that the seed germination of phytochrome A-overexpressing genotypes would be strongly inhibited under prolonged far-red irradiation, whereas seed of the transgenic tobacco which, subsequent to a saturating WL pulse was irradiated by WL+FR, showed a higher level of germination under continuous irradiation by low R:FR ratio than following a 15 min pulse from the same light-source (Fig. 8.2b); thus the (presumed) accumulation of elevated levels of total phytochrome A resulted in the seed being less subject to irradiance-dependent inhibition relative to WT seed. Contradiction of the proposed FR-HIR inhibition of germination is, perhaps, supported by more careful comparison with the HIRs studied in seedling photoresponses. In contrast to classical FR-HIRs (Mancinelli, 1980) intermittent FR treatments are as effective as continuous FR in inhibiting seed germination (Mancinelli *et al.,* 1967). Also at odds with previous characterisation of HIRs in seedlings, where the response is synergistic with the low energy reactions (e.g. Beggs *et al.*, 1980), the implication in seed germination is that the high irradiance reaction is antagonistic to the low fluence induction response. Photomorphogenetic responses in the seedlings of the oat-*phyA*-transformed tobacco demonstrate an irradiance-dependent response to continuous FR presumably mediated by the heterolgoous phytochrome A, and which has been proposed as analagous to the FR-HIR of WT etiolated seedlings (McCormac *et al.,* 1992b). One possible interpretation of the germination behaviour of the transgenic tobacco seed is that the overexpressed phytochrome A mediated an induction response that was dependent on continuous FR irradiation. If a FR-HIR-type reaction were to be implicated in stimulating germination in the transgenic seed, it might be expected that the difference in germination under continuous irradiation and in response to a 15 min pulse

would be maximal under FR, however germination of the transgenic seed under continuous broad-band FR did not significantly exceed that resulting from a FR pulse. On the basis of the established photophysiological behaviour in both WT (Beggs *et al.,* 1980) and transgenic (McCormac *et al.*, 1992b) seedlings it therefore seems unlikely that the stimulatory effect of the heterologous phytochrome was a direct action of P_{fr}; however, different kinetic properties of phytochrome A in imbibed seeds and young seedlings cannot be excluded. Alternatively, while oat-phytochrome A appears to be active in regulating the photomorphogenetic responses of transgenic tobacco seedlings (McCormac *et al.,* 1991; 1992b), within the context of the tobacco seed, the heterologous phytochrome may not be able to substitute for tobacco phytochrome A function. In this scenario, high levels of a foreign phytochrome molecular species may serve to interfere with endogenous phytochromes, perhaps blocking the normal action of the tobacco phytochrome A in FR-mediated inhibition of germination by competing for a reaction partner to form an inactive complex.

In seed of the WT and oat-*phyA*-transgenic tobacco germination was induced above the dark level by brief pulses of R (666nm). The dose response of this R action in the transgenic seed batch demonstrated VLF and LF components across a similar fluence range to that observed in the WT batch, but at subsaturating fluences the germination level in the transgenic seed was reduced relative to WT (Fig 8.3). Some measure of variation in the absolute level of germination in response to a given inductive light treatment has been reported between different seed batches of WT *Arabidopsis* (Cone and Kendrick, 1985). However, the magnitude of the difference observed here between the WT and transgenic seed is not readily attributed simply to innate differences in P_{fr}-requirement of the two batches, especially since all seed was harvested and stored under identical conditions. Both transgenic and WT seed showed sensitivity of the germination response to the wavelength of irradiation in the R -> FR spectrum, consistent with a relationship with P_{fr}/P_{tot} (Figs. 4, 5 and 6). A saturating pulse (1 mol m^{-2}), considered sufficient to establish

photoequilibrium, at wavelengths approaching the FR end of the spectrum appeared less efficient in stimulating germination of the transgenic tobacco seed than in WT (Fig. 8.4). Thus, under subsaturating fluences of 666nm light or saturating fluences of 670-730nm light (conditions establishing the low energy reactions of phytochrome) the response of the oat-*phyA*-transformed tobacco seed, compared to WT, did not indicate any inductive action of the (presumed) enhanced levels of total phytochrome A-P_{fr}. In fact, the indication was for reduced photo-effectiveness in the transgenic response; this could, perhaps, be viewed as supportive of the earlier suggestion that the presence of the heterologous oat phytochrome served to impede normal action of endogenous phytochromes. The action spectra for the WT and transgenic seed (Fig. 8.6) both suggested the general absorption characteristics of the red-absorbing form of phytochrome (P_r). The band width of the transgenic R-action peak was slightly narrower than for WT, but such variation has been reported amongst batches of WT *Arabidopsis* (Cone and Kendrick, 1985) and so does not necessarily reflect a direct effect of the transformed genotype. The shape of the experimental action spectrum can be significantly influenced by the transmission properties of screening pigments in the seed coat (Cone and Kendrick, 1985; Taylorson and Hendricks, 1971). Such screening effects may influence the sharpness of the action maxima (Cone and Kendrick, 1985); however no direct measurements of seed pigment were made for the WT and transgenic seed used in the experiments reported here and so the significance of such a screening effect cannot be assessed. An explanation for the apparent shoulder of activity at the shorter wavelengths, 550-650nm, seen in the action spectrum of the transgenic seed, seems unlikely to be due to direct phytochrome absorption. A speculative interpretation may be the possibility of photon energy transfer of shorter wavelengths for absorption by phytochrome via fluorescence of seed tissue pigments, including chlorophyllous pigments; higher levels of chlorophyll are displayed in the light-grown plants of the transgenic tobacco line (Cherry *et al.,* 1991) and it is conceivable that increased levels of chlorophyllous pigments also exist in the seed embryo

tissues.

In contrast to the photoresponsiveness of the transgenic tobacco seed, the induction of dark-germination in seed batches transgenic *Arabidopsis* appeared more amenable to interpretation in terms of phytochrome action. The level of dark-germination of WT seed batches was observed to be sensitive to the R:FR quality of the light-source under which the seed was matured. This result has also been previously reported (McCullough and Shropshire, 1970; Getten-Haynes and Klein, 1974) and is interpreted as indicating that P_{fr} accumulated in the dry seed functions to stimulate germination when the seed imbibes in darkness. The ability of long wavelength FR irradiation, delivered to the imbibed seeds, to inhibit this dark-germination confirmed the requirement for P_{fr}. Seed batches of transgenic *Arabidopsis* which contained either a heterologous rice-*phyB*-cDNA or homolgous *Arabidopsis-phyB*-cDNA (and hence are proposed to contain elevated levels of total phytochrome B) showed increased levels of dark germination relative to WT seed which had been harvested under conditions establishing low P_{fr}/P_{tot} (Table 8.1); inhibition of germination by FR irradiation of the imbibed ABO seed confirmed this to be dependent on P_{fr} (Table 8.2). The simplest interpretation of this result is that overexpressed phytochrome B-P_{fr} in the transgenic seed alleviated the effect of low levels of native seed P_{fr}. Seed of the *Arabidopsis-PhyB*-transformed line, harvested under low R:FR, germinated fully in darkness whereas the inhibitory effect of this pretreatment on WT seed was only partially alleviated in the rice-*phyB*-transformed seed (Table 8.1); this result may be explained by the lower levels of expression of the rice transgene, seedlings of RBO containing 2.5-fold WT levels of total phytochrome B compared to 15-fold levels in seedlings of ABO (Wagner *et al.*, 1991). Very low levels of dark germination in the *hy3* mutant, even in seed matured under WL, corresponded to a deficiency in the phytochrome B molecular species. Thus in *Arabidopsis* lines comprising WT, transgenic and mutant genotypes, the dark germination behaviour could be correlated

with levels of phytochrome B-P_{fr}. A direct role for the light-stable molecular species phytochrome B is therefore indicated in the induction of germination, and this proposal is consistent with the results of Casal *et al.* (1991) which indicated that light promotion of seed germination in *Datura ferox* is under the control of a type II-like phytochrome. Studies of seed germination in the *hy3* mutant of *Arabidopsis* have demonstrated that, although more light was required for photoconversion than WT, R/FR reversible induction of germination was qualitatively normal (Cone and Kendrick, 1985) despite the deficiency in phytochrome B (Somers *et al.*, 1991; Reed *et al.,* 1993). This therefore suggests that a second molecular species of phytochrome can also mediate photoinduction of germination.

In seed batches of the transgenic *Arabidopsis* lines containing introduced oat-*phyA*-cDNA, no notable effect of the heterologous phytochrome on the absolute or relative levels of dark germination was observed (Table 8.1), i.e. the (presumed) presence of enhanced levels of total phytochrome A did not appear to compensate for low levels of endogenous seed P_{fr}. From the responses of the *phyB*-transformed seed, it appears likely that the introduced cDNA was expressed under the control of the CaMV 35S promoter in cells where it was able to control germination. On this evidence, it therefore seems equally likely that the phytochrome A-encoding sequences introduced into transgenic *Arabidopsis* lines were also expressed in the seed tissues; whether the heterologous polypeptide was stable such that phytochrome A was accumulated to the expected high levels, however, remains uncertain. Thus, the inability of the germination behaviour of the oat-*phyA*-transformed seed to indicate any inductive role for phytochrome A may genuinely suggest differential roles for phytochromes A and B in seed germination, or alternatively may be attributed to inactivity of the foreign protein.

In addition, interpretation of all transgenic responses, even assuming the accumulation of transgenically-encoded physiologically-active phytochrome, is subject to the reservation that overexpression of individual phytochrome species may interfere with

a possible feedback regulation of endogenous phytochromes.

These observations emphasise the need for further molecular charaterization of the phytochrome accumulated in transgenic seed. The physiological analysis of transgenic seed overexpressing the homologous species of phytochrome A, coupled with mutant genotypes deficient in phytochrome A, should further clarify the role of phytochrome A in photo-induction and photo-inhibiiton of germination; roles which the results presented here have failed to provide direct support for, and thereby suggest some measure of doubt.

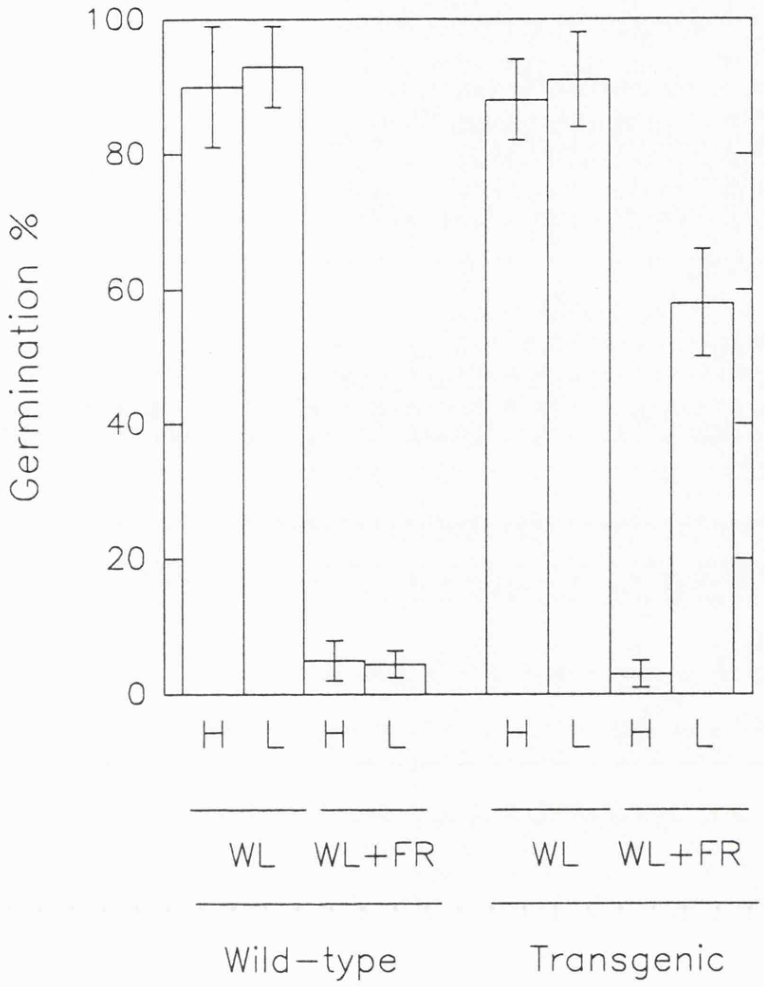
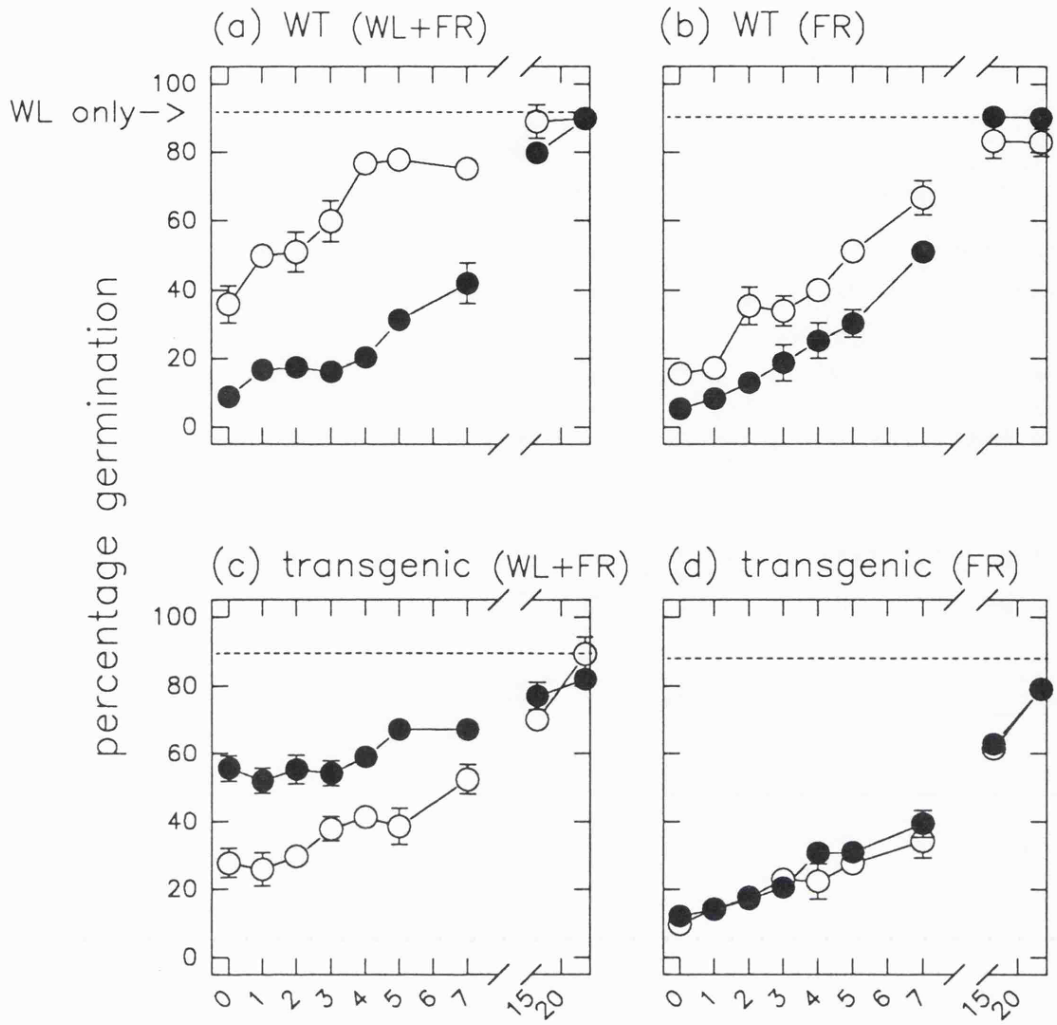
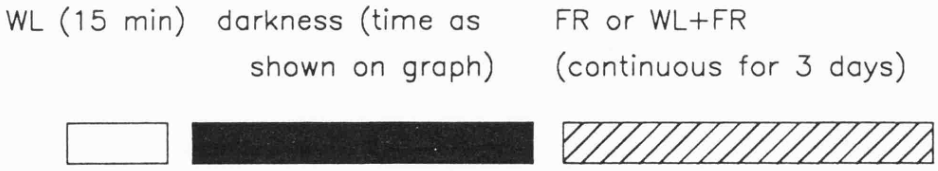


Figure 8.1 Induction of germination under continuous irradiation by white light (WL) and a white light source supplemented with far-red radiation (WL+FR). Seed of wild-type (WT) and oat-*phyA*-transformed genotypes of tobacco were sown onto 1% (w/v) aqueous agar and immediately placed under the light sources. Red (R): far-red (FR) ratios of 6.8 (WL) and 0.07 (WL+FR) were delivered at total photon fluence rates 400-700nm of 160 μmol m^{-2} s^{-1} (H) or 60 μmol m^{-2} s^{-1} (L). Under WL+FR, FR fluence rates (integrated over the 700-800nm wavelength interval) were 500 μmol m^{-2} s^{-1} (H) and 185 μmol m^{-2} s^{-1} (L). Germination was assessed by scoring radicle emergence after 7 days under continuous irradiation.

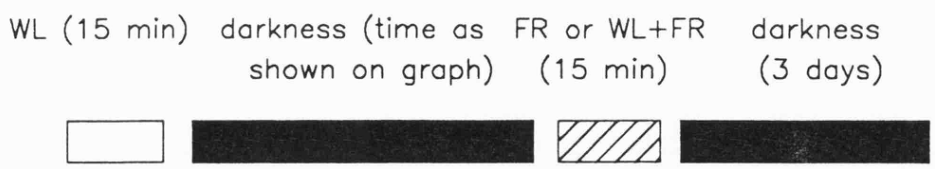
Figure 8.2 Inhibition of germination under continuous irradiation by low red:far-red ratio light. Dark-imbibed seed of wild-type (**a**, **b**) and oat-*phyA*-transformed (**c**, **d**) genotypes of tobacco received a 15 min pulse of white light and were returned to darkness for the period shown. At various times subsequent to this germination-induction treatment, seeds were transferred from darkness to either far-red supplemented white light (WL+FR) (**a**, **c**) or broad-band far-red (FR) (**b**, **d**), and these light treatments were delivered either as a 15 min pulse followed by return to darkness (*open symbols*) or as continuous irradiation throughout the following germination period (*solid symbols*). A schematic summary of the light regime is shown below. Germination levels stimulated in response to a WL pulse only are shown by a dashed line. Germination was scored after 3 days by assessing radicle emergence. Error bars show standard error of the mean



Light regimes



(continuous irradiation; solid symbols)



(15 min pulse; open symbols)

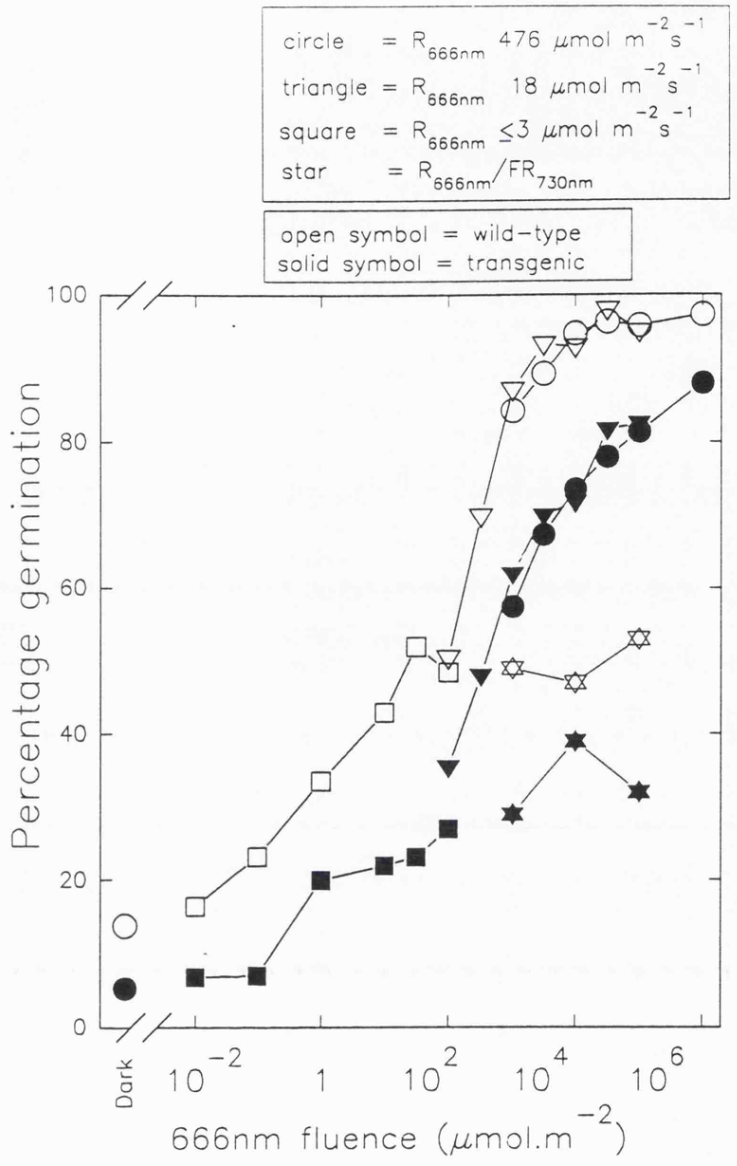


Figure 8.3 Fluence-response curves for induction of seed germination by 666nm light. Seed of wild-type (*open symbols*) and oat-*phyA*-transformed (*solid symbols*) genotypes of tobacco were allowed to imbibe in darkness for 1 day. Seeds were irradiated with various fluences of light of 666nm wavelength and then either immediately returned to darkness (*circle, triangle and square symbols*) or irradiated for a further 15 min by 730nm light (*star symbols*) before returning to darkness. Different fluence rates of 666nm light were used for R-induction of germination [circle = 476 μmol m^{-2} s^{-1}; triangle = 18 μmol m^{-2} s^{-1}; square = 3 μmol m^{-2} s^{-1} (10-10^2 μmol m^{-2}) or 0.02 μmol m^{-2} s^{-1} (10^{-2}-1 μmol m^{-2})]. All seeds were incubated in darkness for 4 days before scoring for radicle emergence. Standard errors were always less than 10% and have been ommitted for clarity.

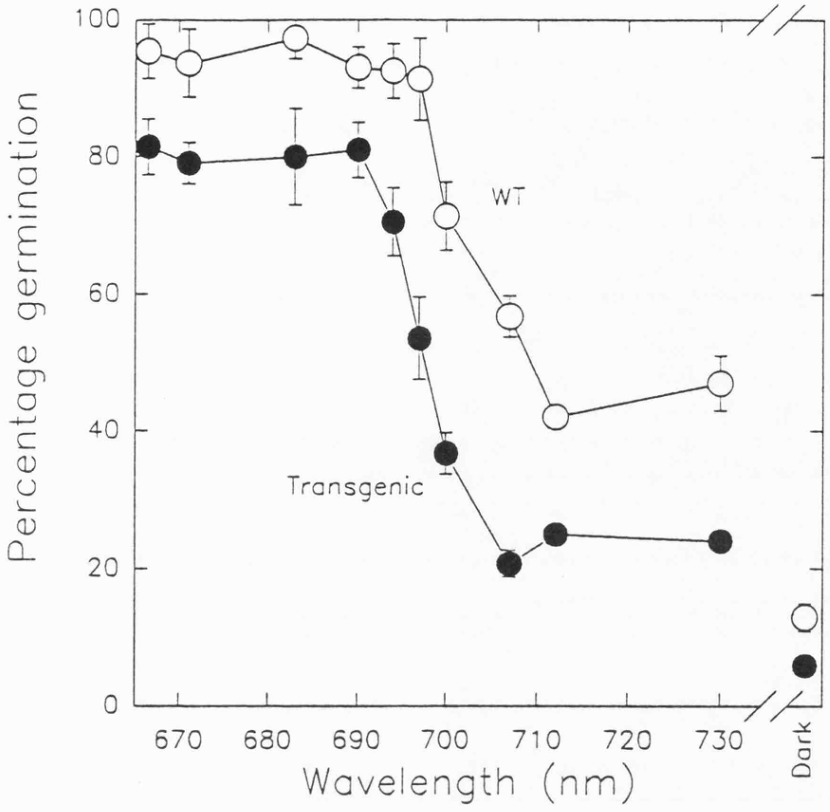


Figure 8.4 Germination induction by radiation in the 670-730nm waveband establishing photoequilibrium. Preimbibed seed of wild-type (*open symbols*) and oat-*phyA*-transformed (*solid symbols*) genotypes of tobacco received a saturating, 15 minute, pulse of light at the wavelength shown. Seeds were subsequently left to germinate in darkness for 4 days. Error bars show standard error of the mean.

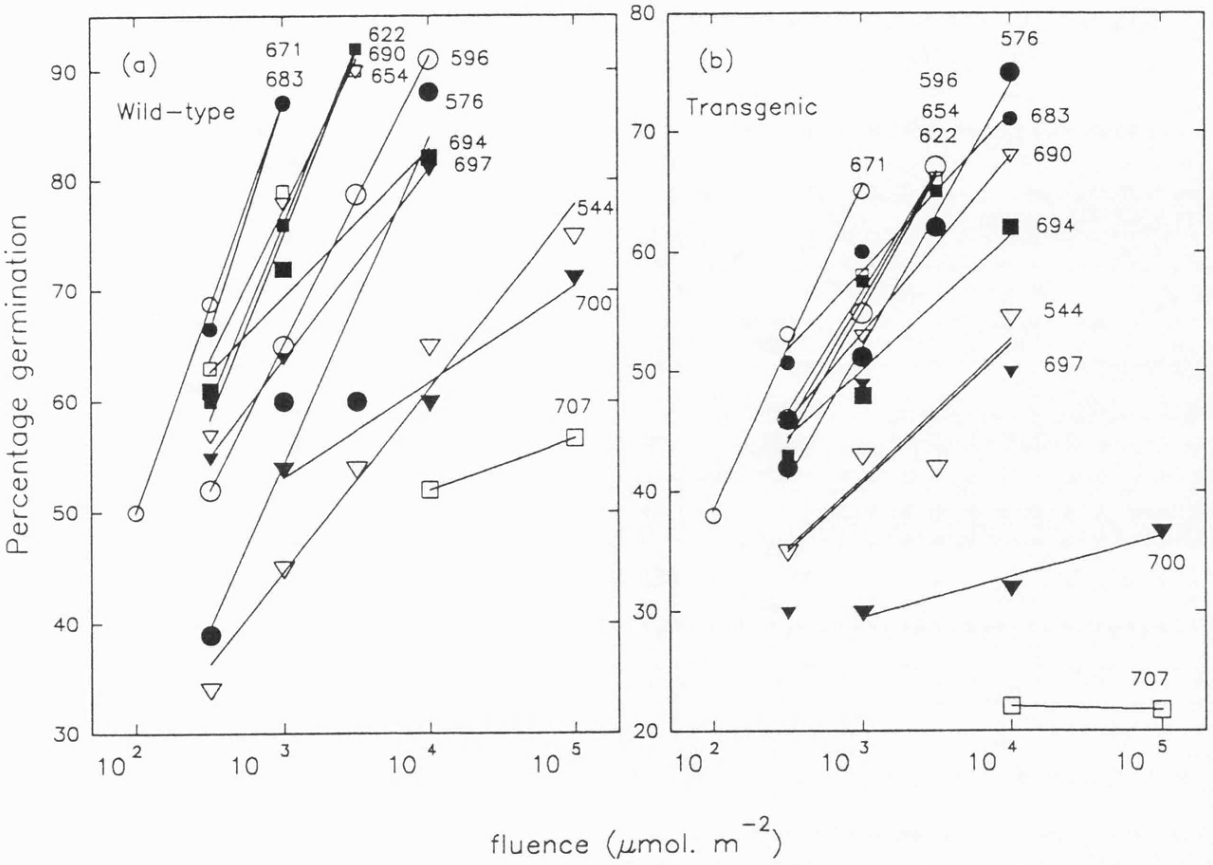


Figure 8.5 Fluence-response curves for the induction of germination with different wavelengths in the red spectrum. Preimbibed seeds of wild-type (**a**) and oat-*phyA*-transformed (**b**) genotypes of tobacco were irradiated with various fluences of light at wavelengths (nm) of 544 (*large open triangle*); 576 (*large solid circle*); 593; 596 (*large open circle*); 607; 617; 622 (*small solid square*); 649; 654 (*small open square*); 664; 666; 671 (*small open circle*); 683 (*small solid circle*); 690 (*small open triangle*); 694 (*large solid square*); 697 *(small solid triangle*); 700 (*large solid triangle*); 707 (*large open square*); 712; 730; irradiation time was maintained constant (2 min) and total fluence was varied by changing fluence rate. Following irradiation, seeds were left to germinate in darkness for 4 days. Some data points and all error bars are ommitted for clarity.

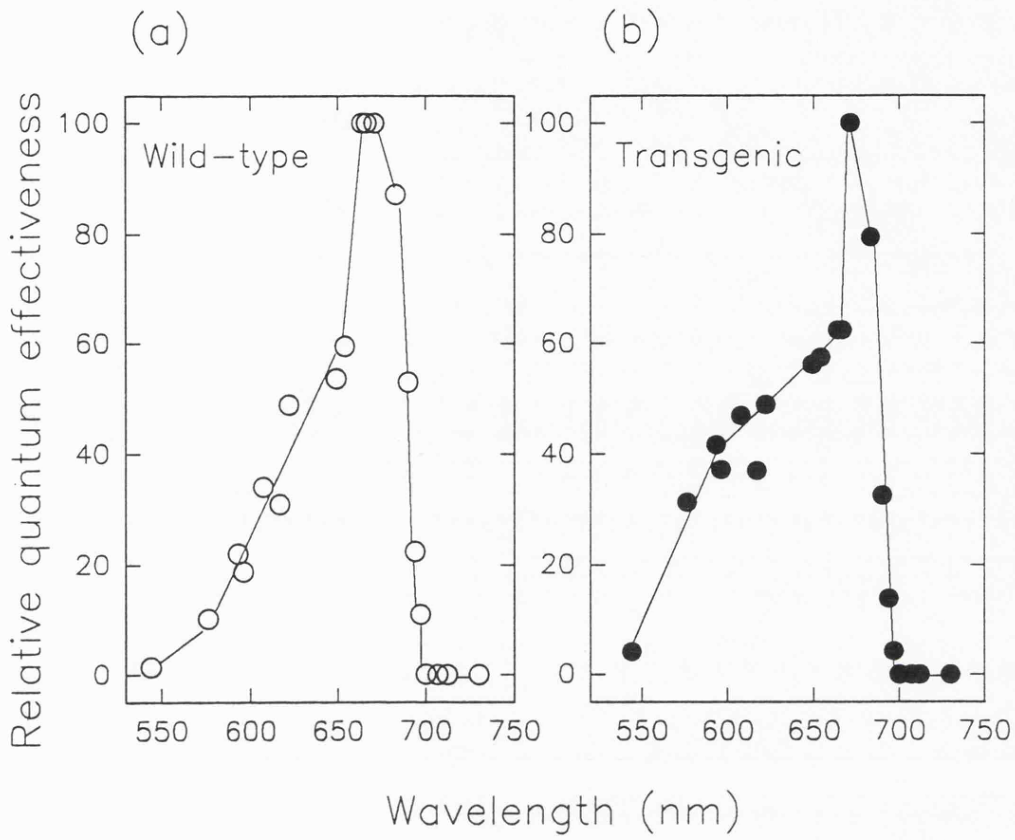


Figure 8.6 Action spectra in the region 550-730nm for the induction of seed germination in wild-type (**a**) and oat-*phyA*-transformed (**b**) genotypes of tobacco. Relative quantum effectiveness for induction of 70% (wild-type) / 55% (transgenic) germination were determind from fluence-response curves (see figure 8.5). Peak effectiveness is normalized to 100%.

Table 8.1 Dark germination of wild-type (WT), mutant and transgenic *Arabidopsis* seeds harvested from plants grown under high or low R:FR ratio. 13K7 and 21K15 were transgenic lines which expressed introduced oat-*phyA*-cDNA; ABO was a transgenic line expressing introduced *Arabidopsis-phyB*-cDNA; RBO was a transgenic line expressing introduced rice-*phyB*-cDNA; all transgenic lines were isogenic with WT ecotype Nossen. *hy3* was a mutant line deficient in the phytochrome B molecular species; isogenic WT ecotype was Landsberg *erecta*. Seeds were sown, within three weeks of harvest, on 1% (w/v) aqueous agar and maintained in the dark for 6 days. Germination was scored by assessing radicle emergence. Data are derived from six replicates, each of fifty seeds, and the numbers in brackets are the standard errors of the means.

Line	% Germination of seeds derived from plants grown under:	
	High R:FR (6.8)	Low R:FR (0.07)
WT (Nossen)	74.2 (3.8)	11.2 (2.0)
13K7	82.8 (2.5)	14.5 (3.2)
21K15	77.6 (6.5)	21.4 (3.6)
ABO	98.6 (1.2)	99.0 (1.1)
RBO	89.5 (3.7)	54.0 (4.2)
WT (Landsberg)	67.5 (5.4)	9.5 (0.8)
hy3	2.5 (0.2)	2.0 (0.2)

Table 8.2 Effect of 10 min 749 nm (FR) light on the germination of imbibed seed of wild-type (WT) and *Arabidopsis-phyB*-cDNA-transformed (ABO) *Arabidopsis*; seed was derived from plants grown under high R:FR.

Line	Imbibition time in dark before FR	% Germination
WT	no FR	74.6 (7.5)
WT	1.5 h	0
WT	3.0 h	0
ABO	no FR	98.6 (1.3)
ABO	1.5 h	0
ABO	3.0 h	0

Chapter 9

Expression of oat-*phyA*-cDNA in the *aurea* mutant of tomato fails to compensate for the *au* lesion.

Introduction

A mutant line of tomato (*Lycopersicon esculentum* Mill.) was isolated as a mutant requiring gibberellin (GA) for germination but which, in contrast to GA-deficient mutants, displayed a long-hypocotyl growth-habit of the light-grown seedlings (Koornneef *et al.,* 1981). Genetic analysis mapped this recessive *aurea* (*au*) mutation to chromosome 1. A mutant at the *au* locus with a similar phenotype has also been derived from the progeny of tissue cultured tomato plants (Lipucci di Paola *et al.,* 1988). The precise location and effect of the *au* lesion is not yet clear. Spectrophotometric and immunological studies (Koornneef *et al.*, 1985; Parks *et al.*, 1987) have established that the total phytochrome of etiolated seedlings of *aurea* is less than 5% of that of wild-type (WT); the mutant is therefore deficient in the light-labile phytochrome pool, presumably the apoprotein product of the *phyA* gene. The *aurea* mutant contains, however, 30-50% of the spectrophotometrically-active phytochrome content of WT plants in the light-grown tissues (Adamse *et al.*, 1988c; Lopez-Juez *et al.,* 1990b) suggesting that it accumulates light-stable phytochrome species.

Light-labile phytochrome of etiolated plants is believed to act as a photosensory trigger for young seedlings emerging into the light environment. The multiplicity of physiological changes associated with the transition from the etiolated to de-etiolated state include inhibition of elongation growth-rate, chlorophyll accumulation and strong up-regulation of expression of a number of genes notably those encoding for components of

the photosynthetic complexes. As might be expected for a photoreceptor-deficient mutant, the photoresponses of the etiolated seedlings of the *aurea* mutant display a reduced sensitivity to red light (R) compared to WT; inhibition of hypocotyl growth under continuous R is weak (Koornneef *et al.,* 1985), potentiation of chlorophyll accumulation by very low fluences of R is lost (Ken-Dror and Horowitz, 1990) and the phytochrome-mediated increase in the abundance of the mRNA encoding chlorophyll *a/b* binding protein (*cab*) is severely restricted in the mutant as compared with WT tomato (Sharrock *et al.*, 1988).

Light-grown plants of *aurea* display apparently normal growth-promotion in response to end-of-day far-red (FR) (Adamse *et al.*, 1988c; Lopez-Juez *et al.*, 1990b) and reductions in R:FR ratio (Whitelam and Smith, 1991). Such responses strongly support the spectrophotometric evidence that the mutant contains a light-stable phytochrome population. The *aurea* lesion is associated with a distinctive phenotype of the light-grown plants compared to WT. An elongated growth habit of the mutant is often observed under white light (Whitelam and Smith, 1991) and the characteristic yellow-green colouration of *aurea* results from a severe deficiency in levels of chlorophyll and anthocyanin (Koornneef *et al.,* 1985). Levels of transcriptional expression of the *rbcS* gene also appear partially depleted in the light-grown plants of *aurea* relative to WT (Sharrock *et al.*, 1988). The apparent persistance of a partially etiolated habit in the light-grown plants of *aurea* has generally been considered attributable to the deficiency in phytochrome; this conclusion, however, is contradicted by the evidence from the *hy1* and *hy2* mutants of *Arabidopsis thaliana*. The *hy1* and *hy2* long-hypocotyl mutants are deficient in biosynthesis of the phytochrome chromophore (Parks and Quail, 1991) and hence contain much reduced levels of spectrophotometrically-active phytochrome including the phytochrome A species. Whilst the *hy1* and *hy2* mutants show reduced levels of light-mediated gene-expression in the etiolated seedlings, plants growing under high fluence rate white light contain WT levels of

cab and *rbcS* transcripts, and also normal levels of anthocyanin pigmentation (Chory *et al.,* 1989b); this result was interpreted to suggest that, in green plants, phytochrome does not exert an important effect on photogene expression.

A cDNA of the structural *phyA* gene, derived from etiolated oat tissues, has been introduced into wild-type tomato where it is expressed under the control of the CaMV 35S promoter to produce a physiologically-active population of foreign phytochrome (Boylan and Quail, 1989). These transgenic plants exhibit the opposite phenotype to *aurea* i.e., the etiolated seedlings show strong inhibition of hypocotyl growth under R and the light-grown plants display high levels of pigmentation and a dwarf stature. Introduction of an oat-derived cDNA encoding phytochrome A into the *aurea* background is described here with the aim of compensating for the native photoreceptor-deficiency and hence restoring the wild-type phenotype.

Materials and methods

Plant material and transformation

A full-length cDNA of oat-*phyA* was subcloned into a binary system vector to generate plasmid pRFY1 (see Chapter 4 of this thesis, *Materials and methods: Plasmid constructions*); in this construct the cDNA was flanked 5' by the promoter region and 3' by the downstream polyadenylation signals from the CaMV 35S transcript. The plasmid was transferred into *Agrobacterium tumefaciens* (strain 2260) by a triparental conjugation, as described in Chapter 4 of this thesis (*Materials and methods: Plasmid constructions*).

Seeds of the *aurea* tomato mutant (obtained from Dr. M. Lipucci Di Paola, University of Pisa, Italy) were surfaced sterilised in a 10% (v/v) solution of domestic bleach and were grown aseptically on moist vermiculite with a 1/2 strength solution of MS salts (Murashige and Skoog, 1962). Cotyledon explants were inoculated with an overnight culture of *Agrobacterium* in a method adapted from McCormick *et al.* (1986). Inoculated explants were incubated on MS medium containing 30 g l^{-1} sucrose, 7g l^{-1} agar, pH 5.8, for 2 days at 28°C. Subsequently, explants were transferred to MS medium containing 30 g l^{-1} sucrose, 2.5 mg l^{-1} 6-benzylaminopurine (BAP), 1 mg l^{-1} indole-3-acetic acid (IAA) and 7 g l^{-1} agar, pH 5.8, which encouraged shoot regeneration in the explants (Fig. 9.1); the medium was supplemented with Augmentin (400 mg l^{-1}) to inhibit further bacterial growth and kanamycin (100 mg l^{-1}) to select positively transformed callus/ shoots. Multiplication of shoots from desirable transformants was achieved by transferring primary shoots to fresh shoot-inducing media which encouraged further callus formation and subsequently shoot production. Rooting of shoots was achieved on MS medium containing 30g l^{-1} sucrose and 7g l^{-1} agar, pH 5.8, supplemented with 100 mg l^{-1} kanamycin. For physiological studies these plantlets were transferred to potting compost. The isogenic wild-type plants were of the cultivar UC-105.

RNA analysis

Total RNA was extracted from light-grown leaf tissue of primary transformants, as described in detail in Chapter 4 of this thesis (*Materials and methods, RNA extractions*). The extracts were run on denaturing RNA gels and were blotted onto a nylon membrane. Blots were probed with a 650 bp *Sac* 1/*Nco* 1 fragment derived from the 5' end of oat-*phyA*-cDNA; details of hybridisation procedures are given in Chapter 4 of this thesis (*Materials and methods, RNA gel analysis*).

Phytochrome extraction and immunoblot analysis

Plants were irradiated with far-red light for 15 minutes and placed in darkness for 4 days (25^{o}C). Under dim green lighting, crude extracts were made and the proteins concentrated by ammonium sulphate precipitation according to Pratt *et al.* (1991). The proteins were resolved on an 8% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membrane. The total protein profile was visualised on the blot by reversible staining with 0.2% ponceau in 3% trichloroacetic acid. The blots were probed using a mixture of monoclonal antibodies which cross-reacted with both monocot. and dicot. phytochrome species. The extract of the oat-*phyA*-transformed tobacco line, included in the immunoblot analysis, was prepared from etiolated seedlings of the "Y_{12}" line, as described in detail in Chapter 4 of this thesis.

Chlorophyll and anthocyanin measurements

Chlorophyll was extracted from fully expanded leaves by immersing leaf discs into N,N-dimethylformamide for a minimum of 2 days; extractions were performed at 4^{o}C in darkness. Absorption of the extracts was measured at 646nm and 664nm and total

chlorophyll levels were calculated according to Moran (1982). Anthocyanin was extracted from the shoot tips by boiling for 5 min in propan-1-ol: HCl: H_2O (18:1:81) and the extracts were partitioned, after Folch *et al.* (1957), by adding equal volumes of chloroform followed by centrifugation. The absorbance of the top phase was measured at 535nm.

Results

Leaves were harvested from the light-grown shoots of primary transformants, total RNA was extracted and blots of the RNA gels were probed with a radiolabelled fragment of oat-*phyA*-cDNA. As controls, RNA from wild-type tomato and etiolated oat tissues was included. Figure 9.2 shows a strong signal corresponding to a band in the etiolated oat tissue whereas no hybridisation was detected in the wild-type tomato, so demonstrating the specificity of the probe. Ten independent kanamycin-resistent transformants of *aurea* were analysed of which two (aurea.tr1 and aurea.tr2 - Fig. 9.2) showed clear levels of hybridisation with the oat-cDNA; the position of the band indicated that the transcript was full-length with respect to the native etiolated oat message.

Blots of total-protein extracts were probed with a mixture of monoclonal antibodies which detected the native phytochrome of etiolated oat and also the phytochrome accumulated in dark-adapted plants of wild-type tomato (Figure 9.3). As a further control, the extract from etiolated seedlings of a transgenic tobacco line, which expressed the polypeptide product of the same CAMV 35S/ oat-*phyA*-cDNA construct as used for the *aurea*-transformations, was also included; two bands were detected in this transgenic tobacco sample, corresponding to the native phytochrome (approx. 120Kd) and oat-*phyA*-encoded phytochrome (approx. 124 Kd). Extracts from the dark-adapted plants of *aurea* did not react with the antibodies. Extracts of transformed plants of *aurea,* which had been identified as accumulating the mRNA transcript of the introduced oat-*phyA*-cDNA (Fig. 9.2), did not contain any detectable levels of phytochrome; thus, the polypeptide product of the oat-*phyA*-mRNA did not appear to accumulate in the transgenic *aurea* plants (Fig. 9.3).

The physiological characteristics of the light-grown plants of oat-*phyA*-cDNA-transformed *aurea* were compared with those of non-transformed *aurea* and wild-type. In plants grown under continuous illumination by white light, internodes of

the transgenic *aurea* plants were elongated relative to wild-type tomato (Table 9.1; Fig. 9.4) and the leaves were visibly depleted in chlorophyll (Table 9.1; Fig. 9.4). Measurement of anthocyanin levels in the shoot tips (Table 9.1) also revealed that the transgenic plants retained the pigmentation-deficiency associated with the light-grown plants of the *aurea* mutant. Therefore, the phenotype of the transgenic *aurea* plants did not resemble wild-type.

Discussion

Expression of a foreign cDNA encoding phytochrome A in plants of the *aurea* mutant did not result in the accumulation of the heterologous phytochrome protein and was unable to restore the wild-type phenotype. These results suggest that the *aurea* mutation cannot be complemented by a *phyA* allele. Doubts as to the location of the *aurea* lesion with respect to the phytochrome structural gene have been raised through previous molecular analysis of the phytochrome deficiency in *aurea*. Northern analysis has indicated that the phytochrome mRNA product is accumulated to normal levels in plants of *aurea* and functions in an *in vitro* translation system to produce an apparently normal protein product (Sharrock *et al.*, 1988). The deficiency in immunodetectable phytochrome *in vivo*, and also the failure to accumulate the oat-encoded polypeptide in the transgenic *aurea* plants, is most likely explained by an instability of the phytochrome protein *in planta*. The information available regarding the mapping of the phytochrome genes in tomato, although limited, also suggests that the *aurea* lesion is at a location distinct from the structural gene of phytochrome.

The implication, therefore, is that the phytochrome deficiency of the *aurea* tomato mutant is the result of an aberration in some secondary factor which influences polypeptide stability; it seems unlikely that this factor involves the stabilization of the apoprotein through attachment of a chromophore group as spectrophotometrically-active forms of light-stable phytochrome appear to accumulate in *aurea* (Adamse *et al.*, 1988a) and the apoprotein product of the *phyA* gene accumulates stablely in oat and pea, even in the event of gabaculine-mediated inhibition of chromophore biosynthesis (Jones *et al.*, 1986; Elich and Lagarias, 1987). A possible explanation for phytochrome-instability in *aurea* could be an aberration in the proteolytic turnover mechanism that degrades phytochrome following conversion of the P_r form to the P_{fr} form (Shanklin *et al.*, 1987) or, alternatively, a (hypothetical) protein required for stabilization of the polypeptide

could be deficient. It is not possible, therefore, to disregard the possibility that the accumulation of other photoreceptor or transduction chain proteins may also be impaired in *aurea*. Interpretation of the photoresponses of this mutant must be made with reservations subject to the precise characterisation of the *aurea* lesion. This conclusion is further emphasised by the recently isolated *hy8* mutant class of *Arabidopsis* which is selectively deficient in phytochrome A (probably due to a lesion in the *phyA* structural gene) but, in contrast to *aurea*, displays an essentially normal phenotype in the light-grown plants (Parks and Quail, 1993).

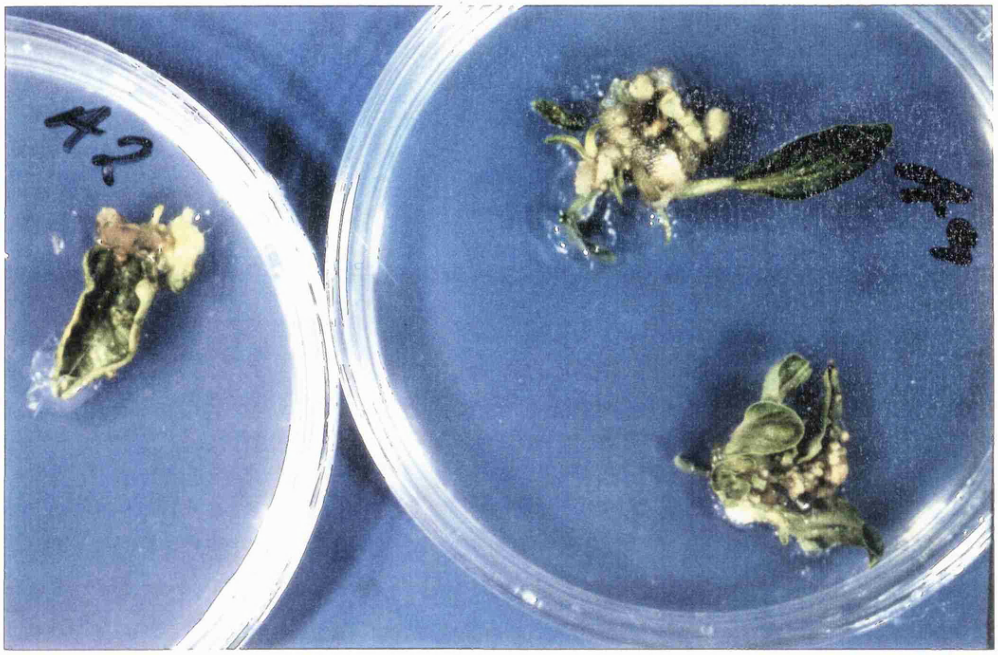


Figure 9.1 Shoot regeneration in cotyledon explants of the *aurea* mutant of tomato. The shoot-inducing medium contained MS salts supplemented with 2.5 mg l^{-1} 6-BAP and 1 mg l^{-1} IAA.

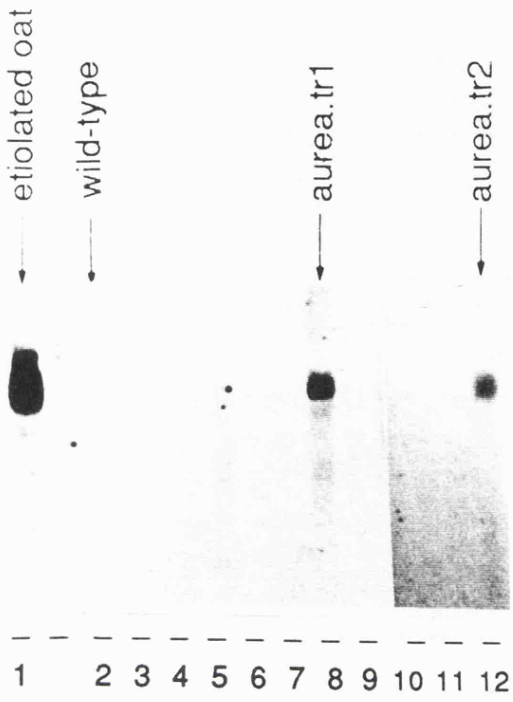


Figure 9.2 RNA gel analysis of total RNA extracts from transformed shoots of the *aurea* mutant of tomato. Blots were probed with a 650 bp fragment of oat-*phyA*-cDNA which detected the native *phyA*-mRNA of etiolated oat tissues (lane **1**) but did not hybridise with total RNA of light-grown plants of wild-type tomato (lane **2**). From the extracts of the light-grown plants of ten independently transformed plants of *aurea* (lanes **3-12**), two hybridised with the probe (aurea.tr1 and aurea.tr2; lane 8 and 12). Lane 1 contained approx. 7 µg of total RNA, lanes 2-12 each contained approx. 30 µg of total RNA.

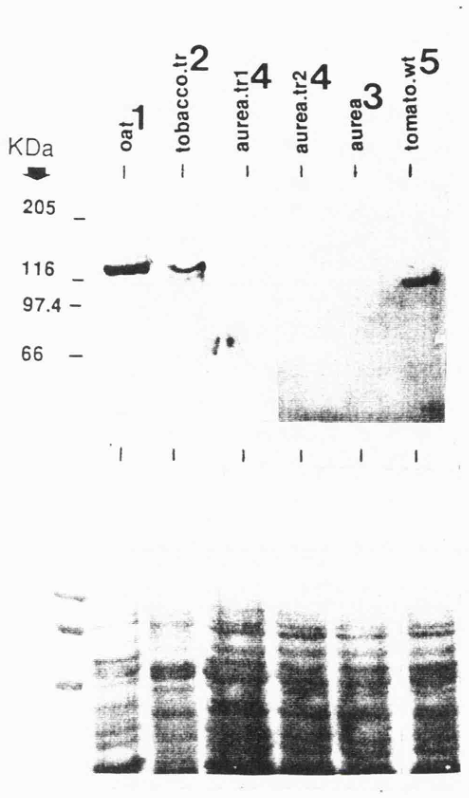


Figure 9.3 *Top* : Immunoblot analysis of protein extracts from dark-adapted plants of transformed *aurea*. Protein extracts were separated on an 8% SDS-polyacrylamide gel, blotted onto nitrocellulose and probed with anti-phytochrome monoclonal antibodies. Extracts from etiolated oat[1] and dark-adapted plants of wild-type tomato[5] showed the monoclonal antibodies to detect both monocot. and dicot. encoded phytochromes; in extracts from etiolated seedlings of transgenic tobacco[2], detection of phytochrome encoded by the introduced oat-*phyA*-cDNA (upper band of doublet) confirmed the oat-cDNA to code for an immunoreactive polypeptide. Extracts of *aurea* [3] and of oat-*phyA*-cDNA-transformed *aurea*[4] (aurea.tr1 and aurea.tr2) did not contain immunodetectable levels of either wild-type or oat-encoded phytochrome.

Below : Total protein profile for each lane.

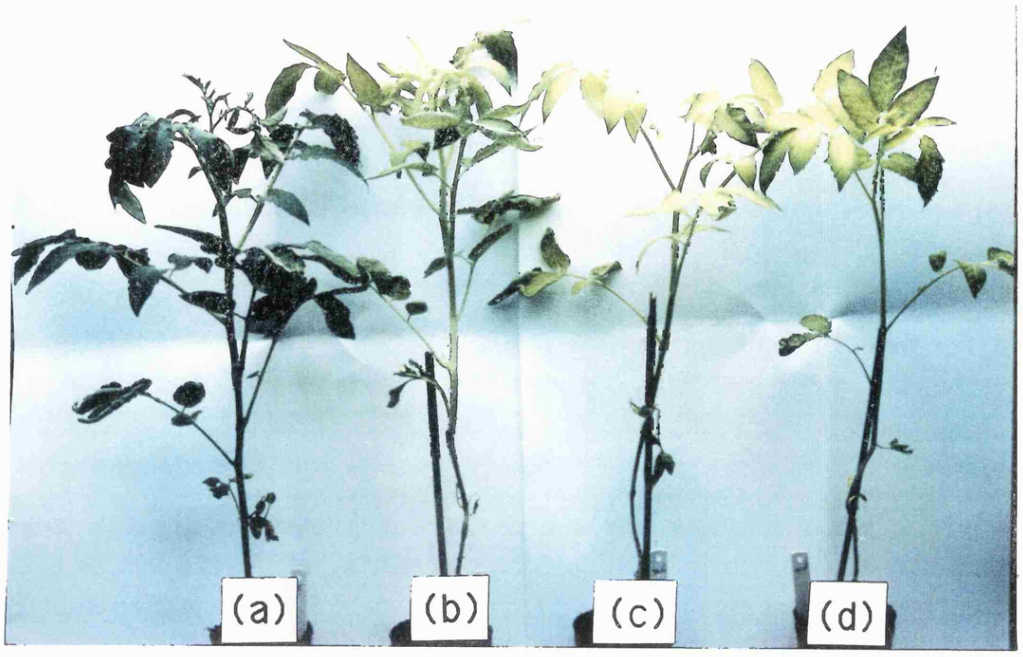


Figure 9.4 Light-grown plants of (**a**) wild-type tomato; (**b**) *aurea* mutant of tomato; (**c**), (**d**) primary transformants of *aurea* which had been shown to accumulate the mRNA product of the introduced oat-*phyA*-cDNA.

Table 9.1 Phenotypic characteristics of light-grown plants of oat-*phyA*-transformed *aurea* (aurea.tr1, aurea.tr2) compared to non-transformed *aurea* and wild-type tomato.
Values are the mean (± S.E.) of a minimum of three measurements.

	Internode length (mm)	Chlorophyll content of leaves ($\mu g \cdot mg\ fr\ wt^{-1}$)	Anthocyanin content in shoot tips ($A_{535} \cdot g\ fr\ wt^{-1}$)
Wild-type	36.7 (± 2.2)	5.51 (± 0.11)	1.02 (± 0.10)
aurea	45.8 (± 4.7)	1.24 (± 0.27)	0.02 (± 0.10)[a]
aurea.tr1	47.6 (± 4.1)	1.18 (± 0.34)	0.03 (± 0.04)[a]
aurea.tr2	48.0 (± 3.8)	1.57 (± 0.04)	0.02 (± 0.18)[a]

[a] virtually undetectable.

Chapter 10

Expression of oat-*phyA*-cDNA in transgenic plants of horseradish (*Armoracia lapathithfolla* L.).

Introduction

Plants which are placed in a competative environment for light employ one of two opposing strategies: shade tolerance or shade avoidance. Of the crop plants used in commercial agriculture, the vast magority demonstrate shade-avoidance tactics. The shade-avoidance strategy basically consists of a marked increase in extension growth of internodes and petioles, growth which acts as a sink for the major redistribution of resources and growth potential away from the leaves and storage organs. The growth-strategy of the shade-avoidance syndrome is therefore associated with reductions in leaf, storage organ and flower/fruit development (Kasperbauer and Karlen, 1986; Kasperbauer, 1987; Keiller and Smith, 1989).

An important corollary of the induction of shade-avoidance is mediated by the red (R):far-red (FR) ratio of radiation reflected from neighbouring plants (Ballaré *et al.*, 1987; 1990). The extension growth rate via the phytochrome-mediated perception of neighbours appears to show a quantitative relationship with plant proximity (Smith *et al.*, 1990). Shade avoidance does not discriminate between plant species and crop plants grown at high density exhibit the shade-avoidance response to a greater or lesser degree, leading to a depletion of seed or storage-organ yield. Reducing plant density would alleviate this problem in the individual plant but would be uneconomic usage of land. A means to disable the shade-avoidance responses of crop plants would provide the potential for large

increases in harvestable yield. In transgenic plants of tobacco and *Arabidopsis* it has been shown that persistence of phytochrome A in light-grown plants effectively eliminates the extension growth responses to low R:FR (McCormac *et al.*, 1991; Whitelam *et al.*, 1992; Chapters 3, 4 & 5 of this thesis). Transgenic manipulation of the photoreceptor population appears, therefore, to be a promising approach.

The most dramatic illustration of the diversion of assimilates away from the agronomically-desirable organ is provided by the development of root crops such as sugarbeet (*Beta vulgaris* L.) and radish (*Raphanus sativus* L.) under simulated shade conditions (Appendix 10.i). Such species would therefore provide an ideal system in which to test for the enhancement of yield in plants which display a disabled shade-avoidance syndrome. Unfortunately, despite attempts to develop a transformation/regeneration system for radish (Appendix 10.ii), routine protocols for the introduction of genes into plants of the major commercial species of root crop are not currently available. For horseradish (*Armoracia lapathifolia* L.), however, a quick and efficient transformation system is available via *Agrobacterium rhizogenes*-mediated generation of hairy roots (Noda *et al.*, 1987); horseradish was therefore used here as a preliminary system for assessing the effect of a heterolgous phytochrome population on the distribution of assimilates under low R:FR light conditions.

Materials and methods

Transformation and regeneration of plants

A full-length cDNA of oat-*phyA* was subcloned into a binary system vector to generate plasmid pRFY1 (see Chapter 4 of this thesis, *Materials and methods: Plasmid constructions*); in this construct the cDNA was flanked 5' by the promoter region and 3' by the downstream polyadenylation signals from the CaMV 35S transcript. The plasmid was mobilized from *E. coli* strain XL1-blue into *Agrobacterium rhizogenes* (strain 9402) using pRK2013 in *E. coli* strain HB101 for plasmid mobilization, in a triparental conjugation. The *Agrobacterium* acceptor strain contained a chromosomally-located rifampicin resistance gene. Conjugates were selected after 24 h of growth at 28^{o}C on non-selective medium, by plating onto MS medium containing 30 g l^{-1} sucrose, 10 g l^{-1} agar and supplemented with 100 mg l^{-1} kanamycin and 200 mg l^{-1} rifampicin.

Using a protocol adapted from Noda *et al.* (1987), surface-sterilised leaf discs of horseradish were inoculated with an overnight culture of *Agrobacterium*. Inoculated explants were incubated for 2-3 days on 1% (w/v) agar. Subsequently, explants were cultivated on phytohormone-free MS medium containing 30g l^{-1} sucrose and 7g l^{-1} agar, pH 5.8. Augmentin (400 mg l^{-1}) was added to inhibit bacterial growth. In contrast to the report of Noda *et al.* (1987), uninoculated leaf discs also produced root out-growths which could not be reliably distinguished from the roots emerging from the inoculated discs. It was therefore necessary to include some means of selection for transformed roots. Roots emerging from the explants were excised when they were at least 1cm in length and placed on fresh medium containing a range of concentrations of kanamycin. Lateral root growth and bud formation under cool white fluorescent lighting was assessed on these various selection levels.

Regeneration of multiple plantlets from a single transformant was achieved by

placing surface-sterilised leaf discs on to MS medium containing 20 mg l^{-1} kanamycin. Kanamycin-resistent root out-growths were allowed to produce shoots. Shoots were transferred to potting compost for physiological studies. WT leaf discs were also incubated on MS medium, but without kanamycin supplement. The resulting plantlets were treated in the same way as the transgenics.

Procedures for RNA extraction and RNA gel analysis were the same as described in *Materials and methods* of Chapter 4 of this thesis.

Light sources for R:FR ratio treatments were provided by an arrangement of filters which has been described in Chapter 3 of this thesis *(Materials and methods; "low PAR cabinets")*.

Petiole extension growth was measured as the ratio of petiole length (measured from the base of the petiole to the base of the leaf laminar) in proportion to the leaf laminar length (measured from the base to the tip of the laminar).

Results

Excised sections of root out-growths from the *Agrobacterium*-inoculated and uninoculated leaf discs of horseradish were grown on media containing 0, 10, 25 or 50 mg l^{-1} kanamycin. Non-transformed roots from the uninoculated leaf discs showed much reduced lateral branching compared to the hairy roots derived from inoculated discs, even in the absence of antibiotic selection (Fig. 10.1a, b). In the presence of 10 mg l^{-1} kanamycin, or higher, the growth of non-transformed roots was almost completely inhibited and no bud formation occurred (Fig. 10.1a). Over 50% of the roots from the inoculated leaf-discs, however, continued to grow vigorously in the presence of 10 mg l^{-1} and 25 mg l^{-1} kanamycin; 50 mg l^{-1} significantly reduced growth of all roots (Fig. 10.1b). High levels of shoot regeneration from kanamycin-resistant roots was obtained on medium containing up to 25 mg l^{-1} kanamycin. A kanamycin level of 20 mg l^{-1} was chosen as optimal for the selection of transformed roots.

RNA gel blots of the light-grown leaves of horseradish plants which had been regenerated from kanamycin-resistant roots were probed with a 650 bp *NcoI*/*SacI* fragment of oat-*phyA*-cDNA. This identified two independent plants which were expressing high levels of transcript of the introduced cDNA (HR.tr1 and HR.tr2 - Fig. 10.2).

Transformed plants which accumulated oat-*phyA*-mRNA were grown under low R:FR (achieved by adding FR radiation to a background of white light). Leaf growth of the individual transgenic plants was compared with WT and with a kanamycin-resistant transformant which had failed to hybridize with the oat-*phyA*-cDNA probe in the RNA gel analysis. The transgenic plants expressing oat-*phyA*-mRNA showed shortened petioles under low R:FR compared to plants not expressing the heterologous phytochrome (Fig. 10.3A). However, transformation of plants of *Brassica napus* with the Ri T-DNA of *A. rhizogenes* has been reported to confer an altered phenotype, including wrinkled leaves and shortened internodes (Guerch *et al.*, 1987). It was therefore necessary to show that the

growth effect of the transgenic genotype of the horseradish plants was a reflection of the response to R:FR. In order to statistically compare the response of the transgenic plants under the high and low R:FR ratio light sources, multiple plantlets were generated asexually via the *in vitro* culture of leaf explants; kanamycin resistent root out-growths produced shoots that were isogenic with the original transgenic parent plant. WT plants were also generated in this manner. Plantlets were transferred to potting compost and grown under continuous irradiation by white light (WL) (R:FR = 6.8) or FR-supplemented WL (WL+FR) (R:FR = 0.2). Leaf-growth of transgenic plants under high R:FR was not obviously different from that of WT plants (Fig. 10.4). However, under low R:FR the petiole extension growth of the transgenic plants was significantly inhibited relative to WT (Fig. 10.3B 10.4). WT plants responded to the reduction in R:FR ratio by an increase in petiole extension growth (measured as an increase in the length of the petiole in proportion to the length of the leaf laminar; Fig. 10.4). Transgenic plants expressing oat-*phyA*-cDNA did not respond to reduced R:FR ratio by any elongation of petiole growth relative to plants under WL (Fig. 10.4). In fact, a slight inhibition of the elongation growth of the transgenic plants was measured under the FR-supplemented light source; such a FR-mediated inhibition of growth has also been reported for the growth-responses of light-grown plants of transgenic tobacco (McCormac *et al.,* 1991) and *Arabidopsis* (Whitelam *et al.*, 1992).

Plants of WT and transgenic horseradish were grown under continuous irradiation from WL or WL+FR light sources for about 6 months, and subsequently were harvested and the root total fresh weight recorded (Table 10.1). The root yield of WT plants grown under low R:FR ratio was reduced relative to plants under WL by approx. 20%. In transgenic plants expressing an introduced oat-*phyA*-cDNA no significant effect of the reduction in R:FR ratio was measured for root fresh weight. However, transgenic root-growth was reduced relative to wild-type plants (Table 10.1).

Discussion

Plants of wild-type horseradish, when growing under conditions simulating the low R:FR light environment experienced by plants growing in competition with overhead or neighbouring plants, responded by increased growth of the petioles. Such increases in extension growth as part of the shade-avoidance syndrome are associated with a concommitant reduction in the development of storage organs (Appendix 10.i) due to diversion of the growth resources (Kasperbauer and Karlen, 1986; Kasperbauer, 1987; Keiller and Smith, 1989). Transgenic plants of horseradish expressing introduced oat-*phyA*-cDNA did not show an elongated phenotype under low R:FR; thus, the response to competitive shade appeared to be disabled as the result of expression of a heterologous phytochrome A population. The storage-root of horseradish is largely a fibrous growth (as opposed to a discrete swollen organ as, for example, in radish), and this characteristic largely precluded the direct measurement of the effect of the transgenic genotype on root yield. However it is conceivable that, by removing the sink for assimilates through suppression of extension growth under low R:FR, the supply of assimilates may be maintained to the storage organ in plants receiving reflected FR from neighbouring plants, and the relative effects of R:FR ratio on root fresh weight yield that were measured in the WT and transgenic horseradish plants is tentatively considered to supports this. However root-growth of transgenic plants, under all light conditions, was reduced compared to that of WT plants; plants regenerated from *A. rhizogenes*-generated hairy roots have been reported to display abnormal phenotypes including altered root-growth characteristics i.e. increased adventitious rooting (Tepfer, 1984) and a tendancy in transfromed plants of horseradish for the proliferation of fibrous roots instead of tuberous roots (Noda *et al.*, 1987). Thus the use of an *A. rhizogenes* transformation system has an inherent effect on root-growth which appeared deleterious to root-yield in the plants of horseradish.

Engineering crop plants to be effectively blind to shade effects from neighbouring

plants may confer substantial advantages in terms of increased allocation of assimilates to harvestable yield components. However without extensive trials, including in-field situations in which end-of-day irradiation effects will also be an important factor (Kasperbauer, 1971; Smith, 1982), it cannot be predicted whether any debilitating effects will be incurred. The evidence indicates that phytochrome A is not normally responsible for proximity perception in wild-type plants. In transgenic plants, the overexpressed phytochrome A population appears to exert its effect by predominating over the activities of the phytochrome species which mediate R:FR ratio perception in WT plants. Overexpression of phytochrome B in transgenic plants of *Arabidopsis* has been shown to reduce, but not eliminate, the extension growth responses of the shade avoidance syndrome (McCormac *et al.*, 1993; Chapters 4 & 5). Manipulation of the light-stable phytochrome population, normally predominant in the light-grown plants, may prove more appropriate for a programme of agronomic development.

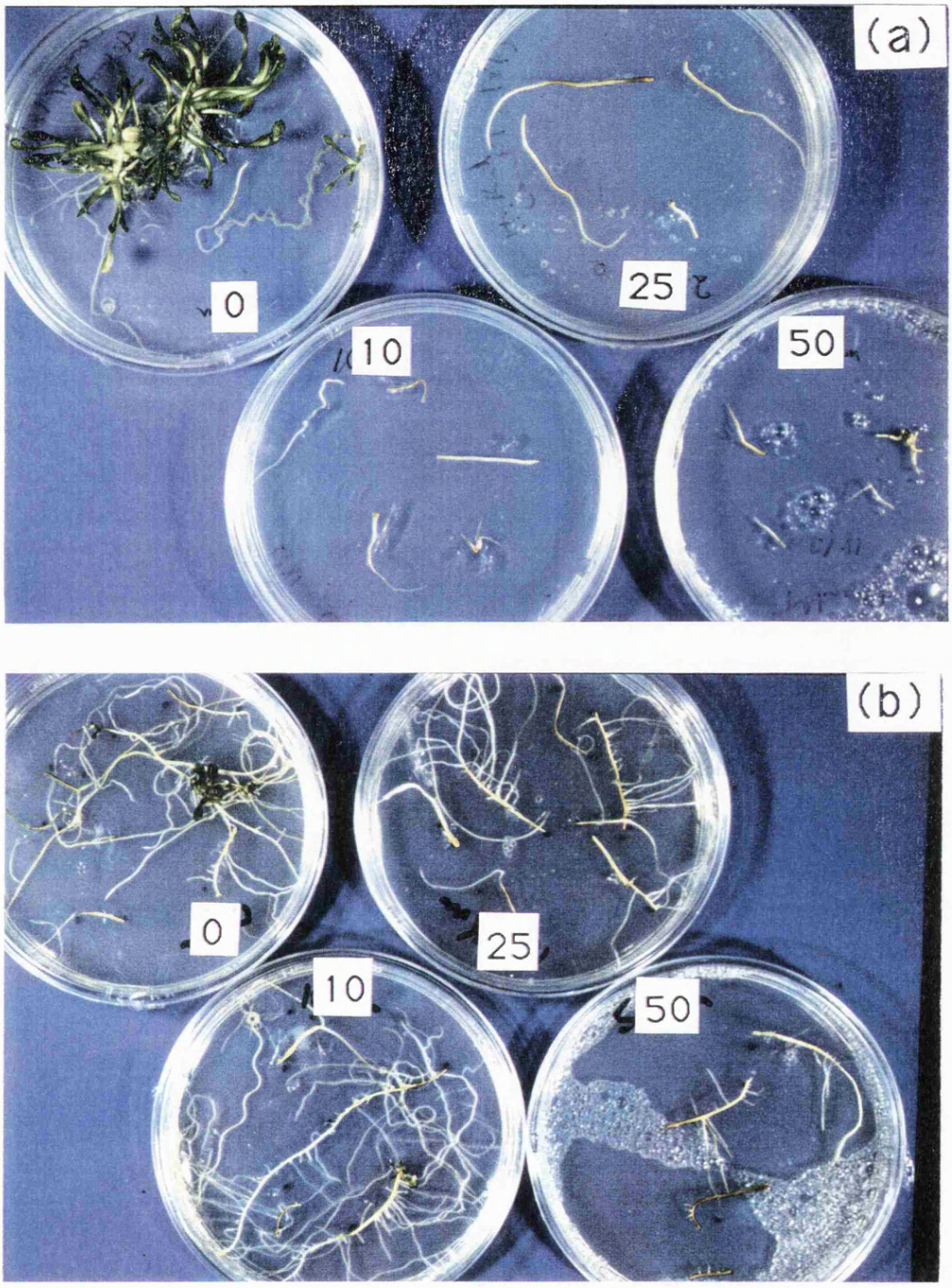


Figure 10.1 A comparison of the antibiotic-resistance of the growth of wild-type and *Agrobacterium rhizogenes*-transformed roots. Root out-growths were excised from leaf discs of horseradish which (**a**) were uninoculated or (**b**) had been inoculated with *A. rhizogenes*. Root-sections were grown under low intensity white light on phytohormone-free medium containing kanamycin at concentrations of 0, 10, 25 and 50 mg l^{-1}.

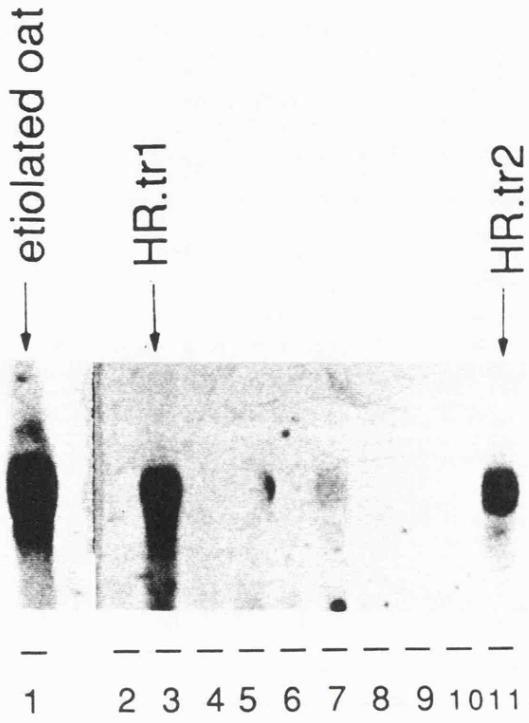


Figure 10.2 RNA gel blot analysis of horseradish plants regenerated from *A. rhizogenes*-transformed roots. Extracts of total RNA from etiolated oat seedlings (lane **1**) and light-grown plants of transgenic horseradish (lanes **2-11**) were probed with a fragment of cDNA specific for oat-*phyA*-mRNA. Lane 1 contained approx. 10 µg total RNA; lanes 2-11 each contained approx. 30 µg total RNA.

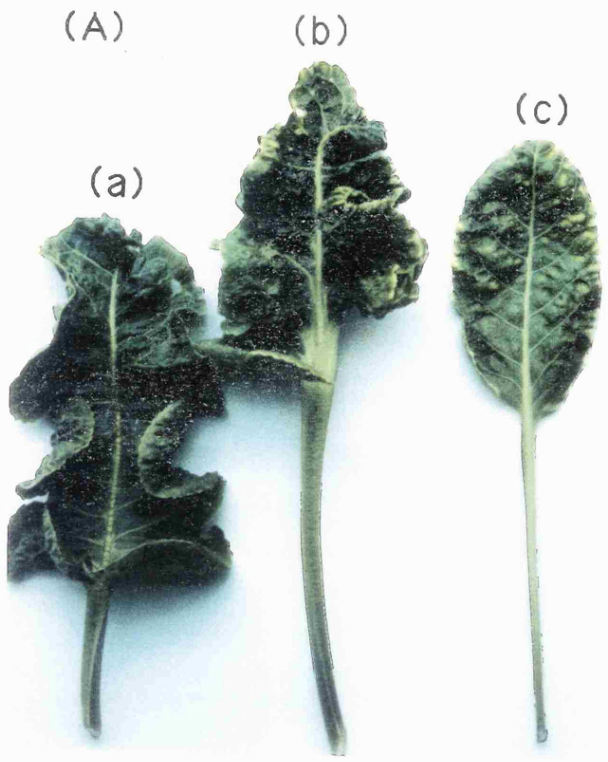


Figure 10.3A Petiole growth of plants of horseradish growing under low R:FR ratio. Leaves shown are from (**a**) wild-type plants, (**b**) plants regenerated from roots transformed with *A. rhizogenes* but which did not express oat-*phyA*-cDNA, (**c**) plants regenerated from roots transformed with *A. rhizogenes* and which accumulated oat-*phyA*-mRNA.

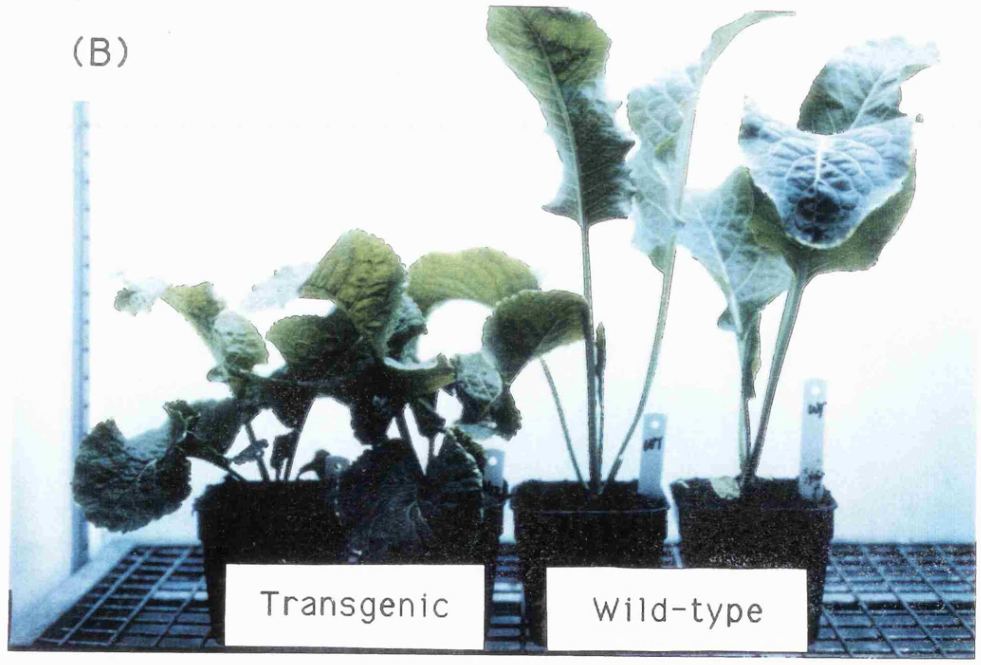


Figure 10.3B Comparison of the growth-habit of wild-type and transgenic plants under low R:FR ratio. Isogenic transgenic plants were regenerated from root out-growths of *in vitro* cultured leaf explants, taken from a single original transformed plant expressing oat-*phyA*-cDNA. The WT plants were also regenerated from cultured root pieces. Plants were grown under continuous irradiation by white light enriched with far-red radiation (R:FR = 0.2).

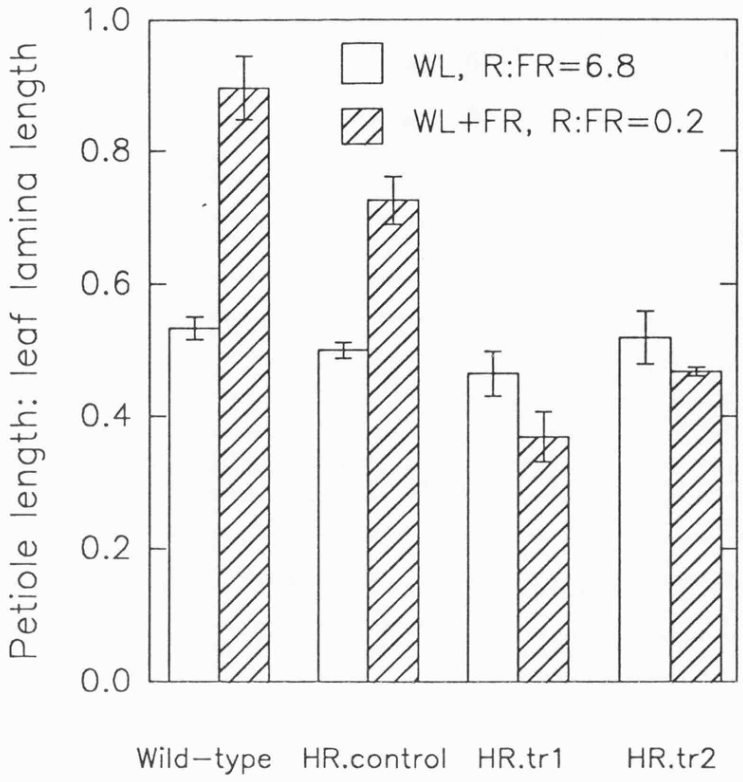


Figure 10.4 Petiole extension growth in wild-type and transgenic plants of horseradish in response to a reduction in R:FR ratio. The measured petiole length is shown in proportion to the length of the leaf laminar in the individual leaves. Isogenic sets of plants of WT and transgenic genotypes were regenerated from root out-growths of *in vitro* cultured leaf-explants taken from a single original plant. *HR. control* was a set of plants which had been regenerated from *A. rhizogenes*-transformed roots but which did not express oat-*phyA*-cDNA. *HR.tr1* and *HR.tr2* were regenerated from two independent transgenic plants which expressed introduced oat-*phyA*-cDNA. Plants were grown under white light (WL) (R:FR=6.8) or WL enriched with far-red (WL+FR) (R:FR=0.2).

Table 10.1 Root yield in plants of wild-type and transgenic horseradish; transgenic plants had been transformed via *Agrobacterium rhizogenes* and expressed an introduced oat-*phyA*-cDNA. Plants were grown for about 6 months under continuous irradiation by white light (WL) (R:FR=6.8) or white light supplemented with far-red (WL+FR) (R:FR=0.2).

Total root fresh weight (g) [mean of six plants (SEM)]

	WL	WL+FR
wild-type	22.0 (1.9)	17.4 (2.0)
transgenic	16.0 (1.2)	15.4 (0.8)

Appendix 10.i

Root yield in radish plants grown under low R:FR ratio

Plants of radish (*Raphanus sativus* L.) were grown under continuous irradiation by white light (R:FR=6.8) or white light enriched with far-red radiation (R:FR=0.2). Plants under the low R:FR ratio light source showed increased extension growth of the internodes and petioles (Fig. 10.ia) and the fresh weight yield of the radish storage-root was significantly reduced (Fig. 10.ia; Table 10.ia). The overall result was a decrease in the ratio of fresh weight yield of storage root versus shoot growth under the FR-enriched light source (Table 10.ia).

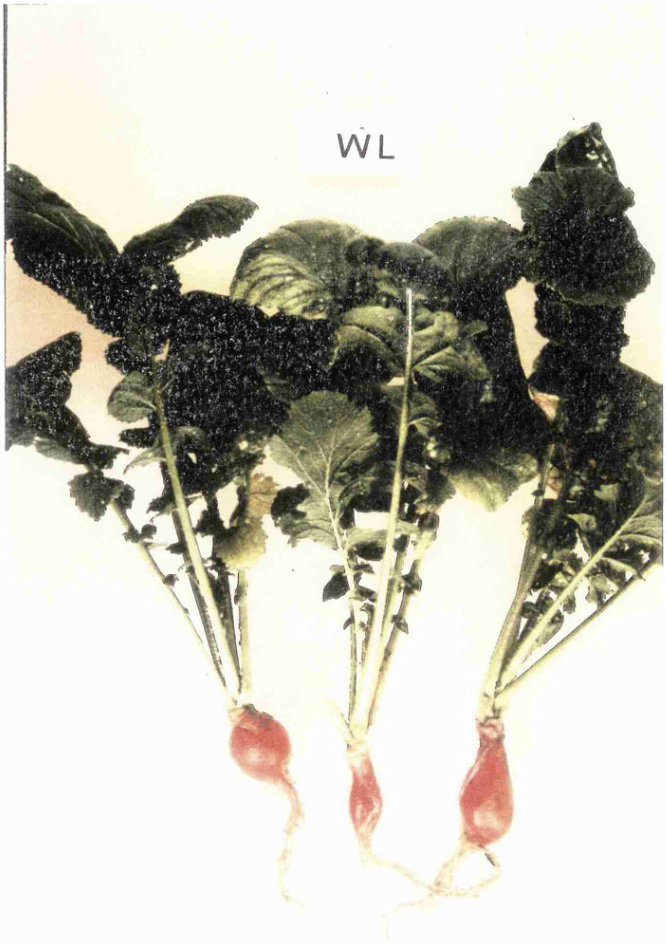
Table 10.ia Yield of radish storage-root under low red (R):far-red (FR) ratio.

	Root yield (g fresh wt.)	Root: shoot yield
WL	5.20 (1.10)	0.90 (0.219)
WL+FR	1.74 (0.50)	0.18 (0.094)

Radish variety, Scarlet globe

WL, white light, R:FR=6.8

WL+FR, white light enriched with far-red, R:FR=0.2



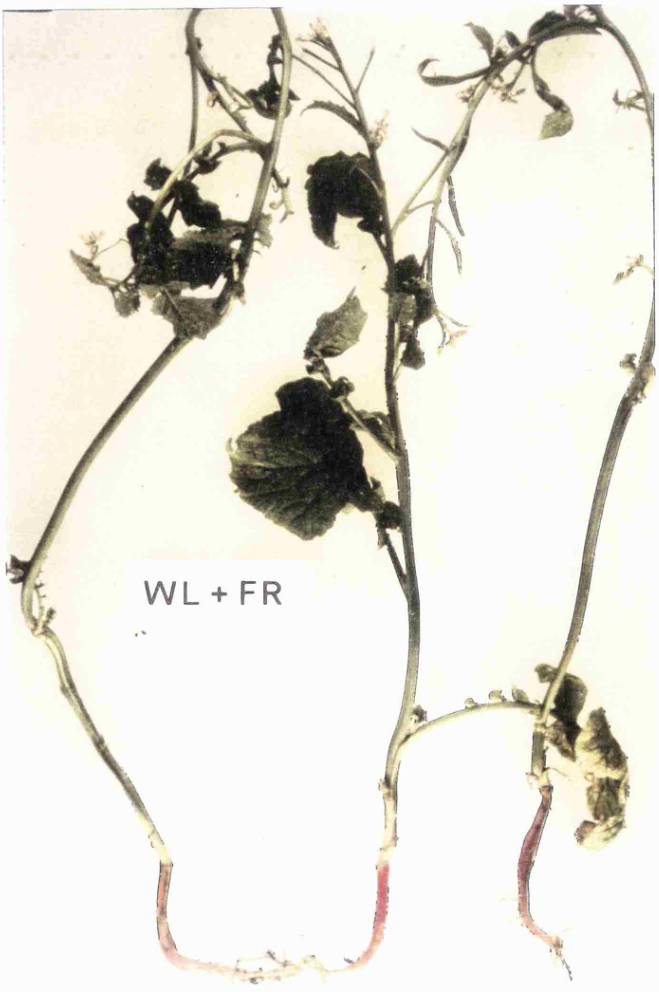


Figure 10.ia Radish (*Raphanus sativus* L.) plants grown under low R:FR ratio exhibit increased extension growth of the shoots, and development of the storage root is impaired. Plants were grown under continuous irradiation by white light (WL) (R:FR=6.8) or white light with added far-red (WL+FR) (R:FR=0.2).

Appendix 10.ii

Shoot regeneration of radish (Raphanus sativus L.) from in vitro culture of explants

The introduction of foreign genes into plants via standard *Agrobacterium tumefaciens*-mediated techniques involves the regeneration of autonomous plants from a single transformed cell. Most *A. tumefaciens*-mediated transformations of explants involve a phase of callus growth followed by phytohormone-induced differentiation of adventitious shoot buds. The optimum hormone environment for shoot induction varies according to plant species. Since no published protocol exists for transformation of radish tissues a range of media, containing various hormone supplements, were tested for their ability to induce shoot regeneration in radish seedling explants. Hypocotyl sections showed poor callus growth on all media tested and therefore seemed unpromising as an explant for transformation. Excised cotyledons/petioles showed good growth of friable callus, suitable for *Agrobacterium*-infection of cells, on medium containing BAP:NAA 4.0:0.25 or 4.0:1.0 (Table 10.iia). Even following prolonged periods of growth (> 3 months), however, explants showed no signs of shoot formation. Pronounced blackening of tissues occurred in the cultured explants, especially petioles, indicating the accumulation of phenolic compounds; it is possible that these componds may have impeded the growth of adventitious shoots.

Shoot-regeneration from Agrobacterium rhizogenes transformed roots of radish

Radish cotyledon explants were inoculated with an *A. rhizogenes* strain containing plasmid pRFY1, as described for horseradish transformations (Chapter 10 of this thesis, *Materials and methods, Transformation and regeneration*). Kanamycin-resistant root out-growths (selected on 20 mg l^{-1} kanamycin) were subjected to various hormone treatments that had been reported to successfully induce shooting in *Nicotiana tabacum* (Taylor *et al.*, 1985) and *Brassica napus* (Guerche *et al.*, 1987) (Table 10.iib). Root explants could be induced to form clumps of callus cells, but no apical bud structures subsquently resulted under the culture conditions as detailed in Table 10.iib.

Table 10.iia Shoot regeneration from *in vitro*-cultured explants of radish.

Explant	Medium (MS salts, 30 g l^{-1} sucrose 7g l^{-1} agar, pH 5.6)			Tissue growth	
	BAP	NAA	Kinetin	Callus	Shoots
Hypocotyl	0.25	_	_	poor	none
	1.00	_	_	"	"
	4.00	_	_	"	"
	1	0.25	_	"	"
	4	0.25	_	"	"
	4	1.00	_	"	"
	4	0.25	2.00	"	"
Cotyledon/ Petiole	0.25	_	_	poor	none
	1.00	_	_	"	"
	4.00	_	_	"	"
	1	0.25	_	some	"
	4	0.25	_	V.good	"
	4	1.00	_	good	"
	4	0.25	2.00	some	"

Radish variety, Scarlet globe
NAA; Naphthalene acetic acid: BAP; 6 Benzylaminopurine.
Seeds were surfaced sterilized in 20 % v/v domestic bleach for 20 min and grown aspectically in moist vermiculite. Explants were taken from young (approx. 1 week old) seedlings.

Table 10.iib Shoot regeneration from *A. rhizogenes*-transformed roots of radish.

Pretreatment	Medium	Hormone supplement	Callus growth	Reference (plant species)
_	MS salts, 30g l^{-1} sucrose 7 g l^{-1} agar, pH 5.8	_	no	Taylor *et al.*, 1985. (*N. tabacum*)
	" "	1mg l^{-1} kinetin	no	
_	MS salts, 10g l^{-1} sucrose 10g l^{-1} mannitol 10g l^{-1} glucose 7g l^{-1} agar, pH 5.8	1.0mg l^{-1} NAA 0.5mg l^{-1} IPA 0.5mg l^{-1} BAP	no	Guerche *et al.*, 1987 (*B. napus*)
15 days in MS (liquid) with 3 mg l^{-1} 2,4 D	" "		yes	

NAA; Naphthaleneacetic acid: BAP; 6 Benzylaminopurine:
IPA; N^6-(Δ^2 Iso-pentenyl)-adenine: 2,4 D; 2,4 Dichloro-phenoxy-acetic-acid.

Chapter 11

Expression of oat-*phyA*-cDNA in a suspension cell culture of transgenic tobacco: a single-cell system for the study of phytochrome function.

Introduction

The plant photoreceptor phytochrome has been the subject of intensive physiological and molecular studies (see Casal and Smith, 1989; Furuya, 1989; Smith and Whitelam, 1990 for reviews). These studies have, to date, concentrated on the processes occuring in whole plants. Interpretation of the results arising from such a complex system is not always simple because, for example, of tissue-specific diversification of response (eg. Casal and Smith, 1988b; Schopfer *et al.*, 1982; Whitelam and Johnson, 1980). Experiments which require the uptake of an exogenously-applied substance may be restricted by the efficiency and uniformity of distribution within the intact plant (eg. Elich *et al.* 1989; Parks *et al.,* 1989). Callus suspension cultures offer an homogeneous and accessible tissue. The kinetics of the phytochrome system of a number of cell cultures have been studied (Gottmann and Schäfer, 1982b) and physiological experiments have demonstrated biological activity of phytochrome in cell suspension cultures of *Petroselinum hortense* (Duell-Pfaff and Wellmann, 1982; Ohl *et al.,* 1989). However, such systems have not proved favourable to further exploitation in studies of phytochrome function. As a single-cell system for transgene expression, cell cultures have been used successfully for the study of heterologous plant proteins (e.g. Matsuoka *et al.*, 1990). Transgenic plants of

tobacco expressing an oat (*Avena sativa* L.) *phyA*-cDNA (encoding the phytochrome A molecular species) under the control of CaMV 35S (a constitutive viral promoter) have been shown to synthesize a physiologically-active population of heterologous phytochrome (Keller *et al.*, 1989; Cherry *et al.*, 1991; McCormac *et al.*, 1991; McCormac *et al.*, 1992b). Cell cultures derived from oat-*phyA*-transformed plants are described here as a potential model system for the study of phytochrome structure and function.

The biological activity of phytochrome is dependent on the spectral properties of the chromophore group, relating to the interphotoconversion of the red-absorbing (P_r) and far-red-absorbing (P_{fr}) forms of phytochrome. Biogenesis of the spectrally-active phytochrome molecule requires two convergent biosynthetic pathways: one for synthesis of the apoprotein encoded by the phytochrome gene and a second for the synthesis of the chromophore moiety. In the cell system described here chromophore biosynthesis would be dependent on the expression of endogenous genes. The spectral properties of the phytochrome pool accumulated in the transgenic cell cultures are compared with those of the corresponding phytochrome species synthesised in etiolated seedlings.

In addition to the spectral activity of the molecule, *in vivo* biological function of phytochrome requires the host cells to possess the facility for transduction of the phytochrome signal. The phytochrome transduction chain remains uncharacterised but it has been established that phytochrome-mediated photoregulation of several nuclear genes, including the chlorophyll a/b binding protein (*cab*) and the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS*), acts to control levels of transcriptional expression (Apel, 1979; Mösinger and Schäfer, 1984; Tobin and Silverthorne, 1985; Wehmeyer *et al.*, 1990). Photoregulation of gene expression in the transgenic tobacco callus culture was measured in order to demonstrate regulatory activity of the heterologous phytochrome in the single cell system.

Materials and methods

Plant material and growth of callus suspension cultures

Plants were grown of wild-type tobacco (*Nicotiana tabacum* cv. xanthi) and of a homozygous isogenic line which had been transformed with oat-*phyA*-cDNA under control of the cauliflower mosiac (CaMV) 35S promoter, as described by Keller *et al.* (1989) and Cherry *et al.* (1991). Leaves were excised from mature plants and surface sterilised in a 10% (v/v) solution of domestic bleach for 15 minutes. Leaf discs were placed onto solid culture medium and incubated under low intensity white fluorescent lights at 25^{o}C. Culture medium consisted of Murashige and Skoog salts supplemented with 9.5 mg l^{-1} pyridoxine, 9.9 mg l^{-1} thiamine, 4.5 mg l^{-1} nicotinic acid, 0.25 mg l^{-1} kinetin, 2 mg l^{-1} 2,4-D, 2 g l^{-1} casein hydrolysate and 30 g l^{-1} sucrose, pH 5.8; solid media contained 1% (w/v) agar. For cultures of the transformed genotype, the medium was supplemented with 100 mg l^{-1} kanamycin. Outgrowths of friable callus were transferred to liquid medium in sterile flasks and continuously orbitally shaken to propagate an homogeneous suspension cell culture (Fig. 11.1). Suspension cell cultures were subcultured every 7 days by diluting the cultures 1 in 4 with fresh medium. Callus growth on solid medium was much slower and subculturing was every 4 weeks.

Measurement of phytochrome

Suspension cultures were extracted approximately 5 days after the last subculture. Cells were filtered through 1 layer of miracloth and 4 layers of muslin and excess liquid gently removed. The cells were homogenised at 4 ^{o}C in buffer (250 µl g^{-1} fresh weight) containing 80% (v/v) ethylene glycol in 0.2M Tris-HCl (pH 7.8) containing 0.28M ammonium sulphate, 0.02M EDTA, 0.04M sodium sulphite and 0.01M PMSF

(phenylmethylsulfonyl fluoride). The supernatant was clarified by centrifugation and boiled for 4 min with a half volume of 3x-strength sodium dodecyl sulphate (SDS) sample buffer. Equal volumes were separated on 8% SDS-polyacrylamide gel and electroblotted onto nitrocellulose (NC-extra, Sartorius AG, D-3400, Göttingen, Germany). Uniformity of protein loadings was confirmed by reversible staining of the blots with 0.2% ponceau in 3% trichloroacetic acid. Blots were incubated with monoclonal antibodies which had been raised against etiolated oat phytochrome. Bands were visualised by secondary incubation with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin antibodies followed by 5-bromo-4-chloro-3-indolyl phosphatase and nitroblue tetrazolium substrates. The developed blots were scanned using a GS Hoefer densitometer (Hoefer Scientific Instruments, San Fransisco, CA., USA) and the relative band intensities quantified by integration of peak areas.

Measurement of rbcS and cab proteins

Cells were harvested and finely ground in liquid nitrogen and lyophilized. The dried tissue was resuspended in a detergent-free buffer at 4^{o}C (14 ml g^{-1} dry weight) of 0.05M Tris-HCl (pH 8.0) containing 5 mM sodium sulphite, 1 mM PMSF, 5 mM ϵ-amino-n-caproic acid, 2 mM iodoacetamide and 1 mM benzamidine. Following centrifugation, the supernatant (supernatant 1) was collected and boiled for 4 min with SDS sample buffer. The remaining pellet of insoluble material was resuspended in SDS-sample buffer, boiled for 4 min and the supernatant clarified (supernatant 2). Supernatant 1 and supernatant 2 were separated on 15% and 12% SDS-polyacrylamide gels respectively and electroblotted onto nitrocellulose. Blots of supernatant 1 were incubated with polyclonal antibodies raised against ribulose bisphosphate carboxylase (large and small subunits) from spinach. Blots of supernatant 2 were incubated with polyclonal antibodies raised against the chlorophyll a/b binding protein of mustard. Bands

were visualised by secondary incubation with a conjugated goat anti-rabbit immunoglobulin antibody, as described above for the measurement of phytochrome.

Reagents for chromophore biosynthesis

4-amino-5-hexynoic acid (AHA) was a gift from The Merrell Dow Research Institute (Merrell Dow Pharmaceuticals, 2110E Galbraith Road, Cincinati, Ohio 45215); a 100 mM stock solution was made in distilled water and filter sterilised. 5-aminolevulinic acid hydrochloride (ALA) was obtained from Sigma (London) Chemical Co.; a 50 mM stock solution was made in distilled water and filter sterilised. Crude biliverdin was obtained from Sigma; a stock solution was made by dissolving in a minimum volume of 1M NaOH followed by dilution with water to 50 mM biliverdin (0.05 M NaOH). Control treatments received an equivalent volume of 0.05M NaOH.

In vitro digestion of phytochrome

Suspension cultures were grown for 5 days in darkness. The cells were collected, under a dim green light, and homogenised in buffer (250 $\mu l\ g^{-1}$ fresh weight) containing 50% (v/v) ethylene glycol in 0.2M Tris-HCl (pH 8) containing 0.02M EDTA and 0.1M β-mercaptoethanol at $4^{o}C$. The supernatant was clarified and divided into 2 samples: one sample received a saturating pulse of 663nm irradiation (red) and the other a saturating pulse of 730 nm irradiation (far-red). A stock solution of 10 mg ml^{-1} trypsin-bovine type XI (Sigma) was made in 25% (v/v) ethylene glycol in 25 mM Tris-HCl (pH 8) buffer containing 1 mM EDTA, and added to the irradiated samples to a final concentration of 0 (control), 5, 15, 25 or 50 mg l^{-1}. The samples were incubated on ice in darkness for 1 hour. The reaction was quenched by the addition of PMSF to a final concentration of 5 mM and the samples were boiled for 4 min with SDS-sample buffer. Phytochrome peptide

fragments were detected by incubation with two distinct monoclonal antibodies raised against etiolated oat phytochrome, using immunblotting techniques described above for the measurement of phytochrome .

Results and discussion

Accumulation of phytochrome in transgenic cell cultures

Crude protein extracts from dark-grown suspension cultures of wild type and transgenic genotypes were probed for phytochrome using monoclonal antibodies. No phytochrome was detected in extracts from the callus cells of wild-type tobacco (Fig. 11.2). In extracts from the suspension culture of the transgenic callus a protein of approximately 124 kDa reacted with the anti-phytochrome antibodies, and monoclonals specific for oat-encoded phytochrome confirmed this to be the product of the heterologous oat-*phyA*-cDNA (Fig. 11.2). Thus, apparently full-length heterologous phytochrome was synthesised in the transgenic cell cultures. However, stable accumulation of the immunoreactive apoprotein of phytochrome can occur even in the absence of the phytochrome chromophore to produce a spectrally-inactive molecule (Jones *et al.*, 1986).

It is well established that, *in vivo*, the P_{fr} form of phytochrome A is degraded far more rapidly than P_r (Shanklin *et al.*, 1987; Cherry *et al.*, 1991), and therefore the proteolytic turnover of the phytochrome pool might be expected to be altered if the molecule was photochemically deficient. Previously dark-grown suspension cultures of the transgenic callus, when transferred to continuous irradiation by broad band red (R; 600-700nm = 4 μmol m^{-2} s^{-1}), exhibited a time-dependent loss of immunodetectable levels of phytochrome (Fig. 11.3). The estimated half life for levels of total phytochrome under R (approx. 4 hours) approximated to that measured for the oat-encoded protein in the etiolated seedlings of the transgenic tobacco (Cherry *et al.*, 1991; McCormac *et al.*, 1992b). In contrast, continuous far-red irradiation (FR; 700-800nm = 40 μmol m^{-2} s^{-1}) did not lead to significant removal of the phytochrome pool (Fig. 11.3); a relationship of levels of total phytochrome with the wavelength of prolonged irradiation is also observed in the seedlings of etiolated tobacco (McCormac *et al.*, 1992b). Following

removal of phytochrome under R irradiation, the transgenic suspension cultures received 15 min FR and were transferred to darkness. In darkness re-accumulation of phytochrome (presumably through *de novo* synthesis) occurred within 24 hours (Fig. 11.4). The *in vivo* proteolytic turnover of phytochrome in the cell cultures was therefore apparently normal, suggesting that the phytochrome population was able to undergo photochemical conversion to a form similar to P_{fr} which was recognized and degraded preferentially by an endogenous protein turnover system (Shanklin *et al.*, 1987), and that synthesis of phytochrome occurred in the P_r form.

Phytochrome is also known to be degraded differentially *in vitro* depending on its structural form as P_r or P_{fr} (Grimm *et al.*, 1988; Lagarias and Mercurio, 1985; Vierstra and Quail, 1982). A crude extract of a dark-grown culture of transgenic cells was irradiated with a saturating pulse of 663nm (red) or 730nm (far-red) light, and subsequently incubated with trypsin at concentrations between 5 and 50 mg l^{-1}. Differences in the resulting phytochrome digestion fragments from the red-irradiated (P_{fr}) and far-red-irradiated (P_r) samples were observed (Fig. 11.5); a terminal cleavage of the intact 124 kDa phytochrome to produce a 114 kDa species occurred readily in the P_r digestion mixture and to a notably lesser degree in the P_{fr} digestion mixture. Digestion of the P_{fr} form also yielded peptide species approximating to 84/82 and 69/67 kDa (Lagarias and Mercurio, 1985) which were not detected in P_r digestion mixtures (Fig. 11.5). Differential sensitivity of the phytochrome pool to *in vitro* proteolysis in response to prior irradiation treatments is indicative of light-induced conformational changes. The pattern of peptide fragments which were yielded by trypsin digestion coincide with those from previous reports of *in vitro* proteolysis of native oat phytochrome (Lagarias and Mercurio, 1985), indicating that no major conformational aberrations exist in the heterologous phytochrome of the transgenic suspension cultures. These findings strongly suggest that, within the cultured cells, the pool of

oat-*phyA*-cDNA-encoded phytochrome is able to form a functional association with a R/FR-sensitive chromophore group.

Chromophore biosynthesis in transgenic callus cells

The chromophore moiety associated with phytochrome molecules in suspension culture cells has been shown to confer conformational and spectral properties which closely resemble those of native phytochrome in etiolated seedlings. The relation of the chromophore group synthesised in the callus cultures to the tetrapyrrole normally associated with phytochrome was investigated further by a comparison of the biosynthetic pathways.

Formation of 5-aminolevulinic acid (ALA) is the first committed step in the biosynthesis of phycobilins, including phytochrome (Brown, Houghton and Vernon,1990). Synthesis of ALA in plants can be inhibited by 4-amino-5-hexynoic acid (AHA) and this results in a decrease in the levels of spectrally-active phytochrome (Elich and Lagarias, 1988). Suspension cultures of transgenic callus, when grown in the presence of 100 μM AHA, accumulated phytochrome which was stable even under R-irradiation (Fig. 11.6). This demonstrates that the action of AHA resulted in a phytochrome molecule which was unable to undergo a photochemical conversion to the P_{fr} form, as recognised by the endogenous protein turnover system. *In vitro* digestion of phytochrome extracted from AHA-treated suspension cultures revealed an identical pattern of proteolytic cleavage of the red and far-red irradiated forms (Fig. 11.7). Thus, phytochrome which was accumulated in the absence of ALA biosynthesis did not respond to R/FR irradiation with any significant conformational change. These results demonstrate a requirement for synthesis of ALA in order for the accumulated phytochrome to demonstrate spectral sensitivity .

When cultures were grown in the simultaneous presence of exogenous AHA

(100μM) and ALA (500 μM) phytochrome was accumulated which was light-labile (Fig. 11.8) and showed differential proteolytic cleavage in the R- and FR-irradiated forms (Fig. 11.9). Exogenously applied ALA was therefore able to reverse the AHA-induced block to the accumulation of spectrally-active phytochrome. These results are consistent with a role for ALA as an essential intermediate in the biosynthesis of the phytochrome chromophore in the suspension cell system. Cell cultures growing under AHA-inhibition could similarly be induced to accumulate spectrally-active phytochrome (as indicated by the ability of the resulting phytochrome population to photoconvert to a form recognized by the Pfr-specific protein turnover system) by the exogenous supply of biliverdin (Fig. 11.10). This is consistent with the results from whole plant systems which suggest that biliverdin (specifically biliverdin IXα) is a late intermediate in chromophore biosynthesis (Elich *et al.*, 1989; Elich and Lagarias, 1987; Parks and Quail, 1991). At least two common intermediates therefore exist for synthesis of the phytochrome chromophore in whole plants and the cell cultures, thereby supporting the proposal that the single-cell system faithfully reproduces the biosynthetic pathway normally operative in plants.

The full length phytochrome extracted from the AHA-inhibited callus cultures was highly susceptible to *in vitro* digestion; incubation with 50 g l^{-1} trypsin yielded a single (as detected by the monoclonal antibodies used here) low molecular weight peptide species (Fig. 11.7). The pattern of proteolytic cleavage therefore differed from both the untreated P_r and P_{fr} species (Fig. 11.5). The *hy1* and *hy2* mutants of *Arabidopsis* possess an endogenous phytochrome population which is photochemically deficient due to defective chromophore biosynthesis (Parks and Quail, 1991). As observed for the phytochrome accumulated in AHA-inhibited cell-cultures, the phytochrome pools of these two photoreceptor mutants demonstrate patterns of *in vitro* proteolysis which are largely uninfluenced by prior irradiation with R or FR, and which are different from both P_r and P_{fr} of wild-type *Arabidopsis* (Parks *et al.*, 1989). The chromophore prosthetic group

therefore appears to be important to the overall conformation of the phytochrome molecule as well as inducing light-mediated changes.

Photoregulation of gene expression

Dark-grown callus cultures (growing on solid substrate) were placed on fresh media and grown for two weeks under various light conditions. Under continuous white light, both wild-type and transgenic cultures accumulated detectable levels of rbcS and cab proteins; although levels of cab and rbcS protein (as a proportion of total protein levels) were lower in WT cells than transgenic (Fig. 11.11b). Irradiation by broad band R or FR light sources, however, did not result in significant levels of these gene-products in the wild-type cells (Fig. 11.11a). This finding is consistent with previous studies of single-cell cultures in which weak levels, at best, of gene expression were stimulated by the action of P_{fr} alone (Ohl *et al.*, 1989). In the transgenic cultures, continuous irradiation by R (600-700nm = 4 μmol m^{-2} s^{-1}) or FR (700-800nm = 40 μmol m^{-2} s^{-1}) resulted in high levels of accumulation of the rbcS protein (Fig. 11.12). R delivered as 15 min pulses (six times per day at 2 hour intervals) also resulted in rbcS accumulation; the effect of R pulses was reversed by an immediately subsequent FR pulse (Fig. 11.12). Thus, photocontrol of rbcS expression demonstrated R/FR reversibility, and a FR-response which was dependent on continuous irradiation. These observations are consistent with the low fluence and high irradiance response modes, respectively, of phytochrome regulation, as are operative for photocontrol of gene expression in etiolated seedlings (Kaufman *et al.*, 1984; Wehmeyer *et al.*, 1990). Accumulation of cab proteins in transgenic callus cells occurred under white light and R irradiation (Fig. 11.12). Cultures grown under continuous FR did not contain significant levels of cab, probably due to the instability of the polypeptide under conditions which do not allow chlorophyll synthesis (Apel, 1979).

In the above set of experiments callus tissue cultured on solid medium was used as this offered a convenient form for handling under the various light treatments. However, due to the slow rate of cell multiplication in such cultures, accumulation of the protein to detectable levels required prolonged periods of growth (>1 week). In etiolated seedlings phytochrome mediated regulation of gene expression acts to stimulate levels of transcription and is therefore a rapid response (Wehmeyer *et al.*, 1990). In actively dividing suspension cultures significant levels of the rbcS protein product were shown to accumulate within 1 day following transfer from darkness to continuous FR (Fig. 11.13). It therefore seems likely that phytochrome-mediated photoregulation in the suspension cell system also acts to control an early event in gene expression, possibly affecting transcript abundance.

Conclusions

It has been demonstrated here that suspension cell cultures of tobacco expressing a transgenic phytochrome-encoding cDNA can assemble a pool of spectrally-active phytochrome which appears indistinguishable from that synthesised in the tissues of intact plants. The properties of the heterologous phytochrome protein indicated that, although the WT cell cultures do not accumulate significant levels of phytochrome, they retain the capacity for substantial chromophore biosynthesis. Phytochrome turnover in the cell cultures appeared normal, suggesting that the appropriate degradation activity is also retained in the de-differentiated cells. Within the transgenic single cell system rapid changes in gene expression were observed in response to the light environment, and in a manner which was consistent with the mode of phytochrome-mediated photoregulation observed in etiolated seedlings of tobacco.

The ability of this single cell system to retain the essential characteristics of the phytochrome system of intact plants suggests that it may have applications in studies of phytochrome at the molecular level. The suspension cultures proved amenable to experiments involving the uptake of exogenously-applied substances, and techniques such as biotin-labelling (Horn and Heinstein, 1990; 1992) have the potential to render the cells accessible to even large and/or hydrophillic molecules, including proteins. Recent work has shown that expression of a transgene encoding a functional antibody against phytochrome can impair the biological activity of endogenous phytochrome (Owen *et al.*, 1992). The direct introduction of site-directed monoclonal antibodies into a system such as the transgenic cell culture described here could provide a powerful model for the mapping of phytochrome structure in relation to function.

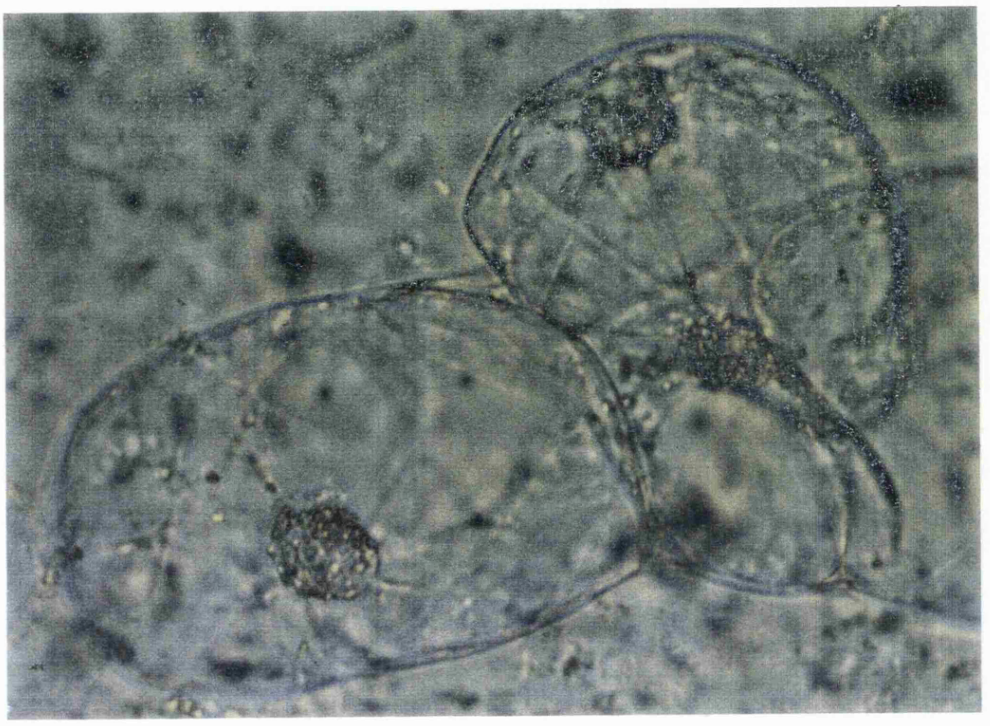


Figure 11.1 Single cells from a 5 day old suspension culture of transgenic tobacco callus. Cultures were grown in liquid MS medium supplemented with vitamins and containing 2 mg l^{-1} 2,4-D (see *materials and methods*). No stain was applied to the cells shown in this figure.

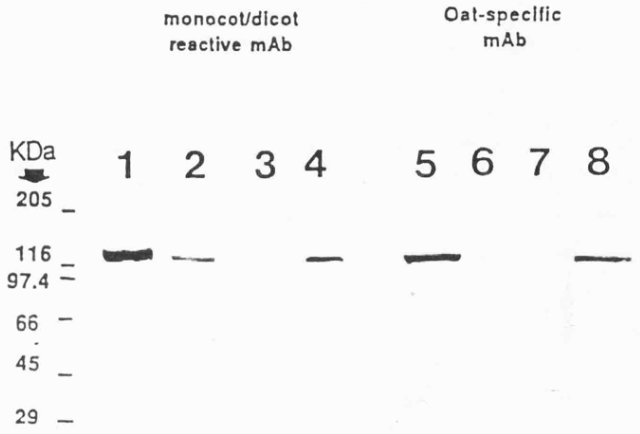


Figure 11.2 Immunoblot analysis of phytochrome content of cell cultures. Crude protein extracts from dark-grown tissues of oat seedlings (lanes **1** and **5**), wild-type tobacco seedlings (lanes **2** and **6**), wild-type tobacco suspension culture (lanes **3** and **7**), and transgenic tobacco suspension culture (lanes **4** and **8**) were probed with monoclonal antibodies reactive with both monocot. and dicot. phytochromes (lanes 1-4) or specific for oat-encoded phytochrome (lanes 5-8).

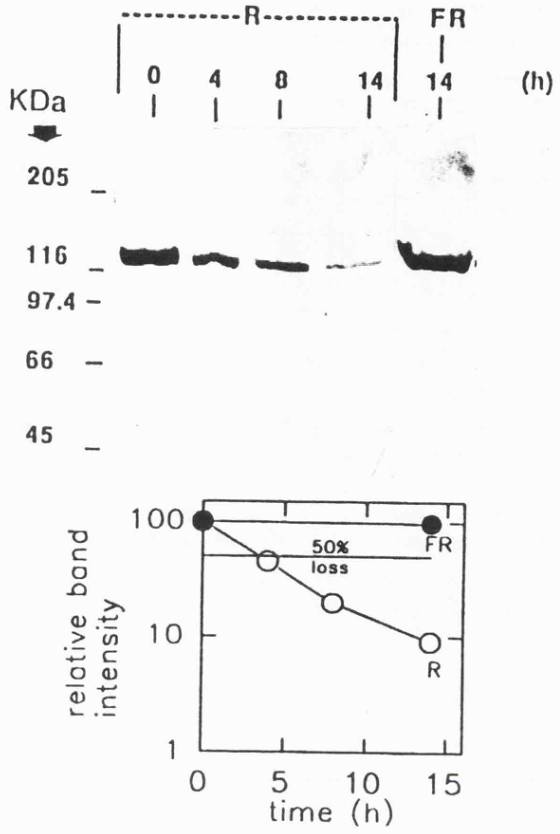


Figure 11.3 Effect of prolonged irradiation by red (R) or far-red (FR) light on levels of total phytochrome in suspension cultures of transgenic tobacco. Previously dark-grown cultures were transferred to the light treatments and crude extracts made at time intervals from the start of irradiation. Samples were separated on an 8% SDS-polyacrylamide gel and blots were probed with a mixture of monoclonal antibodies raised against etiolated oat phytochrome.

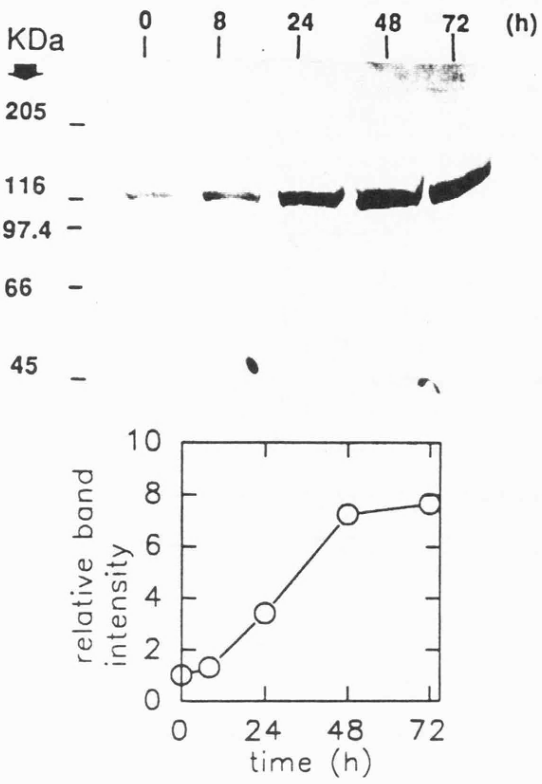


Figure 11.4 Dark-accumulation of phytochrome in suspension cultures of transgenic tobacco. Cultures which has been irradiated by continuous red light for 14 h were given a 15 min far-red pulse and then grown in darkness. Crude extracts were made at time intervals following the transfer to darkness. Samples were separated on an 8% SDS-polyacrylamide gel and blots were probed with a mixture of monoclonal antibodies raised against etiolated oat phytochrome.

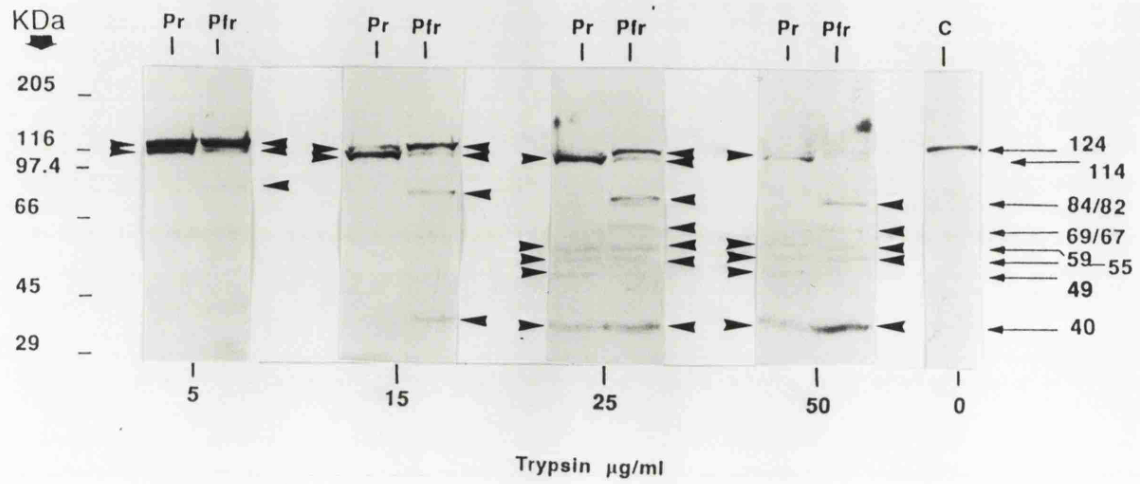


Figure 11.5 Differential proteolytic cleavage of phytochrome in the red-irradiated (P_{fr}) and far-red-irradiated (P_r) forms. Crude extracts of phytochrome from dark-grown suspension cultures of transgenic tobacco callus were irradiated with a saturating pulse of red or far-red light and incubated, in darkness, with the concentrations of trypsin shown for 1 h at 4 ^{o}C. A control sample (C) was incubated without trypsin. Peptide fragments were separated on an 8% SDS-polyacrylamide gel and blots were probed with a mixture of monoclonal antibodies raised against etiolated oat phytochrome.

Figure 11.6 Effect of 4-amino-5-hexynoic acid (AHA) on red-light-mediated destruction of phytochrome in suspension cultures of transgenic tobacco. Cultures were grown for 4 days in darkness in the presence of AHA at the concentrations shown. On the fifth day cultures were either maintained in darkness (D) or transferred to continuous irradiation by a red light source (R). After 24 h irradiation, crude extracts were made and probed with a mixture of monoclonal antibodies raised against etiolated oat phytochrome.

Figure 11.8 5-aminolevulinic acid (ALA) rescues the lability of phytochrome under red light in cell cultures inhibited by 4-amino-5-hexynoic acid (AHA). Transgenic suspension cultures were grown for 4 days in darkness in the presence, simultaneously, of 100 μM AHA and the concentrations shown of ALA. On the fifth day cultures were either maintained in darkness (D) or transferred to continuous irradiation by a red light source (R). After 24 h irradiation, crude extracts were made and probed with a mixture of monoclonal antibodies raised against etiolated oat phytochrome.

Figure 11.10 Biliverdin rescues the lability of phytochrome under red light in callus cultures inhibited by 4-amino-5-hexynoic acid (AHA). Transgenic cell suspension cultures were grown for 4 days in darkness in the presence, simultaneously, of 100 μM AHA and the concentrations shown of biliverdin. On the fifth day cultures were either maintained in darkness (D) or transferred to continuous irradiation by a red light source (R). After 24 h irradiation, crude extracts were made and probed with a mixture of monoclonal antibodies raised against etiolated oat phytochrome.

Figure 11.6

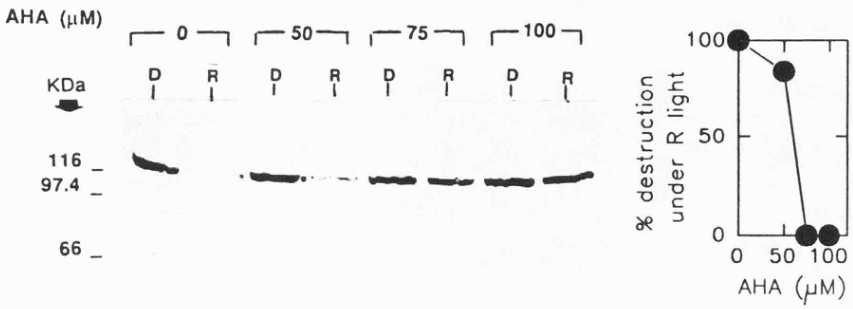


Figure 11.8

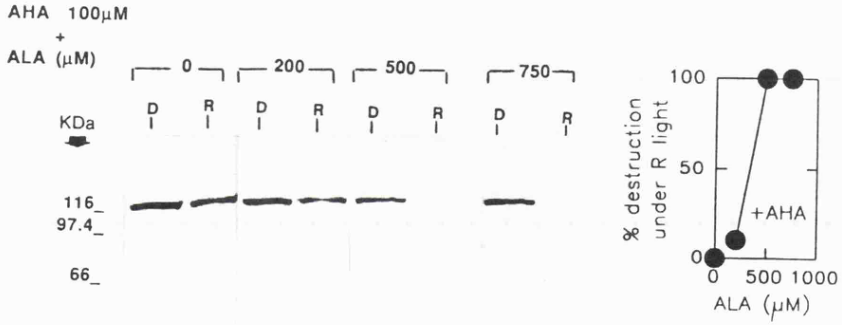


Figure 11.10

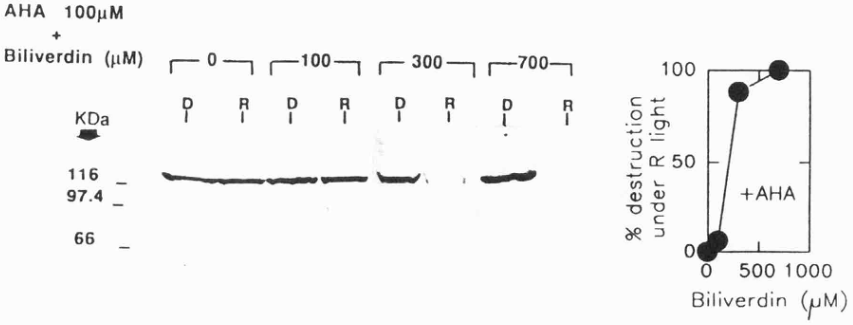


Figure 11.7 Proteolytic cleavage of phytochrome extracted from suspension cultures treated with 4-amino-5-hexynoic acid (AHA) was identical in the red-irradiated (P_{fr}) and far-red-irradiated (P_r) forms. Cultures were grown in darkness in the presence of 100 μM AHA. Crude extracts of phytochrome were irradiated with a saturating pulse of red or far-red light and incubated, in darkness, with the concentrations of trypsin shown for 1 h at 4 ^{o}C. A control sample (C) was incubated without trypsin. Peptide fragments were separated on an 8% SDS-polyacrylamide gel and blots were probed with a mixture of monoclonal antibodies raised against etiolated oat phytochrome.

Figure 11.9 5-aminolevulinic acid (ALA)-mediated rescue of red/far-red sensitivity of proteolytic cleavage in 4-amino-5-hexynoic acid (AHA)-inhibited cell cultures. Transgenic suspension cultures were grown for 4 days in darkness in the presence, simultaneously, of 100 μM AHA and 500 μM ALA. Crude extracts were irradiated with a saturating pulse of red light (P_{fr} = red-irradiated form) or far-red light (P_r = far-red-irradiated form) and incubated, in darkness, with the concentrations of trypsin shown for 1 h at 4 ^{o}C. A control sample (C) was incubated without trypsin. Peptide fragments were separated on an 8% SDS-polyacrylamide gel and blots were probed with a mixture of monoclonal antibodies raised against etiolated oat phytochrome.

Figure 11.7

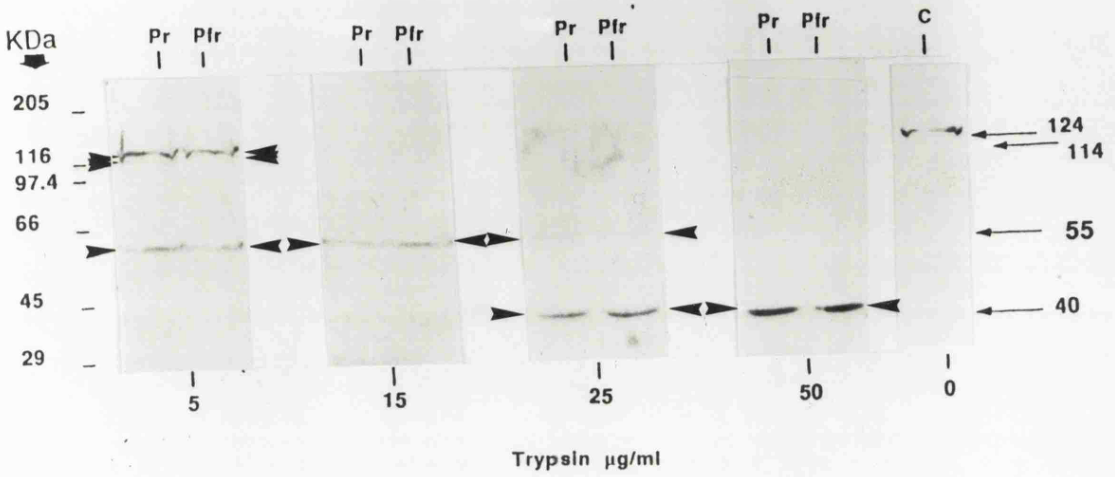
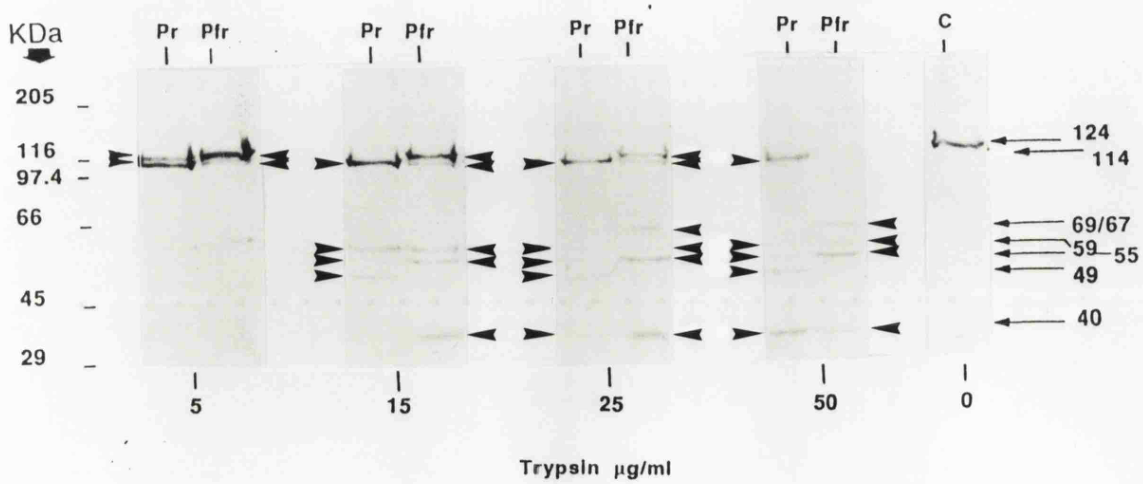


Figure 11.9



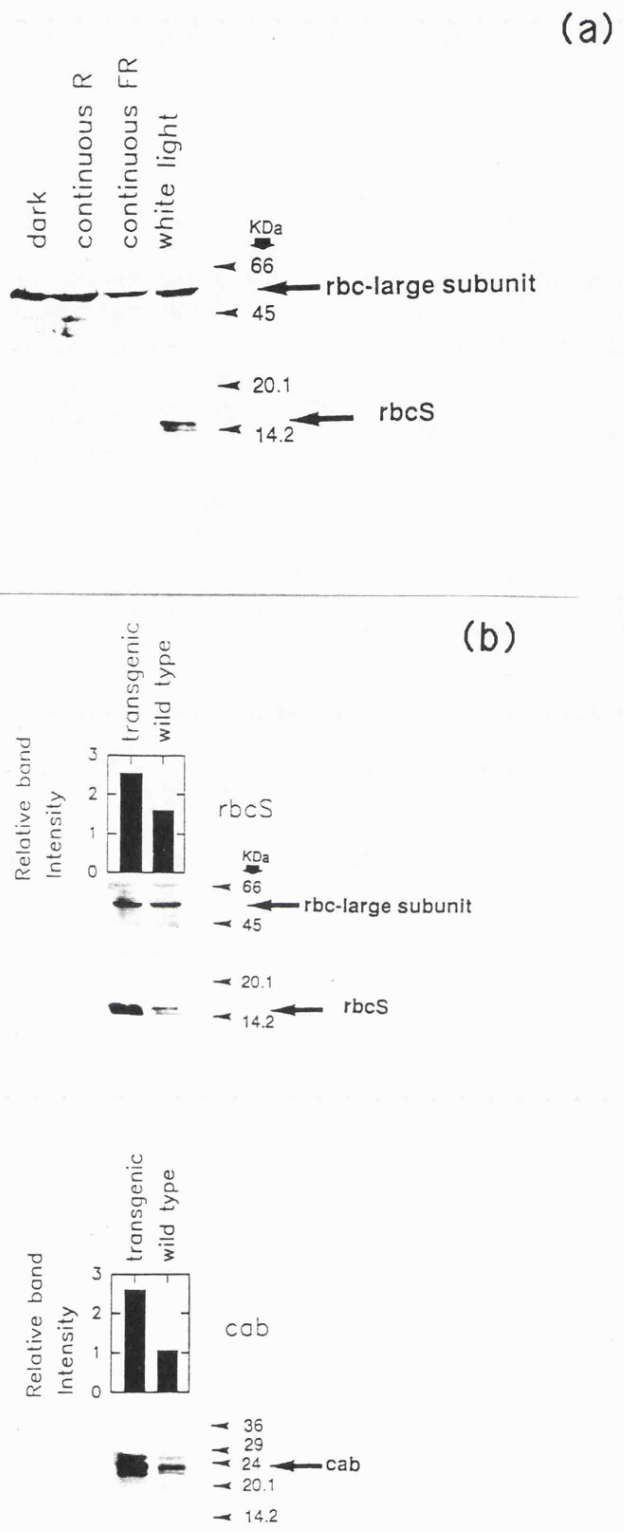


Figure 11.11 (a) Accumulation of ribulose-1,5-bisphosphate carboxylase polypeptide large subunit and small subunit (rbcS) in cultures of callus cells from wild-type tobacco. Cultures were grown on solid medium for 2 weeks in darkness (dark) or under continuous irradiation by red light (R), far-red light (FR) or white light.

(b) Accumulation of ribulose-1,5-bisphosphate carboxylase polypeptide large subunit and small subunit (rbcS) and chlorophyll *a/b* binding protein (cab) in cultures of callus cells of wild-type tobacco and transgenic tobacco expressing introduced oat-*phyA*-cDNA. Cultures were grown on solid medium for 2 weeks under continuous white light. Equal levels of total protein were loaded in each lane.

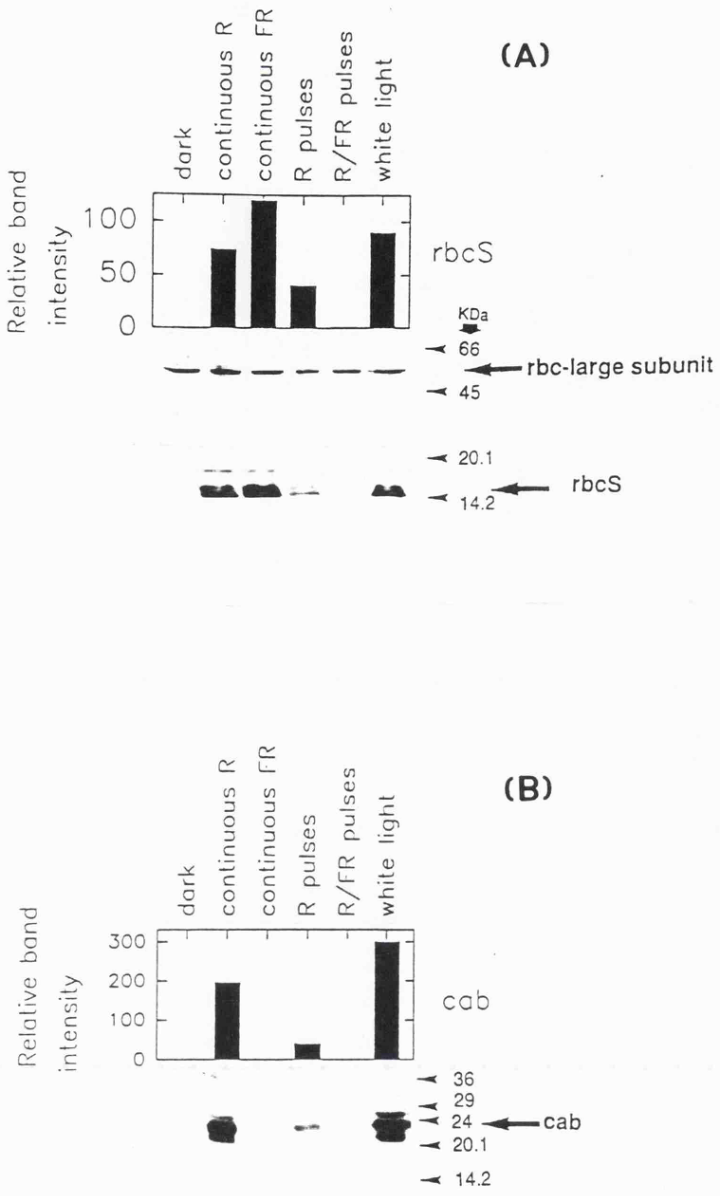


Figure 11.12 Light-mediated accumulation of the protein-products of *rbcS* **(A)** and *cab* **(B)** genes in cell cultures of transgenic tobacco. Callus tissue was maintained in complete darkness or was grown under continuous irradiation by red (R), far-red (FR) or white light, or given multiple pulse treatments of red or red followed by far-red (R/FR) light. Extracts of soluble proteins were separated on a 15 % SDS-polyacrylamide gel and the blot was probed with polyclonal antibodies raised against spinach ribulose-1,5-bisphosphate carboxylase (large and small subunits) (A). Non-soluble material was extracted in SDS-containing buffer; proteins were separated on a 12% SDS-polyacrylamide gel and the blot was probed with polyclonal antibodies raised against mustard chlorophyll *a/b* binding proteins (B). Equal levels of total proteins were loaded in each lane.

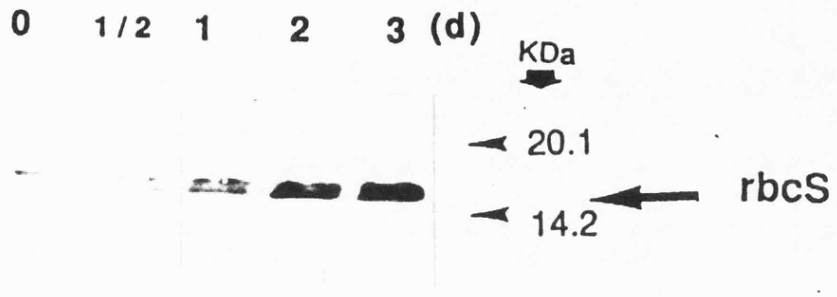


Figure 11.13 Time-course of accumulation of the protein-product of *rbcS* gene in cell cultures. Transgenic suspension cell cultures were transfered from darkness to continuous far-red irradiation. Protein extracts were made after 0, 1/2, 1, 2 and 3 days (d) of irradiation and were separated on a 15 % SDS-polyacrylamide gel; the blot was probed with polyclonal antibodies raised against spinach ribulose-1,5-bisphosphate carboxylase. All lanes were loaded with equal levels of total protein.

Chapter 12

General discussion

Direct molecular evidence that the phytochrome apoprotein is encoded by a family of several divergent gene has been provided from relatively recent studies with *Arabidopsis* (Sharrock and Quail, 1989), tomato (Sharrock *et al.,* 1988), rice (Dehesh *et al.*, 1991; Y.-C. Wang, M.-M. Cordonnier-Pratt and L.H. Pratt, unpublished results, cited in: Reed *et al.*, 1992), *Pinus palustris* (J. Silverthorne, unpublished results, cited in Reed *et al.*, 1992), and oat (Y.-C. Wang, M.-M. Cordonnier-Pratt and L.H. Pratt, unpublished results, cited in: Reed *et al.*, 1992); in *Arabidopsis* five such genes have been identified and are designated *phyA, phyB, phyC, phyD* and *phyE* (Sharrock and Quail, 1989). This finding is of key importance to studies of phytochrome function, raising questions regarding in what way the individual family members relate to the physiologically- and biochemically-defined species of the photoreceptor, and whether they have discrete photosensory roles in regulating plant photomorphogenesis. Molecular and genetic analysis currently in progress is beginning to answer these questions. Microsequencing of the purified Type I phytochrome polypeptide of etiolated seedlings has established unequivocally that this species of the photoreceptor is encoded by the *phyA* gene subfamily (Grimm *et al.*, 1988; Jones and Quail, 1989) and hence designated phytochrome A. Phytochromes B and C are of low abundance and photostable and, therefore, have the properties of the physiologically and biochemically defined Type II phytochrome of green plants (Somers *et al.,* 1991; Lopez-Juez *et al.*, 1992). Phytochromes D and E have yet to be analysed.

Evidence for discrete roles for different phytochrome species from studies with mutant and transgenic plants

Analysis of photomorphogenetic mutants, both conventional photoreceptor-deficient genotypes and transgenic plants overexpressing an introduced phytochrome species, has provided the first evidence linking a defined individual phytochrome species and a specific photoresponse. The *hy3* mutant of *Arabidopsis* has recently been shown to possess an aberrant *phyB* gene and hence is deficient specifically in the phytochrome B molecular species (Somers *et al.*, 1991; Reed *et al.*, 1993). This mutant displays a severely reduced responsiveness to continuous red light (R), manifested as long hypocotyl growth under R (Koornneef *et al.*, 1980; McCormac *et al.*, 1993). These results clearly establish the role of phytochrome B as necessary for perception of continuous R, and conversely neither native phytochromes A, C, D nor E can elicit response to continuous R (at least in the absence of phytochrome B). In contrast, continuous far-red irradiation (FR) induces normal inhibition of hypocotyl elongation in etiolated seedlings of the *hy3* mutant (Koornneef *et al.*, 1980; McCormac *et al.*, 1993). This result would seem to suggest that phytochrome B is not required for the FR-high irradiance response (Mancinelli, 1980) of wild-type etiolated seedlings. The implication is, therefore, that this response-mode is mediated by one or more of phytochromes A, C, D or E. This result is supported by the *lh* mutant of cucumber and the *ein* mutant of *Brassica* which have both been shown to lack wild-type levels of the phytochrome B-homologous polypeptide, and display a similar photoresponse profile to *hy3* (Lopez-Juez *et al.*, 1992; Devlin *et al.*, 1992). This scenario is also consistent with the behaviour of the *hy1* and *hy2* mutants of *Arabidopsis*; being deficient in phytochrome chromophore biosynthesis (Parks and Quail, 1991) these mutants are likely to be deficient in levels of photochemically-active phytochrome of all species A-E. Like *hy3*, the *hy1* and *hy2* mutants lack responsiveness to continuous R but, in contrast to *hy3*, also lack responsiveness to continuous FR which can best be explained

by the additional deficiency in phytochrome(s) A, C, D or E. Transgenic *Arabidopsis* seedlings overexpressing phytochrome B exhibit enhanced sensitivity to continuous R (Wagner *et al.,* 1991; McCormac *et al.*, 1993) but, consistent with the behaviour of endogenous phytochrome B deduced from the *hy3* mutant, responsiveness to continuous FR was not significantly different from wild-type (McCormac *et al.*, 1993), thus underscoring the lack of involvement of this particular photoreceptor species in the irradiance-dependent response of etiolated seedlings to FR. The behaviour of transgenic seedlings of *Arabidopsis* and tobacco overexpressing the phytochrome A species under continuous FR was clearly different from wild-type and also from phytochrome B-overexpressing transgenic seedlings; the elevated levels of total phytochrome A in the dark-grown plants was associated with an enhanced reponsiveness to continuous FR (Whitelam *et al.*, 1992; McCormac *et al.*, 1992b). Conversely, the recently isolated *hy8* mutant class of *Arabidopsis* which is specifically deficient in the phytochrome A photoreceptor fails to display a FR-HIR in the etiolated seedlings (Parks and Quail,1993). Taken together, these data suggest the interpretation (although do not prove) that the capacity to mediate the FR-HIR may be an intrinsic property of phytochrome A. This conclusion is consistent with circumstantial evidence obtained from earlier studies of wild-type plants which have shown that the responsiveness of etiolated seedlings to continuous FR declines rapidly as the result of red or white light pretreatments (Beggs *et al.*, 1980; Holmes and Schäfer, 1981), suggesting that this response is under the control of the physiologically-defined photolabile Type I phytochrome pool. Interpretation of the integrated results from conventional mutant and transgenic genotypes therefore suggests that endogenous phytochromes A and B have reciprocal and independent photosensory roles in seedling development, mediating responsiveness to continuous R and continuous FR, respectively, and that these separate photosensory roles are intrinsic properties of the individual photoreceptor molecules.

Distinct photoregulatory behaviour of phytochromes A and B is also reflected in the

shade-avoidance responses of light-grown plants. Transgenic plants overexpressing phytochrome A under continuous irradiation by light of low R:FR ratio showed severely impaired shade-avoidance elongation-growth responses, and in fact light enriched with far-red radiation caused an inhibition of growth. Conversely, *phyA* mutants of *Arabidopsis* are seen to display wild-type-like responses in the green plants to reductions in R:FR ratio (Whitelam *et al.,* 1993). Thus, the phytochrome A photoreceptor is clearly not functional in mediating the normal shade-avoidance responses of wild-type plants. The *phyB*-mutant of *Arabidopsis* (*hy3*) has been shown to display much attenuated hypocotyl- and petiole-elongation shade-avoidance responses to reduced R:FR ratio (Whitelam and Smith, 1991), suggesting a requirement for this photoreceptor species in R:FR ratio perception in light-grown plants. However, recent evidence has shown that some responses to low R:FR, such as specific stem weight and leaf area, are unaffected by the *hy3* mutation (Robson *et al.*, 1993), implying that a second photoreceptor is responsible. Transgenic plants overexpressing phytochrome B showed a qualitatively normal relationship of increasingly elongated growth under conditions of decreasing R:FR ratio. As might be expected, assuming the popular view that growth is directly related to concentration of P_{fr}, growth under a given R:FR ratio was inhibited in the transgenic plants relative to wild-type. Superficially these results appear compatible with the hypothesis of phytochrome B as the photoreceptor for R:FR ratio perception. Attempts to quantitatively relate growth-responses to calculated levels of phytochrome B-$[P_{fr}]$ however, were unable to confirm this relationship. Whilst this result does not present any substantial evidence to contradict the role of phytochrome B in the shade-avoidance photophysiology, it could be viewed to encourage investigations of the possible interactive roles of the other, more minor photostable phytochrome species, and further questions the assumption of P_{fr} as the sole active form (Smith, 1981; 1983). Current advances in expression of members of the phytochrome family in transgenic plants and micro-organisms may eventually allow the direct investigation of their

photoconversion kinetics and pathways, studies which, for logistical reasons, have so far been restricted to phytochrome A accumulated in etiolated tissues (e.g. Smith and Fork, 1992); an understanding of the photoreactions of Type II phytochrome may help to resolve the apparent paradox regarding the relationship of response with P_{fr} levels.

Etiolated seedlings under continuous FR irradiation display inhibition of hypocotyl elongation, opening and expansion of the cotyledons and induction of incipient chloroplast development (Mohr, 1977). Such developmental changes might represent initiation of the necessary transition from heterotrophic to autotrophic development. One situation outside of the laboratory environment in which the FR-HIR might conceivably have a physiological role is for etiolated seedlings emerging from the soil under a leafy canopy. It might be expected that such an inhibitory effect of FR light on elongation growth would be transient, and as phytochrome A levels were removed, the shade-avoidance regulatory role of the light-stable phytochrome pool would assume dominance and enhanced far-red light levels would lead to accelerated elongation-growth. Such a scenario would appear consistent with the time-dependent change in the observed responses of de-etiolating wild-type seedlings to FR-supplementation of background white light (McCormac *et al.*, 1992a) and the persistence of FR-mediated growth-inhibiton in light-grown transgenic plants constitutively overexpressing phytochrome A (McCormac *et al.*, 1992a; 1992b; Whitelam *et al.*, 1992). Thus, phytochromes A and B would appear to have transiently antagonistic roles in regulating early seedling photomorphogenesis. The roles of phytochromes C, D and E are, as yet, unclear.

Some considerations in the interpretation of physiological responses of transgenic and mutant plants in terms of the action of phytochrome in the wild-type plant

The evidence accumulated so far for the physiological responses of transgenic plants overexpressing individual members of the phytochrome family indicates that the

heterologous phytochromes faithfully reproduce the photoresponse-modes mediated by the corresponding endogenous phytochrome species. Whilst transgenically-overexpressed phytochrome A-function produced an aberrant photophysiology with respect to the temporal pattern of responsiveness (i.e. persistence of FR-mediated growth-inhibition in the light-grown plant) the important point is that the introduced *phyA* gene-product behaved in the same way as endogenous phytochrome A, rather than Type II phytochromes, presumably through the normal Type I signal transduction chain. The apparent single inconsistency appears, at present, to be hypersensitivity of phytochrome A-overexpressing plants to continuous R. The data indicate that phytochrome A possesses the intrinsic capacity to mediate responsiveness to continuous R even though endogenous phytochrome A does not appear to exercise this capacity in the wild-type seedlings. When overexpressed, therefore, phytochrome A seems to appropriate a role normally performed by phytochrome B. The reason for this apparantly aberrant activity is unknown but could be due to high levels of the introduced phytochrome A sustained in the light-grown seedlings of the transgenic plants, in contrast to wild-type levels which are rapidly removed in light (Boylan and Quail, 1991), and hence would be aberrant only in the sense of temporal expression patterns. Another possible, speculative, explanation is the potential for ectopic expression of the heterologous photoreceptor, driven by the constitutive cauliflower mosaic virus 35S promoter, in tissue-types foreign to the endogenous phytochrome A; there is no direct evidence, as yet, to support or contradict this hypothesis.

Thus, although initial physiological results suggest that the introduced phytochrome of transgenic plants functions to mediate response in an essentially normal manner with respect to the transduction pathways and final responses as mediated by the homologous endogenous phytochromes of wild-type plants, a number of fundamental concerns regarding the precise nature of the transgenic plant system require to be addressed before physiological data can be subjected to unequivocal interpretation. The use

of heterologous 5' promoter sequences to drive expression of the introduced cDNA presents a strong likelihood of ectopic expression of the heterologous phytochrome, both with respect to light conditions of growth (as mentioned above for *phyA*) and also, possibly, with regard to cell-type. If the introduced phytochome accumulates in cells which do not contain the endogenous phytochrome species it may elicit aberrant photoresponses, perhaps appropriating roles normally performed by other photoreceptors. Alternatively, the cells may lack the facility for transduction of the phytochrome signal, or the phytochrome polypeptide may be inactive or unstable in the abnormal cellular environment; in this situation it would be difficult to relate measurements of phytochrome levels to quantitative response, but the essential characteristics of the phytochrome-mediated photoresponses might be expected to be retained. Within cell-types which are appropriate for normal expression of phytochrome genes, it still remains to be established whether the introduced phytochrome is stable such that it accumulates to the levels measured from whole plant extracts, especially in the case of introduced sequences from a heterologous plant source.

Another consideration in transgenic plants expressing an introduced phytochrome is whether the overexpressed phytochrome species has any "feedback" effect on the levels of one or more endogenous phytochromes. Transgenic plants expressing high levels of an introduced phytochrome A species appear to retain normal levels of spectrally-active endogenous phytochrome, and therefore it seems unlikely that the chromophore is limiting. However, it may be that abnormally high levels of an introduced phytochrome competes with endogenous phytochrome for reaction partners. In the case of transgenic plants expressing introduced phytochrome sequences from a heterologous plant source, there may be some loss of the possible specificity for different reaction partners displayed by the various endogenous phytochrome family members; if a heterologous phytochrome species did not maintain such distinctions then considerable confusion could arise regarding the respective transduction pathways, and thereby responses, activated by the

individual phytochromes.

It may be argued that attempts to interpret transgenic plant photophysiological responses, before proper consideration of the above potential for abnormalities, is somewhat premature. However, physiological analysis of conventional mutant plants, deficient in some component of the phytochrome system, have shown that results from such studies, even in the absence of precise molecular characterization of the nature and hence consequences of the mutation, can provide stimulating (even if speculative) interpretation. Mutant genotypes which are identified as deficient in immunodetectable levels of a phytochrome polypeptide product may retain a low level of the phytochrome, below the limits of the sensitivity of the extraction/detection assay, and furthermore such reduced levels may reflect an overall reduction throughout the plant or may be due to specific lack of expression of the *phy* gene in certain cell-types. Even photoreceptor-deficient mutants which are clearly identified as containing a mutation in the phytochrome structural gene are subject to potential complications similar to some of those discussed for transgenic plants. For example, as argued for the transgenic overexpression of phytochrome, the deficient photoreceptor species in mutant plants may be involved in some form of feedback regulation of other phytochromes. The possible interaction in the wild-type plant of the mutated phytochrome form with the other molecular species may also complicate interpretation of the photophysiology of the phytochrome-deficient plant, for example a certain level of competition between photoreceptor species for a reaction partner in the wild-type plant may be important to normal photoregulation of response.

It therefore appears unwise to consider either transgenic or mutant plants as exclusive systems for the study of phytochrome function. Coupling complementary studies of photoreceptor-deficient mutants and photoreceptor-overexpressing transgenic plants, however, has already provided the first direct evidence of discrete photoregulatory roles, and has the potential to demonstrate unequivocally that the diverse array of phytochrome

responses that occur throughout plant development are in reality the consequence of a diverse array of discrete phytochromes. In the reductionist approach which is adopted in studies of plant physiology, namely through controlling as many variables as possible in order to individually analyze the component elements of plant processes, transgenic technology should be viewed as contributing another facet.

Potential applications of transgenic plants in studies of phytochrome function in natural plant communities and in crop-plant species

Transgenic plants will also be especially useful in attempts to assess the evolutionary advantage of the possession of the various forms of phytochrome using well established population genetic methods. Typical morphological responses to competition can be induced by changes in the R:FR not only of direct incident light, but also of light reflected by neighbours even before direct shading occurs (Ballaré *et al.*, 1987; 1990; 1991). It has been argued that sensitivity to reflected R:FR light, before actual shading, is adaptive because it enables plants to anticipate and avoid competition for light. This is an ecologically important hypothesis in view of the interest in the evolution of phenotypic plasticity. Plasticity of a given trait can only be considered adaptive if it results in high relative fitness in all environments; in the case of plastic responses to reflected light quality it is therefore necessary to demonstrate that: (a) the morphology induced by low R:FR reflected from neighbours increases relative fitness at high density, and (b) the same morphology is selected against in uncrowded plants (Schmitt and Wulff, 1993). For example if this scenario were correct, transgenic plants overexpressing phytochrome A, displaying impaired shade-avoidance elongation-growth, would be expected to show least relative fitness under conditions of crowding from competitive genotypes, whereas long hypocotyl phytochrome B-deficient mutants would be disadvantaged in situations of low competition for light. The role of phytochrome in etiolated wild-type plants with regard to

adapative fitness is not so readily speculated upon, and this has led to some suggestions that phytochrome A is redundant in seedling photomorphogenesis, and the FR-HIR is an anomaly observed only under artificial laboratory conditions. In evolutionary terms this might seem to suggest phytochrome A as the more primitive member of the phytochrome family, perhaps disfunctional in higher plants. But in contradiction to this view is: (a) the level of conservation in the *phyA* structural sequence between plant species including across the monocot/dicot line, and (b) the closer resemblance of the phytochromes of the evolutionarily lower ferns (*Selaginella*), moss (*Ceratodon*) and green alga (*Mougeotia*) to the Type II phytochromes of angiosperm species (Hanelt *et al.*, 1992; Winands *et al.*, 1992). The various and complementary mutant and transgenic lines in which wild-type phytochrome-mediated responses are variously impaired or exagerated, could provide the basis for experiments to test explicitly the impact of particular phytochrome responses, and indeed individual phytochrome species, on fitness in natural populations.

Since overexpression of an introduced phytochrome species has predictable effects on the photophysiological development of transgenic plants, there may also be a commercial application in crop-improvement programmes. It has been proposed that, if a way could be found to eliminate, disable or markedly reduce the shade-avoidance responses of crop plants grown at high density, potentially large increases in harvestable yield would be expected to be achieved (Smith, 1992; Decoteau *et al.,* 1988; 1989; 1990). A number of purely speculative approaches to achieving this have been discussed in detail by Smith (1992) who concluded that, of the theoretical alternatives, transgenically-engineered "myopia" to perception of neighbouring plants through overexpression of phytochrome is currently the most realistic suggestion.

At present, the gene constructs used for the overexpression of phytochome are somewhat crude, in the sense that a constitutive viral promoter is used to drive expression of the heterologous phytochrome (Boylan and Quail, 1989; 1991; Keller *et al.*, 1989; Kay *et al.*, 1989b). Endogenous *phyA* genes are known to exhibit varying degrees of

light-inhibition of expression (e.g. Colbert *et al.*, 1983; 1985; Sharrock and Quail, 1989) and all *phy* genes are speculated to be subject to some form of tissue-specific regulation. Refinement of transgene-expression to reflect these traits, or alternatively to over-ride a particular aspect of the regulation profile, may be a future step which would render transgenic plants more elegant tools for manipulating phytochrome function both in the natural environment and with the aims of modern agriculture. For example, in a transgenically-engineered crop-plant program, restricting expression of blindness-to-neighbours to the adult plants could be a means to prevent the young seedlings in the field from becoming over-grown by weed species.

Results from transgenic plants have facilitated the isolation of novel phytochrome mutants

Regardless of the precise interpretation of the phytochromes' photosensory roles, it becomes clear from responses of *phyB*-deficient mutants and phytochrome-overexpressing transgenic plants that mutants defective in the activity of the photoreceptor mediating the irradiance-dependent FR-response would retain phytochrome B and therefore remain responsive to continuous R. This prediction might readily explain why previous exhaustive screens for long-hypocotyl phenotypes when performed under white light failed to detect mutations in phytochromes other than phytochrome B. Based on these assumptions, a two-step photobiological screen was able to be devised involving sequential screening of mutagenised populations of *Arabidopsis* etiolated seedlings under continuous FR and continuous R, and has proved to be a highly effective means for the isolation of a novel *hy* locus defining the, previously elusive, *phyA*-null class of mutants (Nagatani *et al.*, 1993; Parks and Quail, 1993; Whitelam *et al.*, 1993). Studies of the photophysiological relationship of transgene expression and response have therefore suceeded in defining an alternative strategy for the isolation of one specific class of photoreceptor-deficient mutants; transgenic plants

overexpressing phytochrome C, which are currently being produced, may prove similarly helpful in the isolation of mutants in the *phyC* gene. Because extensive screens for specific loss of etiolated FR-responsiveness have failed to isolate mutants which are deficient in any phytochrome species other than phytrochrome A, this appears consistent with the hypothesis that phytochrome A is the sole photoreceptor for the FR-HIR.

The use of transgenic plants in structure-function studies of phytochrome

The short hypocotyl phenotype caused by phytochrome A or phytochrome B overexpression can be exploited for structure-function studies of the photoreceptor because this trait provides a biological assay for monitoring the activity of *in vitro* mutagenised *phy* sequences. Several workers have introduced mutated phytochrome genes into transgenic plants in order to compare the effect of overproduction of the mutated phytochrome with the effect of overproduction of the corresponding wild-type phytochrome. These experiments allow identification of residues or domains that affect the phenotype caused by overexpression of phytochromes. Mutation of the chromophore-attachment site, as might be expected, resulted in a protein that caused no phenotype when expressed in *Arabidopsis* (Boylan and Quail, 1991). In another case, a phytochrome gene in which several serine residues from the NH_2-terminal had been mutated to alanine residues caused a more extreme short plant phenotype than the wild-type phytochrome gene did (J. Stockhaus and N.-H. Chua, unpublished results, cited in: Reed *et al.*, 1992). In an initial analysis of mutagenised oat phytochrome A in transgenic tomato it was found that, with one exception, constructs individually containing deletions from various regions across the length of the polypeptide failed to induce the dwarf-phenotype, either through failure to accumulate sufficient levels of the mutated monocot molecule or through disruption of activity (Quail, 1991). A 68 kDa NH_2-terminal fragment of oat phytochrome A which forms the chromophore-bearing domain was expressed in transgenic *Arabidopsis* and resulted in an

elevated spectrophotometrically-measurable level similar to that found in seedlings that overexpressed authentic full-length oat-*phyA*-polypeptide, demonstrating that the 68 kDa polypeptide was photochemically active. Expression of this mutant sequence failed to elicit the short hypocotyl phenotype under R, indicating that the mutation interfered with the biological activity of oat phytochrome A (Boylan and Quail, 1991). However, this mutation is remarkable in that enhanced sensitivity to continuous FR, as exhibited in dark-grown seedlings overexpressing wild-type oat phytochrome A, was also seen in the transgenic seedlings containing the truncated sequence (M.T. Boylan and P.H. Quail, unpublished results). This result would appear to suggest that the regulatory-activities of the oat phytochrome A molecule to continuous R and continuous FR can be physically dissected along the polypeptide. In all such studies more refined point mutational analysis is required to begin to discriminate between specific effects on functionally critical sequences and non-specific effects on global molecular structures.

Conventional mutants deficient in specific individual phytochrome species are now available in *Arabidopsis* for both the *phyA* and *phyB* photoreceptors and would provide the most favourable genetic background for transformation with mutagenised sequences, rescue of the wild-type phenotype being the assay for biological activity. Studies of mutant and transgenic plants have identified specific photoregulatory roles for phytochrome A and phytochrome B, hence optimal light regimes can be devised for *in vivo* study of structure and function for each photoreceptor species; for example, hypocotyl growth under continuous FR and continuous R would, respectively, provide a sensitive and specific assay for the biological activity of *in vitro* mutagenised phytochrome A and phytochrome B molecules.

Although *in vitro* mutagenised sequences have the advantage of allowing site-specific changes to be induced, natural mutants can provide similar insights into phytochrome structure in relation to function. For example the *hy8-3* mutant allele of *Arabidopsis* results in an elongated phenotype of the etiolated seedlings specifically under

continuous FR, as exhibited by phytochrome A-deficient mutants, but normal polypeptide levels of phytochrome A are accumulated which are spectrophotometrically-active (Parks and Quail, 1993). The *hy8-3* mutant is considered very likely to map to the *phyA* structural gene and if this is correct, the mutant gene encodes a photoreceptor molecule which is indistinguishable from wild-type with respect to photochemical activity and competence for P_{fr}-specific *in vivo* degradation, but which lacks photoregulatory activity. Sequence analysis therefore should identify a residue in this ethylmethane sulfonate-induced mutant that is specifically critical to the regulatory action of phytochrome A (as distinct from photoperception by the molecule).

Mutant and transgenic plants in elucidation of phytochrome transduction pathways

Real progress in understanding the mechanisms through which phytochromes function must ultimately be dependent upon the elaboration of a reliable cell-free system in which phytochrome exerts its controlling effect. In the absence of a functional *in vitro* system, genetic approaches are starting to provide interesting results regarding the pathways responsible for transduction of the signal from the individual phytochrome species. Some evidence for distinct transduction pathways for Type I and Type II phytochromes is provided by the pattern of gene expression in the *Arabidopsis hy5* photomorphogenetic mutant. The *hy5* mutant is believed to be deficient in some component affecting the transduction pathway transmitting photostable phytochrome signals and hence displays reduced responsiveness to red and white light (Koornneef *et al.*, 1980). Expression of *cab* genes in *Arabidopsis* shows phytochrome-mediated photoregulation in the etiolated seedlings but becomes unresponsive to phytochrome-control in wild-type light-grown plants and is also unaffected by the *phyB* (*hy3*) mutation (Chory *et al.*, 1989b; Sun and Tobin, 1990); the hypothesis is that a light-labile phytochrome species (Type I) is

responsible. In seedlings of the *hy5* mutant, photoregulation of expression of the *cab* genes is apparently normal (Sun and Tobin, 1990), suggesting that the deficient component in the transduction of the Type II phytochrome signal does not interact with the pathway of Type I phytochrome.

Transgenic plants have displayed similar potential for characterising transduction partners. For example expression of the 68 kDa NH_2-terminal oat phytochrome A fragment in transgenic *Arabidopsis* (Boylan and Quail, 1991; M.T. Boylan and P.H. Quail, unpublished results) apparently demonstrated the dissection of continuous R and continuous FR perception/regulation by the heterologous phytochrome to two disinct domains. Such major deletion effects, however, may have caused a global effect on a site in the NH_2-terminal domain leading to a knockout of the binding of this site to a transduction-pathway component. However, it does seem to suggest either: (a) R- and FR-mediated photoregulation requires distinct sites on the phytochrome A molecule, and hence interact with different secondary reaction partners or, (b) the COOH-terminal deletion causes partial disruption of a single NH_2-terminal site and the continuous R and continuous FR pathways exhibit different affinities for the same, disrupted site. Both interpretations infer the use of different transduction pathways in mediating the same ultimate response of inhibition of hypocotyl elongation, when the heterologous phytochrome A perceives continuous R or continuous FR. Mutagenesis of lines containing point-mutated phytochrome genes, either endogenous or introduced, will permit screening for second site suppressers of the phenotype conferred by the altered phytochrome, and therefore has the potential to identify a signal transduction partner for the photoreceptor.

Concluding remarks

In conclusion, the isolation of genes encoding different molecular species of phytochrome, together with the use of genetic transformation techniques, has pioneered a new and

potentially powerful area of phytochrome research. Studies of transgenic plants overexpressing wild-type *phy* sequences have already provided evidence towards determining the precise photophysiological role for each phytochrome species. Transgenic techniques, including reverse genetics, may prove especially important in the study of the minor phytochrome species for which conventional mutations are currently lacking. Transforming with wild-type genes for overexpression of a particular phytochrome species and conversely, introduction of gene-specific anti-sense cDNA to identify the precise effects of inhibiting expression of one or more individual phytochrome sequences, will be of particular interest in assessing the capacity of the various light-stable phytochrome species to complement the actions of one another. Studies of the epistatic interactions of natural mutations in unidentified transduction chain components with engineered phytochrome mutations would help in determining the relationships between the individual phytochrome pathways and the different signal transduction components, and could significantly aid in the identification of such secondary photoactivated molecules.

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