UNIVERSITY OF SOUTHAMPTON

Manipulation of Growth and Development in Tobacco Through Targeted Inactivation of the Phytochrome Chromophore

Keara Ann Franklin

Doctor of Philosophy

BIOLOGY

FACULTY OF SCIENCE

September 2001

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

BIOLOGICAL SCIENCES

Doctor of Philosophy

MANIPULATION OF GROWTH AND DEVELOPMENT IN TOBACCO THROUGH TARGETED INACTIVATION OF THE PHYTOCHROME CHROMOPHORE

By Keara Ann Franklin

The photo-regulatory activity of the plant photoreceptor phytochrome requires covalent attachment of the plastid-synthesised linear tetrapyrrole, phytochromobilin. Constitutive expression of the mammalian enzyme biliverdin reductase (BVR) in plants inactivates chromophore biosynthesis, resulting in a loss of all phytochromes throughout development. Extensive molecular, biochemical and physiological analyses of tobacco plants (*Nicotiana tabacum* cv. Maryland Mammoth), expressing BVR targeted to both cytosol and plastid, have been used to investigate the consequences of total phytochrome deficiency on the growth and development of a short-day plant.

Constitutive expression of BVR in tobacco resulted in reduced germination, elongated hypocotyls, smaller cotyledons and decreased chlorophyll content in light-grown seedlings. Impaired photomorphogenic responses under continuous far-red and red wavelengths are consistent with deficiencies in both phytochrome A and phytochrome B activities. Mature plants with plastid-targeted BVR displayed elongated internodes, altered leaf morphology and reduced chlorophyll levels, consonant with deficiencies in multiple phytochromes. Photoperiodic sensitivity was reduced in transgenic plants with all lines flowering early in photoperiods above 10.5 hours. This result is consistent with an inhibitory role for phytochrome in controlling flowering in short-day plants. Cytosol-targeted BVR lines showed an intermediate phenotype between that of plastid-targeted BVR lines and wild-type plants, a result consistent with a partial reduction in photoactive holophytochrome. Targeting of BVR to plastids also resulted in perturbations of the tetrapyrrole pathway and altered plastid morphology. A severe protochlorophyllide deficiency was recorded in dark-grown seedlings, despite increased levels of ALA synthesis. Transcription of Mg-chelatase subunits was unaffected in these plants and feeding studies revealed no post-transcriptional impairment of Mg-chelatase activity. In contrast, a marked inhibition of ALA synthesis was observed in light-grown seedlings, an effect resulting in pronounced cotyledon bleaching. Decreased levels of chlorophyll a/b binding protein (*CAB*) transcript and reduced granal stacking were also observed. Altered expression patterns of tetrapyrrole synthesis enzymes were recorded in plastid-targeted BVR seedlings grown both in the dark and the light and are consistent with a role for tetrapyrroles in plastid-nuclear signalling.

Contents

Acknowledgements

I would primarily like to thank my supervisors, Dr. Matthew Terry, Professor Brian Thomas and Dr. Steve Jackson for providing direction and guidance throughout the course of this study.

I acknowledge Professor Clark Lagarias and Dr. Beronda Montgomery for donating the transgenic seed used in this work, sharing unpublished data and useful discussion. cDNA clones provided by Professor Bernhard Grimm were also greatly appreciated. I extend my gratitude to following people for their assistance and expertise: Pat James, Christine Richardson, Rosemary Bell, Dr.s Alex McCormac, Martin Landsberger, Phil Linley, Mark Milford and Anton Page. Extra thanks to Alex, whose honest and pragmatic advice ensured the completion of this thesis within three years.

I am grateful to friends and colleagues at both HRI and Southampton, for helping make the last three years more enjoyable. Thanks also to my family and non-scientist friends for providing support, reality and regular reminders as to whose taxes have paid for my post-graduate education. Finally, I would like to thank the herd (and Matt) for providing an endless source of much-needed distractions over the last three years.

This work was funded by the BBSRC and Horticulture Research International.

Chapter 1. Introduction

Throughout their life cycle, plants are exposed to both differing quantities and qualities of light. The possession of an efficient harvesting system to optimise photosynthetic activity during such fluctuations is therefore imperative to plant survival. In addition to its role as an energy source, light signals also serve to provide plants with information about the surrounding environment (Smith 1995). Efficient perception, interpretation and transduction of such signals allow plants to synchronise their development with seasonal changes and compete effectively with neighbours for limited resources. The ability to utilize light of varying wavelengths and fluence enables plants to respond exquisitely to changing light conditions, thus minimising the effects of environmental perturbations. Three major classes of photoreceptor have been identified for light perception in higher plant tissues; phytochrome, which exists in two photoconvertible isomeric forms Pr and Pfr, absorbing red (R) and far-red light (FR) respectively (Smith 2000) and the UV-A/blue light (B)-absorbing cryptochromes (Ahmad and Cashmore 1996a) and phototropins (Christie *et al* 1998). In addition, there is evidence for a UV-B-absorbing photoreceptor (Kendrick and Kronenberg 1994). It has also been suggested that the chlorophyll precursor, protochlorophyllide, bound to NADPH: protochlorophyllide oxidoreductase (POR), may act as a photoreceptor during plant development (Smith 1975b).

Phytochrome exists as a homodimer of two independently photoreversible subunits. Each subunit consists of a 120-127 kDa polypeptide covalently attached to a linear tetrapyrrole chromophore via a thioether linkage (Lagarias and Rapoport 1980). Each phytochrome molecule is tripartite in structure, consisting of two interactive carboxy domains that dimerize to form a base from which extend the two globular amino terminal domains. Structural analyses of the apoprotein component have revealed the amino terminal domain to include the chromophore-binding site and all structural requisites necessary for photoreversibility and differential light lability (Jones *et al* 1985, Wagner *et al* 1996). Mutational analyses of the amino terminus have revealed two functional domains, one necessary for conformational stability and the other involved in attenuating phytochrome responses (Jordan *et al* 1995a). Determinants required for the dimerisation of phytochrome molecules are believed to be located in the carboxy terminus (Cherry *et al* 1993, Edgerton

and Jones 1992,1993). The carboxy terminal domain is also required for the transfer of perceived informational signals to downstream transduction pathway components (Quail *et al* 1995). A "core region" has been located at the proximal end this domain, within which, missense mutations leading to a loss of phytochrome activity are clustered (Quail 1997). A region also exists at the extreme carboxyl terminus which bears some similarity to bacterial sensory histidine kinases (Schneider-Poetsch 1992).

Interactions between the chromophore and polypeptide components enable phytochrome to assume two spectrally distinct forms: Pr, which has an absorbance maximum at 666-668nm, and Pfr, which has an absorbance maximum at 730nm. Phytochrome is synthesised as Pr, which is biologically inactive for most phytochrome-mediated responses. Activity is acquired upon phototransformation to the Pfr isomer (Kendrick and Kronenberg 1994). The chromophore in Pr is thought to be protonated and in an extended conformation. Spectral differences between Pr and Pfr are believed to arise from a cis-trans isomerisation at the C15 bridge between rings C and D of the chromophore and conformational changes in chromophore-protein interactions (Rüdiger *et al* 1983). After saturating red irradiation, a photoequilibrium is established with approximately 86% of the total phytochrome in the Pfr form (Vierstra and Quail 1983, Lagarias *et al* 1987).

1.1 Phytochrome synthesis

1.1.1 Apoprotein synthesis

Biochemical evidence indicates the existence of two physiologically and spectrally distinct pools of phytochrome in plant tissues (Abe *et al* 1989). One pool is synthesised in the Pr form in darkness and decreases rapidly in the light as the labile Pfr form, while the other is relatively stable as Pfr and is present at a relatively constant level, irrespective of light conditions (Furuya 1991). The existence of five phytochrome genes (A-E), in *Arabidopsis thaliana*, was first demonstrated by Sharrock and Quail (1989) and all of these have now been sequenced. Phytochromes A,B,C and E are evolutionarily divergent proteins, sharing only 46-53% sequence identity, while *PHYD* encodes an apoprotein that shares 80% sequence identity with *PHYB* (Clack *et al* 1994). Molecular phylogenetic analysis supports the occurrence of four major duplication events in the evolution of phytochrome genes. An

initial duplication is believed to have separated *PHYA* (light labile in the Pfr form) and *PHYC* (light stable in the Pfr form (Somers *et al* 1991)) from *PHY B/D/E* (all light stable in the Pfr form (Hirschfeld *et al* 1998)). Subsequent separation of *PHYA* from *PHYC* and *PHY B/D* from *PHYE* resulted in three sub-families: *A/C*, *B/D* and *E* (Smith 2000). At least five different *PHY* genes exist in tomato, including two versions of the *PHYB* gene (*B1* and *B2*) and a gene (*PHYF*) believed to belong to the *PHYC* sub-family (Alba *et al* 2000). In the monocotyledonous plant *Sorghum bicolor*, sequences homologous to the *PHYA*, *PHYB*, and *PHYC* genes of *Arabidopsis* and a phytochrome sequence homologous to the *PHYF* gene of tomato have been isolated (Mathews and Sharrock 1997).

Similar studies in the allotetraploid *Nicotiana tabacum* have so far revealed two *PHYA*- like genes, *A1* and *A2*, (one per diploid genome) and a *PHYB*-like gene (*B1*) (Adam *et al* 1997). *Nt PHYA1* shows 96% homology to *Nt PHYA2* and high (70, 83 and 92% respectively) homology to *PHYA* sequences in *Arabidopsis*, pea and potato. Although no mutants are currently available, it is expected that the functions of tobacco phytochrome A proteins are similar or identical to those of *Arabidopsis* phyA. The isolated *Nt* phytochrome B-like protein displays 78% sequence similarity to its *Arabidopsis* counterpart and is stable in light-grown tissues (Adam *et al* 1997). The possibility exists that tobacco, like tomato, contains more than one *PHYB*-like gene, although the exact number has yet to be determined. Despite an array of apoprotein structures, current evidence indicates that all higher plant phytochromes use the same chromophore (Terry 1997).

1.1.2 Chromophore synthesis

The involvement of the tetrapyrrole pathway in higher plant chromophore synthesis was first suggested by Bonner (1967), who demonstrated that radioactive labelling of the tetrapyrrole precursor 5-aminolevulinic acid (ALA) was incorporated into photoreversible phytochrome. Gardner and Gorton (1985) later showed a reduction in spectrophotometrically detectable phytochrome in etiolated seedlings treated with gabaculine, a known inhibitor of ALA biosynthesis. Subsequent investigation revealed such a reduction to be reversed by the exogenous application of ALA (Jones *et al* 1986) and biliverdin IXα (Elich and Lagarias 1987). Such evidence is consistent with the proposal that chromophore is synthesised *de novo* in cells to form spectrally functional phytochrome.

A lack of coordinate regulation between chromophore and apoprotein synthesis supports the hypothesis of post-translational attachment of chromophore to pre-formed apoprotein, and contends the idea of a feedback mechanism controlling apoprotein levels in response to chromophore synthesis.

Support for the identity of phytochromobilin as the higher plant precursor of chromophore synthesis was provided by Terry and Lagarias (1991). Holophytochrome assembly was used as an *in vitro* asssay to present evidence for the existence of phytochromobilin synthase in isolated plastids of *Cucumis sativus*. The linear tetrapyrrole, biliverdin IXα, formed from the oxygenation of heme (Weller *et al* 1996), was converted to the free phytochrome chromophore, phytochromobilin, which assembled with oat apophytochrome to yield a photoactive holoprotein. The spectral properties of the synthetic phytochrome were indistinguishable from the natural photoreceptor and the stimulation of biliverdin conversion by NADPH and ATP suggested the reaction to be enzymatic (Terry and Lagarias 1991). Proof for the role of phytochromobilin as the native precursor for chromophore biosynthesis was provided by Cornejo *et al* (1992). Methanolysis of solvent-extracted *P.cruentum* cells was shown to release 2(*R*), 3(*E*) phytochromobilin in addition to the expected phycobilins, phycocyanobilin and phycoerythrobilin. This pigment was subsequently shown to spontaneously assemble with recombinant oat apopytochrome to form photoactive holophytochrome, which contained a covalently-bound chromophore and was spectrally indistinguishable from native oat phytochrome. Kinetic high performance liquid chromatography (HPLC) analyses have subsequently demonstrated that that the (3Z) isomer of phytochromobilin is synthesized prior to the (3E) isomer in oat etioplasts (Terry *et al* 1995). It is therefore proposed that (3Z) phytochromobilin is the immediate product of biliverdin IXα reduction by phytochromobilin synthase. This implicates the existence of an isomerase to catalyse the conversion of the (3Z) phytochromobilin to the (3E) isomer, the immediate precursor of the phytochrome A chromophore (Terry *et al* 1995). Heme oxygenase genes have been cloned in a variety of higher plants, including *Arabidopsis* (Muramoto *et al* 1999, Davis *et al* 1999, 2001), tomato (Davis *et al* 2001) and rice (Izawa *et al* 2000).Recent cloning of the *Arabidopsis HY2* gene revealed it to encode

phytochromobilin synthase, a ferredoxin dependent biliverdin reductase (Kochi *et al* 2001). Genes encoding phytochromobilin isomerase enzymes remain to be identified.

1.1.3 Holophytochrome assembly

Holophytochrome assembly is an autocatalytic process, believed to occur in the cytoplasm (Lagarias and Lagarias 1989). Terry and Lagarias (1991) have demonstrated that there is no requirement for the phytochrome apoprotein to interact with plastids in order to receive a chromophore. How holophytochrome assembly is regulated *in vivo* is not known, but cofactors ATP and NADPH are not required (Lagarias and Lagarias 1989). An array of processing events occur after or during translation of phytochrome mRNA in the cytoplasm. These include cleavage of the N-terminal methionone, acetylation of the now N-terminal serine, dimerization of the phytochrome monomers, insertion of the bilin chromophore, phosphorylation and ubiquitination (Whitelam 1991).

1.2 Phytochrome degradation

The quantitative dependence of phytochrome responses on absolute Pfr levels requires controlling mechanisms to ensure that appropriate cellular levels of photoreceptors are maintained (McCormac *et al* 1993b). Phytochrome A (phyA) is regulated at the levels of gene transcription, mRNA stability, protein availability and protein turnover (Casal *et al* 1997, Hennig *et al* 1999). The most influential of these controls is the rapid proteolysis of the chromoprotein, following photoconversion of Pr to Pfr (Clough and Vierstra 1997). Quail *et al* (1973) recorded that the Pfr form of phyA in pumpkin cotyledons has a half- life of only 1-2 hours, whereas that of the Pr form is two orders of magnitude higher. Pfr degradation is sensitive to temperature and metabolic inhibitors, indicatative of an energy requiring enzymatic process. Although Pfr is the predominantly degraded molecule, Pr molecules cycled through Pfr can also be destroyed, suggesting that phytochromes are modified upon phototransformation, which earmark them for degradation, even after conversion to Pr (Stone and Pratt 1979).

The rapid relocation of phyA from a uniform and disperse cytoplasmic distribution as Pr to discrete aggregates upon conversion to Pfr may represent an initial step in chromoprotein turnover (Clough and Vierstra 1997). This process of "sequestering" is believed to represent

a mechanism whereby excess Pfr is removed, and placed in a form accessible to the degradation machinery (Speth *et al* 1987). The thermal reversion of Pfr back to Pr, termed "dark reversion" clearly protects a fraction of Pfr from proteolysis. Fusion protein studies showing the nuclear localisation of phyA molecules upon illumination also revealed the rapid formation of fluorescent spots in the cytosol (Kircher *et al* 1999). Such spots are reminiscent of the previously described sequestered areas of phytochrome (Speth *et al* 1987). The authors propose that only a fraction of cytoplasmic phytochrome molecules are transported into the nucleus upon illumination and sequestering of phyA does not fulfil an important role in the nuclear import mechanism.

One proposed mechanism of phytochrome destruction involves the ubiquitin/ 26S proteosome pathway. Ubiquitin-protein conjugates are selectively recognised by the 26S proteosome, a large proteolytic complex that degrades the target protein but releases ubiquitin intact (Clough and Vierstra 1997). Clough *et al* (1999) used domain swap experiments to demonstrate that the intact carboxy-terminal domain is essential for phyA degradation, while the amino-terminal domain is responsible for selective recognition and ubiqitination of Pfr. A number of regions have been mapped that are preferentially exposed in Pfr and could therefore serve as degradation recognition sites. One of these is the PEST domain, a motif common among short-lived proteins, consisting of two positively charged groups between which proline, glutamic acid, serine and threonine residues are located in high molecular proportion (Rogers *et al* 1986). It has been proposed that PEST domains become phosphorylated which, in turn, enhance their recognition by the ubiquitin/26S proteosome pathway (Kornitzer *et al* 1994). The close proximity of the PEST domain to the phytochrome chromophore suggests that the natural reorientation of the chromophore during photoconversion exposes this sequence to degradative machinery within the cell.

In dicotyledonous plants, degradation is first order with respect to phytochrome, while in monocotyledonous plants, zero order kinetics are observed, even at reduced Pfr levels (Schäfer *et al* 1975). In etiolated plants, the rapid degradation of Pfr upon transfer to light predominantly represents the destruction of phyA. However, analysis of Pfr degradation in etiolated seedlings of *Amaranthus caudatus L.* revealed biphasic kinetics (Brockmann and Schäfer 1982). Such biphasic rates imply the existence of a pool of additional

phytochromes, showing greater stability in the Pfr form than phyA. It is envisaged that the rate of turnover of light stable phytochromes represents a balance between synthesis and degradation, resulting in a stable equilibrium.

1.3 Physiological functions of the phytochromes

Phytochromes enable plants to monitor the intensity, quality and direction of incident light. Such information is used to modulate a number of physiological responses, including seed germination, seedling establishment, plant architecture and the onset of flowering. During seedling development, light stimulates the opening and expansion of cotyledons and the synthesis of chlorophyll. The parallel formation of photosynthetic chloroplasts provides plants with the capacity for light harvesting and photoautotrophic growth.

1.3.1 Germination

Seed germination depends on many environmental circumstances, including position in the soil profile, soil disturbance and extent of canopy cover. In many species, these responses are mediated, in part, by phytochrome perception of the light environment (Botto *et al* 1996, Shinomura *et al* 1996). To reach the light, seedlings developing under soil are required to maximise axis elongation at the expense of leaf development. Having attained soil emergence, leaf and plastid development can then take precedence (Smith and Whitelam 1990). Imbibed seeds and etiolated seedlings display three different modes of phytochrome action, characterised by different fluence response curves, R/FR reversibility and fluence rate dependence. These are the very low fluence response (VLFR), the low fluence response (LFR) and the high irradiance response (HIR).

Phytochrome A is primarily responsible for enhancing the photosensitivity of seedlings before they emerge from the soil (Vierstra 1993). The FR-HIR inhibition of seed germination may be ecologically relevant as a means of delaying germination under chlorophyllous vegetation, or leaf litter (Smith and Whitelam 1990). The phyA-mediated VLFR promotion of germination allows seeds to take opportunistic advantage of potentially beneficial soil disturbances.

1.3.2 Photomorphogenesis

Dark-grown seedlings follow a developmental program termed skotomorphogenesis, and display a characteristic phenotype, including elongated hypocotyls, closed and unexpanded cotyledons and non-photosynthetic etioplasts (Terzachi and Cashmore 1995). Upon transfer to light, hypocotyl growth is inhibited, cotyledons are expanded and etioplasts undergo rapid morphological changes to form photosynthetically-active chloroplasts. Depletion of phyA upon de-etiolation reduces the inhibitory action of low R:FR ratios, and enables phytochrome B (phyB) -regulated hypocotyl extension to occur. It has been suggested that such elongation is mediated by an interacting mechanism involving both phytochrome and gibberellins (López-Juez *et al* 1995). Increased hypocotyl extension under low R:FR ratios, is termed the "shade avoidance syndrome", and is mediated primarily by phyB (Robson *et al* 1993).

1.3.3 Plastid Development

Etioplasts contain prolamellar bodies, paracrystalline structures composed largely of lipids and NADPH:protochlorophyllide oxidoreductase (POR) in its photosensitive ternary complex (Thomas 1997). Upon illumination, the prolamellar body disperses in a sequence of events termed "tube transformation" (Smith 1975b) and initiates a series of defined processes which result in the development of the mature chloroplast. Dispersion of the prolamellar body is followed by an extrusion of tubular material into the stroma. *De novo* replication of membrane material ensues to form functional grana. Membrane replication cannot occur until tube extrusion is complete and is therefore subject to a lag phase of 2-3 hours after the start of irradiation (Smith 1975b). A similar lag phase is observed for chlorophyll biosynthesis.

1.3.3.1 Chlorophyll synthesis

5-aminolevulinic acid (ALA) is the earliest, well-characterised precursor of chlorophyll biosynthesis and is formed, in higher plants, from the intact carbon skeleton of glutamic acid in a process requiring three enzymatic reactions and $tRNA^{Glu}$ (Beale 1990). Glu-$tRNA^{Glu}$ is converted by the action of glutamyl-tRNA reductase (GluTR) to glutamate-1-semialdehyde, with the subsequent release of $tRNA^{Glu}$ (Kumar *et al* 1996a). ALA is formed by the

transamination of glutamate-1-semialdeyde by glutamate-1-semialdehyde 2,1 aminotransferase (GSA-AT). Two molecules of ALA are condensed by ALA dehydratase to form the pyrrole porphobilinogen. The tetrapyrrole hydroxymethylbilane is produced from four molecules of porphobilinogen via the action of porphobilinogen deaminase. Ring closure and simultaneous isomerisation of the acetyl and propionyl groups at the pyrrole ring D lead to the formation of uroporphyrinogen III (Urogen III), which undergoes successive decarboxylations and oxidations to produce protoporphyrin IX, the branchpoint intermediate of the pathway (von Wettstein *et al* 1995, Reinbothe and Reinbothe 1996). The chelation of Fe^{2+} into the porphyrin macrocycle yields protoheme, while the chelation of Mg^{2+} produces Mg-protoporphyrin, which is then esterified to Mg-proto-monomethyl ester. The latter is converted to protochlorophyllide (Pchlide), which is reduced upon illumination to chlorophyllide *a* (Chlide *a*), the immediate precursor of chlorophyll. The enzyme chlorophyll synthase esterifies Chlide *a* with the isoprenoid phytyl diphosphate to produce chlorophyll (Tanaka *et al* 1999). A schematic representation of the tetrapyrrole pathway is shown in Figure1.

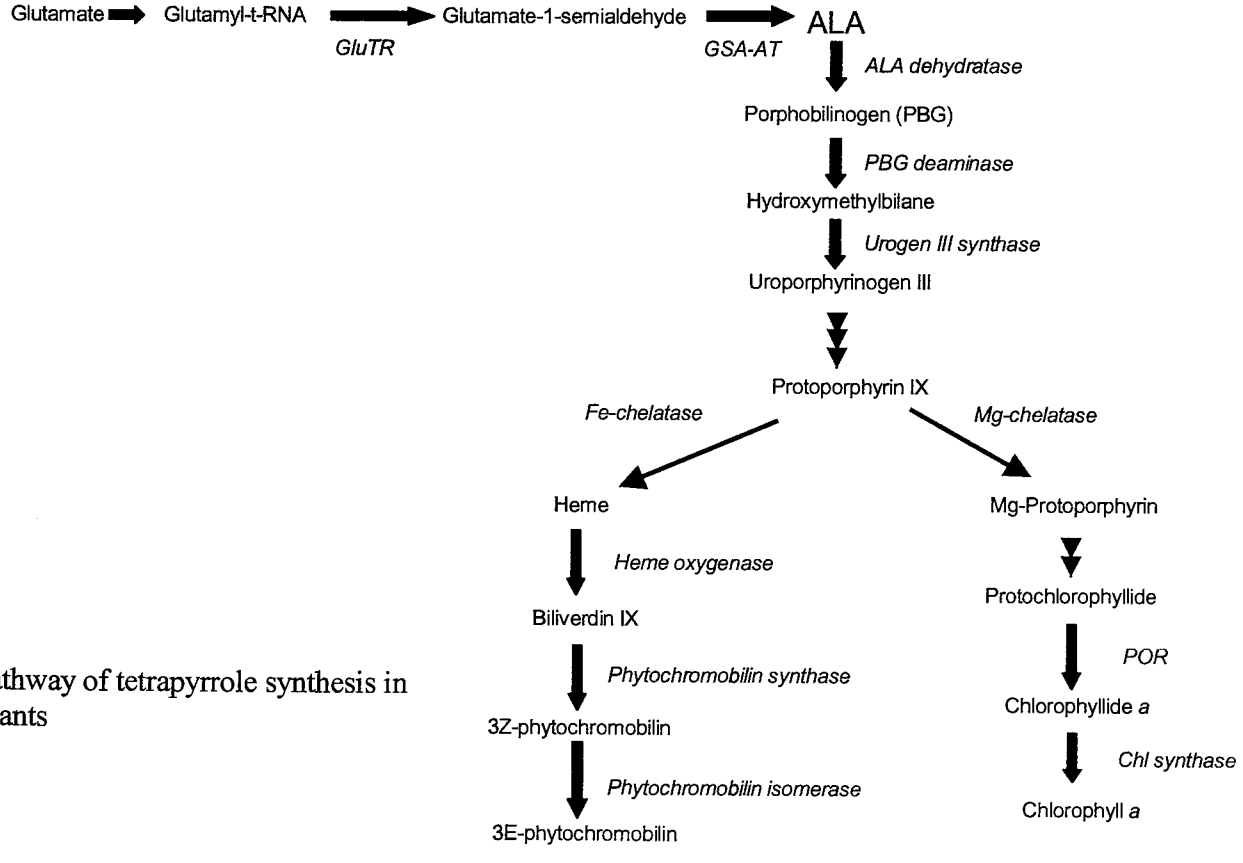


Fig. 1 Pathway of tetrapyrrole synthesis in higher plants

Chlorophyll *b* is produced from the oxygenation of Chlide *a* (Tanaka *et al* 1998). The gene encoding chlorophyllide *a* oxygenase (*CAO*) has been cloned from a variety of species and is highly conserved between cyanobacteria and higher plants (Tomitani *et al* 1999). Formation of chlorophyll *b* takes place stoichiometrically at the cost of Chlide *a*, and has been reported to depend on the action of phytochrome (Oelze-Karow and Mohr 1978). It is envisaged that a threshold level of chlorophyll *a* must accumulate before chlorophyll *b* biosynthesis can commence (Thomas 1997).

1.3.3.2 Regulation of the tetrapyrrole pathway

Much of the control of chlorophyll synthesis occurs at the level of ALA synthesis, although the precise nature of this control has yet to be unequivocally demonstrated. Light appears to stimulate ALA formation (Huang *et al* 1989), whereas heme and the Pchlide ternary complex perform an inhibitory role (Beale and Weinstein 1990, Terry and Kendrick 1999). Papenbrock *et al* (2000) also suggest the existence of a regulatory mechanism in response to metabolic flux through the Mg-porphyrin branch of the pathway. Exogenously applied ALA is metabolised in the dark to produce Pchlide, the immediate precursor of chlorophyll *a*, suggesting that the pathway between ALA dehydratase and POR is constitutive (Thomas 1997).

Sisler and Klein (1963) demonstrated that a brief pulse of light given to dark grown bean seedlings eliminates the lag in chlorophyll accumulation that normally occurs when plants are transferred to continuous white light. Similar observations have since been reported in a variety of species, including pea (Horwitz *et al* 1988), *Arabidopsis* (Lifschitz *et al* 1990), and tomato (Pauncz *et al* 1992). Termed "potentiation of greening", this process is most effectively enhanced by red light (R), and partially reversible by far-red light (FR). Levels of FR responsiveness can, however, depend on seedling age (Sisler and Klein 1963). The role of phytochrome in this process is further supported by similarities between the action spectrum of potentiation and absorption spectrum of Pr (Horwitz *et al* 1988, Briggs *et al* 1988). Biphasic fluence-response curves for potentiation of greening suggest that phytochrome regulation of this process may occur through LF and VLF response modes (Ken-Dror and Howitz 1990). Similar results have been demonstrated for *Arabidopsis* (Lifshitz *et al* 1990).

The observations that exogenous ALA eliminates the lag phase, inhibition of protein synthesis prevents entry into the rapid phase, and exogenous ALA permits chlorophyll synthesis in the presence of protein synthesis inhibitors suggest that the enzyme system responsible for ALA synthesis is formed *de novo* (Castelfranco 1983). Despite demonstrating a Pfr-mediated increase in ALA dehydratase activity, Kasemir and Masoner (1975) concluded that this resulted from an intensification of plastid development, and is probably not significant to the phytochrome control of chlorophyll biosynthesis. Similar work in etiolated mustard seedlings revealed a Pfr-mediated increase in Pchlide (Masoner and Kasemir 1975). The authors propose that ALA synthesis in the dark is regulated by Pchlide through feedback inhibition, and the effect of illumination is to remove this inhibition in addition to increasing the capacity of the ALA forming system. Biochemical investigation of cucumber cotyledons revealed GluTR activity to increase upon illumination, and parallel ALA synthesis throughout greening, suggesting this enzyme to be rate determining in this species (Masuda *et al* 1996). The cloning of *HEMA* genes encoding glutamyl-tRNA reductase in *Arabidopsis* (Ilag *et al* 1994, Kumar *et al* 1996b), cucumber (Tanaka *et al* 1996) and barley (Bougri and Grimm 1996, Tanaka *et al* 1997) has enabled molecular analysis of this process.

The discovery of two *HEMA* genes in both *Arabidopsis* and cucumber, showing different spatial expression patterns has lead to the proposal that different members of this gene family perform different developmental roles. *HEMA1* is expressed primarily in the hypocotyl and cotyledons of developing seedlings and is believed to accumulate in response to demand of chlorophyll biosynthesis (Ilag *et al* 1994, Tanaka *et al* 1996). Reporter gene studies have shown *HEMA1* expression to be light regulated through phytochrome, subject to gating from chloroplast signals (McCormac *et al* 2001). The constitutive expression of antisense *HEMA1* mRNA in *Arabidopsis* resulted in plants displaying reduced levels of ALA, chlorophyll, and non-covalently bound hemes (Kumar and Söll 2000). *HEMA2*, however, is expressed primarily in roots and flowers and is believed to accumulate in response to demand of other tetrapyrroles, such as heme. Two *HEMA* genes, expressed primarily in leaves and up-regulated during illumination have been discovered in barley (Bougri and Grimm 1996). A third member of this family, expressed in roots has been

identified by Tanaka *et al* (1997) and is believed to be regulated by requirements for heme biosynthesis. In contradiction of *Arabidopsis* work by Ilag *et al* (1994), GSA-AT was shown not to contribute to the light-regulated increase of ALA biosynthesis in cucumber (Masuda *et al* 1996), or barley (Bougri and Grimm 1996).

The rate of greening in the phytochrome chromophore-deficient *aurea* mutant of tomato was shown to be slower than wild-type (WT), but the extent (relative to dark control) of potentiation by the red pulse was similar (Ken-Dror and Horwitz 1990). The *aurea* mutant responded only in the low fluence range and was shown to produce less Pchlide in the dark. This is likely to result from feedback inhibition on glutamyl-tRNA reductase by heme (Terry and Kendrick 1999). The latter has also been observed in chromophore-deficient transgenic *Arabidopsis* (Montgomery *et al* 1999), but is unlikely to result from an accumulation of heme.

Potentiation of greening in long hypocotyl (*hy*) mutants of *Arabidopsis* by R was similar to wild-type in all mutants studied (Lifshitz *et al* 1990). Reversibility by FR was, however, greatly decreased in the chromophore deficient *hy1* and *hy2* strains. Taken together, these results and those of *aurea* (Ken-Dror and Horwitz 1990), suggest that residual levels of phytochrome in these mutants is sufficient to initiate potentiation of greening, but not to enable FR reversibility. Pre-treatment of etiolated wheat leaves (Beevers *et al* 1970), and cucumber cotyledons (Fletcher *et al* 1971) with cytokinins abolishes the lag phase of light induced chlorophyll synthesis. It appears that exogenous cytokinins act by stimulating the formation of the ALA synthesising system (Castelfranco 1983). Masuda *et al* (1994) demonstrated that the rate of ALA synthesis by plastids isolated from benzyladeneine (BA) treated cotyledons was twice that of untreated controls. Western and northern analysis revealed stimulation of ALA synthesis by BA to result from increased levels of $tRNA^{Glu}$ within plastids. Work by Bougri and Grimm (1996), suggested that cytokinins activate gene expression of both GluTR and GSA-AT in etiolated barley seedlings. As cytokinins appear to mimic the effect of pre-illumination, potentiation of greening provides a means of examining the involvement of these hormones in phytochrome signal transduction. Mutant studies have revealed a phyA-dependent FR block of greening in etiolated tomato and *Arabidopsis* cotyledons transferred to white light (van Tuinen *et al* 1995b, Barnes *et al*

1996a). Ultrastructural analyses have revealed this block to result from severe repression of *POR* genes, coupled with irreversible plastid damage. Over-expression analysis in transgenic *Arabidopsis* has subsequently confirmed a unique photoprotective role for POR proteins in the developing seedling (Sperling *et al* 1997). A single red pulse has also been shown to result in a block of greening in the *high-pigment-1* mutant of tomato, mediated by phytochrome(s) operating through the LFR (Yang *et al* 1998).

1.3.3.3 Regulation of NADPH:protochlorophyllide oxidoreductase (POR)

NADPH: protochlorophyllide oxidoreductase (POR) catalyses the only known light requiring step of chlorophyll biosynthesis, the reduction of Pchlide to Chlide (Armstrong *et al* 1995). Paradoxically, although light is an essential cofactor of this reaction, POR mRNA and protein levels are drastically down regulated upon illumination (Mapleston and Griffiths 1980, Forreiter *et al* 1990), an effect attributed to differentially regulated isoforms of the enzyme (Armstrong *et al* 1995). Striking similarities exist between the regulation and expression of *Arabidopsis PORA* and *PORB* genes and the angiosperm phytochrome genes *PHYA* and *PHYB* (Armstrong *et al* 1995). POR-mediated photoreduction of Pchlide to Chlide and photoconversion of phytochrome from Pr to Pfr form occur simultaneously during the illumination of etiolated seedlings, and together activate the interconnected processes of plant photomorphogenesis. Phytochrome has also been shown to negatively regulate transcription of total POR mRNA in barley (Apel 1981). Selective abolition of PORA expression by FR treatment at the levels of mRNA and protein correlates with the almost complete absence of Pchlide-F_{655} (phototransformable form), a strong reduction in POR enzyme activity and a subsequent inability of wild-type seedlings to green normally (Frick *et al* 1995). Similarities between expression of *POR* and *PHY* genes may represent similar evolutionary constraints and selective pressures on both these proteins.

A novel POR isoform (PORC) has recently been identified in *Arabidopsis thaliana,* displaying increased expression upon illumination of etiolated seedlings and constant expression in both immature and mature tissues (Oosawa *et al* 2000). Such evidence suggests that chlorophyll biosynthesis and plastid development are regulated by three POR isoforms, subject to differential control by light and development.

1.3.3.4 Chlorophyll a/b binding protein (*CAB*) expression

The *CAB* family of nuclear genes encode the apoproteins of the light harvesting complex of photosystem II (PSII). The expression of *CAB* genes is tightly controlled by both light and plastid signals (López-Juez *et al* 1998). Other controlling factors include the circadian clock (Miller and Kay 1996), plant hormones (Flores and Tobin 1986, Bartholomew *et al* 1991) and sucrose (Dijkwel *et al* 1997). *CAB* transcript abundance in pea shows a biphasic fluence response curve, suggesting that *CAB* is regulated in the LF and VLF ranges. PhyA acts to induce *CAB* expression in both VLF and HIR modes in R and FR respectively (Hamazato *et al* 1997, Cerdan *et al* 1997, 2000). phyB induces *CAB* in R in the LF range, and shows photo-reversibility by FR (Hamazato *et al* 1997, Cerdan *et al* 1997). In contradiction to the work of Horwitz *et al* (1988) in maize, Mösinger *et al* (1988) presented similar fluence response curves for both potentiation of greening and *CAB* accumulation in barley. Subsequent kinetic analyses, however, eliminated a causal relationship between the two processes.

Evidence from Adam *et al* (1991) indicates that both the circadian clock and phytochrome regulate *CAB* transcription, and suggests that the *CAB-1* promoter contains cis-regulatory elements for light induced and circadian clock responsive expression. Miller and Kay (1996) propose that the circadian clock acts negatively on *CAB* expression, antagonising phototransduction pathways during the subjective night in a process termed gating. It can be concluded that *CAB* expression patterns in light-grown plants reflect endogenous timing information, light signals from the environment and several interactions between these regulators (Miller and Kay 1996).

1.4 Photoperiodic induction

In many plant species, responses to light are influenced by the time of day in which the light is perceived. Sensitivity to the timing of light and darkness, termed photoperiodism, enables plants to adapt to seasonal changes in their environment. The ability to anticipate and consequently prevent the adverse effects of a particular seasonal environment confers considerable selective advantage to the plant (Thomas and Vince-Prue 1997). The concept of photoperiodism was first introduced by Garner and Allard (1920, cited in Sage 1992),

who observed that flowering and many other responses in plants could be accelerated by either long or short days. Supporting evidence was later provided by the groups of Parker and Borthwick (Parker *et al* 1946, Borthwick *et al* 1952). In photoperiodically sensitive species, metabolism proceeds in one of two alternative states depending on the relationship between the day-length received and a threshold or "critical" day-length (Thomas and Vince-Prue 1997). The timing of sexual or vegetative reproduction often only occurs when days are sufficiently short (short-day plants; SDP) or long (long-day plants; LDP). Plants insensitive to photoperiodic induction are termed "day neutral". Photoperiodic perception mechanisms are not the same in SDP and LDP (Jackson and Thomas 1997).

1.4.1 Flowering

1.4.1.1 Short-day plants (SDP)

Whether or not SDP flower in light/dark cycles is dependent primarily upon the length of the dark rather than the light period (Thomas 1991). A light treatment (night-break) given during the perceived "dark period" can prevent flowering in SDP (Borthwick *et al* 1952), the timing of which is crucial in many sensitive species. Detection of light/dark transitions is precise and believed to involve loss of Pfr signal (Thomas 1991). The requirement of Pfr for the flowering response during the inductive dark period appears to contradict observations that a R night break, producing a few percent Pfr can prevent flowering in SDP. This apparent paradox can be resolved if photoperiodic timekeeping is controlled by two different pools of phytochrome, with different stabilities as Pfr. Action spectra revealed two separate actions of phytochrome in the floral induction of the SDP *Lemna paucicostata* (Lumsden *et al* 1987). The authors propose that the induction of flowering in SDP involves the simultaneous interaction of two different processes, mediated by two different pools of phytochrome. The first involves phytochrome stable in the Pfr form, the removal of which by FR results in an inhibition of flowering. The second is associated with the night break reaction in which the red light-induced formation of Pfr inhibits flowering. Dark reversion of Pfr to Pr by a subpopulation of light stable phytochrome molecules provides an explanation for the detection of end of day (Thomas 1991). The inhibitory effects of night-break R on floral initiation in SDP can be nullified by subsequent exposure to FR. Preliminary evidence suggests a role for phytochrome C (phyC) in determining sensitivity to

a night-break in SD tobacco (Halliday *et al* 1997). phyB is involved in determining the degree of sensitivity to photoperiod in SDP, while little direct evidence exists for a role of phyA (Jackson and Thomas 1997). Few mutants have been identified in SDP with regard to photoperiodism. *Nicotiana tabacum* is a tetraploid of two diploid species, *N. sylvestris*, a LDP, and *N. tomentosiformis*, a SDP. The cultivar Maryland Mammoth (MM) is a SDP as a result of a recessive, single gene mutation in the long-day response mechanism originating from the *N.sylvestris* parent (Metzger and Zheng 1998). The Sorghum ma_3 mutant shows reduced photoperiodic control of flowering, resulting from a frame-shift mutation in the *PHYB* gene (Childs *et al* 1997). The recently identified *se5* mutant of rice shows an early flowering phenotype and is completely deficient in photoperiodic response. Molecular analysis of the mutated gene has revealed a putative heme oxygenase, which would be expected to cause a deficiency of total phytochrome in these plants (Izawa *et al* 2000).

1.4.1.2 Long-day plants (LDP)

The role of phytochrome in the photoperiodic induction of LDP is less well understood. Action spectra for night-break promotion of flowering in LDP have shown a maximum effectiveness of R and reversibility by FR (Downs 1956). When LD are obtained by extending SD with continuous light, a mixture of R and FR are more effective than R alone (Jackson and Thomas 1997). Late flowering was observed in *Arabidopsis phyA* mutants under conditions where SD of 8 hours were extended by 8 hours of low fluence rate incandescent light (Johnson *et al* 1994). Action spectra performed on *Arabidopsis* plants at different times during a day-night cycle showed diurnal cycling in sensitivity to exposure to R and FR (Metzger and Zheng 1998). It is possible that R and FR affect different aspects of flowering in LDP, with R promoting floral initiation, and FR promoting further development of the primordium and inflorescence (Metzger and Zheng 1998). Action spectra for the day-extension promotion of flowering in the LDP *Triticum aestivum* revealed action maxima at both 660 and 716nm, thus resembling spectra for the phyA-mediated FR-HIR (Carr-Smith *et al* 1989). The role of phyA in the promotion of flowering in LDP is supported by analysis of the phyA-deficient mutant of pea, *fun1*, which exhibits reduced photoperiodism and an inability to detect day extensions (Weller *et al* 1997a).

The delayed flowering response shown in transgenic *Arabidopsis* and tobacco, over-expressing *PHYB,* suggests a role for phyB in the inhibition of flowering, but is not believed to be involved in daylength measurement (Jackson and Thomas 1997). The early flowering phenotype of *Arabidopsis* mutants, deficient in phyB supports such a role (Reed *et al* 1993). In addition to phyB, light stable phytochromes have been shown to regulate flowering in response to light quality. Mutant studies have revealed that stable phytochromes, other than phyB are involved, or at least able to compensate for phyB in the inhibition of flowering in *Arabidopsis* (Koornneef and Peters 1997). Characterisation of the *Arabidopsis* early flowering mutant, *pef1* revealed perturbations in both phyA and phyB mediated signalling pathways (Ahmad and Cashmore 1996b).

Strong sensitivity of the *Cruciferae*, but not in other LDP, to induction by B implies the action of a separate B-absorbing receptor (Thomas and Vince-Prue 1997). The inductive effect of B on floral initiation in the cruciferous LDP *Arabidopsis* was first reported by Mozely and Thomas (1995). Genes for the B receptors cryptochromes 1 and 2 (*CRY1* and *CRY2*) have been cloned from *Arabidopsis* and extensively characterised (Lin *et al* 1996, 1998). Guo *et al* (1998) suggest a role for the B photoreceptor cry2 in the photoperiodic control of *Arabidopsis* flowering. The authors propose that phytochromes mediate the R-dependent inhibition of flowering, whereas cry2 mediates the B-dependent inhibition of phytochrome function. Analysis of the *cry2/phyB* double mutant has provided support for such a hypothesis (Mockler *et al* 1999).

1.4.2 Tuberisation

A role for phyB in the photoperiodic induction of the SDP *Solanum tuberosum ssp. andigena* has been demonstrated by Jackson *et al* (1996). Lowering of *PHYB* levels, using an antisense approach resulted in a loss of control of tuberisation by photoperiod. Grafting experiments have implicated phyB in the production of a graft transmissible inhibitor (Jackson *et al* 1998). The identity of this inhibitor remains to be elucidated, although accumulating evidence implicates a role for gibberellins (Jackson and Prat 1996). Spraying of wild-type (WT) plants with GA_3 mimics the effects of antisensing *PHYB*, with respect to increased stem elongation and reduced chlorophyll levels. Loss of photoperiodic inhibition of tuber formation, however, is mimicked by inhibiting gibberellin biosynthesis.

The authors propose that different gibberellin species control stem elongation and tuberisation in potato. The onset of sexual or vegetative reproduction in photoperiodically sensitive species is believed to depend on relative levels of both inducing and inhibiting substances, the identities of which remain to be elucidated, but are controlled, in part, by phyB.

1.5 Phytochrome signal transduction

The function of phytochrome at the molecular level remains to be unequivocally demonstrated, but recent studies have revealed that phyA and phyB are serine-threonine kinases (Yeh and Lagarias 1998, Fankhauser *et al* 1999). Pioneering work by Chua and colleagues has implicated roles for heterotrimeric G proteins, calcium ions, calmodulin and cyclic guanosine 5'-phosphate (cGMP) in signal transduction (Neuhaus *et al* 1993, Bowler *et al* 1994). Microinjection of putative signalling intermediates into subepidermal hypocotyl cells of the chromophore-deficient mutant, *aurea,* of tomato has provided the first evidence of a branched pathway in phytochrome signalling. It has been demonstrated that phytochrome activates anthocyanin biosynthesis through cGMP and induces partial chloroplast development through calcium acting through calmodulin. Complete chloroplast development, however, requires a combination of calcium and cGMP. The direct involvement of phytochrome in the transduction process is apparently restricted to steps upstream of the putative G protein (Quail *et al* 1995, Okamoto *et al* 2001). The signalling pathway of UV-B, leading to activation of chalcone synthase (CHS) in *Arabidopsis* cell suspension cultures involves calcium, and therefore differs from the cGMP-dependent phytochrome pathway (Christie *et al* 1996). This suggests the utilisation of different signalling pathways by different photoreceptors. Microbeam irradiation experiments on *CAB* expression by Bischoff *et al* (1997) demonstrate signal transfer between neighbouring cells, disputing the proposal by Bowler *et al* (1994) that the phytochrome signal transduction pathway is cell autonomous.

The isolation of mutants within the signal transduction chain, pioneered by Chory and co-workers, has provided insight into the regulation of phytochrome responses. Recessive *det* (de-etiolated) *cop* (constitutive photomorphogenic) and *fus* (fusca) mutants of *Arabidopsis*

exhibit developmental characteristics of light-grown plants when grown in complete darkness (Chory *et al* 1989a, Chory and Peto 1990 Deng *et al* 1991, Misera *et al* 1994). The *lip* (light independent photomorphogenesis) mutant of pea is phenotypically similar and has been shown to result from an internal duplication within the pea homologue of *COP1* (Sullivan and Gray 2000). The recessive nature of these defects implies that the gene products are required for repressing photomorphogenesis in the absence of light, possibly in a large multi-protein complex (Chory *et al* 1996). One of the primary functions of the phytochrome signalling cascade may be to de-repress light-regulated development by inactivating DET/COP/FUS proteins. Fusion protein studies have shown enrichment of COP-1 in the nucleus in darkness and movement into the cytosol in the light (von Arnim and Deng 1994). The relatively slow exclusion process suggests that COP-1 may act in the maintenance of the light program rather than as a molecular switch for photomorphogenesis. Sequence analyses of the COP-1 polypeptide have revealed a novel structure containing a conserved zinc binding motif and a domain homologous to the WD-40 repeat motif of G_β proteins (Deng *et al* 1992). Such a structure would enable COP-1 to function as a negative transcriptional regulator. Many of the phenotypes of *det1* mutants can be mimicked in the dark by exogenous cytokinin application, raising the possibility that DET1 product may have similar action to cytokinins (Furuya 1993). The existence of pleiotropic *COP* and *DET* loci supports the existence of signalling steps after the convergence of signals from multiple photoreceptors.

The identification of phytochrome binding proteins PSK1, NDPK2, and PIF3 in *Arabidopsis*, has provided evidence for the direct regulation of gene expression in the nucleus, mediated through the phytochrome signal transduction chain. PSK1 is a kinase substrate protein, capable of binding to the C-terminal domains of both phyA and phyB (Fankhauser *et al* 1999). Located in the cytosol, PSK1 is phosphorylated in a phytochrome dependent manner and negatively regulates phytochrome signalling. The putative transcriptional regulator PIF3 has been shown to bind non-photoactive carboxy terminal fragments of both phyA and B. Recent evidence has now shown PIF3 to bind photoactive phyB *in vitro* upon light-induced conversion to the active form (Ni *et al* 1999). Isolation of the *poc1* mutant, perturbed in phytochrome signalling by a T-DNA insertion into the

promoter of PIF3, provided additional evidence for the role of PIF3 as a positively acting transcription factor (Halliday *et al* 1999). Subsequent investigation revealed PIF3 to bind to a G-box DNA motif present in a variety of light-regulated promoters (Martínez-García *et al* 2000). The identification of PIF3 as a member of the bHLH class of transcriptional regulators further supports this proposal. Antisense experiments have implicated a role for PIF3 in both phyA and phyB signal transduction (Halliday *et al* 1999).

The interaction of phytochromes with nuclear proteins was confirmed by the recent discovery that both phyA and phyB undergo light-dependent nuclear localisation (Sakamoto and Nagatani 1996, Yamaguchi *et* al 1999, Kircher *et al* 1999). Fusion protein studies using green fluorescent protein (GFP) have shown nuclear import of phyA to operate via the VLFR and phyB via the LFR. Such observations suggest the light-regulated nucleocytoplasmic partitioning of phyA and phyB to be of crucial importance in phytochrome signalling. Other phytochrome binding proteins include the nucleodiphosphate kinase NDPK2, a Pfr-binding positive signaling component, believed to be involved in the FR-HIR (Choi *et al* 1999). PSI2 acts as a negative regulator of phyA and B signalling and is proposed to de-sensitize phyA and phyB by phosphorylation (Genoud *et al* 1998). The utilisation of *Arabidopsis* mutants with altered light perception has revealed further components of light signalling pathways, specific to either phyA or phyB signalling. The nuclear-localised negative regulator SPA1 has been shown to be specific to phyA signal transduction (Hoecker *et al* 1999). In addition, FHY1, FHY3, FIN2, FIN 219, FAR1, RSF1, PAT and EID1 proteins have been identified as integral components of the phyA signal transduction pathway (Whitelam *et al* 1993, Barnes *et al* 1996b, Soh *et al* 1998, Hsieh *et al* 2000, Hudson *et al* 1999, Fankhauser and Chory 2000, Bolle *et al* 2000, Büche *et al* 2000). Putative signalling components specific to phyB include PEF2, PEF3 (Ahmad and Cashmore 1996b) and RED1 (Wagner *et al* 1997).

The transition from skotomorphogenesis to photomorphogenesis therefore involves the perception of light by cytosolic photoreceptors, the transduction of the signal to the nucleus and the inactivation of the COP/DET/FUS repressor system. When repression is relieved, cell type specific positive regulators such as HY5 can act to induce gene expression and development (Oyama *et al* 1997, Chattopadhyay *et al* 1998, Hardke and Deng 2000).

In addition, the expression of light-regulated nuclear genes is also controlled by a pathway involving signalling from the chloroplast to the nucleus, defined by five genes, *GUN1-GUN5* (Susek and Chory 1992, Susek *et al* 1993). The recent molecular cloning of *GUN5* has revealed it to encode a subunit of Mg-chelatase, implicating an important role for tetrapyrroles in such signalling (Vinti *et al* 2000). A schematic representation of phytochrome signal transduction is shown in Figure 2.

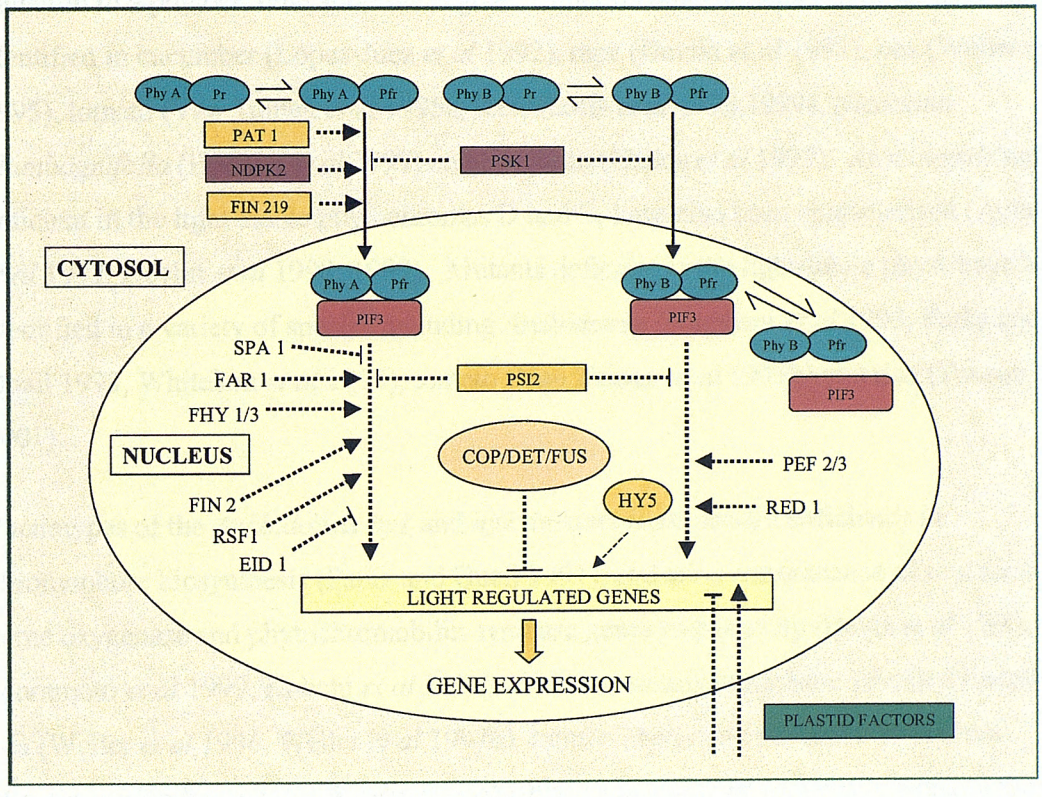


Fig. 2 Schematic representation of phytochrome signal transduction in plant cells (adapted from von Arnim 1999). Red light-induced conversion of phytochrome to its Pfr form triggers translocation to the nucleus, where it binds to the nuclear-localised binding protein PIF3. The phytochrome-PIF3 complex binds to G-box motifs in light-regulated promoters to mediate gene expression. The cytosolic phytochrome binding protein PSK1 negatively regulates both phyA and B signalling, as does the nuclear-localised protein PSI2. Positive regulatory components are represented by arrows, and negatively regulatory components by bars. The light-induced inactivation of the COP/DET/FUS repressor system enables positive regulators such as HY5 to promote gene expression. The exact nature of plastid-nuclear signalling is currently unknown.

1.6 Mutant and transgenic approaches

Understanding of phytochrome function has been significantly enhanced by the identification and characterisation of phytochrome-deficient mutants in a variety of plant species (Whitelam and Harberd 1994). Screens for *Arabidopsis* mutants showing increased hypocotyl growth in white light revealed five complementation groups, corresponding to *hy1-5* loci (Koornneef *et al* 1980). Of these, only the phyB-deficient *hy3* plants contained a mutation in a phytochrome apoprotein gene. Other mutants deficient in phyB have been identified in cucumber (López-Juez *et al* 1992), rape (Devlin *et al* 1992), pea (Weller *et al* 1995), tomato (Van Tuinen *et* al 1995a, Kerckhoffs *et al* 1996,1999), *Nicotiana plumbagnifolia* (Hudson *et al* 1997) and Sorghum (Childs *et al* 1997). *Arabidopsis* mutants, deficient in the light-stable phytochromes D and E, have also been characterised (Aukerman *et al* 1997, Devlin *et al* 1998, 1999). Mutants deficient in the light-labile phyA have been identified in a variety of species including *Arabidopsis* (Nagatani *et al* 1993, Parks and Quail 1993, Whitelam *et al* 1993), tomato (Van Tuinen *et al* 1995b) and rice (Takano *et al* 2001).

Phenotypes of the *Arabidopsis hy1* and *hy2* mutants result from a deficiency in chromophore biosynthesis (Parks and Quail 1991), and are a consequence of mutations in heme oxygenase and phytochromobilin synthase genes respectively (Davis *et al* 1999, Muramoto *et al* 1999, Kohchi *et al* 2001). Similar investigations have revealed mutants of pea (Weller *et al* 1996, Weller *et al* 1997b), tomato (Terry and Kendrick 1996) and *Nicotiana plumbaginifolia* (Kraepiel *et al* 1994) to be deficient in specific steps of the chromophore biosynthetic pathway. The "leaky" nature of many of these mutants, however, has precluded unequivocal definition of individual phytochrome functions.

The use of transgenic technology to introduce phytochrome genes in sense orientation (for over-expression), and antisense orientation (for reduced expression), in conjunction with a mutant based approach, has provided further insight into regulatory mechanisms and revealed a refined picture of individual phytochrome functions. The creation of plants that over-express individual phytochromes has been used to identify exaggerated light-mediated responses. Constitutive expression of oat *PHYA* in tobacco resulted in substantial dwarfing,

increased pigmentation and delayed leaf senescence (Cherry *et al* 1991, Jordan *et al* 1995b). A pronounced growth inhibition in FR was also observed (McCormac *et al* 1991, 1992). Over-expression of *PHYB* resulted in increased responsiveness to R in *Arabidopsis* seedlings (McCormac *et al* 1993b). Similar work by Halliday *et al* (1997) provided the first experimental evidence of a biological function for phyC in the response to night-break treatments. When grown under continuous FR, acceleration of leaf expansion and hook opening were observed in potato over-expressing *PHYA*, both of which were delayed in antisense plants (Heyer *et al* 1995). Advancements in genetic manipulation also include the intracellular expression of synthetic antibody derivatives for the "immunomodulation" of plant cell components (Owen *et al* 1992). Using this approach, tobacco plants transformed with a gene encoding a synthetic derivative of an anti-phytochrome monoclonal antibody were shown to display abberant light-controlled germination.

The use of transgenic technology to define roles for individual phytochromes does however, present problems, particularly when over-expressing genes. The majority of transgenic approaches have used heterologous systems, introducing complications arising from different primary structures and differing stabilities of heterologous trans-gene products. Over–expressed phytochrome may adopt some or all of the functions of other family members (Whitelam and Harberd 1994). Unnaturally high levels of over-expressed phytochrome may also interact with signal transduction components, normally used by other photoreceptors (Batschauer 1998). The ectopic expression of phytochrome genes in cells and tissues that may not normally express a particular phytochrome cannot, therefore, be taken as a true reflection of phytochrome function in the WT plant. The overlapping functions of phytochrome species, coupled with the highly artificial conditions employed in the majority of laboratory experiments (Vince-Prue 1991) require that evidence be treated with circumspection, and conclusions be drawn with care.

A novel approach for modulating phytochrome synthesis has been developed by Lagarias and co-workers, involving the targeted expression of mammalian biliverdin reductase (BVR) in plants (Lagarias *et al* 1997). BVR is a small monomeric enzyme of 33 kDa that catalyses the reduction of biliverdin IXα to bilirubin (Singleton and Laster 1965, Kutty and Maines 1981), a compound unable to assemble with apophytochrome *in vitro* (Terry *et al*

1993). The immediate precursor of the phytochrome chromophore, phytochromobilin, is also a substrate. Plastid targeting of BVR in *Arabidopsis* resulted in plants showing aberrant photomorphogenesis throughout their life cycle (Lagarias *et al* 1997, Montgomery *et al* 1999). Light-grown, transgenic seedlings displayed elongated hypocotyls, smaller cotyledons, inhibition of hook opening and reduced chlorophyll content. Mature plants exhibited elongated internodes, altered leaf morphology, reduced chlorophyll levels and early flowering, consonant with deficiencies in multiple phytochromes. Elongated internodes and early flowering were also observed in cytosol-targeted lines (Montgomery *et al* 1999). A schematic representation of targeted BVR expression within plant cells is shown in Figure 3.

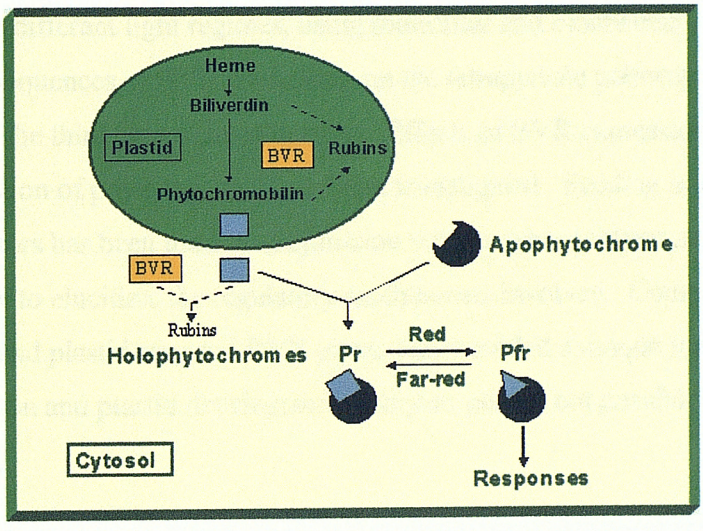


Fig. 3 Schematic representation of targeted BVR expression in plant cells. In cytosol-targeted lines, BVR competes with apoprotein for phytochromobilin, resulting in a partial deficiency of photoactive holophytochrome. Plastid-targeting (achieved by the translational fusion of a chloroplast transit peptide sequence of the soybean small subunit of ribulose bisphosphate carboxylase) enables metabolism of both biliverdin and phytochromobilin within the plastid, resulting in a severe chromophore deficiency.

1.7 Aims of Project

The aim of this project was to gain further insight into the role of phytochrome in the light-regulated development of a SDP. Constitutive, targeted expression of mammalian BVR has been used to inactivate total phytochromes throughout development in Maryland Mammoth tobacco, a species for which no phytochrome-deficient mutants have been reported. The project aimed to address three principle questions. The first involves the role of phytochrome in plant morphology and flowering response to photoperiod. Extensive physiological characterisation has been performed on both seedlings and mature plants to assess the effects of multiple phytochrome deficiency on plant development. The second involves the role of phytochrome in the regulation of chlorophyll biosynthesis. Enzymes and intermediates in the tetrapyrrole pathway have been quantified in WT and transgenic plants, grown under different light regimes, using molecular and biochemical techniques. The regulatory consequences of BVR expression on the tetrapyrrole pathway and plastid development forms the third area of investigation. Effects of BVR expression, other than through the elimination of phytochrome, have been investigated. Feeding of tetrapyrroles and pathway inhibitors has been used in conjunction with enzyme analysis and transmission electron microscopy to elucidate the regulatory mechanisms involved. Comparative analysis of cytosol and plastid-targeted BVR plants has provided a unique insight into tetrapyrrole regulation and plastid development in higher plants, not possible using classical genetic approaches.

Chapter 2. Materials and methods

2.1 Plant material

2.1.1 Seedling analysis

All seedling analyses were performed using facilities at the University of Southampton. *Nicotiana tabacum* cv. Maryland Mammoth (MM) seed obtained from homozygous transgenic lines expressing mammalian biliverdin reductase (*BVR*) under a constitutive (35S) promoter, was provided by Professor J.Clark Lagarias (University of California-Davis, USA). Lines containing BVR targeted to both cytosol and plastid were used for analysis (4 cytosol-targeted lines and 5 plastid-targeted lines were provided). Plants were constructed using an *Agrobacterium*-mediated leaf disc transformation with 35S::pBVR and 35S::cBVR constructs in the binary transformation vector pBIB-KAN (Lagarias *et al* 1997, Montgomery *et al* 1999, 2001). Seeds were surface sterilised in 5% (v/v) bleach solution and germinated on Murashige and Skoog media, 0.7% (w/v) agar, adjusted to pH 5.9 (Murashige and Skoog 1962). For Norflurazon (4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-m-toyl)-3(2H)-pyridazinone) feeding experiments, media was supplemented with 5μM Norflurazon (Sandoz, US, supplied by Professor John Gray, University of Cambridge). A stock concentration of 1 mM was initially prepared in ethanol. Control media contained an equal volume of ethanol. Germination was synchronised by chilling seeds for 3 days in darkness before transfer to a specified light regime at 23°C. White light was provided at a fluence rate of 100 μmol m^{-2} s^{-1} by 70 W type 84 fluorescent tubes (Philips, Eindhoven, Netherlands) with a 16 h photoperiod. Red (R), far-red (FR) and blue (B) light was provided with a 16 h photoperiod in temperature-controlled growth chambers containing light-emitting diodes (LED) (Percival Scientific Inc., Boone, IA, USA). R (620-700 nm, peak 666 nm) was provided at a fluence rate of 70 μmol m^{-2} s^{-1} and B (440-520 nm, peak 470 nm) at a fluence rate of 10 μmol m^{-2} s^{-1}. Irradiance was recorded using a LI-COR LI-250 meter (LI-COR Inc., Lincoln, Nebraska, USA). FR (700-780 nm, peak 740 nm) was provided at a fluence rate of approximately 10 μmol m^{-2} s^{-1} (estimated using manufacturers data). Two additional neutral density filters (#116 and #172; Lee filters, Andover, UK) were used in the FR cabinet to attenuate wavelengths greater than 700nm.

2.1.2 Mature plant analysis

With the exception of ALA assays, all mature plant analysis was performed using facilities at Horticulture Research International (Wellesbourne, UK). Seeds were surface sterilised in 5% (v/v) bleach solution and germinated on Murashige and Skoog media containing 2% (w/v) sucrose and 0.7% (w/v) agar, adjusted to pH 5.9. Germinating seedlings were maintained at 25°C with a 16 h photoperiod of 70 μmol m^{-2} s^{-1} white light from 70 W type 84 fluorescent tubes (Philips, Eindhoven, Netherlands). Irradiance was recorded using a Skye SKP200 meter (Skye Instruments, Llandrindod Wells, UK). Seedlings were planted into 3 inch pots containing Levington M2 bedding compost and sand (2:1), and acclimatised for 14 days. Illumination was provided by a bank of Philips 100W type 84 fluorescent tubes at 85 μmol m^{-2} s^{-1}. Temperature was maintained at 25°C with 70% humidity. Plants were subsequently transferred to 5 inch pots and grown under varying photoperiods in controlled growth chambers (Fitotron SGC 2279, Sanyo Gallenkamp plc, Japan), with a day and night temperature of 22°C. Lighting was provided by 70 W fluorescent tubes (Philips, Eindhoven, Netherlands), supplemented with incandescent lighting, at a fluence rate of 350 μmol m^{-2} s^{-1}. Carbon dioxide concentration was maintained at atmospheric levels (350 ppm). Plants were watered three times a week with a N:P:K (2:1:4) nutrient solution (0.5 g/l) to prevent dessication. Leaf material was harvested into liquid nitrogen and stored at -70°C until required.

2.2 Physiological analyses

Seedling hypocotyl, plant height and internode lengths were measured using a ruler. Height measurements were calculated from the basal leaf to the plant apex. Leaf area measurements were recorded using a leaf area meter (Delta-T devices Ltd, Cambridge, UK) according to the manufacturer's instructions. Flowering times were calculated as the time between planting into soil and the opening of the first flower. Unless stated otherwise, the recorded value represents the mean of ten plants for all physiological analyses. Bars represent standard error values.

2.3 Pigment analysis

2.3.1 Relative chlorophyll content

Relative chlorophyll content of mature leaves was obtained non-destructively using the chlorophyll meter SPAD-502 (Minolta UK, Milton Keynes). Each value was obtained by averaging readings from ten sampling positions on individual leaves. A calibration graph was produced using 115 leaf discs to convert SPAD readings to absolute chlorophyll concentrations. These were obtained following pigment extraction and quantification *in vitro* (see below).

2.3.2 Chlorophyll *a*, Chlorophyll *b* and carotenoid determinations

Leaf /seedling samples of 0.2g were ground to a fine powder in liquid nitrogen, homogenised in 1 ml ice-cold 80% (v/v) acetone and incubated for 1 h at -20°C. Pigment concentrations in the supernatant were determined spectrophotometrically following centrifugation at 11000 *g* for 5 min at 4°C in a Sorvall RMC 14 centrifuge (Sorvall, Stevenage, UK). The absorbances of the supernatant were measured at 470, 646 and 663nm, in a Hitachi UV/VIS spectrophotometer (model u-2001, Hitachi, Tokyo, Japan) using 80% acetone as a blank. Chlorophyll *a* and *b* were determined using the following equations from Hill *et al* (1985) and carotenoid concentrations using equations of Lichtenthaler *et al* (1983).

Chl $a = (12.15 \times A_{663}) - (2.55 \times A_{646})$

Chl $b = (18.29 \times A_{646}) - (4.58 \times A_{663})$

Carotenoids $= ((1000 \times A_{470}) - (3.27 \times \text{chl } a) - (104 \times \text{chl } b))/229$

2.4 Analysis of proteins by western blotting

2.4.1 Phytochrome extraction

Frozen leaf /seedling samples of 0.2 g were homogenised for 2 min in 200 µl extraction buffer (100 mM Tris/HCl (pH 8.3) containing 0.14 M $(NH_4)_2SO_4$, 4 mM phenylmethylsulfonylfluoride, 2.5 µM leupeptin, 10 mM EDTA, 2 mM

iodoacetamide, 49 mM β-mercaptoethanol and 50% (w/v) glycerol), and 20 mg insoluble polyvinylpyrrolidone (Sigma-Aldrich Ltd, Poole, UK) using an Ultra-Turrax T8 blender (IKA Labortechnik, Germany). The extract was centrifuged at 11000 *g* for 10 min and the supernatent collected. Polyethylenimine was added to a final concentration of 0.04% (w/v) (López-Juez *et al* 1992), the extract vortexed and precipitation of acidic materials allowed for 15 min. Following centrifugation at 11000 *g* for 15 min, phytochrome in the supernatant was precipitated by adding 0.725 volumes of saturated ammonium sulphate solution. Precipitation was allowed for 1 h and the precipitate collected by centrifugation at 11000 *g* for 15 min. Pellets were resuspended in 65 µl sample buffer (100 mM Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% glycerin, 10% (v/v) β-mercaptoethanol). Proteins were solubilised and denatured by incubating for 1 min at 100°C.

2.4.2 Total protein extraction

Frozen leaf/seedling samples of 0.4g were ground to a fine powder in liquid nitrogen and mixed with 1 ml ice-cold LE buffer (50 mM lithium phosphate, pH 8.0, containing 120 mM ß-mercaptoethanol, 1 mM sodium moniodoacetate, 1 mM phenylmethylsulphonylfluoride and 5% (v/v) glycerol). The mixture was thawed and 20% (w/v) lithium dodecylsulphate (LIDS) added to a final concentration of 0.5%. Proteins were solubilised and denatured by incubating for 1 min at 100°C, cell debris removed by centrifugation for 15 min at 11000 *g* and the supernatent stored at -20°C for electrophoretic analysis.

2.4.3 Protein quantification

Total protein was quantified in samples using a Coomassie brilliant blue assay method. This assay was found to provide increased accuracy with small volumes of sample. A standard curve was constructed by spotting 10 µl aliquots of protein standards on to 2x2 cm squares of Whatman filter paper. Samples were prepared by diluting bovine serum albumin (BSA; Sigma, UK) with sample buffer (phytochrome extraction) or LE/LIDS (total protein extraction) to produce concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 1.4, 1.8 and 2.0 µg μl^{-1}. Each sample was applied in duplicate, including two reagent blanks. Protein aliquots of 10 µl were applied similarly. Samples were air-dried and fixed in 7% (v/v) acetic acid, before immersion in freshly prepared Coomassie brilliant

blue stain solution for 15 min with shaking. Stain solution contained 0.5% (w/v) Coomassie brilliant blue in methanol-water-glacial acetic acid (4.5/4.5/1; v/v/v). Filter papers were destained repeatedly in water/methanol/glacial acetic acid (68/25/7; v/v/v) and rinsed in 7% (v/v) acetic acid. After air drying, individual squares were eluted with 3 ml of methanol/water/ammonia (66/44/1 v/v/v). The absorbance of each was read at 590 nm against the eluate from the reagent blank. Construction of a standard curve from protein standards allowed the addition of a linear regression line. The equation of this line enabled protein samples to be quantified from the absorbance of their corresponding eluate at 590 nm.

2.4.4 SDS Polyacrylamide gel electrophoresis

Total protein preparations were separated by SDS gel electrophoresis in 12% (w/v) polyacrylamide using a Mini-Protean II apparatus (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Each gel was 0.75 mm thick and comprised a 5 cm deep resolving gel (12.5% (w/v) acrylamide pH 8.8) and a 2 cm deep stacking gel (4.5% (w/v) acyrlamide, pH 6.8). The resolving gel was prepared by mixing 7 ml water, 8 ml acrylamide monomer (30% (w/v) pre-mix Acrylogel; BDH, Poole, UK), 5 ml 1.5M Tris/HCl, pH 8.8, 100 µl 10% (w/v) SDS, 20 µl tetramethylethylene (BDH, UK) and 100 µl 10% (w/v) ammonium peroxidosulphate. The stacking gel was prepared similarly using 5.8 ml water, 1.5 ml monomer, 2.5 ml 0.5 M Tris/HCl, pH 6.8, 50 µl 10% (w/v) SDS, 10 µl tetramethylethylene and 50 µl 10% (w/v) ammonium peroxidosulphate. Phytochrome preparations were separated similarly on a 7.5% (w/v) resolving gel. A 10 µl aliquot of appropriately diluted sample was injected into each well, alongside a range of molecular weight markers (Bio-Rad, UK). Electrode buffer contained 25 mM Tris/HCl, 3.5 mM SDS and 0.2 M glycine. Gels were run at a constant 200 V for 45 min (total protein), or 100 V for 2 h (phytochrome), at 4°C.

Proteins were fixed by shaking in 7% (v/v) acetic acid for 10 min before staining with Coomassie brilliant blue stain solution (as described in section 2.4.3) for a further 15 min. Gels were subsequently destained using the destain solution described previously, placed in gel dryer solution (45% (v/v) methanol, 10% (v/v) acetic acid, 1% (v/v) glycerol) for 30 min. Gels were dried using a Bio-Rad 583 gel dryer according to the manufacturer's instructions (Bio-Rad, UK). Dried, stained gels were

used to assess uniformity of loadings. Samples of 40 µl were loaded on gels for blotting.

2.4.5 Immunoblotting procedure

Electrotransfer of SDS gel-separated proteins to a nitrocellulose membrane was carried out in a Bio-Rad Mini-Protean II transblot apparatus, according to the manufacturer's instructions. Pre-stained molecular mass markers were used to assess the efficiency of protein transfer and provide approximate size standards (low-range, Bio-Rad, UK). Gels, membranes and filter papers were all soaked in ice-cold transfer buffer (20% (v/v) methanol containing 20 mM Tris and 0.15 M glycine) at 4°C, for 30 min prior to blotting. Gels were blotted at 30 V overnight at 4°C or at 45 V for 2 h at room temperature. After transfer, the membrane was washed in Tris-Buffered saline (TBS) (10 mM Tris/HCl, 0.14 M NaCl, pH 7.5). Excess protein binding capacity was blocked by incubation in a 3% solution of non-fat, spray-dried skimmed milk in TBS for 4h at 4°C.

2.4.6 Immunodetection of individual peptides-colourimetric method

For the detection of NADPH:protochlorophyllide oxidoreductase (POR), a colourimetric detection procedure was used. For detection of the less abundant phytochrome and heme oxygenase proteins, the more sensitive technique of chemiluminescence was employed.

2.4.6.1 NADPH: protochlorophyllide oxidoreductase (POR)

A polyclonal wheat anti-POR antibody, provided by Professor W.T. Griffiths (University of Bristol, UK), was diluted 1:10,000 in a 3% (w/v) solution of BSA in TBS (Rowe and Griffiths 1995). Blots were incubated in primary antibody with shaking for 1 h. After thorough washing with TBST (0.1% (w/v) polyethylenesorbitan /Tween 20 in TBS) to remove excess primary antibody, the membrane was incubated in a similarly diluted alkaline phosphatase conjugated secondary antibody for 1 h. This consisted of a phosphatase-conjugated anti-rabbit immunoglobulin (Sigma-Aldrich, UK).

Following further washes, antigen-primary antibody-secondary antibody complexes were detected with a colorimetric phosphatase reaction using nitro blue tetrazolium

chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates. Blots were shaken in 10ml detection solution (0.1 M Tris/HCl, pH 9.0, 0.1 M NaCl, 5 mM $MgCl_2$), containing 30 µl 50 mg/ml NBT (Promega, Madison, WI, USA) and 15 µl 50 mg/ml BCIP (Promega, USA), until banding was visible. After developing, blots were rinsed in TBS and stored in water.

2.4.7 Immunodetection of individual peptides-chemiluminescence method

Gels were run and blotted using the same method as for colorimetric detection. Immunodetection of phytochrome peptides was achieved using a Boehringer Mannheim (BM) chemiluminescence western blotting kit, according to the manufacturer's instructions (Boehringer Mannheim, Sussex, UK). The BM chemiluminescence western blotting system uses peroxidase–labelled secondary antibodies and the substrate luminol. In the presence of hydrogen peroxide, horseradish peroxidase catalyses the oxidation of diacylhydrazides like luminol. An activated intermediate reaction product is formed, which decays to the ground state by emitting light, a process enhanced by 4-iodopenol. Chemiluminescence detection is therefore 1-3 orders of magnitude more sensitive than colorimetric methods. Excess protein binding capacity was blocked by incubating membranes in a 1% (v/v) solution of kit blocking reagent, diluted in TBS (50 mM Tris/HCl, 0.15 M NaCl, pH 7.5) for 4 h at 4°C. After rinsing in TBS, membranes were incubated for 1 h in a 1:1000 dilution of primary antibody in 0.5% kit blocking reagent at room temperature.

Excess primary antibody was removed by washing for 2 x10 min in TBST and 2 x10 min washes in 0.5% (v/v) kit blocking reagent. Membranes were incubated in a 40 mU/ml dilution of kit horseradish peroxidase conjugated secondary antibody for 30 min at room temperature. Excess secondary antibody was removed by 4x10 min washes in TBST. Proteins were detected by placing the membrane in a clean petri dish and applying 10 ml kit detection solution for 1 min. Following incubation, excess detection solution was drained and the membrane blotted and placed between two acetate sheets. This was then placed inside a film cassette with a sheet of Kodak film. An initial exposure of 2 min was used, followed by a longer exposure of up to 1 h. All films were developed immediately. Blots and gels were scanned using an Alphaimager™ 1220 (Alpha Innotech Corporation, San Leandro, CA, USA).

Comparative analysis of optical density, using Alphaease™ software (Alpha Innotech Coporation, USA) enabled signal quantification on blots and standardisation to the large subunit of ribulose bisphosphate carboxylase (rubisco) on gels.

2.4.7.1 Phytochome A

For phytochrome A analysis, plants were initially dark adapted for seven days, prior to harvesting. The P-25 anti-phytochrome A antibody, donated by Professor L. Pratt (University of Georgia,USA) was used for peptide detection (Shimazaki and Pratt 1985).

2.4.7.2 Phytochrome B

The monoclonal antibody mAT1, raised against tobacco phyB (donated by Professor A.Nagatani, University of Tokyo, Japan), was diluted to 10 μg/ml in hybridisation solution (50 mM Tris, pH 7.5, 200 mM NaCl, 0.5% (v/v) blocking solution) (López-Juez *et al* 1992).

2.4.7.3 Heme oxygenase

Antiserum raised against the HY1 protein of *Arabidopsis thaliana* (Provided by Dr. T.Kohchi, Nara Institute of Science and Technology, Nara, Japan) was used for heme oxygenase (HO) detection (Muramoto *et al* 1999).

2.5 Northern Analysis

2.5.1 Total RNA extraction

Total RNA was extracted using the method of Logemann *et al* (1987). Samples of leaf tissue (0.8g) were ground to a fine powder with liquid nitrogen in a pestle and mortar. The ground plant material was decanted into a polypropylene centrifuge tube containing 4 ml Z6 buffer (30 mM 2-(N-Morpholino)ethanesulfonic acid (MES), 20 mM EDTA, 8 M guanidium chloride, pH 7.0), 200 μl β-mercaptoethanol and 4 ml chloroform/IAA stabilised saturated phenol (Biogene Ltd, Cambridge, UK). The mixture was homogenised for 2 min using a blender (Ultra Turrax T8, IKA Labortechnik, Germany). The resulting emulsion was centrifuged at 20000 *g* for 20 min at 4°C in a Sorvall RC-5B refrigerated centrifuge (Sorvall, Stevenage, UK), and the lower, organic phase discarded. The upper aqueous phase was transferred to a

fresh centrifuge tube containing 175 µl acetic acid and 2.45 ml 100% ethanol. After vortexing, RNA was precipitated by incubating at -20°C for 1 h. Total RNA was pelleted by centrifugation at 15000 *g* for 20 min at 4°C in a Sorvall RMC 14 centrifuge (Sorvall, Stevenage, UK). The resulting pellet was re-dissolved in 1 ml 3 M sodium acetate, pH 5.2, and transferred to an Eppendorf tube. Further centrifugation (11000 *g*, 10 min) and ethanol washes (70% (v/v)) were performed to purify the pellet, which, after drying, was re-dissolved in 200 µl H_2O. RNA concentration was determined by measuring the absorbance of the diluted sample at 260 nm. A 4 µl aliquot of each sample was diluted to 400 µl with H_2O in a quartz cuvette and its absorbance read on a "gene-quant" RNA calculator (Pharmacia, Cambridge, UK). A similar procedure was used to isolate RNA from seedling samples of 0.2g and pellets dissolved in 50 µl H_2O. Samples were stored at -70°C until required.

2.5.2 Northern blotting

Samples of 20 µg of total RNA were diluted with 1.625 volumes of sample buffer (15% (w/v) 10xMOPS (N-morpholino propane-sulphonic acid), 18.5% (v/v) formaldehyde, 61.5% (v/v) formamide, 1.5% (w/v) EtBr, 3.5% (v/v) loading dye (0.15% (w/v) bromophenol blue, 0.5% (w/v) SDS, 30% (w/v) 0.5 M EDTA, 50% (v/v) glycerol) and fractioned in a 1.5% (w/v) agarose gel containing 1% (v/v) MEN (0.2 M MOPS, 50 mM NaOAc, 10 mM EDTA) and 16.5% (v/v) formaldehyde. For the detection of lower abundance transcripts (Mg-chelatase subunits and ferrochelatase), 40 µg RNA was loaded on gels. Running buffer contained 20 mM MOPS, 5 mM NaOAc and 1 mM EDTA. All samples were denatured at 65°C for 5 min prior to loading. Gels were run at 100 V for 2 h and viewed under a UV transilluminator to check uniformity of loadings.

RNA was transferred to Hybond-N membrane (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, UK) by the technique of northern blotting. A membrane, cut slightly larger than the gel and 3 MM Whatman filter paper sheets (Whatman International Ltd, Maidstone, UK) were saturated in 20x SSC (3 M NaCl, 0.3 M Na_3citrate). Two filter paper sheets were placed over a glass platform, their ends immersed in a tray of 20x SSC to act as wicks. The gel was placed upside down on the filter paper and the area to be blotted defined with parafilm to ensure buffer

absorption through the gel. The membrane was placed on top of the gel, along with four sheets of filter paper and a suitably absorbent material. The whole blot was weighed down with a 250 g weight and left overnight. RNA was fixed to blots by placing them, RNA side down, on a UV transilluminator for 3 min.

2.5.3 Isolation of probe DNA

2.5.3.1 *BVR* (Biliverdin Reductase)

The gene fragment used for northern analysis was isolated from *E.coli* DH5α containing a 950 bp *BVR* fragment cloned into the *Nco*I site of plasmid RKB55 (Provided by Professor J.C. Lagarias, University of California-Davis). *E.coli* was grown overnight on LB media (casein peptone 10 g/l, yeast extract 5 g/l, sodium chloride 10 g/l, pH 7.0) supplemented with agar (15 g/l) and ampicillin (50 mg/l), at 37°C. A single colony was inoculated into LB media supplemented with ampicillin (50 mg/ml), grown overnight at 37°C and a plasmid miniprep performed to isolate the RKB55 plasmid.

A 1 ml sample of culture was centrifuged at 5000 *g* for 2 min to pellet cells. The supernatant was removed and pellet resuspended in 100 µl of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris/HCl, pH 8.0). A 200 µl aliquot of solution II (0.2 M NaOH, 1% (w/v) SDS) was added and the tube inverted six times to mix the contents. Tubes were placed on ice for 5 min to lyse cells, and 150 µl solution III (3 M potassium acetate, pH 4.8) added. Following centrifugation for 10 min at 11000 *g*, supernatants were mixed with an equal volume of chloroform/IAA stabilised saturated phenol (Biogene ltd, Cambridge, UK) and vortexed for 1 min. Centrifugation was repeated and the aqueous phase transferred to 1 ml 100% (v/v) ethanol. DNA was precipitated by incubation at -20°C for 30 min. Following centrifugation at 11000 *g* for 10 min, pellets were washed in 70% (v/v) ethanol, dried and resuspended in 20 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

Plasmid DNA was digested with *Bam*HI for 1 h at 37°C and run out on a 1.5% agarose gel at 100 V for 30 min. The digested fragment was run onto DEAE cellulose (DE81 Whatman paper washed for 30 min in 2.5 M NaCl) and eluted into 400 µl TE

buffer, pH 7.5 containing 1.5 M NaCl. Elution was carried out for 30 min at 56°C. BVR DNA was then purified as above.

2.5.3.2 *HEMA* (Glutamyl-tRNA reductase)

All cDNA clones encoding tetrapyrrole biosynthetic enzymes were provided by Professor B.Grimm (Humboldt University, Berlin, Germany). *E.*coli, harbouring the pBluescript SK vector, was cultured and plasmid DNA isolated as described previously. A full length cDNA clone, encoding the tobacco *HEMA* gene, was excised from pBluescript SK, by digesting with *Eco*RI at 37°C for 1 h. *HEMA* DNA was run out on a 0.8% (w/v) agarose gel at 40 V for 1 h and the fragment excised. Fragment purification was achieved using a "geneclean III" kit (BIO 101 Inc., CA, USA) according to the manufacturers instructions.

2.5.3.3 *GSA* (Glutamate-1-semialdehyde aminotransferase)

A full length cDNA clone, encoding the tobacco *GSA* gene (Höfgen *et al* 1994), was excised from pBluescript SK by digesting with *Eco*RI at 37°C for 1 h and purified as above.

2.5.3.4 *CHLD*, *CHLH* and *CHLI* (Magnesium chelatase, subunits D,H and I)

Full length cDNA clones, encoding genes for the three subunits of tobacco Mg-chelatase (Papenbrock *et al* 1997) were excised from pBluescript SK by digesting with *Eco*RI at 37°C for 1 h and purified as above.

2.5.3.5 *CAB* (Chlorophyll a/b binding protein)

A 500 bp *Arabidopsis CAB* fragment (Lhcb1*2), supplied by Dr. E. Lopez-Juez (Royal Holloway, London, UK), was excised from the plasmid pAB180 (Karlin-Neumann *et al* 1988), by digesting with *Bam*HI and *Sac*I at 37°C for 1 h and purified as above.

2.5.3.6 Ferrochelatase

A full length cDNA clone encoding plastidic tobacco ferrochelatase was used for northern analysis. Plasmid DNA (pBluescript SK) containing the ferrochelatase gene was electroporated into *E.coli* and purified using procedures described below.

2.5.3.6.1 Preparation of competent cells

Cells were prepared for electroporation using the method of McCormac *et al* (1998). The XL1-Blue strain of *E.coli* was chosen for electroporation. Cells were diluted 1:1000 with LB media and tetracycline added to a final concentration of 5 µg/ml. Following overnight growth at 37°C, cells were further diluted to a concentration of 1:100 in LB media and placed at 37°C, with shaking until the optical density of the culture was between 0.6 and 0.8 OD units. Cultures were chilled on ice for 5 min and centrifuged at 500 *g* for 5 min to pellet cells. Salts were removed from the pellet by resuspension in 5 ml ice cold 10% (v/v) glycerol and centrifugation at 500 *g* for 5 min. Following further washes, pellets were resuspended in 400 µl 10% (v/v) glycerol and stored in aliquots of 80 µl at –70°C.

2.5.3.6.2 Electroporation

Plasmid DNA (ca. 1µg) was added to 80 µl competent cells and placed in an ice-cold 0.2 cm cuvette. Electroporation was carried out in a Bio-Rad gene pulser set to 2.5 KV (Bio-Rad, UK). Following the pulse, cells were resuspended in 0.5 ml LB media, mixed and transferred to an Eppendorf tube before incubation at 37°C for 2 h. Cells were then plated onto LB plates containing 50 mg/l ampicillin and grown overnight at 37°C. Single colonies were inoculated into 20 ml LB containing 50 mg/l ampicillin and cultured overnight at 37°C. Plasmid DNA was isolated and the Ferrochelatase fragment excised by digesting with *Eco*RI at 37°C for 1 h, before purification as described previously.

2.5.3.7 *18S*

A flax 18S rRNA fragment was used as a control probe on all northern blots. Fragment DNA was excised from the plasmid pAT 153 by digesting with *Bam*HI for 1 h at 37°C and purified as described previously (Goldsbrough and Cullis 1981).

2.5.4 Probe labelling and hybridisation

Prehybridisation of each membrane was carried out at 42°C for 4 h in 50% (v/v) formamide, 0.25 M sodium phosphate, 0.25 M NaCl, 1 mM EDTA, 10% (v/v) polyethylene glycol (PEG-6000) and 7% (w/v) SDS, containing 100 µg/ml denatured salmon sperm DNA. This was followed by hybridisation with the radiolabelled DNA

at 42°C for 16 h. ^{32}P (Amersham-Pharmacia Biotech, Little Chalfont, UK) was incorporated into DNA using the RediprimeII random prime labelling system (Amersham-Pharmacia Biotech, UK). A 25 ng sample of DNA (estimated from intensity of band on gel) was added to TE buffer, pH 8.0, to produce a final volume of 50 µl and incubated for 5 min at 100°C to denature the DNA before being quickly transferred to ice. DNA was added to the rediprime tube with 5 µl (^{32}P dCTP) and incubation allowed for 20 min at 37°C. The labelling reaction was stopped by the addition of 5 µl 0.2 M EDTA and DNA denatured by heating to 100°C for 10 min.

Membranes were washed twice in 5 x SSC, 0.1% (w/v) SDS for 15 min and once in 3x, 1x and 0.2x SSC, 0.1% SDS (w/v), each for 15 min at 42°C, wrapped in plastic film and placed in a phosphorimager cassette for up to 24 h. Images were analysed using a phosphorimager SI (Molecular Dynamics Inc., Little Chalfont, UK). Comparative analysis of optical density, using "Image Quant" software (Molecular Dynamics Inc., UK) enabled signal quantification and standardisation with control blots. Membranes were subsequently autoradiographed at -70°C using Kodak film.

2.5.5 Stripping of northern blots

Blots were stripped of radioactivity by shaking in 0.005 M Tris-HCl, pH 8.0, 2 mM EDTA and 0.1x Denhardt solution (0.004% (v/v) BSA, 0.004% (w/v) ficoll, 0.004% (w/v) polyvinyl pyrrolidone) at 65°C for 3 h.

2.6 Holophytochrome quantification

A dual wavelength spectrophotometric assay was used to quantify *in vivo* levels of photoactive holophytochrome in 11 day-old etiolated seedlings. To reduce potential contamination from carotenoid pigments, seedlings were grown on MS media supplemented with 5 µM Norflurazon, a treatment known not to adversely affect phytochrome levels (Thomsen *et al* 1993). Samples of 100 seedlings were lightly packed into a disposable plastic cuvette of path-length 0.5 cm. Cuvettes were kept chilled throughout measurements to prevent the thermal conversion of Pfr to Pr. Samples were irradiated with FR for 60 seconds, then R for 30 seconds, in triplicate cycles, using a Perkin Elmer-557 dual wavelength, double beam spectrophotometer. The difference in absorbance of the R-irradiated form (A_{660nm}-A_{730nm}) and the FR-

irradiated form (A_{660nm}-A_{730nm}) was taken as an estimate of photoactive holophytochrome.

2.7 Aminolevulinic acid (ALA) extraction and quantification

Owing to sporadic aberrancy in germination and growth rate of dark-grown transgenic seedlings, uniformity of developmental stage was maximised by performing initial hypocotyl measurements on all seedlings. Only transgenic seedlings ± 10% of WT hypocotyl length were assayed for ALA and protochlorophyllide. All biochemical analyses were performed on 11-day-old seedlings (time 0 = transfer to light regime from cold treatment). Samples of 50 seedlings (30 seedlings were used for light-grown material) were incubated under white light (100 µmol $m^{-2}s^{-1}$) in 4 ml 0.1 M potassium phosphate buffer, pH 7.0 ± 40 mM levulinic acid (Sigma, UK), a competitive inhibitor of ALA dehydratase, for 6 h at 23°C (Beale and Castelfranco 1974). Etiolated seedlings were incubated in darkness, and all procedures performed under a dim green safelight. For assays of young plant tissue, 3 leaf discs (diameter 1cm) were removed from leaf number 4 (counting from the plant apex) and incubated under white light (100 µmol $m^{-2}s^{-1}$) for 6 h at 23°C. Similar discs were simultaneously assayed for chlorophyll. For leaf disc analysis, plants were grown under a fluence rate of 100 µmol $m^{-2}s^{-1}$ white light with a 16 h photoperiod. Light was provided by 70 W cool white fluorescent tubes (Philips, Netherlands).

Tissue samples were blotted onto filter paper and ground to a fine powder in liquid nitrogen before homogenisation in 1 ml 20 mM potassium phosphate buffer, pH 7.0 for 2 min using a blender (Ultra Turrax T8, IKA Labortechnik, Germany). Samples were then incubated on ice for 20 min. Following centrifugation at 11000 *g* for 5 min, 500 µl supernatant was added to 100 µl ethylacetoacetate and samples incubated at 100°C for 10 min. This treatment results in the condensation of two ALA molecules to form a pyrrole with a free α-position (Beale 1978). After cooling on ice, 500 µl modified Ehrlich's reagent (1.8% (w/v) dimethylbenzaldehyde in 74.6% (v/v) glacial acetic acid, 12.6% (v/v) perchloric acid, 0.31% (w/v) $HgCl_2$) was added and samples centrifuged at 11000 *g* for 5 min. Absorbance was read at 526, 553 and 600 nm using a Hitachi UV/VIS spectrophotometer (model u-2001, Hitachi, Japan) and the concentration of ALA determined using a molar extinction coefficient of 7.45×10^4

$M^{-1}cm^{-1}$ (Mauzerall and Granick 1956). The difference in ALA between control samples and samples incubated in LA was taken as the ALA synthesised during the incubation period and hence the ALA synthesising capacity of the tissue.

2.8 Porphyrin extraction and quantification

Samples of 50 dark-grown seedlings were homogenised in 0.75 ml ice-cold acetone/0.1 M NH_4OH (90/10, (v/v)), under a dim green safelight using a blender (Ultra Turrax, IKA Labortechnik, Germany). Following centrifugation at 11000 g for 2 min, supernatants were retained and pellets re-extracted in 0.75 ml solvent. Supernatants were combined and an equal volume of hexane added to remove esterified products. After thorough mixing, the upper phase was discarded and the wash step repeated. The lower phase of each sample was transferred to a fresh tube and the volume made up to 1 ml with solvent. Relative protochlorophyllide levels were measured by fluorescence spectroscopy following excitation at 440 nm in a Hitachi fluorescence spectrophotometer, model F-2000 (Terry and Kendrick 1999). The emission maximum of protochlorophyllide in this solvent was 639 nm. Relative levels of Mg-protoporphyrin (including Mg-protoporphyrin metyl ester which was indistinguishable in this assay) were estimated from its emission peak at 590 nm, following excitation at 410 nm (Terry and Kendrick 1999).

2.9 ALA / inhibitor feeding

ALA (Sigma, UK) was added to MS media from a 1 M stock in H_2O. Seeds were planted directly onto media and germinated in darkness at 23°C. Control media contained an equal volume of H_2O. For higher concentrations, samples of 50 (11 day old) etiolated seedlings were incubated in 0.1 M phoshate buffer containing ALA, for 6 h at 23°C. The iron chelator 2'2'-bipyridyl (Merck, Darmstadt, Germany) was dissolved in ethanol to produce a 1 M stock. Samples of 75 etiolated seedlings (11day old) were incubated in 0.1 M potassium phosphate buffer ± 10 mM bipyridyl in darkness for 6 h at 23°C. Tissue was washed in buffer, blotted onto filter paper and porphyrins extracted as described above.

2.10 Transmission Electron Microscopy (T.E.M)

2.10.1 Tissue fixation

Tissue pieces (approximately 1 mm^2) of leaf/cotyledon were infiltrated in fixative (3% w/v glutaraldehyde, 4% (v/v) formaldehyde, 0.1 M PIPES buffer, pH 7.2) at room temperature for 1 h. Etiolated material was fixed under a dim green safe-light. PIPES buffer was prepared by dissolving piperazine-NN'-bis-2-ethanesulphonic acid in 10 M NaOH (Hayat 1986). Following two 10 min rinses in PIPES buffer, tissue was post-fixed in 1% osmium tetroxide, 0.1 M PIPES buffer, pH 7.2 for 1 h at room temperature. After two further 10 min washes in PIPES buffer, tissue was dehydrated in a graded ethanol series (10 min washes of 30% (v/v), 50%, 70%, 95% followed by two 20 min washes in 100%). Incubation with histosol (10 min) and 50:50 histosol:resin (12 h) preceded infiltration with polyvinyl Spurr resin for 6 h (Page 1999). Tissue samples were transferred to BEEM capsules, embedded in fresh resin and polymerised for 24 h at 60°C.

2.10.2 Section Viewing

Semi-thin (1µm) leaf/cotyledon cross sections, cut with glass knives (7801A knifemaker, LKB, Stockholm, Sweden) on an ultramicrotome (Ultracut E, Reichert-Jung, Austria) were used for light microscopy prior to electron microscopy (EM). Owing to increased density of samples, etiolated tissue sections were cut with a diamond knife. Light microscopy sections were stained with a 1:1 aqueous mixture of 1% (w/v) toludine blue in 1% (w/v) sodium borate. Stain was applied to sections on a microscope slide and heated at 100°C for 5 seconds before viewing. Ultrathin sections between 50 and 100 nm (silver/gold colouration) for EM were collected on copper grids and counter-stained with 2% (w/v) uranyl acetate for 15 min, followed by lead citrate solution (Reynolds 1963) for 3 min. The latter was applied in the presence of NaOH pellets to absorb CO_2, a compound known to adversely affect staining. Uranyl acetate and lead citrate bind both nucleic acids and proteins, providing contrast and defining organelle constituents. In addition to its fixative properties, osmium tetroxide also acts to define phospholipid membranes. Samples were examined in a Hitachi H7000 electron microscope at 75 KV.

Chapter 3. Seedling Development

3.1 Introduction

Phytochromes perform a multifunctional role throughout seedling growth, regulating the processes of germination, apical hook opening, hypocotyl elongation, cotyledon expansion and plastid development. The most abundant phytochrome species in etiolated seedlings is phyA. The decrease of this photoreceptor upon illumination enables specific perception of light, allowing seedlings to react rapidly upon soil emergence (Casal *et al* 1997, Hennig *et al* 1999). Early detection of light by phytochrome, as the tip of the seedling approaches the surface of the soil, initiates the inhibition of shoot extension and promotes the interconnected processes of leaf expansion and plastid development. It has been proposed that phytochrome in etiolated seedlings performs an "antenna" function, acting to detect the presence, rather than the spectral quality of light (Smith and Whitelam 1990). The high levels of accumulated phytochrome in etiolated seedlings and the relatively low fluence rates of light required to saturate de-etiolation responses support such an idea. In light-grown plants, growth responses are directly related to the photoequilibrium (P_{fr}/P_{tot}), established by the R:FR quantum ratio of the actinic radiation (Morgan and Smith 1976, 1978). The predominant constituents of the phytochrome pool in green plants are light-stable phytochromes. The physiological transition from skotomorphogenesis to photomorphogenesis is therefore associated with a dynamic change in both the total abundance and relative composition of the phytochrome pool.

Compositional differences between the phytochrome pools of etiolated and light-grown seedlings and the characterisation of physiologically distinguishable modes of phytochrome action (Smith and Whitelam 1990) have enabled the screening of large seedling populations for deficiencies in individual phytochromes and the subsequent isolation of mutants in a variety of species. Analysis of de-etiolation responses under continuous R and FR therefore provides a diagnostic assessment of seedling phytochrome composition. Failure to display FR high irradiance responses (HIR), as characterised by insensitivity to prolonged FR irradiation with respect to hypocotyl elongation and the promotion of cotyledon expansion, has provided a basis for the identification of plants deficient in phyA (Whitelam *et al* 1993). Normal de-etiolation responses have been observed, however, in *phyA* seedlings of *Arabidopsis* grown

under prolonged R and white light (Nagatani *et al* 1993, Parks and Quail 1993, Whitelam *et al* 1993). PhyA also plays a role in the regulation of hypocotyl elongation in light-grown seedlings in response to alterations in the R:FR ratio. It has been suggested that phyA action may antagonize the action of phyB in the regulation of elongation growth under low R:FR ratios (Johnson *et al* 1994). The inability of *Arabidopsis phyA* seeds to respond to the promotive effects of FR, or very low doses of R, on germination suggests that phyA operates to promote seed germination in VLFR mode (Shinomura *et al* 1996, Botto *et al* 1996). It is therefore apparent that phyA regulates seedling development not only through the FR-HIR, but also mediates inductive responses to pulses of R.

Etiolated seedlings, deficient in phyB, characteristically display a marked insensitivity to prolonged R with respect to the inhibition of hypocotyl elongation and leaf development (Koornneef *et al* 1980, Devlin *et al* 1992, Reed *et al* 1993). Such phenotypes suggest a major role for phyB in the perception of prolonged R. *Arabidopsis phyB* mutants also display defects in chlorophyll synthesis and plastid development (Chory 1992). A lack of hypocotyl elongation growth responses to end of day (EOD) FR treatments in these seedlings suggest a role for PfrB in the inhibition of elongation growth upon the transition to darkness (Nagatani *et al* 1991). A major role for phyB in regulating germination following brief R treatments has been demonstrated in seeds of *Arabidopsis* (Shinomura *et al* 1994). Analysis of transgenic plants, over-expressing a *PHYB* cDNA, provides further support for such a role. Seeds showed high dark germination frequencies that were not influenced by the R:FR ratios under which the parent plants were grown (McCormac *et al* 1993a). In contrast, seeds of the *Arabidopsis phyB* mutant always showed very low dark germination (McCormac *et al* 1993a).

Pronounced responses to R given as a prolonged treatment or as multiple pulses in seeds of the *Arabidopsis phyA phyB* double mutant indicate the action of another R-absorbing photoreceptor in the photocontrol of seed germination (Whitelam and Devlin 1996). When grown under R, this mutant displayed poorly developed cotyledons, reduced induction of *CAB* gene expression and reduced potentiation of chlorophyll induction (Reed *et al* 1994). Such phenotypes were not observed in either single mutant, suggesting the presence of either phytochrome to be sufficient for full

responsiveness to pulses of red light. In summary, both phyA and phyB are involved in the regulation of seed germination and de-etiolation of dark-grown seedlings. PhyA regulates plant responses to FR irradiation, whereas phyB performs a predominant role in responses to R. However, many responses require synergism and in some cases, mutual antagonism between the actions of phyA and phyB, in addition to input from other members of the phytochrome family.

Despite a diversity of apoprotein sequences, all plants use a single linear tetrapyrrole chromophore, phytochromobilin, for the production of photoactive phytochrome (Terry *et al* 1993). Mutants in both committed steps of the chromophore biosynthesis pathway have been identified in a number of plant species (Terry 1997). Seedlings of these plants exhibit phenotypes consistent with multiple phytochrome deficiencies, including reduced seed germination, decreased chlorophyll and abberant photomorphogenic behaviour under continuous R (Rc) and FR (FRc) (Koornneef *et al* 1980, 1985, Chory *et al* 1989b, 1992, Kraepiel *et al* 1994, van Tuinen *et al* 1996, Weller *et al* 1996, 1997b). Constitutive expression of mammalian biliverdin reductase (BVR), in transgenic *Arabidopsis*, yielded similar phenotypes with defects in chlorophyll accumulation being observed only in plastid-targeted lines (Lagarias *et al* 1997, Montgomery *et al* 1999). Experiments described here explore the functional role of phytochromes in the seedling development of short-day tobacco (*Nicotiana tabacum* cv Maryland Mammoth), a species for which no chromophore-deficient mutants have been reported. Levels of photoactive holophytochrome have been quantified in etiolated cytosol and plastid-targeted BVR seedlings and photomorphogenic behaviour under different wavelengths of continuous irradiation analysed. Germination frequencies and pigment accumulation in transgenic seedlings have also been investigated.

3.2 Results

3.2.1 Holophytochrome Quantification

Photoactive holophytochrome was quantified *in vivo*, in etiolated 11-day-old seedlings, using a dual wavelength spectrophotometric assay. Seedlings were grown in the presence of 5μM Norflurazon to avoid potential contamination from carotenoid pigments. Wild-type (WT) seedlings grown without Norflurazon displayed slightly reduced phytochrome levels (data not shown), confirming that Norflurazon feeding has no adverse effect on phytochrome accumulation and possibly improves assay sensitivity. Total phytochrome is represented by $\Delta(\Delta A_{660 - 730\ nm})$: The photo-reversible change in absorbance difference ($A_{660\ nm} - A_{730\ nm}$) between R-irradiated and FR-irradiated samples. Both cytosol (C2+C4) and plastid (P3+P6)-targeted BVR seedlings showed approximately twenty-fold reductions in photoreversible holophytochrome when compared with WT (Fig.4). Consequently, comparative quantification of holophytochrome levels between these lines was beyond the detection limits of the spectrophotometer.

3.2.2 Seedling morphology

Seedlings of WT, cytosol-targeted BVR line 2 (C2) and plastid-targeted BVR line 3 (P3) were grown on MS media under continuous irradiation of different wavelengths to assess the effects of BVR expression on seedling morphology. Photographs were taken at 21 days, when differences between lines were most pronounced. Figure 5a shows seedlings grown in complete darkness. No obvious morphological differences were observed between lines under these conditions. When grown under continuous white light, however, differences were clearly visible (Fig.5b). Cytosol and plastid-targeted BVR lines displayed increased hypocotyl elongation and reduced chlorophyll, consonant with multiple phytochrome deficiency. The cotyledon "bleaching" effect, observed in plastid-targeted BVR seedlings, is investigated in chapters 5 and 6.

Seedlings grown under continuous R are shown in Figure 5c. WT seedlings were phenotypically similar to those grown under white light, but cytosol-targeting of BVR resulted in increased hypocotyl extension, reduced cotyledon expansion and elongation of the first internode.

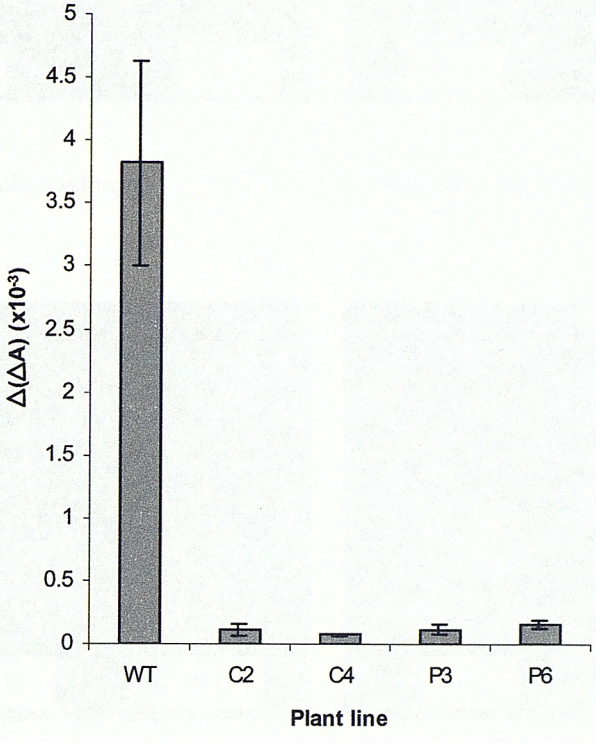


Fig. 4 Holophytochrome quantification in 11-day-old seedlings, grown in complete darkness on 5µM Norflurazon. WT= wild-type, C2= cytosol-targeted BVR line 2, C4= cytosol-targeted BVR line 4, P3= plastid-targeted BVR line 3, P6= plastid-targeted BVR line 6. Total holophytochrome is represented by $\Delta(\Delta A_{660 - 730\,nm})$: The photo-reversible change in absorbance difference ($A_{660\,nm} - A_{730\,nm}$) between R-irradiated and FR-irradiated samples. n=3 ± S.E.

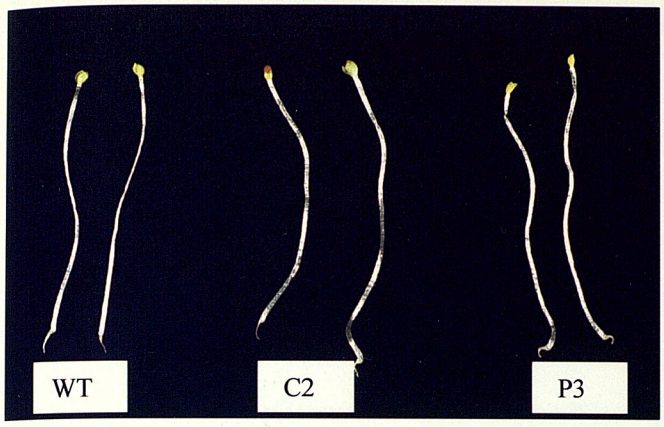


Fig. 5a. Dark-grown 21 day-old seedlings

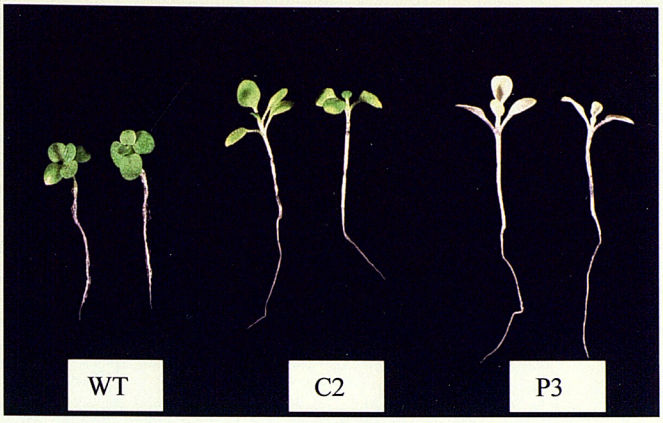


Fig. 5b. White light-grown 21 day-old seedlings

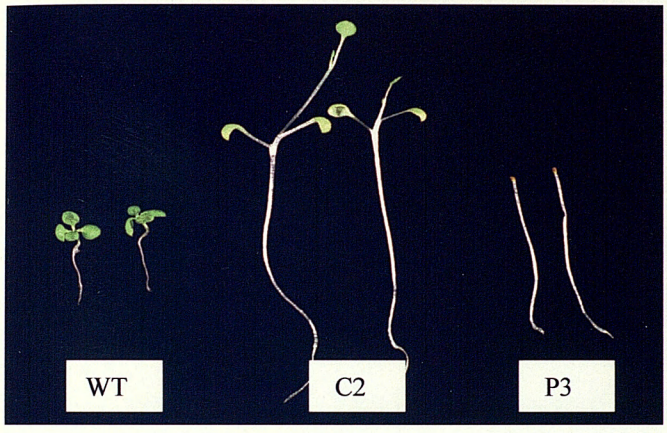


Fig. 5c. Red light-grown 21 day-old seedlings

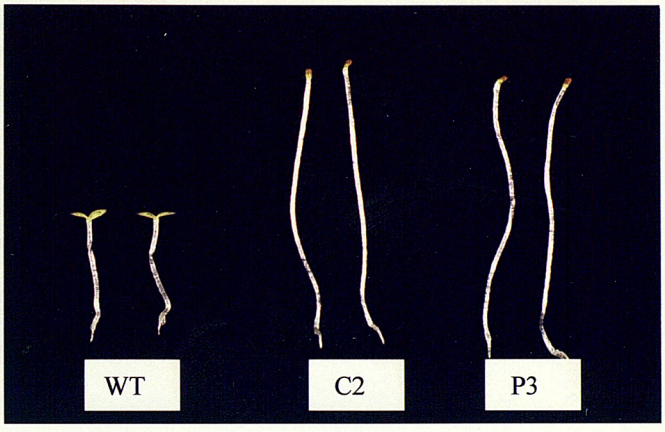


Fig. 5d. Far-red light-grown 21 day-old seedlings

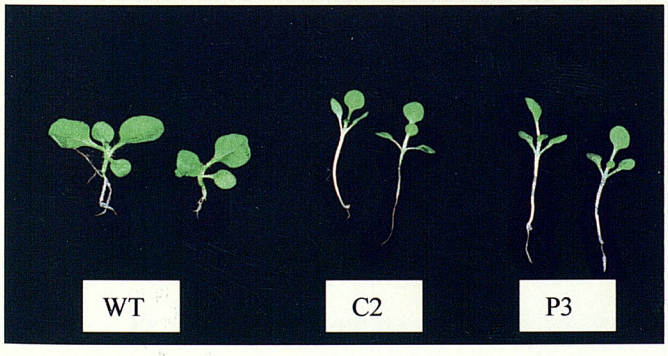


Fig 5e. Blue light-grown 21 day-old seedlings

Figure 5 (a-e). Seedling morphology under continuous irradiation of different wavelengths. White light was provided at a fluence rate of 100 $\mu molm^{-2}s^{-1}$, red light at 70 $\mu molm^{-2}s^{-1}$, far-red light at 10 $\mu molm^{-2}s^{-1}$ and blue light at 10 $\mu molm^{-2}s^{-1}$. WT = wild type, C2 = cytosol targeted BVR line 2 and P3 = plastid targeted BVR line 3.

Such a result is consistent with a deficiency in phyB. Plastid-targeting of BVR resulted in a complete inability to de-etiolate and a phenotype comparable to that of dark-grown seedlings. Such a phenotype suggests a severe deficiency of phyB. When grown under continuous FR (Fig.5d), WT seedlings displayed reduced cotyledon expansion and an absence of chlorophyll, confirming that the light source used in this experiment did not cause Pchlide reduction. Both cytosol and plastid-targeted BVR lines showed a complete inability to de-etiolate, a result consistent with a severe deficiency of phyA. Seedlings grown under continuous B are shown in Figure 5e. Increased hypocotyl elongation, reduced leaf expansion and chlorophyll deficiency were observed in both cytosol and plastid-targeted BVR lines. Photobleaching was not observed in plastid-targeted BVR seedlings, as under white light, but this may be due to the reduced fluence rate used.

3.2.3 Hypocotyl elongation

Mean hypocotyl lengths of 21 day-old seedlings, grown under continuous irradiation of different wavelengths, are shown in Figure 6a. No apparent difference was observed between lines when grown in complete darkness. Phytochrome-mediated inhibition of hypocotyl extension was observed in WT seedlings grown under continuous R. This inhibition was abolished in transgenic lines, which showed hypocotyl lengths similar to those of dark-grown seedlings. Partial inhibition of hypocotyl extension was displayed by WT seedlings grown under continuous FR. This inhibition was severely reduced in both cytosol and plastid-targeted BVR seedlings. Under continuous white and blue light, inhibition of hypocotyl extension was observed in WT seedlings, and was reduced in transgenic lines. Overall, cytosol and plastid targeting of BVR appear to have similar effects on hypocotyl elongation in all light regimes tested.

3.2.4 Germination

Percentage germination of dark-grown seedlings at 14 days, with and without cold pre-treatment is shown in Figure 6b. No differences in percentage germination were observed for light-grown seedlings (data not shown). Dark germination was not affected in cytosol-targeted BVR lines, while plastid-targeting resulted in a reduction of approximately 50%. This could subsequently be overcome by cold pre-treating seeds for 3 days, although 100% germination was never achieved.

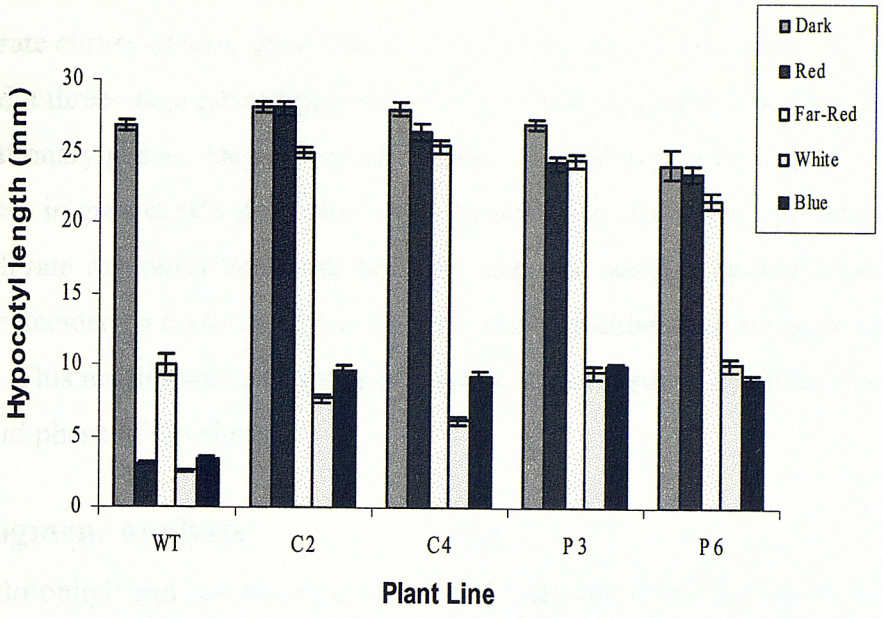


Fig. 6a Mean hypocotyl lengths of 21 day-old seedings grown under continuous irradiation of different wavelengths. Dark-grown transgenic seedlings were phenotypically indistinguishable from wild-type. Elongated hypocotyls were observed in all transgenic lines, under all wavelengths used. WT= wild-type, C2= cytosol-targeted BVR line 2, C4= cytosol-targeted BVR line 4, P3= plastid-targeted BVR line 3, P6= plastid-targeted BVR line 6. n=50 ± S.E..

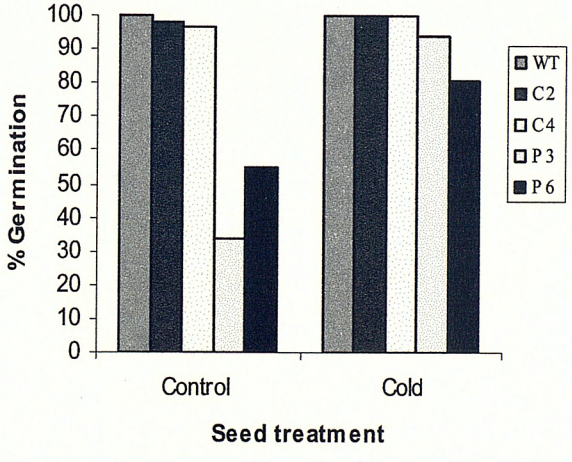


Fig. 6b Percentage germination of dark-grown seedlings at 14 days with and without a 3-day-cold treatment. WT= wild-type, C2= cytosol-targeted BVR line 2, C4= cytosol-targeted BVR line 4, P3= plastid-targeted BVR line 3, P6= plastid-targeted BVR line 6.

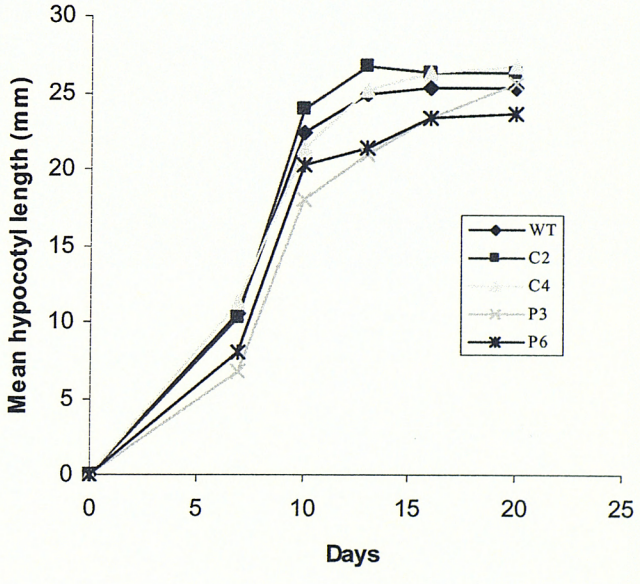


Fig. 6c Growth rates of dark-grown seedlings, as measured by hypocotyl length. WT= wild-type, C2= cytosol-targeted BVR line 2, C4= cytosol-targeted BVR line 4, P3= plastid-targeted BVR line 3, P6= plastid-targeted BVR line 6. Time 0 = transfer from cold treatment to dark incubator. n=50. Standard errors were always less than 15% and have been omitted for clarity.

3.2.5 Seedling growth rates

Growth rate curves of dark-grown seedlings are presented in Figure 6c. All lines displayed a three-stage growth pattern, with an initial lag phase, a rapid growth period and a stationary phase. Despite minor variation in final hypocotyl length, no overall differences in growth rate were observed between lines. However, sporadic aberrancy in growth rate was observed during the early stages of seedling development. It was therefore decided to perform all biochemical and molecular analysis on seedlings at 11 days. This maximised uniformity of growth, while ensuring seedlings were still in their rapid phase of development.

3.2.6 Pigment analysis

Mean chlorophyll and carotenoid contents of 11 day-old white light-grown seedlings are displayed in Figures 7a and 7b respectively. Cytosol-targeting of BVR resulted in a partial deficiency of chlorophyll, whilst the effects of plastid-targeting were more severe. Carotenoid accumulation appeared to parallel that of chlorophyll in all lines. The enhanced photosensitivity of plastid-targeted BVR seedlings grown under white light can therefore be partially attributed to the severe carotenoid deficiency observed in these seedlings.

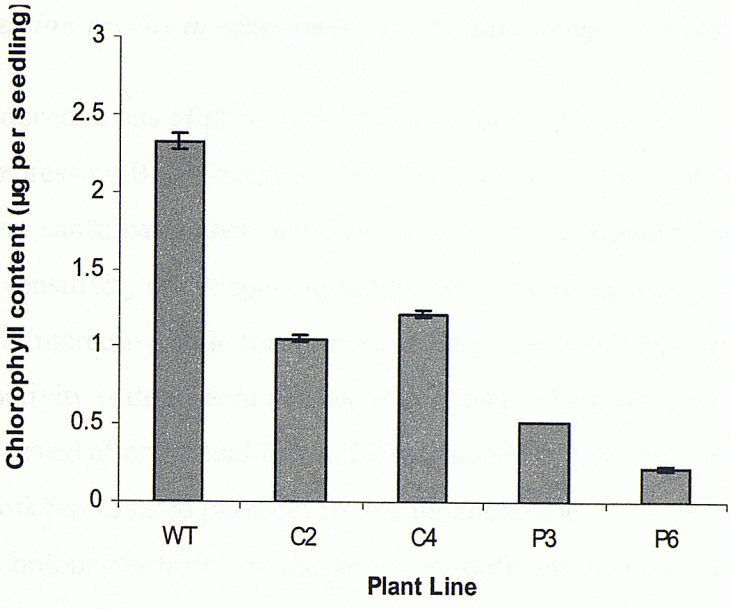


Fig. 7a. Mean chlorophyll content of 11-day-old white light-grown seedlings. WT=wild-type. C2= cytosol-targeted BVR line2, C4= cytosol-targeted BVR line 4, P3= plastid-targeted BVR line 3, P6= plastid-targeted BVR line 6. n=3 ± S.E..

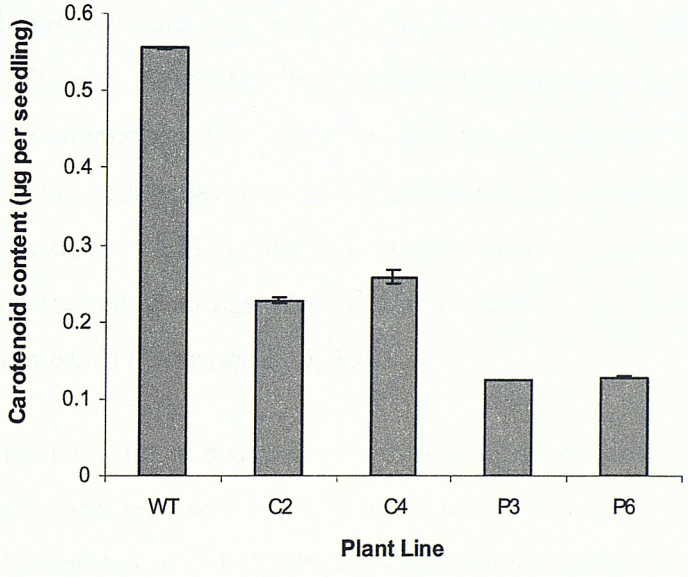


Fig. 7b. Mean carotenoid content of 11-day-old, white light-grown seedlings. WT=wild-type. C2= cytosol-targeted BVR line2, C4= cytosol-targeted BVR line 4, P3= plastid-targeted BVR line 3, P6= plastid-targeted BVR line 6. n=3 ± S.E..

3.3 Discussion

BVR expression results in reduced levels of photoactive phytochrome

Severely reduced levels of photoactive holophytochrome were recorded in etiolated seedlings expressing BVR, irrespective of subcellular localisation (Fig.4). A precise comparative quantification between cytosol and plastid-targeted lines was, however, beyond the sensitivity of the spectrophotometer. The reversible photoisomerisation between two interconvertible forms is a crucial property of phytochrome, on which its biological activity is dependent (Butler *et al* 1964). The pronounced absorbance changes observed after FR and R irradiation therefore provided confidence that measurements represented photoreversible phytochrome. Reduced levels of photoactive holophytochrome in chromophore-deficient mutants of *Arabidopsis* (Parks and Quail 1991), pea (Weller *et al* 1996,1997), and tomato (Koornneef *et al* 1985) were beyond detection using similar spectrophotometric techniques. Trace levels have, however, been quantified in the *pew* mutants of *Nicotiana plumbagnifolia* (Kraepiel *et al* 1994). The extreme phenotype of seedlings expressing BVR suggests that these plants possess an equal, if not greater, phytochrome deficiency than the characterised chromophore-deficient mutants. The possibility exists, therefore, that tobacco seedlings are more amenable to *in vivo* phytochrome quantification than other species. Results may, however, simply reflect differences in experimental technique and spectrophotometer sensitivity between authors. The growth of seedlings on Norflurazon in this assay may also have increased accuracy by reducing potentially contaminating carotenoid pigments. The slight increase in measurable phytochrome in Norflurazon-treated seedlings, observed here (data not shown), has been reported previously in mustard (Thomsen *et al* 1993).

The predominant species of phytochrome measured in etiolated seedlings is phyA. This is much less stable *in vivo* as Pfr than Pr, with a half-life of approximately 1h in the Pfr form (Colbert *et al* 1983). Phototransformation to the Pfr form arises through conformational changes in the chromophore. Reduced turnover of phyA upon illumination is therefore consistent with a deficiency in phytochrome chromophore and has been widely used in the diagnosis of chromophore-deficient mutants (Parks and Quail 1991, Weller *et al* 1996, 1997a). Stability of phyA apoprotein was also

observed in *Arabidopsis* seedlings expressing BVR in the plastid compartment (Lagarias *et al* 1997). A similar experiment in tobacco proved repeatedly unsuccessful, a result eventually attributed to primary antibody quality. Visualisation of holophytochrome within polyacrylamide gels through zinc staining (Berkelman and Lagarias 1986) proved equally unproductive. Problems are likely to have resulted from the relatively low yields of protein obtained from seedling extractions.

BVR expression results in hypocotyl elongation and abberant photomorphogenic responses

Seedlings expressing BVR displayed elongated hypocotyls under continuous irradiation of different wavelengths (Figs.5b-e, 6a). The sub-cellular targeting of BVR to the plastid resulted in quantitatively similar responses, suggesting that phytochrome levels in both cytosol and plastid-targeted lines are below the threshold required to inhibit hypocotyl elongation. The role of phyB in the perception of continuous R is well documented (Koornneef *et al* 1980, McCormac *et al* 1993b, Somers *et al* 1991). The severely reduced responsiveness to R, as manifested by long hypocotyl growth in BVR lines, implies a deficiency of photoactive phyB in these seedlings. This phenotype is displayed in phyB-deficient mutants of *Nicotiana plumbagnifolia* (Hudson *et al* 1997), tomato (van Tuinen *et al* 1995a), pea (Weller *et al* 1995), *Arabidopsis* (Somers *et al* 1991), cucumber (López-Juez *et al* 1992) and *Brassica rapa* (Devlin *et al* 1992). The inability of plastid-targeted lines to de-etiolate under continuous R suggests a greater deficiency of phyB in these seedlings.

WT seedlings grown under continuous FR displayed an inhibition of hypocotyl elongation and the opening and expansion of cotyledons, a phenotype consistent with the phyA-mediated HIR. Such a response was not observed in either cytosol or plastid-targeted BVR lines, implicating a deficiency of phyA in these plants. Mutants of *Arabidopsis* (Nagatani *et al* 1993, Parks and Quail 1993, Whitelam *et al* 1993), tomato (van Tuinen *et al* 1995b), pea (Weller *et al* 1997) and rice (Takano *et al* 2001), found to be specifically deficient in phyA, display similar phenotypes. Persistant growth inhibition under FR was observed in transgenic tobacco and *Arabidopsis* over-expressing phyA, supporting a role for this phytochrome species in FR-mediated responses (Whitelam *et al* 1992, McCormac *et al* 1992). A pronounced increase in hypocotyl elongation and retarded cotyledon expansion under R and FR wavelengths

were also observed in *BVR*-transformed *Arabidopsis* seedlings (Lagarias *et al* 1997, Montgomery *et al* 1999), and a variety of chromophore-deficient mutants (reviewed in Terry 1997). Together, these observations suggest that BVR acts to reduce the total phytochrome pool through targeted inactivation of the chromophore component.

The B-mediated hypocotyl elongation, displayed in both cytosol and plastid-targeted BVR seedlings, is consistent with results observed in plastid-targeted lines of *Arabidopsis* (Lagarias *et al* 1997). It is possible that such differences result, at least in part from the B-mediated conversion of Pr to Pfr. The role of phyA in B sensing has been documented in a variety of species, including tomato (Weller *et al* 2001) and *Arabidopsis* (Neff and Chory 1998, Chun *et al* 2001). It is also likely that co-action exists between R and B-sensing systems, although the exact nature of this co-action remains under dispute. Seedling experiments in a variety of species suggest that B acts to determine the plants responsiveness to Pfr (Mohr 1994). Such an idea is supported by the work of Oelmüller and Kendrick (1991) who showed that photomorphogenesis in the chromophore-deficient *aurea* mutant of tomato can be attributed, primarily, to light absorbtion in the B/UV-A region of the spectrum. The authors propose a co-action model, whereby a B/UV-A photoreceptor works in concert with residual levels of phytochrome to enable survival of the mutant. A contradictory model of co-action has been proposed by Ahmad (1999) based on the interaction of cryptochrome 1 (CRY1) and phyA *in vitro* (Ahmad *et al* 1998). The author suggests the role of phytochrome in the B-dependent inhibition of hypocotyl elongation is to enhance the activity of CRY1 and CRY2 soley.

Despite controversy over the exact nature of co-action, authors agree that B-mediated de-etiolation involves the interaction of both phytochrome and cryptochrome signalling (Yanovsky *et al* 1995, Ahmad *et al* 1997, Casal and Mazzela 1998). In *Arabidopsis*, it has also been shown that additional phytochromes can substitute in synergistic interactions with cryptochrome in the absence of phyA and phyB (Ahmad and Cashmore 1997, Casal and Mazella 1998). It is likely that the deficiency of total phytochromes in plants expressing BVR impairs B sensing, resulting in the increased hypocotyl lengths observed. The prominent increase in hypocotyl elongation in these plants is, however, in contradiction to work on the chromophore-deficient mutants of *Arabidopsis* (Koornneef *et al* 1980) and pea (Weller *et al* 1997b). As chromophore-

deficient mutants are regarded as "leaky", it is possible that the severity of phytochrome deficiency in seedlings expressing BVR exposes a role for blue light in seedling photomorphogenesis (Lagarias *et al* 1997).

Exogenous application of biliverdin was shown to restore some phenotypic aspects of chromophore deficiency in mutants of *Nicotiana plumbagnifolia* (Kraepiel *et al* 1994) and *Ceratodon purpureus* (Lamparter *et al* 1997, Esch and Lamparter 1998). Parks and Quail (1991) also demonstrated that light-induced inhibition of hypocotyl elongation was restored in *hy1* and *hy2* mutant seedlings grown on either biliverdin or the cyanobacterial phytochromobilin analogue, phycocyanobilin. Attempts were made to rescue *BVR*-transformed tobacco seedlings with both biliverdin and phycocyanobilin under continous FR. A small recovery of phenotype was observed in cytosol-targeted BVR lines with both substrates (data not shown), although the technique proved generally ineffective. It is likely that the uptake of both substrates was limited and the chromophore-deficiency in plastid-targeted lines was too severe to enable recovery of phenotype. Simple feeding techniques have also proved unsuitable for analysis of pea and tomato mutants (Terry 1997).

Plastid-targeting of BVR results in reduced dark germination

Although light-mediated germination appeared unaffected, dark germination was severely reduced in plastid-targeted lines (Fig.6b). Impaired germination has also been reported in *Arabidopsis* expressing BVR in the plastid (Lagarias *et al* 1997) and certain chromophore mutants (Terry 1997). Germination behaviour within a seed batch depends on a number of interacting factors. These include genotype and the environmental conditions of seed maturation, harvest and storage (McCormac *et al* 1993a). The quality, duration and fluence rate of irradiation perceived by seeds during germination is also critical. The promotory effect of light is believed to be mediated through phytochrome in its Pfr form. In tobacco, pulses of R have been shown to induce dark germination in a fluence-rate dependent manner (McCormac *et al* 1993a). At higher fluences, induction was reversed by pulses of FR, suggesting the response to comprise of both VLF and LF components. As no impairment of light-mediated germination was observed in either cytosol or plastid-targeted BVR seed, it can be assumed that the residual pool of phytochrome in all lines is sufficient to promote this response. Germination in the dark, however, must be mediated via the

Pfr formed during seed maturation. As growth conditions and harvesting procedures were uniform for all plants, the pronounced germination impairment observed in plastid-targeted BVR seeds must represent the increased severity of phytochrome deficiency in these lines. Over-expression of *PHYB* in *Arabidopsis* enhanced dark germination, a result not observed for *Arabidopsis* or tobacco plants over-expressing *PHYA* (McCormac *et al* 1993a). Dark-germination was also severely reduced in the *phyB* mutant of *Arabidopsis*. Such studies suggest that dark germination is promoted, primarily by phytochrome B in its FR-absorbing form, thus inferring a greater deficiency of this phytochrome in plastid-targeted lines. Percentage germination was increased in these seedlings by a cold pre-treatment of three days. The promotion of dark germination is therefore a complex network of signalling pathways, mediated in part, but not exclusively, by Pfr status.

All transgenic lines displayed similar growth curves to WT seedlings when germinated in the dark (Fig. 6c). Despite sporadic aberrations, final hypocotyl length was not affected by BVR expression. Such data is consistent with phytochrome regulating hypocotyl inhibition in its Pfr form.

BVR expression results in pigment deficiency in light-grown seedlings

When grown under continuous white light, a severe chlorophyll deficiency was observed in transgenic seedlings, a phenotype that was more pronounced for plastid-targeted lines (Fig.7a). The regulatory mechanisms underlying this chlorophyll deficiency are discussed in chapters 5 and 6. Although often regarded as a distinguishing characteristic, chlorophyll deficiency in chromophore-deficient plants is variable and depends on the species, step in the pathway that is blocked and conditions under which the plants are grown (Terry 1997). The increased sensitivity and subsequent "photobleaching" of plastid-targeted BVR lines under elevated fluence rates is consistent with results in *Arabidopsis* (Lagarias *et al* 1997, Montgomery *et al* 1999). A similar phenotype has also been reported in the chromophore mutants *yg-2* and *au* of tomato (Van Tuinen *et al* 1996). Photobleaching can result from an accumulation of toxic tetrapyrrole intermediates, or the uncoordinated excitation of photosystems I and II. Regulatory mechanisms therefore exist to prevent such photooxidative damage. Carotenoids quench free radicals and are central to the dissipation of excess energy via the xanthophyll cycle. The marked

carotenoid deficiency recorded in seedlings expressing BVR (Fig.7b) is therefore likely to impair photoprotective mechanisms in these plants.

Fluorescent differential display has recently been used in *Arabidopsis* to identify genes up-regulated by phyA (Kuno *et al* 2000). Two genes believed to fulfil photo-protective roles in seedling development have been identified. The *Drt 112* gene is believed to be involved in the protection of chloroplast DNA from photodamage and the photosystem II component *psbs*, is envisaged to protect against light stress. Both are induced via the VLFR. The deficiency of phyA in transgenic seedlings may also, therefore, reduce expression of genes involved in light tolerance, thus contributing to the photooxidative damage observed.

Chapter 4. Mature Plant Development

4.1 Introduction

Phytochromes regulate two principal adaptive phenomena in light-grown plants. These are proximity perception, leading to shade avoidance reactions and photoperiodic perception, leading to the induction of flowering and tuberisation in some species (Smith 1995). Fluctuations in the spectral quality of daylight are detected via perception of the ratio of R to FR wavelengths (Holmes and Smith 1975). Such fluctuations occur daily (sunset and sunrise) and under vegetation canopies. Selective attenuation of R wavelengths by chlorophyllous tissues results in a significant decrease in the R:FR quantum ratio of transmitted light detected by plants lower in the canopy. The ability to detect neighbouring vegetation confers significant ecological advantage to plants. Neighbour detection initiates shade avoidance reactions, therefore enabling plants to compete for light (Smith 1992). Such reactions result in enhanced internode and petiole extension growth, increased apical dominance, reduced branching and retarded leaf development (Robson *et al* 1993). These physiological adaptations are accompanied by changes in the distribution of assimilates between leaves, stems and roots (Keiller and Smith 1989), and serve to elevate young leaves to a better-lit stratum in the canopy.

The roles of individual phytochromes in regulating these responses have been largely inferred from studies of mutant and transgenic plants. Mutants of tomato and *Arabidopsis*, deficient in phyA, display an almost indistinguishable phenotype from WT plants when grown in white light (van Tuinen *et al* 1995b, Whitelam *et al* 1993). Such phenotypes suggest that the regulation of mature plant morphology is predominantly regulated by light-stable phytochromes. Constitutive expression of an oat *PHYA* gene in a variety of species resulted in a light exaggerated phenotype, with plants displaying reduced stem elongation, decreased apical dominance and enhanced pigmentation when grown under natural light conditions (Keller *et al* 1989, Kay *et al* 1989, Boylan and Quail 1991, Cherry *et al* 1991). Tobacco plants expressing an introduced oat *PHYA* gene also showed pronounced growth inhibition under low R:FR ratios. This effect was shown to override the WT shade avoidance response and has been suggested to be due to retention of the FR-HIR, mediated by unusually high levels of phyA in the light-grown transgenic plants (McCormac *et al* 1991,1992).

Jordan *et al* (1995b) suggested this dwarf phenotype to result, primarily, from a reduction in active gibberellins. Such over-expression studies confirm the importance of phytochrome in regulating stem elongation and leaf development in mature plants and reveal the ability of phyA to adopt functions predominantly regulated by light-stable phytochrome species.

The End Of Day (EOD) response has been widely used to assess the phytochrome status of mature plants. Removal of Pfr by an EOD-FR treatment results in a qualitatively similar response to lowering of the Pfr level during the light period by reduction of the R:FR photon ratio. Treated plants display elongated internodes and petioles when compared to untreated controls. An inability to respond to EOD-FR treatments is therefore diagnostic of a deficiency in light stable phytochromes. Deficiency of phyB has been shown to result in elongated stems, petioles and leaves, and increased apical dominance in light-grown plants (Koorneef *et al* 1980, Chory *et al* 1989, Reed *et al* 1993). The phyB-deficient *lv* mutant of pea was shown to lack EOD responses to FR and displayed internode elongation in white light and R, a phenotype attributed to increased cell length (Nagatani *et al* 1990). Whilst phyB appears to be the predominant contributor to EOD FR-mediated elongation growth, such behaviour has been detected in *Arabidopsis* phyB-null mutants, indicating the involvement of other phytochromes in these responses (Devlin *et al* 1996). Recent work using mutants of *Arabidopsis* suggest phytochromes D and E to fulfil such a role (Devlin *et al* 1998,1999). The strong shade avoidance response of the *phyA phyB1 phyB2* triple mutant of tomato is comparable to that shown in the *phyA phyB phyD* mutant of Arabidopsis and provides further support for a role for additional phytochromes (phyE/F), in mature plants (Weller *et al* 2001).

Photoperiodic perception occurs in the leaves, but reproductive changes occur in shoot apices in response to the movement of signals throughout the plant (Jackson and Thomas 1997). Current evidence suggests a role for phyA in the photoperiodic detection mechanism of LDP, an idea supported by analysis of phyA-deficient mutants (Weller *et al* 1997a). Light stable phytochromes are thought to fulfil a role in the inhibition of flowering, but are not believed to be involved in daylength measurement (Jackson and Thomas 1997, Koornneef and Peters 1997, Devlin *et al* 1998,1999). Conversely, in SDP, phyB and C are believed to be involved in

determining sensitivity to photoperiod, while little direct evidence exists for a role for phyA (Jackson and Thomas 1997, Halliday *et al* 1997).

Phytochromes therefore perform a variety of discrete and overlapping regulatory functions throughout the development of light-grown plants. Mutations in genes encoding chromophore biosynthetic enzymes result in a deficiency of total phytochromes, with plants displaying characteristic phenotypic traits. Mutant plants are taller than WT under R, FR and white light and display increased apical dominance and retarded leaf development (Koornneef *et al* 1980, López-Juez *et al* 1990, Weller *et al* 1997b). Reduced levels of chlorophyll are always observed, which are accompanied by an increase in the chlorophyll *a*:*b* ratio of leaves (Koornneef *et al* 1985, Chory *et al* 1989b, Weller *et al* 1996,1997b). Perturbations in flowering behaviour vary between species. The *hy* mutants of *Arabidopsis* flower early (Whitelam and Smith 1991), while the *pcd1* mutant of pea is late flowering (Weller *et al* 1996). One feature of chromophore-deficient mutants is that they are able to recover later in development, a phenomenon complicating analysis of mature plants. Recovery of phenotype is believed to result from the "leaky" nature of mutations and/or the presence of differentially regulated isoforms of chromophore biosynthetic enzymes (Terry 1997, Davis *et al* 1999, Muramoto *et al* 1999). The constitutive expression of biliverdin reductase (BVR) in transgenic plants can overcome this problem, enabling the consequences of total phytochrome deficiency to be studied throughout plant development (Lagarias *et al* 1997). The targeting of BVR to different cellular compartments has subsequently provided a method of manipulating the severity of phytochrome deficiency in these plants (Montgomery *et al* 1999).

Experiments described here explore the consequences of total phytochrome deficiency throughout the light-regulated development of a SDP. *BVR* expression and resulting apophytochrome levels have been quantified in *Nicotiana tabacum* cv. Maryland Mammoth, expressing BVR in both the cytosol and plastid compartments. Internode elongation, leaf development and flowering behaviour under different photoperiods have been analysed. The effect of fluence rate on the development of transgenic plants has also been investigated.

4.2 Results

4.2.1 Plant phenotypes

Figure 8a shows WT and cytosol-targeted BVR tobacco plants, one week after transfer to soil. Cytosol-targeted BVR plants show slightly increased internode elongation, but little phenotypic difference is visible between these and WT plants at this developmental stage. However, when BVR is targeted to the plastid, the site of chromophore biosynthesis, phenotypic differences are considerably more pronounced (Fig.8b). Plastid-targeted *BVR*-transformed plants show significantly increased internode extension, decreased leaf area: length ratios and severe chlorosis. These characteristics are equally visible after 11 weeks (Fig.8c,d). At this stage, however, cytosol-targeted plants show an intermediate phenotype between plastid-targeted BVR and WT plants, consonant with a partial reduction in photoactive phytochrome species. Plants of all three groups at 1 week and 11 weeks after transfer to soil are represented in Figures 8e and 8f respectively.

4.2.2 Analysis of *BVR* expression

BVR expression was quantified in transgenic plants using northern analysis. Total RNA was extracted from equivalent leaves of each line at 68 days, blotted onto nylon membrane and probed with a radio-labelled *BVR* fragment. Blots were developed on a phosphorimager (Fig.9a). They were subsequently stripped and re-probed with an *18S* ribosomal control probe (Fig.9b), quantified and standardised to control signals (Figure 9c). The increased molecular mass of bands in plastid-targeted lines results from the plastid signal peptide sequence in these constructs. No *BVR* expression was observed in WT plants. Transgenic lines displayed equal levels of transcription at this developmental stage. No antibody was available to quantify BVR protein in these plants.

4.2.3 Analysis of phytochrome apoprotein

Expression of phytochrome apoproteins A and B was investigated using western analysis. Blots displaying PHYA abundance in WT and transgenic lines are presented in Figures 10a and 10b. As all lanes were equally loaded, results appear to show similar levels of apoprotein in WT and transgenic lines. However, owing to fluctuations between replicate blots and weak signal intensity, it could not be unequivocally demonstrated whether levels of this apoprotein are affected by BVR

Fig. 8a Wild-type (WT) and cytosol-targeted BVR plants (NtMcBVR2) one week after transfer to soil. Plants were grown under white light, with a 8 h photoperiod, at a fluence rate of 350 $\mu molm^{-2}s^{-1}$.

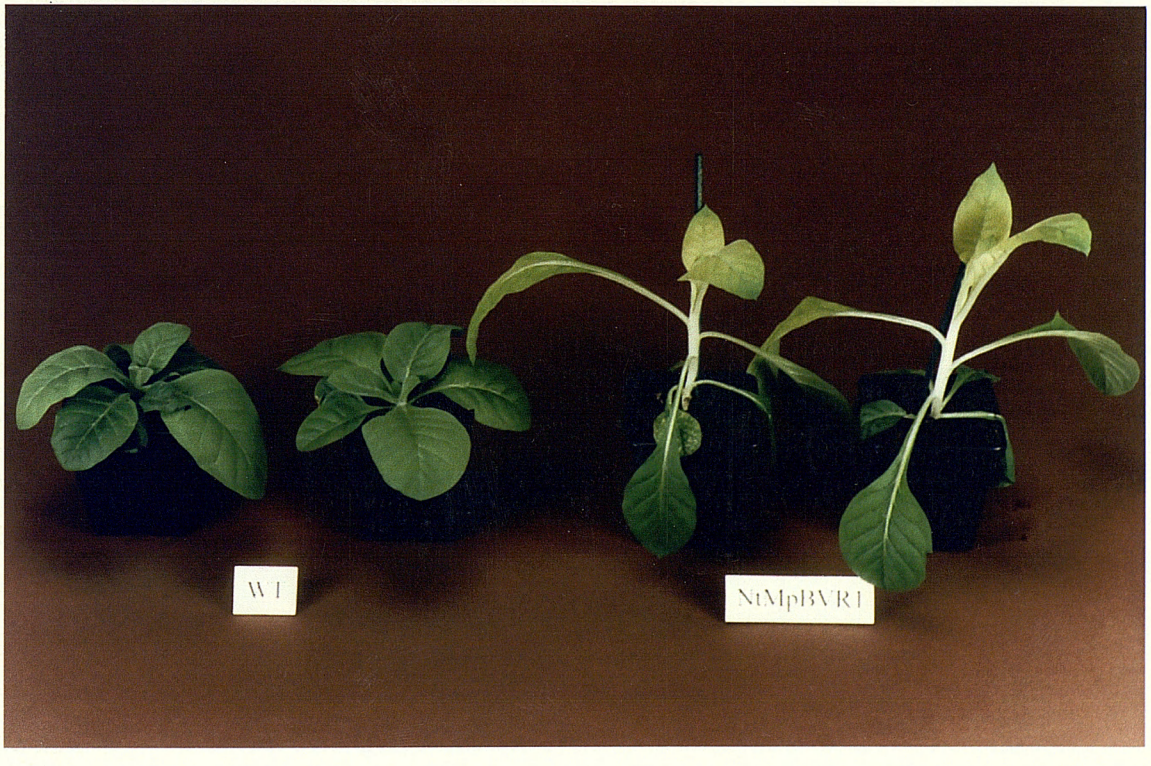


Fig. 8b Wild-type (WT) and plastid-targeted BVR plants (NtMpBVR1) one week after transfer to soil. Plants were grown under white light, with a 8 h photoperiod, at a fluence rate of 350 $\mu molm^{-2}s^{-1}$.

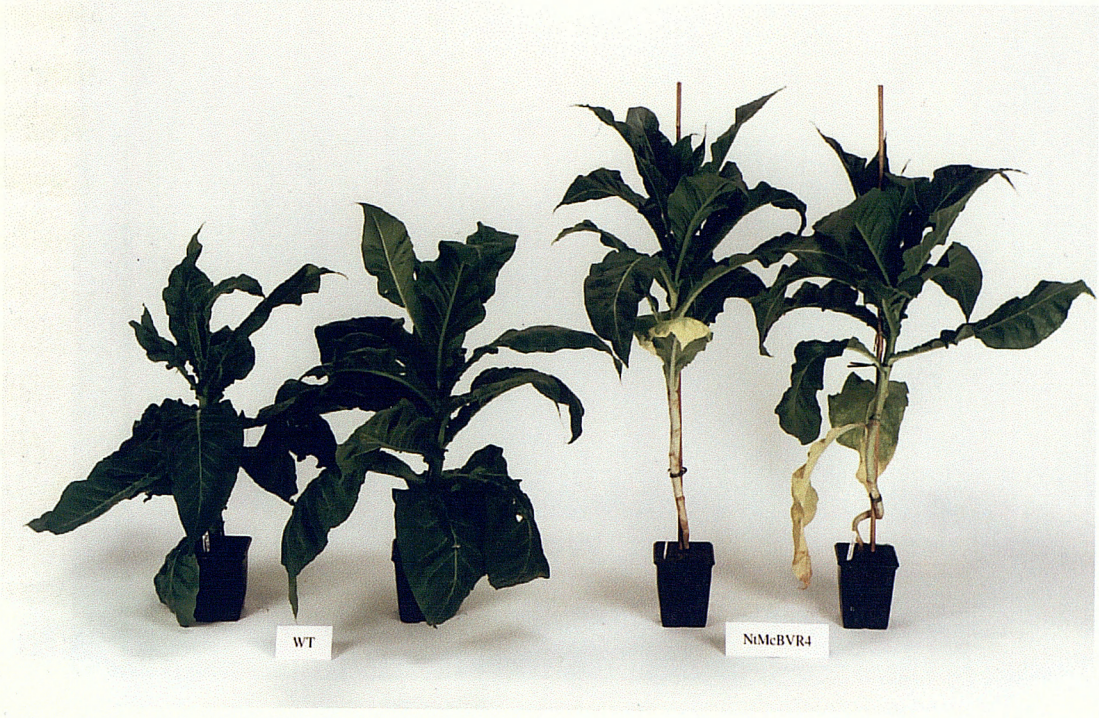


Fig. 8c Wild-type (WT) and cytosol-targeted BVR (NtMcBVR4) plants, 11 weeks after transfer to soil. Plants were grown under white light, with a 8 h photoperiod, at a fluence rate of 350 $\mu molm^{-2}s^{-1}$.

Fig. 8d Wild-type (WT) and plastid-targeted BVR (NtMp BVR4) plants, 11 weeks after transfer to soil. Plants were grown under white light, with a 8 h photoperiod, at a fluence rate of 350 $\mu molm^{-2}s^{-1}$.

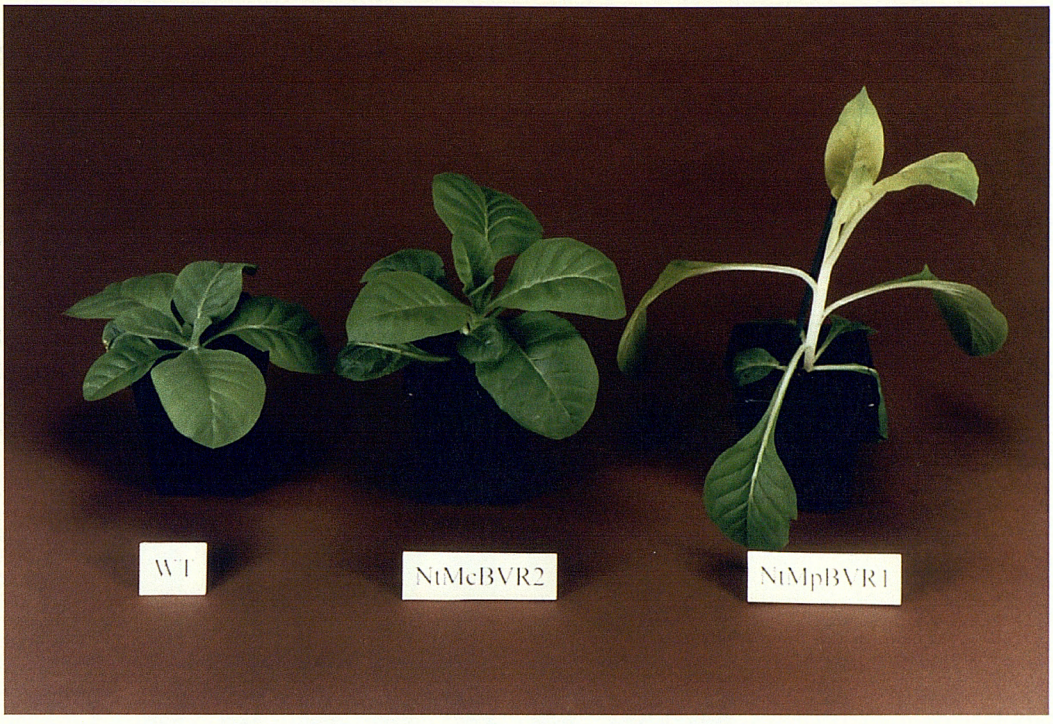


Fig. 8e Wild -type (WT), cytosol-targeted BVR (NtMcBVR2) and plastid-targeted BVR (NtMpBVR1) plants, 1 week after transfer to soil. Plants were grown under white light, with a 8 h photoperiod, at a fluence rate of 350 $\mu molm^{-2}s^{-1}$.

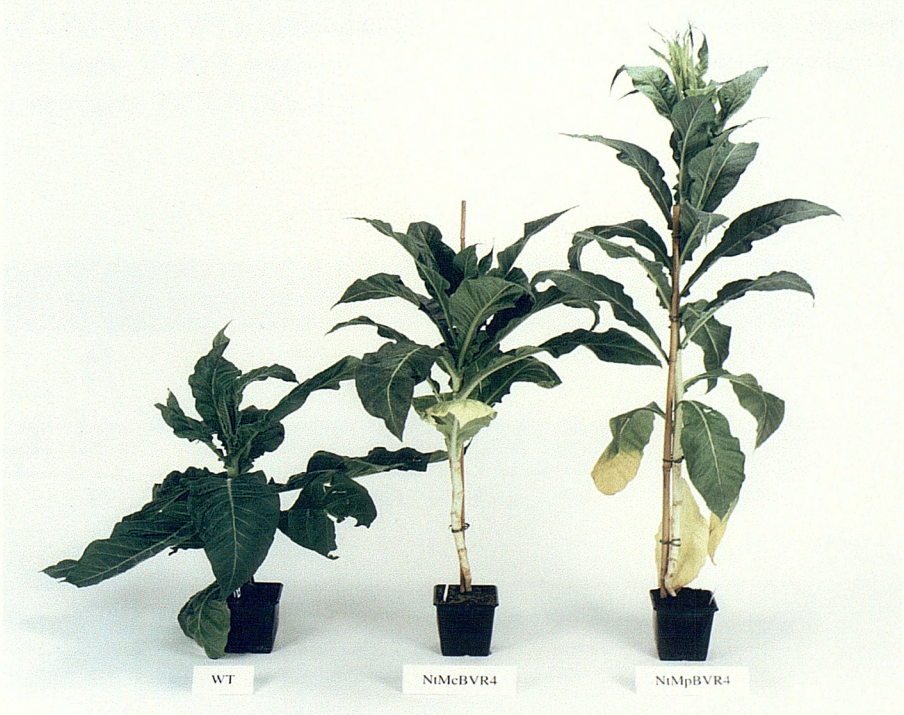


Fig. 8f Wild-type (WT), cytosol-targeted BVR (NtMcBVR4) and plastid-targeted BVR (NtMpBVR4) plants, 11 weeks after transfer to soil. Plants were grown under white light, with a 8 h photoperiod, at a fluence rate of 350 $\mu molm^{-2}s^{-1}$.

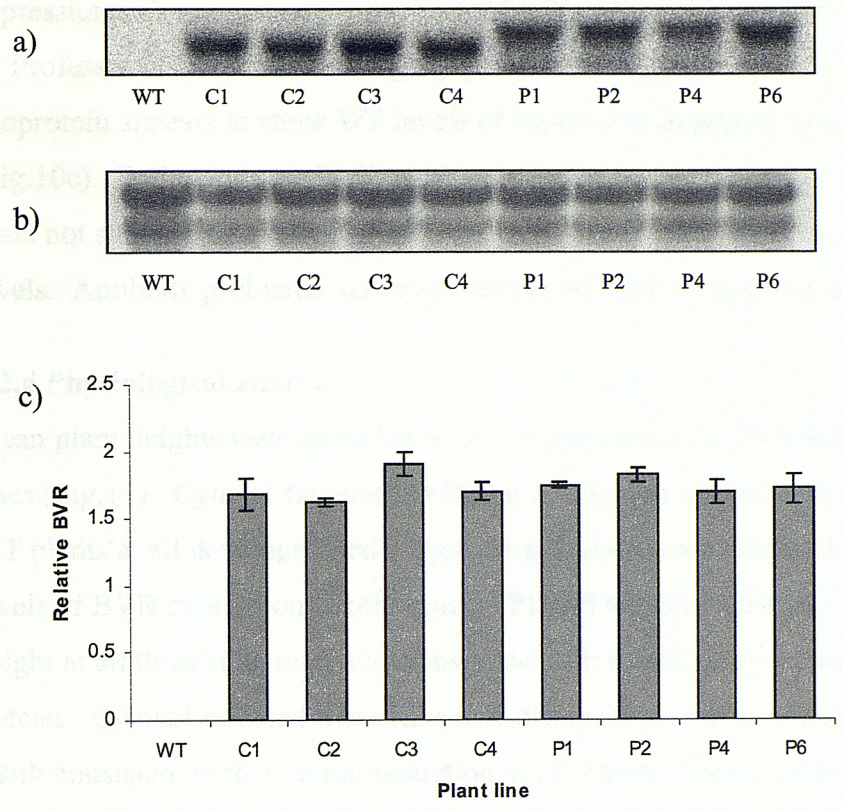


Fig. 9a,b,c Northern analysis of a)*BVR* and b)*18S* in mature (68 day-old) tissue samples of wild-type (WT), cytosol-targeted BVR (C1-4) and plastid-targeted BVR (P1-6) lines. c) *BVR* signal was quantified and standardised with respect to *18S*. Bars represent the S.E of two replicates.

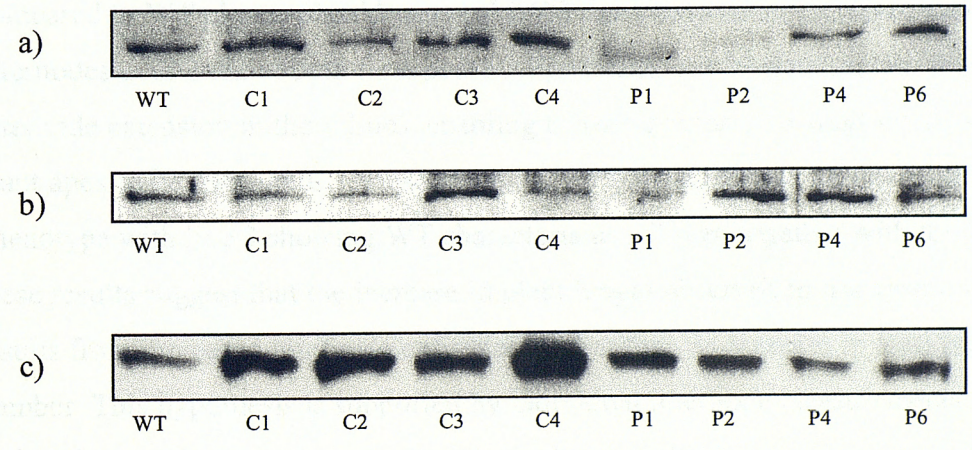


Fig.10 Western analysis of phytochrome A (a,b) in dark-adapted mature (68 day-old) leaf material, and phytochrome B (c) in light-grown mature (68 day-old) leaf material. Each is the result of a separate extraction.

expression. The experiment was repeated with different antiserum (AS32, provided by Professor G.Whitelam, University of Leicester, UK), with no success. PHYB apoprotein appears to show WT levels of expression in plastid-targeted BVR lines (Fig.10c). Following standardisation with the large subunit of Rubisco on stained gels (data not shown), cytosol-targeted BVR lines displayed a small increase in PHYB levels. Antibody problems, however, prevented further repetition of this experiment.

4.2.4 Physiological analyses

Mean plant heights were recorded at 24, 48 and 68 days in WT and all transgenic lines (Fig.11). Cytosol-targeted BVR line 3 (C3) was phenotypically comparable to WT plants at all developmental stages, despite northern analysis showing similar levels of BVR expression to other lines. Plastid-targeted lines showed increased plant height at all three time intervals, consonant with a deficiency in multiple phytochrome species. Cytosol-targeted lines 1,2 and 4 displayed an intermediate phenotype, a result consistent with a partial reduction in photoactive holophytochrome. Surprisingly, BVR protein and activity levels were higher in cytosol-targeted than plastid-targeted lines (Montgomery *et al* 2001).

Mean leaf numbers at 24 and 68 days are displayed in Figure 12. At both time points, no significant difference was observed in leaf number between WT and transgenic plants. Mean internode numbers (over 2mm) are represented in Figure 13. When compared to WT plants, plastid-targeted BVR lines showed an increased number of internodes over 2mm at both time points. This result can be attributed to increased internode extension in these lines, enabling a greater number of short internodes at the plant apex to be measured. Cytosol-targeted BVR lines showed an intermediate phenotype with line 3 showing WT characteristics. Taken together with the leaf data, these results suggest that the increase in plant height observed in transgenic lines results from increased internode extension, rather than an increase in total internode number. This hypothesis is supported by individual internode measurements (Fig.14), which show an increase in mean length of all internodes in plastid-targeted BVR lines, and cytosol-targeted lines 2 and 4.

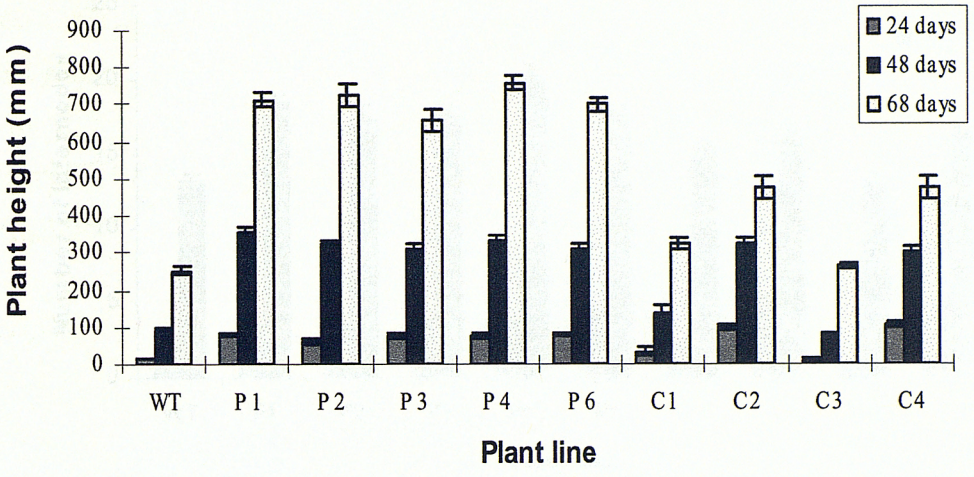


Fig.11 Mean plant heights in wild-type (WT), plastid-targeted (P1-6) and cytosol-targeted (C1-4) and BVR lines at 24, 48 and 68 days. n=10 ± S.E.

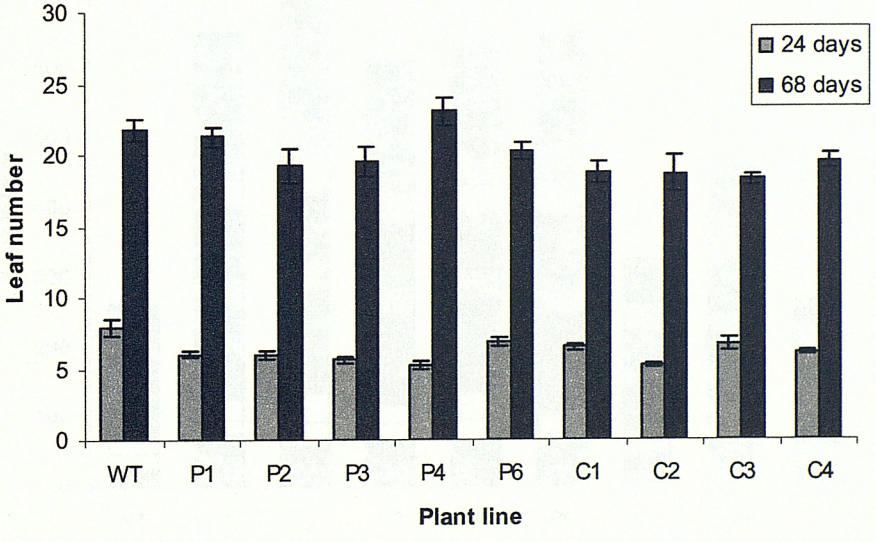


Fig.12 Mean leaf numbers in wild-type (WT), plastid-targeted (P1-6) and cytosol-targeted (C1-4) BVR lines after 24 and 68 days. n=10 ± S.E.

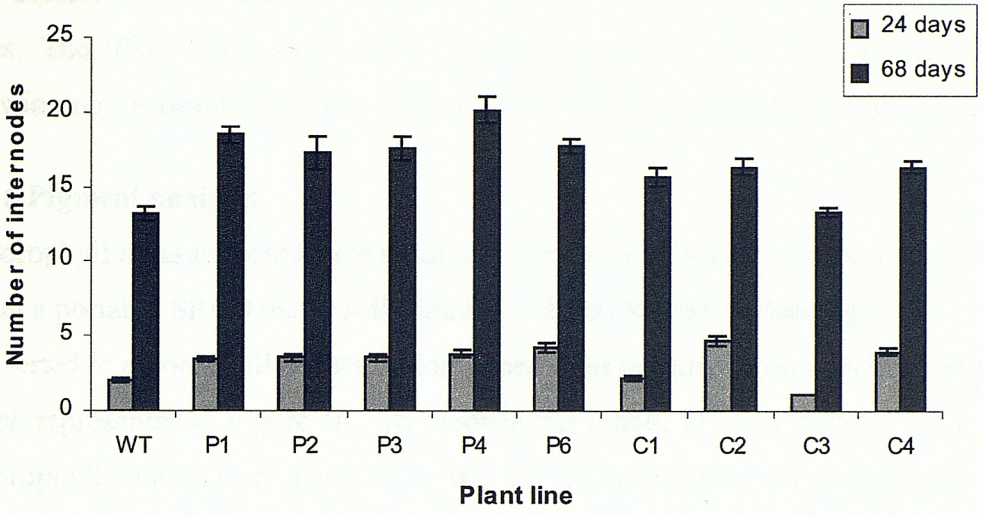


Fig.13 Mean number of internodes over 2mm in wild-type (WT), plastid-targeted (P1-6) and cytosol-targeted (C1-4) BVR lines, after 24 and 68 days. n=10 ± S.E.

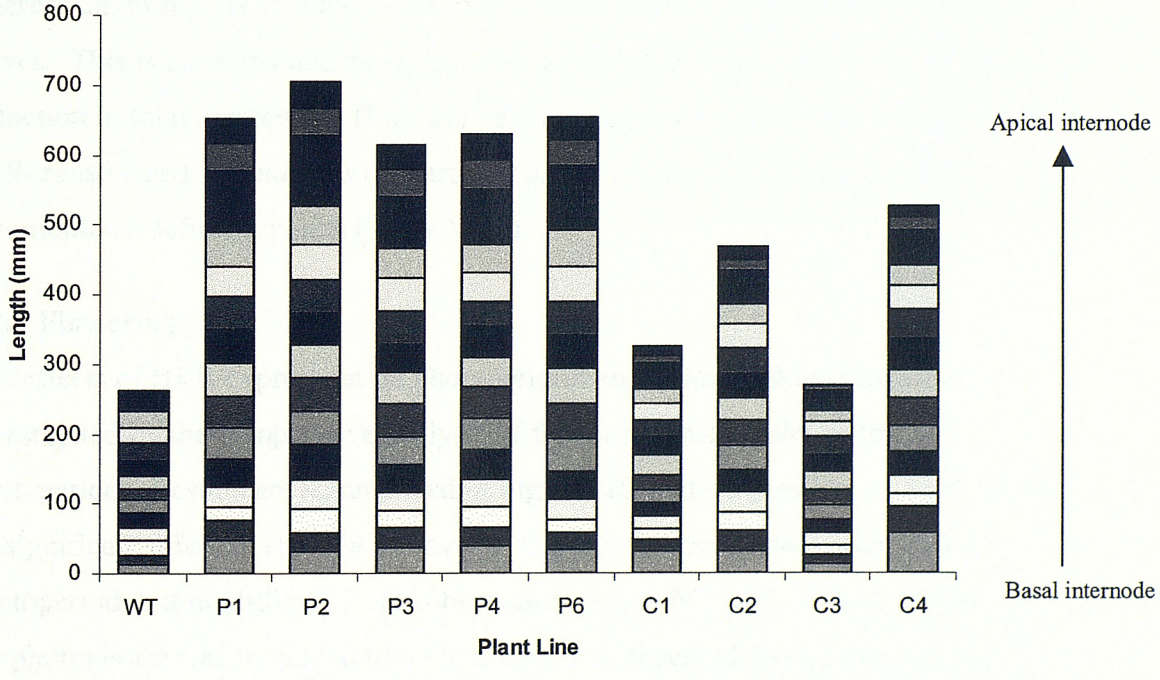


Fig.14 Mean internode lengths in wild-type (WT), plastid-targeted (P1-6) and cytosol-targeted (C1-4) BVR lines after 68 days. Each band represents the mean internode length of ten plants.

4.2.5 Leaf morphology

Plants were grown in controlled growth chambers under white light, at a fluence rate of 350 $\mu molm^{-2}s^{-1}$. Under these conditions, plastid-targeting of BVR protein resulted in a decreased leaf area:length ratio, a phenotype not observed in cytosol-targeted lines. The effect was more prominent in older leaves, with leaf no. 5 in line P4 showing no measurable decrease. Data is represented graphically in Figure 15.

4.2.6 Pigment analysis

Chlorophyll measurements were recorded, non-destructively, throughout growth using a portable SPAD meter (Minolta UK, Milton Keynes). Readings were converted to chlorophyll concentrations (measured by extraction) using the calibration graph represented in Figure 16. To produce this graph, 115 leaf discs of varying chlorophyll content were measured with a SPAD meter before their pigments were extracted in 80% acetone and quantified spectrophotometrically. A regression coefficient of 0.9216 suggests a significant correlation. Mean leaf chlorophyll concentrations of leaves 5 and 9 are represented in Figure 17. With the exception of line C4, cytosol-targeting of BVR appears not to disrupt chlorophyll biosynthesis, whereas targeting this enzyme to the plastid results in severe chlorosis of younger leaves. This is accompanied by an increase in chlorophyll *a*:*b* ratio (Fig.18) and a reduction in total carotenoids (Fig. 19). Severe chlorosis has also been observed in *BVR*-transformed *Arabidopsis* (Lagarias *et al* 1997), and is a characteristic of chromophore-deficient plants (Terry 1997).

4.2.7 Flowering

The effects of BVR expression on photoperiodic induction of flowering were investigated by the comparative analysis of flowering times under different photoperiods. Results are summarised in Figures 20a,c,d. Expression of BVR showed no significant effect in short (8 h) days. All plants flowered later under a 10.5 h photoperiod, but no difference was observed between WT and transgenic lines. As the photoperiod was increased to 13 h, differences appeared more pronounced. The earliest flowering times were observed in plastid-targeted BVR lines, with all transgenic plants flowering earlier than WT. When grown under long (16 h) days, flowering times in WT plants were significantly increased. Plastid-targeted BVR lines flowered considerably earlier in these conditions with cytosol-targeted BVR

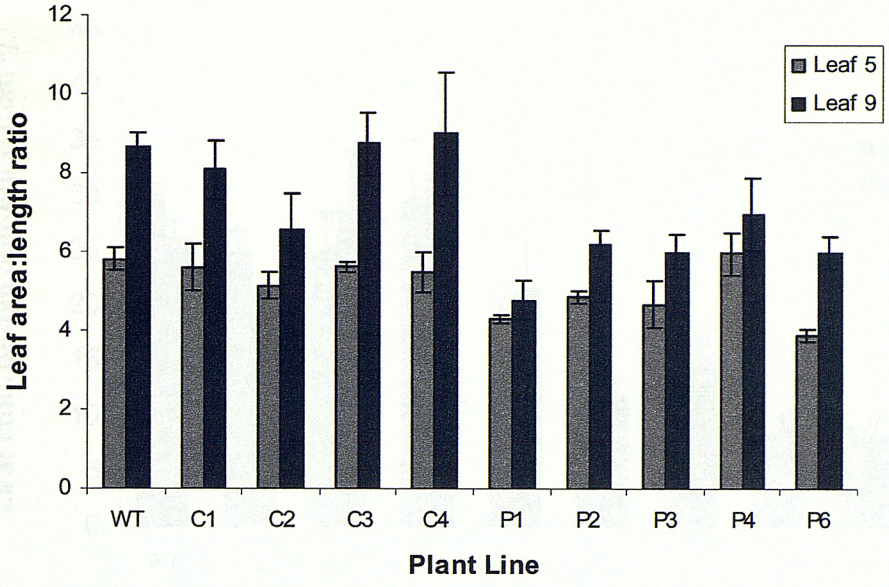


Fig. 15 Mean leaf area: length ratios of wild-type (WT), cytosol-targeted (C1-4) and plastid-targeted (P1-6) BVR lines at 40 days. Leaf numbers were counted from the plant apex. n=10 ± S.E.

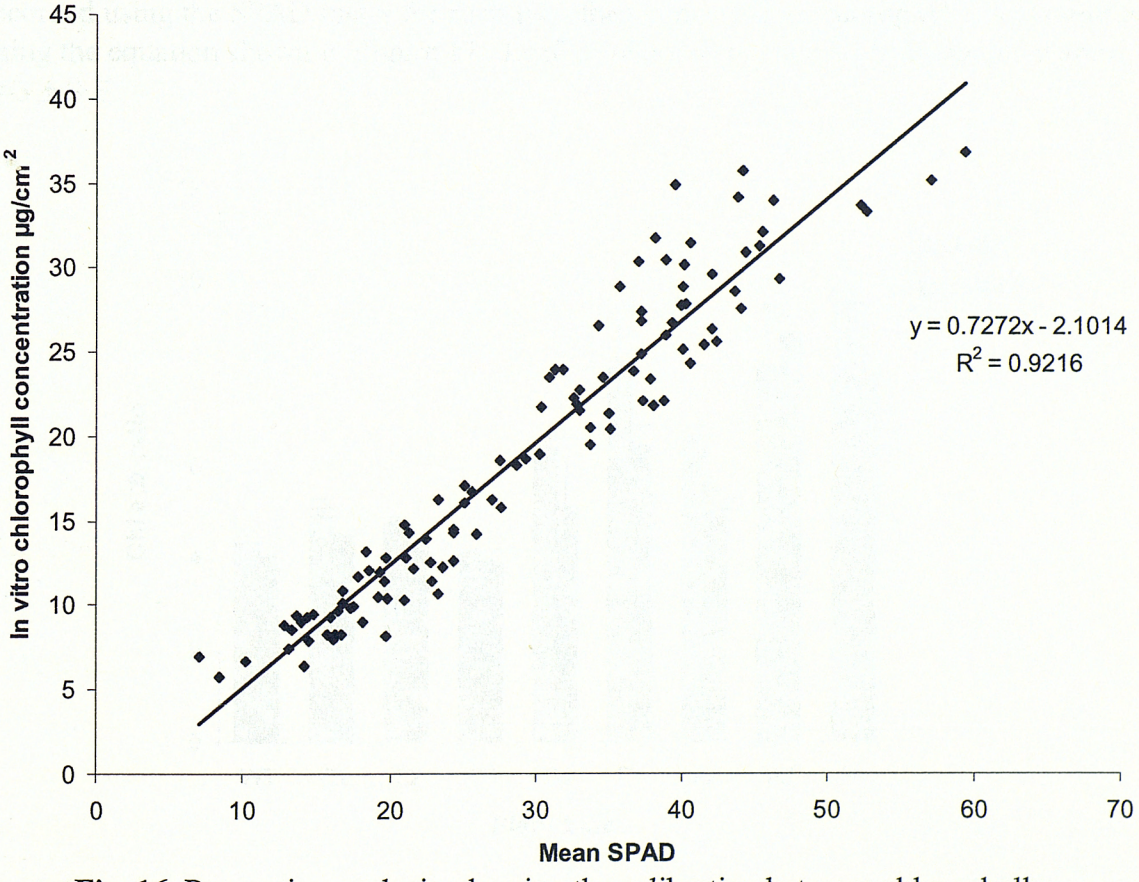


Fig. 16 Regression analysis, showing the calibration between chlorophyll concentrations recorded *in situ* by the SPAD meter and chlorophyll concentrations following acetone extraction. Leaf discs were measured with the SPAD meter before extraction in acetone and spectrophotometric quantification.

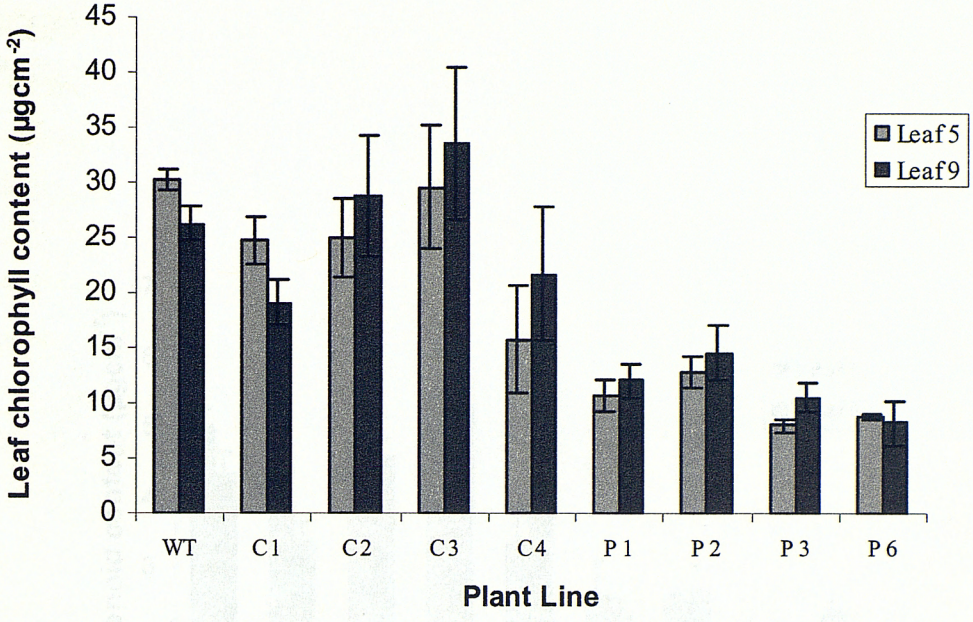


Fig. 17 Mean leaf chlorophyll concentrations of leaves 5 and 9 in wild-type (WT), cytosol-targeted (C1-4) and plastid-targeted (P1-6) BVR lines at 40 days. Measurements were recorded using the SPAD meter for each leaf, then converted to chlorophyll concentrations using the equation shown in Figure 17. Leaf numbers were counted from the plant apex. n=3 ± S.E.

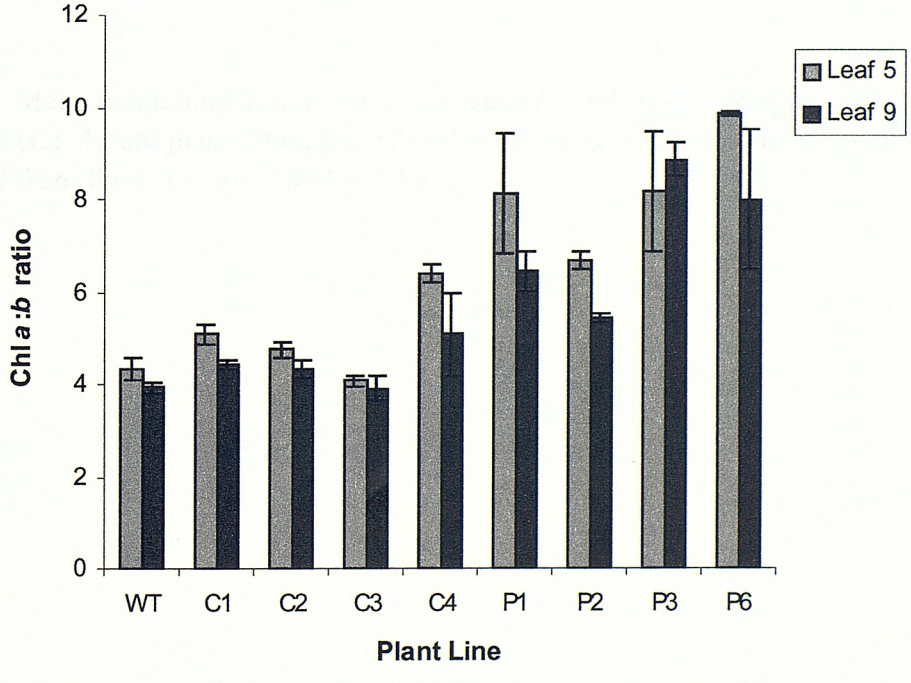


Fig. 18 Chlorophyll *a*:*b* ratio in leaves 5 and 9 of wild-type (WT), cytosol-targeted (C1-4) and plastid-targeted (P1-6) BVR lines at 40 days. Leaf numbers were counted from the plant apex. n=3 ± S.E.

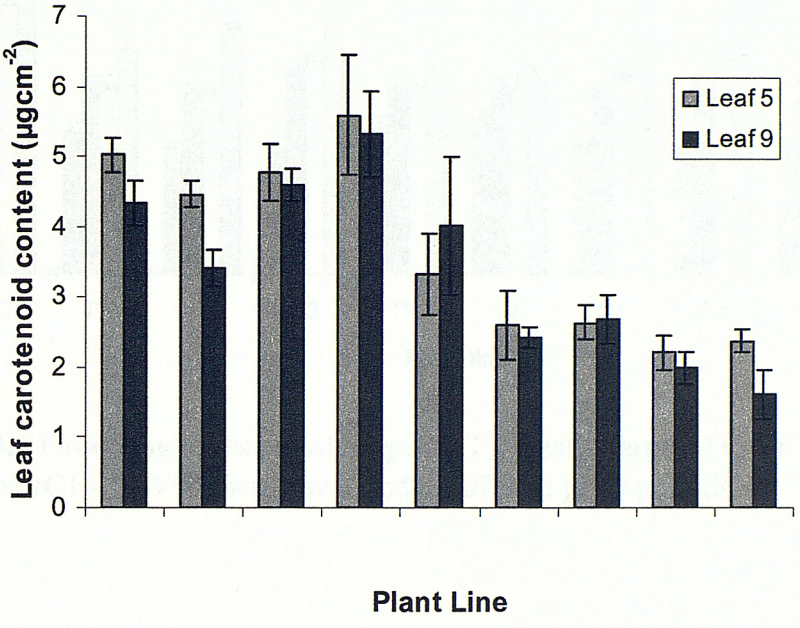


Fig. 19 Mean carotenoid concentration in leaves 5 and 9 of wild-type (WT), cytosol-targeted (C1-4) and plastid-targeted (P1-6) BVR lines at 40 days. Leaf numbers were counted from the plant apex. n=3 ± S.E.

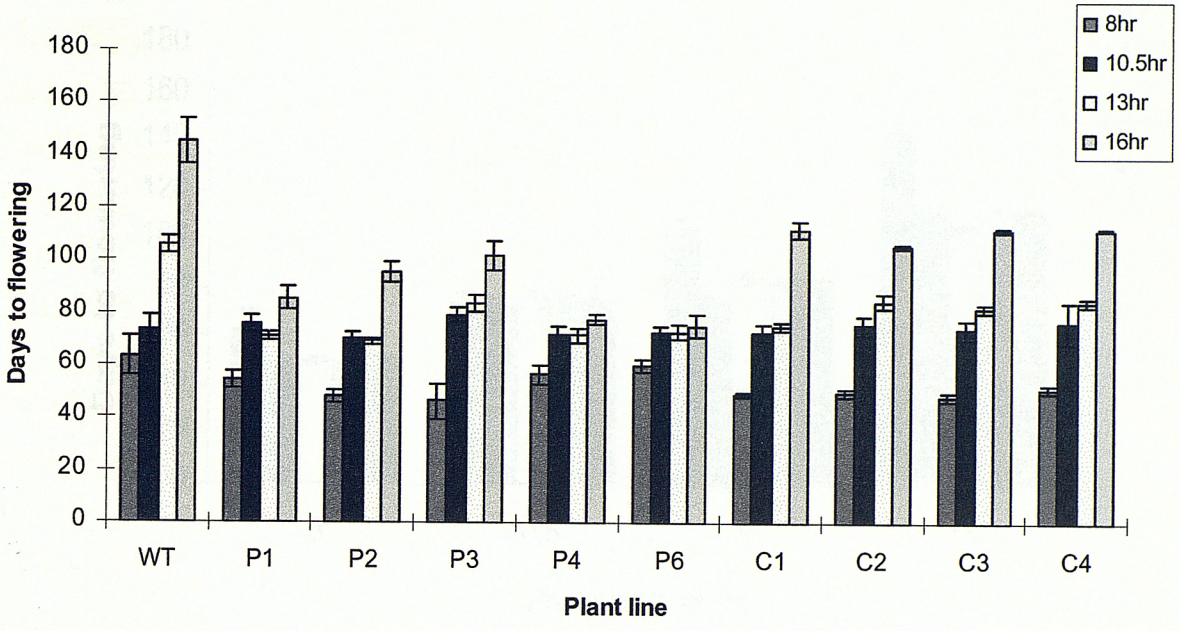


Fig.20a. Flowering times of wild-type (WT), plastid-targeted (P1-6) and cytosol-targeted (C1-4) BVR lines grown under different photoperiods. n=10 ± S.E.

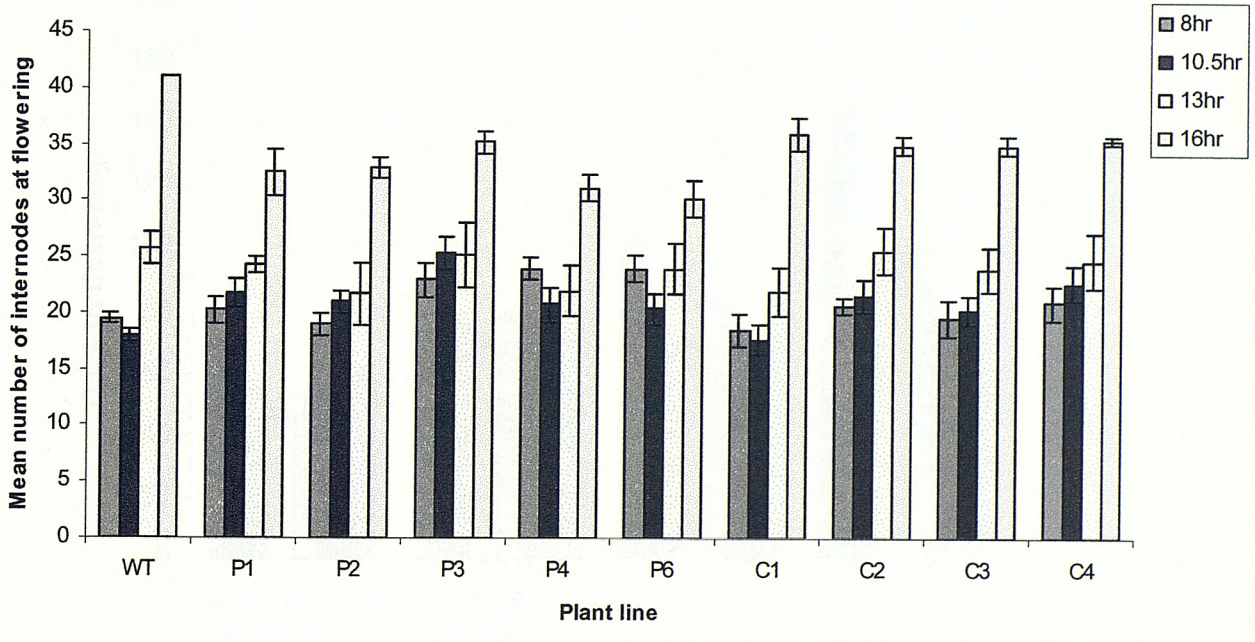


Fig.20b. Mean number of internodes at flowering of wild-type (WT), plastid-targeted (P1-6) and cytosol-targeted (C1-4) BVR lines grown under different photoperiods. n=10 ± S.E.

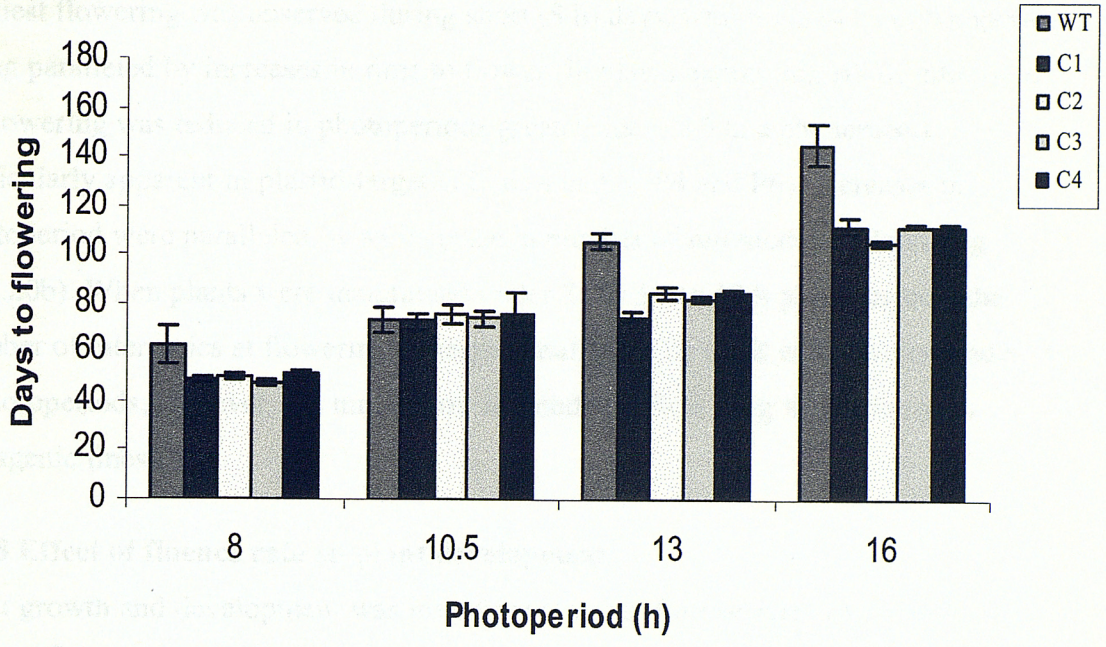


Fig.20c. Flowering times of wild-type (WT), and cytosol targeted lines 1-4 (C1-C4) grown under different photoperiods. n=10 ± S.E.

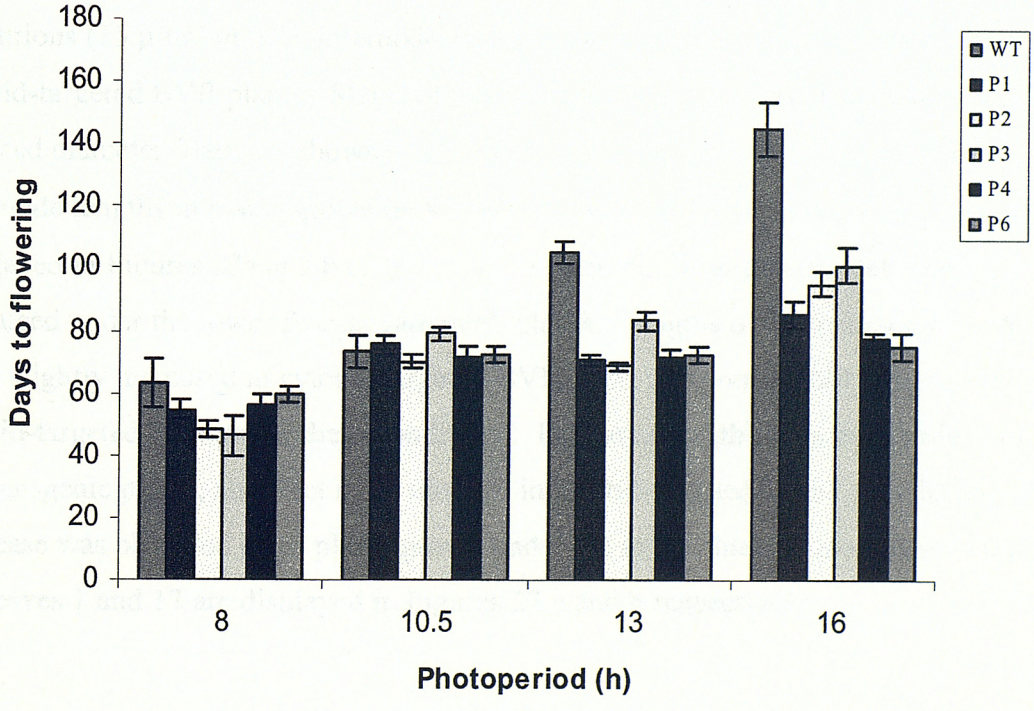


Fig.20d. Flowering times of wild-type (WT), and plastid- targeted lines 1-6 (P1-P6) grown under different photoperiods. n=10 ± S.E.

lines showing an intermediate response. The phytochrome-mediated photoperiodic control of flowering in MM tobacco is easily visualised in the WT data (fig. 20c,d). Earliest flowering was observed during short (8 h) days, with increases in photoperiod being paralleled by increases in time to flower. In plants expressing BVR, inhibition of flowering was reduced in photoperiods greater than 10.5 h, a phenomenon particularly apparent in plastid-targeted lines 4 and 6 (P4 and P6). Increases in photoperiod were paralleled by an increase in number of internodes at flowering (Fig.20b). When plants were maintained under 8, 10.5 and 13 h photoperiods, the number of internodes at flowering appeared unaffected by BVR expression. Under 16 h photoperiods, however, the number of internodes at flowering was reduced in transgenic lines.

4.2.8 Effect of fluence rate on plant development

Plant growth and development was investigated under fluence rates of 15 and 100 μmol $m^{-2}s^{-1}$. Seedlings were germinated as described previously and plants transferred to controlled growth chambers maintained at 22°C. A photoperiod of 16 h was provided by 40 W fluorescent tubes. The phenotype of transgenic plants was shown to be dependent on the light regime used (Fig.21 a,b,c). Under low fluence conditions (15 μmol $m^{-2}s^{-1}$), internode extension was increased in both cytosol and plastid-targeted BVR plants. Stems of plastid-targeted lines showed a considerably reduced diameter (data not shown), and leaves a reduced area:length ratio. Mean internode lengths in plants grown under fluence rates of 100 and 15μmol $m^{-2}s^{-1}$, are displayed in Figures 22a and b respectively. Fewer leaves and internodes were produced under the lower fluence rate in all plants. Lengths of individual internodes were slightly increased in cytosol-targeted BVR lines, and considerably increased in plastid-targeted lines under these conditions. Leaf area:length ratios were reduced in all transgenic plants, an effect more obvious in plastid-targeted lines. An additional decrease was observed in all plants grown under the lower fluence rate. Mean values for leaves 7 and 12 are displayed in Figures 23 a and b respectively.

Fig. 21 a,b,c Wild-type (a), cytosol-targeted BVR line 2 (b) and plastid-targeted line 3 (c), plants maintained under white light of different fluence rates for 7 weeks . Transgenic plants maintained under a fluence rate of 15 $\mu molm^{-2}s^{-1}$ displayed reduced leaf area:length ratios and increased internode extension. Plastid-targeted BVR lines also displayed reduced stem diameter under these conditions.

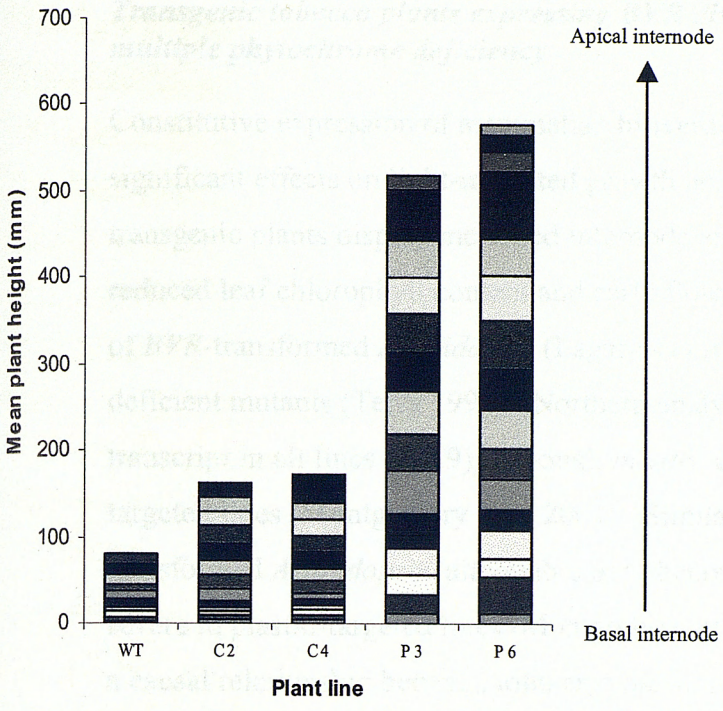


Fig. 22a Mean internode lengths of wild-type (WT), cytosol-targeted BVR lines 2 and 4 (C 2+C4), and plastid-targeted BVR lines 3 and 6 (P3+P6) maintained for 7 weeks under a fluence rate of 100 $\mu molm^{-2}s^{-1}$white light. Each band represents the mean internode length of five plants.

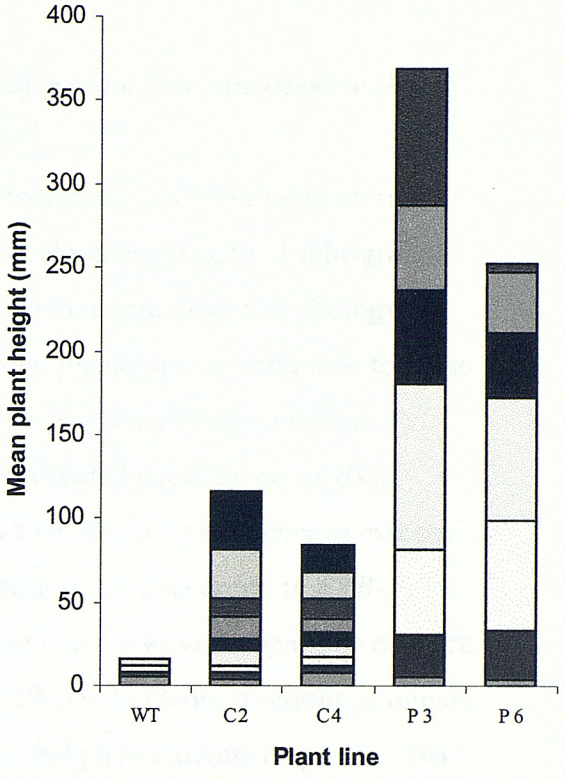


Fig. 22b Mean internode lengths of wild-type (WT), cytosol-targeted BVR lines 2 and 4 (C2+C4), and plastid-targeted BVR lines 3 and 6 (P3+P6) maintained for 7 weeks under a fluence rate of 15 $\mu molm^{-2}s^{-1}$white light. Each band represents the mean internode length of five plants.

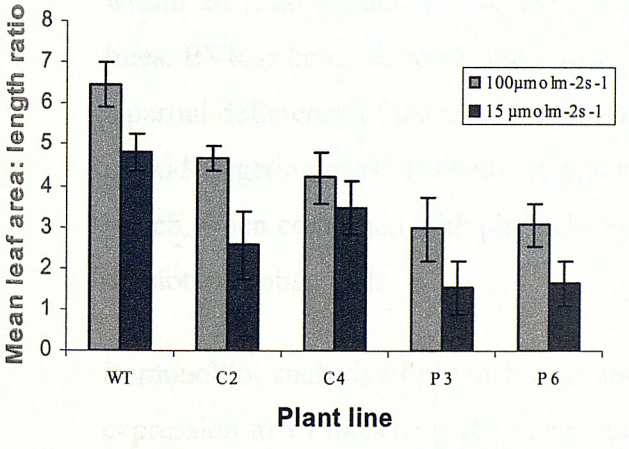


Fig.23a Mean leaf area:length ratios of leaf no.7 (leaves counted from plant apex) in wild-type (WT) cytosol-targeted BVR lines 2 and 4 (C2+C4), and plastid-targeted BVR lines 3 and 6 (P3+P6) maintained for 7 weeks under fluence rates of 100 and 15 $\mu molm^{-2}s^{-1}$white light. n=5 ± S.E.

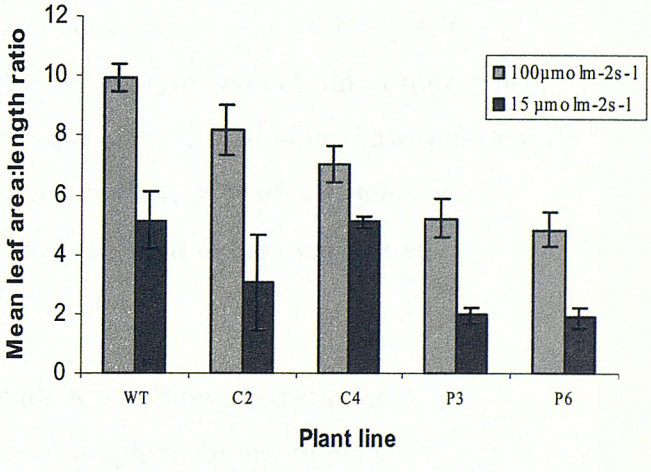


Fig.23b Mean leaf area:length ratios of leaf no. 12 (leaves counted from plant apex) in wild-type (WT) cytosol-targeted BVR lines 2 and 4 (C2+C4), and plastid-targeted BVR lines 3 and 6 (P3+P6) maintained for 7 weeks under fluence rates of 100 and 15 $\mu molm^{-2}s^{-1}$white light. n=5 ± S.E.

4.3 Discussion

Transgenic tobacco plants expressing BVR display phenotypes consistent with multiple phytochrome deficiency

Constitutive expression of mammalian biliverdin reductase (BVR) in tobacco has significant effects on light-mediated growth and development (Fig.8). Light-grown transgenic plants display increased internode extension, altered leaf morphology, reduced leaf chlorophyll content and early flowering, phenotypes comparable to those of *BVR*-transformed *Arabidopsis* (Lagarias *et al* 1997) and many chromophore-deficient mutants (Terry 1997). Northern analysis revealed equal levels of *BVR* transcript in all lines (Fig.9), although *in vitro* enzyme activity was higher in cytosol-targeted lines (Montgomery *et al* 2001). Similar results were recorded in *BVR*-transformed *Arabidopsis*, although plant phenotypes were always comparable or more severe in plastid-targeted lines (Montgomery *et al* 1999). Such observations eliminate a causal relationship between total enzyme activity and physiological response. No correlation was observed between BVR activity (Montgomery *et al* 2001) and severity of phenotype in the lines used here. It appears, therefore, that the sub-cellular targeting of BVR to the plastid compartment has a more influential effect on plant phenotype than total enzyme activity. This is likely to result from an increased efficiency of chromophore inactivation in these lines. It is hypothesised that plastid-targeting of BVR results in the metabolism of both biliverdin and phytochromobilin within the plastid, causing a severe chromophore deficiency. In cytosol-targeted lines, BVR is believed to compete with apoprotein for phytochromobilin, resulting in a partial deficiency of photoactive holophytochrome. It is also possible, however, that plastid-targeting of BVR results in additional perturbations of plastid metabolism which, when combined with phytochrome deficiency, result in the exaggerated phenotypes observed.

Immunoblot analysis of phytochrome apoproteins A and B revealed similar expression in all lines (Fig.10). The reduction of holophytochrome in plants expressing BVR must therefore result from induced chromophore deficiency, rather than destabilisation of apophytochromes. The poor quality of phyA immunoblots has been attributed to weak antibody homology.

BVR expression results in increased internode elongation throughout development

The internode elongation displayed in transgenic lines (Fig.14) is consistent with the loss of multiple phytochrome activities (Devlin *et al* 1998). A similar phenotype is observed in chromophore-deficient mutants during the early stages of growth. These mutants appear to "recover" during development, with mature *aurea* plants containing 70% of WT holophytochrome levels (López-Juez *et al* 1990). Two possibilities exist to explain this recovery. If mutant enzymes are "leaky", then recovery can be attributed to chromophore accumulation throughout development. It is also likely that more than one gene exists for each chromophore biosynthetic enzyme. The latter has been proposed to explain the developmental recovery of the tomato heme oxygenase mutant, *yg-2*, the analogous *hy1* mutant of *Arabidopsis* and the *pcd* mutants of pea (Terry 1997, Weller *et al* 1997b, Muramoto *et al* 1999, Davis *et al* 1999, 2001). Data presented here show plants expressing BVR to display internode elongation throughout their life cycle, thus providing the first quantitative evidence of severe total phytochrome deficiency throughout plant development.

Cell expansion is regulated by both light and hormones. Ethylene, abscisic acid (ABA) and cytokinins all act to inhibit stem elongation, while brassinosteroids, gibberellins and auxin have a promotory effect. Several studies suggest that phytochrome may regulate stem elongation rates by depleting auxin within the epidermis, and thus constraining growth of the entire stem (Neff *et al* 2000). Ectopic expression of phyA in tobacco was shown to correlate with reduced gibberellin (GA) levels and severe repression of stem elongation (Jordan *et al* 1995b). Mutants deficient in phyB have also been shown to exhibit altered responsiveness to, or metabolism of gibberellins (Weller and Reid 1993, López-Juez *et al* 1995, Foster and Morgan 1995). Phytochromes have since been shown to regulate the transcription of GA biosynthetic genes (Kamiya and Garcìa-Martìnez 1999). Although beyond the scope of this study, hormone quantification in different tissues of phytochrome-deficient plants would provide further insights into the mechanisms involved in such regulation.

BVR expression results in retarded leaf development and reduced pigment accumulation in transgenic plants

One of the most characteristic phenotypic traits in *BVR*-transformed plants is an impairment of normal leaf development. This is manifested as a reduction in area: length ratio and a severe chlorophyll deficiency (Figs.15, 17). When BVR is targeted to the cytosol, however, these traits are absent in mature plants. Retarded leaf development is a characteristic of chromophore-deficient plants (Koornneef *et al* 1980, López-Juez *et al* 1990, Weller *et al* 1997b), and was also observed in *BVR*-transformed *Arabidopsis* (Lagarias *et al* 1997).

The reduction of chlorophyll levels and increased chlorophyll *a*:*b* ratio observed in *BVR*-transformed tobacco were fluence-rate dependent only when BVR was targeted to plastids (Montgomery *et al* 2001). It is likely that metabolic perturbations, resulting from the localisation of BVR within plastids, contribute to this phenotype. Detailed analysis of tetrapyrrole regulation in these plants is discussed in chapters 5 and 6. An increased chlorophyll *a*:*b* ratio was also observed in chromophore mutants of *Arabidopsis* and pea (Koornneef *et al* 1985, Weller *et al* 1996, 1997b). Photobiological studies in tobacco revealed a decrease in chlorophyll content of leaves treated with an end-of-photoperiod FR pulse (Casal *et al* 1990). The ratio of chlorophyll *a*:*b* in these plants, however, remained unchanged. In *BVR*-transformed plants, however, low levels of Pfr are present throughout the whole greening period and may therefore contribute to the differences observed. The additional metabolic consequences of targeting BVR to the plastid compartment further complicates comparative analysis.

Chlorophyll *b* is formed from the oxygenation of Chlide *a* (Tanaka *et al* 1999). The majority of chlorophyll *b* is associated with the light-harvesting complexes of photosystem II (PSII). Plants respond to fluctuating light conditions by altering the ratio of photosystems I and II. This maintains a balanced energy distribution and prevents photooxidative damage. The chlorophyll *a*:*b* ratio measured in leaf pigment extractions (Fig.18) may therefore reflect the photosystem ratio within plastids at the time of harvesting. A deficiency in PSII Chlorophyll *a*/*b* binding proteins would serve to reduce the light-harvesting capacity of PSII, thus preventing over-excitation of the PSII reaction centre in a situation where the available light exceeds the capacity

for utilization (Becker *et al* 1992). The functionality of photosystems in plants expressing BVR could be investigated by measuring chlorophyll fluorescence.

A commensurate decline in carotenoid and chlorophyll levels was observed in leaves of plastid-targeted BVR lines (Fig.19). Carotenoids fulfil an important photoprotective role throughout development and are synthesised exclusively in plastids (reviewed by Rau 1983). A recognised correlation exists between the regulation of chlorophyll and carotenoid levels in angiosperms (Hartel and Grimm 1998). The decline of both chlorophyll and carotenoids in leaves of plastid-targeted BVR plants represents a reduction in pigment-protein complexes, which bind carotenoids and require chlorophyll for stabilisation. The possibility exists that the absence of Mg-tetrapyrroles in plastid-targeted BVR lines results in an over-accumulation of geranyl geranyl diphosphate. This compound is a common precursor to both carotenoids and the phytol side chain of chlorophyll (Tanaka *et al* 1999). Such an accumulation could therefore negatively feedback on carotenoid biosynthesis, resulting in the observed deficiency.

The effect of BVR expression on leaf development could be further studied using infrared gas analysis and chlorophyll fluorescence. Quantum yield measurements of O_2 evolution, CO_2 fixation and photosystem II, using chlorophyll-deficient mutants of tobacco and maize revealed plants to possess high capacities for photosynthesis (Edwards *et al* 1993). The chlorophyll-deficient *aurea* mutant of *Nicotiana tabacum* has also been shown to possess greater photosystem I activity, higher photosynthetic rates at saturating irradiances and higher carboxylation efficiency than WT plants (Santrucek *et al* 1992). Only minor perturbations in photosynthetic activity were observed in the chromophore-deficient *aurea* mutant of tomato (López-Juez *et al* 1990, Becker *et al* 1992, Smith *et al* 1993), despite a severe leaf chlorophyll deficiency and a reduction in chloroplast thylakoids (Koornneef *et al* 1985). As photosynthetic efficiency is observed to decrease with leaf age, the high photosynthetic activity displayed in chlorophyll-deficient mutants has been attributed to retarded leaf development in these plants (López-Juez *et al* 1990, Siffel *et al* 1993). Photosynthetic measurements would therefore enable comparative analysis of leaf development between *BVR*-transformed tobacco and chromophore-deficient mutants.

BVR expression reduces photoperiodic sensitivity in transgenic plants

Maryland Mammoth tobacco is a well-characterised SDP. It has been proposed that phyB is responsible for inhibition of flowering under non-inductive conditions in WT plants. PhyC is believed to interact with the *MM* gene to inhibit flowering in long days (Halliday *et al* 1997). It can be concluded from flowering experiments that MM tobacco has a critical daylength between 10.5 and 13 h (Fig.20a,c,d). Such data is consistent with an inhibitory role for phytochrome in controlling flowering in SDP. Expression of BVR reduces the phytochrome-mediated inhibition observed in WT plants, resulting in earlier flowering times in photoperiods greater than 13 h. Reduced inhibition of flowering by long days is most apparent in plastid-targeted lines 4 and 6. Despite showing equal levels of *BVR* expression and activity to other lines, cytosol-targeted line 3 is phenotypically comparable to WT plants throughout development. Flowering data, however, shows this line to behave similarly to other cytosol-targeted lines in every photoperiod. One possibility is that the threshold level of phytochrome varies between different responses and the reduction of phytochrome in this line, although not sufficient to alter internode extension, alters flowering response to photoperiod. No published evidence exists to support this assumption. Levels of *BVR* expression may also differ between internode and leaf tissue in this line, although earlier results suggest there not to be a correlation between BVR activity and physiological response. Another possibility is that the species of active phytochrome reduced by BVR expression are different in this line. However, it is likely that both internode elongation and flowering response are mediated primarily by phyB.

When grown under long days in glasshouse conditions at 28°C, MM plants showed no evidence of flowering after 8 months, whereas plastid-targeted BVR lines had flowered after 4 months (Montgomery *et al* 2000). The inability of WT plants to flower under long day conditions is in apparent contradiction to the results presented here. However, it is likely that temperature differences between experiments are critical, as Roberts (1939) reported flowering of MM plants under long days at reduced temperatures. Differences in plant growth conditions, such as fluence rate, light quality, nutrient application and watering regimes are also likely to have contributed to the variation observed.

MM tobacco is generally regarded as a qualitative short-day plant. The data presented here, however, show a quantitative flowering response at temperatures below 22 °C. Under these conditions, flowering in long days is delayed rather than prevented. The phytochrome deficiency induced by plastid-targeting of BVR is therefore sufficient to release the qualitative inhibition of flowering by long-day photoperiods. An impairment of normal flowering time has also been observed in chromphore-deficient mutants of *Arabidopsis* and pea and is consistent with a deficiency in photoactive holophytochrome (Whitelam and Smith 1991, Weller *et al* 1996). The *hy1* mutant of *Arabidopsis* shows early flowering but retains photoperiodic sensitivity to night-break treatments, suggesting that plants possess sufficient phytochrome levels to retain this response (Reed *et al* 1994). The reduced photoperiodic sensitivity observed in plastid-targeted lines could therefore result from an increased phytochrome deficiency in these plants.

Alternatively, results may reflect differences in signalling pathways between long and short-day species. The recently identified *Se5* mutant of rice, a SDP, displayed early flowering behaviour and was insensitive to extended light treatment with fluorescent lamps (Izawa *et al* 2000). Molecular analysis of this mutant indicated that it lacks heme oxygenase, implicating a chromophore deficiency in these plants (Terry 1997, Weller *et al* 1997b, Muramoto *et al* 1999, Davis *et al* 1999, 2001). As in *BVR*-transformed tobacco, increasing photoperiod exacerbated the difference between mutant and WT responses. In the model SD species *Pharbitis* and *Xanthium*, flowering response is determined almost exclusively by the length of the dark period rather than the length and quality of the light period (Thomas 1991). A night-break treatment given during an extended dark period can prevent flowering, although the length, fluence and timing of such treatments is crucial (Thomas 1991). Night-breaks delayed the flowering of *N. tabacum* cv. Hicks containing the *MM* gene, a response enhanced by the overexpression of phyA, B or C (Halliday *et al* 1997). The role of phytochrome in the flowering response of *BVR*-transformed plants could therefore be further investigated by analysis of responsiveness to night-break treatments. One further possibility is that the early flowering behaviour observed in chromophore-deficient plants results from stress, caused by photosynthetic deficiency in chlorotic leaves. This hypothesis could be addressed by analysis of photosynthetic capacity using infrared gas analysis.

An attempt was made to investigate the roles of individual phytochromes in regulating photoperiodic sensitivity in MM tobacco. Leaf discs of WT plants were transformed with antisense constructs of phytochromes A and B, under a constitutive promoter (prepared by S. Jackson, HRI Wellesbourne). Despite showing strong expression of the antisense genes, resulting transgenic plants showed no decrease in levels of phytochrome transcripts or proteins, and were phenotypically indistinguishable from WT plants under continuous R and FR (data not shown). These plants were therefore not subject to further analyses. It is possible that the tetraploid nature of tobacco makes antisense technology an inappropriate method of manipulating phytochrome levels and an alternative approach, such as virally–induced gene silencing may prove more successful in the future (Baulcombe 1999, 2001).

The phytochrome-deficient phenotype of plants expressing BVR is exaggerated by growth under a reduced fluence rate of white light

In addition to wavelength, photoperiod and temperature, results presented here also suggest that the fluence rate of light under which plants are grown can considerably affect phenotype. Under a low fluence rate of white light (15 $\mu molm^{-2}s^{-1}$), transgenic plants displayed elongated internodes and reduced leaf area:length ratio (Figs. 21-23). This effect was most severe for plastid-targeted lines, which also showed reduced stem diameter and decreased stature. It is possible that the latter result from photosynthetic deficiency in these lines. One possible function of the cryptochromes is to enhance the effectiveness of Pfr (Mohr 1994). Studies in the chromophore mutant, *aurea*, showed B to sensitise the mutant to residual levels of phytochrome. This allowed normal gene expression and survival of the plant. The possibility exists therefore, that under the low fluence rate of white light used here, B wavelengths were limiting. This would result in an inability of plants to sensitise residual levels of phytochrome, thus enhancing the deficiency. Work on *Sinapsis alba* (Casal and Smith 1988a,b) demonstrated the promotion of internode elongation by low phytochrome equilibria in the internode to be enhanced by B perceived by the leaves. The authors suggest that B sets the responsivity of the internode to Pfr. The limiting fluence rate of B used here may therefore exacerbate an existing phytochrome deficiency in *BVR*-transformed plants, resulting in the exaggerated phenotype observed.

Chapter 5. Etioplast development

5.1 Introduction

In the absence of light, angiosperm proplastids develop into etioplasts, organelles distinguishable by their characteristic internal membrane system termed prolamellar bodies (PLBs). These are comprised of highly regular "paracrystalline" networks of tubular membranes, from which prothylakoid membranes extend (Rascio *et al* 1976, Sundqvist and Ryberg 1989). The paracystalline structure of PLBs is dependent on interactions between pigment-protein complexes and membrane lipids. The predominant protein in PLBs is protochlorophyllide oxidoreductase (POR) (Ikeuchi and Murakami 1982, Shaw *et al* 1985, Ryberg *et al* 1986), which forms photoactive ternary complexes with protochlorophyllide (Pchlide) and NADPH. Mutant studies have revealed that Pchlide synthesis is a prerequisite for the development of highly regular PLBs (Mascia 1978, Mascia and Robertson 1978).

Paradoxically, although light is essential for chlorophyll biosynthesis, illumination exerts a rapid and dramatic negative regulation on POR at the levels of enzyme activity and protein accumulation (Kay and Griffiths 1983). This striking contradiction can be explained by the existence of distinct and differentially regulated POR genes (Armstrong *et al* 1995, Reinbothe *et al* 1996). The authors suggest that PORA is confined to the very early stages of transition from etiolated to light growth, while PORB sustains chlorophyll biosynthesis in green tissues. The recently identified PORC displays increased expression upon the illumination of etiolated seedlings and is consistently expressed in immature and mature tissues (Oosawa *et al* 2000). In addition to the biosynthesis of chlorophyll, another possible role for PORA might be to serve as a buffer against photo-oxidative damage caused by the illumination of previously etiolated, Pchlide containing tissues (Reinbothe *et al* 1996, Runge *et al* 1996). Such a function would be consistent with strong expression of PORA in etiolated angiosperm seedlings and the rapid disappearance of this mRNA during the early stages of white-light induced chlorophyll accumulation (Armstrong *et al* 1995, Holtorf *et al* 1995). This proposal does not, however, preclude a continuous presence and activity of PORA in green plants, albeit at a reduced level. The dependence of PORA transport into plastids on its substrate Pchlide has been previously documented (Reinbothe *et al* 1995). Such dependence was proposed to

result in an inability of the plastid to sequester pPORA upon illumination, thus providing a regulatory mechanism controlling Pchlide and PORA supply to the prolamellar body simultaneously. Research has since shown PORA to be imported into plastids using the general import route, independent of Pchlide (Aronsson *et al* 2000). A recent and controversial hypothesis suggests that Pchlide *a* and Pchlide *b* form supramolecular complexes with NADPH, PORA and PORB in the prolamellar body (Reinbothe *et al* 1999). These complexes, termed LHPP (light harvesting-POR-protochlorophyllide) complexes, are proposed to collect sunlight for rapid chlorophyll *a* biosynthesis and simultaneously dissipate excess light energy to Pchlide *b*. An absence of detectable Pchlide *b* in etiolated tissue does, however, question existence of such a complex (Scheumann *et al* 1999, Armstrong *et al* 2000).

In plants, the first committed precursor of tetrapyrroles, 5-aminolevulinic acid (ALA), is synthesied from glutamate in three steps, involving chloroplast-encoded $tRNA^{Glu}$ (Kannangara *et al* 1988, Kumar *et al* 1996a). The enzyme glutamyl-tRNA reductase (GluTR), catalyses the reduction of Glu-$tRNA^{Glu}$ to glutamate-1-semialdehyde (GSA). The subsequent transamination of GSA by the enzyme glutamate-1-semialdehyde 2,1 aminotransferase (GSA-AT) produces ALA. Condensation of two molecules of ALA by the enzyme ALA dehydratase yields the pyrrole porphobilinogen. The first circular tetrapyrrole, uroporphyrinogen III, formed from four porphobilinogen molecules, provides a central template from which all biologically functional tetrapyrroles are made. The successive decarboxylation and oxidation of uroporphyrinogen III produces protoporphyrin IX, the major branch point intermediate of the pathway. At this point, chelation of Fe^{2+} by ferrochelatase produces heme, while chelation of Mg^{2+} by Mg-chelatase is the first step in the formation of chlorophyll (von Wettstein *et al* 1995, Grimm 1998). Phytochromobilin is produced from the oxidation and successive reductions of heme (Terry *et al* 1995, Weller *et al* 1996).

The branching of biosynthetic pathways enables plants to co-ordinate the synthesis of related compounds from common precursors during an integrated developmental programme. Such metabolic plasticity requires precise monitoring of the relative flux of intermediates through branch points and implies the existence of tightly controlled regulatory mechanisms. The chelatase branch point must be subject to exquisite

regulation for a number of reasons. Firstly, different tetrapyrroles are needed in quite different amounts in different parts of the plant. For example, chlorophyll is only found in photosynthetic cells, principally in leaves and stems, whereas heme is required in every cell. Regulation of the pathway is also required to avoid the accumulation of photo-toxic tetrapyrrole intermediates. Both heme and the Pchlide ternary complex are well-documented negative regulators of ALA biosynthesis in dark-grown seedlings (Beale and Weinstein 1990, Terry and Kendrick 1999). Metabolic flux through the Mg-porphyrin branch of the pathway has also been proposed to perform a regulatory function in controlling the transcription of nuclear-encoded plastid proteins (Papenbrock *et al* 2000).

The work described here examines the consequences of BVR expression on plastid development, other than through the elimination of phytochrome. Expression patterns of tetrapyrrole biosynthetic enzymes in dark-grown transgenic plants have been studied using northern and western analysis. Biochemical quantification of tetrapyrrole intermediates has been used in conjunction with inhibitor and precursor feeding studies to investigate pathway flux. The effect of BVR expression on etioplast morphology has also been examined using transmission electron microscopy.

5.2 Results

5.2.1 Protochlorophyllide quantification

Protochlorophyllide (Pchlide) was spectrophotometrically determined in 11 day-old dark-grown seedlings (Fig. 24). Results were highly repeatable, with a 15-20% decrease being observed in cytosol–targeted BVR lines and a 50-55% decrease in plastid-targeted lines. Such results are consistent with the reduced chlorophyll levels observed when dark-grown seedlings are transferred to light (data not shown). Pchlide levels were paralleled by a concomitant decrease in its binding enzyme POR (Fig. 25 a,b). The antibody recognised a band of approximately 37 kDa, a mass consistent with that previously reported for mature POR proteins in other species (Lebedev and Timko 1998, Terry *et al* 2001). PORA and B are not differentially distinguishable using immuno-detection.

5.2.2 ALA synthesis

Seedlings were incubated in phosphate buffer ± levulinic acid (LA), a competitive inhibitor of ALA dehydratase (Beale and Castelfranco 1974), for 6 h at 23°C. ALA was quantified spectrophotometrically and the difference between samples incubated with and without LA taken as the amount of ALA synthesised during the incubation period. Differences between replicate data were minimised by measuring hypocotyl length before harvesting to standardise the developmental stage of seedlings, the most variable factor in ALA measurements. Results for dark-grown seedlings are shown in Figure 24. ALA synthesis is expressed as percentage wild-type (%WT) to standardise differences between experiments. Expression of BVR resulted in an increase in ALA synthesis, a result more prominent in plastid-targeted lines. Despite replicate assays showing a similar trend, there was considerable fluctuation in the degree of percentage increase between experiments.

5.2.3 Enzyme analysis

Transcripts encoding enzymes of the tetrapyrrole biosynthetic pathway were quantified in 11 day-old dark-grown seedlings using northern analysis. Blots were subsequently stripped and re-probed with an *18S* control probe to standardise loadings. A representative blot is presented for each enzyme, with a graph displaying quantified data from replicate experiments. Gene expression is represented as %WT

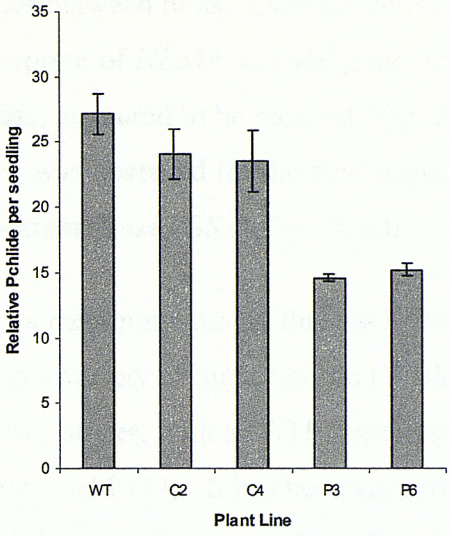
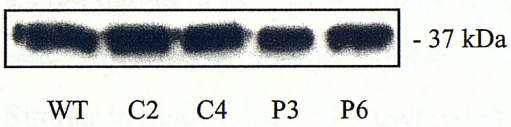


Fig. 24 Mean relative protochlorophyllide levels in dark-grown wild-type (WT), cytosol-targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days, as measured by fluorescence spectrophotometry. n=5 ± S.E.

a)



b)

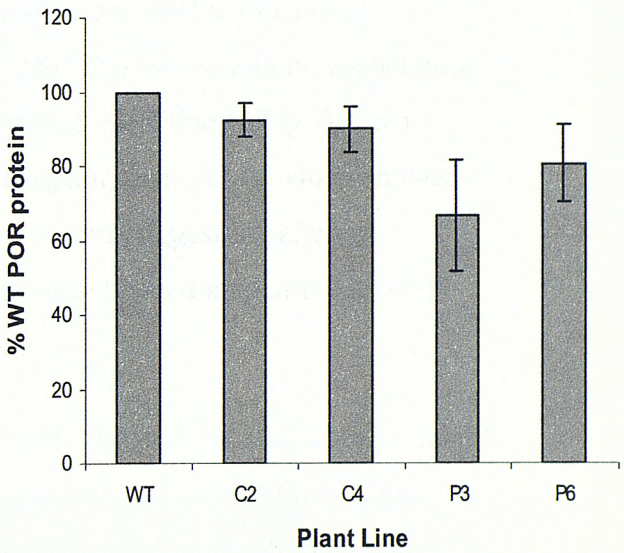


Fig. 25 a) Western analysis of POR expression in dark-grown wild-type (WT), cytosol-targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days. Signals were quantified with respect to LSU Rubisco and mean values displayed in b). n=3 ± S.E.

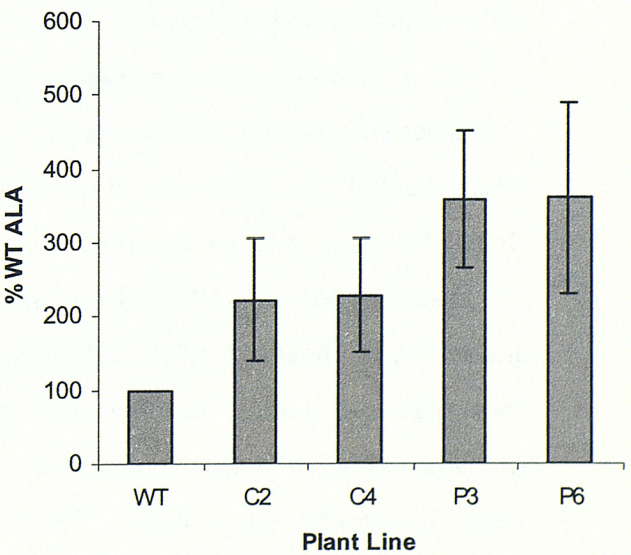


Fig. 26 ALA synthesised during 6 h LA incubation period in 11-day-old, dark-grown wild-type (WT), cytosol-targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings.100% activity corresponds to 3.95 +/- 0.88 nmol/g tissue. n=5 ± S.E.

to standardise differences between blots. Despite the increase in ALA synthesis in transgenic lines, transcription of *HEMA*, encoding the ALA biosynthetic enzyme glutamyl-tRNA reductase, appeared to be reduced (Fig. 27 a,b). A similar result, though less pronounced, was observed for the gene encoding glutamate-1-semialdehyde 2,1 aminotransferase (*GSA*)(Fig. 28 a,b).

Magnesium chelatase is a membrane bound three-subunit enzyme. All of the subunits have now been cloned in a variety of higher plants (Walker and Willows 1997). The reaction takes place in two stages, with an ATP-dependent activation, followed by chelation of Mg^{2+} (Gräfe *et al* 1999). It has been suggested that the activation stage and variations in subunit levels may play a role in determining flux through the branchpoint (Walker and Willows 1997). In plants transformed with BVR, however, no significant differences in levels of subunit transcripts were observed in dark-grown seedlings at 11 days (Fig. 29 a-d). No antibodies were available to analyse accumulation of the protein subunits. A small (15-25%) increase in Ferrochelatase transcript was repeatedly recorded in plastid-targeted BVR line 6 (Fig. 30 a,b). Similar increases were not observed in other transgenic lines, all of which showed transcript levels comparable to WT. Altogether, results suggest the altered tetrapyrrole flux in BVR-transformed seedlings not to be mediated at the level of chelatase transcription.

Northern analysis of heme oxygenase expression proved problematic, as the available probes, isolated from *Arabidopsis* and pea (Muramoto *et al* 1999, Linley *et al*, manscript in preparation), were insufficiently homologous to the tobacco gene to enable hybridisation. Western analysis, however, using an antibody raised against the *HY1* gene of *Arabidopsis* (Muramoto *et al* 1999) proved more successful (Fig. 31 a,b). Two bands of molecular masses 27 and 34 kDa were observed in plastid-targeted lines, whereas a single band of 27 kDa was observed in all other lines. These masses are consistent with the masses of heme oxygenase, with and without its plastid signal peptide. It is therefore possible that in plastid-targeted BVR plants, the increased levels of heme oxygenase protein observed exceed the catalytic capacity of processing enzymes. It is also possible that these represent two isoforms of the tobacco enzyme, as knowledge in this area is currently limited. Signals were quantified with respect to the large subunit of Rubisco and expressed as %WT. Cytosol-targeted lines appeared

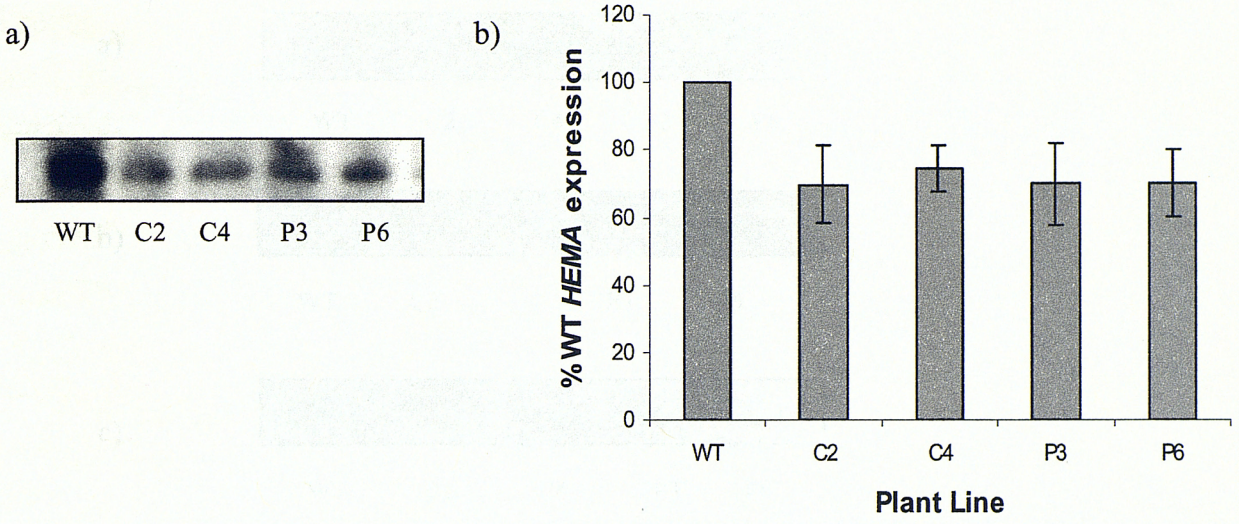


Fig.27 a) Northern analysis of *HEMA* expression in dark-grown wild-type (WT), cytosol- targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days. Signals were quantified with respect to *18S* and mean values displayed in b). n=3 ± S.E.

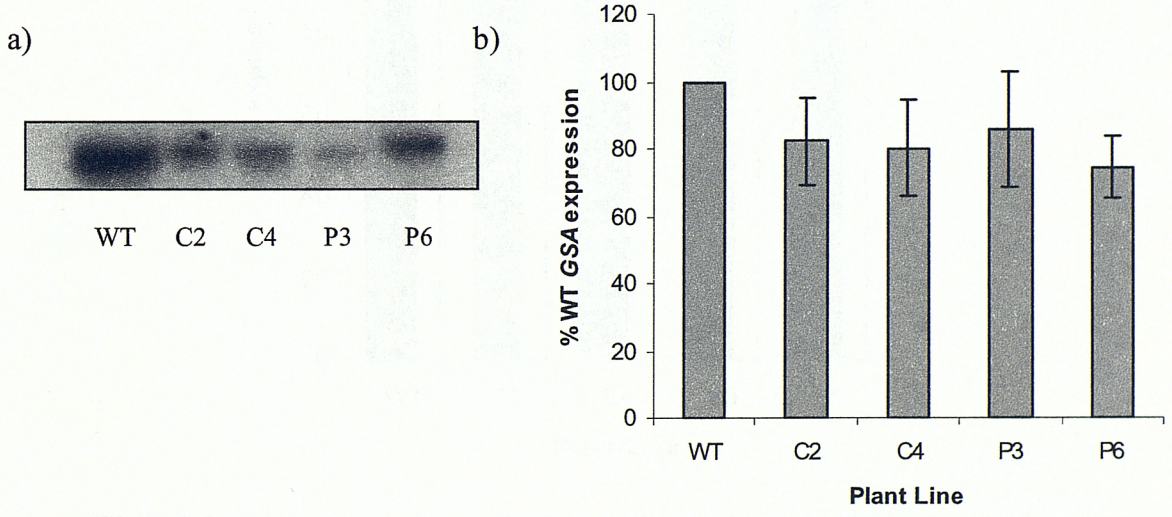


Fig.28 a) Northern analysis of *GSA* expression in dark-grown wild-type (WT), cytosol- targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days. Signals were quantified with respect to 18S and mean values displayed in b). n=3 ± S.E.

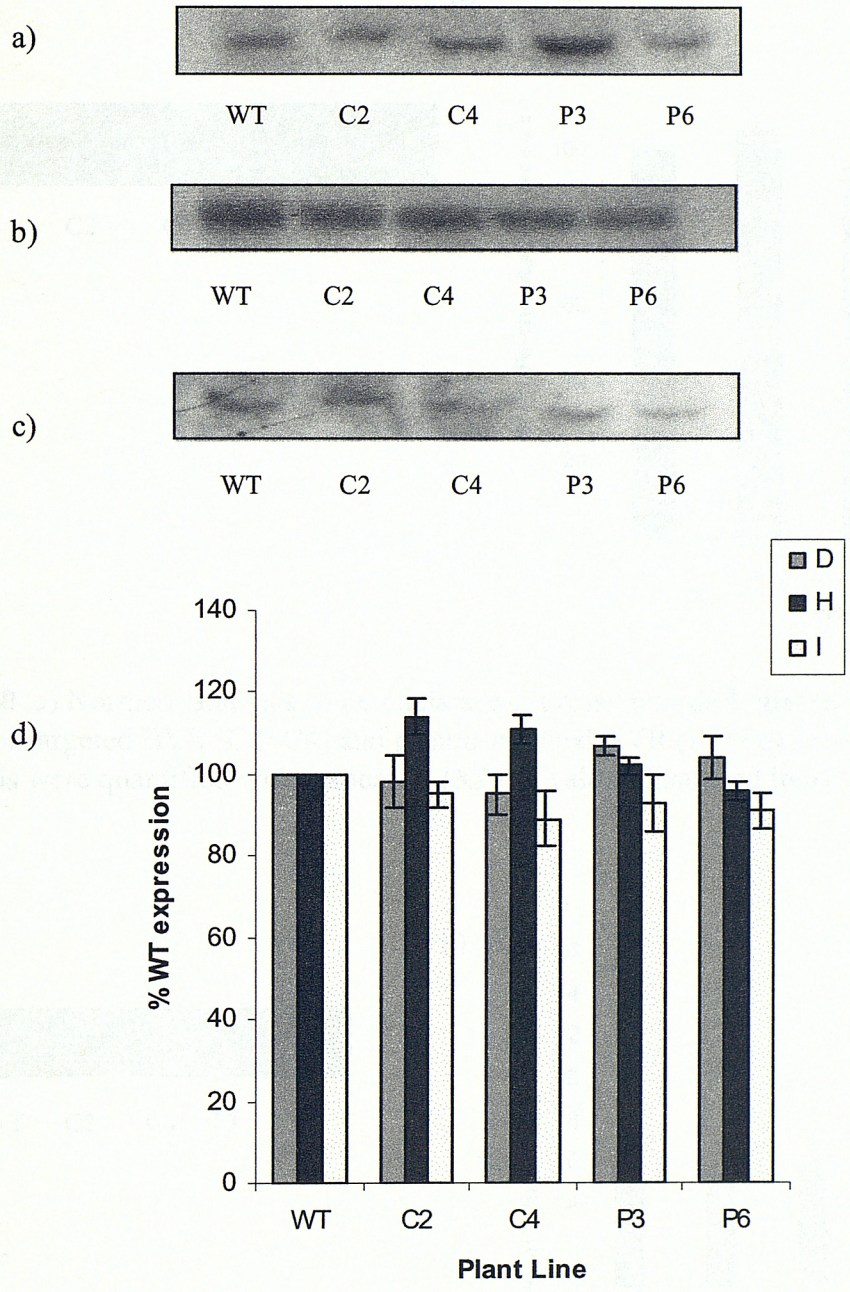


Fig. 29 Northern analysis of *CHLD* (a), *CHLH* (b) and *CHLI* (c) expression in dark-grown wild-type (WT), cytosol- targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days. Signals were quantified with respect to *18S* and values displayed in d). n=3 ± S.E.

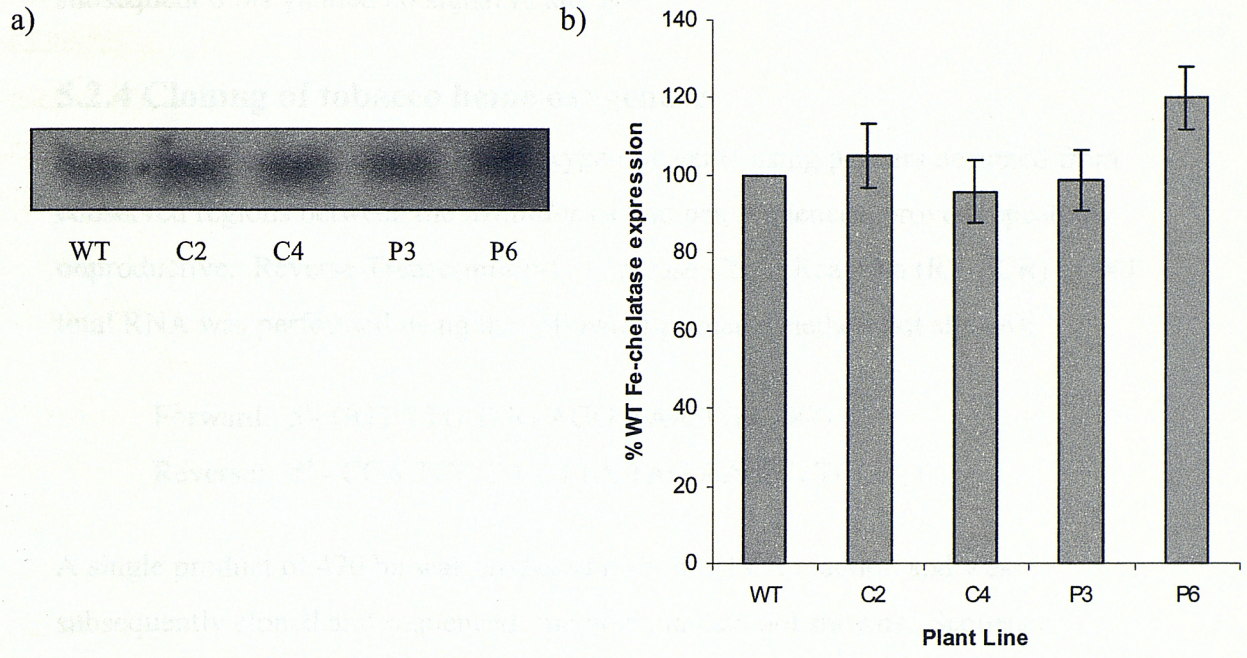


Fig. 30 a) Northern analysis of Fe-chelatase expression in dark-grown wild-type (WT), cytosol-targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days. Signals were quantified with respect to *18S* and values displayed in b). n=3 ± S.E.

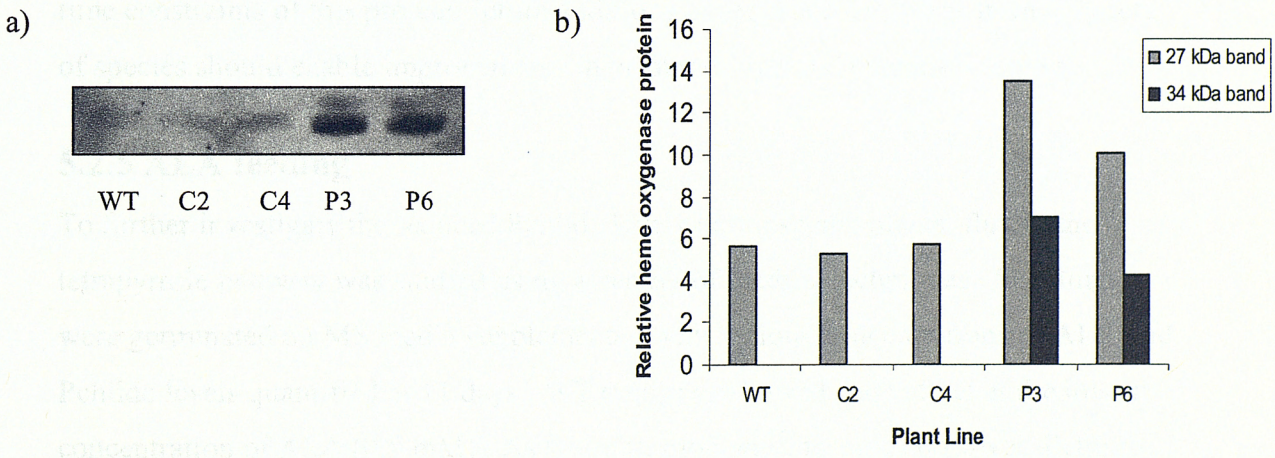


Fig. 31 a) Western analysis of heme oxygenase expression in dark-grown wild-type (WT), cytosol-targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days. Signals were quantified with respect to LSU Rubisco and values displayed in b). n=1.

unaffected by BVR expression, whereas plastid-targeted lines displayed increased levels of both bands. This data is, however, the result of a single experiment as subsequent blots yielded no signal in any line.

5.2.4 Cloning of tobacco heme oxygenase

Attempts to clone the tobacco heme oxygenase gene, using primers designed from conserved regions between the *Arabidopsis* and pea sequences, proved repeatedly unproductive. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) of WT total RNA was performed using the following primers (method not shown):

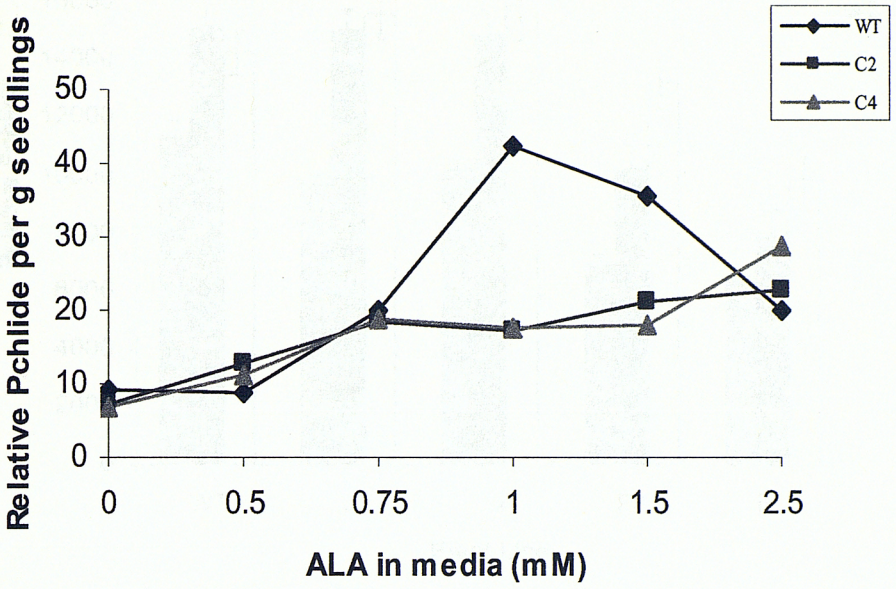
Forward: 5'- GGT TTG TGG AGG AAA TGA GC - 3'
Reverse: 5' - CCA TCC CAT TTA TAG AA(CT) TCC – 3'

A single product of 470 bp was produced from one PCR reaction and was subsequently cloned and sequenced (method and data not shown). Sequence alignment of the cloned DNA revealed > 99% similarity to the heme oxygenase gene of pea, a result eventually attributed to contamination of the original PCR mix (P.Linley-personal communication). Efforts to clone heme oxygenase from *Nicotiana plumbagnifolia*, using primers designed against conserved regions within multiple plant species, has been equally unsuccessful (Davis *et al* 2001). Although beyond the time constraints of this project, future additions to sequence databases from a variety of species should enable improvements in primer design and eventual cloning.

5.2.5 ALA feeding

To further investigate the reduced Pchlide levels in transgenic plants, flux in the tetrapyrrole pathway was studied using a variety of feeding techniques. Seedlings were germinated on MS media supplemented with various concentrations of ALA and Pchlide levels quantified at 11 days. WT seedlings showed little effect at the lowest concentration of ALA (0.5 mM). As concentration was increased however, Pchlide synthesis increased with maximum levels accumulating at 1mM (Fig. 32a). At concentrations between 1 and 2.5 mM, Pchlide levels decreased, but were still elevated with respect to control seedlings. In contrast, cytosol-targeted BVR lines showed increased Pchlide synthesis at 0.5 mM ALA. As the concentration of ALA was increased, levels of Pchlide increased, but at a reduced rate compared to WT. A peak of Pchlide accumulation was not observed for the range of concentrations used.

a)



b)

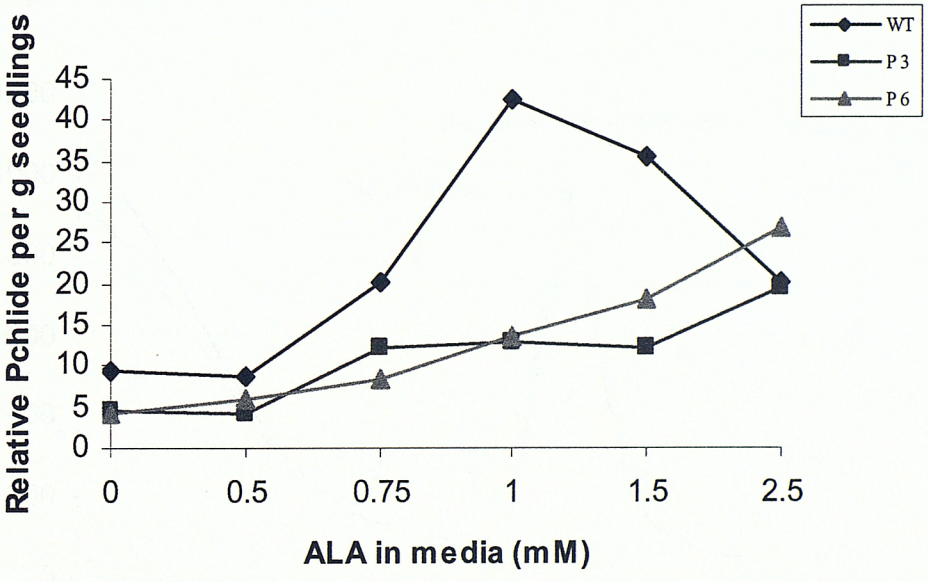


Fig. 32 Relative protochlorophyllide (Pchlide) in dark-grown seedlings grown on MS media containing different concentrations of ALA, as measured by fluorescence spectrophotometry. a) Wild-type (WT) and cytosol targeted BVR lines 2 and 4 (C2+C4) b) Wild type (WT) and plastid-targeted lines 3 and 6 (P3+P6). These data are the result of a single experiment.

Plastid-targeted BVR lines behaved similarly to WT at the lowest concentration of ALA applied (Fig. 32b). At higher concentrations, however, Pchlide increased, but at a reduced rate to WT. When feeding concentrations of ALA higher than 2.5 mM, seedling growth was retarded and large quantities of protoporphyrin IX accumulated (data not shown). For this reason, short incubations in higher concentrations of ALA were subsequently used to assess the Pchlide synthesising capacity of transgenic seedlings.

Incubation in 3 mM ALA resulted in elevated Pchlide synthesis in all lines (Fig. 33). When the concentration was increased to 5 mM, WT seedlings displayed slightly reduced Pchlide, suggesting that the capacity of the seedling to synthesise this pigment had been exceeded. Further increases in pigment accumulation were, however, observed in transgenic seedlings at the higher concentration of precursor. It can therefore be concluded that at concentrations of ALA which saturate the Pchlide synthesising capability of WT seedlings, plants transformed with *BVR* possess the capacity for further pigment accumulation. Cytosol-targeted BVR lines displayed WT levels of Pchlide when incubated in both concentrations of ALA, while Pchlide levels in plastid-targeted lines remained below those of WT seedlings. Despite the higher concentrations used for incubation, seedlings grown on media containing ALA always displayed greater levels of relative Pchlide. It is likely that this represents the increased period of time seedlings were given to accumulate pigment and prevents comparative analysis of absolute levels between the two experiments. No protoporphyrin IX accumulation was recorded in any feeding experiment. The presence of protoporphyrin IX would infer that the activity of branch enzymes was impaired, thus inducing a "bottleneck" effect.

5.2.6 Inhibitor feeding

To further investigate flux through the Mg branch of the tetrapyrrole pathway in transgenic plants, samples of 75 etiolated seedlings were incubated in the iron chelator 2'-2'-bipyridyl for 6 h. Such treatment not only restricts flow through the heme branch of the pathway, but also impedes the conversion of Mg-protoporphyrin to Pchlide (Duggan and Gassman 1974). Feeding of 2'-2' bipyridyl was therefore used to alleviate BVR-induced effects on tetrapyrrole metabolism, enabling comparative measurements of Mg-chelated porphyrins to provide estimates of flux

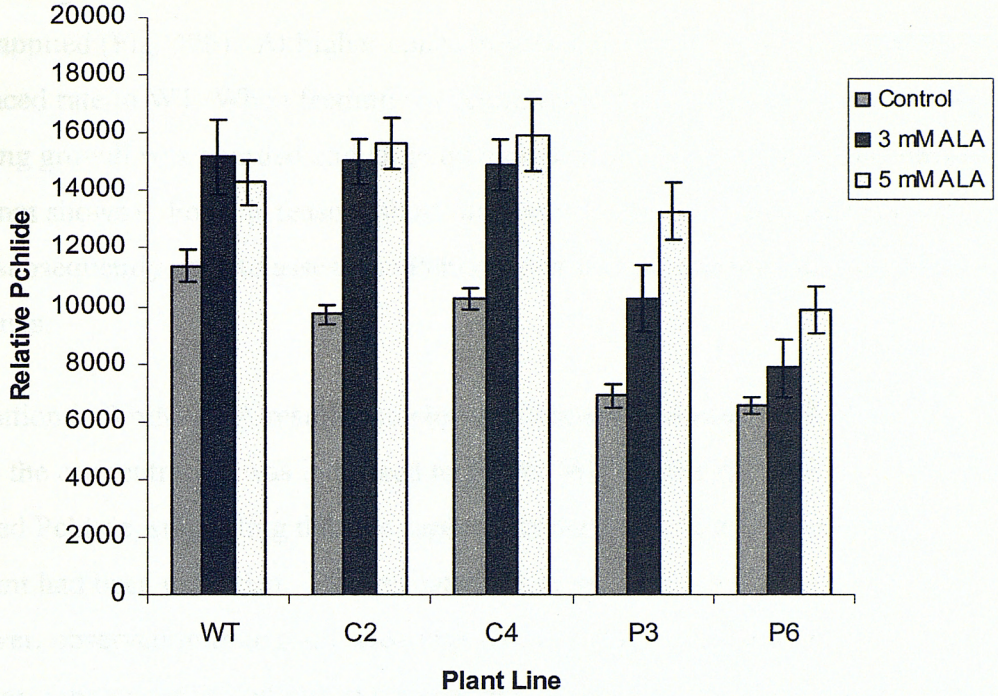


Fig. 33 Protochlorophyllide quantification (as measured by fluorescence spectrophotometry) in dark-grown seedlings incubated for 6 h in different concentrations of ALA. WT= wild-type, C2+C4= cytosol-targeted lines 2 and 4, P3+P6= plastid-targeted lines 3 and 6. n=3 ± S.E.

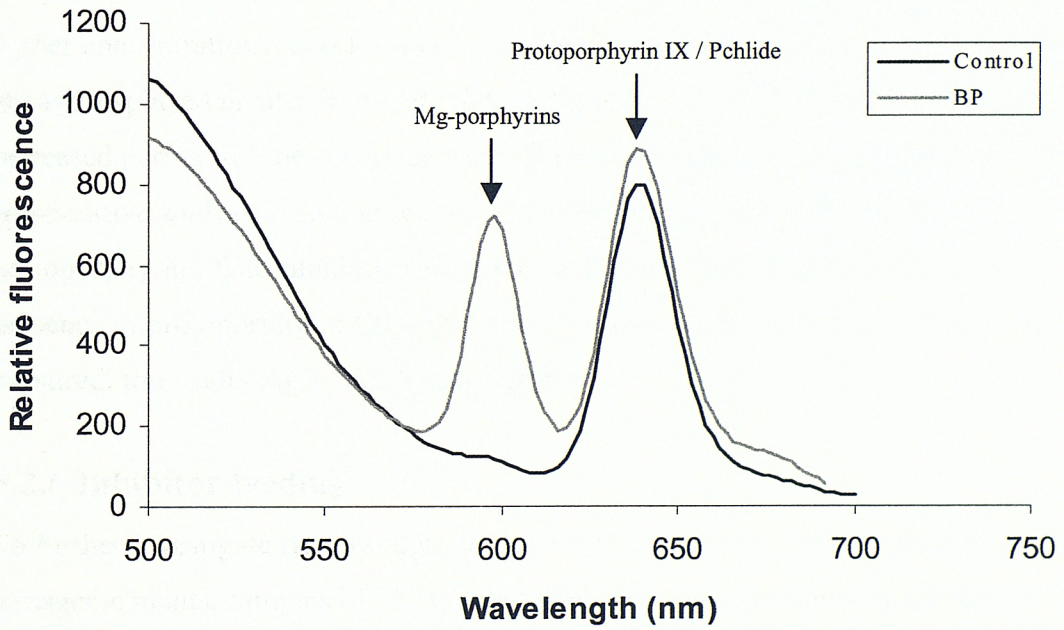


Fig. 34a) Representative fluorescence emission spectra, following excitation at 410nm, from dark-grown wild-type seedlings incubated for 6 h in K-phosphate buffer ± 10 mM 2'-2' -bipyridyl (BP).

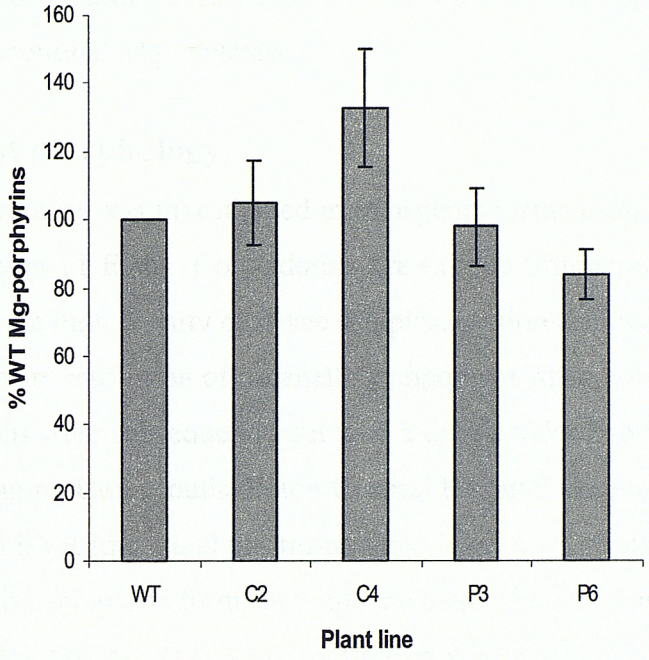


Fig. 34 b) Mg-porphyrin quantification in dark-grown seedlings incubated for 6 h in 10 mM 2'-2'-bipyridyl. WT= wild-type, C2+C4= cytosol-targeted lines 2 and 4, P3+P6= plastid-targeted lines 3 and 6. n=3 ± S.E.

through the Mg-branch in all lines. The accumulation of Mg-porphyrins upon feeding of 2'-2'-bipyridyl can be visualised spectrofluorometrically by the presence of a peak at approximately 590 nm when exciting at 410 nm (Fig.34 a). When levels of Mg-porphyrins were quantified in WT and *BVR*-transformed plants treated with 2'-2'-bipyridyl, equal accumulation was recorded in all lines. Such data suggest the decreased levels of Pchlide observed in *BVR*-transformed seedlings not to result from a reduction in functional Mg-chelatase.

5.2.7 Etioplast morphology

Etioplast ultrastructure was investigated in transgenic plants using transmission electron microscopy (T.E.M). Cotyledons were excised from etiolated seedlings at 11 days. Owing to the high density of tissue samples, sections cut with glass knives provided inadequate resolution of organelle components (data not shown). Transverse cotyledon sections were subsequently cut with a diamond knife by Dr. A.Page (Biomedical Imaging Unit, Southampton General Hospital, Southampton, UK). Cytosol-targeted BVR line 2 and plastid-targeted BVR line 3 were used for analysis. Two representative etioplasts from each line are displayed in Figure 35. Highly regular prolamellar bodies (PLBs) were visible in WT samples (Fig. 35 a,b) and displayed a wide-type structure, similar to those observed in tomato (Terry *et al* 2001). No striking differences were observed in etioplasts from cytosol-targeted BVR lines (Fig. 35 c,d), although reduced section quality prevented detailed comparative analysis with WT plants. Etioplasts from plastid-targeted BVR lines showed increased electron density and less PLB structure was generally observed (Fig. 35 e,f).

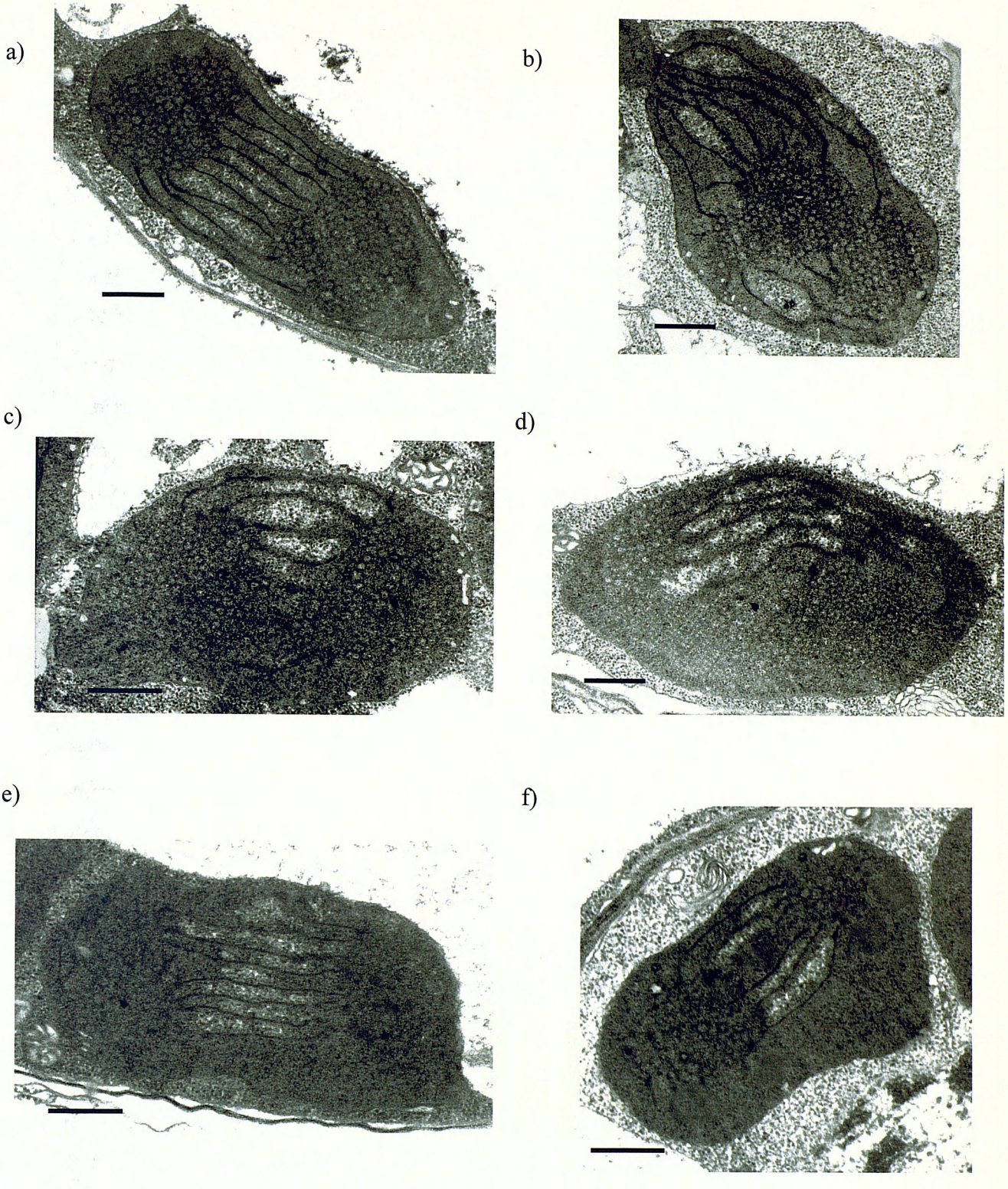


Fig. 35 Electron micrographs showing etioplast ultrastructure of WT (a+b), cytosol-targeted BVR (c+d) and plastid-targeted BVR (e+f) cotyledons from 11-day-old dark-grown seedlings. Bar represents 1µm.

5.3 Discussion

One of the most distinctive phenotypes of seedlings expressing BVR is a marked chlorophyll deficiency in white light-grown cotyledons. Similar deficiencies have been observed in *BVR*-transformed Arabidopsis (Lagarias *et al* 1997, Mongomery *et al* 1999) and a variety of chromophore-deficient mutants (Terry 1997), thus supporting the well-documented role of phytochrome in regulating tetrapyrrole biosynthesis. Investigation of tetrapyrrole metabolism in the *aurea* (*au*) and *yellow-green-2* (*yg-2*) mutants of tomato also revealed the possibility of reduced ALA synthesis through a heme-mediated, phytochrome-independent, inhibitory mechanism (Terry and Kendrick 1999). It is, however, unlikely that the reduced chlorophyll content of seedlings expressing BVR results from heme accumulation. The severity of chlorophyll deficiency in plastid-targeted BVR lines questions whether such a phenotype can be attributed solely to an increased phytochrome deficiency in these plants. It is possible that the presence of BVR within the plastid induces additional perturbations on the tetrapyrrole biosynthetic pathway. Dark-grown seedlings were therefore subject to extensive molecular and biochemical analyses to remove the light-mediated effects of phytochrome deficiency. Using this approach, additional consequences of BVR localisation within the plastid could be investigated.

A summary of results regarding the regulation of the tetrapyrrole pathway in dark-grown, *BVR*-transformed tobacco is presented in Figure 36 a,b. Cytosol-targeted lines displayed reduced levels of *HEMA* and *GSA* transcripts, despite showing increased ALA synthesising capacity. A small Pchlide deficiency was also observed, although chelatase transcription and heme oxygenase protein levels were unaffected. When BVR was targeted to the plastid, a much greater deficiency of Pchlide was recorded. As in cytosol-targeted lines, seedlings displayed reduced levels of *HEMA* and *GSA* transcript, but increased ALA synthesising capacity. Chelatase transcription was unaffected, but an increase in heme oxygenase protein was observed.

Dark-grown seedlings expressing BVR display reduced levels of protochlorophyllide and POR protein

Dark-grown transgenic seedlings consistently displayed decreased levels of the chlorophyll precursor, Pchlide, an effect exacerbated by plastid-targeting (Fig. 24). A similar deficiency was also recorded in chromophore-deficient mutants of tomato (Terry 1997, Terry and Kendrick 1999), *Arabidopsis* and pea (Terry *et al* 2001). In *BVR*-transformed *Arabidopsis*, plastid-targeted lines displayed a similar reduction, but cytosol lines remained unaffected (Montgomery *et al* 1999). The possibility that differences resulted from retarded growth in transgenic seedlings was eliminated by only using seedlings ± 10% of WT hypocotyl length for analysis. One possible explanation is that Pchlide accumulation is regulated in dark-grown plants by phytochrome in its red light-absorbing form (Pr). A reduction in total seed phytochrome content would therefore lead to a deficiency of Pchlide, and the intermediate phenotype displayed by cytosol-targeted lines in tobacco, would represent a partial reduction of holophytochrome in these plants. WT levels of Pchlide were however observed in cotyledons of the phyA/B1-deficient *fri,tri* double mutant of tomato (Terry and Kendrick 1999), suggesting the inhibition of Pchlide synthesis in *BVR*-transformed plants to operate through a phytochrome-independent mechanism. Analysis of the *phyAphyB1phyB2* triple mutant of tomato yielded a similar result for cotyledons, but surprisingly revealed an approximate 40% Pchlide reduction in dark-grown hypocotyls (Terry *et al* 2001). The authors propose a possible regulatory role for Pr in hypocotyl etioplast development, but conclude that the Pchide deficiency in cotyledons results primarily from a phytochrome-independent mechanism. As whole seedlings were used for pigment extraction, a regulatory role for Pr in tobacco Pchlide synthesis cannot be excluded.

The reduced Pchlide synthesis observed in transgenic lines displayed a strong correlation with the loss of POR protein (Fig. 25 a,b). A similar correlation was recorded in chromophore-deficient mutants of tomato and *Arabidopsis* (Terry *et al* 2001, Montgomery *et al* 1999, López-Juez *et al* 1998) and is consistent with the well-documented role of POR in binding the potentially phototoxic Pchlide within the etioplast (Griffiths 1978, Apel *et al* 1980). The import of POR into etioplasts has been previously reported to depend on the presence of Pchlide (Reinbothe *et al* 1995).

A decline in Pchlide concentration may therefore be expected to restrict POR import, leading to increased turnover of the extra-plastidic protein. Recent reports, however, showing POR to be imported into the plastid independently of Pchlide preclude such a scenario (Dahlin *et al* 2000, Aronsson *et al* 2000). Increased intra-plastidic degradation of mature protein in the absence of its substrate may therefore provide an alternative explanation for the decreased levels observed.

The possibility also exists that POR is down-regulated through the kinase activity of accumulated phytochrome apoprotein in transgenic plants (J.Clark Lagarias-personal communication). The reduced levels of total Pchlide recorded would therefore represent turnover of the pigment in the absence of its binding protein. Quantification of Pchlide in POR-deficient plants, however, revealed levels similar to WT, thus contending such a hypothesis (T.Masuda- personal communication). The idea that eukaryotic phytochromes are protein kinases, related to the cyanobacterial two component light sensory system, was first demonstrated by Lagarias and colleagues (Yeh *et al* 1997). Purified recombinant phytochromes from a higher plant and a green alga were subsequently shown to exhibit serine/threonine kinase activity and autophosphorylation *in vitro* (Yeh and Lagarias 1998). Attachment of phycocyanobilin and phytochromobilin to *Avena sativa L.* apophytochrome in the dark was shown to inhibit autophosphorylation, with the apoprotein displaying two-fold greater phosphorylation than either bilin adduct (Yeh and Lagarias 1998). Such data is consistent with the notion of phytochrome apoprotein possessing intermediate kinase activity between that of Pr and Pfr but has, however, yet to be demonstrated *in vivo*. Northern analysis of *PORA* transcript would provide valuable insight into the nature of the decrease of this protein in *BVR*-transformed tobacco. The possible kinase activity of phytochrome apoprotein in transgenic plants could be further analysed by crossing experiments. Should phytochrome apoprotein show kinase activity, *in vivo*, then crossing of a BVR-expressing plant with an apoprotein mutant or apoprotein over-expressing line might be expected to rescue or exacerbate the Pchlide/POR-deficient phenotype, respectively.

Seedlings expressing BVR display increased ALA synthesis in the dark

Despite reduced levels of Pchlide, seedlings expressing BVR displayed increased ALA synthesis, an effect more prominent in plastid-targeted lines (Fig. 26). Such an up-regulation is also in contradiction to the observed transcript abundance of ALA biosynthetic enzymes (Fig. 27-28). Both cytosol and plastid-targeted lines displayed decreased levels of *HEMA* and *GSA* transcript to WT seedlings, suggesting the observed increase in ALA synthesis not to be mediated at the transcriptional level. Analyses in the chromophore-deficient *hy1* and *hy2* mutants of *Arabidopsis* revealed no alterations in ALA synthesis or *HEMA1* transcription in dark-grown seedlings grown under identical conditions (McCormac and Terry -unpublished data). Recognised inhibitors of ALA synthesis in dark-grown seedlings include heme and the Pchlide/POR/NADPH ternary complex (Duggan and Gassman 1974, Masuda *et al* 1990, Beale and Weinstein 1991, Pontoppidan and Kannangara 1994). Etiolated seedlings of the chromophore-deficient mutants *au* and *yg-2* have been observed to display decreased ALA synthesis (M.J. Terry- unpublished data), an effect attributed to feedback regulation by accumulated heme (Terry and Kendrick 1999). Decreased levels in *au* were, however, only apparent following a light flash (M.J. Terry- unpublished data).

The increased ALA synthesising capacity of *BVR*-transformed seedlings therefore presents an apparent paradox. It is possible that flux is increased through both branches of the pathway and that Pchlide turnover in the absence of POR accounts for the reduced levels measured. An alternative hypothesis is that porphyrins are being "pulled" through the heme branch of the tetrapyrrole pathway in transgenic plants and degraded to rubinoid products. Such a scenario would reduce flux through the Mg-branch, consequently reducing levels of Pchlide. The constant metabolism of heme and simultaneous decrease in levels of Pchlide and its binding protein POR, would release inhibition of ALA synthesis, resulting in the increases observed. This hypothesis provides a conceivable explanation for the phenotype of plastid-targeted BVR lines. The "intermediate" phenotype observed in cytosol targeted-lines is, however, more difficult to explain and would appear to suggest that phytochromobilin metabolism in the cytosol exerts a degree of "pull" through the heme branch of the tetrapyrrole pathway in the plastid. Alternatively, Pr, or the phytochrome

chromophore, phytochromobilin, may fulfil as yet, unidentified regulatory roles in etioplast development. The phenotypes of plants expressing BVR would therefore represent deficiencies in either or both of these compounds.

Perturbations in tetrapyrrole pathway flux do not result from altered transcription of genes encoding chelatases in dark-grown seedlings expressing BVR

The characterisation of Mg and Fe-chelatase enzymes from a variety of species has furthered insight into tetrapyrrole branchpoint regulation in recent years. The development of reliable *in vitro* assays for both enzymes has enabled activities to be monitored in isolated plastids (Walker and Willows 1997, Smith *et al* 1999). Application of different pathway inhibitors results in the accumulation of specific tetrapyrrole intermediates. Such experiments have revealed an inverse correlation between Mg-chelatase activity and Mg-protoporphyrin levels, implying regulation at the level of product inhibition (Yaronskaya *et al* 1993, Averina *et al* 1996). Flux is also believed to be controlled, in part, by ATP and differential enzyme kinetics. Photosynthetically-produced ATP is required for Mg-chelatase activity but, in contrast, is inhibitory to Fe-chelatase. The low K_m value of Mg-chelatase is also believed to enable this enzyme to out-compete Fe-chelatase for the common substrate, protoporphyrin IX. The plants requirement for chlorophyll in sunlight demands a channelling of flux through the Mg branch of the pathway and has lead to the proposal that chelatase enzyme activity displays diurnal cycling, with heme production taking precedence during the night (Pöpperl *et al* 1998, Papenbrock *et al* 1999).

Higher plant Mg-chelatase is comprised of three subunits, D, H and I. Subunits H and I are believed to be involved in catalysis, with D fulfilling an activation role, being present only in reduced quantities (Walker and Willows 1997). A more recent model implicates a role for subunit D during the chelation step (Gräfe *et al* 1999), but both propose that variations in subunit transcription could provide a possible regulatory mechanism controlling branchpoint flux. Transcript levels of all three subunits were therefore quantified in dark-grown seedlings expressing BVR using northern analysis (Fig. 29 a-d). No overall significant difference was observed in transgenic lines. Although Mg and Ferrochelatase catalyse comparable reactions, they do not show any structural or catalytic similarities (Grimm 1998). Ferrochelatase is a monomeric

protein of approximate molecular mass 55 kDa. In *Arabidopsis*, two isoforms of the enzyme have been isolated. Ferrochelatase I (FCI) was imported into both chloroplasts and mitochondria and is believed to be required for the production of heme for export to the endoplasmic reticulum and peroxisomes (Chow *et al* 1998). Ferrochelatase II, however, was imported solely into chloroplasts and is believed to be required predominantly for the synthesis of photosynthetic cytochromes (Chow *et al* 1998). The two isoforms showed 69% sequence similarity and associated with both the thylakoid and envelope membranes of the chloroplast. Only one isoform is currently available from tobacco (B.Grimm- personal communication). Transcript levels of this gene showed no alteration in dark-grown *BVR*-transformed seedlings (Fig.30 a,b). In summary it appears that although there are substantial changes in tetrapyrrole pathway flux in transgenic plants, these are unrelated to transcription of Mg and ferrochelatase genes.

Pchlide deficiency in dark-grown seedlings expressing BVR does not result from a reduction of functional Mg-chelatase.

The possibility that reduced levels of Pchlide in etiolated transgenic seedlings resulted from a reduction of functional Mg-chelatase was investigated biochemically through feeding studies. When WT seedlings were fed ALA, there was an increase in Pchlide synthesis that paralleled the increase in ALA concentration, with a maximum increase at 1mM (Fig. 32 a,b). At concentrations greater than this, the relative increase in Pchlide synthesis declined suggesting that the synthesising capacity of seedlings had been exceeded. ALA concentrations greater than 2.5 mM proved detrimental to seedling growth, with the accumulation of large quantities of protoporphyrin IX. Such data is consistent with observations in pea, in which increased concentrations of exogenously applied ALA resulted in inhibition of Mg-protoporphyrin IX methyl transferase and Mg-chelatase activities (Yaronskaya *et al* 1993, Averina *et al* 1996). The authors propose that such a mechanism exists to control the accumulation of excessive amounts of chlorophyll precursors. Both cytosol and plastid-targeted BVR lines behaved similarly, showing increased Pchlide synthesis with ALA feeding, but maintaining a reduced rate to WT seedlings. These results are consistent with the hypothesis that the Mg branch of the pathway is competing for porphyrins with the heme branch in transgenic plants. No peak in Pchlide synthesising capacity was

observed in either cytosol or plastid-targeted lines, although surprisingly, concentrations of ALA above 2.5 mM adversely affected seedling growth. It is possible that the increased acidity of media in these samples resulted in damaging, pleiotropic effects on plant development. For this reason, the Pchlide synthesising capacity of transgenic seedlings at higher concentrations of exogenous ALA was investigated by incubating the seedlings in buffered solutions for 6 h.

Consistent with previous data, incubation experiments revealed transgenic seedlings to possess the capacity for further Pchlide synthesis, at concentrations of ALA that saturated the WT response (Fig. 33). The effective restoration of Pchlide levels to those comparable with WT seedlings provides preliminary evidence that the pathway between protoporphyrin IX and Pchlide is unaffected in cytosol-targeted BVR lines. Such data support the proposal that porphyrin flux through the Mg-branch of the pathway is limited by competition from the heme branch in these plants. Reduced levels of Pchlide were, however, measured in plastid-targeted BVR lines at all concentrations of applied ALA. Incubation of seedlings in the iron chelator 2′-2′-bipyridyl enabled investigation of Mg-chelatase activity in all plants. Through "blocking" the heme branch in etiolated seedlings, it was envisaged that perturbations of the pathway induced by biliverdin metabolism would be alleviated for the duration of the experiment, thus enabling comparative analysis of flux through the Mg-branch in transgenic lines. The use of iron chelators to restrict flow through the heme branch of the tetrapyrrole pathway is well documented (Duggan and Gassman 1974, Beale and Weinstein 1991, Averina *et al* 1996, Terry and Kendrick 1999). The observed similarity in Mg-porphyrin accumulation between all lines (Fig. 34 b) provides further support that the Pchlide deficiency observed in *BVR*-transformed seedlings does not result from post-trascriptional impairment of Mg-chelatase activity.

In conclusion, the reduced levels of Pchlide measured in etiolated seedlings expressing BVR can be explained by either competition from the heme branch, reduced synthesis of Pchlide from Mg-protoporphyrin, or increased turnover of the pigment in the absence of POR. The latter would result in a greater proportion of non-bound (ie. non-phototransformable) Pchlide in transgenic samples. This hypothesis could therefore be investigated using light flash experiments to measure levels of phototransformable Pchlide in all lines.

Plastid-targeting of BVR results in increased levels of heme oxygenase protein in the dark

Heme oxygenase (HO), catalyses the oxidation of heme to biliverdin (BV) with the release of $Fe^{2+}$and carbon monoxide (Ortiz de Montellano and Wilks 2001). The *HY1* gene of *Arabidopsis* encodes a plastid heme oxygenase (AtHO1) required for phytochromobilin synthesis (Muramoto *et al* 1999, Davis *et al* 1999). Western analysis, using an antibody raised against *HY1,* revealed an apparent increase of this protein in plastid-targeted BVR seedlings grown in the dark (Fig. 31 a,b). This is, however, the result of a single experiment as replicate studies failed to produce a signal in any line. It is likely that problems resulted from the low yields of protein extracted from etiolated samples. The production of concentrated protein pellets from such tissue may therefore be preferential to using crude extracts in future experiments. The inability of *Arabidopsis* and pea *HO1* cDNA to hybridise with tobacco mRNA prevented transcriptional analysis. It therefore remains in question whether the increase in HO protein represents amplified gene expression. Such an up-regulation would provide significant support for a phytochrome-independent, tetrapyrrole-related regulatory mechanism controlling nuclear gene transcription in these plants. As HO activity is documented to be inhibited by product accumulation (Ortiz de Montellano and Wilks 2001), it is possible that the steady metabolism of biliverdin, by BVR, in transgenic plants relieves such inhibition, therefore increasing enzyme activity. Such an increase could serve to stabilise a protein that would otherwise be degraded (Thomas 1997). The increased levels of HO protein observed on western blots may therefore represent an increased stability of the enzyme, rather than an up-regulation of transcription.

Plastid-targeting of BVR results in altered etioplast morphology

Etioplasts are distinguishable by their internal membrane system, the prolamellar bodies (PLBs). These display a quasi-crystalline lattice structure and result from the synthesis of thylakoid lipids in darkness without the parallel synthesis of thylakoid proteins (Gunning and Steer 1996). POR is the major protein component of PLBs (Ryberg and Dehesh 1986, Ryberg and Sundqvist 1988) and both PORA and PORB are believed to be involved in their formation (Sperling *et al* 1997). The reduced

levels of POR in *BVR*-transformed plants (Fig. 25) may therefore be expected to adversely affect etioplast development. No obvious differences in etioplast ultrastructure were visible in cotyledons of cytosol-targeted BVR lines (Fig. 35 c,d). Such observations are consistent with the small reduction in POR protein recorded in these plants. Etioplasts from plastid-targeted BVR cotyledons, however, displayed increased electron density and reduced PLB structure (Fig. 35 e,f). Comparable etioplasts were observed in the chromophore-deficient *au* mutant of tomato, which displayed similar deficiencies of Pchlide and POR (Terry and Kendrick 1999, Terry *et al* 2001). It is possible that the observed structural differences arise from an accumulation of membrane material in the absence of sufficient POR, or merely represent a retardation of organelle development. An increased number of sections would need to be viewed to substantiate these conclusions.

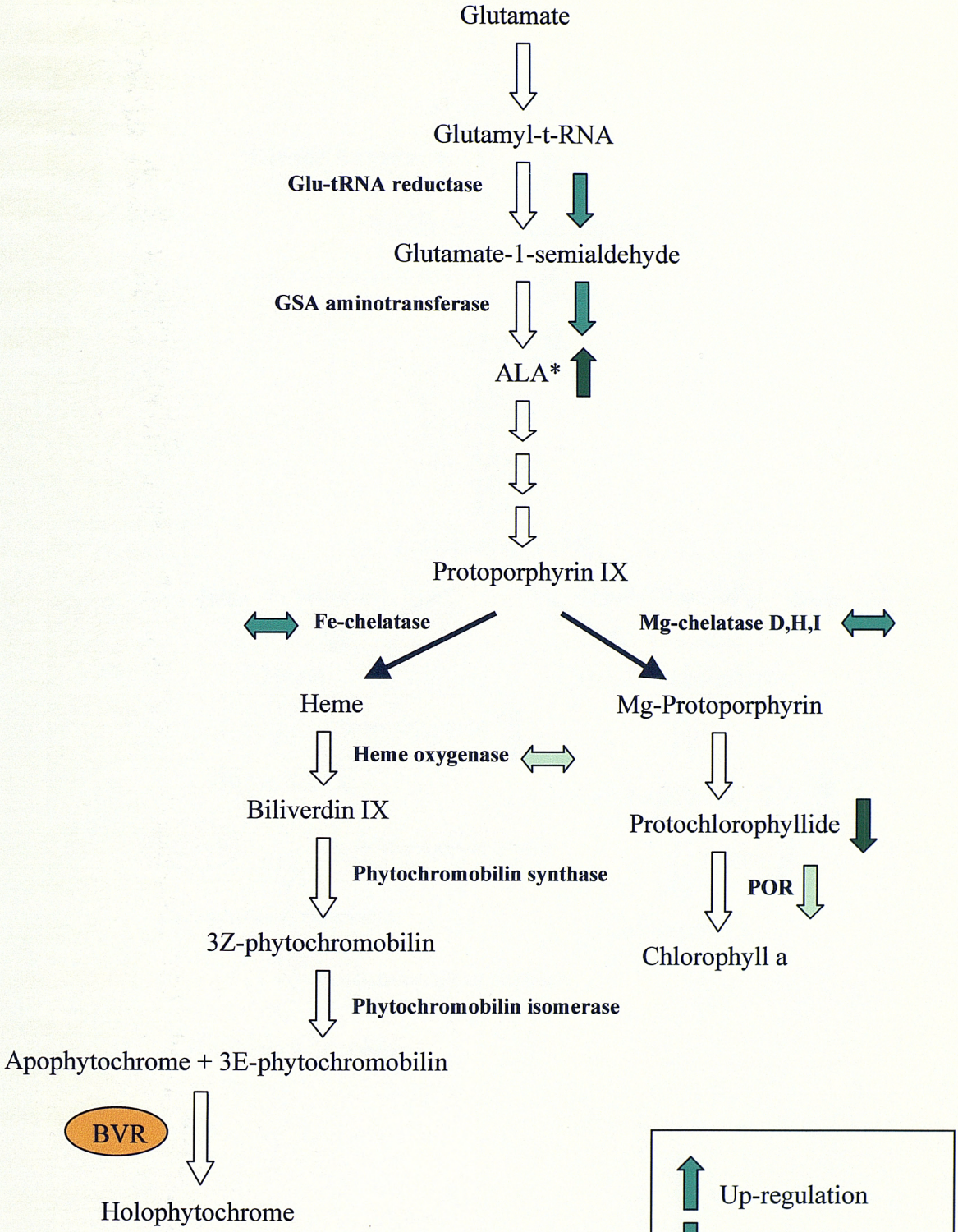


Fig. 36a) Regulation of the tetrapyrrole biosynthetic pathway in dark-grown cytosol-targeted BVR seedlings.

* ALA represents synthesising capacity.

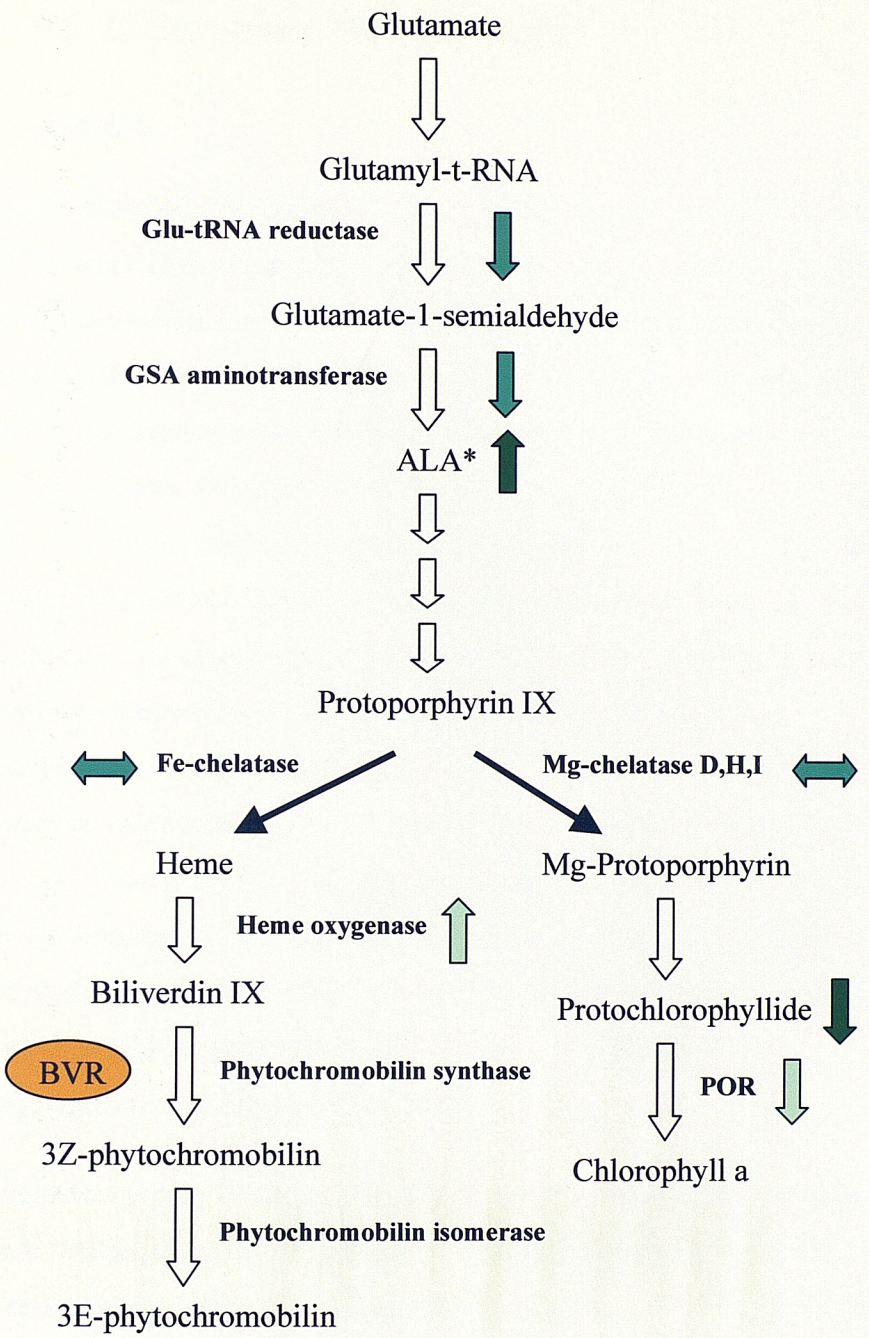


Fig. 36b) Regulation of the tetrapyrrole biosynthetic pathway in dark-grown plastid-targeted BVR seedlings.

* ALA represents synthesising capacity.

Chapter 6. Chloroplast Development

6.1 Introduction

The transition from skotomorphogenesis to photomorphogenesis involves an integrated network of developmental changes, the most pronounced of which are the synthesis of chlorophyll from protochlorophyllide and concomitant formation of photosynthetic chloroplasts. Upon illumination, Pchlide is transformed to Chlide and PORA levels are drastically reduced through repression of gene expression and light-stimulated proteolysis (Mapleston and Griffiths 1980, Armstrong *et al* 1995, Reinbothe *et al* 1996). Light absorption results in the trans addition of hydrogen to the C17 and C18 positions of the pigment to produce Chlide (Griffiths *et al* 1996). The addition of a phytol side chain to Chlide, catalysed by chlorophyll synthase, yields chlorophyll *a*, which is located to thylakoid membranes. The phototransformation of Pchlide initiates the dispersal of PLBs to tubules or vesicles, a highly complex procedure associated with protease and lipase activities (Scheer 1991, Sundqvist and Ryberg 1989). The disaggregation of newly formed Chlide-POR complexes is therefore fundamental in the etioplast to chloroplast transition. The light-mediated reduction of Pchlide to Chlide and the simultaneous activation of photoreceptor signal transduction pathways, together trigger plant photomorphogenesis (Armstrong *et al* 1995).

The multifunctional role of tetrapyrroles during plant growth and the lethality of photo-toxic intermediate accumulation makes the regulation of this pathway a subject of much research. In light-grown plants, ALA synthesis is tightly regulated through the co-ordinated action of multiple signals. These include light, mediated through phytochrome (Huang *et al* 1989, Kasemir 1983, McCormac *et al* 2001), the circadian clock (Kruse *et al* 1997), cytokinin (Masuda *et al* 1994) and the developmental state of the plastids (Kumar *et al* 1999, McCormac *et al* 2001). The majority of plastid proteins are encoded by nuclear genes and translated in the cytosol before import into plastids. Growing evidence supports a role for tetrapyrrole compounds in the transcriptional regulation of nuclear-encoded plastid proteins, although the exact nature of this regulation is currently unclear. Plastid-derived signals enable nuclear gene expression to be coordinated with the developmental state of plastids and are

thought, at some stage, to integrate with light signalling by phytochrome (Mochizuki *et al* 1996, López-Juez *et al* 1996,1998, McCormac *et al* 2001). A strict correlation between responses to light and plastid signal has been reported in a variety of promoter deletion constructs from photosynthetic and tetrapyrrole biosynthetic genes (Kusnetsov *et al* 1996, McCormac *et al* 2001). Such data suggest that both signalling pathways operate through the same *cis*-acting elements.

The identities of plastid signalling molecules remain elusive, although current evidence suggests at least one to be tetrapyrrole related. The existence of such signals has been inferred from gene regulation studies on photo-bleached seedlings. The herbicide Norflurazon is a non-competitive inhibitor of the carotenoid biosynthetic enzyme phytoene desaturase (Chamovitz *et* al 1991) and can photobleach plastids, leading to the loss of light-induced expression of photosynthesis-related genes (Thomsen *et al* 1993). In the absence of carotenoids, chlorophyll absorbs light to form excited triplet states that react with oxygen to form free radicals. Without carotenoids, plants can initiate chloroplast biogenesis, but are rapidly photo-oxidised (Oelmüller 1989). Phytochrome synthesis is unaffected in Norflurazon-treated plants, suggesting the effects on gene expression to result from the absence of a plastid signal (Thomsen *et al* 1993). Mutants in the plastid-nuclear signalling pathway, termed *gun* mutants (*genomes uncoupled)*, have been identified in *Arabidopsis* (Susek *et al* 1993). Such mutants have the ability to up-regulate genes encoding light harvesting chlorophyll binding proteins (*Lhcb* – previously termed *CAB*) upon transfer to light, in the presence of Norflurazon and therefore absence of functional plastids. The recent cloning of *GUN5* has revealed that the gene encodes subunit H of Mg-chelatase, while *gun1* is believed to be non-tetrapyrrole related (Vinti *et al* 2000, Mochizuki *et al* 2001). The *Arabidopsis* mutant *hy1*, deficient in plastidic haem oxygenase, has also been shown to display a *gun* phenotype (Vinti *et al* 2000). The recently identified *Arabidopsis* mutant, *laf6*, deficient an ATP-binding cassette protein was isolated by its reduced responsiveness to continuous FR (Møller *et al* 2001). Increased accumulation of protoporphrin IX in the mutant has lead to the proposal that this compound may act as a light-specific signalling factor in plastid-nuclear communication (Møller *et al* 2001). In addition, tetrapyrrole intermediates have been proposed to perform a regulatory role in nuclear gene transcription in barley (La Rocca *et al* 2001), cress (Oster *et al* 1996) and the unicellular alga

Chlamydomonas reinhardtii (Johanningmeier 1988, Kropat *et al* 1997, 2000). Altogether, current research supports a model whereby one tetrapyrrole intermediate, or an altered ratio between two intermediates, can account for at least one plastid-derived signal regulating nuclear gene expression. Understanding of spatial and temporal branch point regulation in controlling flux in the tetrapyrrole pathway is therefore likely to be crucial to our understanding of plastid signalling.

This work investigates the combined consequences of phytochrome deficiency and BVR targeting on chloroplast development in transgenic plants. The ALA synthesising capacity of light-grown seedlings has been studied in addition to the transcriptional regulation of ALA biosynthetic enzymes. A parallel study of ALA synthesis and chlorophyll accumulation in developing leaves has also been undertaken. Branch point regulation in light-grown seedlings has been investigated through northern and western analysis of pathway enzymes and chloroplast morphology studied using transmission electron microscopy. The role of tetrapyrroles in plastid-nuclear signalling has been further explored through analysis of *CAB* expression in photo-bleached transgenic seedlings.

6.2 Results

6.2.1 ALA synthesis

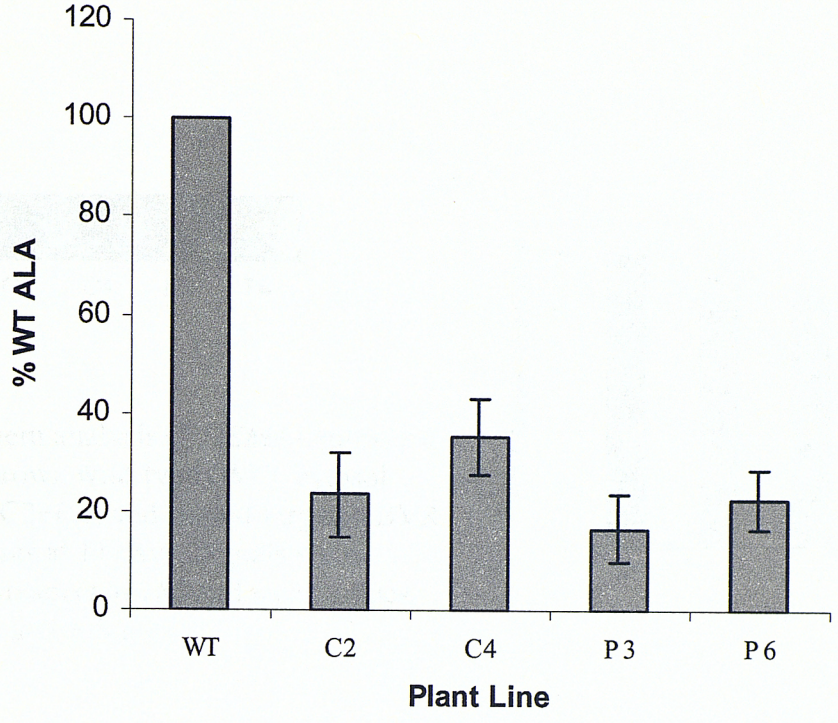
ALA synthesis was quantified in 11 day-old light-grown seedlings as described previously. All transgenic seedlings showed a reduction in ALA synthesis, an effect more marked for plastid-targeted lines (Fig. 37a). ALA was also quantified in dark-grown seedlings transferred to white light for 24 h (Fig. 37b). A similar trend was observed, although the difference between cytosol and plastid-targeted lines was more pronounced. Such data suggests that the reduction in ALA synthesising capacity observed in light-grown seedlings does not result primarily from accumulative plastid damage.

6.2.2 Enzyme analysis

Transcripts encoding enzymes of the tetrapyrrole biosynthetic pathway were quantified in 11 day-old light- grown seedlings using northern analysis. Blots were stripped and re-probed with an *18S* control probe to standardise loadings. A representative blot is presented for each enzyme, with a graph displaying quantification data. Gene expression is again represented as %WT to standardise differences between blots. Expression of *HEMA* and *GSA* transcripts, encoding the ALA biosynthetic enzymes GluTR and GSA-AT, appeared to be equal to WT in all transgenic lines tested (Fig. 26-27). Such data suggest that the reduced ALA synthesis observed in these seedlings is not the result of transcriptional repression. When dark-grown seedlings received a 24 h light treatment, however, transgenic lines displayed reduced levels of these transcripts (Fig. 40-41). Expression of *HEMA* was up-regulated upon transfer to light in all lines tested (Fig 40). This up-regulation was, however, impaired in transgenic plants. The light-regulation of *GSA* transcription was similar to that for *HEMA*, although less pronounced. With the exception of cytosol-targeted BVR line 2 (C2), all plants showed a small increase in *GSA* transcription upon transfer to light (Fig 41).

As in etiolated plants, transcription of genes encoding magnesium chelatase subunits appeared relatively unaffected by BVR expression in light-grown seedlings (Fig. 42 a,b). A small (15-25%) increase in subunit D was, however, repeatedly observed in plastid-targeted lines. A similar increase in Fe-chelatase was also recorded in these

a)



b)

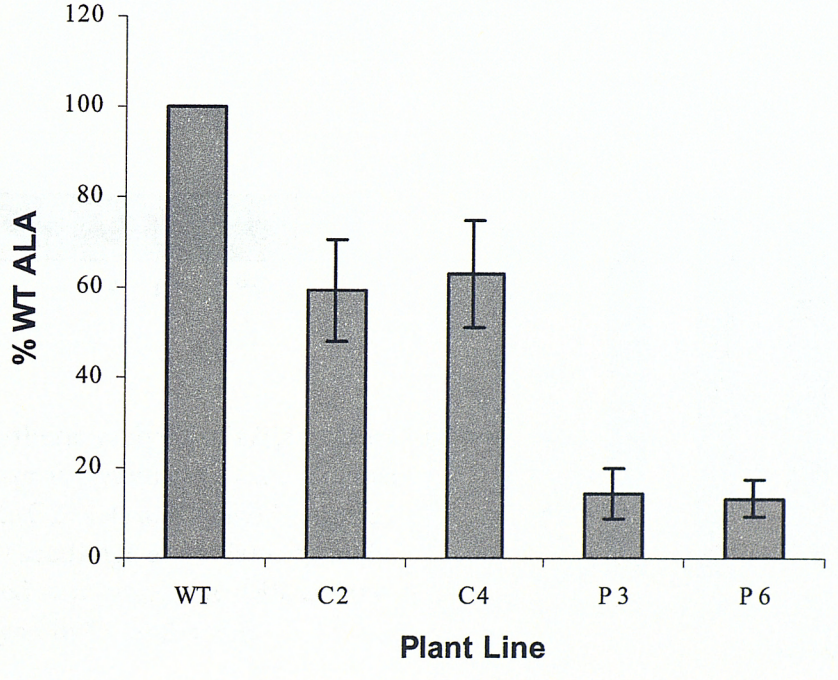
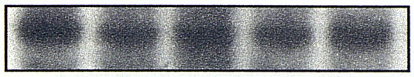


Fig. 37 ALA synthesised during a 6 h LA incubation period in a) 11-day-old white light-grown seedlings and b) 10-day-old dark-grown seedlings, transferred to white light for 24 h. WT= wild-type, C2+C4= cytosol-targeted BVR lines 2+4 and P3+P6= plastid-targeted BVR lines 3+6. White light was provided at a fluence rate of 100 $\mu molm^{-2}s^{-1}$. 100% activity corresponds to 22.5 +/- 3.1 nmol/g tissue. n=5 ± S.E.

a)



b)

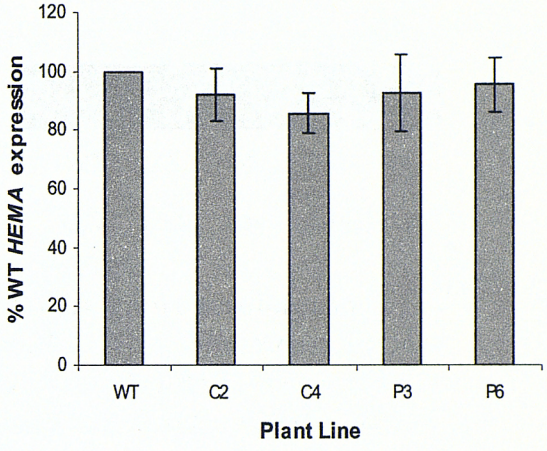
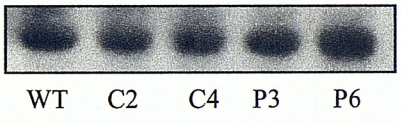


Fig.38 a) Northern analysis of *HEMA* expression in white light-grown wild-type (WT), cytosol-targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days. Signals were quantified with respect to *18S* and mean values displayed in b). n=3 ± S.E.

a)



b)

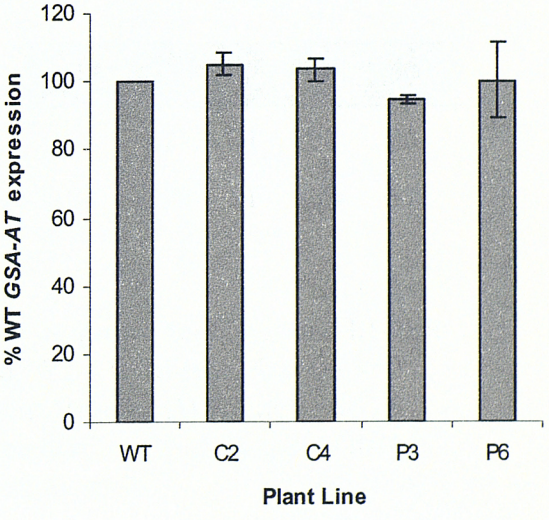


Fig.39 a) Northern analysis of *GSA* expression in white light-grown wild-type (WT), cytosol-targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days. Signals were quantified with respect to 18S and mean values displayed in b). n=3 ± S.E.

a)

WT	C2	C4	P3	P6
1	0.85	0.78	0.72	0.70

b)

WT	C2	C4	P3	P6
2.2	1.12	1.34	0.88	0.94

Fig.40 Northern analysis of *HEMA* expression in a) dark-grown seedlings and b) dark-grown seedlings transferred to white light for 24 h. WT= Wild-type, C2+C4 = cytosol-targeted BVR lines 2 and 4. P3+P6 = plastid-targeted BVR lines 3 and 6. Signals were quantified with respect to *18S* and standardised to the value of dark-grown WT samples. Mean values are displayed below. n=2.

a)

WT	C2	C4	P3	P6
1	0.88	0.76	0.68	0.72

b)

WT	C2	C4	P3	P6
1.4	0.78	1.1	0.74	0.78

Fig.41 Northern analysis of *GSA* expression in a) dark-grown seedlings and b) dark-grown seedlings transferred to white light for 24 h. WT= Wild-type, C2+C4 = cytosol-targeted BVR lines 2 and 4. P3+P6 = plastid-targeted BVR lines 3 and 6. Signals were quantified with respect to *18S* and standardised to the value of dark-grown WT samples. Mean values are displayed below. n=2.

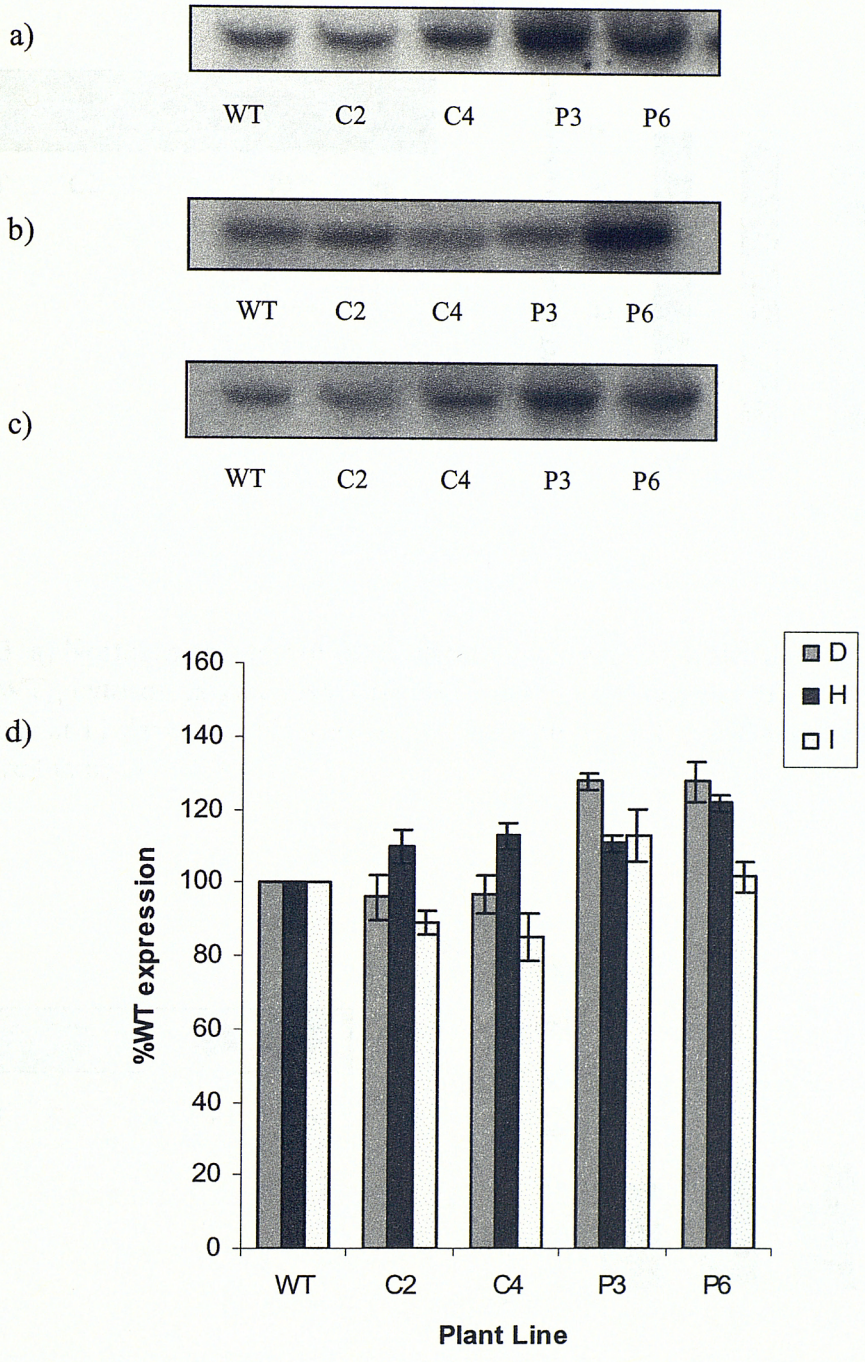


Fig. 42 Northern analysis of *CHLD* (a),*CHLH* (b) and *CHLI* (c) expression in white light-grown wild-type (WT), cytosol-targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days. Signals were quantified with respect to *18S* and mean values displayed in d). n=3 ± S.E.

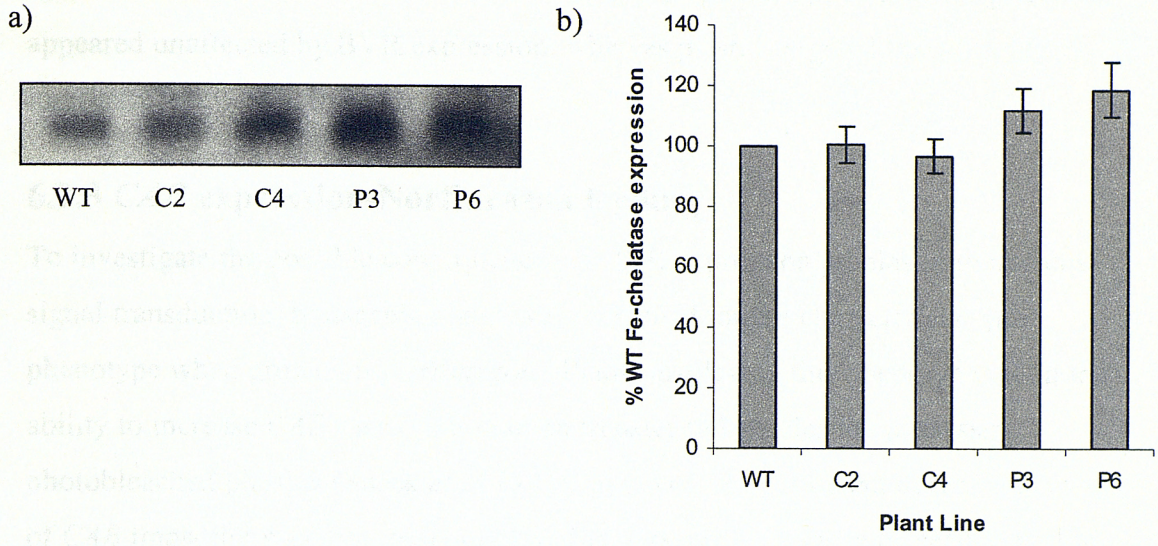


Fig. 43 a) Northern analysis of Fe-chelatase expression in white light-grown wild-type (WT), cytosol- targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days. Signals were quantified with respect to *18S* and mean values displayed in b). n=3 ± S.E.

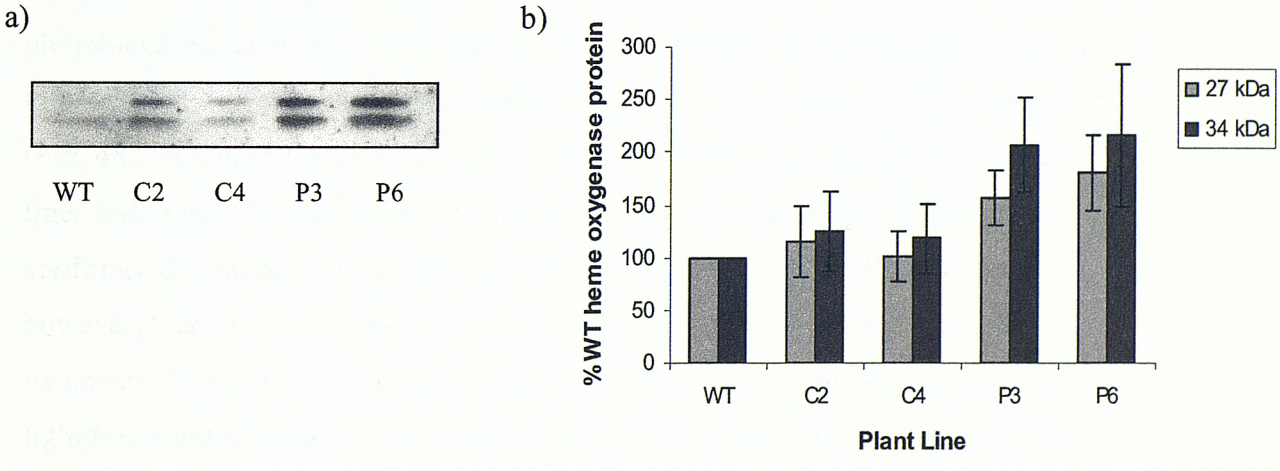


Fig. 44 a) Western analysis of heme oxygenase expression in white light-grown wild-type (WT), cytosol-targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days. Signals were quantified with respect to LSU Rubisco and mean values displayed in b). n=3 ± S.E.

plants (Fig. 43 a,b). Heme oxygenase protein levels were also quantified in light-grown seedlings, using western blotting. As in dark-grown seedlings, two bands of molecular masses 27 and 34 kDa were observed (Fig. 44 a,b). Cytosol-targeted lines appeared unaffected by BVR expression, whereas plastid-targeted lines displayed increased levels of both bands.

6.2.3 *CAB* expression-Norflurazon feeding

To investigate the possible consequences of BVR expression on plastid to nucleus signal transduction, transgenic plants were screened for the characteristic *gun* phenotype when grown on Norflurazon. Plants displaying this phenotype retain the ability to increase *CAB* transcription upon transfer to light despite possessing photobleached plastids (Susek *et al* 1993). A small, but consistent decrease in levels of *CAB* transcript was repeatedly observed in dark-grown transgenic seedlings (Fig. 45 a,b). A similar, but more marked decrease was also observed in light-grown plants (Fig. 46 a,b), consistent with deficiency in phyB (López-Juez *et al* 1998). To assess whether BVR expression induced a *gun* phenotype (Susek *et al* 1993), seedlings were grown on MS media supplemented with the carotenoid synthesis inhibitor, Norflurazon. Visualisation of ethidium bromide-stained gels revealed an absence of lower molecular weight RNA in herbicide-treated light-grown seedlings (Fig. 47 a,b). It is likely that this represents an absence of plastid-encoded transcripts in photobleached seedlings. Upon transfer to light for 24 h, an up-regulation of *CAB* transcription was observed in WT seedlings grown in the absence of Norflurazon (Fig. 48). Reduced levels of this transcript were observed in cytosol-targeted BVR lines following the same treatment, but given the decreased levels in dark-grown seedlings, displayed an equal up-regulation. Plastid-targeted BVR seedlings, however, were unable to up-regulate *CAB* transcription during the given light treatment. It is possible that such a response is delayed in these plants, and a longer light treatment is required. When grown on Norflurazon, no *CAB* transcript was detected in any line, confirming that BVR-transformed tobacco does not display the characteristic *gun* phenotype.

a)

WT C2 C4 P3 P6

b)

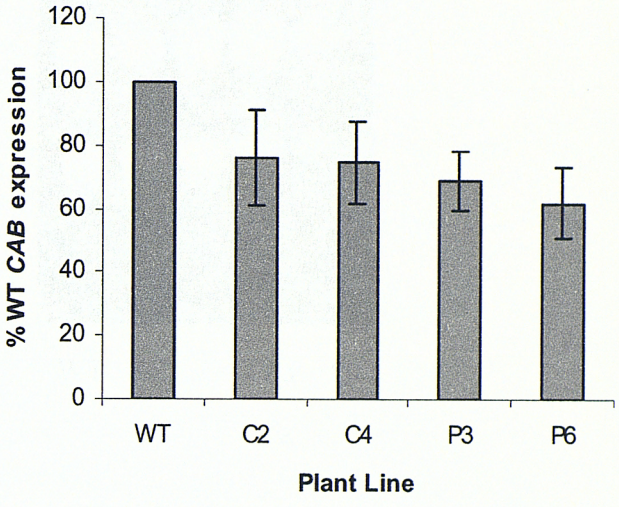


Fig. 45 a) Northern analysis of *CAB* expression in dark-grown wild-type (WT), cytosol-targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days. Signals were quantified with respect to *18S* and mean values displayed in b). n=3.

a)

WT C2 C4 P3 P6

b)

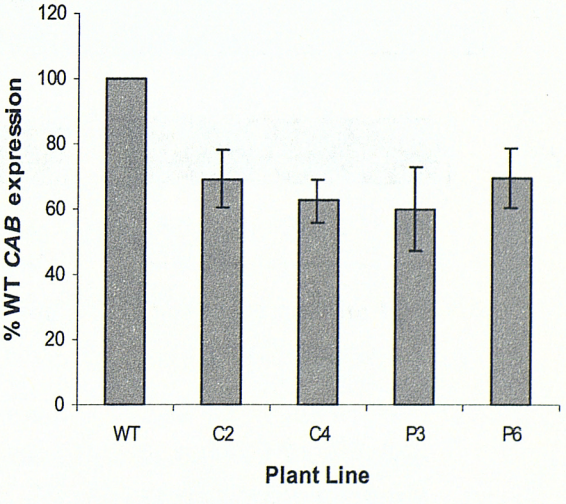


Fig. 46 a) Northern analysis of *CAB* expression in white light-grown wild-type (WT), cytosol-targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings at 11 days. Signals were quantified with respect to *18S* and mean values displayed in b). n=3.

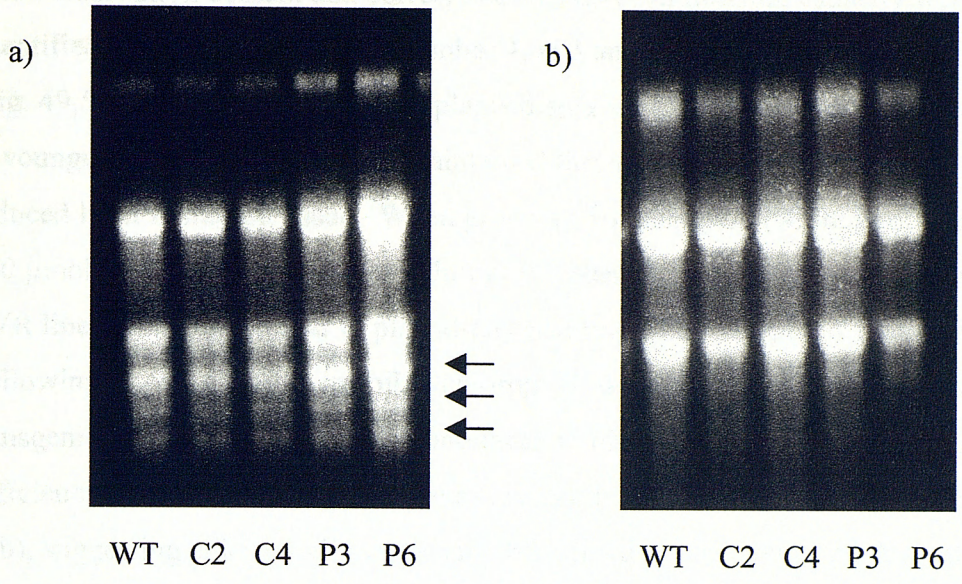


Fig. 47 EtBr-stained RNA gels of 11-day-old seedlings grown on 5 µM Norflurazon in a) the dark and b) white light (16 h days, 70 $\mu molm^{-2}s^{-1}$). An absence of plastid RNA (←) was observed in light-grown samples. WT= wild-type, C2+C4= cytosol-targeted BVR lines 2+4 and P3+P6= plastid-targeted BVR lines 3+6.

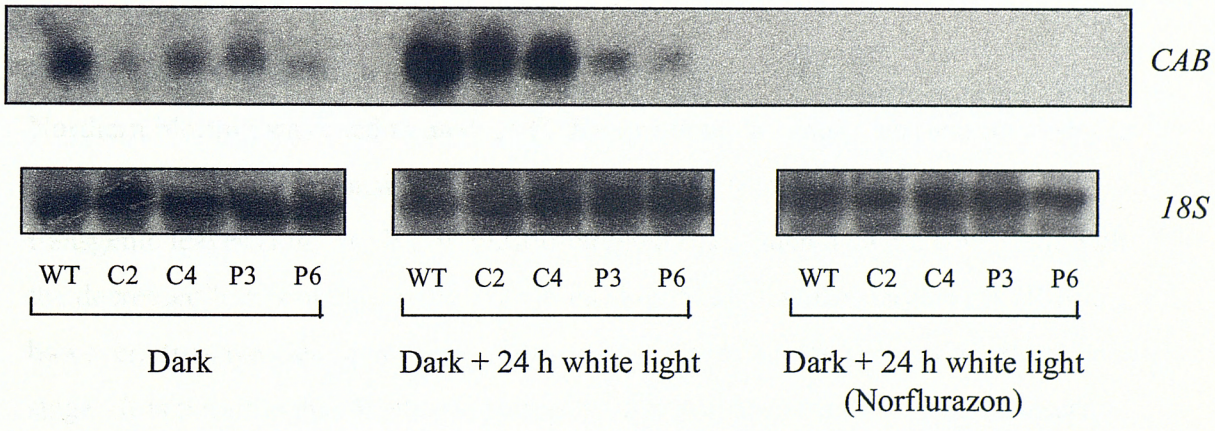


Fig. 48 Northern analysis of *CAB* and *18S* expression in wild-type (WT), cytosol-targeted BVR (C2+C4) and plastid-targeted BVR (P3+P6) seedlings grown in the dark for 11 days, 10 days + 24 h white light, and 10 days +24 h white light with 5 µM Norflurazon. This experiment was repeated 3 times with similar results.

6.2.4 ALA synthesising capacity – young leaf discs

Mean chlorophyll content and corresponding ALA synthesising capacity were quantified in leaf discs from leaf number 4, at 3 and 5 weeks from transfer to soil (Fig. 49,50). Plastid-targeted BVR plants displayed a marked chlorophyll deficiency in younger leaves. This trait was maintained throughout development albeit at a reduced level in mature tissue. When grown at Southampton (white fluorescent tubes, 130 $\mu molm^{-2}s^{-1}$, no incandescent lighting) the phenotype of young cytosol-targeted BVR lines was more similar to plastid-targeted lines than WT plants (data not shown). Following 3 weeks growth in soil, a chlorophyll deficiency was observed in all transgenic plants, an effect more pronounced in plastid-targeted lines (Fig. 49a). This deficiency was paralleled by a severe reduction in ALA synthesising capacity (Fig. 49b), suggesting chlorophyll synthesis to be limited at the level of ALA synthesis in these plants. Following 5 weeks growth in soil, chlorophyll levels in leaf number 4 were similar to WT in cytosol-targeted BVR plants, but still significantly reduced in plastid-targeted lines (Fig. 50a). An increase in ALA synthesis was, however, observed in all transgenic lines (Fig. 50b). Such data support the proposal that reduced chlorophyll levels in young leaves result, at least in part, from decreased ALA synthesis and that recovery of ALA synthesis later in development correlates with recovery of leaf chlorophyll content.

6.2.5 *CAB* expression – young leaf discs

Northern blotting was used to analyse *CAB* expression in tissue from leaf number 4 at 5 weeks in soil. Blots consistently displayed reduced levels of *CAB* transcript in transgenic leaves (Fig. 51a,b). In plastid-targeted lines, such data are consistent with the decreased levels of chlorophyll recorded (Fig. 50a). Cytosol-targeted BVR lines, however, displayed leaf chlorophyll contents comparable to WT at this developmental stage. It is possible that levels of chlorophyll binding proteins differ from transcript abundance in these plants. It was not possible to test this as no antibodies were available for western analysis.

6.2.6 Chloroplast Morphology

Chloroplast ultrastructure was investigated in transgenic plants using transmission electron microscopy (T.E.M). Tissue pieces were excised from cotyledons at 11 days, and from leaf no. 4 at 5 weeks in soil. All plants were grown under white light at a

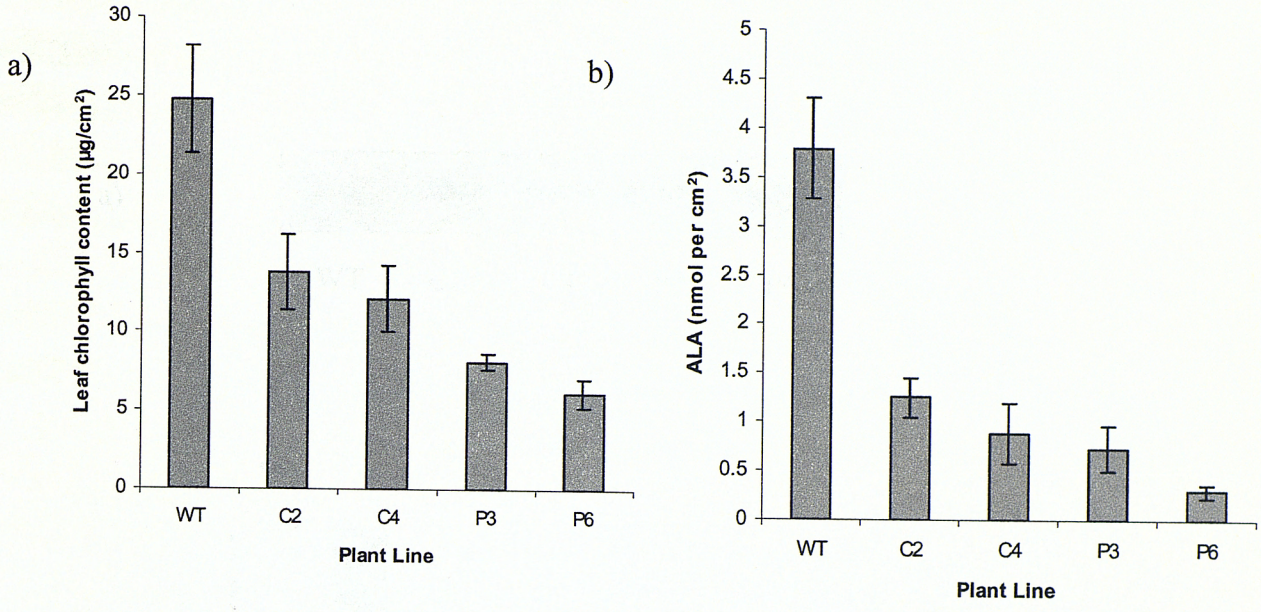


Fig. 49 Chlorophyll content (a) and ALA synthesising capacity (b) of leaf discs from leaf no.4 at 3 weeks in soil. Leaves were counted from the plant apex. WT= wild-type, C2+C4= cytosol-targeted BVR lines 2+4, P3+P6= plastid-targeted BVR lines 3+6. n=3 ± S.E.

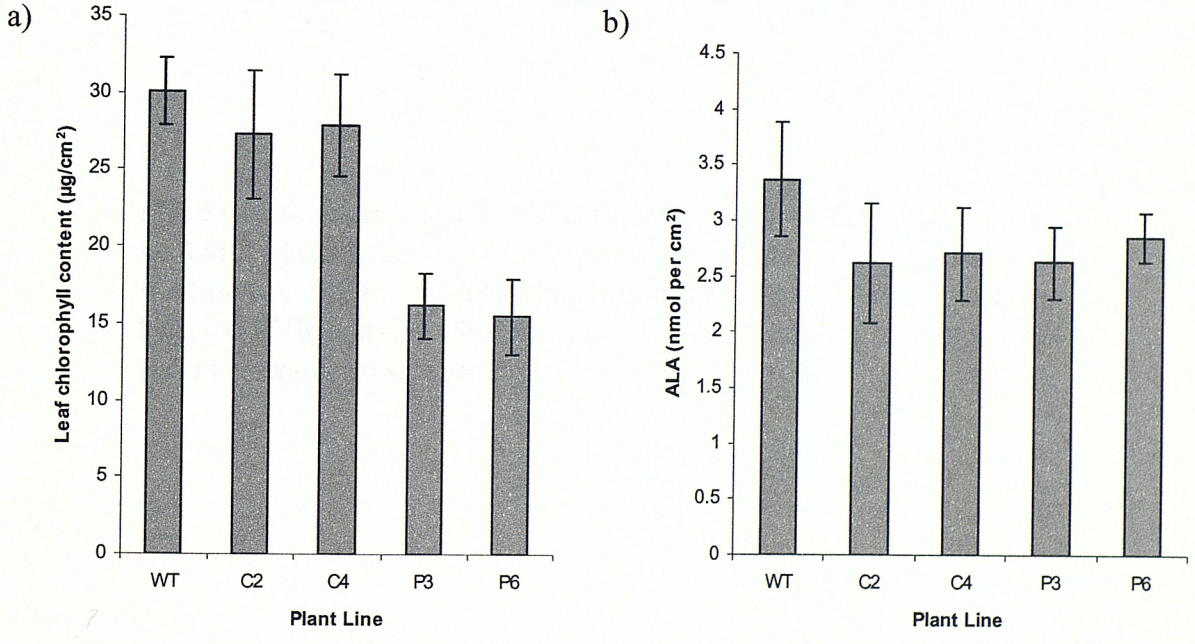


Fig. 50 Chlorophyll content (a) and ALA synthesising capacity (b) of leaf discs from leaf no.4 at 5 weeks in soil. Leaves were counted from the plant apex. WT= wild-type, C2+C4= cytosol-targeted BVR lines 2+4, P3+P6= plastid-targeted BVR lines 3+6. n=3 ± S.E.

a)

WT C2 C4 P3 P6

b)

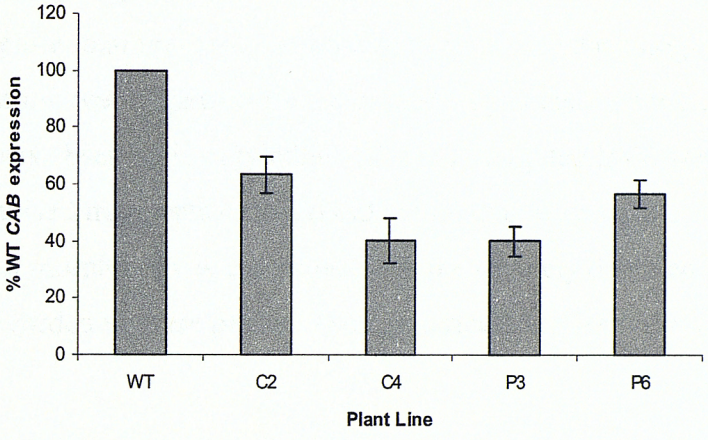
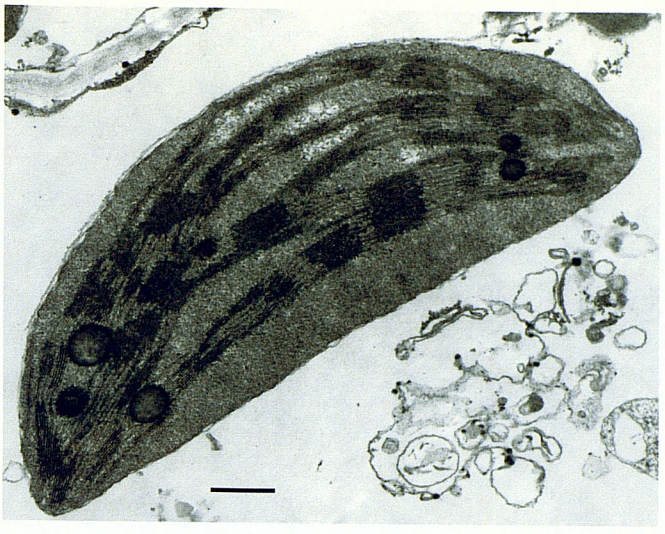


Fig. 51 a) Northern analysis of *CAB* expression in leaf tissue from leaf no.4 at 5 weeks in soil. Leaves were counted from the plant apex. WT= wild-type, C2+C4= cytosol-targeted BVR lines 2+4, P3+P6= plastid-targeted BVR lines 3+6. b) Signals were quantified with respect to 18S and mean values displayed in b). n=3 ± S.E.

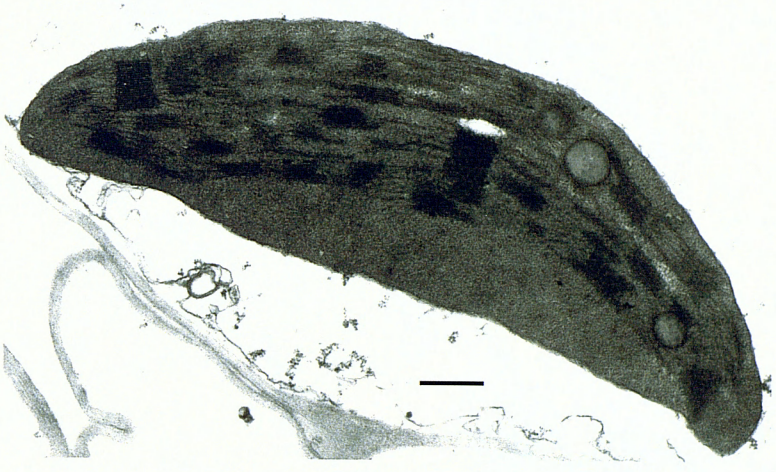
fluence rate of 100 $\mu molm^{-2}s^{-1}$ with an 8 h photoperiod. Plants from cytosol-targeted BVR line 2 and plastid-targeted BVR line 3 were used for analysis. A representative chloroplast from each line at 11 days and 5 weeks in soil are displayed in Figures 52 and 53 respectively. A WT chloroplast at 11 days is shown in Figure 52a. Ordered rows of thylakoid membranes and neatly stacked grana are clearly visible. Despite reduced chlorophyll levels (Fig 7a), plastid ultrastructure in cotyledons of cytosol-targeted BVR plants was similar to WT (Fig. 52b). Large spherical inclusions were observed in many plastids of WT and cytosol-targeted BVR cotyledons. The size and density of these inclusions makes it unlikely that they are starch grains, while dark peripheral staining suggests the presence of an external membrane or lipid layer. It is possible, therefore, that these inclusions represent enlarged plastoglobuli, although darker colouration would generally be expected. The most striking chloroplast morphology was observed in cotyledons of plastid-targeted BVR seedlings (Fig. 52c). Short, disorganised fragments of thylakoid membrane were visible, with no granal stacking. Such morphology is consistent with the severely depleted levels of chlorophyll recorded in these plants. Dark spherical vesicles represent groups of plastoglobuli.

Mature chloroplasts from WT leaves were similar to those from cotyledons, with the absence of spherical inclusions (Fig. 53a). Regular rows of thylakoid membranes and stacked grana were visibly evident. Similar chloroplasts were observed in leaves from cytosol-targeted BVR plants, although large spherical inclusions were still apparent (Fig. 53b). Chloroplasts from leaves of plastid-targeted BVR plants displayed orderly rows of thylakoid membranes and some granal stacking (Fig. 53c). The number of grana in these chloroplasts were, however, always less than in WT and cytosol-targeted BVR lines (data not shown). This morphological "recovery" is consistent with increased ALA synthesis and chlorophyll production later in development.

a)



b)



c)

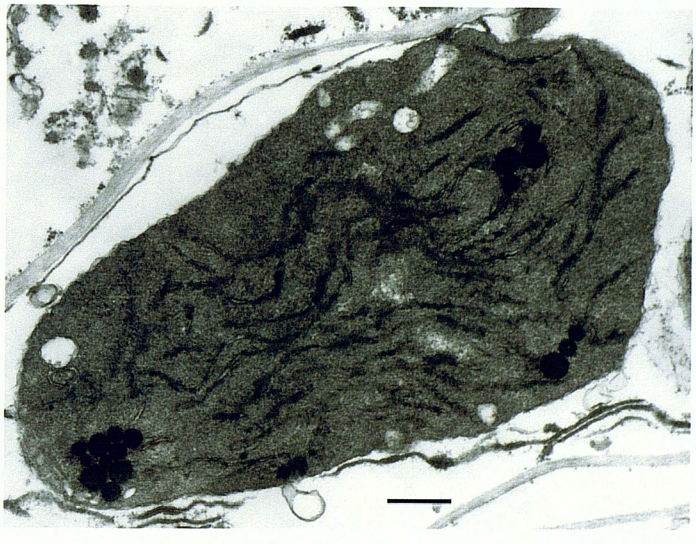
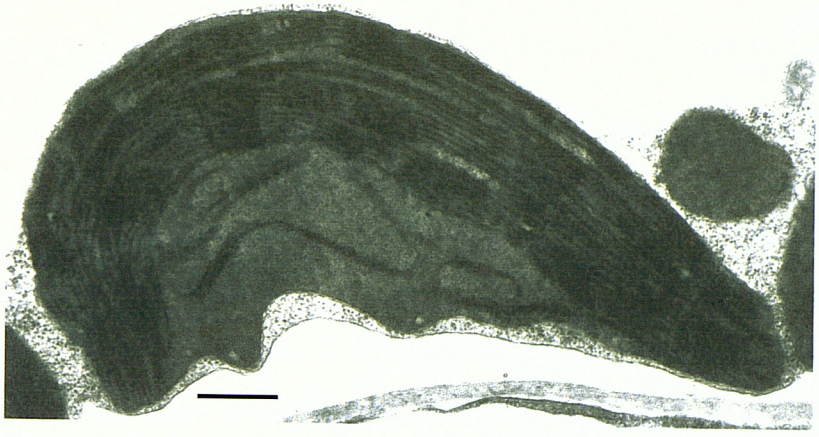


Fig. 52 Electron micrographs showing chloroplast ultrastructure of a) WT, b) cytosol-targeted BVR and c) plastid-targeted BVR cotyledons from 11-day-old white light-grown seedlings. Bar represents 1μm.

a)



b)

c)

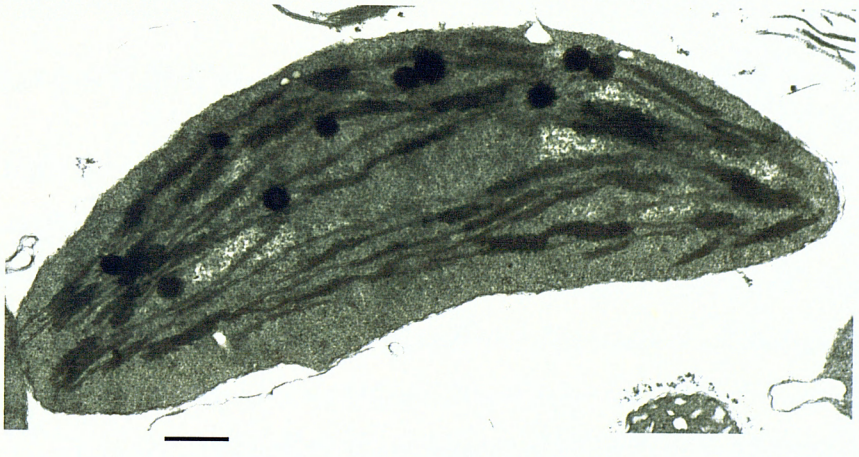


Fig. 53 Electron micrographs showing chloroplast ultrastructure of a) WT, b) cytosol-targeted BVR and c) plastid-targeted BVR leaf tissue. Leaf pieces were excised from leaf no. 4 (counting from plant apex) after 5 weeks growth in soil. Bar represents 1μm.

6.3 Discussion

This study has revealed the expression of BVR in tobacco plastids to induce phytochrome-independent perturbations of the tetrapyrrole pathway in dark-grown seedlings. The combined consequences of BVR-targeting and phytochrome deficiency were investigated through analysis of chloroplast development in light-grown seedlings and young leaves. This enabled the integration of phytochrome dependent and independent regulatory mechanisms in controlling plastid development to be examined.

A summary of the results regarding the regulation of the tetrapyrrole pathway in light-grown *BVR*-transformed tobacco is presented in Figure 54 a,b. Light-grown, cytosol-targeted BVR lines showed no change in levels of *HEMA* and *GSA* transcripts, despite showing a marked decrease in ALA synthesising capacity. Chelatase transcripts and heme oxygenase protein were also unaffected, although seedlings displayed a pronounced chlorophyll deficiency. When BVR is targeted to plastid, a more severe reduction in ALA synthesis was recorded. As in cytosol-targeted lines, no change was observed in the transcription of *HEMA* and *GSA*, suggesting this decrease not to be mediated at the transcriptional level. Despite a small, repeatable increase in levels of *CHLD*, Mg-chelatase transcription appeared unaffected in these plants. A small but consistent increase in ferrochelatase transcript was observed in plastid-targeted lines in addition to increased levels of heme oxygenase protein.

Seedlings expressing BVR display reduced ALA synthesis in the light

Unlike the situation in dark-grown plants, *BVR*-transformed seedlings grown under white light displayed severely reduced ALA synthesis (Fig. 37a). A similar result was observed for dark-grown seedlings transferred to white light for 24 h , although the difference between cytosol and plastid-targeted BVR lines was more pronounced (Fig. 37b). Transgenic seedlings accumulated less chlorophyll than WT plants following 24 h white-light treatment. Plastid-targeted BVR lines were, however, significantly less "bleached" than seedlings grown in continuous white light (data not shown). Such observations suggest that reductions in ALA synthesising capacity do not result primarily from accumulative plastid damage in transgenic plants. Current evidence suggests that phytochrome regulates the tetrapyrrole pathway at the levels of

ALA synthesis and protochlorophyllide reduction (Beale and Weinstein 1991). The severity of inhibition of ALA synthesis in plastid-targeted BVR lines may therefore be the result of reduced holophytochrome in these seedlings.

Studies in a variety of species have revealed a white light requirement for maximal expression of *HEMA1*, suggesting this enzyme to be a principal regulatory target (Ilag *et al* 1994, Bougri and Grimm 1996, Tanaka *et al* 1996). Supporting evidence was provided by antisense expression of *HEMA1* in *Arabidopsis*, which resulted in severely reduced ALA synthesis (Kumar and Söll 2000). Reporter gene studies in *Arabidopsis* have since revealed *HEMA1* expression to be light-regulated at the transcriptional level by phytochromes acting in both the FR-HIR and LFR response modes (McCormac *et al* 2001). Such evidence implicates a role for both phyA and phyB in regulating ALA synthesis. A light-mediated regulatory mechanism has been proposed for *GSA* expression in *Arabidopsis* (Ilag *et al* 1994) and soybean (Sangwan and O'Brian 1993), although direct evidence for the roles of specific phytochrome species is currently lacking (A.C. McCormac-personal communication).

Light-grown seedlings expressing BVR displayed no differences in *HEMA* or *GSA* transcript abundance (Fig. 38-39), suggesting the observed reduction in ALA synthesis not to be mediated at the transcriptional level. Conversely, studies in *Arabidopsis* have revealed a close correlation between levels of *HEMA1* transcript and ALA synthesising capacity in similarly aged, white-light grown plants (Kumar and Söll 2000). When dark-grown seedlings were transferred to white light for 24 h, an increase in *HEMA* expression was observed in all lines (Fig. 40). The level of this increase in WT seedlings was less than that observed for *HEMA1* in *Arabidopsis* (McCormac *et al* 2001), barley (Bougri and Grimm 1996) and cucumber (Tanaka *et al* 1996). This could however, represent the time of harvesting, as maximum expression in barley was shown to occur at 4 h following illumination (Bougri and Grimm 1996). A small increase in *GSA* transcription was observed in WT seedlings upon transfer to light (Fig. 41). This increase was less marked than that observed for *HEMA*, and may reflect plastid integrity rather than a phytochrome-regulated response (A.C. McCormac-personal communication).

The light-mediated induction of *HEMA* transcription in transgenic lines was less pronounced than that observed in WT seedlings and could represent phytochrome

deficiency in these plants (Fig. 40-41). Analysis of *HEMA* induction following R and FR pulses, together with a time course of transcript abundance in white light would provide further insight into the role of phytochrome in regulating this gene in tobacco. It is also possible that enzyme stability was adversely affected in transgenic plants. Attempts to quantify GluTR protein levels in light-grown seedlings were repeatedly unsuccessful. Serum raised against the tobacco protein (provided by Professor B.Grimm, Humboldt University, Berlin, Germany) yielded no signal in any line (data not shown). Western analysis of GSA-AT, using serum raised against the tobacco protein (provided by Professor B.Grimm, Humboldt University, Berlin, Germany) showed considerable variation between replicates, thus precluding interpretation (data not shown).

Plastid-targeting of BVR results in altered chelatase transcription

Regulation of tetrapyrrole pathway flux in light-grown seedlings was investigated at the molecular level through northern analysis of chelatase transcripts. No clear differences in levels of Mg or Ferrochelatase were observed in cytosol-targeted BVR lines (Fig. 42a-d). Plastid-targeted BVR lines displayed a small (15-25%), but consistent, increase in *CHLD* transcript. *CHLH* and *CHLI*, meanwhile, remained relatively unaffected. Subunit D is documented to perform a role in the activation of catalysis, but opinion remains divided as to its function in the catalytic complex (Walker and Willows 1997, Gräffe *et al* 1999). Expression of Ferrochelatase also appeared consistently increased (10-20%) in plastid-targeted BVR lines. Northern analysis is, however, a rather crude method of transcript quantification and it is difficult to ascertain whether such small differences represent metabolically significant changes in gene expression. Accurate quantitative analysis of this nature may therefore be better served by a technique such as quantitative real-time PCR (Grove 1999).

Plastid-targeting of BVR results in increased levels of heme oxygenase protein in the light

As in etiolated seedlings, increased levels of heme oxygenase protein were observed in plastid-targeted BVR lines grown under white light. It is not currently known whether this represents increased stability of the protein, or increased gene expression

(discussed in chapter 5). The inability of available cDNA sequences to hybridise with tobacco RNA prevented analysis at the transcriptional level. A modest (17%) increase in heme oxygenase 1 (*HO1*) transcription was observed in etiolated *Arabidopsis* seedlings transferred to white light for 24 h (Davis *et al* 1999). Recent studies using stem tissue of pea have revealed a similar result (P.J.Linley, M. Landsberger and M.J. Terry- unpublished data). A significant down regulation of this gene was, however, observed in cotyledon tissue of the same seedlings. Such data present the possibility of a tissue-specific, phytochrome-mediated regulatory component controlling heme oxygenase expression. As whole tobacco seedlings were used for protein quantification in this study, the consequences of phytochrome deficiency in transgenic lines can only be speculated. Further investigation into the regulatory mechanisms controlling higher plant heme oxygenase expression should enable future judicious interpretation of these data.

Seedlings expressing BVR do not show the "genomes uncoupled" phenotype

A number of *gun* (*genomes uncoupled*) mutants, perturbed in plastid to nucleus signal transduction, have been characterised in *Arabidopsis* (Susek and Chory 1992, Susek *et al* 1993, Vinti *et al* 2000). The recent identification of *gun5* as a mutation in the gene encoding Mg-chelatase, subunit H, has confirmed the significance of tetrapyrroles in this signalling pathway (Mochizuki *et al* 2001). Tobacco plants expressing antisense constructs of Mg-chelatase, subunit H, were shown to display a marked chlorophyll deficiency (Papenbrock *et al* 2000). Further investigation revealed reductions in ALA synthesis, protoporphyrin IX and heme. The reduced ALA synthesising capacity observed in these plants was attributed to reduced levels and activity of GluTR and ALA dehydratase. The authors propose a regulatory mechanism that co-ordinates the synthesis of ALA at the levels of gene expression and enzyme activity in response to metabolic flux through the Mg branch of the pathway. Such a regulatory mechanism would prevent the accumulation of tetrapyrrole precursors, and consequently photo-oxidative damage. Mg-chelatase activity is maximal at the beginning of illumination and diminishes during the dark period, when ferrochelatase takes precedence (Papenbrock *et al* 1999). The results presented here are in partial accordance with the observations of Papenbrock and colleagues. Discrepancies arise in the nature of such porphyrin-mediated regulation,

as no transcriptional down-regulation of *HEMA* was observed in light-grown BVR-transformed plants. Inconsistencies between groups could result from differences in the age of tissue used for analysis. Results described here relate to light-grown seedlings, whereas Papenbrock and colleagues used mature leaf tissue for all experiments (Papenbrock *et al* 2000). Overall, current evidence suggests flux through the chelatase branchpoint to be of regulatory importance to the expression and activity of nuclear-encoded plastid proteins in light-grown plants.

CAB expression was analysed in dark and light-grown seedlings expressing BVR. In dark-grown transgenic lines, levels of *CAB* transcript were reduced (Fig. 45 a,b). A similar reduction was observed in etiolated seedlings of *aurea* (Sharrock *et al* 1988), which also displayed a deficiency of light harvesting chlorophyll a/b binding polypeptides (Ken-Dror and Horwitz 1990). WT levels of *CAB* mRNA were observed in *phyB* and *hy1* mutants of *Arabidopsis* grown in complete darkness (López-Juez *et al* 1998). These plants were, however, grown on media containing sucrose, therefore precluding direct comparison. Results support the existence of a tetrapyrrole-mediated, phytochrome-independent regulatory mechanism controlling *CAB* expresssion, which is disrupted in *aurea* and *BVR*-transformed tobacco.

When grown under white light, a reduction in *CAB* expression was recorded in all transgenic lines (Fig. 46 a,b). A similar decrease was observed in light-grown seedlings of the *Arabidopsis* mutants *hy1* and *phyB* (López-Juez *et al* 1998), suggesting the deficiency observed in BVR-transformed seedlings to result, at least in part, from a deficiency in phytochrome B. The role of phytochrome in regulating *CAB* transcription is well documented (Horwitz *et al* 1988, Karlin-Neumann *et al* 1988, Hamazato *et al* 1997, López-Juez *et al* 1998). The ability of cytosol-targeted BVR seedlings to up-regulate *CAB* transcription upon transfer to light (Fig. 48), suggests these plants contain sufficient phytochrome to mediate this response. A mild reduction in *CAB* accumulation following a red pulse was observed in *phyB* mutants of *Arabidopsis*, with a much more severe reduction being observed in chromophore-deficient *hy1* and *aurea* plants (Sharrock *et al* 1988, Chory *et al* 1989b, Reed *et al* 1994, López-Juez *et al* 1998). In accordance with these and previously described physiological data (chapters 3 and 4), the inability of plastid-targeted BVR seedlings

to up-regulate *CAB* transcription upon transfer to light is consistent with a severe phytochrome deficiency.

When grown under high-fluence rates of white light in the presence of Norflurazon, plants suffer rapid photo-oxidation of chloroplast components and a loss of plastid signalling (Sagar *et al* 1988, Oelmüller 1989). *CAB* expression was therefore analysed in Norflurazon -treated transgenic seedlings, to investigate the transcriptional effects of altered tetrapyrrole pathway flux. Norflurazon-mediated photobleaching resulted in an absence of plastid-encoded transcripts in all lines (Fig. 47). Despite this, total RNA yields were similar to control plants (data not shown). The absence of *CAB* transcript in light-grown BVR-transformed seedlings, grown in the presence of Norflurazon, demonstrated that these plants do not display a characteristic *gun* mutant phenotype (Susek *et al* 1993). Such a discovery does not, however, eliminate the possibility that plastid to nucleus signal transduction is disrupted. The reduced levels of *HEMA*, *GSA* and *CAB* transcripts in dark-grown transgenic seedlings support such a proposal. Further support for a phytochrome-independent plastid signal mediating the nuclear transcription of plastid proteins is provided by the possible up-regulation of heme oxygenase transcription in both dark and light-grown plastid-targeted BVR seedlings. Differences in the behaviour of seedlings expressing BVR and the *hy1* mutant of *Arabidopsis* may be species specific. Similar analyses of BVR-transformed *Arabidopsis* (Lagarias *et al* 1997, Montgomery *et al* 1999), and chromophore-deficient mutants of tomato (Terry and Kendrick 1996) and *Nicotiana plumbagnifolia* (Kraepiel *et al* 1994) would therefore yield valuable information.

It is likely that multiple "plastid signals" exist in angiosperms, with roles in both the activation and repression of nuclear gene expression. Reporter gene studies revealed ALA feeding to reduce phytochrome activation of the *Lhcb* promoter in *Arabidopsis* (López-Juez *et al* 1999). The authors suggest the possibility of a chlorophyll precursor acting as a repressor of *Lhcb* transcription, with a role for phytochrome in the removal of this repression. The discovery that BVR expression induces gross perturbations of pathway flux in addition to severe phytochrome deficiency makes identification of such a factor problematic in these plants. In a genetic screen for photomorphogenic mutants in *Arabidopsis*, Møller and colleagues isolated a line with

a *Ds* transposon inserted into a gene for a chloroplast protein (Møller *et al* 2001). Termed *long after far-red 6* (*laf6*), mutant plants displayed impairments in FR-mediated hypocotyl growth inhibition. Expression of the light-regulated genes chalcone synthase, ferredoxin $NADP^+$ oxidoreductase and *CAB* was also attenuated in *laf6* seedlings, in response to high-fluence FR. The *LAF6* gene was subsequently revealed to encode an ATP-binding cassette protein (ABC1), possibly involved in solute transport. Biochemical investigation revealed an accumulation of protoporphyrin IX in mutant plants, leading to the proposal that ABC1 functions to import protoporphyrin IX from the envelope, where it is synthesised, to the stroma, where chlorophyll and heme synthesis take place. The possibility that protoporphyrin IX itself acts as a transcriptional repressor is in disagreement with work using *Chlamydomonas*, which showed no effect of protoporphyrin IX feeding on nuclear gene expression (Kropat *et al* 1997). Mutants, defective in the synthesis of Mg-protoporphyrin IX, were unable to induce the expression of nuclear heat shock protein HSP70 upon transfer to light, a response subsequently rescued by feeding Mg-protoporphyrins (Kropat *et al* 1997, 2000). Direct comparison of this data with that of *laf6* is, however, precluded by the use of white light in these experiments and an absence of phytochromes in *Chlamydomonas*.

Overall, it appears that higher plant plastid to nucleus signal transduction involves a complicated integration of multiple components. It can be concluded that at least one component involves tetrapyrrole intermediates (Vinti *et al* 2000, Jarvis 2001), highlighting the developmental importance of chelatase branchpoint regulation in controlling tetrapyrrole pathway flux.

Recovery of chlorophyll content in developing leaves correlates with a recovery of ALA synthesis

Plants expressing BVR display a characteristic phenotype throughout their life cycle. The recovery of chlorophyll synthesis in mature cytosol-targeted BVR lines suggests that inhibitory mechanisms present in young plants have been overcome at this developmental stage. Internode data, however, shows these plants to possess phytochrome deficiency througout their life cycle (Fig. 14). Such data thereby infers that recovery of chlorophyll in these plants is not the result of holophytochrome accumulation. The chlorophyll deficiency observed in mature plastid-targeted lines is

still significant, but not as severe as that displayed in seedlings. Such observations suggest that regulation at this developmental stage is mediated, not by phytochrome, but by feedback mechanisms within plastids.

As in seedlings, the pronounced chlorophyll deficiency in young leaves of plants expressing BVR is paralleled by a severe reduction in ALA synthesis (Fig.49 a,b). The recovery of chlorophyll synthesis throughout development in all transgenic lines corresponds to a recovery in ALA synthesising capacity (Fig.50 a,b). These data suggest that chlorophyll synthesis in younger leaves is limited, at least partially, by the synthesis of its precursor, ALA. A decrease in *CAB* transcription was also observed in young leaves of cytosol and plastid-targeted lines (Fig. 51 a,b), and is consistent with the reduced phytochrome content of these plants. Mature plants of the chromophore-deficient mutants *hy1, hy2* and *aurea*, were all shown to display WT levels of *CAB* under high fluence white light, despite reduced levels of chlorophyll (Chory *et al* 1989a, Becker *et al* 1992). The authors propose that phytochrome plays a primary role in gene expression in etiolated plants, with a modulatory role being adopted in mature tissue. Results here support the proposal that the tobacco tetrapyrrole pathway is controlled by a complex network of regulatory mechanisms, showing different temporal precedence throughout development. It is therefore likely that multiple, overlapping mechanisms exist to ensure tetrapyrrole biosynthesis is adapted to the prevailing conditions.

Expression of BVR results in altered chloroplast morphology

Although reduced chlorophyll levels were observed, cytosol-targeting of BVR had little effect on chloroplast morphology in white light-grown cotyledons (Fig. 52 b). A dramatic effect was, however, observed in chloroplasts from cotyledons of plastid-targeted BVR plants. Chloroplasts were distended and displayed randomly organised pieces of thylakoid membrane, with no granal stacking (fig. 52 c). Such morphology is similar to that of a chloroplast in part transition from an etioplast (Gunning and Steer 1996). Lipoidal bodies, termed plastoglobuli, were also prominent. These are normally observed in senescing chloroplasts, and are associated with thylakoid disassembly (Zavaltea-Mancera *et al* 1999). It is therefore possible that in the absence of chlorophyll and light harvesting chlorophyll binding protein (Lhcb), thylakoid membrane material is degraded via the formation of plastoglobuli.

Chromophore-deficient mutants of *Arabidopsis* and tomato have been shown to possess reduced numbers of chloroplasts (Chory *et al* 1989b) and have less granal stacking per chloroplast (Koornneef *et al* 1985, Chory *et al* 1989b, Neuhaus *et al* 1993, Vinti *et al* 2000). Disorganised thylakoid structure was also observed in the chlorophyll-deficient *chlorina* mutants of wheat (Falbel *et al* 1996). Reductions in both plastid size and granal stacking were observed in the *CAB*-deficient *cue* mutants of *Arabidopsis* (López-Juez *et al* 1998), whereas a more extreme phenotype was recorded in the albino *cla-1* mutant (Mandel *et al* 1996). An absence of *CAB* expression in these plants was accompanied by arrested chloroplast development and vesiculation of thylakoid membranes. The *Arabidopsis* mutant *gun-1*, defective in plastid-nuclear signalling, de-etiolated more slowly but displayed fully developed chloroplasts after 48 h in the light (Mochizuki *et al* 1996). Taken together, results suggest that chlorophyll synthesis and *CAB* expression are essential for chloroplast development. The severe deficiency of both these components in cotyledons of plastid-targeted BVR plants can therefore account for the abnormal morphology observed. The recovery of chlorophyll synthesis later in development is consistent with the partial granal stacking observed in mature chloroplasts (Fig. 53) and suggests a gradual accumulation of light harvesting chlorophyll *a*/*b* binding proteins in these plants.

a)

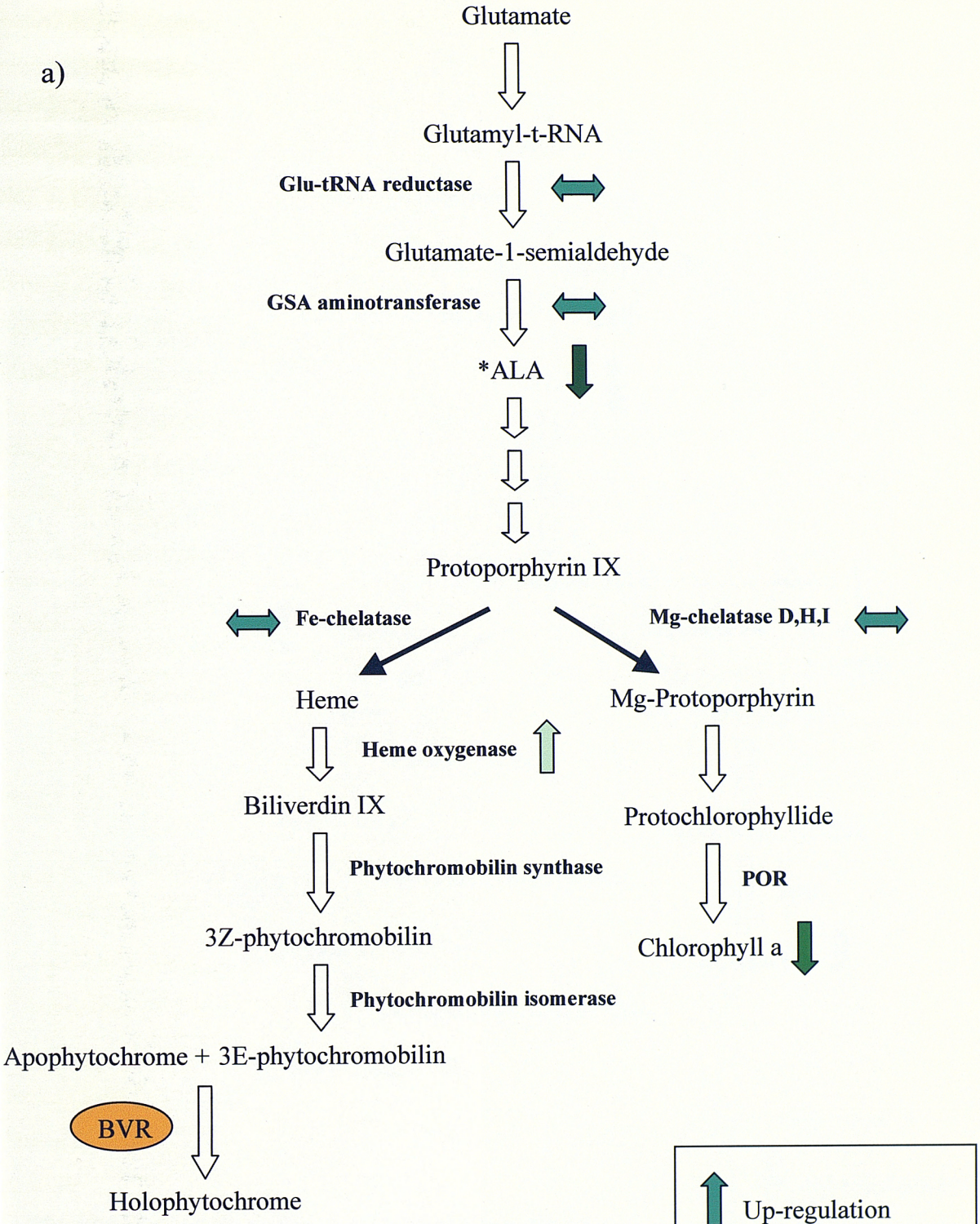


Fig. 54 a) Regulation of the tetrapyrrole biosynthetic pathway in white light-grown cytosol-targeted BVR seedlings.

* ALA represents synthesising capacity.

b)

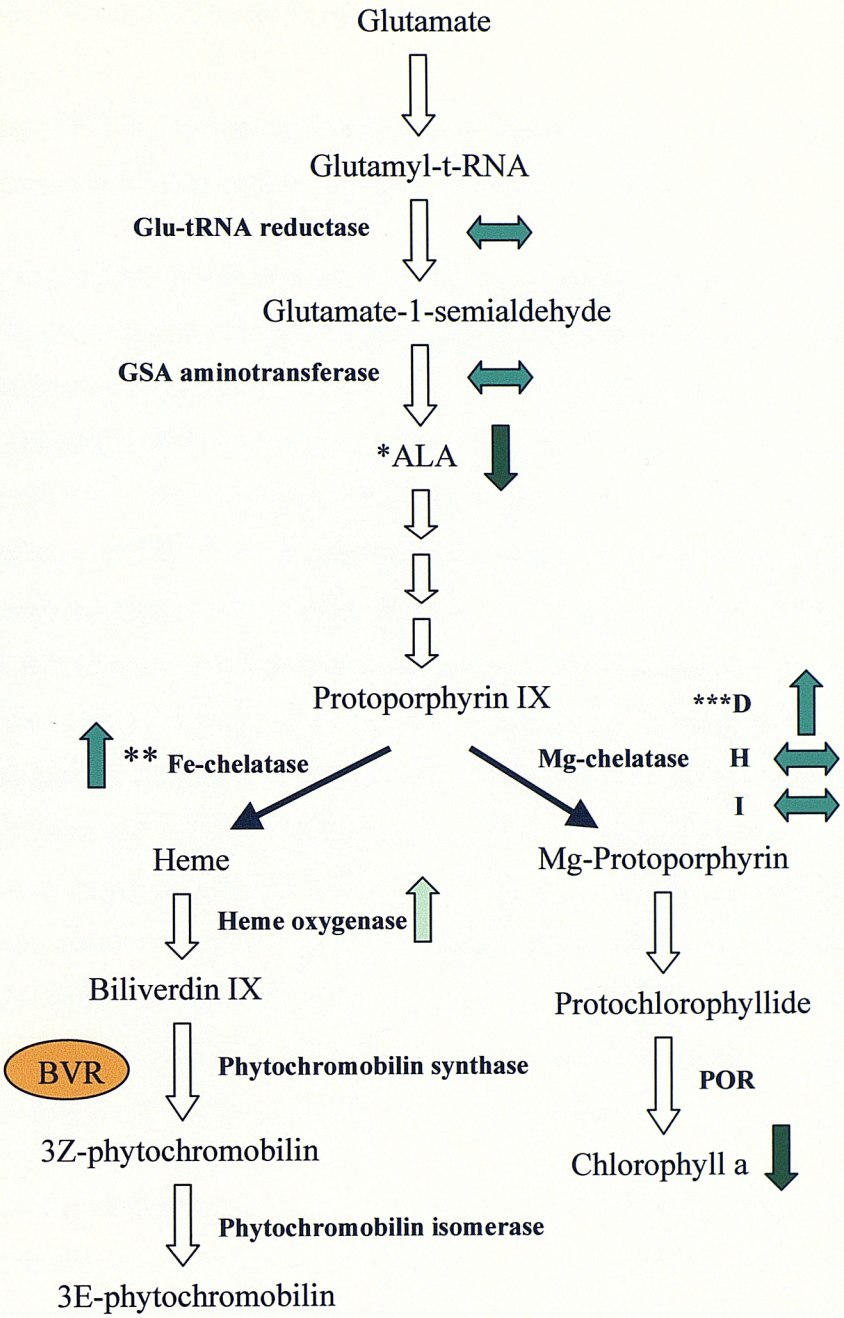


Fig. 54 b) Regulation of the tetrapyrrole biosynthetic pathway in white light-grown plastid-targeted BVR seedlings

* ALA represents synthesising capacity
** A small (10-20%) increase was observed
*** A small (15-25%) increase was observed

Chapter 7. General Discussion

Expression of BVR in tobacco results in plants displaying phenotypes consistent with multiple phytochrome deficiency throughout their lifecycle

Constitutive expression of mammalian BVR in *Nicotiana tabacum* cv. Maryland Mammoth has pronounced effects on light-mediated growth and development. Unlike phytochrome chromophore-deficient mutants, transgenic plants displayed phenotypes consistent with chromophore-deficiency throughout their lifecycle (Kraepiel *et al* 1994, Koornneef *et al* 1995, Terry and Kendrick 1996, Van Tuinen *et al* 1996, Weller *et al* 1996,1997b). Similar results were observed in transgenic *Arabidopsis* expressing BVR (Lagarias *et al* 1997, Montgomery *et al* 1999). Recovery of WT phenotype in chromophore-deficient plants, mutated in heme oxygenase, has been attributed to transcription of additional isoforms of the enzyme, later in development (Davis *et al* 2001). Reduced germination, elongated hypocotyls, smaller cotyledons and decreased chlorophyll content were all observed in light-grown seedlings expressing BVR. The impaired photomorphogenic responses observed under continuous R and FR wavelengths are consistent with deficiencies in both phyA and phyB activities (Koornneef *et al* 1980, Whitelam *et al* 1992,1993 McCormac *et al* 1993b, Reed *et al* 1994). Seedling analysis in *BVR*-transformed lines also reveals a role for B in tobacco seedling morphogenesis.

With the exception of hypocotyl elongation, which was qualitatively the same, independent of subcellular BVR localisation, phenotypes were always more severe for plastid-targeted lines. This effect did not appear to be due to differences in BVR expression, since quantitative levels of enzyme were higher for cytosol-targeted plants (Montgomery *et al* 2001). This suggests that either plastid-targeting of BVR expression is more effective at reducing holophytochrome levels and that different photomorphogenic responses have different phytochrome "thresholds", or that additional metabolic consequences arise from BVR localisation within the plastid. Quantification of photoactive holophytochrome revealed equally severe deficiencies in etiolated seedlings of cytosol and plastid targeted lines, although accurate comparative analysis was beyond the sensitivity of the available spectrophotometer.

Mature plastid-targeted BVR plants displayed elongated internodes, altered leaf morphology and reduced chlorophyll levels, consonant with deficiencies in multiple phytochromes. Exaggerated phenotypes were observed under a low fluence rate of white light and are consistent with a possible role for B in mature plant development. Photoperiodic sensitivity was reduced in transgenic plants, with all lines flowering in photoperiods above 10.5 h. This result is consistent with an inhibitory role for phytochrome in controlling flowering in short-day plants. Cytosol-targeted BVR lines displayed an intermediate phenotype between that of WT and plastid-targeted lines. This result correlates with a partial reduction in photoactive holophytochrome in these plants.

Targeting of BVR to the plastid results in additional perturbations of tetrapyrrole biosynthesis and plastid development

Leaf development was severely impaired in plastid-targeted BVR lines. Mature plants displayed reduced leaf area:length ratios and decreased levels of chlorophyll and carotenoids. The reduction in leaf chlorophyll content was paralleled by an increase in chlorophyll *a*:*b* ratio. As with flowering behaviour, such differences can be attributed to reduced holophytochrome levels in these plants. It is likely, however, that localisation of BVR in plastids also disrupts tetrapyrrole biosynthesis, resulting in abnormal pigment accumulation. Detailed analysis of transgenic lines has revealed perturbations of the tetrapyrrole pathway and altered plastid morphology. A summary of tetrapyrrole regulation in plastid-targeted BVR plants is presented in Figure 55. It is possible that the steady metabolism of biliverdin by BVR within plastids acts to "pull" porphyrins through the heme branch of the pathway, reducing flux through the Mg-branch, and consequently (proto)chlorophyll levels. The fate of accumulated bilirubin is currently unknown but is likely to accumulate in cell vacuoles (A.F. McDonagh- personal communication).

In dark-grown seedlings, the constant metabolism of heme and concomitant decrease in Pchlide:POR:NADPH ternary complex could act to relieve inhibition on ALA biosynthesis, resulting in the increases observed (Duggan and Gassman 1974, Masuda *et al* 1990, Beale and Weinstein 1991, Potoppidan and Kannangara 1994). An elevated flux through the heme branch of the pathway is consistent with the increased levels of heme oxygenase protein observed in plastid-targeted BVR lines. The

decreased levels of Pchlide in cytosol-targted BVR lines are, however, difficult to explain using such a hypothesis. An alternative possibility is that accumulated apoproteins in transgenic plants display kinase activity and reduce POR levels through transcriptional repression and/or increased protein turnover (J.Clark Lagarias-personal communication). The decreased levels of Pchlide in transgenic seedlings would therefore represent increased degradation of the pigment in the absence of its binding protein. Kinase activity of apophytochrome has yet to be demonstrated *in vivo*, but tetrapyrrole analysis of etiolated transgenic plants showing chromophore deficiency and altered apophytochrome levels should yield valuable information.

The decreased levels of *HEMA* and *GSA* transcripts in dark-grown transgenic seedlings are in apparent contradiction to the increased rates of ALA synthesis observed. It would therefore appear that in etiolated plants, the effect of removing biochemical inhibition has a greater overall influence on ALA synthesis than the transcriptional regulation of biosynthetic enzymes. The possibility that altered enzyme expression in these lines results from phytochrome deficiency cannot be discounted. Multiple experiments have suggested an active role for phytochrome in its Pr form (Liscum and Hangarter 1993, Reed *et al* 1993,1994, Shinomura *et al* 1994, Saefkow *et al* 1995), although many of the published experimental protocols include R pre-treatment or growth under continous FR. These conditions are likely to result in some Pfr production and therefore confound interpretation of results. The reduced Pchlide recorded in etiolated hypocotyls of tomato mutants, however, involved no such pre-treatment and support a role for Pr in etioplast development (Terry *et al* 2001).

A significant inhibition of ALA biosynthesis was recorded in white light-grown transgenic seedlings, although northern analysis suggests this not to be mediated at the transcriptional level. Improved antibody quality would enable future investigation into protein translation. The "intermediate" response of cytosol-targeted BVR lines supports a role for phytochrome in regulating ALA synthesis at this developmental stage. The apparent "recovery" of chlorophyll levels in these plants, however, suggests such regulation not to be significant later in development. It is likely that the reduced levels of *CAB* in transgenic plants result from phytochrome deficiency. Depletion of light harvesting chlorophyll binding proteins in light-grown seedlings

could result in photo-oxidative free radical damage. Biliverdin has strong anti-oxidant properties (Stocker *et al* 1987, McDonagh 2001). An absence of this pigment in chloroplasts of plastid-targeted BVR plants could therefore serve to exacerbate free-radical damage and possibly impede ALA synthesis. The inhibition of ALA synthesis observed in etiolated seedlings transferred to light could therefore be explained by reduced *HEMA* and *GSA* transcript abundance, in addition to an indirect inhibition of ALA biosynthesis through free radical damage. A gradual accumulation of light harvesting chlorophyll binding proteins in plastid-targeted BVR plants would be consistent with the gradual recovery of chlorophyll synthesis and grana formation during development. Evidence presented here is consistent with the work of Papenbrock and colleagues (2000) and supports the existence of a regulatory mechanism that co-ordinates ALA synthesis in the light, in response to flux through the Mg-branch of the pathway. The exact nature of this regulation, however, remains unclear.

Analysis of tetrapyrrole regulation in plants expressing BVR supports a role for tetrapyrroles in plastid-nuclear signalling

Flux through the Mg-branch of the tetrapyrrole pathway is widely acknowledged to be of regulatory importance to the transcription of nuclear-encoded plastid proteins in both *Chlamydomonas* and a variety of higher plants (Kropat *et al* 1997, 2000, Vinti *et al* 2000, Møller *et al* 2001, Jarvis 2001). The altered expression patterns of tetrapyrrole biosynthetic enzymes, observed in dark-grown *BVR*-transformed seedlings, cannot easily be attributed to phytochrome deficiency and support the existence of a plastid-derived signal controlling nuclear gene expression. The nature of such a tetrapyrrole signal, however, remains elusive. The reduction of *HEMA* and *GSA* transcript in dark-grown, cytosol-targeted BVR lines suggests the presence of phytochromobilin in the cytosol to be of possible regulatory significance. It is also possible that the hypothesised tetrapyrrole-mediated "plastid signal" relates, not to the accumulation of a single intermediate, but to critical ratios of tetrapyrrole precursors in the same and/or different branches of the pathway (Oster *et al* 1991, Vinti *et al* 2000). The latter regulatory mechanism would enable plants to monitor flux through both the heme and Mg-branches and adjust nuclear transcription accordingly. The recently observed increases in ferrochelatase activity upon wounding and pathogen

attack of tobacco leaves (Smith *et al* 1999), suggest significant metabolic plasticity within the tetrapyrrole pathway under conditions of stress. The authors propose that increased ferrochelatase activity is required to provide heme cofactors for catalase and peroxidase enzymes, both of which are required for the metabolism of H_2O_2. The ability to co-ordinate nuclear gene expression with flux through the tetrapyrrole pathway would enable plants to exquisitely adapt their plastid physiology to the prevailing environmental conditions, thus conferring considerable advantage. The majority of studies investigating plastid to nucleus signal transduction have focussed on a very limited set of nuclear genes during the dark-light transition of etiolated seedlings. The non-conformity of *BVR*-transformed plants to the characterised *gun* phenotype attests the limited scope of such screening procedures and implies that wider analysis will be required to ultimately elucidate signalling pathways. An *in planta* method of flux estimation through different branches of the tetrapyrrole pathway would enable the analysis of mutant and transgenic plants, perturbed in pathway flux, to provide insight into the relationship between tetrapyrrole ratios and nuclear transcription.

Variation between seedling samples has presented problems throughout this work. Results have demonstrated that uniformity of developmental stage is critical to enable comparative analysis between experiments. Differences in stability and turnover of both mRNA and protein make northern and western analysis crude methods of quantification, although sufficient replication should have substantiated conclusions. The convincing repeatability of certain assays provides confidence that results represent the biochemical situation *in planta*, although the ectopic expression of a heterologous trans-gene product represents a highly unnatural situation. Unlike other transgenic approaches, however, the expression of a mammalian gene without a homologue in plants should have reduced pleiotrophy by specifically targeting tetrapyrrole biosynthesis and consequently holophytochome assembly.

Overall, the holistic approach employed in this study has provided explanations for the observed phenotypes of *BVR*-transformed tobacco at the molecular, biochemical and physiological levels. Results have confirmed the importance of phytochrome at all stages of plant development and are consistent with BVR-transformed plants displaying multiple phytochrome deficiency. The inability of antisense gene

expression to reduce transcription of individual phytochromes in tobacco prevented parallel analyses of these plants. Results from the present study, and that of *Arabidopsis* (Montgomery *et al* 1999), have revealed that the subcellular localisation of BVR is critical in determining plant phenotype. Plastid-targeting of the enzyme resulted in exaggerated "phytochrome-deficient" responses and additional perturbations of plastid development. The integrated approach taken in this work has provided a unique insight into the regulation of tetrapyrrole biosynthesis in tobacco. Such analysis has confirmed the importance of plastid integrity on plant growth and provided further support for a role for tetrapyrroles in plastid-nuclear signalling. Future work could include detailed investigation of tetrapyrrole metabolism in a variety of chromophore-deficient mutants. The cloning of tobacco heme oxygenase should enable transcriptional analysis of this gene in *BVR*-transformed plants. Pchlide quantification following light flash treatments and northern analysis of *POR* expression could also be performed. Crossing of chromophore-deficient plants with those showing altered apoprotein accumulation should provide valuable insight into the putative kinase activity of apophytochrome in dark-grown seedlings. The expansion of current mutant screening procedures and future integration of molecular and biochemical data should yield the identities of plastid-derived signalling molecules, thus elucidating the mechanisms of plastid-nuclear communication.

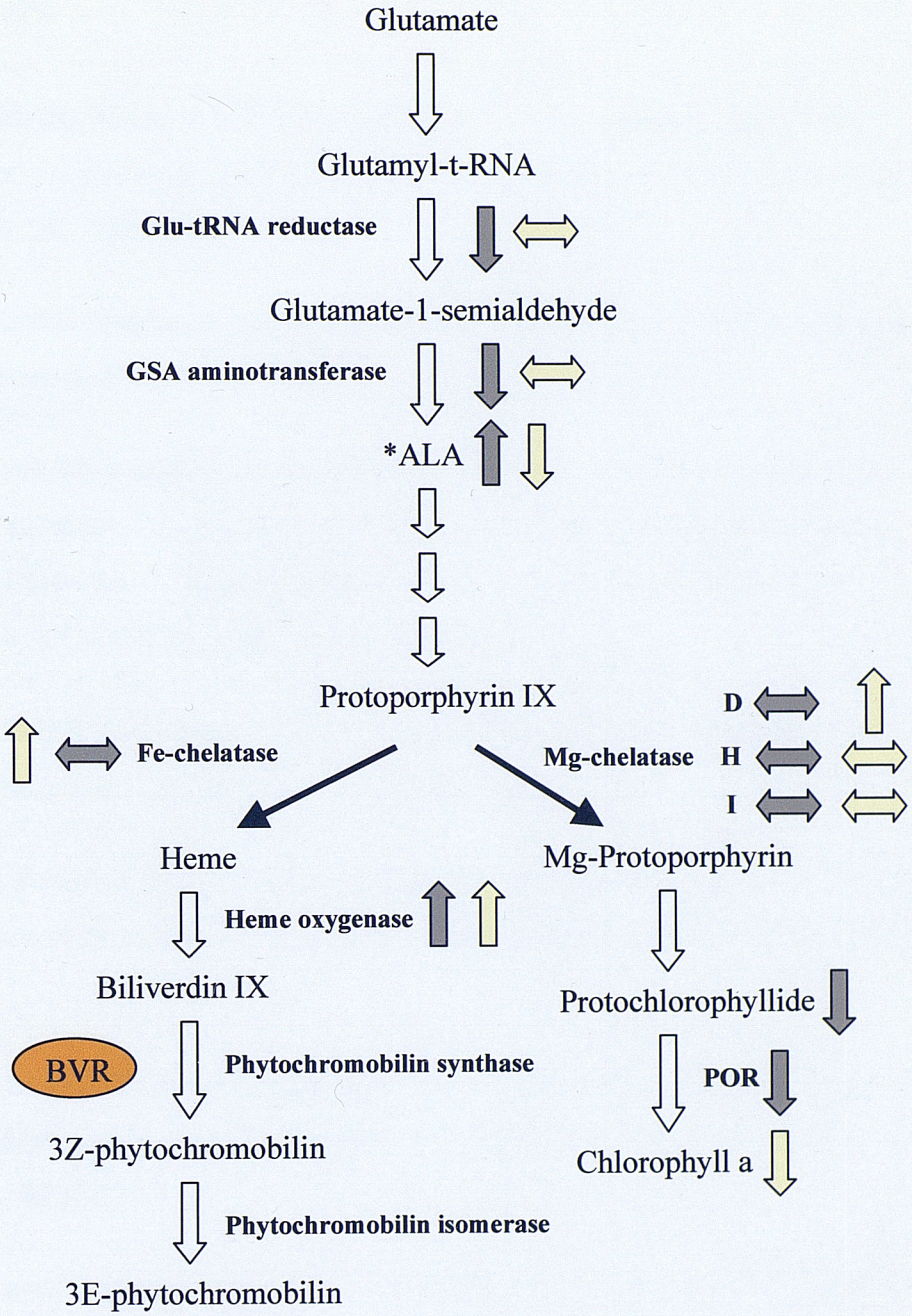


Fig. 55 Regulation of the tetrapyrrole biosynthetic pathway in dark and white light-grown plastid-targeted BVR seedlings.

* ALA represents synthesising capacity

Enzyme quantification data represent mRNA levels, with the exception of POR and heme oxygenase, which represent protein levels.

References

Abe H, Takio K, Titani K and Furuya M (1989) Amino terminal amino acid sequences of pea phytochrome II fragments obtained by limited proteolysis. *Plant Cell Physiol.* 30, 1089-1097.

Adam E, Kozma-Bognar L, Schäfer and Nagy F (1997) Tobacco phytochromes: genes, structure and expression. *Plant Cell Enivron.* 20, 678-684.

Adam E, Szell M, Pay A, Fejes E and Nagy F (1991) Circadian clock and light regulated transcription of the wheat *CAB-1* gene in wheat and transgenic tobacco plants. In: *Phytochrome properties and biological action.* Eds B.Thomas and C.B.Johnson, 191-199, Springer-Verlag, Berlin.

Ahmad M (1999) Seeing the world in red and blue: insight into plant vision and photoreceptors. *Curr. Opin. Plant Biol.* 2, 230-235.

Ahmad M, Jarillo J, Smirnova O, and Cashmore AR (1998) The CRY1 blue light photoreceptor of *Arabidopsis* interacts with phytochrome A *in vitro*. *Mol. Cell* 1, 939-948.

Ahmad M and Cashmore AR (1997) The blue light receptor cryptochrome 1 shows functional dependence on phytochrome A or phytochrome B in *Arabidopsis thaliana*. *Plant J.* 11, 421-427.

Ahmad M and Cashmore AR (1996a) Seeing blue: The discovery of cryptochrome. *Plant Mol.Biol.* 30, 851-861.

Ahmad M and Cashmore AR (1996b) The *pef* mutants of *Arabidopsis thaliana* define lesions early in the phytochrome signalling pathway. *Plant J.* 1103-1110.

Alba R, Kelmenson PM, Cordonnier-Pratt MM and Pratt LH (2000) The phytochrome gene family in tomato and the rapid differential evolution of this family in angiosperms. *Mol. Bio. Evol.* 17, 362-373.

Apel K (1981) The protochlorophyllide holophytochrome of barley (*Hordeum vulgare L .*): Phytochrome induced decrease of translatable mRNA coding for the NADPH:protochlorophyllide oxidoreductase. *Eur. J. Biochem.* 120, 89-93.

Apel K, Santel H-J, Redlinger TE and Falk H (1980) The protochlorophyllide holochrome of barley (*Hordeum vulgare* L.). Isolation and characterisation of the NADPH:protochlorophyllide oxidoreductase. *Eur. J. Biochem.* 111, 251-258.

Armstrong GA, Apel K and Rüdiger WH (2000) Does a light-harvesting protochlorophyllide *a/b*-binding protein complex exist? *Trend. Plant. Sci.* 5, 40-44.

Armstrong GA, Runge S, Frick G, Sperling U and Apel K (1995) Identification of NADPH:protochlorophyllide oxidoreductases A and B: A branched pathway for light dependent chlorophyll biosynthesis in *Arabidopsis thaliana. Plant Physiol.* 108, 1505-1517.

Aronsson H, Sohrt K and Soll J (2000) NADPH:Protochlorophyllide oxidoreductase uses the general import route into chloroplasts. *Biol. Chem.* 381, 1263-1267.

Aukerman MJ, Hirschfield M, Wester L, Weaver M, Clack T, Amasino RM and Sharrock RA (1997) A deletion in the PHYD gene of the *Arabidopsis Wassilewsija* ecotype defines a role for phytochrome D in red/far-red light sensing. *Plant Cell* 9, 1317-1326.

Averina NG, Yaronskaya EB, Rassadina VV and Walter G (1996) Response of magnesium chelatase activity in green pea (*Pisum sativum L.*) leaves to light, 5-aminolevulinic acid and dipyridyl supply. *Photochem. Photobiol.* 36, 17-22.

Barnes SA, Nishizawa NK, Quaggio RB, Whitelam GC and Chua N-H (1996a) Far-red light blocks greening of *Arabidopsis* seedlings via a phytochrome A-mediated change in plastid development. *Plant Cell* 8, 601-615.

Barnes SA, Quaggio RB, Whitelam GC and Chua N-H (1996b) *fhy1* defines a branch point in phytochrome A signal transduction pathways for gene expression. *Plant J.* 10, 1155-1161.

Bartholomew DM, Bartley GE, Scolnik PA (1991) Abscisic acid control of *RBCS* and *Cab* transcription in tomato leaves. *Plant Physiol.* 96, 291-296.

Batshauer A (1998) Photoreceptors of higher plants. *Planta* 206, 479-492.

Baulcombe D (1999) Viruses and gene silencing in plants. *Arch .Virol.* 15,189-201.

Baulcombe D (2001) RNA silencing - Diced defence. *Nature* 409, 295-296.

Beale SI and Weinstein JD (1991) Biochemistry and regulation of photosynthetic pigment formation in plants and algae. In *Biosynthesis of Tetrapyrroles.* Ed. PM Jordan, Elsevier, Amsterdam, the Netherlands, pp 155-235.

Beale SI (1990) Biosynthesis of the tetrapyrrole pigment precursor δ-aminolevulinic acid from glutamate. *Plant Physiol.* 93, 1273-1279.

Beale SI and Weinstein JD (1990) Tetrapyrrole metabolism in photosynthetic organisms. *In* HA Dailey, ed., *Biosynthesis of heme and chlorophylls.* McGraw-Hill, New York, 287-391.

Beale SI (1978) δ-aminolaevulinic acid in plants: Its biosynthesis, regulation and role in plastid development. *Ann. Rev. Plant. Physiol.* 29, 95-120.

Beale SI and Castelfranco PA (1974) The biosynthesis of δ-aminolaevulinic acid in higher plants 1. Accumulation of δ-aminolaevulinic acid in greening plant tissue. *Plant Physiol.* 53, 291-296.

Becker TW, Foyer C and Caboche M (1992) Light regulated expression of the nitrate reductase and nitrite reductase genes in tomato and in the phytochrome deficient *aurea* mutant of tomato. *Planta* 188, 39-47.

Beevers L, Loveys B, Pearson JA and Wareing PF (1970) Phytochrome and hormonal control of expansion and greening of etiolated wheat leaves. *Planta* 90, 286-294.

Berkelman TR and Lagarias JC (1980) Visualisation of bilin-linked peptides and proteins in polyacylamide gels. *Anal. Biochem.* 156, 194-201.

Bischoff F, Miller AJ, Kay SA and Furuya M (1997) Phytochrome induced intercellular signalling activates *cab*:luciferase gene expression. *Plant J. 12*, 839-849.

Bolle C, Koncz C and Chua N-H (2000) PAT1, a new member of the GRAS family, is involved in phytochrome A signal transduction. *Genes Dev.* 14, 1269-1278.

Bonner BA (1967) Incorporation of delta aminolevulinic acid into the chromophore of phytochrome. *Plant Physiol.* 42, 511.

Borthwick HA, Hendricks SB and Parker MW (1952) The reaction controlling floral initiation. *Proc. Natl. Acad. Sci USA* 38, 929-934.

Botto JF, Sanchez RA, Whitelam GC and Casal JJ (1996) Phytochrome A mediates the promotion of seed germination by very low fluences of light and canopy shade light in *Arabidopsis*. *Plant Physiol.* 110, 439-444.

Bougri O and Grimm B (1996) Members of a low-copy number gene family encoding glutamyl-tRNA reductase are differentially expressed in barley. *Plant J.* 9, 867-878.

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

Boylan MT and Quail PH (1991) Phytochrome A over-expression inhibits hypocotyls elongation in transgenic *Arabidopsis*. *Proc. Natl. Acad. Sci USA* 88, 10806-10810.

Briggs WR, Mosinger E and Schafer E (1988) Phytochrome regulation of greening in barley-effects on chlorophyll accumulation. *Plant Physiol.* 86, 706-710.

Brockmann J and Schäfer E (1982) Analysis of Pfr destruction in *Amaranthus caudatus L.* evidence for two pools of phytochrome. *Photochem. Photobiol* 35, 555-558.

Büche C, Poppe C, Schäfer E and Kretsch T (2000) *EID1*: A new *Arabidopsis* mutant hypersensitive in phytochrome A-dependent high-irradiance responses. *Plant Cell* 12, 547-558.

Butler WL Hendricks SB and Siegleman HW (1964) Action spectra of phytochrome *in vitro*. *Photochem. Photobiol.* 3, 521-528.

Carr-Smith MD, Johnson CB and Thomas B (1989) Action spectrum for the effect of day extensions on flowering and apex elongation in green light-grown wheat (*Triticum aestivum L.*). *Planta* 179, 428-432.

Casal JJ and Mazzella MA (1998) Conditional synergism between cryptochrome 1 and phytochrome B is shown by analysis of *phyA*, *phyB hy4* simple, double and triple mutants in *Arabidopsis*. *Plant Physiol.* 118, 19-25.

Casal JJ, Sanchez RA and Yanovsky MJ (1997) The function of phytochrome A. *Plant Cell Environ.* 20, 813-819.

Casal JJ, Whitelam GC and Smith H (1990) Phytochrome effects on the relationship between chlorophyll and steady-state levels of thylakoid peptides in light-grown tobacco. *Plant Physiol.* 94, 370-374.

Casal JJ and Smith H (1988a) Persistent effects of changes in phytochrome status on internode growth in light-grown mustard: Occurrence, kinetics and locus of perception. *Planta* 175, 214-220.

Casal JJ and Smith H (1988b) The loci of perception for phytochrome control of internode growth in light-grown mustard: Promotion by low phytochrome photoequilibria in the internode is enhanced by blue light perceived by the leaves. *Planta* 176, 277-282.

Castelfranco PA (1983) Chlorophyll biosynthesis-Recent advances and areas of current interest. *Ann .Rev. Plant Physiol.* 34, 241-278.

Cerdan PD, Staneloni RJ, Ortega J, Bunge MM, Rodriguez-Batiller MJ, Sanchez RA and Casal JJ (2000) Sustained but not transient phytochrome A signaling targets a region of an Lhcb1*2 promoter not necessary for phytochrome B action. *Plant Cell* 12, 1203-1211.

Cerdan PD, Staneloni RJ, Casal JJ, Sanchez RA (1997) A 146 bp fragment of the tobacco Lhcb 1*2 promoter confers very-low-fluence, low-fluence and high-irradiance responses of phytochrome to a minimal CaMV 35S promoter. *Plant Mol. Biol.* 33, 245-255.

Chamovitz D, Pecker I and Hirschberg J (1991) The molecular basis of resistance to the herbicide norflurazon. *Plant Mol. Biol.* 16, 967-974.

Chattopadhyay S, Ang L-H, Puente P and Deng X-W (1998) *Arabidopsis* bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. *Plant Cell* 10, 673-684.

Cherry JR, Hondred D, Walker JM, Keller JM, Hershey HP and Vierstra RD (1993) Carboxy-terminal deletion analysis of oat phytochrome A reveals the presence of separate domains required for structure and biological activity. *Plant Cell* 5, 565-575.

Cherry JR, Hershey HP and Vierstra RD (1991) Characterisation of tobacco expressing functional oat phytochrome: Domains responsible for the rapid degradation of Pfr are conserved between monocots. and dicots. *Plant Physiol.* 96, 775-785.

Childs KL, Miller FR, Cordonnier-Pratt MM, Pratt LH, Morgan PW and Mullet JE (1997) The Sorghum photoperiod sensitivity gene ma_3 encodes a phytochrome B. *Plant Physiol.* 113, 611-619.

Choi G, Yi H, Lee J, Kwon Y-K, Soh MS, Shin B, Luka Z, Hahn T-R and Song P-S (1999) Phytochrome signalling is mediated through nucleoside diphosphate kinase 2. *Nature* 401, 610-613.

Chory J, Chatterjee M, Cook RK, Elich T, Faukhauser C, Li J, Nagpal P, Neff M, Pepper A, Poole D, Reed J and Vitart V (1996) From seed germination to flowering, light controls plant development via the pigment phytochrome. *Proc. Natl .Acad. Sci USA* 93, 12066-12071.

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

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

Chory J, Peto CA, Reinbaum R, Pratt LH and Ausubel F (1989a) *Arabidopsis thaliana* mutant that develops as a light grown plant in the absence of light. *Cell* 58, 991-999.

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

Chow KS, Singh DP, Roper JM, Walker AR and Smith AG (1998) Two different genes encode ferrochelatase in *Arabidopsis*: mapping, expression and subcellular targeting of the precursor proteins. *Plant J.* 15, 531-541.

Christie JM, Reymond P, Powell GK, Bernascon P, Raibekas AA, Liscum E and Briggs WR (1998) *Arabidopsis* NPH1, A flavoprotein with the properties of a photoreceptor for phototropism. *Science* 282, 1698-1701.

Christie JM and Jenkins GI (1996) Distinct UV-B and UV-A blue light signal transduction pathways induce chalcone synthase gene expression in *Arabidopsis* cells. *Plant Cell* 8, 1555-1567.

Chun L, Kawakami A and Christopher DA (2001) Phytochrome A mediates blue light and UV-A dependent chloroplast gene transcription in green leaves. *Plant Physiol.* 125, 1957-1966.

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

Clough RC, Jordan-Beebe ET, Lohman KN, Marita JM, Walker JM, Gatz C and Vierstra RD (1999) Sequences within both the N and C-terminal domains of phytochrome A are required for Pfr ubiquitination and degradation. *Plant Journal* 17, 155-167.

Clough R.C and Vierstra RD (1997) Phytochrome degradation. *Plant Cell Environ.* 20, 713-721.

Colbert JT (1988) Molecular biology of phytochrome. *Plant Cell Environ.* 11, 305-318.

Colbert JT, Hershey HP and Quail PH (1983) Autoregulatory control of translatable phytochrome mRNA levels. *Proc. Natl. Acad. Sci. USA* 80, 2248-2252.

Cornejo J, Beale SI, Terry MJ and Lagarias JC (1992) Phytochrome assembly. *J .Biol. Chem.* 267, 14790-14798.

Dahlin C, Aronsson H, Almkvist J and Sundqvist C (2000) Protochlorophyllide-independent import of two NADPH:Pchlide oxidoreductase proteins (PORA and PORB) from barley into isolated plastids. *Physiol. Plant.* 109, 298-303.

Darr SC, Somervine SC, Amizen SC (1986) Monoclonal antibodies to the light harvesting chlorophyll a/b binding protein complex of photosystem II. *J. Plant Physiol.* 103, 733-740.

Davis SJ, Bhoo SH, Durski AM, Walker JM and Vierstra RD (2001) The heme oxygenase family required for phytochrome chromophore biosynthesis is necessary for proper photomorphogenesis in higher plants. *Plant Physiol.* 126, 656-669.

Davis SJ, Kurepa J, Vierstra R (1999) The *Arabidopsis thaliana* HY1 locus required for phytochrome-chromophore biosynthesis encodes a protein related to heme oxygenases. *Proc. Natl. Acad. Sci USA* 96, 6541-6546.

Deng X-W, Matsui M, Wei N, Wagner D, Chu AM, Reidmann KA and Quail PH (1992) *Cop1*, an *Arabidopsis* regulatory gene encodes a protein with both a zinc binding motif and a G_{β} homologous domain. *Cell* 71, 791-801.

Deng X-W, Casper T and PH Quail (1991) *COP1*: a regulatory locus involved in light-controlled development and gene expression in *Arabidopsis*. *Genes. Dev* 5, 1172-1182.

Devlin PF, Robson PRH, Patel SR, Goosey L, Sharrock RA and Whitelam GC (1999) Phytochrome D acts in the shade-avoidance syndrome in *Arabidopsis* by controlling elongation and flowering time. *Plant Physiol.* 119, 909-915.

Devlin PF, Patel SR and Whitelam GC (1998) Phytochrome E influences internode elongation and flowering time in *Arabidopsis*. *Plant Cell* 10, 1479-1487.

Devlin PF, Halliday KJ, Harberd NP and Whitelam GC (1996) The rosette habit of *Arabidopsis thaliana* is dependent upon phytochrome action: novel phytochromes control internode elongation and flowering time. *Plant J.* 10, 1127-1134.

Devlin PF, Rood SB, Somer SE, Quail PH and GC Whitelam (1992) Photophysiology of the elongated internode (*ein*) mutant of *Brassica rapa*. The *ein* mutant lacks a detectable phytochrome B-like polypetide. *Plant Physiol.* 100, 1442-1447.

Dijkwel PP, Huijser C, Weisbeek PJ, Chua N-H, Smeekens SCM (1997) Sucrose control of phytochrome A signaling in *Arabidopsis*. *Plant Cell* 9, 583-595.

Downs RJ (1956) Photoreversibility of flower initiation. *Plant Physiol.* 31, 279-284.

Duggan J and Gassman M (1974) Induction of porphyrin synthesis in etiolated bean leaves by chelators of iron. *Plant Physiol.* 53, 206-215.

Edgerton MD and Jones AM (1993) Subunit interactions in the carboxy-terminal domain of phytochrome. *Biochemistry* 32, 8239-8245.

Edgerton MD and Jones AH (1992) Localisation of protein-protein interactions between subunits of phytochrome. *Plant Cell* 4, 161-171.

Edwards GE, Johnson E, Lal A and Krall JP (1993) Quantum yields of photosystem-II and photosynthesis in an aurea mutant of tobacco (C3) and an oil yellow mutant of maize (C4) which have high capacities for photosynthesis despite low chlorophyll contents. *Plant Cell Physiol.* 34, 1205-1212.

Elich TD and Lagarias JC (1987) Phytochrome chromophore biosynthesis. *Plant Physiol.* 84, 304-310.

Esch H and Lamparter T (1998) Light regulation of phytochrome content in wild-type and aphototropic mutants of the moss *Ceratodon purpureus*. *Photochem. Photobiol.* 67, 450-455.

Falbel TG, Meehl JB and Staehelin LA (1996) Severity of mutant phenotype in a series of chlorophyll-deficient wheat mutants depend on light intensity and the severity of the block in chlorophyll synthesis. *Plant Physiol.* 112, 821-832.

Fankhauser C and J Chory (2000) *RSF1*, an *Arabidopsis* locus implicated in phytochrome A signalling. *Plant Physiol.* 124, 39-45.

Fankhauser C, Yeh K-C, Lagarias JC, Zhang H, Elich TD and Chory J (1999) PKS1, a substrate phosphorylated by phytochrome that mediates light signalling in *Arabidopsis*. *Science* 284, 1539-1541.

Fletcher RA and McCullagh D (1971) Benzyladenine as a regulator of chlorophyll synthesis in cucumber cotyledons. *Can. J. Bot.* 49, 2197-2201.

Flores S and Tobin EM (1986) Cytokinin modulation of *Lhcp* messenger-RNA levels -the involvement of post-transcriptional regulation. *Plant Mol Biol.* 11, 409-415.

Forreiter C, Van Cleve B, Schmidt A and Apel K (1990) Evidence for a light dependent negative control of NADPH-protochlorophyllide oxidoreductase in angiosperms. *Planta* 183, 126-132.

Foster KR and Morgan PW (1995) Genetic regulation of development in *Sorgum bicolour*. IX. The Ma_3^R allele disrupts diurnal control of gibberellin biosynthesis. *Plant Physiol.* 108, 337-343.

Frick G, Apel K and Armstrong G (1995) Light dependent protochlorophyllide oxidoreductase, phytochrome and greening in *Arabidopsis thaliana*. In: *Photosynthesis, from light to biosphere.* Vol III, 893-898. Kluwer Academic Publishers.

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

Furuya M, Ito N, Tomizawa K-I and Schäfer E (1991) A stable phytochrome pool regulates the expression of the Phytochrome I gene in pea seedlings. *Planta* 183, 218-221.

Gardner G and HL Gorton (1985) Inhibition of phytochrome synthesis by gabaculine. *Plant Physiol.* 77, 540-543.

Genond T, Miller AJ, Nishizawa N, Kay S, Shäfer E, Nagatani A and Chua N-H (1998) An *Arabidopsis* mutant hypersensitive to red and far-red light signals. *Plant Cell* 10, 889-904.

Goldsbrough PB and Cullis CA (1981) Characterisation of the genes for ribosomal RNA in flax. *Nucleic Acids Research* 9, 1301-1309.

Gräfe S, Saluz H-P, Grimm B and Hänel F (1999) Mg-chelatase of tobacco: The role of the subunit CHL D in the chelation step of protopophyrin IX. *Proc. Natl. Acad. Sci.USA* 96, 1941-1946.

Griffiths WT, McHugh T and Blankenship RE (1996) The light intensity dependence of protochlorophyllide photoconversion and its significance to the catalytic mechanism of protochlorophyllide reductase. *FEBS Lett.* 398, 235-238.

Griffiths WT (1978) Reconstitution of chlorophyllide formation by isolated etioplast membranes. *Biochem J.* 174, 681-692.

Grimm B (1998) Novel insights in the control of tetrapyrrole metabolism of higher plants. *Curr. Opin. Plant Biol.* 1, 245-250.

Grimm R, Eckerskorn F, Lottspeich R, Zenger C and Rüdiger W (1998) Sequence analysis of proteolytic fragments of 124 kilodalton phytochrome from etiolated *Avena sativa L.* conclusions on the conformation of the native protein. *Planta* 174, 396-401.

Gunning BS and Steer MW (1996) Plastids (8): The Greening Process: From Etioplast to Chloroplast. In *Plant Cell Biology.*Eds. BES Gunning and MW Steer, Jones and Bartlett Publishers, London.

Guo H, Yang H, Mockler TC and Lin C (1998) Regulation of flowering time by *Arabidopsis* photoreceptors. *Science* 279, 1360-1363.

Halliday KJ, Hudson M, Ni M, Quin M and Quail PH (1999) *Poc1*, an *Arabidopsis* mutant perturbed in phytochrome signalling because of a TDNA insertion in the promoter of *PIF3*, a gene encoding a phytochrome bHLH protein. *Proc. Natl. Acad. Sci USA* 96, 5832-5837.

Halliday KJ, Thomas B and Whitelam GC (1997) Expression of heterologous phytochromes A,B or C in transgenic tobacco plants alters vegetative development and flowering time. *Plant J.* 12, 1079-1090.

Hamazato F, Shinomura T, Hanzawa H, Chory J and **Furuya M (1997)** Fluence and wavelength requirements for *Arabidopsis CAB* gene induction by different phytochromes. *Plant Physiol.* 115, 1537-1540.

Hardtke C and X-W Deng (2000) The cell biology of the COP/DET/FUS proteins regulating proteolysis in photomorphogenesis and beyond. *Plant Physiol.* 124, 1548-1557.

Hartel H and Grimm B (1998) Consequences of chlorophyll deficiency for leaf carotenoid composition in tobacco synthesising glutamate-1-semialdehyde aminotransferase antisense RNA: dependency on developmental age and growth light. *J. Exp. Bot* 49, 535-546.

Hayat MA (1986) Basic techniques for transmission electron microscopy. Academic Press. London.

Hennig L, Büche, Eichenberg K and Schäfer E (1999) Dynamic properties of endogenous phytochrome A in *Arabidopsis* seedlings. *Plant Physiol.* 121, 571-577.

Heyer AG, Mozely D, Landschutze V, Thomas B and Gatz C (1995) Function of phytochrome A in potato plants as revealed through the study of transgenic plants. *Plant Physiol.* 109, 53-61.

Hill CM, Pearson SA, Smith AJ and Rogers LJ (1985) Inhibition of chlorophyll synthesis in *Hordeum vulgare* by 3-amino-2,3-dihydrobenzoic acid (gabaculin). *Biosci. Rep.* 5, 775-781.

Hirschfeld M, Tepperman JM, Clack T, Quail PH and Sharrock RA (1998) Coordination of phytochrome levels in *phyB* mutants of *Arabidopsis* as revealed by apoprotein-specific monoclonal antibodies. *Genetics* 149, 523-535.

Hoecker U, Tepperman JM and Quail PH (1999) SPA1- a WD-repeat protein specific to phytochrome A signal transduction. *Science* 284, 496-499.

Hoecker U, Xu Y and Quail PH (1998) SPA1, a new genetic locus involved in phytochrome A-specific signal transduction. *Plant Cell* 10, 19-33.

Höfgen R, Axelsen KB, Kannangara CG, Schüttke I, Pohlenz H-D, Willmitzer L, Grimm B and Von Wettstein (1994) A visible marker for antisense mRNA expression in plants: Inhibition of chlorophyll synthesis with a glutamate-1-semialdehyde aminotransferase antisense gene. *Proc. Natl. Acad. Sci. USA* 91, 1726-1730.

Holmes MG and Smith H (1975) The function of phytochrome in plants growing in the natural environment. *Nature* 254, 512-514.

Holtorf H, Reinbothe S, Reinbothe C, Bereza B and Apel K (1995) Two routes of chlorophyllide synthesis that are differentially regulated by light in barley (*Hordeum vulgare L.*). *Proc. Natl. Acad. Sci USA* 92, 3254-3258.

Horwitz BA, Thompson WF and Briggs WR (1988) Phytochrome regulation of greening in *Pisum*-chlorophyll accumulation and abundance of mRNA for the light harvesting chlorophyll a/b binding proteins. *Plant Physiol.* 86, 299-305.

Hsieh H-L, Okamoto H, Wang M, Wang M, Ang L-H, Matsui M, Goodman H and Deng X-W (2000) *FIN219*, an auxin-regulated gene, defines a link between phytochrome A and the downstream regulator COP1 in light control of *Arabidopsis* development. *Genes. Dev.* 14, 1958-1970.

Huang L, Bonner BA and Castelfranco PA (1989) Regulation of 5-aminolevulinic acid (ALA) synthesis in developing chloroplasts II. Regulation of ALA synthesising capacity by phytochrome. *Plant Physiol.* 90, 1003-1008.

Hudson M, Ringli C, Boylan MT and Quail PH (1999) The *FAR1* locus encodes a novel nuclear protein specific to phytochrome A signalling. *Genes. Dev.* 13, 2017-2027.

Hudson M, Robson PRH, Kraepiel Y, Caboche M and Smith H (1997) *Nicotiana plumbagnifolia hlg* mutants have a mutation in a *PHYB*-type phytochrome gene: they have elongated hypocotyls in red light but are not elongated in adult plants. *Plant J.* 12, 1091-1101.

Ikeuchi M and Murakami S (1982) Behaviour of the 36,000-dalton protein in the internal membranes of squash etioplasts during greening. *Plant. Cell Physiol.* 23, 575-583.

Ilag L.L, Kumar A.M and Söll D (1994) Light regulation of chlorophyll biosynthesis at the level of 5-aminolevulinate formation in *Arabidopsis*. *Plant Cell* 6, 265-275.

Izawa T, Oikawa T, Tokutomi S, Okuno K and Shimamoto K (2000) Phytochromes confer the photoperiodic control of flowering in rice (a short-day plant). *Plant J.* 22, 391-399.

Jackson SD, James P, Prat S and Thomas B (1998) Phytochrome B affects the levels of a graft transmissible signal involved in tuberisation. *Plant Physiol.* 117, 29-32.

Jackson SD and Thomas B (1997) Photoreceptors and signals in the photoperiodic control of development. *Plant Cell Environ.* 20, 790-795.

Jackson SD , Heyer A, Dietz J and Prat S (1996) Phytochrome B mediates the photoperiodic control of tuber formation in potato. *Plant J.* 9, 159-166.

Jackson SD and Prat S (1996) Control of tuberisation in potato by gibberellins and phytochrome B. *Physiol. Plant.* 81, 571-577.

Jarvis P (2001) Intracellular signalling: the chloroplast talks! *Curr. Biol.* 11, R307-R310.

Johanningmeier U (1988) Possible control of transcript levels by chlorophyll precursors in *Chlamydomonas. Eur. J. Biochem.* 177, 417-424.

Johnson E, Bradley M, Harberd NP and Whitelam GC (1994) Photoresponses of light grown phytochrome A mutants of *Arabidopsis. Plant Physiol.* 105, 141-149.

Jones AM, Allen CD, Gardner G and Quail PH (1986) Synthesis of phytochrome apoprotein and chromophore are not coupled obligatorily. *Plant Physiol.* 81, 1014-1016.

Jones AM, Vierstra RD, Daniels SM and Quail P (1985) The role of separate molecular domains in the structure of phytochrome from etiolated *Avena sativa L. Planta* 164, 501-506.

Jordan ET, Cherry JR, Walker JM and Vierstra RD (1995a) The amino-terminus of phytochrome A contains two distinct functional domains. *Plant Journal* 9, 243-257.

Jordan ET, Hatfield PM, Hondred D, Talon M, Zeevardt JAD and Vierstra RD (1995b) Phytochrome A over expression in transgenic tobacco. *Plant Physiol.* 107, 797-805.

Kamiya Y and Garcìa-Martìnez JL (1999) Regulation of gibberellin biosynthesis by light. *Curr .Opin. Plant. Biol.* 2, 398-403.

Kannangara CG, Gough SP, Bruyant P, Hoober JK, Kahn A and von Wettstein D (1988) $tRNA^{Glu}$ as a cofactor in delta-aminolevulinate biosynthesis: steps that regulate chlorophyll synthesis. *Trends. Biochem. Sci.* 13, 139-143.

Karlin-Neumann GA, Sun L and Tobin EM (1988) Expression of light harvesting chlorophyll a/b protein genes is phytochrome regulated in etiolated *Arabidopsis thaliana* seedlings. *Plant Physiol.* 88, 1323-1331.

Kasemir H (1983) Light control of chlorophyll accumulation in higher plants. In *Encyclopedia of Plant Physiology, New series.* Vol.16B, Eds Shropshire W and Mohr H, Springer-Verlag, Berlin. pp 662-686.

Kasemir H and Masoner M (1975) Control of chlorophyll synthesis by phytochrome II-The effect of phytochrome on aminolevulinate dehydratase in mustard seedlings. *Planta* 126, 119-126.

Kay SA, Nagatani A, Keith B, Deak M, Furuya M, and Chua N-H (1989) Rice phytochrome is biologically active in transgenic tobbaco. *Plant Cell* 1, 775-782.

Kay SA and Griffiths WT (1983) Light induced breakdown of NADPH-Protochlorophyllide oxidoreductase *in vitro*. *Plant Physiol.* 72, 229-236.

Keiller D and Smith H (1989) Control of carbon partitioning by light quality mediated by phytochrome. *Plant Science* 63, 25-29.

Keller JM, Shanklin J, Vierstra RD and Hershey HP (1989) Expression of a functional monocotyledonous phytochrome in transgenic tobacco. *EMBO J.* 8, 1005-1012.

Kendrick RE and Kronenberg GHM (1994) *Photomorphogenesis in plants* 2nd ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Kendrick RE, Kerckhoffs LHJ, Pundsnes AS, Van Tuinen A, Koornneef M, Nagatani A, Terry MJ, Tretyn A, Cordonnier-Pratt M-M, Hauser B and Pratt LH (1994) Photomorphogenic mutants of tomato. *Euphytica* 79, 227-234.

Ken-Dror S and Horwitz BA (1990) Altered phytochrome regulation of greening in an *aurea* mutant of tomato. *Plant Physiol.* 92, 1004-1008.

Kerckhoffs LHJ, Kelmenson PM, Schreuder MEL, Kendrick CI, Kendrick RE, Hanhart CJ, Koornneef M, Pratt LH and Cordonnier-Pratt MM (1999) Characterization of the gene encoding the apoprotein of phytochrome B2 in tomato, and identification of molecular lesions in two mutant alleles. *Mol. Gen. Genet.* 261, 901-907.

Kerckhoffs LHJ, Van Tuinen A, Hauser BA, Cordonnier-Pratt MM, Nagatani A, Koornneef M, Pratt LH and Kendrick RE (1996) Molecular analysis of tri-mutant alles in tomato indicates the Tri locus is the gene encoding the apoprotein of phytochrome B1. *Planta* 199, 152-157.

Kern R, Gasch A, Deak M, Kay SA and Chua N-H (1993) Phytochrome B of tobacco, a new member of the phytochrome family. *Plant Physiol.* 102, 1363-1364.

Kircher S, Kozma-Bognor L, Adam E, Harter K, Schäfer E and Nagy F (1999) Light quality-dependent nuclear import of the plant photoreceptors A and B. *Plant Cell* 11, 1445-1456.

Kohchi T, Mukougawa K, Frankenberg N, Masuda H, Yokota A and Lagarias JC (2001) The *Arabidopsis HY2* gene encodes phytochromobilin synthase, a ferredoxin-dependent biliverdin reductase. *Plant Cell* 13, 425-436.

Koornneef M and Peters AJM (1997) Floral transition mutants in *Arabidopsis*. *Plant Cell Environ.t* 20, 779-784.

Koornneef M, Cone JW, Deekens RG, O'Herne-Roberts EG, Spruitt CJP and Kendrick RE (1985) Photomorphogenic responses of long hypocotyl mutants of tomato. *J. Plant Physiol.* 120, 153-165.

Koornneef M, Rolff E and Spruitt CJP (1980) Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana* L. Heyhh. *Zeitschrift für pflanzenphysiologie* 100, 147-160.

Kornitzer D, Rayboy B, Kulka RG and Fink GR (1994) Regulated degradation of the transcription factor Gen 4. *EMBO J.* 13, 6021-6030.

Kraepiel Y, Jullien M, Cordonnier-Pratt MM and Pratt L (1994) Identification of two loci involved in phytochrome expression in *Nicotiana plumbagnifolia* and lethality of the corresponding double mutant. *Mol. Gen. Genet.* 242, 559-565.

Kropat J, Oster U, Rüdiger W and Beck CF (2000) Chloroplast signalling in the light induction of nuclear HSP70 genes requires the accumulation of chlorophyll precursors and their accessibility to cytoplasm/nucleus. *Plant J.* 24, 523-531.

Kropat J, Oster U, Rüdiger W, Beck CF (1997) Chlorophyll precursors are signals of chloroplast origin involved in light induction of nuclear heat-shock genes. *Proc. Natl. Acad. Sci. USA* 94, 14168-14172.

Kruse E, Grimm B, Beator J and Kloppstech K (1997) Developmental and circadian control of the capacity for aminolevulinic acid synthesis in green barley. *Planta* 202, 235-241.

Kumar AM and Söll D (2000) Antisense *HEMA1* RNA expression inhibits heme and chlorophyll biosynthesis in *Arabidopsis*. *Plant Physiol.* 122, 49-55.

Kumar AM, Chaturvedi S and Söll D (1999) Selective inhibition of *HEMA* gene expression by photo-oxidation in *Arabidopsis thaliana*. *Phytochemistry* 51, 847-850.

Kumar AM, Schaub U, Söll D and Ujwal ML (1996a) Glutamyl-transfer RNA: At the crossroad between chlorophyll and protein biosynthesis. *Trends. Plant. Sci.* 1, 371-376.

Kumar MA, Casankovszki G and Söll D (1996b) A second and differentially expressed glutamyl-tRNA reductase gene from *Arabidopsis thaliana*. *Plant Mol. Biol.* 30, 419-426.

Kuno N, Muramatsu T, Hamazato F and Furuya H (2000) Identification by large-scale screening of phytochrome-regulated genes in etiolated seedlings of *Arabidopsis* using a fluorescent differential display technique. *Plant Physiol.* 122, 15-24.

Kusnetsov V, Bolle C, Lübberstedt T, Sopory S, Herrmann RG and Oelmüller R (1996) Evidenec that the plastid signal and light operate via the same *cis*-acting elements in the promoters of nuclear genes for plastid proteins. *Mol. Gen. Genet.* 252, 631-639.

Kutty RC and Maines MD (1981) Purification and characterisation of biliverdin reductase from rat liver. *J. Biol. Chem* 256, 3956-3962.

Lagarias DM, Crepeau MW, Maines MD and Lagarias JC (1997) Regulation of photomorphogenesis by expression of mammalian biliverdin reductase in transgenic *Arabidopsis* plants. *Plant Cell* 9, 675-688.

Lagarias JC and Lagarias DM (1989) Self-assembly of synthetic phytochrome holoprotein *in vitro*. *Proc. Natl. Acad. Sci USA* 86, 5778-5780.

Lagarias JC, Kelly JM, Cyr KL and Smith WO (1987) Comparitive photochemical analysis of highly purified 124 kilo-dalton oat and rye phytochromes *in vitro*. *Photochem. Photobiol.* 46, 5-13.

Lagarias JC and Rapport H (1980) Chromopeptides from phytochrome. The structure and linkage of the Pr form of the phytochrome chromophore. *J. Amer. Chem. Soc* 104, 4821-4828.

Lamparter T, Esch H, Cove D and Hartmann E (1997) Phytochrome control of phototropism and chlorophyll accumulation in the apical cells of protonemal filaments of wild-type and an aphototropic mutant of the moss *Ceratodon purpureus*. *Plant Cell Physiol.* 38, 51-58.

La Rocca N, Rascio N, Oster U and Rüdiger W (2001) Amitriole treatment of etiolated barley seedlings leads to deregulation of tetrapyrrole synthesis and to reduced expression of *Lhc* and *RbcS* genes. *Planta* 213, 101-108.

Lebedev N and Timko MP (1998) Protochlorophyllide photoreduction. *Photosynthesis Res.* 58, 5-23.

Lichtenthaler HK and Wellburn AR (1983) Determination of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochem. Soc. Trans.* 11, 591-592.

Lifschitz S, Gepstein S and Horwitz BA (1990) Phytochrome regulation of greening in wild type and long hypocotyl mutants of *Arabidopsis thaliana. Planta* 181, 234-238.

Lin C, Yang H, Guo H, Mocker T, Chen J and Cashmore AR (1998) Enhancement of blue light sensitivity of *Arabidopsis* seedlings by a blue light receptor cryptochrome 2. *Proc. Natl. Acad. Sci. USA* 95, 7686-7699.

Lin C, Ahmad M and Cashmore AR (1996) *Arabidopsis* cryptochrome 1 is a soluble protein mediating blue light-dependent regulation of plant growth and development. *Plant J.* 10, 893-902.

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

Logemann J, Schell J and L Willmitzer (1987) Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* 163, 16-20.

López-Juez E, Baynton CE, Page AM, Pyke KA, Robertson S and Vinti G (1999) Multiple light and plastid signals control chloroplast development in *Arabidopsis*. In *Photosynthesis: Mechanisms and Effects*. Ed. G Garab, Dordrecht: Kluwer, The Netherlands. Pp 2805-2808.

López-Juez E, Jarvis RP, Takeuchi A, Page AM and Chory J (1998) New *Arabidopsis* cue mutants suggest a close connection between plastid and phytochrome regulation of nuclear gene expression. *Plant Physiol.* 118, 803-815.

López-Juez E, Streatfield S and Chory J (1996) Light signals and autoregulated chloroplast development. In *Regulation of plant growth and development.* Eds. Briggs WR, Heath RL and Tobin EM. Bethesda: American society of Plant Physiologists, pp 144-152.

López-Juez E, Kobayashi M, Sakurai A, Kamiya Y and Kendrick RE (1995) Phytochrome, gibberellins and hypocotyl growth. *Plant.Physiol.* 107, 131-140.

López-Juez E, Nagatani A, Tomizawa K.-I, Deak M, Kern R, Kendrick RE and Furuya M (1992) The cucumber long hypocotyl mutant lacks a light-stable PHYB-like phytochrome. *Plant Cell* 4, 241-251.

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

Lumsden PJ, Saij H and Furuya M (1987) Action spectra confirm two separate actions of phytochrome in the induction of flowering in *Lemna paucicostata 441. Plant Cell Physiol.* 28, 1237-1242.

Mandel MA, Feldmann KA, Herrera-Estrella L, Rocha-Sosa M and León P (1996) *CLA1*, a novel gene required for chloroplast development, is highly conserved in evolution. *Plant J.* 9, 649-658.

Mapleston RE and Griffiths WT (1980) Light modulation of the activity of protochlorophyllide oxidoreductase. *Biochem. J.* 189, 125-133.

Martínez-García JF, Huq E and PH Quail (2000) Direct targeting of light signals to a promoter element-bound transcription factor. *Science* 288, 859-863.

Mascia PN (1978) An analysis of precursors accumulated by several chlorophyll biosynthetic mutants of maize. *Mol. Gen. Genet.* 161, 237-243.

Mascia PN and Robertson DS (1978) Studies of chloroplast development in four maize mutants defective in chlorophyll biosynthesis. *Planta* 143, 207-214.

Masoner M and Kasemir H (1975) Control of chlorophyll synthesis by phytochrome I-The effect of phytochrome on the formation of 5-aminolevulinate in mustard seedlings. *Planta* 126, 111-117.

Masuda T, Ohta H, Shioi Y and Takamiya K-I (1996) Light regulation of 5-aminolevulinic acid synthesis system in *Cucumis sativus*: light stimulates activity of glutamyl-tRNA reductase during greening. *Plant Physiol. Biochem.* 34, 11-16.

Masuda T, Tanaka R, Shioi Y, Takamiya K-I, Kannangara GG and Tsuji H (1994) Mechanism of benzyladenine-induced stimulation of the synthesis of 5-aminolevulinic acid in greening cucumber cotyledons: Benzyladenine increases levels of plastid $tRNA^{glu}$. *Cell. Physiol.* 35, 183-188.

Masuda T, Kouji H and Matsunaka S (1990) Diphenyl ether herbicide-decreased heme contents stimulate 5-aminolevulinic acid synthesis. *Pest. Biochem. Physiol* 36, 106-114.

Mathews S and Sharrock RA (1997) Phytochrome gene diversity. *Plant Cell Environ.* 20, 666-671.

Mauzerall D and Granick (1956) The occurrence and determination of δ-Aminolevulinic acid and porphobilinogen in urine. *J. Bio. Chem.* 219, 435-446.

McCormac AC, Fischer A, Kumar AM, Söll D and Terry MJ (2001) Regulation of *HEMA1* expression by phytochrome and plastid signal during de-etiolation in *Arabidopsis thaliana. Plant J.* 25, 549-561.

McCormac AC, Elliot MC and Chen DF (1998) A simple method for the production of highly competent cells of *Agrobacterium* for transformation via electroporation. *Mol. Biotech.* 9, 155-159.

McCormac AC, Smith H and Whitelam GC (1993a) Photoregulation of germination in seed of transgenic lines of tobacco and *Arabidopsis* which express an introduced cDNA encoding phytochrome A or phytochrome B. *Planta* 191, 386-393.

McCormac A, Wagner D, Boylan MT, Quail PH and Whitelam GC (1993b) Photoresponses of transgenic *Arabidopsis* seedlings expressing introduced phytochrome B-encoding cDNA's:evidence that PHYA and PHYB have distinct photoregulatory functions. *Plant J.* 4, 19-27.

McCormac A, Whitelam G and 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 AC, Cherry JR, Hershey HP, Vierstra RD and Smith H (1991) Photoresponses of transgenic tobacco plants expressing an oat phytochrome gene. *Planta* 185, 162-170.

McDonagh AF (2001) Turning green to gold. *Nat. Struc .Biol.* 8, 198-200.

Metzger JD and Zheng Z-L (1998) The role of phytochrome in photoperiodic timing mechanisms. *J. Jap. Soc. Hort. Sci.* 67, 1128-1133.

Miller AJ and Kay SA (1996) Integration of circadian and phototransduction pathways in the network controlling *cab* gene transcription in *Arabidopsis. Proc .Natl. Acad. Sci. USA* 93, 15491-15496.

Misera S, Mueller AJ, Weiland-HeideckerU and Juergens G (1994) The *FUSCA* genes of *Arabidopsis* : negative regulators of light responses. *Mol. Gen. Genet.* 244, 242-252.

Mochizuki N, Brusslan JA, Larkin R, Nagatani A and Chory J (2001) *Arabidopsis genomes uncoupled 5* (*GUN5*) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction. *Proc. Natl. Acad. Sci. USA* 98, 2053-2058.

Mochizuki N, Susek R and Chory J (1996) An intracellular signal transduction pathway between the chloroplast and nucleus is involved in de-etiolation. *Plant Physiol.* 112, 1465-1469.

Mockler TC, Guo H, Yang H, Duong H and Lin C (1999) Antagonistic actions of *Arabidopsis* cryptochromes and phytochrome B in the regulation of floral induction. *Development* 126, 2073-2082.

Mohr H (1994) Coaction between pigment systems. In *Photomorphogenesis in Plants*. 2nd ed. Eds. RE Kendrick and GHM Kronenberg (Dordrecht, the Netherlands: Kluwer Academic Publishers). 353-373.

Møller SG, Kunkel T, Chua N-H (2001) A plastidic ABC protein involved in intercompartmental communication of light signalling. *Genes. Dev.*15, 90-103.

Montgomery BL, Franklin KA, Terry MJ, Thomas B, Jackson SD, Crepeau MW and Lagarias JC (2001) Biliverdin reductase-induced phytochrome chromophore deficiency in transgenic *Nicotiana tabacum* cv.Maryland Mammoth. *Plant Physiol.* 125, 266-277.

Montgomery BL, Yeh K-C, Crepeau MW and Lagarias JC (1999) Modification of distinct aspects of photomorphogenesis via targeted expression of mammalian biliverdin reductase in transgenic *Arabidopsis* plants. *Plant Physiol.* 113, 611-619.

Morgan DC and Smith H (1978) The relationship between phytochrome photoequilibria and development in light-grown *Chenopodium album L. Planta* 142, 187-193.

Morgan DC and Smith H (1976) Linear relationship between phytochrome photoequilibrium and growth in plants under stimulated natural radiation. *Nature* 262, 210-212.

Mosinger E, Batschauer A, Apel K, Schafer E and Briggs WR (1988) Phytochrome regulation of greening in barley-effects on mRNA abundance and on transcriptional activity of isolated nuclei. *Plant Physiol.* 86, 706-710.

Mozely D and Thomas B (1995) Developmental and photobiological factors affecting photoperiodic induction in *Arabidopsis thaliana Heynh. Landsberg erecta. J. Ex .Bot.* 46, 173-179.

Muramoto T, Kohchi T, Yokota A, Hwang I and Goodman HM (1999) The *Arabidopsis* photomorphogenic mutant *hy1* is deficient in phytochrome chromophore biosynthesis as a result of a mutation in a plastid heme oxygenase. *Plant Cell* 11, 335-347.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15, 473-497.

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

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

Nagatani A, Reid JB, Ross JJ, Dunnewij KA and Furuya M (1990) Internode length in *Pisum*. The response to light quality, and phytochrome type I and II levels in *lv* plants. *J. Plant Physiol.* 135, 667-674.

Neff MM, Fankhauser C and Chory J (2000) Light: an indicator of time and place. *Genes. Dev.* 14, 257-271.

Neff MM and Chory J (1998) Genetic interaction between phytochrome A, phytochrome B and cryptochrome 1 during *Arabidopsis* development. *Plant Physiol.* 118, 27-36.

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

Ni M, Tepperman JM and Quail PH (1999) Binding of phytochrome B to its signalling partner PIF3 is reversibly induced by light. *Nature* 400, 781-784.

Oelmüller R and Kendrick RE (1991) Blue light is required for the survival of the tomato phytochrome-deficient *aurea* mutant and the expression of four nuclear genes coding for plastidic proteins. *Plant Mol. Biol.* 16, 293-299.

Oelmüller R (1989) Photo-oxidative destruction of chloroplasts and its effects on nuclear gene expression and extraplastidic enzyme levels. *Photochem. Photobiol* 49, 229-239.

Oelze-Karow H and Mohr H (1978) Control of chlorophyll b biosynthesis by phytochrome. *Photochem. Photobiol.* 27, 189-193.

Okamoto H, Matsui M and Deng X-W (2001) Overexpression of the heterotrimeric G-protein α-subunit enhances phytochrome-mediated inhibition of hypocotyl elongation in *Arabidopsis*. *Plant Cell* 13, 1639-1656.

Oosawa N, Masuda T, Awai K, Fusada N, Shimada H, Ohta H and Takamiya K (2000) Identification and light-induced expression of a novel gene of NADPH-Protochlorophyllide Oxidoreductase isoform in *Arabidopsis thaliana*. *FEBS Lett.* 474, 133-136.

Ortiz de Montellano PR and Wilks A (2001) Heme oxygenase structure and mechanism. *Advances in Inorganic Chemistry* 51, 359-407.

Oster U, Brunner H and Rüdiger W (1996) The greening process in cress seedlings.5. Possible interference of chlorophyll precursors, accumulated after thujaplicin treatment, with light-regulated expression of Lhc genes. *Photochem. Photobiol.* 36, 255-261.

Oster U, Blos I and Rüdiger W (1991) The greening process in cress seedlings III. Age-dependent changes in the capacity of the tetrapyrrole pathway. *Z. Natuturforsch.* 46, 1052-1058.

Owen M, Gandecha A, Cockburn B and Whitelam GC (1992) Synthesis of a functional anti-phytochrome single chain F_v protein in transgenic tobacco. *Biotechnology* 10, 790-794.

Oyama T, Shimura Y and Okada K (1997) The *Arabidopsis HY5* gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyls. *Genes Dev.* 11, 2983-2995.

Page AM (1999) The cytoskeletal architecture of trypanosomes. Ph.D Dissertation, university of London.

Parker MW, Hendricks SB, Borthwick HA and Scully NJ (1946) Action spectrum for the photoperiodic control of floral initiation of short-day plants. *Bot. Gaz* 108, 1-26.

Parks BM and Quail PH (1993) *hy8*, a new class of *Arabidopsis* long hypocotyls mutants deficient in functional phytochrome A. *Plant Cell* 3, 39-48.

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

Papenbrock J, Mock H-P, Tanaka R, Kruse E and Grimm B (2000) Role of magnesium chelatase activity in the early steps of the tetrapyrrole biosynthetic pathway. *Plant Physiol.* 122, 1161-1169.

Papenbrock J, Mock HP, Kruse E, and Grimm B (1999) Diurnal and circadian rhythms in tetrapyrrole biosynthesis: antagonistic maxima of magnesium chelatase and ferrochelatase. *Planta* 208, 264-273.

Papenbrock J, Gräfe S, Kruse E Hänel F and Grimm B (1997) Mg-chelatase of tobacco: identification of ChlD cDNA sequence encoding a third subunit, analysis of the interaction of the three subunits with the yeast two-hybrid system, and reconstitution of the enzyme activity by co-expression of recombinant *CHLD*, *CHLH* and *CHLI*. *Plant J.* 12, 981-990.

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

Pauncz Y, Gepstein S and Horwitz BA (1992) Photocontrol of the accumulation of plastid polypeptides during greening of tomato cotyledons- potentiation by a pulse of red-light. *Plant Physiol.* 100, 1934-1939.

Pontoppidan B and Kannangara CG (1994) Purification and characterisation of barley glutamyl-tRNA reductase, the enzyme that directs glutamate to chlorophyll biosynthesis. *Eur. J. Biochem* 225, 529-537.

Pöpperl G, Oster U and Rüdiger W (1998) Light-dependent increase in chlorophyll precursors during the day-night cycle in tobacco and barley seedlings. *J. Plant Physiol.* 153, 40-45.

Quail PH (1997) An emerging molecular map of the phytochromes. *Plant Cell Environ.* 20, 657-665.

Quail PH, Boylan MT, Parks BM, Short TW, Xu Y and Wagner D (1995) Phytochromes: photosensory perception and signal transduction. *Science* 268, 675-680.

Quail PH, Schäfer E and Marmé D (1973) Turnover of phytochrome in pumpkin cotyledons. *Plant Physiol.* 52, 128-131.

Rascio N, Orsenigo M and Arboit D (1976) Prolamellar body transformation with increasing cell age in the maize leaf. *Protoplasma* 90, 253-259.

Rau W (1983) Photoregulation of carotenoid biosynthesis. In *Biosynthesis of Isoprenoid Compounds*. Eds. JW Porter and SL Spurgoen, Chichester, Wiley, pp123-157.

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

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

Reinbothe C, Lebedev N and Reinbothe S (1999) A protochlorophyllide light-harvesting complex involved in de-etiolation of higher plants. *Nature* 397, 80-84.

Reinbothe S and Reinbothe C (1996) Regulation of chlorophyll biosynthesis in angiosperms. *Plant Physiol.* 111, 1-7.

Reinbothe S, Reinbothe C, Lebedev N and Apel K (1996) PORA and PORB, two light-dependent protochlorophyllide-reducing enzymes of angiosperm chlorophyll biosynthesis. *Plant Cell* 8, 763-769.

Reinbothe S, Runge S, Reinbothe C, vanCleve B and Apel K (1995) Substrate dependent transport of the NADPH:Protochlorophyllide oxidoreductase into isolated plastids. *Plant Cell* 7, 161-172.

Reynolds ES (1963) The use of lead citrate at high pH as an elecron-opaque stain in electron microscopy. *J. Cell Biol.* 17, 209-213.

Roberts RH (1939) Further studies of the effects of temperature and other environmental factors upon the photoperiodic responses of plants. *Journal of Agricultural Research* 59, 699-709.

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

Rogers S, Wells R and Rechsteiner M (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* 234, 364-368.

Rowe JD and Griffiths WT (1995) Protochlorophyllide reductase in photosynthetic prokaryotes and its role in chlorophyll synthesis. *Biochem J.* 311, 417-424.

Rüdiger W, Thummler F, Cmiel E and Schneider S (1983) Chromophore structure of the physiologically active form (Pfr) form of phytochrome. *Proc. Natl. Acad. Sci. USA* 80, 6244-6248.

Runge S, Sperling U, Frick G and Armstrong GA (1996) Distinct roles for NADPH:Protochlorophyllide oxidoreductases POR A and B during greening in higher plants. *Plant J.* 9, 513-523.

Ryberg M and Sundqvist C (1988) The regular ultrastructure of isolated prolammellar bodies depends on the presence of membrane-bound NADPH-protochlorophyll oxidoreductase. *Physiol. Plant.* 73, 218-226.

Ryberg M and Dehesh K (1986) Localisation of NADPH-Protochlorophyllide oxidoreductase in dark-grown wheat (*Triticum aestivum*) by immuno-electron microscopy before and after transformation of the prolamellar bodies. *Physiol. Plant.* 66, 616-624.

Saefkow RL, Alliston TN and Shinkle JR (1995) Absence of PHYB inhibits hypocotyl elongation in dark-grown LH cucumber seedlings: an active role for PrB. *Plant Cell Environ.* 18, 831-835.

Sagar AD, Horwitz BA, Elliot R.C, Thompson WF and Briggs WR (1988) Light effects on several chloroplast components in norflurazon-treated pea seedlings. *Plant Physiol.* 88, 340-347.

Sage LC (1992) *Pigment of the imagination.* Academic Press, San Diego.

Sakamoto K and Nagatani A (1996) Nuclear localisation activity of phytochrome B. *Plant J.* 10, 859-868.

Santrucek J, Siffel P, Lang M, Lichtenthaler HK, Schindler C, Synkova H, Konecna V and Szabo K (1992) Photosynthetic activity and chlorophyll fluorescence parameters in aurea and green forms of *Nicotiana tabacum*. *Photosynthetica* 27, 529-543.

Sangwan I and O'Brian MR (1993) Expression of the soybean (*Glycine max*) glutamate-1-semialdehyde aminotransferase gene in symbiotic root nodules. *Plant Physiol.* 102, 829-834.

Schäfer E, Lassig T-U and Schopfer P (1975) Photocontrol of phytochrome destruction in grass seedlings: the influence of wavelength and irradiance. *Photochem. Photobiol* 22, 193-202.

Scheer H (1991) Chlorophyll proteins. In *Chlorophylls*. Ed. Scheer H, CRC Press Inc, Florida, USA.

Scheumann V, Klement H, Helfrich M, Oster U , Schoch S and Rüdiger W (1999) Protochlorophyllide *b* does not occur in barley etioplasts. *Febs Lett.* 445, 445-448.

Schneider-Poetsch HAW (1992) Signal transduction by phytochrome: Phytochromes have a module related to the transmitter modules of bacterial sensor proteins. *Photochem. Photobiol.* 56, 839-846.

Sharrock RA and Quail PH (1989) Novel phytochrome sequences in *Arabidopsis thaliana*: structure, evolution and differential expression of a plant regulatory photoreceptor family. *Genes Dev.* 3, 534-544.

Sharrock RA, Parks BM, Koornneeff M and Quail PH (1988) Molecular analysis of the phytochrome deficiency in an aurea muant of tomato. *Mol .Gen. Genet.*. 213, 9-14.

Shaw P, Henwood J, Oliver R and Griffiths WT (1985) Immunogold localisation of protochlorophyllide oxidoreductase in barley etioplasts. *Eur. J. Cell. Biol.* 39, 50-55.

Shimazaki Y, Pratt LH (1985) Immunochemical detection with rabbit polyclonal and mouse monoclonal antibodies of different pools of phytochrome from etiolated and green *Avena* shoots. *Planta* 164, 333-344.

Shinomura T, Nagatani A, Manzawa H, KubotaM, Watanabe M and Furuya M (1996) Action spectra for phytochrome A and B-specific photoinduction of seed germination in *Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA* 93, 8129-8133.

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

Siffel P, Santrucek J, Lang M, Braunova Z, Simkova H and Lichtenthaler HK (1993) Age-dependence of photosynthetic activity, chlorophyll fluorescence parameters and chloroplast ultratructure in *aurea* and green forms of *Nicotiana tabacum su/su* mutant. *Photosynthetica* 29, 81-94.

Singleton JW and Laster L (1965) Biliverdin reductase of guinea pig liver. *J. Biol. Chem.* 240, 4780-4789.

Sisler E.C and Klein WH (1963) The effect of age and various chemicals on the lag phase of chlorophyll synthesis in dark grown bean seedlings. *Physiol. Plant.* 16, 315-322.

Smith H (2000) Phytochromes and light signal perception by plants: an emerging synthesis. *Nature* 407, 585-591.

Smith AG, Cornah JE, Roper JM and Singh DP (1999) Compartmentation of tetrapyrrole synthesis in plant cells. In *Plant carbohydrate biochemistry.* Eds. JA Bryant, MM Burrell and NJ Kruger. Bios Scientific Publishers, Oxford.

Smith H (1995) Physiological and ecological function within the phytochrome family. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 46, 289-315.

Smith H, Samson G and Fork DC (1993) Photosynthetic acclimation to shade: probing the role of phytochromes using photomorphogenic mutants of tomato. *Plant Cell Environ.* 16, 929-937.

Smith H (1992) Light quality, photoperception and plant strategy. *Ann. Rev. Plant Physiol.* 33, 481-518.

Smith H and Whitelam GC (1990) Phytochrome, a family of photoreceptors with multiple physiological roles. *Plant Cell Environ.* 13, 695-707.

Smith H (1975a) Introduction. In: *Phytochrome and Photomorphogenesis.* McGraw-Hill. UK.

Smith H (1975b) The photo-control of chlorophyll synthesis. In: *Phytochrome and Photomorphogenesis.* McGraw-Hill. UK.

Soh MS, Hong SH, Hanzawa H, Furuya M and Nam HG (1998) Genetic identification of *FIN2*, a far-red light specific signalling component of *Arabidopsis thaliana. Plant J.* 16, 411-419.

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

Speth V, Otto V and Schäfer E (1987) Intracellular localisation of phytochrome in oat coleoptiles by electron microscopy. *Planta* 168, 299-304.

Sperling U, van Cleves B, Frick G, Apel K and Armstrong GA (1997) Over-expression of light-dependent PORA and PORB in plants depleted of endogenous POR by far-red light enhances seedling survival in white light and protects against photooxidative damage. *Plant J.* 12, 649-658.

Stone HJ and Pratt LH (1979) Characterization of the destruction of phytochrome in the red- absorbing form. *Plant Physiol.* 63, 680-682.

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN and Ames BN (1987) Bilirubin is an antioxidant of possible physiological importance. *Science* 235, 1043-1046.

Sullivan JA and Gray JC (2000) The pea *Light-Independent Photomorphogenesis 1* mutant results from a partial duplication of *COP1*, generating an internal promoter and producing two distinct transcripts. *Plant Cell* 12, 1927-1937.

Sundqvist C and Ryberg M (1989) The distribution and structural role of NADPH-protochlorophyllide oxidoreductase in isolated etioplast inner membranes. *Photosynthetica* 23, 427-438.

Susek RE, Ausubel FM and Chory J (1993) signal transduction mutants of *Arabidopsis* uncouple nulcear *CAB* and *RBCS* gene expression from chloroplast development. *Cell* 74, 787-799.

Susek RE and Chory J (1992) A tale of two genomes: role of a chloroplast signal in coordinating nuclear and plastid genome expression. *Aust. J. Plant. Physiol.* 19, 387-399.

Takano M, Kanegae H, Shinomura T, Miyao A, Hirochika H and Furuya M (2001) Isolation and characterisation of rice phytochrome A mutants. *Plant Cell* 13, 521-534.

Tanaka R, Oster U, Kruse E, Rüdiger W and Grimm B (1999) Reduced activity of geranyl geranyl reductase leads to loss of chlorophyll and tocopherol and to partially geranylgeranylated chlorophyll in transgenic tobacco plants expressing antisense RNA for geranylgeranyl reductase. *Plant Physiol.* 120, 695-704.

Tanaka A, Ito H, Tanaka R, Tanaka NK, Yoshido K and Okada K (1998) Chlorophyll *a* oxygenase (*CAO*) is involved in chlorophyll *b* formation from chlorophyll *a*. *Proc. Natl. Acad. Sci. USA* 95, 12719-12723.

Tanaka R, Yoshida K, Nakayashiki T, Tsuji H, Inokuchi H, Okada K and Tanaka A (1997) The third member of the *hemA* gene family encoding glutamyl t-RNA reductase is primarily expressed in roots in *Hordeum vulgare*. *Photosyn. Res.* 53, 161-171.

Tanaka R, Yoshida K, Nakayashiki T, Masuda T, Tsuji H, Inokuchi H and Tanaka A (1996). Differential expression of two *hemA* mRNAs encoding glutamyl-tRNA reductase proteins in greening cucumber seedlings. *Plant Physiol.* 110, 1223-1230.

Terry MJ, Ryberg M, Raitt CE and Page AM (2001) Altered etioplast development in phytochrome chromophore-deficient mutants. *Planta* In Press.

Terry MJ and Kendrick RE (1999) Feedback inhibition of chlorophyll synthesis in the phytochrome chromophore-deficient *aurea* and yellow-green-2 mutants of tomato. *Plant Physiol.* 119, 143-152.

Terry MJ (1997) Phytochrome chromophore-deficient mutants. *Plant Cell Environ.* 20, 740-745.

Terry MJ and Kendrick RE (1996) The *aurea* and yellow-green-2 mutants of tomato are deficient in phytochrome chromophore synthesis. *J. Biol.Chem.* 271, 21681-21686.

Terry MJ, McDowell MT and Lagarias JC (1995) (3Z)- and (3E)-phytochromobilin are intermediates in the biosynthesis of the phytochrome chromophore. *J. Biol. Chem.* 270, 11111-11118.

Terry MJ, Maines MD and Lagarias JC (1993) Inactivation of phytochrome and phycobiliprotein-chromophore precursors by rat liver biliverdin reductase. *J. Biol. Chem.* 268, 26099-26106.

Terry MJ and Lagarias JC (1991) Holophytochrome assembly. *J. Biol. Chem.* 266, 22215-22221.

Terzachi WB and Cashmore AR (1995) Seeing the light in plant development. *Curr. Biol.* 5, 466-468.

Thomas B and Vince-Prue D (1997) *Photoperiodism in Plants.* Academic Press. London.

Thomas B (1991) Phytochrome and photoperiodic induction. *Physiol. Plant.* 81, 571-577.

Thomas H (1997) Chlorophyll, a symptom and a regulator of plastid development. *New. Phytol.* 136, 163-181.

Thomsen B, Oelze-Karow H, Schuster C and Mohr H (1993) Stimulation of appearance of extraplastidic tetrapyrroles by a photo-oxidative treatment of the plastids. *Photochem. Photobiol.* 58, 711-717.

Tomitani A, Okada K, Miyashita H, Matthüss HCP, Ohno T and Tanaka A (1999) Chlorophyll *b* and phycobilins in the common ancestor of cyanobacteria and chloroplasts. *Nature* 400, 159-162.

Van Tuinen A, Hanhart CJ, Kerckhoffs LHJ, Nagatani A, Boylan MT, Quail PH, Kendrick RE and **Koornneef M (1996)** Analysis of phytochrome-deficient *yellow-green-2* and *aurea* mutants of tomato. *Plant J.* 9, 173-182.

Van Tuinen A, Kerckhoffs LHJ, Nagatani A, Kendrick RE and Koorneef M (1995a) A temporarily red light-insensitive mutant of tomato lacks a light-stable B-like phytochrome. *Plant Physiol.* 108, 939-947.

Van Tuinen A, Kerckhoffs LHJ, Nagatani A, Kendrick RE and Koorneef M (1995b) Far-red light insensitive, phytochrome A-deficient mutants of tomato. *Mol. Gen. Genet.* 246, 133-141.

Vierstra RD (1993) Illuminating phytochrome functions. *Plant Physiol.* 103, 679-684.

Vierstra RD and Quail PH (1983) Photochemistry of 124 kilodalton *Avena* phytochrome *in vitro*. *Plant Physiol.* 72, 264-267.

Vince-Prue D (1991) Phytochrome action under natural conditions. In: *Phytochrome Properties and Biological Action.* Eds B. Thomas and CB Johnson. Springer-Verlag, Berlin.

Vinti G, Hills A, Campbell S, Bowyer JR, Mochizuki N, Chory J and López-Juez E (2000) Interactions between *hy1* and *gun* mutants of *Arabidopsis* and their implications for plastid-nuclear signalling. *Plant J.* 24, 883-894.

Von Arnim AG (1999) Phytochrome in the limelight. *Trend. Plant Sci.* 4, 465-466.

Von Arnim AG and Deng X-W (1994) Light inactivation of *Arabidopsis* photomorphogenetic repressor COP1 involves a cell-specific regulation of its nucleocytoplasmic partitioning. *Cell* 79, 1035-1045.

Von Wettstein D, Gough S and Kannangara CG (1995) Chlorophyll biosynthesis. *Plant Cell* 7, 1039-1057.

Walker CJ and Willows RD (1997) Mechanism and regulation of Mg-chelatase. *Biochem. J.* 327, 321-333.

Wagner D, Hoecker U and Quail PH (1997) RED1 is necessary for phytochrome B-mediated red light specific signal transduction in *Arabidopsis*. *Plant Cell* 9, 731-743.

Wagner D, Fairchild CD, Kuhn RM and Quail PH (1996) Chromophore-bearing NH_2- terminal domains of phytochromes A and B determine their photosensory specificity and differential light lability. *Proc. Natl. Acad. Sci. USA 93,* 4011-4015.

Weller JL, Perrotta G, Schreuder MEL, van Tuinen A, Koornneef M, Giuliano G and Kendrick RE (2001) Genetic dissection of blue-light sensing in tomato using mutants deficient in cryptochrome 1 and phytochromes A, B1 and B2. *Plant J.* 25, 427-440.

Weller JL, Schreuder MEL, Smith H, Koornneef M and Kendrick RE (2000) Physiological interactions of phytochromes A, B1 and B2 in the control of development in tomato. *Plant J.* 24, 345-356.

Weller JL, Murfet IC and JB Reid (1997a) Pea mutants with reduced sensitivity to far-red light define an important role for phytochrome A in day-length detection. *Plant Physiol.* 114, 1225-1236.

Weller JL, Terry MJ, Reid JB and Kendrick RE (1997b) The phytochrome deficient *pcd2* mutant of pea is unable to covert biliverdin IXα to 3(Z) phytochromobilin. *Plant J.* 11, 1177-1186.

Weller JL, Terry MJ, Rameau C, Reid JB and Kendrick RE (1996) The phytochrome deficient *pcd1* mutant of pea is unable to convert heme to biliverdin IXα. *Plant Cell* 8, 55-67.

Weller JL, Nagatani A, Kendrick RE, Murfet IC and Reid JC (1995) New *lv* mutants of pea are deficient in phytochrome B. *Plant Physiol.* 108, 525-532.

Weller JL and Reid J (1993) Photoperiodism and photocontrol of stem elongation in two photomorphogenic mutants of *Pisum sativum L. Planta* 189, 15-23.

Whitelam GC and Devlin PF (1997) Roles for different phytochromes in *Arabidopsis* photomorphogenesis. *Plant Cell Environ.* 20, 752-758.

Whitelam GC and Devlin PF (1996) Novel phytochromes control germination and end-of-day far-red light responses of *Arabidopsis thaliana.* In *Current topics in Plant Physiology*, vol. 17. Regulation of Plant Growth and Development by Light. Eds. WR Briggs, RL Heath and E Tobin. ASPP Press, Rockville, MD,USA, pp 171-179.

Whitelam GC and Harberd NP (1994) Action and function of phytochrome family members revealed through the study of mutant and transgenic plants. *Plant Cell Environ.* 17, 615-625.

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

Whitelam GC, McCormac AC, Boylan MT and Quail PH (1992) Photoresponses of *Arabidopsis* seedlings expressing an introduced oat phyA cDNA: Persistence of etiolated plant phenotype responses in light-grown plants. *Photochem. Photobiol.* 56, 617-621.

Whitelam GC (1991) Post translational modification of phytochrome. In: *Phytochrome Properties and Biological Action.* Eds B. Thomas and CB Johnson. Springer-Verlag, Berlin.

Whitelam GC and Smith H (1991) Retention of phytochrome-mediated shade avoidance responses in phytochrome-deficient mutants of *Arabidopsis*, cucumber and tomato. *J. Plant Physiol.* 139, 119-125.

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

Yamaguchi R, Nakamura M, Mochizuki N, Kay S and Nagatani A (1999) Light-dependent translocation of a phytochrome B-GFP fusion protein to the nucleus of transgenic *Arabidopsis*. *J. Cell Biol.* 145, 437-445.

Yang Y-Y, Peters JL, Kendrick RE, Cordonnier-Pratt M-M and Kamiya Y (1998) A single red light pulse leads to a block of greening in the *high-pigment-1* mutant of tomato. *Planta* 206, 685-688.

Yanovsky MJ, Casal JJ and Whitelam GC (1995) Phytochrome A, phytochrome B and HY4 are involved in hypocotyl growth responses to natural radiation in *Arabidopsis*: weak de-etiolation of the *phyA* mutant under dense canopies. *Plant Cell Environ.* 18, 788-794.

Yaronskaya EB, Shalygo NV, Rassadina VV and Averina NG (1993) Changes of S-adenosyl-L-methionine:magnesium protoporphyrin IX methyltransferase activity in wheat after treatment with 5-aminolevulinic acid. *Photosynthetica* 29, 243-247.

Yeh KC and Lagarias JC (1998) Eukaryotic phytochromes: Light-regulated serine/threonine protein kinases with histidine ancestry. *Proc. Natl. Acad. Sci. USA* 95, 13970-13981.

Yeh KC, Wu S-H, Murphy JT, and Lagarias JC (1997) A cyanobacterial phytochrome two-component light sensory system. *Science* 277, 1505-1508.

Zaveleta-Mancera HA, Thomas BJ, Thomas H and Scott IM (1999) Regreening of senescent *Nicotiana* leaves II. Redifferentiation of plastids. *J. Ex. Bot.* 50, 1683-1689.